

**ΠΑΝΕΠΙΣΤΗΜΙΟ ΘΕΣΣΑΛΙΑΣ**  
**ΣΧΟΛΗ ΓΕΩΠΟΝΙΚΩΝ ΕΠΙΣΤΗΜΩΝ**  
**ΤΜΗΜΑ ΓΕΩΠΟΝΙΑΣ ΙΧΘΥΟΛΟΓΙΑΣ ΚΑΙ ΥΔΑΤΙΝΟΥ**  
**ΠΕΡΙΒΑΛΛΟΝΤΟΣ**  
**ΠΡΟΓΡΑΜΜΑ ΜΕΤΑΠΤΥΧΙΑΚΩΝ ΣΠΟΥΔΩΝ ΜΕΣΟΓΕΙΑΚΗ**  
**ΥΔΑΤΟΚΑΛΛΙΕΡΓΕΙΑ**

**ΜΕΤΑΠΤΥΧΙΑΚΗ ΔΙΠΛΩΜΑΤΙΚΗ ΕΡΓΑΣΙΑ**

**«Ανίχνευση της μυκοτοξίνης Δεσοξυνιβαλενόλης (DON) σε  
δημητριακά που προορίζονται για κατανάλωση από εντατικά  
εκτρεφόμενους ιχθύες»**

**Μαρία Βλαχάβα**

**ΒΟΛΟΣ 2024**

**«Ανίχνευση της μυκοτοξίνης Δεσοξυνιβαλενόλης (DON) σε δημητριακά που προορίζονται για κατανάλωση από εντατικά εκτρεφόμενους ιχθύες»**

**«Deoxynivalenol (DON) in grains intended for consumption by intensively farmed fish»**

Τριμελής Εξεταστική Επιτροπή:

1) Ε. Γκολομάζου, Αν. Καθηγήτρια, Προστασία-Ευζωία Ιχθύων, Τμήμα Γεωπονίας Ιχθυολογίας και Υδάτινου Περιβάλλοντος, Σχολή Γεωπονικών Επιστημών, Πανεπιστήμιο Θεσσαλίας, Επιβλέπουσα.

2) Π. Παναγιωτάκη, Καθηγήτρια, Υδατοκαλλιέργειες, Τμήμα Γεωπονίας Ιχθυολογίας και Υδάτινου Περιβάλλοντος, Σχολή Γεωπονικών Επιστημών, Πανεπιστήμιο Θεσσαλίας, Μέλος.

3) Γ. Ρήγος, Διευθυντής Ερευνών, Ινστιτούτο Θαλάσσιας Βιολογίας, Βιοτεχνολογίας και Υδατοκαλλιεργειών (ΙΘΑΒΒΥΚ), Μέλος.

## ΕΥΧΑΡΙΣΤΙΕΣ

Θα ήθελα να εκφράσω τις ειλικρινείς μου ευχαριστίες σε όλους όσους συνέβαλαν στο να φέρω σε πέρας την παρούσα Μεταπτυχιακή Διπλωματική Εργασία. Ιδιαίτερα θα ήθελα να ευχαριστήσω την Επιβλέπουσα της εργασίας αυτής, κα Ελένη Γκολομάζου για την πολύτιμη βοήθειά της και τη διαρκή υποστήριξή της, τόσο κατά τη διεξαγωγή του πειράματος όσο και κατά τη συγγραφή της παρούσας εργασίας, καθώς και τα υπόλοιπα μέλη της εξεταστικής επιτροπής μου, αποτελούμενη από τους κο Γεώργιο Ρήγο και την κα Παναγιώτα Παναγιωτάκη, για τις χρήσιμες συμβουλές τους και την καθοδήγησή τους καθ' όλα τα στάδια διεκπεραίωσης της εργασίας. Τέλος, θα ήθελα να εκφράσω τις ευχαριστίες μου στην οικογένειά μου, για την αμέριστη συμπαράσταση, βοήθεια και προ πάντων κατανόηση και ανοχή καθ' όλο το χρονικό διάστημα των σπουδών μου.

## ΠΕΡΙΛΗΨΗ

Διάφορα είδη μυκήτων παράγουν δευτερογενείς μεταβολίτες που ονομάζονται μυκοτοξίνες, οι οποίες βρίσκονται στα συστατικά των ιχθυοτροφών και μπορεί να έχουν επιβλαβείς επιπτώσεις στις διαχειριστικές πρακτικές της ιχθυοκαλλιέργειας.

Σε αυτή τη μελέτη χρησιμοποιήθηκε η φασματοσκοπία εγγύς υπερύθρου, Fourier-Transform Near Infrared Spectroscopy (FT-NIR) για να πραγματοποιηθεί φασματοσκοπική ανάλυση δημητριακών (πρώτες ύλες φυτικής προέλευσης που προορίζονται για ιχθυοτροφές) που προήλθαν από Έλληνες προμηθευτές. Η χρήση της μεθόδου FT-NIR καταδεικνύει ότι αποτελεί ένα χρήσιμο εργαλείο ανάλυσης της ανίχνευσης της μυκοτοξίνης Δεσοξυनिβαλενόλης (DON), παρέχοντας σαφή πλεονεκτήματα έναντι των συμβατικών αναλυτικών τεχνικών, όπως η ταχύτητα, η σχέση κόστους-απόδοσης, η ασφάλεια και η ταυτόχρονη ανάλυση πολλών παραμέτρων. Μετρώντας τη φασματική απορρόφηση από τις οργανικές ενώσεις που υπήρχαν στα δείγματα, εκτιμήθηκε έμμεσα η συγκέντρωση της μυκοτοξίνης DON.

Τα αποτελέσματα, εκφρασμένα ως μέσες και μέγιστες συγκεντρώσεις επιμόλυνσης της DON στους τέσσερις δημητριακούς σπόρους (σιτάρι, καλαμπόκι, κριθάρι και σόγια), παρέμειναν στατιστικά σημαντικά χαμηλότερα από τα ανώτατα επιτρεπόμενα όρια. Τα ευρήματα αυτά αποδεικνύουν τις υψηλές προδιαγραφές της ελληνικής υδατοκαλλιέργειας.

Η μυκοτοξίνη DON μετρήθηκε σε δείγματα δημητριακών σπόρων (n=124). Η DON ήταν παρούσα στο 33,9% των δειγμάτων και η μέγιστη συγκέντρωση που παρατηρήθηκε ήταν 2338 µg/kg σε δείγματα σιταριού. Επιπλέον, οι πρώτες ύλες σιταριού, καλαμποκιού και κριθαριού, αλλά όχι της σόγιας, εμφάνισαν θετικότητα στη μυκοτοξίνη DON. Είναι

αξιοσημείωτο ότι οι συγκεντρώσεις της DON και στα 4 δημητριακά, ως συστατικά των ιχθυοτροφών, παρέμειναν πολύ κάτω από τα ανώτατα επιτρεπόμενα όρια (MPL), επιβεβαιώνοντας την ασφάλεια των ιχθυοτροφών που χρησιμοποιούνται στην Ελλάδα σύμφωνα με τη κείμενη νομοθεσία.

Συμπερασματικά, δεδομένου ότι η παρουσία μυκοτοξινών, ακόμη και σε χαμηλές συγκεντρώσεις, μπορεί να είναι επικίνδυνη για την υγεία των ψαριών και των καταναλωτών, είναι επιτακτική ανάγκη η συνεχής επιτήρηση του επιπέδου των μυκοτοξινών στα συστατικά των ιχθυοτροφών.

Λέξεις- κλειδιά: μυκοτοξίνη, DON, ιχθυοτροφές, σιτηρά

# ΠΕΡΙΕΧΟΜΕΝΑ

ΕΥΧΑΡΙΣΤΙΕΣ .....	I
ΠΕΡΙΛΗΨΗ .....	II
1. Introduction .....	1
1.1 Aquaculture .....	1
1.1.1 Greek Aquaculture .....	3
1.2 Fish nutrition requirements and aquafeeds.....	4
1.3 Mycotoxins .....	9
1.3.1 Classification and limits .....	9
1.3.2 Mycotoxins in aquafeeds.....	13
1.3.3 Deoxynivalenol (DON).....	16
1.3.4 Effects of DON on Fish Species.....	19
1.4 Methods of DON detection .....	29
1.4.1 Sampling and Sample Preparation Methods .....	29
1.4.2 Sampling Error .....	30
1.4.3 Sample Pre-Treatment (Extraction and/or Clean-Up).....	31
1.5 Instrumental Analysis.....	33
1.5.1 Chromatographic Methods—Detection Systems .....	33
1.5.2 Immunological Methods (Enzyme-Linked Immunosorbent Assay-ELISA).....	36
1.5.3 Biosensors .....	38

1.5.4	Spectroscopic Methods FT-NIR.....	39
1.6	Aim of the study.....	42
2.	Materials and Methods.....	44
2.1	Aquafeed ingredient samples and preparation for analysis.....	45
2.2	Statistical analysis.....	47
3.	Results.....	49
4.	Discussion.....	52
5.	Conclusions.....	56
6.	Bibliography.....	58
7.	Abstract.....	68

## 1. Introduction

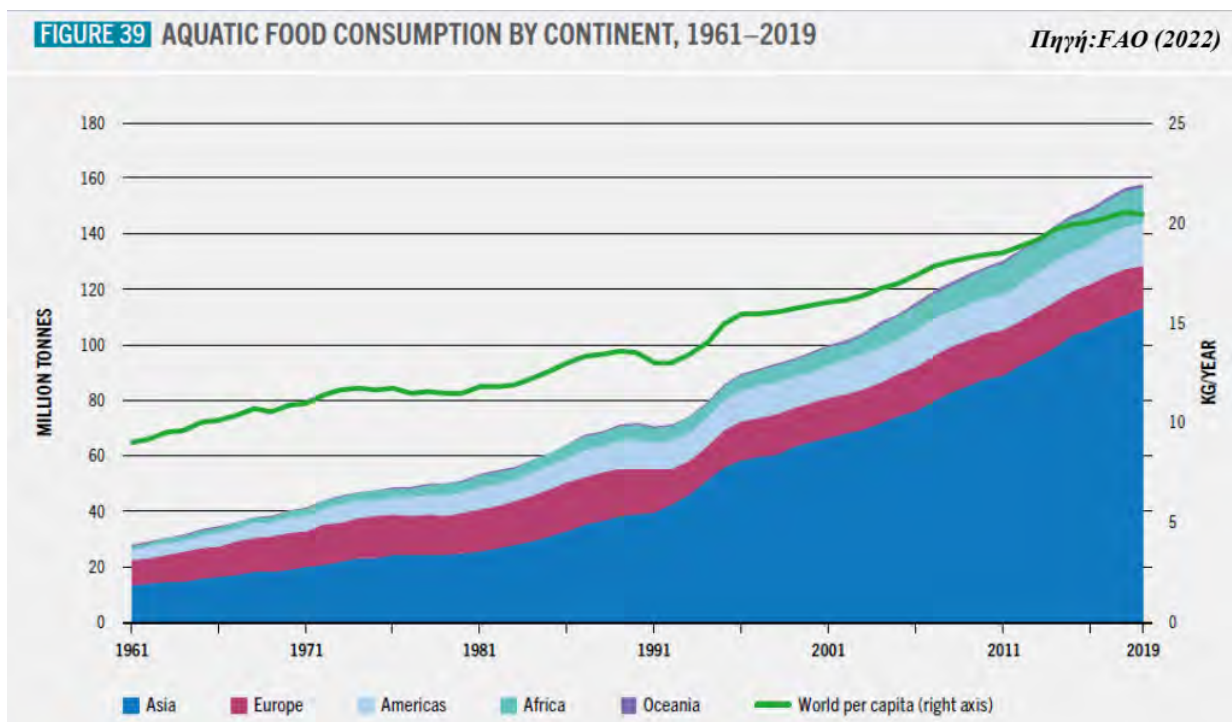
### 1.1 Aquaculture

In the last decades aquaculture industry has been growing rapidly (Figure 1) exceeding the annual growth rates of poultry, pork, dairy and beef industries, having an average growth rate of 5.3% between 2001 and 2018 (Vardali et al. 2023). Nowadays, aquaculture provides half of the fish consumed globally (Galkanda-Arachchige et al. 2020), as shown in Figure 2 and by 2030 aquaculture is predicted to supply 62% of the global fish consumption (Hellenic Organisation of Aquaculture Producers 2022). In terms of nutrients, fish currently provide 16% of the animal protein consumed globally, and this proportion is expected to rise due to increased consumer demand for high-quality seafood (Albrektsen et al. 2022).

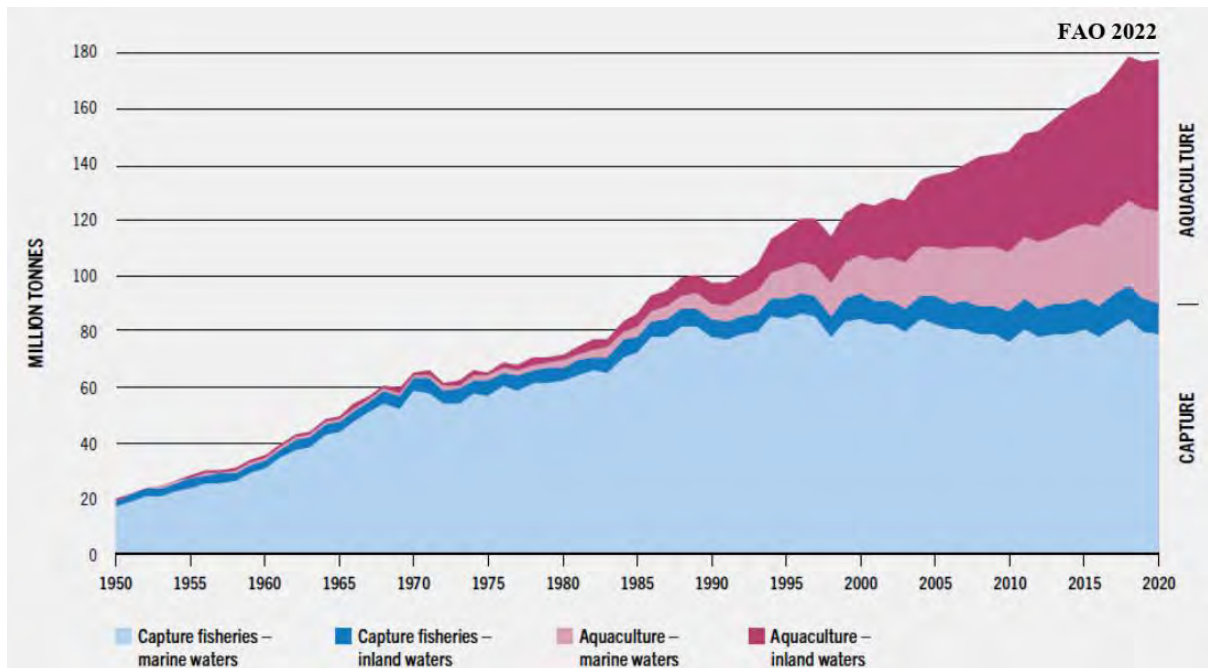
On the other hand, fish stock depletion, rapid increase in global population heading to 10 billion by 2050, high demand for seafood products, and international trade have contributed to the tremendous aquaculture industry development during the past decades. The current and future challenges of the aquaculture industry are to encounter the increasing fish demand and to achieve food security goals within environmental boundaries and the dramatic climate change reality.

