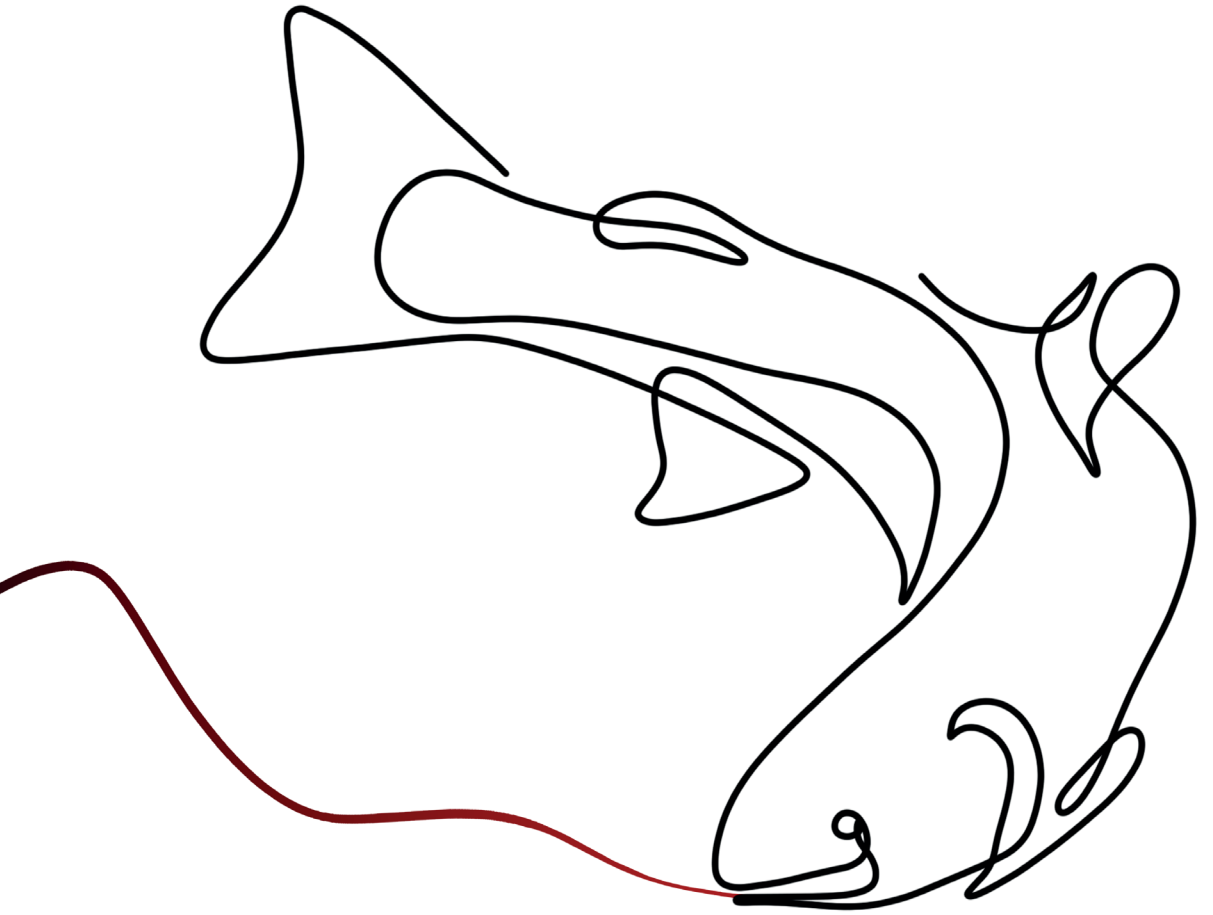
Mycotoxins in aquafeeds:
the impact on rainbow trout



Paraskevi (Vivi) Koletsi

Propositions

1. Deoxynivalenol (DON) impairs rainbow trout performance regardless of being fed on an optimal or sub-optimal diet.
(This thesis)

2. The EU recommended upper limit for deoxynivalenol (DON) in aquafeed is too high.
(This thesis)

3. Artificial intelligence will never surpass human intelligence.

4. “Health nudges” are effective means to alter consumers' behaviour towards a healthier lifestyle.

5. Foreign scholarships are vital to maintain diversity in academic environments.

6. It is impossible to regulate “toxicity” in human relationships.

Propositions belonging to the thesis, entitled
Mycotoxins in aquafeeds: the impact on rainbow trout
Paraskevi Koletsi, 15 June 2023

Mycotoxins in aquafeeds:
the impact on rainbow trout

Paraskevi (Vivi) Koletsi

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Mycotoxins in aquafeeds:
the impact on rainbow trout

Paraskevi (Vivi) Koletsi

Thesis

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Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
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Paraskevi (Vivi) Koletsi

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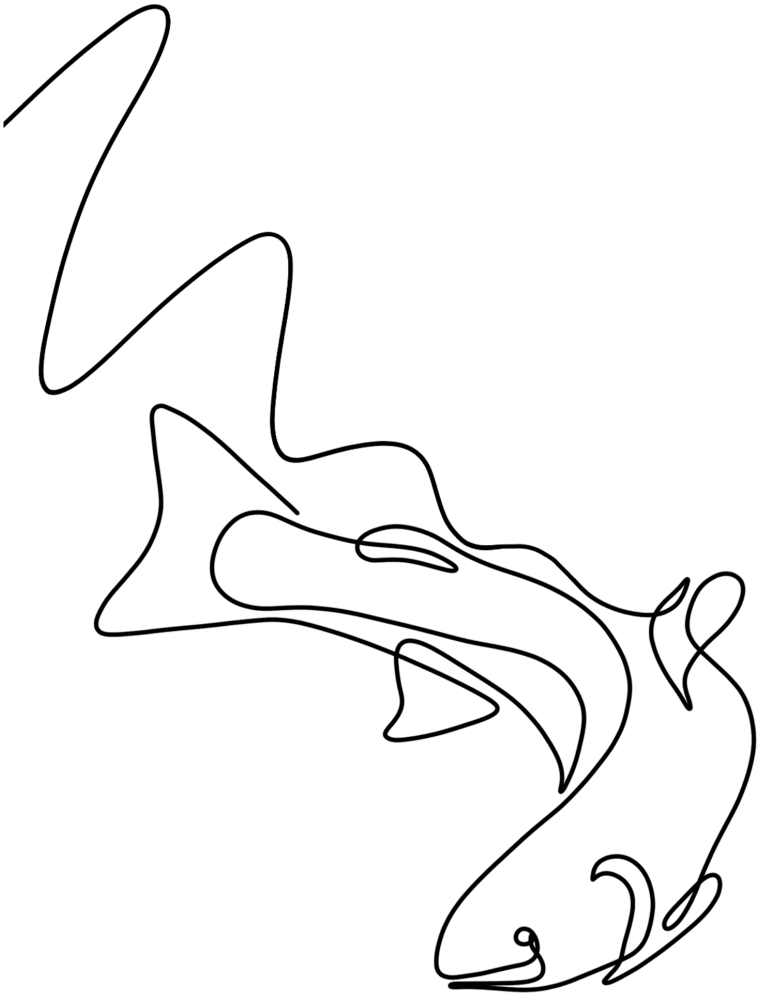
To my mother, Rebecca

Contents

Chapter 1

General Introduction



1.1 Aquaculture

According to projections, the present global population of 7.5 billion people may reach up to 8.4-8.7 billion in 2030, 9.4–10.2 billion in 2050, and 9.6–13.2 billion in 2100 (Stavi et al., 2022). To tackle food security concerns, also correlated with the growing human population, the United Nations formulated Sustainable Development Goal 2 (SDG2) "Zero Hunger" in 2015, aiming to eradicate hunger by 2030 (Hasegawa et al., 2019). Despite many initiatives to implement the Zero Hunger SDG, the proportion of the global population that is facing hunger has been estimated to remain the same, 8% in 2030, while the COVID-19 pandemic might widen food insecurity even further (UNICEF, 2022; Villarreal, 2022). As the global population keeps rising while natural resources become limited, aquaculture is emerging as an effective sector for producing food and already supplying more than half of the fish consumed worldwide (Ameixa et al., 2020; Morón-Elorza, 2021).

Indeed, aquaculture has a crucial role towards achieving the Zero Hunger goal by being the fastest-growing food production sector worldwide (Anderson et al., 2017). The global aquaculture production of fish and shellfish increased from 29 Mt in 1997 to 80 Mt in 2017, and the sector experienced a diversification in farmed species and production systems (Naylor et al., 2021). Following these developments, the global rate of fish consumption rose by about 3.1% per year from 1990 to 2018, outpacing other animal-derived protein foods (FAO, 2020). Aquatic animal foods have nutritional benefits that often outweigh those of terrestrial-derived meat: high protein content on an edible weight, low calorie density, high level of long-chain omega-3 polyunsaturated fatty acids, and high mineral and vitamin content (Tacon et al., 2020). Developing countries, where seafood is the principal source of animal protein contributing to food security (Anderson et al., 2017; Belton et al., 2018), harbour over 90% of aquaculture operations.

Despite the already rapid development of aquaculture, scientists predict that aquaculture production has to expand even more by 2050, to meet the demand for affordable and readily available protein (Boyd et al., 2022). Aquaculture growth will demand an associated growth of aquafeed production, because two-thirds of finfish and crustacean production depends on commercially formulated feeds (Boyd et al., 2022; Naylor et al., 2021). Aquafeeds typically include marine ingredients, mainly fishmeal and fish oil from forage fish, which alone cannot be sufficient enough to supply the aquafeed industry and sustain aquaculture growth (Cottrell et al., 2021). In fact, annual landings of forage fish rather than increased, reduced from 23 Mt in 2000 to 16 Mt in 2017 (Naylor et al., 2021), and according to predictions, catches will not rise in the future (Boyd et al., 2022). Limited marine sources, therefore, set a bottleneck to the growth of the aquafeed industry and have already generated the need to reduce the dependence of aquaculture feeds on wild-caught fish to sustain aquaculture growth (Cottrell et al., 2020). Consequently, the aquaculture industry already had to make a shift towards other feed ingredients considered more sustainable, mainly animal by-products and terrestrial plant materials (Naylor et al., 2021).

Although the aquafeed industry already actively is evaluating the use of several novel aquafeed ingredients or by-products from fisheries and aquaculture, insects, single-cell proteins

(microalgae, bacteria or yeasts), macroalgae or food waste (Hua et al., 2019), the feasibility of their implementation is still questioned mainly due to scalability issues, low volume and/or high production cost (Hua et al., 2019; Pelletier et al., 2018). Instead, terrestrial plant alternatives have already been shown suitable to replace a large proportion of the marine ingredients, to a large extent in commercial diets for omnivorous and carnivorous fish species, and completely in (research) diets for some omnivorous and herbivorous fish species (Klinger and Naylor, 2012). Yet, arguing against plant-based alternatives is that their ingredients usually lack certain amino acids, can have relatively low quantities of protein, insufficient long-chain omega-3 polyunsaturated fatty acids (if any), often have a high content of carbohydrates that are challenging for carnivorous fish to digest, and may contain antinutritional factors (Colombo, 2020; Hardy, 2010). Although processing technologies already brought an increase in their nutritional value resulting in improvements in digestibility and growth of fish receiving diets with processed plant ingredients (Drew et al., 2007), crop materials still have several nutritional and biological constraints for the aquafeed industry to overcome (Francis et al., 2001; Glencross, 2016). Among the constraints of using plant ingredients for aquafeed is the presence of anti-nutritional factors and contaminants naturally occurring in crops, including mycotoxins.

1.2 Mycotoxins

1.2.1 Research history

By definition, mycotoxin is a toxin produced by fungi, derived from the Greek word "mykes" (fungus) and the Latin word "toxicum" (poison) (Goldblatt, 1972). Mycotoxicosis is the syndrome caused by ingestion of fungal compounds by humans and animals, diagnosis of which is difficult because of the absence of distinguished clinical symptoms in exposed individuals (Richard, 2007). In humans, historically, the first reported mycotoxicosis might be ergotism during the Middle Ages linked to the consumption of food containing specific fungal (ergot)-contaminated rye and other grains (Goldblatt, 1972; Grzybowski et al., 2021; Richard, 2007) (Figure 1.1). As a result, thousands of people in Europe died from a disease known back then as "St. Anthony's fire" or “holy fire". The term "fire" came from the fact that ergotism caused blood vessels to constrict strongly, leading to necrosis in peripheral body parts, primarily the hands and feet. Between the years 591 and 1789, ergot was responsible for more than 130 epidemics in Europe, although its true nature remained unknown until the 18th century. In animals, mycotoxicosis incidence was first reported in England in 1960 after an outbreak of a disease causing the death of 100.000 turkeys (Richard, 2008). Initially, because the etiology was unknown, it was known as the turkey “X” disease, but shortly after in 1961, recognition of the consumption of aflatoxin-contaminated peanut meal imported from Brazil was recognized as the disease's cause (see Figure 1.1). In fact, it was the discovery of aflatoxin that caused the breakthrough that led to the start of modern mycotoxicology.

In the early 60s, mycotoxicosis was not only reported for farmed turkey but also for farmed rainbow trout (Richard, 2008), although it was only later that hepatoma carcinoma outbreaks in rainbow trout farms in the USA were recognised as outcomes of the presence of aflatoxin-contaminated cottonseed meal in the fish feed. Subsequent experimental research confirmed

that aflatoxin can cause severe health effects in rainbow trout, including hepatocarcinoma (Halver, 1967; Halver, 1968; Jackson et al., 1968; Sinnhuber et al., 1968). In the following decade, several experimental studies were carried out that recognised the sensitivity of rainbow

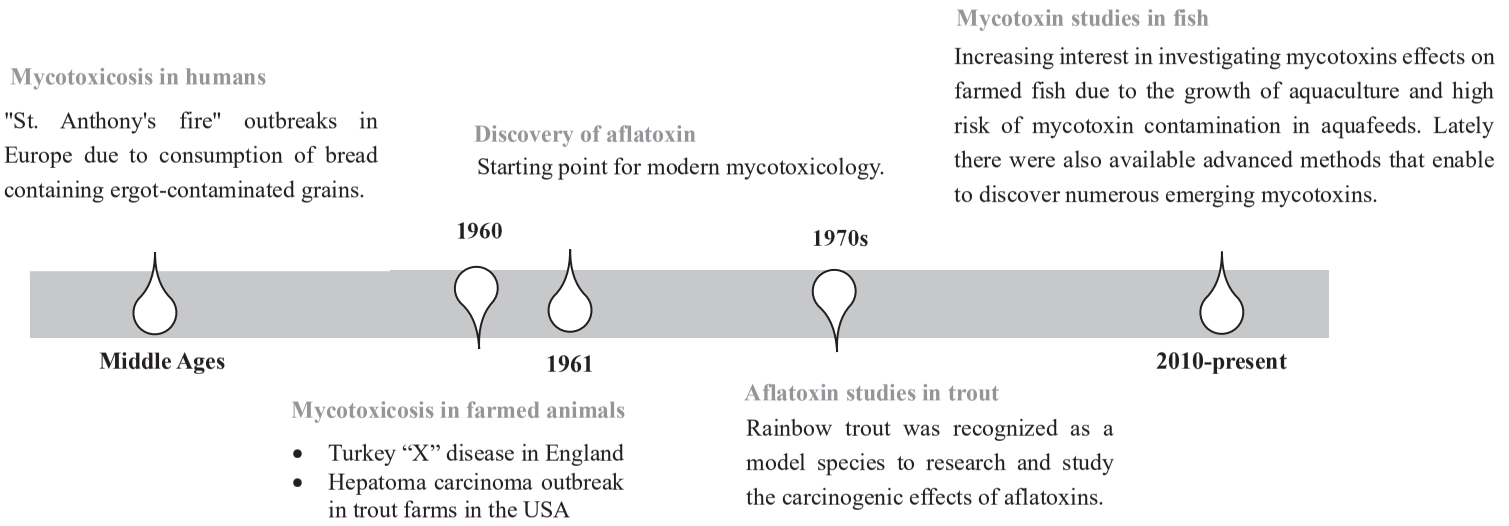


Figure 1.1| Milestones in the recognition of mycotoxicosis over time.

trout to relatively low doses of aflatoxin (Ayres et al., 1971; Canton et al., 1975; Lee et al., 1971; Sinnhuber et al., 1974; Wales et al., 1978). These findings have made trout a highly relevant animal species to investigate the mechanism of action of aflatoxins in fish, and animal model species for relations with human cancer (Richard, 2008; Williams et al., 2009).

1.2.2 Emerging feed safety issue for aquaculture

Over the past decades, the interest in mycotoxicosis in fish species has grown with the discovery of new mycotoxins (thanks to the development of screening techniques for simultaneous determination of mycotoxins e.g., liquid chromatography and mass spectrometry) and with the increasing relevance of mycotoxins for aquaculture. The latter relevance, of course, has been linked to the industry's ongoing growth and diversification, but also by the industry's growing attention for mycotoxin contamination as increasing risk factor. Higher inclusion of plant-based ingredients in aquafeeds driven by the urge to replace marine ingredients in a circular economy, but also climate change and trade globalization all have become key factors increasing the risk of contamination with mycotoxins in the supply chain (Figure 1.2). Indeed, a small-scale field survey in Asia and Europe (Gonçalves et al., 2018a) reported on the ubiquitous presence of mycotoxins in aquafeeds, bringing to light mycotoxins as an emerging feed safety concern in aquaculture.

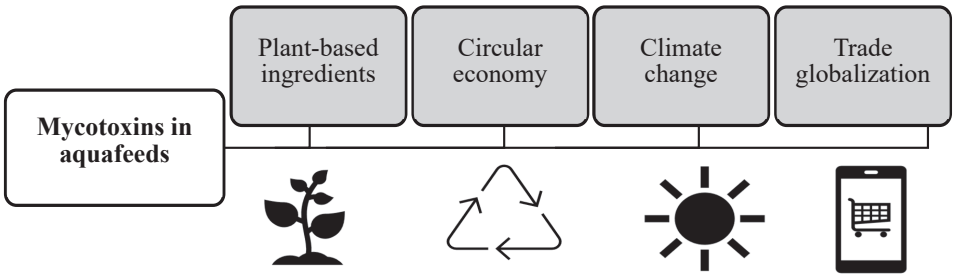


Figure 1.2 | Factors involved in the risk of mycotoxin contamination in aquafeeds.

Plant-based ingredients

The current growth of the aquaculture sector undoubtedly (also) relied on the utilisation of plant-based ingredients that served as more sustainable alternatives to the expensive marine-based ingredients of limited availability, including their use for diets suitable for carnivorous fish. For example, in Norway, the inclusion of marine-based materials in Atlantic salmon feeds has been reduced strongly from 90% in 1990 to just 22% in 2020 (Figure 1.3) (Aas et al., 2022). Contrarily, at the same time, the inclusion of plant-based ingredients climbed to 61% in 2020 (Figure 1.3). Mycotoxins can highly contaminate the crops needed for the plant-based ingredients, either in the field (pre-harvest) or afterwards (post-harvest) during transportation and storage of commodities (Bryden, 2012).

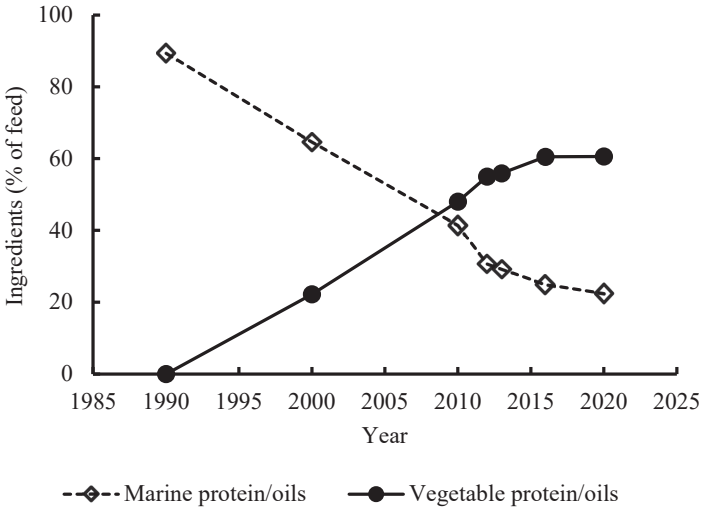


Figure 1.3 | Inclusion (% feed) of marine (protein/oils) and plant (protein/oils) ingredients in the diets of Atlantic salmon produced in Norway from 1990 to 2020.

Circular economy

Without a doubt, the evolution of commercial use of raw materials for aquafeed demonstrates a move away from being mainly marine- to being predominantly terrestrial-based. At present, the aquafeed sector tends to move toward a more circular approach and aims to use more sustainable raw materials (Campanati et al., 2022; Colombo and Turchini, 2021), also embracing new aquafeed materials produced by circular bio-economy frameworks for potential use in aquafeed diets. Among the relatively new aquafeed ingredients, crop-based residues are well established; they were already used in the countries of the European Union for feed production in 2014-2015; e.g., about 20 million tons of cereal by-products per year (Pinotti et al., 2016). In the United States, 20–25% of the wheat kernel is a by-product of flour production destined for human consumption, which is completely used by the animal feed industry (Gatlin Iii et al., 2007). This trend brings an associated risk; data have shown that milling processes may reduce mycotoxin concentration in those fractions intended for human consumption, but rather concentrate mycotoxins up to eight times higher in their by-products used in animal feeds (Cheli et al., 2013). Consequently, it is increasingly necessary to screen cereal residuals before using them in animals feeds since they may be heavily contaminated with mycotoxins. But it is not only by-products from cereals; all promising new by-products such as those from fruit processing (Dawood et al., 2022), agricultural activities and waste (Tu Nguyen et al., 2022), and food wastes (Wong et al., 2016) should be evaluated for their potency to be substrates carrying mycotoxins.

Climate change

Globally, climate change is manifested especially through elevated average yearly temperatures, increased CO_2 levels and extreme weather conditions (drought or flood). These manifestations are all expected to affect the growth patterns of fungi and therefore the production of mycotoxins (Medina et al., 2017). This realization has led to concerns about upcoming food and feed security, and led to predictive models estimating the impact of climate change on mycotoxin production in cereals, in Europe (Van der Fels-Klerx et al., 2016). By the year 2040, DON contamination in wheat is expected to increase mainly in North-Western Europe, in some regions increased levels may be up to 3-fold compared to the original concentrations (van der Fels-Klerx et al., 2012a). Within the next 100 years, in a scenario of a 2 °C elevated temperature, the risk of aflatoxin B1 (AFB1) contamination in corn will increase mainly in Southern regions of Europe such as Spain, Italy and the Balkans because of optimal growth conditions for *Aspergillus flavus* (Battilani et al., 2016). In general, as a consequence of climate change Europe may witness an extension of AFB1 distribution from tropical to previously considered temperate areas, and a similar extension of *Fusarium graminearum* from Southern to Central and Northern Europe (Moretti et al., 2019). For instance, in the Netherlands, the main strain of *Fusarium* fungi in wheat always had been *F. culmorum*, but after the year 2000, *F. graminearum* became more dominant (Waalwijk et al., 2003). In Luxembourg, a shift from *F. graminearum* to *F. culmorum* and vice versa has been reported, indicating plasticity of *Fusarium* strains (Beyer et al., 2014). Overall, although exact proliferations under field conditions remain unpredictable, these studies provide several different examples confirming that in times of climate change, Europe should expect an increased presence and distribution of fungi on cereals and other crops, and the associated risk of contaminations with mycotoxins.

Globalization of the trade

The globalization of trade is leading to encountering unpredictable mycotoxin patterns in imported agricultural commodities, including aquafeed (Binder et al., 2007). For the relatively cold and wet parts of Northern Europe, trade globalization increases the risk of mycotoxins, simply because aflatoxin B1 is produced by *Aspergillus* fungi which thrive in warm and dry climate areas. One such example for the agricultural sector occurred in the Netherlands in 2013 (Focker et al., 2021) when aflatoxin M1 was discovered in cow milk, linked to aflatoxin B1-contaminated maize imported from Eastern Europe and included in the feed of the animals consumed. Although a similar clear example has not been reported for aquaculture, the aquafeed sector certainly is aware of increased risks (Gonçalves et al., 2020a). For example, in Europe, increased risks exist for soybean, with soybean meal as a typical example of an imported plant-based ingredient for aquafeed (Silva et al., 2018). According to the Food and Agriculture Organization of the United Nations (FAO), the top producers of soybean meal in 2021 were Brazil, the United States and Argentina (FAOSTAT, 2021), in warm and dry climate regions. Regulations should ensure that traded raw materials adhere to the safety standards of the importing countries and are put in place for timely procedures, with the aim to test for mycotoxins at various stages in the supply chain.

1.3 Mycotoxins in animal feeds

The most common fungal species associated with mycotoxin contamination in animal feeds belong to the genera *Aspergillus*, *Fusarium*, *Penicillium* and *Alternaria*, and less frequently also to the genera *Claviceps* and *Stachybotrys* (Bryden, 2012; Santos Pereira et al., 2019; Streit et al., 2012). Fungi can produce one or more mycotoxins simultaneously to enhance their pathogenic process and compete for growth with other organisms (e.g., other fungi, bacteria), processes mostly driven by external stimuli (mainly environmental changes in temperature, water activity and pH) (Reverberi et al., 2010). The development of advanced methodologies (e.g., based on liquid chromatography coupled to tandem mass spectrometry: LC-MS/MS) have enabled the quantification and characterization of more than 500 mycotoxins, with new ones being discovered regularly (Haque et al., 2020; Sulyok et al., 2020). Although given to date's climate change scenario new mycotoxins might keep emerging in the food and feed chains (Medina, 2023), currently, the most important mycotoxins for the animal feed industry are aflatoxins (aflatoxins B1, B2, G1, G2), trichothecenes (e.g., deoxynivalenol), fumonisins (fumonisin B1, B2, B3), zearalenone, and ochratoxins (e.g., ochratoxin A) (Magnoli et al., 2019), summarized in Table 1.1.

Table 1.1 | Main mycotoxin-producing fungal genera, species and their main associated mycotoxins in animal feeds.

Fungi[1]	**Mycotoxins[2]**
Fusarium sporotrichioides, F.graminearum, F.culmorum, F.poae, F.roseum, F.tricinctum, F.acuminatum	Deoxynivalenol
Fusarium moniliforme, F.proliferatum	Fumonisin B1, B2, B3
Fusarium graminerum, F.culmorum, F.crookwellense	Zearalenone
Penicillium verrucosum	Ochratoxin A
Aspergillus flavus, A.parasiticus, A.momius	Aflatoxins B1, B2, G1, G2
Aspergillus clavatus	Ochratoxin A

[1]Fungal species retrieved from (Yiannikouris and Jouany, 2002) [2]Important mycotoxins for the feed industry retrieved from (Magnoli et al., 2019).

Traditionally, plant-based ingredients for poultry, pigs, cattle, and fish feed are mainly cereals (wheat, barley, oats, triticale, rye, sorghum, millet), corn and soybean meal (Magnoli et al., 2019). These ingredients and derived final feeds are usually contaminated with multiple mycotoxins, as confirmed by large and periodic field surveys (Binder et al., 2007; De Boevre et al., 2012; Kovalsky et al., 2016; Rodrigues and Naehrer, 2012; Streit et al., 2013; Twarużek et al., 2021). Yet, exact summaries of mycotoxin contaminations remain challenging since such surveys cover different ingredients, different animal species, and different geographic areas and periods. The two largest sets of mycotoxin data relevant to aquaculture are available from surveys of European and Asian aquafeed samples (Gonçalves et al., 2018a) and finished fish feeds and ingredients from East Africa (Marijani et al., 2017). The current thesis sought to fill

this knowledge gap by first conducting a survey on mycotoxin contamination in wheat, corn, soybean, and fish feed samples in Europe.

1.4 Thesis aim and outline

The main objective of this thesis was to better understand the impact of mycotoxins on the performance and health of rainbow trout (*Oncorhynchus mykiss*) in aquaculture.

In this introduction (**Chapter 1**) I provide a brief background to this PhD study, including the main factors that increase the risk of mycotoxin contamination in aquafeeds, as well as the main objective of this study and a short outline of the thesis.

As also mentioned above, the current thesis first sought to fill the knowledge gap on mycotoxin occurrence and contamination patterns in aquafeeds and their impact on different fish species, by conducting a survey on mycotoxin contamination in different crops and in fish feed. For this reason, first, in **Chapter 2**, a survey analysis of mycotoxin contamination data in plant-based ingredients and aquafeeds in Europe was performed. This survey identified deoxynivalenol (DON) as one of the mycotoxins with the highest occurrence in ingredients and aquafeeds. Therefore DON was selected as the toxin of interest for further investigations. In the same chapter, a systematic review analysed the current knowledge about DON effects on fish species and a meta-analysis was performed to predict the impact of DON on feed intake and growth of fish species. For rainbow trout, enough data was available to conduct a separate meta-analysis at the species level. This analysis highlighted as interesting subjects for further investigation in rainbow trout, if host growth response would be directly affected by the intake of DON-contaminated feed, and if host growth responses would be different when DON comes from pure sources, or from naturally contaminated ingredients. Based on the survey analysis in Chapter 2, three *in vivo* experiments were designed.

In all three *in vivo* experiments, DON was selected as the toxin of interest, and rainbow trout was selected as the fish species of interest because of its relatively high sensitivity to the toxic effects of DON. A strong effort was made to keep the experimental design the same and consistent over the three *in vivo* studies regarding the size of the fish, housing and sampling procedures, duration of the exposure and read-out parameters. Each experiment lasted eight weeks: six weeks of restrictive exposure followed by two weeks of *ad libitum* feeding. The restrictive feeding periods and thus exposure to DON were a strategy to measure the direct impact of DON on growth. The *ad libitum* feeding periods were necessary, in particular, to reveal potential feed refusal. In all studies, the **DON impact on performance was measured using the same parameters. Health metrics were also included, with the liver and gastrointestinal tract (GIT) as potential target organs of DON action. Parameters to read out the potential impact of DON on fish health were based on semi-quantitative histopathological analysis of liver and GIT. This common experimental design was applied to** answer a number of specific questions, as follows:

The first *in vivo* experiment, described in **Chapter 3,** aimed to assess the dose effects of a single exposure to DON. Two sources of DON (natural and pure) were tested at two DON levels (low

and high). The second *in vivo* experiment, described in **Chapter 4,** aimed to evaluate if the impact of DON on rainbow trout performance and health would be influenced by sub-optimal conditions, particularly in the GIT. In salmonids, soybean meal (SBM) can induce enteritis and diets based on SBM can be considered sub-optimal diets. The potential interaction of DON with dietary composition was assessed in two groups; marine-based diets ("optimal quality") and SBM-containing diets ("sub-optimal quality"). The third *in vivo* experiment, described in **Chapter 5,** aimed to investigate the potential combined effects of co-exposure of DON with other *Fusarium*-produced mycotoxins, the latter selected based on their co-occurrence in aquafeeds as reported in the survey in Chapter 2.

In **Chapter 6**, the discussion of this thesis, I first critically discuss the limitations of *in vivo* mycotoxin studies such as described in chapters 3, 4 and 5, and I provide recommendations on how to manage the large variability in such *in vivo* experiments. Subsequently, I discuss the implications of the main findings of this thesis for aquaculture practice, with an emphasis on mycotoxin management and control in the aquaculture industry. I conclude with practical advice to fish feed manufacturers and regulatory authorities, aiming to protect not farmers' profitability but also fish health and welfare.

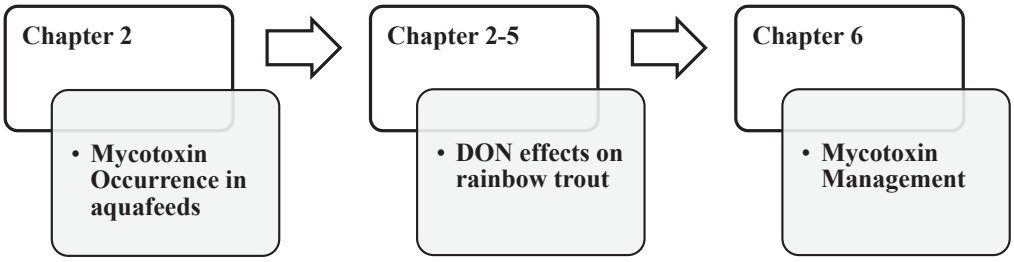


Figure 1.4 | Thesis outline with the flow of the three main research components. It starts with the survey on mycotoxin occurence in aquafeeds, follows with the effects of DON on rainbow trout and implements the current knowledge to manage mycotoxins, in research and commercial perspective.

Chapter 2

The Occurrence of Mycotoxins in Raw Materials and Fish Feeds in Europe and the Potential Effects of Deoxynivalenol (DON) on the Health and Growth of Farmed Fish Species

This chapter has been published as:

Koletsi, P., Schrama, J. W., Graat, E. A., Wiegertjes, G. F., Lyons, P., & Pietsch, C. (2021). The Occurrence of Mycotoxins in Raw Materials and Fish Feeds in Europe and the Potential Effects of Deoxynivalenol (DON) on the Health and Growth of Farmed Fish Species—A Review. *Toxins*, *13*(6), 403.

DOI: 10.3390/toxins13060403

Abstract

The first part of this study evaluates the occurrence of mycotoxin patterns in feedstuffs and fish feeds. Results were extrapolated from a large data pool derived from wheat (n = 857), corn (n = 725), soybean meal (n = 139) and fish feed (n = 44) samples in European countries and based on sample analyses by liquid chromatography/tandem mass spectrometry (LC-MS/MS) in the period between 2012–2019. Deoxynivalenol (DON) was readily present in corn (in 47% of the samples) > wheat (41%) > soybean meal (11%), and in aquafeeds (48%). Co-occurrence of mycotoxins was frequently observed in feedstuffs and aquafeed samples. For example, in corn, multi-mycotoxin occurrence was investigated by Spearman's correlations and odd ratios, and both showed co-occurrence of DON with its acetylated forms (3-AcDON, 15-AcDON) as well as with zearalenone (ZEN). The second part of this study summarizes the existing knowledge on the effects of DON on farmed fish species and evaluates the risk of DON exposure in fish, based on data from *in vivo* studies. A meta-analytical approach aimed to estimate to which extent DON affects feed intake and growth performance in fish. Corn was identified as the ingredient with the highest risk of contamination with DON and its acetylated forms, which often cannot be detected by commonly used rapid detection methods in feed mills. Periodical state-of-the-art mycotoxin analyses are essential to detect the full spectrum of mycotoxins in fish feeds aimed to prevent detrimental effects on farmed fish and subsequent economic losses for fish farmers. Because levels below the stated regulatory limits can reduce feed intake and growth performance, our results show that the risk of DON contamination is underestimated in the aquaculture industry.

2.1 Introduction

Aquaculture, in contrast to capture fisheries that have remained stable over the last decades, continues to grow and contribute to the increasing food supply for human consumption, reaching worldwide production of 80 million metric tonnes (Mt) in 2016 (FAO, 2018). To sustain its growth, the aquaculture industry is highly dependent on commercial feed sources (Naylor et al., 2009; Tacon and Metian, 2015; Troell et al., 2014). Indeed, the production of aquafeeds increased from 8 Mt in 1995 to 48 Mt in 2015 (FAO, 2018). A recent global feed survey revealed that the annual growth of aquafeed production for 2018 was 4% (Alltech, 2019), and was projected to reach 65 Mt in 2020 (Fry et al., 2016). However, the inclusion rate of traditionally used finite and expensive marine protein and fat sources from wild-caught fish (i.e., fishmeal and fish oil) in the diets of farmed fish species will continue to decline and the industry has already shifted to crop-based ingredients to meet the rising demand for aquafeeds (Fry et al., 2016; Naylor et al., 2009; Naylor et al., 2021). For instance, collective data from the Norwegian salmon (*Salmo salar*) industry reflect the change in modern aquaculture diet composition and confirm the reduced dependency on fishmeal derived from wild-caught fish; while in 1990 salmon diets consisted of 90% marine ingredients, already in 2013 their inclusion rate was less than 30%, which increased the share of plant protein sources to 37% (Ytrestøyl et al., 2015). Plant-based ingredients increasingly replace marine-based ingredients and, therefore, an enhanced level of understanding of the nutritional quality of raw materials derived from plant sources is becoming increasingly important for aquafeeds.

Plant-based feed ingredients currently used in aquafeeds as substitutes for marine ingredients include soybean meal, rapeseed/canola meal, maize/corn, wheat bran and wheat (Troell et al., 2014). Even in diets for carnivorous species like Atlantic salmon, the main protein and lipid sources used within the feed in 2012 were derived from crops, such as soybean meal (21.3% average inclusion rate) and rapeseed oil (18.3% average inclusion rate), with the main starch source being wheat (9.9% average inclusion rate) (Ytrestøyl et al., 2015). However, in contrast to marine ingredients that contain well-balanced protein contents to meet the amino acid requirements of aquatic farmed animals, the continuing transition towards higher inclusion of plant-based ingredients poses a real challenge for aquafeed producers due to nutritional limitations (Hardy, 2010; Turchini et al., 2009). The higher inclusion of less-expensive plant sources may introduce a series of anti-nutritional factors (e.g., protease inhibitors, phytates, saponins, glucosinolates, tannins, non-starch polysaccharides) and/or increase the occurrence of animal feed contaminants; factors that might affect the quality and safety of aquafeeds (Francis et al., 2001; Glencross, 2016; Kokou and Fountoulaki, 2018; Softeland et al., 2014; Tacon and Metian, 2008). Frequently occurring natural feed contaminants are mycotoxins, which are mainly detected in plant-based feedstuffs (Boevre et al., 2012; Kovalsky et al., 2016; Pettersson and Fink-Gremmels, 2012; Rodrigues and Naehrer, 2012; Streit et al., 2012). Increasingly (Gonçalves et al., 2017; Gonçalves et al., 2018a; Greco et al., 2015; Marijani et al., 2017; Pietsch et al., 2013), the presence of mycotoxins is reported in aquafeeds.

2.1.1 Mycotoxin-Producing Fungi

Mycotoxins are secondary metabolites produced by fungi that invade crops in the field during plant growth and/or fungi that colonize the crops before harvest and predispose the commodity

to mycotoxins after harvest during drying, transportation and storage (Streit et al., 2012; Tola and Kebede, 2016). Common toxigenic genera are *Aspergillus*, *Penicillium*, *Fusarium, Alternaria* and *Claviceps* which proliferate with climatic conditions considered favourable (close to their preferred temperature and moisture) (Bennett and Klich, 2003; Bryden, 2012; Sweeney and Dobson, 1998). The global distribution of mycotoxigenic fungi is temperature-dependent; *Penicillium* spp. are common in cool climates, *Aspergillus* spp. in the tropics and *Fusarium* spp. in temperate areas (Magan and Aldred, 2007). Fungal growth requirements for minimal and optimal water activity (a_w) differ among genera. *Fusarium* and *Alternaria* are plant pathogens and hygrophilic (1.00 a_w), meaning they proliferate in substrates with high water availability and, therefore, predominate in the fields at pre-harvest. *Aspergillus* and *Penicillium* are xerophilic (< 0.95 a_w), meaning they can proliferate at low water availability and are the main mycotoxigenic fungi post-harvest, during storage (Mannaa and Kim, 2017). Post-harvest measures such as proper storage conditions can possibly prohibit the growth of xerophilic fungi (Neme and Ibrahim, 2017) but pre-harvest conditions such as a continuously changing climate (Paterson and Lima, 2010) cannot be controlled, for which reason the presence and growth of hygrophilic fungi from the fields remains unpredictable.

The occurrence of mycotoxigenic fungi, however, does not necessarily lead to the production of mycotoxins. For instance, *Aspergillus* spp. were detected in aquafeed samples but not the corresponding mycotoxins (Almeida et al., 2011). Such observations reinforce questions of "How, why and when do fungi produce mycotoxins?" These respective questions largely remain unanswered since most research is focused on the toxicological aspects of mycotoxins and their effects on host organisms (Alkhayyat and Yu, 2014). Mycotoxin production may be triggered after environmental abiotic stimuli (light, nutrient, pH) and biotic interactions of different microbes (i.e., fungal–bacterial or fungal–fungal) that lead to up-regulation of biosynthetic gene clusters to secure the ecological niche of fungi in hostile environments by exhibiting antimicrobial functions (Venkatesh and Keller, 2019). Indeed, incubation of commercial fish feeds under different storage conditions can influence fungal growth and mycotoxin production. Specifically, the application of warm (temperature ~27 °C) and humid (~70% relative humidity) conditions may trigger the release of ochratoxin A (OTA), with variations due to distinct hotspots with optimal conditions for fungal growth and production of mycotoxins (Pietsch et al., 2020). Therefore, the presence of mycotoxigenic fungi under storage conditions does not necessarily mean the presence of mycotoxins in aquafeeds.

2.1.2 Classification of Fusarium Mycotoxins: "Traditional", "Emerging" and "Masked"

Fusarium species as soil-borne microbes are the most common pathogens in cereal crops flourishing in a wide geographic range, also in Europe (Bottalico and Perrone, 2002; Perincherry et al., 2019). The toxicologically most important *Fusarium* mycotoxins are trichothecenes, zearalenone (ZEN) and fumonisins (FUM) (Nesic et al., 2014). ZEN occurs more commonly than its metabolites. FUM group is represented by fumonisin B_1 (FB_1), B_2 (FB_2), and B_3 (FB_3), FB_1 being the most abundant member (Bennett and Klich, 2003). Trichothecenes can be divided into four types (A, B, C, D); the concerns regarding type A and type B trichothecenes are higher due to their higher toxicity and occurrence in crops (Marin et al., 2013; Nathanail et al., 2015) Known mycotoxins that belong to type A trichothecenes are

T-2/HT-2 toxin, diacetoxyscirpenol (DAS) and neosolaniol (NEO). Among the type A trichothecenes, T-2 toxin is the most toxic mycotoxin regardless of the exposed animal species, is soluble in non-polar solvents (e.g., ethyl acetate and diethyl ether) and is rapidly metabolised to HT-2 toxin (Adhikari et al., 2017; Dohnal et al., 2008; Li et al., 2011). Known mycotoxins that belong to type B trichothecenes are DON, nivalenol (NIV), fusarenon X (FX) and fusaric acid (FA). Among the type B trichothecenes, worldwide (Berthiller et al., 2013; Marin et al., 2013)DON is the most commonly found mycotoxin in cereal grains.

Besides the "traditional" *Fusarium* mycotoxins described above, *Fusarium* species produce other metabolites called "emerging" mycotoxins such as fusaproliferin (FUS), beauvericin (BEA), enniatins (ENNs), and moniliformin (MON) (Jestoi, 2008). Furthermore, *Fusarium* mycotoxins can occur as plant-derived derivatives which are often not detectable during routine mycotoxin analyses and, therefore, called "masked" mycotoxins, after having been biologically modified by plant defense mechanisms after crop infection (Berthiller et al., 2013; Kovalsky et al., 2016). The most commonly-detected masked mycotoxin conjugates are β-linked glucose-conjugates of trichothecenes: DON-3-glucoside (DON3Glc), nivalenol-3-glucoside (NIV3Glc), HT-2 glucoside (HT2Glc), and ZEN-14-glucoside (ZEN14Glc) (Gratz, 2017). Masked mycotoxins are derived from conjugation reactions following a glucosidation reaction, but can also involve glucuronidation or sulfatation (Phase II of plant metabolism), and are usually less harmful than the parent mycotoxins (Berthiller et al., 2013; Zhang et al., 2019). However, masked forms might be "reactivated" during animal digestion by the action of gut microbiota, which may cleave the polar group and consequently liberate the parent toxin (Berthiller et al., 2013). The concept of toxin reactivation has been confirmed for DON3Glc and NIV3Glc in rats (Nagl et al., 2012; Schwartz-Zimmermann et al., 2019) and for DON3Glc and ZEN14Glc in pigs (Binder et al., 2017; Nagl et al., 2014). To avoid confusion (Rychlik et al., 2014), one should not only distinguish free mycotoxins from masked mycotoxins, but also from matrix-associated and other modified mycotoxins. To further emphasize the distinction, acetylated derivatives of DON such as 15-acetyl DON (15AcDON) and 3-acetyl DON (3AcDON) are fungal metabolites (free mycotoxins). These toxins are commonly detected along with DON in feedstuffs and animal feeds (De Boevre et al., 2012). In other words, mycotoxins can be present in many forms.

In Europe, AFB_1 is the only mycotoxin regulated by the Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances; for fish species the maximum allowed concentration in feed materials is 20 µg/kg (ppb), and for complete feed is 10 ppb (Table 2.S1) (Commission, 2002). For other mycotoxins, including important *Fusarium* mycotoxins such as DON, ZEN, T-2 and HT-2 toxin, FB_1 and FB_2, the EC has established only recommended limits for their presence in feedstuffs and feed (Table 2.S1) (Commission, 2006a; Commission, 2012; Commission, 2013). Among these recommended limits only those for FB_1 and FB_2 refer directly to fish species. In addition, European Commission (EC) regulations/recommendations are based on the occurrence of a single mycotoxin, although feeds are usually contaminated by numerous mycotoxins simultaneously that might, in some instances, result in synergistic effects (Smith et al., 2016).

The present study aims to extrapolate from a large dataset and thus highlight the potential threat of mycotoxins to European aquaculture by a) unravelling mycotoxins patterns in both, fish feeds and in the commonly used plant-based feed ingredients: wheat, corn and soybean meal; b) updating the current state of knowledge on the effects of DON and the risk of DON exposure on important farmed fish species; c) predicting the effects of DON on fish performance; and d) providing practical advice for fish farmers and fish feed manufacturers.

2.2 Materials and Methods

2.2.1 Survey

Field surveys regarding mycotoxin occurrence on plant-based ingredients and feeds for aquaculture are not readily available, or at least not at the same extent as those for terrestrial animal feeds and food. Consequently, researchers have no other option than to use inconsistent data from published literature or extrapolate information based on assumptions. This bottleneck has been acknowledged and discussed in a recent publication (Pietsch, 2020) and aimed to assess the risk for mycotoxin contamination in fish feeds in Europe. To overcome this data gap, we report mycotoxins occurrence data obtained from the database of the Alltech 37+ mycotoxin laboratory (ISO/IEC 17025:2005 accredited), Dunboyne, Ireland. Specifically, the database includes all submitted wheat (n = 857), corn (n = 725), soybean meal (n = 139) and aquafeed (n = 44) samples from European countries between 2012–2019. All samples were analysed by the liquid chromatography-tandem mass chromatography (LC-MS/MS) analytical method for detection and quantification of 43 mycotoxins. Mycotoxins' occurrence was defined considering positive samples, thus samples above the limits of quantification (LOQs) for each mycotoxin. Average and maximum concentration for each toxin were calculated for the positive tested samples. The results are given separately for each matrix. Limits of detection (LODs) and LOQs are available for each toxin and given in Table 2.S3.

On top of that, the probability of the mycotoxin contamination patterns (DON association with other toxins) is elucidated by applying logistic regression analysis (Hosmer Jr et al., 2013). Results are expressed as odds ratio (OR) with the 95% confidence interval. Statistical Analysis Software (SAS) version 9.4 was used. In our case, the OR represents the odds that DON will occur given a particular presence of "Toxin X", compared to the odds of DON occurring in the absence of Toxin X. The OR determines if the presence of "Toxin X" is a risk factor for the presence of DON, and expresses the magnitude of the risk (OR = 1: exposure does not affect odds of outcome, OR > 1: exposure associated with higher odds of outcome; OR < 1: exposure associated with lower odds of outcome).

Comparisons of our results with other surveys are briefly discussed since it has been considered that different methods of analysis and different detection limits can generate variability and thus incomparable data. Also, each step of the testing process (sampling, sample preparation and analytical steps) is associated with errors (Whitaker, 2006). In the current survey, mycotoxin analyses were performed consistently in terms of the analytical method, although not randomly. Samples had been submitted for analyses by stakeholders associated with the feed industry from European countries and, therefore, generated data might be associated with a degree of bias due

to the suspicion of toxin contamination by these stakeholders. In general, taking into account the facts above, mycotoxin results should always be reported with an estimate of the uncertainty.

2.2.2 Systematic Review

A systematic review was conducted by compiling data from scientific articles included research *in vivo* experiments which evaluated the single effects of DON on fish species. In total, 112 articles were retrieved through database searching on Google Scholar, ScienceDirect, Scopus, Scielo, and PubMed using the keywords: deoxynivalenol (DON/vomitoxin) and fish. Initially, we screened the titles and afterwards the abstracts and by using the PRISMA diagram introduced by (Moher et al., 2009). Thus, we selected 28 relevant articles referring to six different species; rainbow trout (13 studies) salmon (3) carp (9) tilapia (2) catfish (1) and zebrafish (1), which were assessed as full-texts and included in the qualitative synthesis summarising the effects of DON per species. The list with the studies used is available in Table 2.S4.

