# Towards an effective improvement of fish health and welfare in aquaculture with standards for protocols for both the evaluation of mycotoxins and the on-farm assessment of fish welfare

Inaugural dissertation
of the Faculty of Science,
University of Bern

presented by

**Linda Tschirren**

from Niedermuhlern, BE

Supervisor of the doctoral thesis:
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Centre for Fish and Wildlife Health
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**Dr. Dominik Refardt**
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The Dean
Prof. Dr. Zoltan Balogh

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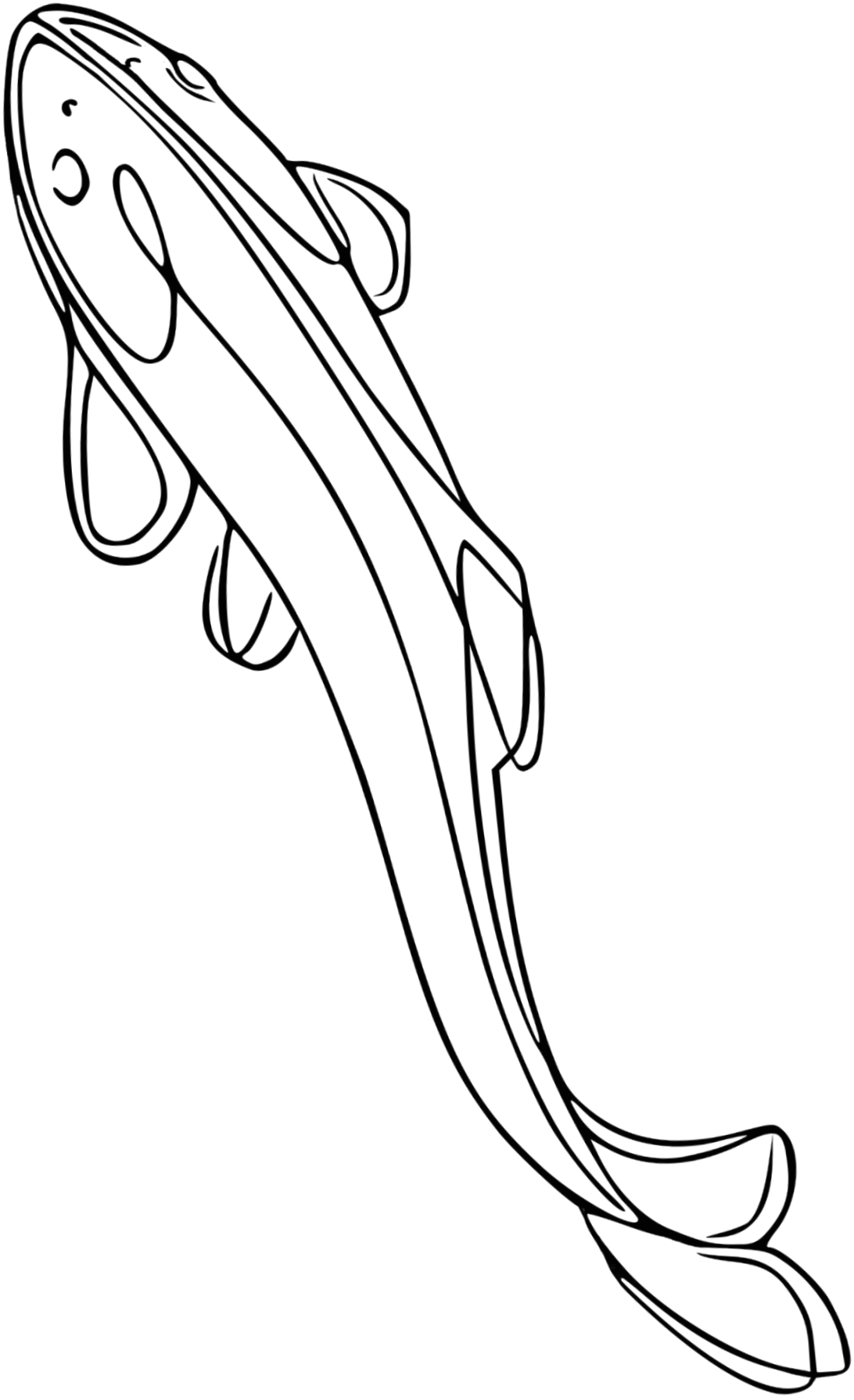


## Summary

Applied research in fish health and welfare misses methodological standards in certain areas and, hence, reasonable standardisation can assist effective research and facilitate improvements of fish health and welfare in aquaculture. This thesis reveals that this process of standardisation requires profound understanding of the system at hand in order to balance advantages and drawbacks of standards. By testing and developing standards for protocols this thesis contributes to improvements in fish health and welfare in the promising food production sector of aquaculture.

**Standards** bring continuity across space and time, yet they adapt and evolve and hence standardisation is an ongoing process. In applied research, where theoretical standardisation directly encounters practical complexity, applicability may force compromises on ideal standards that render standardisation an act of balance and reasoning. Especially in young fields of research and industry, such as aquaculture, the procedure of standardisation therefore must be preceded by the question about advantages and disadvantages of standards.

**Aquaculture** faces challenges like digitalisation, biosecurity and sustainability as well as increasing expectations regarding the production of environmentally sustainable food with an animal-friendly husbandry. Yet fish farming falls short of a profound understanding of the needs of fish and especially the ability to measure and assess fish health and welfare. The applied research in fish health and welfare is a rising and interdisciplinary field, that may benefit from better standards and improved standardisation.

**Fish health** is fundamental for an environmentally, economically and ethically successful farming and can be measured with standardised protocols. One such protocol, the fish embryo acute toxicity test, was used in chapter 1 of this thesis to investigate the effects of mycotoxins during embryogenesis. Mycotoxicosis increasingly affects aquaculture due to contaminated fish feeds, which necessitates expedient and efficient research. The chapter shows that this standardised protocol can benefit the investigation of mycotoxicosis in fish. The chapter further reveals a considerable variability in toxicological datasets, which impedes the necessary hazard characterisation of mycotoxins. Chapter 2 therefore explores potential factors contributing to this variability and formulates specific suggestions that can contribute to an improved standardisation in hazard characterisation trials. The chapter emphasises that and how better standards can benefit hazard characterisation processes for mycotoxins in aquaculture.

**Fish welfare**, contrary to fish health, lacks established and proven methods for a standardised assessment. As fish welfare is crucial for fish farming, chapter 3 elaborates on the development of a standardised protocol for on-farm fish welfare assessment. The model presented is comprehensive, applicable and developable and includes a user-friendly software application. This more standardised method can facilitate the harmonised on-farm evaluation of fish welfare which will promote improvements within the industry, will assist the fact-based discussion about fish welfare in aquaculture, will enable expedient advancements in regulations and will contribute towards a fish-friendly meat production.

# Introduction

# Advancement of fish health and welfare in aquaculture through standardisation

### *Reasonable standardisation in applied research is central*

An essential base of human societies is the creation of a common reality that is rooted in shared uniformities, rules and standards (Epstein & Timmermans 2018). While standards are generally viewed as inherently aspirational, the process of standardisation has the intrinsically demeaning connotation of diminishing flexibility and derogating uniqueness (Timmermans & Epstein 2010). Standardisation indeed forcibly imposes order on the world (Busch 2011) and with it is capable of ruling chaos and arbitrariness (Epstein & Timmermans 2018). Such standardised frameworks are imperative in natural sciences, where the ambition of gaining knowledge about underlying processes and general principles faces an immense complexity that obscures the patterns and rules nature follows (Auyang 1998). In the field of biology, the understanding of these rules and the revelation of such patterns is facilitated by standardisation as the explicit reduction of complexity. Here, models are intended to represent a simplified and therefore workable version of reality, and correlations as well as causations are investigated in specific and therefore tractable contexts (Gunawardena 2014). In biological research, one approach to break-down complexity is the standardisation of methodology. Here, standardisation of protocols enables the repeatability of trials, standardisation of methods allows for the comparison of results across treatments, studies and beyond and standardisation of methodologies facilitates the interpretation as well as extrapolation of data (Zutphen *et al.* 2001).

While standardisation brings continuity across space and time, it simultaneously changes over both (Epstein & Timmermans 2018), in other words, standards adapt and evolve and therefore standardisation is an ongoing process. Especially in applied research theoretical standardisation directly encounters practical complexity. Here, practicability as well as applicability may force compromises on ideal standards that render standardisation an act of balance, reasoning and self-reflection (CIOMS 2002). Setting and upholding standards therefore is a tedious and resource intensive process and hence should only be endeavoured if costs and benefits of uniformity are balanced (de Vries & Veurink 2017). Where standards already exist the question for their usefulness is worth revising (Würbel 2000), while in new fields of science and industry the procedure of standardisation has to be preceded by the question (Fig. 1) whether a standard will improve the current state (Holmes *et al.* 2010). One such young and complex field with yet little standardisation is aquaculture.

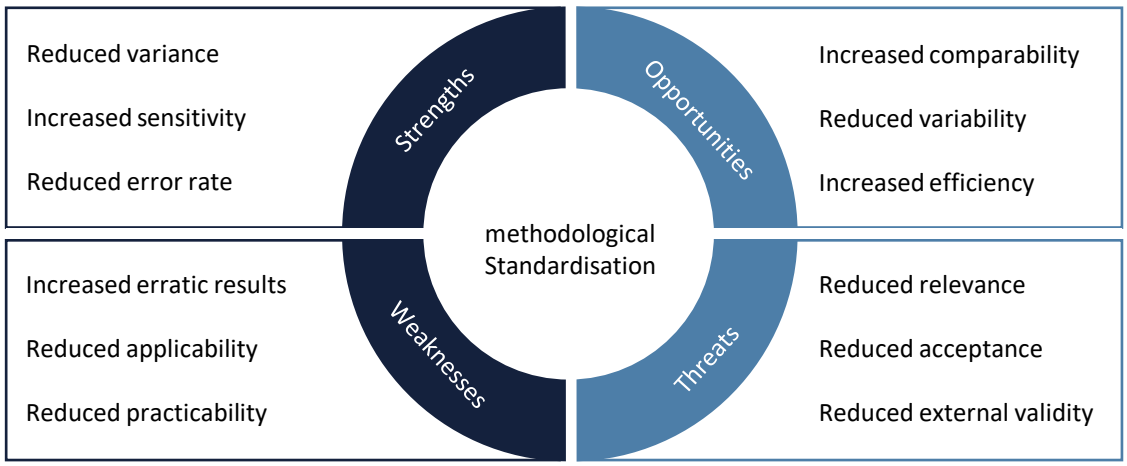



**Figure 1:** An assessment of potential costs and benefits of standardisation, e.g. in form of a SWOT analysis, should precede the establishment of standards.

### *Good fish health and welfare in aquaculture are crucial*

Historically, fish first and foremost came from fisheries and only millennia after terrestrial agriculture fish farming rose to lead a shadowy existence (Nash 2010). Since the 18th century, however, modern aquaculture developed rapidly and has now become a globally relevant source of food produced in diverse systems with various animal species (Tidwell 2012).

Past and feasible future technical advances and possibilities predestine aquaculture as an ecologically and economically sustainable type of meat production (Boyd *et al.* 2020). The industry will therefore grow and increasingly contribute to global food security (FAO 2018). As a growing industry aquaculture faces new challenges like digitalisation, biosecurity and sustainability (FAO 2020). Especially in Europe the population increasingly demands not only an environmentally sustainable food production but also an animal-friendly husbandry (European Commission 2020). However, aquaculture is lagging behind in its development in terms of knowledge about the needs of the animals farmed (de Mori 2019). Compared to fish husbandry, other branches of meat production have better developed control protocols, more trained professionals and a better basic knowledge of the animals (Keeling 2005). This is especially true for the ability to measure, assess and improve fish health and welfare, both being doubtlessly fundamental for an environmentally, economically and ethically successful farming (Segner *et al.* 2019).

The health and welfare of fish are intertwined, but they are not the same. Typically, fish health encompasses the physiological status of the animal and is a purely function-based approach (Segner *et al.* 2012). Fish welfare, however, is evolving both as a concept and as a term (Lawrence 2008; Kristiansen & Bracke 2020). Latest with the introduction of the 5 freedoms (Webster 2005) the term welfare included more than health. For fish first pain (Sneddon *et al.* 2003*a*; Sneddon 2020) and later stress (Madaro *et al.* 2020) was encompassed into the framework of welfare pushing the concept beyond physiological health. This raised issues of ethics (Bovenkerk & Meijboom 2020) and propelled further research (Huntingford & Kadri 2009) investigating the concepts of awareness (van den Bos 2020), consciousness (Fernö *et al.* 2020) and perception (Kristiansen & Fernö 2020) in teleosts. The field developed fast from being merely disease

and water quality focused (Meyer 1991) to a broad and more comprehensive branch of science that included animal perception and behaviour (Kiessling *et al.* 2012). Nowadays the field of fish welfare is multidisciplinary and includes law and ethics, behaviour and genomics, biology and physiology (Kristiansen *et al.* 2020). Prospectively the field of fish welfare will gradually include the industry and authorities, with the FAO (Food and Agriculture Organization of the United Nations) and its European Inland Fisheries and Aquaculture Advisory Commission (EIFAAC) as well as the European Commission and its EFSA (European Food Safety Authority) acknowledging the importance of the topic (European Commission 2018; Segner *et al.* 2019). As a rising field of applied research and at the interface with a young and diverse industry, fish health and welfare may benefit from improved standardisation.

## *Improvement of fish health and welfare in aquaculture through standardisation*

One aspect of applied research, which may improve fish health and welfare through standardisation, is the development of standardised experimental methods and protocols. Here, the benefits of increased reliability, accuracy and transparency of the data can be worth the costs implied by the standardisation process (Holmes *et al.* 2010) and the advantages of increased sensitivity of the experiment may outweigh the drawbacks of reduced external validity of the results (Richter *et al.* 2009). This thesis uses three specific examples to ask and answer the question whether methodological standardisation in applied research promotes advancements in fish health and welfare in science as well as the industry. For each example, the introduction will present the problem at hand, the corresponding chapter will outline a potential solution, while the discussion will review the usefulness of the proposed standardisation:

- Chapter 1 examines whether a specific protocol, the fish embryo acute toxicity test, can be used for the characterisation of toxicological effects of anti-nutritive substances such as mycotoxins in fish. The chapter confirms that the use of this standardised protocol can indeed benefit the investigation of mycotoxicosis in aquaculture. Further, the work shows the scarcity of and variability in data about the effects of mycotoxins in teleosts.
- Chapter 2 therefore investigates, which methodological and biological factors of experimental protocols cause this increased variability in toxicity data. The chapter reveals that better standardisation can indeed benefit the hazard characterisation process for mycotoxins in fish. Furthermore, the work shows the diversity of endpoints used to assess fish health and welfare.
- Chapter 3 thus explores how fish welfare can be assessed and presents a comprehensive model for fish welfare assessment. The chapter emphasises that the use of a standardised on-farm assessment protocol can indeed benefit fish welfare. Further, the work outlines possibilities to validate and develop the model for future purposes.

# A standard protocol for the efficient evaluation of mycotoxins in aquaculture (Chapter 1)

## *Mycotoxins in aquaculture are harmful*

A major aspect of animal husbandry, and hence fundamental for animal health and welfare, is the quantitatively and qualitatively appropriate nutrition. Qualitative aspects not only include the suitable absolute and relative amounts of macro- and micronutrients but also the absence of anti-nutritive substances. An emerging group of such anti-nutritive substances are mycotoxins, facultative metabolites produced by members of the fungus kingdom in different environments and under diverse conditions (Cheeke 1998). The toxic effects of these metabolites are relevant in human and animal nutrition due to direct ingestion (Abnet 2007) as well as potential biomagnification (Gonçalves *et al.* 2020). The probability of mycotoxin contamination of animal feed is increased by the use of plant-based ingredients, which are prone to fungal infection (Gonçalves *et al.* 2020). Despite this, industries such as the aquaculture sector increasingly use plant-based ingredients in feeds (Tacon *et al.* 2011; Jannathulla *et al.* 2019) since they are more environmentally sustainable and cost effective. Thus, the fish-farming industry is increasingly affected by feed-related mycotoxicosis (Goncalves *et al.* 2017; Matejova *et al.* 2017).

Mycotoxicosis due to contaminated fish feed threatens fish health and welfare and causes losses due to acute toxicity as well as chronic exposure (Anater *et al.* 2016). While the focus so far was mainly on effects on fish health, e.g. increased mortalities or reduced growth performance and disease resistance (Gonçalves *et al.* 2018*b*), more recent works include impaired fish welfare as a consequence of mycotoxicosis (Mantovani 2010; Gonçalves *et al.* 2020). The aquaculture industry understands the negative impact of mycotoxins on fish health and welfare and tackles the problem on three levels (Gonçalves & Muccio 2019): (I) Minimization of the initial contamination by preventing fungal growth through improved plant resistance, better field and pesticide management, as well as optimizing storage conditions; (II) Minimization of the effects of contaminated feed through additional production steps intended to remove or destroy the mycotoxins, or through additives and binders in the feed to reduce the toxicity; (III) Monitoring of mycotoxin occurrence through standardized and representative sampling and analyses of feed. Simultaneously, the scientific community contributes the underlying information needed to help prevent and contain losses. Basic science studies the molecular mode of actions of mycotoxins (Rotter & Prelusky 1996), while applied research investigates adverse and beneficial effects of the mycotoxins (Woźny *et al.* 2019) and explores potential counter measures for mycotoxicosis (Fadl *et al.* 2020). Given the economic importance of mycotoxicosis in aquaculture (Gonçalves & Kovalsky 2013) timely progress in research is desirable. Hence, the use of a model fish species might facilitate more efficient research on this topic.

## *Model organisms in aquaculture research are contestable*

When standardisation is used in science to break down complexity, model organisms play a relevant role. Each branch of the tree of life has its typical representatives, and the biological similarities allow for

the extrapolation and generalisation of insights gained with these few specific species. The use of model organisms allows for a more efficient, more focused and more strategic progress in science (Ankeny & Leonelli 2011). And while *in silico* and *in vitro* alternatives are on the rise, the use of whole organisms will remain part of science until the fundamental biological mechanisms are fully understood (Hunter 2008). However, using model species has drawbacks, e.g. a self-enforcing impoverishment of studied species (Farris 2020) or an increasing endangerment of reproducibility (Richter *et al.* 2009) and external validity (Würbel 2000). Hence, model organisms may be utilised in applied research where their advantages propel progress, while the full genetic and phenotypic diversity of various species should be used where appropriate.

For teleosts, an especially diverse taxonomic group, one of the most prevalent model organisms is the zebrafish *Danio rerio* (Parichy 2015). This wide-spread species is used in many fields of science since decades as it is robust in handling, sturdy in husbandry, has a short generation time and a high fecundity, an external fertilisation and no parental care and is transparent during embryogenesis (Dooley & Zon 2000). However, using the zebrafish as a model organism in the field of applied aquaculture research has been suggested only recently (Ribas & Piferrer 2014), possibly due to its irrelevance for industrial fish farming. Yet, it is exactly this field with a broad diversity of fish species where a model species could facilitate research. Particularly, specific protocols, which are tailored to the species, widely used and highly standardised such as fish toxicity tests defined by the Organisation for Economic Cooperation and Development (OECD 2012), may benefit mycotoxin research in aquaculture.

## *A standard protocol for mycotoxin research in aquaculture*

For investigating anti-nutritive substances such as mycotoxins both the screening for their occurrence as well as the investigation of their toxicokinetics and toxicodynamics are key. In both cases, a model organism such as the zebrafish, can be advantageous not least because standardised protocols are available, e.g. the fish acute toxicity test (OECD 2019), fish early-life stage toxicity test (OECD 2013*a*) or the fish embryo acute toxicity test (OECD 2013*b*). In chapter 1 of this thesis the toxicity of ochratoxin A, a common mycotoxin, is tested using a fish embryo acute toxicity test with zebrafish and the results are compared to existing literature (Fig. 2). It is then discussed whether the applicability of this specific protocol and the reliability of the results support the use of this standardised protocol for the investigation of mycotoxicosis in aquaculture.

Standardised protocols affect the variability of data not only within but also between trials. Chapter 1 elaborates on the use of an established protocol in a new field of research and with it uncovers a considerable data variability in current literature, an issue investigated in chapter 2.

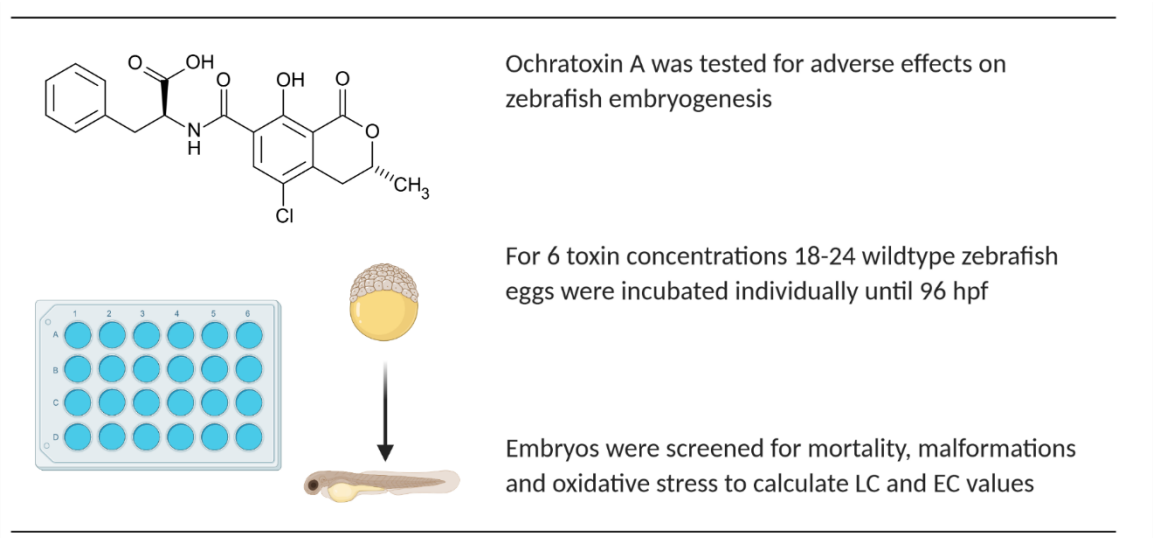



**Figure 2:** Graphical abstract of chapter 1. BioRender.com

## Standardised protocols to reduce data variability in mycotoxin research (Chapter 2)

### *Mycotoxin hazard characterisation is challenging*

International feed and feed ingredient standards lack fish-specific contamination limits for most mycotoxins (European Commission 2006, 2016). This leaves aquaculture and with it the health and welfare of farmed fish at risk for mycotoxicosis (Gonçalves *et al.* 2020). Authorities struggle to setup regulations for mycotoxins for fish due to an impeded hazard characterisation, with the EFSA stating in 2017 that "very limited toxicity data are available for fish for zearalenone" (Knutsen *et al.* 2017*a*). And even if more data is available the definition of threshold values seems difficult, with the EFSA mentioning that for farmed fish "the hazard characterization of deoxynivalenol was overall more difficult than for other farm animals because of the designs and analysis methods chosen by the authors and a large variation in experimental protocols and endpoints measured" (Knutsen *et al.* 2017*b*). These difficulties are reflected in reviews about effects of mycotoxins in fish, which highlight the limited number of studies (Matejova *et al.* 2017), the diversity in study designs (Gonçalves & Muccio 2019), the variation in protocols and fish species used (Anater *et al.* 2016; Oliveira & Vasconcelos 2020) and the variety of endpoints assessed (Pietsch 2020). Together, these issues result in a level of variability in toxicity data that composes an obstacle for data interpretation and, therefore, hazard characterisation, risk assessment and the definition of contamination limits.

### *Standardisation for data variability reduction is debatable*

The variability in toxicity metadata sets might be caused by differences between the individual studies and hence can be gradually decreased through the standardisation of protocols applied. The dose-response-relationship values, which are reported in hazard characterisation studies, strongly depend on methodological (Hrovat *et al.* 2009), biological (Dutra Costa *et al.* 2020) and technical (Gustafson *et al.* 2012) characteristics in a given experiment. Therefore, differences in those characteristics are major sources of variability in toxicological data (Busquet *et al.* 2014). The general principle is to avoid all sources of variability where possible, account for them when they cannot be avoided (or are desirable) and report them when neither is possible. However, rigorous standardization in the past gave rise to the reproducibility crisis (Voelkl & Würbel 2016) where little technical and environmental (Richter *et al.* 2009) as well as biological (Voelkl *et al.* 2020) variation leads to low external validity of data and poor reproducibility of results. In other words, too much standardisation in toxicological trials can lead to erratic and false positive results. Therefore, reasonable standardisation processes will improve data comparability while ensuring reproducibility and validity.

### *Reduction of variability in mycotoxin hazard characterisation trials with teleosts*

For a reasonable standardisation of protocols it is crucial to understand which variations in a study potentially give rise to which amount of variability in the data, i.e. to be able to estimate the relative importance of different sources of variability. In chapter 2 the relative importance of four different sources of variability in toxicological trials on mycotoxins in fish are investigated (Fig. 3). Based on a literature survey the amount of data variability explained by two methodological factors (*identity of mycotoxin* and *mycotoxin application method*) and two biological factors (*fish species* and *endpoint assessed*) were calculated. The transferability of the insights from this metadata set on an individual toxicological study is tested by conducting a laboratory trial with a defined mycotoxin application method (fish embryo acute toxicity test), one varying methodological factor (*identity of mycotoxin* i.e. ochratoxin A, deoxynivalenol and zearalenone) and two varying biological factors (*fish species* i.e. zebrafish, pikeperch and rainbow trout, and *endpoint assessed* i.e. lethality and morphological malformations). Together, the literature survey and the toxicity test facilitate the formulation of specific suggestions on how to improve on data variability in mycotoxin research. It is discussed which steps of standardisation can benefit mycotoxin research in aquaculture and assist future hazard characterisations and risk assessments.

The first two chapters outline the potential advantages of standardisation on the specific example of investigating the adverse effects of mycotoxins on fish health and welfare. It becomes apparent that while there are methods to measure the health, there are little techniques to assess welfare. Chapter 3 therefore elaborates on the welfare of farmed fish and possible benefits of a standardised fish welfare assessment method.

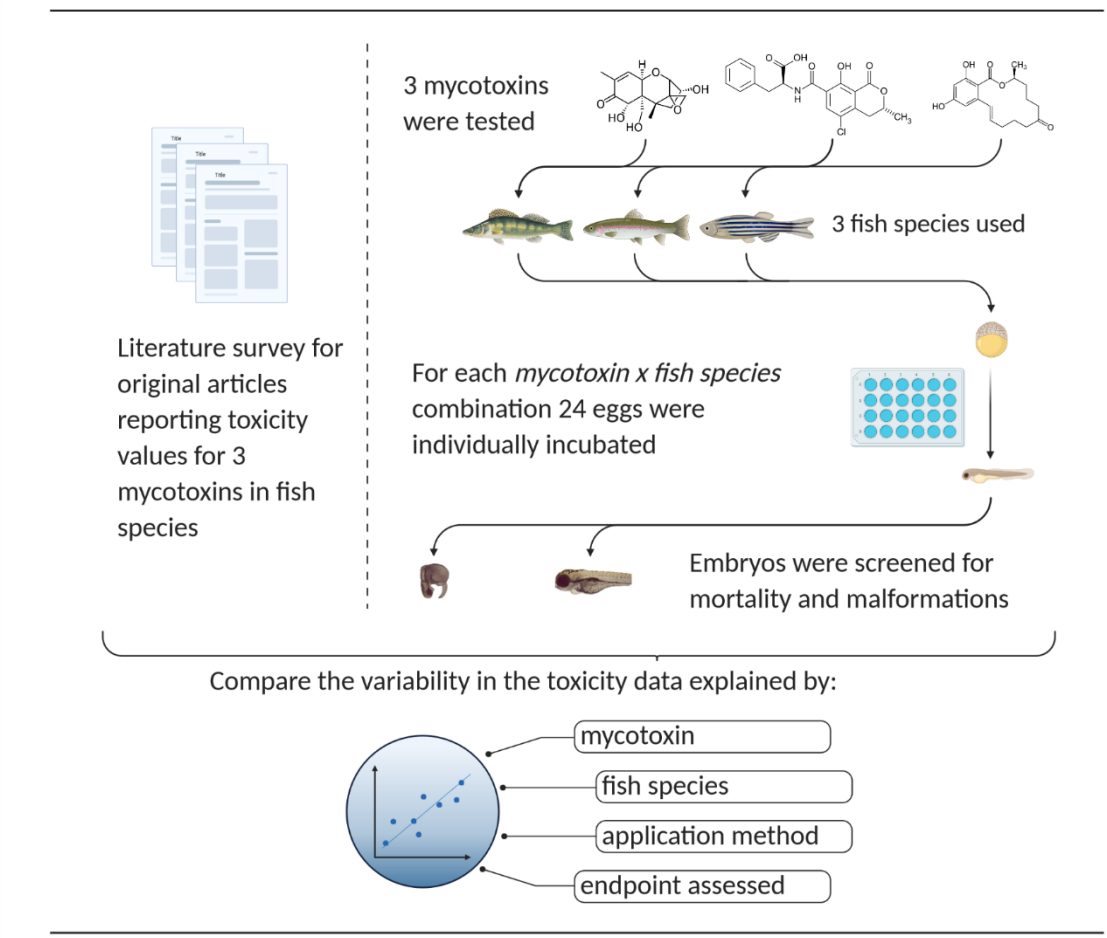



**Figure 3:** Graphical abstract of chapter 2. BioRender.com

# A model for the standardised on-farm assessment of fish welfare (Chapter 3)

## *On-farm assessment of fish welfare is important*

The evaluation of fish welfare is a convoluted topic with very limited applicable and proven methodology for the aquaculture industry. This field of knowledge is progressing fast, driven by scientific insights (Kristiansen *et al.* 2020), industrial pressure (Richards *et al.* 2013) and public demand (Zander & Feucht 2018). Teleosts were only ascribed pure nociception in the early 2000 (Sneddon *et al.* 2003*a*), followed by the capability of experiencing fear (Braithwaite & Boulcott 2007) and the conclusion of fish being sentient creatures (Brown 2015). The practical consequences of these insights for research, aquaculture industry and societal ethics are considerable (de Mori 2019). However, applicable implementations show a time lag with the first on-farm assessment method for farmed fish being published only in 2013 (Stien *et al.* 2013). The reason for this delay is that, in contrast to theoretical concepts, practical applications need verification in the field, iterative improvements and user acceptance. These latter

processes are resource intensive. Nevertheless, both a functioning on-farm welfare assessment for the aquaculture industry as well as a laboratory-suited welfare evaluation for research is needed (Stien *et al.* 2020), especially since healthy and well fish are as key for an environmentally, economically and ethically successful farming (Segner *et al.* 2019) as they are for a reliable science (Sørensen *et al.* 2020).

### *Standardization of overall fish welfare assessment is contentious*

While the necessity of an on-site health and welfare assessment is unquestionable, the degree of appropriate standardisation is a point of discussion. In a field with dozens of different farming systems (Tidwell 2012) and numerous farmed species (FAO 2020) the costs of uniformity are to be considered. Since the relevance of different welfare indicators varies between husbandry systems and threshold values for these indicators can be species-specific (Noble *et al.* 2018, 2020), a full standardisation might be in conflict with the validity, reliability and applicability of a welfare assessment method. However, a more harmonised assessment of welfare in fish will allow to evaluate the current state, to compare and contrast potential improvements, to measure progress and to have a fact-based discussion about the relevance of the topic in general. A fruitful approach can be to standardise where possible, i.e. where indicators of welfare or prerequisites for welfare are consistent across farming systems and fish species, while to individualise where needed, i.e. where indicators and prerequisites are known to be explicit. This compromise asks for a new protocol for welfare assessment with adaptable and developable levels of standardisation.

### *A new model for on-farm fish welfare assessment in aquaculture*

Fish health and welfare is a complex state to evaluate and, hence, there are several requirements that an assessment protocol must fulfil. Comprehensiveness allows for an assessment of overall wellbeing, transparency facilitates the understanding amongst experts, adaptability assists the inclusion of more systems and species, developability enables the incorporation of new knowledge and applicability grants the practical use on-site. None of the attempts for such protocols found wide-spread use in the industry so far (Stien *et al.* 2013; Pettersen *et al.* 2014; Kleingeld *et al.* 2016; Noble *et al.* 2018, 2020; Müller-Belecke 2019; Studer *et al.* 2020), which indicates that these protocols do not fulfil one or more of the requirements. Nevertheless, these works are opportunities to considerably advance this field of science and industry by building upon their advantages and improving on their shortcomings.

Chapter 3 describes the development of a new fish welfare assessment protocol in five steps: (I) the definition of fish welfare in the context of allostasis (Korte *et al.* 2007) and the creation of an ontology of the current knowledge using semantic data modelling, (II) the evaluation and selection of welfare parameters, (III) the establishment of a scoring system, (IV) the development of an equation to calculate welfare grades, and (V) the development of a software application (Fig. 4). It is then discussed how the use of this standardised protocol can benefit on-farm fish welfare assessment in the aquaculture industry.

This thesis, as a whole, emphasises specific areas where applied research on fish health and welfare misses methodological standards and how reasonable standardisation can facilitate effective improvements of fish health and welfare in aquaculture in the future.

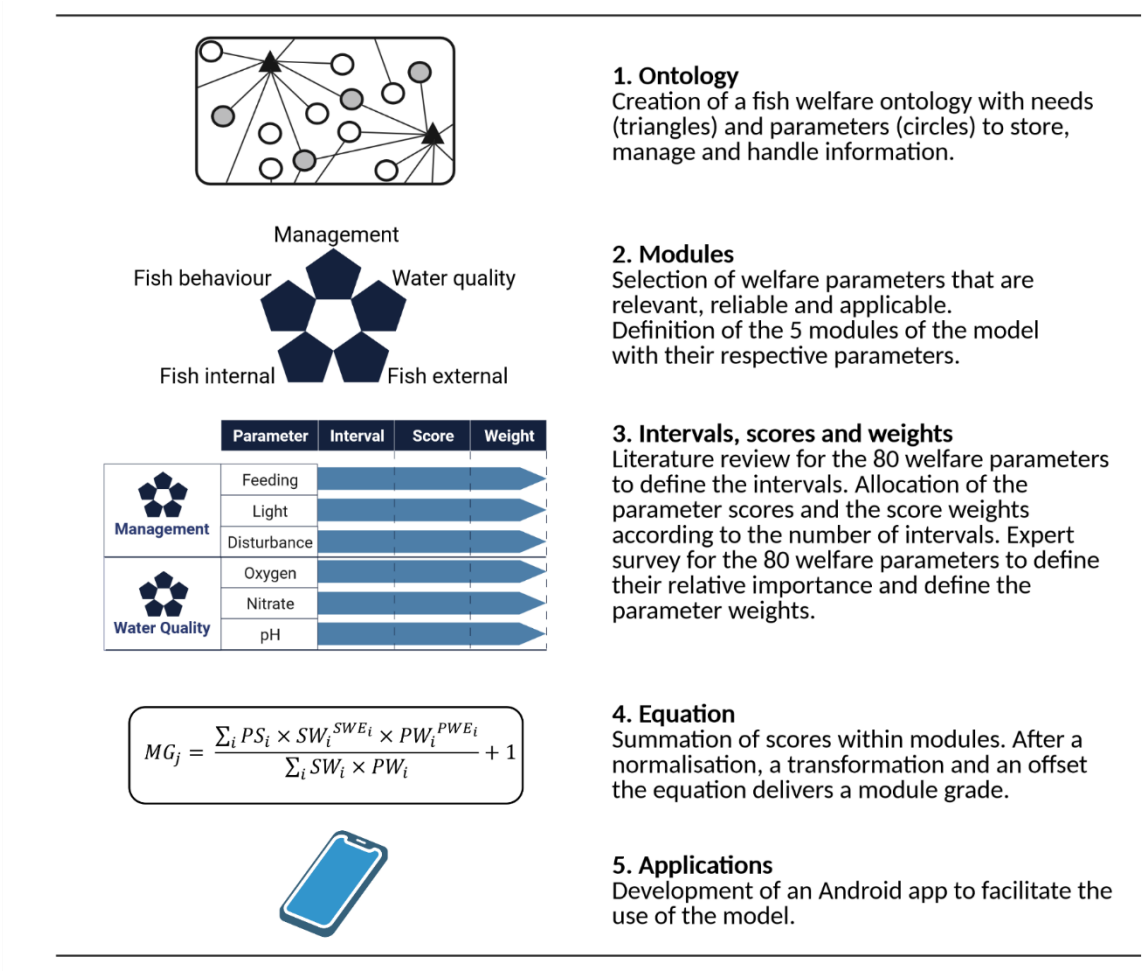
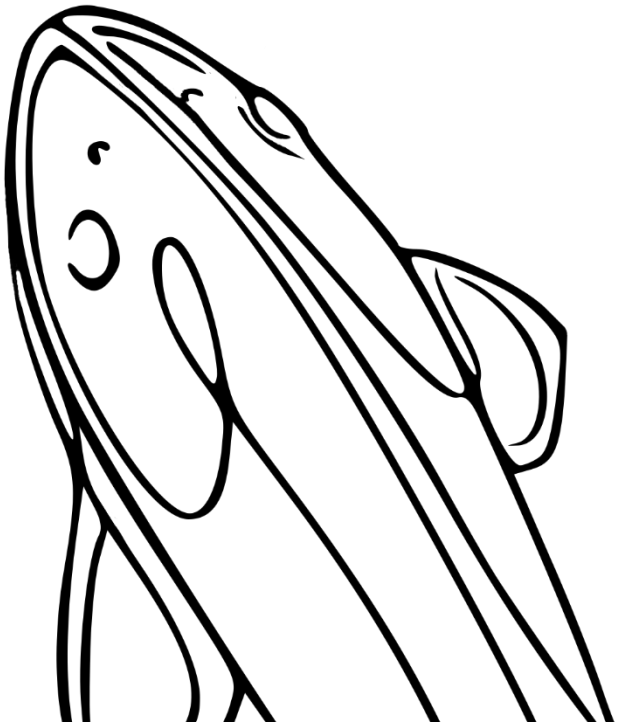
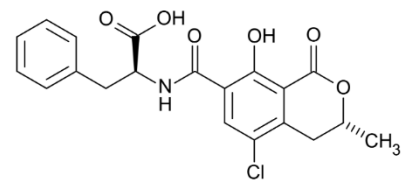



**Figure 4:** Graphical abstract of chapter 3. BioRender.com

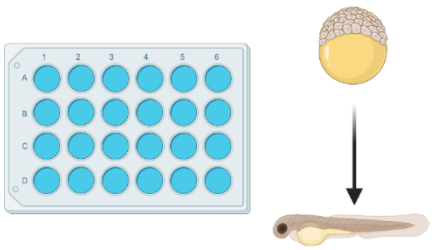


# Chapter 1

## Toxicity of ochratoxin to early life stages of zebrafish (*Danio rerio*)




Ochratoxin A was tested for adverse effects on zebrafish embryogenesis



For 6 toxin concentrations 18-24 wildtype zebrafish eggs were incubated individually until 96 hpf

Embryos were screened for mortality, malformations and oxidative stress to calculate LC and EC values

# Toxicity of ochratoxin to early life stages of zebrafish (Danio rerio)

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**Abstract**: Ochratoxin A is a known contaminant in fish feed but its effect on fish health remains rather unknown. A study was conducted to investigate the effects of different concentrations of ochratoxin on early life stages of zebrafish (*Danio rerio*). The tests with ochratoxin A showed a correlation between the exposure to mycotoxin and the amount of damage. The mortality rate and the incidents of embryonal damage was increased by increasing ochratoxin concentrations. The calculations resulted in a lethal concentration for 50 % of the embryos ($LC_{50}$) of 0.36 mg/l and a concentration at which 50 % of the animals showed impairment ($EC_{50}$) of 0.29 mg/l after 96 h of exposure. During the test, reduced heart rates were also observed revealing a clear dose-response relationship. The $EC_{50}$ determination for this endpoint was 1.26 mg/l after 72 h of exposure. The measurement of oxidative stress was proven to be the most sensitive system to indicate OTA effects on the zebrafish embryos with an $EC_{50}$ value of 0.067 mg/l after 72 h of exposure. The test validity was given because the control test with 3,4-Dichloroaniline showed a $LC_{50}$ value of 2.88 mg after 96h of exposure which is comparable to the available reference values. According to the current knowledge, these experimental doses did not exceed the environmental concentrations of this ochratoxin A. However, this study raises concerns about the effects of ochratoxin on fish.

**Keywords**: mycotoxin, embryo toxicity, heart rates, oxidative stress

**Paper:** Published in Toxins 2018, 10, 264; doi:10.3390/toxins10070264

## *Introduction*

Ochratoxin A (OTA) is mainly produced by fungi of the genus *Aspergillus* and *Penicillium*. As a consequence of their world-wide occurrence, OTA has been found in feed ingredients and feeds at variable contamination levels which are assumed to be caused by differences in humidity and temperature during crop growth and during storage of feed ingredients and compounded feeds (Binder *et al.* 2007; Duarte *et al.* 2010; Rodrigues & Naehrer 2012). The contamination with OTA has to be taken seriously since OTA is assumed to be more stable in the environment than, for example aflatoxins (Moss 2002; Duarte *et al.* 2010). Contaminated feed products lead to the introduction of OTA in the food chain and a risk for humans is assumed (Manning *et al.* 2003). The presence of OTA in the food chain also resulted in detectable OTA levels in humans (Reddy & Bhoola 2010). In addition, waste water is produced at different steps during wine production and winery effluents have been shown to contain considerably high concentrations of OTA (Vlyssides *et al.* 2005; Nogueira *et al.* 2007) which might be an additional point source of OTA for aquatic environments. The effects of OTA in vertebrates are therefore of great concern.

In higher vertebrates, toxic effects of OTA are mainly observed in the kidney and liver and OTA was also reported to be teratogenic and immunotoxic (Duarte *et al.* 2011). In rodents, carcinogenic effects have also been observed (Boorman 1989). In animals, therefore, a higher susceptibility to disease and more secondary infections have been observed (Duarte *et al.* 2011). Embryotoxicity has been shown in amphibians and rats, mice, hamsters and chickens (Hood *et al.* 1976; Brown *et al.* 1976; Arora *et al.* 1983; Wiger & Størnier 1990; O'Brien *et al.* 2005). In addition, exposure of zebrafish embryos to OTA resulted in a variety of severe abnormalities, such as deformities, reduced growth and hatching rates and lethality at concentrations as low as 0.1 mg/l exposure medium (Haq *et al.* 2016). Furthermore, the injection of rainbow trout with OTA resulted in kidney and liver damage and the calculation of a lethal concentration for 50 % of the animals ($LC_{50}$) after 96 h of exposure to OTA of 4.7 mg/kg body weight (Doster *et al.* 1974). Dietary exposure of channel catfish (*Ictalurus punctatus*) and sea bass (*Dicentrarchus labrax*) to OTA led to reduced weight gains, poorer feed conversion rates, lower survival and changes of haematocrit values (Manning *et al.* 2003; El-Sayed *et al.* 2009) in these fish species but not in Atlantic salmon (*Salmo salar* (Bernhoft *et al.* 2017)). In addition, histopathological damage in the liver and posterior kidney and changes of immune parameters were observed in channel catfish (Lovell 1992; Manning *et al.* 2003; Zahran *et al.* 2016). Similar studies on Nile tilapia (*Oreochromis niloticus*) showed that increasing dietary OTA levels resulted in decreased growth, feed utilization and nutrient composition of the carcass (Srour 2004). In contrast to the investigations on fish, a study on shrimp reported no pronounced negative effects of OTA at levels of up to 1 mg/kg (Supamattaya *et al.* 2005). In naturally contaminated feeds, OTA commonly occurs together with other mycotoxins (Hauptman *et al.* 2014) but interactions with other toxins have not been reported.

The present study investigated effects of OTA to describe possible threshold values for fish embryo toxicity for this mycotoxin and targets of OTA in zebrafish.

## *Results*

**Effects on zebrafish embryo development**

As expected, the embryos after 24 h of exposure still use the reserves from the yolk sac and do not yet have pigmentation. This exposure duration causes significant damage due to OTA exposure at concentrations higher than 1.25 mg/l ($p = 0.000$). The detrimental effects of OTA on zebrafish embryos followed a polynomic relationship after 24 h of exposure ($y = 0.4595x^2 + 18.254x - 1.6657$; $r^2 = 0.97$) and a concentration at which 50 % of the embryos showed any detrimental effects ($EC_{50}$ value) including death was identified at 2.65 mg/l (Figure 1A, Table 1). The calculation of a $LC_{50}$ value at this point in time resulted in a high level with low reliability ($LC_{50}$ = 24.22 mg/l; $y = -0.0106x^2 + 1.6564x + 3.6737$; $r^2 = 0.21$). After 48 h, body pigmentation in normal embryos starts; the eye is dark and the yolk sac is further depleted. However, the detrimental effects on the embryos led to a significant developmental retardation at concentrations higher than 0.31 mg/l ($p = 0.011$, Figure 1B) and the effects showed an $EC_{50}$ value (integrating all detrimental effects on the embryos including death) of 0.73 mg/l ($y = -28.917x^2 + 121.81x - 23.178$; $r^2 = 0.99$) after 48 h of exposure. The single embryo that showed a conspicuous development in the solvent control had a yolk sac-oedema (Figure 1B), which was no longer detectable after 72 h and 96 h of exposure. At 48 h of exposure, a more reliable $LC_{50}$ value of 2.57 mg/l OTA was obtained ($y = 3.2147x^2 - 7.919x + 8.3314$, $r^2 = 0.88$). After 72 h of exposure, OTA concentrations higher than 0.31 mg/l resulted in significant damage to the embryos ($p = 0.000$, Figure 1C). At this point, an $EC_{50}$ value (integrating all detrimental effects on the embryos including death) of 0.55 mg/l was calculated ($y = -24.578x^2 + 119.68x - 9.2249$, $r^2 = 0.95$) and 50 % of the animals were found to be dead at 3.32 mg/l OTA ($y = 3.0667x^2 + 4.2717x + 2.0125$, $r^2 = 0.98$).

| Time Point | $EC_{50}$ (mg/l) | $LC_{50}$ (mg/l) | Ratio $LC_{50}$ to $EC_{50}$ |
|---|---|---|---|
| 24 h | 2.65 | 24.22 | 9.14 |
| 48 h | 0.73 | 2.57 | 3.52 |
| 72 h | 0.56 | 3.32 | 5.93 |
| 96 h | 0.29 | 0.36 | 1.24 |

**Table 1:** Summary of the calculated $LC_{50}$ and $EC_{50}$ values for the respective test durations for embryos exposed to different ochratoxin A concentrations, n = 24 for each treatment group.