The growing demand for global food production requires the transformation of the aquaculture sector and better use of available resources and space. Sustainable food production requires efficient use of available resources and a reduction in environmental impact and greenhouse gas emissions (Albrektsen et al. 2022). Land-based food

production has the largest climatic impact, and therefore the food production sector cannot continue to grow unless more sustainable production methods are applied. Oceans have a great potential for increased food production; however, an increase in aquatic food production must come from farmed fish – aquacultures, since 85% of the world's fish species are already maximally exploited. Food and Agriculture Organization (FAO 2020) has predicted that cultivated aquatic species will provide around 53% of the world's supply of seafood by 2030. A projected increase of 26 million metric tons (MT) in global aquaculture production by 2030 will require an additional 40 million MT of feed (Albrektsen et al. 2022).



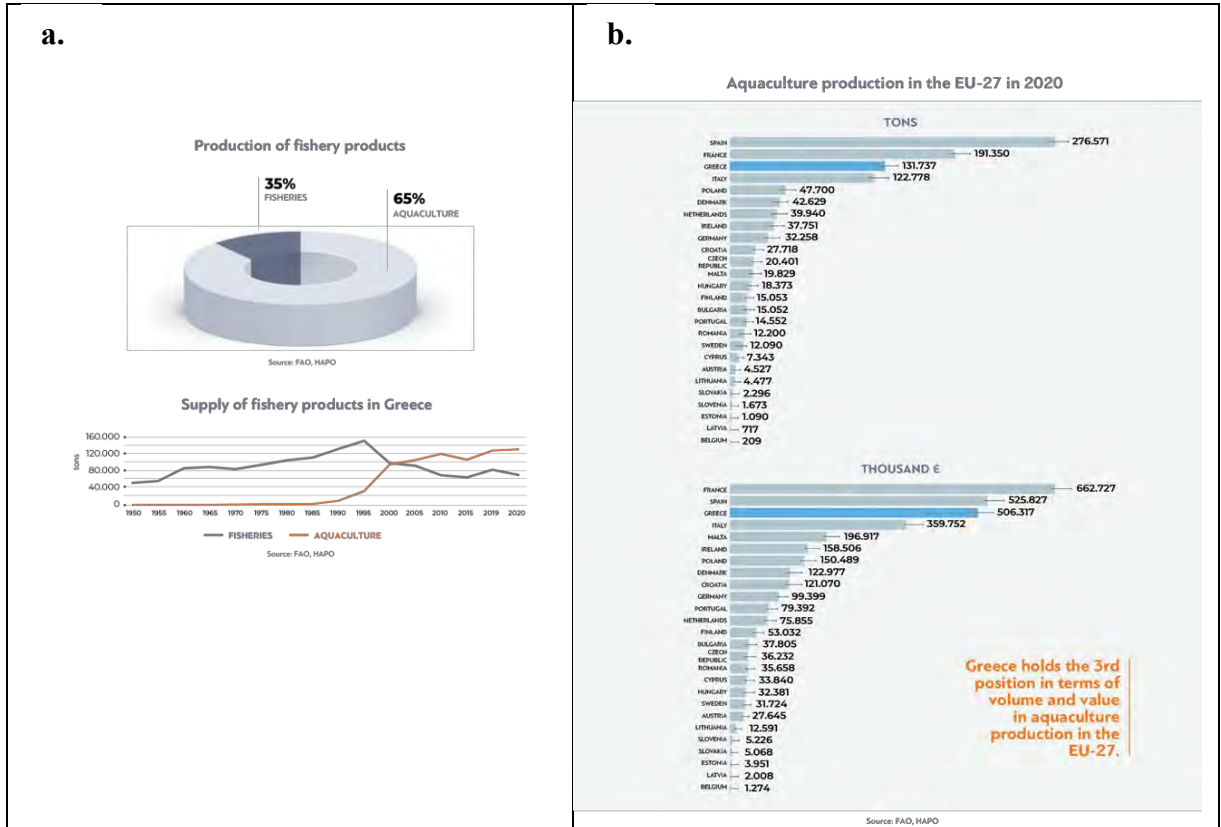
*Figure 1: Aquatic food consumption by continent from 1961-2019 (FAO 2023).*



*Figure 2: Global aquatic production, from 1950 to 2020 (FAO 2023).*

### 1.1.1 Greek Aquaculture

Figure 3 depicts the position of Greece in terms of fishery and aquaculture production on a national (a.) and European level (b.). Since 2003 the supply of aquaculture products in Greece exceeds that of fishing. According to the latest FAO data (FAO 2020) for Greece, 65% of domestic fish production came from aquaculture and the remaining 35% from fishing. Today Greek fish farming production exceeds 120,000 tons, and Greece holds one of the leading positions in the production of Mediterranean species (mainly sea bream and sea bass) at the European and international levels (Greek Aquaculture: Annual Report 2022).



**Figure 3:** Production and supply of fishery products in Greece (a); aquaculture production between the 27 EU countries (b) (Greek Aquaculture: Annual Report 2022).

## 1.2 Fish nutrition requirements and aquafeeds

Fishmeals and fish oils originally come from wild pelagic fish and are the main dietary sources of protein and lipid respectively. Fishmeal and fish oil provide high-quality standards to the end fish-product but on the other hand, their production depends on wild fish stocks that have been widely criticized. In addition, the natural resources are decreasing in parallel with their increasing demand, which has an impact on their cost, causing severe inflation in their global prices, thus developing a need for reducing their inclusion levels in aquafeeds (Karapanagiotidis et al. 2022).

Commercial fish and shrimp feeds contain 25–50% crude protein, which is the principal and most expensive component in these feeds and one of the major nutrients required for the maintenance and growth of animals. Fishmeal (FM) has traditionally been the main protein source used in aquaculture feed accounting for 68% of global fishmeal production (Mallison 2013). This is not only due to its excellent amino acid profile, palatability and digestibility but also because fishmeal is a source of nucleotides, essential fatty acids, phospholipids, minerals and fat and water soluble vitamins (Tacon & Metian 2008). Hardy in 2010 claimed that the demand will soon exceed the world production of fishmeal based on the expected growth rates of aquaculture production and fishmeal utilization in feeds. However, recently Oliva-Teles et al. have shown a steady decline in fishmeal inclusion levels in aquaculture feeds (Oliva-Teles et al. 2015, Galkanda-Arachchige et al. 2020).

Worldwide, intensive research has been initiated to develop alternative sources of protein and essential n-3 long-chain polyunsaturated fatty acid (LC-PUFA) for use in aquaculture feeds due to the overexploitation of the wild fish populations and the strong link to the destruction of rainforests for soy production. Future feed resources are expected to include low-trophic species produced or cultivated in the ocean, such as mesopelagic fish and zooplankton (krill, calanus and amphipods), polychaetes, macroalgae and crustaceans. Ingredients are also produced from fish by-products, and land-based production, such as microbial ingredients (bacteria, yeast and microalgae), insects and animal by-products [ABPs; poultry meal, meat and bone meal, blood meal and hydrolyzed feather meal (HFM)]. Finally, resources resulting from other commercial production, such as biodiesel, brewing and distillation industries, as well as by-products from the agriculture industry, can also be refined and used as feed ingredients (Oliva-

Teles et al. 2015, Albrektsen et al. 2022). All the alternative aquafeeds are categorized in Table 1.

To this direction, new processing technologies and refining methods are used to produce ingredients of high nutritional quality with reduced levels of anti-nutritional factors [(ANFs) such as protease inhibitors, phytate, and saponins] and free from contaminants so as to develop sustainable and safe seafood production (Albrektsen et al. 2022).

*Table 1: Alternative fishmeal and fish oil sources.*

<b>Fishmeal sources</b>	<b>Fish oil sources</b>
Mesopelagic fish and zooplankton (krill, calanus and amphipods), polychaetes, macroalgae and crustaceans	
Plant protein	Plant oils
Land Monogastric Animal by-products, poultry meal, meat and bone meal, blood meal and hydrolysed feather meal	
Insect protein	Insect oils
Fish by-products (seafood processing by-products and by-catches)	
Microalgae protein dependent on photosynthesis	Microalgae n-3 fatty acids EPA + DHA
Bacteria, yeast and microalgae fish feeds dependent on photosynthesis	
Microbial ingredients using organic acids or CH <sub>4</sub> , H <sub>2</sub> and CO <sub>2</sub> gas from industrial waste	
Agriculture industry by-products	
Biodiesel, brewing and distillation industries by-products	

Plant-based protein sources have been generally used to partially substitute fish meal in fish diets. These protein sources are advantageous by having a high content of available

protein, continuous availability, environmental sustainability, and affordable prices. Plant feedstuffs are the major dietary protein sources for omnivorous and herbivorous fish and are second to fishmeal in diets for carnivorous species (Oliva-Teles et al. 2015). Plant-based feed ingredients also provide dietary carbohydrates which can be a source of energy for fish and shrimp; depending on their ability to utilize dietary carbohydrates for energy depending on the species and their natural diet. Furthermore, plant ingredients are sources of starch which is necessary as a binder and facilitates extruded pellet expansion. Plant-based feed ingredients used in aquafeeds as substitutes for marine ingredients include among others, soybean meal, rapeseed/canola meal, maize/corn, wheat bran, wheat, and barley (Hardy 2010).

Corn gluten is also a promising ingredient in fish feed, due to its high nutrient content and its increased availability as a bioethanol production by-product. Corn gluten and wheat gluten are high in protein, low in fiber, rich in vitamins B and E, and do not contain any antinutritional factors. Sunflower meal is highly palatable and has low antinutritional factors. Soybean meal is one of the most interesting alternatives to fishmeal because of the advantages of easy supply, low price, and increased protein and amino acid composition. However, soybean meal has been found to induce a variety of histological and functional changes in the gastrointestinal tracts of several species, such as subacute enteritis of the distal epithelial mucosa including morphological alteration and inflammation (Abbasi et al. 2020, Szczepański et al. 2022).

As opposed to the above-mentioned benefits of plant-based sources, animal diets based on plant proteins can be often associated with challenges such as reduced feed intake, growth performance, and intestinal function. Some ingredients of plant origin have certain characteristics, such as high carbohydrate content, deficiency in some essential

amino acids, low palatability, as well as content in some anti-nutritional factors that limit their use. Both the quality and safety of aquaculture diets can be affected by anti-nutritional compounds including phytates, protease inhibitors, saponins, glycosylates, and tannins, resulting from the inclusion of plant dietary sources (Turchini et al. 2009, Hardy 2010).

Fish diets may be also contaminated by mycotoxins, also derived from plant-based raw feedstuffs, such as cereal grains (Hooft et al. 2011, Gonçalves et al. 2020, Vardali et al. 2023).

## 1.3 Mycotoxins

### 1.3.1 Classification and limits

Mycotoxins are secondary metabolites that are produced by several fungal species and are frequently present in livestock-feeding agricultural products. Livestock and consumers are at risk for health problems due to these toxins. Fungi can contaminate agricultural raw materials while they are being grown, before harvest, or while being stored in unsuitable humidity and/or temperature conditions. Mycotoxins are naturally occurring contaminants that can be harmful to consumers and livestock. Several European and international organizations have addressed this issue by recognizing the significance of mycotoxins, setting regulatory limits, and suggesting acceptable thresholds for specific mycotoxins (Vardali et al. 2023). These include the European Commission (EC, Brussels, Belgium), the US Food and Drug Administration (FDA, Silver Spring, MD, USA), the Food and Agriculture Organization of the United Nations (FAO, Rome, Italy), and the World Health Organization (WHO, Geneva, Switzerland). A scientific expert committee jointly convened by WHO and FAO, named JECFA, serves actually as the international body responsible for evaluating the health risk from all natural toxins including mycotoxins.

Several toxigenic mycotoxins have been identified and those of major significance in animal feeds are primarily produced by the five fungal genera of *Aspergillus*, *Fusarium*, *Penicillium*, *Claviceps*, and *Alternaria*. Approximately 400 compounds have been recognized as mycotoxins (Gonçalves et al. 2020). When mycotoxins are accidentally fed

to animals alone or in combination with other substances, they can have a negative impact on their health (Gonçalves et al. 2018a).

The aflatoxins (AFs) such as aflatoxin B1, B2, G1, G2, and M1 are human and animal health hazards according to the International Agency for Research on Cancer (IARC, 2012). Moreover, ochratoxins (OTA), and fumonisins (FBs) B1, B2, and B3 have been evaluated as possible human carcinogens (WHO, 1993). Other mycotoxins that have also been deemed to be major dangers include trichothecenes (TCs) type A (HT-2 toxin and T-2 toxin) and B (deoxynivalenol-DON), zearalenone (ZEN), Fusarium mycotoxins, ergot alkaloids (EAs), Alternaria toxins (ATs) and patulin (PAT) (Vardali et al. 2023).

AFB1, DON, ZEN, and FB1 belong to the most frequent contaminants of animal feeds (Lee & Ryu 2016).

Generally speaking, mycotoxin contamination can have a significant negative impact on animals' health, leading to reduced growth and productivity, immunotoxicity, toxicity liver problems, and functional abnormalities. Because ZEN interferes with animal reproduction through its estrogenic activity, it can cause reproductive issues like hyperestrogenism, sterility, or even abortions (Vardali et al. 2023).

Incubation of commercial fish feeds under different storage conditions can influence fungal growth and mycotoxin production. Specifically, the application of warm temperature around 27 °C and relative humidity around 70%, can be suitable conditions for triggering the release of ochratoxin A (OTA), with variations due to distinct hotspots with optimal conditions for fungal growth and mycotoxin production. Thus, the presence of mycotoxigenic fungi under storage conditions does not necessarily mean the presence of mycotoxins in aquafeeds (Pietsch 2020).

### 1.3.1.1 *Classification of Fusarium Mycotoxins*

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) (Nathanail et al. 2015). 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 (Zhang et al. 2019). The most commonly detected masked mycotoxin conjugates are  $\beta$ -linked glucose-conjugates of trichothecenes: DON-3-glucoside (DON3Glc), nivalenol-3-glucoside (NIV3Glc), HT-2 glucoside (HT2Glc), and ZEN-14-glucoside (ZEN14Glc). To avoid mistakes, we should not only separate in different categories free mycotoxins from masked mycotoxins but also 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. To sum up, mycotoxins can be present in many forms (traditional, emerging and free) (Koletsis et al. 2021).

### 1.3.1.2 *Limits*

In Europe, AFB1 is the only mycotoxin regulated by 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. For other mycotoxins, including important Fusarium mycotoxins such as DON, ZEN, T-2 and HT-2 toxin, FB1 and FB2, the European Commission (EC) has established only recommended limits for their presence in feedstuffs and feed. Among these recommended limits only those for FB1 and FB2 refer directly to fish species. In addition, EC regulations/recommendations are based on the occurrence of a single mycotoxin, although feeds are usually contaminated by numerous mycotoxins concurrently, which might result in synergistic effects (Koletsis et al. 2021).

