DEVELOPMENT OF A BIOMARKER AND CLAY BASED REMEDIATION STRATEGY FOR POPULATIONS AT RISK FOR FUMONISIN TOXICOSIS

A Dissertation

by

ABRAHAM GAY ROBINSON II

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2012

Major Subject: Toxicology

Development of a Biomarker and Clay Based Remediation

Strategy for Populations at Risk for Fumonisin Toxicosis

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ABSTRACT

Development of a Biomarker and Clay Based Remediation Strategy for Populations at Risk for Fumonisin Toxicosis. (May 2012)

Abraham Gay Robinson II, B.S., Prairie View A&M University

Chair of Advisory Committee: Dr. Timothy D. Phillips

Fumonisin B_1 is the most prevalent congener of the fumonisin mycotoxins produced by *Fusarium verticilliodies* and is considered by many to be the most toxic. Fumonisin B_1 has been classified by IARC as a class 2B carcinogen. This is primarily due to evidence suggesting increased exposure to FB_1 through contaminated foodstuffs is responsible for increased incidence of esophageal cancer in regions of China and South Africa. Fumonisin B_1 exposure has also been implicated in the increased incidence of neural tube defects along the Texas/Mexico border. Therefore the principal goals of this research were to 1) Identify sorbent materials that would be compatible with the chemical characteristics of fumonisin B_1 and evaluate their ability to sequester the toxin using established in vitro techniques; 2) evaluate urinary FB_1 as a biomarker of exposure from a fumonisin contaminated diet; 3) utilize urinary FB_1 as a diagnostic tool to evaluate the efficacy of NS in reducing biomarkers of FB_1 bioavailability in a Ghanaian population suspected to be co-exposed to aflatoxins and fumonisins.

Isothermal analysis and an alternative animal model were examined and compared to previously published results to determine the sorbent toxin interaction activity in vitro as a predictor of in vivo efficacy. An HPLC method for detection and quantitation of urinary FB_1 was developed based on methods previously adapted for primary amine and biomarker analysis. Urinary FB_1 was evaluated as an HPLC detectable biomarker using a rodent model. Calcium and sodium montmorillonite clays were selected to interact with the positive charge on FB_1 at low pH and sorb the molecule. Ferrihydrite was selected to interact with the negative charge on the FB_1 molecule at neutral to high pH. While both polarities of sorbent were effective, montmorillonite clays demonstrated a higher capacity for sorption of FB_1 than ferrihydrite. These in vitro results were confirmed in a rodent model where urinary FB_1 was reduced 27% in NovaSil treated rats vs. controls. Finally, in a Ghanaian population co-exposed to aflatoxins and fumonisins, urinary FB_1 was significantly reduced at 2 time points when the NovaSil treatment was compared to placebo.

DEDICATION

To my wife Karla, whose love, beauty, patience, kindness, and motivation have been the safe harbor in what many times was a tumultuous journey through my personal and academic pursuits; to my parents who instilled in me a solid work ethic and made me believe that nothing was impossible if I worked hard enough; to Dr. Deborah Kochevar, who often times saw in me what I did not see in myself and pushed me to the limits of what I could accomplish and further, and to my friends who have provided perspective and guidance when I was so lost that I could not see beyond what was in front of me.

Some people come into our lives and quickly go. Others stay awhile, make footprints on our hearts and we are never, ever the same.

- Anonymous

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CHAPTER I

INTRODUCTION

1.1 Fumonisin B_1 – The problem defined

Mycotoxins are structurally diverse compounds produced by fungi that contaminate various agricultural commodities under pre-and post- harvest conditions. Tropical weather conditions including: high temperatures and humidity, excessive rains during harvest and flash floods lead to fungal proliferation and production of mycotoxins. Pre-harvest, harvest, and post-harvest practices and conditions; methods of transportation, marketing and processing; and insect damage all contribute to mycotoxin contamination (Wagacha, 2008). Although there are over 300 known mycotoxins, only a few, particularly those affecting cereals (maize, wheat, barley, oats, sorghum, rice) and groundnuts, are considered a significant risk to human health (Hussein and Brazel, 2001). These include aflatoxins, tricothecenes, fumonisins, ochratoxin, zearalanone, patulin and ergot alkaloids. (Pitt, 2000) (Table 1) This work will focus on the fumonisin toxins, specifically fumonisin B_1 (FB_1), a mycotoxin produced by *Fusarium verticillioides* and *F. proliferatum*. Although the term "fumonisin" refers to more than 15 different structural analogs of fumonisin toxin (Marin et. al., 2004), this term will be reserved for describing only the B_1 isoform,

