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Course Manual

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Central Marine Fisheries Research Institute
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MYCOTOXINS IN FEEDS AND THEIR IMPACT ON FISH AND SHRIMP

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Introduction

Mycotoxins are a group of ubiquitous chemical compounds, widely diverse in their structures and toxic effects, produced by a variety of fungi. The name mycotoxin combines the Greek word for fungus 'mykes' and the Latin word 'toxicum' meaning poison.

Although mycotoxins are fungal metabolites, all fungal metabolites are not necessarily mycotoxins. Any feed or raw material and grains stored for more than a few days is a target for mould growth and mycotoxin formation if conditions are favourable. Mycotoxins can occur both in tropical areas and in temperate regions of the world, depending on the species of fungi. The toxins can accumulate in maturing corn, cereals, soybeans, sorghum, peanuts, and other food and feed crops in the field, during transportation and storage under conditions favorable for the growth of the toxin-producing fungi. Mold growth in feedstuffs reduces starch and fat content, lowering digestible energy levels. More importantly it introduces the risk of mycotoxins at levels that are deleterious to aquatic organisms. These mycotoxins are potent inhibitors of protein synthesis, specifically immunosuppressors.

Fungi and the formation of mycotoxins

Over 400 different mycotoxins have been identified. They include aflatoxins (B_1,B_2,G_1,G_2), Fusarium toxins (zearlenones, trichothecenes-vomitoxin, T_2, 'ochratoxins (A&B), cyclopiazonic) and patulin, saframine, fumonisins and citrinin.

Each mycotoxin is produced by one or more very specific fungal species (Table 1). In some cases one species can form more than one mycotoxin. For example the aflatoxins can be formed by *Aspergillus flavus, Aspergillus parasiticus* and limited other Aspergilli, while ochratoxin A is considered to be mainly the product of *A. ochraceus* in tropical regions and *P. verrucosum in* temperature areas. Fungi may grow well under a given set of conditions but not necessarily produce mycotoxins. The fungus produces much more aflatoxin on peanuts than on soybeans, although it grows equally well on both crops. Fungal growth and aflatoxins contamination are the consequence of interactions among the fungus, the host and the environment. The appropriate combination of these factors determines the infestation and colonization of the substrate and the type and amount of aflatoxin produced. Water stress, high temperature stress and insect damage of the host plant are the major determining factors in mold infestation and toxin production.

Safe Levels of Mycotoxins

Even with the best quality-control systems in the world, animal producers often find themselves owning mycotoxin-contaminated grain or feed. The safe level will depend on at least the following factors:

- Chemical class and chemical structure of the mycotoxin. For example, aflatoxin B1 is reported to be the most potent naturally occurring carcinogenic substance

known, its toxicity can be reduced dramatically by changing one chemical bond in the structure of the molecule.

- Presence of other Mycotoxins: A number of studies have demonstrated that mycotoxins occur simultaneously in field situations. This simultaneous occurrence can profoundly affect the toxicity of the mycotoxins present.
- Species and strain of the animals involved. Rainbow trout are highly sensitive to aflatoxins than other fish and shellfish.
- Health status of the animals involved: Nutritiona and environmental stress, physiological state and disease status will independently and collectively determine the response of a given animal to a specific mycotoxin level or complex of mycotoxins.
- Criteria by which effects are determined: At a given dose, aflatoxin reduces weight gain in growing animals, but disease resistance in the same animal may be reduced by about half that dose.
- Duration of exposure: The exact mycotoxin tolerance levels given elsewhere in this publication assume that animals are exposed for a limited period. Obviously, the risks of harm to animals from mycotoxins increase as exposure time increases.

Available Mycotoxin Data on feeds

The mycotoxins of most concern, based on their toxicity and occurrence, are aflatoxin, deoxynivalenol (DON or vomitoxin), zearalenone, fumonisin, T-2 toxin, and T-2-like toxins (trichothecenes). In a recent survey of suspect feed samples in USA some amount of aflatoxin, deoxynivalenol, or fumonisin was found in over 70 percent of the samples tested. Over a 10-year period, data collected from suspect samples analyzed at the North Carolina State University (NCSU) Mycotoxin Laboratory show: that 20 parts per billion (ppb) or more aflatoxin occurred in 34 percent of corn samples tested; deoxynivalenol was detected in over 60 percent of poultry and dairy feeds tested; zearalenone was present in 15 to 20 percent of feedstuffs tested; and T-2 toxin was present in about 5 percent of the feeds tested. Fumonisin, a mycotoxin often associated with horse deaths, is thought to occur very frequently.