*Table 2: Legal mycotoxins limits in animal feed ingredients and (fish) feeds set by European Commission (Table S1 in Koletsis et al. 2021).*

Undesirable substance	Products intended for animal feed	Maximum content in µg/kg (ppb) relative to a feed with a moisture content of 12 %
<b>DIRECTIVE 2002/32/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 7 May 2002 on undesirable substances in animal feed (EC, 2002)</b>		
<b>Aflatoxin B1</b>		
	Feed materials	20
	Complementary and complete feed	10
<b>COMMISSION RECOMMENDATION of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding (EC, 2006)</b>		
<b>Deoxynivalenol</b>		
	Cereals and cereal products with the exception of maize by-products	8000
	Complementary and complete feedingstuffs with the exception of pigs, calves (< 4 months), lambs and kids	5000
<b>Zearalenone</b>		
	Cereals and cereal products with the exception of maize by-products	2000
<b>Ochratoxin A</b>		
	Cereals and cereal products	250
<b>Fumonisin B1 + B2</b>		
	Maize and maize products	60000
	Complementary and complete feedingstuffs for fish	10000
<b>COMMISSION RECOMMENDATION of 27 March 2013 on the presence of T-2 and HT-2 toxin in cereals and cereal products (EC, 2013)</b>		
<b>T-2 &amp; HT-2 Toxin</b>		
	Cereals and cereal products with the exception of oat bran	500
<b>COMMISSION RECOMMENDATION of 15 March 2012 on the monitoring of the presence of ergot alkaloids in feed and food (EC, 2012)</b>		
<b>Ergot alkaloids</b>		
	Feed containing unground cereals	1000

### 1.3.2 Mycotoxins in aquafeeds

Up until the 1960s, the majority of mycotoxin research on animal performance and health had been conducted on terrestrial livestock species. However, an outbreak of aflatoxicosis in trout prompted studies on the effects of mycotoxins on aquaculture species. The high cost of fish meal and the need to find more affordable protein sources, like plant protein or other commercially available by-products (e.g. dried distillers grains and soluble; DDGS), made this even more crucial. Nowadays, the major fish feed ingredients are plant-derived raw materials.

*Aspergillus*, *Penicillium*, and *Fusarium* sp. are typically responsible for producing the majority of mycotoxins that can impair the growth and general health of animals reared in aquaculture. Toxic metabolites produced by these fungi are known to be either carcinogenic (e.g. aflatoxin B1, ochratoxin A, fumonisin B1), oestrogenic (zearalenone), neurotoxic (fumonisin B1), nephrotoxic (ochratoxin), dermatotoxic (trichothecenes) or immuno-suppressive (aflatoxin B1, ochratoxin A and T-2 toxin) (Gonçalves et al. 2018a, Gonçalves et al. 2020).

#### *Aflatoxins*

Aflatoxins are difuranocoumarin derivatives produced by a polyketide pathway by many strains of *Aspergillus flavus* and *A. parasiticus*. *Aspergillus flavus* is principally a common contaminant in agriculture. The main aflatoxins commonly found in aquaculture feedstuffs are AFB1), AFB2, aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2). These mycotoxins occur especially in subtropical and tropical areas contaminating mainly feedstuffs with high starch and lipid content, such as cottonseed, corn, peanut, wheat and

soya bean. AFB1 is one of the most potent carcinogens known, classified as a group I carcinogen by the International Agency for Research on Cancer (International Agency for Research on Cancer (IARC) 1993) and highly hepatocarcinogenic. The biological effects of AFB1 in these aquatic species are thought to be directly linked to the toxin's concentration in feed and to the animal's age and species (Gonçalves et al. 2018a).

#### *Zearalenone (ZEN)*

Zearalenone (ZEN) is a phytoestrogenic compound and a metabolite principally related to several *Fusarium* species. *Fusarium graminearum* is the fungus mainly responsible for the oestrogenic effects commonly found in farm animals. These species are regular contaminants of cereal crops worldwide. ZEN resembles 17 $\beta$ -estradiol, the principal hormone produced by the vertebrate's ovaries. As a hormone mimicking substance, ZEN can bind to oestrogen receptors in target cells. It has been shown that the oestrogenic properties of ZEN give rise to several reproductive disorders in exposed livestock mammals, including decreased libido, anovulation, infertility or neoplastic lesions. Similarly in oviparous animals, including fish, ZEN mimics the action of natural oestrogen, 17 $\beta$ -estradiol, by binding to cells' oestrogen receptors (ERs) and activating responsive genes that encode vitellogenin or zona radiate protein – major structural elements of the oocyte (Gonçalves et al. 2018a).

#### *Trichothecenes (T-2 toxin, DON, Nivalenol)*

Trichothecenes are compounds mainly produced by several *Fusarium* species (e.g. *F. sporotrichioides*, *F. graminearum*, *F. poae* and *F. culmorum*), but can also be produced by members of other genera such as *Myrothecium* and *Trichothecium*. Trichothecenes include T-2 toxin, diacetoxyscirpenol (DAS), deoxynivalenol (known as DON or

vomitoxin) and nivalenol. Both T-2 toxin and DAS are the most toxic. The trichothecenes are extremely potent inhibitors of eukaryotic protein synthesis, interfering with initiation, elongation and termination stages. DON is one of the most frequently found mycotoxins in cereal grains worldwide (Hooft et al. 2011). Common clinical symptoms of DON toxicity include reduced growth and feed intake, vomiting, diarrhoea, gastrointestinal haemorrhaging, inflammation and alteration of the immune response. In aquaculture species, DON ingestion has been associated with highly significant decreases in growth, feed intake, feed efficiency and protein and energy utilization (Hooft et al. 2011, Gonçalves et al. 2018b).

#### *Fumonisin (FB1)*

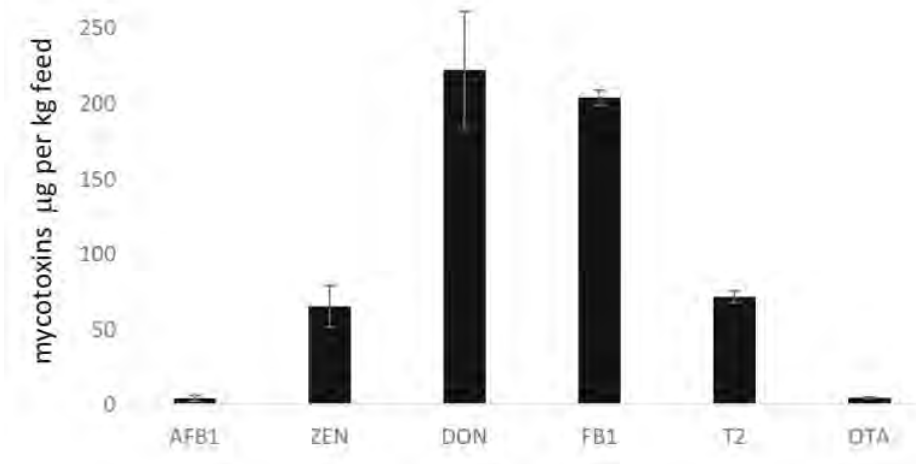
Fumonisin inhibit the sphinganine (sphingosine) N-acyltransferase (ceramide synthase), a key enzyme in lipid metabolism, resulting in a disruption of this pathway. Sphingolipids are important for the membrane and lipoprotein structure and also for cell regulations and communications (second messenger for growth factors). They are mainly produced by a number of *Fusarium* species. The most abundantly produced mycotoxin of the *Fusarium* family is fumonisin B1 (FB1), shown to promote tumours in rats and porcine pulmonary oedema. In aquaculture species, fumonisin B1 has been associated with reduced growth rate, feed consumption and feed efficiency ratio and impaired sphingolipid metabolism (Gonçalves et al. 2018a).

#### *Ochratoxins (OTA)*

Ochratoxins are metabolites of both *Aspergillus* and *Penicillium* species. Ochratoxin A (OTA) is the most toxic compound of this group, known for nephrotoxic and hepatotoxic effects in livestock. Indeed, OTA has already been traced in meat, milk, and dairy

products and other animal-derived swine products. In aquaculture species, has been reported that OTA may cause severe degeneration and necrosis of the kidney and liver leading to inferior weight gains, poorer feed conversion rate, lower survival and haematocrit (Gonçalves et al. 2018a).

In Figure 4 Pietsch depicted the contamination of commercially available fish feeds for multiple mycotoxins (Pietsch 2020).



**Figure 4:** Contamination of aquaculture feeds and naturally contaminated experimental fish feeds with the mycotoxins AFB1, ZEN, DON, FB1, T-2 and OTA reported in the literature in (Pietsch 2020).

### 1.3.3 Deoxynivalenol (DON)

Animal feed samples often contain multiple mycotoxins (75–100%), especially when more than one plant feed ingredient is included in the diet formulations (Koletsis et al., 2021). In addition, it has been evident that DON is the most prevalent mycotoxin in aquafeeds in European aquaculture (Koletsis et al. 2023). Overall, in the European fish feed samples of the study of Koletsis et al., DON and other mycotoxins with a

regulated/guidance value were compliant with the EC limits (2021). However, these limits are not tailored to fish and do not consider species specificities.

#### 1.3.3.1 *DON in wheat*

While only a small percentage of the total production and nearly all of the milling by-product (wheat bran) is utilized as an ingredient in fish and terrestrial animal feeds, the majority of wheat produced is milled and intended for human consumption. Because fungi primarily grow on the outside of the kernels, wheat bran has a higher relative concentration of mycotoxins. 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. These nutritional characteristics are the main reason that traditional formulation software restricts its inclusions in fish feed diets, especially for carnivorous fish species (Gatlin et al. 2007).

Analyses showed DON to be the most frequently reported toxin, detected in 41% of the samples, followed by FB1 (27%). Mean 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, set at 8000 µg/kg. The occurrence of DON in European wheat samples showed an average contamination level of 418 µg/kg with a maximum of 6219 µg/kg (Koletsis et al. 2021). 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 & Naehrer 2009). Moreover, DON has been reported 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 came from 8 years of field surveys which displayed that DON contamination in wheat was mainly affected by year, climatic conditions and geographical region. In contrast, agronomic practices did not influence DON contamination in wheat (Koletsis et al. 2021).

### 1.3.3.2 *DON in Corn*

Corn gluten meal (CGM) is a byproduct of wet milling corn that has a high level of digestibility and an appropriate crude protein content of 60%. It is therefore frequently used as a source of protein in fish diets; however, because of its lysine deficiency, diets are typically supplemented with synthetic amino acids or combined with other protein sources to meet the nutritional needs of the animals. (Tacon & Metian 2015). Fusarium toxins appear to be among the most frequent mycotoxins present in corn. Among the Fusarium mycotoxins with a guidance level, FB1 was found in 70% of the samples, followed by FB2 (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%), FB3 (41%), 15AcDON (20%), 3AcDON (14%) and FX (10%) and of Fusarium emerging mycotoxin MON (10%). In all cases, the maximum DON level exceeded the EC guidance level of 8000 µg/kg, whereas for ZEN all samples were below the EC guidance level (<2000 µg/kg). (Koletsis et al. 2021).

### 1.3.3.3 *DON in Soybean Meal*

Soybean meal (SBM) is one of the most commonly used plant-protein components to replace fishmeal in aquafeeds, although its inclusion is restricted due to its low crude protein level (48%), limited methionine content, and the presence of anti-nutritive factors such as saponins (Hardy 2010). Average values for SBM inclusion have been estimated at 21.3% for salmon diets and, estimated at 15.5% in trout diets, 19.2% in sea bass/sea bream diets and 13.5% in carp diets (Tacon et al. 2011). So far, this is the first study that analyzed and reported FA occurrence in SBM. Following FA (42%), Koletsi reported a common occurrence of FB1 (26%), T-2 toxin (23%). 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. Overall in most studies, contamination levels were always below the EC recommended limit (<8000 µg/kg). Overall, SBM showed relatively low mycotoxin contamination levels compared to contamination levels in wheat and corn (Koletsi et al. 2021).

### 1.3.4 Effects of DON on Fish Species

DON is the most prevalent mycotoxin, and has also been characterized as the most high-risk mycotoxin in aquafeeds. Therefore, the effects of DON on different fish species have been reviewed to evaluate fish health susceptibility. In parallel, data were collected to quantify the risk of exposure in fish. Koletsi et al., used a meta-analytical approach to

explore the extent to which DON affected feed intake and growth performance (Koletsis et al. 2021).

Also in Koletsis et al., studies were reviewed with exposure to DON through restrictive feeding regimes. Some studies exposed fish to “natural” DON (derived from naturally contaminated feed ingredients and other toxins that might be present in the aquafeed) or to “pure” DON (extracted and purified to exclude the presence of other toxins) (Koletsis et al. 2021).

#### 1.3.4.1 *Salmon*

There have been three reported in vivo studies that examined the effects of DON in Atlantic salmon (*Salmo salar*). These studies all used identical experimental conditions, including age (12 months post-smoltification), exposure (8 weeks), and toxin source (pure DON) (Bernhoft et al. 2017, Bernhoft et al. 2018, Moldal 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.

A recent study in salmon (Moldal 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.

#### 1.3.4.2 *Rainbow Trout*

Primary scientific reports on the effects of DON on rainbow trout (*Salmo gairdneri*) were published in the 1980s (Woodward et al. 1983).

Remarkably, no follow-up research was published for almost 30 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 harmful effect on growth performance (Hooft et al. 2011). Apart from growth performance, exposure to 1400 µg/kg DON significantly 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 displayed reduced crude protein content, although no change was observed in the apparent digestibility of crude protein and energy. Histological examination of the liver revealed congestion and subcapsular edema with a fibrinous network in rainbow trout exposed to  $\geq 1400$  µg/kg DON and multifocal areas of fatty infiltration and phenotypically altered hepatocytes in trout

exposed to 2600  $\mu\text{g}/\text{kg}$  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  $\mu\text{g}/\text{kg}$ ). Fish fed the DON diet showed significantly reduced growth rate, feed efficiency, protein and energy utilization efficiencies and whole body crude protein compared to the fish pair-fed the control diet. This observation suggested 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 (Hooft et al. 2011).

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 low feed intake (Ryerse et al. 2015, Ryerse et al. 2016). However, the studies differed in trout size ( $\sim 24$  g (Hooft et al. 2011) and  $\sim 103$  g (Ryerse et al. 2016)). Authors concluded that young trout are more susceptible to metabolic effects of DON.

Following the study of Hooft et al., in 2011, later studies confirmed a significant reduction in feed intake ( $\geq 4100$   $\mu\text{g}/\text{kg}$ ) upon feeding with diets with increasing levels of natural DON (500, 4100, 5900  $\mu\text{g}/\text{kg}$ ) (Ryerse et al. 2016) and ( $\geq 3100$   $\mu\text{g}/\text{kg}$ ) by testing diets with 100, 3100, and 6400  $\mu\text{g}/\text{kg}$  natural DON (Ryerse et al. 2015). Subsequently, follow-up experiments on rainbow trout investigated the effects of a commercial anti-mycotoxin additive (Hooft & Bureau 2017), as well as potential synergy among Fusarium toxins present in naturally contaminated trout feeds (Hooft et al. 2019a).