2.2.3 Risk Assessment

The same articles retrieved from the systematic review were also screened for "no observed adverse effect level" (NOAEL)—the highest dose tested against the control—and the "lowest observed adverse effect level" (LOAEL), the lowest dose tested with a statistically significant effect. We excluded a carp study (Kövesi et al., 2020) from the analysis since the level of DON used (200,000 µg/kg) was extremely high and no more realistic LOAEL levels had been included. To assess the risk of DON exposure in 5% of a fish population, we calculated the critical concentration 5% (CC5) based on the mycotoxin levels in the feeds in the open-source software R (R development core team 2006) based on 146 data points for LOAEL in fish and 111 data points for NOAEL from the same studies (Table 2.S5). The missing NOAEL levels were calculated by using the linear regression model implemented earlier by (Pietsch, 2020). Afterwards, log CC5 were estimated based on the predicted NOAEL data sets using the Bayesian modelling as described by (Pietsch, 2020) and datapoints are available in Table 2.S6. The probability of CC5 estimate was given graphically by kernel density plot, which evaluated 100 equally spaced points that cover the range of the dataset. Also, boxplots were generated that show the distribution of the estimated CC5. Both, kernel density plots and boxplots were created in the software MATLAB (R2019b). The analyses have been performed for four different datasets separately: all fish species (n = 146), rainbow trout (n = 56), salmonids, (n = 67) and all fish species excluding rainbow trout (n = 90).

2.2.4 Meta-Analysis

To answer our research questions regarding the effects of DON on feed intake and growth performance in fish, a meta-analytical approach was performed in alignment with similar studies have done before in pigs and poultry (Andretta et al., 2012; Andretta et al., 2011; Kipper et al., 2020; Pastorelli et al., 2012; Remus et al., 2014). Out of the 28 articles initially used in the systematic review, 12 experiments reported in 11 studies (rainbow trout; 7, salmon; 2, tilapia; 2, carp; 1 experiment included in the meta-analysis (Table 2.S7). We used the following

selection criteria: (1) Only *in vivo* studies were selected. (2) In the selected studies DON concentration in the feed (μg/kg) was reported. (3) The studies aimed to investigate the single effects of DON in fish species. (4) Only studies that implemented satiation feeding were selected so that we could evaluate the effect of the toxin on feed intake capacity. (5) Average daily feed intake (g/fish/day) and average daily weight gain (g/fish/day) should be reported. A study could be included in our dataset only if both feed intake and growth data were available because apart from the effects of dietary DON on feed intake and growth, we also investigated the potential association between feed intake and growth. (6) Each study should include a control diet. (7) Exclusion of treatments that contained a feed additive against mycotoxin (e.g., mycotoxin binder) could moderate the effects of DON. (8) Inclusion of control and treatments diets with the presence of other toxins than DON. In these cases, naturally contaminated ingredients were used and the exclusion of other toxins was impossible. Consequently, the authors decided not to exclude these studies. (9) Inclusion of data on the response variables from different time points within a study, when there were reported.

Each line in our dataset was linked to a different treatment (control or DON treatments) coded as non-challenged (control) and challenged (DON treatments). The analysed independent (predictor) variable was the experimental concentration of DON (μg/kg) in the diets. The dependent (response) variables were average daily feed intake (feed intake) and average daily weight gain (growth). To reduce the variation effect among studies (fish species, experimental duration, fish size etc.), data were standardised by transforming feed intake and growth data relative to their controls; feed intake (% control) and growth (% control). For each study, additional information about the animal (fish species, number of fish per treatment, initial and final body weight) and the experimental conditions (duration of the exposure in days, feeding frequency etc.) was recorded in the database (Table 2.S7).

The meta-analysis was performed for all species collectively (63 data points), and rainbow trout separately (35 data points). Initially, in both databases the quality of the data was assessed graphically by scatter plots and the Spearman's rho correlation test. Outliers were not removed as they might reflect pathological effects and the high variability in the experimental animals received a DON-contaminated diet. Afterwards, the effect of dietary DON on feed intake (% control) and growth (% control) was preliminarily assessed by regression analysis. For all cases, the outcomes showed that the DON impact on the dependent variables was explained better ($>R^2$) by a quadratic relationship over a linear. However, a quadratic relationship could not physiologically explain the effect of DON on either feed intake or growth and, therefore, an exponential trendline was chosen. Finally, by using SAS software® (version 9.4, SAS Institute, Cary, NC) and the NLIN (non-linear regression) procedure, exponential equations were estimated to describe the relation between predictor (x) and response variables (y):

$$(y) = a * e^{b(x)} \quad (1)$$

where (y) is the feed intake (% control) or growth (% control); (x) concentration of DON (mg/kg) in the feed; (a) the value for (y) when DON concentration is 0; and (b) the regression coefficient.

The pseudo-R2 to assess model fit was calculated as:

$$\text{Pseudo-}R^2 = 1 - (\text{SSerror}/\text{SStotal(corrected)}) \quad (2)$$

where SSerror is the error sums of squares and SStotal the total sum of squares.

For rainbow trout data, it was evaluated if the feed intake and growth response vary significantly between two types of DON; natural vs. pure by testing the difference in their regression coefficients. Finally, the relationship between feed intake (% control) and growth (% control) response in all fish species and rainbow trout was assessed by linear regression.

2.3 Results

2.3.1 Survey of Feed Ingredients and Aquafeeds

2.3.1.1 Wheat

Wheat as an Ingredient in Aquafeeds

A total of 266.1 Mt of wheat was produced in 2019 in Europe, where wheat is the main cereal crop (FAOSTAT., 2020; Tacon et al., 2011). Wheat productivity, as for other crops, is dependent upon an optimal range of both temperature and precipitation (Hatfield and Dold, 2018). Based on the predictions of several mathematical models, it is estimated that the production of wheat will fall by 6% for each °C of further temperature rise and thus future wheat productivity could become uncertain (Asseng et al., 2015). Also, fungal growth is dependent on environmental factors such as temperature, but also pH, water availability, nutrients and light and, therefore, it is rational to assume that climate change will affect wheat production through a direct effect on fungal and mycotoxin presence (Thielecke and Nugent, 2018). The majority of wheat produced is milled and destined for human consumption, while only a portion of the total production and almost all of the milling by-product (wheat bran) is used as an ingredient in feeds for both terrestrial animals and fish (Gatlin Iii et al., 2007). Fungi mainly grow in the outer part of the kernels, and consequently, the relative concentration of mycotoxins is higher in wheat bran (Jouany, 2007). The essential amino pattern in wheat and its by-products is unbalanced, so that these feed ingredients are primarily incorporated in aquafeeds as the main starch source, to function as binders that improve water stability of the pellets (Gatlin Iii et al., 2007). These nutritional characteristics are the main reason that traditional formulation software restricts its inclusions in fish feed formulations, especially for carnivorous species (Lall et al., 2015). For instance, the average inclusion of wheat was reported as low as 9.9% for salmon in Norway (Ytrestøyl et al., 2015) or 10.6% for trout feeds in France, Greece, Denmark, Norway and UK (Tacon et al., 2011). Also for marine species farmed in Europe the average inclusion is low at 7.5% while, in contrast, in feeds for herbivorous/omnivorous tilapia, the average wheat inclusion can be as high as 19.9% (Tacon et al., 2011) (Table 2.S2). Thus, wheat inclusion rate varies within feeds for different fish species.

Mycotoxins in Wheat

From our analysis of n = 857 wheat samples from European countries, 42 distinct mycotoxins were retrieved, including regulated toxins, mycotoxins with guidance levels, masked as well as emerging mycotoxins (Table 2.1). Interestingly, 80% of the tested samples were positive for at least one mycotoxin, and in 63% of the analysed samples more than one mycotoxin was found. Average mycotoxin co-occurrence was four, and the maximum number of different toxins present in one sample was 14. Mycotoxin co-occurrence in wheat has been reported in previously published surveys (Alkadri et al., 2014; Curtui et al., 1998; Rafai et al., 2000; Škrbić et al., 2011) although the figures cannot be directly compared since only a few toxins were analysed, and incidence of co-occurrence is presented only for either animal feed samples (Rodrigues and Naehrer, 2012), or for all matrixes analysed (Monbaliu et al., 2010). Finally, data from 8 years of field surveys revealed a co-occurrence of DON and ZEN, and between DON/ZEN and their modified forms in cultivated wheat in the Netherlands (Van der Fels-Klerx et al., 2021).

Our analysis showed DON to be the most frequently reported toxin, detected in 41% of the samples (348/857 positive samples), followed by FB_1 (27%) and FX (23%). Average and maximum values of toxin contamination for the analysed toxins are given in Table 2.1. Average DON contamination was 470 µg/kg, with 8872 µg/kg being the highest level of DON detected in a sample from Lithuania in 2017. The Lithuanian sample was the only sample that exceeded the critical limit in cereals recommended by EC, currently set at 8000 µg/kg (Table 2.S1). Highly comparable to our findings, a recent report on the occurrence of DON in wheat samples from Europe (Gonçalves et al., 2017) mentioned an average contamination level of 418 µg/kg with a maximum of 6219 µg/kg. The most extreme value of DON so far reported for wheat/wheat bran was 49000 µg/kg found in Central Europe, with an average contamination of 848 µg/kg (Rodrigues and Naehrer, 2012). Furthermore, DON characterized as the most frequent mycotoxin in cultivated wheat in The Netherlands, which occurred on average in 54% of the samples with a mean DON contamination of 228 µg/kg (Van der Fels-Klerx et al., 2021). These data come from 8 years of field surveys and revealed that DON contamination in wheat was mainly affected by year and region. In contrast, agronomic practices (fungicides against *Fusarium* spp, crop rotation, resistant wheat cultivars) did not have an influence on DON contamination in wheat. Most commonly, DON levels in wheat appear to be governed by climatic conditions and below the critical limit.

Other important *Fusarium* toxins, like ZEN and T-2 toxin in wheat, were detected in 5% and 7% of the cases, respectively, with only one sample containing 551 µg/kg T-2 toxin, slightly above the critical limit set by the EC (Table 2.S1). The emerging mycotoxins BEA and MON were present in only 1% of the analysed samples with a maximum contamination level of 14 and 24 µg/kg, respectively. Although ENNs have been reported as the most frequent toxins in Romanian wheat grains and flour samples (Stanciu et al., 2017), in our current study wheat samples were not analysed for ENNs. Also, masked mycotoxin DON3Gluc (13%) was found in 53 wheat samples harvested in Serbia, although at low contamination levels from 17 to 83 µg/kg (Škrbić et al., 2011). We detected DON3Gluc in only 7% of the samples, with a maximum value of 1072 µg/kg, suggesting “traditional” DON being most frequent in wheat.

Table 2.1 | Mycotoxins occurrence in wheat (n = 857), corn (n = 725) and soybean meal (n = 139) samples[1].

	Wheat			Corn			Soybean Meal		
Mycotoxin	Occurrence[2] (%)	Mean (µg/kg)	Maximum (µg/kg)	Occurrence[2] (%)	Mean (µg/kg)	Maximum (µg/kg)	Occurrence[2] (%)	Mean (µg/kg)	Maximum (µg/kg)
15-acetyl-deoxynivalenol (15-AcDON)	4	51	217	20	133	1667	1	13	13
3-acetyl-deoxynivalenol (3-AcDON)	7	28	101	14	46	406	.	.	.
Aflatoxin B_1 (AFB_1)	2	2	6	4	12	148	6	1	2
Aflatoxin B_2 (AFB_2)	3	9	51	4	19	92	2	4	5
Aflatoxin G_1 (AFG_1)	1	3	14	2	12	67	2	19	51
Aflatoxin G_2 (AFG_2)	3	3	14	8	7	60	1	2	2
Alternariol	12	30	247	3	20	110	9	27	109
Beauvericin (BEA)	1	5	14	5	56	552	4	11	27
Citreoviridin	0.1	1172	1172	0.1	33	33	.	.	.
Citrinin	1	9	17	0.3	10	18	5	84	224
Cyclopiazonic acid	1	19	44	2	16	73	2	19	30
Deoxynivalenol (DON)	41	470	8872	47	826	10020	11	85	543
Diacetoxyscirpenol (DAS)	1	39	81	3	26	187	.	.	.
DON-3-Glucoside (DON3Glc)	7	137	1072	7	202	851	4	59	62
Ergocristin(in)e	0.4	95	189	.	.	.	.	.	.
Ergocryptin(in)e	0.5	10	25	.	.	.	.	.	.
Ergometrin(in)e	4	23	361	2	8	34	5	4	9
Ergosin(in)e	0.2	35	46	.	.	.	.	.	.
Ergotamin(in)e	3	119	1891	4	7	102	4	4	6
Fumonisin B_1 (FB_1)	27	561	9122	70	2234	49347	26	371	1462
Fumonisin B_2 (FB_2)	14	59	590	54	262	7944	19	83	424

Table 2.1 - Continued

Mycotoxin	Wheat			Corn			Soybean Meal		
	Occurrence[2] (%)	Mean (µg/kg)	Maximum (µg/kg)	Occurrence[2] (%)	Mean (µg/kg)	Maximum (µg/kg)	Occurrence[2] (%)	Mean (µg/kg)	Maximum (µg/kg)
Fumonisin B_3 (FB_3)	4	67	417	41	189	3203	6	50	159
Fusarenon X (FX)	23	91	1267	10	96	604	12	65	196
Fusaric acid (FA)	5	54	337	67	266	4327	42	89	754
Gliotoxin	2	292	811	1	247	879	.	.	.
HT-2 toxin	4	44	456	9	190	2643	4	155	561
Lysergol	3	4	8	2	2	6	8	3	9
Methylergonovine	6	3	11	5	5	30	7	3	14
Moniliformin (MON)	1	14	24	10	171	1103	.	.	.
Mycophenolic acid	2	39	228	4	79	478	1	297	297
Neosolaniol (NEO)	6	18	79	8	48	589	8	26	158
Nivalenol (NIV)	1	275	453	4	661	1660	3	231	291
Ochratoxin A (OTA)	11	6	45	9	24	648	12	3	7
Ochratoxin B	5	3	9	6	4	53	6	3	6
Patulin	1	128	183	1	102	183	2	101	106
Penicillic acid	.	.	.	3	297	2156	.	.	.
Roquefortine C	5	3	26	10	4	71	10	2	5
Sterigmatocystin	6	4	21	8	2	5	12	2	4
T-2 toxin	7	46	551	14	81	757	23	49	348
Verruculogen	8	15	367	5	65	802	3	10	17
Wortmannin	4	39	474	2	124	508	1	25	28
Zearalanone	0.2	463	606	1	137	555	.	.	.
Zearalenone (ZEN)	5	64	738	16	165	1282	14	81	354

[1] Mean and maximum values were calculated for the positive samples. [2] In case that a toxin was not detected in any of the samples (below the detection limits of analysis), the symbol "." is used and represents 0% occurrence.

2.3.1.2 Corn

Corn as an Ingredient in Aquafeeds

The European production of corn reached 132.8 Mt in 2019, corresponding to 11.6% of the total corn produced that year globally (FAOSTAT., 2020). Corn gluten meal (CGM) is a product derived from the wet-milling processing of corn, with an adequate crude protein content of 60% which is highly digestible. Therefore, it is often used as a protein source in fish diets, although due to its deficiency in lysine, diets are usually supplemented with synthetic amino acids or combined with other protein sources to meet the animals` nutritional requirements (Hardy, 2010). Corn itself can be included in the diets of omnivorous species (Gatlin Iii et al., 2007) such as Nile tilapia (*Oreochromis niloticus*) and common carp (*Cyprinus carpio*) at average inclusion levels of 27–30% (Tacon et al., 2011). For carnivorous species (trout, salmon) and marine species (European sea bass, *Dicentrarchus labrax*, gilthead sea bream, *Sparus aurata*), CGM is often used (Gatlin Iii et al., 2007; Hardy, 2010). Its inclusion rate can, therefore, be lower in diets for rainbow trout (*Oncorhynchus mykiss*) at 7.5% and for sea bass and sea bream at 8.8% (Gatlin Iii et al., 2007; Tacon et al., 2011). Corn inclusion rate in aquafeed therefore varies with the exact product (corn versus corn gluten meal) and with the fish species.

Mycotoxins in Corn

From our analyses for regulated toxins, mycotoxins with guidance levels, masked and emerging mycotoxins based on n = 725 corn samples from Europe we could reveal the presence of 40 different mycotoxins (Table 2.1). According to the survey results, at least one mycotoxin was found in 95% of all analysed corn samples, whereas in the majority (88%) of samples multiple mycotoxins were detected. The highest number of mycotoxins that were simultaneously present in a single corn sample was 17, while the average number of mycotoxins co-occurring in corn was 6.

A comparison of our mycotoxin co-occurrence dataset with other studies might not be directly informative due to the inconsistency of the available information presented in the literature, as discussed previously for the wheat data. Yet, the presence of numerous mycotoxins is a phenomenon well described in literature (Gonçalves et al., 2017; Kos et al., 2020; Kovalsky et al., 2016; Scudamore et al., 1998; Streit et al., 2012). *Fusarium* toxins appear to be among the most frequent mycotoxins present in corn (Table 2.1). Among the *Fusarium* mycotoxins with a guidance level, FB_1 was found in 70% of the samples, followed by FB_2 (54%), DON (47%), ZEN (16%) and T-2 toxin (14%). Data analysis also showed a high frequency of *Fusarium* mycotoxins without any regulated or recommended limit; FA (67%), FB_3 (41%), 15AcDON (20%), 3AcDON (14%) and FX (10%) and of *Fusarium* emerging mycotoxin MON (10%). Besides the *Fusarium* mycotoxins, a *Penicillium*-derived mycotoxin, roquefortine C was detected in 10% of the corn samples. In comparison, a three-year survey of corn samples in Europe (Rodrigues and Naehrer, 2012) for aflatoxins (AFLAs) (31%), ZEN (30%), DON (72%), FUM (60%) and OTA (10%) estimated high frequencies of FUM, DON and ZEN similar to our observations. A recent survey (Kos et al., 2020) combined the yearly presence of mycotoxins in corn harvested in Serbia between 2012–2015 with meteorological data and thus

linked observed differences in mycotoxin patterns to different weather conditions. For instance, the high occurrence of AFLAs in 2012 could be related to the prolonged drought reported that year and the high occurrence of DON and ZEN in 2014 could be linked to extreme precipitation. Regardless of the year and weather conditions, FUM were dominating (76–100%) in the corn samples. Unfortunately, in our study, it was not possible to correlate mycotoxins with meteorological data since our database was generated from samples originating from various locations in Europe.

In our survey of corn samples, AFB_1 (4%) and OTA (9%) did not often occur, although in five cases AFB_1 was above the regulated limit of 20 µg/kg, and in one sample OTA exceeded the recommended limit of 250 µg/kg. Of interest, a predictive model on the occurrence of AFB_1 under a climate scenario of 2 °C increase due to global warming within the next 100 years shows that this toxin will become a serious food and feed safety concern in corn, even in temperate areas like Europe (Battilani et al., 2016). In our survey of corn samples, the maximum level for DON was 10,020 µg/kg and the maximum level for ZEN was 1282 µg/kg. Others have reported values for DON = 26121 µg/kg and ZEN = 849 µg/kg (Rodrigues and Naehrer, 2012), or DON = 4000 µg/kg and ZEN = 10,000 µg/kg (Kovalsky et al., 2016) or DON = 19,180 µg/kg and ZEN = 8888 µg/kg (Gonçalves et al., 2017). In all cases, the maximum DON level exceeded the EC guidance level of 8000 µg/kg. In our database, only three samples were detected with a DON level above this limit, whereas for ZEN all samples were below the EC guidance level (<2000 µg/kg). Similarly, levels of the most frequently occurring toxin in our corn samples, FB_1 were below the EC recommendation (60,000 µg/kg). Occurrence of T-2 and HT-2 toxin were collectively examined with 9% positive samples above the guidance of 500 µg/kg and a maximum level of 3340 µg/kg. Other frequently detected toxins cannot be assessed for risk levels since there is not regulatory or guidance limit by the EC. Maximum contamination levels of FA (4327 µg/kg), FB_3 (3203 µg/kg), 15AcDON (1667 µg/kg), 3AcDON (406 µg/kg) and FX (604 µg/kg) detected in our samples are difficult to compare because other surveys have not analysed corn for these toxins. Only (Kovalsky et al., 2016) discussed the presence of the emerging toxin MON in corn samples from Southern Europe but reported generally low concentrations (<100 µg/kg). The same study reported the highest MON values (400 µg/kg) in South Africa, whilst our dataset showed a maximum of 1103 µg/kg with an average MON contamination of 171 µg/kg in European corn samples, which is relatively low.

2.3.1.3 Soybean Meal

Soybean Meal as an Ingredient in Aquafeeds

Soybean meal (SBM) is one of the most commonly used plant-protein ingredients to substitute fishmeal in aquafeeds (Oliva-Teles et al., 2015), although its inclusion is restricted due to its low crude protein level (48%), limited methionine content, and the presence of anti-nutritive compounds such as saponins (Hardy, 2010). Average values for SBM inclusion have been estimated at 21.3% for salmon diets (Ytrestøyl et al., 2015) and, based on extrapolation (Tacon et al., 2011), estimated at 15.5% in trout diets, 19.2% in sea bass/sea bream diets and 13.5% in carp diets. In trout, SBM appears to increase the permeability of the distal intestinal epithelium

and limit the capacity of this region to absorb nutrients (Nordrum et al., 2000), whereas inclusion of untreated SBM up to 30–45% resulted in histopathological alterations in the intestine, described as reduced numbers of absorptive vacuoles and numbers of goblet cells (Heikkinen et al., 2006). Similarly, in Atlantic salmon, inclusion of 30% SBM caused pathological effects in the distal intestine, described as reduced height of tissue folds and reduced vacuolization (Krogdahl et al., 2010). In common carp, dietary inclusion of 20% SBM induced intestinal inflammation which diminished after a few weeks of feeding, implying the ability of carp to adapt to SBM ingestion (Urán et al., 2008a). In marine sea bass, inclusion of 30% SBM in the diet did not adversely affect growth, gut histology, or blood parameters (Bonvini et al., 2018). Also tilapia can tolerate high inclusion levels of SBM, with average inclusion rates of 30.9% (Table 2.S2) (Tacon et al., 2011). Tilapia fingerling growth and health do not seem to be compromised by total replacement of fishmeal by SBM (55% inclusion with supplementation of 0.5% L-lysine) (El-Saidy and Gaber, 2002). Overall, the effects of SBM inclusion in diets depend on the fish species.

Mycotoxins in Soybean Meal

We analysed 139 SBM samples in total for regulated, emerging and masked mycotoxins, in addition to those with a guidance level. Results showed that 33 individual toxins were detected in SBM (Table 2.1). At least one mycotoxin was detected in 87% of the analysed SBM samples and in the greater portion (75%) of these positive samples more than one mycotoxin occurred. On average, co-occurrence of mycotoxins was four, with a maximum of 12 different mycotoxins. We report higher values than an earlier study of European SBM samples in 2015 (Gonçalves et al., 2017), which reported 58% positive samples and 32% co-occurrence in (only) 19 SBM samples. Similar to the high (75%) percentage of co-occurrence we report, a study of soya used for animal feed production in Italy also reported 72% of the samples contained at least two mycotoxins (Gutleb et al., 2015). Co-occurrence of several mycotoxins, therefore, appears common.

Of all mycotoxins in SBM, the ones produced by *Fusarium* fungi were the most common (Table 2.1), with FA being the most represented toxin in our samples (42%). To our knowledge, this is the first study that analysed and reported FA occurrence in SBM. Following FA (42%), we report common occurrence of FB_1 (26%), T-2 toxin (23%) and FX (12)%. Besides these mycotoxins produced by *Fusarium* fungi, also *Penicillium*/*Aspergillus*-derived mycotoxin OTA (12%) and *Aspergillus*-derived sterigmatocystin toxin (12%) were found frequently. Relatively low sample numbers (11%) were positive for DON, with a maximum contamination level of 543 µg/kg. An earlier study in 2004 reported similarly low occurrence (9.1%, 110 µg/kg) in Serbia, but was based on only 11 analysed samples. In strong contrast, other surveys reported DON as the most prevalent toxin in SBM with a maximum contamination level of 930 µg/kg (Gonçalves et al., 2017), maximum contamination levels of 714 and 908 µg/kg in samples from Central and Southern Europe (Rodrigues and Naehrer, 2012), or average DON contamination levels of 2600 µg/kg with a maximum of 6400 µg/kg in Italian soya (Gutleb et al., 2015). Despite the inconsistency in DON contamination levels, possibly related to sampling differences (method, geographic location, climatic conditions), contamination levels were always below the EC recommended limit (<8000 µg/kg). In our database, only one SBM sample

originating from Germany in 2017 was contaminated with T-2 and HT-2 toxin levels (560 µg/kg) that exceeded the EC guidance value. Overall, SBM showed relatively low contamination levels compared to contamination levels in wheat and corn. SBM is a co-product of oil extraction from soybeans and exposed to high temperatures during the processing step of toasting and perhaps heat treatment helps eliminate mycotoxins from SBM (Karlovsky et al., 2016). More extensive screening may reveal more consistent values for DON contamination of SBM in the future.

2.3.1.4 Probability of Mycotoxin Co-Occurrence in Feedstuffs: The Case of Corn

Our results demonstrate that corn represents a matrix with the highest risk of mycotoxin contamination, but the precise explanation for this is unclear. The reason that corn serves as such a prime host for fungal growth might be related to host genotype (Bottalico and Perrone, 2002). Whereas corn defense systems can respond to fungal pathogens through the expression of defense-related genes, expression of such genes seems to be low in susceptible corn varieties (Lanubile et al., 2015). In general, corn acts as a host to multiple fungi (Giorni et al., 2019) and thus multiple mycotoxin contaminations may prevail in corn fields.

Indeed, previous research confirmed DON occurrence in corn samples to be correlated with other toxins although specific co-occurrence patterns were only hypothesized but not identified (Streit et al., 2012). A recent search in literature (Palumbo et al., 2020) suggested that DON + FUM had the highest probability (74.4%) of co-occurrence in European corn samples, but also concluded that further research is needed to identify co-occurrence patterns of multiple mycotoxins based on field investigations. In our samples, DON frequently co-occurred with other *Fusarium* mycotoxins; FA (32%), FB_2 (26%), 15AcDON (19%), ZEN (14%), 3AcDON (12%), DON3Gluc (7%) and FX (6%). A test for significance (Spearman, $p < 0.05$) confirmed a correlation between DON-positive samples and associated toxins with a concentration above the detection limit. A significant moderate correlation ($r > 0.5$ and $p < 0.0001$) was revealed for the following mycotoxin combinations: DON + 3AcDON ($r = 0.57$), DON + 15AcDon ($r = 0.62$), DON + ZEN ($r = 0.64$). We also investigated the concept of mycotoxin co-occurrence in feedstuffs as the likelihood of association between DON and other toxins (“Toxin X”) detected in corn, and data were expressed as odds ratio (OR). Results from the OR test showed that exposure to specific toxins is associated with at least two times higher odds of DON occurrence ($OR > 2$ and $p < 0.05$): DON3Gluc, 15-AcDON, NIV, 3AcDON, ZEN, alternariol, roquefortine C, sterigmatocystin, HT-2 toxin, T-2 toxin. The association of DON with the other 39 toxins detected in corn is displayed in Table 2.2 The toxins in Table 2.2 are ordered from the highest to the lowest significant OR, followed by the toxins for which the OR was not found significant. Only for $OR < 1$, the toxins are ordered from the lowest to the highest value because in these cases, when “Toxin X” (BEA, MON, AFB_2) is present there are fewer odds for the presence of DON. In other words, when “Toxin X” is absent there is a higher risk for the presence of DON.

In corn, DON is more likely to co-occur with other mycotoxins when it is present in its acetylated (3-AcDON, 15-AcDON), modified forms (DON3Gluc). Also, there are higher odds that DON co-occurs with some *Fusarium* toxins (ZEN, T-2 toxin, HT-2 toxin, NIV), while fewer odds with other *Fusarium* toxins (BEA, MON) and aflatoxin B_2 (AFB_2). Available data on the *Fusarium* species and their mycotoxins from maize ear rot in Europe are used to discuss

our observations. For the correlation of DON with the chemotypes ZEN, NIV and DON, associated forms might occur because they can all be produced by the strains *F. graminearum* and *F. culmorum* (Logrieco et al., 2002). By contrast, the negative association of DON with the following toxins might be because they are produced by different fungi; BEA (*F. subglutinans* and *F. proliferatum*), MON (*F. avenaceum, F. proliferatum* and *F. subglutinans*), AFB_2 (*A. flavus*) (Logrieco et al., 2002). T-2 and HT-2 toxin are mainly produced by different strains than DON: *F. sporotrichioides, F. acuminatum* (Logrieco et al., 2002), although we hypothesize that the positive correlation between these chemotypes could be explained by a positive interaction between their fungi. In general, information about the interactions between individual fungal strains is not always available, and we cannot always expect that observed correlations are an outcome of a similar relationship between the relevant mycotoxin-producing fungi (Obradovic et al., 2018). For example, the latter study found a significant positive correlation between AFB_1 and FUM levels, but not between the incidences of *A. flavus* and *F. verticillioides*. Thus, mycotoxin production might be driven more by climatic conditions than by the distribution of their corresponding mycotoxin-producing fungi.

Table 2.2 | Odds ratio (OR) of the association between deoxynivalenol (DON) and contamination with other toxins in corn.

"Toxin X"	Category	Frequency [1]		% DON [2]	Odds ratio (OR)	95% CI	Wald *p*-Value [3]
		n	%				
DON-3-Glucoside (DON3Glc)	present	53	7.3	98.1	69.6	9.6–505.9	***
	absent	672	92.7	42.7	Ref.		
15-acetyl-deoxynivalenol (15-AcDON)	present	142	19.6	96.5	51.7	20.8–128.2	***
	absent	583	80.41	34.7	Ref.		
Nivalenol (NIV)	present	30	4.1	96.7	36.0	4.9–265.9	***
	absent	695	95.9	44.6	Ref.		
3-acetyl-deoxynivalenol (3-AcDON)	present	98	13.5	91.8	17.1	8.1–35.8	***
	absent	627	86.5	39.7	Ref.		
Zearalenone (ZEN)	present	116	16.0	90.5	15.3	8.0–29.1	***
	absent	609	84.0	38.4	Ref.		
Sterigmatocystin	present	57	7.9	68.4	2.7	1.5–4.7	***
	absent	668	92.1	44.9	Ref.		
Roquefortine C	present	72	9.9	68.1	2.7	1.6–4.5	***
	absent	653	90.1	44.4	Ref.		

Table 2.2 - Continued

"Toxin X"	Category	Frequency [1]		% DON [2]	Odds ratio (OR)	95% CI	Wald *p*-Value [3]
		n	%				
Alternariol	present	20	2.8	70.0	2.7	1.04–7.2	*
	absent	705	97.2	46.1	Ref.		
HT-2 Toxin	present	67	9.2	64.2	2.2	1.3–3.7	**
	absent	658	90.8	45.0	Ref.		
T-2 toxin	present	103	14.2	62.1	2.1	1.3–3.2	***
	absent	622	85.8	44.2	Ref.		
Fusarenon X (FX)	present	70	9.7	60.0	1.8	1.1–3.0	*
	absent	655	90.3	45.3	Ref.		
Neosolaniol (NEO)	present	57	7.9	59.7	1.8	1.01–3.1	*
	absent	668	92.1	45.7	Ref.		
Fumonisin B_1 (FB_1)	present	505	69.7	50.3	1.6	1.2–2.2	**
	absent	220	30.3	38.6	Ref.		
Fumonisin B_3 (FB_3)	present	296	40.8	53.0	1.5	1.1–2.1	**
	absent	429	59.2	42.4	Ref.		

Table 2.2 - Continued

"Toxin X"	Category	Frequency [1]		% DON [2]	Odds ratio (OR)	95% CI	Wald *p*-Value [3]
		n	%				
Beauvericin (BEA)	present	36	5.0	25.0	0.36	0.17–0.78	**
	absent	689	95.0	47.9	Ref.		
Moniliformin (MON)	present	70	9.7	31.4	0.48	0.29–0.83	**
	absent	655	90.3	48.4	Ref.		
Aflatoxin B_2 (AFB_2)	present	29	4.0	31.0	0.49	0.22–1.1	#
	absent	696	96.0	47.4	Ref.		
Gliotoxin	present	5	0.7	80.0	4.6	0.51–41.3	NS
	absent	720	99.3	46.5	Ref.		
Zearalanone	present	8	1.1	75.0	3.5	0.69–17.3	NS
	absent	717	98.9	46.4	Ref.		
Lysergol	present	12	1.7	66.7	2.3	0.69–7.7	NS
	absent	713	98.3	46.4	Ref.		
Diacetoxyscirpenol (DAS)	present	25	3.5	60.0	1.7	0.77–3.9	NS
	absent	700	96.6	46.3	Ref.		

Table 2.2 - Continued

"Toxin X"	Category	Frequency [1]		% DON [2]	Odds ratio (OR)	95% CI	Wald *p*-Value [3]
		n	%				
Methylergonovine	present	35	4.8	57.1	1.6	0.78–3.1	NS
	absent	690	95.2	46.2	Ref.		
Ochratoxin A (OTA)	present	62	8.6	56.5	1.5	0.91–2.6	NS
	absent	663	91.5	45.9	Ref.		
Ergotamin(in)e	present	30	4.1	56.7	1.5	0.73–3.2	NS
	absent	695	95.9	46.3	Ref.		
Verruculogen	present	37	5.1	54.1	1.4	0.70–2.6	NS
	absent	688	94.9	46.4	Ref.		
Aflatoxin G_1 (AFG_1)	present	13	1.8	53.9	1.3	0.45–4.0	NS
	absent	712	98.2	46.6	Ref.		
Aflatoxin B_1 (AFB_1)	present	27	3.7	51.9	1.2	0.57–2.7	NS
	absent	698	96.3	46.6	Ref.		
Aflatoxin G_2 (AFG_2)	present	61	8.4	50.8	1.2	0.71–2.0	NS
	absent	664	91.6	46.4	Ref.		

Table 2.2 - Continued

"Toxin X"	Category	Frequency [1]		% DON [2]	Odds ratio (OR)	95% CI	Wald *p*-Value [3]
		n	%				
Fusaric acid (FA)	present	485	66.9	48.0	1.2	0.86–1.6	NS
	absent	240	33.1	44.2	Ref.		
Ochratoxin B	present	44	6.1	50.0	1.1	0.62–2.1	NS
	absent	681	93.9	46.6	Ref.		
Ergometrin(in)e	present	12	1.7	50.0	1.1	0.37–3.6	NS
	absent	713	98.3	46.7	Ref.		
Citreoviridin	present	1	0.1	100.0	1.1	0.06-∞	NS [4]
	absent	724	99.9	46.8	Ref.		
Mycophenolic acid	present	31	4.3	48.4	1.1	0.52–2.2	NS
	absent	694	95.7	46.7	Ref.		
Fumonisin B_2 (FB_2)	present	394	54.3	47.0	1.0	0.76–1.4	NS
	absent	331	45.7	46.5	Ref.		
Wortmannin	present	17	2.3	47.1	1.0	0.39–2.7	NS
	absent	708	97.7	46.8	Ref.		

Table 2.2 - Continued

"Toxin X"	Category	Frequency [1]		% DON [2]	Odds ratio (OR)	95% CI	Wald *p*-Value [3]
		n	%				
Patulin	present	5	0.7	20.0	0.28	0.03–2.5	NS
	absent	720	99.3	46.7	Ref.		
Citrinin	present	2	0.3	0.0	0.47	0.0–4.0	NS[4]
	absent	723	99.7	46.9	Ref.		
Cyclopiazonic acid	present	16	2.2	43.8	0.88	0.33–2.4	NS
	absent	709	97.8	46.8	Ref.		
Penicillic acid	present	24	3.3	45.8	0.96	0.43–2.2	NS
	absent	701	96.7	46.8	Ref.		

[1] n refers to the number of samples where "Toxin X" is present or absent, and % is the percentage of frequency relative to the total number of corn samples. [2] Percentage of cases that DON exists with "Toxin X" present, and cases with "Toxin X" absent. [3] Wald Chi-Square Test: Not significant (NS): $p \geq 0.1$, # $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. [4] Estimated with Exact Logistic Regression.

2.3.1.5 Aquafeeds

Mycotoxins in Aquafeeds

All feed samples analysed (*n* = 44) were contaminated with at least one mycotoxin (Table 2.3). A total of 75% of the samples contained more than one mycotoxin simultaneously, and on average a range of 3 to 9 out of a possible total of 24 mycotoxins was found in aquafeed samples. Likewise, another study of aquafeed samples from Asia (*n* = 31) and Europe (*n* = 10) revealed that in 76% of the samples more than one toxin co-occurred (Gonçalves et al., 2018a). Our data confirm the general observation that animal feed samples often contain multiple mycotoxins (75–100%), especially when more than one plant feed ingredient is included in the diet formulations (Streit et al., 2012).

Table 2.3 | Mycotoxins occurrence in aquafeed samples [1] (n = 44).

Mycotoxin	Occurrence (%)	Mean (µg/kg)	Maximum (µg/kg)
15-acetyl-deoxynivalenol (15-AcDON)	5	82	127
Aflatoxin B_1 (AFB_1)	5	2	4
Aflatoxin G_2 (AFG_2)	2	6	6
Alternariol	14	21	51
Deoxynivalenol (DON)	48	136	469
Diacetoxyscirpenol (DAS)	2	9	9
DON-3-Glucoside (DON3Glc)	18	98	155
Ergometrin(in)e	7	4	5
Ergotamin(in)e	20	38	125
Fumonisin B_1 (FB_1)	36	628	4923
Fumonisin B_2 (FB_2)	27	120	778
Fumonisin B_3 (FB_3)	11	86	223
Fusarenon X (FX)	2	28	28
Fusaric acid (FA)	55	41	265
Gliotoxin	2	92	92
HT-2 toxin	2	43	43
Lysergol	9	10	23

46

Table 2.3 - Continued

Mycotoxin	Occurrence (%)	Mean (µg/kg)	Maximum (µg/kg)
Ochratoxin A (OTA)	2	3	3
Penicillic acid	11	41	58
Sterigmatocystin	2	1	1
T-2 toxin	2	46	46
Verruculogen	9	560	636
Wortmannin	2	20	20
Zearalenone (ZEN)	2	348	348

[1] Mean and maximum values were calculated for the positive samples.

The most representative toxins belong to the *Fusarium* group; FA (55%), DON (48%), FB_1 (36%), FB_2 (27%) and the masked mycotoxin DON3Gluc (18%). For instance, an *Aspergillus*-produced mycotoxin, verruculogen, was present in only 9% of the samples, but with an average contamination level of 560 µg/kg and maximum contamination 636 µg/kg. None of the previous aquafeed mycotoxin surveys had analysed and thus reported the presence of verruculogen. Surprisingly, information is also lacking for FA even if it was the most frequent toxin in our samples with a maximum concentration of 265 µg/kg. Similarly, the existence of DON3Gluc, FB_2 and penicillic acid was not previously reported in published data on aquafeed samples. Overall, it was recommended to analyse aquafeed samples for masked mycotoxins like DON-3-glucoside due to their potential to be metabolized to the parent toxin by commensal lactic acid bacteria in the gastrointestinal tract (Berthiller et al., 2011).

Typically, DON has been described as the most common mycotoxin in animal feeds (Streit et al., 2012) and fish feeds (Gonçalves et al., 2018a). In our study, the average contamination level of DON was 136 µg/kg and the maximum contamination level of DON was 469 µg/kg. Earlier, DON had been identified in commercial aquafeeds with an average contamination of 166 µg/kg and a maximum of 282 µg/kg in 2014 (Gonçalves et al., 2018a). A pilot survey that included 11 samples of different commercial carp feeds from Central Europe detected ZEN in all samples (average contamination 67.9 µg/kg, maximum 511 µg/kg) and DON in 80% of the samples (average contamination 289 µg/kg, maximum 825 µg/kg) (Pietsch et al., 2013). By contrast, out of the 44 samples in the present study, only one sample was positive to ZEN with a concentration of 348 µg/kg. We also observed that in DON positive samples, FA was present in 62% of the cases, FB_1 in 48% and DON3Gluc in 24% of the cases. Our findings address, for the first time, DON contamination in aquafeeds along with other toxins. Previous research studies have not evaluated the toxicological effects of these mycotoxin mixtures on different fish species. Even if detected DON3Gluc concentration was low (average 98 µg/kg, maximum 155 µg/kg) it might potentially increase the total bioavailable DON in the intestinal lumen of the animals. Likewise, high levels of FA were not detected (average 41 µg/kg, maximum 265

μg/kg), although in combination with DON it appeared to induce synergetic effects in pigs (Smith et al., 1997). Overall, in our European fish feed samples, DON and other mycotoxins with a regulated/guidance value were compliant with the EC limits. Nevertheless, these limits are not customized to fish and importantly do not consider species sensitivities. In the following sections, fish susceptibility to DON will be evaluated based on in vivo dose-response exposure studies and take into account differences in species sensitivities.

2.3.2 Effects of Deoxynivalenol (DON) on Fish Species

As previously mentioned in Section 2.1, mycotoxins are readily present in plant ingredients: corn > wheat > soybean meal and in aquafeeds. In terms of occurrence and toxicity, DON has been characterized as the most high-risk mycotoxin in aquafeeds. Therefore here, by a systematic review we will summarize DON effects on different fish species. In parallel, data were collected in order to quantify the risk of exposure in fish. Finally, by employing a meta-analytical approach, the extent to which DON affects feed intake and growth performance was evaluated. Details on the studies used for this systematic review and meta-analysis are given in Tables S4 and S7, respectively.

2.3.2.1 Systematic Review

Like all trichothecenes, DON binds to ribosomes inducing a “ribotoxic stress response” that activates mitogen-activated protein kinases (MAPKs). The latter are components of a signaling cascade that regulate cellular processes; proliferation, differentiation, stress response and apoptosis (Iordanov et al., 1997; Plotnikov et al., 2011) and mediate inflammatory responses by altering the binding activities of specific transcription factors that lead to induction of cytokine gene expression (Xie et al., 2018). Additionally, DON causes oxidative stress in cells by damaging mitochondria function, either by excessive release of free radicals including reactive oxygen species (ROS) which induce lipid peroxidation or by decreasing the activity of antioxidant enzymes (Wu et al., 2014). Oxidative stress via the mitochondrial pathway can also induce apoptosis via MAPKs by the caspase-mediated cellular apoptosis pathway (Ren et al., 2020; Wu et al., 2014). Predominantly, rapidly proliferating cells with a high protein turnover such as immune cells, hepatocytes and epithelial cells of the digestive tract are affected by DON (Mayer et al., 2017; Pinton and Oswald, 2014). Earlier studies in mammals have demonstrated how the mechanism of action of DON affects gut functions (integrity, absorption, immunity), liver functions and the immune system (Akbari et al., 2017; Liew and Mohd-Redzwan, 2018; Oswald et al., 2005; Pestka, 2010; Pinton and Oswald, 2014). In contrast, earlier studies in fish mainly focused on indirect impacts of DON on productivity, e.g., feed intake, feed efficiency and growth performance (Anater et al., 2016; Kipper et al., 2020). Therefore here, when available, we also review the direct biological effects of DON in different fish species. The majority of the studies we reviewed exposed fish to DON through experimental satiation feeding regimes. We will indicate in our systematic review when fish were exposed to DON through restrictive feeding regimes. Also, we will mention if the studies we reviewed exposed fish to “natural” DON (derived from naturally contaminated feed ingredients and other toxins might be present in the aquafeed) or to “pure” DON (extracted and purified to exclude the presence of other toxins). Finally, we will describe the metabolic fate of DON in fish.

Salmon

In total, three *in vivo* studies have been reported that investigated the effects of DON in salmon, and all employed similar experimental conditions; exposure (8 weeks), age (12 months post-smoltification) and source of the toxin (pure DON) (Bernhoft et al., 2017; Bernhoft et al., 2018). Reduced growth performance (feed intake and weight gain) was observed in salmon fed the highest DON-containing diet (6000 µg/kg), but not in the low-DON group (2000 µg/kg) (Bernhoft et al., 2017). In a follow-up study by (Bernhoft et al., 2018), more dietary DON doses were used; 0, 500, 1000, 2000, 4000 and 6000 µg/kg. In this case, negative effects on growth performance appeared already in salmon receiving 4000 µg/kg DON; a significant decrease in feed intake was visible after 4 weeks and a reduced condition factor after 3 weeks of exposure. Salmon treated with the highest DON dose (6000 µg/kg) showed reduced weight gain after 3 weeks, and reduced body length and increased relative liver weight after 6 weeks of exposure. After 8 weeks of DON exposure, triglycerides were reduced at 1000 µg/kg, cholesterol, total proteins and albumin, bile acids, packed cell volume at 2000 µg/kg and alkaline phosphatase at 6000 µg/kg.

The most recent study in salmon (Bernhoft et al., 2018) tested a DON dose of 5500 µg/kg DON against a control treatment. Their findings confirmed impaired salmon performance (reduced feed intake, weight gain, and feed efficiency), and demonstrated for the first time a potential alteration of intestinal integrity and immunity after DON exposure. Specifically, they noted lower relative expression of proteins regulating paracellular permeability between adjacent intestinal epithelial cells, the tight junction proteins (TJPs). Also, an increased relative gene expression of immune markers (suppressors of cytokine signaling, SOCS); SOCS1 (expressed in pyloric caeca and distal intestine) and SOCS2 (expressed in the distal intestine) suggested altered immune regulation to prohibit intestinal damage and inflammation. In all intestinal segments, increased cell proliferation (base on immunohistochemical staining of PCNA, proliferating cell nuclear antigen) was noted in DON-treated salmon, interpreted as a local response to restore intestinal integrity. The total number of goblet cells was unaffected by DON exposure.

Rainbow Trout

The first scientific information about the effects of DON on rainbow trout was published in the 1980s . A dose-response exposure study (1000 to 13000 µg/kg) on juvenile trout for 4 weeks showed that increasing levels of DON resulted in reduced feed intake, weight gain and feed efficiency. Regression analysis suggested that for doses >5000 µg/kg each additional 1000 µg/kg of DON would suppress feed intake by 9% and weight gain by 11%, and for doses >7500 µg/kg each additional 1000 µg/kg of DON would suppress feed efficiency by 6%. In a preliminary experiment as part of the same study, after exposing trout to extremely high DON doses (>20000 µg/kg) for 4 weeks the authors reported a dramatic drop in feed intake within 5 days and a refusion of pellet ingestion. Of interest, after switching back to feeding non-contaminated diets for four more weeks, feed intake and growth recovered, implying the ability of rainbow trout to adapt to DON, at least after a short-term (4 weeks) exposure.

Surprisingly, no follow-up research was published for 28 years, until a comprehensive article (Hooft et al., 2011) defined rainbow trout as a fish species highly sensitive to DON. The authors showed that increasing levels of natural DON (300, 800, 1400, 2000, 2600 µg/kg) in diets of juvenile rainbow trout for 8 weeks, had a detrimental effect on growth performance, mirroring the effects described earlier even at considerably lower DON doses. At the top of growth performance, exposure to 1400 µg/kg DON significant reduced nitrogen (g/fish) and energy (kJ/fish) retention and their retention efficiencies (%). In addition, body composition analysis of trout fed a contaminated diet with 2600 µg/kg DON showed reduced crude protein content, although no change was observed in the apparent digestibility of crude protein and gross energy. Histological examination of the liver revealed congestion and subcapsular edema with a fibrinous network in rainbow trout exposed to ≥1400 µg/kg DON and multifocal areas fatty infiltration and phenotypically altered hepatocytes (pyknotic and karyolytic) in trout exposed to 2600 µg/kg DON. Moreover, to explore DON effects not related to differences in feed intake, authors employed an additional treatment; fish pair-fed the control diet the same amount of feed consumed by fish fed the highest DON dose (2600 µg/kg). Fish fed the DON diet showed significantly reduced growth rate (thermal growth coefficient; TGC), feed efficiency, protein and energy utilization efficiencies and whole body crude protein compared to the fish pair-fed the control diet. This observation suggests that reduced growth performance is not fully attributed to a reduced feed intake, but also metabolic disturbances related to the direct effects of DON on the cellular level. In contrast to (Hooft et al., 2011), in other experiments pair-feeding showed that suppressed weight gain in fish fed DON-contaminated diets might arise from depressed feed intake (Ryerse et al., 2016; Ryerse et al., 2015). However, the studies differed in trout size (~24g (Hooft et al., 2011) and ~103g (Ryerse et al., 2016)). Apart from the indirect effects on feed intake, DON toxicity may be age-dependent, with young trout being more vulnerable to metabolic effects of DON. Following the study in 2011 (Hooft et al., 2011), later studies confirmed a significant reduction in feed intake (≥4100 µg/kg) upon offering diets with increasing levels of natural DON (500, 4100, 5900 µg/kg) (Ryerse et al., 2016) and (≥3100 µg/kg) by testing diets with 100, 3100, and 6400 µg/kg natural DON (Ryerse et al., 2015). Moreover, the latter study in a sub-experiment measured reduced feed intake at the two tested DON doses (3300 µg/kg natural DON and 3800 µg/kg pure DON).