Mycotoxins and Animal Health

Mycotoxins produce a wide range of harmful effects in animals immunotoxic effect of mycotoxins are well established. Decreased humoral response, suppression of phagocytosis, decreased anti body production and delayed hypersensitivity, inhibition of DNA Synthesis, inhibition of lymphocytes transformation, depression of stimulation of B and T cell responses are inhibition of protein synthesis are some of the notable symptoms reported in animals.

The economic impact of reduced animal productivity, increased incidence of disease due to immunosuppression, damage to vital organs, and interferences with reproductive capacity is many times greater than the impact caused by death due to mycotoxin poisoning.

Deoxynivalenol (DON) is the most commonly detected *Fusarium* mycotoxin. DON has been associated with reduced milk production in dairy cattle, vomiting by swine consuming contaminated feed or their refusal to eat feed containing the toxin, and inhibiting reproductive performance and immune function in several animal species. Zearalenone mimics the effect of the female hormone estrogen and, at low doses, increases the size or early maturity of reproductive organs. At higher doses zearalenone

interferes with ovulation, implantation, and the viability of newborn animals.T-2 toxin and its chemical relatives cause irritation, hemorrhage, and necrosis throughout the digestive tract, depress the regenerative process in the bone marrow and spleen, impair immune system function, and cause changes in reproductive organs. Affected animals show signs of weight loss, poor feed utilization, lack of appetite, vomiting, and (in severe cases) death. Fumonisin is a mycotoxin which has only recently been discovered. Thus it has not been extensively studied. Nonetheless, it is known that in most animals fumonisin impairs immune function, causes liver and kidney damage, decreases weight gains, and increases mortality rates.

Aflatoxins

Aflatoxins are toxic metabolites produced by the common fungi *Aspergillus flavus* and *Aspergillus parasiticus* These fungi are ubiquitous and under favourable conditions can grow on a wide variety of raw materials and compound feeds. Aflatoxins are polycylic, unsaturated compounds consisting of a coumarin nucleus flanked by a reactive bifuran system on one side and either a pentanone or a six-membered lactone on the other. The toxin was given the name aflatoxin by virtue of its origin (Afla from *Aspergillus flavus*). Aflatoxins have been associated with various diseases, such as aflatoxicosis in livestock including fish, domestic animals and humans throughout the world.

The moulds grow and produce toxins under proper conditions, which include adequate substrate (carbohydrates), moisture in the substrate ≥ 13 %, relative humidity ≥ 70%, adequate temperature and oxygen. *Aspergillus flavus* produces aflatoxins in starchy cereal grains (for example, corn, wheat, sorghum, oats, barley, millet, and rice) starting at a moisture content of about 18 percent. The critical moisture content for soybeans is 15 to 15.5 percent and for peanuts 8 to 9 percent. The upper limit of moisture for growth of *A. flavus* for aflatoxin production is about 30 percent. *A. flavus* will grow slowly below 54°F and most rapidly at 98°F but will not produce aflatoxin at temperatures below 54°F or above 108°F. Under optimum conditions for growth, *A. flavus* can produce some aflatoxin within 24 hours and a biologically significant amount in a few days.

The toxins produced by the mould are classified as nephrotoxins, hepatoxins, neurotoxins based on their haematological effects and digestive disorders they cause. Aflatoxins may be a cause of disease in fish and shrimp culture because shrimp culture facilities are typically located in humid tropical or semitropical environments, providing conditions favourable for the growth of *Aspergillus spp* and the production of aflatoxins in stored feeds.

There are eighteen compounds in the aflatoxin family, some of these occur naturally in feed stuffs and feed, others are metabolites formed in the animal body after ingestion of the contaminated feed. Aflatoxin types B_1, B2 G_1 and G2 are named according to the fluorescent colour when thin layer chromatographic preparation are viewed under UV light. According to National feed legislation in USA, corn and groundnut products that are to be used for feeding diary and immature animals including fish cannot contain more than 20ppb of aflatoxin.