At 96 h of exposure, there was a pronounced increase in mortality. Significant effects on zebrafish development were observed at OTA concentrations higher than 0.16 mg/l ($p = 0.000$). At this point in time, an $EC_{50}$ value (integrating all detrimental effects on the embryos including death) of 0.29 mg/l was calculated ($y = -0.9341x^2 + 2.2807x - 0.0455$; $r^2 = 0.96$), whereas the $LC_{50}$ value occurred at 0.36 mg/l OTA ($y = -88.12x^2 + 198.92x - 9.9937$, $r^2 = 0.94$). The ratio between the $EC_{50}$ and $LC_{50}$ values at each time point did not show a stable ratio (Table 1).

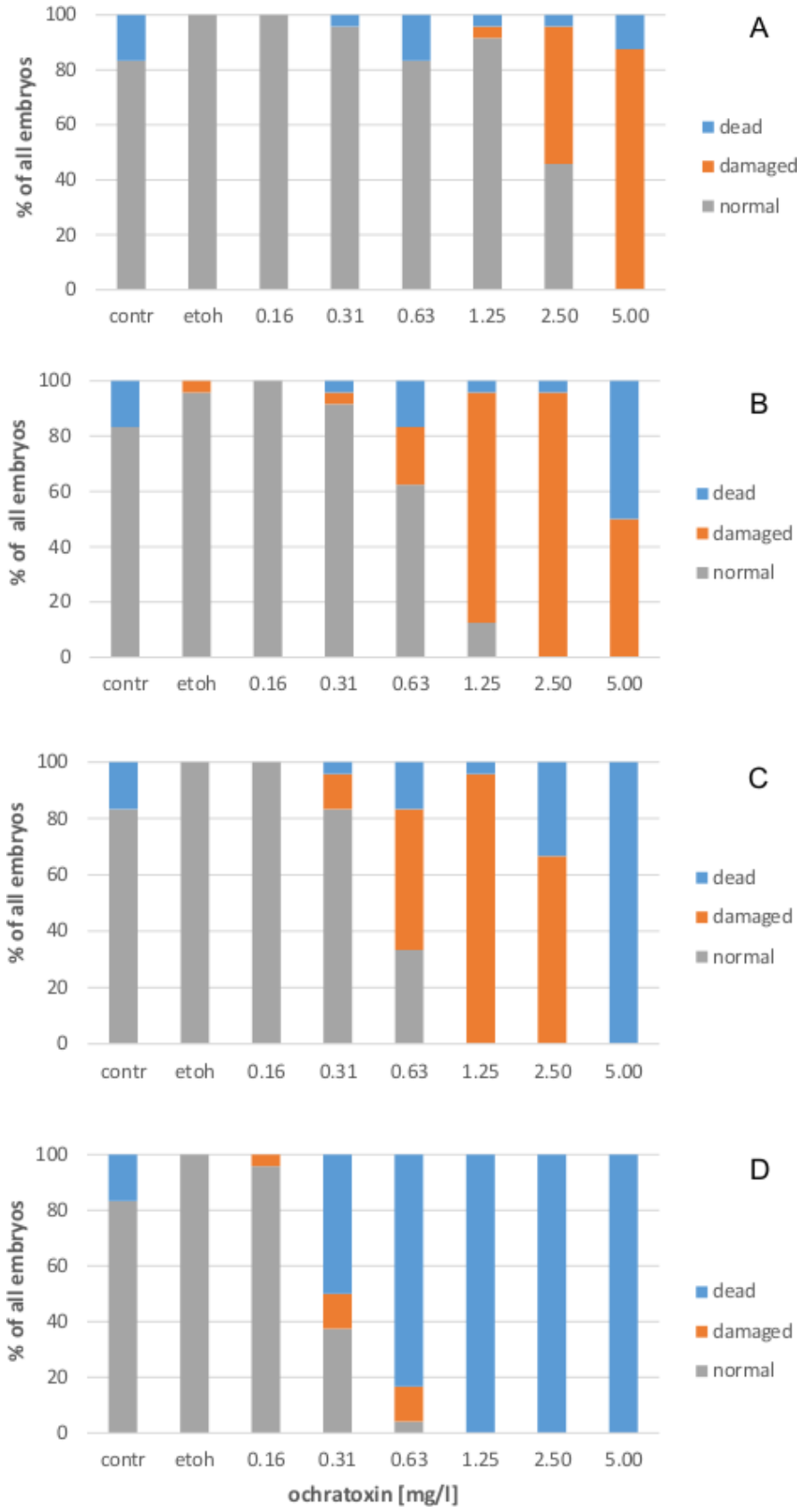


**Figure 1:** Concentration-dependent effects of OTA on zebrafish embryos after 24 (A), 48 (B), 72 (C) and 96 h (D) of exposure.

Figure 2 shows embryos in different developmental stages with OTA treatment. After 24 h of exposure to 5 mg/l OTA, most embryos showed pronounced retardation of development. These embryos showed a developmental stage (Figure 2A) that should have been accomplished 16.5 ± 0.9 h (mean ± SEM) earlier according to a previous study (Braunbeck & Lammer 2006). No further development of these embryos was observed at later points in time and all embryos treated with 5 mg/l OTA were dead after 72 h of exposure. The embryos treated for 24 h with 2.5 mg/l OTA and 1.25 mg/l OTA showed a growth retardation of 3.7 ± 1.3 h and 0.6 ± 0.6 h (means ± SEM), respectively, whereas all remaining fish showed no under-development at this time point. At 48 h of exposure, 92.2 % of the embryos categorized as damaged embryos (Figure 1B) were underdeveloped. At 72 h of exposure, different levels of growth retardation were also observed at the lower OTA concentrations (Figure 2B, C). In total, 75.5 % of all embryos listed in Figure 1C showed retardation of the development, whereas 7 % of the damaged embryos displayed effect on the blood circulation without showing under-development at the same time and the remaining embryos had oedema or had not yet hatched. As an example, the embryo shown in Figure 2B had not developed any pigmentation after 72 h of exposure to 0.63 mg/l OTA and showed a level of development comparable to a zebrafish embryo of less than 30 h after fertilization (Braunbeck & Lammer 2006). A high number of embryos showed deficiencies in blood circulation and the heart development. An example of this is an embryo is shown in Figure 2C that was treated with 0.31 mg/l OTA for 72 h.

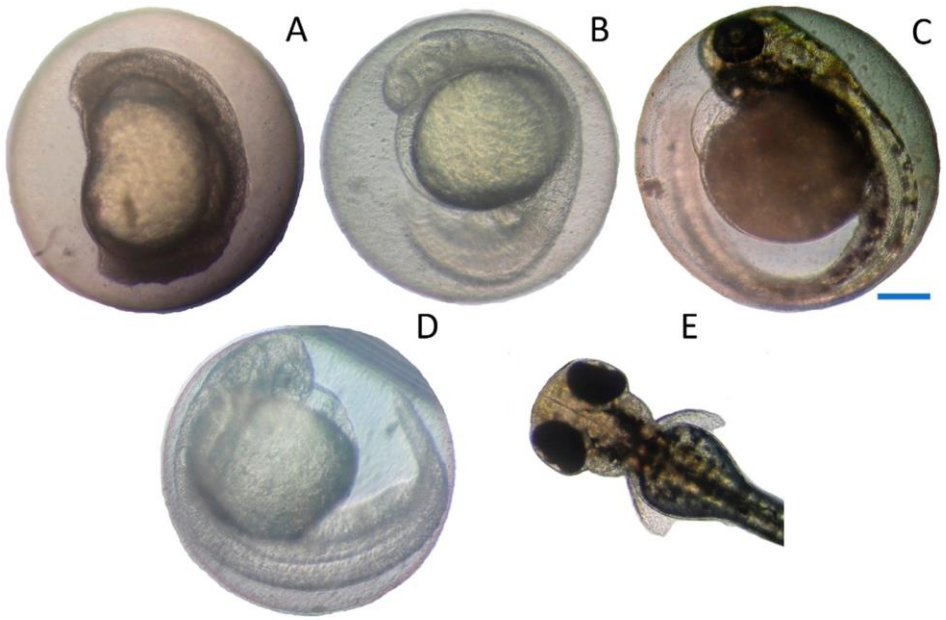


**Figure 2:** Zebrafish embryos after 24 h of exposure to OTA at a concentration of 5 mg/l (A) and after 72 h of exposure to 0.63 mg/l OTA (B) and to 0.31 mg/l OTA (C) showing the retardation of development compared with animals of normal development at 24 h (D) and the anterior part of a larvae hatched at 72 h (E); scale bar 200 µm.

While this embryo showed appropriate development of the head and tail and also possessed pigmentation of the eye and the remaining body, the yolk sac exhibited turbidity and most importantly, the heart appeared to be smaller and thinner with malformed chambers and the presence of a pericardial oedema and there was no heartbeat (Figure 2C).

The hatching occurred in the control and solvent control animal until 72 h of exposure (Table 2). The hatching success was significantly reduced upon exposure to 0.61 mg/l OTA or higher OTA concentrations ($p = 0.000$). The inhibition of hatching reached an $EC_{50}$ value of 0.44 mg/l OTA at 72 h of exposure ($y = 52.936x^2 - 154.9x + 108.14$, $r^2 = 0.92$) and an $EC_{50}$ value of 1.76 mg/l OTA after 96 h of exposure ($y = 14.583x^2 - 106.25x + 191.67$, $r^2 = 1.00$). At concentrations of 1.25 mg OTA or higher no embryo was hatching.

| Treatment | 72 h | 96 h |
|---|---|---|
| contr | 83.3% (100%) | 83.3% (100%) |
| etoh | 95.8% (95.8%) | 100% (100%) |
| 0.16 mg/l OTA | 95.8% (95.8%) | 100% (100%) |
| 0.31 mg/l OTA | 79.2% (82.6%) | 37.5% (81.8%) |
| 0.63 mg/l OTA | 16.7% (21.1%) | 4.2% (33.3%) |
| 1.25 mg/l OTA | 0% (0%) | 0% (0%) |
| 2.50 mg/l OTA | 0% (0%) | 0% (0%) |
| 5.00 mg/l OTA | 0% (0%) | 0% (0%) |

**Table 2:** Summary of the hatching success for the respective test durations reported for all exposed embryos in the different treatment groups (n = 24 for each) and displayed as the percentage of the embryos still alive at this time point in brackets.

**Heart rates**

After 48 h of exposure, heartbeats of the embryos were assessed (Figure 3). The solvent ethanol had no effect on the heart rates in the zebrafish embryos. Figure 4 shows that the heart rate per minute was also significantly reduced in embryos exposed to OTA for 72 h ($p = 0.002$; $EC_{50}$ of 1.26 mg/l, $y = -20.353x^2 - 74.484x + 176.56$; $r^2 = 0.96$). After 72 h of exposure, no embryo exposed to 2.5 mg/l OTA or higher showed any heartbeat.

**Oxidative stress**

The measurement of oxidative stress in zebrafish embryos showed a significant increase of the emitted fluorescence units with increasing OTA concentrations ($p = 0.000$, Figure 5) which were found to be significantly different from the solvent control-treated embryos at concentrations of more than 0.078 mg/l OTA. The measurements of oxidative stress yielded an $EC_{50}$ value of 0.067 mg/l OTA ($y = -46890x^2 + 22883x + 181.71$; $r^2 = 0.96$).

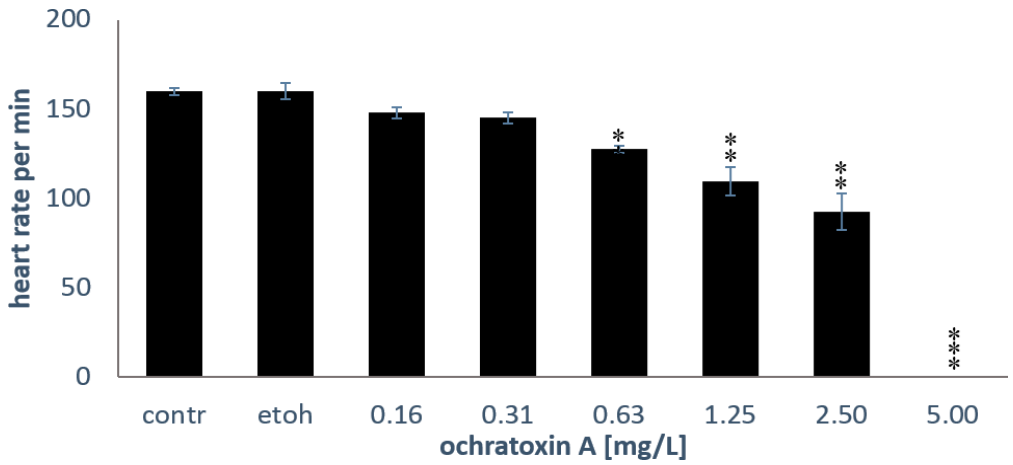


**Figure 3:** Heart rate of embryos. The mean ± SEM of six embryos is shown after 48 h of exposure; the asterisks (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$) indicate significant differences to the solvent control (etoh), Mann-Whitney U-tests with Bonferroni corrections for multiple comparisons, n = 6 embryos per treatment.

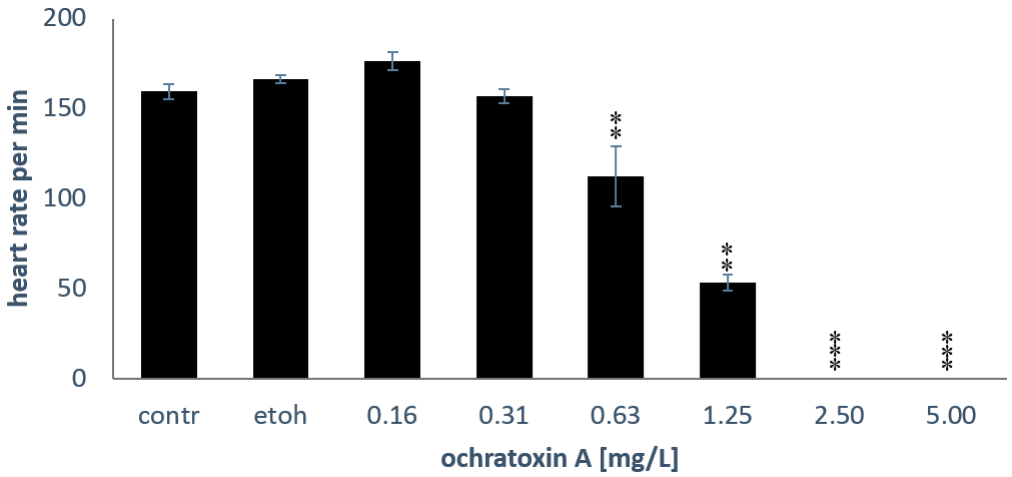


**Figure 4:** Heart rate of embryos. The mean ± SEM of six embryos is shown after 72 h of exposure; the asterisks (**: $p < 0.01$; ***: $p < 0.001$) indicate significant differences to the solvent control (etoh), Mann-Whitney U-tests with Bonferroni corrections for multiple comparisons, n = 6 embryos per treatment.

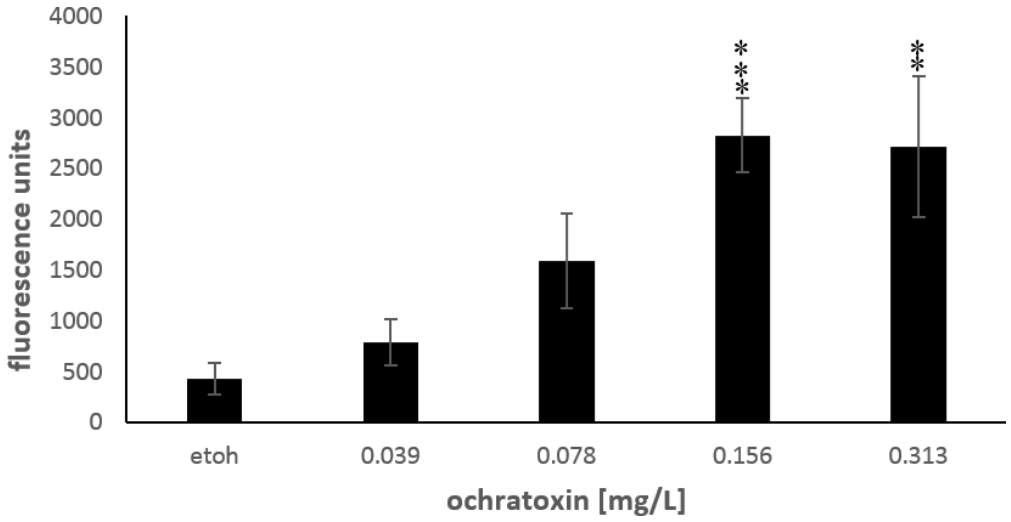


**Figure 5:** Concentration-dependent effects of ochratoxin A on oxidative stress in zebrafish embryos at 72 h of exposure displayed as fluorescence units emitted by the cell-permeant dye 2',7'-dichlorodihydrofluorescein diacetate ($H_2$DCF-DA) upon excitation at 480 nm; asterisks (**: $p < 0.01$; ***: $p < 0.001$) indicate significant differences to the solvent control (etoh), Mann-Whitney U-test with Bonferroni corrections for multiple comparisons, n = 8–14 embryos per treatment.

**3,4-Dichloroaniline**

Zebrafish embryos were sensitive to 3,4-Dichloroaniline (3,4-D) and showed impairment of development (Figure 6). The respective $LC_{50}$ and $EC_{50}$ values are displayed in Table 3. As expected, the exposure to the reference compound 3,4-D resulted in mortality of the fish embryos (Table 3). After 24 h of exposure, significant damage occurred in embryos that had been treated with 4 mg/l 3,4-D ($p = 0.000$) but no significant increase of lethal damages. A $LC_{50}$ value of 9.06 mg/l OTA was noted at this time point. After 48 h of exposure, damaged embryos occurred resulting in an $EC_{50}$ value of 2.09 mg/l ($y = 19.444x^2 - 19.444x + 5.5556$, $r^2 = 1.00$), whereas an $LC_{50}$ value of 9.06 mg/l ($y = 1.0101x^2 - 4.3434x + 6.4646$, $r^2 = 0.56$) was noted. Exposure of the embryos to 3,4-D for 72 h and 96 h further decreased the $EC_{50}$ and $LC_{50}$ values (Table 3).

| Time Point | $EC_{50}$ (mg/l) | $LC_{50}$ (mg/l) | Ratio $LC_{50}$ to $EC_{50}$ |
|---|---|---|---|
| 24 h | 3.47 | 9.06 | 2.61 |
| 48 h | 2.09 | 9.06 | 4.59 |
| 72 h | 2.22 | 6.08 | 2.74 |
| 96 h | 1.59 | 2.88 | 1.81 |

**Table 3:** Summary of the $LC_{50}$ and $EC_{50}$ values for the respective test durations for embryos exposed to 3,4-Dichloroaniline, n = 18.

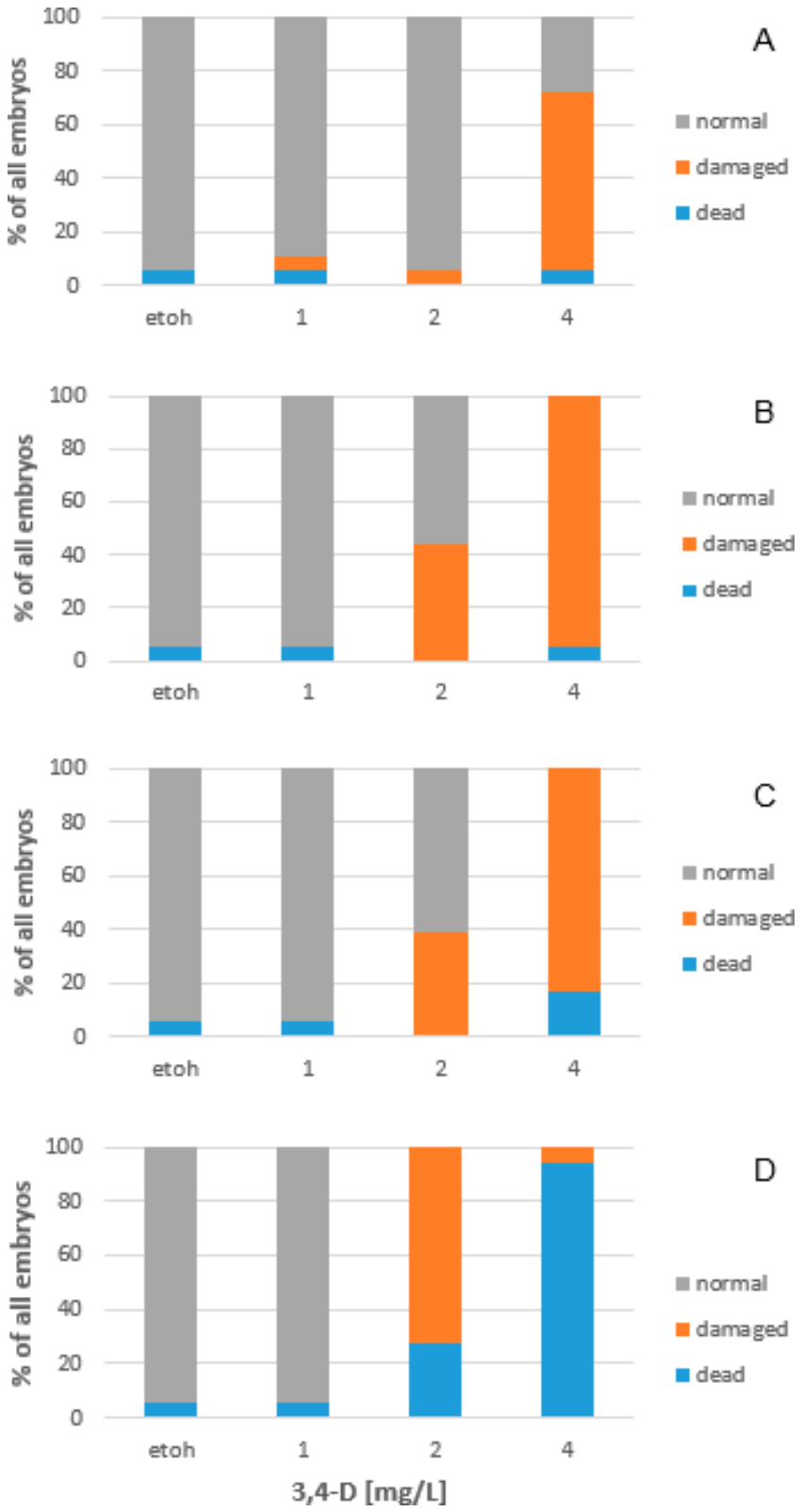


**Figure 6:** Concentration-dependent effects of 3,4-D on zebrafish embryos after 24 (A), 48 (B), 72 (C) and 96 h (D) of exposure.

Compared to OTA the reference compound 3,4-D caused different damages in the embryos. At 24 h, the exposure to 3,4-D resulted in underdeveloped embryos (18.8 % of the damaged embryos) and deformations of embryos (75 % of the damaged embryos showing mainly missing eyes and deformations of the head). Only one damaged embryo showed underdevelopment and deformations. At 48 h of exposure to 3,4-D, 34.6 % of the embryos listed as damaged in Figure 5 showed yolk sac oedema and 46.2 % of the embryos showed deformations and 19.2 % of the impaired embryos showed both, oedema and deformations. The incubation of the embryos for 72 h with 3,4-D, 38.1 % of the embryos listed as damaged in Figure 6 showed yolk sac oedema and 42.9 % of the embryos showed deformations, whereas 19.0 % of the impaired embryos showed both, oedema and deformations. At this time point, only 11.1 % of all embryos showed impairment of the heartbeat. At 96 h of exposure 92.8 % of the impaired but still alive embryos showed oedema and only 7.1 % deformations. The hatching success was significantly reduced in embryos treated with 4 mg/l 3,4-D after 72 h of exposure and significantly impaired by exposure to 2 mg/l 3,4-D after 96 h of exposure.

## *Discussion*

**Toxicity of OTA to fish embryos**

Based on previous studies (Peckham *et al.* 1971; Kuiper-Goodman & Scott 1989), the $LC_{50}$ for OTA in different higher vertebrate species ranges from 2 to 58 mg/kg body weight. However, fish species appear to be more sensitive to OTA than higher vertebrates. If this is also true for the different structures of OTA (i.e., other ochratoxins or of their metabolites (Heussner & Bingle 2015)) remains unknown. The $LC_{50}$ in adult seabass (*Dicentrarchus labrax* L.) after oral exposure was found to be at 9.23 mg OTA per kg diet after 96 h of exposure which was calculated to be equal to an exposure value of 0.28 mg/kg body weight (El-Sayed *et al.* 2009). Investigations on rainbow trout showed a $LC_{50}$ of 5.53 mg OTA $kg^{-1}$ body weight in this fish species after intraperitoneal injection (Doster *et al.* 1974). A study on the embryo toxicity in zebrafish yielded even lower $LC_{50}$ values (Haq *et al.* 2016). However, this study did not indicate the possible toxicological mechanisms that might be involved in OTA toxicity in zebrafish embryos. Therefore, low doses of OTA were again used in the present study to calculate more accurate $LC_{50}$ values and describe the targets of OTA in fish embryos more in detail.

Increasing OTA concentrations clearly resulted in increased mortality of zebrafish embryos. Concentrations higher than 0.63 mg/l led to 100 % mortality after 96 h of exposure. A $LC_{50}$ value of 0.36 mg/l OTA was calculated after 96 h of exposure. Different effects on the zebrafish embryos were observed at even lower OTA concentrations and an $EC_{50}$ value of 0.29 mg/l was obtained. Most of the embryos treated with OTA concentrations higher than 1.25 mg/l showed early retardation of development. In addition, it was observed that especially blood circulation and the heart development and heart rate were negatively affected by OTA exposure. In previous studies, cardiac abnormalities due to OTA exposure have only been described in rats and chickens (Okutan *et al.* 2004; Jameel 2011). Besides these effects, embryos exposed to lower OTA concentration more often showed yolk sac oedema and died at later points in time.

The present study showed higher $EC_{50}$ and $LC_{50}$ values than previously reported (Haq *et al.* 2016) and especially the values for 24 h of exposure were found to be higher than those described by others (Haq *et al.* 2016). However, the study of Haq and the co-authors (Haq *et al.* 2016) used no independently incubated embryos but reared 5 animals together in one replicate for their incubations and did not correct for multiple comparisons between the different treatments. Due to these experimental flaws, the present study is assumed to yield more realistic information on significant threshold levels for OTA toxicity in zebrafish. What is also noticeable when comparing the present study and the previous report (Haq *et al.* 2016) is that the present experiments yielded less deformations of the embryos. Moreover, the previously observed factor of 10 between $LC_{50}$ and $EC_{50}$ (Haq *et al.* 2016) was not confirmed by the present study. The reason for this might be the start of exposure that started at <2 h post fertilization in the study conducted by Haq et al. (Haq *et al.* 2016) but at 3 h post fertilization in the present study.

OTA has been described as a frequent contaminant of diverse food and feed ingredients and can also be detected in processed animal feeds (Binder *et al.* 2007; Duarte *et al.* 2011; Rodrigues & Naehrer 2012). Maximum allowable levels for OTA have been established in Europe (20 µg/kg) and also in other regions (Van Egmond & Jonker 2004). The concentrations in finished animal feeds are often low but might be detrimental for fish if highly contaminated ingredients are used for feed production. High concentrations of OTA have been found in commonly used ingredients of fish feed in some cases, for example in corn (up to 1850 µg/kg, (Rafai *et al.* 2000)), wheat (up to 1024 µg/kg, (Czerwiecki *et al.* 2002)) soybean and sunflower products (up to 350 and 240 µg/kg, respectively (Rafai *et al.* 2000)). In addition, inappropriate storage conditions for 6 weeks may lead to considerable amounts of OTA in commercial fish feeds (up to 400 µg/kg, unpublished results of the authors). In addition, approximately 0.090 mg/l OTA have been reported in effluents from wine production and the time for biodegradation was relevant for the present study (Nogueira *et al.* 2007). The present study, together with previous studies (El-Sayed *et al.* 2009; Haq *et al.* 2016), demonstrated that even very low amounts of this mycotoxin can have detrimental effects on fish.

**Detection of reactive oxygen species (ROS)**

The measurement of oxidative stress in the zebrafish embryos showed a very low $EC_{50}$ value of 0.067 mg/l OTA after 72 h of exposure. The detection of ROS in the embryos is therefore a very sensitive method to confirm that OTA is detrimental for zebrafish embryos. Oxidative stress has also been observed in OTA-treated rodents (Schaaf *et al.* 2002; Sorrenti *et al.* 2013; Tao *et al.* 2018), although a link between ROS occurrence and detrimental effects, for example, renal toxicity was found to be low in an additional study on rats (Zhu *et al.* 2016). The mechanism by which OTA is able to increase ROS formation has not been fully explored so far. One possible explanation for increased ROS production and depletion of intracellular antioxidants in cells (Kamp *et al.* 2005) might be the formation of a phenoxyl radical from OTA by peroxidases (Longoria *et al.* 2008).

The presence of glutathione may lead to reconversion of the phenoxyl radical to OTA which generated a superoxide anion radical (El Adlouni *et al.* 2000). Superoxide anion radicals can lead to further oxidative stress by forming hydrogen peroxide and possible induction of a Fenton reaction and the subsequent formation

of hydroxyl radicals resulting in further oxidative damage. Oxidative stress due to OTA exposure has been reported to lead to damage to lipids, proteins and DNA (Tao *et al.* 2018).

**Positive controls**

The present study used 3,4-Dichloroaniline (3,4-D) as a positive control and at a concentration of 2.88 mg/l, a mortality of 50 % was observed after 96 h of exposure. This 3,4-D concentration therefore fulfilled the criteria for the early life stage test based on the revised OECD guideline (European Commission 2014). In addition, the observed effects of 3,4-D on the embryos was comparable to the effects on survival and development in fish embryos and larvae (Nagel *et al.* 1991; Zhu *et al.* 2013), although the zebrafish embryos in the present study showed less pronounced effect on the heart function and the skeletal development than the early life stages of the rare minnow (*Gobiocypris rarus*, (Zhu *et al.* 2013)).

**Conclusions**

OTA strongly interferes with the development of the early life stages of zebrafish. The toxicity assays revealed that OTA induced dose-dependent mortality in zebrafish embryos resulting in a $LC_{50}$ value of 0.36 mg/l after 96 h of exposure. This confirms that OTA has detrimental effects on fish. The $EC_{50}$ was calculated to be 0.29 mg/l for damage in the embryos. In addition, a correlation between the OTA concentrations and the decrease of the heart rates was observed. The assay for ROS production in OTA-treated embryos showed increased oxidative stress in treatments higher than 0.078 mg/l and was the most sensitive endpoint for detrimental effects of OTA in zebrafish embryos. The assays conducted indicate that OTA-related production of ROS contributed to the detrimental effects of this mycotoxin on zebrafish embryos. This indicates that the detection of ROS can be a useful tool to detect cellular influences of OTA on fish before potential morphological impairment occurs. However, the exact cellular mechanism(s) of action of OTA on cellular functions in different organs still remains to be investigated and further research is needed to provide an organ-wide description of possible effects of this mycotoxin.

## *Materials and Methods*

**Chemicals**

All chemicals were obtained from Sigma-Aldrich (Buchs, Switzerland) unless indicated otherwise. The OTA (Sigma Cat. No. 01877; produced by *Petromyces albertensi*, lot No. 067M4011V) and 3,4-Dichloroaniline (Sigma Cat. No. 437778; lot No. 13509KQV) were solubilized in pure ethanol before use.

**Preparation of exposure medium**

ISO water (DIN Norm 38415-6 T6 2001) containing calcium chloride 2-hydrate (294 mg/l), magnesium sulfate-7-hydrate (123.3 mg/l), sodium hydrogen carbonate (63 mg/l) and potassium chloride (5.5 mg/l) was adjusted to a pH of 7.4, sterile filtered and adjusted to a temperature of 27 °C before use. OTA was solubilized in pure ethanol and added to the ISO medium at serial concentrations ranging from 5 mg/l to 0.039 mg/l (leading to a final ethanol concentration of 0.1 % ethanol).

**Exposure of fish embryos**

The zebrafish eggs were obtained from the EAWAG (Dübendorf, Switzerland), whereas the ROS measurements were performed with zebrafish eggs obtained from the ZHAW brood stock that originated from EAWAG zebrafish adults. The test was conducted according to the DIN norm 38415-6. For each exposure concentration, 18–24 eggs were exposed and negative controls containing ethanol as a solvent were included. The solvent concentration did not exceed 0.1 % in each of the treatments. The eggs were incubated at 27 °C and a light/dark cycle of 16h:8h in a Multitron Pro incubator (Infors AG, Bottmingen, Switzerland). The eggs were incubated with different ochratoxin concentrations at 3 h post fertilization in sterile ISO water containing $mgSO_4$, $CaCl_2$, $NaHCO_3$ and KCl at a pH of 7.4. 3,4-Dichloroaniline (3,4-D) was used at concentrations between 1 and 4 mg/l as a positive control, as recommended by the revised OECD guideline as a new criterion for early life stage tests with zebrafish. At a concentration of 4 mg/l this substance should result in at least a mortality of 30 % after 96 h of exposure (European Commission 2014). Determination of development of all embryos using a microscope (Leica Type 090-135.006, Leica Microsystems (Switzerland) AG, Heerbrugg, Switzerland) was conducted at 24, 48, 72 and 96 h post fertilization.

**Assessment of development**

For the experiment 4 plates with 4 embryos for each treatment were incubated for 96 h at 27 °C whereby each plate contained the respective control exposures with eggs exposed to ISO water only and a solvent control containing the same ethanol content as the ochratoxin treatments (0.1 %). For the subsequent experiment evaluating ROS, 4 plates with 6 embryos for each treatment were incubated for 72 h at 27 °C. Mortality, developmental stage, including the presence of the eyes, somites and the movement of the tail and possible occurrence of oedema (Table 4) were noted after 24 h of exposure using a microscope (Leica-Microscope Type 090-135.006, Leica Microsystems (Switzerland) AG, Heerbrugg, Switzerland). After 48 h and 72 h, pigmentation and blood flow were also assessed. Exact staging of the embryos was done according to Braunbeck and Lammer (Braunbeck & Lammer 2006). A dead embryo was noted if the material in the egg appeared to be at various stages of decomposition or coagulated. At the time points 48 and 72 h post fertilization the heartbeats of each living embryo were assessed for 20 to 30 sec manually under the microscope and heartbeats per minute were calculated.

| Time point | Endpoint |
|---|---|
| 24 h | eye visible, somites, tail movements |
| 48 h | pigmentation, blood circulation, heartbeats, oedema, embryo movements |
| 72 h | pigmentation, blood circulation, heartbeats, oedema, embryo movements, hatching |
| 96 h | pigmentation, blood circulation, heartbeats, oedema, embryo movements, hatching |

**Table 4:** Endpoints used for assessment.

**Measurement of reactive oxygen species (ROS)**

After 72 h of exposure, ROS production was measured using the fluorescent dye 2´,7´-dichlorodihydrofluorescein diacetate ($H_2$DCF-DA) as has previously been done in cell cultures of mammals and fish (Gauron *et al.* 2013; Pietsch *et al.* 2014*c*). After exposure to OTA, the embryos were killed by an overdose MS-222 (150 mg/l in $NaHCO_3$-buffered ISO medium), transferred to an opaque, flat-bottom 96-well plate and the medium was replaced by 100 µL of $H_2$DCF-DA solution (final concentration: 5 µM in ISO medium) was added to each well. The plate was incubated for 30 min at room temperature in the dark. Thereafter, fluorescence at excitation and emission wavelengths of 480 nm and 535 nm, respectively, was measured with a plate reader (Infinite M200, Tecan Instruments, Männedorf, Switzerland).

**Calculation of the 50 % level for effects and lethality**

The half maximal effective concentration ($EC_{50}$) was determined by plotting the responses of the embryos (including death) against the test concentrations. The relationship between the concentrations and the resulting effects followed polynomic equations which are reported for each duration of exposure separately including the respective correlation coefficients. For damages such as deformations and the occurrence of oedema, calculations of the concentration at which 50 % of the effects were observed were conducted separately from the heartbeat calculations and the measurements of oxidative stress. The concentration at which 50 % of the embryos were found to be dead ($LC_{50}$) was calculated similarly.

**Statistics**

Chi-square statistics were employed to compare the incidence of damage to the embryos by using the Monte Carlo approximation (using 10,000 simulations, with confidence interval of 99 %) to the Pearson chi-square test. The heart rates and fluorescence units were compared by using Mann Whitney U-tests and Kruskal Wallis tests (SPSS version 24 for Windows; SPSS Inc., Chicago, IL, USA). Differences between treatment groups were considered statistically significant when $p < 0.05$ to which the according Bonferroni corrections for multiple comparisons were applied.

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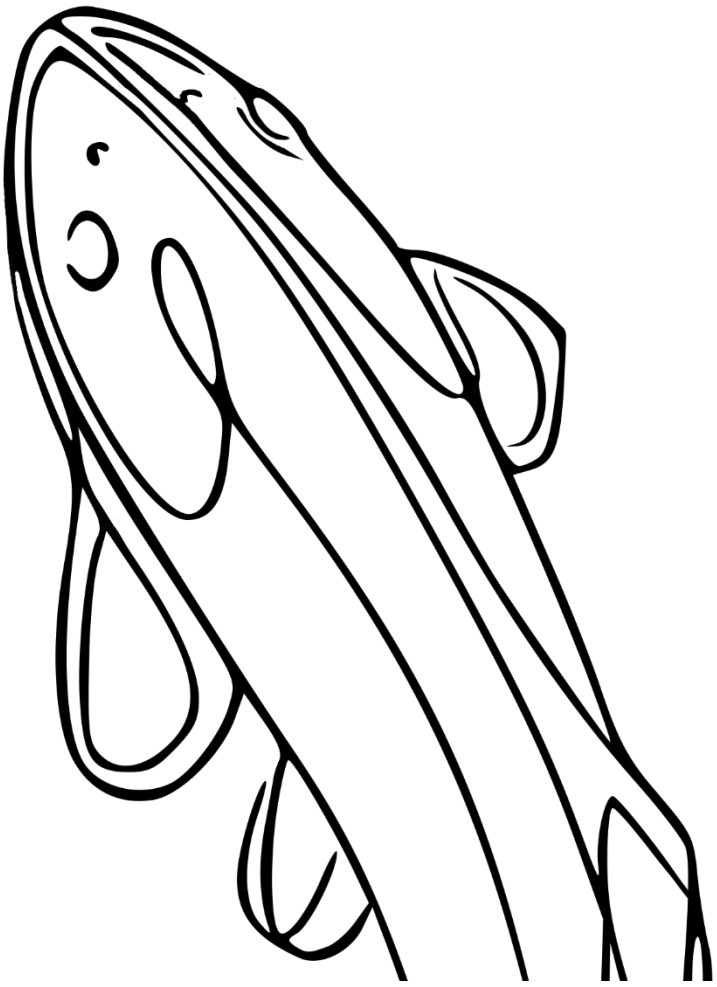
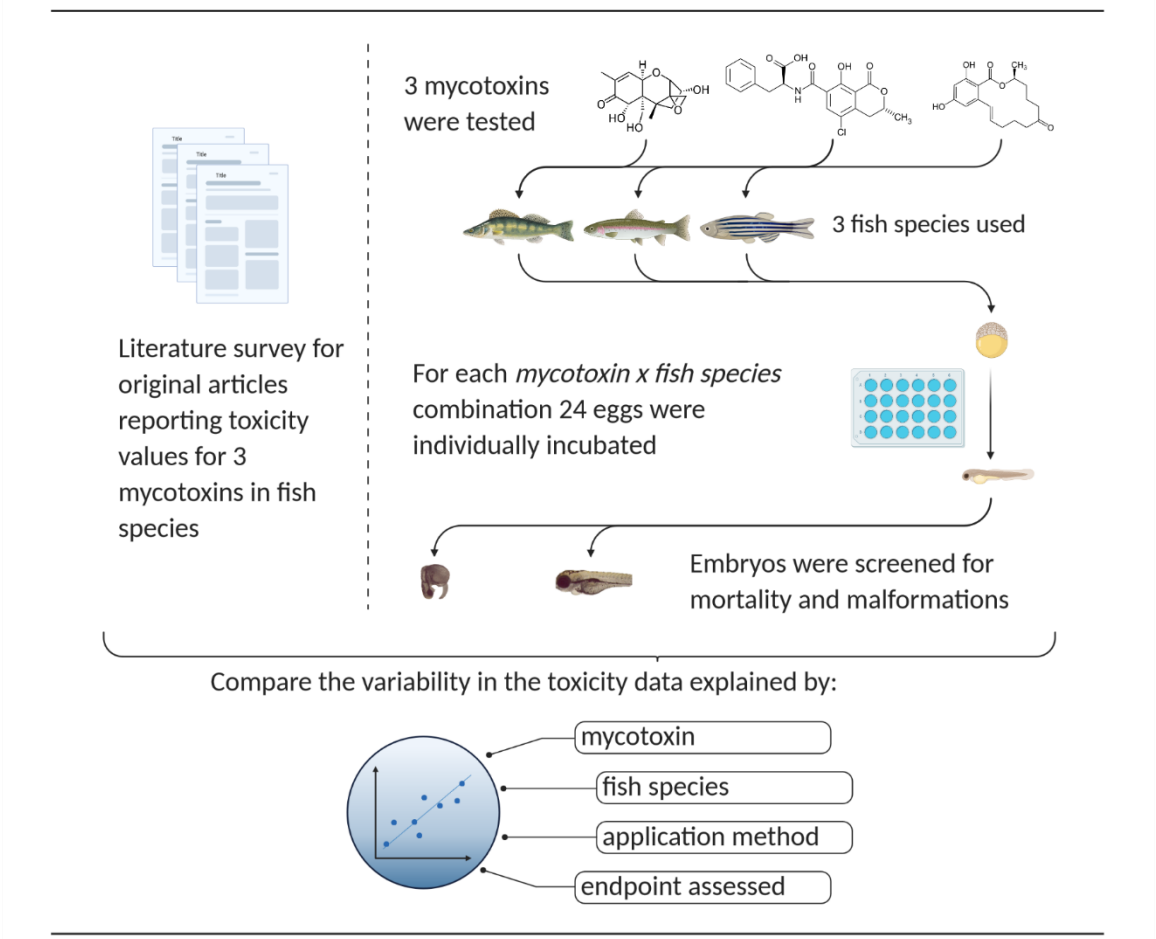


# Chapter 2

## Sources of data variability in mycotoxin embryotoxicity trials with fish: An example with deoxynivalenol, ochratoxin A and zearalenone



# Sources of data variability in mycotoxin embryotoxicity trials with fish
## An example with deoxynivalenol, ochratoxin A and zearalenone

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**Abstract:** Current recommendations of the European Commission for contamination limits in animal feed do not state fish specific values for many mycotoxins. This leaves an important food production sector, the aquaculture industry, without satisfactorily established regulations for fish feed and feed ingredients and therefore potentially vulnerable to damages due to mycotoxicosis. The joint efforts of the industry, government and science for an expedient risk assessment of these toxic fungal metabolites are impeded by methodological disunity in toxicological trials causing variability in the data, which represents a challenge for a solid hazard characterisation. To contribute to a better understanding of existing fish specific hazard characterisation data on mycotoxins potential sources of data variability and their relative importance were investigated with the example of deoxynivalenol, ochratoxin A and zearalenone. A literature survey, where the amount of data variability explained by two methodological factors (identity of the mycotoxin and mycotoxin application method) and two biological factors (fish species and endpoint assessed) was calculated, revealed the statistical relevance of these factors. The relative importance of the two biological factors was further examined in fish embryo acute toxicity tests investigating lethal and sublethal effects of deoxynivalenol, ochratoxin A and zearalenone on pikeperch, rainbow trout and zebrafish. Together, the analysis of the literature survey and the fish embryo acute toxicity tests indicate that the four methodological and biological factors assessed (mycotoxin, application, endpoint and species) are of comparable relevance for resulting toxicological values such as effect or lethal concentrations. For the typically mycotoxin-centric hazard characterisation process this implies that the effects of application, endpoint and fish species need to be increasingly considered. Specific suggestions are brought forward that can assist the improvement of data quality in mycotoxin research in order to facilitate future hazard characterisation and risk assessment for aquaculture.

**Keywords:** fish embryo acute toxicity test, mycotoxin, pikeperch, rainbow trout, zebrafish

**Paper:** in preparation

## *Introduction*

### Mycotoxins in aquaculture

Sustainability goals lead food production sectors such as the aquaculture industry to invest in plant-based alternatives for feed ingredients on a global scale (Hardy 2010; Tacon *et al.* 2011; Jannathulla *et al.* 2019). While plant-based feed ingredients are environmentally friendly and cost effective, they are also prone to fungal infection (Goncalves *et al.* 2020), which increases the probability of contamination of fish feed with toxic fungal metabolites, so-called mycotoxins. Thus, fish farming is increasingly affected by mycotoxicosis (Matejova *et al.* 2017; Gonçalves *et al.* 2018*b*). The toxic effects caused by mycotoxins threaten fish health and welfare due to acute toxicity as well as chronic exposure (Anater *et al.* 2016) and cause losses for the aquaculture sector in both cases. The industry therefore invests in the minimization of initial contamination of feed ingredients, the reduction of effects of contaminated feed and the global monitoring of mycotoxin occurrence (Gonçalves & Muccio 2019).