Subsequently, follow-up experiments on rainbow trout followed that investigated, next to the effects of DON on performance, nitrogen and energy balances and carcass composition, effects of a commercial anti-mycotoxin additive (Hooft and Bureau, 2017), potential synergy among *Fusarium* toxins present in naturally contaminated trout feeds (Hooft et al., 2019a), the impact of diet composition on detoxification capacity, and species sensitivity in a comparison with tilapia (Hooft et al., 2019b). Trout fingerlings (initial weight; 1.8 g) exposed to natural DON for 12 weeks showed reduced feed intake, weight gain, TGC, reduced nitrogen retention efficiency (≥1000 µg/kg), and reduced retained nitrogen (≥1500 µg/kg) (Hooft and Bureau, 2017). None of these effects could be reversed by the inclusion of a commercial feed additive, suggesting that anti-mycotoxin products developed for homeothermic species might not be as effective in cold-blooded species, such as trout. In another study (Hooft et al., 2019a), diets with graded levels of pure DON (0, 700, 1400 and 2100 µg/kg) or natural DON (0, 2100, 4100 and 5900 µg/kg) were offered to rainbow trout (initial weight; 50.3 g) for a period of 8 weeks.

Regardless of the DON source (pure/natural), deleterious effects were present, and similar trends of reduced retained nitrogen, recovered energy, nitrogen retention efficiency (≥2100 µg/kg pure/natural DON), and energy retention efficiency (>2100 µg/kg natural DON) were found. The same study (Hooft et al., 2019a) was the first to use histological examination to show harmful effects of DON on the gastrointestinal tract after feeding 2100 µg/kg pure or 5900 µg/kg natural DON. Last but not least, the most recent work of these authors (Hooft et al., 2019b) investigated if increased levels of digestible starch (12% vs. 24%) in rainbow trout diets contaminated with 100, 700 and 1300 µg/kg natural DON could help enhance DON detoxification to deoxynivalenol-glucuronide (DON-GlcA) via increased glucuronidation capacity. This did not seem to be the case because, regardless of the starch level, rainbow trout exhibited impaired growth performance, disturbances in nitrogen and energy balances and carcass composition, suggesting that the higher supply of carbohydrates from starch, which presumably increases the hepatic glycogen content, did not directly lead to DON detoxification.

Further studies had also confirmed the impact of DON on rainbow trout productivity; either by using low DON doses (1100 and 2700 µg/kg) (Gonçalves et al., 2019) or high (4700 and 11400 µg/kg) (Gonçalves et al., 2018b). Notably, the latter study provided new insights into the direct effects of DON by measuring proteolytic enzyme activity and relevant gene expression in the head kidney, liver, brain and gastrointestinal tract. Experimental DON doses of 4700 and 11400 µg/kg indeed affected the activities of proteolytic enzymes (pepsin, trypsin and chymotrypsin), although it remained unclear if the observed changes in enzyme activity were directly related to the toxin itself or a result of reduced feed intake. Surprisingly, gene expression of the neuropeptide Y precursor (*npy*) in the brain was up-regulated for doses ≥4700 µg/kg DON, whereas the opposite would have been expected for this appetite-stimulating precursor. Less surprising maybe, another gene in the brain of which the expression is also related to feed intake and growth control (growth hormone-releasing hormone/pituitary adenylate cyclase-activating polypeptide PACAP; *adcyap1a*) was down-regulated. Also in the liver, expression of genes related to growth control (insulin-like growth factors; *igf1, igf2*) were down-regulated. Finally, some other studies addressed the effects of DON on health, immune function and oxidative stress (Matejova et al., 2014; Matejova et al., 2015). When 1-year-old trout were exposed for 23 days to ~2000µg/kg DON, plasma biochemical parameters; glucose, cholesterol and ammonia were decreased (Matejova et al., 2014), pro-inflammatory cytokine TNF-α in the head kidney was up-regulated (Matejova et al., 2015) and altered activities of antioxidant enzymes were observed (Matejova et al., 2015). Overall, the sensitivity of rainbow trout productivity to DON is well defined, although further research is needed to explore the direct mechanism of action of the toxin in this species.

Carp

Globally, carp is the most important fish species in terms of total mass production, with grass carp (*Ctenopharyngodon idellus*), silver carp (*Hypophthalmichthys molitrix*) and common carp (*Cyprinus carpio*) listed as first, second and fourth in the list of most intensively farmed fish species in 2018 (FAO, 2020). Contrary to other species, DON research in carp did not focus mainly on performance but rather targeted its mechanisms of action at the cellular level, and DON effects on health.

A series of studies mostly performed by Pietsch and colleagues in common carp (Pietsch and Burkhardt-Holm, 2015; Pietsch et al., 2015; Pietsch et al., 2014a; Pietsch et al., 2014b) investigated the effects of pure DON on immunity, oxidative stress and liver health. Feeding low doses of DON (352, 619 or 953 µg/kg) for 6 weeks (Pietsch et al., 2014a), led to increased oxidative stress in several tissues (953 µg/kg dose). As also described for trout (Hooft et al., 2011), fat aggregation in hepatocytes was observed at DON levels ≥619 µg/kg, assumed to be a result of the ribotoxic effect of DON on the synthesis of protein-lipid transporters (lipoproteins) (Tiemann et al., 2006). Concentrations of serum protein (albumin) in carp were reduced at DON levels of 619 and 953 µg/kg (Pietsch et al., 2014a). Taken together, this implies a negative role of DON on nutrient metabolism. Potentially, DON affects also anaerobic metabolism since the activity of lactate dehydrogenase (LDH) varied in different tissues of DON-exposed carp. For instance, LDH activity increased in head and trunk kidney (≥352 µg/kg), decreased in muscle (953 µg/kg), but LDH activity and consequently lactate concentration increased in serum (953 µg/kg), indicating activation of gluconeogenesis to maintain glucose levels. An additional study measured reduced cell viability and immune function of unstimulated or bacterial lipopolysaccharide (LPS)-stimulated leucocytes derived from the head kidney (Pietsch et al., 2014b), indicative of cytotoxic effects of DON on immune cells.

DON might have immunostimulatory or immunosuppressive properties, depending on dose, frequency and duration of the exposure, as shown in mammals (Pestka, 2008). Thus, DON studies in carp (Pietsch and Burkhardt-Holm, 2015; Pietsch et al., 2015) also evaluated duration of exposure to DON after acute (7, 14 days) and sub-chronic (26, 54 days) exposure. Short-term (acute) exposure to 953 µg/kg DON resulted in activation of pro-inflammatory cytokines and anti-inflammatory cytokines. Reduced ROS production, and increased nitric oxide (NO) production in trunk kidney leucocytes after LPS stimulation confirmed a potential immunostimulatory capacity of DON. Longer-term (sub-chronic) exposure resulted in increased mRNA expression of immune-relevant genes in the trunk kidney, while in other organs mRNA expression levels of the same genes returned to the basal levels. Thus, sub-chronic (26 days) exposure to DON appeared to lead to pro-inflammatory responses and to anti-inflammatory responses, to prevent damage from permanent inflammation. Using the same experimental set-up (control vs. 953 µg/kg DON) (Pietsch and Burkhardt-Holm, 2015), measuring liver enzyme activities and histological changes indicated a suppression with time of the biotransformation and antioxidative capacity influenced by exposure to DON.

Two more studies investigated the effect of pure DON on oxidative stress (Kovesi et al., 2020; Pelyhe et al., 2016) in common carp. Dietary application of 5960 µg DON per kg feed for 4 weeks did not impair lipid peroxidation in the hepatopancreas (Pelyhe et al., 2016). A single, high (1750 µg DON /kg body weight) oral dose given by gavage (Kovesi et al., 2020) equivalent to 200,000 µg DON/kg of feed aimed to evaluate short-term (1-day experiment; sampling at 8, 16 and 24 h) responses that could reveal potential DON effects on lipid peroxidation and parameters of the glutathione redox system in the liver. As mentioned above, DON research in carp often focused on mechanisms of action and effects on growth performance were not studied (Kovesi et al., 2020; Pelyhe et al., 2016), or showed no significant effect of DON (Pietsch and Burkhardt-Holm, 2015; Pietsch et al., 2015; Pietsch et al., 2014a; Pietsch et al.,

2014b). Because these studies applied restricted feeding protocols rather than satiation feeding, DON effects on the growth performance of common carp may not be fully conclusive. Notably, juvenile grass carp fed with a DON level of ≥636 µg/kg (Fan et al., 2018; Huang et al., 2019; Huang et al., 2020) showed poor growth performance and body malformation. Finally, there is one study that referred to increased mortality (16.7%, twice higher than the control) associated with exposure to DON (5960 µg/kg) of common carp (Pelyhe et al., 2016).

DON research on grass carp also focused on unravelling the mechanism of action of the toxin, by addressing effects on oxidative stress and cell apoptosis, and new information was generated on the effects on gut and gill integrity. Investigations on juvenile grass carp (Fan et al., 2018; Huang et al., 2019; Huang et al., 2020) fed until satiation on diets with graded levels of pure DON (27, 318, 636, 922, 1243 and 1515 µg/kg) for 60 days, reported oxidative damage in the intestine after feeding ≥318 µg/kg and reported down-regulation of mRNA levels coding for antioxidant enzymes. In addition, for DON doses ≥636 µg/kg, increased lipid and protein peroxidation in grass carp intestine were noted. Intestinal tissue damage was also confirmed at the molecular level by detecting decreased relative mRNA expression of barrier-forming TJPs, indicating impaired gut integrity already at relatively low doses of 318 µg/kg DON (see Table 2.S4). Following a 60-day growth experiment, grass carp were challenged with *Aeromonas hydrophila* to investigate the effects of DON on intestinal immune function (Huang et al., 2019). At doses ≥636 µg/kg, DON exposure impaired innate and adaptive immune responses in the intestine.

Zebrafish

Zebrafish (*Danio rerio*) is a well-recognized animal model species for human research and now more frequently is also highlighted as an animal model for other fish species, for example to investigate host–microbe immune interactions and fish health (López Nadal et al., 2020) and investigate toxicological effects of mycotoxins *in vitro* (Juan-García et al., 2020). Indeed, zebrafish could represent an ideal animal model to study biological effects of DON on fish. Surprisingly, we could find only one *in vivo* study on the toxicity of DON in zebrafish (Sanden et al., 2012). In this study, although the application of increasing concentrations of 0, 100, 500, 1500, 2000 and 3000 µg/kg pure DON for 45 days to zebrafish (30 days post-hatch) using a restrictive feeding regime showed no effects on growth performance, other effects on sensitive endpoints in biotransformation, oxidative stress, behaviour and reproduction were described. Fecundity, measured as the mean number of eggs produced by individual females, was increased in zebrafish fed with DON 1500 µg/kg, but decreased in zebrafish fed the highest DON dose (3000 µg/kg). To the best of our knowledge, the effects of DON on the fecundity of fish had not been reported before. Effects of DON on behaviour were also examined. A trend for higher swimming activity was found in offspring of zebrafish parents that had been fed the highest DON dose. Nonetheless, freshly fertilized embryos (96, 100 and 120 h) treated with DON (0.01–100 mM) showed no behavioural alterations related to locomotion (Khezri et al., 2018). No matter what, the first results that come from this single study are sufficiently interesting to warrant further examination of the effects of DON on fish biology using the zebrafish as animal model.

Tilapia

Although Nile tilapia (*Oreochromis niloticus*) is the third most important fish species in term of aquaculture, with an annual production of 4.5 million Mt in 2018 (FAO, 2020), research efforts into the effects of DON have not been proportional. The relative lack of effort could be related to Nile tilapia primarily being cultivated in tropical and subtropical areas (Nobrega et al., 2020) while DON is the main contaminant in crops present in temperate regions. Indeed, there have been more research efforts on the threats posed by AFB_1, which is one of the most prevalent mycotoxins in tropical latitudes. To date, only two studies published the effects of natural DON on tilapia (Hooft et al., 2019b; Tola et al., 2015). In the first study, red tilapia (*Oreochromis niloticus* × *O. mossambicus*) fingerlings were exposed for eight weeks to graded low doses of DON (70, 310, 500, 920 and 1150 µg/kg) along with exposure to ZEN (10, 90, 210, 370 and 980 µg/kg) (Tola et al., 2015). Because in this study ZEN levels were relatively high, interpretation of the results is more difficult due to confounding effects of the combined exposure. Consumption of increasing doses of both, DON and ZEN led to a significant linear decrease in growth performance measured as feed intake, weight gain, feed efficiency and thermal daily growth coefficients. Furthermore, the ingestion of highly contaminated diets was linked with either linear or quadratic increase in the percentage of mortalities; an endpoint that had not been reported earlier in studies on DON in fish. Despite the increase in mortality, although lesions were observed in some mycotoxin-treated fish, no significant histopathological alteration in the liver was found and no effects were noted in hematological and biochemical parameters in the blood. In the second study, tilapia were exposed to graded levels of corn naturally contaminated with DON (Hooft et al., 2019b). Exposure of Nile tilapia fingerlings to either a low-starch (12%) or high-starch (24%) diet containing graded levels of natural DON (100, 700 and 1300 µg/kg) and fed until satiation for 10 weeks did not lead to any significant changes in growth performance. Overall, studies on the effects of DON in tilapia have been few and inconclusive.

Catfish

The effects of DON on channel catfish (*Ictalurus punctatus*) have been investigated by only a single study in fingerlings using the following doses 0, 3300, 5500, 7700 and 8800 µg/kg (Manning et al., 2014). After feeding high doses of DON for 7 weeks, these catfish did not experience negative effects on growth performance such as either impaired weight gain or reduced feed efficiency. In fact, and surprisingly, feed conversion in catfish fed with a high dose of DON (8800 µg/kg) was more efficient than in catfish fed with a low dose of DON (3300 µg/kg). Even more surprising, DON seemed to have a protective role against bacterial infection with *Edwardsiella ictaluri* because catfish fed with high doses of DON (> 5500 µg/kg) showed reduced mortality after challenge. Of interest, in an early study of digesta of nine different freshwater fish species (sampled in their natural habitat) on the presence of microbes having the ability to transform trichothecenes to less toxic forms (Guan et al., 2009), there was one catfish species (*Ameiurus nebulosus*) that stood out from the rest for having a microbial community (culture C133) able to completely transform DON to its less toxic metabolite de-epoxy-deoxynivalenol (DOM-1) after incubation for 96 h at 15 °C. Catfish are omnivorous fish species that naturally feed on plant sources, which could imply they strategically developed

through an evolutionary process mycotoxin-transforming microorganisms to help detoxify plant toxins. Undoubtedly, catfish species appear highly tolerant to DON.

Metabolic Fate of DON

Toxicity of DON can be reduced via biotransformation of DON to DOM-1 by anaerobic bacteria in the rumen or intestine, or via oxidation to 3-keto DON along with isomerization to 3-epi DON (3-β-hydroxy) by aerobic bacteria (McCormick, 2013). After intestinal absorption, DON can be metabolised in the liver by conjugation mainly to glucuronic acid, sulfate or sulfonate resulting in more hydrophilic and less toxic forms that can be excreted by the animal's body (Payros et al., 2016). Also in fish, glucuronidation can transform DON to DON-3-glucuronide (DON-3-GlcA), at least in *in vitro* studies of liver microsomes of carp and trout (Maul et al., 2012). An *in vivo* experiment with rainbow trout confirmed metabolisation of DON to the less toxic DON-3-sulfate, possibly explaining the absence of clinical signs with high doses of DON (Gonçalves et al., 2018b). In general, also in fish, DON and its metabolites are readily excreted via the bile and thereby finally via the faeces (Gonçalves et al., 2018b). Studies have shown almost negligible accumulation of DON in the muscle of salmon (Bernhoft et al., 2017; Nácher-Mestre et al., 2015), carp (Pietsch et al., 2014b) and gilthead sea bream (Nácher-Mestre et al., 2015), indicating little risk to humans after consumption of farmed fish fillet. Yet, although crucial for more detailed understandings of the effects of DON on different fish species and potential detoxification strategies, research on the toxicokinetics of DON and its metabolic fate in fish remain scarce.

2.3.2.2 Quantifying the Risk of DON Exposure in Fish

CC5 values are critical concentrations that affect 5% of a (fish) population. Probabilities and distributions of the estimated CC5 values are displayed in Figure 2.1 as log 10[concentration of the toxin] with kernel density and box plots. Our risk assessment of DON in fish feed, performed on a large number of fish species (n = 146), indicated CC5 values of 43–79.4 µg/kg (mean 59 µg/kg). A previous risk assessment of DON in fish feed based on 39 data points (Pietsch, 2020), predicted more variable and higher CC5 values of 23.8–272.3 µg/kg (mean 114.8 µg/kg). In an attempt to gain more detailed information on species-specific sensitivity, we estimated CC5 values for three subgroups; rainbow trout (n = 56), salmonids (n = 67) and all fish species excluding rainbow trout (n = 90). This approach led to a threshold for DON in fish feed for only rainbow trout of 43.7 µg/kg (24–75.2 µg/kg), lower than the 74.1 µg/kg mean value for all salmonids (45.7–116.3 µg/kg). The exclusion of rainbow trout from the complete dataset led to intermediate CC5 values of 53.9 µg/kg (36.1–79.3 µg/kg). More studies would be needed to generate more data points and extrapolate robust predictions for individual fish species.

2.3.2.3 A Meta-Analytical Approach

Our systematic review showed that DON can impair feed intake and growth performance in fish, and our risk assessment revealed critical DON thresholds that might threaten 5% of a fish population. In the next section, we describe the results of a meta-analysis aimed to estimate to what extend DON affects feed intake and growth in rainbow trout, and farmed fish in general,

using quantitative data from *in vivo* studies. For most of these studies, it remained unclear whether instances of impaired growth were an outcome of the observed reduced feed intake or related to increased maintenance requirements due to DON effects at cellular level. Thus, correlation between feed intake and growth data was also studied.

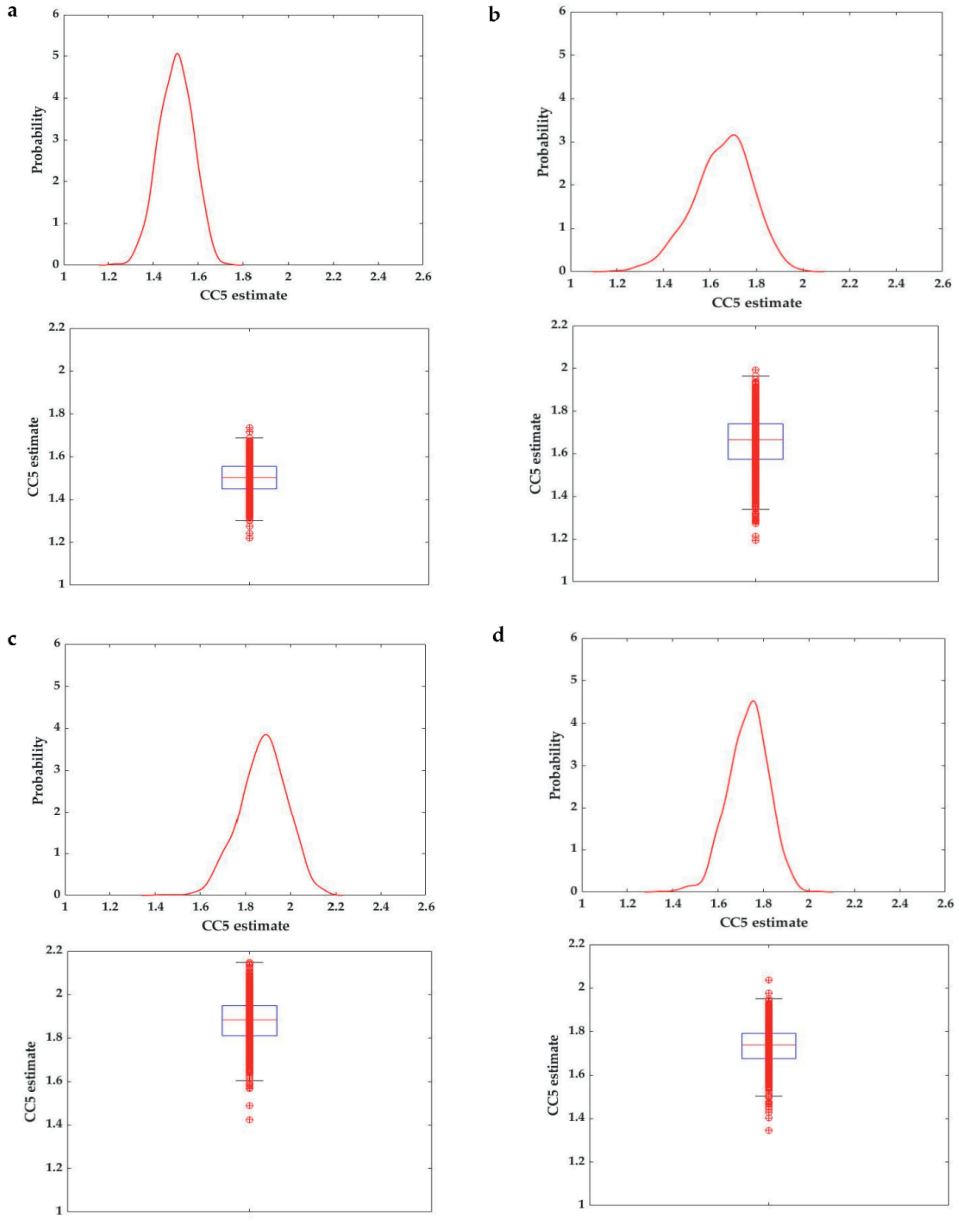


Figure 2.1 | Kernel density plot with the probability of estimated log critical concentration 5% (CC5), and boxplots of log CC5 for DON exposure in (a) fish species, n = 146, (b) rainbow trout, n = 56, (c) salmonids, n = 67 and (d) all fish species excluding rainbow trout, n = 90.

Effects of Dietary DON on Feed Intake and Growth

The number of *in vivo* studies found eligible for our meta-analysis (requirement details in Section 4.4) in all fish species was 11 studies, with a total of 63 data points. Data points were coded as control (n = 18) or challenged (n = 45). Doses in DON-challenged fish ranged from 310 µg/kg to 11412 µg/kg, with a mean of 2575 µg/kg. Control treatments were not always free of DON and ranged from 0 to 300 µg/kg, with a mean of around 71 µg/kg. Duration of DON challenge was on average 56 days (8 weeks). Out of the 11 studies, seven studies (35 data points) referred to rainbow trout, allowing for a separate meta-analysis. In the data subset addressing only rainbow trout, doses in DON-challenged fish (n = 25) ranged from 700 to 11,412 µg/kg, with a mean of approximately 3000 µg/kg, and duration of DON challenge was on average 8 weeks. Table 2.4 summarizes the characteristics of the two datasets used in our meta-analysis. Detailed characteristics of each study (number of experimental animals per treatment etc.) and data on exposure effects on feed intake and growth can be found in Table 2.S7.

Table 2.4 | Descriptive data [1] of control and DON-challenged fish in the two meta-analyses combining information on all fish species (n = 63), or only rainbow trout (n = 35).

All Fish Species	**Control**	**Challenged**
Initial body weight (g)	30.90 ± 6.74	27.85 ± 3.96
DON dose (µg/kg)	70.61 ± 22.03	2575.04 ± 383.32
Feed intake (g/fish)	1.30 ± 0.17	0.98 ± 0.08
Growth (g/day)	1.27 ± 0.19	0.86 ± 0.08
Rainbow Trout	**Control**	**Challenged**
Initial body weight (g)	29.25 ± 9.95	29.41 ± 5.70
DON dose (µg/kg)	97.40 ± 36.19	2994.52 ± 581.40
Feed intake (g/fish)	1.51 ± 0.30	1.05 ± 0.14
Growth (g/day)	1.54 ± 0.33	1.00 ± 0.14

[1] Mean ± standard error.

Feed intake and growth data collected in both meta-analyses were converted to relative values compared to their control and expressed as feed intake (% control) and growth (% control). The effect of dietary DON challenge on relative feed intake and growth was assessed by regression analysis. Exponential curves had the most logical fit and explained the greatest degree of variation in effects on feed intake and growth caused by dietary DON intake. Graphs and estimated equations derived from the exponential model are given in Figure 2.2.

Our results indicate that each additional mg/kg of DON in the aquafeeds leads to an exponential decrease in feed intake (% control) and growth (% control) independent of fish species, and

also for trout specifically. The curves in Figure 2.2 show a rapid and exponential decline in relative feed intake and growth already for low doses of DON, followed by a slower decline at higher doses. These results indicate a more severe impact on feed intake and growth at low DON doses, while at higher doses the impact will level off. The most striking result from our analysis is that already at doses below the EC recommendation limit (5000 µg/kg) there are adverse effects on feed intake and growth. Fish exposed to a diet with 5000 µg/kg DON show reduced feed intake of only 52% of that of the control group and reduced growth of 39% of the control fish (Figure 2.2). Even stronger for rainbow trout, fish exposed to a diet with 5000 µg/kg DON showed a predicted feed intake of only 43% of control values and predicted growth of only 36% of the control. It is relevant that the values for feed intake and growth are more acute predictions for trout, as can be observed by comparing the exponents in the equations (Figure 2.2). In general, with each additional mg/kg of DON, fish show a decline in feed intake of 13.2%, whereas rainbow trout show a stronger decline in feed intake of 18.8%. Similarly, with each additional mg/kg of DON, fish show a decline of 16.5% in growth, whereas rainbow trout show a stronger decline of growth of 20%. Taken together, our results suggest that the current EC recommendation limit might not be sufficiently low to guarantee optimal feed intake and growth in farmed fish species exposed to DON. Our results also suggest that rainbow trout is relatively sensitive to DON.

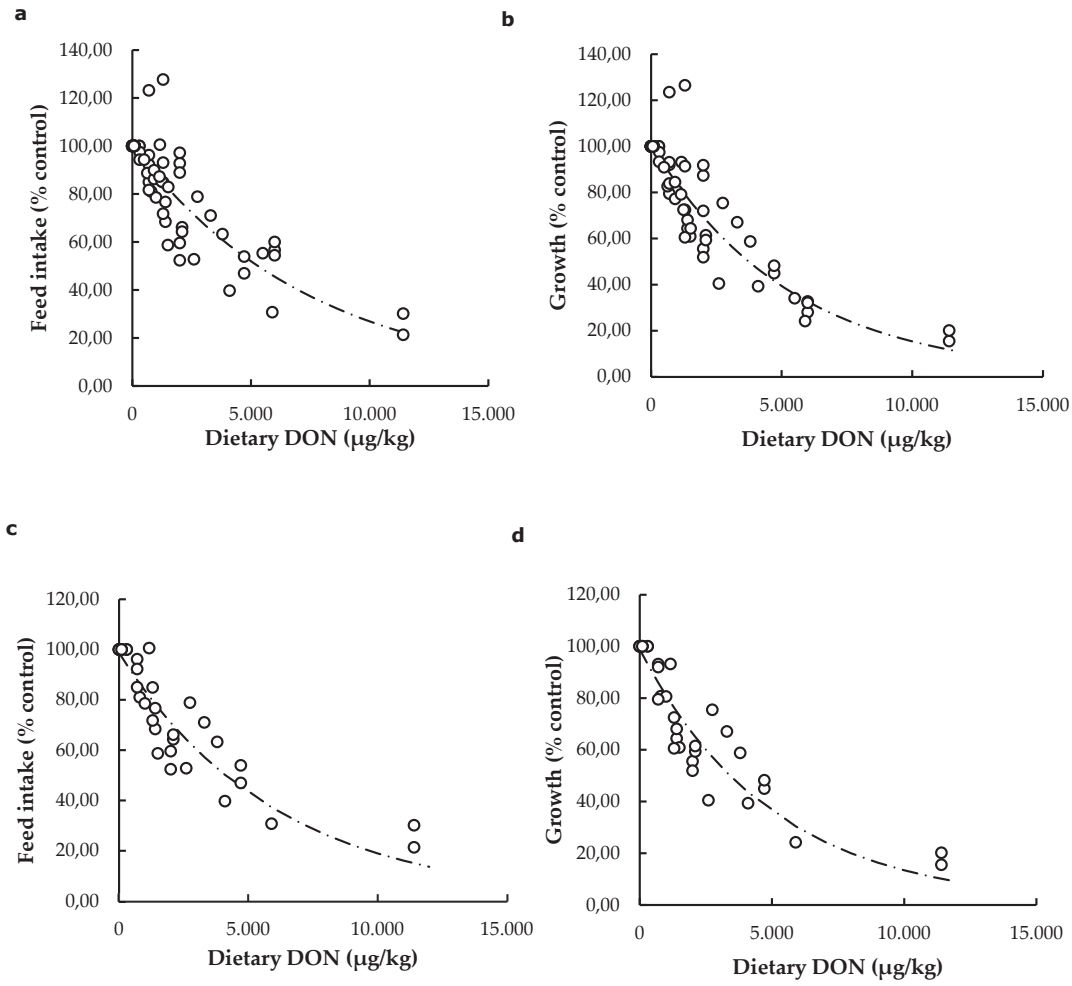


Figure 2.2 | Effect of dietary DON concentration on feed intake (**a**) and growth (**b**) for all fish species in the dataset (n = 63) and for rainbow trout only (c and d; n = 35). Feed intake and growth values are expressed as percentage (%) of feed intake and growth seen in the control groups of the respective studies. The estimated relationships for all fish species were: (**a**) feed intake = 100.4 (± 2.2) $e^{-0.132\ (\pm 0.013)\ \times\ DON}$, pseudo-$R^2$ = 0.74; **(b)** growth= 99.0 (± 2.6) $e^{-0.165\ (\pm 0.016)\ \times\ DON}$, pseudo-$R^2$ = 0.85. The estimated relationships for rainbow trout were: (**c**) feed intake= 101.1 (±2.3) $e^{-0.188\ (\pm 0.016)\ \times\ DON}$, pseudo-$R^2$ = 0.81; (**d**) growth= 98.9 (±2.6) $e^{-0.200\ (\pm 0.018)\ \times\ DON}$, pseudo-$R^2$ = 0.87. In all prediction equations above, DON concentration in the feed is expressed in mg/kg.

Different Types of DON in Rainbow Trout: Natural vs. Pure

In the data subset addressing experimental studies in rainbow trout ($n = 35$), DON challenge by experimental diet could be the result of two different contamination routes. In most of the cases ($n = 25$), DON was added to the diets in natural form by including naturally contaminated plant-based ingredients. Fewer studies ($n = 10$) investigated the impact of DON by testing pure DON purchased as a commercially available powder. Although in theory this could affect outcomes, a recent comparison of natural and pure DON (2100 µg/kg) exposure of rainbow trout found no difference between these two contamination routes (Hooft et al., 2019a). Further research into this comparison is complicated by the challenge to formulate comparable diets containing identical levels of natural and pure DON.

Regression analysis showed that regardless of the dietary source of DON (natural/pure), feed intake and growth (% control) of trout decreased exponentially with each mg/kg of DON added to the feed (Figure 2.3). The regression coefficients for natural and pure DON, however, were highly significantly different ($p < 0.0001$). The decline in feed intake for natural DON (22.1%) was much steeper than for pure DON (12.9%). Likewise, the decline in growth was much steeper for natural DON (26%) than for pure DON (15.7%). These findings strongly suggest that feed naturally contaminated with DON has a more severe impact on feed intake and growth of rainbow trout than feeding with contaminations of pure DON. As discussed above, natural DON is derived from naturally contaminated plant ingredients and usually co-exists with other toxins. In growing pigs, a meta-analysis of effects of individual mycotoxins showed a reduction of feed intake (14%) and growth (17%), but much larger reductions after exposure to multiple mycotoxins of 42% for feed intake and 45% for growth (Andretta et al., 2016). We support the hypothesis that also in fish the occurrence of multiple mycotoxins might lead to synergisms that could explain more severe effects of aquafeed contaminated with natural DON, in comparison to effects in studies using aquafeeds with pure DON.

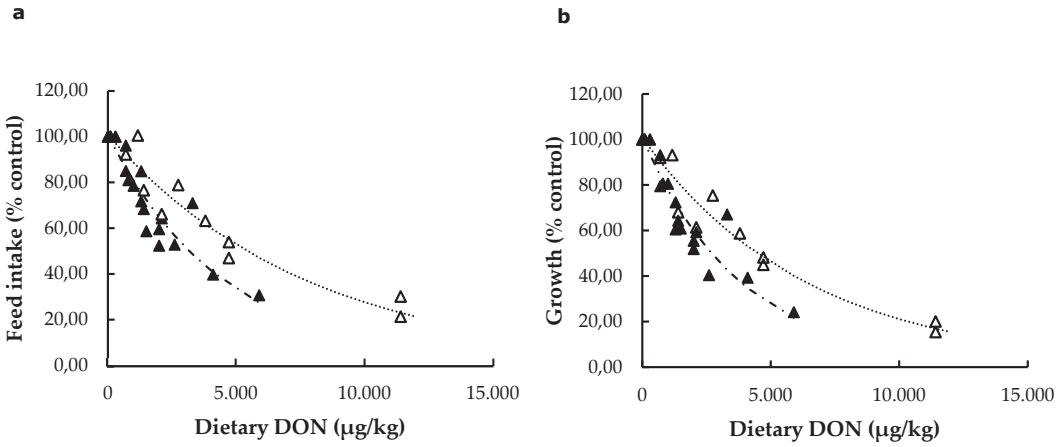


Figure 2.3|Effects of natural dietary DON (▲) and pure DON (△) on feed intake (**a**) and growth (**b**) in rainbow trout (n = 35). Feed intake and growth values were expressed as percentage (%) of the feed intake and growth of the control treatment in the respective studies. The estimated relationships were: (**a** ▲) feed intake = 100.9 (± 1.5) $e^{-0.221\ (\pm\ 0.016)\ \times\ DON}$ pseudo-R^2 = 0.91; (**a** △) feed intake = 100.9 (± 1.08) $e^{-0.129\ (\pm\ 0.008)\ \times\ DON}$ pseudo-R^2 = 0.96 and (**b** ▲) growth = 101.1 (±1.5) $e^{-0.260\ (\pm 0.018)\ \times\ DON}$ pseudo-R^2 = 0.92; (**b** △) growth= 100.9 (± 1.1) $e^{-0.157\ (\pm\ 0.009)\ \times\ DON}$ pseudo-R^2 = 0.97. In all prediction equations above, DON is the concentration in the feed expressed in mg/kg.

Relationship between Feed Intake and Growth

To date, little attention has been paid to the potential interference between reduced feed intake caused by DON and observed reductions in growth in fish. Only a few studies in rainbow trout added to their experimental design an additional control treatment, in which fish received the same amount of feed consumed by the group challenged with the highest dose of DON (pair-fed). One study showed significantly impaired growth in the DON treated group against the control (Hooft et al., 2011), but other studies (Ryerse et al., 2016; Ryerse et al., 2015) did not find significant effects. The outcomes from these pair-fed investigations, therefore, are not fully conclusive either. For that reason, we used the data collected in our meta-analysis to further explore the correlation between feed intake and growth response. Regression analysis showed a linear relationship between relative feed intake (% control) and growth (% control) for all fish species, including trout only (Figure 2.4). According to our model, 94% of the variation in fish growth (98% for trout only) can be explained by the feed intake response. Our data strongly indicate that the impact of DON on fish growth is mostly driven by feed intake. This conclusion, however, is affected by the experimental design of the studies included in our dataset employing satiation feeding strategies, resulting in differences in feed intake. Only if experimental groups are exposed to equal amounts of feed can future experiments aim to unravel the direct effects of DON on fish growth.

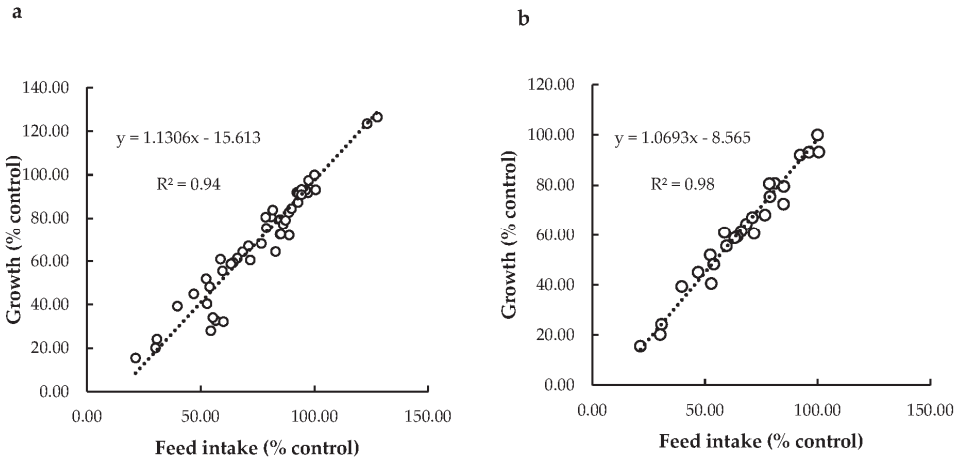


Figure 2.4 | Relationship between feed intake (% control) and growth (% control) in: (a) all fish species in the dataset (n = 63); (b) rainbow trout (n = 35) fed diets with DON.

2.4 Discussion

We aimed to unravel the profile of mycotoxins present in feed ingredients and thus in fish feeds in Europe, despite the lack of consistent, randomly collected field data. Our study included data from samples submitted by industry, and thus we make the assumption that we cannot fully exclude bias associated with suspicious materials also submitted for analysis. Nonetheless, the current study generated a large set of data and showed patterns related to mycotoxin contamination that are highly relevant to the animal and fish feed industry. We found DON occurrence in 44 European fish feed samples with an average contamination of 136 µg/kg and maximum contamination of 469 µg/kg. So far, comparable data on DON contamination have been derived from much smaller data sets analyzing 11 samples of commercial carp feed (average contamination 289 µg/kg, maximum 825 µg/kg) (Pietsch et al., 2013), or 10 samples of commercial fish and shrimp feeds (166 µg/kg and 282 µg/kg) (Gonçalves et al., 2018a). The much larger number of samples in our data set logically produced more reliable outcomes. Is DON occurrence in feed always detrimental for the fish? Not necessarily. Potentially, high temperatures (>150 °C) during the extrusion process might significantly reduce FUM and ZEA and moderately reduce AFLAs, but extrusion may only slightly reduce contamination with DON in finished feeds (Bullerman and Bianchini, 2007; Karlovsky et al., 2016). For instance, extrusion temperatures above 150 °C only led to a slight reduction of ~20% in DON levels in wheat grits (Wu et al., 2011). Overall, complete elimination of mycotoxin is not feasible during feed extrusion and, therefore, prevention of mycotoxin contaminated feeds is of utmost importance for feed manufacturers.

The current survey revealed a risk of association of DON with other *Fusarium* toxins, including emerging and masked mycotoxins. An earlier study based on literature data (Smith et al., 2016) reported common combinations of different mycotoxins in European cereal samples and

addressed their combined risks on different animals, but not fish. Only one study investigated the combined effects of DON with AFB_1 on the fish cell line BF-2, and combined effects of DON with ZEN on zebrafish larvae (Zhou et al., 2017). The results implied the existence of effects synergetic between DON + AFB_1 but antagonistic between DON + ZEN. Future research is needed to investigate similar effects and more diverse combinations of mycotoxins in *in vivo* feeding experiments. Furthermore, emerging and masked mycotoxins generally are not detectable in routine controls in feed mills, and no regulatory/recommendation limits exist (Berthiller et al., 2013). Thus, feed producers might consider subjecting their raw materials to periodical state-of-the-art mycotoxin analyses performed by external, certified labs to screen the full spectrum of mycotoxins present. Even then, commercial fish feeds when stored under warm (25 °C) and humid conditions (>60% relative humidity) for a month, can release OTA (Pietsch et al., 2020). Thus, to prevent fungal growth and potential mycotoxin contamination after feed production, aquafeed producers and fish farmers have to ensure proper storage conditions.

To the best of our knowledge, this is the first comprehensive study that has attempted to summarize the effects of DON in different fish species using a systematic review approach. Based on our review, we see no evidence for bioaccumulation of DON in fish tissues (Bernhoft et al., 2017; Nácher-Mestre et al., 2015; Pietsch et al., 2014b) and see no reason to raise concerns with respect to consumer health. However, consumption of DON-contaminated feeds by fish, even at levels below the EC recommendation limit (5000 µg/kg), can result in adverse although non-lethal effects on fish such as impaired feed intake, growth performance, immunity, detoxification capacity, and tissue damage and oxidative stress. By collecting all reported adverse effects of DON, our review extended a previous risk assessment (Pietsch, 2020) and allowed for a new and updated estimation of critical DON levels for rainbow trout, defined as at risk of affecting 5% of a fish population (CC5). This renewed information could have a direct and practical implication for aquafeed producers when designing their mycotoxin management plans.

Undoubtedly the number of studies investigating single effects of DON on farmed fish species has been increasing, but the data have not been collectively used to assess feed intake and growth performance responses. Our meta-analysis provided new insights into aquaculture nutrition that suggest an exponential relation exists between decreases in feed intake and growth response, and increasing levels of DON (mg/kg) in aquafeeds. These adverse effects of DON appear more severe when natural DON is used for feed formulation instead of pure forms of this toxin, as in experimental studies. Other meta-analyses for pigs and poultry similarly showed negative effects on feed intake and growth performance of mycotoxins, including DON (Andretta et al., 2011; Kipper et al., 2020; Pastorelli et al., 2012). In summary, our study predicts that the current average contamination of 136 µg DON per kg fish feed leads to 3.5% reduction in feed intake and 3.7% reduction in growth of trout. In a worst-case scenario (maximum DON contamination level of 469 µg/kg), we predict an even greater reduction of 9.9% in growth of trout. *Fusarium* fungal growth, DON contamination and risks of reduced feed intake and growth cannot always be predicted, or ignored. To prevent loss of production therefore, particularly when using diets with high inclusion of plant ingredients for more

sensitive species such as rainbow trout, feed manufacturers may consider adding anti-mycotoxin products to aquafeeds and altogether eliminate the risk of mycotoxin exposure.

Another important outcome of our meta-analysis is the attribution of reduced growth performance of DON-challenged fish to reduced feed intake. Feed refusal is a common symptom in animals that have consumed DON and might simply be a response to poor organoleptic characteristics of the contaminated feeds (Akande K.E et al., 2006) or be considered a natural defence mechanism to minimize risks associated with exposure to the toxin. The mechanism through which DON reduces feed intake may be associated with a direct action on the brain or may be indirect through the secretion of gut hormones (Terciolo et al., 2018). The latter phenomenon remains unexplored in fish, however. In the future, direct effects of DON on fish growth should be studied without confounding effects caused by reduced feed intake. Indeed, to better investigate direct effects of DON on fish growth, future experimental designs need to overcome differences in feed intake between experimental groups by pairwise and equal feeding.

Taken together, mycotoxin contamination is an emerging concern for European aquaculture and requires a multidisciplinary approach. Diverse expertise is needed and, therefore, collaboration and communication of stakeholders from the whole value chain and scientific support from fields such as fish nutrition, toxicology, health and welfare, microbiology, feed processing and technology and plant sciences are crucial. Our findings suggest a strong impact of dietary DON on feed intake and fish growth, and regulatory authorities should reconsider their current DON recommendation limit to ensure economic profitability and protect fish welfare.

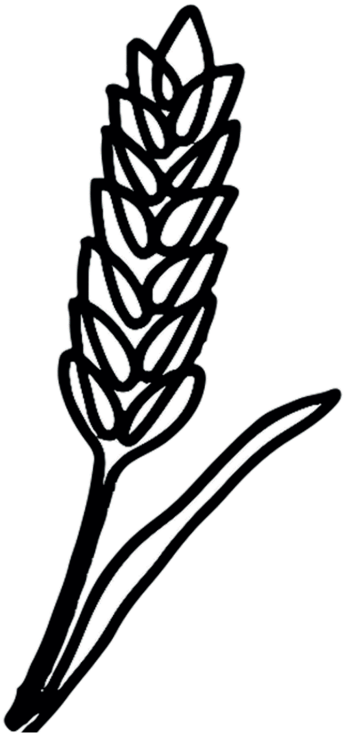
Supplementary Materials
The following are available online at:
https://www.mdpi.com/article/10.3390/toxins13060403/s1

Table 2.S1: Legal mycotoxins limits in animal feed ingredients and (fish) feeds set by European Commission, **Table 2.S2:** Inclusion of wheat, corn and soybean meal in trout, tilapia, marine fish and carp diets, **Table 2.S3:** Limit of detection (LOD) and limit of quantification (LOQ) for all mycotoxins detected in wheat, corn, soybean meal and aquafeeds, **Table 2.S4:** Summary of DON studies used for systematic review and risk assessment, **Table 2.S5**: Data points for lowest observed adverse effect level (LOAEL) and no observed adverse effect level (NOAEL) used in the risk assessment, **Table 2.S6:** Predicted CC5 datapoints, **Table 2.S7:** Summary of DON studies used for the meta-analysis.

Acknowledgments
The authors acknowledge Alltech 37+ mycotoxin lab for generating the survey data and Alltech Mycotoxin Management Team (Nick Adams, Alexandra Weaver, Alex Yannikouris) for providing the data to the authors.

Chapter 3

Time- and Dose-Dependent Effects of Dietary Deoxynivalenol (DON) in Rainbow Trout (*Oncorhynchus mykiss*) at Organism and Tissue Level



This chapter has been based on the publication:

Koletsi, P., Wiegertjes, G. F., Graat, E. A., Lyons, P., & Schrama, J. (2022). Time-and Dose-Dependent Effects of Dietary Deoxynivalenol (DON) in Rainbow Trout (Oncorhynchus mykiss) at Organism and Tissue Level. *Toxins*, *14*(11), 810.

DOI: 10.3390/toxins14110810

Abstract

This study with juvenile rainbow trout evaluated the effects of dietary exposure to deoxynivalenol (DON) at industrially relevant doses (up to 1.7 mg/kg) on growth performance, the liver, and the gastrointestinal tract. Fifteen groups of 30 fish each were given one of five dietary treatments in triplicate: (1) control diet (CON; DON < 100 µg/kg feed), (2) naturally DON-contaminated diet (ND1) with a DON content of 800 µg/kg in the feed, (3) ND2 with a DON content of 1300 µg/kg feed, (4) a pure DON-contaminated diet (PD1) with 900 µg/kg of DON in the feed, and (5) PD2 with DON at a concentration of 1700 µg/kg in the feed. The feeding trial lasted eight weeks: six weeks of restrictive feeding followed by two weeks of *ad libitum* feeding. Exposure to DON during restrictive feeding for six weeks did not affect the growth performance of trout but did lead to a reduction in retained protein in fish fed with higher doses of DON in the ND2 and PD2 groups. During the two following weeks of *ad libitum* feeding, feed intake was similar among all groups, but body weight gain was lower in the ND2 and PD2 groups and feed efficiency was higher in PD2 (week 8). Histopathological assessment revealed liver damage, including altered nuclear characteristics and haemorrhages, in groups fed higher doses of natural DON (ND2) after just one week of restrictive feeding. Liver damage (necrosis and haemorrhage presence in ND2*)* was alleviated over time (week 6) but was again aggravated after *ad libitum* exposure (week 8). In contrast, gastrointestinal tract damage was generally mild with only a few histopathological alterations, and the absence of an inflammatory cytokine response was demonstrated by PCR at week 8. In conclusion, *ad libitum* dietary exposure of rainbow trout to either natural or pure DON resulted in reduced growth (dose-dependent), while restrictive exposure revealed time-dependent effects of natural DON in terms of liver damage.

3.1 Introduction

Mycotoxins have been well-documented as frequent natural contaminants in aquafeeds in Europe, where deoxynivalenol (DON) is the most prevalent toxin (Barbosa et al., 2013; Gonçalves et al., 2018a; Koletsi et al., 2021; Pietsch et al., 2013; Rokvić et al., 2020). The risk of contamination has been highlighted in the global aquaculture sector and is becoming increasingly relevant given the increased use of plant-derived ingredients in fish feeds (Barbosa et al., 2013; Gonçalves et al., 2018a; Greco et al., 2015; Koletsi et al., 2021; Marijani et al., 2017; Mwihia et al., 2020; Pietsch et al., 2013; Rokvić et al., 2020; Tolosa et al., 2014). Plant-based raw materials can have a high nutritional value within diets for farmed fish but conversely represent potential substrates for the growth of mycotoxin-producing fungi (Bryden, 2012). Climate change forecasts suggest that climate-driven fungal growth factors could exacerbate the potential for mycotoxin production in crops and increase the risk of contamination levels in animal feeds (Chhaya et al., 2021; Medina et al., 2017; Thielecke and Nugent, 2018). Therefore, research into the potential effects of diets contaminated with mycotoxins such as DON on fish performance and health is timely.

DON is produced by *Fusarium* fungi that contaminate crops prior to harvest, and at this stage, it is difficult to apply prevention strategies (Richard-Forget et al., 2021). Additionally, DON is a heat-stable toxin, meaning that high temperatures during feed extrusion cannot eliminate DON and guarantee its absence from the final fish feeds (Wu et al., 2017). Rainbow trout is amongst the most sensitive species to this toxin (Koletsi et al., 2021). In rainbow trout, exposure to DON (≥800 µg/kg) under *ad libitum* feeding affects fish in a manner similar to terrestrial animals and is manifested through reduced feed intake and weight gain (Gonçalves et al., 2019; Gonçalves et al., 2018c; Hooft and Bureau, 2017; Hooft et al., 2011; Hooft et al., 2019a; Hooft et al., 2019b; Ryerse et al., 2015). Moreover, DON suppresses the retention efficiency of dietary nitrogen and energy in rainbow trout at doses ≥1300 µg/kg (Gonçalves et al., 2018c; Hooft and Bureau, 2017; Hooft et al., 2011; Hooft et al., 2019a; Hooft et al., 2019b). Histopathological investigations have described liver damage in trout after exposure to DON doses ≥1400 µg/kg and revealed congestion and subcapsular edema with a fibrinous network, fatty infiltration, phenotypically altered hepatocytes (Hooft et al., 2011), vacuolation of hepatocytes, necrosis, scattered haemorrhages (Gonçalves et al., 2019), and a decrease in the number of mitotic cells (Hooft et al., 2019a). Thus far, histopathological assessments have primarily been conducted via qualitative or semi-quantitative methods (Hooft and Bureau, 2021), meaning that quantitative studies of potential damage to the liver and/or intestine caused by DON are largely unreported, especially in rainbow trout.