The effects of aflatoxin vary considerably from species to species. Common effects on animals are poor growth, liver damage, impaired blood clotting, decreased immune responsiveness and increased mortality. Aflatoxin is a potent toxin and hepatocarcinogen. Aflatoxin is acutely toxic to a number of cell types, invertebrates and

vertebrates. The metabolism of aflatoxin was recently outlined. The toxic and mutagenic effects of aflatoxin, apparently require its conversion to metabolites. Some of which show high affinity for cellular constituents such as DNA. Hepatotoxicity and carcinogenecity are the major characteristics of aflatoxin. Among the organs, liver has been found to be target organ in poisoning due to aflatoxin. Many of the effects of the aflatoxin are related to their reaction with nuclear proteins, so that they interfere with protein formation and maintenance of cellular integrity. Metabolic activation of AFB_1 to the 8, 9 epoxide is required for expression of its carcinogenic activity.

Acute toxicity of AFB_1 to various groups of marine animals

Animal	LD_{50}(mg/Kg)
Rainbow trout	0.5
Brine shrimp	14
Copepods (*Cyclops fuscus*)	1
Penaeid shrimp (*P.stylirostris*)	100.5

Effect of Aflatoxin in shrimps

Species	Size	Dosage	Duration	Affects/Symptoms
Penaeus stylirostris	2.6±0.5g	25-160μg/g b.wt injection	10 days	24 hrs median lethal dose 100.5 mg/Kg 96 hrs 49.5 mg/Kg
P.vannamei	0.5g	50-300 mg/Kg	28 days	Histopathological lesions in hepatopancreas, mandibular organ, haemotopoictic organs
P.vannamei	1.61 ±0.19g	50-15000 ppb	21 days	Animals in 15000ppb died within 14 days. In 3000ppb- lost weight FCR varied directly in the high AfB_1. Growth rate decreased, Hepatopancreas and Antennal gland affected.
P.vannamei	1.5g	0-900 ppb	56 days	Abnormal Hepatopancreas and Antennal gland. FCR and growth affected at ≈ 400ppb
P.vannamei	Juveniles	100ppb	171 day grow out	Moderate aflatoxicosis
P.monodon	Juveniles	26.5-202.8 μg/Kg	60 days	Hepatopancreas,Antennal gland,lympoid organs affected, poor growth. >50μg/Kg- Haemocytic infiltration, fibrosis observed.

Effects of Mycotoxins in Fish

Several workers noticed high incidence of hepatic tumors in farmed rainbow trout, where groundnut meal or cottonseed meal formed part of fish feed. Aflatoxin was found to be extremely carcinogenic to trout. Aflatoxin at the level 0.01 ppb in feed could

produce neoplasm in trout. Embryonated eggs bathed in aflatoxin containing water at 1mg/ litre for 15 minutes to 1 hour produced hepatomas in 60-70% of trout hatched out of these eggs. When aflatoxin was fed at higher levels to trout it induced an acute toxic syndrome with massive focal hepatic necrosis, branchial oedema and generalized haemorrhagic syndrome. The presence of fish protein concentrate in feed augmented tumorogenic activity of aflatoxin B_1 in trout. Aflatoxin contamination of feed in addition to hepatoma produced hepatic cirrhosis, cystic liver degeneration and cholangioma. In trout livers aflatoxin B_1 was converted to several metabolites. They are electrophilic 8, 9 epoxide, aflatoxin M_1 aflatoxicol and polar conjugates. The carcinogenicity depended on the formation of electrophilic 8, 9 epoxides, which formed adducts with DNA.

In tilapia carcinogenicity produced wide range of neoplasm like renal tubular carcinoma, lymphoma and hepatoma. Liver showed lipoid degeneration, focal necrosis and loss of architecture. Spleen and kidney showed extensive necrosis of parenchyma. There was reduction in serum protein values. The haemocrit showed severe fall in total RBC, PCV and haemoglobin values. Total leukocyte count decreased in proportion to the amount of toxin and duration of exposure.