International authorities establish food and feed safety regulations based on comprehensive risk assessment processes (European Union 2019) which include four major steps: hazard identification, hazard characterization, exposure assessment and risk characterization (FAO 2015). Gathering the hazard and exposure data needed for a risk assessment is incumbent upon government, industry and science and in the case of mycotoxins in fish feed, this risk assessment includes several trophic levels, global feed ingredient trading and various toxic agents (Glencross *et al.* 2020). The hazard identification reveals six types of mycotoxins (Glencross *et al.* 2020) all of which are facultative metabolites produced by fungi in different environments and under diverse conditions (Cheeke 1998). Their toxic effects are relevant in nutrition due to direct ingestion (Abnet 2007) and potential biomagnification (Goncalves *et al.* 2020). The hazard characterisation highlights that the dose-response relationships and mode of actions are mycotoxin-specific (Ueno *et al.* 1977; Fuchs & Hult 1992; Rotter & Prelusky 1996) and adverse effects can result in considerable damage to fish in husbandry (Gonçalves & Muccio 2019). The exposure assessment underlines mycotoxicosis as a relevant fish health and welfare issue (Pietsch 2020). The final risk characterisation, as an evaluation of hazard and exposure data (Mantovani 2010), is the basis for contamination limits and feed standards, which to this date lack fish-specific regulations for most mycotoxins (European Commission 2006, 2016).

The reason for this lack is an impeded hazard characterisation (Knutsen *et al.* 2017*a*, *b*) due to difficulties with limited numbers of studies (Matejova *et al.* 2017), different study designs (Gonçalves & Muccio 2019), diverse protocols and fish species used (Anater *et al.* 2016; Oliveira & Vasconcelos 2020) and various endpoints assessed (Pietsch 2020). Together, these issues result in a level of variability in hazard data that complicates data interpretation. The preferred methods to handle this data variability for meta-analyses during hazard characterisations are a mathematical incorporation of sources of variability (Ciallella & Zhu 2019), the removal of studies with outlier data (Steinmetz *et al.* 2014) or the selection of high quality studies only, e.g. by removing studies with low scores on the Klimisch scale (Klimisch *et al.* 1997; Schneider *et al.* 2009). The first two solutions require a dataset size that is not given for hazard data of mycotoxins in

fish and the latter method results in a reduction to only a few (Knutsen *et al.* 2017*a*) or no (Knutsen *et al.* 2017*b*) available fish related studies. Hence, a manual evaluation of individual studies (Przybylak *et al.* 2012) may be more expedient in this case but requires information about both the hazard values reported and potential sources of data variability.

**Data variability in toxicological trials**

Hazard values from toxicological studies are typically variable since they are a simple description of a complex process. These toxicological values aim at describing the dose-response-relationship in a quantifiable manner (Fent 2013). The dose, i.e. the amount of mycotoxin in the target tissue, depends on the exposure (the dose or concentration during as well as the duration of the exposure) and the toxicokinetics of the toxin. The response, i.e. the adverse reaction of the targeted organism, includes the structural or functional alterations mostly characterized by their nature, severity, durability and their molecular, physiological and biological effects on the tissue, the organ and the organism. Therefore, dose-response-relationship values strongly depend on methodological, biological and technical characteristics in a given experiment (Hrovat *et al.* 2009; Gustafson *et al.* 2012; Busquet *et al.* 2014), which explains why differences in those characteristics are major sources of variability in toxicological data.

Within studies this data variability is met with diverse measures, e.g. standardisation of protocols, adherence to GLP (good laboratory practise), methods for statistical considerations and transparent reporting. However, to understand and prevent main sources of variability across studies it is crucial to understand, which methodological, biological and technical variations potentially give rise to which amount of variability in the data. We therefore investigated the relative importance of different sources of data variability in mycotoxin trials in fish with a literature survey, where the amount of data variability explained by two methodological factors (*identity of the mycotoxin* and *mycotoxin application method*) and two biological factors (*fish species* and *endpoint assessed*) was calculated. We then tested the transferability of the insights from the literature survey on an individual toxicological study by conducting a laboratory trial with a defined *mycotoxin application method* (fish embryo acute toxicity test), a varying methodological factor (*identity of the mycotoxin* i.e. deoxynivalenol, ochratoxin A and zearalenone) and two varying biological factors (*fish species* i.e. zebrafish (*Danio rerio*), pikeperch (*Sander lucioperca*) and rainbow trout (*Oncorhynchus mykiss*) and *endpoint assessed* i.e. lethality and morphological malformations). Together, the literature survey and the toxicity test assisted the formulation of specific suggestions on how to improve on data variability in mycotoxin research for aquaculture in order to facilitate future hazard characterisations and risk assessments.

## *Methods*

### Literature survey

A literature search was conducted on *Web of Science* (November 2020) and included peer-reviewed original articles about effects of mycotoxins in fish. The search was limited to three of the most relevant mycotoxins (Gonçalves & Muccio 2019), namely deoxynivalenol (DON), ochratoxin A (OTA) and zearalenone (ZEN), which all had approximately the same number of search results (81–86 papers). Similar searches in toxicological databases, e.g. US EPA ECOTOX or Acute Toxicity Database, yielded no or single digit results for these mycotoxins in combination with fish as a study species and hence were not included in this study. If the papers reported specific mycotoxin concentrations that resulted in adverse effects in the trial on living fish, i.e. LOAECs (lowest observed adverse effect concentrations), they were included in the analysis. Each mycotoxin concentration reported was attributed four different categorical variables: identity of *mycotoxin* (DON, OTA, ZEN), taxonomic group of the fish *species* (eight genera), mycotoxin *application* method (dosed (injection, gavage), exposed (exposure in medium) or ingested (feeding)), and *endpoint* assessed (lethal (lethal concentrations, mortality), morphological (growth performance, malformations), or physiological (histological, behavioural, biochemical or molecular markers, fecundity parameters)). Data were analysed using ANOVA-functions in R 4.0.3. with a Bonferroni correction of the alpha level due to multiple testing (p-value < 0.01), while post-hoc tests were done with Games-Howell tests with a Holm-Correction.

### Fish embryo acute toxicity test

*Reagents*

Mycotoxins (Sigma-Aldrich, Buchs, Switzerland. Cat. No.: OTA: O1877, ZEN: Z2125, DON: D0156) were solubilized in 99 % ethanol (Sigma-Aldrich Cat. No.: 02851). ISO medium (ISO 1996) was adjusted to a pH of 7.6 ± 0.2. Each mycotoxin was used in a two-fold serial dilution with five concentration steps (C1–C5) as recommended by the OECD 236 guideline. The range of the concentrations (Tab. 1) was derived from the literature and intended to cover previously reported $LC_{50}$ values. 3,4-dichloroaniline (DCA, Sigma-Aldrich Cat. No.: 437778) at 4 mg/l and ISO medium (ISO) were used as positive and negative control, respectively. Additionally, a solvent control with 0.1% ethanol (ETH, Sigma-Aldrich Cat. No.: 02851) was used. For the euthanasia of the embryos, MS222 (Sigma-Aldrich Cat. No.: E10521) was used at 4 mg/l.

*Egg exposure*

Eggs from the three species were obtained after fertilisation (Rainbow trout: Güfisch, Satteins, Austria; pikeperch: Basis57, Erstfeld, Switzerland; wildtype zebrafish: EAWAG, Dübendorf, Switzerland). From every fish species, 24 fish eggs were exposed to every treatment (3 mycotoxins x 5 concentrations + 3 controls = 18 treatments), yielding a total of 432 samples per fish species run in one trial.

| Test solution | C1 [mg/l] | C2 [mg/l] | C3 [mg/l] | C4 [mg/l] | C5 [mg/l] | Solvent [ETH % v/v] | ISO medium |
|---|---|---|---|---|---|---|---|
| OTA | 0.05 | 0.10 | 0.20 | 0.4 | 0.8 | 0.1 | + |
| ZEN | 0.13 | 0.25 | 0.50 | 1.0 | 2.0 | 0.1 | + |
| DON | 0.31 | 0.63 | 1.25 | 2.5 | 5.0 | 0.1 | + |
| DCA | 4.00 | - | - | - | - | 0.1 | + |
| ETH | - | - | - | - | - | 0.1 | + |
| ISO | - | - | - | - | - | - | + |

**Table 1:** Mycotoxin concentrations used in the FET test. Deoxynivalenol (DON), ochratoxin A (OTA), zearalenone (ZEN), negative control (ISO), solvent control (ETH), positive control (DCA), (+) = used as basic medium.

Due to species-specific requirements, adaptions to the standard fish embryo acute toxicity (FET) protocol (OECD 2013*b*) were made for rainbow trout and pikeperch. Eggs were incubated during the exposure at species-specific light:dark cycles and temperature conditions: 0h:24h at 10 °C for rainbow trout, 0h:24h at 11 °C for pikeperch, and 14h:10h at 26 °C for zebrafish. Pikeperch clutches hatch asynchronously over four days with similar developmental stages irrespective of the hatching process (personal observation). In order to synchronize their hatching, the ambient temperature was increased (1 °C every 12 h) towards the end of the incubation phase (0–11 dpf (days post fertilization): 11 °C, 12 dpf: 13 °C, 13 dpf: 15 °C). Due to different egg diameters (zebrafish: 0.8–1.6 mm (Uusi-Heikkilä *et al.* 2010; Graf *et al.* 2011), pikeperch: 0.5–1.4 mm (Lappalainen *et al.* 2003), rainbow trout: 3.4–5.6 mm (Springate & Bromage 1985; Serezli *et al.* 2010)), eggs were incubated individually in either 24-well plastic plates (zebrafish, pikeperch) with 2 ml of test solution per well or 12-well plastic plates (rainbow trout) with 6 ml of test solution, leading to egg/medium volume ratios of around 0.0003 (pikeperch and zebrafish) and 0.06 (rainbow trout). To ensure a more uniform exposure to the mycotoxin, rainbow trout eggs were incubated in a semi-static trial, with weekly exchanges of the test solutions (OECD 2013*b*), while zebrafish and pikeperch eggs were incubated in a static trial with no replacement of the solutions. This takes the different developmental times of the fish species into account (measured in ATU: accumulated temperature units or degree-days) with zebrafish hatching 70–100 ATU after fertilisation (OECD 2013*b*), pikeperch after 80–100 ATU (Oprea *et al.* 2014) and rainbow trout after 260–340 ATU (Robison *et al.* 1999). Exposure began at 6 hpf (hours post fertilization) for zebrafish and at 3 dpf for pikeperch and rainbow trout (Fig. 1, Appendix A).

*Endpoints*

Endpoints were assessed four times in regular intervals spread across the embryonic development of the zebrafish at 24 hpf, 48 hpf, 72 hpf and 96 hpf (OECD 2013*b*). For the other two fish species they were done at 4 dpf, 8 dpf, 11 dpf and 13 dpf for pikeperch and 17 dpf, 31 dpf, 38 dpf and 44 dpf for rainbow trout (Fig. 1).

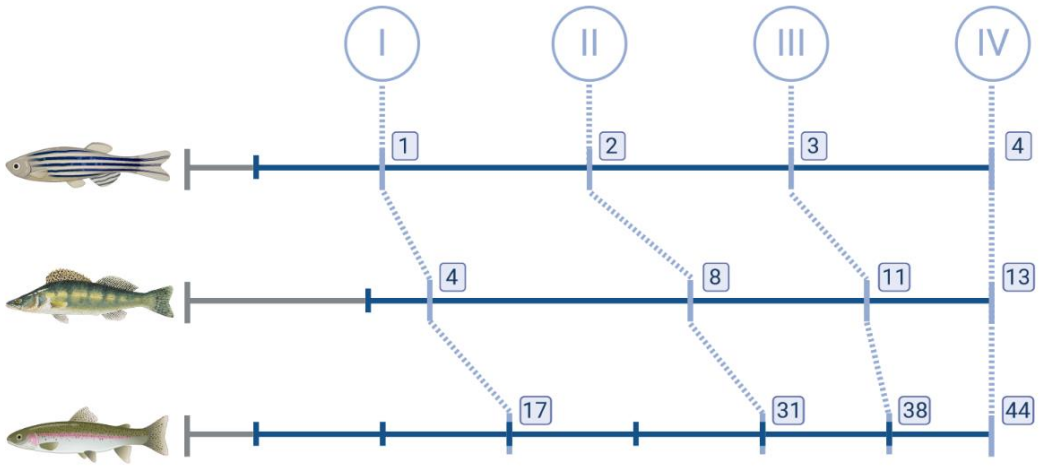



**Figure 1:** Timeline of the fish embryo acute toxicity test from egg fertilisation till hatching. Grey = unexposed, dark blue = exposure time, dark blue ticks = exchange of test solutions, light blue ticks = assessments, circles = assessment numbers, squares = days post fertilisation. Note that timelines have different durations.

Assessment I-III were used for time series data within the species only, comparisons across fish species were based on assessment IV, which represents a comparable point of development amongst all three species, namely the hatching. Assessments of the embryos were carried out by viewing the embryos in the wells under a stereo microscope (Leica MDG41, Wetzlar, Germany) and with species-specific adaptions to the protocol. In addition to the assessment of four lethal endpoints (coagulation, tail detachment, somite formation and heartbeat), as indicated by the OECD protocol, six sublethal parameters were evaluated for pikeperch and zebrafish: blood circulation at assessments II–IV and pericardial edema, yolk sack edema, head malformation, tail malformation and heart malformation at the final assessment IV (Tab. 2). Embryos that were alive but showed one or several of these endpoints in any severity were defined as malformed during the assessment. For this the zebrafish and pikeperch embryos were euthanized and immediately placed on a 5 % agar plate with corresponding moulds to hold the embryos in place for dorsal and lateral imaging.

The opacity of chorion of rainbow trout eggs hindered a visual assessment of the embryo during development. Therefore, only coagulation was taken as endpoint during the incubation phase (assessments I–III), while all four standard endpoints were evaluated for assessment IV. Finally, unhatched eggs of all three species were manually hatched, i.e. dechorionated with forceps under a microscope (Barrett *et al.* 2001), and imaged at assessment IV. Hatching was used to define the hatching rate but not assessed as additional sublethal endpoint.

| Endpoint | Score | Assessment | | | |
|---|---|---|---|---|---|
| | | I | II | III | IV |
| *standard endpoints of the OECD guideline 236* | | | | | |
| coagulation/decomposition | dead | z/p/t | z/p/t | z/p/t | z/p/t |
| no detachment of tail | malformed | z | z/p | z/p | z/p/t |
| malformation of somites | malformed | z | z/p | z/p | z/p/t |
| no heartbeat | dead | - | z/p | z/p | z/p/t |
| *additional sublethal endpoints* | | | | | |
| no blood circulation | malformed | - | z/p | z/p | z/p |
| pericardial edema | malformed | - | - | - | z/p |
| yolk sack edema | malformed | - | - | - | z/p |
| head malformation | malformed | - | - | - | z/p |
| tail malformation | malformed | - | - | - | z/p |
| heart malformation | malformed | - | - | - | z/p |
| not hatched | - | - | - | - | z/p/t |

**Table 2:** Endpoints used for the assessments I–IV during the fish embryo acute toxicity test. z = zebrafish, p = pikeperch, t = rainbow trout.

*Statistics*

For each of the nine combinations (3 fish × 3 mycotoxins) four dose-response curves (one per assessment point) were plotted using ISO and the five concentrations C1–C5 to visualize the mortalities over time with increasing mycotoxin concentration. EC (effect concentration, for pikeperch and zebrafish) and LC (lethal concentration, for all three fish species) values were calculated for the levels of 10 and 50 % based on the final assessment IV using the function *lc.function* (Savi *et al.* 2017) with the software R. For the calculation of LC, the embryos were categorized in alive (normal or malformed) and dead, while for the effect concentrations the categories were normal and affected (dead or malformed). To evaluate the sensitivity of the *species* and the influence of the *endpoint*, two-sided pairwise tests for equal proportions were done on the sum of affected embryos of all concentrations applied.

## *Results and Discussion*

### Literature survey

*Literature dataset*

The literature search resulted in 199 papers about DON, OTA and ZEN, with 63 papers (see Appendix D) reporting a total of 113 specific mycotoxin concentrations with adverse effects (LOAECs). Four datapoints were excluded from the dataset: first, two untypically low values for ZEN reported by the only paper on fathead minnows (Johns *et al.* 2011) and second, two untypically high values for DON reported due to an unusual application method (Khezri *et al.* 2018). A graphical representation of the remaining 109 values that groups the concentrations reported into the four factors of interest (*mycotoxin*, *application*, *endpoint* and *species*) shows several peculiarities of the dataset (Fig. 2). First, the number of studies, which directly apply the mycotoxins into the organism (dosed), is low compared to the other two application methods (15 out

of 109 values). Further, exposure trials were exclusively conducted with zebrafish (31 values). Additionally, the concentrations reported by exposure trials for ZEN show comparatively high variability (4 orders of magnitude compared to ca. 1 for OTA and DON). Furthermore, the dataset is unbalanced with many combinations of the four factors being represented by only single data points or not at all. This prevents a statistical analysis with a full-factorial four-way ANOVA. The dataset allows, however, to test each factor with a separate one-way ANOVA and interpreting the sum of squares as a measurement for the amount of variability accounted for by that factor (Tab. 3). To improve normality, the four ANOVAs were fitted to log-transformed data, each having 108 degrees of freedom and a sum of square of 87.68.

*Methodological factors - Mycotoxin*

The identity of the mycotoxin, expectedly, is relevant (p-value < 0.001, Fig. 3 left). However, it only accounts for 14.7 % of the variability. Pairwise Games-Howell tests show differences for DON:ZEN ($p = 0.01$) and DON:OTA ($p < 0.0001$), making DON significantly less toxic. This sequence in toxicity (OTA ≥ ZEN > DON) is in line with the respective recommendations of maximal contaminations in animal feed by the EU (European Commission 2006). The three mycotoxins show different levels of absolute toxicity potentially due to their different bioavailability, toxicokinetics and toxicodynamics. Deoxynivalenol is a trichothecene, which mainly acts as inhibitor of protein synthesis in the target tissues, where it shows, amongst others, immunotoxic, neurotoxic and hematopoietic effects (Gonçalves & Muccio 2019). During feeding trials, DON has primarily an anorexic effect that leads to reduced growth performance and decreased feed efficiency (Gonçalves *et al.* 2018*a*; Hooft *et al.* 2019*a*). Similarly, ochratoxin A interferes with protein synthesis and is known to be mainly nephrotoxic and hepatotoxic, yet can show teratogenic, embryogenic and carcinogenic effects (O'Brien *et al.* 2005; Gonçalves & Muccio 2019). Contrarily, zearalenone is estrogenic and acts as an endocrine disruptor (Gonçalves & Muccio 2019). As a mycoestrogen, zearalenone, as well as its derivatives, has diverse adverse effects on the reproductive system, is teratogenic as well as immunotoxic (Pietsch 2017; Khezri *et al.* 2018). To conclude, the identity of the mycotoxin does, as expected, affect the LOAECs and hinders an extrapolation of toxicity values across mycotoxins. Hence, focused studies on emerging mycotoxins (Tolosa *et al.* 2014) and effects of mixtures (Matejova *et al.* 2017) are necessary for risk characterisations.

*Methodological factors – Application*

The application method (categorised as dosed, exposed and ingested) affects toxicity values (p-value < 0.0001, Fig. 3 middle) and with 30.5 % explains twice as much variability as does the identity of the mycotoxin. More specifically, ingestion leads to higher LOAEC values, i.e. is less toxic than exposure (Games-Howell tests, $p < 0.0001$). Statistically, this effect is mainly driven by the lower values of exposure for OTA, where exposure generally results in an increase of toxicity by one magnitude, and by a lower average value of exposure for ZEN, where particularly small values increase the average toxicity (Fig.2).

Biologically, there are several potential explanations for an effect of the application method on toxicity values. First, differences in chemical characteristics of the mycotoxin may cause alterations in the bioavailability depending on the application method. For example, increased molecular weight can reduce

passing through the chorion (Pelka *et al.* 2017), which would increasingly affect exposure trials compared to feeding trials the larger the mycotoxin molecule is. However, with 296 g/mol (DON), 404 g/mol (OTA) and 318 g/mol (ZEN), all mycotoxins are below the size of 3000 g/mol that would restrict chorion passage (Pelka *et al.* 2017). Further, polarity may result in a method-dependent altered bioavailability due to leaking of hydrophilic mycotoxins from feed into water (Greeff-Laubscher *et al.* 2020) as opposed to the attachment of hydrophobic molecules to plastic dishes (Wang *et al.* 2018), which are often used in exposure trials. However, with a logKow of -0.7 (DON), 4.7 (OTA) and 3.6 (ZEN), neither behaviour would explain the higher toxicity of OTA in exposure trials. Furthermore, inconsistencies in nominal vs. effective mycotoxin concentrations (issues with solubility or precipitation in exposure studies (Ball *et al.* 2014)) and doses (problems with feed formulations or leakage into water in feeding trials (Pietsch *et al.* 2014*b*)) may cause variances in bioavailability. Second, there may be alterations to the toxicokinetics depending on the application method. Specifically the uptake of the mycotoxins via different tissue membranes may cause variability when comparing feeding and exposure trials (Hogstrand 2000). Further, tendencies in life stages used for different methods (juveniles and adults for feeding trials and embryos for exposure studies) might affect the toxicokinetics (Voslářová *et al.* 2006) in a method dependent manner. Lastly, the effect of the application methods may root in a discrepancy when comparing the units [mg/l medium] and [mg/kg feed], rendering the methods incomparable.

In conclusion, since mycotoxins are in fish feed or potentially solved in the system water, the two relevant routes of uptake are voluntary ingestion and inevitable medium exposure. Therefore, other application methods such as injection or gavage add little insight for hazard characterisation studies and should be refrained from. Additionally, the environmental persistence of mycotoxins may cause accumulation of solved mycotoxins in closed water cycles (Bucheli *et al.* 2008). This may have consequences for the increasingly used recirculating aquaculture systems (Goncalves *et al.* 2020) and predicts an increased relevance of exposure trials for future risk characterisations in aquaculture.

*Biological factors - Endpoint*

While morphological and especially physiological endpoints are stated as being more sensitive than lethal endpoints (Krzykwa *et al.* 2018; Hedgpeth *et al.* 2019), the choice of endpoints had no relevance ($p = 0.75$, Fig. 3 right) when evaluated in the context of the given literature dataset (Tab. 3, Fig. 4). Explanations for this missing effect of *endpoint* as a factor in the metadata set may be found at two levels. First, peculiarities of sublethal endpoints may increase the variance in the data, which would mask an existing effect. For example, the sensitivity of physiological endpoints depends on whether (increased sensitivity) or not (decreased sensitivity) the assessed parameters are part of the mode of action of the mycotoxin in focus (Brotzmann *et al.* 2021). Further, sublethal endpoints are more difficult to assess than lethal endpoints, with the latter describing a more defined state of not being alive or viable (Schweizer *et al.* 2017). Second, a potentially existing effect may be underreported, due to studies reporting only either lethal (El-Sayed *et al.* 2009) or sublethal (Wu *et al.* 2020) effects. Further, studies may report equal toxicity values for physiological, morphological and lethal endpoints either due to too large concentration intervals tested

(Manning *et al.* 2005; Gonçalves *et al.* 2018*a*) or due to an indeed not existing effect. However, the effect of the endpoints assessed was detectable within some studies (Manning *et al.* 2003; Bakos *et al.* 2013; Khezri *et al.* 2018), which accentuates the importance of the choice of endpoints when conducting a toxicological study or interpreting specific data.

In summary, it is advisable to first include endpoints widely used in the field, which increase comparability among trials, e.g. lethal endpoints or systemic markers. Particular endpoints specific for e.g. the mycotoxin, fish species, aquaculture system or trial purpose can then be included to increase the sensitivity of the toxicological study.

*Biological factors – Species*

Eight different fish species (categorised at the genus level) were reported, and their identity is a significant factor (p-value < 0.0001, Fig. 4) that accounts for 44.9 % of the variability in the metadata set, i.e. three times as much variability as does the mycotoxin. Reasons for this effect may be statistical. For example, while zebrafish show a high variance in toxicity values, probably due to their exclusive use in exposure trials, the rest of the species have a variance of approximately one magnitude (Fig. 4). Further, interactions of the factor *species* with *mycotoxin* as well as *application*, i.e. the exclusive use of certain species for specific *applications* or *mycotoxins*, distorts the statistical analysis (Fig. 2). This may misleadingly increase the relevance of the *species* as a factor for this metadata set. Or, however, the effect of the *species* on the toxicity values is indeed biological, i.e. caused by actual species-specific difference in toxicokinetics (Hagelberg *et al.* 1989; Malekinejad *et al.* 2006).

This raises the issue of the most accurate fish species for toxicological trials, especially for risk assessment processes, where ideally sensitive species are used for the definition of safety limits such as MATCs (maximal acceptable toxicant concentrations) (Fent 2013). Due to these species and toxin specific sensitivities uncertainty/safety factors from 10 to 1000 are typically applied to environmental safety limits (Spurgeon *et al.* 2020) and threshold intake levels (Dorne & Renwick 2005).

*Implications from the literature survey*

As a whole, the literature survey, a comparison of the relevance of two methodological and two biological factors for data variability, revealed that the fish species is most important, followed by the application method and only then the identity of the mycotoxin weighs in. In contrast, the choice of endpoints has no influence when comparing LOAEC values across studies. The biological factors tested suggest a strong influence of the *species* and a negligible relevance of *endpoint*. However, they may be affected by peculiarities of the literature dataset (section 3.1.5.) or bias in reporting (section 3.1.4.) and, therefore, were further investigated in a fish embryo acute toxicity test (section 3.2.). The conclusions for the methodological factors imply that: (I) the typically mycotoxin-centric hazard characterisation analysis should select studies based on their application method, i.e. excluding injection and gavage trials as well as continuing to separate medium exposure and feeding trials; and (II) the analyses should remain mycotoxin-specific, which makes separate risk assessment processes necessary for emerging mycotoxins and potentially mixtures.

A comparison of the gathered metadata with contamination regulations reveals that farmed fish are not sufficiently protected. The current recommendations of the European Commission for mycotoxin contamination limits in animal feed do state values for deoxynivalenol, ochratoxin A and zearalenone (European Commission 2006, 2016). However, there are no fish specific values included and the only limits available for all three mycotoxins are for pigs. While the limit for OTA (0.05 mg/kg feed) is below the relevant doses indicated in the fish specific literature, the limits for ZEN (0.25 mg/kg feed) and DON (0.9 mg/kg feed) do not protect farmed fish (Fig. 2). This discrepancy between the regulations and the hazard characterisation for DON was acknowledged in a more recent report (Knutsen *et al.* 2017*b*) where the risk assessment indicated potential chronic adverse effects for farmed fish. An evaluation of the mycotoxin contamination levels in fish feeds in Europe (Pietsch 2020), as part of an exposure assessment, reveals that neither DON (0.22 mg/kg feed) nor OTA (0.01 mg/kg feed) or ZEN (0.05 mg/kg feed) may be an imminent threat in terms of an acute toxicity. Yet, these values indicate a safety factor of less than ten, and hence may not exclude the above mentioned chronic adverse effects. Therefore, more research and especially long-term toxicological studies are needed for a fish specific hazard characterisation and risk assessment of mycotoxins in animal feed.

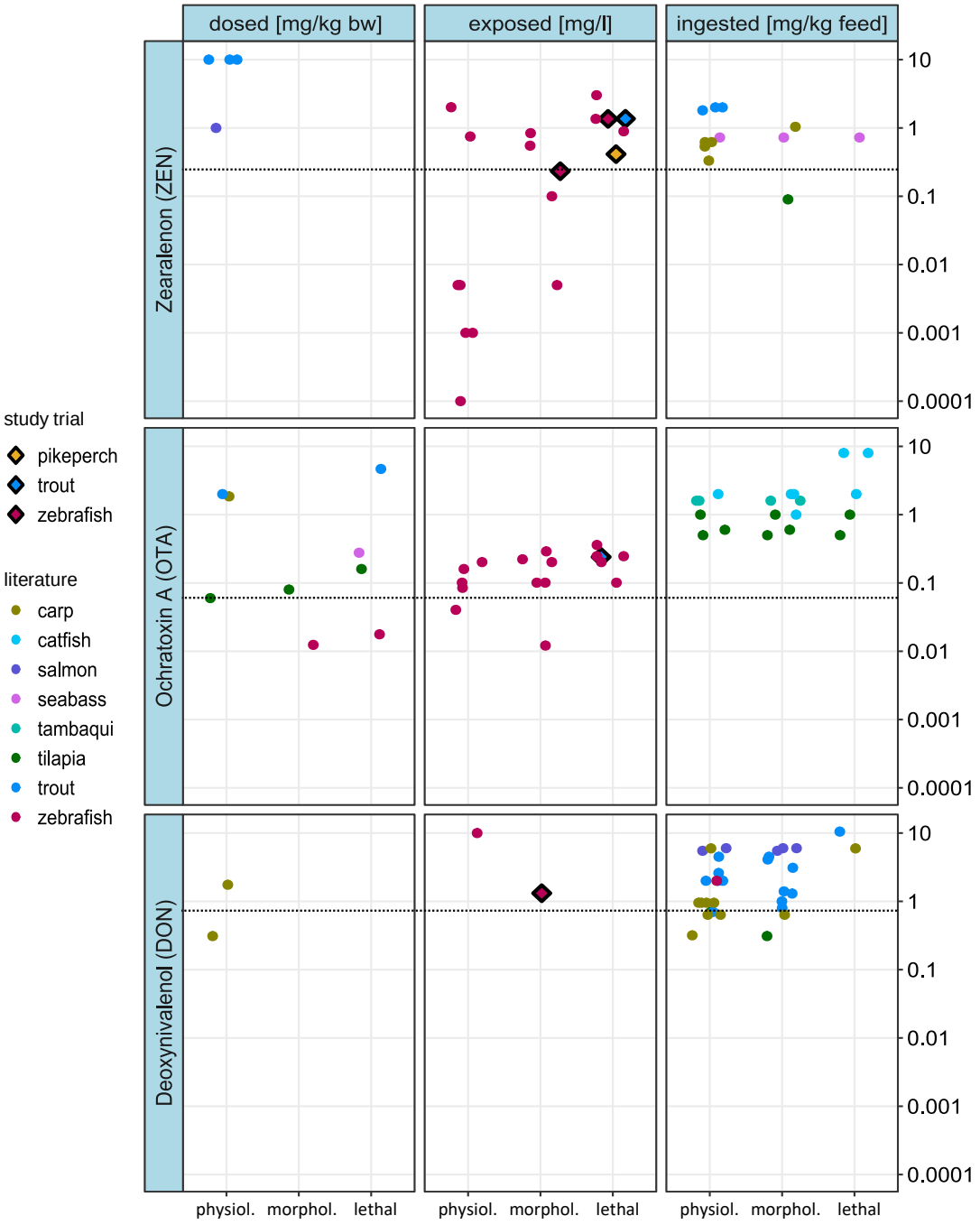



**Figure 2:** Lowest observed adverse mycotoxin concentrations reported in the literature grouped by the factors *mycotoxin* (DON, OTA, ZEN), *application* (dosed, exposed, ingested), *endpoint* (physiological, morphological, lethal) and *species* (see legend), bw = bodyweight. The six toxicity values that were calculated on a level comparable to a LOAEC ($LC_{10}$ and $EC_{10}$) in the FET test in this study (diamonds) as well as the EU regulations limits for pigs (dotted lines) are plotted for comparison.

| ANOVA | factor | Df | Sum Sq | % explained | Mean Sq | P-value |
|---|---|---|---|---|---|---|
| Mycotoxin | Mycotoxin | 2 | 12.85 | 14.7 | 6.423 | < 0.001 |
| | Residuals | 106 | 74.84 | 85.3 | 0.706 | - |
| Application | Application | 2 | 26.76 | 30.5 | 13.378 | < 0.0001 |
| | Residuals | 106 | 60.93 | 69.5 | 0.575 | - |
| Endpoint | Endpoint | 2 | 0.48 | 0.5 | 0.242 | 0.75 |
| | Residuals | 106 | 87.20 | 99.5 | 0.823 | - |
| Species | Species | 7 | 39.39 | 44.9 | 5.627 | < 0.0001 |
| | Residuals | 101 | 48.29 | 55.1 | 0.478 | - |

**Table 3:** Tables of the separate one-way ANOVAs that were fitted to the dataset.

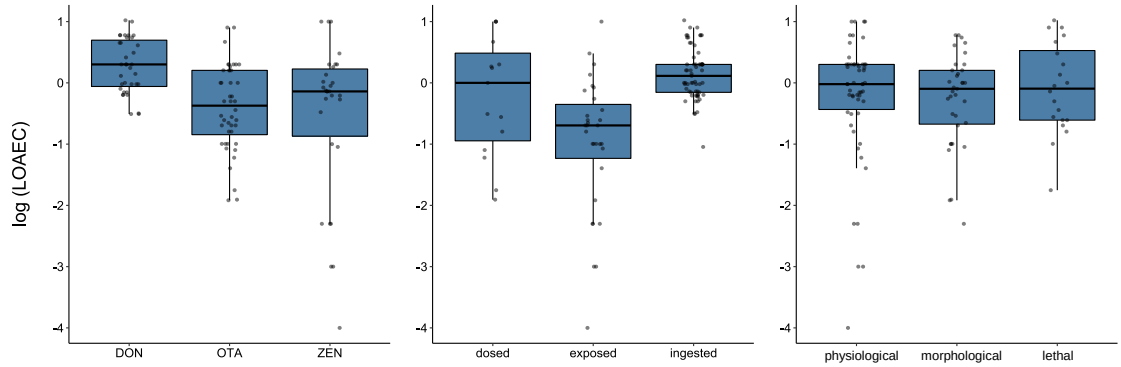



**Figure 3:** Lowest observed adverse mycotoxin concentrations reported in the literature individually grouped by the factors *mycotoxin* (left), *application* (middle), *endpoint* (right).

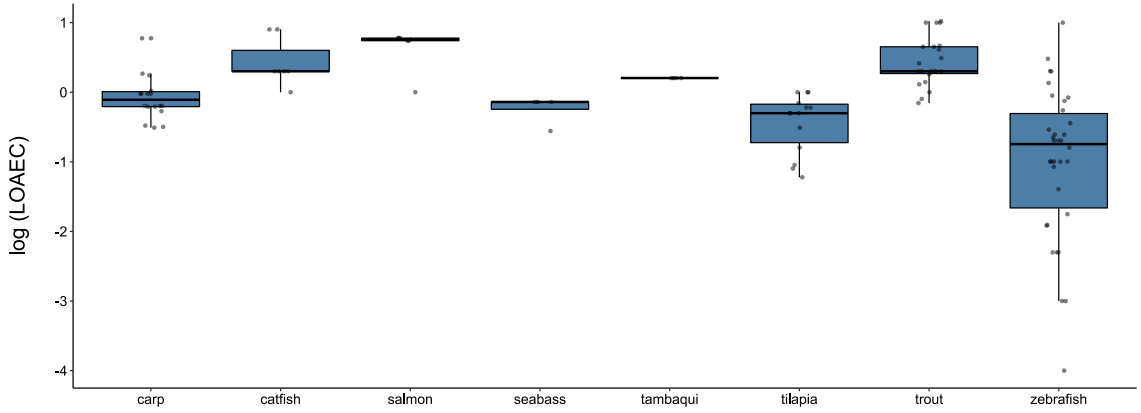



**Figure 4:** Lowest observed adverse mycotoxin concentrations reported in the literature grouped by the fish species used in the studies.

**Fish embryo acute toxicity test**

*Evaluation of the species-specific FET testing protocol*

The mortality rates of the control groups at the end of the incubation phase (assessment IV) confirmed the validity of the FET tests (Fig. 7). The negative control group showed low mortalities (ISO: pikeperch: 0 %, rainbow trout: 17 %, zebrafish: 4 %) that were comparable to the solvent control (ETH, pikeperch: 0 %, rainbow trout: 25 %, zebrafish: 4 %). The positive control (DCA), intended to represent a $LC_{100}$, had a lethal effect on zebrafish and rainbow trout (mortality > 95 %), while the pikeperch showed no mortality but a high malformation rate (96 %). This validation of the FET test according to the quality control criteria of the guideline (OECD 2013*b*) is important since the trials were done with untypical fish species (for an in-depth discussion see Appendix A). Noteworthy is the increased background mortality of the rainbow trout caused by an estimated rate of unfertilised eggs of 17 % (see Appendix A). Overall, the fish embryo acute toxicity tests were successfully conducted with all three fish species and confirm previous findings that this standardized protocol can be adapted to different teleost species (Jeffries *et al.* 2014; Mosneang *et al.* 2015; Krzykwa *et al.* 2018).

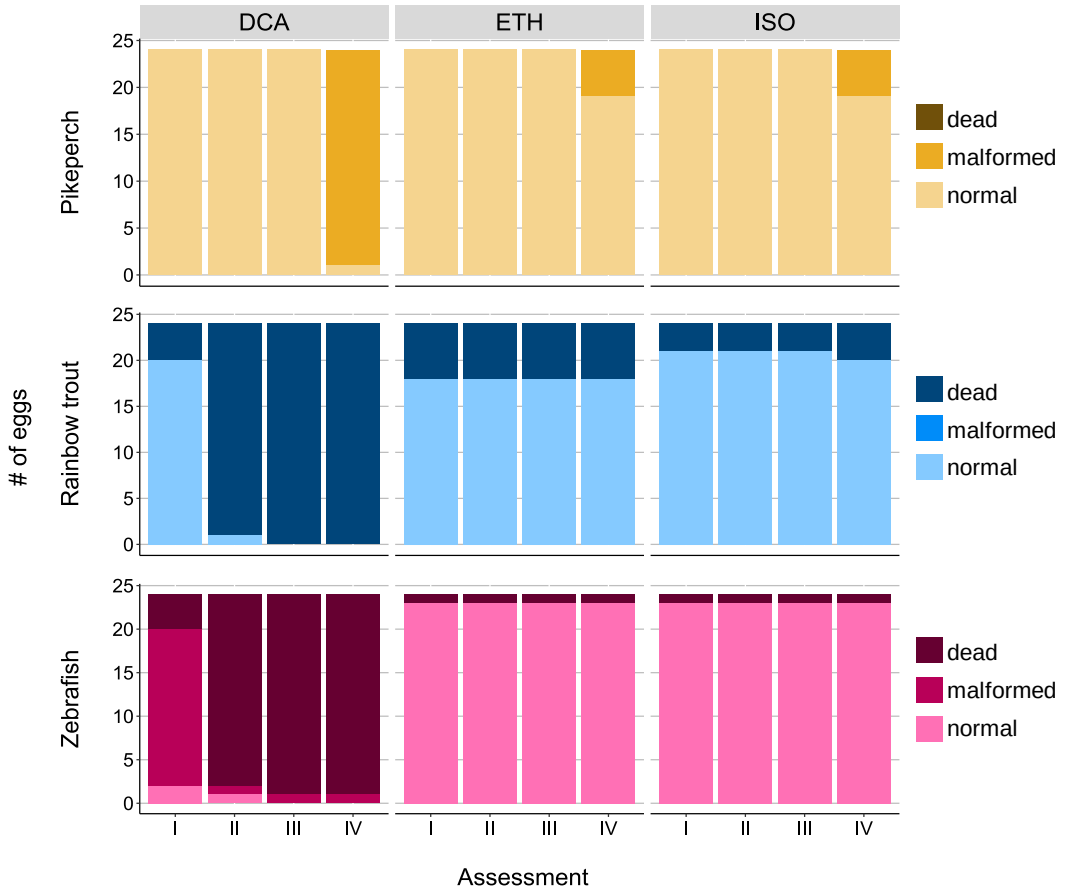



**Figure 7:** Number of dead, malformed and normally developed embryos of the positive (DCA), the solvent (ETH) and the negative (ISO) control groups for the three fish species. Number of exposed eggs per treatment group was 24. Assessments I-IV represent a timeline with sampling points according to Fig. 2.

*Lethal and effect concentrations - Deoxynivalenol*

Deoxynivalenol reduces feed intake and affects sublethal endpoints before the mycotoxicosis results in lethality (Ryerse *et al.* 2015; Gonçalves *et al.* 2018*a*), which confirms the relevance of the endpoint assessed within a study (Tab. 5). The tested range of concentrations (0.25-5 mg/l) did not result in sufficient mortalities to calculate lethal concentrations in any of the three fish species (Fig. 8, left). There are only two feeding studies that report increasing mortality at 6 and 10.5 mg/kg feed (Pelyhe *et al.* 2016*a*; Gonçalves *et al.* 2018*a*), while egg exposure did not affect mortality rates up to concentrations of 5 mg/l (present study), 40 mg/l (Zhou *et al.* 2017) or even 29.6 g/l (Khezri *et al.* 2018).

The concentration range proposed in the guideline (OECD 2013*b*) is insufficient when comparing variability-inducing factors such as *species* and *endpoint*. On one hand, the rate of affected embryos did not exceed 40 % for zebrafish. Therefore, the $EC_{50}$ could not be estimated, while the $EC_{10}$ was calculated to be 1.3 mg/l (Fig. 9, left). On the other hand, at least 30 % of pikeperch were affected in all treatments. Hence, the $EC_{10}$ could not be estimated, while the $EC_{50}$ was 0.67 mg/l. The LOAECs for sublethal endpoints in previous feeding trials range from 0.3 mg/kg feed in tilapia (Tola *et al.* 2015) to 6 mg/kg feed in salmon (Bernhoft *et al.* 2018). Also studies using the same application method (medium exposure) and fish species (zebrafish) report values ranging from 1.3 mg/l (present study) to 10 mg/l (Zhou *et al.* 2017). This highlights the need for larger concentrations ranges during testing.

*Lethal and effect concentrations - Ochratoxin A*

For ochratoxin A the three fish species showed different i.e. species-dependent lethal concentrations. For zebrafish, the tested C1 of 0.05 mg/l matched the $LC_{50}$ (Fig. 8, middle). Pikeperch were more sensitive and LC values could not be calculated as all concentrations (0.05-0.8 mg/l) caused a 100 % mortality. The rainbow trout embryos were more robust with $LC_{10}$ and $LC_{50}$ values of 0.24 mg/l and 0.53 mg/l, respectively. Similarly, previous feeding trials showed a lower sensitivity of catfish towards OTA compared to tilapia with increased mortality at 2-8 mg/l (Manning *et al.* 2003, 2005; Zahran *et al.* 2016) and 0.5-1 mg/l (Diab *et al.* 2018; Fadl *et al.* 2020) respectively.

The mycotoxin concentration range that was indicated by the existing literature to cause malformations in exposure trials (0.01-0.3 mg/l (Haq *et al.* 2016; Wu *et al.* 2016, 2018, 2020; Tschirren *et al.* 2018; Khezri *et al.* 2018)) did not result in usable proportions of sublethally affected embryos. No effect concentrations could be calculated as all ochratoxin A concentrations administered affected all embryos tested in pikeperch as well as zebrafish (Fig. 9, middle). Generally, relevant effect or lethal concentrations can differ considerably between similar studies, e.g. the $LC_{50}$ of 0.05 mg/l (present study) vs. 0.36 mg/l (Tschirren *et al.* 2018), despite both studies using the same *mycotoxin* (OTA), *species* (zebrafish), *application* (FET test) and *endpoint* (mortality). This confirms the necessity of using larger concentrations ranges during testing.

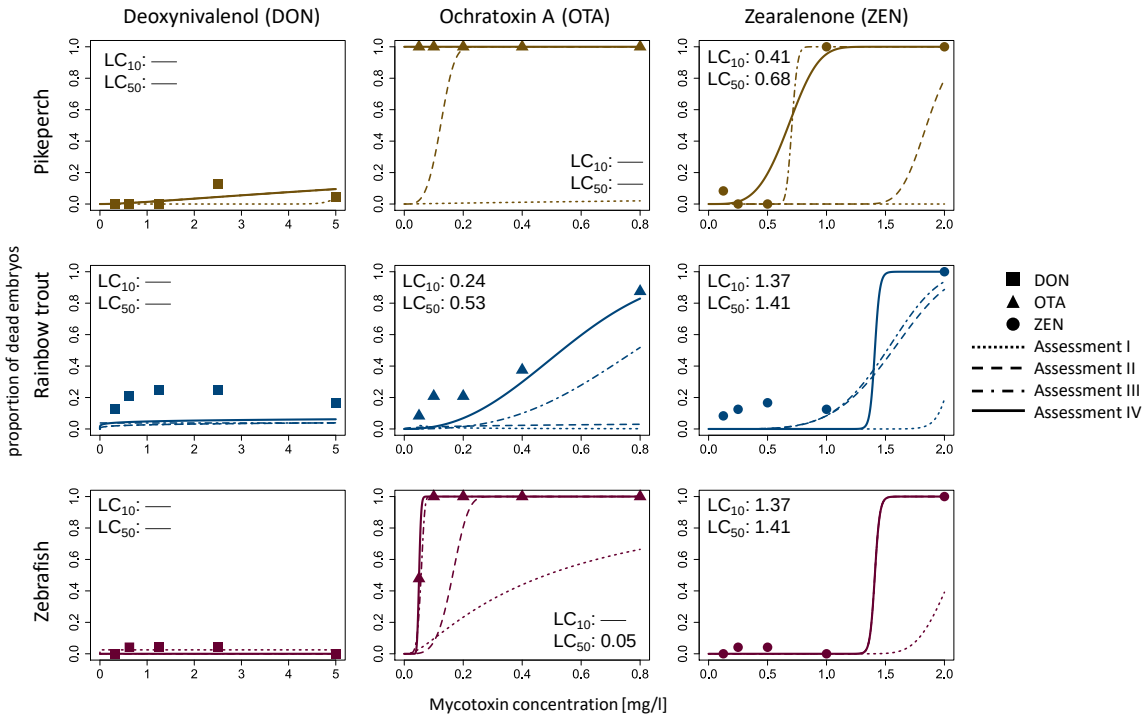



**Figure 8:** Calculations for lethal concentrations (LC) in mg/l for 10 % and 50 % of the exposed embryos. For assessment I – III the calculated dose-response curves are shown, for assessment IV the calculated curve as well as the data points are shown (C1, C2, C3, C4, C5), data in Appendix B.

