Università degli Studi di Padova

Dipartimento di Biomedicina Comparata ed Alimentazione
Dipartimento di Medicina Animale, Produzioni e Salute

Corso di Laurea Magistrale interfacoltà in
Biotecnologie per l'Alimentazione

Tesi di Laurea

EVALUATION OF ADVERSE EFFECT OF DEOXYNIVALENOL AND ZEARALENONE ON FARMED NILE TILAPIA (Oreochromis niloticus)

Relatore: **Dott. Leonardo Alberghini**
Dipartimento di Medicina Animale, Produzioni e Salute

Correlatori: **Prof.ssa Antonella Dalle Zotte**
Dipartimento di Medicina Animale, Produzioni e Salute
Dott.ssa Judit Szabó-Fodor
Institute of Physiology, Biochemistry and Animal Health
Kaposvár University, Hungary

Laureando: Alberto Braggio
Matricola: 1058647

Anno Accademico 2014-2015

RINGRAZIAMENTI

The research of this Master Thesis paper is the result of a working stage conduced at the Kaposvàr University in Hungary, where I had the opportunity to practice and assist to new activities, enriching thus my knowledge and experience.
This experience could not have been possible, were it not for my Erasmus flow responsible, Antonella dalle Zotte, who helped and advised me before and after the project. The help the Szabó-Fodor family gave me, advising and revisioning my work ad thesis, teaching me how to use the statistic software and enriching the knowledge on the thesis subjects was necessary for my personal and professional growth, thus they deserve a heartly thanks.
Special thanks are to address to Dr. Melinda Kovács, who coordinated the experiment, but I also would like to thank the rest of the laboratory staff, specially Andràs Bonai for his friendship, disposability and the microbiology experiences performed.

Ringrazio il Professor Antonio Frangipane per avermi permesso di utilizzare il microscopio millimetrato, indispensabile per l'analisi delle uova. Ringrazio inoltre il Dott. Alberghini per avermi aiutato e consigliato in merito a tesi e presentazione.

The Erasmus experience granted me the chance to meet extraordinary people coming from different Countries and Continents. I warmly remember Randel Antona and Dr. Munene Kitama, the soon-to-be PhD Mariam Kachlek and all the international students. I'd like to thank the hungarian friends too, particularly Laslò Kacsala and Zoltan Weber, for introducing me to their culture and habits.

Ringrazio inoltre la mia famiglia per avermi sempre motivato a continuare ed impegnarmi, Erica per il supporto, l'amore e l'indispensabile aiuto stilistico.
Ringrazio gli amici che si sono allontanati, quelli che si sono avvicinati e soprattutto quelli che mi sono stati sempre vicino per avermi sopportato e supportato.

Infine un grazie di cuore lo dedico al nostro anno di corso a BAL, per le stupende persone con cui sono entrato in contatto, per l'ambiente di studio stimolante, per le amicizie e per le conoscenze non solo didattiche che ci siamo potuti scambiare.

A tutti voi,
Grazie, Thank you, Köszönöm.

ABSTRACT

Tilapia is one of the most farmed fishes all over the world, having a good Food Conversion Rate (FCR), high fillet yield along with flavorless meat. These features are the base of its success among the consumers of the middle and low class, especially in the US, who are not used to fish flavor, and the producers, who have an easily and cheaply reared fish. For ethical and economical reasons fish feed, usually made with fish oil and meat, is currently being modified and replaced with a vegetable protein and lipid source. In this process new hazards come to light for aquacultured fish, such as mycotoxicosis. Mycotoxins are secondary metabolites produced by many mold strains, and new toxins are discovered every year. The best known and most common mycotoxins derive from *Fusarium* and *Aspergillus* strains, with aflatoxins and fumonisins being considered the most toxic. Among the *Fusarium*-produced mycotoxins there are zearalenone(ZEA) and deoxynivalenol (DON) too, both produced mainly by the same mold strain (*F.graminearum*); these two toxins are some of the most common in nature, affecting grains crops all over the world.

Being tilapia one of the most farmed fish while DON and ZEA are two of the most often occurring toxins. This study aims to evaluate the negative effect that a legally acceptable contamination of the feed may have on productive and reproductive parameters as well as animal health issues.

A 6 week experiment started with 7 month-old tilapias being reared in 460 l aquaria and fed with contaminated feed (3% of bodyweight). Fish were separated into 4 groups of exposure: Control (diet not contaminated), Treatment 1, Treatment 2 and Treatment 3, exposed to 1ppm, 3 ppm and 5 ppm of mycotoxins respectively.

The results obtained showed a statistically significant difference in live weight between the control group and the others, as well as a difference in lipid profile (some PUFA and palmitic acid), all involved in inflammatory response, but no difference in oxidative stress indicators such as GsH and MDA.

A preliminary study on the correlation between DON concentration and TMA led to the identification of a not significant difference among the groups for the odor profile, with a 80% accuracy on a 90% needed, suggesting that a longer study is needed to corroborate this correlation.

Keywords: Tilapia, DON, ZEA, TMA

RIASSUNTO

Tilapia è il nome comune di una tipologia di pesci appartenenti alla famiglia dei *Cichlidae*, ed è caratterizzata da un buon indice di conversione alimentare, una buona resa in filetto. Le carni si presentano di colore rosa pallido, dall'odore e sapore non molto marcati, caratteristiche che hanno reso questo pesce apprezzato dalla classe media e bassa non abituata al consumo di pesce, specie negli Stati Uniti.
La facilità d'impiego e le caratteristiche organolettiche, assieme alla facilità ed economicità di allevamento, hanno reso questo pesce una delle specie ittiche più allevate al mondo.
Il mangime per la piscicoltura sta subendo in questi anni una rivoluzione dettata sia da ragioni etiche che economiche. Tradizionalmente il mangime per pesci era a base di oli e proteine derivati da pesci stessi, mentre ora si tenta di sostituire tali fonti lipidiche e proteiche con quelle vegetali. Questa sostituzione porta però all'insorgenza di nuovi rischi, precedentemente inesistenti, come ad esempio le micotossine.
Le micotossine sono metaboliti secondari prodotti da varie specie di muffa, ed ogni anno ne vengono scoperte di nuove. Le specie produttrici di micotossine ad oggi più presenti appartengono ai generi *Aspergillus* e *Fusarium*, produttrici di aflatossine e fumonisine, le micotossine ritenute più dannose.
Solitamente le varie specie fungine sono in grado di produrre più tossine, anche contemporaneamente, come nel caso del *Fusarium graminearum*, produttore sia di deossinivalenolo che di zearalenone, che sono tra le micotossine più comunemente riscontrate nei raccolti di cereali di tutto il mondo.
Essendo la tilapia uno dei pesci più allevati ed essendo DON e ZEA fra le micotossine più presenti nelle contaminazioni vegetali, questo studio si pone l'obiettivo di indagare sugli effetti che un'interazione fra questi elementi possa avere sui parametri produttivi e riproduttivi del pesce, con riguardo anche del benessere animale.
Lo studio qui presentato, della durata di 6 settimane, prevedeva l'uso di pesci di 7 mesi allevati in acquari da 460 litri, alimentati con una dieta sperimentalmente contaminata a 1ppm, 3 ppm e 5 ppm più il gruppo di controllo. Le dosi di micotossine rientrano tutte nei limiti previsti dalla legge europea.

I risultati mostrano che uno scarto significativo di peso è presente fra il peso del gruppo di controllo rispetto ai trattamenti, il profilo lipidico differisce fra il controllo ed il gruppo a maggiore esposizione per due grassi PUFA e l'acido palmitico, tutti coinvolti nella risposta immunitaria, mentre non vi è differenza significativa per gli indicatori di stresso ossidativo, come GsH e MDA.
È stata svolta anche un'analisi preliminare per cercare una correlazione fra esposizione a DON ed aumento di presenza di TMA, molecola responsabile del cattivo odore nel pesce. L'analisi statistica ha individuato diversi profili odorosi per ogni gruppo e si è svolta una cross-validazione per determinare se le differenze fossero statisticamente significative. La soglia di accuratezza richiesta per il successo del test è del 90% tuttavia l'analisi di questi dati ha raggiunto solamente l'80%. Tali valutazioni rendono il risultato non statisticamente significativo, l'insuccesso potrebbe essere tuttavia dovuto all'esposizione subcronica della tossina durante l'esperimento.

List of Abbreviations

AA	Arachidonic Acid
AFB	Aflatoxin B1
ANOVA	Analysis of variance
ATA	Alimentary Toxic Aleukia
aw	Activity Water
BF_3	Methanolic Boron Trifluoride
BW	Body Weight
DAS	4,15-Diacetoxyscirpenol
DGLA	Dihomo-Gamma-Linolenic Acid
DMA	DiMethilAmmine
DNA	DeossiRibonucleic Acid
DPA	DocosaPentaenoic Acid
DON	DeOxyNivalenol
•3-Ac-DON	3-Acetyl- Deoxynivalenol
EC	Esophageal Cancer
EFSA	European Food Safety Authority
ELEM	Equine LeucoEncephaloMalacia
ELISA	Enzyme-Linked ImmunoSorbent Assay
ETE	12,13-EpoxiTricothec-9-Ene
FA	FormAldehyde
FAO	Food and Agriculture Organization
FCR	Food Conversion Rate
FE	Feed Efficiency
GsH	Glutathione
GsHPX	Glutathione Peroxidase
GSI	GonadoSomatic Index
HPLC	High Pressure Liquid Cromatoghraphy
IPD	Individual Protection Devices
kb	Kilobase (number of nucleotides 103)
kg BW	Kilograms of Body Weight
LC/MS	Liquid Cromatography/ Mass Spectrometry
LD50	Lethal Dose for the 50th percentile
LOAEL	Lowest Observable Adverse Effect Level
LOD	Limit Of Detection
LOQ	Limit Of Quantification

LSD			Least Significant Difference
MAPS			Medicina Animale, Produzione e Salute
MDA			MalonDiAldehye
MOS			Metal Oxide Sensor
MT			Methyl Testosterone
MTT			3-(4,5-dimethyltiazol-2-Yi)-2,5-Diphenyltetrazolium Bromide
NIV			NIValenol
	•	ANIV	Acetyl NIV
PAF			Platelet Activating Factor
PCR			Polymerase Chain Reaction
	•	DD-PCR	Differential Display PCR
PDA			Potato Dextrose Agar
ppb			Parts Per Billion
ppm			Parts Per Million
PUFA			PoliUnsaturated Fatty Acids
REGG .			Regulations
RNA			RiboNucleic Acid
SCF			Scientific Committee on Food
SCFA			Short Chain Fatty Acids
SPSS			Statistical Package for Social Science
TBA			ThiobarBituric Acid
	•	TBARS	TBA Reactive Substances
TCA			TriCloroAcetic (acid)
TDI			Tolerable Daily Intake
	•	p-TDI	Provisional TDI
	•	t-TDI	Toxicological TDI
TLC			Thin Layer Chromatography
TMA			TriMethilAmmine
	•	TMA-O	TMA-Oxide
TOF			Time Of Flight
TVBN			Total Volatile Basic Nitrogen
TXA2			ThromboXane A2
USA	or US		United States (of America)
USDA			United States Department of Agriculture
ZEA			Zearalenone
	•	ZEN	Zearalenol

1. Introduction:

1.1 Aquaculture importance and general features

Fish is one of the most important food sources in general and protein source in particular. All across the world, every country or region which has access to lakes, rivers, seas or oceans have their own typical recipes, thus giving us evidence of its culinary importance. FAO estimates a worldwide fish consumption of 131 million tons per year, leading to an average per capita consumption of 18.8 kg/year, with China being the most important consumer all over the world with 31.9 kg/year per capita (FAO, 2012).
Fish provides an amount of protein similar to meat, (Soriguer et al, 1996) and being animal proteins they don't lack any amino acids; along with that fish usually has a lower fat content than terrestrial animal, making it a healthier alternative to traditional meat diets. In addition to this, carnivorous cold saltwater fish contain large amounts of long chain Ω-3 PUFA, which are very useful, lowering the risk of stroke and fatal coronary heart diseases (Kromhout et al, 2010).
Due to intensive fishing and new fishing techniques, pollution and introduction of alien species, the fish stocks in our seas and lakes are becoming under-populated, affecting both monetary income of fishermen and the ecological health of rivers, seas, lakes and oceans.
To face this problem some countries forbid fishing activities during some parts/seasons of the year, in turn allowing more fishes to reproduce, and impose a minimum size and number for every species, allowing them to reproduce at least once in their life. These proceedings lead to a lower quantity of fish to be sold, therefore it affects even factory production of frozen fish and partially prepared food. To mitigate these problems and keeping a constant amount of fishes during all the year, without pauperizing wildlife stock, there is now use of aquaculture process, which is breeding fishes in a restricted area of enclosure. This also ensures faster rates of growth due to limited expenditure of energy.
The European Union has also promulgated a Regulation, number 304/2011, to preserve European environment from the introduction of foreign species, by imposing on aquaculture breeders to have an efficient way of avoiding accidental release of species in the ecosystem as well as their feces.
With aquaculture technique it's possible to control the feed intake, fish health, preventing or treating diseases and parasites and having fish all the time of the year; these fish will also be cheaper than the wild ones because of the feed composition

(composed by corn and soybean too), less mortality (due to health care and lack of predation), faster growing (due to maintenance of optimum range of pH, temperature and water impurities such as ammonia and feces) and the lack of need of particular fishing devices or expensive fishing boats.
Aquaculture can be performed in a number of ways; the most common are pond, cage, raceway and re-circulating systems. Ponds are the easiest way to produce fish, its structure being a basin-like depression on the ground with earth levees; it is also possible to use large ponds for caged production, which entails placing the farmed fish in underwater cages (can be either inland or marine water) which naturally provide the water flow changing. With this technique the fish will be easily susceptible to pollution and it will be possible for the release of foreign species in different environment. Raceway culture consist of tank farming, which is more sophisticated and productive than pond and cage cultures, but as a drawback they're even more expensive; they provide a continual replenishment of the cages, allowing fish to live in cleaner water and having therefore less stress and more room to live and grow.

According to FAO the most important species for aquaculture are carps, shrimps, clams, tilapia and salmon. Not all the fishes fit the aquaculture method, for example tuna. A "new" species that has acquired increasing interest in aquaculture is tilapia, which, due to its flavorless meat and cheapness can be appreciated by lower and middle-class people not used to eating fish. Due to its short productive cycle and vegetarian habits it's easily farmed and by now is one of the largest aquaculture production in the world, 3.58 million tons in 2013, with USA as the largest importer in global market.

1.2 Tilapia main features and history

Tilapia is a common name for a large number of species belonging to *Cichlidae*, however the most economically relevant species is the *Oreochromis niloticus*, which inhabits the Nile river, as the name may suggest, and is also found in Ivory Coast. Usually this fish lives in shallow water, where it can find its food, such as phytoplankton, periphyton, aquatic plants, small invertebrates, benthic fauna, detritus and bacterial films associated with detritus. Nile tilapia can filter feed by entrapping suspended particles, including phytoplankton and bacteria, on mucous in the buccal cavity,

although its main source of nutrition is obtained by surface grazing on periphyton mats. In its native region this fish can live in the optimal range temperature, which varies from 31 to 36 Celsius degrees, and grow up to 5 kilos (Rakocy, 2005).