Effects of dietary exposure to DON can be studied using feed ingredients naturally contaminated with DON (“natural” DON) to compose experimental diets or through the addition of pure DON to diets. Feed ingredients with “natural” DON often also contain other types of mycotoxins (Hooft and Bureau, 2017; Hooft et al., 2011; Hooft et al., 2019a; Hooft et al., 2019b; Ryerse et al., 2016), whereas using pure DON excludes co-exposure to other toxins. In a direct comparison between natural and pure DON (at a dose of 2100 µg/kg) in rainbow trout, no differences in growth performance or nutrient utilization efficiency metrics were observed (Hooft et al., 2019a). In contrast, our meta-analysis indicated that natural DON exposure resulted in a more pronounced reduction in feed intake and growth of rainbow trout

than exposure to pure DON (Koletsi et al., 2021). Time-dependent effects remain somewhat understudied in rainbow trout. So far, only one study has assessed the effects of exposure time to natural or pure DON-contaminated diets on rainbow trout, showing time-related histopathological changes in both the pyloric caeca and the liver (Hooft et al., 2019a). Only in common carp have researchers more specifically addressed the time effects of DON (953 µg/kg) (7, 14, 26, and 56 days) (Pietsch and Burkhardt-Holm, 2015; Pietsch et al., 2015). Liver damage and a reduction in the activity of specific biotransformation enzymes were present only until day 26 (Pietsch and Burkhardt-Holm, 2015), while up-regulation of pro- and anti-inflammatory cytokine gene expression in the spleen, liver, and intestine was found only at day 14 (Pietsch et al., 2015).

A critical aspect of evaluating the effects of dietary exposure to DON on growth is the choice of feeding regime: restrictive versus *ad libitum*. Our meta-analysis on rainbow trout, which only assessed studies with *ad libitum* exposure, showed reduced growth as an outcome of reduced consumption of DON-contaminated feed (Koletsi et al., 2021). If DON exposure disturbs appetite, experimental designs using *ad libitum* feeding would generate differences in feed consumption among experimental treatments and thus generate differences in DON intake. Therefore, *ad libitum* feeding designs do not allow for measuring the direct effects of DON on growth performance. Restrictive feeding experiments should thus be more informative for direct DON-related effects on growth performance. Only pair-fed treatments added to *ad libitum* feeding experiments have shown either direct effects of DON on growth performance (Hooft et al., 2011) or no effect (Ryerse et al., 2015). Well-designed studies with dietary exposure of rainbow trout to DON to examine its direct effects on growth are rare.

The purpose of the present study was to elucidate the direct effects of two different types of DON (natural versus pure) on both growth performance and health metrics of rainbow trout (*Oncorhynchus mykiss*) via a detailed histopathological examination of the liver and gastrointestinal tract. Firstly, the experimental design of the study was based on restrictive feeding for six weeks to ensure equal feed intake, with the aim to reveal direct effects of DON rather than indirect effects caused by differences in feed intake. Secondly, the inclusion of an early sampling point at one week permitted an investigation of whether DON-induced effects would change over time between week 1 and week 6. Thirdly, the inclusion of a final experimental period of two weeks of *ad libitum* feeding allowed for the assessment of indirect effects of DON caused by a reduction in feed intake. Finally, we studied the observed dose-dependent reduction of performance parameters and time-dependent liver damage after dietary exposure of rainbow trout to DON.

3.2 Materials and Methods

This study (project number AVD2330020198084) was carried out in accordance with the Dutch law on the use of animals (Act on Animal Experiments) for scientific purposes, and it was approved by the Central Committee on Animal Experiments (CCD) of The Netherlands. The experiment was executed at the experimental facilities of the Alltech Coppens Aqua Centre (Leende, The Netherlands).

3.2.1 *In Vivo* Experimental Procedure

Rainbow trout (*Oncorhynchus mykiss*) were obtained from a commercial trout farm (Mohnen Aquaculture GmbH, Stolberg, Germany) and kept in a recirculating aquaculture system (RAS). Fish with an average weight of 8 g were randomly distributed over 15 tanks with 30 fish per tank. All five treatments (CON, ND1, ND2, PD1, PD2) were tested in triplicate (3 tanks per treatment). Each tank was additionally aerated and had a volume of 120 L. A cooling system maintained the water temperature constant at 14 ± 0.5 °C, and a photoperiod of 17 h light and 7 h dark was applied during the experiment. Water physicochemical parameters were monitored and maintained within the allowed levels: pH: 7.0–8.5, NH_4^+: < 1 mg/L, NO_2^-: < 0.5 mg/L, alkalinity;: 2.0–5.0, and oxygen (O_2): 8 mg/L. Fish were daily checked for signs of abnormal behaviour (e.g., cannibalism, irregular swimming patterns, lethargic and weak individuals hiding at the bottom of the tank for a while), diseases, wounds, and mortalities.

The experiment lasted eight weeks and consisted of two periods: a 6-week restricted feeding period followed by a 2-week *ad libitum* feeding period. Before the start of the experiment, all fish were acclimated to the facilities for a week and fed a standard commercial trout diet. To assess the direct effect of DON, fish were fed restrictively for six weeks according to their metabolic body weight (12 g/kg$^{0.8}$/d) by handfeeding twice per day to ensure fixed daily intakes of DON and an equal amount of feed given to each tank. To evaluate the effect of DON in combination with impacts on feed intake, during the last two weeks of the experiment, fish were fed *ad libitum* twice daily for one hour. Fish had reached satiation when uneaten pellets remained on the bottom of the tank or floating on the water's surface for more than 10 min or when the feeding time of one hour was over. Uneaten pellets were removed by siphoning and counted to determine feed intake.

The day before the start of the experiment (before distribution to tanks), from the initial population, $n = 6$ fish were euthanized for tissue sampling and $n = 20$ fish for determining the initial body composition at time point zero. At the start and at the end of both feeding periods (i.e., weeks 6 and 8), fish were weighed per tank and counted for the calculation of performance parameters. At the end of the restrictive feeding period (week 6), $n = 5$ fish per tank were euthanized and stored at −20 °C for determination of body composition. After one week of restrictive feeding (week 1), at the end of the restrictive feeding period (week 6), and at the end of the *ad libitum* feeding period (week 8), liver and tissue samples from the gastrointestinal tract (pyloric caeca, midgut, and hindgut) were collected from $n = 2$ fish per tank and stored for histopathological examination and gene expression analysis. Additionally, total liver weight and total body length were recorded for all fish sampled for tissues. Overall, handling of the fish was avoided as much as possible, and the fish were euthanized by an overdose of benzocaine (dissolved in water at 0.5 mL/L).

3.2.2 Experimental Diets

Five experimental diets were formulated, of which one was the control diet (CON), which aimed to have as little DON content as possible, and four diets had different concentrations and origins of DON included. Effects induced by natural DON originating from a batch of "contaminated" wheat (further information below) were compared to effects induced by pure DON. The pure DON was produced by extracting and purifying it from a fermentation medium

of *Fusarium graminearum,* purchased from Fermentek Ltd. (Jerusalem, Israel). The two DON concentrations of 800 and 1600 µg/kg are anticipated to be below the threshold level for DON (<5000 µg/kg) advised by the European Commission (Commission, 2006a).

Before feed production, various batches of wheat were analysed for DON to find two batches of wheat: a "clean" for the control diet and a "contaminated" batch. The "contaminated" batch was designed to have the highest possible DON content. DON was quantified by liquid chromatography/tandem mass spectrometry (LC-MS/MS) at the Alltech 37+ mycotoxin laboratory (ISO/IEC 17025:2005 accredited), Dunboyne, Ireland. Next to DON, the "contaminated" DON wheat also contained small amounts of other mycotoxins. The analysed contents in the "contaminated" wheat was for DON 3842 µg/kg, for DON-3-Glucoside (DON3Glc) 124 µg/kg, for *Fusarenon* X (FX) 29 µg/kg and Alternariol 8 µg/kg.

In order to compose a control diet with minimal dietary contamination of mycotoxins, the CON diet was fully fishmeal and fish oil-based with the inclusion of 40% of the "clean" wheat source (Table 3.1). To compose experimental diets with natural DON concentrations of 900 µg/kg (ND1 diet) and 1700 µg/kg (ND2 diet) the "clean" wheat was partially (ND1) or fully (ND2) exchanged for the "contaminated" wheat. To compose experimental diets with pure DON concentrations of 900 µg/kg (PD1 diet) and 1700 µg/kg (PD2 diet), the CON diet was supplemented with the appropriate amount of pure DON. This approach resulted in five experimental diets, all isonitrogenous and isoenergetic. The experimental diets were produced as 2 mm extruded pellets by Research Diet Services (Wijk bij Duurstede, The Netherlands). Following pelleting, diets were analysed for mycotoxin content to confirm the anticipated DON contamination levels. The results confirmed the low occurrence of DON (75 µg/kg) and other toxins in the control diet (Table 3.1). DON levels in the naturally contaminated diets, ND1 and ND2, were slightly lower than anticipated at ~800 and ~1300 µg/kg, respectively, and also contained small amounts of Enniatin A/A1 and Enniatin B/B1. DON concentrations in PD1 and PD2 diets were close to the anticipated levels at ~900 and ~1700 µg/kg, respectively (Table 3.1).

3.2.3 Chemical Analysis of Feeds and Fish

Feed samples were analysed for: dry matter (DM) content by drying at 103 °C until constant weight for 4 and 24 h, respectively (ISO 6496, 1999), crude protein (CP) based on nitrogen × 6.25 using the Kjeldahl method (ISO 5983, 2005), fat after an initial acid-hydrolysis step followed by a petroleum-diethyl ether extraction (ISO 6492, 1999), ash content after incineration at 550 °C for 4 h (ISO 5984, 2002), and gross energy (GE) content with the adiabatic bomb calorimeter method (ISO 9831, 1998). Fish carcass samples were analysed with the same methods for CP and GE. All chemical analyses were performed by Nutricontrol (Veghel, The Netherlands).

Table 3.1 | Ingredient composition, proximate, and mycotoxin analysis of the experimental diets: control (CON), naturally DON-contaminated diets (ND1 and ND2), and pure DON-contaminated diets (PD1 and PD2).

			Experimental Diets		
Ingredient (%)	CON	ND1	ND2	PD1	PD2
Wheat (no DON)	40.00	18.00	-	40.00	40.00
Wheat (DON contaminated)	-	22.00	40.00	-	-
Pure DON	-	-	-	0.00009	0.00016
LT fishmeal	49.02	49.02	49.02	49.02	49.02
Fish oil	9.90	9.90	9.90	9.90	9.90
Mineral and vitamin premix [1]	1.08	1.08	1.08	1.08	1.08
Analysed nutrient composition [2] (%)					
Dry Matter	96.5	94.3	94.0	93.3	95.7
Protein	41.8	41.5	41.3	41.7	41.6
Fat	15.8	16.3	16.2	16.3	16.2
Ash	9.7	9.9	9.8	9.8	9.5
Gross Energy (MJ/kg)	21.62	21.60	21.64	21.62	21.57
Mycotoxins concentration (µg/kg) [2]					
DON [3]	75	763	1349	897	1709
Enniatin A/A1 [4]	13	13.6	14.6	-	-
Enniatin B/B1 [4]	-	9.6	22.5	-	-
T2 Toxin	-	-	4.4	-	-
Ergotamin(in)e	2.9	3.8	-	2.4	-
Ergocryptin(in)e	4.4	-	-	-	-

[1] Commercial premix from Alltech Coppens that meets NRC, 2011 requirements for rainbow trout. [2] On dry matter basis. [3] In the main text, the rounded levels of DON are mentioned: ND1: 800, ND2:1300, PD1: 900 and PD2: 1700 µg/kg. [4] Wheat batches were not screened for Enniatin A/A14 and Enniatin B/B14.

3.2.4 Histopathological Examination of Liver and Gastrointestinal Tract

At the end of weeks 1, 6, and 8, n = 2 fish per tank (i.e., six per treatment) were sampled for histopathological assessment of the liver and gastrointestinal tract (and n = 6 from the initial population before the experiment starts). Although, due to time constraints, only the diets with the highest DON doses (ND2 and PD2) were further analysed and compared with the CON diet. Overall, two pieces from each liver and a piece from each part of the gastrointestinal tract (pyloric caeca, midgut, and hindgut) were placed into embedding cassettes and fixed by immersion in 10% buffered formaldehyde for three days at room temperature. Samples were later transferred to 70% ethanol until dehydration and embedded in paraffin wax according to standard histological procedures. All liver and intestinal tissue blocks were cut into 5 µm thick paraffin sections, mounted onto microscope slides, and stored in an oven at least overnight, followed by staining (details are described below). Pictures were captured with a Leica DM6 microscope (Leica Microsystems, Wetzlar, Germany).

Liver sections were stained with Periodic acid-Schiff's (PAS) reagent to distinguish between lipid- and glycogen-type vacuoles, followed by staining with Crossman's trichrome (Mason) for coloration of connective tissue (collagen). Liver sections were also stained with Haematoxylin and Eosin (H and E) to assess cellular and nuclear morphology. Glycogen accumulation in the hepatocytes was observed as pink-purple areas of PAS-positive material, while lipid accumulation was observed as well-defined white spherical droplets. Glycogen and lipid vacuolisation were scored as follows: low (1) moderate (2), and high (3). PAS-Crossman-stained liver sections were also screened for histopathological aberrations, including signs of haemorrhage and inflammation, the latter identified as infiltrates of nucleated leukocytes, by scoring "Yes" or "No". We categorised and scored necrotic presence as follows: no necrosis (0), mild (1), moderate (2), and severe (3). Liver sections stained with H&E were used to assess nuclear morphology as follows: presence ("Yes") or absence ("No") of "pyknotic", "dislocated", and "pleomorphic" nuclei. All parameters were assessed for 10 single random frames per sampled fish (n = 5 from each liver piece) stained with PAS (× 20 magnification) and H&E (× 10 magnification). Finally, to exclude bias, blind histological assessment of liver samples was carried out by two evaluators. When scores were not in agreement, differences were discussed until consensus was reached.

Gastrointestinal tract sections were stained with Alcian blue (pH 2.5), a stain that is used to visualise acidic epithelial and connective tissue mucins, followed by Crossman to enhance the contrast between goblet cells (GC) and supranuclear vacuoles (SNV). Alcian blue staining revealed a heterogeneous population of mucus-producing cells, identified by a range of blue stain intensity, presumably because GC which secrete a combination of acidic and neutral mucus would be visible as dark blue and GC which secrete acidic mucus would be visible as light blue. All the GC which stained blue were counted, regardless of intensity, around the perimeter of each mucosal fold (MF) and expressed as the number of GC per μm^2 of MF. Eosinophilic granulocytes (EG), if present, were counted as cells with light pink coloured cytoplasm.

For evaluating histological parameters in each section of the gastrointestinal tract, we randomly picked n = 10 well-oriented (simple) fold units and measured the following parameters: (a)

thickness of sub-epithelium mucosa (SM), measured as the distance between the point where neighbouring folds lose contact with each other prior to the collagenous (greenish-blue) layer of connective tissue (b) stratum compactum height (SC); (c) mucosal fold height (MFH); (d) mucosal fold width (MFW); (e) average lamina propria width (LP) from three different areas; (f) average supranuclear vacuoles width (SNV) from two sides; (g) enterocytes width (EW) calculating from MFH, LP and SNV; (h) stratum granulosum height (SG), defined as the layer bordered by SC and muscular layer, (i) muscularis (MS); and (j) MS (consisting of the inner circular (cm) and longitudinal (lm) layer) was determined as the layer between SG and the thin outermost layer of connective tissue, (k) serosa (SE). Pictures were imported into the ImageJ software (version 1.53 q (Schindelin et al., 2012)), and all the above-mentioned histological parameters were measured with the ROI manager function. Our scoring system is an updated quantitative approach based on previously used parameters to semi-quantitatively score soybean-induced enteritis in Atlantic salmon and common carp (Urán et al., 2008a; Urán et al., 2008b). Finally, an example of the measurements on the described parameters (a–k) to evaluate the effects of DON along the gastrointestinal tract (pyloric caeca, midgut, and hindgut) of trout is available in Figure S2.

3.2.5 Gene Expression

Tissue samples from the pyloric caeca and hindgut of rainbow trout (n = 2 per tank, i.e., 6 per treatment), fed the experimental diets CON, ND2, and PD2, were analysed for gene expression analysis at the end of the experiment (week 8). Small (2 mm in size) tissue samples were placed in Eppendorf tubes filled with RNA*later*, stored at room temperature overnight, and then transferred to −20 °C until RNA extraction. Total RNA was extracted using the RNeasy® mini kit (Qiagen), including on-column DNase treatment with a RNase-free DNase set (Qiagen), according to the manufacturer's instructions. Total RNA was stored at −80 °C until use. Before cDNA synthesis, 1 µg RNA was treated with DNase I, Amplification Grade (InvivoGen). cDNA was synthesised using random primers (300 ng) and Superscript III First-Strand Synthesis for RT-PCR following the manufacturer's (InvivoGen) protocol. cDNA samples were diluted (1:20) in nuclease-free water and used for real-time quantitative PCR (RT-qPCR) with ABsolute QPCR, SYBR Green Mix (Thermo Fisher Scientific) in a Rotor-Gene 6000 (Corbett Research). Fluorescence data were retrieved and analysed by Rotor-Gene Q Series software (version 2.1.0 Build 9). Gene expression was measured as a relative expression ratio calculated according to the Pfaffl method (Pfaffl, 2001). Take-off values of experimental samples were calibrated against a common reference (calibrator) and normalised against the reference gene elongation factor (ELF-1α) of rainbow trout. Specific primer sequences for the reference gene and interleukin-1β (IL-1β), interleukin-8 (IL-8), copies of tumor necrosis factor-α (TNF-α andTNF-α3) are in Table 3.2 Primer pairs had been validated as gene copy-specific by sequencing of PCR products prior to this analysis. The following PCR reaction conditions were applied: 95 °C for 15 min, followed by 35 cycles of 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 20 s. To ensure the specificity of amplification, a melting curve analysis was performed with a hold of 60 °C for 1 min and a melting curve temperature ranging from 60 °C to 99 °C with a gradual increase of 0.5 °C every 5 s.

Table 3.2 | Summary of selected genes primer sequences used for RT-qPCR.

Target Gene	Accession Number	Forward Primer Sequence 5'-3'	Reverse Primer Sequence 5'-3'
elf-1α	AF498320.1	TCTACAAAATCGGCGGTA	CCTCAGTGGTGACATTAGC
il-1β	AJ557021.2	CACCACCACCACCAAT	AAGAGGAAGCGAACCG
il-8 +	NM001124362.1	TGTCAGCCAGCCTTG	ACATCCAGACAAATCTCCT
tnf-α	AJ277604.2	GGCTGTGTGGGGTC	GCTTCAATGTATGGTGGG
tnf-α3	HE798146.1	TACCAAGAAACAAGATCACA	TCTGTCCACTCCACTGA

+ there exist 2–3 paralogs for IL-8 with minor nucleotide differences, the primers were chosen to amplify all paralogs

3.2.6 Calculations

The average initial body weight (IBW, g) and the final body weight (FBW, g) per fish were determined by batch-weighing of the tank biomass and dividing by the number of individual fish. Feed intake (FI) was defined as the average amount of feed (g) consumed by a fish, converted based on the DM content of the feed (g/kg).

By using the following formulas, we calculated per feeding period (restricted and *ad libitum*):

$$\text{Weight gain (g)} = \text{FBW} - \text{IBW} \quad (1)$$

$$\text{Growth (g/d)} = \text{weight gain/days} \quad (2)$$

$$\text{Specific growth rate (SGR, \%/d)} = ((\ln \text{FBW} - \ln \text{IBW})/\text{days}) \times 100 \quad (3)$$

$$\text{Feed conversion ratio (FCR) on DM basis} = \text{FI/Weight gain} \quad (4)$$

$$\text{Hepatosomatic index (HSI, \%)} = (\text{liver weight/W}) \times 100 \quad (5)$$

$$\text{Condition factor (K)} = (W/L^3) \times 100 \quad (6)$$

where W is the individual FBW of the tissue sampled fish and L its body length (cm).

$$\text{Retained protein (g/fish)} = \text{FBW} \times \text{FPC} - \text{IBW} \times \text{IPC} \quad (7)$$

where FPC is the protein content (g) in the fish body at the end and IPC the protein content (g) at the start.

$$\text{Protein retention efficiency (\%)} = (\text{Retained protein/CPI}) \times 100 \quad (8)$$

where CPI is the dietary protein intake (g/fish) calculated as average FI of an individual x protein content in the feed.

Similarly for Retained energy (MJ/fish) and energy retention efficiency (%)

$$\text{Retained energy} = \text{FBW} \times \text{FEC} - \text{IBW} \times \text{IEC} \quad (9)$$

where FEC is the gross energy content (MJ) in the fish body and the end and IEC the protein content (g) at the start.

$$\text{Energy retention efficiency (\%)} = (\text{Retained energy/GEI}) \times 100 \quad (10)$$

where GEI is the dietary gross energy intake (MJ/fish) calculated as average FI of an individual x gross energy in the feed.

3.2.7 Statistical Analysis

For growth parameters (nitrogen and energy retention efficiencies), the experimental unit was the tank, and data were expressed as a treatment mean derived from the three replicates. A one-way analysis of variance ANOVA using a general linear model (GLM) was used to evaluate the effect of dietary DON on the dependent variables. HSI, condition factor, and gene expression measurements were performed on individual fish (means derived from six replicates per treatment, two per tank), therefore a generalised linear mixed model was applied with the tank used as a random effect. However, the tank effect was not significant ($p > 0.05$) and therefore not included in the results. When a significant difference was found ($p \leq 0.05$), a Tukey's honestly significant difference (HSD) *post hoc* test with multiple comparisons (95% level of significance) was used to compare treatment means.

For the outcome variables in the gastrointestinal tract and continuous scores in the liver (glycogen and lipid vacuolisation score, and necrosis score), a general linear regression was performed with diet (CON, ND2, PD2) and time (weeks 1, 6, and 8), and their interaction weas included in the model. Before the statistical analysis, the 10 measurements (liver areas and intestinal folds) were averaged per fish. Gastrointestinal tract parameters were analysed separately per part of the intestine (pyloric caeca, midgut, and hindgut). The model residuals were considered normal when skewness and kurtosis were between −2 and 2. The scores were expressed as least square means ($n = 54$, 6 per diet per time point). For the yes/no liver data (nuclei pyknosis and pleomorphism, necrosis, haemorrhage, inflammation), a logistic regression analysis was performed, which included diet, time, and their interaction in the model. The scores were expressed as frequencies (%) ($n = 540$, 60 per diet per time point). As the 10 measurements within a fish are not independent, a random fish effect was included using the exchangeable correlation structure (GEE model). A marginal R^2 for the GEE model was calculated, which is interpreted similarly to the R^2 in ordinary least square regression models (Zheng, 2000).

The IBM Statistical Package for the Social Sciences (SPSS) programme (v 23.0; New York, NY, USA) was used to perform the statistical analyses for growth performance and gene expression analysis. Histological data were analysed with SAS software® (version 9.4, SAS Institute, Cary, NC, USA).

3.3 Results

3.3.1 Performance

No mortality, notable differences in feed acceptance, or abnormal behavioural responses were noted during the experiment. Performance parameters were not significantly different between diets during the restrictive feeding period of six weeks, except in the case of retained protein and protein retention efficiency ($p < 0.01$; Table 3.3). Rainbow trout fed diets ND2 and PD2 retained less protein and had a lower protein retention efficiency compared to trout fed the control (CON) diet. Trout fed the PD1 diet were similar to trout on the CON diet but differed from fish fed the PD2 diet regarding protein gain and protein retention efficiency.

During the subsequent ad libitum feeding period of two weeks, feed intake, HSI (an indicator of relative liver size), and condition factor K did not differ between control and DON-contaminated diets (Table 3.4). However, absolute growth (g/d) and specific growth rate (SGR, % BW/d) were reduced in rainbow trout fed the diets containing the highest DON contamination level (ND2 and PD2), compared to those fed the CON diet, via an *ad libitum* feeding regime ($p \leq 0.01$; Table 3.4). FCR was only increased for fish fed the PD2 diet compared to the CON-fed fish ($p \leq 0.05$; Table 3.4).

Table 3.3 | Effects of DON on the performance of rainbow trout fed the experimental diets: control (CON; DON < 100 µg/kg), naturally DON-contaminated diets (ND1; DON = 800 µg/kg and ND2; DON = 1300 µg/kg), and pure DON-contaminated diets (PD1; DON = 900 µg/kg and PD2; DON = 1700 µg/kg) during a 6-week restrictive feeding period.

	Experimental Diets						
Performance Parameters	CON	ND1	ND2	PD1	PD2	SEM	*p*-Value
Initial BW (g)	8.0	8.0	7.4	8.2	8.1	0.21	NS
Final BW (g)	36.5	36.1	35.5	36.8	35.5	0.31	NS
Growth (g/d)	0.71	0.70	0.70	0.72	0.68	0.008	NS
SGR (% BW/d)	3.80	3.78	3.91	3.76	3.69	0.069	NS
FCR	0.68	0.70	0.70	0.69	0.71	0.008	NS
HSI (%)	4.4	4.4	4.7	4.2	3.8	0.36	NS
Condition factor (K)	2.1	2.0	1.8	2.0	1.9	0.09	NS
Retained protein (g/fish)	4.2 ^{b}	4.0 ^{ab}	3.9 ^{a}	4.1 ^{b}	3.8 ^{a}	0.05	**
Protein retention efficiency (%)	51.0 ^{c}	49.1 ^{ac}	48.1 ^{ab}	50.4 ^{bc}	47.1 ^{a}	0.59	**
Retained energy (MJ/fish)	0.19	0.19	0.19	0.19	0.19	0.002	NS
Energy retention efficiency (%)	45.9	45.8	44.3	45.8	44.6	0.58	NS

BW: body weight, SGR: specific growth rate, FCR: feed conversion ratio on dry matter basis, HSI: hepatosomatic index, SEM: standard error of means, NS: not significant, **: $p \leq 0.01$, values in the row with different superscripts (a, b, c) are significantly different ($p \leq 0.05$) according to Tukey's multiple comparison test.

Table 3.4 | Effects of DON on the performance and feed intake capacity of rainbow trout fed the experimental diets: control (CON; DON < 100 µg/kg), naturally DON-contaminated diets (ND1; DON = 800 µg/kg and ND2; DON = 1300 µg/kg), and pure DON-contaminated diets (PD1; DON = 900 µg/kg and PD2; DON = 1700 µg/kg) during a 2-week *ad libitum* feeding period.

	Experimental Diets						
Performance Parameters	CON	ND1	ND2	PD1	PD2	SEM	*p*-Value
Initial BW (g)	36.6	36.1	35.5	36.6	36.1	0.47	NS
Final BW (g)	67.8 ^{a}	64.2 ^{ab}	61.9 ^{b}	65.1 ^{ab}	63.1 ^{ab}	1.06	*
Feed intake (g/fish/d)	1.71	1.72	1.58	1.71	1.67	0.045	NS
Feed intake (g/kg$^{0.8}$/d)	18.9	19.5	18.9	19.2	19.2	0.48	NS
Growth (g/d)	2.09 ^{a}	1.88 ^{ab}	1.76 ^{b}	1.90 ^{ab}	1.80 ^{b}	0.048	**
SGR (% BW/d)	4.12 ^{a}	3.84 ^{ab}	3.70 ^{b}	3.83 ^{ab}	3.73 ^{b}	0.064	**
FCR	0.79 ^{a}	0.86 ^{ab}	0.85 ^{ab}	0.84 ^{ab}	0.89 ^{b}	0.017	*
HSI (%)	3.9	3.4	3.8	3.0	3.0	0.40	NS
Condition factor (K)	2.0	1.9	1.9	1.9	2.0	0.07	NS

BW: body weight, SGR: specific growth rate, FCR: feed conversion ratio on dry matter basis, HSI: hepatosomatic index, SEM: standard error of means, ns: not significant, *: $p \leq 0.05$, **: $p \leq 0.01$, values in the row with different superscripts (a, b) are significantly different ($p \leq 0.05$) according to Tukey's multiple comparison test.

3.3.2 Health

3.3.2.1 Histopathological Assessment of the Liver

Qualitative observations suggested that liver cells of fish fed the DON-contaminated PD2 diet had a lower degree of glycogen vacuolization (lower degree of pink coloration in PAS-stained hepatocytes; Figure 3.1) compared to fish fed the CON diet. The quantitative assessment of the glycogen vacuolization score of hepatocytes revealed an interaction effect between diet and time (Table 3.5; $p \leq 0.05$); during the restrictive feeding period, while glycogen vacuolization was similar among dietary treatments and remained unaltered over time. However, at the end of the *ad libitum* feeding period, fish fed the PD2 diet had reduced hepatic glycogen vacuolization. Lipid vacuolization was unaffected by diet and did not alter with time. No differences in the lipid vacuolization of liver cells, identified as white spherical droplets in the hepatocytes, were observed (Figure 3.1).

Only a diet effect was present in the models of nuclei characteristics, pyknosis, and pleomorphism (see Figure 3.1 for qualitative indication), and it did not change over time. Averaged over all sampling moments, trout fed ND2 and PD2 diets had an increased level of pyknotic and pleomorphic nuclei, i.e., altered cells, than trout fed a CON diet, without differences between natural (ND2) and pure DON (PD2). During *ad libitum* feeding, only fish fed the ND2 diet had a higher occurrence of pyknotic and pleomorphic nuclei (week 8), indicating that overexposure to natural DON altered the nuclei of cells.

Further qualitative observations of additional pathological signs, including necrosis (recognized as disrupted cell structure), haemorrhage (recognized as accumulated red blood cells outside blood vessels), and inflammation (recognized as accumulated leucocytes), suggested an early and severe effect of ND2 and PD2 at week 1 on liver health (Figure 3.1). This effect seemed to be time-dependent, since at week 6 of restrictive feeding, most of the pathological signs were no longer different from the control, except for some signs of inflammation in the ND2 group. After two weeks of *ad libitum* feeding (week 8), pathological signs seemed to reappear in the livers of trout fed ND2 (signs of necrosis and inflammation) and PD2 (signs of necrosis and haemorrhage) diets (Figure 3.1). Indeed, quantitative assessment (Table 3.5) confirmed an interaction effect between diet and time for the presence of necrosis ($p \leq 0.01$), necrosis score ($p \leq 0.05$), haemorrhage ($p \leq 0.001$) and inflammation ($p \leq 0.05$). Only for fish fed the ND2 diet, the presence of necrosis and haemorrhage decreased while inflammation increased over time (from week 1 to week 6).

For all liver parameters, diet, time, and their interaction explained a rather low proportion of the variance. The ten measurements per fish for the liver parameters are assumed not to be independent. This is shown by the high proportion of unexplained variation due to the effect of fish (>0.5) in pyknosis and pleomorphism (Table 3.5). This effect was moderate for necrosis (22%) and inflammation (16%) and only 4% for haemorrhage.

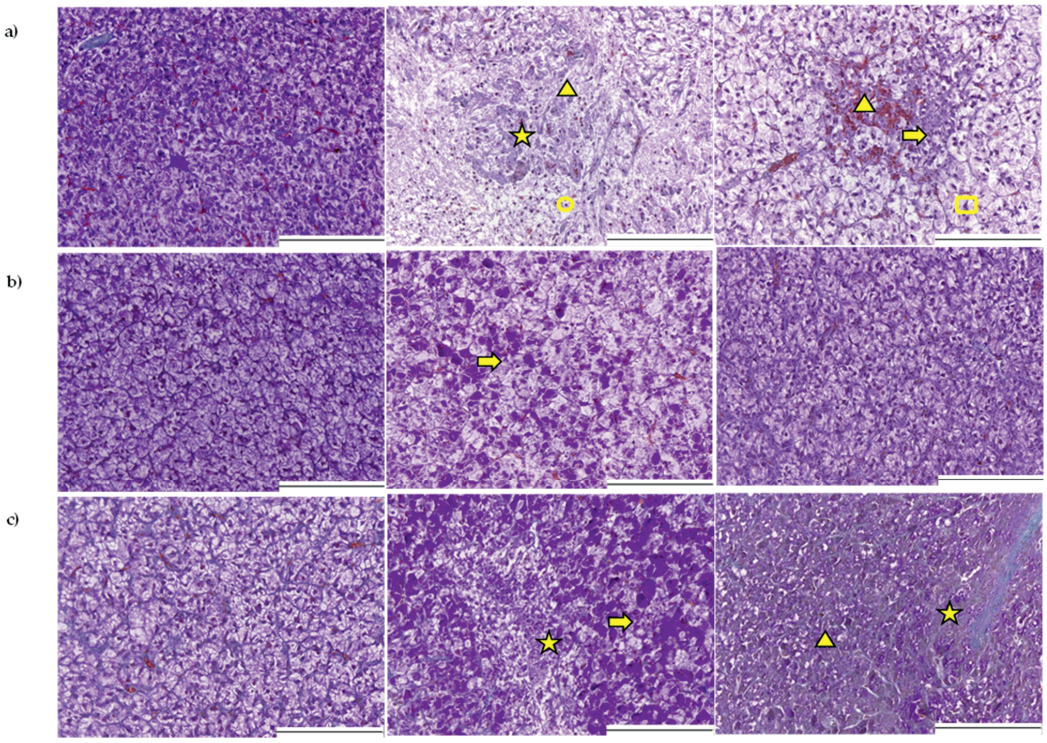


Figure 3.1 | Representative examples of histological sections of the liver from rainbow trout fed diets of control (CON), natural (ND2), and pure DON (PD2), restrictively for one week (**a**), six weeks (**b**), and *ad libitum* for two weeks (**c**). The yellow arrows indicate profound infiltration of presumed *leucocytes*; stars highlight necrotic areas*;* triangles show the presence of a haemorrhage*;* circles indicate pyknotic nuclei; and squares indicate pleomorphic nuclei. Staining: PAS-Crossman; Magnification: ×20; White scale bar = 200 µm.

Table 3.5 | Pathological indicators observed in trout livers with histological assessment after both restricted (week 1 and week 6) and *ad libitum* (week 8) feeding of the experimental diets: control (CON), natural DON (ND2), and pure DON (PD2).

Pathological Indicators	+Week	Experimental Diets			SEM	*p*-Value			R^2 (Fish Effect) ‡
		CON	ND2	PD2		Diet	Time	Diet×Time	
Vacuolization Score									
Glycogen #	1	2.5	2.3	1.8 ^{y}	0.19	***	NS	*	0.42 (NA)
	6	2.0	1.9	1.9 ^{y}					
	8	2.4 ^{b}	2.0 ^{b}	1.0 ^{a,x}					
Lipid #	1	1.6	1.8	1.9	0.13	NS	NS	NS	0.16 (NA)
	6	2.0	1.8	1.9					
	8	1.9	2.0	2.0					
Nuclei characteristics ++									
Pyknotic (%)	1	55 ^{a}	95 ^{b}	93 ^{b}		***	NS	NS	0.36 (0.50)
	6	33	87	78					
	8	23 ^{a}	72 ^{b}	15 ^{a}					
Pleomorphic (%)	1	35 ^{a}	82 ^{b}	82 ^{b}		*	NS	NS	0.29 (0.51)
	6	25	65	68					
	8	18 ^{a}	83 ^{b}	17 ^{a}					
Other indicators									
Necrosis (%)	1	28	62 ^{y}	37 ^{x}		NS	NS	**	0.21 (0.22)
	6	25	22 ^{x}	39 ^{x}					
	8	18 ^{a}	45 $^{a, x, y}$	93 $^{b, y}$					
Necrosis score#	1	0.3	1.2	0.5 ^{x}	0.20	*	NS	*	0.35 (NA)
	6	0.4	0.5	0.5 ^{x}					
	8	0.2 ^{a}	0.7 ^{ab}	1.1 $^{b y}$					

Table 3.5 - Continued

Pathological Indicators	+Week	Experimental Diets			SEM	*p*-Value			R^2 (Fish Effect) ‡
		CON	ND2	PD2		Diet	Time	Diet×Time	
Haemorrhage (%)	1	12 ^{a}	50 $^{b\,y}$	13 ^{a}		***	NS	***	0.15 (0.04)
	6	5	5 ^{x}	12					
	8	3 ^{a}	15 $^{b\,x}$	30 ^{b}					
Inflammation (%)	1	0 ^{a}	2 $^{a\,x}$	13 ^{b}		*	NS	*	0.17 (0.16)
	6	3 ^{a}	45 $^{b\,y}$	9 ^{a}					
	8	5	22 ^{y}	5					

+ Liver samples collected before the start of the experiment (Week 0) were homogeneously characterized with low glycogen and lipid vacuolization (score 1), without nuclei alternations (pyknosis and pleomorphism) and pathological findings (necrosis, haemorrhage, inflammation). ++ Scores of pyknosis and pleomorphism were used as indicators to evaluate cell viability. # Glycogen, lipid vacuolisation, and necrosis scores were analysed with a general linear model ($n = 54$). The other pathological indicators were analysed with a logistic regression model with fish as a random effect ($n = 540$). ‡ The fish effect is the proportion of all unexplained variation due to fish. This was only estimated for pathological indicators analysed by logistic regression. The R^2 is the proportion of variance explained by the model. Standard error of the mean: SEM, Not significant: NS, $p \leq 0.05$: *, $p \leq 0.01$: **, $p \leq 0.001$: ***, Not applicable: NA, a, b and c: different superscripts within rows mean differences between treatments within a week with a $p \leq 0.05$. x, y, z: different superscripts within columns mean differences between weeks within a treatment with a $p \leq 0.05$.

3.3.2.2 Histopathological Assessment of the Gastrointestinal Tract

All measured parameters, indicators for mucosal health, and morphology are listed in Table 3.S1. The thickness of the sub-epithelial mucosa, mucosal fold height, the width of the mucosal fold, lamina propria, stratum granulosum, enterocytes, and muscularis width all increased over time, being highest at week 8 (Table 3.S1). The interaction effects between diet and time were only significant for mucosal fold height in the pyloric caeca (Figure 3.2a; $p \leq 0.01$), mucosal fold width in the hindgut (Figure 3.2b; $p \leq 0.01$) and enterocyte width in the hindgut (Figure 3.2c; $p \leq 0.05$). In the pyloric caeca, the height of the mucosal fold changed over time only within fish fed the ND2 diet. Specifically, mucosal fold height of ND2 in week 8 is significantly different from week 6, and also from the control diet at all weeks and from weeks 1 and 8 in the case of PD2. In the hindgut, a time effect was detected only for mucosal fold and enterocyte metrics in the PD2 diet, where in both cases the widths were significantly higher in week 8 compared to week 1. For the majority of the other histological parameters investigated in the gastrointestinal tract, there were no indications of an effect of diet or an interaction between diet and time (Table 3.S1).

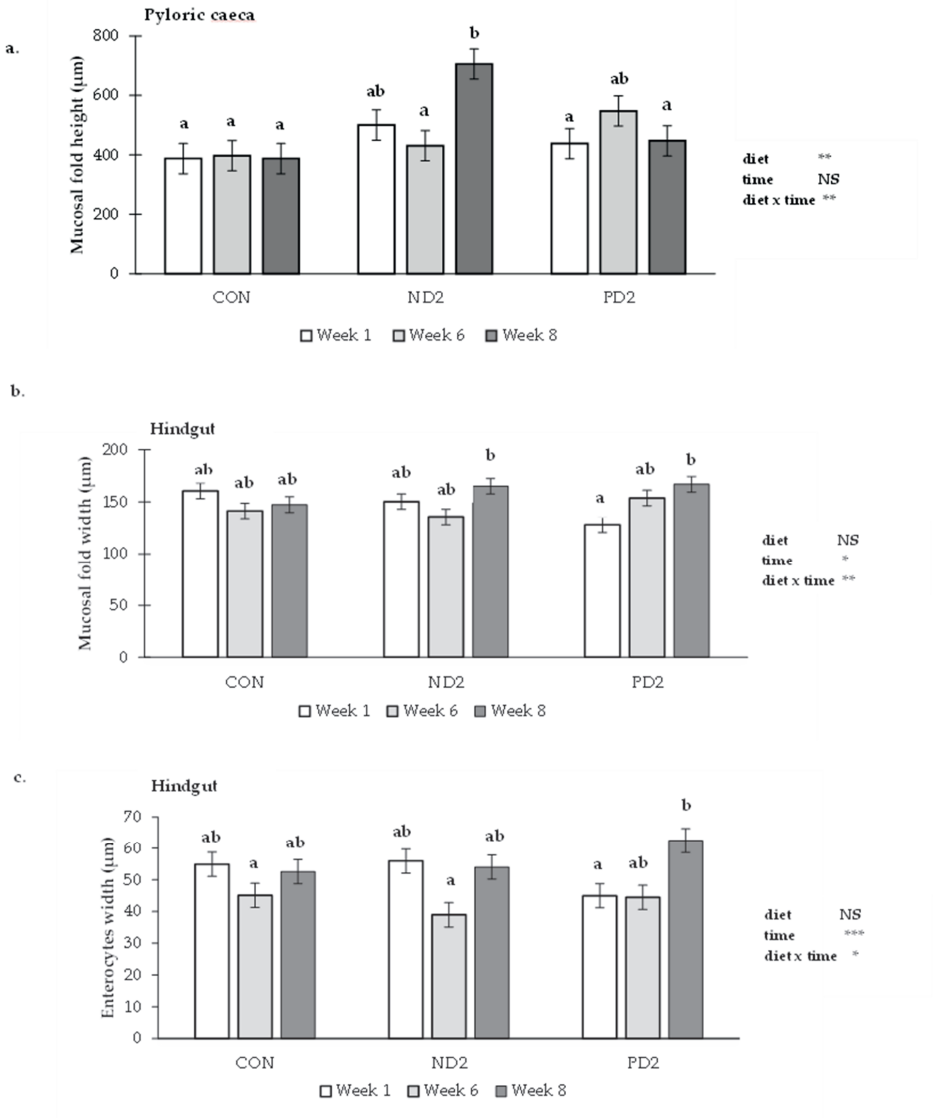


Figure 3.2 | Interaction effects between the experimental diets and exposure time on the (**a**) mucosal fold height in the pyloric caeca, (**b**) mucosal fold width in the hindgut, and (**c**) enterocyte width in the hindgut of rainbow trout. Experimental diets refer to control (CON), natural DON (ND2), and pure DON (PD2), and time to restrictive exposure for 6 days (week 1), 40 days (week 6), and *ad libitum* exposure for 15 days (week 8). The error bars indicate the standard error of the mean; NS: not significant, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$. Treatments lacking a common letter (a, b) are statistically different ($p \leq 0.05$) according to Tukey's multiple comparison test.

3.3.2.3 Assessment of Inflammation by Gene Expression

In order to examine the putative effects of DON on intestinal health in more detail, expression analyses of inflammatory genes were performed by PCR. Gene expression patterns of selected pro-inflammatory cytokines(IL-1*β,* IL-8, and TNF-*α*) were measured in the pyloric caeca and hindgut of rainbow trout at the end of the *ad libitum* feeding period, week 8, where suspected damage would be greatest. Exposure to PD2 resulted in a down-regulation of IL-1*β* in the pyloric caeca (Figure 3.3a), but not an up-regulation. In the same tissue, relative expression of IL-8 and TNF-*α* showed a trend of down-regulation in the PD2 group, although this was not statistically significant. Furthermore, in the ND2 group, gene expression of pro-inflammatory cytokines was also not significantly up- or down-regulated. Gene expression analysis in the hindgut (Figure 3.3b) showed no effect of dietary treatment. Taken together, gene expression analysis confirmed the absence of strong effects of DON in the gastrointestinal tract.

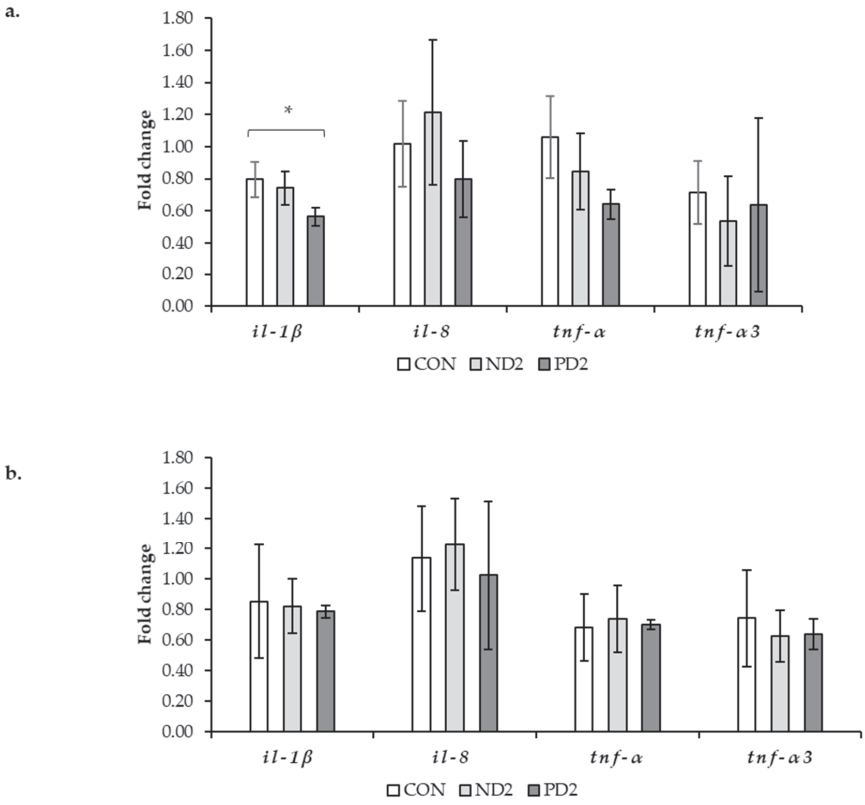


Figure 3.3 | Relative gene expression (fold change) of pro-inflammatory cytokines interleukin-1*β*, interleukin-*8*, and two copies of tumor necrosis factor-*α* at the end of the experimental period (week 8) in (**a**) the pyloric caeca and (**b**) the hindgut of rainbow trout fed the experimental diets: control (CON), natural (ND2), and pure DON (PD2). The number of records used in the statistical analysis per gene ranged from 13 to 14. Primer pairs were gene copy specific; accession numbers of the gene variants amplified can be found in Table 3.2. * refers to a significant difference compared to the CON diet ($p \leq 0.05$).

3.4 Discussion

The purpose of this study was to elucidate the effects of DON on growth performance and on the gut and liver health of rainbow trout. DON was fed at industrially relevant doses of up to 1.7 mg/kg and derived as pure DON or natural DON for inclusion in experimental diets. The study focused on measuring the direct effects of restricted feeding on growth performance and the quantification of histopathological effects in the liver and gastrointestinal tract. Here, we discuss the dose-dependent reduction of growth performance due to pure/natural DON and the time-dependent effects of natural DON on the liver of rainbow trout.

3.4.1 Performance

In the current study, dietary DON inclusion levels of ≥1300 µg/kg had some effect on performance during restricted feeding, but such effects were more pronounced after *ad libitum* feeding. A critical aspect of evaluating the effects of dietary DON exposure on performance is the choice of feeding regime: restrictive *versus ad libitum*. Restrictive feeding should reveal the direct effects of a fixed, daily DON intake, while *ad libitum* feeding should reveal the direct effects of DON plus putative indirect effects on impaired feed intake. Our restrictive feeding experiment induced reductions in retained protein and protein retention efficiency, but only in experimental treatments fed diets containing the highest level of DON contamination (ND2: 1300 µg/kg and PD2: 1700 µg/kg). This reduction is in agreement with an earlier study in trout, although a higher dose of DON (2600 µg/kg) was used (Hooft et al., 2011). Our findings indicate that DON may directly inhibit protein synthesis and/or increase maintenance requirements, which could induce increased protein catabolism and impaired nutrient utilization.

Ad libitum feeding reduced body weight gain in the ND2 and PD2 diets and feed efficiency in PD2. Restrictive feeding did not induce strong effects on growth performance, despite the above-mentioned reduction in retained protein in fish fed with higher doses of DON. The lack of DON-related effects on trout growth during restrictive feeding may be explained by the absolute amount of DON ingested, calculated as estimated daily intake (EDI; µg/g BW/day). There is likely a threshold concentration of DON that individual animals can tolerate, below which no adverse effects would be recorded, which could help explain the stronger effect seen after the *ad libitum* feeding. Indeed, during restrictive feeding, the EDI for trout, which received the highest dose (PD2; 1700 µg/kg), was 0.040 µg DON/g BW/day, while during *ad libitum* feeding the EDI was as high as 0.058 µg DON/g BW/day (Table 3.S2 and Figure 3.S1). *Furthermore, the biomass measurements* at weeks six and three (no reported data) showed no effects of DON, and it is rational to assume that there is no adaptation over time in terms of growth. In future investigations, it may be informative to include an early (week 1) sampling point to determine direct effects of DON on growth performance, similar to observations in pigs (Serviento et al., 2018; Wellington et al., 2020). Alternatively, longer periods of restrictive feeding up to eight weeks of exposure might reveal more prominent direct effects of DON on rainbow trout growth in future studies.

