Mycotoxin contamination of abalone feed: health and safety considerations for the abalone aquaculture industry

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Declaration

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Summary

Abalone farming in South Africa is the largest contributor to the marine aquaculture industry in South Africa and forms part of the worldwide seafood supply chain. Abalone farming has been highly successful, with a consistent positive growth rate over the past decade. However the continued growth rate is dependent on a steady supply of feed resources. Kelp (*Ecklonia maxima*) has been the main feed source for years, but artificial feed has quickly made an impact on this industry, by showing to be more beneficial to farmers in more ways than one. It has become the main feed source on most commercial abalone farms in South Africa, and consists of a variety of grains, often locally sourced, a protein source and vitamins. In these feeds, natural fungal and mycotoxin contamination is therefore unavoidable. The current study has shown that although, natural mycotoxin levels in abalone feed were low, a number of natural toxigenic fungal species were present in feed samples. There are currently no limits for the allowable mycotoxin concentrations in abalone feed, but naturally occurring mycotoxin levels measured during this study, shown in Chapter 2, have all been substantially lower than the allowable mycotoxin contamination for catfish feed reported by the U.S. Food and Drug association (FDA). The toxigenic fungi isolated represented three toxigenic fungal genera namely *Fusarium*, *Aspergillus* and *Penicillium.* In Chapter 3, some of these fungal isolates were shown to be able to produce high levels of mycotoxins when re-inoculated and left to colonise abalone feed. Results in Chapter 4 showed that fumonisin production by *Fusarium verticillioides* were higher at 16 °C than at 26 °C. Furthermore, it was found that fumonisins leach into the water when exposed to seawater. These findings together with the high dilution factor and low levels of naturally occurring fumonisins in abalone feed, indicates that it is unlikely that fumonisins hold a threat to the abalone aquaculture industry. However, it remains unclear what will happen to other mycotoxins when exposed to seawater. From the results of this study, production of other mycotoxins by toxigenic fungi can be expected in poor storage conditions. This dissertation ends with recommendations to abalone farmers to lower the overall risks of fungal and mycotoxin

contamination in abalone feed.

Opsomming

Perlemoen boerdery in Suid Afrika lewer die grootste bydrae tot marine akwakultuur in ons land. Dié industrie vorm deel van die wêreldwye voedselvoorsienningsketting. Perlemoen boerdery is hoogs suksesvol met 'n konstante jaarlikse groei. Die sukses van die industrie is egter afhanklik van konstante voorsiening van voer. Kelp (*Ecklonia maxima*) was vir jare gebruik as die enigste voer, maar kunsmatige voer het vinnig ‘n impak op die industrie gemaak en was voordelig op verskeie vlakke. Dit word nou beskou as die hoofbron van voer op die meeste kommersiële perlemoen plase in Suid-Afrika. Kunsmatige voer bestaan uit ‘n verskeidenheid grane, ‘n proteïenbron en vitamiene. Vir die rede is dit natuurlik dat mikotoksien en swam kontaminasie onvermybaar is. Die studie wys dat natuurlike mikotoksien kontaminasie laag was in die kos, maar dat verskeie toksiese swamspesies teenwoordig was in die monsters. Daar is tans geen maksimum toelaatbare vlakke vir mikotoksiene in perlemoenvoer nie, maar die mikotoksien vlakke wat gedurende die studie gemeet was, (Hoofstuk 2), was laer as die toelaatbare vlakke wat voorgeskryf word deur die U.S. Food and Drug association (FDA) vir katvisvoer. Toksiese swamme wat geïsoleer is vanaf perlemoenvoer, verteenwoordig drie genera naamlik *Fusarium*, *Aspergillus* en *Penicillium.* Hoofstuk 3 wys dat van die swamme die vermoeë het om hoë vlakke mikotoksiene te produseer wanneer dit toegelaat word om perlemoenvoer te koloniseer. Resultate in Hoofstuk 4 toon dat fumonisien-produksie deur *Fusarium verticillioides* op perlemoenvoer hoër was by 16 °C as by 26 °C. Verder is daar gevind dat fumonisiene in die water uit lek wanneer voer aan seewater blootgestel word. Die resultate, tesame met die hoë verdunningsfaktor en lae vlakke natuurlike fumonisienvlakke in die voer is ‘n aanduiding dat dit onwaarskynlik is dat fumonisiene 'n beduidende risiko vir die perlemoenindustrie inhou. Daarteenoor is dit ook onduidelik hoe ander mikotoksiene sal reageer in die teenwoordigheid van seewater. Resultate van die studie toon dat die produksie van ander mikotoksiene op perlemoenvoer verwag kan word, mits opbergingstoestande van voer nie goed gereguleer word nie. Die proefskrif eindig met ‘n paar aanbevelings aan plase om die algehele risiko van mikotoksien en swam kontaminasie in

perlemoen voer te verlaag.

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1 GENERAL INTRODUCTION

1.1 Aquaculture

Aquaculture is the farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants (FAO, 1997). Aquaculture had its beginnings over 4000 years ago when common carp (*Cyprinus carpio*) was kept in ponds to serve as a reliable protein source to humans. Other early aquaculture activities include tilapia farming by the ancient Egyptians, growing fish in ponds by ancient Hawaiins and the Greeks raising eels (Ackefors et al., 1994; Bondad-Reantaso et al., 2005; Costa-Pierce, 1987; Hishamunda and Subasinghe, 2003). During the early days fish were mainly kept in ponds as a food source, but during the 19th century sea fisheries developed and methods to preserve seafood became available (Ackefors et al., 1994; Rabanal, 1988). Marine aquaculture developed during the same era (Hishamunda and Subasinghe, 2003). Fish or more specifically seafood is seen as an excellent source of high quality animal protein, omega fatty acids and highly digestible energy. Abalone is regarded as one of the most highly valued seafood commodities in the world (Clarke, 2004; Naylor et al., 2000; Tacon and Metian, 2008). This is the reason why this industry forms part of the fastest growing food production chain in the world (Bondad-Reantaso et al., 2005; Troell et al., 2004).

Marine aquaculture in South Africa started in 1673 with oyster farming, but only much later the first successful commercial oyster farm was opened in 1948, (FAO, 2016). A rapid development took place in this industry over the years. Currently, it consists of both fresh and marine water culturing each with a number of subsectors. Freshwater subsectors include, trout (*Oncorhynchus mykiss* and *Salmo trutta*), tilapia (*Oreochromis mossambicus*, *Oreochromis niloticus* and *Tilapia rendalli*), catfish (*Clarias gariepinus*), carp (*Cyprinus carpio* and *Ctenopharyngodon idella*), mullet (*Liza richardsonii*), largemouth bass (*Micropterus salmoides*), marron crayfish (*Cherax tenuimanus*), Atlantic salmon (*Salmo salar*) and a number of aquarium species (i.e. koi carp, goldfish, guppies,

etc.), of which rainbow trout are the largest subsector (DAFF, 2012). Marine aquaculture include abalone (*Haliotis midae*), oysters (*Crassostrea gigas*), mussels (*Mytilus galloprovinvialis* and *Choromytilus meridionalis*) and finfish (*Argyrosomus japonicas* and *Seriola lalandi*) of which abalone contributes the most (DAFF, 2012; FAO, 2016). Although the marine aquaculture industry in South Africa has shown an overall growth of 6.35 % from 2001 to 2013 (DAFF, 2014), this industry looks as if it is still in its baby shoes compared to marine aquaculture in countries such as China, Chile and Norway.

1.2 Abalone

1.2.1 General background, biology and distribution of Abalone

Abalone is locally known as 'perlemoen' which originated from the Dutch word 'paarlemoer', meaning 'mother of pearl'(Kgatla, 2015). Abalone are marine gastropods belonging to the Phylum Mollusca and the family Haliotidae (Fallu, 1991; Geiger, 1999). Within this family, the genus *Haliotis* is one of the largest (Geiger, 1999). Adult abalone are commonly found in the intertidal zones, where they use their muscular foot to grip to rock surfaces (Branch et al., 2010). These invertebrate gastropods have soft bodies with a mantle cavity that contains gills, an anterior head with a radula which they use to scrape their food and a large muscular foot. They have a single flattened ear shape shell covering almost the entire body, leaving only the epipodium uncovered (Fallu, 1991; Geiger and Owen, 2012). These shells are rough on the outside but smooth on the inside, serving as protection for their soft bodies.

Roughly a 100 identified species are found across the globe in tropical, temperate and cold waters (Degnan et al., 2006; Geiger, 1999), of which five species are endemic to South Africa. *Haliotis midae, H. parva, H. spadicea, H. queketti, H. speciosa* and *H. pustulata* all occur along specific regions of the South African coastline (Figure 1.1) (Branch et al., 2010; Geiger, 1999; Sales and Britz, 2001) and *H. midae* is the only species that is commercially exploited in South Africa (Sales and

Britz, 2001; Sales, 2001). *Haliotis midae* has a natural distribution from St. Helena Bay on the West coast right through to Port St. Johns on the East coast of Southern Africa (Figure 1.1) (Branch et al., 2010; Greeff, 2012). Water temperatures and seaweed preferences play a role in the geographical distribution of species along the coastline. Although certain species occur in a given habitat, their substrate dependence leads to overlapping in areas where similar species group together in large groups. Some of these species can grow as big as a shell length of 200 mm but it can easily take them 30 years to reach this size (Geiger, 1999; Sales, 2001). Abalone are considered as slow growing and slow moving, which makes them extremely vulnerable to overfishing. These characteristics acted as a driver for abalone cultivation and to this day it still is the only way to keep up with the ever increasing global demand for this highly valuable delicacy (Attwood, 2000; Oakes and Ponte, 1996; Raemaekers et al., 2011).

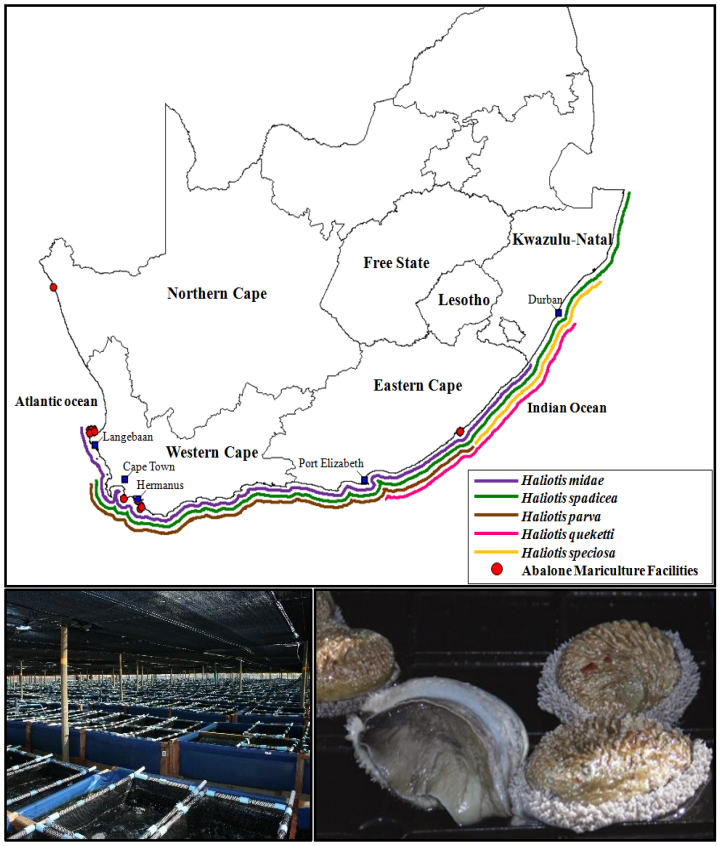

Figure 1.1: Map showing the distribution of endemic Haliotid species along the coastline of Southern Africa and the location of commercial abalone farms. Bottom left to right, typical abalone farm in the Western Cape Province of South Africa and healthy adult abalone (*Haliotis midae*) found on the abalone farms (Branch et al., 2010; Greeff, 2012; Hijmans et al., 2002).

1.2.2 Abalone aquaculture in South Africa

The successful abalone aquaculture industry in South Africa, started in 1949. However, cultivation only started years later in 1981 (Sales and Britz, 2001). The 1990's mark the start of commercial abalone farming in South Africa and by 1997 the first batch of 10 tonnes of abalone were produced (FAO, 2016). Legislated reductions in total allowable catch (TAC) and temporary closures of abalone fishery in 2004/5 along with continued success and development of the abalone aquaculture industry, contributed to the success of this industry in the abalone export market (Hauck and Sweijd, 1999; Raemaekers et al., 2011; Sales and Britz, 2001; Troell et al., 2006). Furthermore, this industry offers the opportunity to control abalone portion size and can therefore, deliver to customer demand (Oakes and Ponte, 1996).

Commercial abalone farms are located along the South African coastline (Figure 1.1), situated close to the shoreline where they have easy access to seawater. Abalone are cultured primarily in large flow-through concrete raceway systems where they are housed in baskets that are pre-moulded or made from oyster mesh. These baskets consists of vertical polycarbonated plates on which abalone attach themselves to. According to South Africa's aquaculture yearbook in 2012, the total contribution towards the overall marine aquaculture in South Africa has increased with 55 %. South African abalone (*Haliotis midae*) is rated one of the best species with an outstanding reputation abroad in terms of brand quality. A total of 1036 tons of abalone was exported in 2011. However, this productive growth rate is dependent on a steady supply of high quality feed. Daily intake of processed feed depends on the species and body size but can be up to 5.7 % of the body weight (Fleming et al., 1996). A total of 1009 tons artificial feed and 6820 tons of kelp were used in 2011 (DAFF, 2012).

1.2.3 Nutrition

Abalone are herbivores throughout their lives and have a diet that change from juvenile to adult

abalone (Knauer, 1994; Lubet, 1994; Takami et al., 2000). This is due to a combination of morphological changes that takes place in their mouths, the radula and their digestive systems (Johnston et al., 2005; Kawamura et al., 2001; Onitsuka et al., 2004) during the different growth stages. These are natural changes that appear, but as abalone grow they can be influenced by diseases, oxygen consumption and when cultured, the stocking density and water salinity. However, growth rate are primarily influenced by the type of feed they eat and its nutritional composition (Britz, 1996a, 1996b; Kruatrachue et al., 2004; Sales and Britz, 2003; Sales et al., 2003a; Serviere-Zaragoza et al., 1997; Viera et al., 2005). Juvenile abalone mostly feed on the macroalgae *Ulva*. Adult abalone show grazing behaviour and feed on pieces of drifting seaweed, which are often trapped by clamping down their foot. Seaweeds form the largest part of their diet. Different species prefer different seaweed. The reason for this is unclear, and could be because of the abundance of a specific seaweed species at a certain location, or due to chemical defences, secondary metabolites and physical defence from the algae or even the nitrogen and protein content (Angell et al., 2012; McShane et al., 1994; Paul et al., 2006). Australasian abalone (*Haliotis rubra, H. laevigata* and *H. roei*) prefer red algae over brown algae. The opposite is found in abalone from the North and Central America (*H. rufescens, H. fulgens* and *H. corrugate*), Japan (*H. discus hannai* and *H. diversicolor*) and New Zealand (*H. iris*), where these species prefer brown algae over red algae (Angell et al., 2012). The latter is also true for South African abalone (*H. midae*) even though they have a specific preference for red kelp (*Plocamium*),and brown kelp (*Laminaria* and *Ecklonia*) (Macey, 2005). *Ecklonia maxima* or better known as kelp, forms the bulk of most of the South African adult abalone diet. This is not only because kelp are the most abundant on the Southern West Coast of South Africa, but also because it is preferred by *H. midae* (Branch et al 2010).

One of the reasons why abalone farms were established along the coast line was the high abundance of kelp that were used as the main food source (Anderson et al., 2006; McBride, 1998; Troell et al., 2006; van der Merwe, 2009). However, this changed over time. The amount of available kelp varies

between different days and seasons. During winter and stormy weather, kelp wash up on the beach causing an excess of available feed but during summer time, days can go by without any available kelp (Francis et al., 2008). Adding to this, rough sea conditions makes it impossible to harvest kelp, leaving abalone to starve for short periods of time (Britz, 1995; Francis et al., 2008). Farms were also location bound because of the wet nature of kelp that made long storage periods and transportation over long distances impractical. (Britz, 1995; Capinpin and Corre, 1996; Viana et al., 1993). These problems limited the industry to expand. If these challenges were not enough, Troell et al, (2006) reported that even though kelp harvesting provides employment to the poorer communities, this resource started to approach the limits of sustainable harvesting in kelp Concession Areas. It is for these reasons that farms started incorporating artificial feeds as main food source (Fleming and Hone, 1996; Fleming et al., 1996; Sales and Britz, 2001; Sales, 2001). Even though kelp will always play an important role in abalone farming, artificial feeds (Figure 1.2) have become the main feed source on industrial abalone farms in South Africa.

Figure 1.2: Examples of different artificial abalone feed currently produced in South Africa. Different formulas and pellet sizes are produced to feed abalone during different life stages.

Macroalgae or kelp have a higher moisture content and lower nutrient levels than artificial feed. This is because artificial feed for abalone consists of a mixture of grains, protein and fatty acids (Britz,

1996a, 1996b; Daume and Ryan, 2004; Sales and Britz, 2001; Troell et al., 2006). Studies have shown that abalone have a combination of preliminary and extracellular digestion with subsequent intracellular digestion and are able to efficiently digest highly concentrated protein and carbohydrates, such as artificial feeds (Britz, 1995; Britz et al., 1994; Mai et al., 1995a, 1995b; Sales et al., 2003b). South African abalone *H. midae* easily adapted to artificial feeds. These feeds showed great success with weaning juveniles of diatoms (Sales and Britz, 2001). This triggered a flurry of research to identify suitable dietary sources to be used as carbohydrates, protein, lipids, essential fatty acids, vitamins and minerals (Britz, 1996a; Britz et al., 1994; Daume and Ryan, 2004; Mai et al., 1995a, 1995b; Serviere-Zaragoza et al., 1997; Viana et al., 1993; Zhang et al., 2004; Zhu et al., 2004). In order for abalone to grow sufficiently they require certain chemical building blocks to create new muscle, including amino acids, components of protein and polyunsaturated fatty acids (Fallu, 1991). Ingredients used in feeds intended for juveniles are different from feed used for adult abalone. Feed for juveniles are mostly made with kelp and fish meal that serves as a protein source (Zhang et al., 2004). While feed used for adult abalone are more complex consisting of a variety of grains, very often locally sourced, fishmeal as the protein source and added vitamins and minerals (Fleming and Hone, 1996; Fleming et al., 1996; Lee, 2004).

Carbohydrates drive all the metabolic processes and serves as the largest energy source for abalone (Fleming et al., 1996). Therefore, carbohydrates make up the largest part of artificial feed, with a total of 30 - 60 %. Wheat flour, maize flour, starch and bran are just some of the grains that are used to serve as the carbohydrate source in artificial feeds. If feed does not have enough carbohydrates, proteins will be used as an energy source. This could be more costly to the feed producer, because proteins are normally more expensive than carbohydrates (Fallu, 1991). When the correct amount of carbohydrates are added to the feed, the more expensive ingredients such as protein will be metabolised into the production of edible flesh (Fallu, 1991). Protein is vital for tissue deposition and optimal growth (Lee, 2004; Mai et al., 1995b). Fishmeal, defatted soybean meal and casein

protein are the three main proteins used (Bautista-teruel and Millamena, 1999).

Feed manufacturers are growing in sync with the abalone farming industry. Increased demand for artificial abalone feed caused the feed manufacturing industry to expand, to produce more than 100 tonnes of feed per month ([1]Personal communication). Fishmeal was the only protein source used in the first formulated feeds, but over time soya were introduced as an alternative. Soya has a lower protein content than fishmeal but it is usually chosen as a protein replacement for fishmeal because it has a high protein content, relative to other plant protein sources, and its amino-acid profile is similar to that of fishmeal. Some farms are using artificial feed in combination with fresh kelp but the majority of the abalone production is with artificial feeds ([1]Personal communication).

Artificial feed may sound like the ideal solution to farmers, as it is convenient, reliable and always available (Britz, 1995; Britz et al., 1994). It is regarded as balanced nutrition to the abalone and with lower food conversions resulting in better growth rates (Britz, 1995; Fleming et al., 1996; Knauer, 1994; Knauer et al., 1996; Lyon, 1995; Viana et al., 1993). Storage and transportation is no longer a problem, thus farm establishment is no longer location bound. This ideal solution is not without drawbacks. When abalone feed on artificial feeds, they no longer ingest beneficial bacteria, associated with natural feeds. This leads to the production of more nutrient-rich faeces which increase the possibility for sabellid (feather duster worm) infection (Macey, 2005; Troell et al., 2006). Feed could become unstable when left in the water for long periods of time causing valuable nutrients to leak into the water and be flushed out of the system (Fleming and Hone, 1996; Sales et al., 2003a; Shipton et al., 2002). In terms of costs, protein could become even more expensive because of a continual decline in fisheries that will influence the availability of fish meal (Fleming et al., 1996; Kruatrachue et al., 2004; Sales, 2004). Finally, fungal growth as a result of high grain content, during transportation and storage can potentially become a significant problem.

[1] Personal conversation with anonymous feed supplier

1.3 Fungi and their associated mycotoxins

Fungi are unicellular or filamentous, multinucleate, eukaryotic organisms. Some species play an important role in the environment where they act as decomposers, while others can act as pathogens on plants and animals. Although fungi have been successfully used in the food industry for many years, some of these fungi have the ability to produce mycotoxins (Balajee et al., 2007; Diaz et al., 2013; Gutiérrez-Correa et al., 2012; Khan et al., 2007; Li et al., 2002; Matsui et al., 1996; Noonim et al., 2009; Rheeder et al., 1992).

Mycotoxins are low weight molecular secondary metabolites (Diaz et al., 2013; Pitt, 2000; Sweeney and Dobson, 1998). These metabolites are toxic to humans and animals (Hussein and Brasel, 2001; Lazicka and Orzechowski, 2010) and can be carcinogenic, neurotoxic, nephrotoxic or immunosuppressive especially when exposure occurs for long periods of time (Frisvad et al., 2004; Gelderblom et al., 1988; Mally and Dekant, 2009; Marnewick et al., 2009; Rheeder et al., 1992; Schoenhard et al., 1981; Shephard, 2008a). Mycotoxicoses are diseases caused by the exposure of foods contaminated with mycotoxins (Ciegler and Bennett, 1980; D'Mello and Macdonald, 1997; Forgacs, 1962). The most common way of exposure in humans and animals is through consumption of feedstuffs or foods that are contaminated with either the fungi, the mycotoxins or both. Mycotoxins occur mostly in the mycelium of fungi but can also be found in spores, making inhalation another route to human and animal infection possible (Placinta et al., 1999). The intensity and action time of mycotoxins are used to classify the symptoms of mycotoxin poisoning. Acute poisonings are visible immediately, while chronic and sub-acute poisoning is caused by a continual infection at low doses, and symptoms are only visible after a period of time (Böhm et al., 2010; Lazicka and Orzechowski, 2010). Although the biggest concerns of mycotoxin contamination generally relate to the effects of chronic life-long exposure, acute human mycotoxicoses due to high levels of mycotoxins have been reported (Azziz-Baumgartner et al., 2005; Doster et al., 1974; Lye et al., 1995; Perrone et al., 2014).

Fungi known to produce mycotoxins are referred to as toxigenic fungi. This group mainly consists of natural occurring fungi and is dominated by *Fusarium, Penicillium* and *Aspergillus* (Pitt, 2000; Placinta et al., 1999; Sweeney and Dobson, 1998). Other toxigenic fungi include genera such as *Alternaria, Claviceps,* and *Stachybotrys* (Bennett and Klich, 2003; Diaz et al., 2013). Originally toxigenic fungi were divided into two groups namely, "field" (or plant pathogenic) and "storage" (or saprophytic) fungi (Diaz et al., 2013; Lazicka and Orzechowski, 2010; Placinta et al., 1999). However, this division can become problematic as it cannot be applied to species such as *Aspergillus flavus,* that is associated with ear- and kernel rot of maize and colonise stored grains under favourable conditions (Atalla et al., 2003; Bennett and Klich, 2003; Campbell and White, 1995; D'Mello and Macdonald, 1997; Hell et al., 2000; Placinta et al., 1999; Williams et al., 2011).

Mycotoxins go hand in hand with agriculture and although there are hundreds of mycotoxins recognised worldwide, there are only a few mycotoxins important in agriculture. These are fumonisins, aflatoxins, ochratoxin A (OTA), nivalenol (NIV), deoxynivalenol (DON) and zearalenone (ZEA) (Biomin, 2015; Diaz et al., 2013; Placinta et al., 1999). Mycotoxin infection can take place before harvesting when fungi acts as a plant pathogen or during and after harvesting when crops are handled, transported and stored (Bush et al., 2004; Hell et al., 2000). When toxigenic fungi colonise crops during storage it creates a perfect opportunity for mycotoxin production (Placinta et al., 1999; Vincelli et al., 1993).

1.3.1 Fusarium

The genus *Fusarium* was first described in 1809 by Link, who recognised the presence of the unique banana shaped conidia as a characteristic (Link, 1809). This is a large group of filamentous fungi, with ~300 phylogenetically distinct species discovered to date, mostly found in soil but are also associated with plants, air and sometimes humans (Crous et al., 2016; Leslie and Summerell, 2006; Marasas et al., 1986, 1985; O'Donnell et al., 2010; Snyder, 1927). They are commonly found in

tropical and subtropical regions. Although *Fusarium* spp. have been associated with a broad range of plant diseases, the biggest concern are the species that produce mycotoxins. To date there are 35 *Fusarium* species known to produce mycotoxins, of which *F. verticillioides* is the most prominent (Beukes, 2015; Marasas et al., 1984). Fumonisins, trichothecenes and zearalenone are the major mycotoxin groups associated with *Fusarium* spp. (Hussein and Brasel, 2001; Marasas et al., 1984; Miller, 2008; Moss, 2002a; Sweeney and Dobson, 1998).

1.3.1.1 Fumonisins

The fumonisins are a group of closely related polar metabolites and were first described in 1988 from a *F. verticillioides* culture, strain MRC 826 (Bezuidenhout et al., 1988; Marasas, 2001). This group of mycotoxins is mainly produced by *Fusarium verticillioides*, *F. proliferatum*, *F. oxysporum*, *F. globosum* and *Alternaria alternata* before and during harvesting (Bhat et al., 2010; Scott, 2012; Shephard et al., 2007). Fumonisins are commonly found in different grains and grain-based products, especially in maize (Berardo et al., 2011; Bush et al., 2004; Kriek and Marasas, 1977; Marasas et al., 1980; Rheeder et al., 1992; Shephard et al., 2007; Vincelli et al., 1993). Six different fumonisin molecules have been identified, namely B_1, B_2, B_3, B_4, A_1 and A_2, of which fumonisin B_1 (FB_1) is the most common. The basic chemical structure of fumonisin is a C-20, diester of propane-1,2,3-tricarboxylic acid and a pentahydroxyicosane containing a primary amino group (Figure 1.3) (Sweeney and Dobson, 1998). Fumonisin B_1 is not only hepatotoxic and hepatocarcinogenic but has also shown to cause inhibition in sphingolipid biosynthesis. Fumonisin B_1 is a competitive inhibitor of the enzyme which links sphinganine to a long chain fatty acid to form ceramide which is itself involved in the biosynthesis of a wide range of complex sphingolipids (Hussein and Brasel, 2001; Moss, 2002a).

In 1970 an outbreak of equine leukoencephalomalacia (ELEM) which was characterized by liquefactive necrotic lesions in the white matter of cerebral hemispheres of horses, occurred in South

Africa. Later on this was shown to be linked to the presence of FB_1 in corn used in feeds (Marasas et al., 1988; Marasas, 2001). It was also found that FB_1 is not only hepatocarcinogenic and hepatotoxic to rats but also causes changes in the cellular membranes, related to fatty acid changes in the major membrane phospholipids (Gelderblom et al., 1996, 1991). In humans it has been linked to oesophageal cancer (Marasas et al., 1980; Marasas, 2001; Rheeder et al., 1992; Viljoen, 2003). In the Transkei region of South Africa it is well known for the community to consume maize as part of their staple diet, and very often home grown maize is contaminated with fumonisin (Rheeder et al., 1992, 2002; Shephard et al., 2013, 2007). This has led to Transkei being one of the leading areas with oesophageal cancer incidences (Marasas et al., 1980; Marasas, 2001; Sydenham et al., 1990).