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This dissertation follows the style of *Toxicological Sciences.*

TABLE 1

Health effects of common mycotoxins

Mycotoxin	Affected Crops	Health Effects
Afaltoxins	Peanuts, corn, wheat, rice, cottonseed, nuts, dairy, products	Heaptotoxicity, bile duct hyperplasia, liver tumors
Fumonisins	Corn	Leukoencephalomalacia, porcine pulmonary edema, esophageal cancer, hepatocellular carcinoma, Neural tube defects, liver cancer
Ochratoxin A	Wheat, barley, oats, corn, peanuts, cheese	Tubular necrosis of kidney, porcine nephropathy, liver damage, teratogenesis, kidney tumors
Patulin	Apples, apple juice, wheat	Brain and lung edema, lung hemorrhage, paralysis of motor nerves, antibiotic, carcinogenesis
Trichothecenes (T-2, DON, diacetoxyscirpenol)	Corn, wheat	Digestive disorders, oral lesions, hemorrhage of stomach, heart, intestines, lung, bladder, kidney, edema
Zearalenone	Corn, hay	Estrogenic effects, e.g., edema of vulva, uterine enlargement, testicular astrophy, abortion

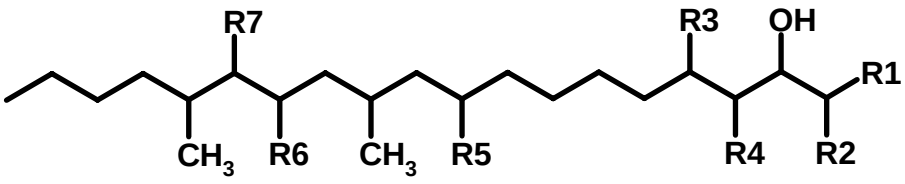
Adapted from the CAST report, 1989

the most common and toxic fumonisin species, unless otherwise specified (Figure 1; Table 2).

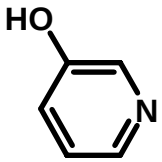
1.2 Historical background

The growth and production of maize or Indian corn likely originated in southern Mexico. Teosinte, a grass that dates back 9000 years, is considered to be the oldest maize and was found by archaeologists near the Central Balsas River Valley near Puebla, Mexico. Archeologists made this discovery based on the original work of Dr. George W. Beadle, a 1958 Nobel Laureate, whose original work comparing the genome of “modern maize” and teosinte lead to the familial link. From there it was a search to find the oldest evidence of teosinte cultivation which lead archeologists to Mexico (Carroll, 2010). Surveys have shown that maize is grown in every suitable agricultural region of the world and that a crop of maize is being harvested somewhere around the globe every month of the year. Maize grows from latitude 58° in Canada and the former Union of Soviet Socialist Republics to latitude 40° in the Southern Hemisphere. Maize crops are harvested in regions below sea-level in the Caspian Plain and at altitudes of more than 4000 m in the Peruvian Andes (FAO, 1992) (Figure 2).

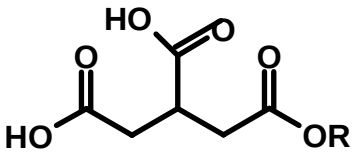
Searches of literature suggest that some of the earliest cultivators of maize (teosinte) were the indigenous people in the Americas in the area now known as Oaxaca, Mexico. Maize was an essential item in Mayan and Aztec civilizations and had an important role in their religious beliefs, festivities and



Fumonisin Backbone



3-Hydroxypyridinium (3HP)



Tricarballylic Acid (TCA)

Figure 1. Two-dimensional structure of the fumonisin back bone, 3-hydroxypyridinium and tricarballylic acid.