In channel cat fish aflatoxin produced, severe anaemia, low haematocrit values and increased mortality. The leukocyte counts were high and necrotic foci were found in the liver. Such foci contained basophilic hepatocytes. Sinusoids of head kidney were dilated and increased haemopoiesis was indicated by the presence of immature eryhrocytes. Intestinal mucosal epithelium accumulated excessive iron pigments. Gastric glands in the stomach were necrotic and infiltrated with macrophages.

Acute toxicity in channel cat fish caused regurgitation of stomach contents. Gills, livers, kidneys, spleens and stomachs of fish were extremely pale. Haemocrits, haemoglobin concentration and erythrocyte counts fell sharply. Leukocyte count also fell. Histological lesions were sloughing of intestinal mucosa, necrosis of the haemopoietic tissues, hepatocytes, pancreatic acinar cells and gastric glands. The spleen showed reduction in volume of the red pulp and reduction in the number of leukocytes in splenic corpuscles. Renal tubules were dilated.

Generally carps were found to be less susceptible to the effects of aflatoxin. However in some studies it was showed that aflatoxin B_1 produced chromosomal aberration in carp kidney cells. The resistance of carp to effects of aflatoxin was probably due to the difference in the enzyme profile of the liver.

The Indian carp *Labeo rohita* was found to be susceptible to aflatoxin B_1 at the rate of 0.4 mg/Kg of feed.. There was pronounced anaemia in the fishes. Total leukocyte count was also depressed. In aflatoxin treated fishes there was fall in total serum protein values as well as albumin levels. Histological studies of the liver indicated fatty change, necrosis of hepatocytes and proliferation of stromal tissue. The thymus, anterior kidney and spleen showed extensive necrosis and loss of haemopoietic elements. The excretory kidney had severe nephrotic changes characterized by tubular epithelial degeneration and necrosis. Glomeruli showed increased cellularity, proliferation of parietal epithelium of Bowman's capsule and periglomerular fibrosis.

Ultrastructural examination revealed severe changes in all organs. In liver hepatocytes exhibited degranulation of rough endoplasmic reticulum (RER), proliferation

of smooth endoplasmic reticulum (SER), and fragmentation, dilatation and vesiculation of both RER and SER. Mitochondrial swelling and separation of desmosomes were also seen. Nuclei became irregular in shape and there was increase in heterochromatin. The thymus and spleen revealed severe damages to lymphoblasts, which was evidenced by dilatation of RER, nuclear invagination and increase in heterochromatin.

Electron microscopic studies of the kidney showed loss of microvilli in epithelial cells, degranulation of RER, swelling of mitochondria, dilatation of endoplasmic reticulum and nuclear envelope. In the interstitial tissue, the cells developed several vesicles in the endoplasmic reticulum. The severe fall in peripheral leukocytes count and destruction of haemopoietic tissue in tilapia and rohu indicated immune suppression. *In vitro* phagocytic activity of the isolated leucocytes from aflatoxin treated rohu revealed severe depression of phagocytic index and percentage of cells showing phagocytosis.

A diet containing 0.4ppb AFB_1 fed to trout over a 14 month period resulted in a 14% incidence of hepatocellular carcinoma. A ten-fold increase resulted in a 60% incidence during the same period. In contrast, Steelhead trout had an incidence of only 6% after 12 months on 8ppb AFB Diets containing 20ppb AFG_1 AFB_2 and AFG_2 tumor incidence of 17,0 and 0% respectively after 16 months in trout.

Fungii and mycotoxin management:

Mycotoxins are generally stable compounds and once formed can persist in foods and feeds for long time. Although prevention is the best way to reduce mycotoxin contamination is unavoidable even after taking precautionary measures highlighted below:

- Feed and raw materials should be be stored under hygienic conditions.
- Farm house should be clean and tidy and should be free from rodents and insects
- Feeds should contain less that 12% moisture.
- Feed utilization practice should be improved and well designed to prevent fungal invasion and consequent mycotoxin contamination.
- Only sufficient feed be procured for particular growth cycle in hatchery/farm. The excess feed remaining to be used for next run should be checked for presence of aflatoxin.
- Definition of and strict adherence to a feed utilization regime based upon regular lot rotation.
- Qualitative analysis for presence of aflatoxins in feeds and feed ingredients.
- Physical removal of fungal infested feeds can be done by ionizing radiation.
- Various chemical agents such as hydrogen peroxide, sodium and calcium hydroxide, chloramines, disinfectant, ammonia and sodium hypochlorite have been reported to destroy aflatoxins in foods and feeds.
- Control of the environment in which toxigenic fungi grow and produce mycotoxins is another means of preventing mycotoxin formation in foods and feeds

Prevention in Silages

Prevention of mycotoxins in silages includes following accepted ensiling practices aimed at inhibiting deterioration primarily through elimination of oxygen. Some silage additives (ammonia, propionic acid, microbial cultures, or enzymatic silage) are shown to

be beneficial in preventing mycotoxins because they are effective at reducing mold growth.