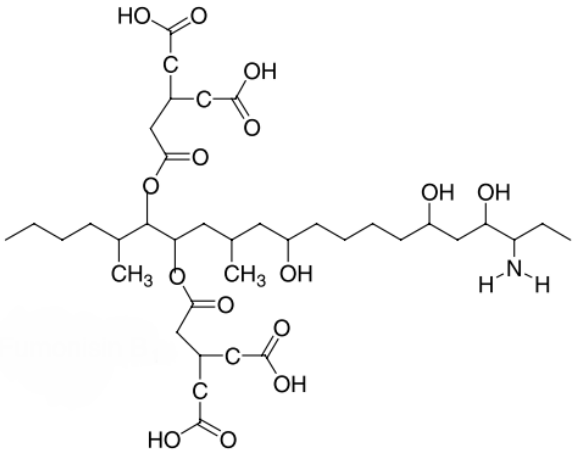

Figure 1.3: Chemical structure Fumonisin B_1 (FB_1) (Murphy et al., 2006).

1.3.1.2 Trichothecenes

Trichothecenes include deoxynivalenol (DON), fusarenon X (Fx), 15-acetyl-deoxynivalenol (15ADON) and nivalenol (NIV). This chemically diverse group of mycotoxins are produced by a variety of *Fusarium* spp., but mostly by *F. graminearum*. It is possible that even in a pure culture, more than one of these mycotoxins are produced (Sweeney and Dobson, 1998). They are all tricyclicsesquiterpenes with a basic 12,13-epoxy-trichothec-9-ene ring system (Sweeney and Dobson, 1998). According to their structures they can be divided into four groups. Type A, which has a functional group other than a ketone at position C-8, while type B has a ketone at Position C-8, type

C which consists of a second epoxy group at C-7, 8 or C-9, 10 and type D, which is the only group to have a macrocyclic ring between C-4 and C-5 with two ester linkages (Mavhunga, 2013; Sweeney and Dobson, 1998). Trichothecenes produced by *Fusarium* spp. are categorized as type A and B and are synthesized by a complex biosynthetic pathway of which DON is an intermediate product and NIV the end product of the pathway. The only difference between these two toxins is one hydroxyl group which is present in NIV and absent in DON (Desjardins and Proctor, 2007; Ichinoe et al., 1983).

Type B trichothecenes include DON, NIV, 15-ADON, 3-ADON and fusarenon X of which DON (Figure 1.4) is the most commonly found (Bily et al., 2004). Deoxynivalinol has been found worldwide in various grain types (Bennett and Klich, 2003; Biomin, 2015; Miller et al., 1983; Richard et al., 2009; Vegi et al., 2011). Deoxynivalenol production takes place in the early stages of the infection process in host plants (Mavhunga, 2013). Deoxynivalenol is also known as vomitoxin due to the acute effects on humans, which include adverse gastro-intestinal symptoms such as nausea, vomiting, diarrhoea, abdominal pain (Moss, 2002a; Pitt, 2000). In 1987, in the Kashmir valley of India, an outbreak of illness involving vomiting and dizziness among approximately 50 000 people was attributed to the consumption of bread made from rain-damaged wheat that contained several trichothecenes, including DON (Bhat et al., 1989). However, regardless of this incident and the severe symptoms when infected with DON, no literature could be found to report any human deaths that have been associated with DON or any suggestions that DON is carcinogenic. It is suggested that DON has a biological effect on animals even when present in very low levels. Deoxynivalenol is known to be immunosuppressant in animals, causing a reduction in the concentration of immunoglobins which predisposes animals to other diseases (Bily et al., 2004). Levels as low as 0.25mg/kg body weight has shown immunosuppressant activity in mice (Moss, 2002a).

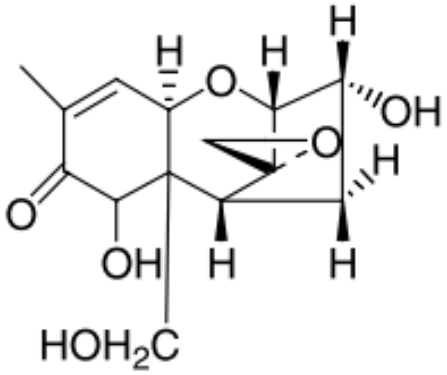

Figure 1.4: Chemical structure deoxynivalenol (DON) (Murphy et al., 2006).

1.3.1.3 Zearalenone

Zearalenone (ZEA) is mostly produced by the species complex *F. graminearum* (Bennett and Klich, 2003; Beukes, 2015; Bily et al., 2004; Logrieco et al., 2002; Pitt, 2000). Other species that produce ZEA include *F. cerealis*, *F. culmorum*, *F. equiseti*, and *F. semitectum.* This non-steroidal estrogenic toxin (Figure 1.5) is biosynthesized through a polyketide pathway (Bennett and Klich, 2003; Zinedine et al., 2007). In contrast to trichothecenes which are produced during early stages of the infection process in host plants, ZEA are produced at the end of the infection process (Miller et al., 1983). Zearalenone is a worldwide problem, including in South Africa and even though it is seen as a mycotoxin mostly produced in the field, high levels have been found in corn-based animal feeds due to improper storage (Dutton and Kinsey, 1996; Kuiper-Goodman et al., 1987). It is hepatotoxic, haematotoxic and considered a promoter for DNA damage and reproductive toxicity (Maaroufi et al., 1996; Marin et al., 2010; Wang et al., 2013). In humans, it is believed to contribute to breast cancer and has been linked to hypo-estrogenic syndromes (Ahamed et al., 2001; Yu et al., 2005). Furthermore, it was suggested that ZEA is a stimulating factor for advanced puberty development in girls, but the Food and Drug association (FDA) do not agree with these suggestions (Kuiper-Goodman et al., 1987; Massart and Saggese, 2010; Pitt, 2000; Zinedine et al., 2007).

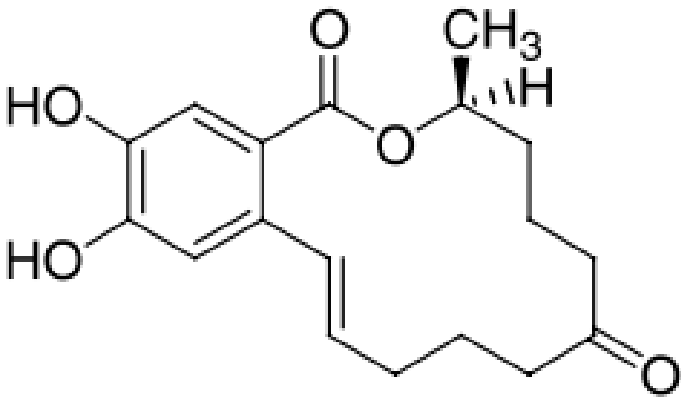

Figure 1.5: Chemical structure zearalenone (ZEA) (Murphy et al., 2006).

Many studies report on the toxic effects that ZEA has on animals, mostly affecting the reproductive system (Arora et al., 1983; Döll and Dänicke, 2011; Döll et al., 2003; Hou et al., 2014; Maaroufi et al., 1996; Schoevers et al., 2012; Zwierzchowski et al., 2008). Pigs are the most sensitive, where disturbances have been found in their reproductive systems, including haemorrhaging and atrophy of the ovaries and testes (Nelson et al., 1994; Pitt, 2000). It has also been found that in prepuberal gilts, the vulva becomes swollen, hyperemic and edematous, the mammary glands are swollen and in severe cases vaginal and rectal prolapse may occur (Nelson et al., 1994).

1.3.2 Aspergillus

Aspergillus belongs to the same family as *Penicillium* namely the *Aspergillaceae* (Houbraken et al., 2014; Samson et al., 2014, 2011). The most important microscopic characteristic to identify species belonging to this genus is the aspergillum-like spore-bearing structure (Bennett, 2010). Species can be found worldwide, in various habitats and even though it is widely used in the industry, some species are known to spoil food and crops (Abbaszadeh et al., 2015; Parra and Magan, 2004; Rajesh et al., 2010; Rodrigues et al., 2011; Soares et al., 2012; Taniwaki et al., 2012; Xu et al., 2013; Zhao et al., 2013). The most common industrial use of this genus is *A. oryzae* in soy sauce (Xu et al., 2013; Zhao et al., 2013). Spores formed by *Aspergillus* spp. are commonly found drifting in air currents, enabling their spread across different environments. The spores can easily be breathed in by humans

and animals, or if they land on a substrate with the right environmental conditions they will germinate (Bennett, 2010). Growth takes place at temperatures between 10 – 43 °C (Parra and Magan, 2004; Ramos et al., 1998; Sweeney and Dobson, 1998). *Aspergillus* is a diverse group containing species that are able to grow on a broad range of substrates. They can occur in the field in soil and on plants before harvesting and after harvesting, during storage (Iheanacho et al., 2014; Keller et al., 1994; Klich, 2007; Ramos et al., 1998). Furthermore, they are able to grow on food and feedstuffs after commercial processing (Bennett, 2010; Cutuli et al., 1991; Diaz et al., 2009; Dutta and Das, 2001; Iheanacho et al., 2014). Grains, nuts and spices with relatively low water content can also be infected by moderately xerophilic species of this genus (Lacey, 1994; Rodrigues et al., 2011; Soares et al., 2012; Taniwaki et al., 2012). Mycotoxin production, mainly in the form of aflatoxins, occurs when certain species colonize crops and feedstuffs (Bennett, 2010; Cole, 1986; Demirel and Sariozlu, 2014; Diaz et al., 2013; Dutta and Das, 2001; Moss, 2002b; Perrone et al., 2014; Shephard, 2008a, 2003; Vincelli et al., 1988).

1.3.2.1 Aflatoxins

There are at least 14 different types of aflatoxins currently known, mainly produced by *Aspergillus flavus* and *A. parasiticus.* The first discovery of aflatoxins were made in the early 1960's in an incident in which almost 100,000 turkeys died after being fed with peanut meal infected with *A. flavus* and contaminated with a compound which fluoresced blue under ultra violet light (Forgacs, 1962; Goldblatt, 1969). Today, this compound is known to be aflatoxin B (Figure 1.6), a secondary metabolite of *A. flavus.* More AF were discovered later and based on chromatography on silicic acid, compounds were separated according to the their fluorescence under ultra violet light, that can be either blue or green and were, therefore, referred to as aflatoxin B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), and G_2 (AFG_2) (Goldblatt, 1969; Nesbitt et al., 1962). Cows and sheep fed with a mixed dose of AFB_1, AFB_2, AFG_1 and AFG_2, were found to excrete a toxic factor in their milk and urine. This toxin was called aflatoxin M, to indicate that its original isolation was from milk (Goldblatt, 1969).

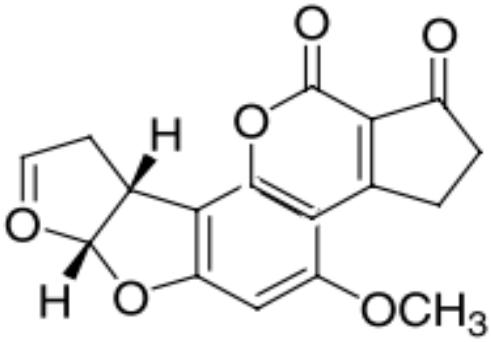

Figure 1.6: Chemical structures of aflatoxin B_1 (AFB_1) (Murphy et al., 2006).

Aflatoxins have always been and still are very commonly found in vegetable products of tropical and sub-tropical origin, especially peanuts and maize (Afsah-Hejri et al., 2013; Darwish et al., 2014; Lazicka and Orzechowski, 2010; Lye et al., 1995; Moss, 2002b; Nyinawabali, 2013; Shephard, 2008b). Although the level of sensitivity to toxins can differ between hosts, there is no doubt that aflatoxins are toxic to both animals and humans, affecting mainly the liver (Ciegler and Bennett, 1980; Cole, 1986; El-Sayed and Khalil, 2009). Interestingly, it could even differ between sexes of the same species, and are generally more toxic to males than to females (Lazicka and Orzechowski, 2010; Moss, 2002b). Aflatoxin B_1 (AFB_1) is one of the most carcinogenic mycotoxins in animals such as rabbits, cats, dogs and pigs, with a LD_{50} (median lethal dose) value of less than 1 mg/kg body weight (Moss, 2002c). Hamsters are some of the most resistant hosts with a LD_{50} of 10.2 mg/kg body weight (Moss, 2002b, 2002c). Acute exposure to AF through the consumption of contaminated food, will cause toxic hepatitis in humans and animals that can lead to jaundice and in severe cases, death. (Krishnamachari et al., 1975; Lye et al., 1995; Mohd-Redzwan et al., 2013; Shephard, 2008a, 2008b, 2003). Apart from previous reported outbreaks of aflatoxicosis and deaths it is furthermore suspected that AF poses serious reproductive health threats to exposed individuals, in both developed and developing countries (Eze and Okonofua, 2015). It was found that a higher percentage of infertile men with AF present in their semen, showed more abnormalities in their sperm count, motility and morphology compared to infertile men with no AF exposure (Ibeh et al., 1994). This should increase

the awareness for AF and the possible repercussions when humans and animals are exposed to AF over a long period of time.

1.3.3 Penicillium

The genus *Penicillium* belongs to the phylum Ascomycota, and is probably one of the most common fungi with a diverse range of habitats, including soil, vegetation, outdoor- and indoor air and food products (Samson et al., 2014). They mainly act as decomposers in nature, where some species cause damage to feedstuffs as pre- and post-harvest pathogens (Frisvad and Samson, 2004; Pitt and Hocking, 1997). This diverse genus consists of species that are extensively used in commercial processes, such as ripening of speciality cheeses like Camembert and blue cheese (Babel, 1953; Cakmakci et al., 2014). Most *Penicillium* spp. produce a variety of secondary metabolites, some of which are toxic (mycotoxins) and others not (Kozlovsky et al., 2009). The mycotoxins produced by these species can be divided into two groups based on the effect they have on the host. The first group is known have a negative impact on the liver and kidney function, while the second group has a destructive effect on the nervous system (Frisvad et al., 2004). The three most important mycotoxins are ochratoxin A (OTA), patulin and citrinin. In this study we mainly focused on OTA, as this toxin is the most toxic of the three compounds and one of the most important mycotoxins present in animal products (Hussein and Brasel, 2001; Mwanza, 2011; Sweeney and Dobson, 1998).

1.3.3.1 Ochratoxins

Ochratoxins were first isolated in 1965 from an *Aspergillus ochraceus* culture and was later also shown to be a secondary metabolite of *Penicillium* spp. in temperate regions (Hussein and Brasel, 2001; van der Merwe et al., 1965a, 1965b). In cold and temperate climates ochratoxin is mostly produced by *P. verrucosum* on grains (Cabañes et al., 2010; Moss, 2002b; Pitt, 1987). In contrast, production of ochratoxin by *A. ochraceus* takes place in warmer temperatures. Ochratoxins occurs worldwide, where environmental conditions are favourable for production (Afsah-Hejri et al., 2013;

Ashiq, 2015; Demirel and Sariozlu, 2014). Three different ochratoxin structures have been identified namely, ochratoxin A (OTA), ochratoxin B (OTB) and ochratoxin C (OTC), of which OTA (Figure 1.7) is the structure most abundantly found and also the most toxic (Hussein and Brasel, 2001). This structure consists of a dihydroisocoumarin moiety linked to its 7-carboxyl group by an amide bond to one molecule of L-β phenylalanine (Figure 1.7) (Hussein and Brasel, 2001; Mwanza, 2011; Sweeney and Dobson, 1998; van der Merwe et al., 1965a). This nephrotoxic mycotoxin produces anaemia in humans and has been suggested as the cause of Balken Endemic Nephropathy (BEN), a fatal human renal disease (Pfohl-Leszkowicz and Manderville, 2007). Several studies have indicated that OTA is also a carcinogen in ruminants (Mally and Dekant, 2009; Petzinger and Ziegler, 2000; Purchase and van der Watt, 1971).

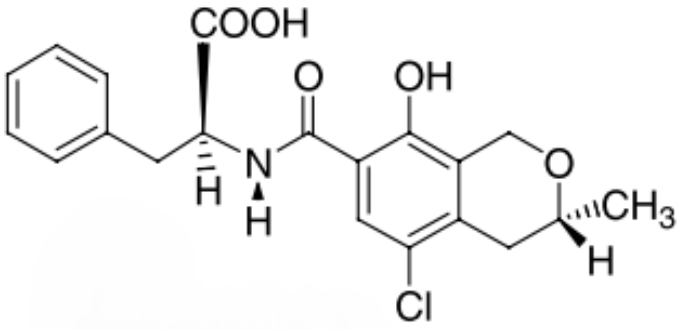

Figure 1.7: Chemical structure ochratoxin A (OTA) (Murphy et al., 2006).

1.4 Factors influencing mycotoxin production

A variety of factors can influence mycotoxin production. D'Mello and Macdonald (1997) categorized, these factors into three groups namely, physical, chemical and biological. The amount of fungal growth and toxin production is therefore not directly related to each other. Excessive fungal growth can take place without producing mycotoxins. Equally, high levels of mycotoxins can be produced at low growth activity. It is for this reason that fungal biomass fails to be an indicator for mycotoxin production.

1.4.1 Physical factors

Physical factors that influence mycotoxin production on plants and crops include time, humidity, temperature and extent of insect damage. Even though there is an overlap between the optimum growth conditions and conditions under which mycotoxin production takes place, these are not identical. Optimal growth conditions for fungi are generally much wider than the optimal conditions for mycotoxin production (Moss, 2002a, 2002b; Sweeney and Dobson, 1998). Table 1.1 lists a number of previous studies highlighting the differences between temperature under which growth takes place and when mycotoxin production takes place, but it is important to note that this can vary between studies, when physical, chemical or biological factors are different. It is even possible to get different results between samples from the same area taken during two or more seasons (Anukul et al., 2014).

Table 1.1: A few examples showing the difference between temperatures favouring growth vs. temperatures under which mycotoxin production takes place.

Species	**Mycotoxins**	**Growth temperature (°C)**	**Mycotoxin production (°C)**	**Reference**
A. ochraceus	OTA	10 – 43	12 - 37	Ramos et al., 1998, Ribeiro et al., 2006
A. flavus, *A. parasiticus*	AF	10 - 43	12 – 40	Pitt and Hocking, 1997, Ribeiro et al., 2006, Koehler et al., 1985
F. verticillioides	FUM	7 - 37	10 - 30	Marín et al., 1999
P. verrucosum	OTA	0 - 31	15 - 25	Cairns-Fuller et al., 2005

FUM = Fumonisins, OTA = Ochratoxin A, AF = Aflatoxins

1.4.2 Chemical factors

Fungicides and fertilizers are two compounds very commonly used in farming that could influence mycotoxin production. Fungicides can be used with great success towards controlling diseases but not mycotoxin production. A few studies have shown that sub-lethal concentrations, may increase mycotoxin production. This is especially true for *Fusarium* phytopathogens (D'Mello et al., 1998). One such example is while tebuconazole and triadimenol are used to treat head blight in wheat, it

stimulates the production of NIV (Gareis and Ceynowa, 1994). Systematic fungicides such as tridemorph, fenpropimorph and fenarimol, showed to not only increase aflatoxin production, but also alters the AFB_1 and AFB_2 ratio (Badii and Moss, 1988). The effect of fungicides on mycotoxin production are not consistent and are influenced by other environmental factors (D'Mello et al., 1998). Furthermore, nitrogen- and carbon sources as well as trace metals, often present in fertilizers, also have an effect on mycotoxin production (Bouras et al., 2009; D'Mello and Macdonald, 1997; Placinta et al., 1999). For instance an increase in nitrogen concentration in fertilizers cause an increase in DON production by *F. graminearum* (Lemmens et al., 2004).

1.4.3 Biological factors

The interaction between a toxigenic fungal species and the substrate is one biological factor influencing mycotoxin production. Other factors include strain specificity, strain variation and finally the instability of toxigenic properties (Alinezhad et al., 2011; Ciegler and Bennett, 1980; Sinha et al., 2001). Diener and Davis (1969), reports on a few studies that were conducted in the 1960's, *A. flavus* were isolated from different crops including peanuts, grains, corn and rice but only 57.7 % of them produced toxins. Where the same substrates and incubation conditions were applied, differences in mycotoxin production were seen as confirmation that production ability differs between strains. It was also noted that overall aflatoxin production is lower on substrates containing high levels of oil such as peanuts and soybeans. Substrates with high carbohydrate levels such as rice and corn support aflatoxin production more than oilseeds. This was presumably attributed to the oil that is not immediately metabolised by *A. flavus.*

1.5 Toxigenic fungi and their associated mycotoxins in artificial feed

1.5.1 Artificial feed for terrestrial animals

Extensive research has been done over the years to determine the impact of mycotoxins on animal

and human health, but mycotoxins are globally still very much part of terrestrial and aquatic animal feeds. Increasing amounts of toxigenic fungi and their associated mycotoxins were detected in feeds and feedstuffs and this raised awareness of the increased risk of fungi and mycotoxin contamination in feed and feedstuffs (Alinezhad et al., 2011; Anukul et al., 2014). Table 1.2 shows a summary of toxigenic fungi and mycotoxins detected in previous studies. Deoxynivalenol and aflatoxins were the most dominant toxins in most of the studies listed. This is similar to a survey conducted in 2015 by an animal health and nutrition company. During this survey agricultural commodities were sampled across 75 countries, and it was found that *Fusarium* related toxins had the highest dominance in most countries. Deoxynivalenol followed by ZEA and fumonisins were shown to be the biggest threat in both the agricultural commodities and finished feed. Aflatoxins and OTA also causes reason for concern (Biomin, 2015).

1.5.2 Artificial feed for aquatic animals

The ever growing aquaculture industry, caused an increase in the demand for a readily available feed supply. A depletion in natural sources encouraged the use of formulated diets. The primary aim of a fish nutritionist is to develop a diet that is practical, sustainable and provides balanced nutrition that supports the overall health of fish (Fleming et al., 1996; Tacon and Metian, 2008). Fish nutritionists mostly rely on agricultural commodities to formulate fish diets. These serve as the carbohydrate sources and in some instances as the protein source (Amaya et al., 2006; Nácher-Mestre et al., 2015; Valenta et al., 2002). One example is warm water channel catfish (*Ictalurus punctatus*) where their commercial diet has evolved over the past decade to include little to no animal protein (Manning, 2005).

Little research has been done on aquatic artificial feeds in terms of toxigenic fungi and their associated mycotoxins, but similar fungi and mycotoxins are expected to be present in any fish feeds, due to the feed composition (Table 1.2). *Fusarium* spp., mostly *F. verticillioides*, were isolated from ingredients

used in shrimp feed, but no fungi were found in the final feeds. This could be attributed to high temperatures used during processing of the feed (Anukul et al., 2014). Ingredients included, corn, soybean meal, and peanut samples. The authors of that study did not screen for any mycotoxins in their samples, instead three different methods were used to determine the toxin production ability of the isolates. The three methods included enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR) and ultra-pressure liquid chromatography-tandem mass spectrometry.

Table 1.2: Summary of toxigenic fungi and mycotoxins found in terrestrial- and aquatic animal feed and feedstuffs.

Samples	**Sample year**	**Location**	**Sample source**	[2]**Dominant Toxigenic fungi**	[2]**Dominant Mycotoxins**	**Reference**
Commercial dog food	2007	Vienna, Austria	Markets, Supermarkets, Pet food markets	-	DON, ZEA, FUM, OTA	Böhm et al., 2010
Animal feeds and feedstuffs	2011	South Korea	National products quality management service	-	FB_1, FB_2	Seo et al., 2013
Feedstuffs	2000, 2001	South West Germany	Randomly	-	DON, NIV, 15-ADON, 3-ADON, ZEA	Schollenberger et al., 2006
Domestic animal feed, Poultry feed, cattle cakes	1985, 1986, 1987	Bihar, India	Poultry farms, Markets, Villages	*A. flavus*, *A. ochraceus*, *A. versicolor*, *P. citrinum*, *F. moniliforme*	AF, ZEA, OTA	Ranjan and Sinha, 1991
Pig feed	2011	North West Croatia	Feed factories	*Penicillium* spp., *Fusarium* spp., *Aspergillus* spp.	DON, ZEA, FUM, OTA, AF	Pleadin et al., 2012
Equine feed	2003, 2004	Rio de Janeiro, Brazil	Equine centres	*Aspergillus* spp., *Penicillium* spp., *Fusarium* spp.	AFB_1, FB_1	Keller et al., 2007
Trout feed and feedstuffs	2009	Iran	Feed manufacturers	*Aspergillus* spp., *Penicillium* spp.	AFB_1	Alinezhad et al., 2011
Shrimp feed, Commercial fish feed, Commercial soya meal	2012	Thailand	Animal feed factories	*F. verticillioides*, *F. oxysporum* , *F. equiesti*	-	Anukul et al., 2014
Commercial fish feed	-	Europe	Random	-	DON, ZEA	Pietsch et al., 2013
Fish feed	2009, 2010	Rio de Janeiro, Brazil	Tilapia farms	*Aspergillus* spp., *Penicillium* spp.	FB_1, AFB_1, OTA	Barbosa et al., 2013

[2] Toxigenic fungi and mycotoxins were listed in decreasing dominance order.

DON = Deoxynivalenol, ZEA = Zearalenone, FUM = Fumonisins, OTA = Ochratoxin A, FB_1 or B_2 = Fumonisin B_1 or B_2, NIV = Nivalenol, ADON = Acetyl-deoxynivalenol, AF = Aflatoxins

Although there were discrepancies between the results they concluded that all their isolates had the ability to produce only fumonisins (Anukul et al., 2014). Therefore, even though no fungi could be found in the final feed, it is unclear whether the final product contained fumonisins that were produced before processing started. In a different study FB_1 was found in 98 % of the commercial fish feed samples, but no record was made of any fumonisin producing fungi present in the feed (Barbosa et al., 2013). While other authors have argued that fungal determination in feed will provide information to which mycotoxin to expect (Anukul et al., 2014; Dalcero et al., 1997; Magnoli et al., 2002), Barbosa et al. (2013) showed that this is not always true. Although *A. flavus* and aflatoxin contamination showed a positive relation, this was not the same for *F. verticillioides* and fumonisin contamination. The authors attributed the high levels of fumonisin contamination to possible contaminated ingredients (Barbosa et al., 2013). These studies prove that the presence of toxigenic fungi and their associated mycotoxins in feeds and feedstuffs are not dependent on each other. Toxigenic fungi can be present without producing mycotoxins. In contrast, mycotoxins can be present without the presence of fungi. This occurs when fungi are removed during feed processing leaving the mycotoxins behind to end up in the finished feed. Therefore, it is important to monitor both toxigenic fungi and their associated mycotoxins in any artificial feed (Anukul et al., 2014).

1.6 Mycotoxins in Aquaculture

An understanding of mycotoxin related-issues within the aquaculture industry is becoming more and more important due to the increasing evidence showing the negative impact of mycotoxins in aquatic species (Encarnação, 2011; Hooft et al., 2011; Manning, 2010, 2005; Santacroce et al., 2008). The first report of mycotoxins in aquaculture was in the 1960's after isolating liver nodules from rainbow trout at fish hatcheries in the United States (Halver, 1969) At first it was only a few reported incidences, but after changing the feed from a wet diet consisting of glandular meats and slaughter house waste products to a dry fish ration, this problem became serious and more frequent. In the 1950's liver tumours were reported from fish farms in Japan, Italy and France. At the same time liver

tumours were found in rainbow trout from many different hatcheries and trout species throughout the United States, all fed with dehydrated commercial feed or prepared fish feeds (Halver, 1969). This was a clear indication for a common responsible source (Halver, 1969). It was only later that aflatoxins in feed were identified as the cause for these liver tumours. *Aspergillus flavus* was the primary source of AF which were introduced into the feed through contaminated cottonseed meal (Halver, 1969). Following these incidents more research took place in attempt to understand the effect of mycotoxins on aquatic animals (Table 1.3). It was found that the effect of mycotoxins on aquatic animals are influenced by the age and size of the animals along with the period of application and concentration of mycotoxins. Smaller animals affected by mycotoxins will exhibit symptoms faster than larger animals (Doster et al., 1974; Lumlertdacha and Lovell, 1995; Manning, 2005; Manning et al., 2003; Schoenhard et al., 1981; Tuan et al., 2003; Yildirim et al., 2000). One example is where smaller channel catfish (*Ictalurus punctatus*) were found to be more sensitive and showed a quicker reduction in weight gain than larger fish. This was the conclusion after a decrease in growth rates were observed in small juveniles after 10 weeks fed with 20 mg FB_1/kg feed and larger juveniles started showing a decrease in growth rate after 12 weeks fed with double the FB_1 concentration in feed (Lumlertdacha et al., 1995). Another example is where feed with naturally DON contaminated wheat, were used to evaluate the effect of DON on Pacific white shrimp (*Liropenaeus vannamei*). Only after 4 weeks, a significant decrease in growth rate were noticed in groups fed with the highest DON contamination (1 ppm) feed and 14 weeks to see a decrease in growth rates for groups fed with the lowest DON contaminated (0.2 ppm) feed (Trigo-stockli et al., 2000).