Trout fingerlings (initial weight; 1.8 g) exposed to natural DON for 12 weeks showed reduced feed intake, weight gain, reduced nitrogen retention efficiency ( $\geq 1000$   $\mu\text{g}/\text{kg}$ ), and reduced retained nitrogen ( $\geq 1500$   $\mu\text{g}/\text{kg}$ ) (Hooft & 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. 2019b), diets with graded levels of pure DON (0, 700, 1400 and 2100  $\mu\text{g}/\text{kg}$ ) or natural DON (0, 2100, 4100 and 5900  $\mu\text{g}/\text{kg}$ ) were offered to rainbow trout (initial weight; 50.3 g) for 8 weeks. Regardless of the DON source (pure/natural), damaging effects were present, and similar trends of reduced retained nitrogen, recovered energy, nitrogen retention efficiency ( $\geq 2100$   $\mu\text{g}/\text{kg}$  pure/natural DON), and energy retention efficiency ( $> 2100$   $\mu\text{g}/\text{kg}$  natural DON) were found. The same study (Hooft et al. 2019b) was the first to use histological examination to show harmful effects of DON on the gastrointestinal tract after feeding 2100  $\mu\text{g}/\text{kg}$  pure or 5900  $\mu\text{g}/\text{kg}$  natural DON.

Further studies have also confirmed the impact of DON on rainbow trout productivity; either by using low DON doses (1100 and 2700  $\mu\text{g}/\text{kg}$ ) (Gonçalves et al. 2019) or high (4700 and 11,400  $\mu\text{g}/\text{kg}$ ) (Gonçalves et al. 2018b).

A recent dose-response exposure study (1000 to 13,000  $\mu\text{g}/\text{kg}$ ) on juvenile trout for 4 weeks showed that increasing levels of DON resulted in reduced feed intake, weight gain and feed efficiency (Koletsi et al. 2023a). Regression analysis suggested that for doses  $> 5000$   $\mu\text{g}/\text{kg}$  each additional 1000  $\mu\text{g}/\text{kg}$  of DON would suppress feed intake by 9% and weight gain by 11%, and for doses  $> 7500$   $\mu\text{g}/\text{kg}$  each additional 1000  $\mu\text{g}/\text{kg}$  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 ( $> 20,000$   $\mu\text{g}/\text{kg}$ ) for 4 weeks, the scientists observed a sharp decline in feed intake and a rejection of pellet ingestion within five days. Interestingly, feed intake and growth returned to normal after four more

weeks of feeding non-contaminated diets, indicating that rainbow trout can, at least temporarily, adapt to DON (Koletsis et al. 2023a).

Finally, some other studies addressed the effects of DON on health, immune function and oxidative stress (Matejova et al., 2017). When 1-year old trouts were exposed for 23 days to approximately 2000 µg/kg DON, plasma biochemical parameters; glucose, cholesterol and ammonia were decreased, pro-inflammatory cytokine TNF- $\alpha$  were up-regulated and altered activities of antioxidant enzymes were observed. 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.

#### 1.3.4.3 *Carp*

Globally, common carp (*Cyprinus carpio*) is the most important fish species in terms of total mass production, with grass carp (*Ctenopharyngodon idellus*), silver carp (*Hypophthalmichthys molitrix*) and common carp) 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 et al. 2014a, Pietsch et al. 2014b, Pietsch & Burkhardt-Holm 2015, Pietsch et al. 2015) 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, led to increased oxidative stress in several tissues (953 µg/kg dose). Concentrations of serum protein

(albumin) in carp were reduced at DON levels of 619 and 953  $\mu\text{g}/\text{kg}$  (Pietsch et al. 2014b). Overall, this suggests 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.

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. 2014a), indicative of cytotoxic effects of DON on immune cells.

Thus, DON studies in carp (Pietsch et al., 2015) (Pietsch & Burkhardt-Holm 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  $\mu\text{g}/\text{kg}$  DON resulted in activation of pro-inflammatory cytokines and anti-inflammatory cytokines. Reduced ROS (reactive oxygen species) production, and increased nitric oxide (NO) production in trunk kidney leucocytes after LPS stimulation confirmed a potential immuno-stimulatory capacity of DON. Using the same experimental set-up (control vs. 953  $\mu\text{g}/\text{kg}$  DON) (Pietsch & 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 (Pelyhe et al. 2016, Kövesi et al. 2020) in common carp. Dietary application of 5960  $\mu\text{g}$  DON per kg feed for 4 weeks did not harm lipid peroxidation in the hepatopancreas (Pelyhe et al. 2016). A single, high oral dose (1750  $\mu\text{g}$  DON /kg body weight) given by gavage (Kövesi et al. 2020) equivalent to 200,000  $\mu\text{g}$  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 ( Pelyhe et al. 2016, Kövesi et al. 2020), or showed no significant effect of DON (Pietsch et al. 2014a, Pietsch & Burkhardt-Holm, 2015). Because these studies applied restricted feeding regimes rather than satiation feeding, DON effects on the growth performance of common carp may not be fully conclusive and further research is needed to draw firmer conclusions. 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).

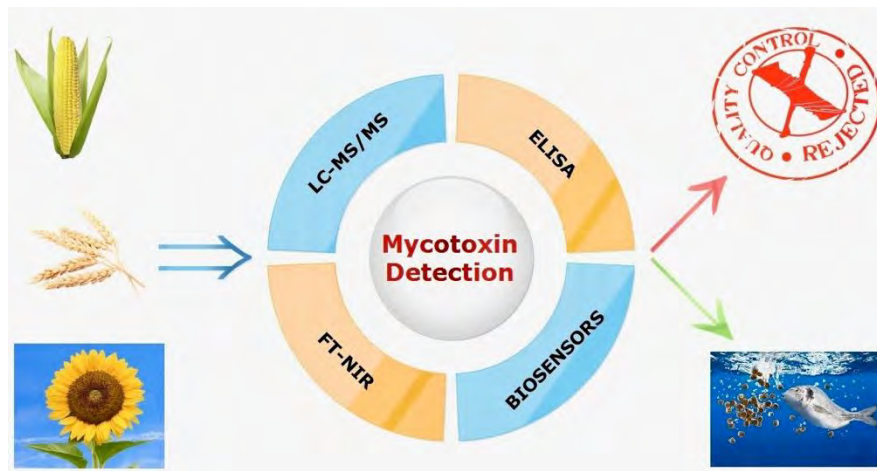
#### 1.3.4.4 *Zebrafish*

The best animal model to investigate the biological effects of DON on fish may be zebrafish. 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. Nevertheless, the preliminary findings from this one study were intriguing enough to justify more research into how DON affects fish biology, by using the zebrafish as animal model.

#### 1.3.4.5 *Tilapia*

To date, only two studies published the effects of natural DON on tilapia (Tola et al. 2015, Hooft et al. 2019). In the first study of Tola et al. (2015) 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). Because in this study ZEN levels were relatively high, interpretation of the results is more difficult due to confounding effects of the combined mycotoxin 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 a linear or quadratic increase in the percentage of mortalities; an endpoint that had not been reported earlier in studies of DON in fish. Despite the increase in mortality, and 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. 2019a). 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, so further studies are needed to assess the effects of DON contamination on Tilapia.



*Figure 5: Methods of mycotoxin detection in aquafeeds (Vardali et al. 2023).*

## 1.4 Methods of DON detection

The potential dangers connected to mycotoxin presence in feed materials have spurred the development of various analytical techniques for mycotoxin detection and measurement in food samples. It is imperative to consistently enhance mycotoxin analytical methodology through expeditious and effective techniques to safeguard aquatic product consumers and adhere to updated regulations. A recent analysis of the methods used to detect mycotoxin concerning the health of humans and animals was carried out in a range of food matrices (Vardali et al. 2023).

This section focuses on the advancements in mycotoxin detection in raw materials derived from plants- which make up most of the ingredients in fish feed- during the previous ten years.

### 1.4.1 Sampling and Sample Preparation Methods

Accuracy and representativeness in sample collection are crucial in the mycotoxin sampling process. There is a slight chance of inaccurate sampling because mycotoxins are not consistently contaminated in products and can be found in barely detectable amounts. To gather representative laboratory samples from each sampling location, a particular protocol is followed. The lots that are chosen for examination need to be suitable. Multiple replicate samples from various locations within the lot are combined to create the final sample (Vardali et al. 2023).

This is achieved by mixing and dividing to obtain a representative sample. Mycotoxin detection is typically done by collecting and testing samples of food or feed. The samples

should be taken randomly from the entire lot and should be collected from different locations within the lot (Turner et al. 2015).

The size of the lot will determine the required sample size, but to prevent contamination, a minimum of 500 g should be collected using sterile and properly sterilized sampling equipment. To stop the mycotoxin from degrading, samples must be kept in airtight containers, kept at the right temperature and humidity, and delivered as soon as possible to the lab. Creating a clean, representative sample that the selected analytical method can accurately analyze is the aim of sample preparation. The particular procedures required for sample preparation vary depending on the kind of sample and the analytical method employed. To obtain an analytical part (test portion), a sample must be ground, homogenized, and subsampled. This analytical portion is then extracted with a solvent, analyzed, and the mycotoxin concentration is determined using a validated analytical approach (European Commission 2006, Vardali et al. 2023).

Sample preparation is an important stage in analytical techniques because it could greatly affect the accuracy and precision of the results.

#### 1.4.2 Sampling Error

Sampling errors can occur in several ways and can greatly affect the accuracy and precision of analytical results. Sampling errors in mycotoxin detection refer to the potential for variability in the results of mycotoxin testing due to the collection and preparation of the sample. These mistakes can also happen if a sample is contaminated during collection or handling, or if it is not representative of the entire lot.

It is crucial to adhere to correct sampling protocols to reduce sampling error. These protocols include selecting multiple samples from various locations within the batch and utilizing the right tools and techniques. Furthermore, samples must be prepared following established protocols and stored appropriately. It is imperative to incorporate quality control measures to guarantee the precision and consistency of the test outcomes (Vardali et al. 2023).

### 1.4.3 Sample Pre-Treatment (Extraction and/or Clean-Up)

#### 1.4.3.1 *Solid–Liquid Extraction (SLE)*

Solid–liquid extraction involves a simple and high-sensitivity process for preparing samples in liquid chromatography–mass spectrometry (LC-MS). The multi-residue analysis ability of the detection apparatus is rather beneficial. The capacity of matrix effects in LC-MS analysis to modify chromatographic signals is accomplished due to co-eluting matrix components, while the ion suppression problem that arises can be overcome by proper sample preparation using specific matrices and internal standards (Razzazi-Fazeli & Reiter 2011). In addition to being simple and sensitive, this procedure's sample preparation is regarded as reliable and has been effectively used to identify mycotoxins in plant samples. All methods used a solvent mixture of acetonitrile/water for the extraction of mycotoxins before LC-MS/MS analysis (Silva et al. 2019). However, the simplicity of the sample preparation process may affect the method's performance characteristics (low recoveries, matrix effect, etc.).

#### 1.4.3.2 *Dispersive-Solid Phase Extraction (d-SPE)*

In the d-SPE technique, sample cleaning is achieved by the use of a solid sorbent in a liquid or dissolved sample which maintains impurities. After separation, the sample is centrifuged for sorbent removal (Anand & Srivastava 2020). The QuEChERS protocol is a commonly applied extraction method that requires small amounts of sample and solvent while at the same time producing high extraction efficiency. The extraction phase is the first step where an organic solvent such as acetonitrile is needed along with a variety of salts to modulate polarity and pH and to facilitate phase separation and recovery of the analyte. Purification is the second stage in the cleaning process. The remaining water and other interfering substances from the matrix are eliminated in this stage. There are several methods using the QuEChERS protocols for the extraction and clean-up of mycotoxins before instrumental analysis (Rausch et al. 2020).

#### 1.4.3.3 *Clean Up by Immunoaffinity Column (IAC)*

The creation of IAC is another technique where specific antibodies for certain mycotoxins are bound to a specifically activated SP support. This method is commonly used to detect Afs, OTA, and FBs. In detail, the support is packed into a cartridge while a suspension is performed in a buffer solution. After the extract or fluid's mycotoxin attaches to the antibody and any contaminants are washed away with water or an aqueous solution, the mycotoxin is desorbed using a miscible solvent (methanol). IAC can be used for further separation and LC quantification (Göbel & Lusky 2004).

#### 1.4.3.4 *Solid Phase Extraction (SPE)*

The SPE reduces matrix-based interferences to concentrate on a target analyte. To properly separate the analytes from the other interfering parties, this adsorbent is selected based on the physicochemical characteristics of the analytes. Dispersive SPE, is a modern technique that requires nanoparticles in a magnetic mode. This detection method has been recently adopted. Notably, the characteristics of the magnetic SPE (mSPE) look like those found in standard SPE. Matrix composition may affect the selection of the adsorbent and elution mixture. In mSPE, continuous contact with the adsorbent is necessary through the dispersion of the magnetic material into the solution containing the target molecules (Ríos et al. 2013).

### 1.5 Instrumental Analysis

Figure 5 summarises the 4 methods of mycotoxin detection in aquafeeds analyzed in the following section.

#### 1.5.1 Chromatographic Methods—Detection Systems

Chromatographic-based methods include liquid chromatography (LC) or gas chromatography (GC) that is coupled with ultraviolet (UV), mass spectrometry (MS), or fluorescence (FLD). The chromatographic methods combined with a UV detector and FLD are usually used for the analysis of a compound or a small number of mycotoxin-related chemicals. The MS method has many benefits such as high sensitivity, selectivity,

and accuracy, compared to the two other methods. Tandem MS (MS/MS), where two MS equipment are coupled together, is a highly sensitive, specific, and reliable tool for detecting contaminants in food and has become the most popular approach for multianalyte analyses. LC-tandem MS (LC-MS/MS) has been increasingly used for the accurate quantitative analysis of mycotoxins in food. A limited number of multi-mycotoxin techniques, particularly for finished fish feeds and shrimp feeds, has been reported. Aquatic feeds are complex matrices consisting of minerals, vitamins, fatty acids, and proteins in high concentrations that are challenging to remove. As a result, choosing an appropriate clean-up step that reduces matrix effects (MEs) and interferences during chromatograph analysis is essential. The literature on exclusive analyses of fish feed ingredients seems insufficient, therefore, the data are mainly based on analyses of raw materials used in all types of animal feed, including fish feed. Most of the methods found in the literature use LC-MS/MS as a detection system. Some of them using various extraction processes before LC-MS/MS analysis for the mycotoxin determination are detailed below. LC-MS/MS method was reported for the analysis of 15 mycotoxins in fish feed. The extraction was achieved by a USE step followed by a clean-up step by a lipid cartridge. The recoveries varied between 25 and 109% for the 15 mycotoxins and LODs ranged between 0.05 and 54  $\mu\text{g}/\text{kg}$ . Furthermore, for the detection of AFB<sub>1</sub>, AFM<sub>1</sub>, T-2, HT-2, DON, OTA, and ZEN in fish feed and shrimp feed, an HPLC-MS/MS method was developed. The samples were extracted with a mixture of acetonitrile and water followed by a defatted step by hexane and a clean-up step with a multi-toxin purification column. LODs ranged between 1.83 and 12.63  $\mu\text{g}/\text{kg}$  and the method was successfully applied in several fish feeds in China. The LODs of the method ranged between 0.15 and 61  $\mu\text{g}/\text{kg}$ . Another method for the simultaneous determination of ZEN

and DON in fish feed was reported based on an HPLC-DAD system and an SLE extraction step followed by clean-up with an IAC column (Pietsch et al. 2013).