TABLE 2

Fumonisin toxin structural analogs

Analog	Side chains to the fumonisin backbone						
	R1	R2	R3	R4	R5	R6	R7
FA_1	CH_3	$NHCOCH_3$	H	OH	OH	TCA	TCA
FA_2	CH_3	$NHCOCH_3$	H	OH	H	TCA	TCA
FA_3	CH_3	$NHCOCH_3$	H	H	OH	TCA	TCA
$PHFA_{3a}$	CH_3	$NHCOCH_3$	H	H	OH	OH	TCA
$PHFA_{3b}$	CH_3	$NHCOCH_3$	H	H	OH	TCA	OH
HFA_3	CH_3	$NHCOCH_3$	H	H	OH	OH	OH
FAK_1	CH_3	$NHCOCH_3$	H	OH	OH	TCA	O
FBK_1	CH_3	NH_2	H	OH	OH	TCA	O
FB_1	CH_3	NH_2	H	OH	OH	TCA	TCA
Iso-FB_1	CH_3	NH_2	OH	H	OH	TCA	TCA
$PHFB_{1a}$	CH_3	NH_2	H	OH	OH	OH	TCA
$PHFB_{1b}$	CH_3	NH_2	H	OH	OH	TCA	OH
HFB_1	CH_3	NH_2	H	OH	OH	OH	OH
FB_2	CH_3	NH_2	H	OH	H	TCA	TCA
FB_3	CH_3	NH_2	H	H	OH	TCA	TCA
FB_4	CH_3	NH_2	H	H	H	TCA	TCA
FB_5							
FC_1	H	NH_2	H	OH	OH	TCA	TCA
N-acetyl-FC_1	H	$NHCOCH_3$	H	OH	OH	TCA	TCA
Iso-FC_1	H	NH_2	OH	H	OH	TCA	TCA
N-acetyl-iso-FC_1	H	$NHCOCH_3$	OH	H	OH	TCA	TCA
OH-FC_1	H	NH_2	OH	OH	OH	TCA	TCA
N-acetyl-OH-FC_1	H	$NHCOCH_3$	OH	OH	OH	TCA	TCA
FC_3	H	NH_2	H	H	OH	TCA	TCA
FC_4	H	NH_2	H	H	H	TCA	TCA
FP_1	CH_3	3HP	H	OH	OH	TCA	TCA
FP_2	CH_3	3HP	H	OH	H	TCA	TCA
FP_3	CH_3	3HP	H	H	OH	TCA	TCA