Trout was shown to be highly sensitive to DON. One study showed that DON caused up to 92 % decrease in weight gain in rainbow trout (*Salmo gairdneri*) when fed maize containing 13.0 mg DON/kg feed over a period of 4 weeks (Woodward et al., 1983). Authors from a different study argued that the concentration of contamination in the feed is not a true reflection of the amount of DON actually consumed by the fish. This is because a decrease in feed intake was noticed. The

effect of DON on regional brain neurochemistry may have contributed to the reduction in feed intake of fish exposed to the increasing dietary concentrations of DON. It was, therefore, suggest that DON is directly and indirectly responsible for the deleterious effects on the nutrient metabolism in rainbow trout (Hooft et al., 2011).

Various studies were performed to determine the effect that fumonisins have on aquatic animals. Although all these studies showed no mortality amongst the animals tested, there was an overall agreement that the kidneys are the most sensitive organs. Furthermore, fumonisins cause a significant decrease in growth rate along with other health problems depending on the species tested (Anukul et al., 2014; Goel et al., 1994; Lumlertdacha et al., 1995; Manning, 2005; Yildirim et al., 2000). Studies over time have showed that although, most fish species show behaviour changes, skin lesions, yellowing of the body surface, eye cataracts, and hemorrhages in the head, some species are more sensitive than others (El-Sayed and Khalil, 2009; Encarnação, 2011; Halver, 1969). Studies indicated that rainbow trout and sea bream are among the more sensitive fish species while channel catfish and Nile tilapia are less sensitive (Centoducati et al., 2009; Halver, 1969; Manning, 2005). However, it should be noted that sensitivity to mycotoxins is different for the different mycotoxins.

An overall conclusion can be made that each aquatic species reacts differently and that the effect and LD_{50} of mycotoxins on different aquatic species have to be determined. This is especially true for farmed aquatic species that have a higher risk of exposure, due to the use of artificial feed. Farmed animals form part of a worldwide food production chain and could hold a risk to humans. Little is known about the possible accumulation of mycotoxins in aquatic animals. However, the results of two studies that tested for accumulation in two different aquatic animals agree that one rule does not apply to all. After feeding AFB_1 contaminated (0.025 – 0.2 ppm) feed to pre-adult shrimp (*Penaeus monodon*) for 60 days no toxins were detected in the tissue (Bautista et al., 1994). In contrast AFB_1 was measured in the edible muscle of sea bass (*Dicentrarchus labrax*) after feeding them with conta-

Table 1.3: Summary of the effect of mycotoxins on aquatic animals.

Host	Mycotoxin	Effect	Reference
Channel catfish (*Ictalurus punctatus*)	FB_1	Decrease in growth rates [3]SA/SO ratio increase in the kidneys, serum and muscle Decrease in bacterial resistance Pathological changes in the liver	Lumlertdacha et al., 1995 Goel et al., 1994; Yildirim et al., 2000
	DON	Decrease in growth rate Decrease in feed conversions	Manning, 2005
Juvenile channel catfish (*Ictalurus punctatus*)	OTA	Lower weight gain Decrease in feed conversions Haematocrit values decreased Multifocal melanomacrophage centres in renal tissue	Manning et al., 2003
Nile tilapia (*Oreochromis niloticus*)	FUM	SA/SO increase in the kidneys, serum and muscle Decrease in bacterial resistance	Tuan et al., 2003
Rainbow trout (*Oncorhynchus mykiss*), (*Salmo Gairdneri*)	DON	Decrease in weight gain Inhibition of protein synthesis Subcapsular edema Morphological changes in the liver	Woodward et al., 1983 Hooft et al., 2011
Rainbow trout (*Salmo gairdneri*)	AFB_1	Hepatocellular carcinomas	Lee et al., 1968; Schoenhard et al., 1981

[3] SA/SO = ratio between sphinganine and sphingosine

FB_1 = Fumonisin B_1, FUM = Fumonisins, DON = Deoxynivalenol, OTA = Ochratoxin A, AFB_1 = Aflatoxin B_1

Table 1.3 continue

Host	Mycotoxin	Effect	Reference
White shrimp (*Liropenaeus vannamei*)	DON	Decrease growth rates	Trigo-stockli et al., 2000
Sea Bream (*Sparus aurata*)	AFB_1	Reduction in cell viability (*In vitro*)	Centoducati et al., 2009
Panaeid shrimp	AFB_1	Lesions of the hepatopancreas Lesions of the mandibular organ Lesions of the hematopoietic organs	Wiseman et al., 1982
Sea bass (*Dicentrarchus labrax* L.)	AFB_1	Accumulates in the edible tissue Behavioural changes Decrease in plasma proteins Increase in alkaline phosphatase activities Serum transaminases Hepatocellular carcinomas	El-Sayed and Khalil, 2009
Juvenile Gibel carp (*Carassius auratus gibelio*)	AFB_1	Supressing growth Toxin accumulation in hepatopancreas Impaired physiological responses	Han et al., 2010

minated feed (0.018 ppm) for 42 days (El-Sayed and Khalil, 2009). Authors of the latter study proposed the high levels of AFB_1 detected in the edible tissue as a high risk to the consumer, since the US Food and Drug Administrator (FDA) has a limited allowance of 5 ppb AFB_1 in human foods.

1.7 Mycotoxin regulations

The European Food Safety Authority (EFSA) regards fumonisins, along with other mycotoxins as a risk to human and animal health, and has therefore published maximum allowable limits of these toxins relative to animal feed with a moisture content of 12 %. The allowable limit for FB_1 and FB_2 in fish feed according to the EFSA is 10 ppm (Food standards agency, n.d.). The U.S. Food and Drug Administration (FDA) also allows a maximum of 10 ppm of total fumonisins in catfish feed (U.S. Food and Drug Adinistration, 2001). According to the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act no 36 of 1947, republished in 2006, the South African national regulations include maximum allowable mycotoxin limits for a variety of animal feeds and ingredients (Table 1.4), but does not state any regulations with regards to allowable mycotoxin limits in aquatic animal feeds (National Department of Agriculture, 2006).

Table 1.4: Maximum allowable mycotoxin limits in animal feeds and ingredients under the South African national regulations, according to the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act no 36 of 1947, republished in 2006 (National Department of Agriculture, 2006).

Mycotoxin	**Feeds and ingredients**	**Maximum allowable limit (ppm) relative to a feed with a moisture content of 12%**
AFB_1	Feed ingredients with the exception of :	0.02
	Groundnut, copra, palm kernel, cotton seed, maize, and products derived from the processing thereof	0.05
	Complete farm feeds for cattle, sheep and goats with the exception of:	0.05
	Dairy cattle,	0.005
	calves and lambs	0.01
	Complete feeds for pigs and poultry (expect young animals)	0.02
	Other complete farm feeds	0.01
DON	Feeding stuffs on full ration basis for:	
	Pigs	1
	Cattle	5
	Calves up to 4 months	2
	Dairy cattle	3
	Poultry	4
FB_1	Horses	5
	Pigs	10
	Beef and poultry	50
OTA	Feeding stuffs on full ration basis for:	
	Sows, pigs, and piglets	0.05
	Poultry	0.2
ZAE	Feeding stuffs on full ration basis for	
	Sows and pigs	0.25
	Young pigs	0.1
	Young cattle and dairy cattle	0.5

AFB_1 = Aflatoxin B_1, DON = Deoxynivalenol, FB_1 = Fumonisin B_1, OTA = Ochratoxin A, ZEA = Zearalenone

1.8 Conclusion

Mycotoxigenic fungi and their associated mycotoxins in feeds pose a serious threat to the seafood industry. Extensive research has been conducted on the effect of mycotoxins on humans and some animals, (Bily et al., 2004; Döll et al., 2003; Goldblatt, 1969; Maaroufi et al., 1996; Marasas, 2001; Nelson et al., 1994; Pitt, 2000; Rheeder et al., 1992; Viljoen, 2003) but not much is known about the effect on farmed aquatic animals. The first mycotoxin related problem in the aquaculture industry was encountered in the early 1900's yet there are still crucial unknowns with regards to

mycotoxigenic fungi and their associated mycotoxins in the aquaculture industry (Halver, 1969). The reason why this problem is so often overlooked is probably because the effect that the mycotoxins have on the animals can be confused with other physiological problems or it could be just because the effects are not dramatic enough. Most of the studies done on fish species, were conducted by a constant exposure of high mycotoxin levels. In contrast, mycotoxin levels in feed are much lower and vary over time, due to different batches of feed that are used. Agricultural commodities used in feed processing are harvested during different seasons, rainfalls and temperatures, therefore, the mycotoxin concentration will vary between feed batches. This will result in a continual fluctuation of mycotoxin concentrations that animals are exposed to, instead of a constant high exposure. This will add to the problem that the effect of mycotoxins is not drastic enough. However, from the limited research that was done on aquatic animals it is known that mycotoxins are responsible for a decrease in weight gain and minor behavioural changes (El-Sayed and Khalil, 2009; Hooft et al., 2011; Tuan et al., 2003). Furthermore, some mycotoxins such as aflatoxins accumulate in the edible tissue of sea bass (El-Sayed and Khalil, 2009). When this happens it broadens the problem as the consumer can now be affected. From the literature and previous studies no information could be found about the effect of mycotoxins on any shellfish species.

1.9 Problem statement

Although it is expected to find mycotoxins in abalone feed as a result of the ingredients that are used, this has not been investigated. The South African act for Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act no 36 of 1947, republished in 2006, does not include maximum allowable limits for mycotoxins in aquatic animal feeds and ingredients. The only authority that regards mycotoxins in aquatic animal feed is the U.S. Food and Drug Administration (FDA), with maximum mycotoxin allowance for materials used in catfish feed (U.S. Food and Drug Adinistration, 2001; Union European, 2006). Neither of these authorities have any guidelines on the maximum allowable mycotoxin levels in abalone feed nor the ingredients used. However, regulations such as

this cannot be established before it is known which toxigenic fungi and associated mycotoxins are present in the abalone industry. Furthermore, because mycotoxin production is influenced by so many factors, it is unknown whether mycotoxins will be produced on abalone feed during storage. Based on the literature and the unanswered questions we hypothesize that toxigenic fungi and their associated mycotoxins are present in ingredients used in the processing of abalone feed and are not removed during feed processing. Adding to this the location of abalone farms promotes the colonisation of toxigenic fungi on feed, during long storage periods. Abalone will consume feed contaminated with low levels of mycotoxins which could potentially accumulate in the tissue and then end up in the food chain, posing a health risk to humans.

1.10 Study objectives

The aims of the project was thus to:

(1) Determine the frequency of fungi in feeds for cultured abalone.

(2) Identify potential mycotoxigenic fungi in the feeds of cultured abalone and compare these to other mycotoxigenic fungi from food and feed commodities,

(3) Determine whether fumonisin production takes place when *F. verticillioides*, previously isolated from abalone feed, colonize abalone feed under different temperatures,

(4) Determine what happens with fumonisins in abalone feed when exposed to sea water,

(5) Provide a solution that could be applied to the aquaculture industry in terms of feed preparation and storage.

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2 THE OCCURRENCE OF NATURAL FUNGI AND THEIR ASSOCIATED MYCOTOXINS IN SOUTH AFRICAN ABALONE (*HALIOTIS MIDAE*) FEED

2.1 Abstract

Abalone feed in South Africa is mainly processed from grains, locally sourced, and a protein source. Therefore, fungal species associated with grains used in the processing of animal feed is a predictable problem. *Fusarium*, *Penicillium* and *Aspergillus* are genera known to produce mycotoxins when colonising grains, and are therefore, the most studied mycotoxigenic genera. In the present study the aim was to investigate the extent of toxigenic fungal contamination and their mycotoxins in ingredients used to process abalone feed as well as artificial feed used on abalone farms and in South Africa. In this study, 248 fungal strains were isolated from feed and raw materials that were collected from three different abalone farms in South Africa and one feed supplier. Morphological and multi-gene phylogenies were used to identify all isolates. *Fusarium, Penicillium* and *Aspergillus* species were isolated. Standard mycotoxin extractions were performed followed by liquid chromatography tandem mass spectrometry (LC-MS/MS) for mycotoxin quantification. Results indicated that low levels of fumonisin B_1 and ochratoxin A occur naturally in artificial abalone feed used in South Africa.

2.2 Introduction

Various animal feedstuffs across the globe are produced from a combination of local and imported grains. Sub-standard grains that are not fit for human consumption, are often used in animal feeds. For this reason, fungal growth on animal feed is a predictable consequence and cannot be ignored (Diaz et al., 2013). Environmental conditions such as temperature and moisture can predispose crops and animal feed to fungal growth. Plant stress and insect damage contribute to the increased the risk of fungal growth before harvesting. Furthermore, crops used in feedstuffs are contaminated by fungi

due to poor harvesting practices, handling, storage and transport conditions (Bhat et al., 2010; Glenn, 2007; Nácher-Mestre et al., 2015; Richard et al., 2009; Toffa et al., 2013).

Naturally occurring toxigenic fungi on grains used in animal feed are mainly dominated by species representing three genera namely *Fusarium, Penicillium* and *Aspergillus.* Other genera also known to have species that produce toxins are *Alternaria, Claviceps* and *Stachybotrys* (Bennett and Klich, 2003; Diaz et al., 2013; Eppley and Bailey, 1973; Ren et al., 1998; Szathmary Cs. et al., 1976). Under certain conditions these fungi are able to produce secondary metabolites, known as mycotoxins which are toxic to the consumer. Fumonisins, trichothecenes, ochratoxins and aflatoxins are some of the most significant mycotoxins produced by the three dominant mycotoxin producing genera (Pitt, 2000). *Fusarium* species can be found in both tropical and temperate regions but grow best under humid conditions where they are able to produce fumonisins and trichothecenes (Bhat et al., 2010; Booth, 1971; D'Mello et al., 1999; Hinojo et al., 2006; Rheeder et al., 2002, 1992). Certain *Fusarium* species are plant pathogens, infecting plants before harvesting and continuing to grow after harvesting (Diaz et al., 2013; Logrieco et al., 2002; Massart and Saggese, 2010). The genus that produced the widest class range of mycotoxins, is the genus *Penicillium,* with ochratoxins, patulin and citrinin most commonly found (Sweeney and Dobson, 1998). *Penicillium* and *Fusarium* species are well adapted in temperate environments with cooler climates and wet conditions (Diaz et al., 2013), while *Aspergillus* species prefer subtropical and tropical regions (Klich, 2002; Lin and Li, 1996). Some species within *Aspergillus* and *Penicillium* are opportunistic pathogens of crops and can be found on most produce. *Aspergillus* contamination in crops can either be experienced during cultivation in the field or during harvesting and storage (Klich, 2007).

Mycotoxins accumulate mostly in the mycelium but can also be found in the spores of fungi (Mavhunga, 2013). Mycotoxins are dangerous to humans (Fung and Clark, 2004; Rheeder et al., 1992; Shephard, 2003), domesticated animals such as chickens, pigs, horses, ruminants (D'Mello and

Macdonald, 1997; D'Mello et al., 1999; Placinta et al., 1999) and aquatic species such as sea bream, sea bass, penaeid shrimp and black tiger shrimp (Centoducati et al., 2009; El-Sayed and Khalil, 2009; Encarnação, 2011; Khoa et al., 2004; Wiseman et al., 1982), resulting in a condition referred to as mycotoxicoses (Ciegler and Bennett, 1980; Forgacs, 1962). There is increasing concern of the effect of mycotoxicoses on animal health (Pleadin et al., 2012).

The occurrence of mycotoxins in animal feeds could not only have a major effect on the animals but could also move up the food chain and affect humans (Arai and Hibino, 1983; Wang et al., 2013). Depending on the process of production of the animal feed, fungi and their mycotoxins can contaminate the end product in various ways. It originates on the farm, infects grains during transport and storage before the production of the feed, or infect the final feed product during transport and storage. In all three of these scenarios, viable fungal spores and mycotoxins will be present in the final product. This makes fungal growth possible when feeds are left in humid storerooms for long periods of time, and potentially produce mycotoxins (Bragulat et al., 2008; Klich, 2007; Mogensen et al., 2009; Parra and Magan, 2004; Ramos et al., 1998).

Fungal contamination in food and animal feed is a worldwide phenomenon. To name a few examples, in Egypt peanut seeds were infected with *Aspergillus*, *Penicillium* and *Fusarium* (El-Magraby and El-Maraghy, 1988), while a study years later in Spain, showed popcorn and maize used for human consumption were predominantly contaminated with *Aspergillus* and *Penicillium* spp. (Alborch et al., 2012). Interestingly, Anukul et al. (2014) found fungal contamination in the ingredients used for shrimp feed but not in the finished feed, suggesting that the fungi might be destroyed during the feed manufacturing process. Furthermore, fish feed from two tilapia farms located in Rio de Janeiro State, Brazil were found to be contaminated with eight different filamentous fungi of which *Aspergillus* and *Penicillium* spp. were the most prominent toxigenic genera (Barbosa et al., 2013). The presence of fungal contamination or mycotoxins in animal feedstuffs may result in health problems for both

workers and animals as well as significant financial losses (Afsah-Hejri et al., 2013; Bhat et al., 2010; Böhm et al., 2010; Glenn, 2007; Vincelli et al., 1988). There is no doubt that feed is one of the main aspects securing the success of an aquatic farm, regardless of the type of animals.

In South Africa, the abalone farming industry has expanded over the last decade and now dominates the marine aquaculture industry (FAO, 2013, 2012; DAFF, 2011). Previously, this industry relied on *Ecklonia maxima* (kelp) as the main food resource, but this resource has reached the limit of sustainable harvesting. The abalone farming industry was forced to find alternatives, resulting in different formulas of commercially produced feeds, some containing *E. maxima* and others produced from local grains with fishmeal as the primary protein source (FitzGerald, 2008; Fleming et al., 1996; Troell et al., 2006). The industry now relies on a combination of harvested *E. maxima* and commercially processed feed and, therefore, it comes as no surprise that fungi, and/or mycotoxins are associated with artificial abalone feed. The first aim of this study, therefore, was to conduct a survey to establish the extent of the presence of toxigenic fungi on artificial abalone feed used on abalone farms in South Africa. Secondly, to identify mycotoxin-producing fungi on abalone feed and the ingredients, from a commercial abalone feed supplier, before distribution to farms, and finally to screen these feed and ingredients for the presence of fumonisins, trichothecenes, ochratoxins and aflatoxins.

2.3 Materials and Methods

2.3.1 Sample collection

In 2013 and 2014 feed stocks from three farms, located along the South African coastline and one supplier of commercially produced feeds in South Africa, were sampled for analysis. In addition, samples were taken at different steps throughout the feed processing procedure (during processing, end product before drying and final feed before packaging) at the supplier. Feed and ingredients were randomly sampled and placed into brown paper bags. Samples used for fungal isolation were kept

one side and samples used for mycotoxin screening were stored at -20 °C.

2.3.2 Fungal isolation

Twenty grams of each sample was taken randomly and placed in sterile moist chambers. To ensure isolation of slow and fast growing fungi, feed were also crushed and dilution series ($10^0 – 10^{-3}$) were prepared with sterile water, 100 µL of feed-water suspension at each concentration was spread on potato dextrose agar (PDA) and malt salt agar (MSA), both containing 0.05 % chloramphenicol and 0.01 % streptomycin. All moist chambers and spread plates were incubated at 26 °C ±1 °C, for 7 - 10 days. After incubation, all fungi were isolated from the moist chambers and spread plates, and pure cultures were obtained on oatmeal agar.

2.3.3 Morphological characterisation

Aspergillus, *Penicillium* and *Fusarium* strains were separated from all the other isolates and grouped separately. For *Fusarium*, single spores were obtained by mixing a needle tip full of actively growing hyphae and spores from a 14 day-old culture on PDA, with 1 mL MilliQ water. Followed by spreading the hyphae/spore suspension on 1.5 % water agar plates. These were left to stand for 2 min before excess water was discarded. After overnight incubation at 25 °C ±1 °C, an Olympus SZX12 stereomicroscope was used to transfer single viable microconidia to PDA plates, plates were incubated at 25 °C ±1 °C. After 48 hours, agar plugs (6 mm diameter) were transferred to PDA and carnation leaf agar (CLA), and incubated at 26 °C ±1 °C for 7 and 14 days, respectively. Morphological characteristics that were examined on day 7 included, colony colour on PDA, presence (and colour) or absence of sporodochia in the centre of the PDA and carnation leaf pieces on CLA plates and thickness of hyphal growth on the PDA plates. Carnation leaf agar plates were used to perform micro-morphology. An Olympus BX50 light microscope was used to look for the presence and absence of sporodochia, macroconidia (number of septa and the shape of the apical and basal

cells), microconidia (shape, long or short chains and the arrangement of phialides) and chlamydospores. Characterisation was done based on the taxonomic keys of Leslie and Summerell, (2006).

Spore suspensions of all *Aspergillus* and *Penicillium* strains were prepared in a semi-solid agar solution containing 0.2 % agar and 0.05 % Tween (Pitt, 1979). Spore suspensions were used to do three-point inoculations on Czapek yeast autolysate agar (CYA) and malt extract agar (MEA), and plates were incubated at 26 °C ±1 °C. After 7 days of incubation, isolates were grouped together based on macro-morphological characteristics. Representatives of each group were chosen for micro-morphological characterisation and DNA analyses. Macro-morphological characteristics for *Penicillium* include growth rate, colony texture, medium buckling, colony colours and presence or absence of sclerotia (Pitt, 1979; Visagie, 2012). An Olympus BX50 light microscope was used to observe the following micro-morphological characteristics of cultures on MEA plates, conidiophore branching, surface texture of the stipes walls, divergence and number of metulae, shape of phialides, and shape and ornamentation of conidia (Pitt, 1979; Visagie, 2012).

Morphological characterisation of *Aspergillus* was done based on Thom and Raper (1945). The macro-morphological characteristics included growth rate, colony colours, exudates, soluble pigment and micro-morphological characteristics included seriation and shape of vesicles, conidial shape and surface texture, surface texture of the stipes walls and the presence or absence of ascospores (Thom and Raper, 1945).

2.3.4 Molecular phylogeny

DNA extractions – Total genomic DNA from all *Fusarium* strains and representatives of the *Penicillium* and *Aspergillus* strains were extracted. For larger morphological groups, more than one representative was used. Hyphae collected from PDA or CYA plates were added to 1.5 mL

Eppendorf tubes containing 500 µL TES buffer and glass beads. Samples were vortexed for 5 min at top speed using a Vortex-2 Genie, model G560E. Subsequently cell lyses extraction was done according to Möller et al. (1992). DNA was stored in 50 µL MilliQ water.

Molecular identification was done based on multi-gene phylogenetic analysis. The ITS1-5.8-ITS2 rDNA region for all the samples were amplified using primer set ITS1 and ITS4 (White et al., 1990). Reaction mixtures (20 µL) consisted of 10µL Kapa ReadyMix (Kapa, Biosystems) and 0.4 µL (0.25 µM) of primers ITS1 and ITS4, respectively. A second gene for each genus was amplified and sequenced in order to assist with identification. In the case of *Fusarium*, the partial EF-1α gene was amplified, using primers EF1 and EF2 (O'Donnell et al., 1998). For all *Penicillium* strains, a partial β-tubulin gene was amplified using primers Bt_2a and Bt_2b (Glass and Donaldson, 1995) and part of the Calmodulin gene in all the *Aspergillus* strains was amplified using the primers CMD5 and CMD6 (Hong et al., 2006) or CF1 and CF2 (Peterson, 2005). Conditions for all the PCR amplifications were as follow, initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 45 s, 45 s annealing (see Table 2.1 for specific annealing temperatures) and 72 °C for 1 min, followed by a final extension at 72 °C for 7 min for the EF-1α primer set or 10 min for all the other primer sets. PCR products were run on a 1 % electrophoresis gel to confirm amplification. Amplicons generated by PCR were sequenced with the Big Dye terminator cycle sequencing premix kit, with an initial denaturing step at 94 °C for 5 min, followed by 25 cycles at 94 °C for 10 s, 55 °C for 10 s, and 60 °C for 4 min. Sequences were mostly done in one direction and only when confirmation was needed, in both directions. An ABI PRISM 310 genetic analyser was used to determine the sequences. Resulting sequences were checked against the NCBI database using the BLAST option and aligned with closely related ex-type sequences. Alignments were done in MAFFT v7.221 using the L-INS-I option. The software package MEGA6 was used to construct neighbour joining trees with bootstrap analysis of a 1000 replicates.

Table 2.1: Primers and their annealing temperatures used for amplification and sequencing.

Locus	Primer name	Annealing temperature (°C)	Primer sequence (5' – 3')	Reference
Internal transcribed standard (ITS)	ITS 1	55 (*alt.52)	TCC GTA GGT GAA CCT GCG G	White et al., 1990
	ITS 4		TCC TCC GCT TAT TGA TAT GC	White et al., 1990
B-tubulin	Bt_2a	55	GGT AAC CAA ATC GGT GCT GCT TTC	Glass and Donaldson, 1995
	Bt_2b		ACC CTC AGT GTA GTG ACC CTT GGC	Glass and Donaldson, 1995
Calmodulin	CMD5	55 (*alt.58)	CCG AGT ACA AGG ARG CCT TC	Hong et al., 2006
	CMD6		CCG ATR GAG GTC ATR ACG TGG	Hong et al., 2006
	CF1	52	GCC GAC TCT TTG ACY GAR GAR	Peterson, 2005
	CF2		TTT YTG CAT CAT RAG YTG GAC	Peterson, 2005
Translation elongation factor 1α	EF1	55	ATG GGT AAG GAR GAC AAG AC	O'Donnell et al., 1998
	EF2		GGA RGT ACC AGT SAT CAT GTT	O'Donnell et al., 1998

* alt.: alternative temperature

2.3.5 Mycotoxin analyses

Feed samples were ground with a blender (400 W blender, Philips, South Africa) for 5 min. Five (5.00) g (±0.02 g) of each sample was transferred into 50 mL centrifuge tubes, and 70 % of analytical grade methanol extraction buffer was added (4 mL:1 g). Samples were placed at an angle (approximately 45 °) in a shaking incubator, incubating for 30 min at 25 °C at 200 rpm. After incubation, tubes were centrifuged for 10 min at 4 °C at 500 rpm. In total 2 mL of the supernatant was filtered into a 2 mL microcentrifuge tube through a Minisart 0.02 µm (regenerated cellulose) RC syringe filter. After overnight incubation at 4 °C, samples were centrifuged for 10 min at 14 000 rpm. The supernatant (1800 µL) was transferred to a 2 mL screw neck amber glass vial and submitted to

the Central Analytical Facility (CAF), Stellenbosch University for toxin quantification using liquid chromatography tandem mass spectrometry (LC-MS/MS). Extractions were done in duplicate. The following standard dilution series of mycotoxins with known concentrations were prepared; (1) Aflatoxins (AFB_1, AFG_1, AFB_2 and AFG_2), (2) fumonisins (FB_1, FB_2 and FB_3), (3) ochratoxin A and (OTA) (4) trichothecenes (DON, NIV, 3ADON, Fx) and zearalenone (ZEA).

2.4 Results

A total of 248 fungal isolates representing different genera, including *Fusarium, Penicillium, Aspergillus, Cladosporium, Rhizopus, Trichoderma* and *Mucor* were isolated in this study, of which 120 were obtained from abalone feed during the 2013 survey. The remaining isolates, include 65 from ingredients and 68 from finished feed. For this study the focus was only on potential mycotoxin producing genera, including *Fusarium, Penicillium* and *Aspergillus,* which represented 52.4 % of the total fungal isolates. Members of *Penicillium* and *Aspergillus* were found on all feed samples, sampled during the survey in 2013, but no *Penicillium* was isolated from the final feed samples, sampled at the supplier in 2014 (Table 2.2).