Ad libitum feeding did not induce differences in feed intake, despite the above-mentioned reduced body weight gain and feed efficiency measurements noted in the ND2 and PD2 groups,

whereas other studies did report reduced feed intake (Gonçalves et al., 2019; Gonçalves et al., 2018c; Hooft and Bureau, 2017; Hooft et al., 2011; Hooft et al., 2019a; Hooft et al., 2019b; Ryerse et al., 2016; Ryerse et al., 2015; Woodward et al., 1983). Also, in our meta-analysis, we predicted that each additional mg/kg of DON in trout feed would lead to an exponential decline in feed intake, with a rate of 18.8% (Koletsi et al., 2021). A possible explanation for the absence of DON-induced feed refusal in the current experiment might be the relatively short duration of two weeks of *ad libitum* exposure. The majority of other studies maximum exposure times of up to eight weeks. Another hypothesis could be derived from the restrictive feeding period of six weeks, prior to the *ad libitum* feeding, during which fish may have adapted to tolerate the consumption of DON-contaminated feeds.

Feed ingredients naturally contaminated with DON often also contain other types of mycotoxins, whereas the use of DON produced under controlled laboratory conditions excludes cross-contamination (Koletsi et al., 2021). In our study, a direct and fair comparison between natural (ND) and pure DON (PD) proved difficult because DON levels in naturally contaminated diets were lower than anticipated. After restrictive feeding, performance was not different between ND2 and PD2. Yet, after *ad libitum* feeding, ND2 (at 1300 µg/kg) had a stronger effect on feed intake (but not significant) than PD2 at 1700 µg/kg. Our observations therefore are not in disagreement with the results of our meta-analysis for trout (Koletsi et al., 2021), which suggested the presence of combined effects due to the potential co-contamination of natural sources of DON with other *Fusarium* toxins.

3.4.2 Liver

The liver is the most studied organ evaluated for DON toxicity in fish species, although there are inconsistent approaches to scoring (semi-quantitative/qualitative) and staining protocols (H&E/PAS) among studies screening for the same pathological indicators in histological assessments (Hooft and Bureau, 2021). In the present study, a semi-quantitative approach was used to evaluate histopathological parameters in the liver, meeting the requirements of fundamental concepts for the semi-quantitative scoring of tissues (Meyerholz and Beck, 2018). A detailed scoring protocol was developed that translated qualitative information from microscopic liver images into data suitable for statistical analyses. This protocol quantified the severity of DON toxicity in trout liver using histopathological parameters and is the first study in trout to measure the toxic effects of DON over time within a restrictive feeding period.

Restrictive DON feeding did not appear to affect glycogen or lipid vacuolization. During restrictive feeding (weeks 1–6), time-related DON effects were noted in the form of haemorrhage, inflammation, and necrosis. Notably, these changes over time were found only after feeding the ND2 diet, implying a more severe toxic response using co-contaminated feed. Furthermore, we identified numerical but not statistically significant differences for the presence of pyknosis and pleomorphism in nuclei in week 6 (higher presence in DON-contaminated diets than the CON diet), most likely due to the low sample size and consequently lower statistical power. Studies comparable to ours have only been performed in common carp, where restrictive exposure to 953 µg/kg DON for six weeks did not induce differences in

hepatic glycogen vacuolization, but did increase lipid accumulation (Pietsch et al., 2014a). Carp showed histopathological indicators of impaired liver functioning on days 14 (hyperaemia, vacuolization, and dilation of sinusoids) and 26 (fat aggregation and dilation of sinusoids), but nothing indicated histopathological liver damage at the end of the experiment (day 56). Based on these common findings, at least in situations of restrictive exposure, it is reasonable to assume early acute responses to DON toxicity could possibly be diminished by physiological adaptation mechanisms.

Ad libitum feeding of DON (weeks 6–8) affected almost all histopathological parameters scored within the liver; glycogen vacuolization, pyknosis, pleomorphism of nuclei, increased presence of necrosis, and haemorrhages. So far, studies in trout (Gonçalves et al., 2019; Hooft et al., 2019a) have not made a distinction between lipid- and glycogen-type vacuolization. At present, although the mechanism of glycogen depletion in hepatocytes of DON-treated fish is not yet fully understood, glycogen vacuolization appears to be a good indicator of DON-induced liver effects. Other experiments in trout using high doses of DON (2700 µg /kg) reported multiple areas of necrosis with scattered haemorrhages (Gonçalves et al., 2019) and phenotypically altered hepatocyte nuclei (pyknosis and karyolysis) in trout fed 2600 µg/kg natural DON (Hooft et al., 2011). The presence of necrosis in the livers of our control-treated fish, although the percentages showed a declining trend along the sampling points, suggests an adaptation to the high-carbohydrate diet. Another points is that we observed dislocated nuclei at the edges of the cells in all sampling points and treatments (data not shown), which is most likely due to high lipid vacuolization and is not necessarily a pathological finding. However, we did not find differences in hepatic lipid vacuolization as was hypothesized; DON would inhibit the lipoprotein synthesis and cause fat accumulation as droplets in the hepatocytes (Tiemann et al., 2006). Perhaps the high carbohydrate content in our diets was the main factor that caused fat accumulation in the hepatocytes of all treatments, including the CON, and therefore I was not possible to detect the DON effects. An early study in trout measured hepatic lipid accumulation after six weeks of DON exposure (2600 µg/kg), although the histological appraisal in that study was conducted through H and E staining, thus rendering a distinction between glycogen and lipid droplets more challenging (Hooft et al., 2011).

Pure DON (PD2 diet) induced a stronger reduction of glycogen vacuolization than natural DON (ND2) and affected necrosis (presence and score at week 8) more than natural DON, but both DON diets had a comparable effect on aggravating haemorrhage presence. The differences between pure and natural DON on the severity of liver damage may have been masked to some extent by the experimental diets because the DON level in the naturally contaminated diet (ND2) was slightly lower than anticipated. As a result, PD2 groups received a higher absolute DON intake during *ad libitum* feeding (PD2 diet: 0.058 µg/g BW/day) than ND2 groups (0.044 µg/g BW/day). Thereby, *ad libitum* access to the feed maximised the absolute DON intake. Eventually, DON causes injury to fish livers through, for example, lipid peroxidation induced by oxidative stress, causing necrotic tissues and pro-inflammatory responses to haemorrhages, at least in common carp (Pietsch and Burkhardt-Holm, 2015; Pietsch et al., 2014a). The mechanisms at work in rainbow trout exposed to DON are yet to be fully unravelled.

3.4.3 Gastrointestinal Tract

The effects of DON on the gastrointestinal tract (pyloric caeca, midgut, and hindgut), if any, were generally mild. DON did not affect intestinal integrity and morphology during restrictive feeding (measured at weeks 1 and 6), and only a few mild alterations were observed after the *ad libitum* exposure. At week 8, the pyloric caeca of trout fed the ND2 diet showed increased mucosal fold heights, possibly an adaptation to a DON-driven impairment in nutrient absorption. The effects were only present in the ND2 and not the PD2 diet, possibly due to the presence of multiple mycotoxins in the naturally contaminated DON sources. The pyloric caeca comprises the first part of the gastrointestinal tract and appears more sensitive to mycotoxins like DON than the midgut and hindgut. Other studies reported an increased number of dead (apoptotic/necrotic) cells and a reduced number of mitotic cells in the pyloric caeca of trout fed DON at 5900 µg/kg (Hooft et al., 2019a) and a reduction in the expression of tight junction proteins (TJPS) in salmon fed DON at 5500 µg/kg (Moldal et al., 2018). Next to the observations in the pyloric caeca, also in the hindgut, some relatively minor effects were seen after *ad libitum* exposure at week 8 compared to week 1, including increased widths of mucosal folds in the ND2 and PD2 groups and increased enterocyte widths for the PD2 group. Similar compensatory morphological changes, i.e., an increase in villus height in the jejunum and ileum, have also been reported for the intestine of chickens exposed to DON (Girgis et al., 2010). Possibly, the general absence of clear pathology might be attributed to the rapid absorption of DON in the upper part of the gastrointestinal tract. A toxicokinetic study in salmon showed that DON reached a peak concentration in the liver one hour after the last feeding and then decreased with a half-life (t1/2) of 6.2 h (Bernhoft et al., 2017). This suggests that toxicokinetic studies into the breakdown of DON in the gastrointestinal tract of rainbow trout would be of immediate relevance.

The absence of clear effects of DON on intestinal health was further detailed by gene expression analysis of the pro-inflammatory cytokines IL-1*β,* IL-8, and TNF-*α* in the pyloric caeca and hindgut. PCR analysis was performed at the end of the *ad libitum* feeding period, at week 8, where, based on the histopathological findings, the suspected damage would be greatest. If anything, cytokine gene expression in the pyloric caeca was downreglated rather than upregulated. Moreover, there was no clear pattern in the regulation of the expression of cytokine genes in the hindgut. Overall, gene expression analysis confirmed the absence of strong effects of DON in the gastrointestinal tract.

Thus far, the effects of DON on pro-inflammatory gene expression in trout appear to have been limited to studies of the spleen and head kidney (Matejova et al., 2015). Although these authors reported an up-regulation of the pro-inflammatory cytokine TNF-*α* in the head kidney of DON-fed trout (1964 µg/kg) after 23 days of exposure, the comparison between a systemic organ like the head kidney and the gastrointestinal tract is difficult to make. In Atlantic salmon (Moldal et al., 2018), comparable to our findings, DON exposure (5500 µg/kg) for 8 weeks also did not induce differences in the gene expression of ILl-1β in pyloric caeca, midgut and hindgut. Most information on the effects of DON on gene expression in the gut comes from a study in carp (Pietsch et al., 2015). Here, study of the proximal intestine of DON-fed carp (953 µg/kg) showed an up-regulation of pro-inflammatory (IFN-γ, TNF-α2, INOS) and also anti-

inflammatory cytokines (IL-10, Arg1, and Arg2), after 14 days of exposure, returning to control levels at later time points (days 26 and 56). Taken together, the absence of cytokine-induced immune responses in the gastrointestinal tract is in alignment with the absence of strong histopathological changes in the same tissue.

3.5 Conclusions

To summarize, our study within a restrictive feeding exposure regime for six weeks showed direct effects of DON on protein gain in rainbow trout regardless of the source of industrially relevant DON levels (natural: 1300 or pure: 1700 µg/kg), below the current European Commission recommendation limit of 5000 µg/kg. Moreover, we revealed time-related DON effects on liver histological parameters, an early response at week 1 and a recovery by week 6. Apparently, rainbow trout exposed restrictively to a certain amount of naturally contaminated DON diet (1300 µg/kg) develop an adaptation mechanism to recover overtime and eliminate necrosis and haemorrhage in the liver. The adaptation process is not fully understood, and therefore further research is recommended on the antioxidant system, detoxification capacity, and toxicokinetics of DON in trout. Moreover, additional research is required in order to shed light on the potential combined effects of *Fusarium* toxins since we observed adaptation over time within the natural DON group. The *ad libitum* access to DON after the restrictive exposure did not impair feed intake but suppressed growth performance. At the end of the *ad libitum* exposure, histopathological damage was detected in the liver but not in the gastrointestinal tract, where no immunomodulating properties of DON were present. The severity of DON that was described during *ad libitum* exposure is an outcome of the absolute amount of DON ingested that led to a threshold dose that trout could not further tolerate. Finally, because DON severity might be species-specific, similar *in vivo* investigations are also recommended in other farmed fish species.

Supplementary Materials

The following are available online at:
https://www.mdpi.com/article/10.3390/toxins14110810/s1
Figure 3.S1: A comparative overview of the estimated daily DON intake (EDI) in salmonid studies; **Figure 3.S2:** Parameters in the gastrointestinal tract used for the quantitative histopathological assessment; **Table 3.S1:** Results on the histological parameters in the gastrointestinal tract of rainbow trout; and **Table 3.S2:** Calculation of EDI in salmonids.

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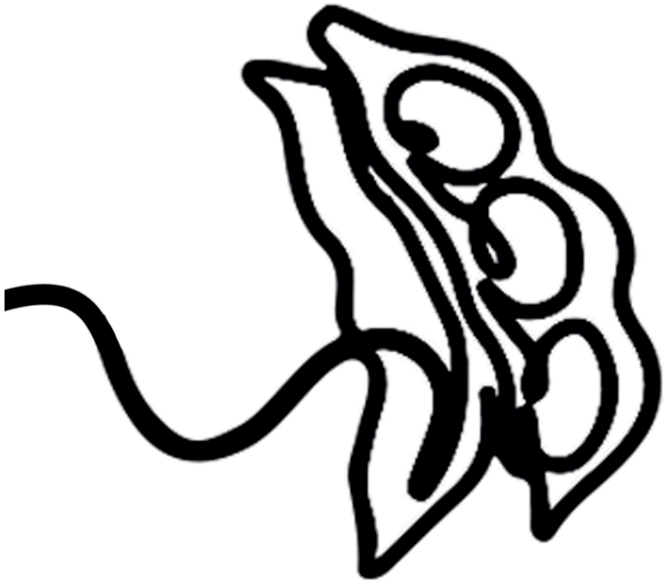
Chapter 4

Are the effects of Deoxynivalenol (DON) on performance, liver and gastrointestinal tract health of rainbow trout (*Oncorhynchus mykiss*) influenced by dietary composition?



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Abstract

This study investigated if dietary composition influences the effects of DON on the health and performance of rainbow trout (*Oncorhynchus mykiss*). Four experimental diets (2x2 factorial design) were formulated which differed in 1) the type of protein source; fishmeal (FM) versus soybean meal-based (SBM) and 2) the DON content of wheat; clean versus naturally contaminated wheat. Triplicate groups of n=30 fish were assigned to each diet: (1) CON-FM; DON= 0 µg/kg feed; (2) DON-FM; DON=1200 µg/kg feed; (3) CON-SBM; DON= 46 µg/kg feed; (4) DON-SBM; DON= 1300 µg/kg feed. The 8 week experiment was divided into two feeding periods: after 6 weeks of restrictive feeding, fish were fed *ad libitum* for 2 weeks. Influences on performance were evaluated by determining growth, protein and energy gain metrics, and on health parameters through the determination of histopathological changes in the liver and gastrointestinal tract (GIT). Restrictive feeding showed negative effects of DON and dietary composition on performance but did not show an interaction between DON and diet composition. Similarly, subsequent *ad libitum* feeding showed effects of DON and/or diet composition on growth, feed efficiency and body biometrics, but no interaction effects. These data confirmed the challenging nature of the SBM-based diet and confirm previously noted negative effects of DON on performance. Neither DON, nor diet composition indicated significant effects on liver health, nor was there an interaction effect. We discuss that the combination of DON and a sub-optimal diet based on SBM could accentuate the effects of DON, in particular concerning GIT functioning. Indeed, the histopathological assessment of mucosal fold width, enterocyte width and goblet cell density indicated significant interaction effects between DON and diet composition in the midgut. Yet, the differences were generally small and interaction effects were restricted to the midgut. The combined information on performance and health suggests that DON is harmful to rainbow trout regardless of diet quality.

4.1 Introduction

Globally, the growth of the aquaculture sector strongly depends on the expansion of aquafeed production (51 Mt in 2017 and 73 Mt expected in 2025) (Naylor et al., 2021; Tacon, 2020). Over the last 50 years, the required increase in production of aquafeeds already has led to the introduction of various alternative ingredients for fishmeal and fish oil, while the latter ingredients have become increasingly scarce and more expensive (Cottrell et al., 2020). Consequently, the ingredient composition of aquafeeds has diversified, with more plant- and animal-based ingredients/by-products of terrestrial origin being included in novel formulae (Naylor et al., 2021). For instance, in Norwegian salmon feeds, the inclusion level of marine ingredients has declined from 89% in 1990 to 41% in 2010 (Ytrestøyl et al., 2015) and even further to 25% in 2016 (Aas et al., 2019) and 22% in 2020 (Aas et al., 2022). The introduction of more plant-based ingredients in aquafeeds has also brought associated risks. Apart from nutritional imbalances, the increasing use of plant-based ingredients is linked to the introduction of anti-nutritional factors, contaminants and mycotoxins (Francis et al., 2001). Due to climate change, environmental conditions might become more favourable for fungus development on crops, leading to an increase in fungus-derived mycotoxin occurrence in plant-based ingredients which could be transferred to finished aquafeeds (Anater et al., 2016; Gonçalves et al., 2020a; Koletsi et al., 2021). This means there is an increasing risk of feeding fish with mycotoxin-contaminated feed.

Ever since mycotoxins have been described as emerging feed contaminants for European aquaculture, it has become evident that deoxynivalenol (DON) is the most prevalent mycotoxin in aquafeeds (Koletsi et al., 2021). Different fish species display different sensitivities to DON. Compared to other fish species, rainbow trout (*Oncorhynchus mykiss*) is very sensitive to DON (Hooft et al., 2011; Koletsi et al., 2021). Despite the sensitivity of fish to DON, comparatively few studies have investigated the impacts of DON on fish (Hooft and Bureau, 2021), in stark contrast to the depth of research on this topic in terrestrial animals. However, a meta-analysis of the available data across several fish species show that feed intake and growth decline exponentially with dietary DON levels (Koletsi et al., 2021). In more detail, DON has been shown, among others, to reduce protein gain in rainbow trout which suggest an inhibition of protein synthesis (Koletsi et al., 2022). DON may also influence fish health. Indeed, DON has been reported to induce liver pathology in several fish species, as indicated by marked alterations in hepatic histological parameters (Hooft et al., 2011; Koletsi et al., 2022; Pietsch and Burkhardt-Holm, 2015; Pietsch et al., 2014a). Gut health is also impacted through the reduction in expression of genes that regulate tight junction proteins in grass carp, indicating a disruption of the intestinal epithelial barrier (Huang et al., 2018). In rainbow trout, the available literature suggests that DON impacts fish health predominantly by inducing changes in the liver with less impact on the intestinal barrier integrity, as evidenced by minimal changes in intestinal histological parameters (Koletsi et al., 2022). Overall, with the increasing risk of feeding fish mycotoxin-contaminated feed there is an associated and thus also increasing risk of introducing DON-induced negative effects on fish performance and health.

Experimentally, studies on the effects of DON have generally targeted the concentration of the mycotoxin instead of focusing on the composition of the diet. Studies with DON in carnivorous

fish have hitherto been performed against a background of optimal quality marine-based diets. Yet, as argued above, ingredient composition of aquafeeds has diversified rapidly to include more plant-based ingredients. This not only introduces the risk of feeding mycotoxin-contaminated ingredients but also introduces a risk that the negative effects of DON can be amplified by sub-optimal diets. Information on the interaction between DON and diet composition is generally lacking. Here, we chose to study the effects of DON against a sub-optimal diet based on soybean meal (SBM).

Traditionally, SBM is the most frequently used plant feedstuff to replace fishmeal in aquafeeds (Oliva-Teles et al., 2022). However, SBM can be challenging for fish, as it can contain antinutritional factors, such as soy saponins (Oliva-Teles et al., 2015), suggested to be the primary inducers of intestinal enteritis in Atlantic salmon (Krogdahl et al., 2015), and its inclusion level is thus often restricted in salmonid diets. Symptoms of SBM-induced enteritis in the distal intestine were first described for Atlantic salmon (Krogdahl et al., 2003; van den Ingh et al., 1991; van den Ingh et al., 1996), but later also for rainbow trout, which seem to endure slightly higher SBM levels before developing enteritis (Heikkinen et al., 2006; Merrifield et al., 2009; Mosberian-Tanha et al., 2016; Romarheim et al., 2008). Symptoms of SBM-induced enteritis in rainbow trout also include increased permeability of the distal intestinal epithelium, which may lead to reduced nutrient uptake and affect performance (Merrifield et al., 2009; Mosberian-Tanha et al., 2016; Nordrum et al., 2000). Enteritis-associated changes such as induced by SBM could possibly influence mycotoxin-induced effects on fish performance and health. Therefore, SBM is often used in nutritional challenge models in salmonid fish species.

The main aim of this study was to assess if dietary composition influences the effect of DON on rainbow trout (*Oncorhynchus mykiss*). Based on the findings that SBM can lead to the disruption of the integrity of the intestinal epithelium in salmonids (Knudsen et al., 2008; Mosberian-Tanha et al., 2016), we hypothesised that the combination of DON and a challenging SBM-based diet could aggravate effects of DON in rainbow trout, in particular with respect to the functioning of the gastrointestinal tract (GIT). To this end, we designed both an ‘optimal quality’ marine-based diet and a ‘sub-optimal quality’ SBM-based diet with and without dietary DON. The effects of dietary DON were investigated through a detailed assessment of both performance and health metrics of rainbow trout fed these experimental diets.

4.2 Materials and methods

This experiment was carried out at the experimental facilities of the Alltech Coppens Aqua Centre (Leende, The Netherlands). The project (number AVD2330020198084) had been approved by the Central Committee on Animal Experiments (CCD) of The Netherlands and all experimental procedures were carried out in accordance with the Dutch law on the use of animals for scientific purposes.

4.2.1 *In vivo* experimental procedure

Experimental methods, system and husbandry procedures, duration of exposure, feeding regimes and practices, and sampling protocols were similar to those described for an earlier study (Koletsi et al., 2022). Briefly, 10 g rainbow trout (*Oncorhynchus mykiss*) were obtained from a commercial trout farm (Mohnen Aquaculture GmbH, Germany) and housed after arrival in a recirculating aquaculture system (RAS). After one week of acclimatization and feeding a standard commercial trout diet, groups of 30 fish were each stocked in one of 12 120-L tanks (triplicates per dietary treatment). Throughout the experiment, water temperature was kept constant at 14 ± 0.5 °C, and a photoperiod of 17 h of light and 7 h of darkness was used. The following water physicochemical parameters were monitored and kept within optimal ranges: pH: 7.0–8.5, NH4+: <1 mg/L, NO2−: <0.5 mg/L, and oxygen (O_2) above 8 mg/L.

4.2.2 Experimental diets

Four experimental diets were formulated according to a 2x2 factorial design (Table 4.1). The first factor aimed to create a contrast in the "quality" of the diets, which was done by replacing 25% of an optimal quality fishmeal LT (FM) by 25% non-GMO soybean meal (SBM; CP >45). The lower crude protein content of SBM compared to FM was not compensated. Crystalline methionine was added to the SBM-diets to achieve a balanced amino acid profile (NRC, 2011). The second factor aimed to create a contrast in dietary DON level. Control (CON) diets were aimed to be of free of the mycotoxin DON, which was achieved via the inclusion of 40% of a "clean" batch of wheat, confirmed free of DON and other toxins by liquid chromatography/tandem mass spectrometry (LC-MS/MS) at the Alltech 37+ mycotoxin laboratory (ISO/IEC 17025:2005 accredited) (Dunboyne, Ireland). DON-contaminated diets contained 40% "naturally contaminated" wheat, analysed for mycotoxin profiles and used in a previous study (Koletsi et al., 2022). Details on ingredient and analysed nutrient content of the four experimental diets (CON-FM, DON-FM, CON-SBM and DON-SBM) are presented in Table 4.1. Final pelleted feeds were analysed with LC-MS/MS to confirm the absence or low occurrence of DON in control, and high DON levels in contaminated diets (Table 4.1). Indeed, the CON-FM diet was free of DON and the CON-SBM diet had a minimal level of DON (46 µg/kg) and also mycophenolic acid (34 µg/kg). Both DON-contaminated diets (DON-FM and DON-SBM) had comparable DON levels of 1200 µg/kg and 1300 µg/kg, respectively, and also low concentrations of other toxins.

The experimental diets were produced by Research Diet Services (Wijk bij Duurstede, The Netherlands) as 2.5 mm extruded pellets. Fish were hand-fed twice daily. During the restrictive feeding period, the total amount of feed given per fish was equal for all treatments in order to have similar DON intake and similar dietary challenge between the experimental diets, following the 2 x 2 factorial design. Feeding equal amounts of feed and (therefore) DON should reveal the direct impact of the toxin on fish while excluding its potential effect on feed intake. The restrictive feeding was performed according to the metabolic body weight of the fish (12 $g/kg^{0.8}/d$). During the *ad libitum* feeding period, fish were fed to apparent satiation for one hour during each feeding event, in order to study the impacts of DON and diet quality on feed intake.

Table 4.1 | Ingredients composition, proximate and mycotoxin analysis of the experimental diets.

	Experimental diets			
	Optimal quality		Sub-optimal quality	
Ingredients Inclusion (%)	CON-FM	DON-FM	CON-SBM	DON-SBM
Wheat 'clean'	40.00	-	40.00	-
Wheat 'contaminated'	-	40.00	-	40.00
LT fishmeal	37.01	37.01	15.13	15.13
Soybean meal (CP>45)	-	-	25.00	25.00
Fish oil	11.86	11.86	10.93	10.93
Blood meal	9.87	9.87	6.93	6.93
Monocalcium phosphate	0.13	0.13	0.70	0.70
Methionine	-	-	0.18	0.18
Choline	0.15	0.15	0.17	0.17
Premixes[1]	0.99	0.99	0.97	0.97
Analysed nutrient composition (%)[2]				
Dry Matter	94.6	94.9	92.4	94.3
Protein	44.7	44.4	38.7	38.9
Fat	17.0	17.0	14.6	14.8
Ash	7.3	7.1	5.7	5.8
Gross Energy (MJ/kg) DM	22.3	22.3	22.8	21.4
Mycotoxin concentration (µg/kg)[2]				
DON	-	1206	46	1329
DON-3-Glucoside	-	36	-	38
Enniatin A/A1	-	-	-	2
Enniatin B/B1	-	15	-	5
Mycophenolic Acid	-	-	34	80

[1]Commercial premix from Alltech Coppens to meet (NRC, 2011) requirements of rainbow trout.

[2]On dry matter basis, the symbol "-" means that the toxin was not present (0.00 µg/kg) or below the detection limit.

[3] In the main text, the rounded levels of DON-contaminated diets are mentioned: DON-FM: 1200, DON-SBM: 1300 µg/kg.

4.2.3 Sampling and analyses

The sampling schedule and subsequent analyses were similar to those described for an earlier study (Koletsi et al., 2022). Briefly, for growth performance measurements, the biomass per tank was recorded at the start of the experiment and at the end of each feeding period, restrictive (week 6) and *ad libitum* (week 8). At week 6, five fish per tank were euthanised and stored at -20°C for body composition analysis. Fish carcass and feed samples were analysed by Nutricontrol (Veghel, The Netherlands) for dry matter, crude protein, fat, ash and gross energy content.

For histopathological analysis, two sections of liver and a section from each segment of the GIT (pyloric caeca, midgut and hindgut) tissue from two fish per tank were collected at the end of

week 1 and week 6 of restrictive feeding and at the end of the *ad libitum* feeding period (week 8). Samples were processed according to the histological procedures described by (Koletsi et al., 2022). Briefly, liver sections were stained separately with Periodic acid-Schiff's (PAS) reagent and with Haematoxylin and Eosin (H&E) and evaluated according to a previously developed scoring system (Koletsi et al., 2022). GIT sections were coloured with Alcian blue (pH 2.5) followed by Crossman, and pictures were imported in ImageJ software (version 1.53q) (Schindelin et al., 2012) to measure mucosal fold width, mucosal fold height, lamina propria width, enterocyte width, supranuclear vacuoles width (SNV) and goblet cell density.

Additionally, from the fish sampled for tissues (n=2 per tank), total liver weight and total body length were recorded. During sampling, handling of the fish was avoided as much as possible while fish were euthanized by an overdose of benzocaine (dissolved in water at 0.5 ml/L). Samples were also collected from the initial population (before the start of the experiment and distribution to tanks, at time point zero) totalling 6 fish for tissue sampling and 20 fish for determining the initial body composition.

4.2.4 Calculations and statistical analysis

Growth performance parameters were calculated as follows: Weight gain (g)= FBW-IBW, FBW is the final body weight (g) and IBW the initial body weight (g); Growth (g/d)= weight gain/days; Specific growth rate (SGR, %/d)= ((ln FBW − ln IBW)/days)x100; Feed conversion ratio (FCR) on DM basis = FI/ weight gain, where FI is the feed intake defined as the average amount of feed (g) consumed by a fish, converted based to DM content of the feed (g/kg); Hepatosomatic index (HSI, %)= (liver weight/ W) *100; and Condition factor (K) =(W/L^3) *100, where W is the individual FBW of the tissue sampled fish and L its body length (cm). Moreover, retained protein and energy and their retention efficiencies in rainbow trout whole body samples were calculated as follows: Retained protein (g/fish)= FBW*FPC - IBW*IPC, where FPC is the protein content (g) in the fish body and the end and IPC is the protein content (g) at the start; Protein retention efficiency (%) = (Retained protein/CPI)x100, where CPI is the dietary protein intake (g/fish) calculated as =average FI of an individual x protein content in the feed. Similarly, for retained energy (MJ/fish) and energy retention efficiency (%); Retained energy= FBW*FEC - IBW*IEC, where FEC is the gross energy content (MJ) in the fish body and the end and IEC is the protein content (g) at the start; Energy retention efficiency (%)=(Retained energy/GEI)x100, where GEI is the dietary gross energy intake (MJ/fish) calculated as = average FI of an individual x gross energy in the feed.

For the statistical analysis of growth performance and protein and energy retention, tanks (n=12) were designated as the experimental units. The effect of dietary DON (CON versus DON) and diet composition (FM versus SBM) and their interaction was tested with a two-way ANOVA using the general linear model (GLM). Model residuals were tested for normality by using the Kolmogorov-Smirnov test and homogeneity of variance was determined by Levene's test. If interaction effects were significant, a Tukey's multiple comparison test was performed, with statistical significance being defined at a p-value≤0.05. The histological parameters of the GIT were analysed separately for each segment (pyloric caeca, midgut and hindgut). Mixed-

effects models were applied; a generalized linear model for the variables in the GIT and a multinomial logistic regression model for the continuous scores in the liver, with toxin (CON versus DON), diet (FM versus SBM), time (week 1, 6 and 8) and their interactions to be included in the model (n=720, 60 per diet per time point), while fish was used as a random effect. The binomial (yes/no) liver data (nuclei pyknosis and pleomorphism, necrosis, haemorrhage, inflammation), were also analysed with mixed model using logistic regression with toxin, diet, time and their interactions as fixed effects and fish as a random effect in the model. The binomial outcomes were expressed as percentages (%) (n=720, 60 per diet per time point). All data was statistically analysed in the IBM Statistical Package for the Social Sciences (SPSS) program (v 23.0; New York, NY, USA).

4.3 Results

4.3.1 Performance

During the entire experiment (eight weeks), no mortalities occurred, and no abnormal behaviour or difficulties in feed acceptance was observed.

Restrictive feeding period

During the restrictive feeding (6 weeks), both growth and FCR were affected by diet quality ($p \leq 0.001$) and by the presence of DON ($p \leq 0.05$). Growth of fish fed with SBM diets was 12% lower than growth of fish fed with FM diets, confirming that the experimental SBM-based diets could be considered sub-optimal. Growth of fish fed with diets containing DON had a 6% lower growth rate than fish fed with the CON diets (Table 4.2). These effects of diet quality and DON were additive, with an absence of an interaction effect ($p > 0.05$). In other words, the reduction in growth, or increase in FCR caused by DON were comparable for both optimal and sub-optimal quality diets.

Measurements of the hepatosomatic index (HSI) did not differ between treatments ($p > 0.05$), indicating there were no main effects on the liver caused by either diet quality, or DON. The condition factor (K), however, was different between diets because affected by diet quality; fish fed the SBM diets had a lower body condition score ($p \leq 0.05$; Table 4.2), confirming the experimental SBM-based diets could be considered sub-optimal. The treatments effects on retained protein and energy paralleled the pattern observed for growth. Both retained protein and retained energy were affected by DON and by diet (the two main effects), but there was no significant interaction (Table 4.2), meaning that the impact of DON on these parameters was similar in the groups fed an optimal- (FM) or a sub-optimal quality diet (SBM). Protein retention efficiency was affected only by DON; trout fed diets contaminated with DON had a 6% lower protein retention efficiency compared to trout fed the control diets ($p \leq 0.01$). The only performance indicator affected by the interaction effect of diet quality and DON ($p \leq 0.05$) was energy retention efficiency; exposure of fish to DON reduced the energy retention efficiency in the FM diets but not in the SBM diets (Table 4.2). These outcomes indicate that a sub-optimal diet such as SBM does not necessarily aggravate DON effects on performance.

Table 4.2 | Effects of dietary DON, diet quality (FM versus SBM) and their interaction on the performance of rainbow trout fed the experimental diets (CON-FM diet; DON= 0 µg/kg, DON-FM; DON=1200 µg/kg, CON-SBM; DON=46 µg/kg and DON-SBM; DON=1300 µg/kg) during a 6-week restrictive feeding period.

	Experimental diets					p-value		
Growth parameters	CON-FM	DON-FM	CON-SBM	DON-SBM	SEM	DON	DIET	DON*DIET
Initial BW (g)	9.91	9.99	9.97	9.90	0.122	NS	NS	NS
Final BW (g)	40.6	39.2	37.1	35.6	0.50	*	***	NS
Growth (g/d)	0.77	0.73	0.68	0.64	0.012	*	***	NS
SGR (%BW/d)	3.53	3.42	3.28	3.20	0.041	*	***	NS
FCR	0.70	0.73	0.79	0.83	0.012	*	***	NS
HSI (%)	2.26	2.30	2.17	2.00	0.23	NS	NS	NS
Condition factor (K)	1.23	1.18	1.15	1.14	0.022	NS	*	NS
Retained protein (g/fish)	4.80	4.49	4.16	3.90	0.078	**	***	NS
Protein retention efficiency (%)	50.3	47.5	49.9	46.8	0.8	**	NS	NS
Retained energy (MJ/fish)	0.239	0.222	0.202	0.194	0.004	**	***	NS
Energy retention efficiency (%)	50.3^{a}	46.6^{b}	41.4^{c}	42.5^{c}	0.74	NS	***	*

FM: fishmeal-based diet, SBM: soybean meal-based diet, BW: body weight, SGR: specific growth rate, FCR: feed conversion ratio on dry matter basis, HSI: hepatosomatic index, SEM: standard error of means, NS: not significant, ***: $p \leq 0.001$, **:$p \leq 0.01$, *: $p \leq 0.05$. Treatments lacking a common letter are statistically different ($p \leq 0.05$) according to Tukey's multiple comparison test.

Ad libitum feeding period

During the 2 weeks of *ad libitum* feeding, daily feed intake was not affected by diet quality, nor by the presence of DON (Table 4.3), and with an average of 1.8-1.9 g/fish/day, feed intake was highly similar among treatments. Although over this relatively short feeding period diet quality did not affect growth, the *ad libitum* feeding did result in DON affecting growth ($p \leq 0.05$); trout fed with DON diets had an 11% lower growth rate than trout fed with CON diets. FCR was affected by both the effects of DON ($p \leq 0.001$) and diet quality ($p \leq 0.001$). FCR was 9% higher in DON compared to CON diets and 9% higher in SBM compared to FM diets, indicating that the two factors (DON and diet quality) contribute equally to a poorer feed efficiency in trout. Both growth and FCR were unaffected by the interaction effect ($p > 0.05$), though numerically the effect of DON on FCR was larger in fish fed the SBM diets compared to the FM diets. This is in line with our hypothesis of a poorer expected performance in trout fed the sub-optimal quality diet.

HSI was reduced by the presence of DON in the diet ($p \leq 0.05$), possibly indicative of DON negatively affecting liver health, but was unaffected by the diet quality ($p > 0.05$). HSI was equal for both FM diets, whilst the addition of DON to the SBM diet numerically reduced HSI (DON-SBM<CON-SBM). Though the interaction effect between DON and diet quality was not statistically significant, the outcome still implies that DON effects on the liver (HSI) were more obvious against the background of a less optimal diet. The condition factor (K), however, was different between diets and was thus affected by diet quality; fish fed the SBM diets had a lower body condition score ($p \leq 0.05$; Table 4.3), confirming the experimental SBM-based diets could be considered sub-optimal. Finally, there was no effect of DON on condition factor, regardless of whether fish were fed a FM- or SBM-based diet ($p > 0.05$).

Table 4.3 | Effects of dietary DON and diet quality (FM versus SBM) on the performance of rainbow trout fed the experimental diets (CON-FM diet; DON=0 µg/kg, DON-FM; DON=1200 µg/kg, CON-SBM; DON=46 µg/kg and DON-SBM; DON=1300 µg/kg) during a 2-week *ad libitum* feeding period.

	Experimental diets					p-value		
Growth parameters	CON-FM	DON-FM	CON-SBM	DON-SBM	SEM	DON	DIET	DON*DIET
Final BW (g)	73.7	70.2	68.6	63.1	1.94	*	*	NS
Growth (g/d)	2.23	2.03	2.07	1.82	0.092	*	NS	NS
SGR (%BW/d)	4.03	3.79	4.02	3.79	0.110	NS	NS	NS
Average daily feed intake (g/fish/day)	1.88	1.84	1.92	1.83	0.062	NS	NS	NS
FCR	0.80	0.86	0.86	0.95	0.015	***	***	NS
HSI (%)	2.3	2.3	2.7	2.0	0.22	*	NS	NS
Condition factor (K)	1.23	1.18	1.15	1.14	0.022	NS	*	NS

FM: fishmeal-based diet, SBM: soybean meal-based diet, BW: body weight, SGR: specific growth rate, FCR: feed conversion ratio on dry matter basis, HSI: hepatosomatic index, SEM: standard error of means, NS: not significant, ***: $p \leq 0.001$, **:$p \leq 0.01$, *: $p \leq 0.05$.

4.3.2 Health

Histopathological assessment of the gastrointestinal tract (GIT)

Qualitative assessment of the histological pictures from the GIT did not reveal obvious clinical alterations. Representative pictures from intestinal folds after *ad libitum* exposure (week 8) are presented in Figure 4.1. Further examples during and after restrictive exposure (week 1 and week 6) are also available (tile scans, Figures 4.S1, 4.S2). Diet quality (i.e., FM versus SBM diets) seemed to cause some histological alterations in the intestine; a slight reduction of the width and a more irregular appearance of the zone with supranuclear vacuoles (SNV) was visible especially in the hindgut (Figure 4.1). Feeding diets with DON did not cause obvious histological changes in the intestine.

Quantitative assessment should allow for detection of statistically significant alterations in the GIT (pyloric caeca, midgut and hindgut) induced by the imposed factors, such as DON, diet and time (Table 4.S1). No significant histological effects of DON intake were observed in the hindgut or the pyloric caeca within the GIT. However, DON-induced alterations were detected in the midgut area, where a main effect of DON over time was present on the width of the area with supranuclear vacuoles (SNV) ($p \leq 0.05$). This suggests that DON may have an effect on the GIT, detected as reduced zone with SNV ($p \leq 0.05$; CON diets: 44.8 µm versus DON diets: 41.0 µm) in the midgut, with the effect being dependent upon the duration of the feeding period. Interaction effects between DON and diet were present and significant for several histopathological parameters measured in the midgut. Both, mucosal fold width and enterocyte width, were reduced by DON against the background of a sub-optimal SBM-based diet, but not FM-based diet ($p \leq 0.05$; Figure 4.2). Also, the number of goblet cells in the midgut was highest in fish fed a CON-SBM diet, and intermediate in fish fed DON, independent of diet quality ($p \leq 0.05$; Figure 4.2).

The main effects of diet quality on the different histopathological parameters measured in the GIT are presented in Table 4.4 and shortly summarized below. Mucosal fold width was reduced in fish fed SBM diets compared to fish fed FM diets, at least in the pyloric caeca and midgut ($p \leq 0.05$). Mucosal fold height was reduced in fish fed SBM diets compared to fish fed FM diets, but only in the midgut ($p \leq 0.05$). Lamina propria width was unaffected by diet quality in all gut segments ($p > 0.05$). As previously highlighted, enterocyte width was unaffected by diet quality, with the exception of the interaction effect between diet quality and DON in the midgut. Moreover, the width of the SNV layer inside the enterocytes was reduced by replacing FM by SBM, in all gut segments ($p \leq 0.05$). Goblet cell density, i.e., the number of goblet cells in the pyloric caeca and hindgut were not influenced by diet (Table 4.4). Some of the histopathological parameters were affected by time (Table 4.S1), but are not discussed here since for almost all parameters investigated, 2 and 3-way interaction effects with time were not significant.

Table 4.4|Main effect of dietary quality (FM versus SBM) on histological parameters in pyloric caeca, midgut and hindgut of rainbow trout fed restrictively for 6 days (week 1) and 40 days (week 6) and *ad libitum* for 15 days (week 8) the experimental diets; CON-FM diet (DON= 0 µg/kg), DON-FM (DON=1200 µg/kg), CON-SBM (DON= 46 µg/kg) and DON-SBM (DON= 1300 µg/kg).

	Dietary Composition			
	FM	SBM	SEM[1]	p-value
Mucosal fold width (µm)				
Pyloric	155.7	144.0	3.60	*
Midgut	137.1	127.2	3.34	*
Hindgut	150.7	141.5	4.23	NS
Mucosal fold height (µm)				
Pyloric	436.5	401.1	20.85	NS
Midgut	372.9	336.4	10.55	*
Hindgut	383.6	375.2	15.22	NS
Lamina propria width (µm)				
Pyloric	16.16	16.00	0.64	NS
Midgut	17.9	17.5	0.60	NS
Hindgut	17.7	17.2	0.65	NS
Enterocyte width (µm)				
Pyloric	85.5	78.4	2.42	*
Midgut	73.7	68.1	2.35	NS
Hindgut	65.0	65.5	2.44	NS
Supranuclear vacuole (SNV) width (µm)				
Pyloric	53.0	48.8	1.50	*
Midgut	44.9	40.8	1.14	*
Hindgut	67.0	57.8	2.95	*
Goblet cell density[2]				
Pyloric	0.038	0.043	0.003	NS
Midgut	0.085	0.100	0.004	**
Hindgut	0.054	0.049	0.004	NS

[1]Pooled standard error of means: SEM (total cases n=720, included cases in pyloric: n=695, midgut: n=600, hindgut: n=550) [2]Calculated per µm fold height, Not significant: NS, p≤0.05: *, p≤0.01: **

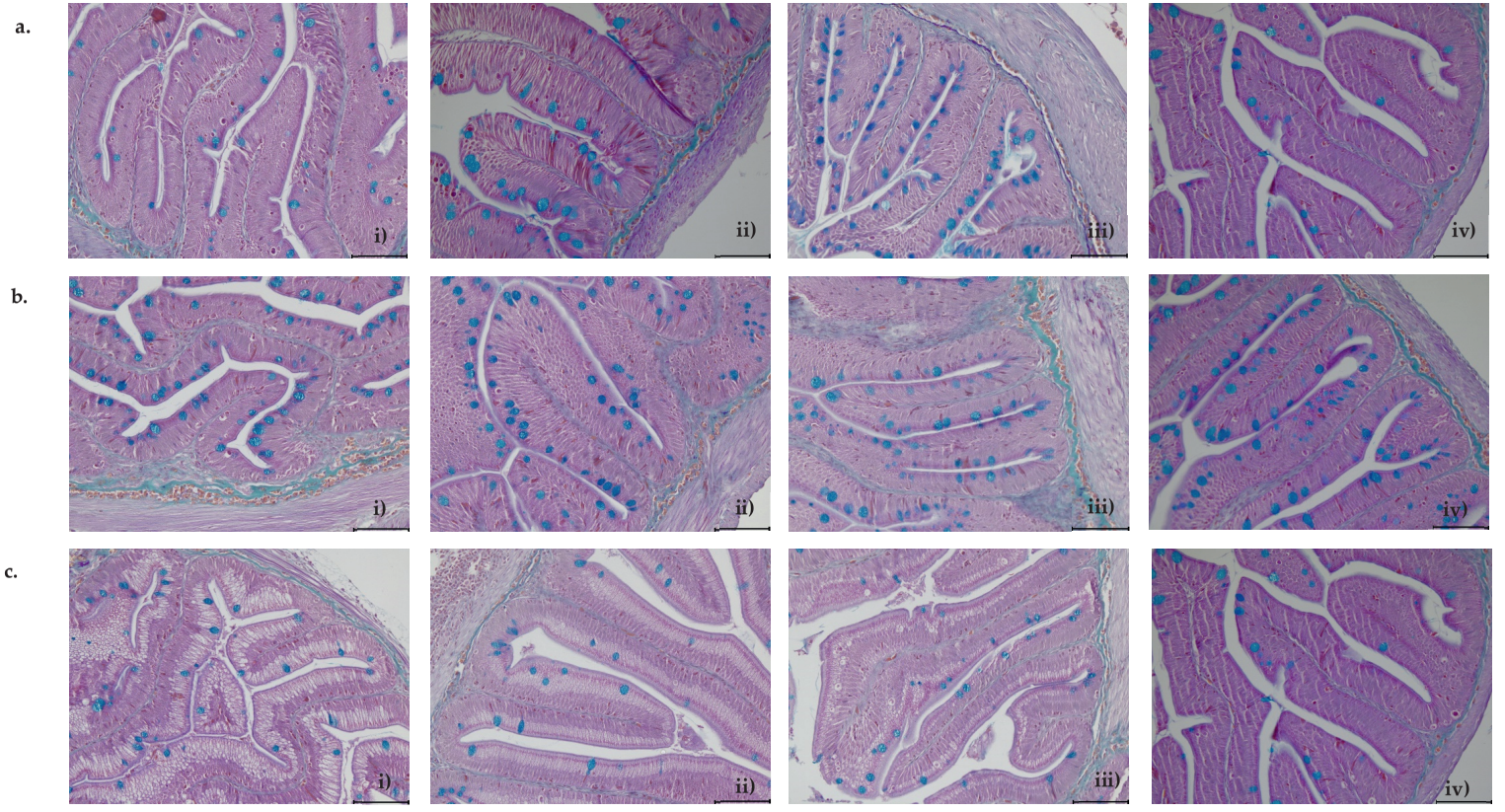

Figure 4.1 | Representative examples of histological sections of the intestinal folds in **a)** pyloric caeca **b)** midgut and **c)** hindgut of rainbow trout fed: **i)** a control fishmeal-based diet without mycotoxins (CON-FM), **ii**) a fish meal based diet with DON (DON-FM), **iii) a** control soybean meal-based diet without mycotoxins (CON-SBM) and iv) a soybean meal-based diet with DON (DON-SBM) *ad libitum* for 2 weeks. Staining: Alcian blue-Crossman; Magnification: x 20; Black s*cale bar = 100 µm.*

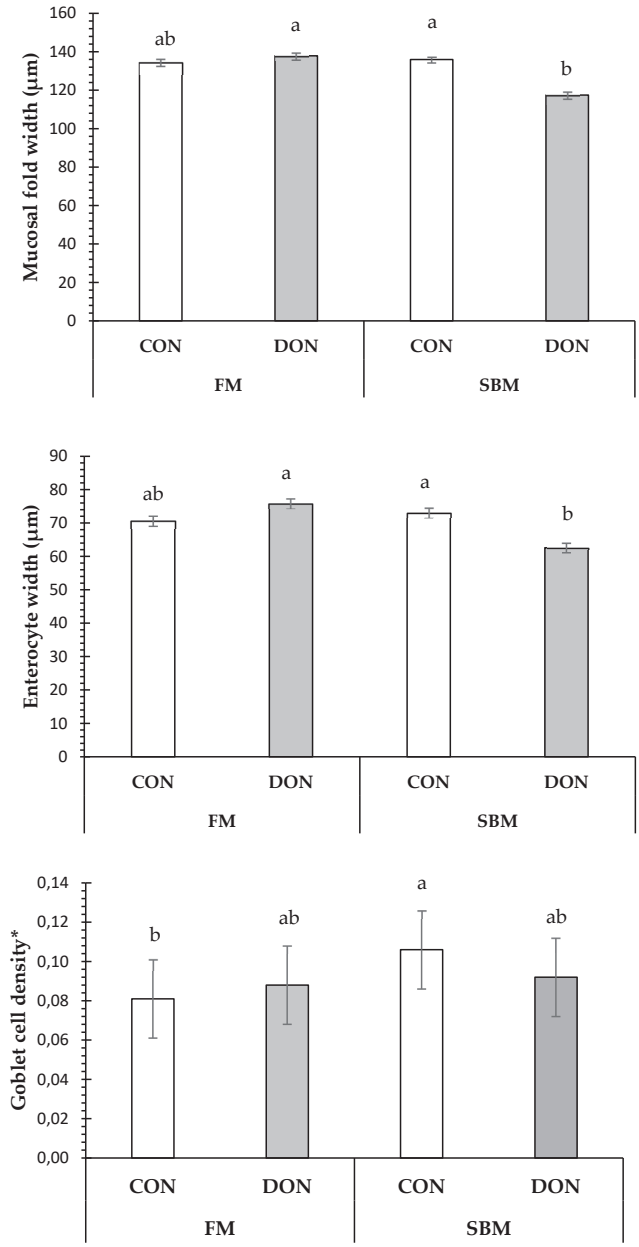

Figure 4.2 | Interaction effects between dietary DON (CON versus DON) and diet quality (FM versus SBM) on mucosal fold width ($p \leq 0.05$), enterocyte width ($p \leq 0.05$) and goblet cell density ($p \leq 0.05$) in the midgut of rainbow trout fed the experimental diets: CON-FM, DON-FM, CON-SBM and DON-SBM restrictively for 6 days (week 1) and 40 days (week 6) and *ad libitum* exposure for 15 days (week 8). *Goblet cell density was calculated as the number of cells per µm fold height. Error bars indicate standard error of means. Treatments lacking a common letter are statistically different ($p \leq 0.05$) according to Tukey's multiple comparison test.