Adapted from Rheeder et al., 2002

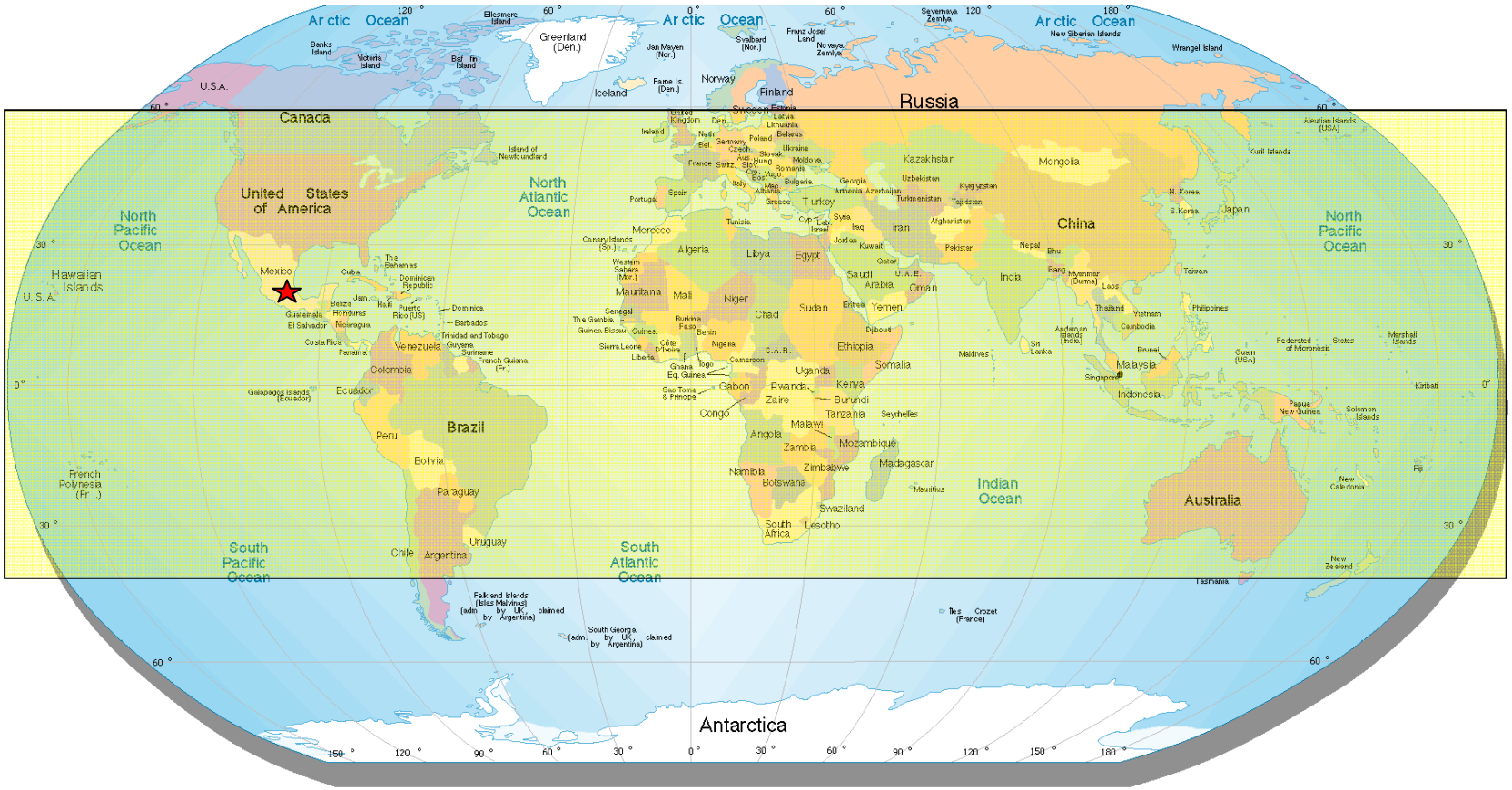


Figure 2. Food and Agriculture Organization of the United Nations global growth of maize. The origin of maize is indicated by the star.

nutrition. They claimed that “flesh and blood were made from maize”. These early civilizations made a type of oven- baked bread from maize that was eaten with fish, grasses and vegetables. This balanced diet likely prevented the symptoms that would later be associated with a diet based predominately on corn consumption (FAO, 1992; Karthikeyan and Thappa, 2002).

The ancient Mayan and Aztec civilizations used lime and ash to create alkaline solutions in order to process maize in a process known as nixtamalization. Nixtamalization was very important in the early Mesoamerican diet, as niacin (vitamin B_3) is not bioavailable in unprocessed corn. Alkaline hydrolysis unbinds and releases niacin for utilization in NAD and NADH production. It is debatable, however, as to whether these early civilizations understood the full implications of their discovery outside of tortilla production (Harper et al, 1958; Christianson et al., 1968; FAO, 1992).

Christopher Columbus discovered cultivated maize in Haiti, where it was known as MAHIZ, a name perhaps originating from the Mayan people responsible for its diffusion. Shortly thereafter, he introduced maize to Europe in the late 15th century, being grown in Spain as early as 1498. Maize spread throughout Europe and became the staple of an unbalanced diet in many poorer countries due to its low cost. Despite its successful cultivation, the critical nixtamalization process was omitted. Without the alkaline processing, maize lacked niacin bioavailability and malnutrition struck many areas where maize consumption had become dominant. This nutritionally deficient disease was first

described by Casal in 1735 as “mal de la rosa” with classic symptoms of dermatitis, diarrhea, and dementia, with death as the eventual outcome (Karthikeyan and Thappa, 2002). By the 19th century, pellagra epidemics were recorded in France, Italy, and Egypt (Marasas, 1996). In 1881, Italian born botanist and mycologist Pier Andrea Saccardo hypothesized that a fungus found on moldy corn was the causative agent in the European pellagra out breaks. Saccardo identified the causative organism as *Oospora verticillioides*. While Saccardos’s hypothesis was ultimately disproven, he helped lay the ground work implicating moldy corn in the pathogenesis of human and animal disease. Nearly 50 years later, in 1922, Goldberger and Tanner first suggested that the symptoms associated with pellagra were caused by an amino-acid deficiency. (Karthikeyan and Thappa, 2002)

In 1904, John Sheldon first identified *Fusarium moniliforme* from moldy maize in feed associated with animal diseases (Sheldon, 1904). Although this anamorph name was used widely by plant pathologists throughout the twentieth century, Manns and Adams were the first to suggest that the *Oospora verticilliodes*, described by Saccardo and *Fusarium moniliforme*, described by Sheldon were identical (Manns and Adams, 1923). In 1924, Wineland described the teleomorph of *F. moniliforme* as *Gibberella moniliformis*. Realizing that these maize contaminants were identical, Nirenberg combined the above to form *F. verticillioides/ G. moniliformis* in 1976, which is the commonly accepted

nomenclature (Kriek, 1981). Research on the production and health effects caused by consumption of moldy corn followed shortly thereafter.