2.4.1 Fusarium

A total of 31 *Fusarium* isolates were obtained from abalone feeds and were identified based on morphology as *F. verticillioides, F. subglutinans, F. chlamydosporum* and *F. oxysporum* (Table 2.3, Appendix Figure A, G - J). Sequence analysis of the ITS region (data not shown) confirmed that all isolates belong to the genus *Fusarium.* A second and less conserved gene was amplified (partial translation elongation factor-1α) to differentiate between species (Figure 2.1), confirming the morphological identification.

Table 2.2: Number of fungal isolates isolated from artificial abalone feed samples.

		Fusarium	***Penicillium***	***Aspergillus***
Survey 2013	**Site 1**	7	1	1
	Site 2	2	16	4
	Site 3	0	5	5
	Site 4	2	8	2
Supplier Feed type 1 - 2014	**During processing**	1	3	8
	Before drying	1	2	3
	Final feed	1	0	5
Supplier Feed type 2 - 2014	**During processing**	1	4	6
	Before drying	0	0	2
	Final feed	0	0	5
Supplier 2014 Ingredients	**Starch 1**	10	3	4
	Starch 2	1	0	5
	Starch 3	5	1	6
	Protein source	0	0	0

2.4.2 Aspergillus

A total of 56 *Aspergillus* isolates were obtained. Morphological characterisation resulted in 8 morphological groups, which was supported by sequence analysis of the ITS region. However, although the use of the ITS region is seen as the official DNA barcode for fungi, in this study it lacked variation in the *Flavi* group, which made identification within this group impossible based only on one marker (Figure 2.2, Table 2.4, Appendix: Figure D - F). Therefore a secondary marker (calmodulin) was used for these isolates. Based on the combined results a total of 12 different species were identified with *A. effusus* the most dominant species followed by *A. flavus.*

2.4.3 Penicillium

Within the 43 *Penicillium* isolates, 8 morphological groups were identified (Appendix: Figure B and C), of which two groups were dominant. Sequence analysis of the ITS (data not shown) was not enough to identify all isolates to species level, and a secondary marker was sequenced. Better species

identification was obtained by aligning the beta-tubulin gene sequence (Figure 2.3, Table 2.5). *Penicillium crustosum* was the most dominant species (15 out of 43 isolates) and 10 isolates were grouped into the *P. chrysogenum* species complex. The other 18 isolates included species such as *P. polonicum, P. aethiopicum, P. melaconidium, P. griseofulvum, P. novae-zeelandiae* and *P. corylophilum*.

2.4.4 Mycotoxin analyses

Screening were done for the presence of 13 mycotoxins in all the feed and starch samples, and only 3 were detected in some of the samples. Fumonisin B_1 (FB_1) was the most dominant toxin detected followed by OTA and FB_2. Table 2.6 shows the average concentration of mycotoxins detected in the samples of the 2014 season. All the starch samples showed positive results for one or more mycotoxins which is in contrast to the protein source, where none of the mycotoxins were detected.

Table 2.3: Morphological characteristics of *Fusarium* isolates.

	Macromorphology (On PDA)			Micromorphology (On CLA)			
	Colony colour	**Sporodachia**	**Hyphal growth**	**Sporodachia**	**Microconidia**	**Macroconidia**	**Chlamydospores**
F. verticillioides	White mycelium with violet pigment when colony aged	Absent	Aerial mycelium	Present, light tan colour	Oval, no septate, long chains, monophialides	3-4 septa, elongate, slightly curved apical cell: curved, basal cell: foot shaped	Absent
F. subglutinans	White mycelium	Absent	Aerial mycelium	Present, light tan colour	Oval, no septate, false heads	3 septa, elongate, thin needlelike, apical cell: curved, basal cell: barely notched	Absent
F. chlamydosporum	White mycelium with dark red to burgundy pigment	Absent	Abundant aerial mycelium	Absent	Oval and obovoid, no septate, in pairs or single from phialidic opening, polyphialides	3- 5 septa, dorsiventral curvature, apical cell: curved, basal cell: notched	Smooth walled, intercalary or terminal, in pairs
F. oxysporum	White to light violet mycelium	Absent	Sparse growth	Absent	Oval, no septate, false heads, monophialides	3 septate, elongate, slightly curved apical cell: tapered basal cell: foot shaped	Smooth walled, intercalary, in pairs or short chains

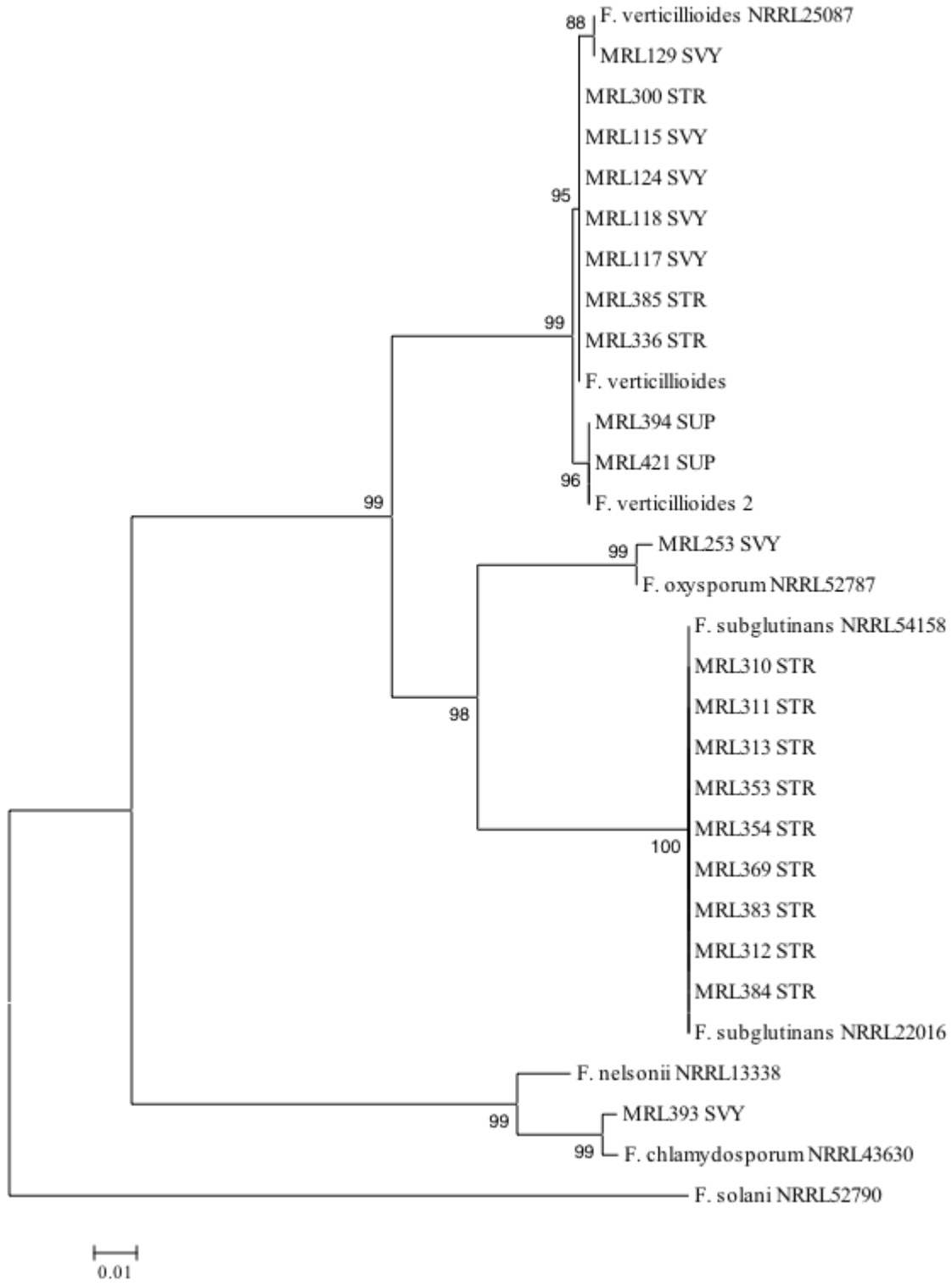

Figure 2.1: Phylogeny of *Fusarium* species isolates based on the analysis translation elongation factor sequences. Numbers within the tree represent the bootstrap values of 1 000 replicates. Isolates isolated from the survey (SVY), isolates from the supplier (SUP) and isolates isolated from the starch samples (STR).

Table 2.4: Limited morphological characteristics of *Aspergillus* spp. on malt extract agar (MEA).

	Macromorphology					Micromorphology			
	Growth rate (mm)/week	**Conidial colour**	**Mycelial colour**	**Exudate**	**Soluble pigments**	**Seriation**	**Vesicle shape**	**Stipe walls**	**Conidia**
A. flavus	65	Green	White	Clear	Absent	Uniseriate	Pyriform	Smooth	Elipsoidal, smooth walls
A. oryzae	65	Dark green	White	Clear	Absent	Uniseriate	Spherical	Finely roughened	Spherical, smooth walls
A. effusus	55	White	Green	Clear	Absent	Uniseriate	Spherical to pyriform	Finely roughened	Ellipsoidal, smooth walls
A. sydowii	24	Blue-green	White	Yellow-brown	Absent	Biseriate	Pyriform	Finely roughened	Ovoid, finely roughened walls
A. tamarii	65	Yellow-green	White	Absent	Absent	Biseriate	Spherical	Finely roughened	Ellipsoidal, rough walls with spikes
A. bridgeri	50	White	White	Absent	Absent	Biseriate	Spherical	Finely roughened	Ellipsoidal, smooth walls
A. niger	55	Dark brown to black	Brown	Clear	Absent	Biseriate	Spherical	Smooth	Spherical, rough walls with spikes
A. tritici	25	White	White	Absent	Absent	Biseriate	Spherical	Finely roughened	Spherical, smooth walls
A. clavatus	42	Blue-green	White	Clear	Absent	Uniseriate	Spathulate	Smooth	Ellipsoidal, smooth walls
A. tennesseensis	20	Dark green	Beige	Absent	Absent	Uniseriate	Pyriform	Smooth	Ellipsoidal, rough walls with spikes
A. amoenus	25	Greyish green	White	Clear	Absent	Biseriate	Pyriform	Smooth	Ellipsoidal, finely roughened
A. cristatum	20	Yellow	White	Absent	Absent	Uniseriate	Pyriform	Finely roughened	Ovoid, finely roughened

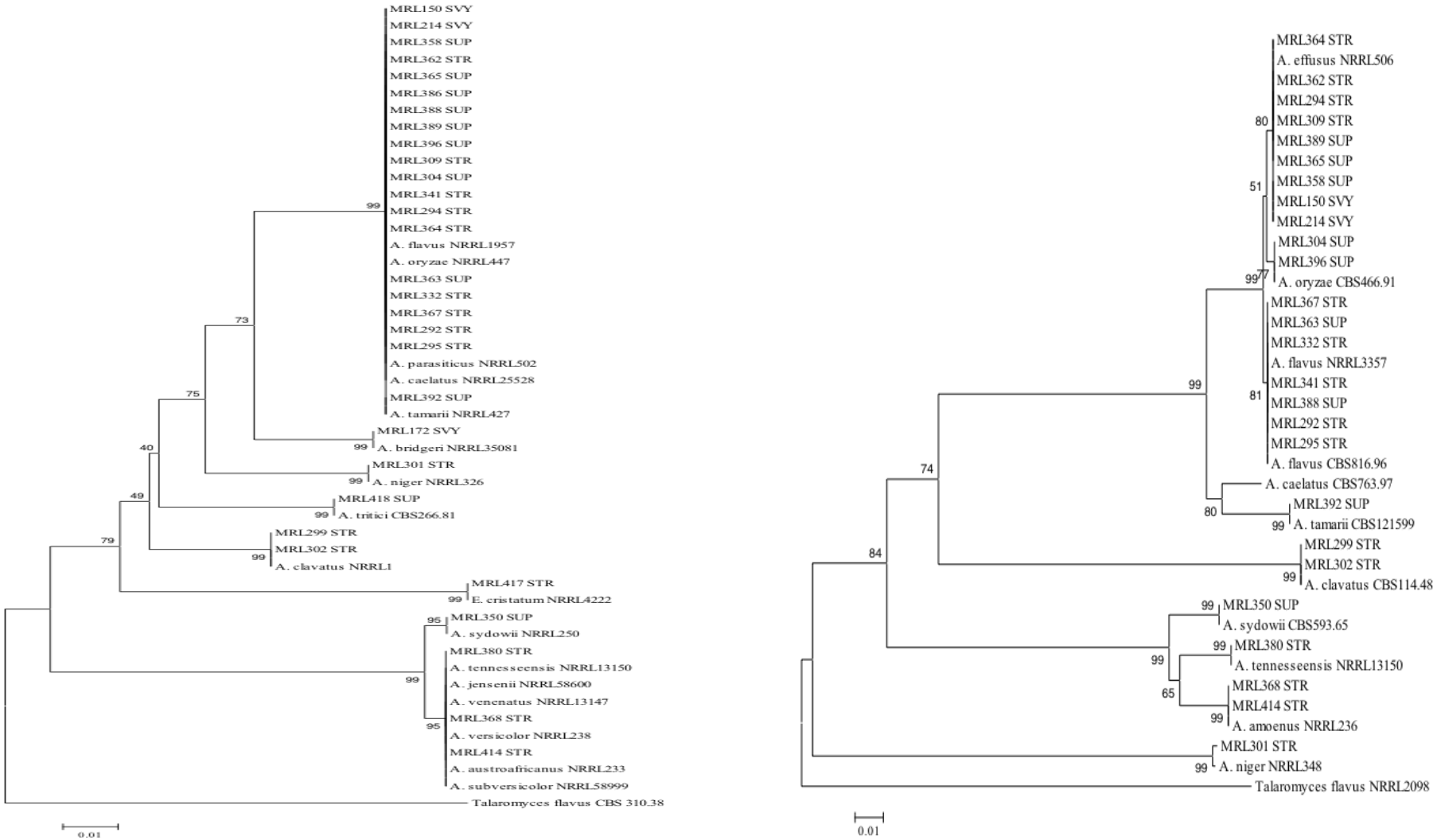

Figure 2.2: Phylogeny of *Aspergillus* species isolates based on the analysis of two gene locus's namely a) internal transcribed spacer and b) partial calmodulin sequences. Numbers within the tree represent the bootstrap values of 1 000 replicates. Isolates isolated from the survey (SVY), isolates from the supplier (SUP) and isolates isolated from the starch samples (STR).

Table 2.5: Limited morphological characteristics of *Penicillium* spp. on malt extract agar (MEA).

	Macromorphology				**Micromorphology**				
	Growth rate (mm)/week	**Texture**	**Colony colour**	**Exudate**	**Conidiophore branching**	**Stipe walls**	**Metula**	**Phialides**	**Conidia**
P. crustosum	45	Velutinous	White	Absent	Biverticillate	Rough	Appressed	Ampulliform	Ellipsoidal, smooth walled
P. chrysogenum	43	Fluccose	White	Absent	Biverticillate	Rough	Divergent and appressed	Ampulliform	Subspheriod, smooth walled
P. polonicum	35	Velutinous	Blue-green	Absent	Biverticillate	Smooth	Appressed	Ampulliform	Subspheriod, smooth walled
P. aethiopicum	30	Velutinous	Dark olive green	Brown	Biverticillate	Smooth	Divergent	Ampulliform	Subspheriod, smooth walled
P. melaconidium	25	Fasciculate	White	Absent	Terverticillate	Smooth and finely rough	Appressed	Ampulliform	Ellipsoidal, smooth walls
P. griseofulvum	27	Velutinous	Light olive green to grey	Absent	Terverticillate, often more complex	Smooth	Divergent	Ampulliform	Ellipsoidal, smooth walls
P. novae-zeelandiae	35	Floccose	Dark olive green	Absent	Terverticillate	Rough	Divergent and appressed	Ampulliform	Spheriod and ellipsoidal, smooth walls
P. corylophilum	25	Velutinous	Olive green	Absent	Biverticillate	Smooth	Divergent	Ampulliform	Spheriod, smooth walls

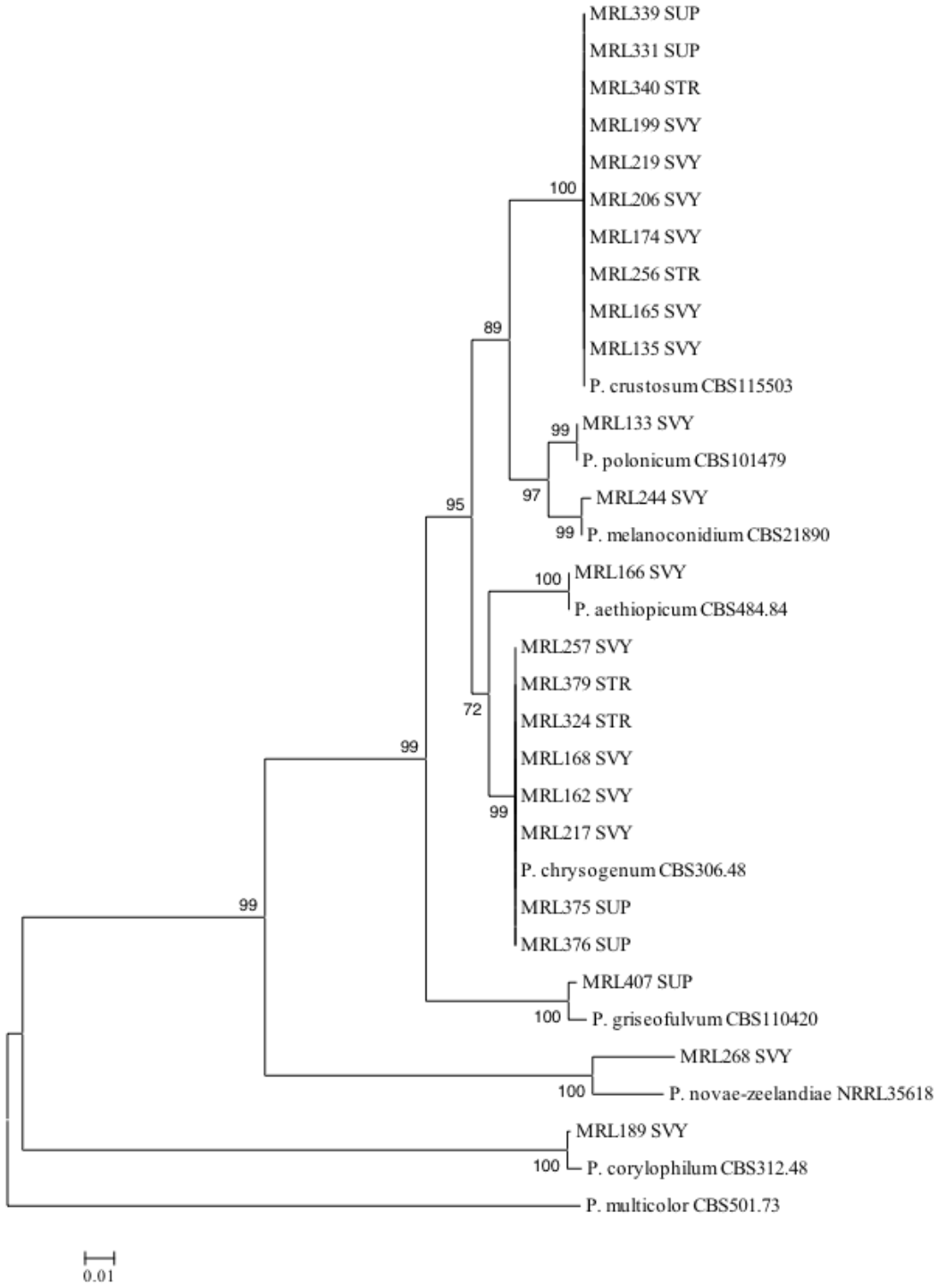

Figure 2.3: Phylogeny of *Penicillium* species isolates based on the analysis of beta-tubulin sequences. Numbers within the tree represent the bootstrap values of 1 000 replicates. Isolates isolated from the survey (SVY), isolates from the supplier (SUP) and isolates isolated from the starch samples (STR).

Table 2.6: Average mycotoxin concentrations (ppm) detected in the ingredients and feed samples from 2014.

	FB_1	**FB_2**	**Ochratoxin A**
Starch 1	0.01844	0.00294	ND
Starch 2	0.01829	ND	ND
Starch 3	0.01787	0.00222	0.00040
Feed type 1	0.01788	ND	0.00017
Feed type 2	0.01771	ND	0.00025

FB_1 or FB2 = Fumonisin B_1 B_2, OTA = Ochratoxin A.
ND = No mycotoxins were detected in these samples.

2.5 Discussion

Local grains (starch), fishmeal, soya bean meal, vitamins and minerals are the main ingredients of artificial abalone feed in South Africa (Troell et al., 2006). Fungal growth and production of secondary metabolites are likely to happen on any grain based artificial feed (Afsah-Hejri et al., 2013; Barbosa et al., 2013; Bragulat et al., 2008; Diaz et al., 2013; Glenn, 2007; Hooft et al., 2011; Labuda and Tancinova, 2006; Pleadin et al., 2012). The fungal presence on abalone feed in this study was therefore not unexpected. Results from this study showed that mycotoxigenic fungi were present in all abalone feed samples collected in 2013, representing all three of the prominent toxigenic genera (*Fusarium*, *Aspergillus* and *Penicillium*) reported from literature. In 2014, *Aspergillus* species were isolated from both feed types from the supplier, while *Fusarium* was isolated from only one feed type. This difference could be attributed to many factors including the quality of the ingredients that were used in 2013 (not tested in this study), and storage conditions. Ingredients were tested in 2014 and a similar cohort of fungal species were found in the starch samples and the final feed products. Therefore, it is possible that that these fungi were introduced to the raw grain products even before the processing stage of the final feed products. Hyphae and spores attach to grains and in most cases cannot be fully removed during processing. The use of contaminated starches can result in fungal contamination of final feed. Although *Penicillium* species were absent in one of the starches tested in this study, all three the dominant genera, namely *Fusarium*, *Penicillium* and *Aspergillus* were present in the remaining two starches (Table 2.2). However, a slight downward trend was observed

in the number of *Fusarium* isolates when comparing the number of isolates in the starches to the final feed products. These findings are similar to a study done on the ingredients used in shrimp feed (Anukul et al., 2014), where three *Fusarium* spp. were isolated from feed. Two species namely *F. oxysporum* and *F. verticillioides,* were common to both studies. *F. oxysporum* and *F. verticillioides* are both plant pathogens (Gordon and Martyn, 1997; Jimenez et al., 1993; Leslie and Summerell, 2006; O'Donnell et al., 1998; Summerell et al., 2003). *Fusarium verticillioides* were present in most of the samples, including samples from the supplier, and the starches. This indicates that *F. verticillioides* likely originated from the raw starch products. Data from this study further suggests that the manufacturing process does not affect the viability of the fungal material, resulting in fungal growth that could take place if moisture and temperature is not controlled. This may be considered a risk factor where fishmeal is replaced with vegetable proteins in aquaculture feeds in order to reduce the cost (Amaya et al., 2006). In this study the protein source tested was free of viable fungi, while the starches, which are plant based, were all contaminated with fungi. Therefore, plant based protein could increase potential fungal contamination in the end product. Furthermore, when low quality agricultural materials that are not fit to use for human consumption are used, there is a higher risk of fungal contamination. *Fusarium* infection already takes place in the field before or during harvesting especially when late harvesting occurs (Logrieco et al., 2002; Nelson et al., 1992), while *Aspergillus* infection tends to take place before and after harvesting. *Aspergillus flavus* can infect crops both before and after harvesting as well as during storage (Diaz et al., 2013). Regardless of when infection took place, mycotoxin production normally occurs as soon as environmental conditions are optimal. Caution should be taken when sourcing grains used for especially abalone feeds, as the effect of mycotoxins on these animals are not yet known and needs further investigation.

Mycotoxins have been causing health related problems in many terrestrial animals and is a major concern in aquaculture (Doster et al., 1974; El-Sayed and Khalil, 2009; Encarnação, 2011; Goel et al., 1994; Hooft et al., 2011; Lee et al., 1968; Manning, 2005; Pietsch et al., 2013; Schoenhard et al.,

1981; Tuan et al., 2003; Wiseman et al., 1982; Yildirim et al., 2000). *Fusarium verticillioides* can produce toxic secondary metabolites namely, fumonisins (Gelderblom et al., 1988; Nelson et al., 1992; Rheeder et al., 1992; Shephard et al., 2007; Sweeney and Dobson, 1998). The presence of FB_1 in the starches is thus an indication that the agricultural commodities used as ingredients were not only infected with *F. verticillioides* at some stage, but conditions also favored mycotoxin production. The presence of fumonisins in abalone feed is definitely not unique as contaminated crops are also used in other grain based feed and food products (Bennett and Richard, 1996). This is a concern because of the severe toxigenic effects of fumonisins associated with animals and humans (Marasas, 2001; Marasas et al., 1980; Rheeder et al., 1992; Schumacher et al., 1995). Many studies have shown that specifically FB_1 interferes in metabolic pathways, and has a detrimental effect on the health and growth of several fish species (Barbosa et al., 2013; Tuan et al., 2003; Wang et al., 1991; Yildirim et al., 2000). The highest fumonisin levels detected in this study was in starch 1, which was 0.0184 ppm. This is substantially lower than the recommended allowance for ingredients used in catfish feed according to the FDA, which is 20 ppm of total fumonisins in maize and maize by-products, provided that these contribute 50 % or less to the final feed, resulting in a final total fumonisin concentration of 10 ppm in the end product (FDA, 2001) (% starch composition in the feed used in this study was kept secret, by request from the supplier). In South Africa, according to the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act no 36 of 1947, republished in 2006, South Africa does not have maximum allowable mycotoxin limits for aquatic animal feeds or ingredients used. However, FB_1 and OTA levels measured is this study in feeds and ingredients were substantially lower than the maximum allowable limits for horse, pig, beef and poultry finished feeds. Maximum FB_1 limits for these feeds are 5 ppm for horse feed, 10 ppm for pig feed, and 50 ppm for beef and poultry feeds. No allowable limits are stated for FB_1 in ingredients. Maximum allowable OTA limits for ingredients used in full ratio in sows, pigs and piglet feeds are 0.05 ppm while the limit for poultry feeds are 0.2 ppm (National Department of Agriculture, 2006). To date, mycotoxin risk assessments to establish maximum allowable mycotoxin levels in abalone feed and ingredients have not yet been

conducted for the South African market.

Species in the genus, *Penicillium* are widespread and can produce a number of mycotoxins as secondary metabolites (Frisvad and Filtenborg, 1989; Kozlovsky et al., 2009; Lund and Frisvad, 2003; Samson et al., 2004). In this study, a wide variety of *Penicillium* species were isolated, with *P. crustosum* as the most common, followed by strains of the *P. chrysogenum* species complex. Both these groups produce volatile and non-volatile metabolites (Frisvad and Filtenborg, 1989; Houbraken et al., 2012; Larsen and Frisvad, 1995). However, they do not produce any of the mycotoxins tested during this study. The low levels of OTA detected in one of the starches and final feed samples, are therefore not because of the presence of *Penicillium* but could rather be attributed to *Aspergillus* contamination. *Aspergillus niger* and *A. ochraceus* are among many *Aspergillus* species able to produce OTA (Abarca et al., 2003; Palumbo et al., 2011; Samson et al., 2004). Both these species were present in the starches sampled in this study. Ochratoxin A in artificial feed is a major concern in aquatic animals because it increases fish mortalities but also contaminates edible tissue of fish and other animals that consume it, resulting in a compromise of the entire food chain (Manning, 2010). *Aspergillus niger* was isolated from only one of the starch samples. In addition, another 11 other *Aspergillus* species were isolated in this study with the majority of isolates from section *Flavi.* This corresponds with previous studies that *Aspergillus,* section *Flavi* is the most commonly found in artificial animal feeds (Alinezhad et al., 2011; Diaz et al., 2009; Keller et al., 2007). In this study, *Aspergillus* were found in the starches, as well as in the final feed products. This indicates that the viability of *Aspergillus* species is not affected during feed manufacturing. Since final feeds are already infected with *Aspergillus,* sporulation will take place when storage conditions favors fungal growth, resulting in a higher risk for mycotoxin production. Aflatoxins and ochratoxins are mostly produced as secondary metabolites when fungi grow on maize during dry conditions (Diener and Davis, 1969; Lund and Frisvad, 2003; Marin et al., 1998). However not all isolates of the same species are able to produce mycotoxins (D'Mello and Macdonald, 1997). A common aflatoxin

producing species is *A. flavus*, but isolates differ in their ability to produce the mycotoxin. Aflatoxin production on poultry feed varied from 1.60 % of the strains isolated from poultry feed and ingredients to 76.0 % of the strains isolated from animal feed in India (Astoreca et al., 2011; Dutta and Das, 2001; Labuda and Tancinova, 2006). Isolates from the latter study produced toxins ranging from 7.5 – 33.5 ppm (Dutta and Das, 2001). Another study reported that 75.0 % of *A. flavus* strains isolated from trout feed, produced aflatoxins on natural media (Cutuli et al., 1991). In the current study, no aflatoxins were found even in samples where *A. flavus* were present. More research is needed to determine why no aflatoxins were found but it can be speculated that, the isolates are not aflatoxin producers or environmental conditions influences aflatoxin production were not optimal. Mycotoxin production are influenced by a variety of factors, including temperature and time, substrate, humidity and moisture, plant damage and oxygen (Diener and Davis, 1969). D'Mello and Macdonald (1997) categorised these factors into three groups namely, physical, chemical and biological.