Prevention of Feed Contamination

Controlling mold growth and mycotoxin production is very important to the feed manufacturer and the farmer. Control of mold growth in feeds can be accomplished by keeping moisture low, feed fresh, equipment clean, and using mold inhibitors. Grains and other dry feeds should be stored at a moisture level of 12 percent or less to discourage mold growth. Aeration of grain bins is important to reduce moisture migration and to keep the feedstuffs dry.

Moisture Control

Moisture is the single most important factor in determining if and how rapidly molds will grow in feeds. Moisture in feeds comes from three sources: feed ingredients, feed manufacturing processes, and the environment in which the feed is stored. To control the moisture content of feeds successfully, moisture from all the three sources must be controlled.

- Since grains are a primary source of the moisture and molds found in feed, the first important step in controlling moisture in feed is to control it in the grains from which the feed is prepared. Since all feed ingredients contain moisture, they should be monitored and their moisture content controlled.

- Grains are commonly ground with a hammer mill to aid in mixing and handling, to improve digestibility, and to improve the pelleting process. This grinding process creates friction, which causes heat to build up. If unchecked, temperature increases greater than 10 degrees Fahrenheit will cause significant migration of grain moisture encouraging mold growth. This is particularly true in cold weather when temperature differences cause moisture to condense on the inside walls of bins. Air-assisted hammer-mill systems reduce heat buildup in the product and, in turn, reduce moisture problems.
- The pelleting process involves mixing steam with the feed, pressing the mixture through a die, and then cooling the pellets to remove heat and moisture. Generally, heat and 3 to 5 percent moisture are added to the feed during the pelleting process in the form of steam. If the pelleting process is done correctly, this excess moisture is removed from the feed before shipment. If, however, this excess moisture is not removed when the pellets are cooled, mold growth will be encouraged. Although pelleting of feed has been shown to reduce mold counts by a factor of 100 to 10,000, many mold spores remain in the feed after it has been pelleted. After pelleting, the remaining spores can grow if conditions are right. Thus the pelleting process delays, but does not prevent, the onset of mold growth and plays only a minor role in efforts to control molds. In addition, pelleted feeds may be more easily attacked by molds than non-pelleted feeds.
- To control mold growth, obvious sources of moisture in the feed handling and storage equipment must be eliminated. These sources may include leaks in feed storage tanks, augers, roofs (either at the barn or at the feed mill), and compartments in feed trucks.

Keeping Feed Fresh

Time is required for both mold growth and mycotoxin production to occur. It is therefore important to have feeds delivered often so that they will be fresh when used. Feeds should generally be consumed within 3 months of delivery.

Equipment Cleanliness

When feed is manufactured and delivered to farms, it may come in contact with old feed that has lodged or caked in various areas of the feed storage and delivery systems. This old feed is often very moldy and may "seed" the fresher feed it contacts, increasing the chances of mold growth and mycotoxin formation. To prevent this problem, caked, moldy feed should be removed from all feed manufacturing and handling equipment.

Use of Mold Inhibitors

The use of chemical mold inhibitors is a well-established practice in the feed industry. The main types of mold inhibitors are (1) individual or combinations of organic acids (propionic, sorbic, benzoic, and acetic acids), (2) salts of organic acids (calcium propionate and potassium sorbate), and (3) copper sulfate. Solid or liquid forms work equally well if the inhibitor is evenly dispersed through the feed. Generally, the acid form of a mold inhibitor is more active than its corresponding salt.

Many factors influence the effectiveness of mold inhibitors.