Histopathological assessment of the liver

Qualitative analysis of the livers for histopathological signs indicated the occasional presence of necrotic areas which seemed inconsistent with treatment, but rather varied greatly within each of the treatment groups; necrosis was observed in groups fed DON, but also in control groups not fed with DON (Figure 4.3), and in groups fed both diet qualities (Figure 4.S3). In line with this high variation within groups, subsequent quantitative analysis of the livers for histopathological signs did not reveal significant effects of DON, diet quality, or time (Table S2). Notably, although necrotic areas may have been present to some extent in all groups, including fish fed CON diets, mean necrosis score was generally low (<0.7), as was the presence of haemorrhages and inflammation in all treatment groups during the whole experiment. The only exception was the time main effect ($p \leq 0.01$) and the significant 3-way interaction effect of DON, diet quality and time on the lipid vacuolisation ($p \leq 0.01$); within the FM group, lipid vacuolization was increased in the DON diet compared to the CON diet by week 8.

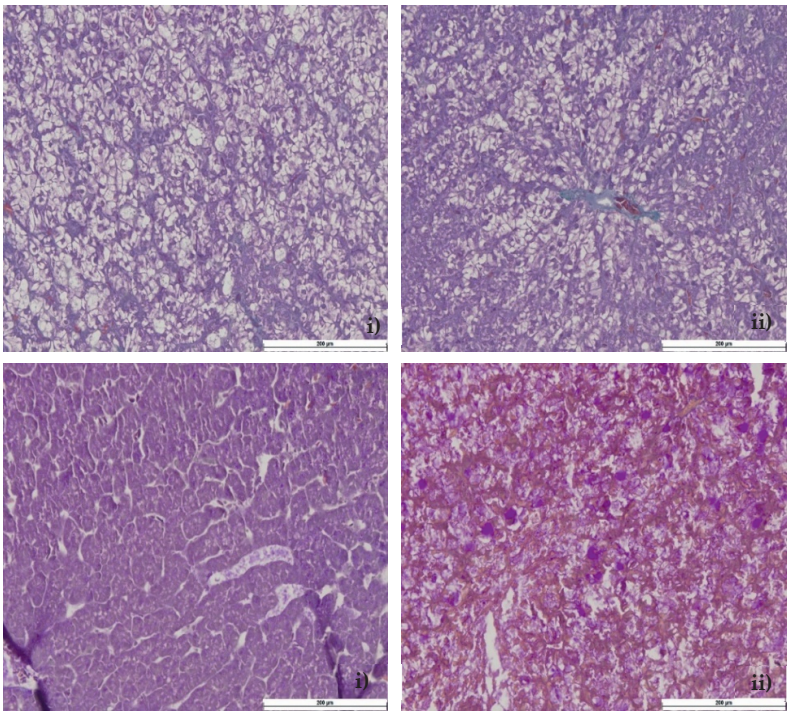

Figure 4.3|Representative examples of histological sections of the liver from rainbow trout fed **i)** a control fishmeal-based diet without mycotoxins (CON-FM) and **ii**) a control fishmeal-based diet with DON (DON-FM). The first row **(a)** shows representative pictures per diet without pathological indication and the second row **b)** examples of livers with necrotic areas. Staining: PAS-Crossman; Magnification: x 20; White s*cale bar = 200 µm.*

4.5 Discussion

Previously, our study in rainbow trout indicated that dietary exposure to DON can reduce growth and have time-dependent effects on fish health in terms of liver damage, and may also negatively impact the integrity of the intestinal barrier (Koletsi et al., 2022). While we observed negative effects on performance and health induced by DON, this previous study was performed with an optimal-quality marine-based diet. It is possible that plant-based could further worsen the observed negative effects of DON. To gain insight in such possible interactions between DON and diet composition, the present study investigated the effects of DON by studying fish performance and health in experimental treatments fed a fishmeal (FM)-based diet versus fish fed a sub-optimal diet based on soybean meal (SBM). The data from our experiment indicate that the impact of DON on growth and liver health did not differ between the FM- and SBM-based diets. This was shown by the absence of an interaction effect between DON and diet quality, suggesting that combined effects of DON and diet composition were additive. The data from our experiment also indicate that the impact of DON on intestinal health did differ between the FM- and SBM-based diets. The interaction effect between DON and diet quality was observed for particular histopathological parameters, for example, dietary DON contamination aggravated a number of SBM-induced enteritis symptoms in the midgut area of the gastrointestinal tract. Before discussing the interaction between DON and diet quality, first the main effects of DON and diet quality on trout performance and health will be discussed.

With regard to performance, during both restrictive and *ad libitum* feeding in our study, exposure to industrially-relevant DON levels (1200-1300 µg/kg) reduced weight gain and feed efficiency of rainbow trout. The current results on a reduced performance during satiation feeding have been reported in various studies in rainbow trout (Gonçalves et al., 2019; Gonçalves et al., 2018c; Hooft and Bureau, 2017; Hooft et al., 2011; Hooft et al., 2019a; Hooft et al., 2019b; Ryerse et al., 2015). In slight contrast to our previous study (Koletsi et al., 2022), which used similar DON levels, growth and FCR were negatively affected in this experiment. Yet, in line with our previous observations (Koletsi et al., 2022), again, impairment of performance during the restrictive feeding period was mirrored by suppressed protein and energy gain. Together, our studies suggest that exposure of rainbow trout to levels of DON that are of practical relevance for aquaculture has a direct effect on growth, possibly through inhibition of protein synthesis. Furthermore, during the restrictive feeding period, the estimated daily intake (EDI, µg/g BW/day) of DON in rainbow trout was 0.028 (DON-FM) and 0.033 (DON-SBM), comparable with the EDI calculated for the naturally DON-contaminated diet (1300 µg/kg), 0.033 (Koletsi et al., 2022). Comparing our earlier study with the present study shows that despite similar exposure levels, the effects of DON can vary, which was also the case for histopathological effects on the liver.

With regard to health, in the present study, DON exposure did not induce histopathological changes in the liver, while using similar levels of DON in our previous study did affect liver health (Koletsi et al., 2022). In most DON studies in trout, the liver is the major organ of study due to its sensitivity to DON (Hooft and Bureau, 2021; Koletsi et al., 2022). Here, even after the *ad libitum* feeding period, hepatic damage was not obvious, despite a relatively high EDI for DON, estimated at 0.040 µg/g BW/day (DON-FM diet), or 0.049 µg/g BW/day (DON-SBM

diet). In our previous study, although the EDI was comparable (0.044 µg/g BW/day DON diet) to the present study, we did observe DON effects on the liver after *ad libitum* exposure to DON (Koletsi et al., 2022). In general, there is high variability in the responses to DON among studies with the same design. The variability might be related to the different life histories of fish batches, the mycotoxin profile in the naturally contaminated ingredients or even the statistical power of the study or duration of the exposure. Regarding DON impact on feed acceptance, in both studies, the present and (Koletsi et al., 2022), no effects on feed intake were present during *ad libitum* exposure. These observations are opposite to the reduced feed intake even at practical relevant DON levels (1000 to 1500 µg/kg) found in a meta-analysis on trout (Koletsi et al., 2021). The absence of a reduction in feed intake by DON may be due to the short duration of the *ad libitum* feeding period. Another reason might be that trout were already adapted to DON exposure prior to the *ad libitum* period since fish were already fed the DON diets during the six weeks of the restrictive feeding period. Studies in pigs have shown early effects of DON on feed intake from the first 7 days (Serviento et al., 2018) to 4 weeks (Wellington et al., 2020) of exposure, whilst later the animals become adapted to the contaminated diets.

In the present study, a sub-optimal diet with 25% SBM inclusion aimed to have a mild response, although it did not affect histopathological parameters linked to inflammation. There was no widening of lamina propria and infiltration of inflammatory cells as has previously been described for salmon (Krogdahl et al., 2003; van den Ingh et al., 1996). Our findings are in contrast to observations in rainbow trout where 40% SBM inclusion caused granulomatous enteritis (Mosberian-Tanha et al., 2018). Our histopathological assessment did however detect a reduced gut mucosal fold width and height, reduced SNV width and an increased goblet cell density, all of which have been previously described as being indicative of enteritis in Atlantic salmon (van den Ingh et al., 1991) . Surprisingly, most of these changes occurred in the midgut and not in the hindgut in the current study. The hindgut is normally the affected part of the intestine by SBM in salmonids (van den Ingh et al., 1991). A recent meta-analysis in salmon (Agboola et al., 2022) showed that the most affected variable in the assessments of SBM enteritis in salmon is the loss of SNV, which was the only parameter that was affected in all intestinal segments of our SBM-treated trout, including the hindgut. The lack of enteritis severity in the hindgut might be related to the 25% inclusion level of SBM, which is lower than 30% when enteritis seems to develop in trout (Refstie et al., 2000). However, the severity is mostly governed by the source of SBM instead of the inclusion level (Agboola et al., 2022; Urán et al., 2009). Indeed, the severity of SBM-induced enteritis seems to have declined over the years (after 2014) due to improved diet formulations, processing methods for SBM that minimize anti-nutritional factors, and the genetic selection of fish that are adapted to plant-based diets (Agboola et al., 2022).

We did not find that diet composition influences the impact of DON on trout, on either the growth performance or health parameters measured. Although this suggests that in rainbow trout DON-induced effects on performance and health may be such that they overrule effects induced/modulated by diet, it could be that the current experimental set-up relied on a sample size of insufficient power to detect interaction effects. Yet, there are no clear indications for a limited power of this study, for example, during the *ad libitum* feeding period, the reduction in

growth at both FM and SBM diets was numerically equal. It does not seem likely that the absence of an interaction effect would be due to low DON exposure in the current study (1200-1300 µg/kg). Although often higher DON doses are tested in trout experiments along with slightly longer periods of *ad libitum* exposure of up to eight weeks (Gonçalves et al., 2018c; Hooft et al., 2011; Hooft et al., 2019a), our data showed that DON exposure did affect growth, FCR, protein and energy gain, which suggests the applied DON levels may have been relatively mild but sufficient to induce changes. In fact, at (very) high DON levels an interaction between DON and diet composition usually is not present, as indicated by a meta-analysis for trout (Koletsi et al., 2021), also because feed intake declines exponentially with increasing DON levels. Indeed, it is most likely that interaction effects with diet quality would be most easily detected against a backdrop of mild effects induced by DON, as in the current study. It is not unlikely that the absence of a clear interaction effect with diet quality could have been due to a relatively small contrast between the experimental diets based on FM, or SBM. It could be that the level and/or type of SBM included in the diet was not challenging enough to affect performance and/or health to a great extent. Indeed, in the current study, 25% inclusion of SBM only led to relatively small changes in the gastrointestinal tract of rainbow trout. Future studies addressing the influence of dietary composition on effects of DON on performance, liver and gastrointestinal tract health of rainbow trout could consider designing experimental diets with a greater contrast in diet quality.

Last but not least, one could hypothesize that interaction effects would be absent because dietary composition (i.e. effects of SBM) affects/targets different parts of the GIT than does exposure to DON. DON is known to be quickly taken up in the pyloric caeca region of the intestine and afterwards is distributed to organs including the liver (Bernhoft et al., 2017). In contrast, SBM-induced enteritis in salmon is mainly present in the distal and not in the upper part of the gastrointestinal tract (van den Ingh et al., 1991). Maybe arguing against this hypothesis are our observations that some histopathological parameters in the GIT (mucosal fold width; enterocyte width) did show an interaction effect between DON and diet composition. Changes in these parameters induced by SBM inclusion in the diets were largest when DON was also present in the diets, suggesting that DON may have enhanced SBM-induced enteritis, although only observed in the midgut. A complete understanding of the potential role of the midgut in developing SBM-induced enteritis and interaction effects with DON in rainbow trout would require further research on the use of plant ingredients in salmonid diets. To date, dietary SBM-induced effects on rainbow trout have only been co-evaluated with other types of challenges common to aquaculture practices, i.e. hypoxia (Mosberian-Tanha et al., 2018) and salinity (Nordrum et al., 2000), but not exposure to DON or other mycotoxins, making our study unique.

In conclusion, this study did not confirm that diet composition influences the impact of DON in rainbow trout, based on metrics of growth performance and liver histology. Regarding intestinal histology, the present research found that DON can alter SBM-induced enteritis symptoms but only in the midgut. Mucosal fold width and enterocyte width were reduced by DON only in the SBM-treated trout. In future studies, it might be worthwhile to further explore the DON exposure and SBM challenge in the midgut by employing an *in vitro* epithelial barrier

model (e.g. RTgutGC cell line from rainbow trout). This approach might allow us to zoom into the cellular level and understand further unknown pathological changes in the midgut of rainbow trout. Overall, our findings show that DON effects on liver and fish performance are similar in trout regardless of the dietary composition. This information is relevant for the industry, perhaps leading to a more flexible formulation of aquafeeds allowing higher inclusion of alternative ingredients other than fishmeal without aggravating mycotoxins effects.

Supplementary Materials

Table 4.S1. Least square means and SEM of histological parameters in pyloric caeca, midgut and hindgut of rainbow trout after feeding restrictively for 6 days (week 1) and 40 days (week 6) and *ad libitum* for 15 days (week 8) the experimental diets; CON-FM diet (DON= 0 µg/kg), DON-FM (DON=1200 µg/kg), CON-SBM (DON= 46 µg/kg) and DON-SBM (DON= 1300 µg/kg).

		Experimental diets					p-value							
	Week	CON-FM	DON-FM	CON-SBM	DON-SBM	SEM[1]	DON	diet	time	DON*diet	DON*time	diet*time	DON*diet*time	Fish effect (%)[2]
Mucosal fold width (µm)														
Pyloric	1	160.4	148.1	130.0	145.3									
	6	154.7	156.2	149.6	158.2	8.82	NS	*	NS	NS	NS	NS	NS	11.6
	8	156.3	158.5	136.3	144.8									
Midgut	1	135.1	141.8	143.8	115.6									
	6	137.1	125.7	126.0	114.8	8.14	NS	*	NS	*	NS	NS	NS	15.5
	8	133.6	149.2	137.8	125.0									
Hindgut	1	148.0	159.8	150.9	152.0									
	6	140.0	147.4	129.9	140.1	10.36	NS	NS	NS	NS	NS	NS	NS	19.0
	8	155.1	154.1	134.7	141.4									
Mucosal fold height (µm)														
Pyloric	1	374.8	372.3	312.9	319.1									
	6	460.4	484.0	488.9	430.6	51.07	NS	NS	**	NS	NS	NS	NS	19.9
	8	420.6	506.8	514.9	340.2									
Midgut	1	321.7	369.7	287.2	291.4									
	6	372.0	394.6	372.0	327.0	25.84	NS	*	**	NS	NS	NS	NS	7.6
	8	402.7	376.9	333.0	407.7									
Hindgut	1	359.2	415.9	448.3	390.8									
	6	336.1	364.9	318.8	398.6	37.28	NS	NS	NS	NS	NS	NS	NS	6.3
	8	388.2	437.1	334.2	360.7									

Table 4.S1 – Continued

		Experimental diets					p-value							
	Week	CON-FM	DON-FM	CON-SBM	DON-SBM	SEM[1]	DON	diet	time	DON*diet	DON*time	diet*time	DON*diet*time	Fish effect (%)[2]
Lamina propria width (µm)														
Pyloric	1	14.7	15.4	14.3	15.6									
	6	15.9	17.6	17.0	16.5	1.56	NS	NS	NS	NS	NS	NS	NS	23.8
	8	17.6	15.8	16.3	16.3									
Midgut	1	16.5	20.4	20.0	15.9									
	6	18.0	15.8	17.0	15.6	1.48	NS	NS	NS	NS	NS	NS	NS	8.9
	8	17.6	19.4	17.8	18.6									
Hindgut	1	18.4	20.2	18.5	16.8									
	6	16.1	17.2	15.9	17.9	1.59	NS	NS	NS	NS	NS	NS	NS	11.4
	8	18.1	16.1	15.3	18.6									
Enterocyte width (µm)														
Pyloric	1	86.4	79.8	67.9	77.7									
	6	81.1	78.6	80.1	81.3	5.92	NS	*	NS	NS	NS	NS	NS	12.5
	8	93.5	93.7	79.7	84.0									
Midgut	1	66.3	75.7	73.9	63.9									
	6	72.4	66.1	63.9	56.8	5.76	NS	NS	**	*	NS	NS	NS	16.1
	8	75.3	86.5	82.2	67.8									
Hindgut	1	56.2	73.3	66.8	75.9									
	6	51.8	66.2	57.4	55.9	5.98	NS	NS	**	0.895	NS	NS	NS	14.2
	8	77.2	65.6	63.5	73.1									

Table 4.S1 – Continued

		Experimental diets					p-value							
	Week	CON-FM	DON-FM	CON-SBM	DON-SBM	SEM[1]	DON	diet	time	DON*diet	DON*time	diet*time	DON*diet*time	Fish effect (%)[2]
Supranuclear vacuole width (µm)														
Pyloric	1	58.0	51.8	46.5	50.7									
	6	56.4	58.3	51.8	60.0	3.67	NS	*	***	NS	NS	NS	NS	14.4
	8	44.8	49.0	40.1	43.5									
Midgut	1	51.0	45.8	48.9	35.4									
	6	46.1	43.7	44.3	41.2	2.78	*	*	*	NS	*	NS	NS	16.4
	8	40.9	42.1	37.4	37.9									
Hindgut	1	70.8	64.5	63.3	57.7									
	6	71.0	63.5	56.6	65.7	7.23	NS	*	NS	NS	NS	NS	NS	38.4
	8	60.1	71.9	54.5	48.8									
Goblet cell density (per µm fold height)														
Pyloric	1	0.031	0.038	0.044	0.050									
	6	0.038	0.035	0.034	0.037	0.008	NS	NS	NS	NS	NS	NS	NS	42.6
	8	0.043	0.041	0.045	0.050									
Midgut	1	0.075	0.088	0.111	0.079									
	6	0.092	0.092	0.106	0.121	0.010	NS	**	*	*	NS	NS	NS	29.4
	8	0.074	0.089	0.105	0.080									
Hindgut	1	0.052	0.047	0.056	0.050									
	6	0.056	0.042	0.043	0.033	0.009	NS	NS	*	NS	NS	NS	NS	31.7
	8	0.069	0.059	0.047	0.064									

[1]Pooled standard error of means: SEM (pyloric: n=695, midgut: n=600, hindgut: n=550)

[2]Fish effect is the proportion of all non-explained variation due to fish

Not significant: NS, p ≤0.05: *, p≤0.01: **, p ≤ 0.001: ***

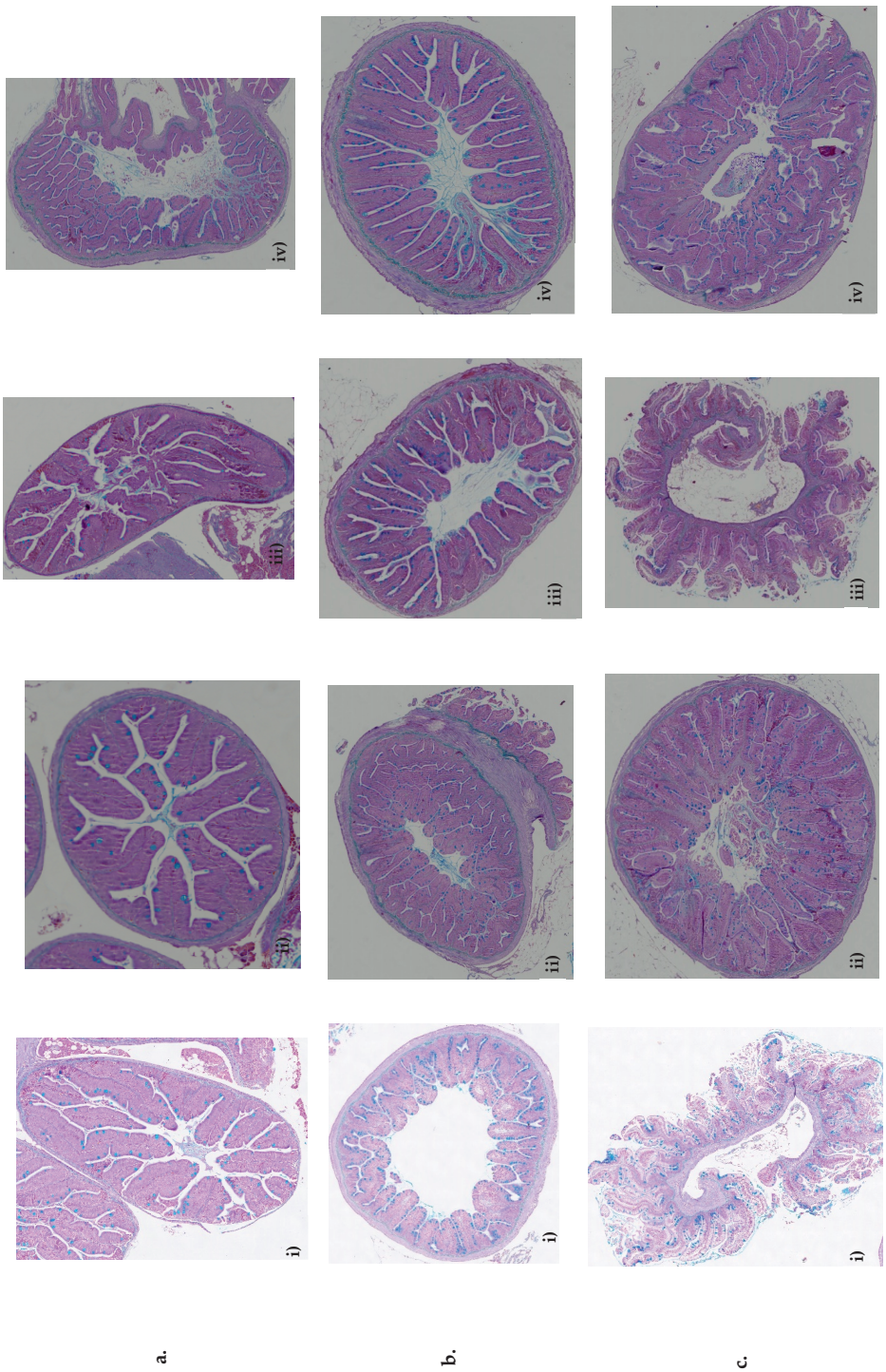

Figure 4.S1. Representative pictures from the gastrointestinal segments in **a)** pyloric caeca **b)** midgut and **c)** hindgut of rainbow trout fed: **i)** a control fishmeal-based diet without mycotoxins (CON-FM) **ii)** or with DON (DON-FM) and **iii)** a control soybean meal-based diet without mycotoxins (CON-SBM) **iv)** or with DON (DON-SBM) restricitively for 6 days (week 1). Staining: Alcian blue followed by Crossman; Magnification: x 20.

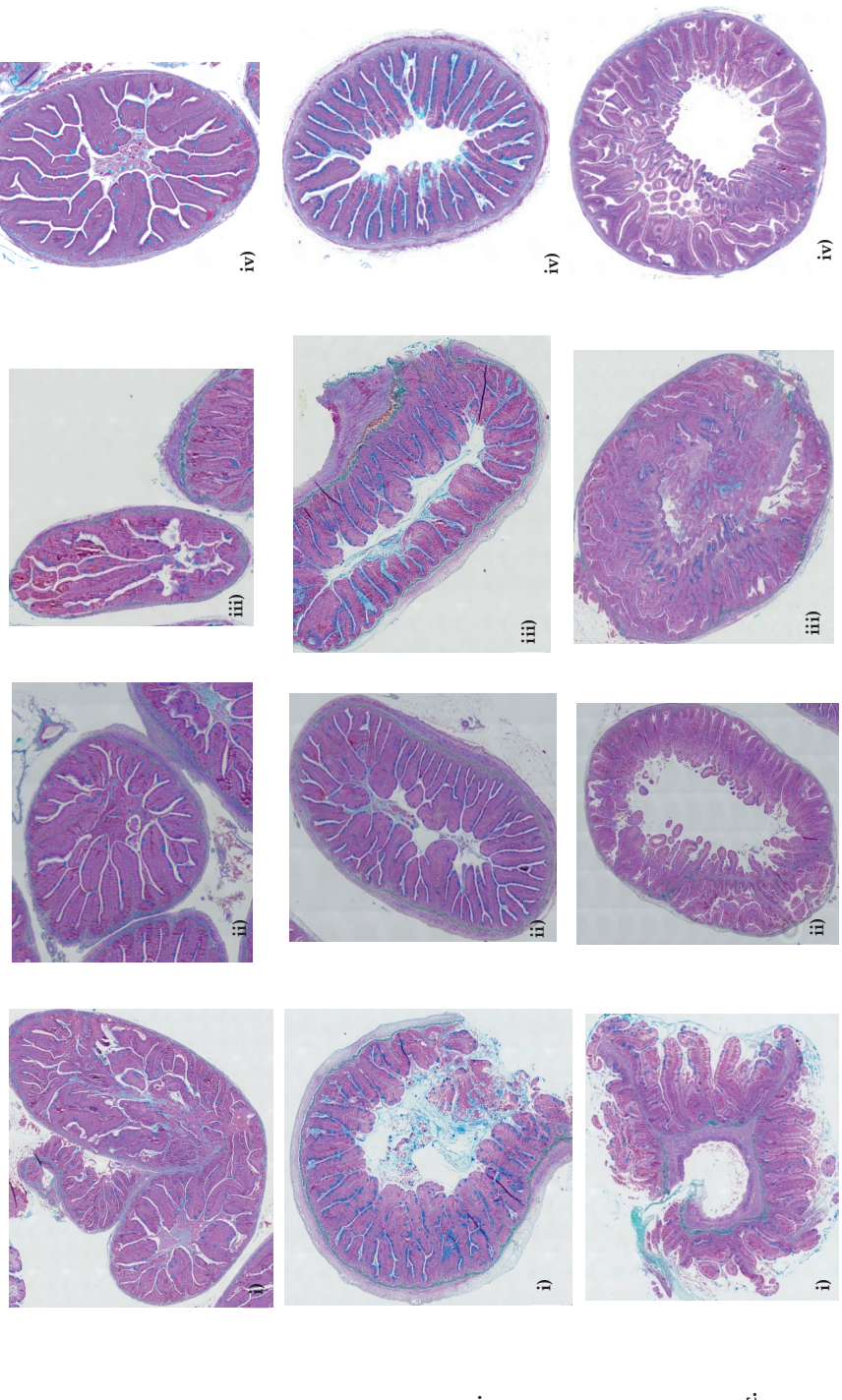

Figure 4.S2. Representative pictures from the gastrointestinal segments in **a)** pyloric caeca **b)** midgut and **c)** hindgut of rainbow trout fed: **i)** a control fishmeal-based diet without mycotoxins (CON-FM) **ii)** or with DON (DON-FM) and **iii)** a control soybean meal-based diet without mycotoxins (CON-SBM) **iv)** or with DON (DON-SBM) restricitively for 40 days (week 6). Staining: Alcian blue followed by Crossman; Magnification: x 20.

Table 4.S2. Histological assessment in trout livers after feeding restrictively for 6 days (week 1) and 40 days (week 6) and *ad libitum* for 15 days (week 8) the experimental diets; CON-FM diet (DON=0 µg/kg), DON-FM (DON=1200 µg/kg), CON-SBM (DON=46 µg/kg) and DON-SBM (DON=1300 µg/kg).

		Treatment				p-value						
	Week	CON-FM	DON-FM	CON-SBM	DON-SBM	DON	diet	time	DON*diet	toxin*time	diet*time	DON*diet*time
Vacuolization Score												
Glycogen[#]	1	1.83	1.96	1.73	1.71	NS	NS	NS	NS	NS	NS	NS
	6	1.83	1.56	1.63	1.88							
	8	2.23	1.82	1.88	1.68							
Lipid[#]	1	1.85	1.60	1.67	2.00	NS	NS	**	NS	NS	NS	**
	6	2.00	2.10	2.00	2.00							
	8	1.75	2.20	2.22	2.07							
Nuclei characteristics												
Pyknotic (%)	1	6.7	20.0	10.0	13.3	--[+]	--	--	--	--	--	--
	6	5.0	1.7	16.7	3.3							
	8	41.7	21.7	11.7	0.0							
Pleomorphic (%)	1	18.3	6.7	3.3	3.3	--[+]	--	--	--	--	--	--
	6	3.3	13.3	0.0	0.0							
	8	1.7	6.7	13.3	0.0							
Pathological indicators												
Necrosis (%)	1	26.7	21.7	15.0	21.7	NS	NS	NS	NS	NS	NS	NS
	6	20.0	33.3	28.3	25.0							
	8	38.3	18.3	21.7	15.00							
Necrosis score[#]	1	0.42	0.35	0.25	0.45	NS	NS	NS	NS	NS	NS	NS
	6	0.20	0.67	0.38	0.40							
	8	0.40	0.18	0.25	0.22							

Table 4.S2 -Continued

		Treatment				p-value						
	Week	CON-FM	DON-FM	CON-SBM	DON-SBM	DON	diet	time	DON*diet	toxin*time	diet*time	DON*diet*time
Haemorrhage (%)	1	0.0	0.0	0.0	0.0	--+	--	--	--	--	--	--
	6	0.00	10.0	6.7	10.0							
	8	8.3	23.3	10.0	8.3							
Inflammation (%)	1	1.7	3.3	3.3	1.7	--+	--	--	--	--	--	--
	6	0.0	0.0	5.0	3.3							
	8	0.0	0.0	0.0	0.0							

[#]Glycogen, lipid vacuolisation and necrosis scores were analysed with a a generalized linear mixed model (fish random effect), by using multinomial logistic regression and the other pathological indicators by using binary logistic regression.

+For nuclei characteristics, haemorrhage and inflammation it was not possible to estimate model coefficients, since one of more treatment-week combinations are 0%. It is not possible to perform exact logistic regression with a random effect in the model.

Total number of observations was n=720, apart from glycogen and lipid valuolisation n=696; Not significant: NS, p≤0.01: **

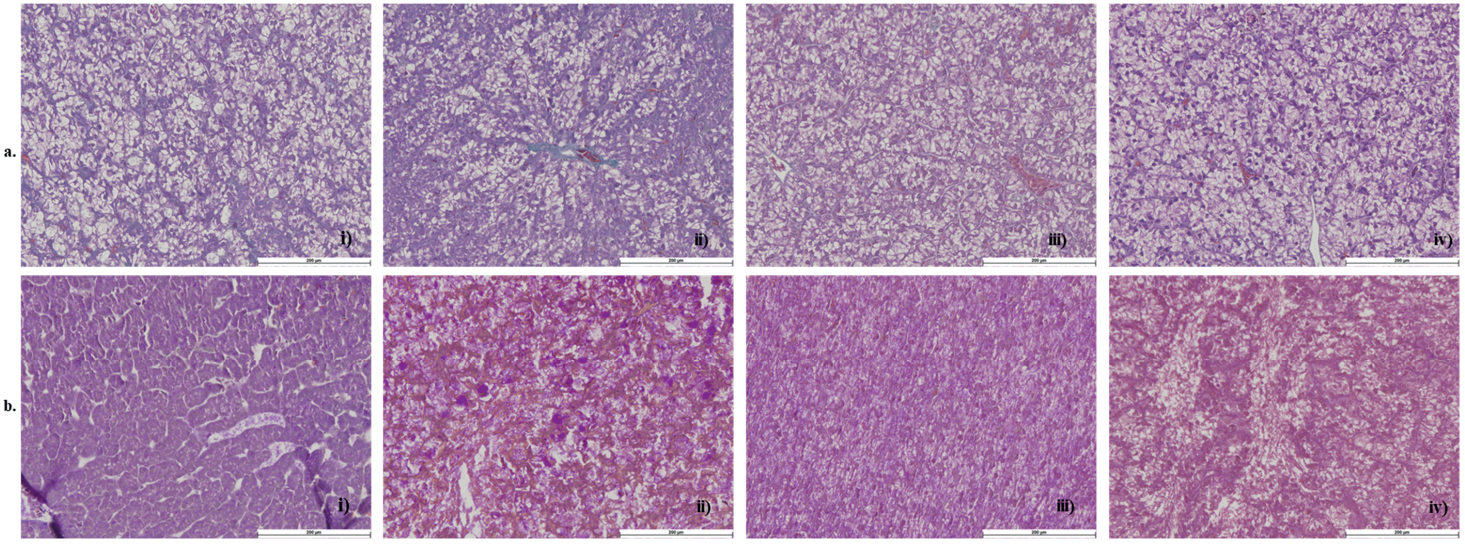

Figure 4.S3. Representative examples of histological sections from of the liver from rainbow trout fed **i)** a control fishmeal-based diet without mycotoxins (CON-FM) **ii**) or with DON (DON-FM) and **iii)** a control soybean meal-based diet without mycotoxins (CON-SBM) iv) or with DON (DON-SBM) ad libitum for 2 weeks. **a)** The first row shows representative pictures per diet without pathological indication and the second row **b)** examples of livers with necrotic areas. Staining: PAS-Crossman; Magnification: x 20; White scale bar = 200 µm.

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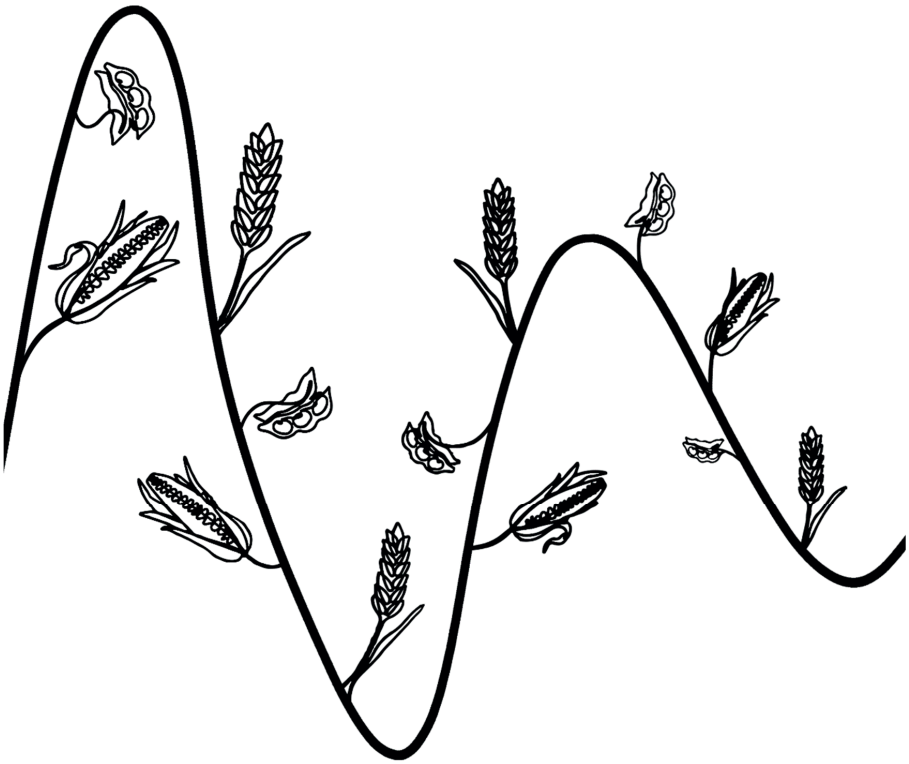
Chapter 5

Individual and combined effects of deoxynivalenol (DON) with other *Fusarium* mycotoxins on rainbow trout (*Oncorhynchus mykiss*) performance and health.



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Abstract

This study assessed whether the toxicological effects of deoxynivalenol (DON) produced by *Fusarium graminearum* in rainbow trout (*Oncorhynchus mykiss*) are altered by the co-exposure to a mixture of toxins produced by *Fusarium verticillioides* (FU_{mix}). This FU_{mix} contained fusaric acid and fumonisin B_1, B_2, and B_3. Four diets were formulated according to a 2x2 factorial design: CON-CON; CON-FU_{mix}; DON-CON; and DON-FU_{mix}. Diets with and without DON contained on average 2700 and 0 µg/kg feed, respectively. The sum of the analysed FU_{mix} toxins was 12700 and 100 µg/kg feed in the diets with and without FU_{mix}, respectively. The experiment consisted of a 6-week restrictive feeding period immediately followed by a 2-week *ad libitum* feeding period. Performance measurements were taken per feeding period. Histopathological parameters in the liver and gastrointestinal tract (pyloric caeca, midgut, and hindgut) were assessed at the end of week 1 and week 6 of the restrictive feeding period and at week 8, the last day of the *ad libitum* feeding period. During both restrictive and *ad libitum* feeding the effects of FU_{mix} and DON on performance were additive (no interaction effect; $p>0.05$). During the restrictive feeding period, exposure to DON ($p\leq0.001$) as well as FU_{mix} ($p\leq0.01$) inhibited growth and increased feed conversion ratio (FCR). During this period, DON exposure decreased the protein ($p\leq0.001$) and energy retention ($p\leq0.05$) in the trout. During the *ad libitum* feeding period, performance was not affected by FU_{mix}, while DON exposure reduced feed intake ($p\leq0.001$) and growth ($p\leq0.001$) and increased FCR ($p\leq0.01$). In general, for both liver and intestinal tissue parameters, no interaction effects between DON and FU_{mix} were observed. In the liver, histopathological analysis revealed mild alterations; increased necrosis score by DON ($p\leq0.01$), increased glycogen vacuolization by FU_{mix} ($p\leq0.05$) and decreased percentage of pleomorphic nuclei by FU_{mix} ($p\leq0.01$). DON had a minor impact on the intestinal histological parameters. Over time, some of the liver (glycogen vacuolization score, pleomorphic nuclei; $p\leq0.01$) and intestinal parameters (mucosal fold and enterocyte width; $p\leq0.01$) were aggravated in fish fed the FU_{mix} contaminated diets, with the most severe alterations being noted at week 8. Overall, the co-exposure to FU_{mix} and DON gave rise to additive effects but showed no synergistic or antagonistic effects for the combination of DON with other *Fusarium* mycotoxins.

5.1 Introduction

The diversity and inclusion level of vegetable/plant ingredients in aquafeeds has increased over the years (Turchini et al., 2019), even for carnivorous fish like salmonids (Aas et al., 2022). This is related to multiple factors, including the continuous expansion of the aquaculture sector (Naylor et al., 2021) and thereby the increasing demand for aquafeeds (Tacon, 2020), the limited availability of fishmeal and fish oil (Naylor et al., 2009) and the competition for ingredients for farmed animal feeds and biofuel production (Kraan, 2013). Next to other adverse antinutritional effects, the use of grains, seeds and their by-products increases the risk of fish and shrimp being exposed to mycotoxins (Francis et al., 2001; Glencross, 2016; Hardy, 2010).

Mycotoxin contamination of crops by fungi can occur pre-harvest in the fields and post-harvest during transportation and storage, depending on climatic conditions (temperature and humidity) (Bryden, 2012). Ongoing climate change and more extreme weather conditions affect pre-harvest fungal proliferation, which increases the risk of mycotoxin contamination (Paterson and Lima, 2010; Perrone et al., 2020; Zingales et al., 2022). Aquafeeds which contain multiple plant-based ingredients, can be contaminated with a mixture of different mycotoxins which are produced by one or several fungi (Smith et al., 2016; Streit et al., 2012). Indeed, surveys at regional and country level have reported multiple mycotoxin contamination in aquafeeds (Europe, (Koletsi et al., 2021); Asia, (Gonçalves et al., 2017); East Africa, (Marijani et al., 2017); Brazil, (Barbosa et al., 2013); Argentina, (Greco et al., 2015); Serbia, (Rokvić et al., 2020); Kenya, (Mwihia et al., 2020)). For instance, in European aquafeeds, 75% of the samples analysed were contaminated with two or more mycotoxins (Koletsi et al., 2021). In this review study, the most prevalent toxins in aquafeeds were identified as *Fusarium*-produced mycotoxins: fusaric acid (55%), deoxynivalenol (DON) (48%), fumonisin B_1 (FB_1) (36%) and fumonisin B_2 (FB_2) (27%). Fumonisins (FB_1, FB_2 and FB_3) and fusaric acid are produced (often as a mixture) by *Fusarium verticillioides* and DON by *Fusarium graminearum* (Thrane, 2014). *F. verticillioides* and *F. graminearum* grow under similar climate conditions in the field (Thrane, 2014). Consequently the occurrence of DON often goes together with the presence of a mixture of *F. verticillioides* toxins.

Compared to terrestrial animals, the toxicological effects of mycotoxins are barely studied in fish (Gonçalves et al., 2020a). The majority of the few fish studies that have been published have often focussed on one single mycotoxin (Anater et al., 2016). Due to its sensitivity, several studies on the toxicological impact of DON have been performed in rainbow trout (*Oncorhynchus mykiss*) (Hooft and Bureau, 2021; Koletsi et al., 2021). With the exception of one study on FB_1 (Carlson et al., 2001), no studies on *F. verticillioides* toxins have been completed in trout. FB_1 altered the metabolism of sphingolipids in rainbow trout (Carlson et al., 2001), but no information was presented regarding its effect on performance. In other farmed fish species, fumonisins impaired growth (seabream, (Gonçalves et al., 2020b); turbot, (Gonçalves et al., 2020c); African catfish, (Gbore et al., 2010); Nile tilapia, (Tuan et al., 2003); channel catfish (Lumlertdacha et al., 1995; Yildirim et al., 2000). Despite its frequent occurrence in European aquafeeds (Koletsi et al., 2021), information on fusaric acid toxicity in farmed fish species is lacking. Finally, information on the interactions between different types of toxins (co-exposure) in fish is minimal. In zebrafish, it was observed that co-exposure to

different combinations of toxins also bring about different toxicological effects. The toxicological effects of FB_1 and aflatoxin B_1 (AFB_1) were additive (no interaction) (Di Paola et al., 2022). Similarly, zearalenone (ZEN) and FB_1 effects were additive (Yang et al., 2021), whereas the effects of AFB_1 and DON were synergistic, and the effects of DON and ZEN, and AFB_1, DON and ZEN were antagonistic (Zhou et al., 2017). To our knowledge, only two *in vivo* feeding experiments were reported on farmed fish species, where synergistic toxicological effects of FB_1 and moniliformin were found in catfish (Yildirim et al., 2000) and AFB_1 and ZEN in rainbow trout (Ghafarifarsani et al., 2021).

Therefore, this experiment aimed to determine whether the toxicological effects of deoxynivalenol (DON) produced by *F. graminearum* are altered by the co-exposure to a mixture of toxins produced by *F. verticillioides* (fusaric acid and FB_1, FB_2 and FB_3) in rainbow trout. This was assessed by measuring growth performance and histopathological parameters in the liver and gastrointestinal tract under restrictive and *ad libitum* exposure.

5.2 Materials and Methods

The current study (project number: AVD2330020198084) was approved by the Central Committee on Animal Experiments (CCD) of The Netherlands. All experimental procedures were carried out following the Dutch law on the use of animals for scientific purposes. The feeding trial was performed at the experimental facilities of the Alltech Coppens Aqua Centre (Leende, The Netherlands).

5.2.1 Experimental design and diets

In the experiment four diets were studied according to a 2x2 factorial design. The first factor was a dietary contrast in *Fusarium graminearum* produced DON. The intended DON exposure levels were 0 and 2000 µg/kg feed on fresh basis (CON- versus DON- diets). The second factor was a dietary contrast in the exposure to a mixture of toxins produced by *Fusarium verticillioides* (FU_{mix}, fusaric acid and FB_1, FB_2 and FB_3). The intended contrast in exposure to this FU_{mix} was aimed to have a FB_1 content of 0 versus 8000 µg/kg feed on fresh basis (CON- versus FU_{mix}-diets). These contrasts in mycotoxins between the four experimental diets were created by exchanging toxin free ingredients with artificially contaminated ingredients (rice and cracked corn for the DON and FU_{mix} exposure respectively). Consequently, the four experimental diets (CON-CON, CON-FU_{mix}, DON-CON and DON-FU_{mix}) were nutritionally identical (isoenergetic and isonitrogenous) and only differed in the mycotoxin profile (Table 5.1). Diets were produced by Research Diet Services (Wijk bij Duurstede, The Netherlands) as 2 mm extruded pellets.

The artificially contaminated ingredients were produced by fermentation with mycotoxin-producing fungi at the Laboratory of Mycotoxins and Mycology, Department of Biological Sciences, College of Agriculture Luiz de Queiroz, University of São Paulo. Rice inoculated with a *F. graminearum* isolate was fermented to produce DON contaminated rice and cracked corn with a *F. verticillioides* isolate to produce the FU_{mix}. Briefly, Erlenmeyer flasks of 500 mL volume were used each containing 100 g of rice or corn. At least 2 hours before the sterilization, 40 mL of distilled water was added to the flask and mixed with rice or corn. The sterilization

was performed at 121 °C for 1 hour (CS -75, Prismalab, Rio de Janeiro, RJ, Brazil). Thereafter the flasks were left to cool down before inoculation. The sterilized ingredients were inoculated with 2 mL of conidia suspension with 10^6 conidium/mL of either *Fusarium graminearum* or *Fusarium verticillioides.* The incubation was carried out for 25 days at a constant temperature of 25 °C in static conditions for the DON and FU_{mix} production. After incubation, the fermented ingredients containing the respective mycotoxins were oven dried at 50 °C. After drying, ingredients were ground in a mill with a 0.85-mm sieve. For the control treatments, non-inoculated rice and/or cracked corn of the same batches were used.

The mycotoxin content of the spiked ingredients and experimental diets were analysed with liquid chromatography/tandem mass spectrometry (LC-MS/MS) at the Alltech 37+ mycotoxin laboratory (Dunboyne, Ireland; ISO/IEC 17025:2005 accredited). The analysed DON content in rice was 768 mg/kg on as is basis and the FB_1 content in corn 220 mg/kg on as is basis. Based on these analysed contents and the targeted contrasts in DON (2000 µg/kg) and FB_1 (8000 µg/kg) between diets, the inclusion levels of clean and contaminated rice and cracked corn was set at respectively 0.26% and 1.60% in the diets (Table 5.1). In the experimental diets, the targeted levels of DON and FB_1 (in the FU_{mix}) were reached, however the DON-CON diet contained some traces of fusaric acid (Table 5.1).

Table 5.1 | Ingredient composition, proximate, and mycotoxin analysis of the experimental diets: without DON or other mycotoxins (CON-CON), without DON but contaminated with a mixture of toxins produced by *Fusarium verticillioides*: fusaric acid and FB_1, FB_2 and FB_3 (CON-FU_{mix}), contaminated with DON alone produced by *Fusarium graminearum* (DON-CON), and co-contaminated with all toxins produced by *F. graminearum* and *F. verticillioides* (DON-FU_{mix}).

	Experimental diets			
Ingredients Inclusion (%)	CON -CON	CON -FU_{mix}	DON -CON	DON -FU_{mix}
Wheat	38.42	38.42	38.42	38.42
Soybean meal	25.00	25.00	25.00	25.00
LT Fishmeal	12.93	12.93	12.93	12.93
Fish oil	11.98	11.98	11.98	11.98
Blood meal	7.94	7.94	7.94	7.94
Clean Cracked Corn	1.60	-	1.60	-
Contaminated Cracked Corn	-	1.60	-	1.60
Clean Rice	0.26	0.26	-	-
Contaminated Rice	-	-	0.26	0.26
Monocalcium phosphate	0.66	0.66	0.66	0.66
DL-Methionine Liquid	0.16	0.16	0.16	0.16
Choline Chloride Liquid	0.18	0.18	0.18	0.18
Premixes[1]	0.88	0.88	0.88	0.88
Analysed nutrient composition (%)[2]				
Dry Matter	94.0	94.4	94.5	94.4
Protein	37.6	37.5	37.6	37.7
Fat	15.8	16.0	15.8	15.9
Ash	6.3	6.2	6.1	6.3
Gross Energy (MJ/kg)	22.6	22.6	22.3	22.4
Mycotoxin concentration (µg/kg)[2,3]				
Deoxynivalenol (DON)	-	-	2809	2495
Fusaric acid	-	2696	183	3281
Fumonisin B_1 (FB_1)	-	7599	-	8557
Fumonisin B_2 (FB_2)	-	1199	-	1163
Fumonisin B_3 (FB_3)	-	485	-	526

[1] Commercial premix from Alltech Coppens to meet (NRC, 2011)requirements of rainbow trout

[2]On dry matter basis. CON-CON diet contained only Enniatin A/A1 0.95 µg/kg.

[3] In the main text, the rounded levels are mentioned. E.g., DON 2800 and 2500 µg/kg. Rounded FU_{mix} totals are respectively 12000, 180 and 13500 µg/kg.