Aspergillus were the most dominant genus detected in all the starches and feed samples, but only one of the associated mycotoxins were detected, namely OTA. This could be due to biological factors, such as temperature and time. Growth temperature does not necessarily promote mycotoxin production (Diener and Davis, 1969). It is possible that *Aspergillus* infection took place during harvesting but environmental conditions during handling and storage never favoured aflatoxin production. Furthermore, the feed tested were sampled from the supplier, thus no time was allowed for fungal germination and colonisation on feed. Grains are normally ideal for *Aspergillus* to produce aflatoxins, therefore it is speculated that more ideal environmental conditions would have favoured mycotoxin production. Diener and Davis (1969) reported on various studies where aflatoxin production on grain products are high, and even takes place on soya beans and soya protein at lower concentrations.

It is important to note that the presence of species belonging to *Fusarium, Aspergillus* and *Penicillium* is not always related to mycotoxin contamination. In this study, in contrast to *Aspergillus, Fusarium* was the least detected, yet FB_1 was the most dominant mycotoxin. This indicates that the starches used in abalone feed favours fungal growth of the genus *Fusarium* and fumonisin production. Feed samples tested in this study were taken directly from the supplier, therefore it can be concluded that the FB_1 contamination in the final feed could be a result of the infected starches, and that the mycotoxin is not removed during the manufacturing process. Therefore, not just fungal isolation and identification should be studied in the agricultural materials to determine the microbiological control of abalone feed manufacturing, but quantification of mycotoxins in finished abalone feed should also be done to determine associated risks.

2.6 References

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3 MYCOTOXIN PRODUCTION BY *FUSARIUM, ASPERGILLUS* AND *PENICILLIUM* ON ABALONE FEED IN SOUTH AFRICA

3.1 Abstract

Mycotoxins are toxic secondary metabolites produced by various filamentous fungi, of which *Fusarium, Aspergillus* and *Penicillium* are the 3 main genera. Abalone feed consist of a combination of grains and protein. The raw grains in these feeds are susceptible to fungal infections, either from the field, or from post-harvest storage and processing conditions. In addition, abalone farms are mainly located along the coastline, where the climate can be humid, creating the optimum conditions for fungal growth, when feeds are stored before and during use. Mycotoxin production is dependent on temperature and available nutrients in the substrate. The ability to produce mycotoxins varies between isolates. In this study the aim was to determine whether fungi previously isolated from abalone feed and ingredients have the potential to produce mycotoxins when inoculated onto sterile abalone feed. A total of 93 fungal isolates were inoculated onto abalone feed, including representatives of *Fusarium, Aspergillus* and *Penicillium*, followed by a 6 week incubation period. Results showed that abalone feed is a good substrate for mycotoxin production, but results are very much strain dependent. Natural controls containing sterile water and non-sterile feed measured very low to no levels of mycotoxins, indicating that mycotoxin production can be influenced by the presence of other natural occurring fungi.

3.2 Introduction

Mycotoxins are low weight molecular secondary metabolites mainly produced in the mycelial structures of certain filamentous fungi, during fungal growth (Bhat et al., 2010; D'Mello and Macdonald, 1997; Leslieand Summerell, 2006; Placinta et al., 1999). These low molecular weight secondary metabolites are toxic to humans and animals when consumed and can even be

carcinogenic, neurotoxic, nephrotoxic or immunosuppressive especially when exposure occurs on a chronic basis (Gelderblom et al., 1988; Hussein and Brasel, 2001; Lazicka and Orzechowski, 2010; Rheeder et al., 1992). The most common way of exposure in humans and animals are through consumption of contaminated feedstuffs or foods (Bhat et al., 2010; Mwanza, 2011; Rheeder et al., 1992). Fungi known to produce mycotoxins are referred to as toxigenic fungi. This group is dominated by three genera namely *Fusarium*, *Penicillium* and *Aspergillus*, and to a lesser extent other genera that include *Alternaria*, *Claviceps*, and *Stachybotrys* (Bennett and Klich, 2003; Diaz et al., 2013). *Fusarium* species represent a number of plant pathogens, and are responsible for infection before and during harvesting, while *Penicillium* and *Aspergillus* more commonly colonize commodities and foods during drying and storage (Diaz et al., 2013; Miller, 2008; Placinta et al., 1999; Sweeney and Dobson, 1998).

Mycotoxins produced by *Fusarium* are widespread contaminants of animal feed, and have been shown to cause biological side effects even when present in low concentrations (Gelderblom et al., 1988; Marasas et al., 1980; Moss, 2002a; Rheeder et al., 1992; Tuan et al., 2003). Fumonisins, trichothecenes and zearalenone (ZEA) are the major mycotoxin groups associated with *Fusarium* spp. (Hussein and Brasel, 2001; Miller, 2008; Moss, 2002a; Sweeney and Dobson, 1998). Fumonisins are mainly produced by *F. verticillioides*. The fumonisin group consists of 4 series namely fumonisin B (FB_1, FB_2 and FB_4), A (FA_1 and FA_2), C (FC_1, FC_2, FC_3 and FC_4) and P (FP_1, FP_2 and FP_3) of which the B series is the most abundant naturally occurring group (Musser et al., 1996; Rheeder et al., 2002; Seo and Lee, 1999). Fumonisin B_1 is normally found in the highest concentrations and is associated with oesophageal cancer in humans (Hussein and Brasel, 2001; Manning, 2005; Rheeder et al., 1992; Sweeney and Dobson, 1998).

Another group of mycotoxins, the trichothecenes, include deoxynivalenol (DON), ZEA, fusarenon X (Fx), 15-acetyl-deoxynivalenol (15ADON) and nivalenol (NIV) (Bennett and Klich, 2003; D'Mello

et al., 1999). This chemically diverse group of mycotoxins are produced by a number of *Fusarium* species, but *F. graminearum* is commonly known to produce these toxins on natural products, such as grains (Beukes, 2015; Garcia et al., 2012; Logrieco et al., 2002; Marasas, 2001; Pitt, 2000). Optimal temperatures for fumonisins and trichothecene production is 25 ±1 °C (Hussein and Brasel, 2001; Moss, 2002a), this falls within the optimal growth temperature for *Fusarium* spp. Trichothecenes are known to be cytotoxic to mammalian cells and potent inhibitors of protein synthesis, causing animals to be predisposed to other diseases (Hussein and Brasel, 2001; Sweeney and Dobson, 1998). In aquaculture, consumption of fumonisin contaminated feed results in a drastic decrease in weight gain and significant elevation in the sphinganine:sphingosine ratios when fed to Channel catfish (*Ictalurus punctatus*) and Nile Tilapia (*Oreochromis niloticus*) (Lumlertdacha and Lovell, 1995; Tuan et al., 2003; Yildirim et al., 2000).

Aflatoxins were first discovered in 1959/1960, when the disease named 'turkey x disease' broke out and caused the death of 100 000 turkeys that were fed with groundnuts infected with *A. flavus* (Cole, 1986). Aflatoxins are made up of several toxic compounds namely B_1, B_2, G_1 and G_2, of which B_1 (AFB_1) is the most abundantly produced by *A. flavus* and *A. parasiticus.* The optimum growth rate of these species and the optimum temperature for aflatoxin production are almost identical, with 10 – 43 °C and 12 – 40 °C, respectively (Sweeney and Dobson, 1998). In humans and animals, aflatoxin B_1 (AFB_1) is one of the most toxic liver carcinogens (Hussein and Brasel, 2001; Moss, 2002b; Sweeney and Dobson, 1998) and hepatocellular carcinomas (HCC) is one of the most common diseases caused by aflatoxins in fish. The susceptibility to aflatoxins varies between species. While warmwater fish such as channel catfish are less susceptible, rainbow trout (*Salmo gairdneri*) are highly susceptible and will be affected by levels as low as 0.4 ppb AFB_1 (Lee et al., 1968; Manning, 2005; Santacroce et al., 2008; Schoenhard et al., 1981). In addition, behavioral changes were noticed and clinical signs, such as skin lesions, yellowing of the body surface, abnormal swimming, haemorrhages in the head and eye cataracts, were observed in sea bass (*Dicentrarchus labrax*), when

they were exposed to AFB_1 (El-Sayed and Khalil, 2009).

Ochratoxins are produced by some species of *Penicillium* and *Aspergillus*, with ochratoxin A (OTA) being the most common. Ochratoxin A is a strong nephrotoxin, teratogen and carcinogen that can be produced by *A. ochraceus* and certain *Penicillium* spp. Production of OTA by some *Penicillium* spp. such as *P. verrucosum* normally takes place at more temperate climates (4 – 31 °C), while production of OTA by *A. ochraceaus* takes place at higher temperatures up to 37 °C (Manning, 2010; Moss, 2002b). Not only does the temperatures differ between these two species for optimal OTA production, but they also have different substrate requirements. *Aspergillus ochraceus* produce optimally when grown on oil seeds such as peanuts and soyabeans while *P. verrucosum* produce OTA optimally when grown on cereal grains like maize, barley and wheat (Klich, 2007; Manning, 2010). However, they are both classified as storage fungi, which implies that they grow best under poor storage conditions, which increase the risk for OTA production. Ochratoxin A can cause a fatal human kidney disease, called Balkan endemic nephropathy (BEN), which is characterize by tubular degeneration and renal lesions (Pfohl-Leszkowicz and Manderville, 2007). Necrosis of kidney tubular and liver cells, along with a reduction in weight gain, are some of the effects OTA has on rainbow trout (Doster et al., 1974; Manning, 2005). Associated risks of mycotoxins in abalone is unknown.

Mycotoxin production can be influenced by several factors, which can be divided into three main groups, namely physical, chemical and biological factors. Physical factors include environmental conditions that influence fungal colonization and growth. This is mainly temperature, relative humidity and insect infestation. Fungicides and fertilizers form part of the chemical factors and biological factors refer to the interactions between toxigenic fungal species and the substrate (D'Mello and Macdonald, 1997). Nutritional factors such as carbohydrate and nitrogen sources,

and trace metals in the substrate all have an effect on mycotoxin production (Luchese and Harrigan, 1993; Placinta et al., 1999). Furthermore, mycotoxin production is influenced by strain specificity, strain variation and instability of toxigenic properties which may vary over time (D'Mello and Macdonald, 1997; Sweeney and Dobson, 1998). This leads to the aim of this study, which is to determine the ability of previously isolated and identified toxigenic fungal species (Chapter 2), to produce mycotoxins on abalone feed under storage conditions.

3.3 Materials and Methods

3.3.1 Sample preparation

Twenty one *Fusarium*, 36 *Aspergillus* and 36 *Penicillium* strains previously isolated from abalone feed, were inoculated onto PDA, MEA and CYA, respectively, from 15 % glycerol stocks that were kept at 4 °C. Cultures were incubated at 26 ±1 °C for 10 days. *Fusarium verticillioides* MRC0826 were included to serve as a positive control for fumonisin production (Rheeder et al., 2002). *Aspergillus flavus* MRC3951 (Thembo et al., 2010) was included as a positive control for the production of aflatoxins and *P. viridicatum* MRC0356 as an ochratoxin producing strain. Negative controls were set up containing sterile feed and water with no fungal inoculum, and natural controls containing sterile water and non-sterile feed, in which no inoculum was included.

Twenty-five (25) grams of abalone feed (Production date 24/04/2015; Batch #114/15) were weighed into 250 mL erlenmeyer flasks containing 30 mL MilliQ distilled water. Flasks were covered with cotton wool and tin foil, left overnight at room temperature and autoclaved the following day. Overnight incubation was done to allow the feed to absorb the water. Sterile flasks were aseptically inoculated with 4 agar plugs (2 mm) from one of the tester strains. Negative controls with no inoculant were included as well as natural controls. Natural controls refer to flasks where feed was added after autoclaving flasks with water, and no additional inoculations were done. This represented the feed left to rot naturally, resulting in a natural fungal community rather than a pure culture

colonizing the feed. Flasks were incubated at 26 ±1 °C for 6 weeks.

3.3.2 Mycotoxin analyses

Toxin extraction adapted from Szécsi et al. (2005) was used to extract the various mycotoxins. Extractions were done by adding 100 mL of 70 % analytical grade methanol extraction buffer (4 mL:1 g feed) into the flasks. Feed was crushed and stirred with a stainless steel spatula and then incubated on a shaker at 180 rpm, for 60 min at 25 °C. Following incubation, 15 mL of the slurry was filtered through a single layer cheesecloth into a 50 mL centrifuge tube. Tubes were centrifuged for 10 min at 4 °C at 500 rpm. Thereafter, 2 mL supernatant were filtered into a 2 mL microcentrifuge tube through a Minisart 0.02 µm (regenerated cellulose) RC syringe filter. After overnight incubation at 4 °C, samples were centrifuged for 10 min at 14000 rpm. After centrifugation, 1800 µL supernatant was transferred to a 2 mL screw neck amber glass vial and sent to the Central Analytical Facility (CAF), Stellenbosch University for toxin quantification using liquid chromatography tandem mass spectrometry LC-MS/MS. Care was taken when aflatoxins were extracted by working in the dark. Standard dilution series with known concentrations of the following toxins were prepared, namely fumonisins (FB_1, FB_2 and FB_3), trichothecenes (DON, NIV, 15ADON, Fx) ZEA, aflatoxins (AFB_1, AFG_1, AFB_2 and AFG_2), and OTA. See Table 3.1 for the range in which concentrations were measured.

Mycotoxins were detected in the sterile control samples (Table 3.2 and Table 3.3). These mycotoxins were not produced under laboratory conditions and were present in the feed when collected from the supplier. Values from analysis of the sterile control samples were considered as being the mycotoxin concentration of feed without any fungal growth. These values were deducted from the mycotoxin values measured in inoculated samples in order to give a true value of the mycotoxin concentration produced by a specific isolate. Provision was made for potential sample carry-over, by deducting

0.05 % (% specified by the autosampler) from values following after readings greater than 0.5 ppm. Sample carry-over is when the liquid of a previous sample elutes upon subsequent samples due to chemical/physical characteristics of the sample, analysis system or both. This takes place especially when very high concentrations of the measuring substance, in this case the mycotoxins are present in a single sample. As a result a subsequent sample is loaded with mycotoxin concentrations and does not reflect the true concentration of the specific sample.

T able 3.1: Mycotoxin measuring limits (ppm) for this study

Mycotoxins	**Lowest measured concentration**	**Highest measured concentration**
FB1	0.01008	201.6
FB2	0.0101	202
FB3	0.00104	20.8
DON	0.0064	100
NIV	0.0064	100
15ADON	0.0064	100
Fx	0.0064	100
ZEA	0.0128	200
AFB1	1.5333 x 10-5	3.8333 x 10-1
AFB2	4.4267 x 10-6	1.1066 x 10-1
AFG1	1.5333 x 10-5	3.8333 x 10-1
AFG2	4.4267 x 10-6	1.1066 x 10-1
OTA	0.016196	0.259146

FB_1 or B_2 = Fumonisin B_1 or B_2, DON = Deoxynivalenol, NIV = Nivalenol, 15ADON = 15-acetyl-deoxynivalenol, Fx = fusarenon X, ZEA = Zearalenone, AFB_1 or AFB_2 or AFG_1 or AFG_2 = Aflatoxin B_1 or B_2 or G_1 or G_2, OTA = Ochratoxin A.

3.4 Results

3.4.1 Fusarium

Fusarium verticillioides MRC826 along with 15 *F. verticillioides* isolates in this study produced FB_1, FB_2 and FB_3 when growing on abalone feed (Table 3.2). No trichothecenes (TCT) were detected for all isolates. Feed (sterile control) used in this experiment showed low natural fumonisin contamination with no TCT's present (Table 3.2). All *F. verticillioides* isolates produced all three groups of fumonisins, with isolate MRL124 producing the highest concentrations. Only some of the *F. subglutinans* isolates produced low levels of fumonisins. Single isolates of *F. chlamydosporum* and *F. oxysporum* produced low levels and no fumonisins, respectively.

Table 3.2: Measured fumonisin concentrations (ppm) produced by *Fusarium* spp. inoculated on abalone feed after 6 weeks of incubation at 26 ±1 °C.

Isolate #	**Species name**	**FB_1**	**FB_2**	**FB_3**	**Total Fumonisins**
MRL115	*F. verticillioides*	0.2961	0.0261	0.0130	0.3353
MRL117	*F. verticillioides*	4.4801	0.6350	1.1401	6.2552
MRL118	*F. verticillioides*	3.1640	0.3072	0.3073	3.7785
MRL124	*F. verticillioides*	8.8747	1.4463	2.1139	12.434
MRL129	*F. verticillioides*	3.9765	0.2536	0.6052	4.8353
MRL253	*F. oxysporum*	ND	ND	ND	ND
MRL300	*F. verticillioides*	1.3383	0.1806	0.1579	1.6768
MRL310	*F. subglutinans*	0.0050	0.0019	0.0013	0.0082
MRL311	*F. subglutinans*	0.0017	0.0013	0.0006	0.0036
MRL312	*F. subglutinans*	0.0070	0.0036	0.0037	0.0143
MRL313	*F. subglutinans*	ND	ND	ND	ND
MRL336	*F. verticillioides*	5.5711	0.9296	0.6713	7.1720
MRL353	*F. subglutinans*	0.0002	0.0003	ND	0.0005
MRL354	*F. subglutinans*	0.0005	0.0002	ND	0.0007
MRL369	*F. subglutinans*	ND	ND	ND	ND
MRL383	*F. subglutinans*	ND	ND	ND	ND
MRL384	*F. subglutinans*	ND	ND	ND	ND
MRL385	*F. verticillioides*	2.2452	0.3131	0.1504	2.7087
MRL393	*F. chlamydosporum*	0.0070	0.0015	0.0017	0.0102
MRL394	*F. verticillioides*	0.5731	0.0646	0.0388	0.6765
MRL421	*F. verticillioides*	0.6911	0.0560	0.0152	0.7623
MRC826(Positive control)	*F. verticillioides*	1.0344	0.1466	0.0130	1.194
Sterile control	-	0.0020	0.0005	0.0002	0.0027
Natural control	-	ND	ND	0.0003	0.0003

ND = No mycotoxins were detected in these samples.

3.4.2 Aspergillus

Only AFB_1 and AFB_2 (Table 3.3) were detected in these samples and no AFG_1 and AFG_2. One *A. oryzae* isolate produced more than 60 ppm AFB_1 and more than 20 ppm AFB_2, which was the highest aflatoxin levels detected in this study. *Aspergillus flavus* MRC3951 produced both AFB_1 and AFB_2 on the feed. Seven out of the nine *A. flavus* isolates from this study produced aflatoxins, ranging from 0.3 – 45.7 ppm AFB_1 and 0.02 – 8.8 ppm AFB_2. Other aflatoxin producing species included *A. effusus* and *A. tennesseensis.*

Table 3.3: Measured aflatoxin concentrations (ppm) produced by *Aspergillus* spp. inoculated on abalone feed after 6 weeks of incubation at 26 ±1 °C.

Isolate #	**Species name**	**AFB_1**	**AFB_2**	**Total Aflatoxins**
MRL172	*A. bridgeri*	ND	ND	ND
MRL292	*A. flavus*	ND	ND	ND
MRL295	*A. flavus*	4.2851	0.5763	4.8614
MRL299	*A. clavatus*	ND	ND	ND
MRL301	*A. niger*	ND	ND	ND
MRL302	*A. clavatus*	ND	ND	ND
MRL303	*A. clavatus*	ND	ND	ND
MRL304	*A. oryzae*	60.6007	29. 2181	89.8188
MRL309	*A. effusus*	0.4810	0.0126	0.4936
MRL332	*A. flavus*	45.6809	8.8260	54.5069
MRL341	*A. flavus*	ND	ND	ND
MRL342	*A. tamarri*	0.3362	0.0096	0.3458
MRL343	*A. tamarri*	1.1261	0.2043	1.3304
MRL344	*A. effusus*	ND	ND	ND
MRL358	*A. effusus*	ND	ND	ND
MRL359	*A. tamarri*	ND	ND	ND
MRL361	*A. tamarii*	ND	ND	ND
MRL362	*A. effusus*	ND	ND	ND
MRL363	*A. flavus*	34.1191	4.0938	38.2129
MRL364	*A. effusus*	ND	ND	ND
MRL365	*A. effusus*	ND	ND	ND
MRL366	*A. tamarii*	ND	ND	ND
MRL367	*A. flavus*	20.5714	2.9474	23.5188
MRL368	*A. amoenus*	ND	ND	ND
MRL380	*A. tennesseensis*	ND	ND	ND
MRL386	*A. flavus*	3.7456	0.1037	3.8493
MRL387	*A. flavus*	1.9922	0.0258	2.0180
MRL388	*A. flavus*	1.1052	ND	1.1052
MRL389	*A. effusus*	0.3780	0.0777	0.4557
MRL390	*A. effusus*	ND	ND	ND
MRL392	*A. tamarii*	ND	ND	ND
MRL396	*A. oryzae*	ND	ND	ND
MRL410	*A. effusus*	ND	ND	ND
MRL414	*A. amoenus*	ND	ND	ND
MRL416	*A. tennesseensis*	0.7873	0.1352	0.9225
MRL417	*A. cristatum*	ND	ND	ND
MRC3951(Positive control)	*A. flavus*	25.7582	7.9090	33.6672
Sterile control	-	0.3655	0.0534	0.4189
Natural control	-	ND	ND	ND

ND = No mycotoxins were detected in these samples.

3.4.3 Penicillium

Ochratoxin A (OTA) was not detected in any of the feed samples inoculated with *Penicillium* isolates in this study. Isolates used in this study included a number of species namely *P. crustosum, P. chrysogenum, P. polonicum, P. melanoconidium, P. aethiopicum, P. griseofulvum, P. novae-*

zeelandiae and *P. corylophilum.* No OTA were detected in either the sterile control or the natural control. *Penicillium viridicatum* MRC356 produced OTA as high as 536.4 ppm on abalone feed. However, this value was above the highest measuring concentration and could, therefore, be regarded as an inaccurate value, but is still regarded as positive.

3.5 Discussion

South African abalone farms are located along the coastline, where the climate is known to be humid, creating the optimum conditions for fungal growth, when artificial feeds are stored (Whitlow and Hagler Jr., 2005). Some fungal genera such as *Fusarium, Aspergillus* and *Penicillium* produce toxic secondary metabolites better known as mycotoxins (Bezuidenhout et al., 1988; Cabañes et al., 2010; Cole, 1986; Cutuli et al., 1991; Gelderblom et al., 1988; Rheeder et al., 1992; Wei and Jong, 1986). Although it is known that only certain species produce mycotoxins, it is strain dependent and is influenced by the growth substrate and environmental conditions (D'Mello and Macdonald, 1997). This study aimed to access the potential of fungal isolates previously isolated from abalone feed and ingredients to produce mycotoxins when re-inoculated onto abalone feed. In total 94 isolates were inoculated onto abalone feed to assess potential mycotoxin production at standardised temperatures over 6 weeks. Artificial abalone feeds are made up of a variation of grains together with protein, making it an ideal substrate, not only for fungal growth but also for mycotoxin production (Burgess et al., 1981; Cassini, 1981; Diener and Davis, 1969). This was indeed the findings for the positive control strains in this study. All three strains that were used as positive controls from each genus were able to produce their respective mycotoxins. This indicates that the incubation temperature and time period used during this study was sufficient for mycotoxin production across all three the genera tested. This is similar to conditions reported by Diener and Davis (1969), showing that 25 – 30 °C is the optimal temperature range for aflatoxin production across a variety of growth substrates (Ribeiro et al., 2006). Furthermore, 25 °C was also shown to be ideal for mycotoxin production by *Fusarium* species (Gelderblom et al., 1988; Rabie et al., 1982).

Fusarium verticillioides is a species well known to produce fumonisins on many different substrates (Beukes, 2015; Bezuidenhout et al., 1988; Bush et al., 2004; Falasconi et al., 2005; Gelderblom et al., 1988; Kriek and Marasas, 1977; Rheeder et al., 2002, 1992). All the *F. verticillioides* strains in this study produced mycotoxins, ranging from 0.33 ppm to 12.43 ppm. The strains with the highest levels were almost 10 times higher than the positive control. The isolate used as a positive control, *F. verticillioides* MRC826, produced surprisingly low levels of fumonisins on abalone feed, with a total fumonisin concentrations of only 1.19 ppm, of which 1.03 ppm were FB_1. This is contrary to a study by Gelderblom et al. (1988) who inoculated the same strain (*F. verticillioides* MRC826) onto maize and incubated at the same temperature for a shorter period of time. They were able to extract up to 2 g FB_1 from 1 kg culture material (2000 ppm). This is a clear indication that even though artificial abalone feed favours fumonisin production in this specific strain, it may not be the best substrate to promote fumonisin production specifically for *F. verticillioides* MRC826. However, the higher production levels of the other *F. verticillioides* isolates tested in this study indicate that artificial abalone feed is definitely a suitable substrate for fumonisin production, and that their capacity varies with strains. In this study, FB_1 was the most abundantly produced toxin. Artificial abalone feed and shrimp feed consist of similar ingredients, thus it would be expected that isolates from these two feeds would perform similarly in terms of mycotoxin productions (Anukul et al., 2014). However FB_1 production by isolates in this study ranged from equal to higher levels than FB_1 production levels that were reported to be produced by *F. verticillioides* strains isolated from shrimp feed (Anukul et al., 2014). Isolates from that study (Anukul et al., 2014) produced FB_1 ranging between 1600 – 3600 ng/g (1.6 ppm – 3.6 ppm). The difference in results could be because toxin production of isolates from Anukul et al. (2014) were tested on sterile maize and not artificial feed, which is therefore not a true reflection of the expected FB_1 production when growing on artificial feed. Furthermore, even though incubation temperatures for the two studies were similar, incubation time in the current study was 4 weeks longer, giving the fungal isolates more time to produce mycotoxins (D'Mello and Macdonald, 1997). During the current study, *F. graminearum* was not amongst the isolates

recovered, and because this species is responsible for the production of TCT's, this may be the reason for the absence of TCT's in all the samples (Beukes, 2015; Bily et al., 2004; Garcia et al., 2012; Miller, 2008; Szécsi et al., 2005).