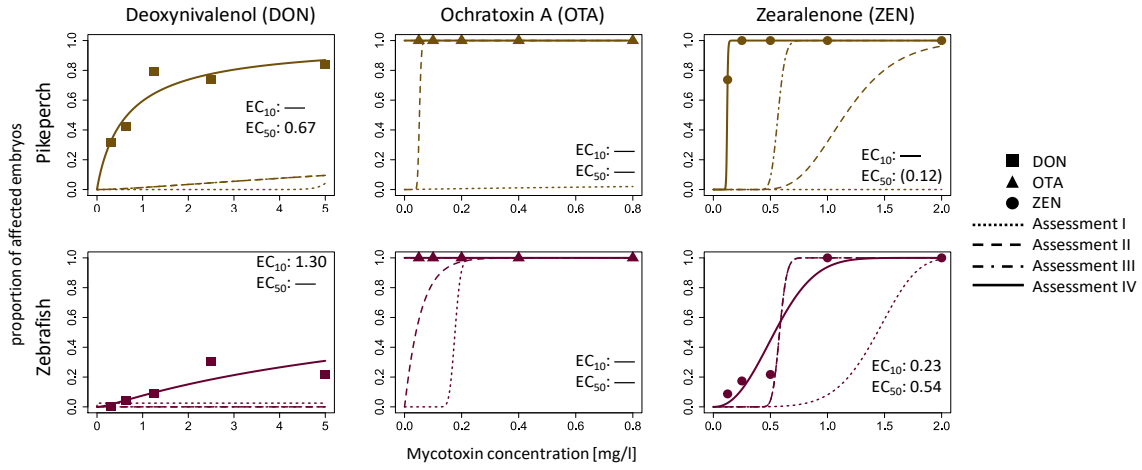



**Figure 9:** Calculations for sublethal effect concentrations (EC) in mg/l for 10 % and 50 % of the exposed embryos. EC values in brackets are outside the tested concentration range. For assessment I – III the calculated dose-response curves are shown, for assessment IV the calculated curve as well as the data points are shown (C1, C2, C3, C4, C5), data in Appendix B.

*Effect and lethal concentrations - Zearalenone*

Zearalenone shows concentration as well as time dependency, which underlines the influence of exposure duration and assessment timing. The dose-response curves confirm that effects increase with higher concentrations and longer exposure (Fig. 8, right), which emphasises the need for standardised and transparently reported exposure and assessment timing. Further, ZEN affects none to all embryos within one concentration step, which leads to steep dose-response curves due to large concentration intervals. For pikeperch values of 0.41 mg/l ($LC_{10}$) and 0.68 mg/l ($LC_{50}$) were calculated, rainbow trout and zebrafish had a $LC_{10}$ of 1.37 mg/l and $LC_{50}$ of 1.41 mg/l. These values are comparable to previous exposure studies with zebrafish, which have reported lethal concentrations of 0.9-3 mg/l (Bakos *et al.* 2013; Zhou *et al.* 2017; Khezri *et al.* 2018).

While sublethal endpoints improve sensitivity within a study, they may result in increased variability for interstudy comparisons. The sublethal endpoints for zearalenone were 0.23 mg/l ($EC_{10}$) and 0.54 mg/l ($EC_{50}$) for zebrafish. In pikeperch the C1 treatment affected 80 %, hence the $EC_{10}$ was not calculated and the $EC_{50}$ of 0.12 mg/l is an extrapolated estimation. These values have a variance comparable to DON and OTA within the study presented. The exposure trials on zebrafish in the literature dataset, however, show a considerable range of LOAECs, which exceeds the reported typical magnitude of one (Gustafson *et al.* 2012). This variance may partly be caused by a different trial setup. These exposure trials reported effects on sublethal endpoints for adult zebrafish at 0.1-5 µg/l medium (Schwartz *et al.* 2010; Muthulakshmi *et al.* 2018*a*), while exposure of embryos showed alterations starting at 0.1-2 mg/l (Zhou *et al.* 2017; Muthulakshmi *et al.* 2018*b*; Khezri *et al.* 2018), with one exception that detected changes in gene expression as early as 5 µg/l (Bakos *et al.* 2013). This considerable difference in EC values depending on the fish's life stage may be partly caused by the generally longer trial duration for adult fish and/or a higher sensitivity of adult fish towards this estrogenic mycotoxin.

In summary, the FET tests have proven suitable to investigate biological factors such as *species* and *endpoint*, which cause data variability in metadata sets. Where LC and EC values could be calculated the results from the FET test in this study (Fig. 8 and 9) were within the expected range of one magnitude for toxicity data (Fig. 2), underlining the flexibility of the protocol applied (Appendix A). An adequate selection of the range and interval of concentrations tested as well as the timing of exposure and endpoint assessment is crucial for a toxicological study (Fent 2013), emphasising that the protocol's suggestions (OECD 2013*b*) may need trial specific adaptations such as larger ranges or different intervals of mycotoxin concentrations used.

*Biological factors - Endpoint*

Assessments based on morphological endpoints yielded lower toxicological values compared to lethal endpoints (Tab. 5) which confirms that sublethal endpoints indeed increase sensitivity of FET testing (Panzica-Kelly *et al.* 2010; Andrade *et al.* 2016). The effective differences, e.g. ZEN in zebrafish ($LC_{50}$ 1.41 mg/l vs. $EC_{50}$ 0.54 mg/l) and pikeperch ($LC_{50}$ 0.68 mg/l vs. $EC_{50}$ 0.12 mg/l), were between factor 3 and 6. A reason for this proximity of the two median concentrations may be that the chosen sublethal endpoints

of developmental malformations represent severe adverse effects. The inclusion of physiological endpoints, e.g. oxidative stress (Tschirren *et al.* 2018) may yield more pronounced differences between EC and LC. A consequence of this proximity of effect and lethal concentrations is that while statistical differences between endpoints are found within a study, this effect disappears in the literature study as the difference is within the typical range of variability in metadata sets of one magnitude (Fig. 2). Additionally, the sensitivity of sublethal endpoints depends on various aspects (section 3.1.4.), which contributes to an increased data variability and result in a negligible effect of *endpoint* as a factor in the metadata set (section 3.1.6.). Hence, while trial specific endpoints add sensitivity and insights to a study, the inclusion of widely used endpoints increase the comparability and the external validity of the results (Schaefer & Myers 2017).

*Biological factors - Species*

The three fish species did not show a consistent sequence of sensitivity towards the three mycotoxins (Tab. 6). While for DON the rainbow trout was most sensitive (rainbow trout > pikeperch = zebrafish), for OTA they were most robust (pikeperch > zebrafish > rainbow trout) with pikeperch being affected earliest, as was the case for ZEN (pikeperch > rainbow trout = zebrafish). The effective differences, e.g. $LC_{50}$ for OTA (zebrafish 0.05 mg/l vs. rainbow trout 0.53 mg/l) or $LC_{50}$ for ZEN (pikeperch 0.68 mg/l vs. zebrafish 1.41 mg/l), ranged from factor 2 to 10 and hence were within the usual range of variability in toxicological metadata sets of one magnitude (Fig. 2). Additionally, methodological problems due to the later onset of exposure of pikeperch or the larger egg/volume ratio for rainbow trout did not result in a detectable systematic effect. Overall, there are species-specific sequences of sensitivity for individual mycotoxins. However, there is no generalization possible about the overall sensitivity of one fish species towards mycotoxins. This explains the statistically relevant effect of the *species* in the literature survey (Tab. 3), without the presences of an overall pattern of sensitivity of fish species (Fig. 2).

| | | Comparison | $P_1$ | $P_2$ | X-squared | p-value |
|---|---|---|---|---|---|---|
| DON | zebrafish | lethal - sublethal | 3/120 | 19/120 | 11.3 | < 0.001 |
| | pikeperch | | 4/120 | 84/120 | 112.0 | < 0.0001 |
| OTA | zebrafish | lethal - sublethal | 108/120 | 120/120 | 10.6 | 0.001 |
| | pikeperch | | 120/120 | 120/120 | NA | NA |
| ZEN | zebrafish | lethal - sublethal | 26/120 | 62/120 | 22.0 | < 0.0001 |
| | pikeperch | | 50/120 | 115/120 | 79.4 | < 0.0001 |

**Table 5:** Pairwise tests for equal proportions of affected embryos (all five mycotoxin concentrations combined with n=24 for each).

| | Comparison | P1 | P2 | X-squared | p-value |
|---|---|---|---|---|---|
| DON | zebrafish – pikeperch | 3/120 | 4/120 | < 1 | 1 |
| | zebrafish – rainbow trout | 3/120 | 24/120 | 16.7 | < 0.0001 |
| | pikeperch – rainbow trout | 4/120 | 24/120 | 14.6 | < 0.001 |
| OTA | zebrafish – pikeperch | 108/120 | 120/120 | 10.6 | < 0.001 |
| | zebrafish – rainbow trout | 108/120 | 42/120 | 75.1 | < 0.0001 |
| | pikeperch – rainbow trout | 120/120 | 42/120 | 112.6 | < 0.0001 |
| ZEN | zebrafish – pikeperch | 26/120 | 50/120 | 10.2 | < 0.01 |
| | zebrafish – rainbow trout | 26/120 | 36/120 | 1.8 | 0.18 |
| | pikeperch – rainbow trout | 50/120 | 36/120 | 3.1 | 0.08 |

**Table 6:** Pairwise tests for equal proportions of lethally affected embryos (all five mycotoxin concentrations combined with n=24 for each).

*Implications from the fish embryo acute toxicity test*

As a whole, the FET tests conducted in this study indicate that the two biological factors, *endpoint* and *species*, have a comparable effect on the variability of LOAECs within a study. The differences in toxicological values caused by different *endpoint* and *species* ranged from factor 2 to 10 and hence were within the typically observed magnitude of variance in toxicological metadata sets (Gustafson *et al.* 2012). The discrepancies to the findings in the literature survey are likely caused by peculiarities of the metadata set. The graphical representation (Fig. 2) indicates statistical interactions between *species* and *mycotoxin* as well as *application*, which may misleadingly increase the relevance of the *species* as a factor for a metadata set. Similarly, statistical interactions of the *endpoint* with *mycotoxin* and *application* might lead to a decrease of the relevance of *endpoint* as a factor.

The conclusions for the biological factors imply that: (I) hazard characterisation studies need to include endpoints that are widely used to increase the comparability and external validity of the results (Schaefer & Myers 2017) as well as endpoints that are specific for the study purpose or the mycotoxin to increase the sensitivity of the study (Hedgpeth *et al.* 2019), and (II) fish species should be used that are representative for the study purpose, e.g. zebrafish for basic research on toxicodynamics (Juan-García *et al.* 2020) and trout, salmon and carp for applied research on mycotoxicosis in aquaculture (Cai *et al.* 2019). Additionally, aspects of endpoints, e.g. assessment methods or biological relevance, as well as characteristics of the fish, e.g. age, condition or strain, must be reported in a transparent and complete manner. The aim of a toxicological study is to not only have relevance on its own but to add value to a larger dataset in the context of a complete hazard characterisation.

**Conclusions**

Together the analysis of the literature survey and the fish embryo acute toxicity test indicate that the methodological and biological factors assessed, i.e. *the identity of the mycotoxin*, *mycotoxin application methods*, *endpoints assessed* and *fish species* used, are of comparable relevance for resulting toxicological

values such as LOAECs. For the typically mycotoxin-centric hazard characterisation process this implies that the effects of the application, endpoint and fish species need to be increasingly considered. Specific suggestions based on the results in this study are (I) the confirmation of mycotoxin-specific risk characterisations including emerging mycotoxins and potentially mixtures, (II) the emphasis of an application-specific selection of a metadata set with the specific exclusion of studies using injection or gavage, (III) the importance of including widely used endpoints to benefit comparability as well as specific endpoint to increase sensitivity, and (IV) the advantage of using trial purpose-specific fish species.

The awareness of the relative importance of sources of variability benefits mycotoxin hazard characterisation by assisting the selection of reliable metadata sets and the improvement of future studies. However, a data variability of one magnitude is likely to be inherent to this kind of data. This has two major consequences: First, the necessity to integrate safety factors of ten or higher when defining contamination limits for fish feed and feed ingredients. And second, the inevitability to analyse the literature in its entirety and base any contamination limits on a possibly variable and broad but valid and representative metadata set. Only an improved and fish specific hazard characterisation and risk assessment for mycotoxins in animal feed will help secure fish health and welfare in aquaculture and grant the thriving of a crucial food industry sector.

# *Appendix*

## Appendix A: Test validity

*Validity of the fish embryo acute toxicity test*

The validation of the FET test according to the quality control criteria of the guideline (OECD 2013*b*) is important, especially when species-specific adaptations were made to the protocol. Overall, the tests presented in this study were successfully conducted with the three species. A more detailed discussion of the six criteria of the OECD 236 guideline can be found below.

*The overall fertilization rate of the fish eggs must be ≥ 70 %*

This criterion ensures the overall quality of the clutch and can be better verified with transparent eggs. When fish eggs are opaque a trade-off between early mycotoxin exposure and fertilisation security emerges. In this study all eggs went through a first screening by the producer before transportation and were screened again before the exposure. The check in the lab showed a successful fertilization of over 95 % for the zebrafish and an exposure starting at 6 hpf with only fertilized eggs could be guaranteed. To ensure the same for the pikeperch, these eggs were only transported at 3 dpf. The check in the lab showed a fertilization rate of over 95 % as well and the exposure was started with a delay at 3 dpf with only fertilized eggs. As for the rainbow trout, the visual inspection was hindered by the opaque chorion and the development at the beginning is comparably slow, therefore the early embryo is difficult to spot. It is only around 14 dpf that the visual detection of embryonal structures can be done reliably (Barrett *et al.* 2001). A delayed exposure has to be balanced against the usual tendency to start the exposure to the mycotoxins as early during the development as possible. The rainbow trout eggs were exposed at 3 dpf as well, however, to account for a potentially higher background mortality rate caused by unfertilized eggs, an additional 120 eggs were incubated in the negative control solution (ISO). This group had 12.5 % dead eggs at assessment I and 17.5 % at assessment IV (data not shown), indicating that up to 17 % of the mortality may be caused by unfertilized eggs.

Overall, all three fish species fulfilled this first criteria of an at least 70 % fertilization rate. While, this OECD guideline criterion aims at setting a standard for the general quality of the clutch, the mycotoxin exposure should be started with fertilised eggs only. The pikeperch eggs might be reliably screened for a successful fertilisation as early as 2 dpf or even 1 dpf (personal observation), which would allow a timely mycotoxin exposure comparable to zebrafish. As for the rainbow trout, the trade-off between early exposure and certainty about the fertilisation will remain, which makes this fish species less suitable for future FET testing. A possible workaround, the dechorionation of the eggs at the start of the exposure, a process that is regularly applied for zebrafish (Panzica-Kelly *et al.* 2010) and was shown to be applicable for pikeperch (Güralp *et al.* 2016), may not be an option for rainbow trout as the method has only been successfully applied to rainbow trout eggs after the eye-stage (Barrett *et al.* 2001; Ciuhandu *et al.* 2005).

*The water temperature must be 26 ± 1 °C*

This criterion ensures stable and appropriate water temperatures and while the stability should be guaranteed in any FET test, the optimal water temperature must be adapted to each fish species. In this study the average temperature targeted was 26 °C for zebrafish, 11 °C for pikeperch and 10 °C for rainbow trout. The variation of the temperature during the incubation was ± 0.5 °C for zebrafish and ± 2 °C for rainbow trout (caused by the handling during the weekly exchange of the exposure solutions). For the pikeperch, this criterion was not met due to the intentional temperature increase (+ 4 °C) in the last two days of the incubation. This change in temperature successfully decreased the otherwise asynchronous hatching of over 3–4 days (Güralp *et al.* 2017) to a time window between 11 dpf (0 % hatched) and 13 dpf (100 % hatched) for all control groups. This adaption of the protocol is advantageous as synchronous hatching is desirable during FET tests, because exposure (Pelka *et al.* 2017) and/or development (Ninness *et al.* 2006) might be altered by the missing chorion and the hatching rate itself is part of the standardisation (see the fifth criterion).

Generally, lower water temperatures for FET tests are especially suitable for ecotoxicological studies in tempered climates or temperature-sensitive chemicals. This makes pikeperch eggs, which were successfully reared between 9–16 °C (Oprea et al. 2014; Güralp et al. 2016, 2017) an interesting alternative to warm water species.

*The negative and the solvent control groups must have a survival rate of ≥ 90 %*

This criterion ensures appropriate trial conditions and embryo quality and partly depends on a successful fertilisation screening. In this study the zebrafish had a mortality of 4 % and the pikeperch 0 % for both control groups. The mortality rate of the rainbow trout was 17 % and 25 % for the respective control group. However, accounting of the background mortality caused by unfertilized eggs of 17 %, the survival rate of the rainbow trout embryos equally fulfils this criterion.

*The survival rate of the positive control must be ≤ 70 %,*

This criterion ensures appropriate trial conditions, handling and assessment and relies on an universally toxic chemical. In this study, the 3,4-dichloroanline had a lethal effect on zebrafish and rainbow trout with a mortality rate of over 95 %. However, the pikeperch showed a high malformation rate (96 %) but no mortality. This may be due to (I) the comparably late start of the exposure (but DCA is not known to only affect fish during the very early development (Schäfers & Nagel 1993)); (II) the comparably shorter duration of the exposure (but the same concentration of DCA previously showed lethal effects for zebrafish within 24 h (Schiwy *et al.* 2020)); (III) a temperature dependent difference of the effects of DCA (but the rainbow trout were incubated at equally low water temperatures and showed considerable mortality) or (IV) a different species-specific toxicity for pikeperch (as has been shown before for DCA (Schäfers & Nagel 1993; Jeffries *et al.* 2014)). The latter cause would require an adaption of the protocol for pikeperch to either a higher standard concentration or a different positive control with a chemical that has a more uniform effect on teleost embryos.

*The hatching rates of the negative and solvent control must be ≥ 80 %*

This criterion ensures appropriate trial conditions and handling as well as embryo quality and depends on a defined and synchronous hatching. In this study, the zebrafish and the pikeperch showed hatching rates of over 95 % in both control groups. The timing of assessment IV for the rainbow trout was defined by the additional negative control group (n=120). Here the first two eggs hatched at 41 dpf, and by day 44 dpf 86 embryos had hatched successfully. Accounting for the 17 % (21 eggs) of background mortality due to unfertilised eggs, 87 % (86/99 eggs) had hatched and hence the final assessment was done on 44 dpf. For the actual negative control group (n=24) 4 eggs had died early on (accounted for by the 17 % of unfertilised eggs), 16 had hatched and 4 were alive but had not yet hatched, resulting in a hatching rate of 80 % (16 out of 20 remaining living eggs) at the time point of assessment IV (data not shown). The key parameter here is the time point of this last assessment, which should be adapted to the species-specific incubation duration. Due to the lack of a standard protocol, the timing of the final assessment was matched with the control groups reaching the hatching threshold of 80 %. It must be considered that individual eggs will hatch considerably later than eggs reared in groups (personal observation).

*The dissolved oxygen saturation in the negative control must be ≥ 80 %*

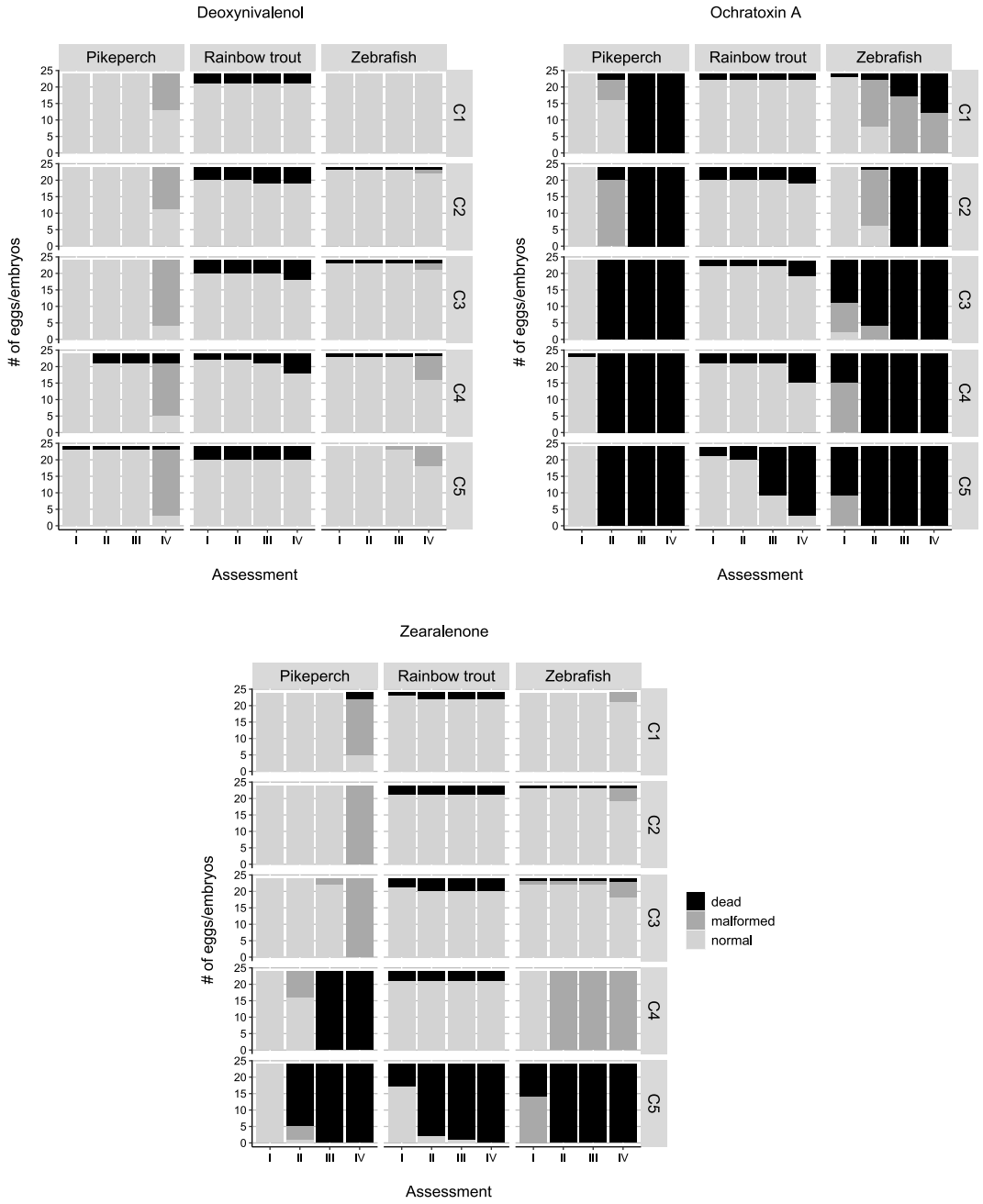
This criterion ensures appropriate trial conditions. The 80 % correspond to 6 mg/l at a water temperature of 26 °C, a criterion that was met in this study. The oxygen concentration was above 7 mg/l throughout the trials for all three species.

*Conclusions*

The toxicity values measured in this study are in line with the existing literature (Fig. 2). Using FET tests can be a valuable method for mycotoxin research in the context of aquaculture and aquatic environments for several reasons. First, non-feeding stages of fish embryos are not protected by European law, which facilitates the conduction of FET trials. Moreover, compared to the acute fish toxicity test (OECD 2019) and the early-life stage toxicity (ELST) test (OECD 2013*a*) the FET test is more in line with the 3R principles. Additionally, the test has shown good correlation with other methods and generally delivers reliable results, which underlines the usefulness of the information gathered (Braunbeck *et al.* 2015). Furthermore, FET tests with diverse species allow the investigation of species-specific toxicokinetics and toxicodynamics, two fields where the test and its standard model species, the zebrafish, have proven valuable in the past (Scholz *et al.* 2008). Further, the increasing occurrence of known fungal toxins as well as new, so-called emerging mycotoxins will make more and faster research necessary in order to understand the effects of the mycotoxins, perform sound risk assessment analysis and govern restrictions and measures (Juan-García *et al.* 2020). Additionally, the FET test is not only valuable on its own but facilitates advancements as part of whole toolbox of methods, e.g. by assisting the range-finding process prior to resource intensive trials or by accelerating toxicological screenings.

**Appendix B: Assessment data**

Number of dead, malformed and normally developed embryos of the 15 mycotoxin treatments for the three teleost species (pikeperch, rainbow trout and zebrafish). Number of exposed eggs per treatment group was 24. Assessments I – IV represent a timeline with sampling points according to Figure 2. C1–C5 correspond to the concentrations given in Tab. 1.



**Appendix C: Assessment data**

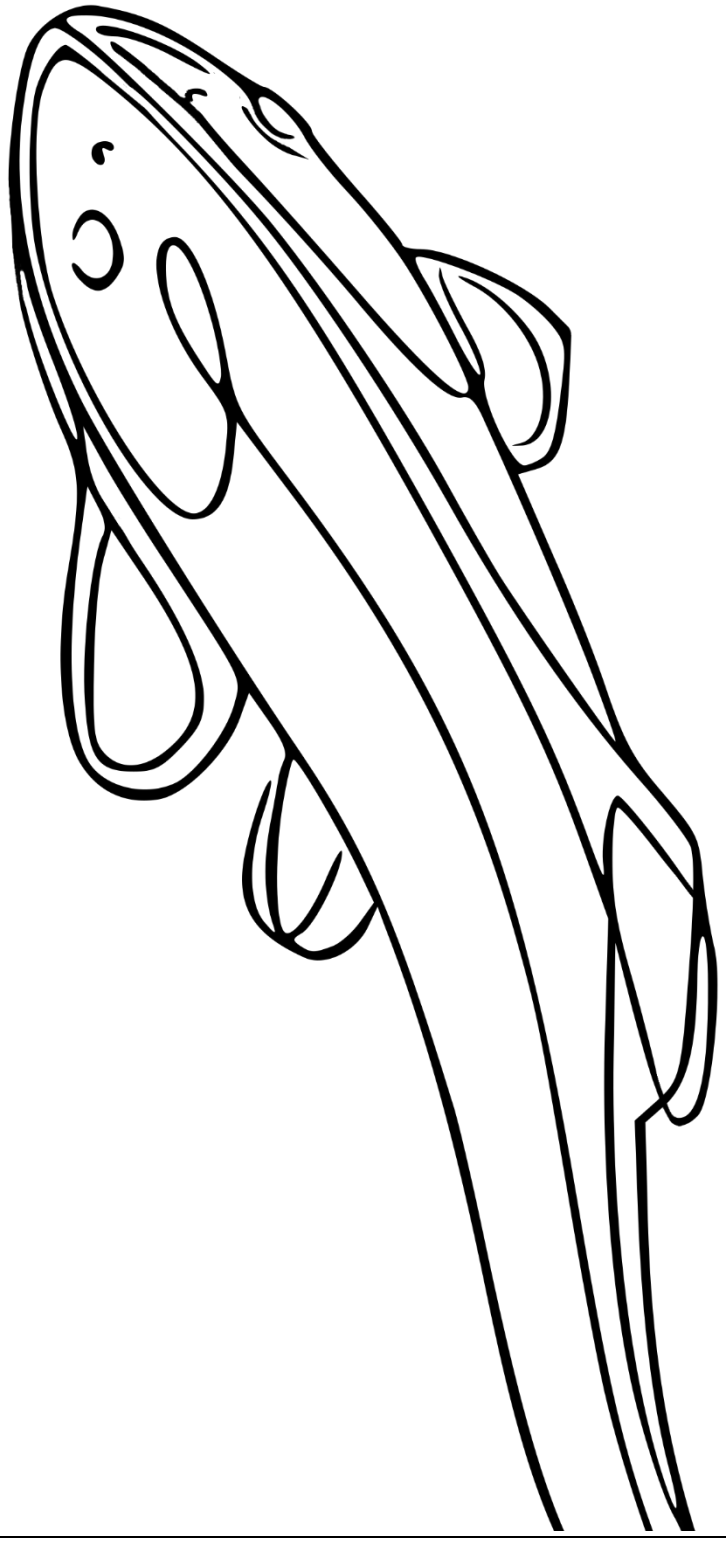
Percentage of embryos per treatment group that were normally developed, malformed or dead. For the malformed individuals, the table then indicates the percentage of fish that had malformations of the head, the heart and the tail or edema in the heart or the yolk sack. For example, the negative control group (ISO) for the pikeperch had 5 malformed embryos, i.e. 21 %. Of those 5 individuals, two had a heart edema, one had a malformed tail, one had a heart edema and a malformation of the tail, and one had malformations of the head and the heart, resulting in 1 fish with a head malformation (20 %), 1 with a heart malformation (20 %), two with a tail malformation (40 %), and three fish with an edema in the heart (60 %). Mycotoxin concentrations are indicated in mg/l medium.

| | | | **Survival** | | | **Malformation** | | | **Edema** | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Normal | Malformed | Dead | Head | Heart | Tail | Heart | Yolk |
| Controls | Pike-perch | ISO | 79 | 21 | 0 | 20 | 20 | 40 | 60 | 0 |
| | | ETH | 79 | 21 | 0 | 0 | 20 | 40 | 100 | 0 |
| | | DCA (4) | 4 | 96 | 0 | 17 | 74 | 57 | 83 | 65 |
| | Zebra-fish | ISO | 96 | 0 | 4 | | | | | |
| | | ETH | 96 | 0 | 4 | | | | | |
| | | DCA (4) | 0 | 4 | 96 | 100 | 100 | 0 | 100 | 0 |
| Deoxynivalenol (DON) | Zikeperch | C1 (0.3) | 54 | 46 | 0 | 45 | 55 | 27 | 18 | 9 |
| | | C2 (0.6) | 46 | 54 | 0 | 62 | 31 | 46 | 15 | 0 |
| | | C3 (1.25) | 17 | 83 | 0 | 45 | 80 | 30 | 25 | 0 |
| | | C4 (2.5) | 21 | 67 | 13 | 69 | 81 | 63 | 56 | 0 |
| | | C5 (5) | 13 | 83 | 4 | 80 | 40 | 10 | 50 | 0 |
| | Zebrafish | C1 (0.3) | 100 | 0 | 0 | | | | | |
| | | C2 (0.6) | 92 | 4 | 4 | 0 | 100 | 0 | 0 | 0 |
| | | C3 (1.25) | 88 | 8 | 4 | 0 | 100 | 0 | 0 | 0 |
| | | C4 (2.5) | 67 | 29 | 4 | 14 | 71 | 43 | 14 | 14 |
| | | C5 (5) | 75 | 25 | 0 | 0 | 100 | 17 | 0 | 0 |
| Zearalenone (ZEN) | Pikeperch | C1 (0.13) | 21 | 71 | 8 | 12 | 47 | 24 | 82 | 0 |
| | | C2 (0.25) | 0 | 100 | 0 | 38 | 92 | 54 | 88 | 4 |
| | | C3 (10.5) | 0 | 100 | 0 | 79 | 96 | 54 | 96 | 38 |
| | | C4 (1) | 0 | 0 | 100 | | | | | |
| | | C5 (2) | 0 | 0 | 100 | | | | | |
| | Zebrafish | C1 (0.13) | 88 | 13 | 0 | 0 | 0 | 33 | 67 | 0 |
| | | C2 (0.25) | 79 | 17 | 4 | 25 | 75 | 0 | 50 | 0 |
| | | C3 (10.5) | 75 | 21 | 4 | 20 | 40 | 60 | 60 | 0 |
| | | C4 (1) | 0 | 100 | 0 | 92 | 96 | 100 | 83 | 0 |
| | | C5 (2) | 0 | 0 | 100 | | | | | |
| Ochratoxin A (OTA) | Pikeperch | C1 (0.05) | 0 | 0 | 100 | | | | | |
| | | C2 (0.1) | 0 | 0 | 100 | | | | | |
| | | C3 (0.2) | 0 | 0 | 100 | | | | | |
| | | C4 (0.4) | 0 | 0 | 100 | | | | | |
| | | C5 (0.8) | 0 | 0 | 100 | | | | | |
| | Zebrafish | C1 (0.05) | 0 | 50 | 50 | 100 | 100 | 100 | 83 | 8 |
| | | C2 (0.1) | 0 | 0 | 100 | | | | | |
| | | C3 (0.2) | 0 | 0 | 100 | | | | | |
| | | C4 (0.4) | 0 | 0 | 100 | | | | | |
| | | C5 (0.8) | 0 | 0 | 100 | | | | | |

**Appendix D: Reference list**

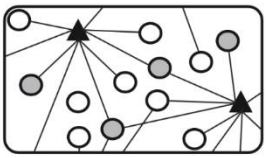
List of references of the literature survey: The literature search resulted in 199 papers about DON, OTA and ZEN, with 63 papers reporting a total of 113 specific mycotoxin concentrations with adverse effects (LOAECs). Four datapoints were excluded from the dataset: first, two untypically low values for ZEN reported by the only paper on fathead minnows (Johns et al. 2011) and second, two untypically high values for DON reported due to an unusual application method (Khezri et al. 2018).

| | |
|---|---|
| **A** | (Abdel-Tawwab et al. 2019)**;** (Arukwe et al. 1999) |
| | (Bakos et al. 2013)**;** (Baldissera et al. 2020a, b)**;** (Bernhoft et al. 2017, 2018) |
| **C** | (Csenki et al. 2019) |
| | (Diab et al. 2018); (Doster et al. 1972) |
| **E** | (El-Marakby et al. 2018); (El-Sayed et al. 2009) |
| | (Fadl et al. 2020) |
| **G** | (Gonçalves et al. 2018a) |
| | (Haq et al. 2016); (Hooft et al. 2011, 2019a, b; Hooft & Bureau 2017); (Huang et al. 2018, 2019, 2020) |
| **K** | (Khezri et al. 2018); (Kovesi et al. 2020) |
| | (Manning et al. 2003, 2005); (Mansour et al. 2011, 2015); (Matejova et al. 2014, 2016); (Moldal et al. 2018); (Muthulakshmi et al. 2018a, b) |
| **P** | (Pelyhe et al. 2016a, b); (Pietsch et al. 2014a, b, 2015a, b; Pietsch & Burkhardt-Holm 2015; Pietsch & Junge 2016; Pietsch 2017) |
| | (Ryerse et al. 2015, 2016) |
| **S** | (Sanden et al. 2012); (Schwartz et al. 2010, 2013); (Šišperová et al. 2015) |
| | (Tola et al. 2015); (Tschirren et al. 2018) |
| **W** | (Wang et al. 2019); (Woźny *et al.* 2008, 2012, 2015, 2019, 2020); (Wu et al. 2016, 2018, 2020) |
| | (Zahran et al. 2016); (Zhou et al. 2017) |



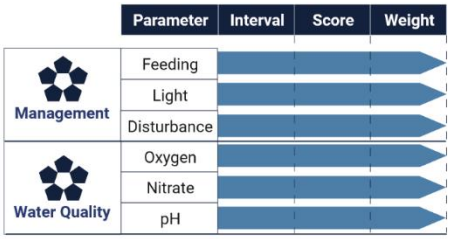
# Chapter 3

## MyFishCheck: A model to assess fish welfare in aquaculture



**1. Ontology**
Creation of a fish welfare ontology with needs (triangles) and parameters (circles) to store, manage and handle information.


**2. Modules**
Selection of welfare parameters that are relevant, reliable and applicable.
Definition of the 5 modules of the model with their respective parameters.




**3. Intervals, scores and weights**
Literature review for the 80 welfare parameters to define the intervals. Allocation of the parameter scores and the score weights according to the number of intervals. Expert survey for the 80 welfare parameters to define their relative importance and define the parameter weights.

$$MG_j = \frac{\sum_i PS_i \times SW_i^{SWE_i} \times PW_i^{PWE_i}}{\sum_i SW_i \times PW_i} + 1$$

**4. Equation**
Summation of scores within modules. After a normalisation, a transformation and an offset the equation delivers a module grade.

**5. Applications**
Development of an Android app to facilitate the use of the model.

# MyFishCheck: A model to assess fish welfare in aquaculture

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**Abstract:** Welfare in animal husbandry includes considerations of biology, ethics, ecology, law and economics. These diverse aspects must be translated into common quantifiable parameters and applicable methods to objectively assess welfare in animals. To assist this process in the field of aquaculture, where such methods are largely missing, we developed a model to assess fish welfare. A network of information was created to link needs, i.e., fundamental requirements for welfare, with parameters, i.e., quantifiable aspects of welfare. From this ontology, 80 parameters that are relevant for welfare, have practicable assessment methods and deliver reliable results were selected and incorporated into a model. The model, named MyFishCheck, allows the evaluation of welfare in five distinct modules: farm management, water quality, fish group behaviour, fish external and fish internal appearance, thereby yielding five individual grades categorising welfare ranging from critical, to poor, to acceptable, and good. To facilitate the use of the model, a software application was written. With its adaptability to different fish species, farming systems, regulations and purposes as well as its user-friendly digital version, MyFishCheck is a next step towards improved fish welfare assessment and provides a basis for ongoing positive developments for the industry, the farmers and the fish.

**Keywords:** aquaculture; fish welfare; ontology; semantic data model; animal welfare assessment

**Paper:** Published in Animals 2021, 11, 145; doi.org/10.3390/ani11010145

## *Introduction*

Awareness of animal welfare in Europe emerged in 18th century literature, where philosophers attributed to animals the capacity to feel (Hume 1777) and to suffer (Bentham 1789). Two centuries later, the scientific community had delivered evidence that some animals were indeed sentient creatures (Griffin 1992; Dawkins 1998, 2000). In the early 2000s, legislation followed these insights by putting the terrestrial farming animals and their welfare under the protection of the law to a new degree (HSMO 2006). Aquaculture has caught up to these standards only recently, with fish being ascribed the ability to perceive pain beyond simple nociception less than two decades ago (Sneddon 2003*a*, *b*; Sneddon *et al.* 2003*a*, *b*; Ashley & Sneddon 2008). This insight, as well as a better understanding of stress physiology in teleosts (Ashley 2007) and ethical, environmental and economical thinking (Huntingford *et al.* 2006), gave rise to the topic of fish welfare (Kristiansen & Bracke 2020).

### Appropriate methodology for fish welfare assessment

While fish have specific characteristics (Huntingford & Kadri 2014), the general concepts of animal welfare apply to terrestrial and aquatic environments alike (Bateson 1991), giving the aquaculture industry a chance to assimilate proven approaches from agriculture. For example, most animal welfare concepts (Fraser *et al.* 1997; Huntingford & Kadri 2008; Lawrence 2008) incorporate the three major philosophical aspects of well-being: (I) the nature-based aspect, i.e., animals are living a natural life where they can express natural behaviour and hence satisfy their so-called behavioural needs; (II) the function-based aspect, i.e., animals are exposed to an environment where their physiological systems can work well; and (III) the feelings-based aspect, i.e., animals are spared negative feelings such as pain or fear while being able to experience positive feelings such as positive anticipation. These holistic concepts of definitions and meanings of animal welfare need to be translated first into measurable parameters and then into applicable protocols to assess fish welfare. This step from a general and sometimes subjective viewpoint to a methodological and objective assessment is crucial (Bovenkerk & Meijboom 2013), since only the latter allows fact-based discussions and facilitates both unbiased comparisons and applicable improvements.

To derive such objective welfare assessments from nature-based, function-based and feeling-based aspects, the animals as well as their environment are evaluated (Bracke 2007), and the information gathered is referenced against known correlations with welfare (Bracke *et al.* 1999*a*; Anonymous 2001). This can be done using risk analysis (Collins 2012), a method focusing on the identification of so-called hazardous critical points of interest or hazard analysis and critical control points (HACCP), and taking the necessary measures to secure these points to provide fish welfare (Müller-Graf *et al.* 2012; van de Vis *et al.* 2012). However, by concentrating on only a threatened or negative welfare status, this method misses the opportunity to incorporate signs of positive welfare (Bracke *et al.* 2008). A more flexible method, which allows the evaluation of indications of a positive and negative welfare status, and therefore is a more complete approach to assess overall welfare in fish, is desirable. Furthermore, methodological fish welfare assessment is interdisciplinary, involving biology, engineering, chemistry, physics, economy, ecology, law

and ethics, predicating the management of information from various sources, of diverse nature and for different purposes. A method that matches all these requirements is semantic data modelling.

**Suitable semantic data models for information management**

Semantic data models are frameworks that are well suited to data and information integration (Embley 2009). They are a method to structure data that includes semantic information, i.e., words that add meaning to pieces of information and the relationship between them. While semantic data modelling has been applied to process data during animal welfare assessment for a number of farming animals (Bracke 2008; Botreau *et al.* 2009; Shimmura *et al.* 2011) including fish (Stien *et al.* 2013), the possibility to manage basic information about fish welfare has not yet been exploited. For example, domain ontologies may be a suitable way to help the field of aquaculture store, access, share and widen fish welfare information. An ontology is an application of conceptual semantic data modelling (Gruber 2009) and is defined as "a formal, explicit specification of a shared conceptualisation. A 'conceptualisation' refers to an abstract model of some phenomenon in the world by having identified the relevant concepts of that phenomenon. 'Explicit' means that the type of concepts used, and the constraints on their use, are explicitly defined. 'Formal' refers to the fact that the ontology should be machine readable, which excludes natural language. 'Shared' reflects the notion that an ontology captures consensual knowledge, that is, it is not private to some individual, but accepted by a group" (Studer *et al.* 1998) (p. 184). In a nutshell, an ontology is a digital network of information about a certain topic or domain. At the core of an ontology are the so-called triples, i.e., the domain's classes and their relationship with each other. For example, "fish diseases" and "water quality" are both classes and have the relationship "are affected by". By adding more triples, a complex network of the domain's information i.e., a representation of the topic can be built (He *et al.* 2012). Such an ontology, created for the domain of fish welfare, can be used as the basis for a more methodological approach to assess fish welfare compared to past attempts.

**Advantages and disadvantages of existing methods**

Previous attempts to assess animal welfare in aquaculture were based on different methods, each with specific advantages. The first semantic data model for aquaculture was developed in 2013 for salmon in sea cages (Stien *et al.* 2013) and subsequently extended by adding more physiological indicators (Pettersen *et al.* 2014). Both publications illustrate notably well the methodology of employing multiple welfare indicators to derive an overall index. However, the species- and system-specific focus limits the developability of the models. A similar model intended for pikeperch in recirculating systems (Müller-Belecke 2019) is available as a user-friendly version based on Microsoft Excel, which facilitates its application on-farm. However, the use of a reduced number of indicators results in a limited comprehensiveness, that may at times lead to an inadequately assessed fish welfare. A different attempt evaluating the potential for welfare in fish husbandry is based on knowledge about wild populations (Saraiva *et al.* 2019*a*). This approach, mainly focused on a nature-based aspect of welfare, underestimates the difference of proximate and ultimate causes of welfare. For example, if large home ranges in nature are due to scarce food sources rather than an intrinsic need or desire to swim long distances, welfare in husbandry may not be impaired

by the reduced space available, given that food is abundant. A noteworthy application of this specific nature-based approach shows the importance of including additional aspects of welfare by revealing that farm management, e.g., education and sensibilisation of personnel, is important for fish welfare (Studer *et al.* 2020). However, another focus of previous assessment attempts was the applicability on-farm, where e.g., the documentation was facilitated by a set of protocols (Kleingeld *et al.* 2016). However, as these protocols are text-based, the standardisation of assessments and hence the possibilities for scientific methodology and on-farm quality controlling are limited. In contrast, the detailed summaries of welfare indicators for salmon (Noble *et al.* 2018) and rainbow trout (Noble *et al.* 2020) in different rearing systems allow crucial high-quality knowledge transfer but do not provide applicable tools for on-site assessment. In conclusion, a comprehensive, standardised and applicable method for the evaluation of fish welfare in aquaculture is still missing.

**Improvement of fish welfare assessment in aquaculture**

Aquaculture is in need of adequate methods for animal welfare assessment and the work presented is a next step towards this goal. The model described below incorporates the specific advantages of the aforementioned welfare assessment attempts in a single application. We focus on three key requirements.

*Comprehensiveness*

(I) We incorporated parameters from function-, nature- and feelings-based welfare concepts. This ensures an inclusive assessment (Huntingford & Kadri 2008) that is unaffected by the potentially incomplete knowledge about welfare or bias of the assessor. (II) We assessed the overall welfare in five modules (farm management, water quality, fish group behaviour, fish external and fish internal appearance). By not abstracting a high-resolution assessment into one overall index, the five distinct module grades facilitate the identification of potential causes of welfare problems. (III) With at least ten parameters per module, we ensured the sufficient coverage of signs of and prerequisites for welfare to allow an interpretation of the welfare state of the fish.

*Applicability*

(I) We ensured the applicability of the model by selecting the parameters based on three characteristics: science-based relevance for welfare, practicability of existing measuring methods and reliability of the results delivered. (II) The model can be used with only a subset of the modules or the parameters, enabling a flexible and purpose-oriented use. Scientists can benefit from a comprehensive model that allows a detailed assessment of fish welfare, while a simplified version of the same model has an increased practicability that assists fish farmers in their daily routines. (III) We provide a user-friendly version of the model by means of a software application. The users can profit from an efficient parameter evaluation and standardised documentation, which is important and should be as easy and intuitive as possible (Folkedal *et al.* 2016).

*Developability*

(I) Parameters that need to be adapted to specific fish species, production systems or local regulations in order to deliver meaningful results are highlighted. This facilitates the future adaptation of the model to other species, systems or countries. (II) We provide access to the digital ontology the model is based on. This enables the inclusion of new knowledge by making it easy to adjust existing needs, parameters or relationships and to add new ones when pertinent.

## *Model development*

The model development consisted of five phases (Figure 1) where first a digital information network, an ontology, for fish welfare was created. On this basis, welfare parameters were selected and grouped into five modules. In a third phase, a literature review and an expert survey were conducted to define the parameter intervals, scores and weights. These were incorporated into a mathematical equation delivering one grade per module. As a last step, two different applications were developed.