Concerning fish feed ingredients, the simultaneous determination of 11 mycotoxins in maize, wheat, and barley was achieved by UHPLC-MS/MS analysis using a simple SLE extraction with acetonitrile/water/formic acid (79/20/1, v/v/v). The UPLC-MS/MS method for the analysis of DON and T-2 in maize and oats was also developed, followed by SLE extraction and an SPE clean-up step. The LODs of the method were between 0.13 and 0.38  $\mu\text{g}/\text{kg}$ . In wheat, corn, rice, and barley, LC-MS/MS was applied for the determination of 38 mycotoxins using a QuEChERS extraction (Rausch et al. 2020). Another LC-MS/MS method was developed for the detection of T-2 and HT-2 toxins in corn and oat using an SLE extraction followed by a clean-up step with an IAC column, achieving very low LODs, ranging between 0.02 and 0.08  $\mu\text{g}/\text{kg}$ .

Only four methods were found using an FLD detection system. For the detection of aflatoxins AFB1, AFB2, AFG1, AFG2, and DON in maize, oat, rice, rye, barley, and wheat an HPLC-DAD-FLD system was developed, after two SLE extractions and a clean-up step by SPE. The LODs of the method were 0.02 to 16.2  $\mu\text{g}/\text{kg}$ . In wheat, corn, oat, barley, and rice, a validated HPLC-FLD system coupled with a photochemical reactor was tested for the simultaneous determination of aflatoxins, OTA, and ZEN. The LODs of the method ranged between 0.004 and 0.5  $\mu\text{g}/\text{kg}$ . Full scan MS was also found in pertinent literature using a TOF-MS and an Orbitrap MS system for the identification of mycotoxins. Orbitrap MS and TOF MS are used to estimate both known and unknown compounds. This is because they can allow detailed discrimination in molecular weight by accurately measuring the mass to five significant digits. A validated UHPLC-ToF-MS method was developed for the determination of nine mycotoxins in maize. The extraction

step proposed was very easy, using two SLE steps with acetonitrile 80% (v/v). The method's LODs ranged between 0.5 and 62.5 µg/kg (Silva et al. 2019). Furthermore, the LC-Orbitrap MS method combined with QuEChERS step was applied for the determination of 20 fusarium toxins in corn, wheat, barley, sunflower, soybean, feeds, and feedstuffs and the LODs of the method were 5 µg/kg. Ten mycotoxins in maize and rice were detected by a full-scan LC-MS method using a second-order calibration method based on an alternating trilinear decomposition (ATLD) algorithm (Liu et al. 2017, Gonzalez-Jartin et al. 2021).

### 1.5.2 Immunological Methods (Enzyme-Linked Immunosorbent Assay-ELISA)

Lateral flow immunoassay, ELISA, and immunosensors are immunochemical detection methods based principally on antibody–antigen binding (Li et al., 2009). Antibodies and antigens belong to some of the most commonly used capture agents in immunoassays for disease treatment, environmental monitoring, and food safety regulation. Their high commercial recognition is, however, not deprived of drawbacks. For example, immunization and purification are necessary for the development of high-quality antibodies. These processes can be difficult, expensive, and laborious. Additionally, the applicability of antibodies is limited due to their sensitivity to pH and temperature variation. Moreover, antibodies can only recognize substances that are immunogenic and immunoreactive. Finally, the chemical conjugation effectiveness of mycotoxins to a protein carrier is limited. Immunosorbent assays and immunosensors require simpler sample pre-treatment in comparison to those used for chromatographic methods and have

the advantages of high throughput and good specificity although, detection results still must be output by instruments. Under the same sample pre-treatment procedure, ELISA assays are however more prone to more errors due to the laborious operation procedure (Li et al. 2018).

For the quantitative and simultaneous detection of different mycotoxins, various immunological methods have been assessed. Detection of ZEN, FB1, DON, and AFB1 in corn, and wheat, has been achieved by a suspension array. Suspension arrays have the advantages of sensitivity, rapidity, and accuracy. Signal responses are observed using red and green laser lights to achieve qualitative and quantitative detections. The LODs of the method were 0.51–6.0 ng/mL for the four mycotoxins (Wang et al. 2013). Multiplex fluorescent immunosorbent assay (FLISA) using quantum dots (QDs)-based immunochemical techniques has been used for multi-contaminated cereal samples, allowing the simultaneous determination of all compounds. The mycotoxins DON, ZEN, AFB1, T-2, and FB1 were allocated to different wells of the same multi-well plate, and the sample was treated before being dispensed over the wells (single-analyte multiplex, SAM). Moreover, multi-contamination with ZEN and AFB1 was determined with the double-analyte multiplex (DAM). Two different specific antibodies were distributed in one single well and the mycotoxins ZEN and AFB1 were determined in wheat and maize, on the condition that their conjugates are labeled with QDs, which are fluorescent in different parts of the spectrum at two different wavelengths (Vardali et al. 2023).

Another sensitive tool for the simultaneous quantitative determination of DON, ZEN, and AFB1 in cereal-based products in one single well of a microtiter plate, has been applied. This one is based on the use of a colloidal quantum dot enrobed into a silica shell (QD@SiO<sub>2</sub>) derivatives as a highly responsive label. Silica-coated quantum dots were

prepared and subsequently modified via co-hydrolysis with tetraethylorthosilicate (TEOS) and various organosilane reagents. The LODs were 1.9–5.4  $\mu\text{g}/\text{kg}$  for the three mycotoxins (Beloglazova et al. 2014).

Moreover, another study proposed the development of a polyvinylidene fluoride (PVDF) membrane-based dot immunoassay for the rapid and simultaneous detection of AFB1, ZEN, DON, OTA, and FB1 in corn, and wheat. The LODs of the method for mycotoxins were 20–1000  $\mu\text{g}/\text{kg}$  (He et al. 2012).

Cereal contamination with ZEN and DON, was identified using Cd-based QDs as labels, while an imprinted BSA was immobilized on a microwell plate. This technique involved putting silica on green- and red-emitting QDs to turn them hydrophilic, before coupling with mycotoxin-protein occurs. The ZEN detection cut-off level varies depending on cereal origin. On the other hand, the cut-off level for DON is considerably lower when compared to its permissible limits (Beloglazova et al. 2014). The multi-mycotoxins (AFB1, ZEN, and T-2 toxin) determination in cereals has also been accomplished using a multicolor immunochromatographic strip (ICS). In this method, three monoclonal antibodies are bound to three different colored nanoparticles to act as immunoassay probes and the three mycotoxins may be quantified at the same time according to color decrease (Xu et al. 2018).

### 1.5.3 Biosensors

Biosensors consist of various elements such as a molecularly imprinted polymer (MIP), an aptamer, a DNA/RNA molecule, an enzyme, a tissue, living cells, and antibodies. A

transducer is also necessary to connect these parts, which transforms the observed physical or chemical changes into a quantifiable signal. Depending on the signal transduction mechanism and the applied recognition elements, three categories of biosensors exist: optical, electrochemical, and piezoelectric. Immunosensors are of the most commonly used analytical methods for mycotoxin detection, although other cutting-edge methods such as MIP-based sensors are available. Antibodies, antigens, and their fragments, are used for biomolecular recognition in immunosensors. The essential premise behind all immunosensors is that the precise binding of the immobilized components in the sample results in the production of an analytical signal that is affected by the concentration of the target analyte (Vardali et al. 2023).

DON detection in crop samples (wheat and maize) has been achieved using a white light reflectance spectroscopy (WLRS) optical immunosensor. It was proved to be a fast and high-sensitivity assay for the assessment of contamination in the whole grain (Anastasiadis et al. 2020).

A portable and reusable optofluidic immunosensor OIP-v2 was developed for rapid and sensitive on-site detection of DON using DON-BSA modified bio-probes as biorecognition elements. The OIP-v2 was used for the detection of DON with high sensitivity, accuracy, and rapidity. The LOD of DON was 0.16 µg/L (Liu et al. 2022).

#### 1.5.4 Spectroscopic Methods FT-NIR

The most promising techniques for detecting mycotoxins are those based on infrared (IR) spectroscopy because they just need small samples and minimal technical know-how.

Furthermore, these methods don't require sample pre-treatment and are inexpensive. The most common method for identifying mycotoxin contamination in crops is spectroscopy (Cen & He 2007).

Mid-infrared (MIR) spectroscopy is specified for molecular vibrations while standard NIR spectroscopy determines the molecular overtones and combined vibrations of chemical bonds. All spectra produced by overtones and mixed vibrations seen in the NIR range are challenging to interpret for specific constituents present in a sample. Chemometrics can be used for direct information extraction from the data, which solves the upcoming necessity of mathematical processing to extract chemicals and linked information in the assessment of NIR and MIR spectra (Vardali et al. 2023).

Three phases are often involved in NIR or MIR spectroscopy using chemometrics: spectral pre-processing, multivariate model construction for calibration, and model transfer (Cen & He 2007). Qualitative and quantitative methodologies are used for NIR and MIR spectroscopic model development. Some examples of qualitative techniques include principal component analysis (PCA), cluster analysis (CA), and linear discriminant analysis (LDA). In contrast, principal component regression (PCR), multiple linear regression, and partial least squares (PLS) are common methodologies for quantitative multivariate calibration (MLR).

The first mycotoxins analyzed by FT-IR were FB1 and FB2 in maize meal (concentrations) as a quick way to distinguish contaminated meals. Based on an optimized feature model for NIR spectroscopy, a quantitative assay for AFB1 in maize has been suggested. The potential of NIR spectroscopy in conjunction with chemometric techniques for the quick and accurate quantitative detection of the AFB1 in maize was

demonstrated using a portable NIR spectroscopy device to evaluate maize samples with varied degrees of contamination. To effectively mine the wavelengths of the NIR spectra, different variable selection algorithms were used. After the screening, the wavelength variables were utilized to create a support vector machine (SVM) and a partial least squares (PLS) test model, respectively, to measure AFB1 in maize. As a result, by using a nonlinear SVM detection model, the characteristics of NIR spectra are beneficial for the rapid and accurate testing of the AFB1 in maize (Deng et al. 2022).

Additionally, NIR and FT-NIR were used to evaluate their applicability and efficiency for the analysis of Brazilian wheat flour samples contaminated with DON, with partial least-squares discriminant analysis (PLS-DA) and principal component analysis-linear discriminant analysis (PC-LDA) used as discriminatory methods. Validation samples through PLS-DA showed correct classification rates in the range of 85–87.5% with an error of 10–15% error. For PC-LDA, the hit rate was over 85% with an error of 10–15% demonstrating that NIR is an excellent alternative method for the classification of wheat flour samples according to DON content (Tyska et al. 2021).

## 1.6 Aim of the study

Understanding the occurrence of mycotoxins, particularly in field conditions, remains crucial even with the implementation of good manufacturing practices. This knowledge gap is particularly pronounced in terrestrial animals, while information regarding mycotoxin presence in fish feeds is limited. The profile of mycotoxin contaminants in fish feeds depends on environmental conditions, geographic origin, and the plant cultivation practices.

This is the first study on Greek aquaculture, a sector that holds the leading position in terms of volume and value in EU aquaculture production, as reported in the Introduction (section 1.1). This research studied the prevalence of mycotoxins that could impact Mediterranean aquaculture, aiming to guide the formulation of effective measures to mitigate adverse effects. Adjusting mycobinding approaches to specific mycotoxin types is crucial, and this knowledge will enable the development of preventive treatment to decrease fungal prevalence in crops. The understanding gained from this study will contribute significantly to the improvement of aquaculture practices and the sustainable maintenance of the Mediterranean aquaculture industry.

The aim of the present study is to assess mycotoxin DON contamination in Greek aquafeed ingredients derived from grains (n=124) so as to evaluate the method of Fourier Transform-Near Infrared Spectroscopy (FT-NIR) spectroscopy as a validated technique for continuous inspection as well as a useful diagnostic tool for animal feed ingredient analysis. Specifically, the DON contamination rates of raw materials (wheat, barley, corn

and soy) used in fish feed production in Greek aquaculture were estimated. The following results will lead to conclusion regarding the guarantee of farmed fish and consumers' health.

## 2. Materials and Methods

Numerous analytical techniques for identifying mycotoxins in aquafeeds and their ingredients have been documented, as analysed in the Introduction (section 1.5). Being the most widely used method for multianalyte analyses, High Performance Liquid Chromatography (HPLC) is extensively used for mycotoxin determination as a highly sensitive, specific, and dependable tool for detecting contaminants in food (Soleimany et al., 2012). Several immunological techniques, such as the Enzyme-Linked Immunosorbent Assay (ELISA), have been also documented together with Biosensors used to analyze mycotoxin levels in a range of aquafeed raw materials (Vardali et al. 2023).

Optical techniques like Near Infrared Spectroscopy (NIR) have significantly attributed in the quest for quick procedures for the measurement of components in food samples (Bevilacqua et al. 2013). These techniques for detecting mycotoxins need small samples and minimal technical know-how. Additionally, unlike conventional chemical analytical methods, these techniques are inexpensive and do not require complicated sample pre-treatment. The most common method for identifying mycotoxin contamination in crops is spectroscopy (McMullin et al. 2015). This technology relies on indirect measurements due to the complexity of the obtained spectral data. When mycotoxin contamination by fungi and subsequent fungal infection arises, the chemical makeup of food used in the manufacturing sector is changed. NIR has the capacity to examine these variations within particular ranges and develop predictive models using qualitative or quantitative techniques (McMullin et al. 2015). Therefore, in order to retrieve the analytical

information from the relevant spectra, methods requiring calibrations using mathematical models and multivariate statistical tools must be used (Roggo et al. 2007).

## 2.1 Aquafeed ingredient samples and preparation for analysis

124 aquafeed ingredient samples (approximately 2 kg per sample) comprising of barley (n=2), soybean meal (n=32), wheat (n=46), and corn (n=44) collected from various Greek suppliers, were analyzed between 2022 and 2023. Following collection, samples were refrigerated for less than 15 days until further analysis.

### *Pre-treatment of samples*

To create a representative laboratory sample, the quartering method was applied to ensure representative sampling (Campos-M & Campos-C 2017). Every sample was placed on a spotless, non-absorbent flat surface upon its arrival in the laboratory. A cone-like shape was created from the aggregate sample. Next, using a quartering divider, the top of the cone was flattened and divided into four equal quarters.

Following the removal of two opposing quarters (making sure that all dust and other tiny particles were also wiped away each time), the remaining pair was combined and formed into a different cone. This process was repeated until the remaining quarters were down to 100 g. A portion of each sample (100 g) was then ground in an ultra-centrifugal mill (ZM 200 Ultra Centrifugal Mill, Retsch GmbH, Haan, Germany) with 1 mm diameter sieve.