Aspergillus flavus is well known to produce aflatoxins, but toxin production is known to be strain dependent (Bennett and Klich, 2003; D'Mello and Macdonald, 1997; Dutta and Das, 2001; Johnston et al., 2012; Klich, 2007; Manning, 2005; Moss, 2002b). Even though all the isolates in this study were treated the same with the same environmental conditions during incubation, two *A. flavus* isolates did not produce aflatoxins while the others produced varied concentrations of aflatoxins, with the highest being a total aflatoxin level of 54.5 ppm (MRL332) detected on abalone feed. This is relative low compared to previous studies where aflatoxin production ranged from less than 10 ppm to >100 ppm in sucrose based liquid growth medium (Geiser et al., 2000; Horn and Dorner, 1999). Even though *A. flavus* and *A. oryzae* have several homologues of aflatoxin biosynthesis pathway genes, genetic defects have led to the silencing of the aflatoxin pathway in *A. oryzae* (Takahashi et al., 2002; Watson et al., 1999). This has been supported by many studies (Barbesgaard et al., 1992; Bennett and Klich, 2003; Geiser et al., 2000; Klich, 2007; Wei and Jong, 1986). Blumenthal (2004) lists a few other toxic secondary metabolites produced by *A. oryzae,* but supports the theory that *A. oryzae* does not produce aflatoxins (Geiser et al., 2000). In contrast, two different studies found that *A. oryzae* originally isolated from wheat grains and meat products, respectively, produced aflatoxins when re-inoculated onto wheat and liquid media, respectively (Atalla et al., 2003; El-Kady et al., 1994). Despite of these findings the overall agreement still is that *A. oryzae* is non toxigenic, and that isolates could have been wrongly identified, such as *A. oryzae* NRRL1988 which was reported to produce aflatoxins but was later re-identified as *A. parasiticus* (Blumenthal, 2004; Fennell and Morse, 1976). This possibility is not unlikely as *A. oryzae* falls within the *A. flavi* clade, and is closely related to toxigenic species (Samson et al., 2014). Considering the effect that the growth substrate and other environmental conditions have on mycotoxin production (D'Mello and Macdonald, 1997), we

suggest that some *A. oryzae* isolates are toxigenic but that they are dependent on the substrate for mycotoxin formation. From this study, one of the two *A. oryzae* isolates tested produced both AFB_1 and AFB_2. Aflatoxin production by *A. oryzae* MRL304 was exceptionally high, the highest out of all the isolates tested. Thus more research on this specific isolate is needed to confirm identification and toxin production.

No OTA was produced by any of the *Penicillium* spp. isolates on abalone feed. Isolates used in this study were unable to produce OTA when colonising abalone feed, in contrast to the strain used as a positive control, *P. viridicatum* MRC356, which produced high levels of OTA. However, *P. viridicatum* MRC356 produced OTA concentrations exceeding the maximum level of detection. This is an indication that if toxigenic *Penicillium* species colonise on abalone feed, ochratoxins can be produced but because the species tested in this study did not produce OTA, they are either potentially non-toxigenic, or the conditions were not optimal for mycotoxin production.

In contrast to the *Penicillium* isolates from this study that appear to be non-toxigenic, results showed that some of the *Fusarium* and *Aspergillus* isolates are toxigenic and can produce potent mycotoxins when grown on abalone feed under certain conditions. Although *Aspergillus* spp. isolated from abalone feed were able to produce aflatoxins when re-inoculated onto abalone feed, no detectable levels of aflatoxins were measured in a previous study (Chapter 2) under natural occurrence in abalone feed and ingredients. This is an indication that aflatoxins could potentially be produced after harvesting of agricultural commodities, and during handling and storage of feed (Bennett, 2010; Dutta and Das, 2001; Klich, 2007). Therefore, when feed is kept fresh and stored correctly it lowers the risk of aflatoxin contamination. This is confirmed by the absence of detectable toxins in the natural controls. These samples were not inoculated with a pure culture but rather left to mould naturally, in order to reflect feed that could be left in storerooms to mould. With more species present and representing many different genera, interactions between species, along with competing for nutrients,

takes place. These two aspects are both part of the biological factors listed by D'Mello, J.P.F., Macdonald (1997) to influence mycotoxin production. More research is needed but it could be speculated that it is possible for these interactions to have a detoxifying effect on the feed, as mycotoxin concentrations measured on sterile feed were higher than on the natural controls. In conclusion, isolates previously isolated from abalone feed are toxigenic and abalone feed as a substrate favours mycotoxin production under sterile conditions. However, when a variety of naturally occurring fungal species colonize the feed it is more likely that none of the mycotoxins tested in this study will be produced, or may be produced at levels that are below detectable limits. Mycotoxin production in this study were shown to be isolate dependent, thus it is recommended that the industry make use of routine screening for possible toxigenic fungal contamination in parallel with mycotoxin contamination on feed.

3.6 References

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4 EFFECT OF SEAWATER ON ABALONE FEED INFECTED WITH *FUSARIUM VERTICILLIOIDES* AND RELATED MYCOTOXINS

4.1 Abstract

Fusarium verticillioides is a toxigenic fungus associated with contamination of a variety of feeds and cereals. Although environmental conditions play a role in mycotoxin production, fumonisins, especially the B series, are the most prevalent mycotoxins produced by *F. verticillioides.* Fumonisin production takes place on feed during long storage periods, and are influenced by temperature. In this study, the fumonisin production by *F. verticillioides* on abalone feed at two different temperatures were tested. Fumonisins are water soluble and even though they are stable in soil and grains, not much is known about their stability in artificial feed when exposed to water, like in aquaculture. Therefore, the second aim of this study was to determine whether fumonisins leach out of abalone feed into the water when exposed to water. Abalone feed inoculated with *F. verticillioides* was incubated for 10 weeks at two different temperatures. It was found that fumonisins were produced in higher concentrations at a lower temperature and that fumonisin production peaks after 5 weeks of incubation. The second aim was achieved by soaking abalone feed, which was incubated for 6 weeks in the presence of *F. verticillioides*, in autoclaved seawater for 24 hours. Fumonisins leached out of feed into the water and lowers the risk to abalone. Due to the high dilution factor, trace amounts of fumonisins get moved into the environment with water as a carrier. Although this is unlikely to pose a threat to the environment, it is advised that farms continue to monitor fumonisins in artificial feed and in cases where water is likely to be recycled.

4.2 Introduction

Although a number of *Fusarium* species are well known plant pathogens, many of them are saprophytic filamentous fungi with a wide distribution in the environment. It has previously been

isolated from a variety of foodstuffs including wheat, maize, nuts, bananas and feedstuffs (Alborch et al., 2012; Burgess et al., 1981; Cassini, 1981; Glenn, 2007; Jimenez et al., 1993; Logrieco et al., 2002; Marin et al., 1998; O'Donnell et al., 1998). The presence of *Fusarium* species in feedstuffs are problematic, not only because these they act as pathogens but also because some of these species produce toxic secondary metabolites (Alberts et al., 1990; Bezuidenhout et al., 1988; Hinojo et al., 2006; Rheeder et al., 2002, 1992; Vesonder and Hesseltine, 1981). These toxic secondary metabolites are referred to as mycotoxins.

Fusarium verticillioides along with other *Fusarium* species are able to produce mycotoxins under certain environmental conditions. Fumonisins, trichothecenes (TCT) and zearalenone (ZEA) are the three most important groups of mycotoxins produced by *Fusarium* species (Desjardins and Proctor, 2007). Although all three of these groups have proven to cause major health problems amongst animals (D'Mello et al., 1999; Gelderblom et al., 1988; Hooft et al., 2011; Kuiper-Goodman et al., 1987; Maaroufi et al., 1996; Manning, 2005; Marasas, 2001; Schoevers et al., 2012), this study focussed on the fumonisin group. This heat stable, water soluble group of mycotoxins consist of six different toxins, namely B_1, B_2, B_3, B_4, A_1 and A_2 of which the B-series is the most commonly found with fumonisin B_1 (FB_1) the most abundantly produced in nature (Alberts et al., 1990; Desjardins and Proctor, 2007; Hussein and Brasel, 2001; Moss, 2002; Sweeney and Dobson, 1998; Yildirim et al., 2000). Fumonisin B_1 and B_2 are associated with oesophageal cancer in humans and are also responsible for leukoencephalomalacia (ELEM) in horses, mules and donkeys, and are toxic and hepatocarcinogenic to rats (Gelderblom et al., 1988; Marasas et al., 1988; Rheeder et al., 1992). Fumonisins are not only a threat to humans and terrestrial animals, but have caused health problems in the aquaculture industry. Goel et al. (1994) reported an alteration of sphingolipid levels in channel catfish kidney, liver, muscle, and serum caused by fumonisin contaminated feed. Furthermore, a significant increase in the ratio between sphinganine and sphingosine was noticed in channel catfish and Nile tilapia, when fed contaminated feed (Manning, 2005; Tuan et al., 2003). Fumonisin B_1

inhibits the sphinganine N-acyltransferase, the enzyme which links the sphinganine to fatty acids in order to form ceramide. Inhibition of this step leads to tissue accumulation of sphinganine, which is the intermediate in the biosynthetic pathway of complex sphingolipids. Sphingolipids form part of cell membranes of cells in the brain and nervous system and regulate cellular growth (Soriano et al., 2005). Therefore, disruption in the sphingolipid biosynthesis in fish can lead to cellular deregulation and cell death (Goel et al., 1994). A decrease in weight gain was reported in channel catfish and Nile tilapia when feed contaminated with fumonisins at concentrations as low as 10 mg/kg feed, was consumed (Manning, 2005; Tuan et al., 2003; Yildirim et al., 2000). Reduction in weight gain of any cultured animals have a negative impact on farm production. Other mycotoxins such as aflatoxins (secondary metabolites of *Aspergillus* species) accumulate in the edible tissue and ovaries of sea bass (*Dicentrarchus labrax* L.) and Gibel carp (*Carassius auratus gibelio*), but it is currently not known whether fumonisins accumulate in any aquatic animal tissue (El-Sayed and Khalil, 2009; Han et al., 2010). Fumonisins are, however, water soluble (Alberts et al., 1990), and it is possible that when aquatic animals are fed under normal conditions they will consume very little to no fumonisins because of potential leaching of the mycotoxins into the water. This is most likely possible when feed is not consumed immediately, but left in the water for the animals to eat on demand.

In South Africa, cultured abalone are primarily grown-out in large flow-through concrete raceway systems and to a lesser extent in recirculation systems. Abalone are housed in baskets that are pre-moulded or made from oyster mesh, in tanks with an average capacity of 2000 L (Mouton, 2010). The feeding ratio for these cultured abalone is ±1 g feed/952.38 mL. However, feed is not always consumed immediately and can stay in the water for up to 32 hours, before it is consumed or flushed by the water system. Most farms have their water completely replaced every two hours, which can potentially remove contaminants from the environment ([1]Personal communication).

[1] Personal communication with anonymous abalone farm

Fumonisin production, just like any other mycotoxins, is influenced by a variety of factors. D'Mello and Macdonald (1997), categorised these factors into three groups, namely physical, chemical and biological. Physical factors refer to the environmental conditions under which fungal colonisation takes place. These environmental factors include temperature, relative humidity and insect infestation. Chemical factors refer to the influence of fungicides and/or fertilizers used while crops are grown. While biological factors are the interactions between the toxigenic fungal species and the substrate, as well as strain specificity and strain variation. It is important to note that mycotoxin production can also happen before harvesting and/or after harvesting during storage (Cassini, 1981; Diaz et al., 2013; Rheeder et al., 1992). Physical factors have the largest influence on mycotoxin production during storage after harvesting. Even though environmental conditions during storage are optimal for fungal growth, it is possible that mycotoxin production will not take place (Alberts et al., 1990). Growth temperatures are normally broader than the temperature range where mycotoxins are produced (Moss, 2002; Sweeney and Dobson, 1998).

Based on a previous study, (Chapter 3) it was determined that mycotoxin levels in abalone feed are low. However, some fungal isolates that were isolated from abalone feed were able to produce mycotoxins when pure cultures were re-inoculated onto sterile abalone feed. This is a concern as abalone farms in South Africa are located along the coast where humidity is high and temperature averages are 16 °C during winter months and 26 °C during summer months. These temperatures fall well within the temperature range for both fungal growth and fumonisin production (Alberts et al., 1990; Booth, 1971; Garcia et al., 2012; Nelson et al., 1994). Therefore, the initial aim of this study was to determine the impact of two different average temperatures (representing winter and summer temperatures in South Africa along the coastline) on fumonisin production by *F. verticillioides*, previously isolated from abalone feed. Secondly, to determine whether or not fumonisins leach from abalone feed when exposed to seawater. This is important in order to determine the fumonisin concentration in feed at the time of consumption. Understanding the changes in mycotoxin levels

when feed contaminated with fumonisins is exposed to water can assist in future risk assessments to determine allowable limits in artificial feed intended for aquatic animals.

4.3 Materials and Methods

4.3.1 Fungal isolates

Three different isolates (MRL117, MRL124, MRL336), all identified as *Fusarium verticillioides* (Chapter 2), were used in this study. The selected isolates produced the highest amounts of fumonisins on abalone feed as growth substrate (Chapter 2). Cultures were stored in 15 % glycerol at 4 °C. Cultures were first inoculated on water agar to confirm viability. Throughout the experiment cultures were kept on water agar. Seven days before inoculation, cultures were transferred onto Potato dextrose agar (PDA) and incubated at 26 °C.

4.3.2 Inoculation and incubation conditions

Two sets of Erlenmeyer flasks were prepared in order to achieve the two objectives of this study.

i) Determining the impact of two different average temperatures on fumonisin production by *F. verticillioides*, previously isolated from abalone feed. Erlenmeyer flasks were prepared with 25 g ± 0.02 g abalone feed and 30 mL MilliQ water. Flasks were plugged with cotton wool and covered with tin foil, left to soak overnight at room temperature and autoclaved the following day. Sterile flasks were aseptically inoculated with 1 agar plug (2 mm) of each culture. Negative controls with no inoculant were included as well as natural controls. Natural controls refer to flasks where feed was added after autoclaving flasks with water, no additional inoculations were done. This represented the feed left to rot naturally, resulting in a natural fungal community rather than a pure culture colonising the feed. Preparation was done every second week over a period of 10 weeks and incubated at 2 different temperatures, 16 °C and 26 °C, respectively. Experiments were

done in triplicate.

ii) Determining whether fumonisins leach from abalone feed when exposed to seawater. A second set of Erlenmeyer flasks were prepared with 4.2 g ± 0.01 g abalone feed and 5 mL MilliQ water. Flasks were prepared as described in the previous experiment. Sterile flasks were aseptically inoculated with 1 agar plug (2 mm) of each culture. Negative and natural controls were included, as described above. All flasks were prepared at the same time and incubated for 6 weeks at 26 °C. Enough flasks were prepared to perform fumonisin extractions on feed before water exposure and after 24 hours of water exposure. Experiments were done in triplicate. After the incubation period flasks were randomly divided into 2 groups with 3 flasks each. One group was labelled as "before water exposure" and the second group was labelled as "water exposure". The same protocol was followed for both the control groups.

4.3.3 Water exposure

Sterile plastic containers were prepared with 4 L autoclaved seawater and plastic pipes (5 mm thick) to pump air through. Flasks labelled as "before water exposure" were set aside for fumonisin extractions and the contents of the flasks labelled as "water exposure" were added into the seawater directly from the Erlenmeyer flasks. Feed was left in the water for 24 hours. Water samples were taken before feed was added (T0). Thereafter, water samples were taken at 2 hours (T1), 8 hours (T2) and 24 hours (T3) after the feed were added into the water. Water samples were taken with a sterile syringe and filtered through a Minisart 0.02 µm (regenerated cellulose) RC syringe filter, into a 2 mL screw neck amber glass vial and sent to the Central Analytical Facility (CAF), Stellenbosch University for fumonisin quantification using liquid chromatography tandem mass spectrometry LC-MS/MS.

4.3.4 Fumonisin extractions

Fumonisin extractions were done according to an adapted method of Szécsi et al. (2005).

i) For the first set of samples, all extractions were performed at the end of the 10 week incubation period.

ii) Flasks labelled as "before water exposure" were extracted at once. The samples that were exposed to seawater for 24 hours was centrifuged at 800 rpm for 10 min, to get rid of the excess water prior to the extractions.

Extractions were done by adding 4 mL:1 g of 70 % analytical grade methanol extraction buffer into the flasks. Feed was crushed and stirred with a stainless steel spatula and then incubated on a shaker at 155 rpm for 60 min at 25 °C. Following incubation, 15 mL (samples used for different incubation temperatures) and 5 mL (samples used in water exposure), of the slurry was filtered through a single layer cheesecloth into a 50 mL centrifuge tube. Tubes were centrifuged for 10 min at 4 °C at 500 rpm. Thereafter, 2 mL supernatant were filtered into a 2 mL microcentrifuge tube through a Minisart 0.02 µm (regenerated cellulose) RC syringe filter. After overnight incubation at 4 °C, samples were centrifuged for 10 min at 14000 rpm. After centrifugation, 1800 µL supernatant were transferred to a 2 mL screw neck amber glass vial and sent to the Central Analytical Facility (CAF), Stellenbosch University for fumonisin quantification using liquid chromatography tandem mass spectrometry LC-MS/MS. A Standard dilution series with known concentrations were prepared for FB_1, FB_2 and FB_3.

4.3.5 Statistical analysis

STATISTICA 13 software was used to perform statistical analyses. To determine the effect of temperature on fumonisin production over 10 weeks, factorial analysis of variance (ANOVA), was performed. Tests for equal variance failed, due to the high amount of zero values. Logarithmic (Ln) transformations were used as follow to transform data closer to normality, Ln(x+1), where x is the fumonisin concentration measured. Bonferroni method was used for post hoc multiple comparisons between time intervals. Significance was assigned to p-values <0.05.

4.4 Results

4.4.1 Different temperature incubations

Over the 70 day incubation period fungal growth could be seen, at both incubation temperatures, on feed inoculated with *Fusarium verticillioides*, and natural controls, while no fungal growth took place on sterile control samples. No fumonisins were detected in the sterile controls. Although growth was visible on the natural controls (Figure 4.1 and 4.2) from as early as 9 days, no fumonisins were detected in any of these samples (Figure 4.3). Fumonisin levels stayed stable in both the sterile and natural controls over 70 days. In contrast, fumonisin production could be measured in samples inoculated with *F. verticillioides* from as early as 9 days. A significant increase in production of FB_1, FB_2 and FB_3 took place at 35 days for both the incubation temperatures (Figure 4.3). The highest total fumonisin levels were measured at 16 °C after 49 days (Table 4.1). The measured concentration was 11.7533 ppm while the highest levels at 26 °C were 4.3225 ppm measured after 35 days (Table 4.1). Overall, fumonisin production was higher at 16 °C than at 26 °C. Fumonisin B_1 was the most abundantly produced fumonisin at both temperatures by the *Fusarium* culture.

4.4.2 Water exposure

Results showed a significant increase in total fumonisins measured in the water at the first measurement taken after contaminated feed were placed in the water. While a significant decrease in fumonisin concentrations in feed were measured after feed was soaked in seawater for 24 hours no fumonisins were detected in the feed that was soaked in seawater for 24 hours (Figure 4.4).

Figure 4.1: Fungal growth on abalone feed incubated at 16 °C, for 10 weeks. From left to right incubation time after 9 days, 4 weeks, 6 weeks, 8 weeks and 10 weeks. From top to bottom, top row is the samples inoculated with *Fusarium verticillioides* culture, middle row are sterile control samples followed by the natural control samples in the bottom row.

Figure 4.2: Fungal growth on abalone feed incubated at 26 °C, for 10 weeks. From left to right incubation time after 9 days, 4 weeks, 6 weeks, 8 weeks and 10 weeks. From top to bottom, top row is the samples inoculated with *Fusarium verticillioides* culture, middle row are sterile control samples followed by the natural control samples in the bottom row.

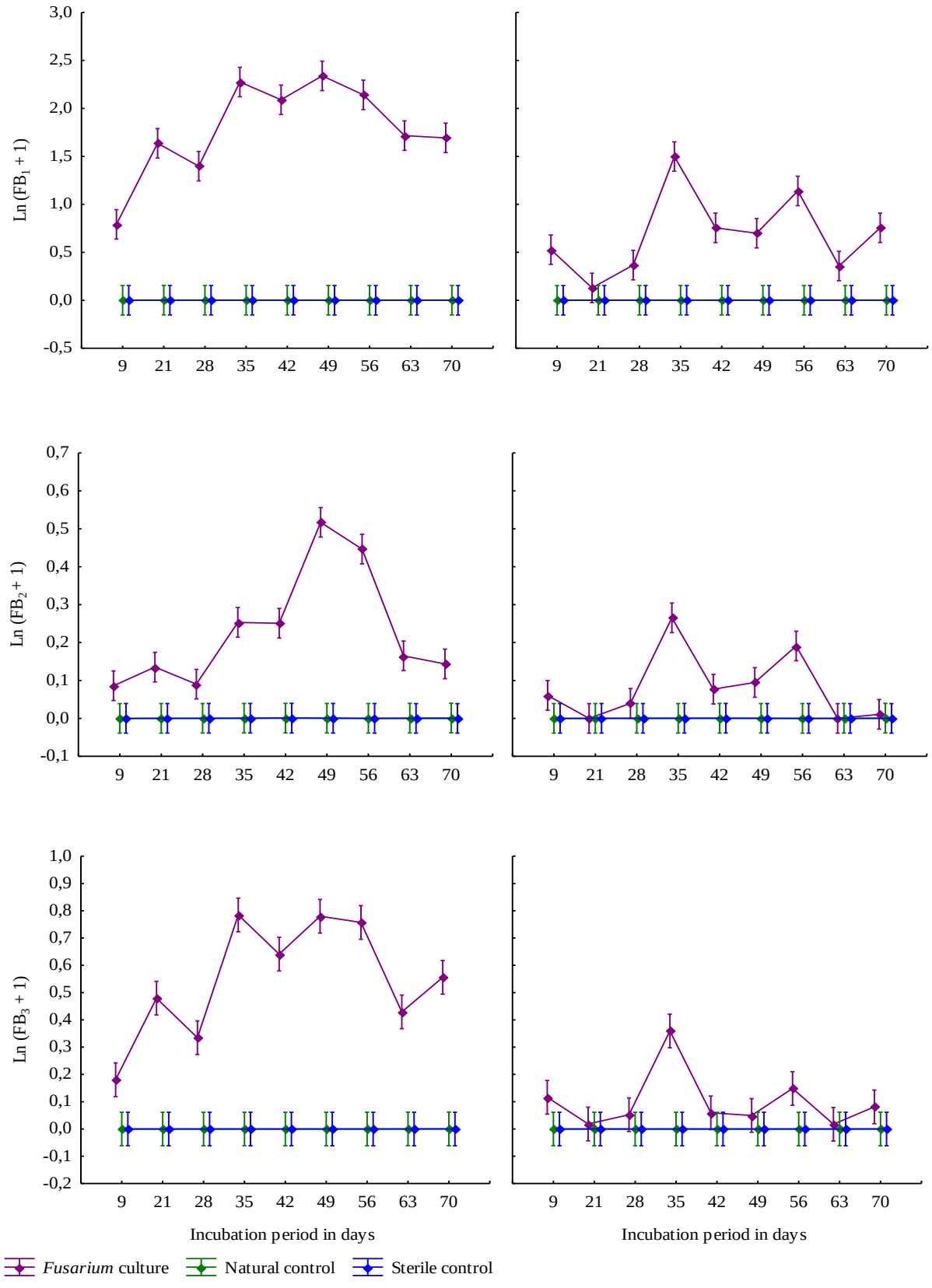

Figure 4.3: Fumonisin production on abalone feed over 10 weeks at 16 °C (left) and 26 °C (right), top to bottom FB_1, FB_2, and FB_3.

Table 4.1: Fumonisin production on abalone feed, by *Fusarium verticillioides*, at 16 °C and 26 °C, over a 70 day incubation period.

Incubation period (days)	FB_1 (ppm)		FB_2 (ppm)		FB_3 (ppm)		Total Fumonisins (ppm)	
	16 °C	26 °C	16 °C	26 °C	16 °C	26 °C	16 °C	26 °C
9	1.2298	0.7708	0.0901	0.0632	0.1983	0.1258	1.5182	0.9598
21	4.1431	0.1386	0.1458	0.0000	0.6185	0.0187	4.9074	0.1573
28	3.0812	0.4684	0.0947	0.0408	0.3994	0.0541	3.5753	0.5633
35	8.8548	3.5762	0.2886	0.3064	1.1970	0.4399	10.3404	4.3225
42	7.2194	1.1939	0.2862	0.0802	0.9019	0.0613	8.4075	1.3354
49	9.8630	1.0196	0.6909	0.0996	1.1994	0.0509	11.7533	1.1701
56	7.7537	2.2176	0.5677	0.2117	1.1606	0.1617	9.4820	2.5910
63	4.6020	0.4415	0.1816	0.0000	0.5444	0.0176	5.3280	0.4591
70	4.5805	1.1579	0.1556	0.0108	0.7579	0.0850	5.4940	1.2537

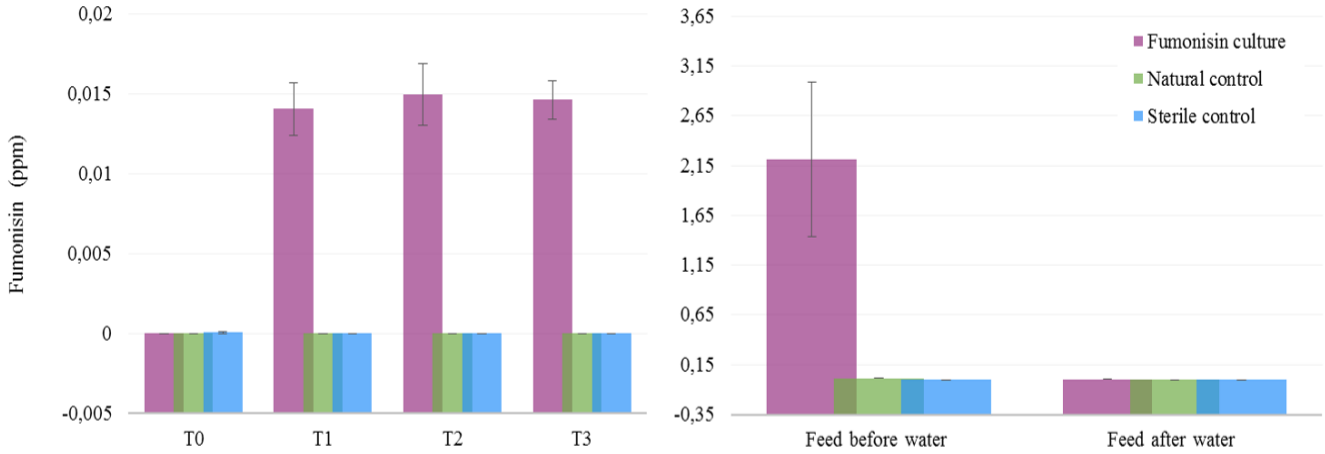

Figure 4.4: Total fumonisin concentrations in water (left) measured at 4 different times and in abalone feed (right) measured before water exposure and after 24 hours of exposure to autoclaved seawater.

4.5 Discussion

Fumonisins are produced by the filamentous fungus, *Fusarium verticillioides*, which is commonly found in animal feed and feedstuffs (Afsah-Hejri et al., 2013; Ashiq, 2015; Bhat et al., 2010; Diaz et al., 2013; Manning, 2005; Placinta et al., 1999; Ranjan and Sinha, 1991). Many authors have stated that fumonisin production is influenced by environmental factors such as substrate, pH, temperature and humidity (D'Mello and Macdonald, 1997; Garcia et al., 2012; Melcion et al., 1997; Parsons, 2008). In a previous study (Chapter 3) it was concluded that *F. verticillioides* isolates, originally

isolated from abalone feed, can produce fumonisins when re-inoculated onto abalone feed and left to colonise. This could be of concern, because abalone farms in South Africa are mainly located along the coast where the humidity is high with average temperatures of 16 °C ± 2 °C and 26 °C ± 2 °C during winter and summer months, respectively. These temperatures fall well within the growth and fumonisin production range of *Fusarium* species and fumonisin production (Alberts et al., 1990). Therefore, it is expected that fumonisin production could take place during winter and summer months on abalone farms when abalone feed are stored for extended periods of time under high moisture conditions. Although environmental moisture conditions influence fungal growth, two different studies found that differences in water activity did not significantly influenced FB_1 and FB_2 production, both these studies tested water activity high enough to promote fungal growth (Hinojo et al., 2006; Marin et al., 1995). It is for this reason that different water activity levels were not included in this study. Furthermore, fumonisin production is not dependent on the amount of growth, yet some growth is necessary for mycotoxin production to take place (Cahagnier et al., 1995).