Figure 1: Tilapia biological features

Body compressed; caudal peduncle depth equal to length. Scales cycloid. A knob-like protuberance absent on dorsal surface of snout. Upper jaw length showing no sexual dimorphism. First gill arch with 27 to 33 gillrakers. Lateral line interrupted. Spinous and soft ray parts of dorsal fin continuous. Dorsal fin with 16 - 17 spines and 11 to 15 soft rays. Anal fin with 3 spines and 10-11 rays. Caudal fin truncated. Color in spawning season, pectoral, dorsal and caudal fins becoming reddish; caudal fin with numerous black bars. (Pulvenis et al., 2009) Picture taken from Americas tilapia alliance

Tilapia farming is actually one of the most ancient fish breeding, as it began more than 4000 years ago with the ornamental ponds in ancient Egypt. Industrial farming started in 1940s with *Oreochromis mossambensis* in Japan, Thailand and Philippines. In 1970s *O. niloticus* was initially carried from Ivory Coast to Brazil, from where it was introduced for US aquaculture. The world's largest producer of Tilapia nowadays is China, where this fish arrived in 1978; in this country many ponds were settled to breed it, leading to a great production of Tilapia fish. Furthermore thanks to hormonal

utilization it was possible to obtain batches of only male fishes, with a better Conversion index and faster growth. With this, the tilapia prices decreased and became an easily suitable food source all over China and south east Asia.
One of the drawbacks of this fish breeding is that they are "maternal mouth brooder", which means that they make the eggs hatch in the female mouth. This obviously leads to a lower egg production than other pond fishes. However this discomfort is easily avoidable by controlling water temperature, because as long as the temperature doesn't fall below 24° Celsius females keep spawning eggs. It should also be provided a period in which the fishes are separated by sex and not allowed to mate because females don't eat during reproductive season (Pulvenis, 2008).

1.3 Production of Tilapia

Usually big farming companies have their own hatchery facilities, so that they can continue having their own fry from the previous generations. The mating process starts at the right water temperature and after the male has made the nest; even though a single male can fertilize several nest, they put 1 male tilapia for every 3 or 4 females, limiting competition. After the mating process females can be separated from the males and they have to be fed with a high energy feed (during this period they eat less or they don't eat at all).
At this point there are two possible strategies, the first one requires more time but less labor; entailing putting the fish out of the fry by use of a mesh six times a day with 5 days intervals up to 8-10 weeks. At the end of this process the pond or tank shall be drained and clean before inserting new brood females because the older fry have a great predatory instinct in younger fry. The other strategy consist of catching every single female tilapia and collecting the eggs from her mouth before they hatch, in order to transport them to a tank in which they will develop until the absorption of vitelline sac. This is a very "fast" technique because it need just a day to be completed, but can be used only where it's economically suitable because of the working hours of the workers, like South East Asia.
In many parts of the world, after this time of egg harvesting, it's usual to provide the fish with a high dose of a male hormone, 17β methyltestosterone (MT), in order to have a sex reversal of the females. The growing rates of male tilapia is almost double that

of the female, and it's due both to egg production and starving during mating and brooding period.
After this phase fingerlings are farmed until they reach a proper size, 30 to 40 g, to enter the growing facilities. In this process they are fed with feed that contains at least 30% proteins at an initial rate of 15% of the total live weight in the pond or tank per day. This amount of feed gradually decrease to 4%, which is the regular intake in commercial breeding. The final commercial size of these fish is up to 1 kg.
Tilapia can fit in every aquaculture method of production, from ponds to recirculation systems, and all with a good production rate. In China and Brazil it has been demonstrated how chicken litter can be efficiently used to grow seaweeds for feeding directly in the pond, reducing feed consumption and therefore management prices, although the quantity should not exceed 500kg/ha/week in order to maintain a suitable amount of oxygen for the fishes.
Thanks to these features these fish can be farmed in almost every part of the world, including lower developed areas, such as Sub-Sahara Africa and South East Asia, providing a considerable protein income for the people (Zajdband, 2012).

1.4 Brief excursus on mycotoxin in general

More than 50 years passed since the outbreak of "turkey X disease" in 1962, and the further discovery of mycotoxins, which led to the development of mycotoxicology, investigating in their effect both *in vitro* and *in vivo*.
Mycotoxins are secondary metabolites not necessary for the fungal development, produced by filamentous *fungi*. In this group fit lots of molecules, many of them are at low molecular weight, and their effect can be extremely various. Some of these toxins can also resist heavy thermal treatment such sterilization, and of course pasteurization as well, contaminating food and feed though it's not moldy or there is not actual evidence of fungal proliferation.
Mycotoxins usually affect cereals, legumes or oilseed crops, and the contamination may occur in every stage of production: growth of the cereal, harvest, stocking or processing the crop. The diffusion of mycotoxin-productive molds vary according to the climate, as it happened in the US, where, during 2 following years in the same area (the Corn Belt) the prevalence of ZEA and DON in crops dropped from 43% and 92%

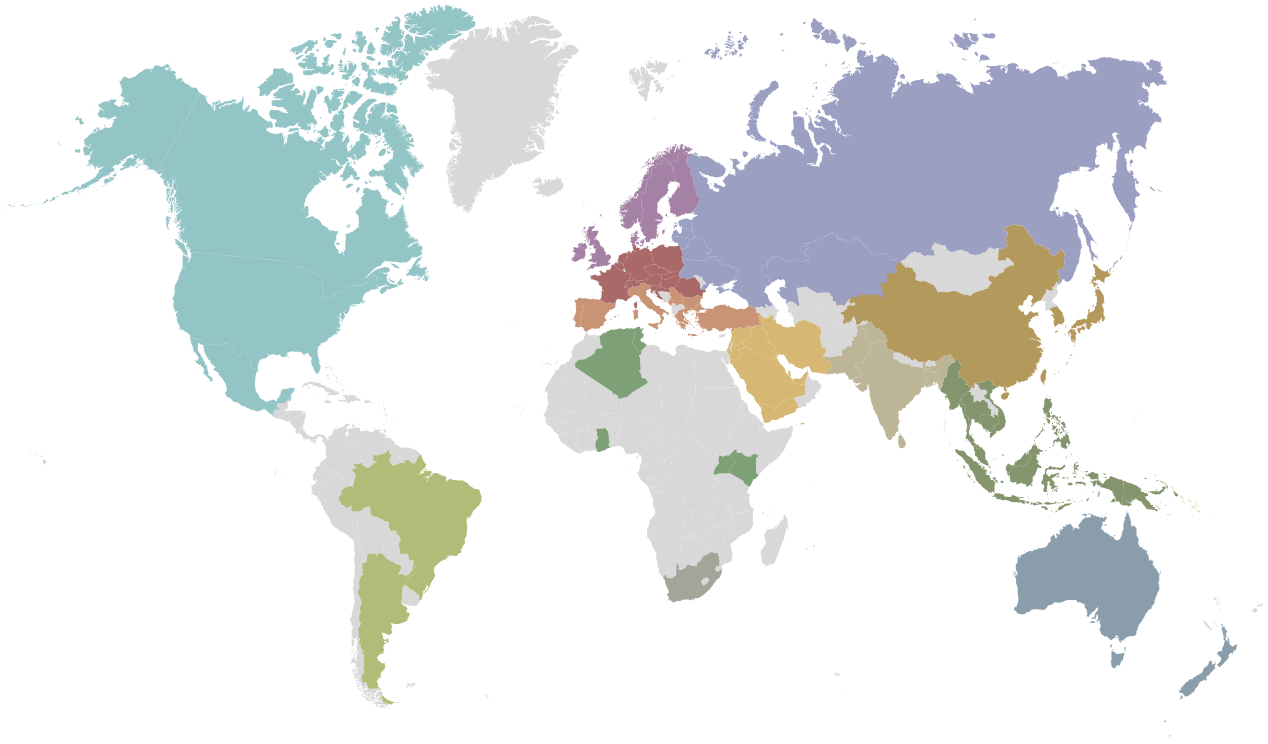
respectively in 2009 harvest to 16% and 62%. The only parameter that changed in that period was the weather, passing from an unusually cold and wet season in 2009 to an uncommonly hot and dry in 2010. Other mycotoxins, such as aflatoxins, were enhanced by the climate change, growing their prevalence from 11% to 32% in the same years, proving that different toxin-producing fungal genus need different climate to develop. Different environmental situation induce different development of the mold strains, as seen in Table 1, and thus all over the world there is a different prevalence of mycotoxicosis and mycotoxin kind (Schatzmayr and Streit, 2013).
Usually media attention focus on the most immediately lethal mycotoxin, such as aflatoxin, as shown in turkey X disease and human deaths, like in Kenya in 2004. However other mycotoxin can have a low or less serious acute effect while can lead to a serious health issue for the chronical effects. Aflatoxin B1 is the most efficient carcinogen molecule known, T2, belonging to tricothecenes, is known as a "molecular gun", interfering with DNA and leading to neoplastic formation, while zearalenone is a mimic of the etrogenic effect. Some molds can also produce ergot alkaloids, which are psychosomatic substances with hallucinogenic effect (the most known and common in Europe is the *Claviceps purpurea*) that can be used even for treating illnesses.
An important feature of mycotoxins is that there is not antidote or vaccine available now to cure a mycotoxin intoxication, making acute and chronic disease impossible to treat, but at least the illness is nor contagious or infectious, being mainly linked to the assumption of contaminated food.

To avoid or prevent the risk of fungal contamination and therefore mycotoxin formation is necessary use correct agricultural techniques, such as crop rotation and tillage, use genetically mold-resistant crops, harvest the cereals at the right temperature and humidity (both of the kernel and environmental), along with the right use of fungicides, which have to be used in the right amount otherwise they'll stress the mold, making it produce mycotoxins. Another important step for the reduction of mycotoxin risk is the fight against parasite insects, such as borer, that shred the caryopsis making easier for the mold to penetrate inside the plant.
Mycotoxin intoxication mainly occur per os, consuming specially through the consumption of directly contaminated vegetables, such as cereals, bean, oilseeds, cocoa, coffee, spices and fruit, yet it's not the only way. Consuming products derived from animals which have been fed with contaminated feed may lead to a carry-over effect; this event is easily recognizable in M1 aflatoxin, which is a B1 aflatoxin metabolite, and in A ochratoxin, which can be observed in swine meat.

It is also possible for workers in feedstuff production and laboratory to be contaminated by respiration of mycotoxins, although should be quite rare if the good manufacturing practice are applied (Richard et al., 2007).
Aspergillus, *Fusarium* and *Penicilium* are the most common source for these toxins, producing the best known mycotoxins, like aflatoxin, tricothecenes, fumonisin, ocratoxin, patulin, zearalenone. The production of these molecules occurs in suboptimal conditions, low temperature and humidity over 15%, and the molecule can be found even when the mold has died. A part from the most known mycotoxins there are even "new" mycotoxins, recently discovered or found only in low quantity, the effects of which are not well known, but can have synergetic effect with other mycotoxin, opening new toxicity pattern or enhancing the effects. To this category belong moniliformin, fumotoxin, and other mold products (Binder, 2007).

Mycotoxin production appears to be strain-specific, although the same stain can produce different toxins; main mycotoxin producer, as mentioned before, belong to *Aspergillus s*pp., *Fusarium* spp., *Penicilium* spp., as seen in Table 2.

Table 1: Global prevalence of agricultural commodities contaminated with mycotoxins



North America		South America		Northern Europe		Central Europe		Southern Europe	
Aflatoxin	**33%**	Aflatoxin	**12%**	Aflatoxin	**32%**	Aflatoxin	**29%**	Aflatoxin	**55%**
Zearalenone	**9%**	Zearalenone	**39%**	Zearalenone	**34%**	Zearalenone	**26%**	Zearalenone	**18%**
Deoxynivalenol	**33%**	Deoxynivalenol	**33%**	Deoxynivalenol	**87%**	Deoxynivalenol	**66%**	Deoxynivalenol	**50%**
Fumonisins	**55%**	Fumonisins	**76%**	Fumonisins	**86%**	Fumonisins	**36%**	Fumonisins	**71%**
Ochratoxin A	**2%**	Ochratoxin A	**2%**	Ochratoxin A	**40%**	Ochratoxin A	**28%**	Ochratoxin A	**46%**

Africa		South Africa		Middle East		Eastern Europe		North Asia	
Aflatoxin	**67%**	Aflatoxin	**0%**	Aflatoxin	**33%**	Aflatoxin	**27%**	Aflatoxin	**14%**
Zearalenone	**26%**	Zearalenone	**0%**	Zearalenone	**9%**	Zearalenone	**23%**	Zearalenone	**57%**
Deoxynivalenol	**67%**	Deoxynivalenol	**10%**	Deoxynivalenol	**33%**	Deoxynivalenol	**56%**	Deoxynivalenol	**79%**
Fumonisins	**78%**	Fumonisins	**92%**	Fumonisins	**55%**	Fumonisins	**49%**	Fumonisins	**47%**
Ochratoxin A	**56%**	Ochratoxin A	**3%**	Ochratoxin A	**2%**	Ochratoxin A	**37%**	Ochratoxin A	**15%**

South Asia		South-East Asia		Oceania	
Aflatoxin	**59%**	Aflatoxin	**59%**	Aflatoxin	**4%**
Zearalenone	**26%**	Zearalenone	**49%**	Zearalenone	**19%**
Deoxynivalenol	**36%**	Deoxynivalenol	**34%**	Deoxynivalenol	**24%**
Fumonisins	**57%**	Fumonisins	**65%**	Fumonisins	**16%**
Ochratoxin A	**55%**	Ochratoxin A	**25%**	Ochratoxin A	**13%**

Data are provided by the Biomin survey on mycotoxin; the percentage is referred to the crops with contamination above the LOQ (Nährer & Kovalsky, 2014)

Table 2: Main mycotoxin-producer molds and their main toxins

Mold strain	Mycotoxin
Aspergillus	
A. flavus	**Aflatoxin B1, B2**
A. parasiticus	**Aflatoxin B1, B2, G1, G2**
A. ochraceus	**Ochratoxin A**
Penicilium	
P. verrucosum	**Ochratoxin A, Citrinin**
P. expansum	**Patulin**
Fusarium	
F. verticilloides, F. proliferatum	**Fumonisin, Moniliformin**
F. roseum graminearum, F. culmorum, F. poae, F. sporotrichioides	**ZEA, Tricothecenes (NIV, DAS, T-2, HT-2, DON)**

A brief summary of the main mycotoxins produced by the main mold strains (Nebbia, 2009)

1.5 *Fusarium* spp. main features

Fusarium is a fungal specie which colonized almost every part of the world, from desert to arctic environment, and they are capable of using many substrates as energy source, thus they inhabit soil, water and other organic substrates, such as plants.
Fusarium has an optimum laboratory growth at 25°C in aerobic situation on Potato Dextrose Agar media, spreading in woolly or cottony flat colonies, as a typical filamentous fungus. The same growing conditions we can find in temperate climate zones, where this mold is a common plant parasite or saprofite both of the roots and of the aerial part (Bolkan et al., 1979).
Regularly *Fusarium* spp. infest soybean, beans, rice and oilseeds, depending on the region in where it's growing, Europe, Asia, Africa or Americas, affecting thus the commodities most people use, with both sanitary and economical consequences.
Though *Fusarium* species are capable of producing a large variety of mycotoxins, with various action pathways, not every specie can synthesize harmful molecules, thus the bare presence of this fungus is not enough to assume that the whole harvest will be contaminated. Mycotoxicosis however is not the only way that this mold has to harm human beings and other animals, it has also been reported that *Fusarium* species are becoming a more common source of opportunistic mycosis (Pitt et al., 1994).
Some *Fusarium* species which parasite plants can produce even hormones (*Giberella fujikuroi*, aka *Fusarium moniliformis,* now *verticilloides*) and other toxin to grow better on the plant and spread more deeply inside the tissues in order to gain more nutrients and reproduce. In this process the plant may accuse injuries that lead to further bacterial or fungal infection, reducing the harvest or spreading illnesses in the whole field. The infection of *Fusarium* often lead to a decreasing in the nutritional value of the grains, due to fungal consumption of energetic compounds, and to an alteration of the kernels, both morphological and organoleptic (Guarro and Gene, 1995).

There are various way in which *Fusarium* can infect the crops, it can be already inside the seed, as a spore that will activate when the condition will be suitable for the development, can infect the flower or the caryopsis in the early stage or either it can be transported by insects, such as borer, that can injury the plant or the seed, making possible to *Fusarium* to infest it. These various way this mold has to infest crops make almost impossible to prevent the spreading. Furthermore infected caryopsis can infect the other stored crops thanks to the release of spores, contaminating virtually the whole harvest.

If the humidity of the seed or the storage room is high and the temperature is stressful for the mold the risk of mycotoxin production will increase. If the production eventually happens and it's very large the harvest has to be destroyed or mixed with uncontaminated seeds in order to lower the risk to an acceptable level for human or animal consumption.