5.2.2 Husbandry

Rainbow trout (*Oncorhynchus mykiss*) with an average initial body weight of approximately 7 g were maintained in a recirculating aquaculture system (RAS) for eight weeks. The housing conditions were similar to those of previous *in vivo* experiments (Koletsi et al., 2022) and Chapter 4. Fish were purchased from a commercial trout farm (Mohnen Aquaculture GmbH, Germany) 1 week prior to the start of the experiment during which they were fed a standard commercial trout diet. Ten tanks were each stocked with 30 fish. Tanks were randomly assigned to one of the experimental diets. The CON-CON and CON-FU_{mix} diets were tested in duplicate

and the DON-CON and DON-FU_{mix} diets in triplicate. Fish were housed at a temperature of 14±0.5°C. The applied photoperiod was 17 hours of light and 7 hours of darkness. Water quality was monitored and maintained within the optimal range for trout. In the outlet water of the tanks, the measured pH ranged from 7.0 to 8.5, NH_4^+ was below 1 mg/L, NO_2^- was below 0.5 mg/L, and oxygen (O_2) was above 8 mg/L. During the first 6 weeks of the experiment, trout were fed restrictively in order to measure the direct impact of toxins. In this period, the feeding level was based on the metabolic body weight of the fish (12 $g/kg^{0.8}/d$). During the last 2 weeks of the experiment, fish were fed *ad libitum for* 1 hour during each meal to determine the potential impact of the tested mycotoxins on feed intake capacity. During the whole experiment, fish were hand-fed twice per day.

5.2.3 Sampling

The sampling scheme and the processing of samples were similar to those applied in previous *in vivo* experiments (Koletsi et al., 2022) and Chapter 4. Briefly, tank biomass measurements were performed at the start of the experiment, the end of the restrictive feeding period (week 6) and the end of the *ad libitum* feeding period (week 8) to calculate growth performance parameters. At the start of the experiment 20 fish from the initial population and at the end of the restrictive exposure (week 6) five fish per tank were euthanised and stored at −20 °C. These samples were used for body composition measurements to calculate protein and energy retention. Additionally, for histopathological analysis, tissue samples from the liver (two sections per fish) and one section of each gastrointestinal tract segment (pyloric caeca, midgut, and hindgut) were collected from six fish of the initial population and from two fish per tank at week 1 and week 6 of restrictive feeding period, and also at the end of the *ad libitum* feeding period (week 8). These tissues samples were placed into embedding cassettes, fixed by immersion in 10% neutral buffered formaldehyde for three days at room temperature and afterwards transferred to 70% ethanol until further processing. Before collecting these tissue samples, body weight, liver weight and body length were recorded in these fish.

5.2.4 Chemical analysis

Fish carcass and feed samples were analysed for dry matter, crude protein and fat, ash content and gross energy by Nutricontrol (Veghel, The Netherlands) as described previously (Koletsi et al., 2022).

5.2.5 Histological analysis

Liver and intestinal tissue samples were dehydrated in a tissue processor and embedded in paraffin wax according to standard histological procedures. Tissue blocks were cut into 5 μm thick paraffin sections, mounted onto microscope slides and stored until further processing. Thereafter, liver sections were stained with two separate techniques: Haematoxylin and Eosin (H&E) to colour the cell nuclei and structure, and Periodic acid-Schiff's (PAS) reagent to distinguish glycogen from lipid vacuolisation. The gastrointestinal tract sections were stained with Alcian blue (pH 2.5) followed by Crossman. All stained slides were pictured with a Leica DM6 microscope (Leica Microsystems, Wetzlar, Germany). Liver pictures (n=10 per fish) were further evaluated using the semi-quantitative scoring system described by (Koletsi et al., 2022).The gastrointestinal tract pictures were imported in ImageJ software (version 1.53q)

(Schindelin et al., 2012). With the ROI manager function of ImageJ, on 10 well-oriented (simple) mucosal fold units per fish (n=10 per fish) the following parameters were measured as previously described (Koletsi et al., 2022): mucosal fold width, mucosal fold height, lamina propria width, enterocyte width, supranuclear vacuoles width and goblet cell density.

5.2.6 Calculations and Statistics

The following performance parameters were calculated separately for each feeding period (six weeks restrictive and two weeks ad libitum feeding) according to well established equations given in detail by (Koletsi et al., 2022): growth (g/d), specific growth rate (SGR, %/d), feed conversion ratio (FCR), hepatosomatic index (HSI, %), condition factor (K), retained protein (g/fish), protein retention efficiency (%), retained energy (MJ/fish), energy retention efficiency (%).

A two-way ANOVA was used to analyse the growth parameters for the effect of DON supplementation, FU_{mix} supplementation and their interaction effect (FU_{mix} and DON). Before ANOVA, Levene's test was used to determine whether the variance of the data was homogeneous. The Kolmogorov-Smirnov test was applied to determine whether the distribution of residuals was normal. For non-normally distributed data, a non-parametric test, Kruskal Wallis, was applied to test the FU_{mix} effect and the DON effect, although this model could not test the interaction effect. Histological data (n=600 per time point) from each segment of the gastrointestinal tract (pyloric caeca, midgut and hindgut) and ordinal parameters in the liver: glycogen and lipid (scores of 1, 2 and 3) and necrosis (scores of 0, 1, 2 and 3) were analysed with a mixed-effect model, multinomial logistic regression using the fish as the random effect. The fixed variables tested were the effects of FU_{mix}, DON, time (week 1, 6 and 8) and their interactions. Liver binomial data (nuclei pyknosis and pleomorphism, necrosis, haemorrhage, inflammation) were expressed as percentages (%) and analysed with a mixed binary logistic regression model including FU_{mix}, DON, time and their interactions as fixed effects and the fish as random effect. Statistical significance was tested at a probability level below 0.05 ($p \leq 0.05$), while p-values between 0.1 and 0.05 ($0.1 > p \geq 0.05$) were defined as close to statistical significance and reported as tendencies. All data were statistically analysed in the IBM Statistical Package for the Social Sciences (SPSS) program (v 23.0; New York, NY, USA).

5.3 Results

5.3.1 Growth performance

During the eight-week experiment, no mortality, abnormal behaviour, or issues with feed acceptance were noted.

Restrictive feeding period

During the restrictive feeding period, growth and FCR were affected by both FU_{mix} exposure ($p \leq 0.01$) and DON exposure ($p \leq 0.001$) (Table 5.2). Trout fed the FU_{mix}-diets had 7% lower growth than those fed the diets without the FU_{mix}. Growth of trout fed the DON-diets was 11%

lower than that of trout fed the diets without DON. The decline in growth due to the presence of DON (0.05 g/d) was identical at diet level with and without the FU_{mix} (Table 5.2), indicating that the effects of DON and FU_{mix} were additive (no interaction). FCR was increased by 7% when diets were contaminated with the FU_{mix} and by 12% when contaminated with DON compared to their controls. During the restrictive feeding period, the HSI was only affected by FU_{mix} ($p \leq 0.01$); being 39% lower in trout fed the diets with the FU_{mix} compared to trout fed the diets without the FU_{mix}. The condition factor was only influenced by DON ($p \leq 0.05$), and was 7% lower in fish fed diets with DON compared to those fed the diets without DON. Finally, DON was also the only factor that affected protein retention ($p \leq 0.001$), protein retention efficiency ($p \leq 0.001$), energy retention ($p \leq 0.05$) and energy retention efficiency ($p \leq 0.05$) (Table 5.2). Trout fed the DON-contaminated diets retained 18% less protein and 6% less energy compared to trout fed diets without DON. FU_{mix} had no impact on metrics of retained protein and energy (Table 5.2).

***Ad libitum* feeding period**

During the *ad libitum* feeding period, the feed intake, growth and FCR of rainbow trout was only influenced by the DON treatment ($p \leq 0.01$; Table 5.3), not by FU_{mix} treatment as well as the interaction. Trout fed the DON contaminated diets had a 22% lower feed intake, 35% lower growth and 21% higher FCR compared to those fed the DON-free diets (Table 5.3). At the end of the *ad libitum* feeding period, both DON and FU_{mix} treatments did not affect the condition factor. Liver weight (HSI) was reduced by 23% in fish fed diets containing the FU_{mix} compared to those fed diets without the FU_{mix} ($p \leq 0.01$; Table 5.3).

Table 5.2 | The effects of FU_{mix} exposure, DON exposure and their interaction on the performance of rainbow trout during a 6-week restrictive feeding period.

Performance parameters[2]	Experimental diets[1]				SEM	p-value		
	CON -CON	CON -FU_{mix}	DON -CON	DON- FU_{mix}		FU_{mix}	DON	FU_{mix} * DON
Initial BW (g)	7.1	7.5	7.3	7.3	0.16	NS	NS	NS
Final BW (g)	26.4	25.5	24.6	23.4	0.22	**	***	NS
Growth (g/d)	0.48	0.45	0.43	0.40	0.01	**	***	NS
FCR	0.79	0.85	0.89	0.95	0.01	**	***	NS
HSI (%)[3]	2.6	1.6	2.3	1.4	0.43	**	NS	--
Condition factor (K)	1.30	1.28	1.22	1.18	0.03	NS	*	NS
Retained protein (g/fish)	3.1	2.9	2.5	2.4	0.07	NS	***	NS
Protein retention efficiency (%)	52.9	50.6	43.6	41.3	1.33	NS	***	NS
Retained energy (MJ/fish)	0.16	0.16	0.15	0.15	0.004	NS	*	NS
Energy retention efficiency (%)	47.2	45.3	43.7	42.7	1.18	NS	*	NS

FU_{mix}, a mixture of toxins produced by *Fusarium verticillioides*: fusaric acid and FB_1, FB_2 and FB_3.

DON, a toxin produced by *Fusarium graminearum*.

[1]CON-CON: diet without DON and without FU_{mix} contamination, CON-$FU_{mix:}$ diet without DON and with FU_{mix} contamination, DON-CON: diet with DON and without FU_{mix} contamination, DON-$FU_{mix:}$ diet with DON and with $FU_{mix:}$ contamination.

The measured levels of DON and FU_{mix} (fusaric acid and FB_1, FB_2 and FB_3) in the diets are given in Table 5.1.

[2]BW: body weight, FCR: feed conversion ratio on dry matter basis, HSI: hepatosomatic index, SEM: standard error of means, NS: not significant, ***:$p \leq 0.001$, **:$p \leq 0.01$, *:$p \leq 0.05$.

[3]Analysed with a non-parametric test (Kruskal-Wallis) for FU_{mix} and DON effect, where "--" FU_{mix} * DON was not applicable. Values presented are means based on n=2 for the diets CON-CON and CON-FU_{mix} and n=3 for the diets DON-CON and DON-$FU_{mix.}$

Table 5.3| The effects of FU_{mix} exposure, DON exposure and their interaction on the performance of rainbow trout during a 2-week *ad libitum* feeding period.

Performance parameters[2]	Experimental diets[1]				SEM	p-value		
	CON -CON	CON -FU_{mix}	DON -CON	DON- FU_{mix}		FU_{mix}	DON	FU_{mix}*DON
Final BW (g)	52.1	51.2	41.2	39.9	1.12	NS	***	NS
Growth (g/d)	1.71	1.70	1.11	1.10	0.07	NS	***	NS
Feed intake (g/fish/d)	1.57	1.60	1.22	1.25	0.04	NS	***	NS
FCR	0.86	0.89	1.04	1.08	0.04	NS	**	NS
HSI (%)	2.1	1.4	2.2	1.9	0.18	**	NS	NS
Condition factor (K)	1.3	1.3	1.3	1.2	0.04	NS	NS	NS

FU_{mix}, a mixture of toxins produced by *Fusarium verticillioides*: fusaric acid and FB_1, FB_2 and FB_3.

DON, a toxin produced by *Fusarium graminearum*.

[1]CON-CON: diet without DON and without FU_{mix} contamination, CON-$FU_{mix:}$ diet without DON and with FU_{mix} contamination, DON-CON: diet with DON and without FU_{mix} contamination, DON-$FU_{mix:}$ diet with DON and with $FU_{mix:}$ contamination.

The measured levels of DON and FU_{mix} (fusaric acid and FB_1, FB_2 and FB_3) in the diets are given in Table 5.1.

[2]BW: body weight, FCR: feed conversion ratio on dry matter basis, HSI: hepatosomatic index, SEM: standard error of means, NS: not significant, ***: $p \leq 0.001$, **: $p \leq 0.01$, *: $p \leq 0.05$.Values presented are means based on n=2 for the diets CON-CON and CON- FU_{mix} and n=3 for the diets DON-CON and DON-$FU_{mix.}$

5.3.2 Histopathological assessment of liver and gastrointestinal tract

Liver

The qualitative assessment of the liver histology did not show severe liver damage. Some examples of these minor changes are given in Figure 5.1; where panel i) shows an unaffected liver; panel ii) a liver with necrotic areas; panel iii) a liver with scattered blood cells; and panel iv) a liver with both necrotic areas and scattered blood cells.

The semi-quantitative assessment (Table 5.4) showed for pyknotic nuclei all scores were 0 in week 8 only for the DON-CON diet. For inflammation all scores during the restrictive feeding period (week 1 and 6) were 0 for all diets. After *ad libitum* feeding (week 8), however, 23% and 27% inflammation spots were found for the FU_{mix} contaminated diets, compared to 7% in the DON-CON diets and 0% in the CON-CON diet. Due to the presence of 0 scores in one or multiple combinations of diets and weeks, the effects of DON and FU_{mix} could not be estimated for pyknotic nuclei and inflammation (Table 5.4).

During the restrictive feeding period (week 1 and 6), the glycogen vacuolization score was similar for all diets. At the end of the *ad libitum* feeding period (week 8), however, the glycogen vacuolization score increased only in the trout fed the diets containing the FU_{mix} (interaction $p \leq 0.001$). The effect of DON on glycogen vacuolization was not present (Table 5.4). Lipid vacuolization did not change over time and was unaffected by both dietary treatments. Regarding the percentage of pleomorphic nuclei, the 3-way interaction effect was present ($p \leq 0.01$), but there were no clear patterns of the effects of DON and FU_{mix} over time (Table 5.4). In livers of DON-fed trout, the risk on the higher order necrosis scores was increased compared to livers of trout not exposed to DON. Necrosis was also present in trout fed the CON-CON and CON-FU_{mix} diet, although with a low average score (ranging from 0.1-0.3) and a lower percentage of liver parts affect. Time also affected liver necrosis score ($p \leq 0.01$), being the highest at week 6 (Table 5.4). The percentage of haemorrhage was not significantly affected by the dietary treatments as well as time ($p > 0.05$; Table 5.4).

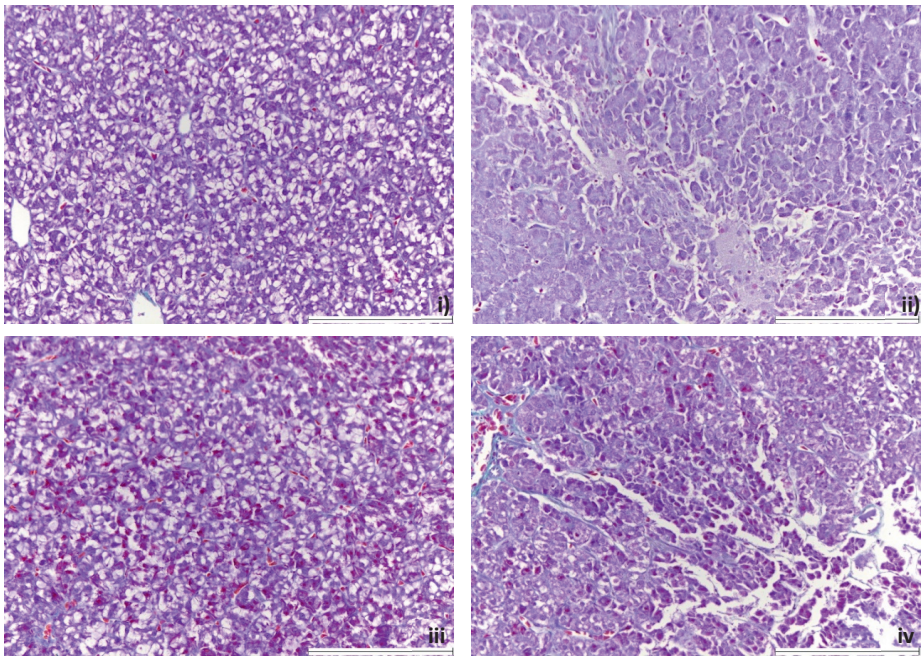

Figure 5.1|Examples of histological sections of the liver at the end of the experiment (week 8) from rainbow trout fed: **i)** CON-CON diet (DON=0, FU_{mix}=0), **ii)** CON-FU_{mix} diet (DON=0, FU_{mix}=12000), **iii)** DON-CON diet (DON=2800, FU_{mix}=180) and **iv)** DON-FU_{mix} diet (DON=2500, FU_{mix}=13500). Staining: PAS-Crossman; Magnification: x 20; White *scale bar*=200 µm.

Table 5.4 | Histological assessment in trout livers with the effects of DON, FU_{mix}, time and their interactions after feeding the experimental diets restrictively for 6 days (week 1) and 40 days (week 6) and *ad libitum* for 15 days (week 8).

		Experimental diets				p-value						
	Week	CON-CON	CON-FU_{mix}	DON-CON	DON-FU_{mix}	FU_{mix}	DON	time	FU_{mix} *DON	FU_{mix} *time	DON*time	FU_{mix} *DON*time
Vacuolization Score												
Glycogen[#]	1	2.0	1.8	1.7	1.6	*	NS	***	NS	***	NS	NS
	6	1.8	1.5	1.7	1.9							
	8	1.9	2.7	2.0	2.5							
Lipid[#]	1	2.0	2.0	1.9	2.0	NS	NS	NS	NS	NS	NS	NS
	6	2.2	2.0	2.1	1.8							
	8	1.7	1.3	1.9	1.8							
Nuclei characteristics												
Pyknotic (%)	1	15	23	5	10	--[+]	--	--	--	--	--	--
	6	18	20	20	3							
	8	25	25	0	20							
Pleomorphic (%)	1	3	43	13	25	**	NS	***	NS	***	NS	**
	6	48	28	47	3							
	8	72	3	62	63							
Pathological indicators												
Necrosis (%)	1	13	8	23	15	NS	**	#	NS	NS	NS	NS
	6	18	20	30	45							
	8	25	15	20	27							
Necrosis score[#]	1	0.2	0.1	0.3	0.2	NS	**	*	NS	NS	NS	NS
	6	0.3	0.3	0.5	0.9							
	8	0.3	0.2	0.3	0.5							
Haemorrhage (%)	1	30	15	52	45	NS	NS	NS	NS	NS	NS	NS
	6	18	35	32	23							
	8	15	13	13	23							

Table 5.4 - Continued

		Experimental diets				p-value						
	Week	CON-CON	CON-FU_{mix}	DON-CON	DON-FU_{mix}	FU_{mix}	DON	time	FU_{mix} *DON	FU_{mix} *time	DON*time	FU_{mix} *DON*time
Inflammation (%)	1	0	0	0	0	--[+]	--	--	--	--	--	--
	6	0	0	0	0							
	8	0	23	7	27							

[#]Glycogen, lipid vacuolisation and necrosis scores were analysed with a a generalized linear mixed model by using multinomial logistic regression and the other pathological indicators by using binary logistic regression in which frequencies were used. In the table, however, descriptive means are shown.

+ For pyknotic nuclei and inflammation it was not possible to estimate model coefficients, since one or more diet-week combinations show 0% affected spots.

Total number of observations was n=600, apart from glycogen and lipid vacuolisation n=585; NS: not significant, ***:p≤0.001, **:p≤0.01, *:p≤0.05, #:p≤0.1

Gastrointestinal tract

The statistical outcome of the semi-quantitative histological assessment in the gastrointestinal tract of rainbow trout response to FU_{mix}, DON, time and their interactions (3-way and 2-way) is presented in Table 5.5, showing mild histopathological changes indicated by a few 2-way significant interactions. Figure 5.2 displays examples of the intestinal folds from pyloric caeca, midgut and hindgut, collected at the end of the experiment (week 8). Similarly in the qualitative analyses (Figure 5.2), no notable histological alterations were observed in the gastrointestinal tract.

The semi-quantitative assessment of the intestinal histology showed that none of the parameters were affected by the 3-way interaction effect between DON, FU_{mix} and time (Table 5.5). The enterocyte width in the midgut was the only intestinal parameter that was affected by the interaction between DON and time ($p \leq 0.05$), which was related to an alteration in the effect of DON between week 1 and week 6 of the restrictive feeding period (Table 5.5). Mucosal fold width in midgut and enterocyte width in midgut and hindgut were affected by the 2-way interaction between time and FU_{mix} ($p \leq 0.01$). These parameters were higher during week 1 and week 6 in fish fed diets containing FU_{mix}, while during week 8 (the end of the *ad libitum* feeding period) these width parameters were reduced in fish fed diets containing FU_{mix} (Table 5.5). The goblet cell density of the pyloric caeca was the only parameter with an interaction effect between DON and FU_{mix} ($p \leq 0.01$). Fish fed diets containing toxins had a similar goblet cell density in the caeca, but these densities were lower compared to the fish fed the toxin free diet (CON-CON; Table 5.5). No interaction effect between DON and FU_{mix} was noted in any of the other intestinal parameters measured. The supranuclear vacuole width in the hindgut was affected by both DON ($p \leq 0.05$) and FU_{mix} ($p \leq 0.01$), without an interaction. Trout exposed to DON as well as FU_{mix} had a reduced supranuclear vacuole width (Table 5.5).

Table 5.5|Effects of DON, FU_{mix}, time and their interactions on histological parameters in pyloric caeca, midgut and hindgut of rainbow trout after feeding the experimental diets restrictively for 6 days (week 1) and 40 days (week 6) and *ad libitum* for 15 days (week 8).

		Experimental diets					p-value						
	Week	CON-CON	CON-FU_{mix}	DON-CON	DON-FU_{mix}	SEM[1]	FU_{mix}	DON	time	FU_{mix}*DON	FU_{mix}*time	DON*time	FU_{mix}*DON*time
Mucosal fold width (µm)													
Pyloric	1	129	127	138	128								
	6	126	141	124	130	8.7	NS	NS	**	NS	NS	NS	NS
	8	169	145	136	149								
Midgut	1	122	131	132	134								
	6	134	150	128	131	6.2	NS	NS	#	NS	**	#	NS
	8	145	130	150	131								
Hindgut	1	130	139	118	129								
	6	184	132	132	120	14.5	#	#	NS	NS	#	NS	NS
	8	156	122	144	133								
Mucosal fold height (µm)													
Pyloric	1	301	317	311	304								
	6	403	399	378	372	28.7	NS	NS	***	NS	NS	NS	NS
	8	490	425	414	452								
Midgut	1	306	379	350	329								
	6	343	371	377	314	28.1	NS	NS	***	#	NS	NS	NS
	8	458	425	410	374								
Hindgut	1	380	433	397	404								
	6	459	390	323	303	52.3	NS	NS	NS	NS	NS	#	NS
	8	432	394	474	486								

Table 5.5 - Continued

		Experimental diets					p-value						
	Week	CON-CON	CON-FU_{mix}	DON-CON	DON-FU_{mix}	SEM[1]	FU_{mix}	DON	time	FU_{mix}*DON	FU_{mix}*time	DON*time	FU_{mix}*DON*time
Lamina propria width (µm)													
Pyloric	1	12	12	14	12								
	6	16	17	14	14	2.0	NS	NS	NS	NS	NS	NS	NS
	8	18	12	13	15								
Midgut	1	15	14	15	15								
	6	18	19	19	18	1.6	NS	NS	**	NS	NS	NS	NS
	8	14	15	17	16								
Hindgut	1	12	13	10	11								
	6	16	16	14	14	1.6	NS	NS	*	NS	NS	NS	NS
	8	13	12	14	13								
Enterocyte width (µm)													
Pyloric	1	66	64	72	66								
	6	65	76	67	74	4.7	NS	NS	***	NS	#	NS	NS
	8	87	80	77	79								
Midgut	1	56	65	66	68								
	6	72	82	60	69	5.0	NS	NS	***	NS	***	*	NS
	8	84	69	87	71								
Hindgut	1	51	65	52	71								
	6	80	69	61	67	7.5	NS	NS	#	NS	**	NS	NS
	8	80	60	76	66								

Table 5.5 - Continued

		Experimental diets					p-value						
	Week	CON-CON	CON-FU_{mix}	DON-CON	DON-FU_{mix}	SEM[1]	FU_{mix}	DON	time	FU_{mix}*DON	FU_{mix}*time	DON*time	FU_{mix}*DON*time
Supranuclear vacuole width (µm)													
Pyloric	1	50	51	51	49								
	6	44	46	42	41	4.5	NS	NS	*	NS	NS	NS	NS
	8	58	52	44	52								
Midgut	1	49	52	44	50								
	6	44	48	42	43	3.7	NS	NS	NS	NS	NS	NS	NS
	8	47	47	46	44								
Hindgut	1	67	59	54	46								
	6	86	45	57	40	8.1	**	*	NS	NS	NS	NS	NS
	8	63	50	54	54								
Goblet cell density (per µm fold height)													
Pyloric	1	0.05	0.04	0.03	0.02								
	6	0.07	0.05	0.04	0.04	0.012	*	*	#	**	NS	NS	NS
	8	0.08	0.02	0.03	0.04								
Midgut	1	0.08	0.07	0.07	0.07								
	6	0.09	0.11	0.12	0.12	0.010	NS	#	***	NS	NS	NS	NS
	8	0.07	0.07	0.09	0.08								
Hindgut	1	0.04	0.06	0.05	0.05								
	6	0.05	0.04	0.04	0.04	0.009	NS	NS	NS	NS	NS	NS	NS
	8	0.04	0.04	0.04	0.05								

[1]Pooled standard error of means: SEM (pyloric: n=544, midgut: n=481, hindgut: n=285)

NS: not significant, ***:p≤0.001, **:p≤0.01, *:p≤0.05, #:p≤0.1

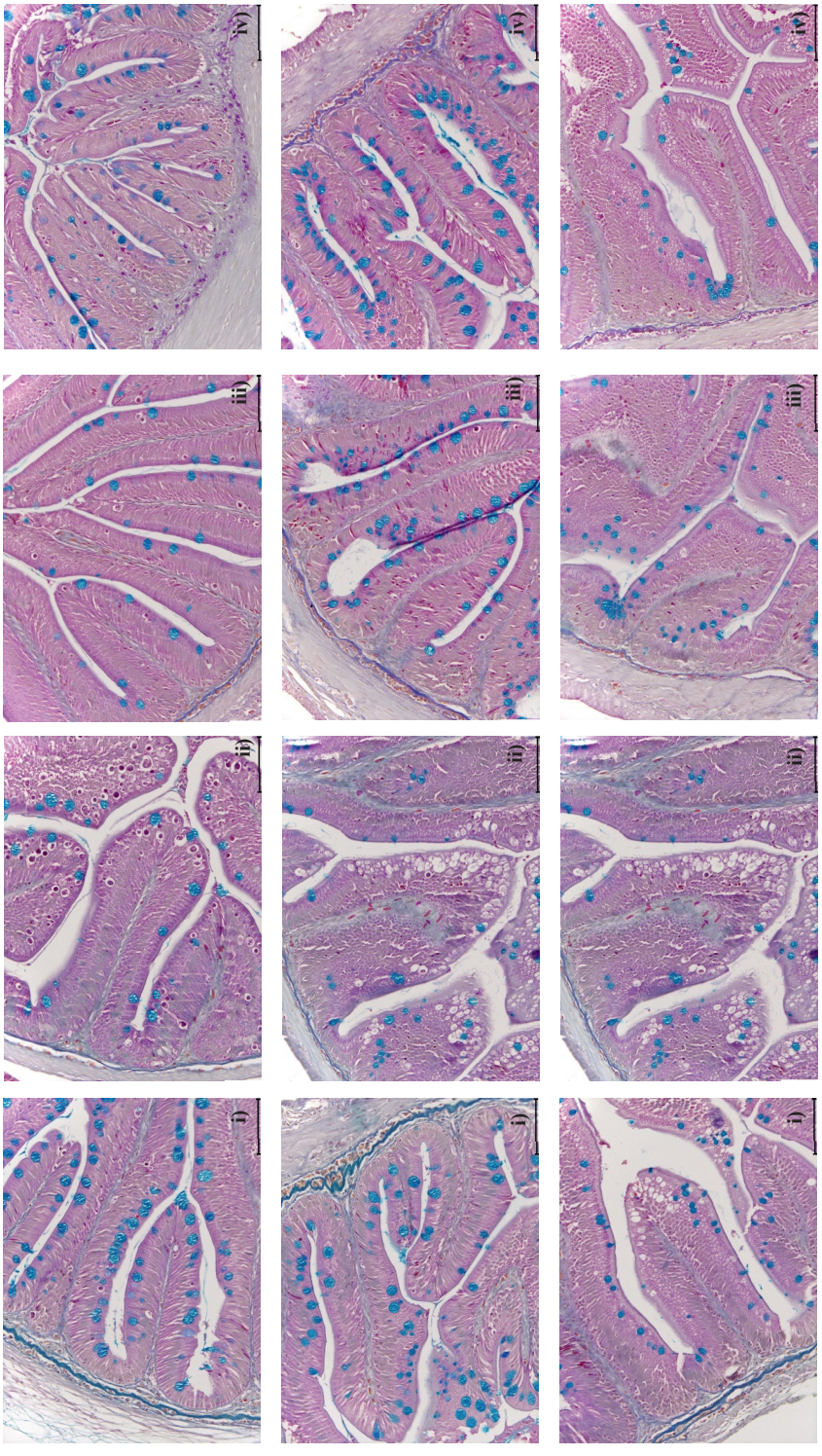

Figure 5.2 | Representative examples of histological sections of the intestinal folds at the end of the experiment (week 8) in **a)** pyloric caeca **b)** midgut and **c)** hindgut of rainbow trout fed: **i)** CON-CON diet (DON=0, FU_{mix}=0), **ii)** CON-FU_{mix} diet (DON=0, FU_{mix}=12000), **iii)** DON-CON diet (DON=2800, FU_{mix}=180) and **iv)** DON-FU_{mix} diet (DON=2500, FU_{mix}=13500).Staining: Alcian blue-Crossman; Magnification: x 20; Black *scale bar* = 100 µm.

5.4 Discussion

The current study investigated, via a 2x2 factorial design, the impact of individual and combined effects of *Fusarium graminearum*- and *Fusarium verticillioides*-produced toxins on rainbow trout performance and liver and gastrointestinal tract health. The first factor was DON contamination produced by *F. graminearum* (DON), and the second factor was the mixture of the toxins: fusaric acid and FB_1, FB_2 and FB_3 (FU_{mix}) produced by *F. verticillioides.* Therefore, the four experimental diets had a contrast without and with DON contamination (CON versus DON) and without and with FU_{mix} contamination (CON versus FU_{mix}).

During the *ad libitum* feeding period, DON exposure reduced feed intake in trout. This is in contrast to previous *in vivo* studies in trout with the same experimental design (Koletsi et al., 2022) and Chapter 4, where no effect of DON on feed intake was present, which might be explained by the higher DON exposure applied in the current study (2700 μg/kg). The current DON dose resulted in an estimated daily intake (EDI) of 0.104 μg DON/g BW/day during the *ad libitum* feeding period, whereas in our earlier studies, the EDI of DON was only 0.044 μg/g BW/day (Koletsi et al., 2022) and 0.049 μg/g BW/day (Chapter 4). Most likely, the differences in DON exposure level may explain the differences between studies regarding appetite. The current observation of a reduced feed intake is in line with studies in trout applying an *ad libitum* feeding period of 8 weeks (Gonçalves et al., 2018c; Hooft and Bureau, 2017; Hooft et al., 2011; Hooft et al., 2019a; Hooft et al., 2019b; Ryerse et al., 2016). The combination of DON level and experimental duration should therefore be considered when investigating statistical differences in feed intake.

The restrictive feeding period revealed a direct impact of DON on performance parameters (growth, FCR, protein and energy retention), not associated with differences in feed intake. The direct impacts of DON have also been described in earlier studies where restrictive feeding was applied (Koletsi et al., 2022) and Chapter 4. In Chapter 4 it was observed that at a DON level of 1300 μg/kg feed, growth, FCR, protein and energy retention were affected, whereas an earlier study at the same DON level only reported an effect on protein retention (Koletsi et al 2022). Again, the variation between studies most likely is due to the DON levels applied.

Considering the higher DON level (2700 μg/kg) applied in the current study, it was expected that alterations in the liver histological parameters would be more severe compared to earlier studies (Koletsi et al., 2022) and Chapter 4. However, in the current study minor changes in liver histology occurred when trout were exposed to DON, being comparable to the observations in Chapter 4. While earlier alterations in liver histology were detected (Koletsi et al., 2022) even at half the DON dose applied in the current study. In other studies applying DON doses above 2000 μg/kg feed, qualitative histopathological changes in the liver of trout were observed (Gonçalves et al., 2019; Hooft et al., 2011). The minor changes in the current study and variability between studies in DON impact on the liver might be linked to factors such as: differences in the power of the study (in the current study 4 or 6 fish were sampled for histology per treatment at each time point); variability inside the tank between fish in EDI of DON due to differences in feed intake; the occurrence of unknown co-exposure with other mycotoxins (over the years the detection methods of mycotoxins have evolved; new toxins are

discovered and analysed); differences in experimental conditions and genetic background and life history of the experimental fish. The minor/mild histological changes induced by DON on gut histology is in line with earlier studies (Koletsi et al., 2022) and Chapter 4. The absence/minor effect of DON on trout intestinal tissues may be related to the more rapid absorption of the toxin in the upper part of the gastrointestinal tract (Bernhoft et al., 2017). While the gastrointestinal tract was consistently unaffected by DON in our studies, alterations in liver histological parameters have been detected before (Koletsi et al., 2022).

Regarding the second factor in this study, FU_{mix}, it is not possible to estimate the contribution of each separate toxin present in the mixture produced by *F. verticillioides* (fusaric acid and FB_1, FB_2 and FB_3) to the total effect of the mixture. Information on fusaric acid and FB_3 effects on fish is absent. In the EU recommendation for toxins, FB_1 and FB_2 are summed with a current limit of 10000 µg/kg (Commission, 2006a). In the FU_{mix} contaminated diets in the current study, the mean FB_1 and FB_2 level was ~9000 µg/kg feed, which is below the current EU recommended limit. Compared to the other toxins produced by *F. verticillioides*, FB_1 is main toxin produced by this fungi, occurring more frequently and most toxic and therefore also most frequently studied (Galeana-Sánchez et al., 2017).

This study is the first to evaluate the sensitivity of rainbow trout to fumonisins. Trout exposed to the FU_{mix} (with a sum of FB_1 and FB_2 being 9000 µg/kg) showed a significant reduction in the growth of 7%, but only during restrictive feeding and not during *ad libitum* feeding. The sensitivity of fish to fumonisins seems to differ strongly between fish species. In studies with a longer *ad libitum* period than the current study, lower fumonisins levels resulted in reduced growth in seabream (FB_1 and FB_2 ≥168 µg/kg; (Gonçalves et al., 2020b)) and in turbot (FB_1 and FB_2 ≥1000 µg/kg; (Gonçalves et al., 2020c)). In other fish species, fumonisins effects on growth were only observed at higher levels (FB_1≥5000 µg/kg in African catfish; (Gbore et al., 2010)) (FB_1 ≥40000 µg/kg in Nile tilapia; (Tuan et al., 2003)) (FB_1≥20000 µg/kg in channel catfish; (Lumlertdacha et al., 1995); (Yildirim et al., 2000)). The disappearance of the FU_{mix} on growth (reduction in growth <1.0%) during the *ad libitum* period, might suggest that trout adapted to FU_{mix} exposure. In other words, the fish may have become less sensitive to the toxic effects of this mixture. However, liver and intestinal histopathological observations do not support this hypothesis of adapting to these toxins. Instead, various histopathological parameters (e.g., increased glycogen vacuolization in the liver and reduced mucosal fold and enterocyte width in the gastrointestinal tract) revealed that FU_{mix} effects aggravated with time, being more severe at the end of the *ad libitum* feeding period. The time (or feeding level) related change in FU_{mix} effects together with the large variability between fish species in sensitivity to *F. verticillioides* toxins warrants further research on this group of toxins to improve the current recommended EU limits. The approach taken in the current study to use a mixture of *F. verticillioides* produced toxins can be advised as approach also for other fish species because feed ingredients with an infestation of *F. verticillioides* are most likely to contain a mixture of fusaric acid and FB_1, FB_2 and FB_3.

The main objective of this study was to investigate the presence of interaction effects (antagonism, synergism or additivity) of FU_{mix} and DON. For performance data during both

feeding periods (restrictive and *ad libitum*), no significant interaction effects were present (Table 5.2 & 5.3), which suggests that the effects of FU_{mix} and DON are additive during co-exposure. Apart from the goblet cell density of the pyloric caeca, all studied histological parameters suggested additivity of FU_{mix} and DON effect. It can be hypothesised that the combination of *Fusarium* spp. toxins, as applied in the current study (FU_{mix} versus DON), do not influence each other's toxicological effects. It has been suggested that combining mycotoxins with structural similarities, comparable modes of action and thus toxicity profiles, increases the likelihood that their effects are additive (Speijers and Speijers, 2004). The absence of a significant interaction effect might also be related statistical power of this study (a too low number of tanks/animals being included into the study). A major toxicological impact FB_1, the most abundant toxin produced by *F. verticillioides*, is an interference with the sphingolipids' metabolism via inhibition of ceramide synthase enzymes (Feijó Corrêa et al., 2018), which results in an alteration of the sphinganine/sphingosine ratio in livers. Therefore, this ratio is used as a biomarker of FB_1 exposure (Riley et al., 1994). It can also be the case that the proper parameters for quantifying FU_{mix} effects were not assessed in the present study in order to reveal interaction effects (e.g., the sphinganine/sphingosine ratio in the liver).

Only few studies in fish addressed co-exposure, thus the comparison between effects of co-exposure to FU_{mix} and DON is only possible with terrestrial animal literature. Feeds and also ingredients are often co-contaminated with multiple toxins (Streit et al., 2012). Next to the limited information on the effects of co-occurrence, also in terrestrial animals, there is a large variability in responses between studies, species as well as the measured parameters (Smith et al., 2016). In pigs, an early study (Smith et al., 1997) found synergism between DON and fusaric acid on growth performance. In contrast, a later study in pigs (Grenier et al., 2011) did not show a interaction effect between DON and fumonisins on growth, but a synergistic action was observed regarding the severity of histopathological lesions in the liver. In ducks, synergism between fumonisins, DON, and ZEN resulted a lower growth, but this was not observed in any of the other factors assessed (Peillod et al., 2021). In another pig study (Bracarense et al., 2012), synergism, antogonism as well as aditivity was observed for the co-exposure to DON and fumonisins depending on the measured parameters. Due to the large variability between as well as within studies, further *in vitro* and *in vivo* research is required to understand and explain the combined mycotoxin effects and to predict their interactions. Such information is needed for regulatory authorities of the animal feed industry in formulating recommended limits for mycotoxin mixtures.

This first rainbow trout study evaluating the combined effects of the most prevalent mycotoxins in aquafeeds produced by *F. graminearum*: DON and *F. verticillioides*: FU_{mix} (fusaric acid and FB_1, FB_2 and FB_3), showed that the co-exposure of FU_{mix} and DON primarily had additive effects on performance (no interaction effects). The exposure to FU_{mix} as well as to DON impaired growth and FCR during the restrictive feeding period. During *ad libitum* feeding, growth and feed intake were reduced by DON exposure, but not by FU_{mix}. There were no toxins interaction effects on histopathological parameters in the liver and gastrointestinal tract. DON exposure in the current study resulted in minor histological changes, and FU_{mix} did lead to minor alteration in liver and intestinal tissue but mainly at the end of the *ad libitum* feeding period.

In conclusion, despite the absence/minor impact on the liver, the current study clearly shows a substantial effect on performance already at a DON exposure of level of 2700 µg/kg feed; e.g., 11 to 35% reduced growth (Table 5.2 & 5.3). This implies that the current EU recommended limit for DON at 5000 µg/kg may need to be reconsidered for fish. Since no other studies in trout have evaluated the effects of the sum of FB_1 and FB_2, a conclusion cannot be drawn about the effectiveness of the EU recommended limit at 10000 µg/kg, although it is suggested future studies to measure the effects of FUmix instead of the sum of FB_1 and FB_2.

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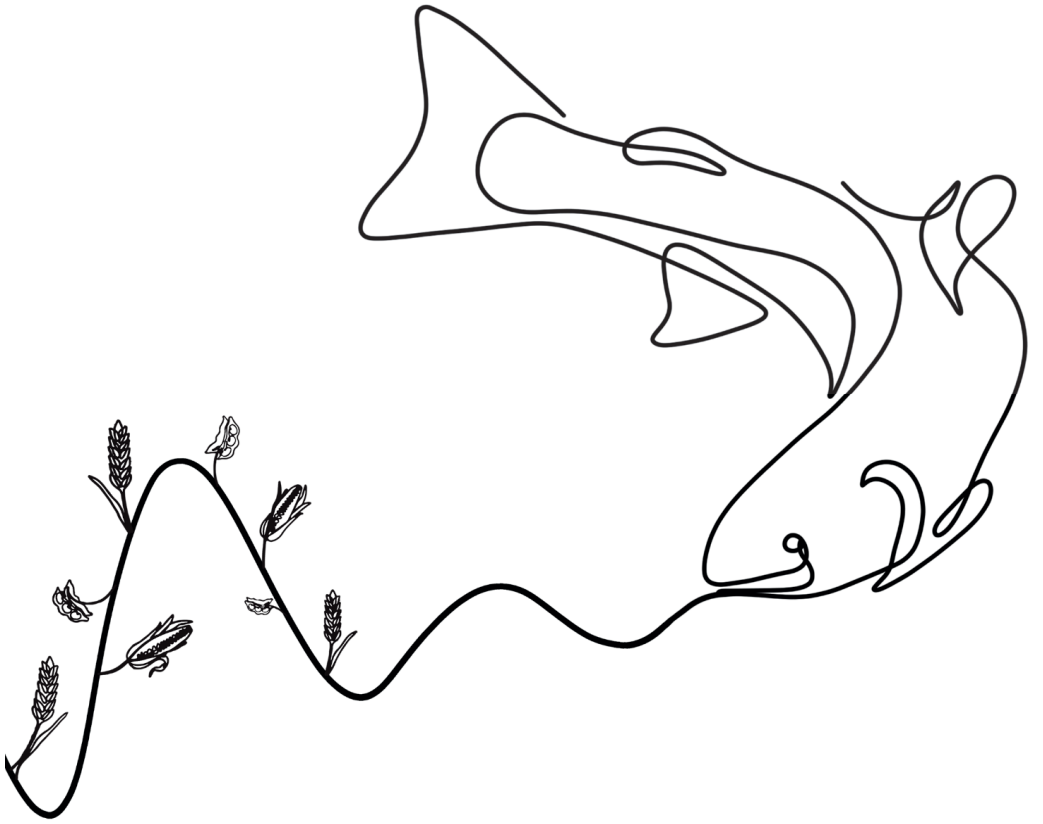
Chapter 6

General Discussion



6. General Discussion

This thesis aimed to better understand the impact of mycotoxins on the performance and health of rainbow trout in aquaculture. Mycotoxins are fungal secondary metabolites with an undefined biological role; they do not contribute to fungal growth and development, but perhaps have a protective role in securing fungal ecological niches (Fox and Howlett, 2008). DON, for example, is a mycotoxin frequently present in aquafeeds in Europe, produced by *Fusarium* fungi and may enhance fungal pathogenicity in crops (Venkatesh and Keller, 2019). Mycotoxin contamination of crops is a worldwide concern because, apart from economic losses for crop farmers and also the animal feed industry, they can have a negative impact on the health and productivity of farmed animals when fed diets contaminated with mycotoxins (Magnoli et al., 2019). The high utilization of ingredients derived from crops in modern fish feed diets has increased the risk of mycotoxin contamination of these diets and thus created a need to explore potential effects of mycotoxins on the performance and health of farmed fish species (Anater et al., 2016). Therefore, in the current thesis rainbow trout (*Oncorhynchus mykiss*) was used as a target species presumed sensitive to the effects of mycotoxins and specifically, to assess the performance and health effects of DON as a mycotoxin frequently occurring in aquafeeds. This General Discussion focuses on key findings from the research chapters 2–5, which described the effects of DON alone, or in combination with factors potentially moderating the effects of DON (e.g., time, diet composition, and co-exposure to other toxins) in order to formulate recommendations for further mycotoxin research in aquaculture and advise feed producers and authorities on how to manage mycotoxin contamination in aquaculture practice.

6.1 Critical reflections on conducting *in vivo* experiments with mycotoxins in fish

Sourcing the mycotoxins used in the experiments

A choice to be made when designing mycotoxin experiments is the source of mycotoxins, which can be from naturally-contaminated ingredients (natural), artificially-contaminated ingredients or purified mycotoxins (pure). The latter is commercially produced after extraction and purification, processes that allow the separation and isolation of specific mycotoxins. Such pure sources are best used in experiments designed to study the effects of a single mycotoxin to provide answers to fundamental questions e.g., elucidating mechanisms of toxicity. Among the advantages of using pure mycotoxins in experiments is precise control over the dose, especially relevant when studying dose-response relationships. In contrast, naturally-contaminated ingredients usually contain more than one mycotoxin, as shown by the survey in **Chapter 2**. As such, naturally-contaminated sources can more closely mimic the practical reality of co-contamination in aquafeeds with multiple mycotoxins. For this reason, when experiments target circumstances more relevant to practical situations (e.g., to test the efficacy of feed additives with a promise to bind several mycotoxins), natural sources should be preferred. Furthermore, as shown by the meta-analysis in **Chapter 2**, rainbow trout exposed to a natural source of mycotoxin (i.e., DON) showed a more severe effect on feed intake and growth than rainbow trout exposed to a pure source of DON. In Figure 6.1, a generalized concept of the dose response of one type of mycotoxin administered in pure or natural form is shown for a performance parameter. In the case of synergism with other naturally occurring toxins, the two lines will differ. With a stronger synergism effect the difference between the lines, indicated by the arrow, will be larger. The extent of the synergism will depend on factors like fish species, age, mycotoxin combinations etc, but also on the dose of tested toxin. The co-exposure of mycotoxins used in **Chapter 5** (DON and a mixture of toxins produced by *Fusarium verticillioides*) did not lead to synergistic responses.

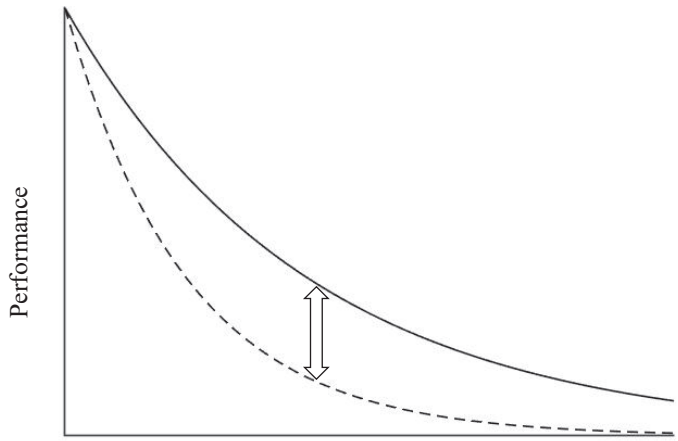


Figure 6.1 | Hypothesized difference (arrow) in response between naturally-contaminated (dashed line) and pure (solid line) mycotoxin sources based on the meta-analysis of effects caused by DON in rainbow trout (Chapter 2).

Although experiments with naturally-contaminated ingredients may provide results more relevant to the industry, there are difficulties associated with their use that might affect the outcome of the experimental studies. Firstly, sourcing a batch of ingredients naturally-contaminated with the desired mycotoxin in sufficient amount and contamination level is challenging and time-consuming. Secondly, because the distribution of mycotoxins in a grain lot is not uniform, sample collection can lead to an underestimated source of error. In general, sampling error (sum of errors in sample collection, sample preparation and analytical determination) is a long-debated issue in mycotoxin research (Miraglia et al., 2005). To minimize the sampling collection error, here the batch of contaminated wheat used to compose the experimental diets used in **Chapters 3-4** was repeatedly grounded and mixed before collecting three random samples for analysis. To minimise the analytical determination error, the analyses of the samples were performed by an accredited laboratory using liquid chromatography and tandem mass spectrometry (LC-MS/MS) for the detection of multiple mycotoxins (Iqbal, 2021). Indeed, here the LC-MS/MS results of the three random samples of contaminated wheat showed homogeneity in contamination levels of DON: 4082, 4215 and 4283 µg/kg. Also for our studies, finding a batch of ingredients contaminated with mycotoxin in sufficient amounts and large enough for several experiments proved challenging: the total amount of the sourced naturally-contaminated wheat was enough only to formulate experimental diets for the experiments in **Chapters 3-4**. As a result, in **Chapter 5**, I had to use artificially-contaminated ingredients (after fermentation with mycotoxin-producing fungi) as an alternative contamination source. Furthermore, because the DON contamination level in the sourced naturally-contaminated wheat was relatively low (on average 4193 µg/kg), in order to aim at a desired final contamination level of 1700 µg/kg in the complete feeds, diets had to be formulated to include a high (40%) inclusion of wheat. Still, the maximum DON concentration in final feeds was lower than targeted (i.e. 1300 µg/kg, see **Chapters 3-4**), which might have been caused by the extrusion process required for the final feed preparation. In summary, the final choice between sourcing mycotoxins from pure, artificially-contaminated ingredients or naturally-contaminated ingredients strongly depends on the research objectives.