### Creating an ontology for fish welfare

An ontology of fish welfare represented the basis for the model. For this, fourteen welfare needs for fish (Table 1) were defined based on current knowledge (Bracke *et al.* 1999*b*; Stien *et al.* 2013; Noble *et al.* 2020). These needs stem from function-based, feelings-based and nature-based welfare aspects and are complementary rather than mutually exclusive requirements. If they are met, a fish is assumed to experience good welfare, while unsatisfied needs can result in suffering (Dawkins 1990). To assess whether a need is met, measurable parameters are necessary. For example, the access to shelter is a quantifiable parameter that is correlated to the need for safety (shelter as a protection from actual or perceived danger), for rest (shelter as a place with lower water current) and for exploration (shelter as a structure for environmental enrichment). Such parameters can be either potential signs of welfare or prerequisites for welfare and health, and they are all correlated to one or more welfare needs. This composition of a need and a parameter, as classes, and their correlation is, in a semantic data modelling context, a triple. We defined over 200 parameters and their correlations (affecting, affected by, or both) to the list of needs. These three kinds of correlation are substantiated, i.e., there is at least reasonable potential for a correlation if not scientific evidence of a correlation or even of a known causation. Using Protégé and Python, all triples were combined into one ontology of “fish welfare”, which aids an understanding of the complex network of needs, parameters and their relationships (available at www.myaquaculturefarm.ch).

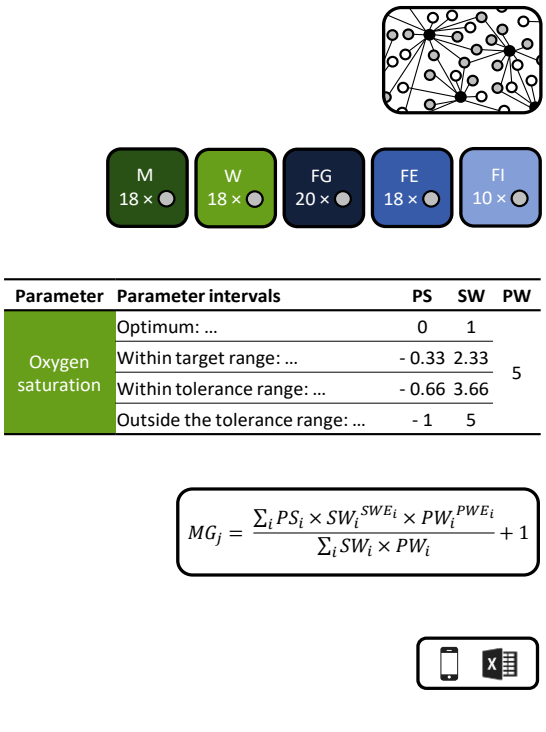


**Figure 1:** Flow chart of the model development process. M (dark green) = module farm management, W (light green) = module water quality, FG (dark blue) = module fish group behaviour, FE (blue) = module fish external appearance, FI (light blue) = module fish internal appearance, SWE = score weight exponent, PWE = parameter weight exponent.

| **Need** | **A fish needs to be…** |
|---|---|
| Respiration | able to perform gas exchange over the gills |
| Osmotic regulation | able to maintain homeostasis of cellular fluids |
| Thermal regulation | able to maintain body temperature for successful metabolism |
| Water quality | spared from abiotic adverse influences (toxins, particles, metabolites, ions, gases) |
| Hygiene | spared from biotic adverse influences (parasites, bacteria, viruses) |
| Health | spared from disease, illness, malfunction, or malformation |
| Body care | able to perform body care |
| Nutrition | able to take up food of right quality and quantity |
| Safety | able to avoid perceived danger and physical injury |
| Movement | able to move freely |
| Social contact | able to have contact to conspecifics |
| Rest | able to rest |
| Exploration | able to seek and find external stimuli |
| Reproduction | able to perform reproductive behaviour when sexually mature |

**Table 1:** Fish welfare needs, adapted from knowledge (Bracke *et al.* 1999*b*; Stien *et al.* 2013; Noble *et al.* 2020), representing function-based, feelings-based and nature-based aspects of welfare. If these needs are met the fish is assumed to experience good welfare.

**Selecting and grouping parameters for the model**

Based on the ontology, welfare parameters were chosen that fulfilled three criteria: (I) they were relevant, i.e., there is scientific evidence of a correlation with fish welfare, the nature of this correlation is known and is documented with defined values, e.g., optimum or tolerance ranges; (II) their assessment is practicable, i.e., measurement on-farm is possible and costs (time, equipment) are reasonable; (III) they are reliable, i.e., there are existing measuring methods giving results that consistently and predictably relate to welfare. As an example, Figure 2 illustrates an extract of the ontology with the need respiration (correlated with 44 parameters, five of which are shown) and nutrition (correlated with 56 parameters, five of which are shown). The correlations are represented as arrows and are incorporated in the ontology only as relationships between needs and parameters (the triples); potential relationships among needs or among parameters are not included. The parameters jaw deformation, TAN (total ammonium nitrogen), cataract, feed type, gill pathogens and ventilation rate all fulfil the three criteria of being relevant, practicable and reliable. The VSI (viscerosomatic index), however, is not practicable on-farm as the proper sampling of fat is tedious, and it is not relevant in the context of this model as the correlation to welfare, especially in terms of optimum threshold values, is not clear yet (Jobling *et al.* 1998; Bandarra *et al.* 2006; Güler & Yildiz 2011; Barnes *et al.* 2014; Voorhees *et al.* 2019). The same is true for the hematocrit; it cannot be defined as relevant here as the connection to welfare is complex, with many physiological processes affecting the number and volume of red blood cells (Jawad *et al.* 2004; Noga 2010). Moreover, appropriate sampling is not practicable with hematocrit values being affected by external stimuli within a few minutes (Skov *et al.* 2011; Phuong *et al.* 2017) making measuring normal or unstressed values on-farm very difficult.

This selection process resulted in 80 welfare parameters that were grouped into five distinct modules based on their measuring methodology (Supplementary File S1). The modules are farm management (M), parameters that describe the farm, the management, or procedures; water quality (W), parameters that describe the quality of the system water; fish group behaviour (FG), parameters that describe behavioural patterns and dynamics of the fish as a shoal; fish external appearance (FE), parameters that describe the external physiological aspects of the individual fish; and fish internal appearance (FI), parameters that describe the physiological aspects of the individual fish obtained by an invasive examination. The modules facilitate several aspects: (I) a more practical grouping of parameters that simplifies the assessment process on-farm; (II) the correlation of only related groups of parameters (such as water temperature and oxygen saturation as compared to, e.g., water temperature and personnel training) that ensures parameter comparability; (III) a usefulness of assessing any given number of modules, which makes the assessment more flexible; and (IV) an indication of which module impairs welfare, what facilitates the detection of problematic parameters. With the welfare parameters chosen, a model was developed that calculates separate welfare grades for every module.

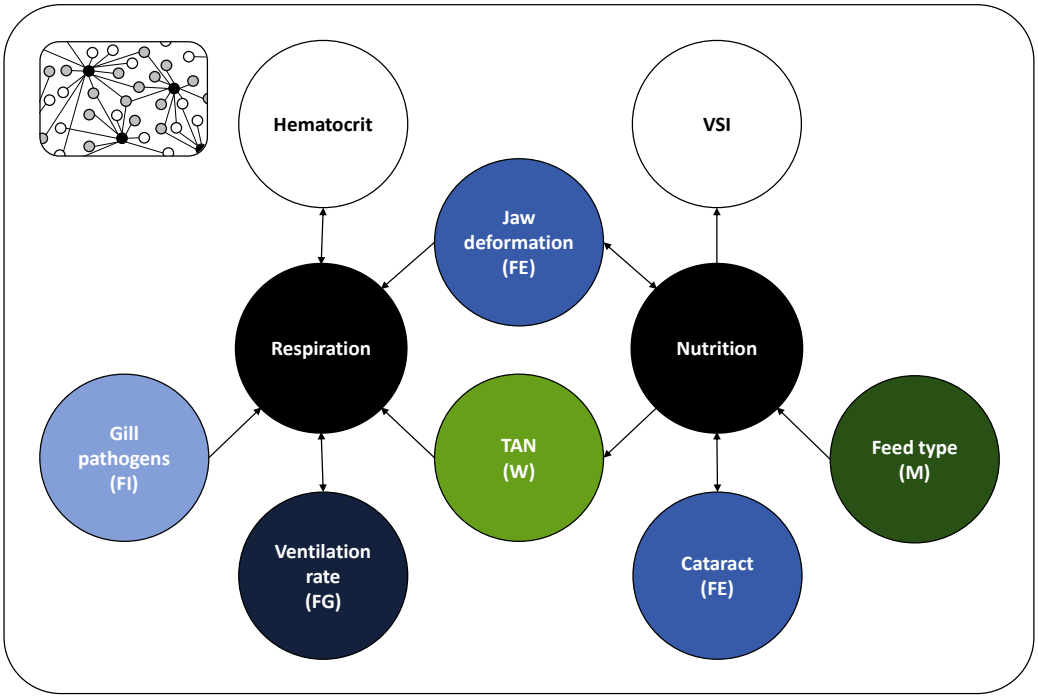


**Figure 2:** The figure represents a part of the fish welfare ontology that in total consisted of 14 needs, over 200 parameters and their relationships. The needs respiration and nutrition (black) with some of their associated parameters are shown here. The parameters given in colour fulfil the three criteria of being relevant, practicable and reliable, and hence are included in the modules of the model. The parameters in white are neither practicable on-farm nor relevant in the context of this model and therefore are not included. M (dark green) = module farm management, W (light green) = module water quality, FG (dark blue) = module fish group behaviour, FE (blue) = module fish external appearance, FI (light blue) = module fish internal appearance, TAN = total ammonia nitrogen, VSI = viscerosomatic index.

**Developing the equation for the model**

The foundation of the mathematical calculation in the model is the concept of allostasis (Sterling & Eyer 1988) and how it applies to animal welfare (Korte *et al.* 2007) and stress in fish (Segner *et al.* 2012; Schreck & Tort 2016; Sopinka *et al.* 2016). Briefly, organisms have evolved to cope with deviations from homeostasis, i.e., stress, and too little as well as too much stress will impair welfare (Schreck 2010). Any stress inflicted on an animal will cause a stress response aimed at restoring a new balance, a process that is costly (Korte *et al.* 2007). As long as these costs, the allostatic load, are below a certain threshold, the animal can cope with the stress. If the load exceeds individual limits, negative effects on welfare and health will follow (Segner *et al.* 2012). The higher the severity, consisting of the intensity, the duration and the frequency of the inflicted stress, the higher the allostatic load. Furthermore, if more than one stressor acts on the animal, the result is a cumulative overall allostatic load (Korte *et al.* 2007). The aforementioned parameters chosen for this model are a combination of signs of past and present welfare, i.e., signs of current optimal allostatic load such as a normal ventilation rate or healthy organs, as well as prerequisites

for present and future welfare, i.e., potential stressors such as water temperature or accurate feed. The equation for the model is based on these characteristics of allostasis and was built in seven steps.

*Parameter intervals and parameter scores (PS)*

The 80 parameters selected are standardised into a scoring system (Botreau *et al.* 2007) so they can be set against each other (Appendix Tables A1–A7). When measured, each parameter falls into a parameter interval, which is based on scientific literature (Supplementary File S2) and can either be numerical (e.g., water temperature is 10–16 °C) or ordinal (e.g., the ventilation rate is reduced, normal or increased). The interval is then assigned to a discretised parameter score (PS) between 0 (no or positive influence on welfare) and −1 (negative influence on welfare).

Some parameters might be policed by local laws, regulations, or industry and label standards. In Switzerland, the law sets minimal standards for the parameters personnel training, treatment journal and mortality documentation, as well as threshold values for stocking density, dissolved oxygen, ammonia, nitrite, pH and water temperature. If these regulations do not reflect the current scientific literature or the common practice, the parameter intervals may be defined depending on the purpose of the model, i.e., internal control for farms vs. scientific survey or experiment.

The number of intervals per parameter partly defines the resolution of the assessment. The more intervals the parameters have, the more fine-scaled the model becomes. However, each interval boundary needs a scientific basis and therefore the availability of relevant literature can limit the number of intervals, e.g., for the module W with four intervals per parameter. Additionally, a large number of similar intervals complicate the assessment as they are harder to choose from, e.g., in the modules FE and FI with four intervals per parameter each. Since the module FG incorporates the aspects of severity as well as abundance, the parameters have six intervals. In contrast, the assessment of the diverse parameters in module M is facilitated by the use of three intervals.

*Parameter weights (PW)*

The parameters are weighted according to their relative importance by assigning them a parameter weight (PW) taking into account that some stressors, e.g., low oxygen inflict more severe or more imminent allostatic loads than others, e.g., high carbonate hardness. These weights were established through an independent evaluation of each parameter's relevance by 20 experts (seven aquaculture engineers, seven fish biologists, and six fish veterinarians) based on their experience and knowledge. The experts assigned the parameters within each module an integer from 1 to 5 (based on the simplest version of Miller's number (Miller 1956) to make the assignment of weights as intuitive as possible), where 1 means less relevance for welfare and 5 represents a parameter that is very relevant to welfare. The medians of this evaluation were taken (Appendix Figures B1–B2) and incorporated into the model as the parameter weights.

*Score weights (SW)*

The parameter scores are weighted with a score weight (SW), again with integers ranging from 1 (for parameter intervals that inflict low or no stress) to 5 (for strong, long or frequent stressors) taking into account that more severe stress results in higher allostatic loads.

**Developing the equation for the model**

*Sum of scores*

The parameter score (PS), the score weight (SW) and the parameter weight (PW) are multiplied, and the weighted products for all parameters within one module are summed up. This considers the cumulative nature of the allostatic loads.

*Normalisation*

The cumulated weighted products are divided by the weighted mean of the module, i.e., the sum of the product of all SW and PW used. This ensures that the result of the equation is valid, even if not all parameters were measured.

*Off-set*

The equation is transformed by adding an offset of 1 to ensure the result is an easy to interpret numeric value between 0 and 1, the module grade (MG). By performing steps 4–6 only within each module, and thus only correlating the related parameters, the model results in one module grade per module.

*Parametric transformation*

The equation was tested with different datasets of parameter values with clear, known impacts on fish welfare (i.e., optimal vs. lethal conditions). Both weights, SW and PW, were supplemented with an exponent, the score weight exponent (SWE) and parameter weight exponent (PWE), respectively. The exponents were adjusted such that the equation consistently reproduced a corresponding module grade for the test datasets. This calibration of a multiclass classification with fixed decision boundaries in combination with a parametric feature transformation was done manually. To simplify the process, both exponents SWE and PWE were kept identical, ensuring that the magnitude of the weights is balanced and none of the weights can overpower the other. SWE = PWE = 1.7 produced the best results for the modules W, FG, FE, and FI. For the module M, PWE was kept at 1.7 but SWE was set to zero, setting the score weights for all intervals to 1 in this module. As the change in severity between the parameter intervals affects the fish's welfare mainly indirectly, a dynamic score weight was not needed for module M.

*Module grades*

The whole calculation (Equation (1)) results in numeric grades for each module ranging from 0 to 1. The Supplementary File S3 provides a step-by-step example of how Equation 1 was used to calculate the module grade based on the information given in Appendix Tables A1–A7. To further increase the intuitive

interpretability of the module grades, one of four semantic attributes were assigned to the grades according to their numerical value:

- [0–0.25): critical welfare: welfare is severely compromised, short- and long-term impairments are expected
- [0.25–0.5): poor welfare: welfare is affected negatively, long-term impairments are expected
- [0.5–0.75): acceptable welfare: given the current knowledge the model is based on, the fish experience acceptable although improvable welfare
- [0.75–1]: good welfare: given the current knowledge the model is based on, the fish are likely to experience good welfare

$$MG_j = \frac{\sum_i PS_i \times SW_i^{SWE_i} \times PW_i^{PWE_i}}{\sum_i SW_i \times PW_i} + 1 \quad (1)$$

**Developing a software application for the model**

Some parameters (ammonia, relative dissolved oxygen, body condition factor) were not measured but calculated as were the module grades. For the model to be readily applicable for research, a version including these calculations for an indoor recirculating aquaculture system with pikeperch based on Microsoft Excel was implemented and is freely available (Supplementary File S4). This file assists scientific users with a ready-to-use model that can be adapted and developed if desired, as well as incorporated in further applications, such as statistical programmes. For the application of the model on-farm, both the automated calculations as well as the documentation and storage of the individual assessments were important. To this end, a software application was created that helped the user by providing (I) a user interface for a digital assessment, (II) methods and protocols for the measurement of the parameters, (III) automated calculation of the module grades, (IV) documentation of past assessments and (V) the possibility to compare past assessments and import or export the data. The first version of this app, suitable for Android devices, is freely available (www.myaquaculturefarm.ch).

**Model validation**

The model was subjected to a first testing on-site at six farms (Table 2) using the Microsoft Excel version of the model including the appropriate specific set of parameters (location, system, species). The time needed for a complete assessment of all parameters was 2.5–3 h. Assessment time mainly depends on the number of fish sampled for the modules FE and FI. This number can be adapted, as fewer fish are sufficient, e.g., for regular internal screenings, while more fish may be sampled for a detailed evaluation. Fewer than three fish will yield unreliable results and more than ten fish will considerably increase the duration of the assessment. For the model testing, five fish were sampled for module FE and FI on each farm (Swiss animal trial license number: LU01/18) and their average score was taken for the model calculations. The data entered in the excel files during the on-site testing as well as the calculated module grades are given in Table 3.

| Farm | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Location | indoor | indoor | outdoor | outdoor | indoor | indoor |
| System | RAS | RAS | FTS | FTS | RAS | RAS |
| Species | RT | RT | RT | RT | PP | PP |
| Purpose | grow-out | grow-out | grow-out | restocking | grow-out | grow-out |

**Table 2:** Characteristics of the six fish farms for the model validation. RAS = recirculating aquaculture system, FTS = flow-through system, RT = rainbow trout, PP = pikeperch.

Farm 1 was a small indoor RAS (recirculating aquaculture system) stocked with rainbow trout. With the feeding rate rather high and the water velocity lower than optimal, the fish showed a high BCF (body condition factor). This, together with an indication of damaged gill tissue, lowered the module FE grade of the farm. Farm 2 was a mid-scale RAS with rainbow trout. The module W grade was lowered mainly by a high value of dissolved carbon dioxide in the system water (due to an underground water source) and an increased nitrite level. Farm 3 was an extensive outdoor FTS (flow-through system) stocked with rainbow trout. The farm had suboptimal documentation processes that lowered the module M grade. The water quality was negatively impacted by an insufficient level of dissolved oxygen, resulting in a poor module W grade. The FE module revealed the slightly too high BCF, considerable deformations of the upper jaws and discolouration of the gills. The gills were also affected on a microscopic level, where they showed swelling of the secondary lamellae and a slight infestation with pathogens, hence the decreased module FI grade. The impaired health of the gills and the low oxygen level of the water resulted in increased ventilation rates and occasional air gulping, which lowered the module FG grade. Additionally, the deformations of the upper jaws further decreased this module grade. Farm 4 was an extensive outdoor FTS with rainbow trout bred to stock surface waters for recreational fisheries purposes. The farm had suboptimal documentation of mortalities, which lowered the module M grade. Farm 5 was a large-scale indoor RAS with pikeperch. The values and scores were within the optimal or target range resulting in good grades of all modules. Farm 6 was a mid-scale indoor RAS stocked with pikeperch. The module M grade was affected by a low water exchange rate, resulting in a low pH, a high EC (electrical conductivity) and increased dissolved carbon dioxide. The module FE grade was lowered by signs of discoloured gills, a slightly lowered BCF and damage to the dorsal fins.

The preliminary testing of the model on-site showed a good applicability of the model in different locations (indoor and outdoor), with different systems (RAS and FTS) and with different fish species (rainbow trout and pikeperch). The model revealed points where the fish welfare was negatively affected and hence offers farm-specific assistance for improvement.

| Farm management | | | | | | |
|---|---|---|---|---|---|---|
| **Farm** | **1** | **2** | **3** | **4** | **5** | **6** |
| Personnel training | 1 | 0 | 2 | 1 | 0 | 0 |
| Daily Check | 0 | 0 | 0 | 0 | 0 | 0 |
| Treatment journal | 0 | 1 | 1 | 1 | 0 | 1 |
| Target value sheet | 1 | 1 | **2** | 1 | 0 | 1 |
| Emergency concept | 1 | 1 | **2** | 1 | 0 | 1 |
| Hygiene concept | 1 | 1 | 1 | 1 | 0 | 1 |
| Mortality documentation | 1 | 1 | **2** | **2** | 0 | 1 |
| Biomass documentation | 1 | 0 | **2** | 1 | 0 | 1 |
| Predator protection | NA | NA | 2 | 1 | NA | NA |
| Plant cleanliness | 0 | 0 | 1 | 0 | 0 | 0 |
| Stocking density | 0 | 1 | 0 | 0 | 1 | 1 |
| Sorting | 0 | 0 | 0 | 1 | 0 | 0 |
| Slaughter | 0 | 0 | 1 | 0 | 0 | 0 |
| Feeding interval/rate | 0 | 0 | 0 | 0 | 0 | 0 |
| Feed type | 0 | 0 | 0 | 0 | 0 | 0 |
| Disturbances | 1 | 1 | 0 | 1 | 0 | 0 |
| Ambient light | 0 | 0 | NA | NA | 0 | 0 |
| Tank light | 0 | 0 | 1 | 1 | 0 | 0 |
| **Module grade** | **0.78** | **0.79** | **0.50** | **0.69** | **0.98** | **0.79** |

**Table 3:** On-site farm testing results using the MyFishCheck model with the final module grades. For the module water quality, the parameter values were presented; for the modules farm management, fish group behaviour, fish external appearance and fish internal appearance, the parameter intervals are given. Parameters mainly responsible for lower module grades are given in bold. NA = data not available as the parameter does not apply in this location or system.

| Water quality | | | | | | |
|---|---|---|---|---|---|---|
| **Farm** | **1** | **2** | **3** | **4** | **5** | **6** |
| Carbonate hardness [CaCO3 in mg/l] | 194 | 310 | 347 | 128 | NA | **28.2** |
| Total suspended solids [TSS in mg/l] | 26 | 10 | 20 | 5 | 12 | 15.9 |
| Ammonium [TAN in mg/l] | 0.04 | 0.79 | NA | NA | 0.03 | 0.21 |
| Ammonia [NH3-N in mg/l] | 0.001 | 0.005 | NA | NA | 0 | 0 |
| Nitrite [NO2-N in mg/l] | 0.04 | **0.12** | NA | NA | 0.01 | 0.05 |
| Nitrate [NO3-N in mg/l] | 6.18 | 7.29 | NA | NA | 6.53 | 73.1 |
| pH [-] | 7.84 | 7.5 | 7.61 | 7.75 | 7.5 | **6.4** |
| Conductivity [µS/cm] | 487 | 711 | 640 | 254 | NA | **8030** |
| Temperature [°C] | 16.9 | 11.5 | 14.8 | 7.4 | 23.7 | 22.8 |
| Oxygen [O2 in mg/l] | 9.57 | 11 | **5.9** | 9.2 | 8.5 | 9.1 |
| Oxygen saturation [O2 in %] | 106 | 108 | **62** | 82 | 108 | 113 |
| Carbon dioxide [CO2 in mg/l] | 6.1 | **21.8** | 5.5 | 1.6 | 2 | **7.5** |
| Total gas pressure [%] | 99 | 102 | 100 | 100 | 100 | 100 |
| Water velocity [body lengths/sec] | 0.3 | 0.3 | 0.3 | 0.4 | 0.3 | 0.3 |
| **Module grade** | **0.80** | **0.59** | **0.31** | **0.75** | **0.95** | **0.64** |

| Fish group behaviour | | | | | | |
|---|---|---|---|---|---|---|
| **Farm** | **1** | **2** | **3** | **4** | **5** | **6** |
| Aggression | 0 | 0 | 1 | 0 | 0 | 0 |
| Territoriality | 0 | 0 | 0 | 0 | 0 | 0 |
| Apathy | 0 | 0 | 0 | 0 | 0 | 0 |
| Isolation | 1 | 0 | 0 | 0 | 0 | 1 |
| Scratching | 0 | 0 | 1 | 0 | 0 | 0 |
| Surfacing | 0 | 2 | 0 | 0 | 0 | 0 |
| Air gulping | 0 | 0 | 1 | 0 | 0 | 0 |
| Ventilation rate | 0 | 0 | **2** | 0 | 0 | 0 |
| Fleeing | 0 | 0 | 0 | 0 | 0 | 0 |
| Fin position | 0 | 0 | 0 | 0 | 0 | 0 |
| Balance | 0 | 0 | 0 | 0 | 0 | 0 |
| Body colour | 0 | 1 | 0 | 0 | 1 | 0 |
| Feeding | 0 | 1 | 0 | 0 | 0 | 0 |
| Jaw deformations | 0 | 0 | **4** | 0 | 0 | 0 |
| Gill cover deformations | 0 | 0 | 2 | 0 | 0 | 0 |
| Spinal deformations | 0 | 0 | 0 | 0 | 0 | 0 |
| Eye injuries | 1 | 1 | 1 | 0 | 2 | 2 |
| Skin injuries | 2 | 2 | 1 | 0 | 0 | 1 |
| Fin injuries | 2 | 2 | 2 | 1 | 2 | 2 |
| Fungal infections | 0 | 0 | 0 | 0 | 0 | 0 |
| **Module grade** | **0.84** | **0.79** | **0.69** | **0.98** | **0.87** | **0.86** |

**Table 3:** Continued

| Fish internal appearance | | | | | | |
|---|---|---|---|---|---|---|
| **Farm** | **1** | **2** | **3** | **4** | **5** | **6** |
| Heart | 0 | 0 | 0 | 0 | 0 | 0 |
| Kidney | 0 | 0 | 0 | 0 | 0 | 0 |
| Spleen | 0 | 0 | 0 | 0 | 0 | 0 |
| Liver | 1 | 0 | 0 | 0 | 0 | 0 |
| Intestines | 1 | 0 | 0 | 0 | 0 | 0 |
| Muscles | 0 | 0 | 0 | 0 | 0 | 0 |
| Reproductive organs | 1 | 0 | 0 | 0 | 0 | 0 |
| Gill lamellae | 1 | 0 | **2** | 0 | 1 | 1 |
| Gill pathogens | 0 | 0 | **1** | 0 | 0 | 0 |
| Body cavity | 0 | 0 | 0 | 0 | 0 | 0 |
| **Module grade** | **0.78** | **1.00** | **0.61** | **1.00** | **0.86** | **0.86** |

| Fish external appearance | | | | | | |
|---|---|---|---|---|---|---|
| **Farm** | **1** | **2** | **3** | **4** | **5** | **6** |
| Standard length [cm] | 19.5 | 19.6 | 11.6 | 25 | 25.2 | 28.7 |
| Total length [cm] | 21.7 | 21.7 | 13.5 | 27.6 | 26.1 | 32.4 |
| Body weight [g] | 132 | 111 | 25.3 | 218 | 154 | 198 |
| Body condition factor [-] | **1.8** | 1.5 | **1.6** | 1.4 | 0.96 | **0.84** |
| Mucus pathogens | 0 | 0 | 0 | 0 | 0 | 0 |
| Spinal deformation | 0 | 0 | 0 | 0 | 0 | 0 |
| Jaw deformation | 0 | 1 | **2** | 0 | 0 | 0 |
| Mouth injury | 1 | 1 | 1 | 0 | 1 | 1 |
| Skin alterations | 1 | 0 | 1 | 0 | 0 | 0 |
| Skin fungus | 0 | 0 | 0 | 0 | 0 | 0 |
| Skin injury | 0 | 0 | 0 | 0 | 0 | 0 |
| Cataract | 1 | 0 | 0 | 0 | 1 | 1 |
| Eye injury | 1 | 1 | 1 | 0 | 1 | 1 |
| Exophthalmia | 0 | 0 | 0 | 0 | 0 | 0 |
| Pectoral fins | 0 | 0 | 1 | 1 | 1 | 1 |
| Ventral fins | 1 | 1 | 0 | 1 | 1 | 1 |
| Anal fin | 0 | 0 | 0 | 1 | 1 | 1 |
| Caudal fin | 0 | 0 | 0 | 0 | 1 | 1 |
| Dorsal fin | 0 | 1 | 1 | 0 | **2** | **2** |
| Gill cover | 0 | 0 | 1 | 0 | 1 | 1 |
| Gills | **1** | **0** | **1** | 0 | 0 | 1 |
| **Module grade** | **0.57** | **0.78** | **0.54** | **0.82** | **0.73** | **0.68** |

**Table 3:** Continued

## *Discussion*

**Implementation of semantic data modelling**

Upcoming new technologies of data management will change future fish farming practices (FAO 2018, 2020) and semantic data modelling may be one of them. Using this method for the fish welfare assessment model presented here revealed several advantages. (I) The approach imposed few constraints on the identification and naming of classes such as needs and parameters and thus, allowed for the inclusion of diverse aspects of fish welfare that were rated with either metric or ordinal values. (II) The concept of the triples, i.e., the defined relationships between classes, and the ontology, i.e., the sum of the triples, enabled the digital management of the complex and interrelated topic that is fish welfare. New insights in the form of new classes or better defined relationships can be added to the current data, making the ontology an adaptable and evolvable concept. (III) The graphical representation of the ontology intuitively depicted classes, i.e., parameters and needs, with comparably numerous or few relationships. Many connections reveal key classes, which facilitated the selection of parameters for certain purposes. Few connections may either indicate less important aspects of fish welfare, what allows for a justified omission of parameters and a desired reduction in complexity, or may expose gaps in knowledge, providing an identification of areas where more research is needed.

**Use of the concept of allostasis**

The concept of allostasis, which was used in this work as a theoretical basis for the mathematical calculation of a module grade, entailed two main advantages. First, the severity of stressors, i.e., the intensity, the duration and the frequency of the inflicted stress can be incorporated into the parameter intervals and translated to parameters scores, which represented the resulting allostatic loads. This can be done for any shape of the stress-effect dynamic landscape (Schreck 2010), enabling a reduction from many different units to only one. Second, the equation developed was based on the sum of scores and considered the cumulative nature of the allostatic loads. Together this represented a successful translation of a holistic concept into applicable and practicable protocols, a process that is crucial for a methodological and objective assessment of animal welfare.

**Subjectivity in the model**

One constraint on developing a model as shown here is the subjectivity that is undoubtedly included when defining the relevant parameters, the limits of the intervals and the weighing of the scores (Botreau *et al.* 2007). This subjectivity, and the danger of biases and misinterpretations that come with it, can be progressively reduced by adding scientific knowledge (Bracke *et al.* 1999*a*). The more these definitions are based on existing information, the more objective the model becomes. The model presented sets out to achieve this by defining 80 parameters out of over 200 based on three criteria (relevant, practicable and reliable), by defining the intervals based on research of the literature and by defining the weights with a survey amongst experts. The latter illustrated the problem and the solution especially well. There is not enough literature on the relative importance of the welfare parameters in the model, therefore, an expert

survey was conducted. The subjectivity was made obvious by the variance of the weights assigned by the experts (Appendix Figure B1). This was considered by calculating the median weight, which introduced transparency and improved objectivity. It must be emphasised that the model is meant to evolve, i.e., the parameters, the weights and the interval limits may need adapting when new knowledge is acquired.

**Validation of the model**

Irrespective of any evolution and adaptation of such models, their scientific verification will remain difficult. Since the model is based on scientific literature, past models and expert opinions, an assessment of the performance of the model based on literature, existing models or expert evaluations represents a circular argumentation or more precisely, a self-dependent justification (Hahn 2011). While a verification is not feasible, a validation is possible, e.g., by demonstrating the operational validity of the model (Irobi *et al.* 2004). Hence, the model can prove its validity over time through a successful application on farms, a general acceptance by experts and a confirmed usefulness by the industry. Main aspects in terms of quantifiable evaluation points for the validation of the model may be the applicability on-site, the repeatability of the results, the robustness towards missing input data as well as the long-term effect on the fish welfare when regularly used on the farm.

**Future development and adaptation of the model**

Part of the future evolution and adaptation of the model presented is the development for further specific applications. The model allows for the exchange of parameters and the adaptation of the limits as well as the number of intervals. This feature will enable the model to be tailored to particular aspects known to alter relevant husbandry conditions and their assessment, e.g., fish species (Dalsgaard *et al.* 2013), live stages, selection line (Goldammer 2015), level of domestication (Saraiva *et al.* 2019*b*) and husbandry system and procedures (Conte 2004), or the field of application, e.g., fish farms, fisheries, or scientific laboratories (Sneddon *et al.* 2016). If new parameters are to be included into the model, they must be investigated for their suitability according to the criteria of being relevant, practicable and reliable. Furthermore, the parameter weights may be set at 3 per default and adapted to any integer from 1 to 5 if evidence for a lower or higher relative importance of the parameter exists. If the boundaries of the parameter intervals are adapted, e.g., to local laws or other fish species, the thresholds set must be based on scientific literature. Furthermore, the number of intervals should be balanced between the desired level of resolution and applicability (which may change depending on the purpose of the assessment) and can but must not be kept the same for all parameters within a given module. This developability of the model facilitates both the expansion of its use as well as adapting when new knowledge is acquired. Additionally, the normalisation in the calculation, done by a division by the weighted mean within the modules, allows the model to function even if not all parameters are assessed. This enables the model to be spontaneously customised to a certain extent, e.g., when parameters cannot be measured due to a lack of equipment or do not apply to a given situation. This makes the model flexible and purpose oriented.

**Value of the new model**

Animal welfare assessment is a continuous process of improvement, a process that started only recently for fish welfare. Previous models were important steps into the right direction, however, they were either comprehensive but not applicable (Noble *et al.* 2018, 2020), applicable but not comprehensive (Kleingeld *et al.* 2016; Müller-Belecke 2019), modular but not adaptable (Pettersen *et al.* 2014; Folkedal *et al.* 2016) or developable but biased (Saraiva *et al.* 2019*a*; Studer *et al.* 2020). By incorporating their advantages and improving on their disadvantages the model described here represents a new attempt to fish welfare assessment. The model is comprehensive and applicable, developable and adaptable, modular and purpose-oriented and as a whole is the next step on the way towards a gradually more sustainable and fish-friendly aquaculture.

**Conclusions**

The MyFishCheck model developed here allows researchers to assess fish welfare based on the full model in a standardised and efficient way. This enables representative surveys of the whole industry, evaluations of measures across farms and the validation of theoretical ideas or lab trials in practice. Initial tests on six different farms showed that the model is applicable on different fish species, different aquaculture systems and different locations. In addition, the available Microsoft Excel version of the model facilitates its use in science. Furthermore, the model allows fish farmers to perform regular controls based on a customised version of the model as part of their quality control management. This enables the documentation of on-farm welfare standards, the tracking of improvements and the tracing of problems. During the testing, the model reliably produced lower module grades where parameters showed negative effects on welfare. Additionally, the app enables the user to perform these single-point evaluations more conveniently and to store, evaluate and compare past assessments. The model represents a next step towards a standardised evaluation of welfare, a digital documentation of assessments and a widespread application of welfare assessments. MyFishCheck will both in its current form as well as in future adaptations serve the field of aquaculture by assisting advancements for the common goal of better fish welfare.

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# *Appendix*

**Appendix A**

The five modules of the model with their 80 welfare parameters incl. the corresponding applications (location, system, species), intervals, scores and weights. The full parameter table including the corresponding literature as well as remarks and explanations can be found in the Supplementary Material (Supplementary File S2). For tables A1-A7: In = indoor, Out = outdoor, RAS = recirculating aquaculture system, FTS = flow-through system, RT = rainbow trout, PP = pikeperch, PS = parameter score, SW = score weight, PW = parameter weight, SWE = score weight exponent, PWE = parameter weight exponent.

| Location System Species | Parameter | Parameter intervals | PS | SW | PW | SWE PWE |
|---|---|---|---|---|---|---|
| In / Out RAS / FTS RT / PP | Personnel training | 0: Apprenticeship/master degree with work experience | 0 | 1 | | |
| | | 1: Apprenticeship/master degree in Aquaculture or "FBA Aquakultur" with work experience | -0.5 | 3 | 3 | |
| | | 2: "FBA Aquakultur" | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Daily check | 0: Daily check with appropriate controls | 0 | 1 | | |
| | | 1: Daily check | -0.5 | 3 | 5 | |
| | | 2: System is checked insufficiently | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Disturbances | 0: No external disturbances | 0 | 1 | | |
| | | 1: Little or slight disturbances | -0.5 | 3 | 3.5 | |
| | | 2: Frequent and / or severe disturbances | -1 | 5 | | |
| Out RAS / FTS RT / PP | Predator protection | 0: Completely protected from predators | 0 | 1 | | |
| | | 1: Partially protected from predators | -0.5 | 3 | 4 | |
| | | 2: Not protected | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Plant cleanliness | 0: The farm is clean and tidy, working materials are clean and disinfected | 0 | 1 | | |
| | | 1: The farm is clean, working materials are clean | -0.5 | 3 | 3 | |
| | | 2: The farm is chaotic and dirty, working materials dirty | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Treatment journal | 0: Medication, extraordinary and routine (disinfection) measures are documented | 0 | 1 | | |
| | | 1: Medication and extraordinary (disinfection) measures are documented | -0.5 | 3 | 3 | |
| | | 2: Medications are documented | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Target value sheet | 0: Target value document and action plan are accessible | 0 | 1 | | |
| | | 1: Target value document and an action plan are known, but not documented | -0.5 | 3 | 4 | |
| | | 2: There are no target values or specific action plan applied | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Emergency plan | 0: An appropriate emergency plan is available and accessible | 0 | 1 | | |
| | | 1: An appropriate emergency plan is known, but not documented | -0.5 | 3 | 4 | |
| | | 2: No emergency plan is available, or it is not appropriate | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Hygiene concept | 0: An appropriate hygiene concept is available and accessible | 0 | 1 | | |
| | | 1: An appropriate hygiene concept is applied, but not documented | -0.5 | 3 | 4 | |
| | | 2: No emergency hygiene is available, or it is not appropriate | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Mortality documentation | 0: All mortalities and their cause are documented and deducted from biomass | 0 | 1 | | 0 |
| | | 1: All mortalities are documented and deducted from the biomass | -0.5 | 3 | 4 | 1.7 |
| | | 2: All mortalities are documented | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Biomass documentation | 0: Biomass/stocking density are documented and recalculated, sporadically interim weighings | 0 | 1 | | |
| | | 1: The biomass and stocking density are documented and sporadically verified with weighings | -0.5 | 3 | 3.5 | |
| | | 2: The biomass is documented | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Sorting | 0: The group is homogeneous | 0 | 1 | | |
| | | 1: The group is slightly heterogeneous, unproblematic | -0.5 | 3 | 3 | |
| | | 2: The group is very heterogeneous, problematic | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Slaughter | 0: Crowding: short / stunning method: effective / killing: fast / no fish shows reflexes | 0 | 1 | | |
| | | 1: Crowding: short / stunning method: effective / killing: delayed / no fish shows reflexes | -0.5 | 3 | 5 | |
| | | 2: Crowding: long / stunning method: effective / killing: delayed / no fish shows reflexes | -1 | 5 | | |
| In / Out RAS / FTS RT | Stocking density | 0: 0–40 kg/m$^3$ | 0 | 1 | | |
| | | 1: 40–60 kg/m$^3$ | -0.5 | 3 | | |
| | | 2: 60–80 kg/m$^3$ | -1 | 5 | 3 | |
| In / Out RAS / FTS PP | | 0: 0–30 kg/m$^3$ | 0 | 1 | | |
| | | 1: 30–50 kg/m$^3$ | -0.5 | 3 | | |
| | | 2: 50–80 kg/m$^3$ | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Feeding interval and rate | 0: 5–6 points | 0 | 1 | | |
| | | 1: 3–4 points | -0.5 | 3 | 3.5 | |
| | | 2: 0–2 points | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Feed type | 0: Feed type and pellet size are adapted to the fish | 0 | 1 | | |
| | | 1: Pellets are too small / big for the animals | -0.5 | 3 | 4 | |
| | | 2: Type and size does not match the fish | -1 | 5 | | |
| In RAS / FTS RT / PP | Ambient light | 0: Light intensity and phases are adjusted | 0 | 1 | | |
| | | 1: Light intensity or light phases are adjusted | -0.5 | 3 | 3 | |
| | | 2: Neither light intensity nor light phases are adjusted | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Tank light | 0: Light intensity and light distribution adapted | 0 | 1 | | |
| | | 1: Light intensity or light distribution adapted | -0.5 | 3 | 3 | |
| | | 2: Neither intensity nor light distribution adapted | -1 | 5 | | |

**Table A1:** Module farm management.

| Location System Species | Parameter | Parameter intervals | PS | SW | PW | SWE PWE |
|---|---|---|---|---|---|---|
| In / Out RAS / FTS RT | Temperature | Optimum: [10–16] | 0 | 1 | 4 | |
| | | Within target range: [6–10) ∪ (16–18] | -0.33 | 2.33 | | |
| | | Within the tolerance range: [4–6) ∪ (18–22] | -0.66 | 3.66 | | |
| | | Outside the tolerance range: [0–4) ∪ (22–35] | -1 | 5 | | |
| In / Out RAS / FTS PP | | Optimum: [20–25] | 0 | 1 | | |
| | | Within target range: [13–20) ∪ (25–28] | -0.33 | 2.33 | | |
| | | Within the tolerance range: [8–13) ∪ (28–30] | -0.66 | 3.66 | | |
| | | Outside the tolerance range: [0–8) ∪ (30–40] | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Oxygen | Optimum: [8–10] | 0 | 1 | 5 | |
| | | Within target range: [7–8) ∪ (10–13] | -0.33 | 2.33 | | |
| | | Within the tolerance range: [6–7) ∪ (13–15] | -0.66 | 3.66 | | |
| | | Outside the tolerance range: [2–6) ∪ (15–30] | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Oxygen saturation | Optimum: [80–120] | 0 | 1 | 5 | |
| | | Within target range: [70–80) ∪ (120–140] | -0.33 | 2.33 | | |
| | | Within tolerance range: [60–70) ∪ (140–160] | -0.66 | 3.66 | | |
| | | Outside the tolerance range: [20–60) ∪ (160–300] | -1 | 5 | | |
| In / Out RAS RT / PP | Ammonium | Optimum: [0–0.5] | 0 | 1 | 4 | |
| | | Within target range: (0.5–1.5] | -0.33 | 2.33 | | |
| | | Within tolerance range: (1.5–5] | -0.66 | 3.66 | | |
| | | Outside the tolerance range: (5–20] | -1 | 5 | | |
| In / Out RAS RT / PP | Ammonia | Optimum: [0–0.01] | 0 | 1 | 5 | |
| | | Within target range: (0.01–0.02] | -0.33 | 2.33 | | |
| | | Within tolerance range: (0.02–0.1] | -0.66 | 3.66 | | |
| | | Outside the tolerance range: (0.1–2] | -1 | 5 | | |
| In / Out RAS RT / PP | Nitrite | Optimum: [0–0.05] | 0 | 1 | 5 | |
| | | Within target range: (0.05–0.1] | -0.33 | 2.33 | | |
| | | Within tolerance range: (0.1–0.5] | -0.66 | 3.66 | | |
| | | Outside the tolerance range: (0.5–5] | -1 | 5 | | |
| In / Out RAS RT / PP | Nitrate | Optimum: [0–50] | 0 | 1 | 2.5 | 1.7 |
| | | Within target range: (50–75] | -0.33 | 2.33 | | 1.7 |
| | | Within tolerance range: (75–150] | -0.66 | 3.66 | | |
| | | Outside tolerance range: (150–500] | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Carbonate hardness | Optimum: [40–150] | 0 | 1 | 3 | |
| | | Within target range: [30–40) ∪ (150–250] | -0.33 | 2.33 | | |
| | | Within tolerance range: [20–30) ∪ (250–400] | -0.66 | 3.66 | | |
| | | Outside tolerance range: [0–20) ∪ (400–500] | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Total suspended solids | Optimum: [0–25] | 0 | 1 | 2 | |
| | | Within target range: (25–50] | -0.33 | 2.33 | | |
| | | Within tolerance range: (50–200] | -0.66 | 3.66 | | |
| | | Outside tolerance range: (200–500] | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | pH | Optimum: [7–7.5] | 0 | 1 | 4 | |
| | | Within target range: [6.5–7) ∪ (7.5–8] | -0.33 | 2.33 | | |
| | | Within the tolerance range: [6–6.5) ∪ (8–8.5] | -0.66 | 3.66 | | |
| | | Outside the tolerance range: [4–6) ∪ (8.5–10] | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Conductivity | Optimum: [500–1000] | 0 | 1 | 2 | |
| | | Within target range: [300–500) ∪ (1000–5000] | -0.33 | 2.33 | | |
| | | Within tolerance range: [200–300) ∪ (5000–15000] | -0.66 | 3.66 | | |
| | | Outside tolerance range: [0–200) ∪ (15000–30000] | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Carbon dioxide | Optimum: [0–5] | 0 | 1 | 3.5 | |
| | | Within target range: (5–20] | -0.33 | 2.33 | | |
| | | Within tolerance range: (20–30] | -0.66 | 3.66 | | |
| | | Outside the tolerance range: (30–100] | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Total gas pressure | Optimum: </= 100 | 0 | 1 | 4 | |
| | | Within target range: (100–103] | -0.33 | 2.33 | | |
| | | Within tolerance range: (103–105] | -0.66 | 3.66 | | |
| | | Outside tolerance range: (105–120] | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Water velocity | Optimum: [0.5–1] | 0 | 1 | 3 | |
| | | Within target range: [0.3–0.5) ∪ (1–2] | -0.33 | 2.33 | | |
| | | Within tolerance range: [0.2–0.3) ∪ (2–3] | -0.66 | 3.66 | | |
| | | Outside the tolerance range: [0–0.2) ∪ (3–5] | -1 | 5 | | |