To evaluate mycotoxin presence it's possible to apply many methods, such as HPLC, TLC and ELISA, depending on the accuracy we want to detect the amount of mycotoxin in the crops. It is possible to use even tandem mass spectrophotometry in order to detect not only the parenteral molecule but also its metabolites or epitopes. This last technology can unequivocabilly recognize the molecules thanks to the TOF, time of flight, that the molecule has once it has been excited, and the regular spectrophotometry analysis.

1.6 Focus on main *Fusarium* mycotoxin

Fusarium metabolites are the most commonly found in Europe, with DON being the most common, with 97% of positive samples containing it. Other metabolites such as ZEA and masked forms of DON happen in over 80% of positive cases (Schatzmayr and Streit, 2013).
These 2 mycotoxins however are not the only produced by *Fusarium* strains, and some are more toxic and therefore healthy relevant for the population.

- **Fumonisin:**

Fumonisin are a relatively newly-discovered class of mycotoxin, being individuated in 1988 in Africa (Marasas et al., 2001), produced mainly by *F. verticilloides* and *F. proliferatum*. Fumonisins are divided into four structurally distinct groups designated fumonisins A, B, C and P. The most important fumonisins are those of the B series, namely FB1 and FB2. Production occur preferably at 20 °C, making temperate zone risky for this mycotoxin.
While its concentration may be high in food and feed, usually reaching ppm values, it's scarcely absorbed during digestion, with studies showing that more than 80% is expelled with feces in its parenteral form, and has a really short half-life (few hours, depending on the host specie). Though its poor absorption fumonisin

is capable to provoke serious injuries due to its conformational analogy to sphinganine, a sphingomielin precursor. The action pathway of this toxin consist in block sphinganine acetyltransferase, arresting production of sphingomielin, resulting in different effects depending on the specie it affect. It is also proven that interfere with sphingosin N-transferase, inhibiting sphingomielin turnover in ceramide.
In horses fumonisin provokes a serious neuronal illness, the leukoencephalomalacia, ELEM, while in pigs it affect lungs, promoting Porcine Pulmonary Edema. Rodents seem to be the most sensitive to this mycotoxin, showing cerebral, hepatic an renal alterations in rabbits, while in mice there are evidences that prove this mycotoxin to be a carcinogen and even at low doses for a long period it can interfere with erythropoiesis. Several studies shown that long time exposure to fumonisin in humans induce esophageal cancer, EC, mostly by the consumption of highly contaminated corn and rice, as shown by report in South Africa, China, Iran and Northern-East Italy, where a positive correlation between consumption of contaminated corn and incidence of esophageal cancer was set.
European Union imposed a safety limit for food in 0.2-2 mg/kg and 5-50 mg/kg for feed, depending on the specie involved, thanks to REGG. 1881/2006 and 576/2006 respectively. According to European Scientific Committee on Food a provisional TDI is to be found in 2μg/kg BW/day for Fumonisin B1,2,3 alone or together. (SCF, 2004)

- **Zearalenone:**

Zearalenone is a toxin produced by *F. sporotrichioides* (synonim for *F. trincitum*), *gibbosum* and *roseum*, especially *F. roseum graminearum* subspecies, from now on referred as *F. graminearum*. Production is usually enhanced by low temperature, 12-14 °C, below the growth temperature of the mycelium, and by high substrate a_w, beyond 0.95. Usually it's not present alone and co-occurs with tricothecenes mycotoxins, such as DON. It is known only one conformation of this toxin, while it can be processed in two different metabolites, α- zearalenol and β-zearalenol.
Zearalenone is absorbed at a very high ratio, around 80-85% and after that it's processed in the liver, where it's hydroxilated, resulting a bioactivation, in α- zearalenol and β-zearalenol. After that it's usually conjugated with glucuronic acid in order to be expelled with bile, but evidence prove that intestinal microbiota can break the bound of glucuronidation, freeing the molecule and permitting its entero-hepatic circle, with further effect on the animal. This toxin act as a hormonal equilibrium disruptive, mimic the effect of 17-β estradiol thanks to its similarity with that molecule. Zearalenone and zearalenol bind the hormonal receptor for estrogen, activating it and making the

complex migrate in the nucleus, leading to the production of the regular signaling pathway for estrogen presence. α- Zearalenol is more efficient in this downfall activation than the other metabolite and the parenteral molecule itself.
Most sensitive terrestrial farming animal is the pig, where this mycotoxin cause vulvar edema, udder hypertrophy and early estrus. Ruminants are less sensitive to zearalenone because of the different biotransformation that this molecule get inside rumen, leading to β-zearalenol formation. The same pattern of biotransformation happens in poultry, which are even less sensitive than cattle, sheep and goats.
Human beings are shown to have similar metabolism to pigs for transformation and entero-hepatic circle, proving that they're very sensitive to this molecule and its metabolites. Main way to get contaminated is the ingestion of infected cereals or spices, but studies about carry-over in milk and eggs show that they can concur in exceeding the TDI, especially if the animals are fed with strongly contaminated feed. The greatest source of contamination for humans are cereals, like barley rye oat and sorghum and their derivate products, especially bread.
Zearalenone outbreaks have happened even in the late 1990s in Hungary, where high concentration of ZEA (up to 100 μg/l) was reported in children exhibiting early puberty. Since there is no evidence that this molecule is carcinogenic European Union classify it in 3B group (Zinedine et al., 2007; Agag et al., 2004). European regulation imposed a limit quantity in food of 50-200 μg/kg for adults, while in food for baby, children and kids the quantity should not exceed 20 ppb. Also the Joint FAO/WHO Committee on Food Additives promulgated a PMTDI of 0.5μg/kg BW/day. No legal limit to the presence of ZEA was set in fish, as the literature on the subject is still too few and controversal. (Recommendment 2006/576/EC) (SCOOP, 2003)

- **Tricothecenes:**

Tricothecenes are a mycotoxin family composed by more than 200 members, which share a common 12,13-epoxitricothec-9-ene group (ETE). They are produced by *Hypocreales* genera *fungi*, like *Fusarium*, and they are toxic both for animals and vegetal.
Thanks to the investigation on historical report symptoms and nutritional behavior analysis of these times, toxicologist can now shed light on mysterious illness outbreak, like the Alimentary toxic Aleukia, ATA, which occurred during the 1930s in URSS. This serious condition was probably caused by moldy grains, which were population staple food. Retrospective analysis of symptoms led to the conclusion that vomit, diarrhea, leukopenia, hemorrhage and hemorrhagic shock were caused by an high contamination by *Fusarium* mycotoxin, probably tricothecenes. Several studies pro-

ved that in eukaryotic cell these toxin impair protein synthesis interfering with the bond of peptyl transferase in 60s ribosomial subunity, formation of free radicals and their accumulation inside the cell, that lead to oxidative stress, and mitochondrial protein synthesis inhibition, disrupting cell metabolism, apopltosis. In plants these effect cause wits and blights, while in terrestrial animals can provoke feed refusal, immunological problems, vomiting, skin dermatitis and hemorrhagic lesions.
Tricothecenes toxins are amphipathic molecules, with a low molecular weight and therefore capable of moving through cell membranes passively. They're thus easily absorbable by the gastrointestinal system, from which they can spread to the organism, affecting mostly rapidly proliferative tissues, leading to vomit, diarrhea and food refusal. Tricothecenes production is regulated by a gene cluster of 26 kb, 2 genes and a locus, identified as Tri genes, whose products are enzymes that lead to the transformation of farnesyl pyrophosphate to the actual mycotoxin (Mc Cormick et al., 2011).
The usual classification of this kind of mycotoxin refer to them as 4 classes group, divided in A,B,C and D. These classes feature different conformation in ETE, A,B and C have different function in C8, while D class has an additional ring connecting C4 and C15. Although this classification has been used since a long time, there are many structural features that are not accounted in the system (*Fusarium*-produced toxin for example have an oxygen function in C3, while other mold strains have not), this suggest that since we have the knowledge of biochemical synthesis and transformation occurring in mycotoxin production and we are capable of using genetics, a genetic-based classification should be more precise and desirable.
Tricothecenes biosynthesis start from farnesyl pyrophosphate, which is ciclysed to thricodiene by trichodiene synthase, regulated by Tri5. This molecule become target of p450 monooxygenase, encoded by Tri4, which add 4 oxygen in C2,C3,C11 and to C 12-13 epoxide, forming thus Isotrichotrid.
Isothricotrid undergoes a non-enzymatic modification with the formation of a pyran ring with oxygen in C2 position, creating isotrichodermol. This molecule has a very high toxic capacity and therefore has to be detoxified in order to prevent the mold itself from dying. The process of detoxification consist in hydroxilating C3, C15 and C4, then acetilating C15 hydroxid group. This molecule is the precursor of DON, NIV and T2 toxin. In NIV formation two pathways can be followed, the first one involving hydroxilation and acetylation of C4 (formation of 3,4,15-triacetoxyscirpenol), hydroxilation of C7 and C8 with further oxidation of C8, the other one consist in hydroxilation of C7 and C8, with further oxidation of C8 and hydroxilation with acetylation of C4. Both these path lead to the formation of 3,4,15 ANIV, which will be hydroxilated in C

3, C4 and C15 to give NIV.

T2 formation depart from 3,4,15-triacetoxyscirpenol, with hydroxilation of C8, followed by the addition of an isovaleryl moiety. The hydroxilation of C3 complete the production of T2 toxin (McCormick et al., 2011).

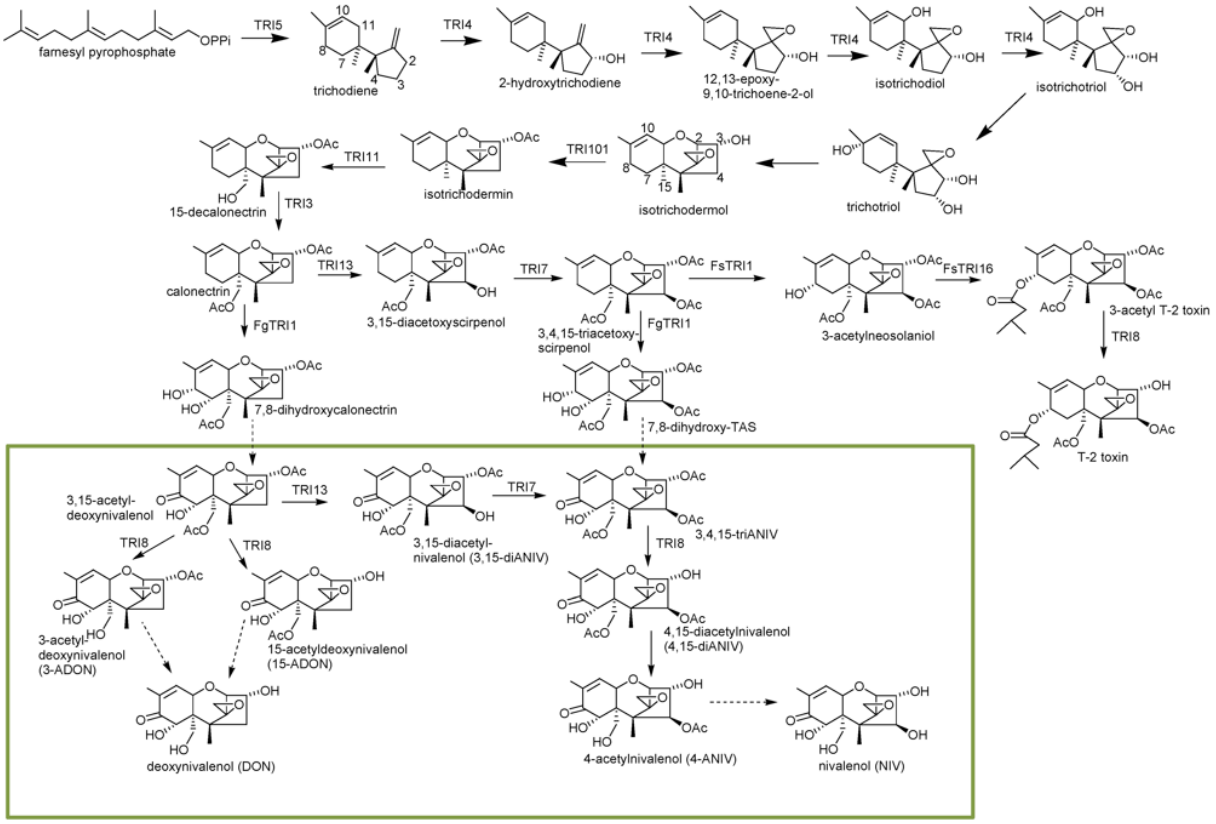


Figure 2: Production of DON, NIV and T2

Synthetization pathway of the main tricothecenes from farnesil pyrophosphate to their definitive form, it's possible to appreciate the modification that occour among the various tricothecenes and their similitude (McCormick,2011).

- **T-2 and HT-2:**

T-2 and is a mycotoxin belonging to type A tricothecenes, and has an esteric function in C8, its toxicity consist in diminution in protein synthesis, lipid peroxidation and disrupted nucleic acids synthesis. This toxin is probably the most toxic of all the tricothecenes, having a t-TDI of just 100 ng /kg BW, with a LD50 of 5-10 mg/kg BW.

Though the literature often refers to this mycotoxin as T-2 most of the toxicity is due to its metabolite, HT-2, which is proven to be more toxic than the parenteral molecule.

The effects of this toxin comprehend necrosis and apoptosis, hematotoxicity and myelotoxicity , affecting consequentially the bone marrow. On high exposure this fungal metabolite may even induce dermal toxicity, reproductive organs dystrophy and development alteration. The most sensitive animal we know is the pig, showing hematological and immunologic effects at 29 μg/kg BW daily, although the most exposed are the milking goats, while the least exposed are the fish.
In vivo studies have been performed for the acute and subchronic exposure to T-2 and HT-2, while the chronic exposure is not investigated since the late '80s (1988 and 1987), when the LOAEL was determined to be 0.1mg/kg BW per day on mice.
Acute toxicity was investigated only in mice, showing evidence of LD50 ranging from 5 to 10 mg/kg BW, inducing lesion on hematopoietic tissue and gastroenteric epithelium, even if administered by parenteral way. If the dose is lower, 4.64 mg/kg BW in the study, death comes during the 24 hours, preceded by bloody diarrhea and food refusal. The group receiving T-2 toxin lower concentration than 2.15 mg/kg BW survived the whole week of experiment, though they showed bloody feces and feed refusal.
Subchronical exposure evidenced histopatological lesion in liver, kidneys and heart, with signs of necrosis and immunotoxicity along with an higher exposure rate.
Immunotoxicity was evidenced even in in vitro studies on human lymphocites using the MTT assay and Tripan Blue exclusion test (SCF, 2001).

- **NIV:**

Nivalenol is a *Fusarium* toxin produced by *F. cerealis* and *F. poae*, but sometimes can be produced by *F. graminearum* strains, occurring in the same products as DON. This mycotoxin is often seen as a contaminant in barley, wheat, rye, corn and their product, such as bread, malt and beer.
Nivalenol effects are similar to DON, yet with a lower LD50 (38.9 mg/kg BW. vs the 78 mg/kg of DON, both for oral administration) and a higher dose to induce emetic effect (180 μg/kg BW vs 30 μg/kg BW). The chronical effects of NIV have been investigated in 2008 by 2 different group of study (Takahashi et al. and Kubosaki et al.) and showed evidence that, at a 3.5 ppm dose for 90 days, erythropoietic system was disrupted, leading to a lower number of erythrocytes and a depression of bone marrow, along with leucopenia.

A toxicological TDI has been established by European Union at 0.7 μg/ kg BW, yet in 2011 EFSA promoted a investigation to see which was the real exposure of animal and humans, yet of all the food and feed samples very few were above the LOD (10 and 6

%, respectively).
Unprocessed grains were the most contaminated and, as several studies reveal, whole bran grains are more contaminated than processed caryopsis, but in milling process a redistribution may occur, contaminating the core of the seed and thus the processed product. As expected the result showed that bran was the most contaminated part of the product. Nivalenol is stable throughout most commercial processing that contaminated cereals will undergo. It is unstable under high temperatures (> 150 °C) and alkaline conditions, and the rate of degradation increases with increased time and/or temperature conditions (CONTAM, 2013).