1.3 Conditions leading to the growth of *Fusarium* fungi – The source of fumonisin B_1

Fumonisin has been known to contaminate wheat and cereal grains, but it is primarily found in maize (Marasas, 1995). Fungi in the genus *Fusarium* are the most common causes of ear and kernel rot diseases of maize. Historically, *F. moniliforme* had been reported as the most common pathogen causing Fusarium ear rot; currently, its synonym *F. verticillioides* is considered the predominant species, and it seems clear that some previous reports of *F. moniliforme* overlooked other fumonisin producing species such as *F. subglutinans* and *F. proliferatum* (Munkvold, 2003). *Fusarium* ear rot is characterized by its diffuse white to pink cotton-like appearance on the ear. In many instances, infected kernels are scattered around the cob among healthy-looking kernels and display white streaks, in a starburst appearance, on their surface. These symptoms of corn infected by *F. verticillioides* are identical to those seen in *F. proliferatum*, and *F. subglutinans* contamination. Occasionally, these symptoms are also associated with contamination by other Fusarium species.

Gibberella ear rot or “red ear rot” usually initiates from the tip of the ear and develops a red or pink mold covering a large proportion of the ear. Usually,

it is caused by *Fusarium graminearum*. *Gibberella* ear rot predominates in cooler areas or those with higher precipitation during the growing season.

Of the 13 species that are able to produce fumonisins, only those belonging to the Liseola section, especially *F. verticillioides* and *F. proliferatum*, have been associated with fumonisin contamination of agricultural products (Table 3). The severity of ear infection has been shown experimentally to be a good indicator of fumonisin accumulation in corn ears inoculated with *F. verticillioides* (Marin et al, 2004). Among all the known fumonisins produced by Fusarium spp., FB_1 is the fumonisin analog most commonly associated with food contamination and disease formation in animals and humans. Environmental conditions are known to have a significant effect on *Fusarium* contamination and toxin production on cereals (Table 4). Two related factors that have an important impact on the activity of *Fusarium* section Liseola are water activity (a_w) and temperature (Doohan et al., 2003). The a_w of a food is defined as the ratio between the vapor pressure of the food itself, when in a completely undisturbed balance with the surrounding air media, and the vapor pressure of distilled water under identical conditions. A water activity of 0.80 means the vapor pressure of the analyte is 80 percent of that of pure water. In most situations, the water activity increases with temperature. Most foods have a water activity above 0.95 and that will provide sufficient moisture to support the growth of bacteria, yeasts, and mold (US FDA,1984).

TABLE 3

Fumonisin-producing *Fusarium* species, analogs produced, and the maximum yields of FB_1, FB_2 and FB_3 reported for each species

Fusarium sp.	Fumonisin analog(s)	Maximum fumonisin level (mg/kg) for:		
		FB_1	FB_2	FB_3
Section Liseola				
F. verticillioides MP-A	FA_{1-3}, FB_{1-5}, iso-FB_1, FAK_1, FBK_1, $FC_{1,4}$, FP_{1-3}, PH_{1a-b}	17900	3000	2300
F. fujikuroi MP-C	FB_1	21	NT	NT
F. sacchari MP-B	FB_1	7	NT	NT
F. proliferatum MP-D	FA_{1-3}, FB_{1-5}, FAK_1, FBK_1, $FC_{1,4}$, FP_{1-3}, PH_{1a-b}	31000	17000	5700
F. subglutinans MP-E	FB_1	230	NT	NT
F. thapsinum	FB_{1-3}	30	5	5
F. anthophilum	FB_{1-2}	610	35	NT
F. globosum	FB_{1-3}	330	4	24
Section Dlaminia				
F. nygamai	FA_{1-3}, FB_{1-5}, FAK_1, FBK_1, $FC_{1,4}$, FP_{1-3}, PH_{1a-b}	7200	530	140
F. dlamini	FB_1	82	NT	NT
F. napiforme	FB_1	480	NT	NT
F. pseudonygamai	FB_{1-2}	Tr	Tr	NT
F. andiyazi	FB_1	Tr	ND	NT
Section Elegans				
F. oxysporum	$FC_{1,3-4}$, N-acetyl-FC_1,iso-FC_1, N-acetyl-iso-FC_1, OH-FC_1, N-acetyl-OH-FC_1	NT	NT	NT
F. oxysporum var. redolens	FB_{1-3}	300	6	0.9
Section Arthrosporiella				
F. polyphialidicum	FB_1	500	NT	NT

NT, not tested. Tr, trace amounts (1 to 4 ng g^{-1}) were detected. ND, not detected (<1 ng g^{-1}). Adapted from Rheeder et al., 2002.