**Table A2:** Module water quality.

| Location System Species | Parameter | Parameter intervals | PS | SW | PW | SWE PWE |
|---|---|---|---|---|---|---|
| In / Out RAS / FTS RT / PP | Aggression | 0: No fish shows dominance or aggression | 0 | 1 | 3.5 | |
| | | 1: Individual fish show dominance behavior | -0.2 | 1.8 | | |
| | | 2: Some fish show dominance behavior | -0.4 | 2.6 | | |
| | | 3: Individual fish show aggression behavior | -0.6 | 3.4 | | |
| | | 4: Some fish show aggressive behavior | -0.8 | 4.2 | | |
| | | 5: Many fish are either dominant or aggressive | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Territoriality | 0: No fish shows territorial behavior | 0 | 1 | 3 | |
| | | 1: Individual fish show territorial behavior | -0.2 | 1.8 | | |
| | | 2: Some fish show territorial behavior | -0.4 | 2.6 | | |
| | | 3: Individual fish show a territorial monopolization of key areas | -0.6 | 3.4 | | |
| | | 4: Some fish show a territorial monopolization of key areas | -0.8 | 4.2 | | |
| | | 5: Some fish show a territorial monopolization of key areas, part of shoal has no access to these | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Scratching | 0: No fish jumps or scratches | 0 | 1 | 4 | |
| | | 1: Individual fish occasionally jump and/or scratch themselves on surfaces | -0.2 | 1.8 | | |
| | | 2: Some fish occasionally jump and/or scratch themselves on surfaces | -0.4 | 2.6 | | |
| | | 3: Individual fish frequently jump and/or scratch themselves on surfaces | -0.6 | 3.4 | | |
| | | 4: Some fish frequently jump and/or scratch themselves on surfaces | -0.8 | 4.2 | | |
| | | 5: Many fish frequently jump and/or scratch themselves on surfaces | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Apathy | 0: No fish show signs of apathy | 0 | 1 | 5 | |
| | | 1: Individual fish show apathetic swimming behavior, react normally to stimulation | -0.2 | 1.8 | | |
| | | 2: Some fish show apathetic swimming behavior, react normally to stimulation | -0.4 | 2.6 | | |
| | | 3: Individual fish show apathetic swimming behavior, do not react to stimulation | -0.6 | 3.4 | | |
| | | 4: Some fish show apathetic swimming behavior, do not respond to stimulation | -0.8 | 4.2 | | |
| | | 5: Many fish show apathetic swimming behavior, do not respond to stimulation | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Isolation | 0: All fish are part of a shoal | 0 | 1 | 3.5 | |
| | | 1: Individual fish stand apart | -0.2 | 1.8 | | |
| | | 2: Some fish stand apart | -0.4 | 2.6 | | |
| | | 3: Individual fish stand apart and/or on the surface | -0.6 | 3.4 | | |
| | | 4: Some fish stand apart and/or on the surface | -0.8 | 4.2 | | |
| | | 5: Many fish stand apart and/or on the surface | -1 | 5 | | 1.7 |
| In / Out RAS / FTS RT / PP | Surfacing | 0: All fish swim normally in the water column | 0 | 1 | 4 | 1.7 |
| | | 1: Individual fish are predominantly lying on the bottom | -0.2 | 1.8 | | |
| | | 2: Some fish are constantly lying on the bottom | -0.4 | 2.6 | | |
| | | 3: Individual fish are increasingly swimming on the surface | -0.6 | 3.4 | | |
| | | 4: Some fish swim mainly on the surface | -0.8 | 4.2 | | |
| | | 5: Many fish swim mainly on the surface | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Air gulping | 0: No fish shows air breathing | 0 | 1 | 4 | |
| | | 1: Individual fish show occasional gasps | -0.2 | 1.8 | | |
| | | 2: Some fish show occasional gasps | -0.4 | 2.6 | | |
| | | 3: Individual fish show frequent gasps | -0.6 | 3.4 | | |
| | | 4: Some fish show constant air gulping | -0.8 | 4.2 | | |
| | | 5: Many fish show constant air gulping | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Ventilation rate | 0: All fish have a normal ventilation rate | 0 | 1 | 4 | |
| | | 1: Individual fish show an increased ventilation rate | -0.2 | 1.8 | | |
| | | 2: Some fish show increased ventilation rate | -0.4 | 2.6 | | |
| | | 3: Individual fish show a greatly increased or slightly reduced ventilation rate | -0.6 | 3.4 | | |
| | | 4: Some fish show a greatly increased or clearly reduced ventilation rate | -0.8 | 4.2 | | |
| | | 5: Many fish show a greatly increased or clearly reduced ventilation rate | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Fleeing | 0: All fish show normal fleeing when stimulated and calm down quickly | 0 | 1 | 3 | |
| | | 1: Individual fish show an increased and/or prolonged fleeing behavior | -0.2 | 1.8 | | |
| | | 2: Some fish show an increased and/or prolonged fleeing behavior | -0.4 | 2.6 | | |
| | | 3: Individual fish show no or constant fleeing behavior | -0.6 | 3.4 | | |
| | | 4: Some fish show no or constant fleeing behavior | -0.8 | 4.2 | | |
| | | 5: Many fish show no or constant fleeing behavior | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Fin position | 0: All fish show a normal and calm fin position | 0 | 1 | 3 | |
| | | 1: Individual fish occasionally have their fins pinched or splayed out | -0.2 | 1.8 | | |
| | | 2: Some fishes occasionally pinch or splay out their fins | -0.4 | 2.6 | | |
| | | 3: Individual fishes have the fins constantly pinched or splayed out | -0.6 | 3.4 | | |
| | | 4: Some fish have the fins constantly pinched or splayed out | -0.8 | 4.2 | | |
| | | 5: Many fishes have the fins constantly pinched or splayed out | -1 | 5 | | |

**Table A3:** Module fish group behaviour (1/2).

| Location System Species | Parameter | Parameter intervals | PS | SW | PW | SWE PWE |
|---|---|---|---|---|---|---|
| In / Out RAS / FTS RT / PP | Balance | 0: All fish show a normal balance and orientation | 0 | 1 | 4.5 | |
| | | 1: Individual fish are sometimes misaligned | -0.2 | 1.8 | | |
| | | 2: Some fish are crooked at times | -0.4 | 2.6 | | |
| | | 3: Individual fish are constantly crooked | -0.6 | 3.4 | | |
| | | 4: Some fish are constantly crooked | -0.8 | 4.2 | | |
| | | 5: Many fish are constantly crooked | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Body color | 0: All the fish show a normal body coloration | 0 | 1 | 3 | |
| | | 1: Single fish have temporarily a conspicuously bright or dark coloration | -0.2 | 1.8 | | |
| | | 2: Some fish have temporarily a conspicuously bright or dark coloration | -0.4 | 2.6 | | |
| | | 3: Individual fish have constantly striking a bright or dark coloration | -0.6 | 3.4 | | |
| | | 4: Some fish constantly have a noticeable light or dark color | -0.8 | 4.2 | | |
| | | 5: Many fish constantly have a noticeable light or dark color | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Feeding | 0: All fish show normal feeding behavior | 0 | 1 | 3 | |
| | | 1: Individual fish show a very hungry, hectic eating behavior | -0.2 | 1.8 | | |
| | | 2: Some fish show a very hungry, hectic eating behavior | -0.4 | 2.6 | | |
| | | 3: Individual fish show a starved, aggressive eating behavior | -0.6 | 3.4 | | |
| | | 4: Some fish show a starved, aggressive eating behavior | -0.8 | 4.2 | | |
| | | 5: Many fish show a starved, aggressive eating behavior | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Jaw deformations | 0: No fish has injuries/deformations of the jaw/snout | 0 | 1 | 3 | |
| | | 1: Individual fish have slight injuries/deformations of the jaw/snout | -0.2 | 1.8 | | |
| | | 2: Some fish have slight injuries/deformations of the jaw/snout | -0.4 | 2.6 | | |
| | | 3: Individual fish have severe injuries/deformations of the jaw/snout | -0.6 | 3.4 | | |
| | | 4: Some fish have severe injuries/deformations of the jaw/snout | -0.8 | 4.2 | | |
| | | 5: Many fish have severe injuries/deformations of the jaw/snout | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Gill cover deformations | 0: No fish has injuries/deformations of the opercula | 0 | 1 | 2 | |
| | | 1: Individual fish have slight injuries/deformations of the opercula | -0.2 | 1.8 | | |
| | | 2: Some fish have slight injuries/deformations of the opercula | -0.4 | 2.6 | | |
| | | 3: Individual fish have severe injuries/deformations of the opercula | -0.6 | 3.4 | | |
| | | 4: Some fish have severe injuries/deformations of the opercula | -0.8 | 4.2 | | |
| | | 5: Many fish have severe injuries/deformations of the opercula | -1 | 5 | | 1.7 |
| In / Out RAS / FTS RT / PP | Spinal deformations | 0: No fish has injuries/deformations of the spine | 0 | 1 | 3 | 1.7 |
| | | 1: Individual fish have a slight injuries/deformations of the spine | -0.2 | 1.8 | | |
| | | 2: Some fish have a slight injuries/deformations of the spine | -0.4 | 2.6 | | |
| | | 3: Individual fish have a severe injuries/deformations of the spine | -0.6 | 3.4 | | |
| | | 4: Some fish have severe injuries/deformations of the spine | -0.8 | 4.2 | | |
| | | 5: Many fish have a severe injuries/deformations of the spine | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Eye injuries | 0: No fish has eye injuries/deformations | 0 | 1 | 3 | |
| | | 1: Individual fish have slight injuries/deformations to the eyes | -0.2 | 1.8 | | |
| | | 2: Some fish have minor eye injuries/deformations | -0.4 | 2.6 | | |
| | | 3: Individual fish have severe injuries/deformations to the eyes | -0.6 | 3.4 | | |
| | | 4: Some fish have severe eye injuries/deformations | -0.8 | 4.2 | | |
| | | 5: Many fish have severe injuries/deformations to the eyes | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Skin injuries | 0: No fish has injuries/deformations of the skin | 0 | 1 | 4 | |
| | | 1: Individual fish have slight injuries/deformations of the skin | -0.2 | 1.8 | | |
| | | 2: Some fish have slight injuries/deformations of the skin | -0.4 | 2.6 | | |
| | | 3: Individual fish have severe injuries/deformations of the skin | -0.6 | 3.4 | | |
| | | 4: Some fish have severe injuries/deformations of the skin | -0.8 | 4.2 | | |
| | | 5: Many fish have severe injuries/deformations of the skin | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Fin injuries | 0: No fish has injuries/deformations of the fins | 0 | 1 | 3 | |
| | | 1: Individual fish have slight injuries/deformations of the fins | -0.2 | 1.8 | | |
| | | 2: Some fish have slight injuries/deformations of the fins | -0.4 | 2.6 | | |
| | | 3: Individual fish have severe injuries/deformations of the fins | -0.6 | 3.4 | | |
| | | 4: Some fish have severe injuries/deformations of the fins | -0.8 | 4.2 | | |
| | | 5: Many fish have severe injuries/deformations of the fins | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Fungal infections | 0: No fish has any fungus | 0 | 1 | 4 | |
| | | 1: Individual fish have fungal infection of the fins | -0.2 | 1.8 | | |
| | | 2: Some fish have fungal infection of the fins | -0.4 | 2.6 | | |
| | | 3: Individual fish have fungal infection of the fins and the body | -0.6 | 3.4 | | |
| | | 4: Some fish have fungal infection of the fins and the body | -0.8 | 4.2 | | |
| | | 5: Many fish have fungal infection of the fins and the body | -1 | 5 | | |

**Table A4:** Module fish group behaviour (2/2).

| Location System Species | Parameter | Parameter intervals | PS | SW | PW | SWE PWE |
|---|---|---|---|---|---|---|
| In / Out RAS / FTS RT / PP | Cataract | 0: Both eyes are clear | 0 | 1 | 3 | |
| | | 1: One lens shows light clouding | -0.33 | 2.33 | | |
| | | 2: Both lenses show light clouding or one lens strong clouding | -0.66 | 3.66 | | |
| | | 3: Both lenses show strong clouding | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Eye injury | 0: No indication | 0 | 1 | 3 | |
| | | 1: One-sided small injury, not inflamed or healing | -0.33 | 2.33 | | |
| | | 2: One-sided injury or both-sided small injury, slightly inflamed | -0.66 | 3.66 | | |
| | | 3: One-sided severe injury or both-sided injury, inflamed | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Exophthalmia | 0: No indication | 0 | 1 | 3 | |
| | | 1: One-sided slight exophthalmia | -0.33 | 2.33 | | |
| | | 2: Both-sided slight exophthalmia or one-sided exophthalmia | -0.66 | 3.66 | | |
| | | 3: Both-sided exophthalmia | -1 | 5 | | |
| In / Out RAS / FTS RT | Body condition factor | 0: 1–1.3 | 0 | 1 | 3 | |
| | | 1: 0.8–1.5 | -0.33 | 2.33 | | |
| | | 2: > 1.5 | -0.66 | 3.66 | | |
| | | 3: < 0.8 | -1 | 5 | | |
| In / Out RAS / FTS PP | | 0: 0.9–1.1 | 0 | 1 | | |
| | | 1: 0.7–1.3 | -0.33 | 2.33 | | |
| | | 2: > 1.3 | -0.66 | 3.66 | | |
| | | 3: < 0.7 | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Spinal deformation | 0: No indication | 0 | 1 | 3 | |
| | | 1: Indication of deformation | -0.33 | 2.33 | | |
| | | 2: Clear deformation | -0.66 | 3.66 | | |
| | | 3: Strong deformation | -1 | 5 | | 1.7 |
| In / Out RAS / FTS RT / PP | Jaw deformation | 0: No indication | 0 | 1 | 3 | 1.7 |
| | | 1: Indication of deformation | -0.33 | 2.33 | | |
| | | 2: Clear deformation | -0.66 | 3.66 | | |
| | | 3: Strong deformation | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Mouth injury | 0: No indication | 0 | 1 | 3 | |
| | | 1: A few small injuries | -0.33 | 2.33 | | |
| | | 2: Several small injuries | -0.66 | 3.66 | | |
| | | 3: One or more large/deep injuries | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Mucus pathogens | 0: No parasites detectable | 0 | 1 | 4 | |
| | | 1: A few parasites | -0.33 | 2.33 | | |
| | | 2: Considerable parasite load | -0.66 | 3.66 | | |
| | | 3: Heavy parasite load | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Skin alterations | 0: No indication | 0 | 1 | 3.5 | |
| | | 1: A few small alterations (tumors, swellings, rashes, bleedings) | -0.33 | 2.33 | | |
| | | 2: Several small alterations (tumors, swellings, rashes, bleedings) | -0.66 | 3.66 | | |
| | | 3: One or more large alterations (tumors, swellings, rashes, bleedings) | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Skin fungus | 0: No indication | 0 | 1 | 4 | |
| | | 1: A few small areas infected | -0.33 | 2.33 | | |
| | | 2: Several small areas infected | -0.66 | 3.66 | | |
| | | 3: One or more large areas infected | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Skin injury | 0: No indication | 0 | 1 | 3.5 | |
| | | 1: A few small injuries or small areas with scale loss | -0.33 | 2.33 | | |
| | | 2: Several small injuries and/or small areas with scale loss | -0.66 | 3.66 | | |
| | | 3: One or more large/deep injuries and/or areas with scale loss | -1 | 5 | | |

**Table A5:** Module fish external appearance (1/2).

| Location System Species | Parameter | Parameter intervals | PS | SW | PW | SWE PWE |
|---|---|---|---|---|---|---|
| In / Out RAS / FTS RT / PP | Gill cover | 0: Both-sided: undamaged opercula | 0 | 1 | 2 | |
| | | 1: One-sided/both-sided: opercula covers min. 2/3 of gill area | -0.33 | 2.33 | | |
| | | 2: One-sided/both-sided: opercula covers min. 1/3 of gill area | -0.66 | 3.66 | | |
| | | 3: One-sided/both-sided: opercula covers less than 1/3 of gill area | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Gills | 0: Both-sided: undamaged, red gills | 0 | 1 | 5 | |
| | | 1: One-sided/both-sided: indications of damaged and/or discolored gill tissue | -0.33 | 2.33 | | |
| | | 2: One-sided/both-sided: several small areas of damaged and/or discolored gill tissue | -0.66 | 3.66 | | |
| | | 3: One-sided/both-sided: extensive areas of damaged and/or discolored gill tissue | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Pectoral fins | 0: Undamaged fins | 0 | 1 | 3 | |
| | | 1: One-sided/both-sided: indications of scar tissue or small/active fin damage | -0.33 | 2.33 | | |
| | | 2: One-sided/both-sided: active fin damage or of fungal infections and/or inflammation | -0.66 | 3.66 | | |
| | | 3: Both-sided: extensive scar tissue / extensive active fin damage / fungal infection or fin loss | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Ventral fins | 0: Undamaged fins | 0 | 1 | 2 | 1.7 |
| | | 1: One-sided/both-sided: indications of scar tissue or small/active fin damage | -0.33 | 2.33 | | 1.7 |
| | | 2: One-sided/both-sided: active fin damage or of fungal infections and/or inflammation | -0.66 | 3.66 | | |
| | | 3: Both-sided: extensive scar tissue / extensive active fin damage / fungal infection or fin loss | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Anal fin | 0: Undamaged fin | 0 | 1 | 2 | |
| | | 1: Indications of scar tissue or small and active fin damage | -0.33 | 2.33 | | |
| | | 2: Active fin damage or indications of fungal infections and/or inflammation | -0.66 | 3.66 | | |
| | | 3: Extensive scar tissue / extensive active fin damage / extensive fungal infection or fin loss | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Caudal fin | 0: Undamaged fin | 0 | 1 | 3 | |
| | | 1: Indications of scar tissue or small and active fin damage | -0.33 | 2.33 | | |
| | | 2: Active fin damage or indications of fungal infections and/or inflammation | -0.66 | 3.66 | | |
| | | 3: Extensive scar tissue / extensive active fin damage / or extensive fungal infection or fin loss | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Dorsal fin | 0: Undamaged fin | 0 | 1 | 3 | |
| | | 1: Indications of scar tissue or small and active fin damage | -0.33 | 2.33 | | |
| | | 2: Active fin damage or indications of fungal infections and/or inflammation | -0.66 | 3.66 | | |
| | | 3: Extensive scar tissue / extensive active fin damage / extensive fungal infection or fin loss | -1 | 5 | | |

**Table A6:** Module fish external appearance (2/2).

| Location System Species | Parameter | Parameter intervals | PS | SW | PW | SWE PWE |
|---|---|---|---|---|---|---|
| In / Out RAS / FTS RT / PP | Heart | 0: Inconspicuous | 0 | 1 | 3 | |
| | | 1: Slight discoloration | -0.33 | 2.33 | | |
| | | 2: Discolored and/or small necrosis and/or small hemorrhages | -0.66 | 3.66 | | |
| | | 3: Severely discolored and/or necrosis and/or hemorrhages | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Kidney | 0: Inconspicuous | 0 | 1 | 3.5 | |
| | | 1: Slight discoloration | -0.33 | 2.33 | | |
| | | 2: Discolored and/or slightly granular | -0.66 | 3.66 | | |
| | | 3: Severely discolored and/or granular | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Spleen | 0: Inconspicuous | 0 | 1 | 4 | |
| | | 1: Slight enlargement | -0.33 | 2.33 | | |
| | | 2: Discolored and/or slightly enlarged | -0.66 | 3.66 | | |
| | | 3: Severely discolored and/or enlarged | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Liver | 0: Inconspicuous | 0 | 1 | 4 | |
| | | 1: Slight discoloration | -0.33 | 2.33 | | |
| | | 2: Discolored and/or slightly enlarged and/or small necrosis | -0.66 | 3.66 | | |
| | | 3: Severely discolored and/or enlarged and/or necrosis | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Intestines | 0: Homogeneously filled with smooth food pulp | 0 | 1 | 3 | |
| | | 1: Unevenly filled with food pulp | -0.33 | 2.33 | | |
| | | 2: Indications of inflammation and change in tissue (discoloring, swelling, tumors) | -0.66 | 3.66 | | |
| | | 3: Inflammation / change in tissue (discolored, tumors, hemorrhages, necrosis) or foreign objects | -1 | 5 | | 1.7 |
| In / Out RAS / FTS RT / PP | Muscles | 0: Normal | 0 | 1 | 3 | 1.7 |
| | | 1: Single small hemorrhages, small vaccination damage | -0.33 | 2.33 | | |
| | | 2: Several small or single extensive hemorrhages and/or clear vaccination damage | -0.66 | 3.66 | | |
| | | 3: Extensive hemorrhages and/or necrosis and/or extensive vaccination damage | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Body cavity | 0: Inconspicuous | 0 | 1 | 3 | |
| | | 1: Slight bleeding into the intestine and/or abdominal fat and/or swim bladder wall | -0.33 | 2.33 | | |
| | | 2: Bleeding into the intestine / abdominal fat / swim bladder wall / slight fluid accumulation | -0.66 | 3.66 | | |
| | | 3: Severe bleeding into the intestine / abdominal fat / swim bladder wall / fluid accumulation | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Reproductive organs | 0: Not developed | 0 | 1 | 2 | |
| | | 1: Slightly developed/enlarged | -0.33 | 2.33 | | |
| | | 2: Developed/enlarged | -0.66 | 3.66 | | |
| | | 3: Ready to spawn | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Gill lamellae | 0: Normal | 0 | 1 | 5 | |
| | | 1: Lamellae slightly swollen | -0.33 | 2.33 | | |
| | | 2: Lamellae swollen, small hemorrhages / necrosis / edema / detachment of epithelium | -0.66 | 3.66 | | |
| | | 3: Lamellae severely swollen, hemorrhages / necrosis / endema / detachment of epithelium | -1 | 5 | | |
| In / Out RAS / FTS RT / PP | Gill pathogens | 0: No parasites detectable | 0 | 1 | 4 | |
| | | 1: A few parasites | -0.33 | 2.33 | | |
| | | 2: Considerable parasite load | -0.66 | 3.66 | | |
| | | 3: Heavy parasite load | -1 | 5 | | |

**Table A7:** Module fish internal appearance.

**Appendix B**

Results of the expert survey. Twenty experts independently evaluated the relevance of each parameter based on their experience and knowledge by assigning them weights from 1 to 5 i.e., {1, 2, 3, 4, 5} with 1 = being less relevant for welfare, and 5 = being very relevant for welfare. For each parameter, the median of these weights is used as the parameter weight in the model (see Appendix Tables A1–A7). Sample size per module—farm management: 20, water quality: 20, fish external appearance: 18, fish internal appearance: 18, fish group behaviour: 18. The parameters body colouration and body cavity were included in the model after the survey and therefore have a default parameter weight of 3.

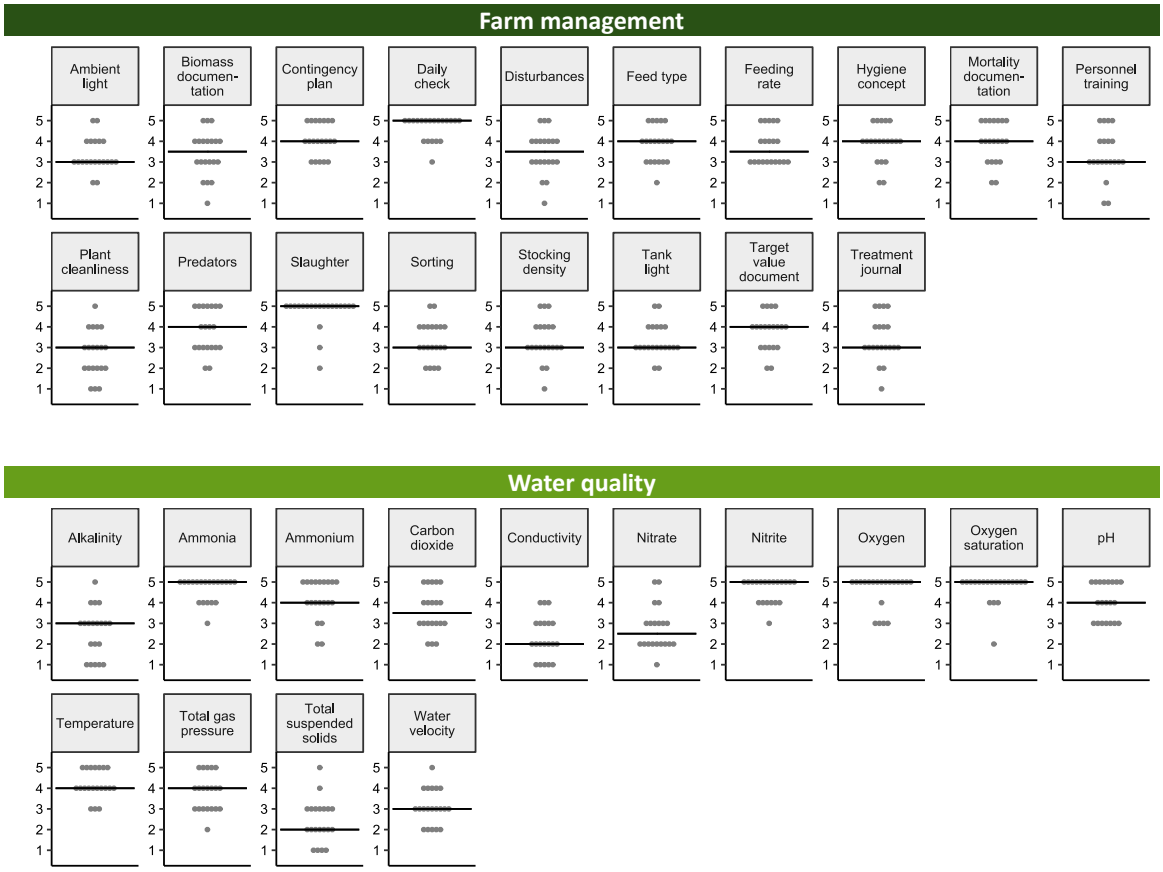


**Figure B1:** Results of the expert survey for the modules farm management and water quality.

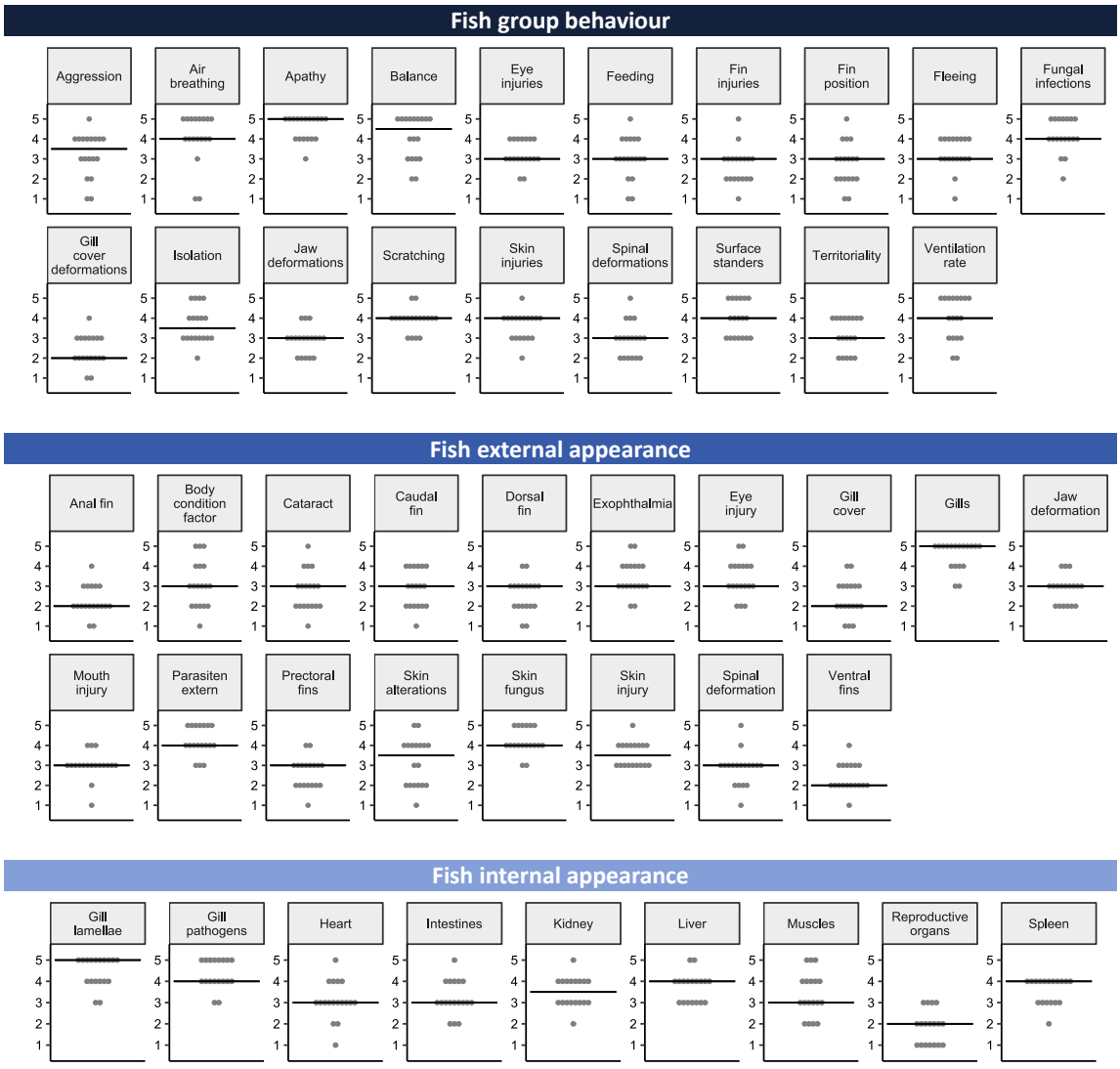


**Figure B2:** Results of the expert survey for the modules fish group behaviour, fish external appearance and fish internal appearance.

## *Supplementary material*

**Supplementary S1:** Parameter selection

Selection of welfare parameters that are relevant, reliable and applicable. Definition of the five modules of the model with their respective parameters. y = yes, n = no.

| Parameter | relevant | practicable | reliable | into model | module |
|---|---|---|---|---|---|
| blood - adrenocorticotropic hormone | n | n | y | n | |
| blood - alanine aminotransferase | n | n | y | n | |
| blood - albumin 1 | n | n | y | n | |
| blood - albumin 2 | n | n | y | n | |
| blood - alcaline phosphatase | n | n | y | n | |
| blood - ammonia | y | n | y | n | |
| blood - ammonium | y | n | y | n | |
| blood - aspartate aminotransferase | n | n | y | n | |
| blood - bile acids | n | n | y | n | |
| blood - calcium | n | n | y | n | |
| blood - cholesterol | n | n | y | n | |
| blood - creatine kinase | n | n | y | n | |
| blood - epinephrine | y | n | y | n | |
| blood - globulin | n | n | y | n | |
| blood - glucose | n | n | y | n | |
| blood - hematocrit | n | n | y | n | |
| blood - lactate dehydrogenase | y | n | y | n | |
| blood - leucocytes | y | n | y | n | |
| blood - nitrite | y | n | y | n | |
| blood - norepinephrine | n | n | y | n | |
| blood - pH | y | n | y | n | |
| blood - potassium | n | n | y | n | |
| blood - serotonin | n | n | y | n | |
| blood - telomere length | n | n | y | n | |
| blood - total bilirubin | n | n | y | n | |
| blood - total protein | n | n | y | n | |
| blood - uric acid | n | n | y | n | |
| individual - anal erosion | y | y | y | y | FE |
| individual - anal infection | y | y | y | as anal fin | |
| individual - caudal erosion | y | y | y | y | FE |
| individual - caudal infection | y | y | y | as caudal fin | |
| individual - dorsal erosion | y | y | y | y | FE |
| individual - dorsal infection | y | y | y | as dorsal fin | |
| individual - pectoral asymmetry | n | y | y | n | |
| individual - pectoral erosion | y | y | y | y | FE |
| individual - pectoral infection | y | y | y | as pectoral fin | |
| individual - ventral asymmetry | n | y | y | n | |
| individual - ventral erosion | y | y | y | y | FE |
| individual - ventral infection | y | y | y | as ventral fin | |

| Parameter | relevant | practicable | reliable | into model | module |
|---|---|---|---|---|---|
| individual - bacterial infection | y | n | n | n | |
| individual - body cavity | y | y | y | y | FI |
| individual - body composition | n | n | n | n | |
| individual - body condition factor | y | y | y | y | FE |
| individual - cardiac activity | y | n | n | n | |
| individual - cardiosomatic index | y | n | y | n | |
| individual - cataract | y | y | y | y | FE |
| individual - exophthalmia | y | y | y | y | FE |
| individual - eye bleeding | y | n | y | n | |
| individual - eye colour | n | n | n | n | |
| individual - eye injury | y | y | y | y | FE |
| individual - eye roll reflex | n | y | y | n | |
| individual - gill cover deformation | y | y | y | y | FE |
| individual - gill cover injury | y | y | y | as gill cover | |
| individual - gill lamellae (secondary) | y | y | y | y | FI |
| individual - gill pathogens | y | y | y | y | FI |
| individual - gills (primarly lamellae) | y | y | y | y | FE |
| individual - gonadosomatic index | n | y | y | n | |
| individual - growth - otoliths | n | n | n | n | |
| individual - growth - scale | n | n | n | n | |
| individual - heart | y | y | y | y | FI |
| individual - hepatosomatic Index | n | n | y | n | |
| individual - intestines | y | y | y | y | FI |
| individual - jaw deformation | y | y | y | y | FE |
| individual - kidney | y | y | y | y | FI |
| individual - liver | y | y | y | y | FI |
| individual - max swim speed | n | n | n | n | |
| individual - metabolic rate | n | n | n | n | |
| individual - mouth injury | y | y | y | y | FE |
| individual - mucus pathogens | y | y | y | y | FE |
| individual - muscles | y | y | y | y | FI |
| individual - pre-rigor mortis time | n | y | y | n | |
| individual - reproductive organs | y | y | y | y | FI |
| individual - rigor mortis time | n | y | y | n | |
| individual - scale loss | y | y | y | as skin alterations | |
| individual - skin alterations | y | y | y | y | FE |
| individual - skin bleeding | y | n | y | n | |
| individual - skin colour | n | n | n | n | |
| individual - skin fungus | y | y | y | y | FE |
| individual - skin injury | y | y | y | y | FE |
| individual - spinal deformation | y | y | y | y | FE |
| individual - spleen | y | y | y | y | FI |
| individual - spleenosomatic index | n | y | y | n | |
| individual - swim endurance | n | n | n | n | |
| individual - tail-grab reflex | n | y | y | n | |
| individual - vaccination damage | y | y | y | as body cavity | |
| individual - viral infection | y | n | n | n | |
| individual - viscerosomatic index | n | n | y | n | |

**Supplementary S1:** Continued.

| Parameter | relevant | practicable | reliable | into model | module |
|---|---|---|---|---|---|
| organs - corticotropin releasing hormone | n | n | n | n | |
| organs - gill cysts | y | n | y | n | |
| organs - gill epithelial sloughing | y | n | y | n | |
| organs - gill hyperplasia | y | n | y | n | |
| organs - gill hypertrophy | y | n | y | n | |
| organs - gill lamellar fusion | y | y | y | as gills | |
| organs - heart cell lysis | y | n | y | n | |
| organs - heart vacuoles | y | n | y | n | |
| organs - heat shock proteins | n | n | n | n | |
| organs - immediate early genes | n | n | n | n | |
| organs - liver cell lysis | y | n | y | n | |
| organs - liver glycogen | n | n | n | n | |
| organs - liver vacuoles | y | n | y | n | |
| organs - mucus composition | n | n | y | n | |
| organs - mucus quantitiy | n | n | n | n | |
| organs - muscle pH | n | n | n | n | |
| organs - oxydative stress | y | n | n | n | |
| plant - ambient light cycle | y | y | y | as ambient light | |
| plant - ambient light intensity | y | y | y | y | M |
| plant - biomass documentation | y | y | y | y | M |
| plant - catching methods | y | y | n | n | |
| plant - cleanliness | y | y | y | y | M |
| plant - emercency plan | y | y | y | y | M |
| plant - crowding duration | y | y | n | n | |
| plant - daily check | y | y | y | y | M |
| plant - disturbances | y | y | y | y | M |
| plant - FCR documentation | y | y | y | as biomass doc | |
| plant - feed contamination | y | n | n | n | |
| plant - feed leftovers | y | y | y | as feeding | |
| plant - feed macro-nutrient composition | y | n | y | n | |
| plant - feed micro-nutrient composition | y | n | y | n | |
| plant - feed storage | n | y | n | n | |
| plant - feed type | y | y | y | y | M |
| plant - feeding interval | y | y | y | y | M |
| plant - feeding rate | y | y | y | as feeding | |
| plant - hygiene concept | y | y | y | y | M |
| plant - mortality documentation | y | y | y | y | M |
| plant - perging duration | y | y | n | n | |
| plant - personnel training | y | y | y | y | M |
| plant - predator protection | y | y | y | y | M |
| plant - slaughter method | y | y | y | y | M |
| plant - sorting interval | y | y | y | y | M |
| plant - sorting methods | y | y | n | n | |
| plant - stocking density | y | y | y | y | M |
| plant - target value document | y | y | y | y | M |
| plant - treatment journal | y | y | y | y | M |
| plant - vaccination | y | y | n | n | |

**Supplementary S1:** Continued.

| Parameter | relevant | practicable | reliable | into model | module |
|---|---|---|---|---|---|
| plasma - coritcosterone | n | n | y | n | |
| plasma - cortisol | n | n | y | n | |
| plasma - cortisone | n | n | y | n | |
| plasma - glucose | n | n | y | n | |
| plasma - lactate | y | n | y | n | |
| plasma - osmolality | y | n | y | n | |
| plasma - total cholesterol | n | n | y | n | |
| plasma - total protein | n | n | y | n | |
| plasma - triglycerides | n | n | y | n | |
| shoal - aggression | y | y | y | y | FG |
| shoal - air breathing | y | y | y | y | FG |
| shoal - apathy | y | y | y | y | FG |
| shoal - balance | y | y | y | y | FG |
| shoal - body colour | y | y | y | y | FG |
| shoal - eye injuries | y | y | y | y | FG |
| shoal - feeding | y | y | y | y | FG |
| shoal - fin injuries | y | y | y | y | FG |
| shoal - fin position | y | y | y | y | FG |
| shoal - fish life stage | y | y | n | n | |
| shoal - fleeing | y | y | y | y | FG |
| shoal - fungal infections | y | y | y | y | FG |
| shoal - gill cover deformations | y | y | y | y | FG |
| shoal - isolation | y | y | y | y | FG |
| shoal - jaw deformations | y | y | y | y | FG |
| shoal - jumping | y | y | y | as scratching | |
| shoal - mortality rate | y | y | n | n | |
| shoal - orientation | y | n | y | n | |
| shoal - scratching | y | y | y | y | FG |
| shoal - skin injuries | y | y | y | y | FG |
| shoal - space use | n | y | n | n | |
| shoal - spinal deformations | y | y | y | y | FG |
| shoal - startling | n | y | y | n | |
| shoal - submission | y | n | n | n | |
| shoal - surfacing | y | y | y | y | FG |
| shoal - territoriality | y | y | y | y | FG |
| shoal - ventilation rate | y | y | y | y | FG |
| tank - acustic level | y | n | n | n | |
| tank - cover | y | y | n | n | |
| tank - enrichment | n | y | n | n | |
| tank - light distribution | y | y | y | as tank light | |
| tank - light intensity | y | y | y | y | M |
| tank - visual protection | n | y | y | n | |

**Supplementary S1:** Continued.

| Parameter | relevant | practicable | reliable | into model | module |
|---|---|---|---|---|---|
| water - alkalinity | n | y | y | n | |
| water - ammonia | y | y | y | y | W |
| water - ammonium | y | y | y | y | W |
| water - BSB | n | n | n | n | |
| water - carboante hardness | y | y | y | y | W |
| water - conductivity | y | y | y | y | W |
| water - coritcosterone | n | n | y | n | |
| water - cortisol | n | n | y | n | |
| water - cortisone | n | n | y | n | |
| water - CSB | n | y | y | n | |
| water - disolved carbon dioxide | y | y | y | y | W |
| water - disolved nitrogen | n | y | y | n | |
| water - disolved oxygen | y | y | y | y | W |
| water - exchange rate | n | y | y | n | |
| water - light absorption | n | n | y | n | |
| water - nitrate | y | y | y | y | W |
| water - nitrite | y | y | y | y | W |
| water - oxygen saturation | y | y | y | y | W |
| water - oyxgen | y | y | y | y | W |
| water - pH | y | y | y | y | W |
| water - phosphate | n | y | y | n | |
| water - salinity | n | y | y | n | |
| water - sulfate | n | y | y | n | |
| water - temperature | y | y | y | y | W |
| water - total gas pressure | y | y | y | y | W |
| water - total suspended solids | n | n | y | n | |
| water - turbidity | n | n | y | n | |
| water - velocity | y | y | y | y | W |

**Supplementary S1:** Continued.

**Supplementary S2:** Parameter Table

Literature review for the 80 welfare parameters to define the intervals. Allocation of the parameter scores (PS) and the score weights (SW) according to the number of intervals. Expert survey for the 80 welfare parameters to define their relative importance resulting in the parameter weights (PW). SWE = score weight exponent, PWE = parameter weight exponent

| | Literature | Remarks | Parameter question | Location System Species | Parameter | Parameter intervals | PS | SW | PW | SWE PWE |
|---|---|---|---|---|---|---|---|---|---|---|
| Farm management | FAWC 1996 [113]; North et al. 2008 [76]; Segner et al. 2019 [99] | Good education, training, and experience of the fish farmers secure fish welfare. In Switzerland, the legal minimum for fis farms is for at least one person to have the "FBA Aquakultur" course | What is the highest training of the personnel in charge? | In / Out RAS / FTS RT / PP | Personnel training | 0: Apprenticeship/master degree with work experience | 0 | 1 | | |
| | | | | | | 1: Apprenticeship/master degree in Aquaculture or "FBA Aquakultur" with work experience | -0.5 | 3 | 3 | |
| | | | | | | 2: "FBA Aquakultur" | -1 | 5 | | |
| | FAWC 1996 [113]; Timmons et al. 2010 [114]; Bregnballe 2015 [115] | Regular controls allow for early detection and prevention of problems that potentially can impair fish welfare. The appropriate interval for controls varies for different parts of th farm's systems system, different farm systems, different life stages of the fish checked? etc. | How are the | In / Out RAS / FTS RT / PP | Daily check | 0: Daily check with appropriate controls | 0 | 1 | | |
| | | | | | | 1: Daily check | -0.5 | 3 | 5 | |
| | | | | | | 2: System is checked insufficiently | -1 | 5 | | |
| | Jentoft et al. 2005 [116]; North et al. 2008 [76]; Noble et al. 2020 [42] | A reduction of disturbances to only the unavoidable level in farming assists good fish welfare. | Are the fish exposed to external disturbances? | In / Out RAS / FTS RT / PP | Disturbances | 0: No external disturbances | 0 | 1 | | |
| | | | | | | 1: Little or slight disturbances | -0.5 | 3 | 3.5 | |
| | | | | | | 2: Frequent and / or severe disturbances | -1 | 5 | | |
| | FAWC 1996 [113]; North et al. 2008 [76]; Noble et al. 2020 [42] | Predation from birds or mammals can impact welfare throug attacks and injuries. | Are the fish protected from predators? | Out RAS / FTS RT / PP | Predator protection | 0: Completely protected from predators | 0 | 1 | | |
| | | | | | | 1: Partially protected from predators | -0.5 | 3 | 4 | |
| | | | | | | 2: Not protected | -1 | 5 | | |
| | Klontz 1991 [95]; North et al. 2008 [76]; Bregnballe 2015 [115] | Proper storage, cleaning and handling of all sorts of material assists a secure functioning of the system and a working hygiene protocol and with this secures fish welfare. | Is the farm and the used material kept clean? | In / Out RAS / FTS RT / PP | Plant cleanliness | 0: The farm is clean and tidy, working materials are clean and disinfected | 0 | 1 | | |
| | | | | | | 1: The farm is clean, working materials are clean | -0.5 | 3 | 3 | |
| | | | | | | 2: The farm is chaotic and dirty, working materials dirty | -1 | 5 | | |
| | North et al. 2008 [76]; BLV 2016 [136] | Documentation of medication and disinfection measures ass the tracking of health and welfare problems and the effects of the measures. In Switzerland, the documentation of any medication is mandatory. | Are chemical measures documented? | In / Out RAS / FTS RT / PP | Treatment journal | 0: Medication, extraordinary and routine (disinfection) measures are documented | 0 | 1 | | |
| | | | | | | 1: Medication and extraordinary (disinfection) measures are documented | -0.5 | 3 | 3 | |
| | | | | | | 2: Medications are documented | -1 | 5 | | |
| | | Written and easily accessible specifics about the system's tar value and corresponding action plans if those values are not met help secure the fish health and welfare. | Are target values and action plans available? | In / Out RAS / FTS RT / PP | Target value sheet | 0: Target value document and action plan are accessible | 0 | 1 | | |
| | | | | | | 1: Target value document and an action plan are known, but not documented | -0.5 | 3 | 4 | 0 |
| | | | | | | 2: There are no target values or specific action plan applied | -1 | 5 | | 1.7 |
| | North et al. 2008 [76]; Bregnballe 2015 [115]; Segner et al. 2019 [99] | Written and easily accessible emergency plans help secure th fish health and welfare. Appropriate emergency plans are system specific. | Is an emergency plan available? | In / Out RAS / FTS RT / PP | Emergency plan | 0: An appropriate emergency plan is available and accessible | 0 | 1 | | |
| | | | | | | 1: An appropriate emergency plan is known, but not documented | -0.5 | 3 | 4 | |
| | | | | | | 2: No emergency plan is available, or it is not appropriate | -1 | 5 | | |
| | Meyer 1991 [124]; Bregnballe 2015 [115]; Noble et al. 2020 [42] | Written and easily accessible hygiene concepts help secure th fish health and welfare. Appropriate hygiene concepts are system specific. | Is a hygiene concept available? | In / Out RAS / FTS RT / PP | Hygiene concept | 0: An appropriate hygiene concept is available and accessible | 0 | 1 | | |
| | | | | | | 1: An appropriate hygiene concept is applied, but not documented | -0.5 | 3 | 4 | |
| | | | | | | 2: No emergency hygiene is available, or it is not appropriate | -1 | 5 | | |
| | Schweiter TSchV 2008 [127]; Ellis et al. 2012 [128]; Kleinegdd et al. 2016 [40] | Documentation of mortalities assists the tracking of health ar welfare problems and the effects of the measures. In Switzerland, the documentation of any mortality is mandator | Are mortalities documented? | In / Out RAS / FTS RT / PP | Mortality documentation | 0: All mortalities and their cause are documented and deducted from biomass | 0 | 1 | | |
| | | | | | | 1: All mortalities are documented and deducted from the biomass | -0.5 | 3 | 4 | |
| | | | | | | 2: All mortalities are documented | -1 | 5 | | |
| | Klontz 1991 [95]; Waynarovich et al. 2011 [96]; Kleinegdd et al. 2016 [40] | Documentation of biomass and stocking density assists the appropriate feeding, good system maintenance and correct timing of husbandry procedures, all of which help to secure welfare. The FCR (feed convertion ratio) is an indicator for fis health and farm management. | Are biomass, stocking density and FCR documented? | In / Out RAS / FTS RT / PP | Biomass documentation | 0: Biomass/stocking density are documented and recalculated (including the FCR) i sporadically verified with intermediate weighings | 0 | 1 | | |
| | | | | | | 1: The biomass and stocking density are documented and sporadically verified with intermediate weighings | -0.5 | 3 | 3.5 | |
| | | | | | | 2: The biomass is documented | -1 | 5 | | |
| | Zenert & Hedrich 2005 [80]; Zake et al. 2004 [141]; Backlandt et al. 2018 [82] | Sorting the shoal helps to maintain a homogeneously sized group, but the procedure also inflicts stress. An appropriate sorting procedure and interval help maintain fish welfare and health in the long-term. | Is the sorting interval appropriate? | In / Out RAS / FTS RT / PP | Sorting | 0: The group is homogeneous | 0 | 1 | | |
| | | | | | | 1: The group is slightly heterogeneous, unproblematic | -0.5 | 3 | 3 | |
| | | | | | | 2: The group is very heterogeneous, problematic | -1 | 5 | | |
| | Robb 2008 [139]; Ellis et al. 2012 [128]; Lines & Spence 2012 [140] | Humane slaughtering includes short crowding times, effectiv and lasting stunning, and fast killing. In Switzerland, fish mu not show any signs of consciousness (reflexes) between stunning and death. | Is the slaughter process humane? | In / Out RAS / FTS RT / PP | Slaughter | 0: Crowding: Short / stunning method: effective / killing: fast / no fish shows refle | 0 | 1 | | |
| | | | | | | 1: Crowding: Short / stunning method: effective / killing: delayed / no fish shows reflexes | -0.5 | 3 | 5 | |
| | | | | | | 2: Crowding: long / stunning method: effective / killing: delayed / no fish shows r | -1 | 5 | | |