What feeding strategy to choose?

In animal experiments, the chosen feeding strategy can be restrictive or *ad libitum*, depending on the specific research questions. *In vivo* studies on the effects of mycotoxins typically involve feeding animals with contaminated diets, making the choice of feeding strategy an important consideration in the experimental design. Most mycotoxin studies carried out in fish have chosen to provide fish with *ad libitum* access to feed, maximising the estimated daily intake (EDI) of the toxin, thereby assessing if the toxin would impact the feed intake capacity. Such studies indeed make sense knowing that mycotoxins such as DON are suspected to reduce appetite. As shown by the meta-analysis (**Chapter 2**), *ad libitum* access to DON appears to reduce feed intake exponentially, leading to correlated growth suppression. Feeding *ad libitum* (to satiation) automatically implies the introduction of variability in feed intake both at individual fish level as well as at tank level. Thus striving for maximum exposure to DON by satiation feeding will lead to variation in the EDI of the toxin, which is shown in Figure 6.2. The increased variation in EDI will also result in a larger variation in the response parameters,

reducing the power of the study. Restrictive feeding strategies will minimise the variation in feed intake and in EDI of the toxin, and thus reduce the variation in response parameters, indicated by the dots in Figure 6.2. Moreover, restrictive feeding allows for studying the direct effects of a mycotoxin on health and growth parameters, independent of its impact on total feed consumption.

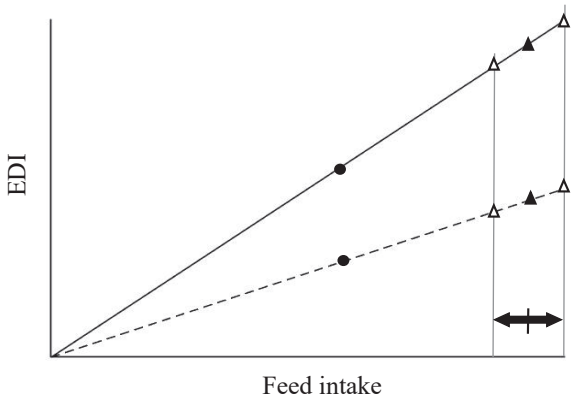


Figure 6.2 | Schematic representation of how variation in feed intake under *ad libitum* (indicated by the arrow) will lead to variability in the estimated daily intake (EDI) of toxins, at two diets with a high (solid line) and a low toxin level (dashed line). The dots represent the feed intake at a restrictive feeding level, the solid triangles the mean feed intake during *ad libitum* feeding with the open triangles representing the minimum or maximum feed intake during *ad libitum* feeding.

Here, we chose for a combined approach of a six-week restrictive feeding period with a subsequent two-week *ad libitum* feeding period for the *in vivo* experiments (**Chapters 3-5**). The choice for a restrictive feeding strategy made our studies different from previous studies in trout because, rather than focusing on the total effect of DON, which combines the effect of DON on feed intake and direct physiological effects, this choice allowed us to study separately from effects on feed intake, the direct impact of DON on health and performance. The addition of a subsequent *ad libitum* feeding period allowed us to also investigate the potential effects of DON on feed intake, and allowed for a comparison of our findings with previous studies in rainbow trout. The *ad libitum* feeding periods, albeit relatively short, confirmed an effect of DON on feed intake, not significant at doses of DON ≤1300 µg/kg (**Chapters 3-4**), but significant at doses of DON >2000 µg/kg (**Chapter 5**). It is likely that *ad libitum* exposures of two weeks are too short to demonstrate clear effects on feed acceptance at lower doses. In retrospect, two separate eight-week experiments each with different feeding strategies could have been designed to answer questions related to both, effects of DON on growth (restrictive feeding) and effects of DON on feed intake (*ad libitum* feeding). Of course, extended periods of animals experimentation come at a financial cost, and ethical cost related to desired reductions in the number of experimental animals (3Rs approach) (Russell and Burch, 1959). To conclude, the 'best' feeding strategy always depends on the research question, targeting

either direct mechanistic effects (restricted feeding) or indirect effects on the animals via changes in feed acceptance (*ad libitum* feeding).

How to deal with variation between studies?

One important challenge when it comes to interpreting and comparing data from different studies is linked to variation in toxin intake by the fish, even when similar exposure doses were applied in well-designed experimental setups with restrictive feeding. Usually, exposure doses are expressed as a concentration of toxin in (µg) or (mg) per kg feed, without further considering absolute feed intake and size of the animals. To allow for a better comparison of the outcomes of the experiments described in **Chapters 3-5** and also other studies published in literature, I calculated the estimated daily intake (EDI) in µg/g BW/day (DON concentration in the feed*mean daily feed intake/average body weight). Figure 6.3 presents a plot of mycotoxin concentrations in the diets as used in **Chapters 3-5** against the calculated EDI. Since in all experiments there was a restrictive and *ad libitum* feeding period, two relationships are plotted. The large difference between both lines implies that EDI can differ largely depending on the feeding strategy, even at a similar dietary toxin level.

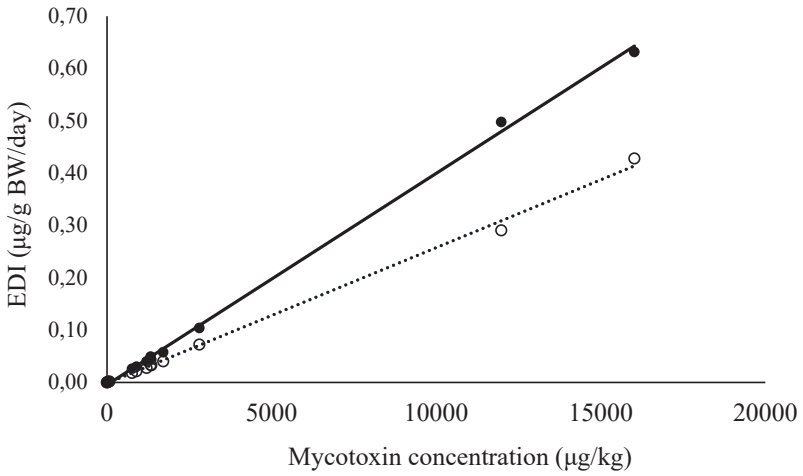


Figure 6.3 | The relationship between dietary mycotoxin concentrations and the calculated EDI combining all experimental diets (both DON and the sum of FU_{mix}) from **Chapters 3-5**. All diets were fed first restrictively for 6 weeks (open circles and dashed line) followed by a 2-week *ad libitum* feeding period (closed circles and solid line).

When is the best moment to sample your fish?

Another element of importance in mycotoxin studies is the sampling procedure, mainly the timing of the sampling points. In fish experiments, growth measurements are often performed at the beginning and at the end of an experiment, but (sometimes) earlier sampling points also provide informative data. For instance, a study on mycotoxicosis in pigs showed an immediate reduction in feed intake and growth, while they recovered after the first week of exposure (Serviento et al., 2018), showing that the timing of the sampling can be an important factor when determining growth parameters. Therefore, future investigations on mycotoxin effects on fish growth should also consider sampling points earlier than at the end of the experiment, e.g. after one week of exposure, particularly when examining effects of toxins known to decrease appetite.

Similarly, for measuring health parameters (e.g., histological assessments: **Chapters 3-5** or changes in gene expression induced by the uptake of DON: **Chapter 3),** the choice of sampling moments matters. For instance, the histological assessments of livers in **Chapter 3** did not show significant effects of DON after six weeks of restrictive exposure, but did show effects after one week. This finding implies an adaptation to DON over time, at least regarding the liver effects, meaning that late samplings can overlook early effects. As in many studies, in mycotoxin studies addressing fish health, what also matters is the time between the last exposure (feeding) and the time of tissue sampling for e.g., gene expression (Moreno et al., 2021; Zhou et al., 2003). For example, in mice, gene expression of pro-inflammatory cytokines in the spleen was significantly elevated at 3 and 6 hours post-exposure to DON but returned to control levels after 9 hours (Zhou et al., 2003). Likewise, in fish, DON exposure has been shown to lead to an up-regulation during the first weeks, to return to control levels by the end of the experiment (Matejova et al., 2015; Pietsch et al., 2015). In retrospect, in **Chapter 3**, the choice of sampling moments for studying immune gene expression, two days after the last feeding and eight weeks after the first feeding, may not have been optimal to reveal significant effects on the gene expression of pro-inflammatory cytokines, or other molecules. This timing was determined mainly to measure effects quantified by histological assessments, and timing optimal for one parameter cannot always be aligned with timing optimal for another parameter of interest. Overall, the best sampling moment depends on the type of analysis, mycotoxin, research questions and experimental design.

How to measure and quantify histopathological changes?

When it comes to histopathological assessments in mycotoxin studies in fish, there are two major evaluation methods that are different but also related: qualitative and semi-quantitative. In general, for studies on liver health, even with the liver being a frequently-examined tissue of interest for DON studies in fish, there is much variation in the exact protocols used to evaluate histopathological changes. There are studies performed qualitative assessments of liver health with conclusions based on sometimes single pathological incidences, including evidence of haemorrhage or necrosis, with no further statistical analysis possible (Gonçalves et al., 2019; Hooft et al., 2011). Other studies have used semi-quantitative scoring systems by translating qualitative assessments of liver health into numerical (i.e. quantitative) data, to allow for

statistical comparisons among groups (Hooft et al., 2019a; Pietsch and Burkhardt-Holm, 2015; Pietsch et al., 2014a). Even when performed on the same tissue sample of a single fish, technical replicates of stained sections can provide quantitative data using simple scoring ranges such as 0 = no alterations, 1 = mild, 2 = moderate, and 3 = severe alterations. This approach has been used to score and statistically test the severity of lesions, fat aggregation, hyperaemia, vacuolization, and dilation of sinusoids in HE/PAS-stained liver sections (Pietsch and Burkhardt-Holm, 2015; Pietsch et al., 2014a), and also to score other liver health parameters such as the number of mitotic cells, and vacuolisation of cells as; 1= absent to slight, 2= mild and moderate to severe = 3 vacuolization (Hooft et al., 2019a).

In this thesis, an extended version of such semi-quantitative protocols was applied **(Chapter 3)** to assess liver health based on histopathological assessments; protocols which are consistently used throughout all studies (**Chapters 3-5**). The extended protocol included six biological (fish) replicates per treatment and ten technical replicates (picture areas) per fish, with the use of two categories of parameters; binomial, and ordinal parameters that received a score. The binomial parameters were scored as yes/no depending on the incidence (or not) of necrosis, haemorrhage, inflammation and nuclei with pleomorphism, or with pyknosis expressed as frequency (%). The ordinal parameters for glycogen and lipid vacuolisation were scored as 1=low, 2=moderate and 3=high, while necrosis was scored as 0=no necrosis, 1=mild, 2=moderate, and 3=severe. Furthermore, the extended protocol included two different stainings: HE staining to evaluate cell architecture (e.g. nuclei characteristics), and PAS staining to distinguish lipid from glycogen vacuolization. In these protocols, PAS staining is highly recommended to reveal the glycogen vacuolization (visible as pink), while the lipid vacuolisation is visible as white droplets. The subsequent statistical analysis of the histological data is where our study differed most from previous studies. In our statistical models, fish was used as random effect since the 10 technical replicates within a fish are not independent observations. With these models, the correlation between the measurements within a fish, and the proportion of variance that is attributable to the fish, could be estimated. It was shown (in most of the parameters) that fish accounted for a considerable proportion (e.g., in the liver parameters in Chapter 3 varying between 4 and 51%, average of 28.6%) of the total non-explained variation. As the magnitude of this intraclass correlation coefficient increases, the power of the study decreases (Donner et al., 1981). The high correlation between measurements within a fish implies that fewer technical replicates could have been used; this will decrease the power only up to a certain limit (Koepsell et al., 1991). To have a substantial increase in the power, however, an increased number of biological replicates (number of sampled fish) is needed (Donner et al., 1981).

Similarly, semi-quantitative determinations of DON effects on gastrointestinal tract health aimed to assess all segments of the GIT; pyloric caeca, midgut and hindgut (**Chapters 3-5**). Parameters measured here were mucosal fold height, mucosal fold width, lamina propria width, supranuclear vacuoles width, the width of enterocytes, and counted numbers of goblet cells, visible as dark blue by Alcian blue staining. In general, well-designed semi-quantitative scoring systems can improve the quality, reproducibility, and accuracy of histopathological investigations compared to mere qualitative (Meyerholz and Beck, 2018). Arguments against

semi-quantitative scoring are the time-consuming process for the persons that do the scoring and risk of lack of objectivity when scoring large numbers of histological slides. A straightforward solution may be to seek (more) assistance from automated tissue processors, automated tissue embedders, motorized microtomes combined with automated section mounting, automated slide staining systems and, if one pleases even automated cover slippers and labelling. All automated steps will reduce the time spent on processing and staining and also may provide more acurate results. In addition, automation has also been implemented in slide scanning and subsequent histological analysis, with automated methods for stain normalization, tissue deformation correction, and image stitching. Ultimately, increasing automation of histological observations will lead to faster and more accurate test results. Here, both for liver and analysis of GIT slides, simple software (Image J) analysis already helped to quantify the parameters measured. More recently, artificial intelligence-aided digital image analysis platforms have become available that may provide an even more accurate road to semi-quantitative scoring (Bencze et al., 2021). Surely, further innovation will come in the form of artificial intelligence for data analysis, especially when massive amounts of data need to be compiled and sorted. Overall, researchers are encouraged to - as much as possible - further automate processes and use artificial intelligence-based software for large-scale histopathological analyses, with the aim to make most-accurate diagnoses.

What next to study?

Ideally, it would help the interpretation of effects on fish such as those induced by DON to have knowledge of the bioaccessibility, which is measured as the percentage of mycotoxin released from feed during digestion, because it represents best the available toxin that can be absorbed through the intestinal epithelium (Dima et al., 2020). Bioaccessibility, expressed as a percentage and determined as the concentration of mycotoxin in the chyme after digestion/concentration of mycotoxin in the feed matrix before digestion (x100), can be best estimated with *in vitro* models (González-Arias et al., 2013). Such estimation would indicate the maximal oral bioavailability; the maximal amount of mycotoxin that can reach the blood after intestinal absorption. Of course, *in vitro* models cannot take into account several factors that may influence the *in vivo* bioaccessibility of a mycotoxin (e.g., feed matrix, contamination level, type of mycotoxin: natural or pure) (González-Arias et al., 2013). Only few studies addressed bioaccessibility. An *in vitro* digestion model estimated the bioaccessibility of aquafeed naturally contaminated with aflatoxin B1 at 85% (Nogueira et al., 2020), while a customised *in vitro* model for Nile tilapia (*Oreochromis niloticus*) revealed more insights about aflatoxin B1 bioaccessibility in contaminated peanut meal (Nogueira et al., 2022): depending on total digestion time (3 to 6 h), the relative amount of substrate (250 to 750 mg), and the amount of rice husk used as an adsorbent, aflatoxin B1 bioaccessibility varied from 36 to 100%. The latter study showed that bioaccessibility was increased with substrate amount and digestion time, while bioaccessibility decreased with the rice husk addition. Such information should stimulate further *in vitro* research on factors affecting other mycotoxins' bioaccessibility from aquafeed, such as DON, which has remains unexplored so far. If DON bioaccessibility is known for a feed, a more precise EDI can be calculated in the *in vivo* studies.

As a next step after studying bioaccessibility, studies into bioavailability; the amount of toxin that is absorbed, reach the systemic circulation and affect the animal, are relevant. For the mycotoxin of interest here; DON, there is no available data on its bioavailability in fish species, because DON toxicokinetics (absorption, distribution, metabolism, and excretion) is less studied compared to terrestrial animals (Sun et al., 2022). What is known, is based on a study on Atlantic salmon (Bernhoft et al., 2017) and rainbow trout (Gonçalves et al., 2018b): aquatic animals seem to quickly absorb DON being detected in the liver already one hour after the last feeding. Furthermore, salmon showed a significantly slower plasma clearance of DON than terrestrial animals, $t_{1/2}$ > 15 h (Bernhoft et al., 2017), which might explain the high sensitivity of salmonids to DON. In the distributed organs, DON also seems to be eliminated slowly in salmon: kidney $t_{1/2}$ >13 h, liver $t_{1/2}$ > 6 h, muscle $t_{1/2}$ > 16 h, brain $t_{1/2}$ > 13 h. DON-3GlcA was the main metabolite found in the liver of salmon, indicating that fish species can metabolise DON through glucuronidation (phase II biotransformation pathway). Also, a study in catfish showed the possibility of the gut microbial community transforming DON to DOM-1 (Guan et al., 2009). More research is needed to fully understand the toxicokinetics of DON in fish, which may facilitate the development of strategies to mitigate effects of DON exposure.

Overall, the variation in findings from mycotoxin research in fish is driven by the number of different mycotoxins studied, mycotoxins co-contamination, their concentrations and contamination route (natural versus pure), duration of exposure, fish history, size, species, and measured variables. Researchers are encouraged to perform meta-analytical studies by using existing information from *in vivo* studies (if available) to detect the common findings. For now, EDI calculations may provide the first approach to handle the large variation between studies.

6.2 How to deal with mycotoxin contamination in practice

Prevention of mycotoxin contamination before harvest

One could argue the best solution to avoid mycotoxicosis in farmed fish would be to exclude plant-based materials from aquafeed formulations. However, with the anticipated growth of aquaculture, current pressure on the use of marine products as protein sources for aquafeed, and ongoing but unfinished development of alternative protein sources, it will likely take a long time to phase out all plant-based materials from fish feed. In other words, the risk of mycotoxin contamination of aquafeeds will be present for several years to come. The second-best solution could be to prevent mycobacterial growth on crops on the fields in the period before harvest. Fungal growth on crops and mycotoxin production is highly affected by environmental conditions, mainly (higher) temperature and (more) water activity (Mannaa and Kim, 2017; Milani, 2013).

On the field, environmental temperature is difficult to control, especially under a climate change scenario(Medina, 2023). Also moisture is not easily managed. This environmental condition is especially relevant for mycotoxins produced by hygrophilous plant-pathogenic *Fusarium sp*. which produce mycotoxins such as deoxynivalenol, T-2 toxin, fumonisins, zearalenone. *Especially F. graminearum, the* dominant causal pathogen of *Fusarium* head blight (FHB) disease in cereals (predominantly wheat), is associated with mycotoxin production (Figlan and Mwadzingeni, 2022), while *Fusarium*-produced mycotoxins are among the most frequent detected in aquafeed samples in Europe.

It may be worthwhile to develop mathematical models that predict fungal growth and mycotoxin production based on meteorological data (empirical models) and long-term climate change scenarios (mechanistic models)(Chhaya et al., 2022), possibly combined with machine learning approaches (Camardo Leggieri et al., 2021), to forecast mycotoxin outbreaks and hotspots, enabling relevant stakeholders to take appropriate actions. Some studies have sought to create empirical models for DON prediction (Franz et al., 2009; Hooker et al., 2002; Mansfield et al., 2005; Van der Fels-Klerx et al., 2012b). Mechanistic models for predictions of aflatoxin B1 have received the most attention (Battilani et al., 2013; Battilani et al., 2016; Van der Fels-Klerx et al., 2019).

The main line of defence against mycotoxin contamination on crops is methods based on good agricultural practices (GAP) (Luo et al., 2018). Among these practices are growing resistant crop varieties, crop rotation, soil cultivation with ploughing, chemical (e.g., fungicides) and biological (e.g., microbial antagonists) control of plant diseases, and insect and weed control (Edwards, 2004). The European Commission advised GAP application to prevent and reduce *Fusarium* toxins in cereals (Commission, 2006b), although farmers should receive further assistance from consultants to ensure proper implementation. GAP application may reduce DON concentration in the harvested crop but cannot always guarantee elimination due to climatic and regional influences (Karlovsky et al., 2016; van der Fels-Klerx et al., 2014), and large data collection is necessary to record responses to GAP, which will allow quantifying their effectiveness (van der Fels-Klerx et al., 2014). Figure 6.4 provides a schematic overview

of prevention and mitigation strategies along the aquafeed supply chain that can help manage mycotoxin contamination in aquaculture.

Prevention of mycotoxin contamination after harvest

After harvest, even when the original plant-based materials are free of fungi, storage fungi especially belonging to *Aspergillus* and *Penicillium* genera (e.g., *A. flavus, A. parasiticus, and P. nordicum*) may proliferate and produce aflatoxins and ochratoxin A (Zadravec et al., 2022). To prevent their growth, good manufacturing practices (GMP) during the handling, storage, processing, and distribution of cereals are recommended (Commission, 2006b) and include that stored materials should not be exposed to environmental factors such as high moisture, heat and/or physical damage, as these conditions may promote fungal growth and mycotoxins production (Neme and Ibrahim, 2017; Park et al., 1999). Aeration can be used to maintain optimal storage temperatures, and desiccants such as calcium chloride ($CaCl_2$) can be applied to maintain low moisture levels (Matumba et al., 2021; Neme and Ibrahim, 2017).

Mitigation of mycotoxins before feed production

If prevention of mycotoxins cannot be realized, there are a number of physical, chemical, thermal and other processing methods which may reduce mycotoxins even after harvest (Luo et al., 2018). i) Sorting methods range from very simple cleaning of grains from dust, husks and broken/damaged kernels, because that is where mycotoxin contamination usually occurs (Karlovsky et al., 2016), to more complex mechanical (e.g., sieving and density separation) and optical sorting at industrial scale to remove e.g., *Fusarium* mycotoxin-contaminated parts from maize (Pascale et al., 2022). ii) A simple chemical decontamination method for mycotoxins on grains could be washing by immersion in water or another washing solution to remove mycotoxins concentrated on the surface of the product (Karlovsky et al., 2016). iii) Milling and dehulling processes can reduce mycotoxins from the outer layer of the grains (Karlovsky et al., 2016; Peng et al., 2018). iv) Thermal processes such as heating, roasting, or drying can be post-harvest strategies to decrease mycotoxins, although their effectiveness might be uncertain for heat-stable mycotoxins, while high temperatures might affect the quality of the product (Karlovsky et al., 2016; Peng et al., 2018). v) At industrial scale, irradiation may partly reduce mycotoxins with non-ionizing (e.g., UV) and ionizing (e.g., gamma rays) radiations, although this application remains restricted due to high costs and public concerns on chemical safety of ionizing irradiation (Karlovsky et al., 2016; Peng et al., 2018). vi) Last, innovative technologies applying magnetic materials and nanoparticles (Luo et al., 2018), and cold atmospheric plasma (Wang et al., 2022) are under investigation for their potential use in decontaminating grains from mycotoxins. Since none of the above-listed methods can completely eliminate mycotoxins by its own, and some may be associated with high operational costs, low practicability, substantial grain mass and nutrient loss, chemical residuals (Peng et al., 2018) etc., it is probably best to combine several methods as part of an overarching GMP approach.

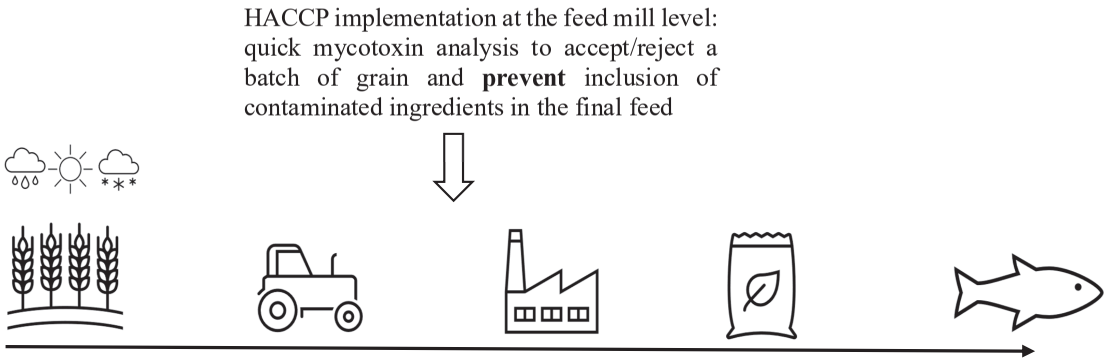


Figure 6.4 | Overview of prevention and mitigation strategies along the aquafeed supply chain to manage mycotoxin contamination in aquaculture.

What can aquafeed producers do before feed production?

First, aquafeed producers could establish and maintain a quality control plan based on a 'Hazard Analysis and Critical Control Point' (HACCP) system to identify hazards, establish controls and monitor these controls to screen plant-based materials for mycotoxin contamination (Fumagalli et al., 2021). For such as control plan, it is useful to have access to updated feed surveys for a hazard analysis and identification of risky plant-based ingredients and their mycotoxin profiles, for example identifying corn as a suspicious ingredient possibly contaminated with DON, co-occurring with other *Fusarium* toxins (see **Chapter 2**). The next step includes the feed mill as the critical control point for mycotoxin screening of plant-based ingredient batches upon arrival in the factory. For a reliable screening of incoming batches, the number of incremental samples is important and depends on the batch weight; a recommended sampling scheme for mycotoxins in grains is available from the European Commission (Commission, 2006c). Quick methods for in-factory mycotoxin testing are available using techniques based on biological binding components (Tittlemier et al., 2021). These rapid tests can detect specific key mycotoxins (not all spectrum) that allow producers to establish corrective actions; e.g., rejecting contaminated batches or applying mitigation strategies. Next to these quick methods, it is also advised that samples are analysed periodically by external laboratories using state-to-art methods, preferably liquid chromatography-tandem mass spectrometry (LC-MS/MS), which allows for the detection of the full spectrum of mycotoxins (Iqbal, 2021).

Critical limits provide a reference to compare mycotoxin concentrations with values found by routine monitoring, and should be part of the HACCP. In general, aquafeed producers may find it difficult to set such critical limits because not all target species will have the same sensitivities to the different mycotoxins. The safest approach is to set the critical limit to fit the most sensitive fish species. To be able to do so, research needs to determine the lowest observed adverse effect level (LOAEL) for different mycotoxins, and for different fish species (see **Chapter 2,** supplementary materials). Although the recommended/regulatory limits set by authorities for animal feed ingredients should correspond with critical limits determined by systematic research, this is not always the case. Here, the outcomes from the meta-analysis (**Chapter 2**), confirmed by *in vivo* experiments (**Chapters 3-5**), indicate the current EC-recommended limit for DON of 5000 µg/kg is too low to ensure optimal performance and health of rainbow trout. This conclusion is supported by the realization that DON often co-occurs with its masked form: DON-3-glucoside, formed by the plant defence system (see **Chapter 2**). This leads to a systematic under-estimation of the limit because the masked form can be cleaved during the animal's digestion, releasing the parent toxin and increasing the total DON toxicity (Berthiller et al., 2011; Nagl et al., 2012; Nagl et al., 2014), an aspect that authorities still need to take under consideration when setting recommended/regulatory limits. In conclusion, my advice for regulatory authorities is to re-consider the current upper limits for DON. My advice for feed producers is to not wait for the authorities and already apply stricter limits. For now, it may be practical to use limits per ingredient-mycotoxin combination, using the maximum inclusion of the ingredient in the recipes and the critical limit of the most sensitive fish species.

Can insects 'clean up' mycotoxin-contaminated batches?

It is nearly impossible to find plant ingredients completely free of mycotoxins; when tested, most samples appear contaminated with at least one mycotoxin (even at low levels), and there are high chances of multiple mycotoxin contamination (**Chapter 2)**. When mycotoxins are detected at low levels often, incoming batches are accepted and feed producers are advised to take further corrective measures. But when mycotoxins are detected at levels above the critical limit, batches should be rejected. Yet, also contaminated grains might keep their economic value as feed ingredients for insects, especially when realizing several insect species themselves are considered novel feed ingredients for aquaculture (Gasco et al., 2023). Some are optimistic about using mycotoxin-contaminated waste streams as feed for insects (Niermans et al., 2021). Several studies have shown that insects can tolerate mycotoxins at high concentrations without clear effects on growth or mortality. This includes observations on yellow mealworm larvae fed with DON-contaminated wheat (Ochoa Sanabria et al., 2019; Van Broekhoven et al., 2017); black soldier fly and yellow mealworm larvae fed poultry feed spiked with aflatoxin B1(Bosch et al., 2017); and black soldier fly and lesser mealworm larvae fed wheat spiked with a mycotoxin mixture of aflatoxin B1, DON, ochratoxin A and zearalenone (Camenzuli et al., 2018). At present, research should be stimulated into species-specificity of the effects of mycotoxin accumulation and biotransformation in the insect body, and into effects of type and concentration of mycotoxin, substrate, and developmental stage of the insect itself. Such research should help to unravel the potential of farmed insects for animal feed ingredients, not only as a valuable proteins source, but as "mycotoxin cleaners".

What can aquafeed producers do during feed production?

Even with HACCP in place, further – corrective - actions by feed producers remain relevant for several reasons. Rapid tests may only detect specific mycotoxins, and other mycotoxins might be present in the materials. Feed extrusion will reduce but not completely eliminate mycotoxins, depending on the mycotoxin type, temperature, moisture and pressure conditions used (Hoffmans et al., 2022). Mycotoxins are quite heat stable, and even high temperatures above 150°C may reduce zearalenone and fumonisins substantially, aflatoxins moderately, but DON only slightly (Bullerman and Bianchini, 2007). Therefore, mycotoxins may still be present after extrusion and sometimes further corrective actions may be required.

The most common corrective action feed producers take against the presence of mycotoxins is the inclusion of feed additives, "substances for reduction of the contamination of feed by mycotoxins: substances that can suppress or reduce the absorption, promote the excretion of mycotoxins or modify their mode of action" (Commission, 2009). These can be novel mycotoxin-detoxifying agents classified by EFSA as mycotoxin-adsorbing agents and mycotoxin-biotransforming agents (Boudergue et al., 2009), but can also be other feed additives such as products with protective antioxidant properties (e.g., vitamins, minerals and mixtures of natural antioxidants such as plant extracts, essential oils, herbs, spices) (Čolović et al., 2019; Da Silva et al., 2018). In the context of this thesis, most interesting are the mycotoxin-adsorbing agents.

Mycotoxin binders are adsorbing agents which are non-nutritive substances acting as “chemical sponges” designed to bind mycotoxins in the gastrointestinal tract forming mycotoxin-adsorbent complexes (Čolović et al., 2019). This mechanism prevents mycotoxins absorption into the bloodstream and distribution to target organs and promotes excretion via the faecal route (Xu et al., 2022). Mycotoxin binders are considered the most common strategy to minimize mycotoxins bioavailability and minimize potential impact of mycotoxins on the animal's health and performance (bioactivity) (Binder, 2007). Adsorbents can be inorganic or organic binders. In general, their binding efficacy depends on several factors: e.g., pH along the gastrointestinal tract, mycotoxin type and concentration, dose of the binder, and feed composition (Čolović et al., 2019). For a long time already, inorganic agents have been used to bind with aflatoxins (Boudergue et al., 2009; Huwig et al., 2001). Yet, their binding efficiency for other mycotoxins is low, they may bind micronutrients and affect the bioavailability of vitamins, amino acids, and minerals in the feed (Jouany, 2007). Organic binders include yeast cell wall fractions (β-D-glucan and mannan oligosaccharides), which can bind sufficiently to a spectrum of mycotoxins (e.g., DON, zearalenone, ochratoxin A, and aflatoxin B1), as demonstrated by *in vitro* and *in vivo* studies in poultry, pigs and ruminants (Xu et al., 2022). Especially the ability to bind a spectrum of mycotoxins make organic binders of highly interesting also for the aquafeed industry and this should be enough to stimulate research into the efficiency of (yeast cell wall-based) organic binders to mitigate mycotoxin-induced effects in aquatic animals.

No single approach will suffice to manage all potential effects of all mycotoxins in aquaculture practice. The feed chain is long and involves many stakeholders, including crop farmers, processors, feed producers, authorities, distributors, and fish farmers. The food chain therefore is complex and is further influenced by societal discussions on the use of plant-based ingredients and by-products, and choice of fish species. A further complicating factor is the lack of updated and sufficient regulation by the authorities, especially relevant for aquatic farmed animals such as rainbow trout which are highly sensitive to mycotoxins. Therefore, an integrated approach is necessary to manage mycotoxins and include application of good agriculture practise, good manufacture practise, and application of Hazard Analysis and Critical Control Points to finally prevent – as much as possible – contamination of aquafeed with mycotoxins. However, GAP, GMP and HACCP are not a panacea against mycotoxin contamination of aquafeeds. Meanwhile, it appears worthwhile to study the ‘protective’ effects of currently available and new organic binders for reducing mycotoxins in aquafeed products and fish.

7.3 Conclusions

According to the findings of the studies carried out in this thesis, the following conclusions can be drawn:

- Among other plant-based ingredients (e.g., wheat and soybean meal), corn is more frequently contaminated with DON, which often co-occurs with other toxins that cannot be detected by commonly used rapid detection methods in feed mills.

- Aquafeeds are often contaminated with more than one mycotoxin, exposing fish to a mixture of toxins. Exposure to multiple mycotoxins has been poorly investigated in fish, so their combined effects remain largely unknown.

- Dietary DON reduces feed intake and growth in several fish species.

- In rainbow trout, performance responses are more severely affected by experimental diets containing ingredients naturally-contaminated with DON than when spiked with pure DON.

- Next to alterations in feed intake, DON also has a direct impact on performance, which is shown when fish including rainbow trout are fed restrictively with DON-contaminated diets.

- DON effects - at least on rainbow trout liver histopathology - can change over time during restrictive feeding.

- DON effects on rainbow trout growth are independent of diet composition; e.g., a sub-optimal diet containing SBM does not necessarily aggravate the DON effects on growth and other performance parameters.

- SBM-induced enteritis symptoms in rainbow trout (e.g., altered mucosal fold width, enterocyte width) are influenced by DON, not in the hindgut but in the midgut, which calls for further assessment.

- DON produced by *Fusarium graminearum* co-exposed with a mixture of toxins (FU_{mix}=fusaric acid, fumonisin B_1, B_2 and B_3) produced by *Fusarium verticillioides* does not show interaction (synergism or antagonism) effects on performance and health of rainbow trout.

- DON does not severely affect the GIT of rainbow trout. It is hypothesized that this is due to the rapid absorption in the proximal intestine followed by detoxification in the liver. To support this hypothesis, more studies on the bioaccessibility and bioavailability of DON in rainbow trout are recommended.

- The estimated daily intake (EDI) of a toxin is a better measure of the exposure dose than dietary toxin concentration.

- DON affects rainbow trout performance at levels below the recommended EU limit of 5000 μg/kg feed.

Appendices

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A

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Summary

Mycotoxins are classified as feed contaminants and are produced by certain species of fungi on crops and on stored commodities used as ingredients or feed for animals. These contaminants are well-known in the livestock industry and their effects such as feed refusal, weight loss and immune suppression have been well investigated in terrestrial farm animals. Only lately, because the inclusion and variety of plant-based ingredients in fish feeds are increasing, there is a growing awareness of mycotoxin contamination in the aquaculture industry. The research into the effects of mycotoxins on fish in aquaculture may be growing but is still limited. **Therefore, this thesis aims to explore the impact of mycotoxins on the performance and health of rainbow trout (*Oncorhynchus mykiss*) in aquaculture**. First, a survey assessed mycotoxin contamination patterns in feed ingredients and aquafeeds. Based on the survey results, deoxynivalenol (DON) was selected as the toxin of interest for this thesis. Secondly, a series of three *in vivo* studies were set up to investigate the effects of DON in rainbow trout and investigate if DON effects would change with potential co-factors (e.g., time, diet composition, and co-exposure to other toxins).

Chapter 1 shortly introduces the aquaculture sector, its continuous growth and its potential role in contributing to hunger elimination. This chapter also explains how the growth of the aquaculture sector largely depends on aquafeeds, which increasingly are formulated to increasing include more plant-based ingredients. However, plant-based materials might introduce into aquafeed and into the aquaculture supply chain, contaminants such as mycotoxins. Chapter 1 summarises a number of milestones in mycotoxin research and factors involved in the increased risk of mycotoxin contamination in fish feeds.

Chapter 2 aimed to reveal mycotoxin contamination patterns in commonly used plant-based feed ingredients and aquafeeds. A survey was carried out on a large dataset of samples of wheat, corn, soybean meal and fish feed, all submitted in European countries between 2012–2019, and all analyzed by liquid chromatography/tandem mass spectrometry (LC-MS/MS). The results showed that deoxynivalenol (DON) was frequently present in corn (in 47% of the samples) > wheat (41%) > soybean meal (11%) and also in aquafeeds (48%), while multiple mycotoxins frequently were detected in the samples. Chapter 2 also summarizes the recent state of knowledge on the effects of DON on several important farmed fish species including, salmon, rainbow trout, carp, tilapia, and catfish. A risk assessment to estimate critical concentrations of DON in the fish feed that affect 5% of fish (CC5), calculated values of 74 µg/kg on average for all salmonids, and 44 µg/kg for rainbow trout. By a meta-analysis, it was estimated that feed intake and growth declined exponentially with dietary DON level. This exponential impact of DON on feed intake and growth was larger in fish exposed to naturally contaminated ingredients, rather than pure DON in experimental diets. Finally, the study in chapter 2 will help to increase awareness in the aquaculture industry of mycotoxin contaminations and provide new insights into the effects of DON on different fish species; information that is relevant for aquafeed producers and regulatory authorities.

Chapter 3 describes the first in a series of *in vivo* experiments studying the effects of DON on rainbow trout. This experiment examined the effects on rainbow trout of two types of DON contamination sources (natural and pure) at two levels (low and high). A diet almost free of mycotoxins was used as control (DON < 100 µg/kg feed), while diets with DON = 800-900 µg/kg feed were defined as low contaminated, and diets with DON = 1300-1700 µg/kg feed defined as highly contaminated. The slight differences in concentrations were caused by the source of DON (natural versus pure). After six weeks of restrictive feeding DON-contaminated diets, there were direct effects on protein gain but not on the growth of rainbow trout fed industrially-relevant concentrations of DON = 1300-1700 µg/kg feed, regardless of DON source. Effects on protein gain were not related to reduced feed intake, because of the restrictive feeding. A subsequent period of two weeks of *ad libitum* feeding of the experimental diets also did not impair feed intake but led to suppressed growth in trout fed with the highest DON contamination levels (DON = 1300-1700 µg/kg). Effects on health in the gastrointestinal tract (GIT): pyloric caeca, midgut and hindgut, as determined by histopathological assessment after 1 and 6 weeks of restrictive feeding, and at week 8 after 2 further weeks of *ad libitum* feeding, showed only mild alterations in the GIT. Cytokines gene expression at week 8 confirmed the absence of a clear inflammatory response. On the other hand, histopathological examination of the liver revealed an early response after only one week of restrictive feeding with the highest (natural/pure) DON-contaminated diets. Some of these responses (e.g., the presence of necrosis and the presence of haemorrhages) had returned to normal by week 6. After a further 2 weeks *of ad libitum* exposure, at week 8, liver health was again affected, regardless of the DON source (natural versus pure). In conclusion, DON affected protein gain and induced time-dependent effects on liver health of rainbow trout and most evident at higher concentrations (DON = 1300-1700 µg/kg feed).

Chapter 4 describes the second in a series of *in vivo* experiments studying the effects of DON on rainbow trout. This experiment addressed the question if the effects of DON on rainbow trout performance and health would be influenced by diet composition. In salmonids, soybean meal (SBM) can induce enteritis and thus, diets based on SBM can be considered sub-optimal. The potential interaction effects between DON and dietary composition were assessed by a 2x2 factorial design that differed in: 1) the type of protein source; fishmeal (FM)-based diets ("optimal quality") versus SBM-containing diets ("sub-optimal quality") and 2) the DON content of wheat; clean versus naturally contaminated with DON ~ 1300 µg/kg feed. A six weeks period of restrictive feeding showed direct effects of DON on growth and performance parameters, which were also present after a subsequent period of two weeks *of ad libitum* feeding. The dietary composition impaired growth and most performance parameters after the restrictive feeding period of six weeks, and feed conversion ratio (FCR) after the subsequent *ad libitum* feeding period of two weeks. These data confirmed the previously described effects of DON on performance and effects influenced by the nature of the SBM-based diet. Regardless of the feeding period (restrictive/*ad libitum*), there were no interaction effects between DON and diet quality on the assessed parameters. Studies into liver health by histopathological assessment indicated that neither DON, diet composition nor their interaction affected the evaluated parameters. Contrary, the histopathological assessment in the gastrointestinal tract (pyloric caeca, midgut and hindgut) revealed a few significant interaction

effects between DON and diet composition. These results indicated that DON altered a few SBM-induced enteritis symptoms including, mucosal fold width, enterocyte width and goblet cell density. However, these interaction effects were present only in the midgut and not in the hindgut, where SBM-induced enteritis symptoms commonly appear in salmonids. This opens up investigations into the role of the midgut in SBM-induced enteritis in rainbow trout and modulations thereof by DON. In conclusion, DON (~1300 µg/kg feed) impaired rainbow trout growth and performance, regardless of diet quality.

Chapter 5 describes the third in a series of *in vivo* experiments studying the effects of DON on rainbow trout. This experiment addressed the question if effects of DON produced by *Fusarium graminearum* would be affected by co-exposing rainbow trout to a mixture of toxins produced by *Fusarium verticillioides* (FU_{mix}: fusaric acid and fumonisin B1, B2, and B3). To test the individual and potential interaction effects between DON and FU_{mix}, four diets were formulated according to a 2x2 factorial design: DON = 0 versus 2700 µg/kg feed, and FU_{mix} = 100 versus 12700 µg/kg feed (values being the sum of the four *Fusarium verticillioides* toxins). During the 6-week restrictive feeding period, both DON and FU_{mix} inhibited growth and increased feed conversion ratio (FCR), while only DON led to decreased protein and energy retention. During the subsequent 2-week *ad libitum* feeding period, DON reduced feed intake and growth and increased FCR, while no effects of FU_{mix} were present. Regardless of the feeding period, the combination of DON and FU_{mix} did not show evidence of interaction effects (either synergistic or antagonistic). Histopathological assessment of the liver and gastrointestinal tract did not reveal clear effects of DON, FU_{mix} or their interaction. In conclusion, this study in rainbow trout, which was the first to examine the combined effects of common mycotoxins in aquafeeds produced by *Fusarium graminearum*: DON, and by *F. verticillioides*: FU_{mix} (fusaric acid and fumonisin B_1, B_2, and B_3), showed effects of co-exposure to DON and FU_{mix} on trout performance were primarily additive (no interaction effects).

Chapter 6 discusses the main findings of the different studies described in the different chapters of this thesis and puts in a broader context the outcomes of these studies, to provide recommendations for optimising mycotoxin research in aquaculture. Based on the experience and knowledge gained, the limitations and challenges encountered by researchers performing *in vivo* studies are discussed and followed by suggestions for improvements. Further, mycotoxin management in aquaculture practice is discussed, next to prevention as well as mitigation strategies for aquafeed producers and regulatory agencies.

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Outside AFI, but still, within Zodiac I would like to share more “thank you”. My office neighbor, my Italian bella **Laura, y**our presence in the corridor was like a ray of sunshine on cold winter mornings. Thank you for bringing so much warmth, and positivity and sharing your anti-age secrets, my skincare mentor! Still on the same floor but in the other corridor, there was my handsome Catalan guy, **Adria** who I deeply thank for listening to my drama stories and he never got tired of it. I am thankful to Adria for partying, shopping, dining and designing ambitious scientific collaborations with zebrafish & mycotoxins which never came true (ma*%&*, what a life!). During the course on Laboratory Animal Science, I had the pleasure to meet the sweetest horse vet, **Julia** who I thank for being a great teammate in that course, and most importantly that remaining a good friend until today. While attending courses for my TSP, I gladly met fellow WIAS PhDs **Isabella**, **Simen** and **Alex**, who I thank for being the best company in and later outside the courses. Last but not least, I thank **Bahadir** for sharing with me at the begging of my PhD the PowerPoint templates for the most eye-catching presentations, and later for designing the cover of my thesis.

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About the author

Paraskevi (Vivi) Koletsi was born on the 29th of September 1991 in Ioannina in Greece. She grew up in Paramythia, a small mountainous town in the Epirus region gratefully "next door" to the mythical Acheron River and the magical coasts of the Ionian Sea. After completing her secondary education, she moved to Thessaloniki and studied Biology (BSc.) at Aristotle University. There, Vivi worked at the population genetics and traceability of animal organisms laboratory, and her thesis was about the phylogeny of the Balkan brown bear (*Ursus artus*). At the same lab, she participated in projects about the traceability of farmed fish species (e.g., seabass and seabream). At that moment, she was introduced to aquaculture and was inspired to continue her studies in this field.

Vivi moved to the Netherlands for her MSc. programme "Aquaculture and Marine Resource Management" at Wageningen University & Research. During this study, she became interested in the field of fish nutrition and, therefore, decided to study for her MSc. thesis the effect of protein source (fishmeal- vs plant-based) and non-starch polysaccharide level on fat digestibility and faecal bile acid loss in rainbow trout (*Oncorhynchus mykiss*). For her MSc. internship Vivi chose to work for a Dutch aquafeed producer, Alltech Coppens. She investigated the mycotoxins in aquafeeds implementing a management plan at the production facility to prevent mycotoxin contamination in the final feed. After her MSc. graduation, Vivi accepted the challenge to continue the research in the field of mycotoxins in the form of a PhD scholarship from Alltech in collaboration with the Aquaculture and Fisheries Group at Wageningen University. During her PhD, she performed experimental work at Alltech Coppens facilities and laboratory work at Wageningen University, where she completed her Education and Training Plan at the end of 2022. Upon finalizing her PhD thesis, Vivi was employed by Alltech Coppens (the Netherlands) to work for Alltech Mycotoxin Management Team (the UK) as a specialist for aquatic animal species.

WIAS Training and Supervision Plan (TSP)

Training and Supervision (2018-2022)	Year	ECTS
The Basic Package		
WIAS Introduction Day	2018	
WIAS Course on Essential Skills	2018	
Scientific Integrity	2020	
Ethics and Animal Sciences	2020	
Subtotal		**3**
Disciplinary Competences *(minimum 2 courses)*		
WIAS research proposal	2018	
Species-specific course: fish	2018	
Advanced Statistics Course Design of Experiments	2018	
Introduction in Laboratory Animal Science	2019	
(Zebra)Fish Immunology/Vaccination Workshop	2019	
AFI Knowledge Exchange Programme (China)	2019	
Nutrient evaluation monogastrics	2019	
Fish Nutrition Workshop	2021	
Subtotal		**16**
Professional Competences *(minimum 2 courses)*		
Project and Time Management	2018	
Supervising BSc and MSc Thesis Students	2018	
Scientific Writing	2021	
Reviewing WIAS PhD Proposal	2021	
WIAS Course The Final Touch: Writing the General Introduction and Discussion	2022	
Subtotal		**5**

Training and Supervision (2018-2022)	Year	ECTS
Presentation Skills *(max 4 credits)*		
Mycotoxins in aquafeeds: Post-harvest measures for aquafeed producers to prevent contamination in the finished diets, AQUA conference, Montpellier, France (poster presentation)	2018	
Sensitivity of rainbow trout (*Oncorhynchus mykiss*) to mycotoxins, WIAS Science Day, Lunteren, the Netherlands (poster presentation)	2019	
Occurrence of mycotoxins in feedstuffs and fish feeds in Europe and the potential effects of deoxynivalenol (DON) on farmed fish species, WIAS Annual Conference (online oral presentation)	2021	
Time- and dose-dependent effects of dietary deoxynivalenol (DON) in Rainbow trout (Oncorhynchus mykiss), EAS conference, Madeira, Portugal (oral presentation)	2021	
Mycotoxins in Aquaculture: Occurrence in feed ingredients and feed - Effects on fish productivity and health. Virtual PRE-CONFERENCE on ANIMAL HEALTH: The World Mycotoxin Forum (online oral presentation)	2021	
Mycotoxins in Aquaculture: Occurrence in feed ingredients and feed - Effects on fish productivity and health. The World Mycotoxin Forum, Parma, Italy (oral presentation)	2022	
Subtotal		**4**
Teaching competences *(max 6 credits)*		
BSc thesis supervision: Isabella Roest	2020	
MSc thesis supervision: Bart van Rijn	2020	
MSc thesis supervision: Marijn de Kool	2022	
Mycotoxins in aquafeeds: effects on fish productivity and health? Fish Nutrition Workshop, Wageningen, The Netherlands (lecture)	2022	
Mycotoxins: emerging feed contaminants in Aquaculture that threat fish productivity and health. AquaHealthClub seminar, ERASMUS Mundus Msc Health Management in Aquaculture (online lecture)	2022	
Subtotal		**6**
Education and Training Total (minimum 30 credits*) *One ECTS credit equals a study load of approximately 28 hours		**34**

Colophon

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