TABLE 4

***Fusarium* fungi and mycotoxin production**

Toxin	Species	Substrates	Optimum production conditions
Type A tricothecenes [T-2 toxin, HT-2 toxin, neosolaniol, diacetoxyscirpenol]	*F. sporotrichioides, F. poae*	Barley, oats, rice, wheat, maize	Moderately warm and humid (20 - 15C, a_w = 0.990)
Type B tricothecenes[deoxynivalenol (DON), 15 acetyl-DON, nivalenol (NIV)]	*F. graminearum, F. culmorum*	Barley, wheat, rice, maize	Warm and humid (25-28C, a_w = 0.97)
Zeralenone	*F. graminearum, F. culmorum*	Wheat, rice, maize	Warm (17-28C) or temp cycles (e.g. 25-28C for 14-15 days, 12-15C for 20-28 days) and humid (a_w = 0.97)
Fumonisins	*F. verticillioides, F. proliferatum, F. subglutinans*	Maize	Cool to warm conditions and humid (15-30C, a_w = 0.98)
Moniliformin	*F. subgultinans, F. verticillioides, F. avenaceum*	Wheat, rye, barley, oats, maize	Warm temperatures (25-30C)

Adapted from Doohan et.al., 2003; a_w = water activity

Studies have shown for a given temperature, a_w seems to dictate the metabolic activity of *F. verticillioides* and *F. proliferatum*. Between 15 - 30°C, an a_w of $\geq$ 0.88 is required for fungal germination, however, an $a_w \geq 0.90$ is required for growth for the fungi. The a_w limit for FB_1 production is $\geq$0.92-0.93, however, *F. verticillioides* and *F. proliferatum* prefer different temperatures for this production, 10-25°C and 10-35°C, respectively (Figures 3 and 4). Generally, optimal conditions for *F. verticillioides* growth and fumonisin production are 20-25°C with a water activity of 0.95-1.0, however the same activity in *F. proliferatum* preferentially occurs at 15°C (Marin et al, 2004).

It is no coincidence that regions worldwide with tropical and subtropical climates report increased prevalence of *Fusarium verticillioides* contamination with some reporting increased incidences of esophageal cancer and neural tube defects attributed to fumonisin B_1 exposure. Maize production methods have a significant effect on the incidence of *Fusarium* contamination leading to corn rot. Maize is typically grown in a short rotation with one or two other crops in crop production areas with little overall crop diversity. As a result, most fields where the maize is grown have some remnants of past corn crop in or on the soil, or such residue is present in nearby fields. This residue is the primary source of inoculum for infections of maize kernels (Smith and White, 1988). *Fusarium* species survive well on maize crop residue as mycelium or other survival structures. These *Fusarium* populations in the soil flourish on senescent maize tissues that are deposited there regardless