- Mold inhibitors cannot be effective unless they are completely and thoroughly distributed throughout the feed. The entire surface of each feed particle should come in contact with the inhibitor and that the inhibitor should also penetrate feed particles so that interior molds will be inhibited.
- The particle size of the carriers for mold-inhibiting chemicals should be small so that as many particles of feed as possible are contacted. In general, the smaller the inhibitor particles the greater the effectiveness.
- Certain feed ingredients may also affect mold inhibitor performance. Protein or mineral supplements (soybean meal, fishmeal, poultry by-product meal, and limestone) tend to reduce the effectiveness of propionic acid. These materials can neutralize free acids and convert them to their corresponding salts, which are less active as inhibitors.
- Dietary fat tends to enhance the activity of organic acids, probably by increasing their penetration into feed particles. Certain unknown factors in corn also alter the effectiveness of organic acid inhibitors.
- When mold inhibitors are used at the concentrations typically recommended, they in essence produce a period of freedom from mold activity. If a longer mold-free period is desired, a higher concentration of inhibitor should be used. The concentration of the inhibitor begins to decrease almost immediately after it is applied as a result of chemical binding, mold activity, or both. When the concentration of the inhibitor is reduced until it is incapable of inhibiting mold growth, the mold begins to use the inhibitor as a food source and grows. In

addition, feeds that are heavily contaminated with molds will require additional amounts of inhibitor to achieve the desired level of protection.

- The widespread use of pelleted feeds in the feed industry is beneficial to the use of mold inhibitors. The heat that the feed undergoes during pelleting enhances the effectiveness of organic acids. Generally, the higher the pelleting temperature, the more effective the inhibitor. Once mold activity commences in pellets, however, it proceeds at a faster rate than in nonpelleted feed because the pelleting process that makes feed more readily digestible by animals also makes it more easily digested by molds.

Inorganic binders

The possible use of inorganic binders (mineral clay products) such as zeolites, bentonite, bleaching clays from refining of canola oil, and hydrated sodium calcium aluminosilicates [HSCAS])to bind mycotoxins, and prevent them from being absorbed by the animal's gut, has received a lot of research attention recently. While one HSCAS product called NovaSil has been shown to bind aflatoxin protecting animals against aflatoxicosis, under FDA regulations these clay products cannot be sold as mycotoxin binders.

Because little is known about the mycotoxins, and because many unidentified mycotoxins exist, aquafarmers should avoid feeding moldy feeds if at all possible.

Decontamination Methods

- Physicals methods, such as sorting and solvent, extraction and pneumatic sorting, ionizing radiation
- Solvent extraction with solvents or aqueous solutions of sodium bicarbonate or calcium chloride has been tried with limited success
- Treatment with chemicals such as hydrogen peroxide, sodium and calcium hydroxide, a chloramines disinfectant, ammonia and sodium hypochlorite have been reported to destroy aflatoxins in foods and feeds
- Use of biological agents such as yeasts also have been reported

Table 1. Some Common Mycotoxins and the Organisms that produce them

Mycotoxin	Organism
Aflatoxin	*Aspergillus flavus, A. parasiticus*
Aflatrem	*Aspergillus flavus*
Citrinin	*Aspergillus carneus, A. terreus, Penicillium citrinum, P. hirsutum, P. verrucosum*
Citreoviridin	*Aspergillus terreus, Penicillium citreoviride*
Deoxynivalenol	*Fusarium moniliforme, F. culmorum, F. avenaceum, F. roseum,* and *F. nivale*

Fumonisin B_1	*Fusarium moniliforme, F. culmorum, F. avenaceum,* and *F. nivale*
Ochratoxin	*Aspergillus ochraceus, Penicillium viridictum*
Oxalic acid	*Aspergillus niger*
Patulin	*Aspergillus clavatus, Penicillium expansum, Botrytis, P. roquefortii, P. claviforme, P. griseofulvum*
Penicillic acid	*Aspergillus ochraceus*
Sterigmatocystin	*Aspergillus flavus, A. nidulans, A. versicolor, Penicillium rugulosum*
T-2 toxin	*Fusarium moniliforme, F. equiseti, F. culmorum, F. solani, F. avenaceum, F. roseum,* and *F. nivale*
Zearalenone	*Fusarium culmorum, F. graminearum, F. oxysporum, F. roseum, F. moniliforme, F. avenaceum, F. equiseti,* and *F. nivale*

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