- **DON:**

Deoxynivalenol is a *Fusarium* toxin, mainly produced by *Fusarium graminearum*, it affects mostly staple grains, such as wheat, barley and corn, and nowadays it's the most detected tricothecene, showing the highest concentration compared to other mycotoxin. The main DON contamination source for human health are the vegetables, especially grains and their products, such as bakery, flour, beer, polenta, porridge, and pasta. This toxin was firstly discovered in Japan in 1972 and referred as Rd-toxin in the publication and its presence was confirmed in North America too the following year, where was identified as an "emetic factor" in moldy grains, forging then DON colloquial name, "vomitotoxin". Due to the high presence in nature this mycotoxin affect even animal feeding, thus impairing farming and breeding performances of farm animals and having their products, such as meat, although at very low dose (Morgavi et al.,2007 and references therein).

As seen in figure 2 DON has an epoxy-sesquiterpenoid form, with hydroxil groups in C3, C7 and C15. Usually it's not found in nature only as parenteral molecule but along with precursor molecules, such as 3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol, which have lower toxic effect. 3-Ac-DON has been shown to have a lower, yet not statistically relevant, LD50 in mice, investigated by intraperitoneal and subcutaneous injection, while 15-Ac-DON was found to have a toxic effect 1.4 times lower than DON. Deoxynivalenol effects are dose-dependent, as most of mycotoxins, and at a high dose it cause emetic effect, even stronger than other tricothecenes considered as more toxic such as T2 and NIV, and if not expelled can even cause shock death, as seen in mice. Necropsy revealed after an high DON dose diet intestinal hemorrhage, bone marrow necrosis, lymphoid tissue necrosis and kidneys and heart lesions, while in vita the animal showed abdominal distress, emesis, increased salivation, malaise, anorexia

and anemia. The low dose effects comprehend cachexia, loss of weight, feed refusal, which can be partial or total, depending on the dose in the diet, leading to strong reduction of growing performances. Piglets fed with 2 to 4 mg/kg DON in fodder were seen to have an impaired feed conversion rate and lower metabolic efficiency. In another experiment pigs we fed with diets ranging from 1 to 12 mg/kg in order to describe the effect this molecule has on the appetite, and it has been seen that since 1-2 mg/kg it affect the appetite, with a dose-dependent magnitude until reaching the complete feed refusal at 12 mg/kg.
For what we know now the most sensitive animal is the pig, which has a metabolic pattern very similar to the human, followed by mice and rats; the least sensitive farm animals appear to be ruminants and poultry, which scientific studies reveal to have a low absorption rate (Petska, 2007).
Histological evidence of trauma due to exposure to DON has been found in many animals. Usually low dose of DON in feed lead to an increased activity and presence of leucocytes, while high doses of this tricothecene induce apoptosis. The most damaged organs are the lymphatic system and gastroenteric system, in which lesions were found when the concentration in feed was higher than 5 mg/kg. Deoxynivalenol is also proven to impair reproductive system, as seen in rats and mice. The latter animal during pregnancy has been seen to reabsorb fetuses when exposed to more than 2.5 mg/kg. Resorption rate was dose-dependent and total resorption was confirmed at 10 mg/kg in diet feed.

Apart from the litter numerosity DON affect even the health of the newborn; concentration higher than 1 mg/kg impair skeletal formation, while the assumption of more than 2 mg/kg during the weaning period lower milk intake, body weight, perinatal mortality and increase skeletal malformation in newborns.
Reproductive effects were investigated even in rat. In an experiment held for 6 weeks rats were fed with various concentration of DON before and during pregnancy.
Though the fetuses and the newborn did not have any skeletal malformation and their weight was regular, treated mothers shown a lighter uterus and a lower body mass the control when the contamination was lower than 5 mg/kg.
When an contamination of 5 mg/kg or higher occurred body weight gain and feed consumption of mothers was suppressed and there was partial litter resorption, with evidence of teratogenic effect.
Evidence show that DON impair even male reproductive system. Rats feed 4 weeks with 5 ppm diet of DON showed lower body weight, sperm and testosterone quantity

Table 3: EFSA limits for DON presence

Commodity	Limit (ppm)
Cereals and cereal products with the exception of maize by-products	8
Maize by-products	12
Complementary and complete feedstuffs	15
Complementary and complete feedstuffs for pigs	0,9
Complementary and complete feedstuffs for calves (< 4 months), lambs and kid	2

Legal limits for DON, expressed in parts per million, in commodities for the European Union (EFSA, 2006)

and a decreased number of spermatozoa than control and lower toxin dose groups. Other effect were the degeneration and abnormal development of gem sperm cells and failure in ejaculation. These effects were encountered only in diets exceeding 1 ppm deoxynivalenol and their intensity was dose-dependent.
Molecular effect of DON have been investigated in rodents and pigs as well as in in vitro cell cultures. DON seems to stimulate serotonine release in the guts, generating a signal pathway that lead to releasing anorexigenic hormones. Even low doses can impair gastric emptying and gut mobility, limiting food intake.
Serotonine is not the only proinflammatory protein over-synthesized with DON presence. Even cytokines are produced in many organs unleashing a “cytokine storm”, responsible of the high-dose intoxication effects. Cytokines can even impair growth, interfering with the growth hormone axis and this could explain why in chronic intoxication growth is that lower compared with the control group. Along with this body is forced to produce suppressor of cytokine signaling, thus impairing inflammatory response and being more vulnerable to chemicals and bacteria.
In a mice experiment has been investigated the food refusal due to DON in diet. It has been noticed that the simple taste of the food taken when DON was in high concen-

tration is sufficient to impair feed consumption. This experiment was run using male rats which received a saccharine solution and after that an intraperitoneal injection of 0. 125 mg/Kg BW of DON or vehicle.
The control mice shown an higher saccharine solution consumption than the treated in a two-bottle preference test. In vitro experiment were performed in clonal cell lines HT-29-D4 and Caco-2, both are human colon adenocarcinoma cell. With the first cell line the active transporters have been investigated, leading to the discovery that glucose and fructose transporter are inhibited after the exposition to DON (Sergent et al., 2006; Maresca et al., 2002). In the Caco-2 cell experiment it has been proven an inflammatory effect in the acute response due to the synthesis of IL-8, a proinflammatory chemokine, and this fact accord to the previously described innate immune system activation from deoxynivalenol.

Chronical consumption of DON induces loss of P-gp proteins, as seen in Caco-2 cell, impairing cell capacity to expel toxic compound like toxins or chemicals, thus lowering immune defense. Along with this, deoxynivalenol lowers the transepithelial electrical resistance, loosing cell junction and therefore enhancing the risk of bacterial invasion as much as chemical penetrations. Also a proteomic experiment on EL4 murine cell set in 2010 proved the effect of DON in inducing overexpression of protein involved in immune system and oxidative stressors, such as H2O2 and fatty acid synthase. This proteomic study explicit the molecular effect and interaction of DON as oxidative stressor and immune system disruptor (Osman et al., 2010).

1.7 A new threat, synergistic effect of mycotoxins

Most of the studies about mycotoxin toxic effects focus only on the effects of a single mycotoxin a time. Recent studies (Alassane-Kpembi et al., 2013) point out that most people are exposed to several mycotoxin at the same time, considering that molds strain can produce more than one mycotoxin at the same time and several fungal contamination may occur on the same product. Along with this consideration a balanced diet, both animal and human, is composed by many components, thus enhancing the risk of synergistic effect of mycotoxins (Streit et al., 2012).
Tricothecenes are the most common mycotoxins in Europe and North America and

the co-occurrence of many of these toxins has been proven during the years. *Fusarium graminearum*, for instance, is capable of producing both deoxynivalenol and zearalenone at the same time, as shown in this very experiment, while *Fusarium verticillioides* is capable of producing both moniliformin and B1 fumonisin (Sharma et al., 2008).
DON and ZEA are even proven to have a synergistic effect on pigs, having an enhanced magnitude of the estrogenic and emetic effects on animal health (Pedrosa et al., 2011), and this may be relate to the usual co-occurrence of these two toxin in the same feedstuff.
It has been even shown that the toxicity of some individual mycotoxins can be increased or decreased when they are present as co-contaminants in feed and food (Huff et al., 1988; Kubena et al., 1995; Streit et al., 2012; Alassane-Kpembi et al., 2013).

1.8 Mycotoxins in aquaculture

Mycotoxin are usually referred as a problem concerning only terrestrial animal farming, as long as these are the traditional farming animal; with the springing and development of aquaculture techniques however this sector has become more and more important in providing food for human beings.
Since the early 1980s some studies were performed in the most economically relevant fish breed, challenging them with mycotoxin in order to investigate any effect on growing performances. Usually these studies involved aflatoxin B1 or fumonisin B1, as they're considered the most dangerous mycotoxins produced.
Aflatoxin is known to provoke hepatocellular carcinoma even in rainbow trout *Oncorhynchus mykiss*, with the same action mechanism as in terrestrial animal, as investigated in 1987 in USA, where the animals were found to develop nodules and hepatocellular lesions (Bailey et al., 1987). Thanks to this fact a limit for AFB1 in the USA was promulgated, setting feed contamination at a 20 ppb maximum level. This limit was sometimes retired from USDA in order to grant the commercialization of feedstuff such as cottonseed or corn, saying that animal meat is not so contaminated to effectively represent a risk for human health.

Also, in 2002 an AFB1 experimental diet was performed on a Nile tilapia (*Oreochromis niloticus*) population in USA (Nguyen et al., 2002). This experiment showed the effects

of various toxin concentration in feed, and the parameters analyzed were FCR, hematocrit and hepatic lesions.
Tilapias fed with a toxin concentration below 2.5 ppm showed no effects and no significant difference than the control group. The 2.5 ppm diet instead showed a lower hematocrit than the control along with an higher Food Conversion Rate, implying that an larger quantity of feed is needed to achieve a weight gain. Furthermore 10 ppm diet showed an even lower hematocrit, a higher FCR and also hepatic lesions, with the vacuolization of hepatocytes and showed a lipid oxidative stress symptom (lipofucsin). Feed intake was impaired by the retching of feed itself in the 10 ppm diet, implying that the magnitude of the contamination was lower than the 4-fold predicted by the feed composition (3.26 times).
As we can imagine the 100 ppm diet effects on fishes were even worse, showing a 60% mortality at the end of the 8 weeks experiment along with liver necrosis, remarkably low hematocrit and weight loss due to feed consumption, implying that this diet is lethal. Feed refusal was more evident with this diet, along with the premature expulsion of feed, so, as previously said for the 10 ppm diet, the average contamination of fishes was of 59.4 mg/kg BW , so significantly different from the 173 mg/kg BW predicted .

Fusarium toxins gained growing importance and attention during the last years due to their worldwide distribution and various effect both on human being and on animals. Main attention is still focused on B1 Fumonisin, which provokes the most severe illnesses, such as leukoencephalomalacia in equines and PPE in swine. In channel catfish (*Ictalurus punctatus*) it has been shown to have a negative effect when given to fingerlings, decreasing weight gain, feed intake and increasing feed conversion rate. These effect are almost absent when the body weight exceeded 30 g, even if there were hepatic lesions encountered during histological investigation. In adult channel catfish feed contaminated with up to 300 ppb for over 5 weeks showed no evidence of toxicity (Manning et al., 2003).
Common carp (*Cyprinus carpio*) is more sensitive to fumonisin, as seen on 1 year old carp experiment, which were fed with diet containing 0.5 to 5 mg/kg BW. In this experiment weight loss and variation of enzymatic parameters. Liver enzymes level increased and lesion of endocrine and exocrine glands were found during histological investigation, as long as interrenal lesions, ischemia and increased cell wall permeability (Petrinec et al., 2004). Zearalenone has been found to have an high affinity with estrogen receptor on rainbow trout, inducing oogenesis within 7 days after admini-

stration. In a 2012 genetic study ongoing with DD-PCR differential expression of regulatory genes controlling blood coagulation, iron storage and cytoskeleton composition has been correlated with ZEN in diet, showing new ways in which this mycotoxin can affect animal welfare (Wožny, 2012). A report showed how, generally, ZEA in vitro didn't show particularly high estrogenic effect, yet in vivo it deeply affect reproductive organs of the same living specie (*Danio rerio*) even after a short-term exposure. (Schwartz et al., 1999)

In vitro experiment on fish cell lines exposed to a 24 hours exposure to Ac-DON showed how acute toxicity is related not only to the toxin but also to the specie, as in terrestrial animals. This fact implies that response to mycotoxin could vary among the breeds of fish which are farmed, and thus further analysis should be performed to clarify the *in vivo* and *in vitro* effects of mycotoxins (Pietsch, 2011).

1.9 TMA and its commercial importance

Trimethilammine (TMA) and trimethilammine oxide (TMA-O) are non-proteic nitrogen compounds usually found in most marine and brackish fishes, and some freshwater fishes. The molecule has a positive redox potential, impairing thus the metabolism of anaerobic bacteria, preserving the fish from infection of bacteria such as *Lactobacillus* spp., *Clostridium* spp. and others. In arctic fishes they increase osmotic concentration, preventing fish blood and meat from freezing.
In vivo the molecule is present in muscles as TMA-O, which is not volatile and has a high molecular weight. After animal death bacterial enzymes reduce the amine oxide to its reduced phase, which is volatile and responsible for off-flavors and fishy smell, spoiling the sensory perception of the consumers.
Freshness evaluation can be achieved by measuring the quantity of TMA in the muscle; usually a fish with a TMA level around 4 or 6 (depending on the species) mg/100g meat can be recognized as fresh, while having a TMA content above 10 mg/100 g meat imply that the fish is smelly and definetly not fresh (this method is usually recommended on fresh product only, as the DMA and FA are the most important chemicals produced in frozen products) (Uniprom, 2001). Even if TMA is usually reported as a seawater fish criteria for freshness, a detectable amount, higher than other freshwa-

ter fish, has been detected in tilapia species in their wild environment, and an experiment in 2002 proved that a high amount of choline could enhance the amount of TMA-O in fish fillet, even though the level was not as high as in seawater teleost such as mackerel (Niizeki et al., 2002).

2. Experimental part

2.1 Aim of the study

Being *Fusarium*-related intoxication some of the most common causes of grains contamination all over the world, and being aquaculture one of the new farming frontiers, this research wants to clarify the effect of deoxynivalenol (DON) and zearalenone (ZEA) on Nile tilapia (*Oreochromis niloticus*) on growth and reproductive performances.

The study was carried out by our group in a frame of the project number TÁMOP-4.2.2.A-11/1/KONV-2012-0053, titled "The examination of nutrient solution markers affecting the success of artificial insemination".

2.2 Material and methods

2.2.1 Experimental design

To investigate the short term effect of DON and ZEA on productive and reproductive parameters in tilapia (*Oreochromis niloticus*) farming a six week long experiment was set by the University of Kaposvàr, using 7 month-old tilapias.

The fish were reared in the Fish Nutrition Department, each group containing 32 individuals per tank, one tank per group.

The three experimental groups differed for the mycotoxins (DON and ZEA) concentration: 1, 3 and 5 mg/kg of dietary inclusion level.. Control diet did not contain any mycotoxin. Preparation of experimental diets has been performed manually, starting from commercial diet, grinded and then added the right amount of fungal culture containing the given levels of DON and ZEA. Fish were fed *ad libitum* along the 6 weeks of the study using the contaminated diet.

Fishes in all tanks received the same amount of feed in relation to bodyweight twice a day in small portions to avoid feed spill and possible DON contamination of the flow-through water in the tanks.