In this study the fumonisin production by *Fusarium verticillioides*, isolated from abalone feed, was evaluated on the feed at two different temperatures, over a period of 10 weeks. Conditions in this study simulated storage temperatures of abalone feed on abalone farms in South Africa. Fungi were able to grow at both temperatures (Figure 4.1 and 4.2). Except for a slight colour change from white to beige, no difference could be noticed in terms of growth characteristics of *F. verticillioides* on abalone feed at the two different temperatures. These results support the findings of Cahagnier et al. (1995), that fumonisin production can be influenced by environmental factors without influencing the fungal growth. Therefore, when growth is visible, regardless of the amount of biomass, it is possible that fumonisin production is in progress, but mycotoxin analyses is needed to confirm the level of contamination (Melcion et al., 1997). Although previous studies reported 25 °C ±2 °C to be optimal temperatures for fumonisin production (Alberts et al., 1990; Melcion et al., 1997), this study showed that fumonisin production was higher at 16 °C than at 26 °C (Figure 4.3). However, the

fumonisin production per unit in this study was not even a fraction of the amount reported by these studies, where fumonisin production on maize measured >14000 ppm and 850 ppm, after 7 and 10 days, respectively (Alberts et al., 1990; Melcion et al., 1997). In contrast to these values, Garcia et al. (2012) reported less than 0.02 ppm fumonisin after *F. verticillioides* was incubated on sterile soya beans for 21 days at different temperatures ranging between 15 °C and 30 °C. Although the fumonisin levels measured in the latter study was lower than the levels measured in our study, it is closer to this study and more comparable. One of the differences between these studies is the growth substrate, which is categorised as a biological factor that influences mycotoxin production. There appears to be a link between the protein content of these substrates and the fumonisin production reported by the different authors, including the results found in this study. Soya beans have the highest protein content of 48 - 50 %, but low levels of fumonisin production were reported on this substrate (Garcia et al., 1997, 2012,). Abalone feed used in this study contains 35 % protein and fumonisin levels were measured between 0.157 – 11.753 ppm, while maize contains only 8 - 11 % protein, but had the highest fumonisin production reported (Alberts et al., 1990; FAO, 2016; Marifeed, 2016; Melcion et al., 1997; South African Grain Laboratory, 2011). Although more research is needed, it could be hypothesised that protein content in the growth substrate, influences the fumonisin production, and that higher protein content reduces fumonisin production. This could possibly be because of the difference in solubility of soya bean protein compared to maize protein (Castro-Rubio et al., 2006). Therefore, the amount and source of protein in abalone feed should be taken into consideration when formulating feed. This is especially important as agricultural commodities used in processing can also be a source for fumonisin contamination (Chapter 2). Furthermore, a significant decrease in fumonisin concentrations measured, was noticed at 16 °C after 56 days. Although the reason for this is unclear, it is hypothesised that fungal growth started decreasing by this time due to nutrition depletion in the growth substrate. A similar significant decrease was noticed at 26 °C, but a week earlier after 42 days. This is the optimal growth rate for *Fusarium* species and it is likely that fungal growth at 26 °C was faster, leading to an earlier nutrition depletion of the growth substrate. It is

assumed that chemical changes take place in the growth substrate over time, due to the fungal usage of nutrients. These changes may represent a developmental transition in signalling metabolites which could play a role in regulating FB_1 synthesis by *F. verticillioides* (Warfield and Gilchrist, 1999).

The European Food Safety Authority (EFSA) regards fumonisins, along with other mycotoxins as a risk to human and animal health and has therefore, published maximum allowable limits of these toxins relative to animal feed with a moisture content of 12 %. The allowable limit for FB_1 and FB_2 in fish feed according to the EFSA is 10 ppm (Food standards agency, n.d.). The U.S. Food and Drug Administration (FDA) also allows a maximum of 10 ppm of total fumonisins in catfish feed (U.S. Food and Drug Adinistration, 2001). In South African, according to the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act no 36 of 1947, republished in 2006, South Africa does not have maximum allowable mycotoxin limits for aquatic animal feeds. However, levels measured is this study were substantially lower than the maximum allowable limits for horse, pig, beef and poultry feeds with a moisture content of 12 %. Maximum FB_1 limits for these feeds are 5 ppm for horse feed, 10 ppm for pig feed, and 50 ppm for beef and poultry feeds (National Department of Agriculture, 2006). To date, mycotoxin risk assessments to establish allowable fumonisin contamination levels in abalone feed and ingredients have not been conducted for the South African market. It is expected that high levels of mycotoxins can decrease the growth rate in abalone, similar to other aquatic animals. However, fumonisin levels measured in this study were substantially lower than any of the above mentioned maximum allowable limits. Abalone feed used in this study had a slightly lower moisture content of 8.5 – 11.5 %. Therefore, if comparing the findings of this study to the current available allowable levels for animal feed, abalone feed tested in this study could be regarded as safe for animal consumption.

Fumonisins are water-soluble, heat-stable and alkaline-resistant aliphatic hydrocarbons with a terminal amine group and two tricarboxylic acid side chains (Bezuidenhout et al., 1988; Desjardins

and Proctor, 2007; Steyn, 1995; Sweeney and Dobson, 1998). Williams et al. (2003), reported that fumonisins are stable in a soil environment, but can be mobilized by rainwater and recovered, chemically intact, in the leachate. It was, therefore, expected that fumonisins will leach into the water when fumonisin contaminated feed is fed to abalone, but not consumed immediately. To our knowledge this is the first study to investigate the effect of water on fumonisin contaminated abalone feed.

Fumonisin B_1 was the most abundantly produced throughout this study (Figure 4.3 and 4.4). This is in agreement with current available literature (Melcion et al., 1997; Moss, 2002; Rheeder et al., 2002; Schumacher et al., 1995; Waśkiewicz et al., 2015). Furthermore, this study showed that FB_1 was the only fumonisin that could be detected in the water even after 24 hours. This correlates with a previous study, showing that when a fumonisin contaminated source comes in contact with water, fumonisins leach from the source into the water and could be detected at levels as low as ng/L level in an aqueous environment (Waśkiewicz et al., 2015). In this study, fumonisin levels in the abalone feed decreased significantly in the water over 24 hours, while FB_1 concentration in the water increased significantly (Figure 4.4). This is a strong indication that fumonisins leach from the feed into water. Although this reduces the concern of abalone consuming fumonisin contaminated feed, it increases the potential risk to the direct surrounding environment. If fumonisin contaminated water gets in contact with soil at any stage, fumonisins could stay behind posing a threat to the surrounding ecosystem (Williams et al., 2003). It is worth noticing that the fumonisin concentrations measured in the water were below 1 ppm. It is possible that the concentrations were this low due to the high dilution factor. The same feed to water ratio used on abalone farms, were used in this study. Considering the high dilution factor, the environmental risk is considered to be exceedingly small and unlikely to be problematic. It should be kept in mind that recycled water could build up fumonisin levels over time. However, it was previously suggested that more studies should be conducted on a wider scale to provide detailed explanation of fumonisin migration in the environment and the risk for the ecosystem and wild fish

in their natural habitat (Waśkiewicz et al., 2015). Therefore, it is recommended to monitor the presence of fumonisins and other mycotoxins, not only on abalone farms but on any animal farm that use artificial feed until the effect that migrating fumonisins or any mycotoxins have on the environment and wild fish, is fully understood and proven to have no damaging effect.

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5 GENERAL DISCUSSION AND CONCLUSION

Mycotoxins are secondary metabolites of filamentous fungi and despite the negative impacts they have on humans and terrestrial animals, not much is known about the effect on aquatic animals. Previous studies aimed to determine the effect on a number of fish species, but no research could be found on the effect that mycotoxins could have on shellfish. Shellfish farms in South Africa are monitored by the South African Molluscan Shellfish Monitoring and Control Programme (SAMSM&CP) for a number of human health hazards (DAFF, 2012). This include biotoxins, microorganisms, heavy metals, pesticides, polychlorinated biphenyls (PCB's) and radionuclides. Currently no monitoring is done for any mycotoxins (DAFF, 2012). The South Africa Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act no 36 of 1947, republished in 2006, state maximum allowable mycotoxin limits for a variety of animal feeds and ingredients, but does not state any regulations with regards to allowable mycotoxin limits in aquatic animal feeds (National Department of Agriculture, 2006).

It is expected that toxigenic fungi and their mycotoxins are present in feed used for aquatic animals due to its composition which is similar to feed used for terrestrial animals (Bondad-Reantaso et al., 2005; Fleming, Van Barneveld, & Hone, 1996). An aquatic environment differs from a terrestrial environment in many ways and so does the type of animal species that are cultured (van der Merwe, 2009). Therefore, research is needed to determine the presence and behaviour of mycotoxins in the aquatic environment. This is especially important in the abalone farming industry because in South Africa alone, this industry has expanded over the last decade and dominates the marine aquaculture industry (DAFF, 2011, 2012; FAO, 2012, 2013).

Natural occurring toxigenic fungi on grains used in animal feed are dominated by three genera namely *Fusarium, Penicillium* and *Aspergillus.* Fumonisins, trichothecenes, ochratoxins and aflatoxins are

some of the most significant mycotoxins produced by the three dominant mycotoxin producing genera (Gelderblom et al., 1988; Klich, 2007; Pitt, 1987; Rheeder et al., 1992; Scott, 1993). The occurrence of mycotoxins in animal feeds does not only have a major effect on the animals, but could move up the food chain and affect the health of humans (Arai & Hibino, 1983; Y. K. Wang et al., 2013). This study shows that artificial abalone feed is no different to other artificial feeds, previously found to be contaminated with natural occurring toxigenic fungi and their associated mycotoxins (Alinezhad et al., 2011; Anukul, Maneeboon, Roopkham, Chuaysrinule, & Mahakarnchanakul, 2014; Barbosa et al., 2013; Böhm et al., 2010; Keller et al., 2007; Pietsch, Kersten, Burkhardt-Holm, Valenta, & Dänicke, 2013; Pleadin et al., 2012; Ranjan & Sinha, 1991; Schollenberger et al., 2006; Seo, Phat, Kim, & Lee, 2013; W. Wang et al., 2012).

During this study, the frequency of toxigenic fungi in artificial abalone feed was determined by isolating toxigenic fungi and determining the presence of their associated mycotoxins in abalone feed and ingredients, in a survey done in 2013 and 2014. A total of 248 fungal isolates belonging to different genera, including *Fusarium*, *Penicillium*, *Aspergillus*, *Cladosporium*, *Rhizopus*, *Trichoderma* and *Mucor* were isolated in this study (Chapter 2) with the focus on the three dominant genera and their associated mycotoxins. *Fusarium*, *Penicillium* and *Aspergillus* represented 52.4 % of the total fungal isolates. Of the 13 mycotoxins that were screened for in the ingredients and finished feed, only 3 were detected, namely fumonisin B_1 and B_2 and ochratoxin A (OTA). Overall, the most abundant toxin detected was FB_1. Starches can get infected with fungi before harvesting or during storage and handling, before reaching the artificial feed manufacturer (Diaz, Whitlow, & Hagler Jr., 2013; Miller, 2008; Placinta, D'Mello, & Macdonald, 1999; Sweeney & Dobson, 1998). Starches tested in this study could support fungal growth and mycotoxin production. All the starches showed positive results for one or more mycotoxins, while none of the mycotoxins could be measured in the protein source. Feed samples and ingredients were collected directly from the supplier. Therefore, the presence of mycotoxins in the starches and final feed is an indication that when infected

starches are processed, mycotoxins are not removed during the manufacturing process.

Previous studies have shown that especially FB_1 interferes in metabolic pathways and has a detrimental effect on the health and growth of fish species (Barbosa et al., 2013; Tuan, Manning, Lovell, & Rottinghaus, 2003; E. Wang, Norred, Bacon, Riley, & Merrill, 1991; Yildirim, Manning, Lovell, & Grizzle, 2000). It can be expected that when high levels of FB_1 are consumed by abalone that it will have a similar effect on the health of abalone. However, when comparing the findings of this study to the current available allowable limits for animal feed, abalone feed tested in this study could be regarded as safe for animal consumption with regards to the fumonisin contamination levels. The FDA has a recommended allowance for a maximum total fumonisins concentration in catfish feed of 10 ppm (U.S. Food and Drug Administration, 2001). In South Africa, according to the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act no 36 of 1947, republished in 2006, South Africa does not have maximum allowable mycotoxin limits for aquatic animal feeds. There are however, maximum allowable FB_1 limits for terrestrial animal feeds with a moisture content of 12 % (National Department of Agriculture, 2006). Abalone feed used in this study had a slightly lower moisture content of 8.5 – 11.5 % and fumonisin levels measured in feed and ingredients were substantially lower with the highest measured level in starch being 0.0184 ppm (Chapter 2). Therefore, when comparing the findings of this study to the current available allowable limits for animal feed, abalone feed tested in this study could be regarded as safe for animal consumption with regards to the fumonisin contamination levels. A recent survey conducted by an animal health and nutrition company, pointed out that deoxynivalenol (DON), along with fumonisins, were present in more than 50 % of the agricultural samples collected across 75 countries. Authors of this report continued by stating that the high levels in fumonisin contamination is reason for concern (Biomin, 2015). This report emphasizes the reality of the possible effect that fumonisins can have on human and animal health (D'Mello & Macdonald, 1997; D'Mello, Placinta, & Macdonald, 1999; Fung & Clark, 2004; Khoa, Hatai, & Aoki, 2004; Placinta et al., 1999; Pleadin et al., 2012; Rheeder et al.,

1992; Shephard, 2003; Wiseman, Price, Lightner, & Williams, 1982). More mycotoxin risk assessments are suggested to establish the maximum allowable fumonisin levels in abalone feed and ingredients for the South African market.

Mycotoxin risk assessments will take time, therefore for the interim while not much is known about the effect of mycotoxins on abalone we suggest that the maximum allowable total fumonisins limits recommended by FDA for catfish feed is used as a guideline. The current study clearly showed that the aquatic medium has an effect on fumonisins, therefore using maximum allowable levels similar to another aquatic animal is more accurate than using maximum allowable levels for terrestrial animals. In terms of other mycotoxins where no limits are available for aquatic animals, we suggest that the supplier use the maximum allowable limits for AFB_1 allowed in ingredients used in feeds for all farmed animals (Table 1.4).

The combination of toxigenic fungi present in abalone feed along with their ability to produce mycotoxins on abalone feed as growth substrate, is a concern (Chapter 3). To add to this, average temperatures during winter and summer months are ideal for toxin production (Chapter 4). Even though mycotoxin production is influenced by a variety of factors (D'Mello & Macdonald, 1997), these three aspects are all factors that cannot be avoided and cannot easily be controlled, thus it should raise concerns within the abalone industry. In this study, we tested whether mycotoxins are produced when toxigenic fungi, isolated from abalone feed, are allowed to colonise feed under two different temperatures. While none of the *Penicillium* isolates produced the toxins screened for in this study, isolates representing *Fusarium* and *Aspergillus* species were able to produce their associated mycotoxins under sterile laboratory conditions on artificial abalone feed. Mycotoxin levels were measured after 6 weeks of incubation at 26 ±1 °C. This incubation temperature was used for two reasons, firstly because it represents the average day temperatures during summer in South Africa where most of the abalone farms are located, and secondly this is the suggested temperature at which

a wide range of mycotoxins can be produced within all three genera tested (Diener & Davis, 1969; Gelderblom et al., 1988; Rabie, Marasas, Thiel, Lübben, & Vleggaar, 1982; Ribeiro et al., 2006). Fumonisins were measured in feed inoculated with *F. verticillioides* that was previously isolated from abalone feed, and concentrations ranged from 0.33 – 12.43 ppm.

Aflatoxins were measured in feed samples that were inoculated with *Aspergillus* isolates, also previously isolated from abalone feed. Aflatoxin production by *Aspergillus flavus* showed to be strain dependent, with two isolates where production was absent and others that produced levels as high as 54.5 ppm (MRL332) on abalone feed. This is in agreement with previous studies that reported aflatoxin production to be strain dependent (Bennett & Klich, 2003; D'Mello & Macdonald, 1997; Dutta & Das, 2001; Johnston et al., 2012; Klich, 2007; Manning, 2005; Moss, 2002).

Even though *A. flavus* and *A. oryzae* have several homologues of aflatoxin biosynthesis pathway genes, genetic defects have led to the silencing of the aflatoxin pathway in *A. oryzae* (Takahashi et al., 2002; Watson, Fuller, Jeenes, & Archer, 1999). This has been supported by previous studies (Barbesgaard, Heldt-Hansen, & Diderichsen, 1992; Bennett & Klich, 2003; Geiser, Dorner, Horn, & Taylor, 2000; Klich, 2007; Wei & Jong, 1986). Despite the ongoing debate on the aflatoxin production ability of *A. oryzae* (Atalla, Hassanein, El-Beih, & Youssef, 2003; Blumenthal, 2004; El-Kady, El-Maraghy, & Zohri, 1994; Geiser et al., 2000), we suggest that some *A. oryzae* isolates could be toxigenic but are highly substrate specific. This suggestion is based on the consideration that the growth substrate influences the mycotoxin production and the results of Chapter 3, where one out of two *A. oryzae* isolates tested in this study produced both AFB_1 and AFB_2. Aflatoxin production by *A. oryzae* MRL304 were exceptionally high (89.8 ppm) and more research is suggested on this specific isolate to confirm identification and toxin production. Apart from the OTA measured in the sample inoculated with the positive control, *P. viridicatum* MRC356, no OTA was detected in the samples inoculated with *Penicillium* isolates. *Penicillium viridicatum* MRC356 produced OTA levels

exceeding the maximum level of detection and although this makes the measurement inaccurate, it is clear that this isolate was able to produce OTA on abalone feed as growth substrate. This is an indication that artificial abalone feed is a favourable growth substrate and promotes the production of OTA. However, because none of the species that were previously isolated from abalone feed produced OTA, it is possible that they are non-toxigenic and pose no threat to the industry in terms of mycotoxins screened for in this study.

Abalone farms in South Africa are mainly located along the West- and East coast, with average temperatures of 16 °C ± 2 °C and 26 °C ± 2 °C during winter and summer months, respectively. The current study support that both these temperatures fall well within the growth and fumonisin production range of *Fusarium* species and fumonisin production (Alberts et al., 1990). Although previous studies reported 25 °C ±2 °C to be optimal for fumonisin production (Alberts et al., 1990; Melcion, Cahagnier, & RichardMolard, 1997), our study showed that fumonisin production was higher at 16 °C than at 26 °C. Water activity was not included as a variable in this study as two previous studies failed to show that water activity significantly influence mycotoxin production (Hinojo et al., 2006; Marin, Sanchis, Vinas, Canela, & Magan, 1995). Therefore, based on the positive results for fumonisin production on abalone feed as growth substrate, it was assumed that when growth is visible, regardless of the amount of biomass, it is possible that fumonisins can be produced, but mycotoxin analyses is needed to confirm the level of contamination.

Apart from temperature that had a slight influence on fumonisin production, we further suggest that protein content in growth substrates could have an effect on fumonisin production. Soya beans have the highest protein content at 48 - 50 % and levels as low as ~0.02 ppm of fumonisin production were reported on this substrate (Garcia et al., 1997, 2012,). Abalone feed used in this study contains 35 % protein (protein source was kept secret by request from the supplier) and fumonisin production levels over 10 weeks were between 0.15 – 11.75 ppm, while maize contains only 8 - 11 % protein,

but had the highest fumonisin production reported of levels exceeding 14000 ppm (Alberts et al., 1990; FAO, 2016; Marifeed, 2016; Melcion et al., 1997; South African Grain Laboratory, 2011). This could possibly be because of the different solubility of soya bean protein compared to maize protein (Castro-Rubio, García, & Marina, 2006). Therefore, a higher protein content seems to reduce fumonisin production during storage after feed processing. When protein levels in artificial abalone feed exceed the optimal levels needed for abalone growth efficiency, the excess protein will either be excreted or metabolized as energy (Coote, Hone, Van Barneveld, & Maguire, 2000; Lee, 2004; Mai, Mercer, & Donlon, 1995). Currently one of the protein sources used is fishmeal, but due to the devaluation of South Africa's currency, imported fishmeal has become very expensive (Sales, 2001), and increasing protein could become expensive. It is for this reason, along with the decrease in availability of fishmeal that the abalone industry has turned to alternative protein sources such as soybean and sunflower meal. This might appear to be a good alternative, as these two ingredients have been shown to have no detrimental effect on abalone growth (Sales, 2001; Shipton, 1999). Furthermore, abalone is well adapted to digest higher levels of protein by breaking it down with proteases that are found throughout their gut system (Erasmus, Cook, & Coyne, 1997; Knauer, Britz, & Hecht, 1996; Picos-García, García-Carreño, & Serviere-Zaragoza, 2000). On the negative side, two studies reported that plant proteins could be a source for mycotoxin contamination, and by using these protein sources it could increase the possibility for mycotoxins to enter into the feed from the ingredients (Nácher-Mestre et al., 2015; Valenta, Danicke, & Bluthgen, 2002). Therefore, the amount and source of protein in abalone feed should be taken into consideration, when formulating not only artificial abalone feed, but any artificial feed.

Research to improve artificial feed is not only based on the nutritional quality and cost-effectiveness of feed, but should include the availability and behaviour of nutrients and toxins in a tank system, along with problems associated with waste removal and aeration in the water (Fleming et al., 1996). Fleming et al. (1996) suggested that the only way to accurately measure the leaching of a specific

content is by comparing the content in feed pellets before and after exposure to water, and ideally under commercial culture conditions. To our knowledge this is the first study to attempt to measure fumonisins in artificial feed before and after water exposure and to determine the effect of water on fumonisin contaminated feed. This was done under controlled conditions in a laboratory with the same feed:water ratio used on abalone farms. The outcome of the fourth objective of this study is in agreement with literature, which is that fumonisins leach from the source, in this case artificial abalone feed, into the water (Bezuidenhout et al., 1988; Desjardins & Proctor, 2007; Steyn, 1995; Sweeney & Dobson, 1998). Fumonisin levels in the abalone feed decreased significantly after 2 hours of exposure to water, while FB_1 concentration in the water increased significantly. This stayed the same throughout the 24 hours of the experiment. Although this reduces the concern of abalone consuming fumonisin contaminated feed, it increases the potential risk to the direct surrounding environment. If fumonisin contaminated water gets in contact with soil at any stage, fumonisins could stay behind posing a threat to the surrounding ecosystem (Williams et al., 2003). It is worth noticing that the fumonisin concentrations in the study were substantially diluted and levels measured in the water were below 1 ppm. However, it was previously suggested that more studies should be conducted on a wider scale to provide a detailed explanation of fumonisin migration in the environment and the risk for the ecosystem and wild fish in their natural habitat (Waśkiewicz, Bocianowski, Perczak, & Goliński, 2015).

In conclusion, similar to previous findings of trout feed, the presence of mycotoxin producing fungi on feed does not necessarily mean the presence of mycotoxins and vice versa (Alinezhad et al., 2011). This is because mycotoxins can come from raw products and find their way into final feed. Alternatively, spores from toxigenic fungi can end up in the final feed and germinate under poor storage conditions followed by mycotoxin production. Therefore, constant monitoring of artificial abalone feed samples is essential prior to their consumption. Especially because mycotoxin and fungal contamination in agricultural commodities can differ between seasons (Ranjan & Sinha, 1991;

South African Grain Laboratory, 2011). Even though fumonisins leach out of feed into the water, this might not be the same for other mycotoxins that are not water soluble.

Based on our findings, our recommendations to the abalone industry agree with the following suggestions, made previously by other authors based on mycotoxin contamination in artificial feed;

- Eliminate fungal contamination in agricultural commodities, used as ingredients, as much as possible, by removing materials that contain fungal growth (Barbosa et al., 2013; Pietsch et al., 2013).
- Avoid using low quality ingredients.
- Ensure to use a trustworthy supply source, if possible request random fungal and mycotoxin screening or use the South African Grain Laboratory NPC as a guideline as to what level of contamination to expect between seasons.
- Until more mycotoxin risk assessments have been conducted to establish the maximum allowable limits of mycotoxins in abalone feedstuffs, we suggest to keep FB_1 limits lower than 10 ppm, which is the maximum allowable FB_1 limits, recommended by the FDA in catfish feeds.
- Furthermore, we suggest that the supplier use the maximum allowable limits according to the South African national regulations, according to the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act no 36 of 1947, republished in 2006 for AFB_1 allowed in ingredients used in feeds for all farmed animals (Table 1.4).
- If continued mycotoxin contamination is suspected in agricultural commodities, the following steps can be taken prior to feed processing. When physical removal of contaminated commodities are not possible, removing of fumonisins in aqueous cooking can be done, fumonisins will get discarded with the water. This might not work with other mycotoxins as not all mycotoxins are water-soluble. Thermal processing at 150 – 200 °C can reduce fumonisins. Another method used is chemical conversion, where fumonisins are hydrolysed

during alkaline cooking (Humpf & Voss, 2004; Manning, 2010). However ideally commodities free of any mycotoxins should be used.

- Use animal protein, this will reduce the risk of additional mycotoxin contamination through agricultural commodities, as opposed to plant protein.
- Control storage conditions.
- Storage containers should be emptied regularly to prevent feed to naturally rot.
- Storerooms should be kept in good condition to avoid rain from entering.

Control of storage conditions of ingredients and final feed should be a priority. Condensation can form on the inside of store rooms and containers during seasonal temperature changes and rainfall (Manning, 2010). This will allow toxigenic fungi in the ingredients or feeds to develop and start producing mycotoxins. More caution should be taken during winter months in South Africa. Most of the abalone farms in South Africa are located in areas with a winter rainfall and from our findings higher levels of fumonisins were produced at lower temperatures. No fumonisins were detected in samples that were left to rot naturally. Therefore, it is possible that fumonisin production will not take place when feed is left to rot naturally, but it is unknown whether other mycotoxins will be produced under natural conditions. Especially because *A. flavus* has shown to be strain dependent. It is possible that contamination of crops can take place during different seasons by different strains of *A. flavus*. Therefore, aflatoxin production during poor storage conditions will not always take place.

Based on this study it is clear that mycotoxin contamination in abalone artificial feed is highly likely. Even though this study focussed mainly on fumonisins produced by *Fusarium verticillioides,* it does not go unnoticed that abalone artificial feed is an ideal growth substrate for other toxigenic fungal species and the production of their associated mycotoxins. Therefore, more mycotoxin risk assessments are suggested to determine associated risks and to establish maximum allowable

mycotoxin levels for ingredients used in artificial feed intended for aquatic animals.