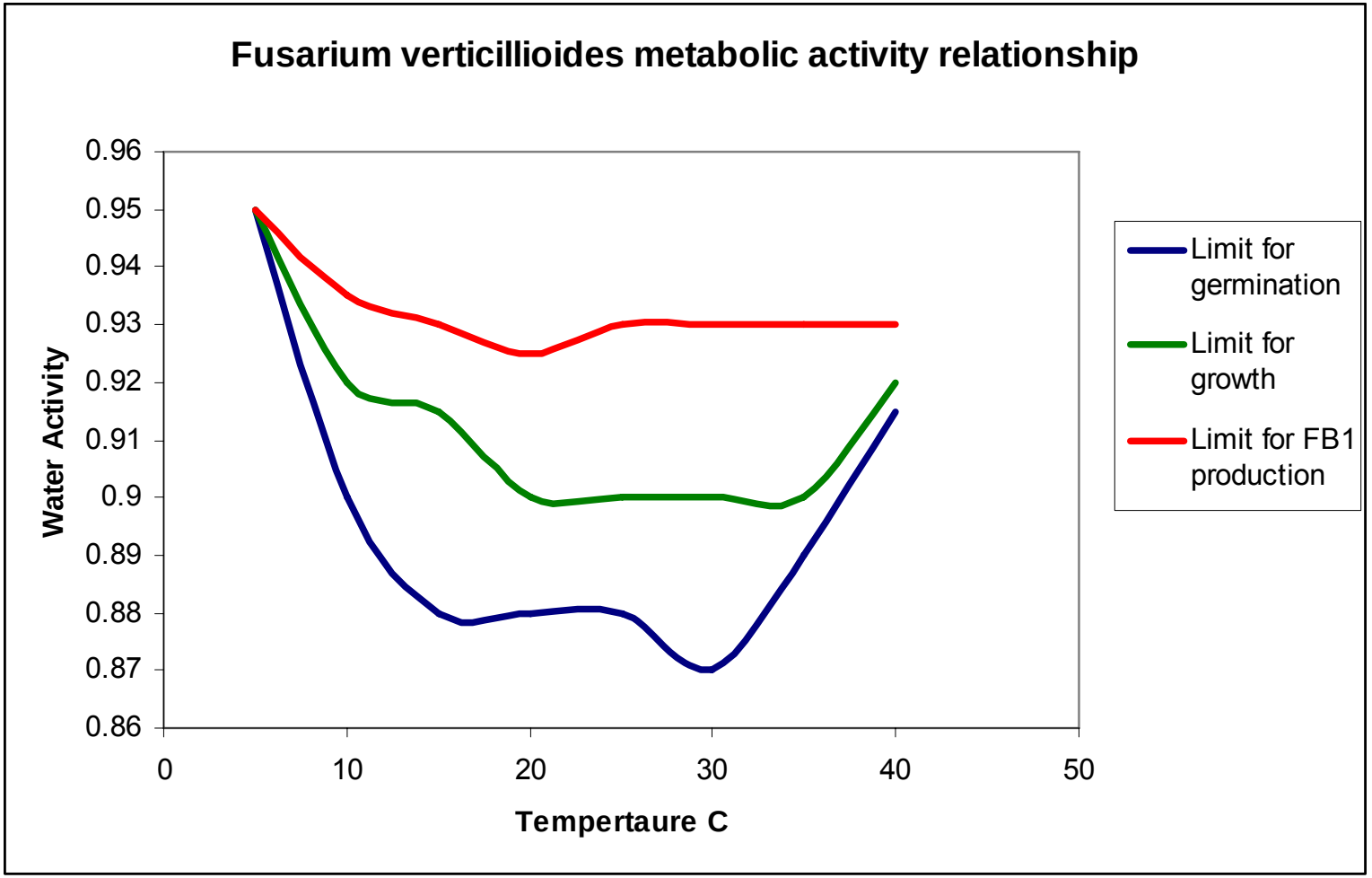


Figure 3. Isopleth showing the combined values of a_w and temperature that limit FB_1 production (1mg FB_1/g mold), growth (0.1mm/day), and germination (10% conidia) of isolates of *F. verticillioides*. An isopleth is a line drawn through all points of equal value for some measureable feature. Adapted from Marin et al., 2004.