2.2.2 Mycotoxin production and quantification:

Toxins were produced using *Fusarium graminearum* strain number IFA 77 (from "Das Interuniversitäre Department für Agrarbiotechnologie", Tulln, Austria) fungal culture (7 days old), which was grown on Potato Dextrose Agar (PDA; Chemika-Biochemica, Basil, Switzerland). Spore suspension was prepared by dislodging colonies from a plate, which were then stored at 10°C in darkness in sterile physiological solution (0.9% NaCl) in 50 ml test tubes.
For toxin production, maize (20 g) was soaked in distilled water (20 ml) at room temperature for 1 hour in baby food jars, which were closed and then autoclaved at 121 °C for 15 minutes in Labo Autoclave MLS-3751/L (Sanyo Electric co., Moriguchi, Japan). After the sterilization a 0.5 ml spore suspension was added to each jar. The cultures were then stored and incubated at 28 °C for 2 weeks. When the incubation time was complete the fungus-infected cereal was dried at room temperature and ground. Toxin was produced in Kaposvàr University, Institute of Physiology, Biochemistry and Animal Health.

2.2.3 Animal feeding and housing

a- Feed:

Pelletted feed was obtained by milling commercial feed, and mixing it with ground corn obtained with the previously described method.
The feed (Table 4) was ground with a grinder used only for this purpose at 0.7 mm and after the homogenization of the feedstuff it was mixed with the fungal culture. This dry compound was then moisted with tap water and then knead until pellettable. The pelletting process was pursued by using a mincer which extruded the wet dough through 6 mm pores, obtaining long stripes of feed (Figure 3).
The stripes were then severed and broken into pieces of 1 cm approximately and then put on a single layer to dry 48 hours under forced air in a chemical cabinet, model at Kaposvàr University, Institute of Physiology, Biochemistry and Animal Health.
All the manipulation of mycotoxin culture and contaminated feed was performed using IPD, such as a single-use laboratory coat, a pair of single-use nitrile gloves, a pair of gum gloves, a filtering mask class FFP2 and a pair of protection glasses, in order to prevent any contact with skin, mucosae and eyes. Once dried the feed was stored in clean glass jars, closed and sealed and stocked in a dark room at room temperature.

A smaller amount of feed was put in plastic jars with sealed tops in order to be easily accessible for the feeding.

A B

Figure 3: Production of the feed

Part of the feed production; in figure 2a is possible to see the extrusion process; the feed was previously milled and supplemented with the toxins, then pushed through 0.6 cm pores. In figure 2b is possible to see the IPD used during the process; every row of feed correspond to a certain concentration (for the trip to the lab and the cabinets every plate was signed with a indelible marker).

The control group was analyzed as well, finding the contamination level under detection limit. The measurements have been processed with the LC-MS/MS previously described, having analyzed the feed 3 times in quintuplicate.
Before feeding them with the experimental feed tilapia were fed in Aller Sturgeon, a high protein fish meal that covers the requirement of small, growing fishes.

Table 4: Feed composition

Commercial name	Producer	Components	Nutritional Values	
Aller Sturgeon	Aller Aqua, Christenfled, Denmark	Fish meal, wheat, wheat gluten, fish oil, corn gluten, yeast, krill meal, vitamins (A, D3, E) and minerals	Crude protein Crude fat NFE Ash Crude Fiber P in dry matter Gross energy Digestible Energy	52 % 12 % 17.9 % 9 % 1.1 % 1.7 % 20.3 MJ/kg 18.6 MJ/kg
Skretting Classic K2P	Skretting, Stavanger, Norway	Soybean meal, wheat, fish meal, rapeseed meal, corn gluten, rapeseed oil, fish oil, vitamins, minerals and amino acids.	Crude protein Crude fat NFE Ash Crude Fiber P in dry matter Gross energy Digestible energy	37 % 10 % 37.3 % 7.1 % 3.6 % 1.3 % 19.7 MJ/kg 17.5 MJ/kg

Composition of the two feed administered to the fish; the one above was given before the start of the experiment and meets the requirements for the initial growth. The second was the base for the experimental diet.

b- Fish farming and management:

Seven-month-old Nile tilapias (*Oreochromis niloticus*) weighting 24.37±7.76 g were divided in 4 groups, of 32 fish each.

Fishes were provided by Szarvasfish Ltd. (Szarvas, Hungary) 50 g tilapia were introduced to the Fish Nutrition Department and reared till sexual maturation. All the tilapias used in the experiment spawned in September from the parental line here above, and were farmed until the right experimental weight. Their daily feed needing was calculated every week as 3% of the whole pond fish weight. The fish weight was calculated since the beginning using an A&D scale " A&D FZ-120i " (A&D Engineering 1756 Automation ParkwaySan Jose, CA. 95131 USA) anesthetizing them with clove oil (*Syzygium aromaticum*) from Medi Natural (Medi Natural Kecskemét 6044,103 Külsónyír) and then drying them on a towel in order to not have high level of water or mucus compromising the accuracy of the weighing process.

c- Farming parameters:

The fishes were farmed in 480 liters glass ponds, non-recirculating system filled with tap water from Kaposvàr aqueduct and kept between 25 and 29 Celsius degrees, with a mean of 26.98±1.15. To preserve animal welfare each day 10% of the water in ponds was removed in order to clean from feces and other debris, and then enough water was pumped in to replace it. Conventional water cleanness and oxygenation was maintained through a "simple inner biofilter unit", assembled in Kaposvàr University, Department of Fish Nutrition, consisting in an oxygenation plastic pipe and airstone formed by empty sponge filled with cylindrical plastic tubes, around 0.7 cm hole diameter and 1.3 cm length and small stones.

Average water temperature range was 27.29 ± 0.84 °C for the control group, 26.61 ± 1.93 for the 1 ppm group and 26.92 ± 0.28 27.10 ± 1.56 for 3 ppm and 5 ppm diet respectively, using 300 watt water heater (L2RH 300, Jager, Aquarium Regier Heizer, Deizisau, Germany). Temperature was checked twice a day with an analytical thermometer in order to check and arrange the temperature to the growth optimum of tilapia.

The farming routine consisted in feeding fishes twice a day, with an higher administration of feed in the morning, temperature checking and pond cleaning during morning time. Every week the ponds were completely emptied and cleaned in order to avoid concentration of mycotoxins in water and algae growth. Along with the cleaning program fish weighing was performed as well in order to have a solid Feed Efficiency (FE) calculation. The experiment started 19th of May 2014 and lasted until 30th June 2014, when fishes were stunned by head percussion. The original protocol planned an over-anesthetization with clove oil, yet this technique would impair further analysis, such as the TMA detection with the electronic nose.

During the sacrifice of the fish at the end of the 6th week of exposure to the toxin the tilapias were stunned, then dried and only then weighted.

Fish dissection was performed in a laboratory room at the Kaposvar University (Guba Sàndor Ut., Kaposvàr, Hungary), where fish were weighted, opened from anus to mouth, sexually classified and gutted. The liver and the sexual organs were weighted as well as the total gut content. They were then skinned and the fillet was weighted as well and then used to perform the TMA content analysis. Fish fillet was obtained by skinning the gutted fish and then removing the flesh from the fish bone.

Samples of egg sacks were taken from 3 females from every group, in order to investigate on the possible effects of the diet contaminated with DON and ZEA.

During the 5th measurement of fish weight a fish jumped from the control aquarium

to the 5 ppm aquarium, thus corrupting the further weight analysis as well as mean and standard deviations.

2.2.4 Lipid quantification and oxidative stress

a- Lipid extraction:

Tissue samples were extracted with the method of Folch et al. (1957). All solvents used were ultrapure-grade by Sigma– Aldrich (Schnelldorf, Germany), and 100 mg/l) 100 mg/l butylated hydroxytoluene was added to the extraction mixture (chloroform/ methanol 2/1 v/v) as antioxidant.

Complex lipids were fractionated after the method of Leray et al. (1997), on short silica gel columns. Extracted lipids were transferred to glass chromatographic columns, containing 300 mg silica gel (230-400 mesh) for 10 mg of complex lipids. Lipids were then transmethylated by the base-catalysed sodium-methoxide method of Christie (1982). Neutral lipids were eluted with 10 ml chloroform for the above fat amount, then 15 ml of a solution of acetone and methanol (9:1 v/v) was added. 10 ml of pure methanol was instead used to elute the total phospholipids, which were evaporated under nitrogen stream and then transmethylated by methanolic boron trifluoride (BF3).

Gas liquid chromatography was performed on a Shimadzu 2100 apparatus, equipped with a SP-2380 (Supelco, USA) type capillary column (30 m x 0.25 mm internal diameter, 0.20 μm film, Cat. No.: 24110-U) and flame ionization detector (FID 2x10-11). Characteristic operating conditions were: injector temperature: 270 °C, detector temperature: 300 °C, helium flow: 28 cm/s. The oven temperature was graded: from 80 to 205 °C: 2.5 °C/min, 5 min at 205 °C, from 205 to 250 °C 10 °C/min and 5 min at 250 °C.

To identify individual FA, an authentic standard (Mixture Me100 (Cat.No: 90-1100, Larodan Fine Chemicals AB, Sweden)) was used. Results were expressed as weight percentage of total fatty acid methyl esters.

b- Lipid peroxidation index:

To evaluate oxidative metabolism and oxidative stress on the cell membranes, analysis were performed to measure the concentration of Malondialdehide (MDA), Glutathione (GsH) and Glutathione peroxidase (GSH-Px).

Aldehydes, such as malondialdehyde (MDA) are meta-stable end-products of lipid peroxidation. During this process the breakdown of the fatty acids occurs and small molecular weight products, such as aldehydes emerge. Among these the primary product is malondialdehyde, which was proposed as a diagnostic marker of *in vivo* lipid

peroxidation (Janero et al., 1990).
In this study, MDA concentration was determined from homogenized samples , which were frozen stored (-70 °C), after the addition of 0.9 ml cold (4 °C) physiological saline per 1 g of tissue, using the Lawrie method (Lawrie, 1974). Fish sample were kept frozen since the slaughter and then homogenized with an Ultra Turrax (Donau Lab AG, Linz, Austria). Estimation of thiobarbituric reactive substances (TBARS) levels was performed by the method of Placer (et al.,1966).
During a nucleophilic addition reaction malondialdehyde and some other small molecular weight primary and secondary end-products of lipid peroxidation formed a yellow-reddish MDA:TBA adduct with 2-thiobarbituric acid (TBA) in acidic environment (pH 2.2) and at high (100 °C) temperature.
Glutathione (GSH) is a tripeptide (L-γ-glutamyl-L-cysteinylglycine) with multiple functions in living organisms. As a carrier of an active thiol group in the form of a cysteine residue, it acts as an antioxidant either directly by interacting with reactive oxygen/nitrogen species (ROS and RNS) and electrophiles or by operating as a cofactor for various enzymes. Glutathione is moderately stable in the intracellular milieus because intracellular peptidases can cleave peptide bonds formed by the α-carboxyl groups of amino acids, but typically not the γ-carboxyl groups. The reduced and oxidized forms of glutathione act in concert with other redox-active compounds to regulate and maintain cellular redox status (Volodymyr et al.,2012). The GSH-Px acts as a scavenger of the peroxides generated during the burst of arachidonic acid (AA) metabolism. Such a mechanism inhibits the biosynthesis of both thromboxane A2 (TXA2) and lipoxygenase products (Perona et al, 1990), thus is an anti inflammatory protein as well as an indicator of oxidative stress.
Tissue samples (0.5 g) were homogenized in nine-fold volume of 0.65% NaCl and the samples were stored at -20 °C until analysis. Glutathione concentration was measured spectrophotometrically after deproteinisation with TCA solution (10% w/v) in alkaline buffer (Tris-HCl, 0.4 M, pH 8.9) using Ellmann's reagent (5,5'-dithiobis-2 nitrobensoic acid, Sigma, St. Louis) according to the method of Sedlak and Lindsay (1968).

Dienes and trienes are a marker for lipid peroxidation, being a structure of lipid hydroperoxides, known as LOOH, which are the early stage of MDA and thus indicating early oxidative stress, and were determined with liquid spectrophotometry, in solution, at the wavelength of 234 nm, while trienes were determined with the same technique at 268 nm (Shahidi et al., 2002).

2.2.5 TMA level measurement

TMA is a volatile compound, a fraction of the TVBN and is one of the molecules responsible for the off-flavour in fish. For the detection of this molecule 30 fish fillet, stored at -70 °C since the slaughtering of the fish, were processed for the quantification of TMA using an electronic nose, Alpha MOS FOX 4000 (Montpellier, France; Figure 4). Fillet processing consisted in long time thawing, 24 hours at +4 °C, in order to not destroy membranes and prevent bacterial growth. During this time the sensitivity of the device was tested with a solution of acetone and 2 with propanol, 1-propanol and 2-propanol respectively.

After this, 1 g homogenized (IKA A11 Basic lab Mill) meat sample was weighed into 20 ml headspace sample vials and was closed with silicone lined metal crimping heads. Sample vials were randomly placed into the analysis plate (Figure 4), but those were one-by-one taken from a fridge (+4 °C) placed upon this plate to avoid adulteration at room temperature, on which the prior measurement sample queue stood.

One full measurement cycle lasts for 20 minutes, but this also includes the purging time, the elimination of the volatile compounds from the electronic sensors allocated into a 18-member sensor array. Thus, only the data during the immediate sample adsorption (120 seconds) on the MOS (metal oxide sensor) was handled as results, all other data are representing the purging delay.

The measurement happens from the over-sample head space. To create a stabilized valor volume over the sample, a pre-incubation is used, with the settings given in Table 5. After this step the auto sample makes the injection via an 5 ml Hamilton syringe into the analyzer. The headspace generation and the syringe temperature can be freely adjusted; current settings are given above in the table.

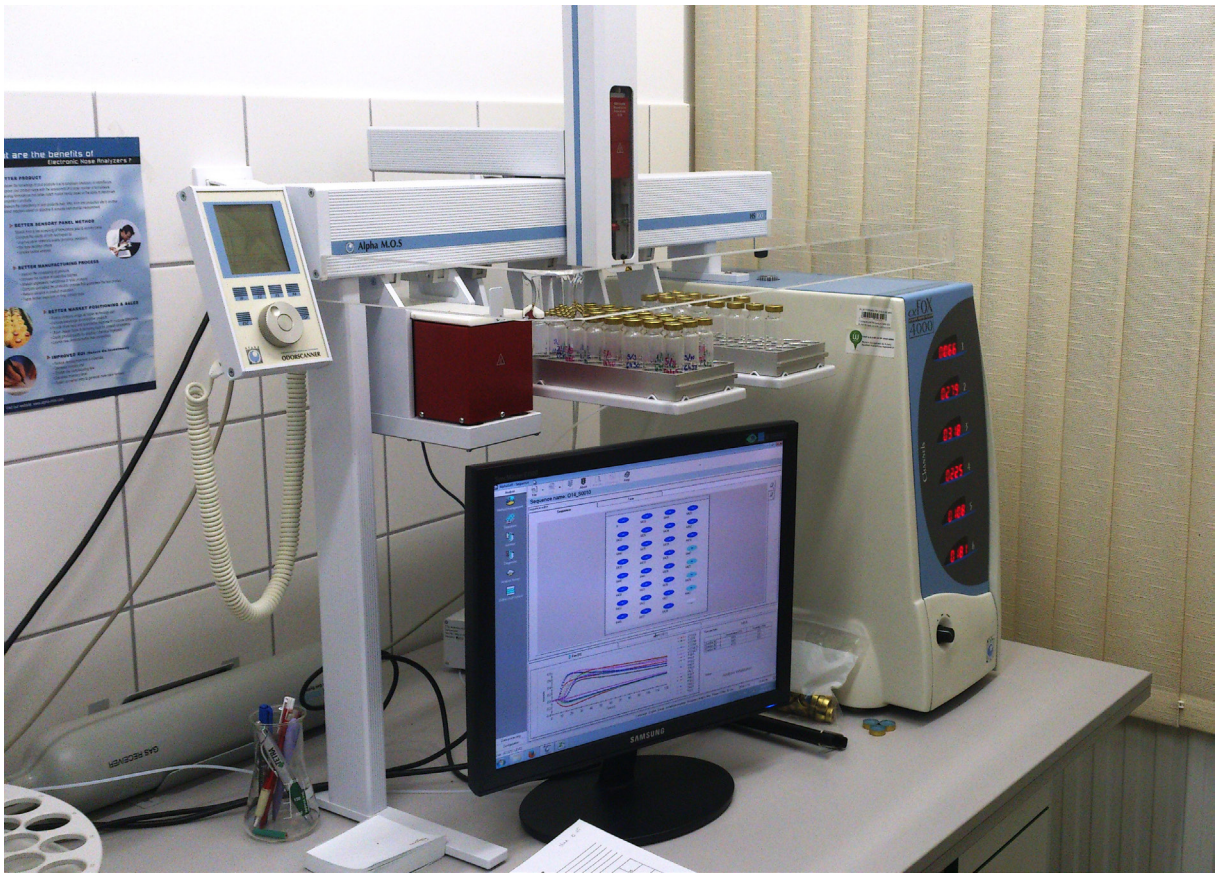


Figure 4: Electronic nose

In the picture it is possible to see the auto sampler above the monitor, while the temperature and the other parameters are constantly checked.