5.1 References

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6 APPENDIX

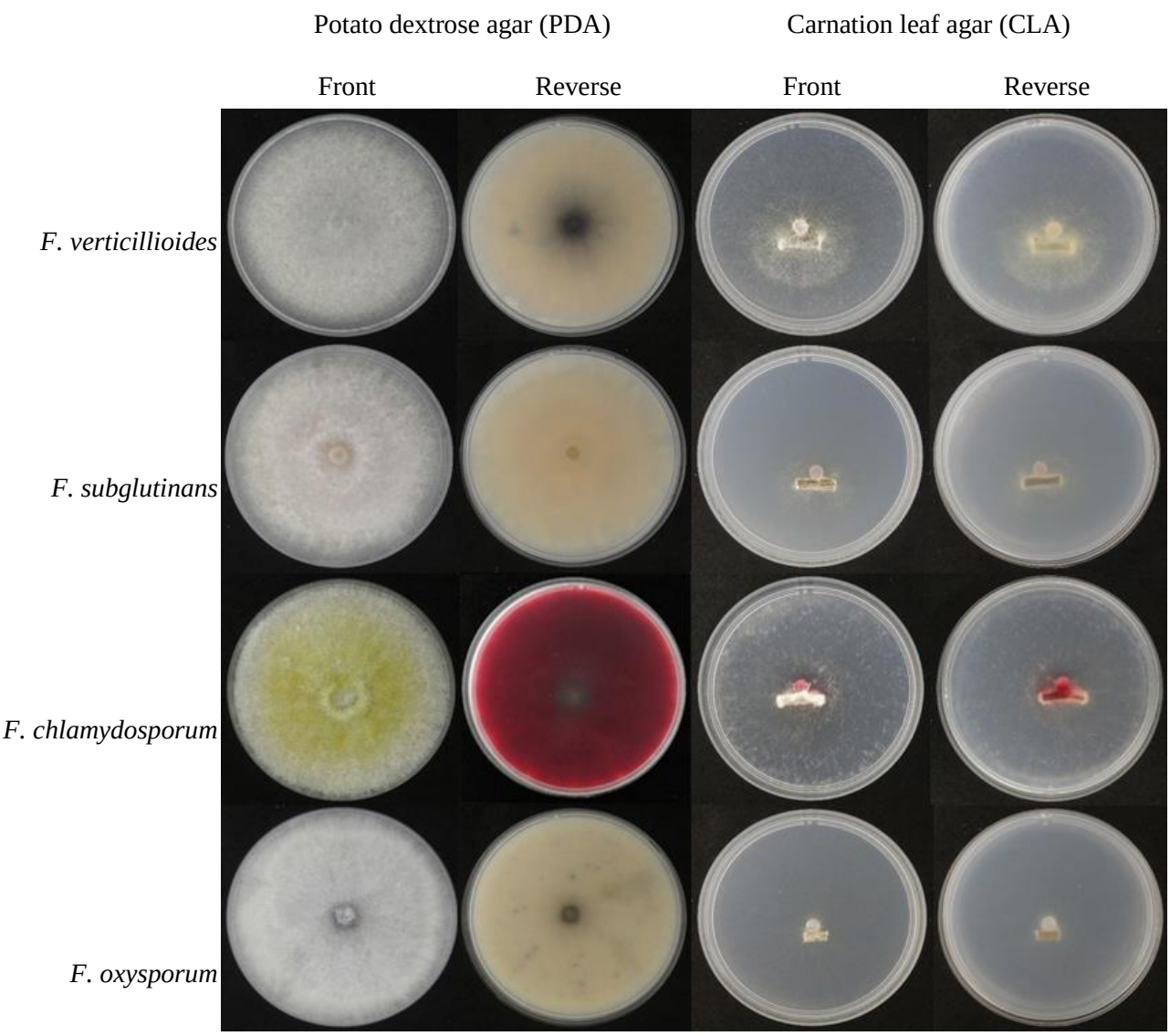

Figure A: Fourteen day old *Fusarium* cultures, used for morphological identifications. From left to right, potato dextrose agar (PDA) front and reverse, followed by carnation leaf agar (CLA) front and revers. From top to bottom, *F. verticillioides*, *F. subglutinans*, *F. chlamydosporum*, *F. oxysporum*.

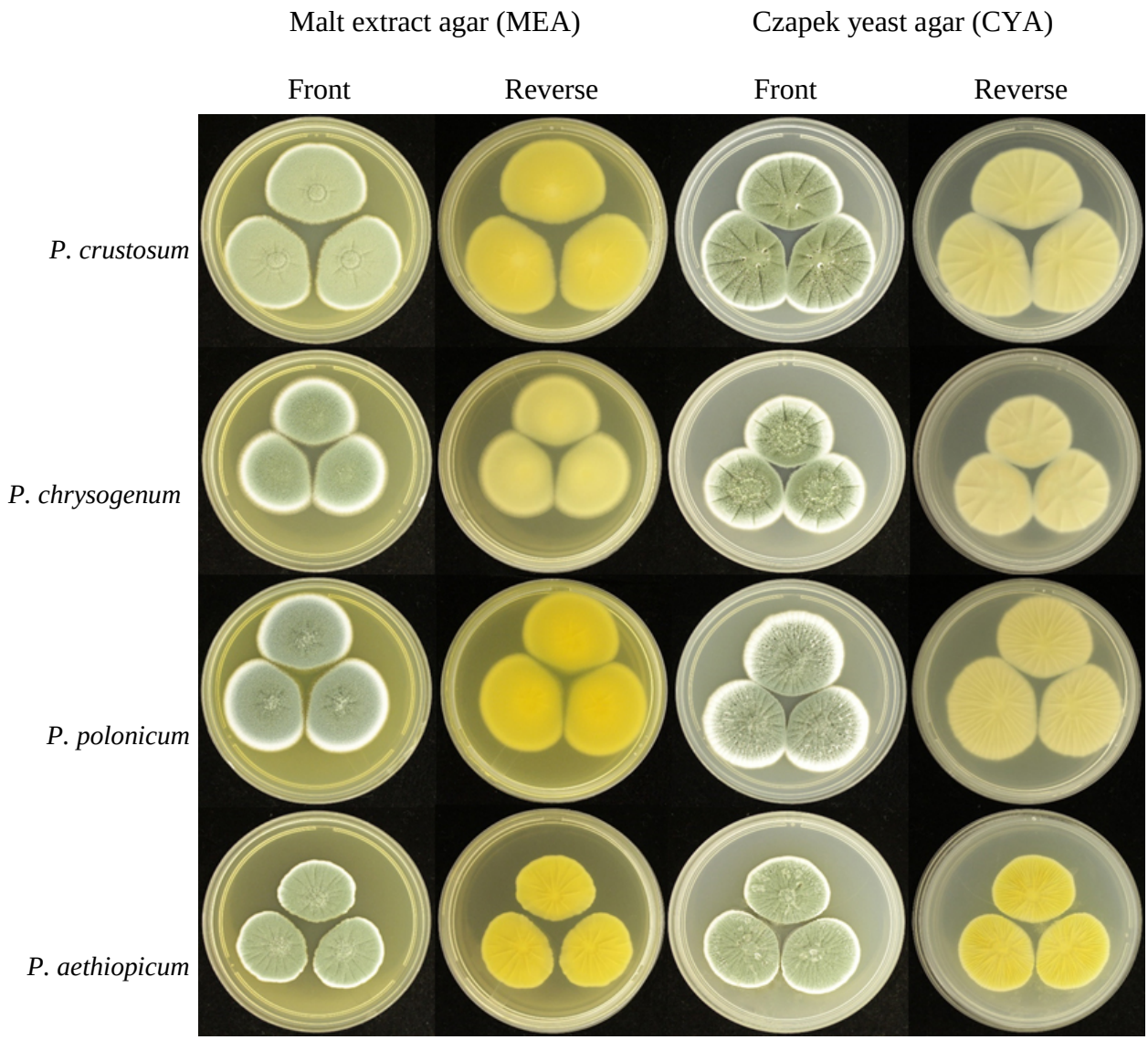

Figure B: Seven day old *Penicillium* cultures, used for morphological identifications. From left to right, malt extract agar (MEA) front and reverse, followed by czapek yeast agar (CYA) front and revers. From top to bottom, *P. crustosum, P. chrysogenum, P. polonicum, P. aethiopicum.*

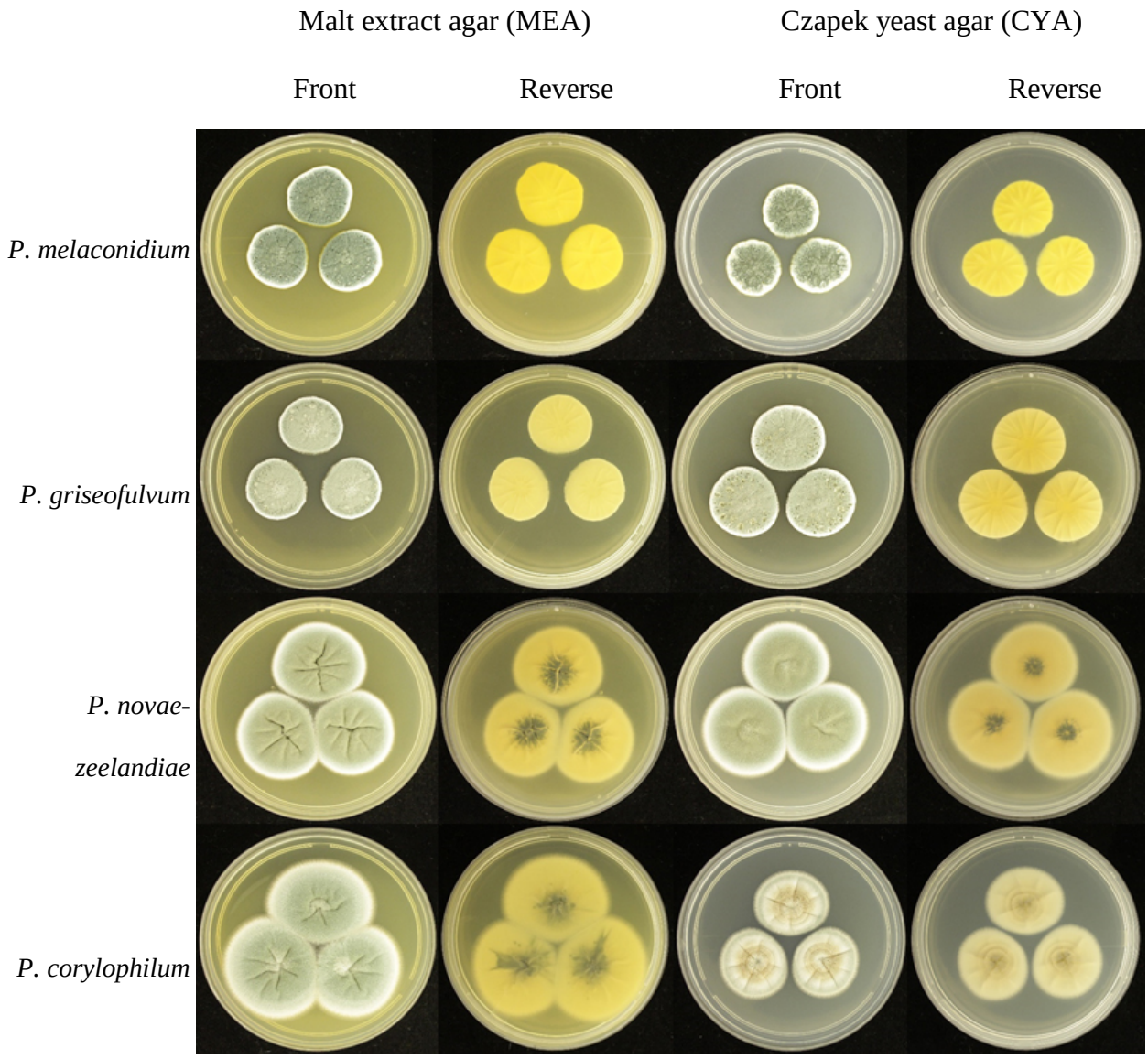

Figure C: Seven day old *Penicillium* cultures, used for morphological identifications. From left to right, malt extract agar (MEA) front and reverse, followed by czapek yeast agar (CYA) front and revers. From top to bottom, *P. melaconidium*, *P. griseofulvum*, *P.novae-zeelandiae*, *P. corylophilum.*

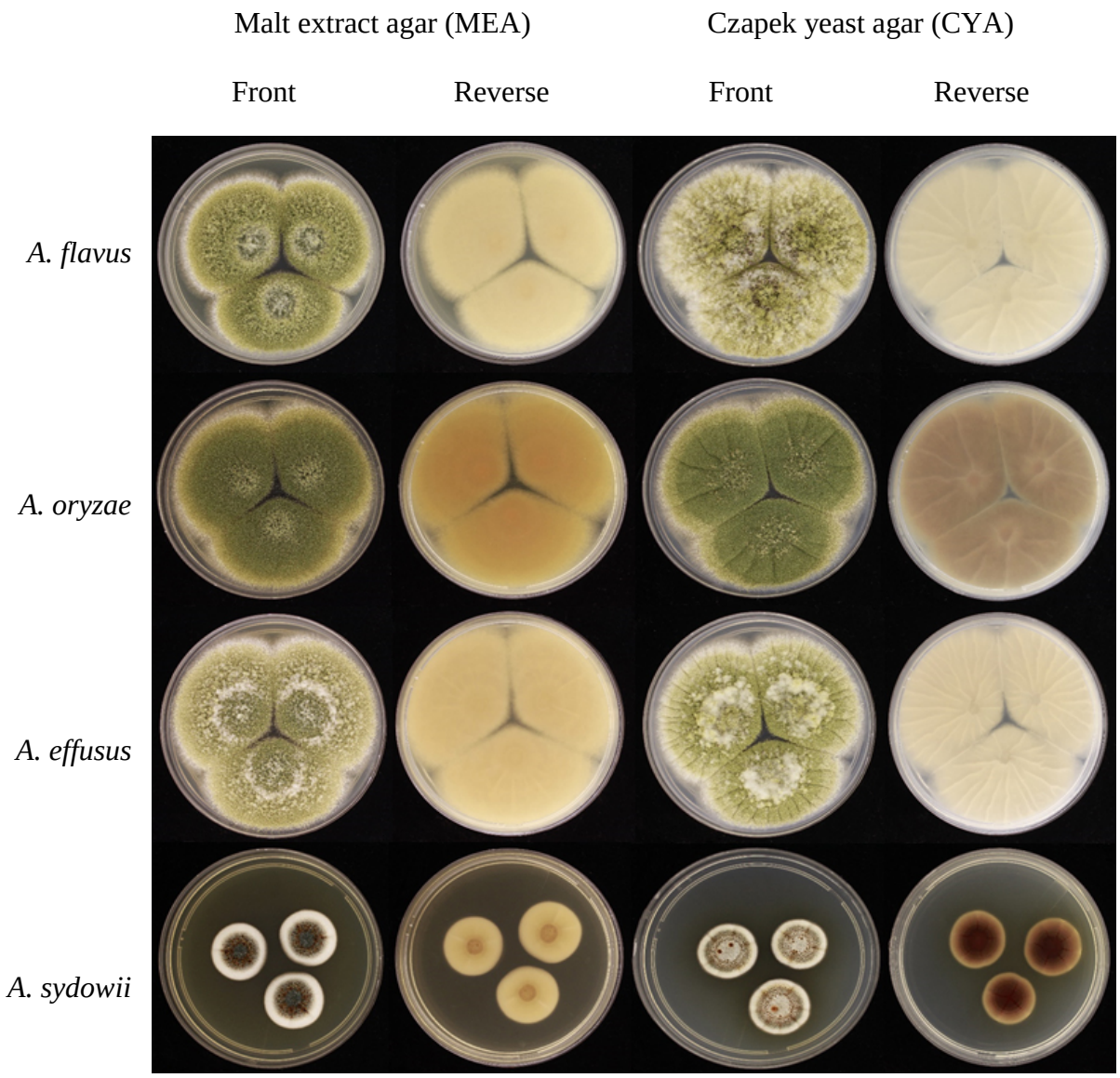

Figure D: Seven day old *Aspergillus* cultures, used for morphological identifications. From left to right, malt extract agar (MEA) front and reverse, followed by czapek yeast agar (CYA) front and revers. From top to bottom, *A. flavus*, *A. oryzae*, *A. effusus*, *A. sydowii*.

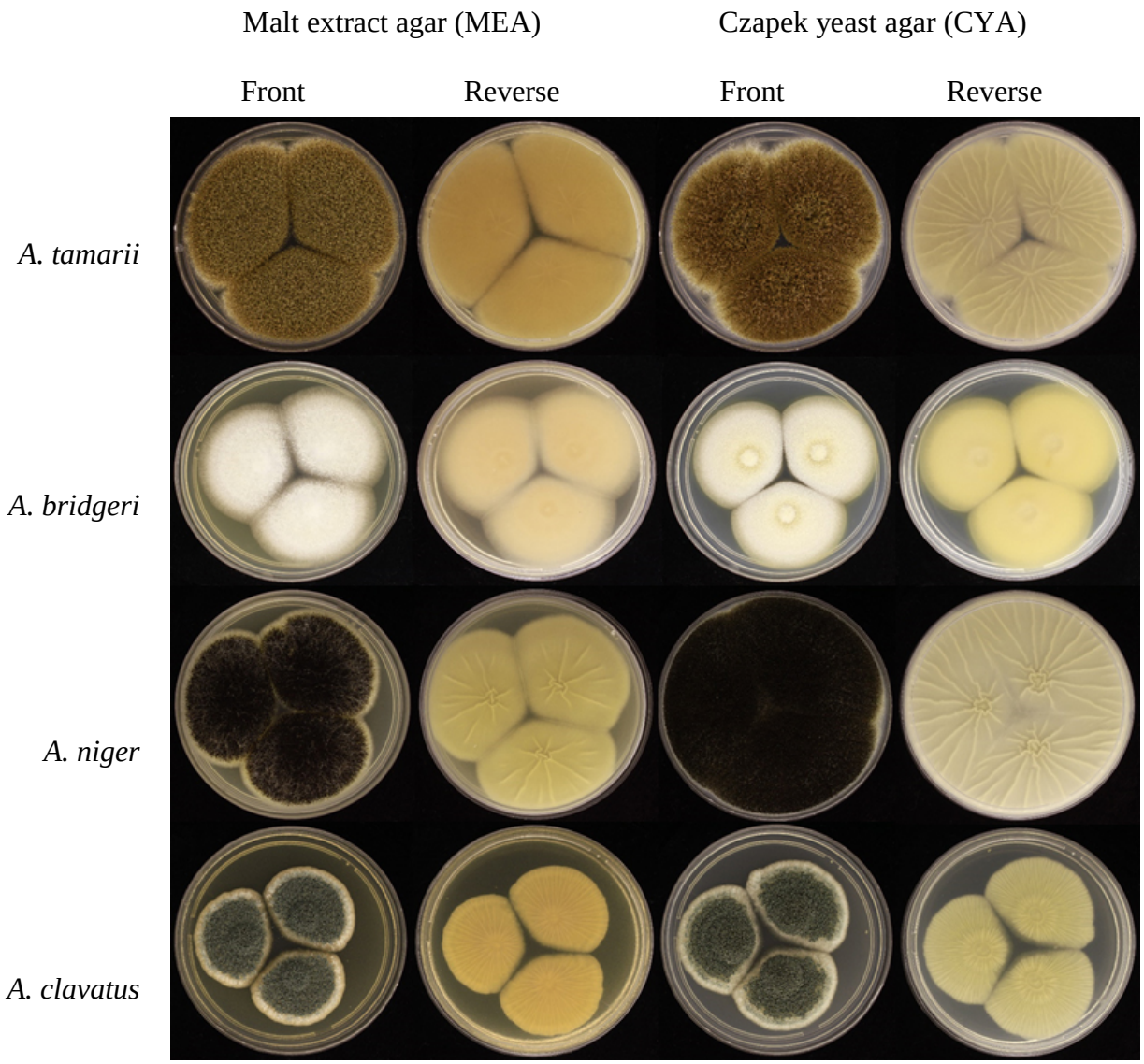

Figure E: Seven day old *Aspergillus* cultures, used for morphological identifications. From left to right, malt extract agar (MEA) front and reverse, followed by czapek yeast agar (CYA) front and revers. From top to bottom, *A. tamari, A. bridgeri, A. niger, A. clavatus.*

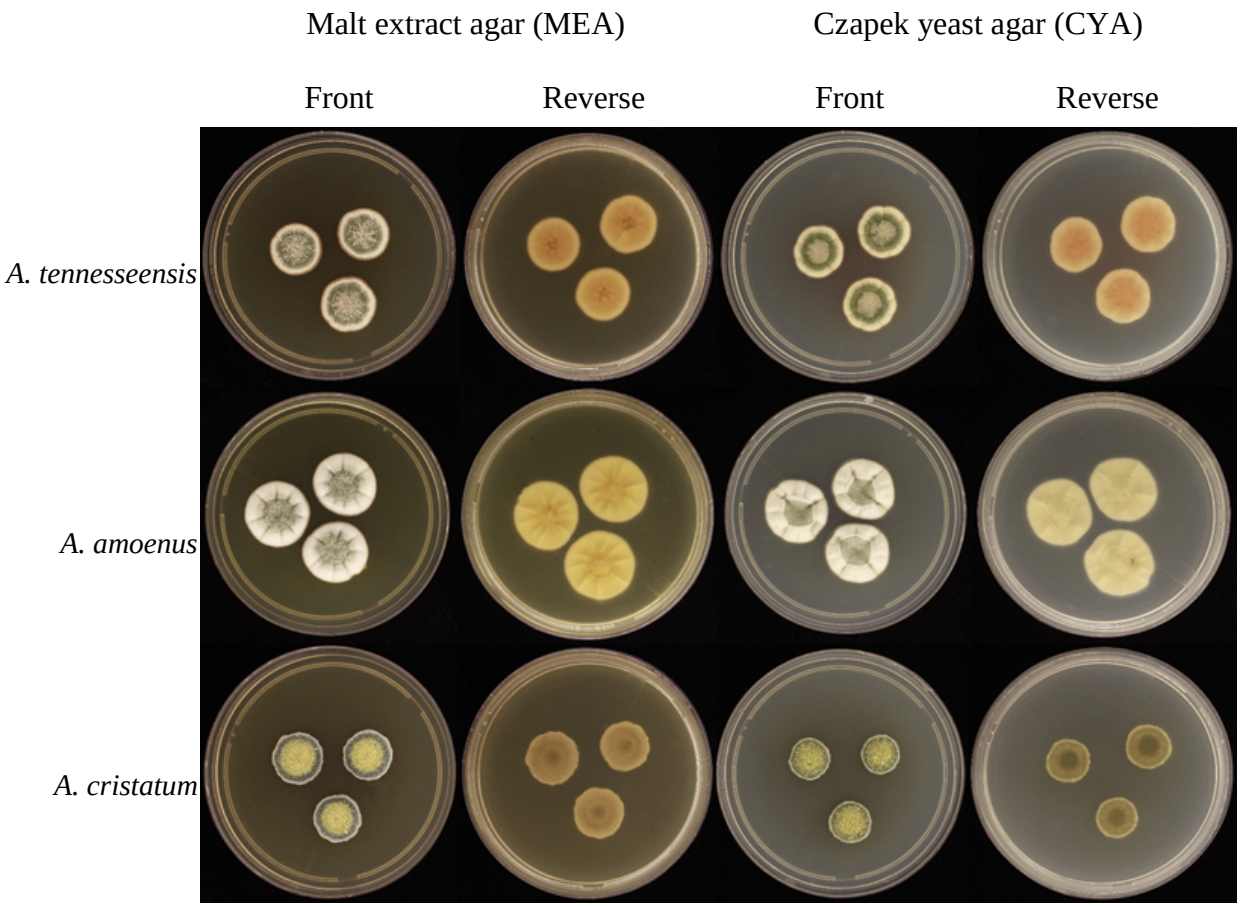

Figure F: Seven day old *Aspergillus* cultures, used for morphological identifications. From left to right, malt extract agar (MEA) front and reverse, followed by czapek yeast agar (CYA) front and revers. From top to bottom, *A. tennesseensis, A. amoenus, A. cristatum.*

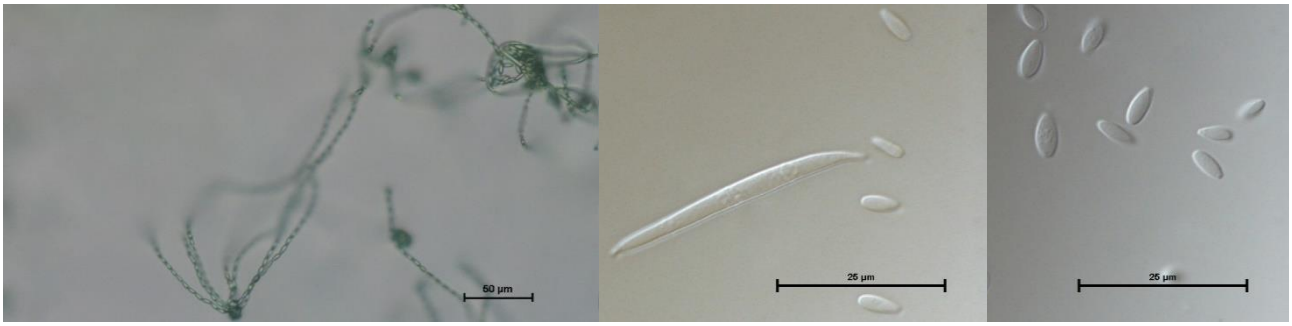

Figure G: *Fusarium verticillioides* after 14 days on carnation leaf agar (CLA). From left to right, microconidia *in situ*, macroconidia, microconidia.

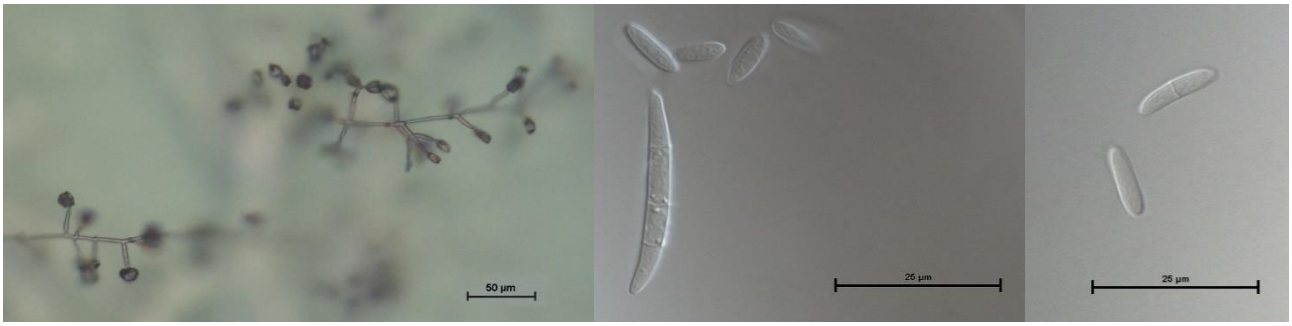

Figure H: *Fusarium subglutinans* after 14 days on carnation leaf agar (CLA). From left to right, microconidia *in situ*, macroconidia, microconidia.

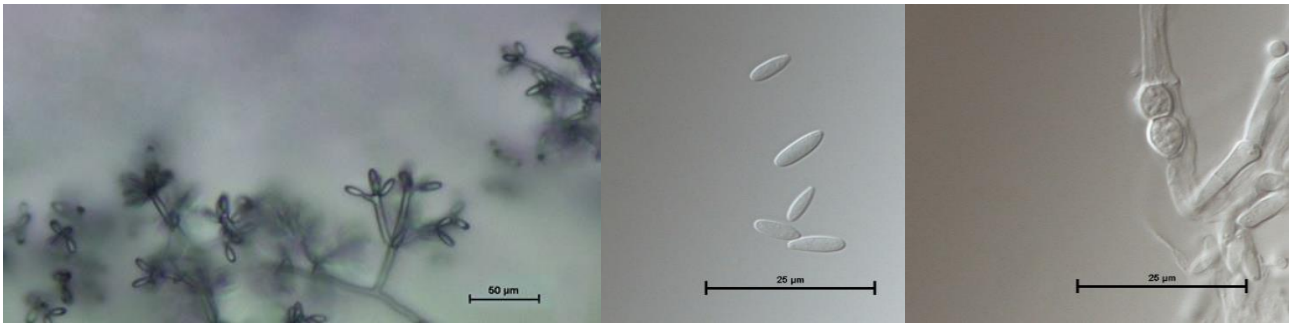

Figure I: *Fusarium chlamydosporum* after 14 days on carnation leaf agar (CLA). From left to right, microconidia *in situ*,microconidia, chlamydospores in hyphae.

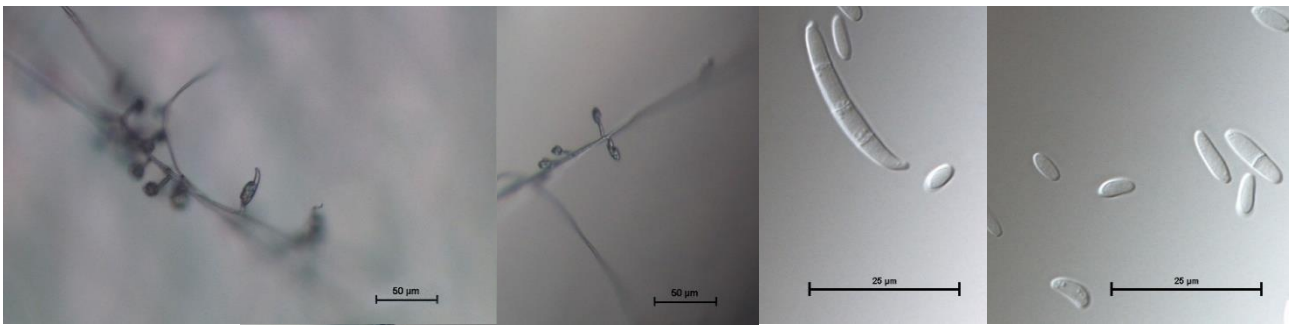

Figure J: *Fusarium oxysporum* after 14 days on carnation leaf agar (CLA). From left to right, microconidia *in situ*, microconidia *in situ*, macroconidia, microconidia.