**Supplementary S2:** Continued.

**Supplementary S2:** Continued.

| | Literature | Remarks | Parameter question | Location System Species | Parameter | Parameter intervals | PS | SW | PW | SWE PWE |
|---|---|---|---|---|---|---|---|---|---|---|
| Farm management | Ellis et al. 2002 [73]; North et al. 2006 [142]; Turnbull et al. 2008 [143]; Skov et al. 2011 [53] | Stocking density is correlated with other welfare parameters such as wa quality and group behaviour. In Switzerland, the density is regulated to a maximum of 80 kg/m³ for salmonids and 100 kg/m³ for cyprinids. Depending on the system too low densities may favour territoriality and, aggression in the shoal. | What is the current stocking density? | In / Out RAS / FTS RT | Stocking density | 0: 0–40 kg/m³ | 0 | 1 | 3 | 0 1.7 |
| | | | | | | 1: 40–60 kg/m³ | -0.5 | 3 | | |
| | | | | | | 2: 60–80 kg/m³ | -1 | 5 | | |
| | Zienert & Heidrich 2005 [80]; Steenfeldt et al. 2010 [144]; Dalsgaard et al. 2013 [68] | | | In / Out RAS / FTS PP | | 0: 0–30 kg/m³ | 0 | 1 | | |
| | | | | | | 1: 30-50 kg/m³ | -0.5 | 3 | | |
| | | | | | | 2: 50-80 kg/m³ | -1 | 5 | | |
| | McCarthy et al. 1992 [120]; Moutou et al. 1998 [121]; Wang et al. 2009 [122]; López-Olmeda et al. 2012 [123] | Feeding rate (kg feed / kg biomass) and interval (amount of feed per feedi and numbers of feedings per day) influence growth, health and social behaviour. | The current feeding interval and rate secures how many points? | In / Out RAS / FTS RT / PP | Feeding interval and rate[1] | 0: 5–6 points | 0 | 1 | 3.5 | |
| | | | | | | 1: 3–4 points | -0.5 | 3 | | |
| | | | | | | 2: 0–2 points | -1 | 5 | | |
| | Geay & Kestemont 2015 [118]; Antony Jesu Prabhu et al. 2015 [119]; Baekelandt et al. 2018 [82] | Feed type (reproduction, rearing, salmonids, percids etc.) and pellet size the feed are appropriate to the fish species and life stage. | Is the feed type and size appropriate? | In / Out RAS / FTS RT / PP | Feed type | 0: Feed type and pellet size are adapted to the fish | 0 | 1 | 4 | |
| | | | | | | 1: Pellets are too small / big for the animals | -0.5 | 3 | | |
| | | | | | | 2: Type and size does not match the fish | -1 | 5 | | |
| | Noble et al. 2005 [77]; Karakatsouli et al. 2007 [78]; Mizusawa et al. 2007 [79] | Ambient light is a relevant external factor for biological processes and therefore fish health and welfare. Appropriate light conditions vary with species and fish life stage. | Is the ambient light appropriate? | In RAS / FTS RT | Ambient light[2] | 0: Light intensity and phases are adjusted | 0 | 1 | 3 | |
| | | | | | | 1: Light intensity or light phases are adjusted | -0.5 | 3 | | |
| | | | | | | 2: Neither light intensity nor light phases are adjustec | -1 | 5 | | |
| | Zienert & Heidrich 2005 [80]; Feiner & Höök 2015 [81]; Baekelandt et al. 2018 [82] | | | In RAS / FTS PP | Ambient light[3] | 0: Light intensity and phases are adjusted | 0 | 1 | | |
| | | | | | | 1: Light intensity or light phases are adjusted | -0.5 | 3 | | |
| | | | | | | 2: Neither light intensity nor light phases are adjustec | -1 | 5 | | |
| | Noble et al. 2005 [77]; Karakatsouli et al. 2007 [78]; Mizusawa et al. 2007 [79] | Light conditions in the tanks influence social and feeding behaviour. Appropriate light conditions vary with fish species, fish life stage and farr system. | Is the light in the husbandry tanks appropriate? | In RAS / FTS RT | Tank light[4] | 0: Light intensity and light distribution adapted | 0 | 1 | 3 | |
| | | | | | | 1: Light intensity or light distribution adapted | -0.5 | 3 | | |
| | | | | | | 2: Neither intensity nor light distribution adapted | -1 | 5 | | |
| | | | | Out RAS / FTS RT | Tank light[5] | 0: Light intensity and light distribution adapted | 0 | 1 | | |
| | | | | | | 1: Light intensity or light distribution adapted | -0.5 | 3 | | |
| | | | | | | 2: Neither intensity nor light distribution adapted | -1 | 5 | | |
| | Luchiari et al. 2006 [145]; Feiner & Höök 2015 [118]; Geay & Kestemont 2015 [81] | | | In RAS / FTS PP | Tank light[6] | 0: Light intensity and light distribution adapted | 0 | 1 | | |
| | | | | | | 1: Light intensity or light distribution adapted | -0.5 | 3 | | |
| | | | | | | 2: Neither intensity nor light distribution adapted | -1 | 5 | | |
| | | | | Out RAS / FTS PP | Tank light[7] | 0: Light intensity and light distribution adapted | 0 | 1 | | |
| | | | | | | 1: Light intensity or light distribution adapted | -0.5 | 3 | | |
| | | | | | | 2: Neither intensity nor light distribution adapted | -1 | 5 | | |

1 ▪ no feed leftovers ▪ good spatial distribution of the feed in the tank ▪ enthusiastic feeding behavior (neither apathetic nor aggressive) ▪ all fish receive enough food (also subdominant fish) ▪ neither overfed nor emaciated fish ▪ is adjusted weekly

2
- Light intensity: the (room) lighting allows safe working of the personnel and a visual inspection of the fish
- Light intensity: the room lighting is not too strong, the fish behaves calmly
- Light phases: any transitions from light/dark phases are long/gentle, fish always remain calm

3
- Light intensity: the (room) lighting allows safe working of the personnel and a visual inspection of the fish
- Light intensity: the room lighting is soft, the fish behave calmly
- Light phases: any transitions from light/dark phases are long/gentle, fish always remain calm

4
- Light intensity: fish are protected from excessive light intensity (either by shading or sufficient water depth or weak ambie nt lighting)
- Light intensity: Light intensity in the tank allows the fish a safe feed intake (they see the feed)
- Light distribution: no or weak light/dark transitions in the pool, group uses the entire water volume

5
- Light intensity: fish are protected from excessive UV radiation (either by shading or sufficient water depth)
- Light distribution: no/weak light/dark transitions in the pool, swarm uses the entire water volume

6
- Light intensity: fish have a weak light intensity (either by shading or sufficient water depth or weak ambient light)
- Light intensity: Light intensity in the tank allows the fish a safe feed intake (they see the feed)
- Light distribution: no or weak light/dark transitions in the pool, group uses the entire water volume

7
- Light intensity: fish are protected from UV radiation (either by shading or sufficient water depth)
- Light distribution: no/weak light/dark transitions in the pool, swarm uses the entire water volume

**Supplementary S2:** Continued.

| | Literature | Remarks | Parameter question | Location System Species | Parameter | Parameter intervals | PS | SW | PW | SWE PWE |
|---|---|---|---|---|---|---|---|---|---|---|
| Water quality | Schurmann et al. 1991 [146]; Bear et al. 2007 [147]; Lewis et al. 2010 [148]; Woynarovich et al. 2011 [96]; Beakes et al. 2014 [149]; Janhunen et al. 2016 [150] | Water temperature affects biological processes and is a key aspect of water quality. Preferences and tolerances are fish species and life stage specific, a intervals need to be adapted accordingly. In Switzerland, the upper limit for the temperature of the system water is 22 for salmonids and 30°C for cyprinids. | Temperature of the system water in [°C] | In / Out RAS / FTS RT | Temperature | Optimum: [10-16] | 0 | 1 | 4 | 1.7 1.7 |
| | | | | | | Within target range: [8-10) ∪ (16 - 18] | -0.33 | 2.33 | | |
| | | | | | | Within the tolerance range: [4-6) ∪ (18 - 22] | -0.66 | 3.66 | | |
| | | | | | | Outside the tolerance range: [0-4) ∪ (22 - 35] | -1 | 5 | | |
| | Willemsen 1978 [151]; Frisk et al. 2012 [152], 2013 [153]Swirplies et al. 2019 [154] | | | In / Out RAS / FTS PP | | Optimum: [20- 25] | 0 | 1 | | |
| | | | | | | Within target range: [13-20) ∪ (25 - 28] | -0.33 | 2.33 | | |
| | | | | | | Within the tolerance range: [8-13) ∪ (28 - 30] | -0.66 | 3.66 | | |
| | | | | | | Outside the tolerance range: [0-8) ∪ (30 - 40] | -1 | 5 | | |
| | Zienert & Heidrich 2005 [80]; Glencross 2009 [135];Labbé et al. 2014 [130]; BLV 2016 [136] | Oxygen is needed for biological processes and is a key aspect of water quality While preferences are similar for most farmed fish species, tolerance ranges fish species and life stage specific and intervals can be adapted accordingly. In Switzerland, the lower limit for oxygen in the system water is 5 mg/l for salmonids and 3.5 mg/l for cyprinids. | Dissolved oxygen ($O_2$) in the system water in [mg/l] | In / Out RAS / FTS RT / PP | Oxygen | Optimum: [8 - 10] | 0 | 1 | 5 | |
| | | | | | | Within target range: [7-8) ∪ (10 - 13] | -0.33 | 2.33 | | |
| | | | | | | Within the tolerance range: [6-7) ∪ (13 - 15] | -0.66 | 3.66 | | |
| | | | | | | Outside the tolerance range: [4-6) ∪ (15 - 30] | -1 | 5 | | |
| | Klontz 1991 [95]; Boyd & Tucker 1998 [137]; Baekelandt et al. 2018 [82] | | Dissolved oxygen ($O_2$) in the system water in [% saturation] | In / Out RAS / FTS RT / PP | Oxygen saturation | Optimum: [80- 120] | 0 | 1 | 5 | |
| | | | | | | Within target range: [70-80) ∪ (120 -140] | -0.33 | 2.33 | | |
| | | | | | | Within tolerance range: [60-70) ∪ (140 -160] | -0.66 | 3.66 | | |
| | | | | | | Outside the tolerance range: [20-60) ∪ (160 - 300] | -1 | 5 | | |
| | Wicks et al. 2002 [86]; Capkin et al. 2009 [87]; Steinberg et al. 2018a [88] | Ammonium is fish toxic and a relevant water quality parameter in RAS. Little data for pikeperch is available. Since ammonium is the main factor for ammonia levels and toxicity of ammonia is similar in trout and pikeperch, th same intervals for ammonium are used for both species. | Total ammonia nitrogen (TAN, $NH_4$-N + $NH_3$-N) of the system water in [mg/l] | In / Out RAS RT / PP | Ammonium | Optimum: [0 - 0.5] | 0 | 1 | 4 | |
| | | | | | | Within target range: (0.5-1.5] | -0.33 | 2.33 | | |
| | | | | | | Within tolerance range: (1.5-5] | -0.66 | 3.66 | | |
| | | | | | | Outside the tolerance range: (5-20] | -1 | 5 | | |
| | Zienert & Heidrich 2005 [80]; Calamari et al. 1981 [83]; Randall & Tsui 2002 [84]; Schram et al. 2014 [85] | Ammonia is highly fish toxic and a relevant water quality parameter in RAS. Ammonia levels are mostly calculated from TAN and pH values. In Switzerland, the upper limit for ammonia in the system water is 0.01 mg/l f salmonids and 0.02 mg/l for cyprinids. | Ammonia ($NH_3$-N) of the system water in [mg/l] | In / Out RAS RT / PP | Ammonia | Optimum: [0 - 0.01] | 0 | 1 | 5 | |
| | | | | | | Within target range: (0.01-0.02] | -0.33 | 2.33 | | |
| | | | | | | Within tolerance range: (0.02-0.1] | -0.66 | 3.66 | | |
| | | | | | | Outside the tolerance range: (0.1-2] | -1 | 5 | | |
| | Russo et al. 1974 [132]; Williams & Eddy 1986 [133]; Kroupova et al. 2008 [134] | Nitrite is highly fish toxic and a relevant water quality parameter in RAS. Pikeperch might be up to 10 times more tolerant to nitrite than salmonids, however conclusive evidence is still missing. In Switzerland, the upper limit for nitrite in the system water is 1.5 mg/l. | Nitrite ($NO_2$-N) of the system water in [mg/l] | In / Out RAS RT / PP | Nitrite | Optimum: [0 - 0.05] | 0 | 1 | 5 | |
| | | | | | | Within target range: (0.05-0.1] | -0.33 | 2.33 | | |
| | | | | | | Within tolerance range: (0.1-0.5] | -0.66 | 3.66 | | |
| | | | | | | Outside the tolerance range: (0.5-5] | -1 | 5 | | |
| | Müller-Belecke et al. 2013 [129]; Labbé et al. 2014 [130]; Steinberg et al. 2018b [131] | Nitrate is fish toxic in high concentrations and a relevant water quality parameter in RAS. Before nitrate concentrations poses an acute risk for the fi they hinder the proper functioning of the biofilter, what leads to an increase nitrite and TAN. | Nitrate ($NO_3$-N) of the system water in [mg/l] | In / Out RAS RT / PP | Nitrate | Optimum: [0 - 50] | 0 | 1 | 2.5 | |
| | | | | | | Within target range: (50-75] | -0.33 | 2.33 | | |
| | | | | | | Within tolerance range: (75-150] | -0.66 | 3.66 | | |
| | | | | | | Outside tolerance range: (150-300] | -1 | 5 | | |

**Supplementary S2:** Continued.

| | Literature | Remarks | Parameter question | Location System Species | Parameter | Parameter intervals | PS | SW | PW | SWE PWE |
|---|---|---|---|---|---|---|---|---|---|---|
| Water quality | Wedemeyer 1996 [105]; Altinok et al. 2006 [107]; Boyd et al. 2016 [108] | Carbonate hardness affects the stability of the water quality and biolo processes of the fish°fH and °dH are convertible into mg/l of CaCO | Carbonate hardness of the system water in $CaCO_3$ [mg/l] | In / Out RAS / FTS RT / PP | Carbonate hardness | Optimum: [40- 150] | 0 | 1 | 3 | |
| | | | | | | Within target range: [30-40) ∪ (150 -250] | -0.33 | 2.33 | | |
| | | | | | | Within tolerance range: [20-30) ∪ (250 -400] | -0.66 | 3.66 | | |
| | | | | | | Outside tolerance range: [0-20) ∪ (400 -500] | -1 | 5 | | |
| | Wedemeyer 1996 [105]; Becke et al. 2018 [157]; Steinberg et al. 2018a [88] | High TSS values are indicators of low water quality and impact the function and health of the gills. | TSS of the system water in [mg/l] | In / Out RAS / FTS RT / PP | Total suspended solids | Optimum: [0 - 25] | 0 | 1 | 2 | |
| | | | | | | Within target range: (25-50] | -0.33 | 2.33 | | |
| | | | | | | Within tolerance range: (50-200] | -0.66 | 3.66 | | |
| | | | | | | Outside tolerance range: (200-500] | -1 | 5 | | |
| | Zienert & Heidrich 2005 [80]; Klontz 1991 [95]; Altinok et al. 2006 [107] | pH affects biological processes of the fish and the biofilter, and is a key aspect of water quality. In Switzerland, the legal allowed range for the pH of the system water is 5.5- 9. | pH of the system water | In / Out RAS / FTS RT / PP | pH | Optimum: [7 - 7.5] | 0 | 1 | 4 | |
| | | | | | | Within target range: [6.5-7) ∪ (7.5 - 8] | -0.33 | 2.33 | | |
| | | | | | | Within the tolerance range: [6-6.5) ∪ (8 - 8.5] | -0.66 | 3.66 | | |
| | | | | | | Outside the tolerance range: [4-6) ∪ (8.5 - 10] | -1 | 5 | | |
| | Brown et al. 2001 [109]; Altinok & Grizzle 2003 [110]; Scott et al. 2008 [111]; Xiong et al. 2019 [112] | Preferences/tolerances for salinity vary considerably among fish speci hence the parameter should be added to the model when assessing fis adapted to salt or brackish water. For freshwater species salinity can b measured via the conductivity. Other ions affect conductivity as well (e. nitrate) and limit upper conductivity levels for welfare before the salini itself impairs welfare. | Conductivity of the system water in [µS/cm] | In / Out RAS / FTS RT / PP | Conductivity | Optimum: [500- 1000] | 0 | 1 | 2 | 1.7 1.7 |
| | | | | | | Within target range: [300-500) ∪ (1000- 5000] | -0.33 | 2.33 | | |
| | | | | | | Within tolerance range: [200-300) ∪ (5000- 15000] | -0.66 | 3.66 | | |
| | | | | | | Outside tolerance range: [0-200) ∪ (15000- 30000] | -1 | 5 | | |
| | Wedemeyer 1996 [105]; Good et al. 2010 [106]; Steinberg et al. 2017 [104] | Elevated levels of dissolved carbon dioxide impair fish health and can occur in RAS as well as FTS. | Dissolved carbon dioxide ($CO_2$) in the system water in [mg/l] | In / Out RAS / FTS RT / PP | Carbon dioxide | Optimum: [0 - 5] | 0 | 1 | 3.5 | |
| | | | | | | Within target range: (5-20] | -0.33 | 2.33 | | |
| | | | | | | Within tolerance range: (20-30] | -0.66 | 3.66 | | |
| | | | | | | Outside the tolerance range: (30-100] | -1 | 5 | | |
| | Weitkamp et al. 1980 [155]; Wedemeyer 1996 [105]; Bohl 1997 [156] | Elevated levels of total gar pressure in the system water impair fish he and can occur in RAS as well as FTS. | TGP in the system water in [% saturation] | In / Out RAS / FTS RT / PP | Total gas pressure | Optimum: < / = 100 | 0 | 1 | 4 | |
| | | | | | | Within target range: (100-103] | -0.33 | 2.33 | | |
| | | | | | | Within tolerance range: (103-105] | -0.66 | 3.66 | | |
| | | | | | | Outside tolerance range: (105-120] | -1 | 5 | | |
| | Jobling et al. 1993 [158]; Lauff & Wood 1996 [159]; Larsen et al. 2012 [160]; Huntingford & Kadri 2013 [161] | Water velocity affects fish welfare (e.g. physiological exercise, group behavior) as well as system functioning (e.g. tank self-cleaning, biofilter efficiency). Optimal and tolerance values are fish species and life stage well as system specific and need to be adapted accordingly. | Water velocity in the fish tank in [body lengths/sec] | In / Out RAS / FTS RT / PP | Water velocity | Optimum: [0.5- 1] | 0 | 1 | 3 | |
| | | | | | | Within target range: [0.3-0.5) ∪ (1 - 2] | -0.33 | 2.33 | | |
| | | | | | | Within tolerance range: [0.2-0.3) ∪ (2 - 3] | -0.66 | 3.66 | | |
| | | | | | | Outside the tolerance range: [0-0.2) ∪ (3 - 5] | -1 | 5 | | |

**Supplementary S2:** Continued.

| | Literature | Remarks | Parameter question | Location System Species | Parameter | Parameter intervals | PS | SW | PW | SWE PWE |
|---|---|---|---|---|---|---|---|---|---|---|
| Fish group behaviour | Ellis et al. 2002 [73]; Ashley 2007 [12]; Martins et al. 2012 [74]; Magnhagen 2015 [75]; Noble et al. 2020 [42] | Dominant (e.g. fin or opercula spreading, approaching) and aggressive (e.g. biting, bumping) behaviours are costly for both the dominant and the submissive individual. They can stress and potentially injuries and hence are a threat to the welfare of the fish. | Is aggressive behaviour detectable when observing the fish group? | In / Out RAS / FTS RT / PP | Aggression | 0: No fish shows dominance or aggression | 0 | 1 | 3.5 | |
| | | | | | | 1: Individual fish show dominance behavior | -0.2 | 1.8 | | |
| | | | | | | 2: Some fish show dominance behavior | -0.4 | 2.6 | | |
| | | | | | | 3: Individual fish show aggression behavior | -0.6 | 3.4 | | |
| | | | | | | 4: Some fish show aggressive behavior | -0.8 | 4.2 | | |
| | | | | | | 5: Many fish are either dominant or aggressive | -1 | 5 | | |
| | | Territoriality can cause aggression and if key areas (inlet, feeder, shelter, shading) are monopolized it may further impair health and welfare by preventing certain fish the access to these recourses. | Is territorial behaviour detectable when observing the fish group? | In / Out RAS / FTS RT / PP | Territoriality | 0: No fish shows territorial behavior | 0 | 1 | 3 | |
| | | | | | | 1: Individual fish show territorial behavior | -0.2 | 1.8 | | |
| | | | | | | 2: Some fish show territorial behavior | -0.4 | 2.6 | | |
| | | | | | | 3: Individual fish show a territorial monopolization of key areas | -0.6 | 3.4 | | |
| | | | | | | 4: Some fish show a territorial monopolization of key areas | -0.8 | 4.2 | | |
| | | | | | | 5: Some fish show a territorial monopolization of key areas, a large part of the shoal has no access to these | -1 | 5 | | |
| | North et al. 2008 [76]; Martins et al. 2012 [74]; Kleingeld et al. 2016 [40] | Jumping out of the water and/or scratching the body on surfaces (often visible as "flashing" when the brighter vent side of the fish shortly shows) are signs of discomfort most cause by pathogens. The behaviors are therefore indicators of reduced health and welfare. | Is scratching detectable when observing the fish group? | In / Out RAS / FTS RT / PP | Scratching | 0: No fish jumps or scratches | 0 | 1 | 4 | 1.7 |
| | | | | | | 1: Individual fish occasionally jump and/or scratch themselves on surfaces | -0.2 | 1.8 | | 1.7 |
| | | | | | | 2: Some fish occasionally jump and/or scratch themselves on surfaces | -0.4 | 2.6 | | |
| | | | | | | 3: Individual fish frequently jump and/or scratch themselves on surfaces | -0.6 | 3.4 | | |
| | | | | | | 4: Some fish frequently jump and/or scratch themselves on surfaces | -0.8 | 4.2 | | |
| | | | | | | 5: Many fish frequently jump and/or scratch themselves on surfaces | -1 | 5 | | |
| | Martins et al. 2012 [74]; Kleingeld et al. 2016 [40]; Noble et al. 2020 [42] | Apathy i.e. the lack of behaviours (e.g. feeding or swimmin especially after external stimuli (e.g. fleeing) are a sing of impaired health and welfare. | Is apathic behavior detectable when observing the fish group? | In / Out RAS / FTS RT / PP | Apathy | 0: No fish show signs of apathy | 0 | 1 | 5 | |
| | | | | | | 1: Individual fish show apathetic swimming behavior, react normally to stimulation | -0.2 | 1.8 | | |
| | | | | | | 2: Some fish show apathetic swimming behavior, react normally to stimulation | -0.4 | 2.6 | | |
| | | | | | | 3: Individual fish show apathetic swimming behavior, do not react to stimulation | -0.6 | 3.4 | | |
| | | | | | | 4: Some fish show apathetic swimming behavior, do not respond to stimulation | -0.8 | 4.2 | | |
| | | | | | | 5: Many fish show apathetic swimming behavior, do not respond to stimulation | -1 | 5 | | |
| | | Fish separating themselves from the shoal is caused by so of physiological stress and is therefore an indication of impaired health and/or welfare. | Is isoloating behavior detectable when observing the fish group? | In / Out RAS / FTS RT / PP | Isolation | 0: All fish are part of a shoal | 0 | 1 | 3.5 | |
| | | | | | | 1: Individual fish stand apart | -0.2 | 1.8 | | |
| | | | | | | 2: Some fish stand apart | -0.4 | 2.6 | | |
| | | | | | | 3: Individual fish stand apart and/or on the surface | -0.6 | 3.4 | | |
| | | | | | | 4: Some fish stand apart and/or on the surface | -0.8 | 4.2 | | |
| | | | | | | 5: Many fish stand apart and/or on the surface | -1 | 5 | | |
| | | Abnormal swimming pattern (e.g. laying on the bottom or drifting at the surface) can be indicators of social (submissive/avoiding behavior) or physiological (buoyancy problems, pain) problems. This parameter is fish species specific as different fish have a different use of the water column and hence the parameter and its intervals should adapted if needed. | Where in the water column are the fish? | In / Out RAS / FTS RT / PP | Surfacing | 0: All fish swim normally in the water column | 0 | 1 | 4 | |
| | | | | | | 1: Individual fish are predominantly lying on the bottom | -0.2 | 1.8 | | |
| | | | | | | 2: Some fish are constantly lying on the bottom | -0.4 | 2.6 | | |
| | | | | | | 3: Individual fish are increasingly swimming on the surface | -0.6 | 3.4 | | |
| | | | | | | 4: Some fish swim mainly on the surface | -0.8 | 4.2 | | |
| | | | | | | 5: Many fish swim mainly on the surface | -1 | 5 | | |

**Supplementary S2:** Continued.

| | Literature | Remarks | Parameter question | Location System Species | Parameter | Parameter intervals | PS | SW | PW | SWE PWE |
|---|---|---|---|---|---|---|---|---|---|---|
| Fish group behaviour | North et al. 2008 [76]; Noga 2010 [52]; Noble et al. 2020 [42] | The gulping of air at the surface is an indication of breathing impairments eithe caused by a malfunction of the gills or low oxygen concentrations in the system water. | Is air gulping detectable when observing the fish group? | In / Out RAS / FTS RT / PP | Air gulping | 0: No fish shows air breathing | 0 | 1 | 4 | |
| | | | | | | 1: Individual fish show occasional gasps | -0.2 | 1.8 | | |
| | | | | | | 2: Some fish show occasional gasps | -0.4 | 2.6 | | |
| | | | | | | 3: Individual fish show frequent gasps | -0.6 | 3.4 | | |
| | | | | | | 4: Some fish show constant air gulping | -0.8 | 4.2 | | |
| | | | | | | 5: Many fish show constant air gulping | -1 | 5 | | |
| | | Chronically altered ventilation (i.e. increased/decreased rates or magnitude o opercula movements) are signs of chronic stress and health impairments. The effects of acute stress (feeding time, external disturbance) have to be conside when defining deviations for "normal" ventilation behaviour. | Is the ventilation rate normal? | In / Out RAS / FTS RT / PP | Ventilation rate | 0: All fish have a normal ventilation rate | 0 | 1 | 4 | |
| | | | | | | 1: Individual fish show an increased ventilation rate | -0.2 | 1.8 | | |
| | | | | | | 2: Some fish show increased ventilation rate | -0.4 | 2.6 | | |
| | | | | | | 3: Individual fish show a greatly increased or slightly reduced ventilation ra | -0.6 | 3.4 | | |
| | | | | | | 4: Some fish show a greatly increased or clearly reduced ventilation rate | -0.8 | 4.2 | | |
| | | | | | | 5: Many fish show a greatly increased or clearly reduced ventilation rate | -1 | 5 | | |
| | Davis 2010 [94]; Martins et al. 2012 [74]; Kleingeld et al. 2016 [40] | Fleeing behavior is normal for all most fish species. A prolonged or missing reaction to external stimuli is a sign of impaired health and welfare. | Is the fleeing behaviour normal? | In / Out RAS / FTS RT / PP | Fleeing | 0: All fish show normal fleeing when stimulated and calm down quickly | 0 | 1 | 3 | |
| | | | | | | 1: Individual fish show an increased and/or prolonged fleeing behavior | -0.2 | 1.8 | | |
| | | | | | | 2: Some fish show an increased and/or prolonged fleeing behavior | -0.4 | 2.6 | | |
| | | | | | | 3: Individual fish show no or constant fleeing behavior | -0.6 | 3.4 | | |
| | | | | | | 4: Some fish show no or constant fleeing behavior | -0.8 | 4.2 | | |
| | | | | | | 5: Many fish show no or constant fleeing behavior | -1 | 5 | | |
| | | An abnormal position of the fins can be an indicator of social of physiological stress. The normal position of fins may be species specific and any assessment abnormalbehaviorsshould be adapted accordingly. | Is the fin position normal? | In / Out RAS / FTS RT / PP | Fin position | 0: All fish show a normal and calm fin position | 0 | 1 | 3 | 1.7 1.7 |
| | | | | | | 1: Individual fish occasionally have their fins pinched or splayed out | -0.2 | 1.8 | | |
| | | | | | | 2: Some fishes occasionally pinch or splay out their fins | -0.4 | 2.6 | | |
| | | | | | | 3: Individual fishes have the fins constantly pinched or splayed out | -0.6 | 3.4 | | |
| | | | | | | 4: Some fish have the fins constantly pinched or splayed out | -0.8 | 4.2 | | |
| | | | | | | 5: Many fishes have the fins constantly pinched or splayed out | -1 | 5 | | |
| | Lee 1893 [92]; MacIntyre et al. 2008 [93]; Davis 2010 [94] | Fish should be able to constantly uphold an upright body position and have prc orientation within the water column. Struggle or failure to do this indicate physiological problems. | Do the fish have good balance? | In / Out RAS / FTS RT / PP | Balance | 0: All fish show a normal balance and orientation | 0 | 1 | 4.5 | |
| | | | | | | 1: Individual fish are sometimes misaligned | -0.2 | 1.8 | | |
| | | | | | | 2: Some fish are crooked at times | -0.4 | 2.6 | | |
| | | | | | | 3: Individual fish are constantly crooked | -0.6 | 3.4 | | |
| | | | | | | 4: Some fish are constantly crooked | -0.8 | 4.2 | | |
| | | | | | | 5: Many fish are constantly crooked | -1 | 5 | | |
| | Ferguson 2006 [98]; North et al. 2008 [76]; Noga 2010 [52]; Segner et al. 2019 [99] | Changes in body coloration (e.g. pale or darkened) can indicate social or physiological stress. The particular body coloration is fish species and life stag specific and can be subject to seasonal changes. These aspects have to be considered when defining deviations for "normal" body coloration. | Are the fish coloured normally? | In / Out RAS / FTS RT / PP | Body color | 0: All the fish show a normal body coloration | 0 | 1 | 3 | |
| | | | | | | 1: Single fish have temporarily a conspicuously bright or dark coloration | -0.2 | 1.8 | | |
| | | | | | | 2: Some fish have temporarily a conspicuously bright or dark coloration | -0.4 | 2.6 | | |
| | | | | | | 3: Individual fish have constantly striking a bright or dark coloration | -0.6 | 3.4 | | |
| | | | | | | 4: Some fish constantly have a noticeable light or dark color | -0.8 | 4.2 | | |
| | | | | | | 5: Many fish constantly have a noticeable light or dark color | -1 | 5 | | |
| | North et al. 2008 [76]; Martins et al. 2012 [74]; Noble et al. 2020 [42] | Fish in husbandry should feed eagerly. Lack of feeding behavior (covered with t parameter "apathy" and "isolation") or overly hectic and aggressive feeding are indicators of suboptimal feeding conditions or impaired health and welfare. Feeding behavior is fish species and life stage specific and is subject to quotidi and seasonal changes. These aspects have to be considered when defining deviations for "normal" feeding behavior. | Is the feeding behavior expressed normally? | In / Out RAS / FTS RT / PP | Feeding | 0: All fish show normal feeding behavior | 0 | 1 | 3 | |
| | | | | | | 1: Individual fish show a very hungry, hectic eating behavior | -0.2 | 1.8 | | |
| | | | | | | 2: Some fish show a very hungry, hectic eating behavior | -0.4 | 2.6 | | |
| | | | | | | 3: Individual fish show a starved, aggressive eating behavior | -0.6 | 3.4 | | |
| | | | | | | 4: Some fish show a starved, aggressive eating behavior | -0.8 | 4.2 | | |
| | | | | | | 5: Many fish show a starved, aggressive eating behavior | -1 | 5 | | |

| Fish group behaviour | Literature | Remarks | Parameter question | Location System Species | Parameter | Parameter intervals | PS | SW | PW | SWE PWE |
|---|---|---|---|---|---|---|---|---|---|---|
| | Kestemont et al. 2007 [126]; North et al. 2008 [76]; Rodger & Phelps 2015 [97]; Policar et al. 2016 [91] | Deformations may inflict pain and/or restrict movement, breathin and feeding and therefore impair fish health and welfare . Information about how many fish are affected, and when and how fast the deformations appeared can assist the identification of the deformations? causes and potential measures for improvement. | How many fish have jaw deformations? | In / Out RAS / FTS RT / PP | Jaw deformations | 0: No fish has injuries/deformations of the jaw/snout | 0 | 1 | | |
| | | | | | | 1: Individual fish have slight injuries/deformations of the jaw/snout | -0.2 | 1.8 | | |
| | | | | | | 2: Some fish have slight injuries/deformations of the jaw/snout | -0.4 | 2.6 | 3 | |
| | | | | | | 3: Individual fish have severe injuries/deformations of the jaw/snout | -0.6 | 3.4 | | |
| | | | | | | 4: Some fish have severe injuries/deformations of the jaw/snout | -0.8 | 4.2 | | |
| | | | | | | 5: Many fish have severe injuries/deformations of the jaw/snout | -1 | 5 | | |
| | | | How many fish have opercula deformations? | In / Out RAS / FTS RT / PP | Gill cover deformations | 0: No fish has injuries/deformations of the opercula | 0 | 1 | | |
| | | | | | | 1: Individual fish have slight injuries/deformations of the opercula | -0.2 | 1.8 | | |
| | | | | | | 2: Some fish have slight injuries/deformations of the opercula | -0.4 | 2.6 | 2 | |
| | | | | | | 3: Individual fish have severe injuries/deformations of the opercula | -0.6 | 3.4 | | |
| | | | | | | 4: Some fish have severe injuries/deformations of the opercula | -0.8 | 4.2 | | |
| | | | | | | 5: Many fish have severe injuries/deformations of the opercula | -1 | 5 | | |
| | | | How many fish have spinal deformations? | In / Out RAS / FTS RT / PP | Spinal deformations | 0: No fish has injuries/deformations of the spine | 0 | 1 | | |
| | | | | | | 1: Individual fish have a slight injuries/deformations of the spine | -0.2 | 1.8 | | |
| | | | | | | 2: Some fish have a slight injuries/deformations of the spine | -0.4 | 2.6 | 3 | |
| | | | | | | 3: Individual fish have a severe injuries/deformations of the spine | -0.6 | 3.4 | | |
| | | | | | | 4: Some fish have severe injuries/deformations of the spine | -0.8 | 4.2 | | |
| | | | | | | 5: Many fish have a severe injuries/deformations of the spine | -1 | 5 | | |
| | | | How many fish have eye injuries? | In / Out RAS / FTS RT / PP | Eye injuries | 0: No fish has eye injuries/deformations | 0 | 1 | | |
| | | | | | | 1: Individual fish have slight injuries/deformations to the eyes | -0.2 | 1.8 | | |
| | | | | | | 2: Some fish have minor eye injuries/deformations | -0.4 | 2.6 | 3 | 1.7 |
| | | | | | | 3: Individual fish have severe injuries/deformations to the eyes | -0.6 | 3.4 | | 1.7 |
| | | | | | | 4: Some fish have severe eye injuries/deformations | -0.8 | 4.2 | | |
| | | | | | | 5: Many fish have severe injuries/deformations to the eyes | -1 | 5 | | |
| | Ashley & Sneddon 2008 [11]; Noble et al. 2012 [117] | Injuries inflict pain and/or restrict movement, breathing, and feec and therefore impair fish health and welfare. Information about hi many fish are affected, and when and how fast the injuries appear can assist the identification of the causes and potential measures improvement. | How many fish have skin injuries? | In / Out RAS / FTS RT / PP | Skin injuries | 0: No fish has injuries/deformations of the skin | 0 | 1 | | |
| | | | | | | 1: Individual fish have slight injuries/deformations of the skin | -0.2 | 1.8 | | |
| | | | | | | 2: Some fish have slight injuries/deformations of the skin | -0.4 | 2.6 | 4 | |
| | | | | | | 3: Individual fish have severe injuries/deformations of the skin | -0.6 | 3.4 | | |
| | | | | | | 4: Some fish have severe injuries/deformations of the skin | -0.8 | 4.2 | | |
| | | | | | | 5: Many fish have severe injuries/deformations of the skin | -1 | 5 | | |
| | | | How many fish have fin injuries? | In / Out RAS / FTS RT / PP | Fin injuries | 0: No fish has injuries/deformations of the fins | 0 | 1 | | |
| | | | | | | 1: Individual fish have slight injuries/deformations of the fins | -0.2 | 1.8 | | |
| | | | | | | 2: Some fish have slight injuries/deformations of the fins | -0.4 | 2.6 | 3 | |
| | | | | | | 3: Individual fish have severe injuries/deformations of the fins | -0.6 | 3.4 | | |
| | | | | | | 4: Some fish have severe injuries/deformations of the fins | -0.8 | 4.2 | | |
| | | | | | | 5: Many fish have severe injuries/deformations of the fins | -1 | 5 | | |
| | Meyer 1991 [124]; Noga 2010 [52]; Klenigeld et al. 2016 [40] | Infections of the body and fins with fungi or moulds are a sign of impaired health and welfare. Information about how many fish an which body parts are affected, and when and how fast the infectio appeared can assist the identification of the causes and potential measures for improvement. | How many fish have fungal or mould infections? | In / Out RAS / FTS RT / PP | Fungal infections | 0: No fish has any fungus | 0 | 1 | | |
| | | | | | | 1: Individual fish have fungal infection of the fins | -0.2 | 1.8 | | |
| | | | | | | 2: Some fish have fungal infection of the fins | -0.4 | 2.6 | 4 | |
| | | | | | | 3: Individual fish have fungal infection of the fins and the body | -0.6 | 3.4 | | |
| | | | | | | 4: Some fish have fungal infection of the fins and the body | -0.8 | 4.2 | | |
| | | | | | | 5: Many fish have fungal infection of the fins and the body | -1 | 5 | | |

**Supplementary S2:** Continued.

| | Literature | Remarks | Parameter question | Location System Species | Parameter | Parameter intervals | PS | SW | PW | SWE PWE |
|---|---|---|---|---|---|---|---|---|---|---|
| Fish external appearance | Ferguson 2006 [98]; North et al. 2008 [76]; Noga 2010 [52]; Pettersen et al. 2014 [36]; Rodger & Phelps 2015 [97]; Kleingeld et al. 2016 [40]; Noble et al. 2020 [42] | The eye is a major organ and any damage to it can affect fish health and welfare. Causes and effects of different eye damages vary depending on the nature of the damage. Hence cataracts, bleedings, injuries and exophthalmia are each separate parameters. Their distinction assists the assessment of the impairment health and welfare and helps identifying the causes and potential measures for improvement. While the inflicted pain or discomfort might be equal amongst fish, the effects of impaired vision are species specific i.e. more impairment is expected in visual predators and highly social species. | Does the fish have clouding of the eye lens? | In / Out RAS / FTS RT / PP | Cataract | 0: Both eyes are clear | 0 | 1 | | |
| | | | | | | 1: One lens shows light clouding | -0.33 | 2.33 | | |
| | | | | | | 2: Both lenses show light clouding or one lens strong clouding | -0.66 | 3.66 | 3 | |
| | | | | | | 3: Both lenses show strong clouding | -1 | 5 | | |
| | | | Does the fish have an injury of the eye? | In / Out RAS / FTS RT / PP | Eye injury | 0: No indication | 0 | 1 | | |
| | | | | | | 1: One-sided small injury, not inflamed or healing | -0.33 | 2.33 | | |
| | | | | | | 2: One-sided injury or both-sided small injury, slightly inflamed | -0.66 | 3.66 | 3 | |
| | | | | | | 3: One-sided severe injury or both-sided injury, inflamed | -1 | 5 | | |
| | | | Does the fish have bulging of the eye? | In / Out RAS / FTS RT / PP | Exophthalmia | 0: No indication | 0 | 1 | | |
| | | | | | | 1: One-sided slight exophthalmia | -0.33 | 2.33 | | |
| | | | | | | 2: Both-sided slight exophthalmia or one-sided exophthalmia | -0.66 | 3.66 | 3 | |
| | | | | | | 3: Both-sided exophthalmia | -1 | 5 | | |
| | | The fins are key for movement and communication and any damage to them can affect fish health and welfare. Causes and effects of different fin damages vary depending on the nature (e.g. rotting, erosion, abrasion, bites) and the location (i.e. pectoral, ventral, anal, caudal, dorsal) of the damage. Hence the fins are each separate parameters. The distinction assists the assessment of the impairment of health and welfare and helps identifying the causes and potential measures for improvement. | Does the fish have damages or deformations of the pectoral fins? | In / Out RAS / FTS RT / PP | Pectoral fins | 0: Undamaged fins | 0 | 1 | | |
| | | | | | | 1: One-sided/both-sided: indications of scar tissue or small/active fin damage | -0.33 | 2.33 | | |
| | | | | | | 2: One-sided/both-sided: active fin damage or indications of fungal infections and/or inflammation | -0.66 | 3.66 | 3 | |
| | | | | | | 3: Both-sided: extensive scar tissue and/or extensive active fin damage (with/without inflammation) or extensive fungal infection or fin loss | -1 | 5 | | |
| | | | Does the fish have damages or deformations of the ventral fins? | In / Out RAS / FTS RT / PP | Ventral fins | 0: Undamaged fins | 0 | 1 | | 1.7 |
| | | | | | | 1: One-sided/both-sided: indications of scar tissue or small/active fin damage | -0.33 | 2.33 | | 1.7 |
| | | | | | | 2: One-sided/both-sided: active fin damage or indications of fungal infections and/or inflammation | -0.66 | 3.66 | 2 | |
| | | | | | | 3: Both-sided: extensive scar tissue and/or extensive active fin damage (with/without inflammation) or extensive fungal infection or fin loss | -1 | 5 | | |
| | | | Does the fish have damages or deformations of the anal fin? | In / Out RAS / FTS RT / PP | Anal fin | 0: Undamaged fin | 0 | 1 | | |
| | | | | | | 1: Indications of scar tissue or small and active fin damage | -0.33 | 2.33 | | |
| | | | | | | 2: Active fin damage or indications of fungal infections and/or inflammation | -0.66 | 3.66 | 2 | |
| | | | | | | 3: Extensive scar tissue and/or extensive active fin damage (with/without inflammation) or extensive fungal infection or fin loss | -1 | 5 | | |
| | | | Does the fish have damages or deformations of the caudal fin? | In / Out RAS / FTS RT / PP | Caudal fin | 0: Undamaged fin | 0 | 1 | | |
| | | | | | | 1: Indications of scar tissue or small and active fin damage | -0.33 | 2.33 | | |
| | | | | | | 2: Active fin damage or indications of fungal infections and/or inflammation | -0.66 | 3.66 | 3 | |
| | | | | | | 3: Extensive scar tissue and/or extensive active fin damage (with/without inflammation) or extensive fungal infection or fin loss | -1 | 5 | | |
| | | | Does the fish have damages or deformations of the dorsal fin? | In / Out RAS / FTS RT / PP | Dorsal fin | 0: Undamaged fin | 0 | 1 | | |
| | | | | | | 1: Indications of scar tissue or small and active fin damage | -0.33 | 2.33 | | |
| | | | | | | 2: Active fin damage or indications of fungal infections and/or inflammation | -0.66 | 3.66 | 3 | |
| | | | | | | 3: Extensive scar tissue and/or extensive active fin damage (with/without inflammation) or extensive fungal infection or fin loss | -1 | 5 | | |