Table 5: Flow chart of the Electronic nose

Component working	Operation	Measure required
Analyser		
	acquisition time	120
	acquisition period	1
	acquisition delay	1080
	flow (ml/min)	150
	injection volume (µl)	**3000**
	injection speed (ms)	**500**
Autosampler		
	incubation time	**180**
	incubation temperature, °C	**60**
	flushing time (purging)	120
	syringe temperature, °C	70
	fill speed	500
	agitation speed (s)	500
	agitation on (number)	5
	agitation off (number)	2

Cycle steps of the electronic nose, divided per function. Where not specified the value in the box is in seconds. The proper analysis process is in the colored box.

2.3 Statistical Analysis

All the data produced during this study and showed further on have been statistically processed with the IBM SPSS Statistic version 20.0 software (SPSS, Inc., Chicago, IL, USA). One way ANOVA was used to determine the differences among the groups for body weight, FE, lipid profile, Gonadosomatic Index, eggs size and antioxidant, in order to correlate the toxin concentration with the effect it might have had on the parameter. The significance limit set for the ANOVA was $\alpha<0.05$, and different subsets in the tables are identified from the different letter of the value. Tukey HSD and LSD posto hoc tests were used to express the results.
The Canonical Discriminant Function was performed on the odor profile to determine whether this was different or not among the groups, revealing thus a different composition in the total volatile compounds.

Weight gain was calculated as

$$\text{Weight gain}(\%) = \frac{(\text{tank weight during the week})_i}{(\text{tank weight during the week})_j} \times 100$$

Where i is the initial week considered and j the following.

Feed Conversion Rate and Feed Efficiency was calculated as

$$FCR = \frac{\text{total feed administred during the week}}{\Delta\,\text{Weight}}$$

$$FE = \frac{\Delta\,\text{Weight}}{\text{total feed administred during the week}}$$

With Δ Weight= $(\text{tank weight during the week})_j - (\text{tank weight during the week})_i$

Eggs size was calculated as

$$\text{Egg volume} = \left(\frac{\text{length}}{2}\right) \mathbf{x} \left(\frac{\pi\,(\text{width})^2}{2}\right)$$

Length and width of the eggs were expressed in μm.

Odor profile was processed with AlphaSoft 12.3 to obtain Figure 6.

3. Results and discussion

3.1 Productive parameters:

Body weight was checked weekly by anesthetizing the fish and drying them with a towel before weighting them during the first weeks of the experiment. The analysis of the single week weight showed no significant difference among the treatments but for the last two weeks of the experiment (Table 6). In the fifth week only the Least Statistical Difference analysis showed statistical differences between control group and the 5 ppm group, the one with the highest exposure.
In the last week instead, the result was more evident and showed significant difference between control group and all the treatment, thus showing and impaired growth due to the treatment itself. To avoid result contamination one-way ANOVA was performed including sex, temperature and towin concentration, each of whom was considered both as a single parameter and a factor among the others. The result was not different from the previous analysis.

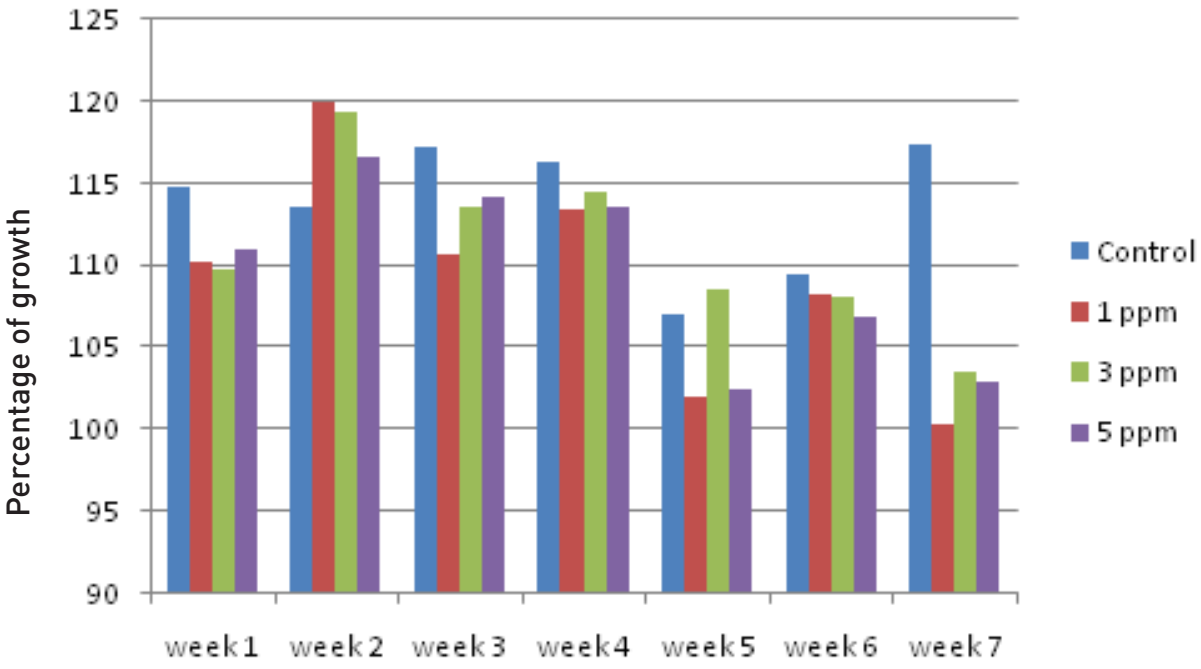


Figure 5: Chart of the Weight Gain

Representation of the weight gain (percentage) during the weeks (abscissa), expressed as final weight/initial weight x 100

Males however showed an higher mean value than the females, probably due to the oogenesis that dispatch lots of energy in the creation of the vitelline sac and other nutrients for the fry. Weight gain itself was measured and statistical differences were not found, using one way ANOVA, among the groups (Figure 5).

Table 6: Body weight of animals in different groups from the 1st week after the feeding of toxin containing diet, expressed in g.

Toxin concentration	No	Mean 1st week	Mean 2nd week	Mean 3rd week	Mean 4th week	No	Mean 5th week	Mean 6th week
0	32	28,28	32,13	37,69	43,33	31	52,06	**60,10b**
1	32	26,71	32,03	35,47	39,80	32	45,06	**44,66a**
3	32	27,09	32,34	36,78	41,59	32	50,18	**51,16a**
5	32	26,60	31,03	35,41	39,79	33	44,65	**45,42a**
Significance		0,84	0,93	0,77	,54		0,06	0,05
Mean Square (Error)							131,588	162,244

For the fifth and sixth week an armonic size of 31,984 was used, for both tables the Alpha was p<0.05. Letters a and b indicate different subsets.

Table 7: Feed Efficiency during the 6 weeks

Toxin concentration	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
0	0.453	0.823	0.775	0.174	0.449	0.829
1	0.668	0.510	0.635	0.094	0.396	0.015
3	0.646	0.662	0.680	0.414	0.384	0.167
5	0.575	0.648	0.645	0.268	0.330	0.141
Significance	0,97	0,94	0,97	0,65	0,34	0,17

Average feed efficiency of the fish during the experiment, sorted per group.

Feed Efficiency was evaluated and compared with the one way ANOVA, in order to prove loss of efficiency in the feed metabolism. The feeding was *ad libitum*, so the basis of the FE was the 3% of the live weight given each day, and the feed excess would be removed on daily basis. The excess could not be measured as it would be moistened by the water and mixed with feces.

Based on the exposed results we can say that an high, yet legal, amount of DON in diet can impair the growth of this fish, even though the weight gain and the FE seem not to be affected during this subchronical exposure level. Probably, yet not certainly, a more extended exposure could have shown effects even on weight gain and FE themselves, being the body weight itself statistically different among the groups only in the last week. These parameters are probably the most interesting for the fish farmers, as they can enhance the economical profit, giving a larger number of growth cycles per year, a shorter growth cycle and a better use of feed.

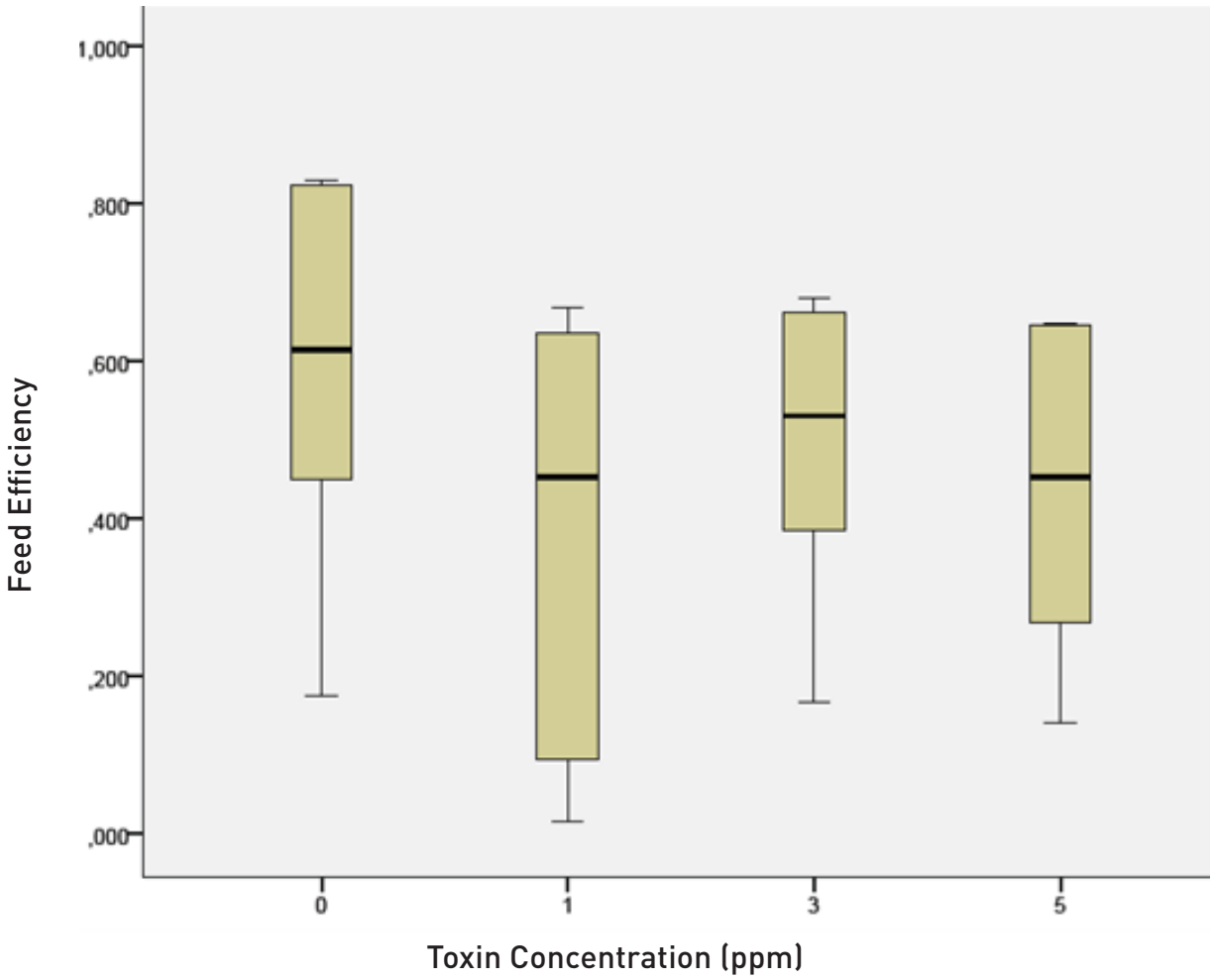


Figure 6: Statistical analysis of the FE

Feed Efficiency of the groups during the weeks, not only there is no a significant difference among the groups in the ongoing weeks, but even the distribution of the data is not correlated to the toxin in the feed.

3.2 Guts and sexual organs

A reliable index used to individuate problems and dysfunction in reproductive system is the GSI, the gonadosomatic index, measured as sexual organ weight on total body weight. Decreased GSI is indicative of decreased hypothalamic, pituitary or gonadal activity and may be used along with an histological analysis to detect which tissue is

mainly affected by the disease that decrease the index itself. Histopatological analysis however did not report evidence of visceral lesions.

Table 8: Gonadosomatic Index

Sex	Toxin concentration	No	GSI
Males	0	9	0,012
	1	9	0,004
	3	10	0,008
	5	10	0,004
	Significance		0,052
Females	0	5	**0,042a**
	1	5	**0,037a**
	3	4	0,080ab
	5	4	**0,109b**
	Significance		<0,05

The comparison between males and females shows a remarkable difference between the two sex, reason why they have to be analyzed separately. The males have a lower GSI due to the higher body weight and lower weight of sexual organs. Letters and b indicate different subsets

Eggs size was measured from a representative amount of fish for each group, yet only the size of the eggs itself was possible to measure, the egg sac being sometimes broken during the fish evisceration. Measurements were performed at the Department of Animal Medicine, Production and Health (Padova University), using a millimetered microscope. The volume was calculated as 2 cones having the same base (which radium was half the width) and the height equal to half the length.
The analysis showed evidence of significant difference among the groups($p<0.05$), being the control group the one with the largest eggs. This result is probably due to the presence of ZEA, which is known to be a mimic-estrogen and may thus have disrupted the oogenesis. The statistical analysis gave evidence of difference among the groups, also with a negative correlation between toxin concentration and eggs size.
A larger egg means larger yolk reserve for the fry and a better FCR along with an higher consumption rate of feed; this beginning advantage in nature is overwhelmed by environmental condition, but under controlled farming parameters could result in better growing performances, reaching selling size in less time. (Brooks et al., 1997).

Table 9: Egg volume (mm3) comparison

Toxin concentration	No	Egg volume
0	90	1763,87a
1	90	903,39b
3	64	607,27c
5	60	514,37c
Significance		<0,05

This table allows us to appreciate the correlation between eggs size decreasing and higher level of exposure. Letter a, b and c indicate different subsets. An armonic size of 73,376 was used to correctly analyze the differences among the groups.

This study showed no evidence of differences among the males of the four groups (Table 8), yet the females of the highest exposure showed significantly lighter gonads than the control group and the 1 ppm group (Table 9), yet this could be due both to an estrogenic activity of ZEA or to the depletion of body weight induced by DON or to an eventual synergistic effect of these two mycotoxin combined.

3.3 Oxidative stress

Being DON a toxin that disrupt the oxidative metabolism an investigation on the most common antioxidant parameter was needed, so glutathione, glutathione peroxidase, conjugated dienes and trienes and plasma malondialdehyde were evaluated in fish liver.

3.3.1 GsH and GsHPx:

This tripeptide (y-glutamylcysteinylglycine), which functions in metabolism, catalysis, transport, and cellular protection, provides cells with their reducing processes. Glutathione deficiency in newborn rats and in guinea pigs is lethal, but death can be prevented by administration of high doses of ascorbic acid (Meister et al., 1992). Glutathione is one of the most efficient antioxidant, its depletion may be caused by high amount of free radicals and liver stress, thus its investigation was necessary to clarify if DON has an acute oxidative effect. The statistical analysis yet showed no difference among the groups, not even indicating a trend of depletion at increasing exposure.

3.3.2 MDA:

Total quantification of MDA did not differ significantly among the groups, and as in the case of GsH and GsHPx was impossible even to identify a trend. The absence of significant difference among the groups proved the lack of oxidative stress effect of DON in this short-term study.