### *Fourier Transform-Near Infrared (FT-NIR) analysis and acquisition*

In the field of mycotoxin detection, Infrared (IR) spectroscopy based methods stand out as among the most promising due to their ability to work with small samples and requiring limited technical expertise. These techniques not only offer cost-effective solutions but also eliminate the need for extensive sample pretreatment, streamlining the detection process. Mycotoxin contamination in crops is routinely identified through spectroscopic approaches, with Near-Infrared (NIR) spectroscopy playing a pivotal role. NIR spectroscopy identifies molecular overtones and combined vibrations of chemical bonds. All spectra are challenging to decipher for specific constituents present in a sample. Chemometrics are instrumental in direct information extraction from the data involving three phases: spectral pre-processing, multivariate model construction for calibration, and model transfer. Qualitative and quantitative methodologies are used for NIR spectroscopic model development.

The quantity of 100 g ground sample was transferred to an FTNIR spectrometer (TANGO FT-NIR spectrometer, Bruker Optics, Ettlingen, Germany) which emitted electromagnetic radiation in the infra-red region to perform spectral acquisition.

For the recording of FT-NIR spectra with diffuse reflectance, the milled samples were placed in a glass vial (52.0 mm x 22 mm x 1.2 mm, Nipro Diagnostics Germany GmbH, Ratingen, Germany) and positioned at the beam outlet of a TANGO NIR spectrometer (Bruker Optics, Ettlingen, Germany). All measurements were performed at room temperature. FT-NIR spectra were then recorded in total reflectance mode in the wave number range between 12500 and 3600  $\text{cm}^{-1}$  at a resolution of 16  $\text{cm}^{-1}$ . The rotating sphere macro sample was the cell type used for reading solid samples; each spectrum was read in approximately 30 s. The background was acquired at the beginning of each measurement series and additionally when indicated by the spectrometer software. The

FT-NIR spectrometer was remotely controlled using TANGO software (Bruker Optics, Ettlingen, Germany). Measured spectra were uploaded to the Olimpo platform (Pegasus Science) and the result of mycotoxin concentration in each sample was acquired. The method's Limits of Detection (LOD) for the acquired mycotoxins were 5 µg/kg for AFB1, 200 µg/kg for the sum of FB1 and FB2, 350 µg/kg for DON, 30 µg/kg for ZEN and 10 µg/kg for OTA.

### *Mycotoxin occurrence*

Mycotoxin occurrence (%) in each aquafeed ingredient was calculated by the ratio of positive samples to all samples analyzed. Aquafeed samples with mycotoxin concentration above the method's LOD were considered as positive whereas mycotoxin concentration below LOD was identified as negative.

## 2.2 Statistical analysis

Statistical analysis of occurrence data for mycotoxin DON involved the utilization of the chi-square ( $\chi^2$ ) statistical test. A comparison of mycotoxin rates was conducted. Evaluation of differences between detectable (positive) and non-detectable (negative) values for DON mycotoxin was performed, with statistically significant differences indicated in the graph through lowercase letters. To accurately identify points of statistical significance, the data analysis involved partitioning the initial  $\chi^2$  into several individual tests (2×2). In the assessment of mycotoxin concentrations in all samples, Analysis of Variance (ANOVA) and Tukey's post hoc analysis were applied as statistical tests to assess differences in mean concentrations of DON among the four raw materials. The

SPSS Statistics 26 package was utilized in all instances, with a significance level set at  $P < 0.05$ .

### 3. Results

In this section are presented the results of the FT-NIR analyses. Table 3 describes the DON mycotoxin - mean and max - concentration ( $\mu\text{g}/\text{kg}$  or ppb), as well as the maximum permitted limit of DON, in the four different raw materials (corn, barley, wheat and soy) of the aquafeeds. The same piece of information is depicted in Figure 6. DON was detected in all examined samples to be much lower than the maximum permitted limits (MLP) OF  $8,000\mu\text{g}/\text{kg}$  of DON. DON was identified in all 4 grains of corn, barley, wheat and soy, with the lowest values found in soy ( $<350 \mu\text{g}/\text{kg}$ ), below the limit of detection of the analytical method. The second lower mean values of DON were found in corn ( $463,5 \mu\text{g}/\text{kg}$ ). Barley exhibited a significantly higher concentration of  $894,5 \mu\text{g}/\text{kg}$  while wheat samples displayed the highest mean concentration value of  $1007,2 \mu\text{g}/\text{kg}$ .

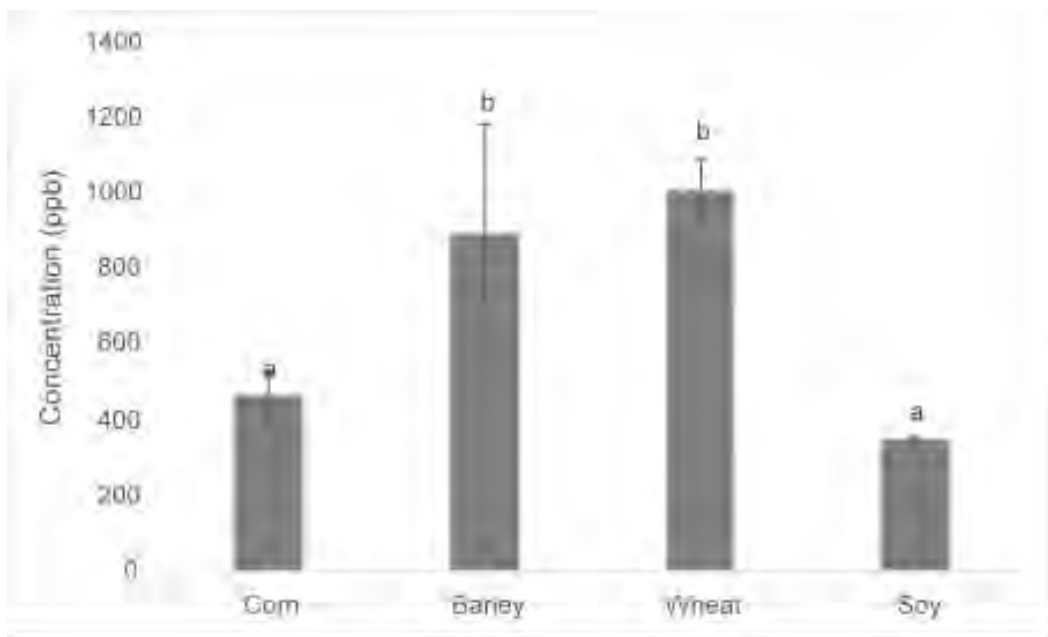
**Table 3:** Mean mycotoxin DON concentration  $\pm$  standard error of positive analyzed samples.

Maximum values in aquafeed ingredients ( $\mu\text{g}/\text{kg}$ ) are presented for corn, barley, wheat, and soy samples, Maximum Permitted Limits (MPL). Mycotoxins not sharing a common superscript letter among aquafeed ingredients are statistically significant different ( $p<0.05$ ).

	Corn		Barley		Wheat		Soy		
	MPL	Mean	Max	Mean	Max	Mean	Max	Mean	Max
<b>DON</b>	8,000	463.5 $\pm$ 63.7	654	894.5 $\pm$ 289.5	1184	1007.2 $\pm$ 82.2	2338	<350	<350
		a		b		b			

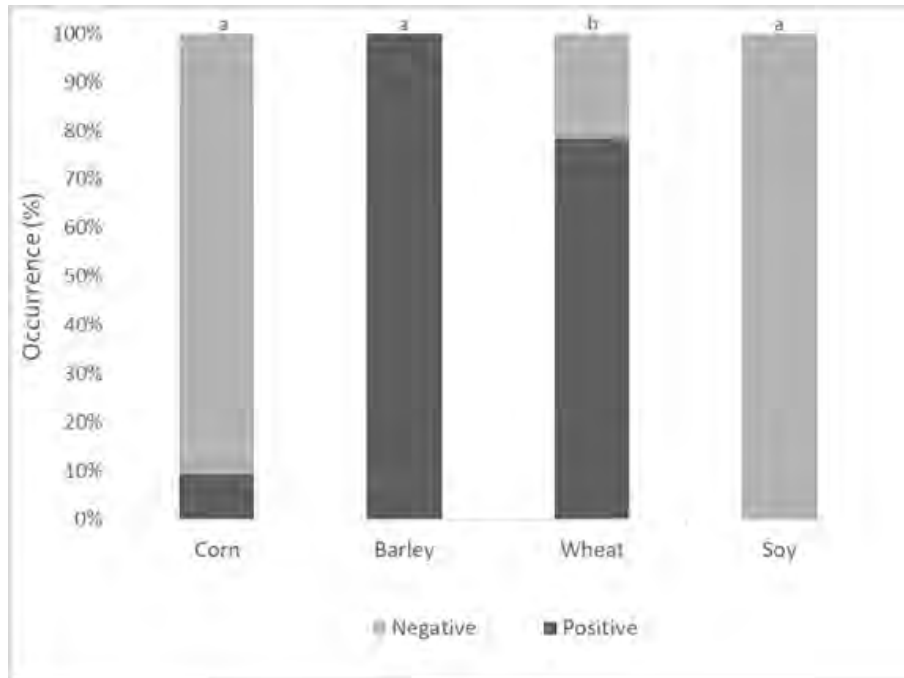
Note: MPL- Maximum Permitted Limits; DON: Deoxynivalenol; a,b: Statistical differences of a specific mycotoxin between different raw materials, ( $p<0.05$ ).

As shown in Figure 6, the mean DON concentration in corn samples (463.5  $\mu\text{g}/\text{kg}$ ) was significantly lower compared to DON mean concentration in barley and wheat samples. In addition, DON mean concentration in soy samples was the lowest measured (<350  $\mu\text{g}/\text{kg}$ ) and significantly lower towards DON values measured in barley and wheat samples (894.5  $\mu\text{g}/\text{kg}$  and 1007.2  $\mu\text{g}/\text{kg}$  respectively).



**Figure 6:** Concentration ( $\mu\text{g}/\text{kg}$  or ppb) of mycotoxin DON (Deoxynivalenol) in corn, barley, wheat and soya samples as aquafeed ingredients.

Figure 7 shows the DON mycotoxin mean occurrence (%) in each of the aquafeed ingredients. DON exhibited a significantly lower occurrence at 9.09% in the analyzed corn samples compared to wheat samples (78.29% mean occurrence). The barley samples (n=2) exhibited 100% contamination with DON mycotoxin. In wheat samples, a significantly higher rate was observed for DON (78.26% mean occurrence) compared to corn- and soy- contaminated samples (9.09% and 0% respectively mean occurrence). In soy samples, DON, was not detected at all.



**Figure 7:** Occurrence (%) of mycotoxin DON in corn, barley, wheat and soya samples as aquafeed ingredients. a, b: denote statistical differences of a specific mycotoxin between different raw materials ( $P < 0.05$ ).

In the following equation total DON occurrence (%) in the total amount of samples (n=124) is calculated as 33.9%.

$$\begin{aligned}
 \text{Total DON occurrence (\%)} &= \frac{\text{SUM (samples per ingredient} \times \text{\% occurrence of DON)}}{\text{total amount of all samples}} \\
 &= \frac{(2 \text{ barley samples} \times 100\%) + (46 \text{ wheat samples} \times 78.29\%) + (44 \text{ corn samples} \times 9.09\%) + (32 \text{ soya samples} \times 0\%)}{124} \\
 &= \mathbf{33.9\%}
 \end{aligned}$$

## 4. Discussion

The inclusion of plant-based protein in fish feed has been identified as a specific contributing factor to the worldwide problem of mycotoxin contamination in animal production, including aquaculture. The problem arises from the fungi that can unexpectedly contaminate feed ingredients. Mycotoxins are present in agricultural products used to feed animals, which puts consumers and farmed animals at risk for health issues.

DON was present in 33.9% of the samples, and the maximum concentration observed was 2338 µg/kg in wheat samples. Moreover, all raw materials displayed positivity for DON mycotoxin. In the review study of Koletsi et al., (2021) DON was prevalent at rates varying from 11% in soy samples to 41 % in wheat and 47% in corn samples (Koletsi et al. 2021).

However, higher average contamination values were observed in analyses of 10 samples of commercial fish and shrimp feeds for DON (165.61 µg/kg), compared to their Asian counterparts (Gonçalves et al. 2018a). Similar data on DON contamination had also been derived from a smaller data set analyzing 11 samples of commercial carp feed (average contamination 289 µg/kg, maximum 825 µg/kg) (Pietsch et al. 2013). Confirming the previous studies, the larger study of Koletsi et al., showed patterns related to mycotoxin contamination that were highly relevant to the animal and fish feed industry. They found DON occurrence in 44 European fish feed samples with an average contamination of 136 µg/kg and a maximum contamination of 469 µg/kg (Koletsi et al. 2021).

Furthermore, DON mean concentration measured in corn samples was 463.5 µg/kg with a maximum of 654 µg/kg. These values were also lower compared to those reported in the recent large study of Koletsi et al., which measured 725 corn samples a mean concentration to be 826 µg/kg (maximum 10,020 µg/kg) for DON.

In our study, in the few barley samples measured (n=2), was found that DON was a prevalent mycotoxin, with a mean concentration of 894.5 µg/kg (maximum 1184 µg/kg). Unfortunately, these values cannot be compared to other sets of published data, as measurements regarding barley samples in aquafeeds are not currently available.

Our wheat samples displayed contamination with DON, with the highest mean concentration of DON among the 4 grain samples, at 1007.2 µg/kg (maximum 2338 µg/kg). Comparative analysis with previously presented European wheat samples in the review study of Koletsi et al. (2021) revealed some higher maximum contamination values for DON but lower mean concentration (470 µg/kg, maximum 8872 µg/kg) in 857 wheat samples.

In our data, for soybean meal samples, concentrations of DON are the lowest among the four grain samples and consistently below the limit of detection of the analytical method (<350µg/kg). Interestingly, soybean meal in aquafeeds had been previously reported (Gonçalves et al. 2020) to be contaminated with DON at higher mean concentrations (230 µg/kg (Greco et al. 2015); 236.2 µg/kg (Pietsch et al. 2013). Moreover, 139 soy samples in the publication of Koletsi, exhibited positivity for DON, with mean concentrations of 85 µg/kg (maximum 543 µg/kg) (Koletsi et al. 2021).

Overall, all results of DON contamination rates (low means and max concentrations of DON) in the four different grains, remained below the maximum permitted limits. To this

direction, in Greek aquaculture the situation appears more advantageous compared to other countries (Gonçalves et al. 2018a, Koletsi et al. 2021). On the other hand, the occurrence of DON contamination in the grain samples examined in the present study, is undoubtedly concerning so as for aquaculture industry to take preventive action measures.

According to Goncalves et al., the results of the most recent mycotoxin occurrence surveys of aquaculture feeds clearly show an increase in mycotoxin occurrence compared to previous surveys. Unfortunately, the authors' data couldn't prove that there is a higher mycotoxin risk now compared to the past. This was because the target mycotoxins analyzed in older studies were not the same and sensitivity detection levels and methodologies have since improved significantly. Nonetheless, it was theoretically expected that an increasing level of plant meals in aquafeeds would lead to increased occurrence of mycotoxins in these feeds, which was observable by the most recent occurrence surveys (Gonçalves et al. 2020).