**Supplementary S2:** Continued.

| | Literature | Remarks | Parameter question | Location System Species | Parameter | Parameter intervals | PS | SW | PW | SWE PWE |
|---|---|---|---|---|---|---|---|---|---|---|
| Fish external appearance | Barnes et al. 2014 [49]; Delic et al. 2016 [100]; Zahedi et al. 2019 [101] | As a ratio of weight and length the body condition factor is a health and welfare indicator for fish that reacts in the mid long-term to suboptimal husbandry conditions. As the value is strongly influenced by the basic body shape, the optimal and tolerance values are fish species and life stage specific and ne to be adapted accordingly. | Fulton's condition factor K [bodyweight/ standardlength*x 100] | In / Out RAS / FTS RT | Body condition factor | 0: 1 - 1.3 | 0 | 1 | | |
| | | | | | | 1: 0.8 - 1.5 | -0.33 | 2.33 | | |
| | | | | | | 2: > 1.5 | -0.66 | 3.66 | | |
| | Molnar et al. 2006 [102]; Zakęś et al. 2012 [103]; Steinberg et al. 2017 [104] | | | In / Out RAS / FTS PP | | 3: < 0.8 | -1 | 5 | 3 | |
| | | | | | | 0: 0.9 - 1.1 | 0 | 1 | | |
| | | | | | | 1: 0.7 - 1.3 | -0.33 | 2.33 | | |
| | | | | | | 2: > 1.3 | -0.66 | 3.66 | | |
| | | | | | | 3: < 0.7 | -1 | 5 | | |
| | Ashley 2007 [12]; Branson & Turnbull 2008 [125]; Noble et al. 2012 [117], 2020 [42]; Rodger & Phelps 2015 [97] | Deformations of the spine may inflict pain and/or restrict movement and therefore affect feeding, behavior, and health and welfare. | Does the fish have spinal deformations? | In / Out RAS / FTS RT / PP | Spinal deformation | 0: No indication | 0 | 1 | | |
| | | | | | | 1: Indication of deformation | -0.33 | 2.33 | 3 | |
| | | | | | | 2: Clear deformation | -0.66 | 3.66 | | |
| | | | | | | 3: Strong deformation | -1 | 5 | | |
| | | Deformations of the jaws may inflict pain and/or restrict feedi and breathing and therefore affect health and welfare. | Does the fish have deformations of the lower or upper jaw? | In / Out RAS / FTS RT / PP | Jaw deformation | 0: No indication | 0 | 1 | | |
| | | | | | | 1: Indication of deformation | -0.33 | 2.33 | 3 | |
| | | | | | | 2: Clear deformation | -0.66 | 3.66 | | |
| | | | | | | 3: Strong deformation | -1 | 5 | | |
| | | Injuries of the mouth and the jaws inflict pain and/or restrict feeding and breathing and therefore affect health and welfare | Does the fish have an injury on the mouth? | In / Out RAS / FTS RT / PP | Mouth injury | 0: No indication | 0 | 1 | | |
| | | | | | | 1: A few small injuries | -0.33 | 2.33 | 3 | |
| | | | | | | 2: Several small injuries | -0.66 | 3.66 | | |
| | | | | | | 3: One or more large/deep injuries | -1 | 5 | | |
| | Ferguson 2006 [98]; North et al. 2008 [76]; Noga 2010 [52] | External pathogens (parasites, fungi, moulds, bacteria) generz Are parasites visible in impair fish health and welfare. Assessing the parasite load in a mucus swop under a mucus in a semiquantitative way helps assessing the health, 40-100 fold tracking counter measures and detecting problems early. | | In / Out RAS / FTS RT / PP | Mucus pathogens | 0: No parasites detectable | 0 | 1 | | |
| | | | | | | 1: A few parasites | -0.33 | 2.33 | 4 | 1.7 |
| | | | | | | 2: Considerable parasite load | -0.66 | 3.66 | | 1.7 |
| | | | | | | 3: Heavy parasite load | -1 | 5 | | |
| | Ferguson 2006 [98]; North et al. 2008 [76]; Noble et al. 2012 [117]; Rodger & Phelps 2015 [97]; Kleingeld et al. 2016 [40] | The skin is a major barrier between the fish and its environme and any damage to it can affect fish health and welfare. Cause and effects of different skin damages vary depending on the nature of the damage. Hence alterations, fungal infections, bleedings, injuries and scale loss are each separate parameter Their distinction assists the assessment of the impairment of health and welfare and helps identifying the causes and potential measures for improvement. | Does the fish have alterations of the skin? | In / Out RAS / FTS RT / PP | Skin alterations | 0: No indication | 0 | 1 | | |
| | | | | | | 1: A few small alterations (tumors, swellings, rashes, bleedings) | -0.33 | 2.33 | 3.5 | |
| | | | | | | 2: Several small alterations (tumors, swellings, rashes, bleedings) | -0.66 | 3.66 | | |
| | | | | | | 3: One or more large alterations (tumors, swellings, rashes, bleedings) | -1 | 5 | | |
| | | | Does the fish have fungi or moulds on the skin? (fins are excluded) | In / Out RAS / FTS RT / PP | Skin fungus | 0: No indication | 0 | 1 | | |
| | | | | | | 1: A few small areas infected | -0.33 | 2.33 | 4 | |
| | | | | | | 2: Several small areas infected | -0.66 | 3.66 | | |
| | | | | | | 3: One or more large areas infected | -1 | 5 | | |
| | | | Does the fish have an injury of the skin or loss of scales? | In / Out RAS / FTS RT / PP | Skin injury | 0: No indication | 0 | 1 | | |
| | | | | | | 1: A few small injuries or small areas with scale loss | -0.33 | 2.33 | 3.5 | |
| | | | | | | 2: Several small injuries and/or small areas with scale loss | -0.66 | 3.66 | | |
| | | | | | | 3: One or more large/deep injuries and/or areas with scale loss | -1 | 5 | | |
| | Branson & Turnbull 2008 [125]; Pettersen et al. 2014 [36]; Noble et al. 2020 [42] | Injuries or deformations of the opercula can impose pain and impair breathing and therefore affect health and welfare. | Does the fish have a damage or deformation of the gill cover/opercula? | In / Out RAS / FTS RT / PP | Gill cover | 0: Both-sided: undamaged opercula | 0 | 1 | | |
| | | | | | | 1: One-sided/both-sided: opercula covers min. 2/3 of gill area | -0.33 | 2.33 | 2 | |
| | | | | | | 2: One-sided/both-sided: opercula covers min. 1/3 of gill area | -0.66 | 3.66 | | |
| | | | | | | 3: One-sided/both-sided: opercula covers less than 1/3 of gill area | -1 | 5 | | |
| | Ferguson 2006 [98]; North et al. 2008 [76]; Pettersen et al. 2014 [36] | Injuries or alterations of the gill's primary lamellae may impos pain and can impair breathing and therefore affect health and welfare. | Does the fish have damaged or discolored gills? | In / Out RAS / FTS RT / PP | Gills | 0: Both-sided: undamaged, red gills | 0 | 1 | | |
| | | | | | | 1: One-sided/both-sided: indications of damaged and/or discolored gill tissue | -0.33 | 2.33 | 5 | |
| | | | | | | 2: One-sided/both-sided: several small areas of damaged and/or discolored gill tissue | -0.66 | 3.66 | | |
| | | | | | | 3: One-sided/both-sided: extensive areas of damaged and/or discolored gill tissue | -1 | 5 | | |

**Supplementary S2:** Continued.

**Supplementary S2:** Continued.

| | Literature | Remarks | Parameter question | Location System Species | Parameter | Parameter intervals | PS | SW | PW | SWE PWE |
|---|---|---|---|---|---|---|---|---|---|---|
| Fish internal appearance | Ferguson 2006 [98]; Ashley 2007 [12]; North et al. 2008 [76]; Noga 2010 [52]; Pettersen et al. 2014 [36] | Healthy and well functioning organs are key for fish health and welfare. Each organ is a parameter as resulting impairments may be organ specific. The ex nature of the damage of an organ assists the identification of the causes and potential measures for improvement. | How does the fish's heart look like? | In / Out RAS / FTS RT / PP | Heart | 0: Inconspicuous | 0 | 1 | 3 | |
| | | | | | | 1: Slight discoloration | -0.33 | 2.33 | | |
| | | | | | | 2: Discolored and/or small necrosis and/or small hemorrhages | -0.66 | 3.66 | | |
| | | | | | | 3: Severely discolored and/or necrosis and/or hemorrhages | -1 | 5 | | |
| | | | How does the fish's kidney look like? | In / Out RAS / FTS RT / PP | Kidney | 0: Inconspicuous | 0 | 1 | 3.5 | |
| | | | | | | 1: Slight discoloration | -0.33 | 2.33 | | |
| | | | | | | 2: Discolored and/or slightly granular | -0.66 | 3.66 | | |
| | | | | | | 3: Severely discolored and/or granular | -1 | 5 | | |
| | | | How does the fish's spleen look like? | In / Out RAS / FTS RT / PP | Spleen | 0: Inconspicuous | 0 | 1 | 4 | |
| | | | | | | 1: Slight enlargement | -0.33 | 2.33 | | |
| | | | | | | 2: Discolored and/or slightly enlarged | -0.66 | 3.66 | | |
| | | | | | | 3: Severely discolored and/or enlarged | -1 | 5 | | |
| | | | How does the fish's liver look like? | In / Out RAS / FTS RT / PP | Liver | 0: Inconspicuous | 0 | 1 | 4 | |
| | | | | | | 1: Slight discoloration | -0.33 | 2.33 | | |
| | | | | | | 2: Discolored and/or slightly enlarged and/or small necrosis | -0.66 | 3.66 | | |
| | | | | | | 3: Severely discolored and/or enlarged and/or necrosis | -1 | 5 | | |
| | Ferguson 2006 [98]; Pettersen et al. 2014 [36]; Noble et al. 2020 [42] | Healthy intestines are a sign of and a prerequisite for good nutrition and hea The exact nature of the damage of the organ assists the identification of the causes and potential measures for improvement. | How do the fish's stomach and intestines look like? | In / Out RAS / FTS RT / PP | Intestines | 0: Homogeneously filled with smooth food pulp | 0 | 1 | 3 | |
| | | | | | | 1: Unevenly filled with food pulp | -0.33 | 2.33 | | |
| | | | | | | 2: Indications of inflammation and change in tissue (discoloring, swelling, tumors) | -0.66 | 3.66 | | |
| | | | | | | 3: Inflammation and/or change in tissue (discolored and/or swollen tissue, tumors, hemorrhages, necrosis) or foreign objects | -1 | 5 | | |
| | Ferguson 2006 [98]; Noga 2010 [52]; Pettersen et al. 2014 [36] | Healthy muscles are key for fish health and welfare. The exact nature of the damage of the tissue assists the identification of the causes and potential measures for improvement. | How does the fish's muscle tissue look like? | In / Out RAS / FTS RT / PP | Muscles | 0: Normal | 0 | 1 | 3 | 1.7 1.7 |
| | | | | | | 1: Single small hemorrhages, small vaccination damage | -0.33 | 2.33 | | |
| | | | | | | 2: Several small or single extensive hemorrhages and/or clear vaccination damage | -0.66 | 3.66 | | |
| | | | | | | 3: Extensive hemorrhages and/or necrosis and/or extensive vaccination damage | -1 | 5 | | |
| | Noga 2010 [52]; Rodger & Phelps 2015 [97]; Noble et al. 2020 [42] | A healthy body cavity is a sign of and a prerequisite for good health. The exa nature of the damage of the organ assists the identification of the causes an potential measures for improvement. | How does the fish's body cavity look like? | In / Out RAS / FTS RT / PP | Body cavity | 0: Inconspicuous | 0 | 1 | 3 | |
| | | | | | | 1: Slight bleeding into the intestine and/or abdominal fat and/or swim bladder wall | -0.33 | 2.33 | | |
| | | | | | | 2: Bleeding into the intestine and/or abdominal fat and/or swim bladder wall / slight fluid accumula | -0.66 | 3.66 | | |
| | | | | | | 3: Severe bleeding into the intestine and/or abdominal fat and/or swim bladder wall / fluid accumu | -1 | 5 | | |
| | North et al. 2008 [76]; Wootton & Smith 2015 [138]; Folkedal et al. 2016 [43] | Under farming conditions (except for reproduction) the development of the gonads and expression of spawning behavior are usually not desired. Due to additional stress and reduced immune system an active reproduction state i included as a welfare parameter. This parameter and its intervals should be adapted depending of the fish species, life stage and purpose of the husban | How far are the ovaries or testes developed? | In / Out RAS / FTS RT / PP | Repro-ductive organs | 0: Not developed | 0 | 1 | 2 | |
| | | | | | | 1: Slightly developed/enlarged | -0.33 | 2.33 | | |
| | | | | | | 2: Developed/enlarged | -0.66 | 3.66 | | |
| | | | | | | 3: Ready to spawn | -1 | 5 | | |
| | Ferguson 2006 [98]; North et al. 2008 [76]; Noga 2010 [52] | Injuries or alterations of the gill's secondary lamellae may impose pain and i impair breathing and therefore affect health and welfare. | How do the fish's secondary gill lamellae look like under a 40-100 fold magnification? | In / Out RAS / FTS RT / PP | Gill lamellae | 0: Normal | 0 | 1 | 5 | |
| | | | | | | 1: Lamellae slightly swollen | -0.33 | 2.33 | | |
| | | | | | | 2: Lamellae swollen, small hemorrhages and/or necrosis and/or edema and/or detachment of epitheli | -0.66 | 3.66 | | |
| | | | | | | 3: Lamellae severely swollen, hemorrhages and/or necrosis and/or edema and/or detachment of epithelium, extensive mucus | -1 | 5 | | |
| | | External pathogens (parasites, fungi, moulds, bacteria) generally impair fish health and welfare. Assessing the parasite load of the gills in a semi-quantitative way helps assessing the health, tracking counter measures and detecting problems ea | Are parasites visible in a gill sample under a 40-100 fold magnification? | In / Out RAS / FTS RT / PP | Gill pathogens | 0: No parasites detectable | 0 | 1 | 4 | |
| | | | | | | 1: A few parasites | -0.33 | 2.33 | | |
| | | | | | | 2: Considerable parasite load | -0.66 | 3.66 | | |
| | | | | | | 3: Heavy parasite load | -1 | 5 | | |

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**Supplementary S3:** Calculation example

A step-by-step example, using the parameter water temperature, of how the equation of the model is used to calculate the module grade based on the information given in table 2 (for the convenience of the reader the necessary excerpt of Table 2 is shown). The steps are:

**1**. Locate the measured value in the intervals, e.g. 18 °C for an aquaculture system with pikeperch

**2.** Obtain the interval's parameter score (PS): -0.33

**3.** Obtain the interval's score weight (SW): 2.33

**4.** Obtain the parameter's parameter weight (PW): 4

**5.** Obtain the corresponding score weight exponent (SWE): 1.7

**6.** Obtain the corresponding parameter weight exponent (PWE): 1.7

**7.** Obtain the product for this parameter: $-0.33 \times 2.33^{1.7} \times 4^{1.7} = -14.67$

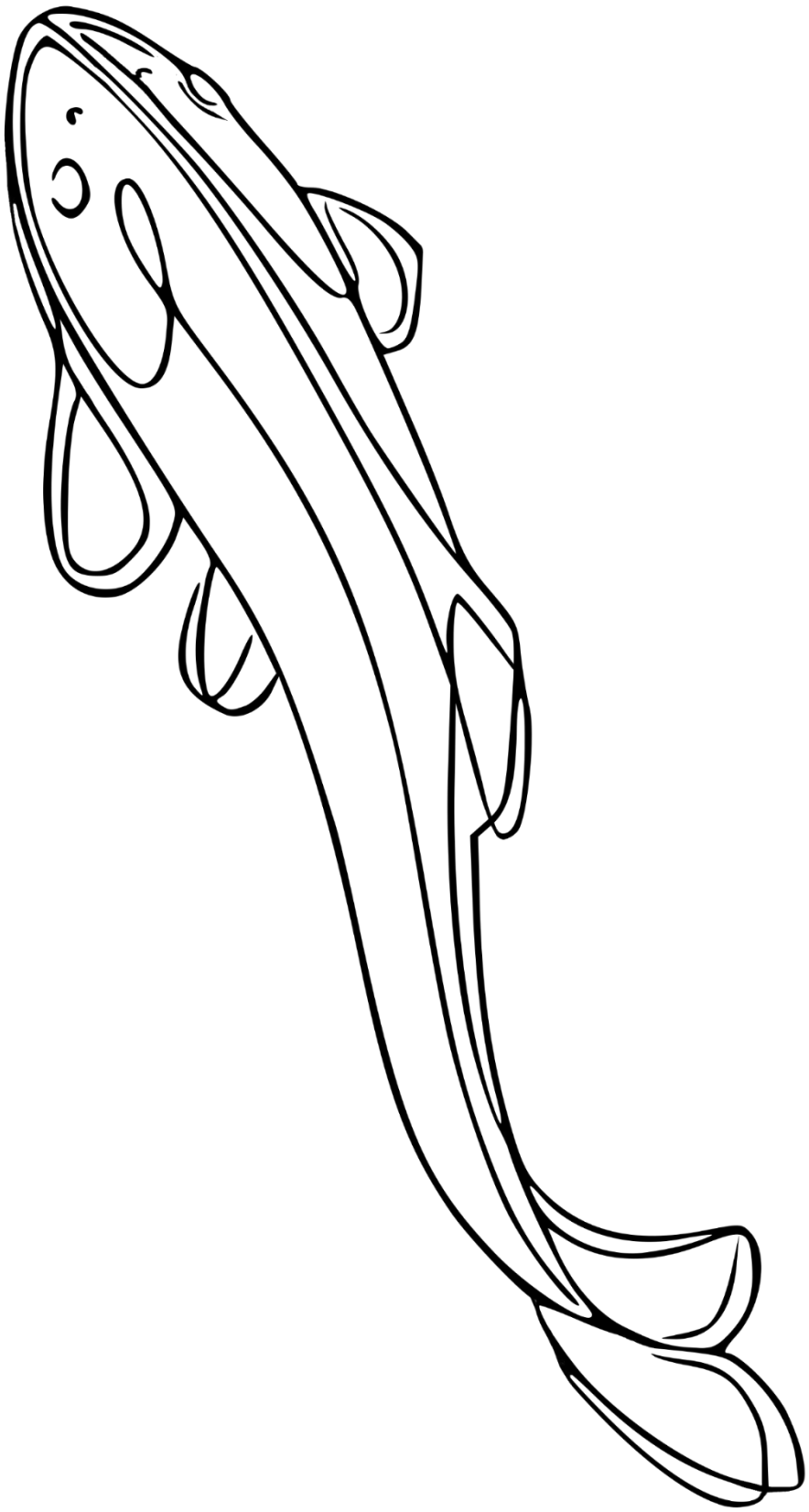
**8.** Repeat 1-6 with all used parameters of the module and add up all the products of used parameters

**9.** Dived by the product of **the used** score and parameter weights

**10.** Add 1

| Parameter question | Location System Species | Parameter | Parameter intervals | PS | SW | PW | SWE | PWE |
|---|---|---|---|---|---|---|---|---|
| Temperature of the system water in [°C] | In / Out RAS / FTS RT | Temperature | Optimum: [10–16] | 0 | 1 | ❹ 4 | ❺ 1.7 | ❻ 1.7 |
| | | | Within target range: [6–10) ∪ (16–18] | -0.33 | 2.33 | | | |
| | | | Within the tolerance range [4–6) ∪ (18–22] | -0.66 | 3.66 | | | |
| | | | Outside the tolerance range: [0–4) ∪ (22–35] | -1 | 5 | | | |
| | In / Out RAS / FTS PP | | Optimum: [20–25] | 0 | 1 | | | |
| | | | ❶ Within target range: [13–20) ∪ (25–28] | ❷ -0.33 | ❸ 2.33 | | | |
| | | | Within the tolerance range [8–13) ∪ (28–30] | -0.66 | 3.66 | | | |
| | | | Outside the tolerance range [0–8) ∪ (30–40] | -1 | 5 | | | |

$$MG_j = \frac{\overbrace{\sum_i \overbrace{PS_i}^{❷} * \overbrace{SW_i}^{❸}{}^{\overbrace{SWE_i}^{❺}} * \overbrace{PW_i}^{❹}{}^{\overbrace{PWE_i}^{❻}}}^{❽}}{\underbrace{\sum_i SW_i * PW_i}_{❾}} + 1 \quad ❼$$



# Discussion

## A standard protocol for the efficient evaluation of mycotoxins in aquaculture (Chapter 1)

### *Toxicity of ochratoxin to early life stages of zebrafish*

The chapter confirmed that the fish embryo acute toxicity test has a good applicability and delivers reliable results. The study examined the effects of ochratoxin A on the embryonic development of zebrafish and compared the results to existing data. Endpoints were assessed at three different levels, mortality (lethal), malformation of body parts (morphological) and reactive oxygen species (ROS) formation using whole-body fluorescence (physiological). These three levels delivered different median effect concentrations after 96 h with a $LC_{50}$ of 0.36 mg/l and $EC_{50}$ of 0.29 mg/l for malformations and 0.07 mg/l for ROS. This pronounced difference in effect concentrations due to different endpoints may cause variability in toxicity data reported. The results were therefore compared to previous work, most notably a study exploring the teratogenicity of ochratoxins in zebrafish embryos (Haq *et al.* 2016). The study used a similar experimental protocol and reported toxicity values at 96 hpf for mortality ($LC_{50}$ = 0.1 mg/l) and body deformations ($EC_{50}$ = 0.01 mg/l). Similarly, other exposure trials with ochratoxin A in zebrafish showed median lethal concentrations of 0.1–0.25 mg/l (Haq *et al.* 2016; Wu *et al.* 2016, 2018; Khezri *et al.* 2018). The chapter hence highlighted three key points: First, the applied protocol generated reproducible results. The repetition of previous studies confirmed that the fish embryo acute toxicity test delivered repeatable and reliable results for this mycotoxin. Second, the metadata indicated variability in toxicological data. The comparison to previous trials confirmed that the typical variability of toxicological metadata sets (Busquet *et al.* 2014) is equally present in ochratoxin datasets. Third, individual exposure improved the statistical power of the protocol. Contrary to previous studies (Haq *et al.* 2016; Wu *et al.* 2016) the embryos were incubated individually, which considerably improved the statistical analysis due to a larger experimental sample size. Overall, the protocol yielded results with good accuracy, yet limited precision.

### *A standard protocol for mycotoxin research in aquaculture*

The fish embryo acute toxicity test, as a standardised protocol, can benefit the investigation of mycotoxicosis in aquaculture. The protocol (OECD 2013*b*) has been thoroughly tested and optimised in the past (Busquet *et al.* 2014) and includes standards where needed (sample size, assessment timing or trial validation criteria), while providing freedom where required (fish strain, additional endpoints or experimental design). The core protocol requires only basic laboratory equipment, yet the method can be adapted to meet scientific high-end demands by adding specific endpoints (Krzykwa *et al.* 2018) or fish strains (Koiwa *et al.* 2019). Due to this applicability and flexibility the protocol is a key method in vertebrate animal trials (Braunbeck *et al.* 2015). Finally, the protocol is in line with the 3R principles while still being a systemic experimental model based on the complexity of a whole organism (OECD 2012).

The zebrafish, as the main model organism for the fish embryo acute toxicity test, is a suitable species for research on mycotoxins including their adverse effects in aquaculture. The species has numerous advantages in husbandry (ZFIN 2021) and has proven invaluable in biomedical research (Lin *et al.* 2016), drug screening (Cassar *et al.* 2020) and toxicology (Padilla & Glaberman 2020). Further, the zebrafish has recently been proposed as a model for fish diseases in aquaculture (Jørgensen 2020) and specifically for the evaluation of adverse effects of mycotoxins (Juan-García *et al.* 2020). It is, however, important to understand and avoid the potential pitfalls of universally used model species as well as over-standardisation. Extrapolation of insights to other species may be limited (Farris 2020) and uniform experimental environments (Richter *et al.* 2009), little biological variation (Voelkl *et al.* 2020) and homogeneity of study samples (Voelkl *et al.* 2018) in rodent research caused the so-called reproducibility crisis (Voelkl & Würbel 2016). Various fields of science face studies with results that suggest a fallaciously high experimental sensitivity and findings with little external validity (Würbel 2000). Zebrafish research can benefit from emerging solutions such as ensuring genetic diversity of trial species, providing environmental enrichment in husbandry, incorporating phenotypic plasticity (Voelkl & Würbel 2016) and improving statistical analysis and reporting (Gosselin 2020).

It is to be expected that future research will focus on three major aspects: the screening and monitoring of mycotoxin occurrence, the detailed study of individual prevalent mycotoxins and the search and confirmation of remedies for mycotoxicosis. In all three aspects standardised protocols tailored to zebrafish can facilitate efficient and expedient research of mycotoxicosis and therefore improve fish welfare in aquaculture. More precisely, the speed and applicability of the fish embryo toxicity test enable an effective screening and monitoring. Further, the existing knowledge and available techniques for model species facilitate detailed investigations about relevant mycotoxins. Finally, the gained insights promote the development of rapid laboratory testing (Lattanzio *et al.* 2019) and on-site test kits, allowing the testing of feed ingredients during feed production as well as feeds directly on fish farms. Together these advantages assist the prevention, diagnosis and treatment of mycotoxicosis and hence promote better health and welfare of farmed fish.

The topic of mycotoxins in aquaculture is complex and therefore calls for a comprehensive risk assessment. However, the variability in toxicological data, as shown in this chapter, impedes the hazard characterisation process. Potential factors contributing to this variability in metadata sets were therefore addressed in chapter 2.

# Standardized protocols to reduce data variability in mycotoxin research (Chapter 2)

## *Sources of data variability in mycotoxin embryotoxicity trials with fish*

Aspects of the methodology and biology of individual studies explained a considerable amount of variability in mycotoxin hazard characterisation datasets on fish. More precisely, the chapter revealed the relative importance of the two methodological factors (*mycotoxin identity* and *application method*) and two biological factors (*fish species* and *endpoint assessed*) on the amount of variability in mycotoxin hazard data. Based on a literature survey the factor *fish species* proved most important, followed by the *application method* and only then the *mycotoxin identity* weighted in. In contrast, the choice of *endpoint assessed* had no influence when comparing toxicological values across studies. This confirmed the limited possibility of extrapolation of results between mycotoxins, highlighted similar limitations when comparing applications methods and indicated considerable constraints when comparing or extrapolating toxicological values across fish species. This strong influence of the *species* as well as the negligible relevance of *endpoint* may, however, have been affected by peculiarities of the literature dataset or biases in reporting. Statistical interactions between *species* and *mycotoxin* as well as *application* may have misleadingly increased the relevance of the *species* as a factor. Similarly, statistical interactions of the *endpoint* with *mycotoxin* and *application* might have led to a decreased relevance of *endpoint* as a factor in the literature survey.

Therefore, the two factors were further investigated in a fish embryo acute toxicity test. The test was conducted with one varying methodological factor, i.e. *mycotoxin identity* (ochratoxin A, deoxynivalenol and zearalenone) and two varying biological factors, i.e. *fish species* (zebrafish, pikeperch and rainbow trout) and *endpoint assessed* (lethality and morphological malformations). The *mycotoxin identity* was not included as a factor to standardize upon, but as a landmark to compare the other factors with. The results indicated that the two biological factors have a comparable effect on the variability of toxicological values within a study. The differences in relevant concentrations caused by different endpoints and species ranged from factor 2 to 10 and hence were within the typically observed magnitude of variance in toxicological metadata sets (Gustafson *et al.* 2012). Hence, the choice of endpoints as well as fish species was similarly important for individual toxicological studies (Benfenati *et al.* 2016) and the discrepancies between the estimated relevance of the factors in the literature survey vs. the toxicity test were likely caused by peculiarities of the metadata set.

While various methodological factors, e.g. environmental conditions (Hrovat *et al.* 2009) or experimental design (Keddig *et al.* 2015), and biological aspects, e.g. developmental stage (Voslářová *et al.* 2006; Dutra Costa *et al.* 2020) or body condition (Zhao *et al.* 2020), were identified before as having an effect on data variability for hazard characterisation, this chapter put four factors in the context of relevance. The result, i.e. the comparable relevance, revealed that the formulated suggestions to reduce data variability (see below) have equal priority and should be addressed with equal effort when conducting or analysing mycotoxin hazard trials.

### *Reduction of variability in mycotoxin hazard characterisation trials with teleosts*

The chapter revealed how increased standardisation can benefit hazard characterisation process for mycotoxins in fish feed. While there are harmonised protocols for fish exposure trials for different life stages and exposure durations (OECD 2012), this is not the case for feeding trials. Despite fish being increasingly important for the food industry (FAO 2020) as well as science (Kinth *et al.* 2013) and feed uptake being a relevant path for the uptake of chemicals, there are no internationally agreed upon protocols for fish feeding trials. This is undoubtedly a cause for the variety of trial designs and methods used, which is impeding data interpretation and trial comparability (Knutsen *et al.* 2017*a*, *b*). The result is a lack of fish-specific mycotoxin contamination limits (European Commission 2006, 2016), which makes an improved risk assessment process for mycotoxins vital (Mantovani 2010) especially for aquaculture feeds (Glencross *et al.* 2020).

Therefore, specific suggestions are formulated in the chapter that can contribute to an improved standardisation in hazard characterisation. First, the typically mycotoxin-centric hazard characterisation analysis should select studies based on their application method, i.e. excluding injection and gavage trials as well as continuing to separate medium exposure and feeding trials. Second, the analyses of metadata sets should remain mycotoxin-specific, which makes separate risk assessment processes necessary for emerging mycotoxins and potentially also for mixtures thereof. Third, toxicological studies should include endpoints that are widely used to increase the comparability and external validity of the results (Schaefer & Myers 2017) as well as endpoints that are specific for the study purpose or the mycotoxin to increase the sensitivity of the study (Hedgpeth *et al.* 2019). Fourth, studies should use fish species that are representative for the study purpose, e.g. zebrafish for basic research on toxicodynamics (Juan-García *et al.* 2020) and trout, salmon and carp for applied research on mycotoxicosis in aquaculture (Cai *et al.* 2019). Fifth, aspects of endpoints (assessment methods or biological relevance) as well as characteristics of the fish (age, condition or strain) should be reported in a transparent and complete manner.

In the light of the reproducibility crisis (Voelkl *et al.* 2018), standardisation is limited in the extend it can and should reduce data variability. A data variability of one magnitude seems to be inherent to toxicological hazard characterisation metadata (Gustafson *et al.* 2012). Therefore, safety factors of 10 or higher should be included for the definition of contamination limits for fish feed and feed ingredients. Additionally, metadata sets should consist of the existing literature in its entirety, which allows contamination limits to be based on possibly variable and broad but valid and representative information. Together, these basic steps of standardisation can benefit mycotoxin research in aquaculture and assist future hazard characterisations and risk assessments.

The emphasis may be on the farmed fish, however, the welfare of the trial animals is important as well. While an improved risk assessment benefits the fish in fish farming, better standards in toxicological trials contribute to increased welfare of fish used during the research. Especially the second 3R principle, the pursuit to reduce, profits from decreased data variability. Less variable trial data allows for the reduction of samples sizes and decreased variability in metadata facilitates a hazard characterisation based on fewer studies. In both cases trial animal numbers are reduced, in line with the 3R guideline.

Typically, toxicological trials focus on fish health, potentially not least due to a lack of methods to assess fish welfare. However, welfare is crucial in both teleost research as well as fish farming. Therefore, a standardised protocol for the on-farm, or in-lab, fish welfare assessment was developed in chapter 3.

## A model for the standardised on-farm assessment of fish welfare (Chapter 3)

### *MyFishCheck: A model to assess fish welfare in aquaculture*

The chapter described the development of a model for on-farm fish welfare assessment that is comprehensive, applicable and developable. For this purpose, fish welfare was defined in the context of allostasis (Korte *et al.* 2007) and semantic data modelling (Embley 2009) was used as a method to gather, manage and correlate existing information. The resulting ontology, a digital network of the current knowledge about fish welfare, facilitated the identification of *needs*, i.e. fundamental requirements for welfare, and *parameters*, i.e. quantifiable aspects of welfare. These parameters were evaluated based on their relevance, reliability and practicability and 80 were selected and grouped into five distinct modules: *farm management*, *water quality*, *fish group behaviour*, *fish external* and *fish internal appearance*. Based on current literature optimal values of these parameters, that promote good welfare, were defined and framed in a scoring system. The developed equation summarized the inputs from the parameters within the modules and yielded five different welfare grades.

The digital application of the model consisted of an easy-to-adapt Microsoft® Excel® file and an easy-to-use Android™ app. The app allowed the fish farmers, the main target audience of the application, a fast access to the model for both creating a new assessment and comparing previous assessments. When using the model on-farm, a high consistency in how parameters are measured, scored and evaluated was important. The digital user interface facilitated this standardised capturing of data as the units and ranges of numeric values as well as the definitions of scores were set. This ensured that the assessments can be compared across time in order to judge changes on a given farm, as well as across space to evaluate different systems or fish species. With its adaptability to different fish species, farming systems, regulations and purposes as well as its user-friendly digital version, the model was a next step towards improved fish welfare assessment and provided a basis for future positive developments for the industry, the farmers and the fish.

### *A new model for on-farm fish welfare assessment in aquaculture*

The model presented is a step into the direction of standardised fish welfare assessment. A more standardised method can facilitate the harmonised on-farm evaluation of fish welfare, which will promote improvements within the industry, assist the fact-based discussion about animal welfare in aquaculture, help expedient advancements in regulations and support a sustainable meat production. So far assessing fish welfare in aquaculture has proven difficult (Stien *et al.* 2020) and no existing protocol or method gained widespread acceptance in the industry. The proposed model is more applicable (Noble *et al.* 2018, 2020), more comprehensive (Kleingeld *et al.* 2016; Müller-Belecke 2019), more adaptable (Stien *et al.* 2013;

Pettersen *et al.* 2014) and more inclusive (Saraiva *et al.* 2019*a*; Studer *et al.* 2020) than preceding attempts and hence is the next step in this process of fish health and welfare development.

A notable strength of the model is the scoring system. It allows for various parameters to be incorporated into one equation that eventually results in one welfare grade. This scoring step is needed as the parameters have different units, different assessment methodologies and different levels of standardisation. The scoring further provides additional flexibility and adaptability for the model since the parameters themselves, the interval limits and the interval number can be adjusted to new knowledge without affecting the use of the equation. While the first two are subject to biological reasoning, the number of intervals is a question of standardisation. With more intervals the model becomes potentially more scientifically accurate but probably also more system- and species-specific. More importantly, this higher resolution requires more available literature since each interval limit needs a scientific basis. Furthermore, more scoring intervals typically reduce the applicability of scoring systems by laypeople. This process of balancing generalisation and specificity led to different interval numbers for the five modules. In conclusion, differential scoring allows the incorporation of parameters with different levels of standardisation and allows a coarser grading where an empirical basis for more detail is weak and a finer grading where specificity is possible and adds value.

The model has yet to be tested on-farm in mid- and long-term application trials. A verification is difficult due to issues with self-dependent justification (Hahn 2011), however, the model can be validated by demonstrating the operational effect of the application (Irobi *et al.* 2004). More precisely, the evaluation of quantifiable aspects like applicability on farm, repeatability of results, robustness towards missing data and the objective long-term effect of its use will reveal if the model and its digital application indeed assist fish health and welfare improvements.

Potential drawbacks of the model, which may be subject to future adaptations and improvements, are the required knowhow for the use of the model and interpretation of the grades. While the app provides specific descriptions for each parameter, the application of the model is certainly facilitated if the user has a basic understanding of fish biology, fish behaviour and aquaculture technology. Further, experts with extensive knowledge in these areas may find the app simplistic but still helpful mainly for the purpose of an assisted documentation process. Therefore, the current model covers the audience of knowledgeable laymen and may need divergent development if the targeted audience changes. Similarly, the interpretation of the modular welfare grades is not covered by the model. While the model aims at providing a standardised help for the assessment of fish welfare, the correct interpretation of the results within the context of a specific situation of aquaculture system is incumbent upon the user. The transparent, modular and adaptable nature of the model will allow its development for further users, systems and species and provides a basis for ongoing positive developments for the industry, the farmers and the fish.

The next crucial step for fish welfare improvements in aquaculture is implementation. While applied research will continue to contribute knowledge and frameworks about the fish and their welfare, it is for the industry to implement these fish welfare concepts. Joint projects of industry and science assist the development of applicable tools, i.e. a loop of use, feedback and revision, which gradually results in

practicable and implemented methods, that will directly affect the welfare of each farmed fish. The European Union, representative for many authorises, included the welfare of farmed fish in their Horizon Europe program, signalling the rising interest in this topic throughout society.

# Advancement of fish health and welfare in aquaculture through standardisation

## *Improvement of fish health and welfare in aquaculture through standardisation*

Applied research on fish health and welfare misses methodological standards in certain areas and reasonable standardisation can assist effective research and therefore facilitate improvements of fish health and welfare in aquaculture. Chapter 1 describes the use of the fish embryo acute toxicity test in mycotoxin research and highlights how the use of an elsewhere established and standardised protocol can benefit emerging topics such as mycotoxicosis in teleost. Chapter 2 elaborates on sources of variability in mycotoxin metadata sets and underlines how the adherence to simplistic standards can benefit challenging fields such as mycotoxin hazard characterisation. Chapter 3 develops a model for the standardised on-farm assessment of fish welfare and demonstrates how stepwise progress can lead to a sensible standardisation of highly complex systems such as fish welfare. The thesis reveals that the process of standardisation requires profound understanding of the system at hand in order to balance costs and benefits of standards (Fig. 1).

## *Fish health and welfare in aquaculture*

While future insights will continue to shape our understanding of fish, the current knowledge is applied in practice. Fish welfare is addressed in the aquaculture industry (van de Vis *et al.* 2020) and frameworks for the assessment and improvement are developed (Stien *et al.* 2020; Tschirren *et al.* 2021). It is to be expected that welfare will gradually gain relevance in the aquaculture industry, as well as science (Utne-Palm & Smith 2020), fisheries (Breen *et al.* 2020), recreational fishing (Ferter *et al.* 2020) and private husbandry (Torgersen 2020). This shift in relevance is less a temporary trend but a fundamental change which will shape fish farming towards a more fish-conscious industry.

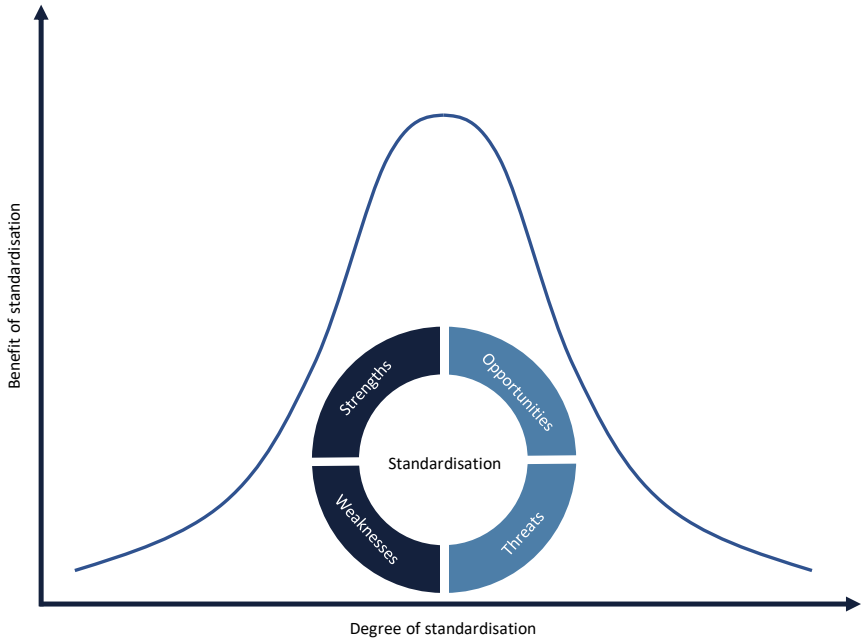



**Figure 1:** An assessment of potential costs and benefits of standardisation, e.g. in form of a SWOT analysis, can assist to optimize the degree of standardisation of a process, a method or a protocol.

## *Standardisation in applied research*

Standardisation has a bell-shaped curve (Fig.1). While optimal and reasonable standardisation yield effective and efficient systems, overregulation results in stiff and slow processes and underregulating fosters erratic and disruptive methods. The optimal balance between flexibility and uniformity is specific to an individual process, method or system. This optimum should be based on fact-driven objectivity where possible, but is admittedly and inevitably subjective to some extent. This approach is nevertheless expedient given that the process of standardisation is accompanied by a transparent reasoning. This allows for the subjectivity to be gradually replaced by objective knowledge as it is gathered in the future.

## *Closing remarks*

It is time to recognise fish welfare as what it is, our duty and the fish's right. The past decades have fundamentally changed how we think about fish and the next decades are the time to act upon this new understanding. In a moment when exploring and investigating turn into implementing and applying this thesis is, hopefully, able to contribute its part. The ongoing sustainability transformation as well as the digital revolution, that is about to take off, pose challenging hurdles and auspicious opportunities for the young, diverse and vivacious industry of aquaculture. The route may not be smooth, but it can and must be walked and I am grateful to be part of it.

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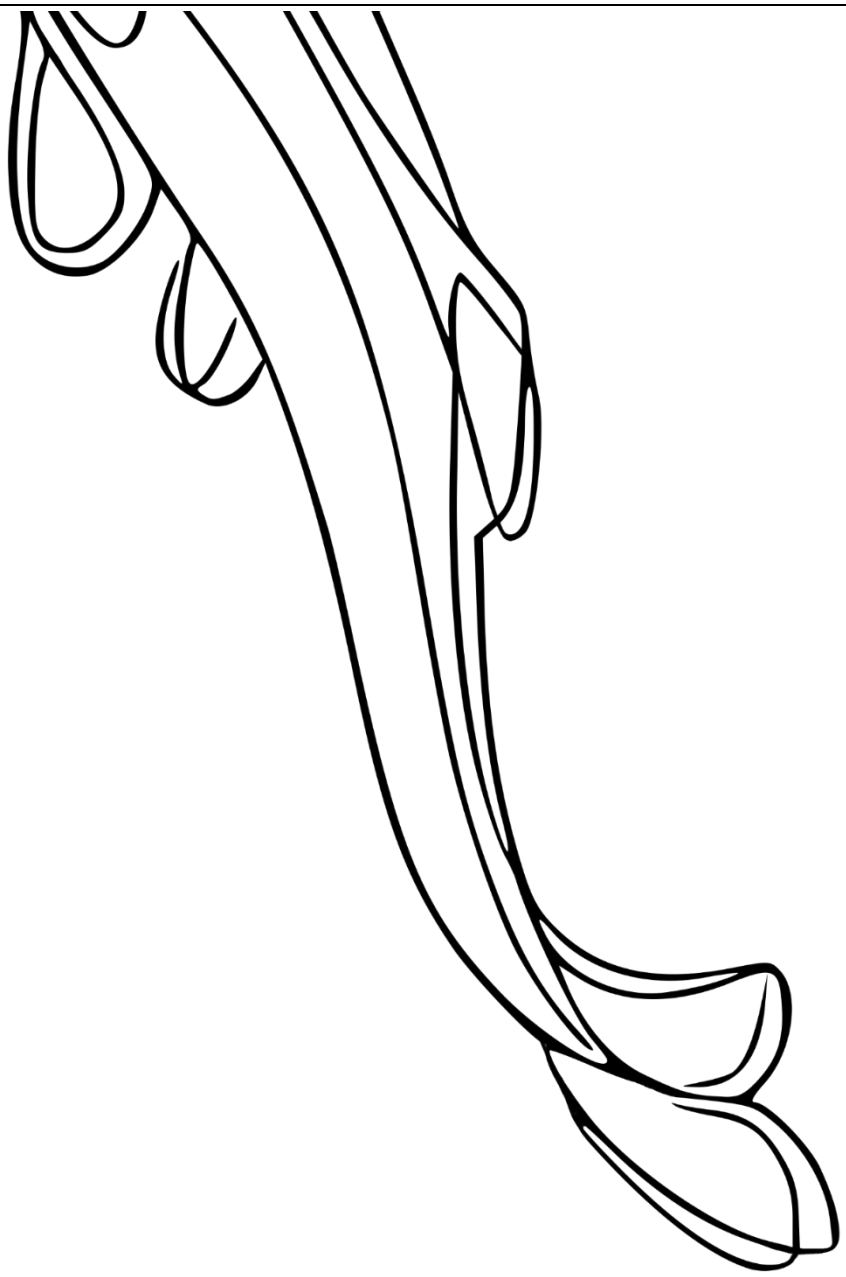

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## *Declaration of consent*

on the basis of Article 18of the PromR Phil.-nat. 19

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| Name: | Linda Tschirren |
| Registration number: | 04-124-350 |
| Study program: | PhD in Ecology and Evolution |
| Title of the thesis: | Towards an effective improvement of fish health and welfare in aquaculture with standards for protocols for both the evaluation of mycotoxins and the on-farm assessment of fish welfare |
| Supervisor: | Prof. Dr. Helmut Segner |

I declare herewith that this thesis is my own work and that I have not used any sources other than those stated. I have indicated the adoption of quotations as well as thoughts taken from other authors as such in the thesis. I am aware that the Senate pursuant to Article 36 paragraph 1 litera r of the University Act of September 5th, 1996 and Article 69 of the University Statute of June 7th, 2011 is authorized to revoke the doctoral degree awarded on the basis of this thesis. For the purposes of evaluation and verification of compliance with the declaration of originality and the regulations governing plagiarism, I hereby grant the University of Bern the right to process my personal data and to perform the acts of use this requires, in particular, to reproduce the written thesis and to store it permanently in a database, and to use said database, or to make said database available, to enable comparison with theses submitted by others.

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## *Doctoral program*