3.3.3 Dienes and Trienes:

The quantification of trienes and dienes showed no significant difference among the groups, and a distribution of quantification not correlated to the amount of toxin in the feed. Antioxidant content was not statistically different among the groups, thus the oxidative stress effect usually reported for DON in cell culture and in vivo was not confirmed. This could be due to the relatively short period of exposure rather than to the actual incapacity of DON to provoke oxidative stress of the higher oxidative tolerance of some kind of fish (Pietsch et al., 2014).

3.4 Lipids analysis

In order to investigate on the metabolic consequences the diet containing DON and ZEA a fatty acid profile was obtained from the fish fillet. For this purpose 6 fillet samples were taken from each group and processed with the Christie method and then processed using the GC/MS. The C16:0, C20:3 n-6 and C22:5 n-3 have significant differences between the control group and the highest exposure one (Tables 10,11 and 12). The C22:5 n-3, (DPA), and C20:3 n-6, (dihomo-gamma-linolenic acid -DGLA-), are polyunsaturated fatty acids (PUFA), involved in regulation of the inflammatory process, as they regulate inflammatory responses through the production of eicosanoids including prostaglandins (PGs), thromboxanes (TXs) and leukotrienes (LTs) (Aslan et al., 2014), thus its increasing is a symptom of the effects of DON on the immune system of the fish. Also, the longer the chain and the more unsaturated is the fatty acid, the more stressful is for the organism to have high quantity of it, as demonstrated in cytometric on lymphocytes assay by Lima (et al., 2002).

Table 10: Effect of dietary toxins concentration on DPA content in fish fillets (% on total lipids)

Toxin concentration	No	C22:5 n-3 percentage
0	6	0,2683a
1	6	0,4750ab
3	5	0,5240ab
5	5	0,7580b
Significance		<0,05

Comparison of the mean percentage of DPA in tilapia at the end of the 6th week; letters a and b in apex indicate different subsets. An armonic size of 5.455 was used to compare different groups.

Table 11: Effect of dietary toxins concentration on DGLA content in fish fillets (% on total lipids)

Toxin concentration	No	C20:3 n-6 percentage
0	6	0,5833a
1	6	0,6983ab
3	5	0,7340ab
5	5	0,8340b
Significance		<0,05

Comparison of the mean percentage of DGLA in tilapia with different level of exposure to DON, letters a and b in apex indicate different subsets. An armonic size of 5.455 was used to compare different groups.

C16:0 on the other side is a SCFA, whose biological effect is demonstrated to be adverse for human and mice cells at high concentration, is present at lower concentration in the 5 ppm group, indicating thus a lower possibility to induce the formation of the C16-PAF, which is a highly effective inflammatory mediator.

In vitro tests on stabilized cell culture of melanoma from human skin SK-Mel 23 and 28 showed the toxic effect of palmitic acid at concentration higher than 200μM, at which it produced DNA fragmentation in treated cell at 24 and 48 hours of exposure (Nogueira et al., 2005).

The reason of this difference may although be because in Perciformes the liver is one of the main sites of lipid storage and palmitate is generally acting as an oxidisable energy source. The dominant palmitate accretion as a result of hepatic lipogenesis is otherwise characteristic for increased energy uptake (Molnar et al., 2012).

Table 12: Effect of dietary toxins DON and ZEA on Palmitic acid content in fish fillets (% on total lipids)

Toxin concentration	No	Palmitate percentage
0	6	15,556a
1	6	16,820ab
3	5	17,365ab
5	5	18,435b
Significance		<0,05

Palmitic acid depletion is proportional to the amount of toxin in the feed. An armonic size of 5.455 was used to compare different groups; letters a and b in apex indicate different subsets.

As previously described, only few fatty acid showed statistical differences among the groups, particularly some long chain fatty acids related to inflammatory status. This parameter indicates that the already evidenced effect of DON in stimulating immune system in terrestrial animals can be found even in aquatic organism.

3.5 TMA levels

TMA is difficult to detect with the electronic nose, so the whole volatile part was analyzed in order to get a total profile. The preliminary study (Figure 6) on the sensibility granted the choice of the reliable sensors on which to base the study.

With the aim of testing the reliability of the method and the analysis, supporting the results, 2 sets of analysis were established. The first one gave every sample a sensory profile, derived from the 17 working sensors of the electronic nose. The second one was a blind analysis that tried to associate every sample to the group predicted with the analysis of the previous analysis. Each sample was tested with each sensor, giving a specific profile to every group by comparing the mean values for each sample, for a complex of smells.

In this way a "group profile" was achieved and the reliability of the system, as well as the efficiency of this test could be proven by a second blind sensing of the samples. In this case an accuracy above 90% could have shown success, identifying 9 times out of every 10 the sample in the right group. The efficiency of this test could not reach above 80%, as seen in table 17 and so it's not possible to say that there actually is a different odor profile per each group, and whether it was it's not possible to ascribe it to the only factor of TMA.

The efficiency of this test could not reach above 80%, as seen in table 17 and so it's not possible to say that there actually is a different odor profile per each group, and whether it was it's not possible to ascribe it to the only factor of TMA.

Odor profile was investigated with the intention of proving that DON may alter the TMA concentration in fish fillet and therefore fish shelf life and freshness, yet the result was not clear enough to prove that actually the mixture of DON and ZEA could change the aromatic profile even though different group profiles could be identified.

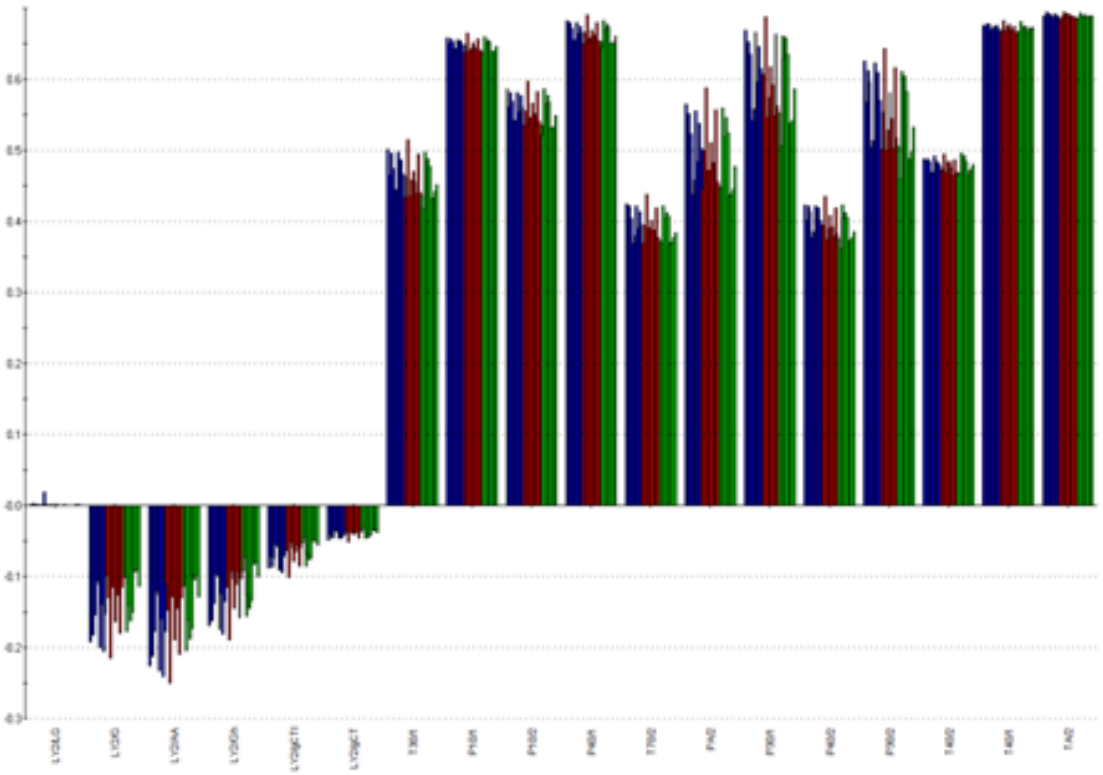


Figure 6 : Odor profile of the fishes, sorted per group (MOS output)

The blue column represent the 1 ppm group, the red one the 3 ppm group and the green the 5 ppm group. AlphaSoft 12.3 was used for the evaluation of the data.

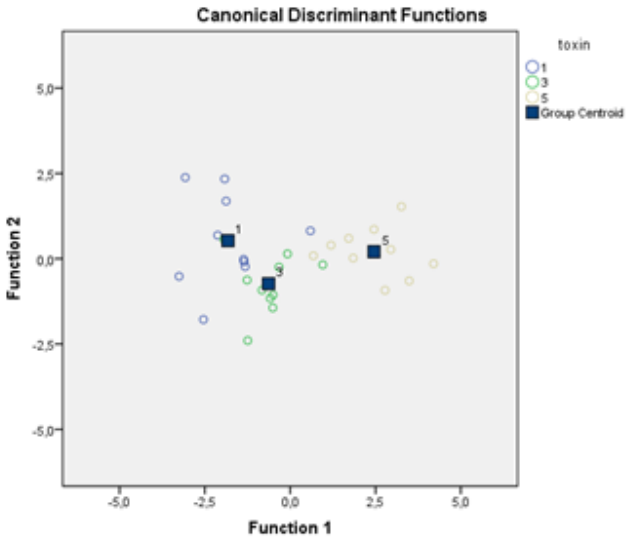


Figure 7: Canonical discriminant Function of the odor profile

This graph represent the correlation of the results achieved with the 2 Discriminant Functions; this visualization allows us to identify differences of patterns in the evaluated profiles.

Table 17: Blind classification results

Test		Toxin concentration	Predicted Group Membership			Total
			1	3	5	
Original	Count	1	7	2	1	10
		3	1	8	1	10
		5	0	1	9	10
	%	1	70,0	20,0	10,0	100,0
		3	10,0	80,0	10,0	100,0
		5	0,0	10,0	90,0	100,0
Cross-validated	Count	1	4	5	1	10
		3	5	4	1	10
		5	0	3	7	10
	%	1	40,0	50,0	10,0	100,0
		3	50,0	40,0	10,0	100,0
		5	0,0	30,0	70,0	100,0

The previously classified samples have to be re examined in a blind way, in order to test the specificity of the test. The most ineffective results are obtained between groups 1 and 3 ppm of exposure, where the error in the cross validation is 50%.

4. Conclusions and future research perspectives

This study aimed to enlighten on the effects of a diet containing a relatively low concentration of DON and ZEA, which are some of the most common mycotoxin, especially in cold and moist environments or reaping season, on a tilapia specie, *O. niloticus*.
This fish, being one of the most farmed fish all over the world, primarily in emerging countries, has a very high economical importance, furthermore the new aim of aquaculture is to replace animal-sourced protein (i.e. fish) with vegetable-source protein, such as soybean and barley.
This change of diet may be more ethically correct (*Salmo salar* , the Atlantic salmon, needs 1.1 to 1.5 kg of dry matter from fish to grow 1 kg BW (Pelletier et al., 2009)), sustainable and economically more efficient, yet opens the gate to new adversity, such as different bacterial contaminations and fungal contamination along with mycotoxicosis and mycotoxin bioactivation (Guan et al., 2009; Pietsch et al., 2013).
The overall analysis proves that a diet containing a high level of DON and ZEA, whether legally allowed, can actually disrupt and alter many farming and productive parameters even if not maintained during the whole growing period. This fact suggests that either a synergistic effect is occurring between DON and ZEA (Perdrosa, 2011) or a 5 ppm dose of DON is sufficient to induce severe effect on the growth gain. Furthermore ZEA, as a mimic estrogenic xenobiotic is proven to disrupt gametes (Kime et al., 1999).
Aquaculture is one of the most recent field of animal production and its potential in providing food is not quantified yet, as the technologies involved are continuously upgraded and there are new farmed fishes every year. A possible future improvement could consist in deep DNA sequencing, providing knowledge about growth, muscle development and yield of fillet, as well as metabolism of toxin and antibiotics. A MAS could slightly improve productivity even in the field of aquaculture.

Usually the effect of a single mycotoxin is investigated, while naturally it occurs that a multiple contamination is present, even from different molds strains or species. The comprehension of how mycotoxins work together could help us redefine the TDI, the NOAEL and other toxicological parameters in order to preserve human and animal health. This investigation on multiple mycotoxin contamination occurring at the same time could also improve the efficiency of animal production, reducing the FCR and enhancing the production rate of some food sources.

Recent studies reveal that not the whole amount of mycotoxin present in crops is detected with the conventional methods, exposing the problem of hidden mycotoxins. These mycotoxins are present in the matrix but usually bound to other components, forming complex molecules that are not detectable with HPLC, TLC or even LC-MS/MS. The toxins however can eventually fulfill their toxic function after digestion, as during this process the bounds that linked mycotoxins to other components are severed (Szabó-Fodor et al., 2014). To evaluate the real amount of toxin that can actually accomplish toxic function in the organism the usual method should be improved by a pre-digestion of each sample.

Eventually a more accurate analysis on the correlation between TMA and DON should be set in order to evaluate if there actually is an effect induced by crescent doses of this toxin in fish, as there are feeble results showing this evidence.
A longer time of exposure could have brought to light more solid results regarding fat composition, odor profile and farming parameters as well. The analysis of these data permit to assert that the highest exposure dose lead to worsen the productive and reproductive performances, giving even the hint of inflammatory status in the organism.

Bibliographic references

Agag B.I. 2004 Mycotoxins In Foods And Feeds 3-Zearalenone. Association University Bull Environmental Research, Volume 7, Number 2, Pages 173-206

Alassane-Kpembi I. , Kolf-Clauw M. , Gauthier T. , Abrami R., Abiola F. A. , Oswald I. P. , Puel O. 2013 New insights into mycotoxin mixtures: The toxicity of low doses of Type B trichothecenes on intestinal epithelial cells is synergistic. Toxicology and Applied Pharmacology, Volume 272, Issue 1, Pages 191-198

Arukwe A. , Grotmol T., Haugen T. B. , Knudsen F. R. , Goksøyr A. 1999 Fish model for assessing the in vivo estrogenic potency of the mycotoxin zearalenone and its metabolites. Science of The Total Environment, Volume 236, Issues 1–3, Pages 153-161

Aslan M. , Aslan I. , Özcan F. , Eryılmaz R. , Ensari C. O. and Bilecik T. 2014 A pilot study investigating early postoperative changes of plasma polyunsaturated fatty acids after laparoscopic sleeve gastrectomy. Lipids in Health and Disease, Volume 13, Page 62

Bailey G., Selivonchick D., Hendricks J. 1987 Initiation, promotion, and inhibition of carcinogenesis in rainbow trout (*Oncorhynchus mykiss*). Environ Health Perspect, Volume 71, Pages 147-153

Binder E.M. , Tan L.M. , Chin L.J., Handl J. , Richard J. 2007 Worldwide occurrence of mycotoxins in commodities, feeds and feed ingredients. Animal Feed Science and Technology, Volume 137, Issues 3–4, Pages 265-282

Bonisławska M. , Formicki K. , Korzelecka-Orkisz A. , Winnicki A. 2001 FISH EGG SIZE VARIABILITY: BIOLOGICAL SIGNIFICANCE. Electronic Journal of Polish Agricultural Universities (EJPAU), Volume 4, Issue 2

Brooks S. , Tyler C. R. , Sumpter J. P. 1997 Egg quality in fish: what makes a good egg? Fish Biology and Fisheries, Volume 7, Issue 4, Pages 387-416

Byelashov A. 2013 DPA: An Up-and-Coming Fatty Acid. http://omega3.supplysideinsights.com/articles/2013/05/dpa-an-up-and-coming-fatty-acid.aspx

Christie W.W. 1982 A simple procedure for rapid transmethylation of glycerolipids and cholesteryl esters. Journal of Lipid Research, Volume 23, Pages 1072-1075

CONTAM (EFSA Panel on Contaminants in the Food Chain) 2013 Scientific Opinion on risks for animal and public health related to the presence of nivalenol in food and feed. EFSA Journal 2013, Volume11, Page 3262

EFSA 2006 Commission Recommendation On The Presence Of Deoxynivalenol, Zearalenone, Ochratoxin A, T-2 And Ht-2 And Fumonisins In Products Intended For Animal Feeding. 2006/576/EC

FAO 2012 World Review Of Fisheries And Aquaculture, Status and trends, part One. Rome.