Mycotoxins affect the whole food chain from fields (grain crops) to aquafeeds, to marine foods to fork. AFB1, DON, ZEN, and FB1 are the four predominant mycotoxins found in animal feeds most frequently (Lee & Ryu 2016), while marine foods contain varying levels of mycotoxins, including AFs, ENs, DON, ZEA, and OTA, all of which are hazardous to human health (Mirza Alizadeh et al. 2022).

Fish health and welfare can be significantly impacted by mycotoxin contamination (increased disease susceptibility, decreased productivity (Koletsi et al. 2021)), and this can also have large financial consequences in the aquaculture industry, as well as health implications for consumers and public health.

It is crucial for the earliest detection of mycotoxins (even at low concentrations), the application of increased monitoring and mitigation strategies, within aquaculture

practices and subsequent treatment or rejection of contaminated feeds. In addition, variability in mycotoxin levels in aquafeed samples across different geographical regions makes this issue even more complex to tackle. Moreover, factors such as climate change and the world trade of commodities make it challenging to estimate the risk of mycotoxins in aquaculture finished feeds.

The increasing globalization of trade commodities and incorporation of imported raw materials into aquafeeds exposes the industry to the potential risk of mycotoxins, which are sometimes not even common for the region. Therefore, mycotoxin contamination needs to take into account the globalization of raw materials, which could already have significant levels of mycotoxins together with the monitoring of finished feeds (Gonçalves et al. 2020).

The European Union (EU) has made mycotoxin contamination in food and feed a top priority, as evidenced by the multiple legislative documents that set maximum recommended levels of contamination (European Commission, 2006, EU 2016). Nonetheless, the observation that the EU has established comparatively high maximum recommendation levels for the detection of mycotoxin in aquafeed ingredients has gained attention, and several scientists suggest stricter legislation to be implemented. In comparison to other countries, our results show lower mean and maximum concentration levels of DON contamination, which highlights the high standards of the Greek aquaculture sector.

## 5. Conclusions

FT-NIR analytical technique was used in a comprehensive study to detect mycotoxin DON in four different types of animal feed ingredients (corn, barley, wheat, and soybean meal) from different Greek suppliers.

Nowadays, mycotoxin contamination is a growing problem for aquaculture in Europe and calls for a multidisciplinary approach. A wide range of expertise is required, so cooperation and communication amongst stakeholders throughout the value chain are essential, as is scientific support from disciplines like toxicology, microbiology, fish nutrition, health and welfare, feed processing and technology, and plant sciences.

DON was present in 33.9% of the 124 samples, and the maximum concentration observed was 2338 µg/kg in wheat samples. Moreover, all raw materials displayed positivity for DON mycotoxin. Our wheat samples displayed contamination with DON, with the highest mean concentration of DON among the 4 grain samples, at 1007.2 µg/kg (maximum 2338 µg/kg). All results expressed as mean and maximum concentrations of DON contamination in the four different grains, remained considerably below the maximum permitted limits. These findings indicate the high standards of the Greek aquaculture industry.

However, it is well known that mycotoxins can seriously impair the health and welfare of farmed animals, both aquatic and terrestrial, even at low concentrations. Therefore, it is thought to be essential to continue monitoring and use an early and rapid method of mycotoxin detection. It is recommended that regulatory bodies should reevaluate the

current DON recommendation limit in order to maintain economic viability and safeguard fish welfare.

## 6. Bibliography

- Abbasi, A., Oujifard, A., Torfi Mozanzadeh, M., Habibi, H., & Nafisi Bahabadi, M. (2020). Dietary simultaneous replacement of fish meal and fish oil with blends of plant proteins and vegetable oils in yellowfin seabream (*Acanthopagrus latus*) fry: Growth, digestive enzymes, antioxidant status and skin mucosal immunity. *Aquaculture Nutrition*, *26*(4), 1131–1142. <https://doi.org/10.1111/anu.13070>
- Albrektsen, S., Kortet, R., Skov, P. V., Ytteborg, E., Gitlesen, S., Kleinegris, D., Mydland, L. T., Hansen, J. Ø., Lock, E. J., Mørkøre, T., James, P., Wang, X., Whitaker, R. D., Vang, B., Hatlen, B., Daneshvar, E., Bhatnagar, A., Jensen, L. B., & Øverland, M. (2022). Future feed resources in sustainable salmonid production: A review. *Reviews in Aquaculture*, *14*(4), 1790–1812. <https://doi.org/10.1111/raq.12673>
- Anastasiadis, V., Raptis, I., Economou, A., Kakabakos, S. E., & Petrou, P. S. (2020). Fast Deoxynivalenol Determination in Cereals Using a White Light Reflectance Spectroscopy Immunosensor. In *Biosensors* (Vol. 10, Issue 11). <https://doi.org/10.3390/bios10110154>
- Beloglazova, N. V., Speranskaya, E. S., Wu, A., Wang, Z., Sanders, M., Gofman, V. V., Zhang, D., Goryacheva, I. Y., & De Saeger, S. (2014). Novel multiplex fluorescent immunoassays based on quantum dot nanolabels for mycotoxins determination. *Biosensors and Bioelectronics*, *62*, 59–65. <https://doi.org/https://doi.org/10.1016/j.bios.2014.06.021>
- Bernhoft, A., Høgåsen, H. R., Rosenlund, G., Ivanova, L., Berntssen, M. H. G., Alexander, J., Eriksen, G. S., & Fæste, C. K. (2017). Tissue distribution and elimination of deoxynivalenol and ochratoxin A in dietary-exposed Atlantic salmon (*Salmo salar*). *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*, *34*(7), 1211–1224. <https://doi.org/10.1080/19440049.2017.1321149>
- Bernhoft, A., Høgåsen, H. R., Rosenlund, G., Moldal, T., Grove, S., Berntssen, M. H. G., Thoresen, S. I., & Alexander, J. (2018). Effects of dietary deoxynivalenol or

- ochratoxin A on performance and selected health indices in Atlantic salmon (*Salmo salar*). *Food and Chemical Toxicology*, *121*, 374–386. <https://doi.org/10.1016/j.fct.2018.08.079>
- Bevilacqua, M., Bucci, R., Materazzi, S., & Marini, F. (2013). Application of near infrared (NIR) spectroscopy coupled to chemometrics for dried egg-pasta characterization and egg content quantification. *Food Chemistry*, *140*(4), 726–734. <https://doi.org/10.1016/j.foodchem.2012.11.018>
- Campos-M, M., & Campos-C, R. (2017). Applications of quartering method in soils and foods. *International Journal of Engineering Research and Applications*, *7*(1), 35–39. <https://doi.org/10.9790/9622-0701023539>
- Cen, H., & He, Y. (2007). Theory and application of near infrared reflectance spectroscopy in determination of food quality. *Trends in Food Science & Technology*, *18*(2), 72–83. <https://doi.org/https://doi.org/10.1016/j.tifs.2006.09.003>
- Deng, J., Jiang, H., & Chen, Q. (2022). Characteristic wavelengths optimization improved the predictive performance of near-infrared spectroscopy models for determination of aflatoxin B1 in maize. *Journal of Cereal Science*, *105*, 103474. <https://doi.org/https://doi.org/10.1016/j.jcs.2022.103474>
- EU. (2016). Commission Recommendation (EU) 2016/ 1319 - amending Recommendation 2006/ 576/ EC as regards deoxynivalenol, zearalenone and ochratoxin A in pet food. In *Off J Eur Union* (Vol. 73, Issue July).
- European Commission. (2006). Commission Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. *Off. J. Eur. Union*, 12–34.
- FAO. (2020). The State of World Fisheries and Aquaculture 2020, Sustainability in Action. In *INFORM* (Vol. 32, Issue 6). American Oil Chemists Society. <https://doi.org/10.4060/ca9229en>
- Galkanda-Arachchige, H. S. C., Wilson, A. E., & Davis, D. A. (2020). Success of fishmeal replacement through poultry by-product meal in aquaculture feed formulations: a meta-analysis. *Reviews in Aquaculture*, *12*, 1624–1636.

<https://doi.org/10.1111/raq.12401>

- Gatlin, D. M., Barrows, F. T., Brown, P., Dabrowski, K., Gaylord, T. G., Hardy, R. W., Herman, E., Hu, G., Krogdahl, Å., Nelson, R., Overturf, K., Rust, M., Sealey, W., Skonberg, D., Souza, E. J., Stone, D., Wilson, R., & Wurtele, E. (2007). Expanding the utilization of sustainable plant products in aquafeeds: A review. In *Aquaculture Research* (Vol. 38, Issue 6, pp. 551–579). <https://doi.org/10.1111/j.1365-2109.2007.01704.x>
- Gonçalves, R. A., Menanteau-Ledouble, S., Schöller, M., Eder, A., Schmidt-Posthaus, H., Mackenzie, S., & El-Matbouli, M. (2019). Effects of deoxynivalenol exposure time and contamination levels on rainbow trout. *Journal of the World Aquaculture Society*, 50(1), 137–154. <https://doi.org/https://doi.org/10.1111/jwas.12542>
- Gonçalves, R. A., Naehrer, K., & Santos, G. A. (2018a). Occurrence of mycotoxins in commercial aquafeeds in Asia and Europe: a real risk to aquaculture? *Reviews in Aquaculture*, 10(2), 263–280. <https://doi.org/10.1111/raq.12159>
- Gonçalves, R. A., Navarro-Guillén, C., Gilannejad, N., Dias, J., Schatzmayr, D., Bichl, G., Czabany, T., Moyano, F. J., Rema, P., Yúfera, M., Mackenzie, S., & Martínez-Rodríguez, G. (2018b). Impact of deoxynivalenol on rainbow trout: Growth performance, digestibility, key gene expression regulation and metabolism. *Aquaculture*, 490, 362–372. <https://doi.org/10.1016/j.aquaculture.2018.03.001>
- Gonçalves, R. A., Schatzmayr, D., Albalat, A., & Mackenzie, S. (2020). Mycotoxins in aquaculture: feed and food. *Reviews in Aquaculture*, 12(1), 145–175. <https://doi.org/10.1111/raq.12310>
- Greco, M., Pardo, A., & Pose, G. (2015). Mycotoxigenic fungi and natural co-occurrence of mycotoxins in rainbow trout (*Oncorhynchus mykiss*) feeds. *Toxins*, 7(11), 4595–4609. <https://doi.org/10.3390/toxins7114595>
- Hardy, R. W. (2010). Utilization of plant proteins in fish diets: Effects of global demand and supplies of fishmeal. *Aquaculture Research*, 41(5), 770–776. <https://doi.org/10.1111/j.1365-2109.2009.02349.x>
- He, Q.-H., Xu, Y., Wang, D., Kang, M., Huang, Z.-B., & Li, Y.-P. (2012). Simultaneous

multiresidue determination of mycotoxins in cereal samples by polyvinylidene fluoride membrane based dot immunoassay. *Food Chemistry*, 134(1), 507–512. <https://doi.org/https://doi.org/10.1016/j.foodchem.2012.02.109>

Hellenic Organisation of Aquaculture Producers. (2022). *Greek Aquaculture: Annual Report 2022*.

Hooft, J., Ferreira, C., Lumsden, J. S., Sulyok, M., Krska, R., & Bureau, D. P. (2019b). The effects of naturally occurring or purified deoxynivalenol (DON) on growth performance, nutrient utilization and histopathology of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, 505, 319–332. <https://doi.org/10.1016/j.aquaculture.2019.02.032>

Hooft, J., Wu, P., Powell, C. D., Lou, Y., Squires, E. J., Cant, J. P., Sulyok, M., Krska, R., & Bureau, D. P. (2019a). A comparative investigation of the effects of feed-borne deoxynivalenol (DON) on growth performance, nutrient utilization and metabolism of detoxification in rainbow trout (*Oncorhynchus mykiss*) and Nile tilapia (*Oreochromis niloticus*) fed diets containing. *Aquaculture*, 505, 306–318. <https://doi.org/https://doi.org/10.1016/j.aquaculture.2019.02.019>

Hooft, J.M., Elmor, A. E. H. I., Encarnaç o, P., & Bureau, D. P. (2011). Rainbow trout (*Oncorhynchus mykiss*) is extremely sensitive to the feed-borne Fusarium mycotoxin deoxynivalenol (DON). *Aquaculture*, 311(1–4), 224–232. <https://doi.org/10.1016/j.aquaculture.2010.11.049>

Hooft, Jamie M, & Bureau, D. P. (2017). Evaluation of the efficacy of a commercial feed additive against the adverse effects of feed-borne deoxynivalenol (DON) on the performance of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, 473, 237–245. <https://doi.org/https://doi.org/10.1016/j.aquaculture.2017.02.019>

International Agency for Research on Cancer. A Review of Human Carcinogens. F. Chemical Agents and Related Occupations: IARC Monographs on the Evaluation of Carcinogenic Risks to Humans; International Agency for Research on Cancer: Lyon, France, 2012; p. 100F.

Karapanagiotidis, I. T., Metsoviti, M. N., Gkalogianni, E. Z., Psoufakis, P., Asimaki, A., Katsoulas, N., Papapolymerou, G., & Zarkadas, I. (2022). The effects of replacing

- fishmeal by *Chlorella vulgaris* and fish oil by *Schizochytrium* sp. and *Microchloropsis gaditana* blend on growth performance, feed efficiency, muscle fatty acid composition and liver histology of gilthead seabream (*Sparus aurata*). *Aquaculture*, 561(April), 738709. <https://doi.org/10.1016/j.aquaculture.2022.738709>
- Koletsis, P. et al. (2023a). Individual and combined effects of deoxynivalenol (DON) with other *Fusarium* mycotoxins on rainbow trout (*Oncorhynchus mykiss*) growth performance and health. *Mycotoxin Res.* 39, 405–420
- Koletsis, P., Graat, E. A. M., Lyons, P., Wiegertjes, G. F., & Schrama, J. W. (2023b). Are the effects of Deoxynivalenol (DON) on performance, liver and gastrointestinal tract health of rainbow trout (*Oncorhynchus mykiss*) influenced by dietary composition? *Aquaculture Reports*, 32. <https://doi.org/10.1016/j.aqrep.2023.101740>
- Koletsis, P., Schrama, J., Graat, E., Wiegertjes, G., 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. In *Toxins* (Vol. 13, Issue 6). MDPI AG. <https://doi.org/10.3390/toxins13060403>
- Kövesi, B., Pelyhe, C., Zándoki, E., Mézes, M., & Balogh, K. (2020). Combined effects of aflatoxin B1 and deoxynivalenol on the expression of glutathione redox system regulatory genes in common carp. *Journal of Animal Physiology and Animal Nutrition*, 104(5), 1531–1539. <https://doi.org/https://doi.org/10.1111/jpn.13343>
- Lee, H. J., & Ryu, D. (2016). *Worldwide Occurrence of Mycotoxins in Cereals and Cereal-Derived Food Products: Public Health Perspectives of Their Co-occurrence*. <https://doi.org/10.1021/acs.jafc.6b04847>
- Li, P., Zhang, Q., & Zhang, W. (2009). Immunoassays for aflatoxins. *TrAC Trends in Analytical Chemistry*, 28(9), 1115–1126. <https://doi.org/https://doi.org/10.1016/j.trac.2009.07.003>
- Li, S., Wang, J., Sheng, W., Wen, W., Gu, Y., & Wang, S. (2018). Fluorometric lateral flow immunochromatographic zearalenone assay by exploiting a quencher system composed of carbon dots and silver nanoparticles. *Microchimica Acta*, 185(8), 388.