Folch J., Less M., and Stanley G.H.S. 1957 A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry, Volume 226, Pages 497–499

Griessler K. , Rodrigues I. , Handl J. , Hofstetter U. 2010 Occurrence of mycotoxins in Southern Europe. World Mycotoxin Journal, Volume 3, Issue 3, Pages 301-309

Guan S. , He J. , Young J. C. , Zhu H. , Li Xiu-Z. , Ji C. , Zhou T. 2009 Transformation of trichothecene mycotoxins by microorganisms from fish digesta. Aquaculture, Volume 290, Issues 3–4, Pages 290-295

Guarro J. , Gené J. 1995 Opportunistic fusarial infections in humans. European Journal of Clinical Microbiology and Infectious Diseases, Volume 14, Issue 9, Pages 741-754

He C.-H., Fan Y.-H. , Wang Y., Huang C.-Y. , Wang Xi-C. , and Z. H.-B. 2010 The Individual and Combined Effects of Deoxynivalenol and Aflatoxin B1 on Primary Hepatocytes of *Cyprinus carpio*. International Journal of Molecular Sciences , Volume 11, Pages 3760–3768

Hooft J. M. , Elmor Abd El Hakeem I. , Encarnação P. , Bureau D. P. 2011 Rainbow trout (*Oncorhynchus mykiss)* is extremely sensitive to the feed-borne *Fusarium* mycotoxin deoxynivalenol (DON). Aquaculture, Volume 311, Issues 1–4, Pages 224-232

Huff W. E., Harvey R. B., Kubena L. F., And Rottinghaus G. E. 1988 Toxic Synergism Between Aflatoxin and T-2 Toxin. Broiler Chickens Poultry Science, Volume 67, Pages 1418-1423

Janero D. R. 1990 Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. Free Radical Biology & Medicine, Volume 9, Pages 515–540

Jozef S., Raymond H. L. 1968 Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Analytical Biochemistry, Volume 25, Pages 192-205

Kime David E. 1999 A strategy for assessing the effects of xenobiotics on fish reproduction. Science of The Total Environment, Volume 225, Issues 1–2, Pages 3-11

Kromhout D., Giltay E.J., Geleijnse J.M. 2010 n-3 fatty acids and cardiovascular events after myocardial infarction. The New England journal of medicine, Volume 363, Pages 2015-2026

Kubosaki A., Aihara M., Park B.J., Sugiura Y., Shibutani M., Hirose M., Suzuki Y., Takatori K. and Sugita-Konishi Y. 2008 Immunotoxicity of nivalenol after subchronic dietary exposure to rats. Food and Chemical Toxicology, Volume 46, Pages 253-258

Lawrie, R.A. 1974 Meat Science, 2nd edn. Pergamon Press, Oxford, UK.

Leray, C., Andriamampandry, M., Gutbier, G., Cavadenti, J., Klein-Soyer, C., Gachet, C. & Cazenave, J.P. 1997 Quantitative analysis of vitamin E, cholesterol and phospholipid fatty acids in a single aliquot of human platelets and cultured endothelial cells. Journal of Chromatographic Biology, Volume 696, Pages 33–42

Lima T.M. , Kanunfre C.C. , Pompéia C. , Verlengia R. , Curi R. 2002 Ranking the toxicity of fatty acids on Jurkat and Raji cells by flow cytometric analysis. Toxicology in Vitro, Volume 16, Issue 6, Pages 741-747

Lushchak V. I. 2012 Glutathione Homeostasis and Functions: Potential Targets for Medical Interventions. Journal of Amino Acids, Volume 2012, Article ID 736837, 26 pages

Manning B. B. , Abbas H. K. , Wise D. J., Greenway T. 2014 The effect of feeding diets containing deoxynivalenol contaminated corn on channel catfish (*Ictalurus punctatus*) challenged with *Edwardsiella ictaluri*. Aquaculture Research, Volume 45, Pages 1782-1786

Marasas W. F. 2001 Discovery and occurrence of the fumonisins: a historical perspective. Environ Health Perspect, Volume 109(Suppl 2), Pages 239–243

Maresca M. , Mahfoud R. , Garmy N. and Fantini J. 2002 The Mycotoxin Deoxynivalenol Affects Nutrient Absorption in Human Intestinal Epithelial Cells. Journal of Nutrition, Volume 132: 9, Pages 2723-2731

McCormick S. P., Stanley A. M. , Stover N. A.and Alexander N. J. 2011 Trichothecenes: From Simple to Complex Mycotoxins. Toxins Volume 3, Pages 802–814.

Molnár T., Gergely and Biró J., Hancz C., Romvári R., Varga D., Horn P., Szabó, A. 2012 Fatty acid profile of fillet, liver and mesenteric fat in tilapia (*Oreochromis niloticus*) fed vegetable oil supplementation in the finishing period of fattening. ARCHIV FÜR TIERZUCHT-ARCHIVES OF ANIMAL BREEDING, Volume 55, Pages 194-205

Morgavi D.P. , Riley R.T. 2007 An historical overview of field disease outbreaks known or suspected to be caused by consumption of feeds contaminated with *Fusarium* toxins. Animal Feed Science and Technology, Volume 137, Issues 3–4, Pages 201-212

Nährer K. and Kovalsky P. 2014 BIOMIN Mycotoxin Survey. A summary of the major threats. BIOMIN Holding GmbH Industriestrasse 21, A-3130 Herzogenburg, AUSTRIA

Nebbia C. 2009 Micotossine, In Residui di farmaci e contaminanti ambientali nelle produzioni animali. Ed. Carlo Nebbia, Pages 453-475 Napoli: EdiSES.

Nguyen A. T., Grizzle J. M. , Lovell R. T. , Manning B. B. , Rottinghaus G. E. 2002 Growth and hepatic lesions of Nile tilapia (*Oreochromis niloticus*) fed diets containing aflatoxin B1. Aquaculture, Volume 212, Issues 1–4, Pages 311-319

Niizeki N. , Daikoku T. , Hirata T. , El-Shourbagy I. , Song X. , Sakaguchi M. 2002 Mechanism of biosynthesis of trimethylamine oxide from choline in the teleost tilapia, *Oreochromis niloticus*, under freshwater conditions. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, Volume 131, Issue 3, Pages 371-386

Nogueira de Sousa A. L., Martins de Lima T., Curi R., de Lauro Castrucci A. M. 2005 Toxicity of fatty acids on murine and human melanoma cell lines. Toxicology in Vitro, Volume 19, Issue 4, Pages 553-560

Osman A. M., Pennings J. L. A. , Blokland M., Peijnenburg A., van Loveren H. 2010 Protein expression profiling of mouse thymoma cells upon exposure to the trichothecene deoxynivalenol (DON): Implications for its mechanism of action. Journal of Immunotoxicology, Volume 3, Pages 147-156

Pedrosa K. , Borutova R. 2011 Synergistic effects of mycotoxins discussed. Feedstuffs Volume 83, Issue 19, Pages 1-3

Pelletier N. , Tyedmers P. , Sonesson U., Scholz A., Ziegler F. , Flysjo A. , Kruse S., Cancino B. and Silverman H. 2009 Not All Salmon Are Created Equal: Life Cycle Assessment (LCA) of Global Salmon Farming Systems. Environmental Science & Technology, Volume 43 , Pages 8730-8736

Perona G., Schiavon R., Guidi G.C., Veneri D., Minuz P. 1990 Selenium dependent glutathione peroxidase: a physiological regulatory system for platelet function. Thromb Haemost, Volume 64, Pages 312-318

Pestka James J. 2007 Deoxynivalenol: Toxicity, mechanisms and animal health risks. Animal Feed Science and Technology, Volume 137, Issues 3–4, Pages 283-298

Pestka James J. 2010 Deoxynivalenol: mechanisms of action, human exposure, and toxicological relevance. Archives of Toxicology Volume 84, Issue 9 , Pages 663-679

Petrinec Z., Pepeljnjak S., Kovacic S., Krznaric A. 2004 Fumonisin B1 causes multiple lesions in common carp (*Cyprinus carpio*). Dtsch Tierarztl Wochenschr, Volume 111, Pages 358-363

Pietsch C. , Bucheli T. D. , Wettstein F. E. , Burkhardt-Holm P. 2011 Frequent biphasic cellular responses of permanent fish cell cultures to deoxynivalenol (DON). Toxicology and Applied Pharmacology, Volume 256, Issue 1, Pages 24-34

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, Volume 5, Pages 184-192

Pietsch C., Schulz C., Rovira P., Kloas W., Burkhardt-Holm P. 2014 Organ Damage and Hepatic Lipid Accumulation in Carp (*Cyprinus carpio L.*) after Feed-Borne Exposure to the Mycotoxin, Deoxynivalenol (DON). Toxins, Volume 6, Pages 756-778

Pitt I.J., Hocking. A.D., Bhudhasamai, K., Miscamble, B.F., Wheeler, K.A. and Tanboon-Ek, P. 1994 The normal mycoflora of commodities from Thailand. 2. Beans. rice. small grains and other commodities. International Journal of Food Microbiology, Volume 23, Pages 35-53

Placer Z.A., Lind L., Cushmann M., Johnson B.C. 1966 Estimation of product of lipid peroxidation (MDA) in biological systems. Analitical Biochemestry, Volume 16, Pages 359–364

Pulvenis de Séligny J.-F. , Gumy A. and Grainger R. Wijkström U. Ababouch L., Cochrane K. , CsirkeJ. , Gueye N. , Jia J. , Nomura I. , Turner J. and Valdimarsson G. 2009 The State of World Fisheries and Aquaculture 2008. FAO Fisheries and Aquaculture Department. Rome

Rakocy, J. E. 2005 Cultured Aquatic Species Information Programme. Oreochromis niloticus. Cultured Aquatic Species Information Programme. In:FAO Fisheries and Aquaculture Department [online]. Rome

Richard J. L. 2007 Some major mycotoxins and their mycotoxicoses—An overview. International Journal of Food Microbiology, Volume 119, Issues 1–2, Pages 3-10

Ryerse I., Lumsden I. 2014 The Effects of Foodborne Deoxynivalenol Exposure in Rainbow Trout (*Oncorhynchus mykiss*) Experimentally Infected with F. psychrophilum. Thesis Pathobiology Master of Science Department of Pathobiology, University of Guelph

Sanden M. , Jørgensen S. , Hemre Gro-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, Volume 50, Issue 12, Pages 4441-4448

SCF 2004 Updated opinion of the Scientific Committee on Food on Fumonisin B1, B2 and B3. SCF/CS/CNTM/MYC/28 Final, In: EUROPEAN COMMISSION HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Schatzmayr G. and Streit E. 2013 Global occurrence of mycotoxins in the food and feed chain: facts and figures. World Mycotoxin Journal, Wageningen Academic Publishers, Volume 6, Number 3, Pages 213-222

Schwartz P. , Thorpe K. L. , Bucheli T. D. , Wettstein F. E. , Burkhardt-Holm P. 2010 Short-term exposure to the environmentally relevant estrogenic mycotoxin zearalenone impairs reproduction in fish. Science of The Total Environment, Volume 409, Issue 2, Pages 326-333

SCOOP (Scientific Cooperation on Questions Relating to Food) 2003 Task 3.2.10 Collection of occurrence data of *Fusarium* toxins in food and assessment of dietary intake by the population of EU member states. Subtask II: zearalenone. European Commission, Directorate General Health and Consumer Protection, Brussels, Belgium, Pages 239-482.

Sedlak J., Lindsay R.H. 1968 Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Analitical Biochemestry, Volume 25, Pages 192–205

Sergent T. , Parys M. , Garsou S., Pussemier L. , Schneider Y.-J., Larondelle Y. 2006 Deoxynivalenol transport across human intestinal Caco-2 cells and its effects on cellular metabolism at realistic intestinal concentrations. Toxicology Letters, Volume 164, Issue 2, Pages 167-176

Shahidi F. and Wanasundara U. N., in C. C. Akoh and D. B. Min 2002 Food Lipids: Chemistry, Nutrition and Biotechnology. Food Lipids: Chemistry, Nutrition and Biotechnology, Marcel Dekker, Inc., New York, Pages 465–487.

Sharma D., Asrani R. K., Ledoux D. R., Jindal N., Rottinghaus G. E., and Gupta V. K. 2007 Individual and Combined Effects of Fumonisin B1 and Moniliformin on Clinicopathological and Cell-Mediated Immune Response in Japanese Quail Poultry. Science, Volume 87, Pages 1039-1051

Shi-Xi D., Li-Xia T., Fu-Jia L., Sheng-Jie J., Gui-Ying L., Hui-Jun Y., Zhen-Yu D., Yong-Jian L. 2010 Toxic effects and residue of aflatoxin B1 in tilapia (*Oreochromis niloticus × O. aureus*) during long-term dietary exposure. Aquaculture, Volume 307, Issues 3–4, Pages 233-240

Soriguer F. ,Serna S. ,Valverde E. ,Hernando J. ,Martín-Reyes A. ,Soriguer M. ,Pareja A. ,Tinahones F., Esteva I. 1997 Lipid, protein, and calorie content of different Atlantic and Mediterranean fish, shellfish, and molluscs commonly eaten in the south of Spain. European Journal of Epidemiology Volume 13, Issue 4 , Pages 451-463

Streit E., Schatzmayr G., Tassis P., Tzika E., Marin D., Taranu I., Tabuc C., Nicolau A., Aprodu I., Puel O., Oswald IP. 2012 Current Situation of Mycotoxin Contamination and Co-occurrence in Animal Feed—Focus on Europe. Toxins. Volume 4 Pages 788-809

Szabó-Fodor J., Dall'Asta C., Falavigna C., Kachlek M. , Szécsi Á., Szabó A. and Kovács M. 2014 Determination of the amount of bioaccessible fumonisin B1 in different matrices after in vitro digestion. World Mycotoxin Journal, in press.

Takahashi M., Shibutani M., Sugita-Konishi Y., Aihara M., Inoue K., Woo G., Fujimoto H. and Hirose M. 2008 A 90-day subchronic toxicity study of nivalenol, a trichothecene mycotoxin, in F344 rats. Food and Chemical Toxicology, Volume 46, Pages 125-135

Uniprom, Fish Promoting Consortium 2001 Indagine Internazionale per l'individuazione di tecnologie e tecniche innovative relative ai prodotti ittici. http://www.uniprom.it/_cd2/ricerca.htm

Wożny M. , Brzuzan P. , Gusiatin M. , Jakimiuk E. , Dobosz S., Kużminski H. 2012 Influence of zearalenone on selected biochemical parameters in juvenile rainbow trout (Oncorhynchus mykiss). Polish Journal of Veterinary Sciences. Volume 15, Issue 2, Pages 221–225

Wożny Maciej , Brzuzan Paweł , Wolinska Lidia , Góra Maciej , Łuczynski Michał K. 2012 Differential gene expression in rainbow trout (*Oncorhynchus mykiss*) liver and ovary after exposure to zearalenone. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, Volume 156, Issues 3–4, Pages 221-228

Zachariasova M. , Dzuman Z. , Veprikova Z. , Hajkova K. ,Jiru M. , Vaclavikova M. , Zachariasova A. , Pospichalova M., Florian M. , Hajslova J. 2014 Occurrence of multiple mycotoxins in European feedingstuffs, assessment of dietary intake by farm animals. Animal Feed Science and Technology, Volume 193, Pages 124-140

Zajdband A. 2012 Seafood Watch Tilapia Nile tilapia (*Oreochromis niloticus*), Blue tilapia (*Oreochromis aureus*), Mozambique tilapia (*Oreochromis mossambicus*), and Hybrid tilapia (*Oreochromis spp.*). Monteray Bay Acquarium Seafood Watch

Zinedine A., Soriano J. M. ,Moltó J. C. , Mañes J. 2007 Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: An oestrogenic mycotoxin. Food and Chemical Toxicology, Volume 45, Issue 1, Pages 1-18