<https://doi.org/10.1007/s00604-018-2916-1>

- Liu, Y., Chen, Y., Xu, W., Song, D., Han, X., & Long, F. (2022). Rapid, Sensitive On-Site Detection of Deoxynivalenol in Cereals Using Portable and Reusable Evanescent Wave Optofluidic Immunosensor. In *International Journal of Environmental Research and Public Health* (Vol. 19, Issue 7). <https://doi.org/10.3390/ijerph19073759>
- Mallison, A. (2013). *Fishery discards and by-products: increasing raw material supply for fishmeal and fish oil*. February, 1–2.
- Matejova, I., Svobodova, Z., Vakula, J., Mares, J., & Modra, H. (2017). Impact of Mycotoxins on Aquaculture Fish Species: A Review. *Journal of the World Aquaculture Society*, 48(2), 186–200. <https://doi.org/10.1111/jwas.12371>
- McMullin, D., Mizaikoff, B., & Krska, R. (2015). Advancements in IR spectroscopic approaches for the determination of fungal derived contaminations in food crops. *Analytical and Bioanalytical Chemistry*, 407(3), 653 – 660. <https://doi.org/10.1007/s00216-014-8145-5>
- Moldal, T., Bernhoft, A., Rosenlund, G., Kaldhusdal, M., & Koppang, E. O. (2018). Dietary Deoxynivalenol (DON) May Impair the Epithelial Barrier and Modulate the Cytokine Signaling in the Intestine of Atlantic Salmon (*Salmo salar*). *Toxins*, 10(9). <https://doi.org/10.3390/toxins10090376>
- Nathanail, A. V., Varga, E., Meng-Reiterer, J., Bueschl, C., Michlmayr, H., Malachova, A., Fruhmann, P., Jestoi, M., Peltonen, K., Adam, G., Lemmens, M., Schuhmacher, R., & Berthiller, F. (2015). Metabolism of the Fusarium Mycotoxins T-2 Toxin and HT-2 Toxin in Wheat. *Journal of Agricultural and Food Chemistry*, 63(35), 7862–7872. <https://doi.org/10.1021/acs.jafc.5b02697>
- Oliva-Teles, A., Enes, P., & Peres, H. (2015). Replacing fishmeal and fish oil in industrial aquafeeds for carnivorous fish. In *Feed and Feeding Practices in Aquaculture*. Elsevier Ltd. <https://doi.org/10.1016/B978-0-08-100506-4.00008-8>
- Pelyhe, C., Kövesi, B., Zándoki, E., Kovács, B., Szabó-Fodor, J., Mézes, M., & Balogh, K. (2016). Effect of 4-week feeding of deoxynivalenol- or T-2-toxin-contaminated

- diet on lipid peroxidation and glutathione redox system in the hepatopancreas of common carp (*Cyprinus carpio* L.). *Mycotoxin Research*, 32(2), 77–83. <https://doi.org/10.1007/s12550-016-0242-1>
- Pietsch, C. (2020). Risk assessment for mycotoxin contamination in fish feeds in Europe. *Mycotoxin Research*, 36(1), 41–62. <https://doi.org/10.1007/s12550-019-00368-6>
- Pietsch, C., & Burkhardt-Holm, P. (2015). Feed-borne exposure to deoxynivalenol leads to acute and chronic effects on liver enzymes and histology in carp. *World Mycotoxin Journal*, 8(5), 619–627. <https://doi.org/10.3920/WMJ2015.1879>
- Pietsch, C., Katzenback, B. A., Garcia-Garcia, E., Schulz, C., Belosevic, M., & Burkhardt-Holm, P. (2015). Acute and subchronic effects on immune responses of carp (*Cyprinus carpio* L.) after exposure to deoxynivalenol (DON) in feed. *Mycotoxin Research*, 31(3), 151–164. <https://doi.org/10.1007/s12550-015-0226-6>
- Pietsch, C., Kersten, S., Burkhardt-Holm, P., Valenta, H., & Dänicke, S. (2013). Occurrence of deoxynivalenol and zearalenone in commercial fish feed: An initial study. *Toxins*, 5(1), 184–192. <https://doi.org/10.3390/toxins5010184>
- Pietsch, C., Michel, C., Kersten, S., Valenta, H., Dänicke, S., Schulz, C., Kloas, W., & Burkhardt-Holm, P. (2014a). In vivo effects of deoxynivalenol (DON) on innate immune responses of carp (*Cyprinus carpio* L.). *Food and Chemical Toxicology*, 68, 44–52. <https://doi.org/https://doi.org/10.1016/j.fct.2014.03.012>
- Pietsch, C., Schulz, C., Rovira, P., Kloas, W., & Burkhardt-Holm, P. (2014b). Organ Damage and Hepatic Lipid Accumulation in Carp (*Cyprinus carpio* L.) after Feed-Borne Exposure to the Mycotoxin, Deoxynivalenol (DON). *Toxins*, 6(2), 756–778. <https://doi.org/10.3390/toxins6020756>
- Rausch, A.-K., Brockmeyer, R., & Schwerdtle, T. (2020). Development and Validation of a QuEChERS-Based Liquid Chromatography Tandem Mass Spectrometry Multi-Method for the Determination of 38 Native and Modified Mycotoxins in Cereals. *Journal of Agricultural and Food Chemistry*, 68(16), 4657–4669. <https://doi.org/10.1021/acs.jafc.9b07491>
- Razzazi-Fazeli, E., & Reiter, E. V. (2011). 2 - Sample preparation and clean up in

- mycotoxin analysis: principles, applications and recent developments. In S. De Saeger (Ed.), *Determining Mycotoxins and Mycotoxigenic Fungi in Food and Feed* (pp. 37–70). Woodhead Publishing. <https://doi.org/https://doi.org/10.1533/9780857090973.1.37>
- Ríos, A., Zougagh, M., & Bouri, M. (2013). Magnetic (nano)materials as an useful tool for sample preparation in analytical methods. A review. *Anal. Methods*, 5(18), 4558–4573. <https://doi.org/10.1039/C3AY40306H>
- Rodrigues, I., & Naehrer, K. (2009). A Three-Year Survey on the Worldwide Occurrence of Mycotoxins in Feedstuffs and Feed. *Toxins*, 4, 663–675. <https://doi.org/10.3390/toxins4090663>
- Roggo, Y., Chalus, P., Maurer, L., Lema-Martinez, C., Edmond, A., & Jent, N. (2007). A review of near infrared spectroscopy and chemometrics in pharmaceutical technologies. *Journal of Pharmaceutical and Biomedical Analysis*, 44(3 SPEC. ISS.), 683 – 700. <https://doi.org/10.1016/j.jpba.2007.03.023>
- Ryerse, I A, Hooft, J. M., Bureau, D. P., Hayes, M. A., & Lumsden, J. S. (2015). Purified deoxynivalenol or feed restriction reduces mortality in rainbow trout, *Oncorhynchus mykiss* (Walbaum), with experimental bacterial coldwater disease but biologically relevant concentrations of deoxynivalenol do not impair the growth of *Flavobacterium psychrophilum*. *Journal of Fish Diseases*, 38(9), 809–819. <https://doi.org/https://doi.org/10.1111/jfd.12295>
- Ryerse, Ian A, Hooft, J. M., Bureau, D. P., Anthony Hayes, M., & Lumsden, J. S. (2016). Diets containing corn naturally contaminated with deoxynivalenol reduces the susceptibility of rainbow trout (*Oncorhynchus mykiss*) to experimental *Flavobacterium psychrophilum* infection. *Aquaculture Research*, 47(3), 787–796. <https://doi.org/https://doi.org/10.1111/are.12537>
- Sanden, M., Jørgensen, S., Hemre, G.-I., Ørnsrud, R., & Sissener, N. H. (2012). Zebrafish (*Danio rerio*) as a model for investigating dietary toxic effects of deoxynivalenol contamination in aquaculture feeds. *Food and Chemical Toxicology*, 50(12), 4441–4448. <https://doi.org/https://doi.org/10.1016/j.fct.2012.08.042>
- Silva, A. S., Brites, C., Pouca, A. V., Barbosa, J., & Freitas, A. (2019). UHPLC-ToF-MS

- method for determination of multi-mycotoxins in maize: Development and validation. *Current Research in Food Science*, 1, 1–7. <https://doi.org/https://doi.org/10.1016/j.crfs.2019.07.001>
- Soleimany, F., Jinap, S., Faridah, A., & Khatib, A. (2012). A UPLC-MS/MS for simultaneous determination of aflatoxins, ochratoxin A, zearalenone, DON, fumonisins, T-2 toxin and HT-2 toxin, in cereals. *Food Control*, 25(2), 647 – 653. <https://doi.org/10.1016/j.foodcont.2011.11.012>
- Szczepański, A., Adamek-Urbańska, D., Kasprzak, R., Szudrowicz, H., Śliwiński, J., & Kamaszewski, M. (2022). Lupin: A promising alternative protein source for aquaculture feeds? In *Aquaculture Reports* (Vol. 26).
- Tacon, A. G. J., Hasan, M. R., & Metian, M. (2011). Demand and supply of feed ingredients for farmed fish and crustaceans : Trends and prospects. In *FAO Fisheries and Aquaculture Technical Paper* (Vol. 564).
- Tacon, A. G. J., & Metian, M. (2008). Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. *Aquaculture*, 285(1–4), 146–158. <https://doi.org/10.1016/j.aquaculture.2008.08.015>
- Tacon, A. G. J., & Metian, M. (2015). Feed matters: Satisfying the feed demand of aquaculture. *Reviews in Fisheries Science and Aquaculture*, 23(1), 1–10. <https://doi.org/10.1080/23308249.2014.987209>
- Tola, S., Bureau, D. P., Hooft, J. M., Beamish, F. W. H., Sulyok, M., Krska, R., Encarnação, P., & Petkam, R. (2015). Effects of wheat naturally contaminated with *Fusarium* mycotoxins on growth performance and selected health indices of red tilapia (*Oreochromis niloticus* × *O. mossambicus*). *Toxins*, 7(6), 1929–1944. <https://doi.org/10.3390/toxins7061929>
- Turchini, G. M., Torstensen, B. E., & Ng, W. K. (2009). Fish oil replacement in finfish nutrition. *Reviews in Aquaculture*, 1(1), 10–57. <https://doi.org/10.1111/j.1753-5131.2008.01001.x>
- Turner, N. W., Bramhmbhatt, H., Szabo-Vezse, M., Poma, A., Coker, R., & Piletsky, S. A. (2015). Analytical methods for determination of mycotoxins: An update (2009–

- 2014). *Analytica Chimica Acta*, *901*, 12–33. <https://doi.org/https://doi.org/10.1016/j.aca.2015.10.013>
- Tyska, D., Mallmann, A., Gressler, L. T., & Mallmann, C. A. (2021). Near-infrared spectroscopy as a tool for rapid screening of deoxynivalenol in wheat flour and its applicability in the industry. *Food Additives & Contaminants: Part A*, *38*(11), 1958–1968. <https://doi.org/10.1080/19440049.2021.1954699>
- Van der Fels-Klerx, H. J., Focker, M., De Rijk, T., & Liu, C. (2021). Mycotoxins in wheat cultivated in the Netherlands: results from eight years of field surveys. *Mycotoxin Research*, *37*(2), 183–192. <https://doi.org/10.1007/s12550-021-00427-x>
- Vardali, S., Papadouli, C., Rigos, G., Nengas, I., Panagiotaki, P., & Golomazou, E. (2023). Recent Advances in Mycotoxin Determination in Fish Feed Ingredients. In *Molecules* (Vol. 28, Issue 6). MDPI. <https://doi.org/10.3390/molecules28062519>
- Wang, Y.-K., Yan, Y.-X., Li, S.-Q., Wang, H., Ji, W.-H., & Sun, J.-H. (2013). Simultaneous Quantitative Determination of Multiple Mycotoxins in Cereal and Feedstuff Samples by a Suspension Array Immunoassay. *Journal of Agricultural and Food Chemistry*, *61*(46), 10948–10953. <https://doi.org/10.1021/jf4036029>
- World Health Organization; International Agency for Research on Cancer. Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins. In IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans; World Health Organization: Geneva, Switzerland, 1993; Volume 56.
- Woodward, B., Young, L. G., & Lun, A. K. (1983). Vomitoxin in diets for rainbow trout (*Salmo gairdneri*). *Aquaculture*, *35*, 93–101. [https://doi.org/https://doi.org/10.1016/0044-8486\(83\)90077-7](https://doi.org/https://doi.org/10.1016/0044-8486(83)90077-7)
- Zhang, Z., Nie, D., Fan, K., Yang, J., Guo, W., Meng, J., Zhao, Z., & Han, Z. (2019). *Critical Reviews in Food Science and Nutrition A systematic review of plant-conjugated masked mycotoxins: Occurrence, toxicology, and metabolism A systematic review of plant-conjugated masked mycotoxins: Occurrence, toxicology, and metabolism*. <https://doi.org/10.1080/10408398.2019.1578944>

## 7. Abstract

Several kinds of fungi produce secondary metabolites called mycotoxins. They can have a detrimental effect on fish farming practices and are commonly found in ingredients for fish feeds.

Fourier-Transform Near Infrared Spectroscopy (FT-NIR) was used in this study to perform a spectroscopic analysis of aquafeed ingredients that were sourced from Greek suppliers. The use of FT-NIR demonstrates that it is a useful analytical tool for contaminant detection, providing clear benefits over conventional analytical techniques, such as speed, cost-effectiveness, safety, and simultaneous analysis of several parameters. By measuring the spectral absorption from the organic compounds present in the samples, the concentration of mycotoxin was indirectly estimated.

All results expressed as mean and maximum concentrations of DON contamination in the four different grains, remained considerably below the maximum permitted limits. These findings demonstrate the high standards of Greek aquaculture industry.

In the examined samples (n=124) deoxynivalenol (DON) mycotoxin was measured. DON was present in 33.9% of the 124 samples, and the maximum concentration observed was 2338 µg/kg in wheat samples. Moreover, wheat, corn and barley raw materials but not soya, displayed positivity for DON mycotoxin. Remarkably, all mycotoxin concentrations in aquafeed ingredients remained well below the maximum permitted limits (MPL), confirming the safety of aquaculture feeds used in Greece in compliance with the relevant legislation.

In conclusion, since the presence of mycotoxins, even in low concentrations, may be hazardous to animal and public health, it is imperative to continuously monitor the ingredients in fish feeds.

Keywords: mycotoxin, DON, fish feeds, wheat