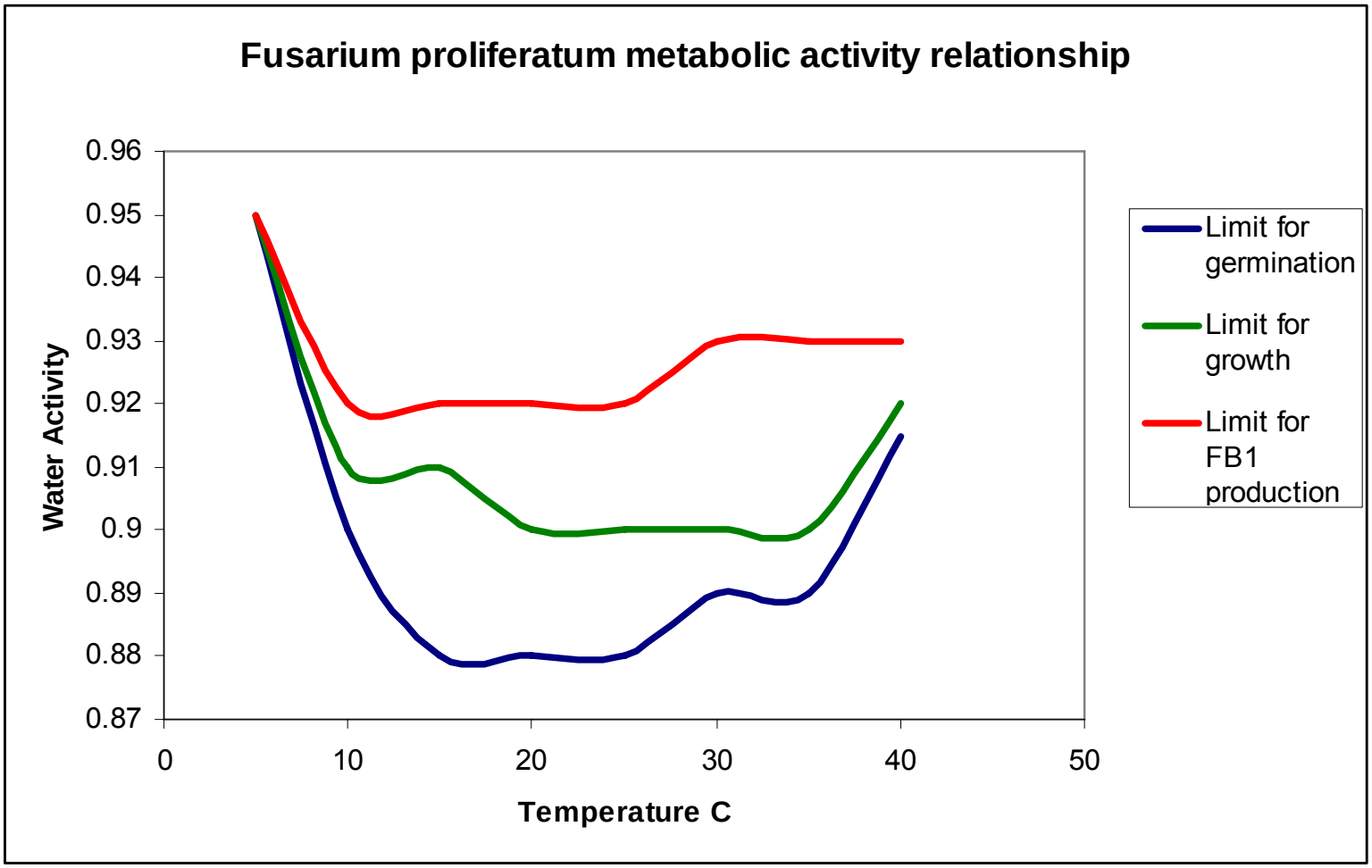


Figure 4. Isopleth showing the combined values of a_w and temperature that limit FB_1 production (1mg FB_1/g mold), growth (0.1mm/day), and germination (10% conidia) of isolates of *F. proliferatum*. An isopleth is a line drawn through all points of equal value for some measureable feature. Adapted from Marin et. al., 2004.

of whether they were previously infected. *Fusarium* species can also colonize senescent tissues of other crop and weed species that are not considered hosts for these pathogens (Munkvold, 2003).

F. verticillioides employs several maize infection pathways including silk infection, insect injury and systemic transmission from seeds or roots to kernels (Munkvold et al.,1997; Sobek and Munkvold, 1999). The relative importance of these infection pathways vary based on geographic region. In the Midwest region of the U.S., severity of *Fusarium* ear rot and symptomless kernel infection are correlated with insect injury, primarily due to *Ostrinia nubilalis*, which appears to be the most important infection pathway in this area (Munkvold, 2003). For reasons that are not entirely known, drought stress and insect damage to crops (especially corn) are inextricably linked. Furthermore, *Fusarium* ear rot severities have been correlated to wound size caused by insect damage in studies of experimental inoculation methods (Miller, 2001).

The relative importance of lepidopteran insects (a large order of insects that includes butterflies and moths) in disease development caused by *F. verticillioides* versus *F. graminearum* can be illustrated by how insect control affects the success of the two pathogens. In the U.S., transgenic Bt maize hybrids, have been very successful in preventing damaged from insects (Bucchini and Goldmann, 2002). These hybrids, which are highly resistant to European corn borer moth injury, have much lower levels of *Fusarium* ear rot and fumonisins (up to 90% reduction), compared to conventional hybrids

(Munkvold et al., 1999). Bt hybrids have also been shown to have lower levels of DON (as a result of reduced *F. graminearum* infection), but the reductions have been more modest (up to 59% reduction) (Schaafsma et al., 2002). A similar situation exists in Europe as reductions in deoxynivalenol, nivalenol and zearalenone were not as great as reductions in fumonisins when these insects were controlled with transgenic insect resistance in the field in France and Spain (Bakan et al., 2002). It is believed that insect damage does not seem to be as critical in *F. graminarium* infection when compared to *F. verticilliodes* infection.

Aside from insect damage, there are other significant pathways of *Fusarium* infection. Secondary to insect injury, infection through silks has been shown to be a significant source of *Fusarium* ear rot and symptomless infection by F. verticillioides (and probably *F. proliferatum* and *F. subglutinans*) (Desjardins et al., 2002; Munkvold et al., 1997; Nelson, 1992). Systemic transmission of the fungus seems to be of lesser importance, but reports of the frequency of this infection method vary widely (Desjardins et al., 1998; Munkvold and Carlton, 1997). There may be environmental conditions under which systemic transmission is more prevalent, but this has not yet been demonstrated (Figure 5).

In addition to these exogenous biotic factors, endogenous biotic conditions may also have an impact on the growth and toxin production of *Fusarium* species. These biotic factors include but are not limited to availability of growth substrates and competition from other organisms that have adapted to be

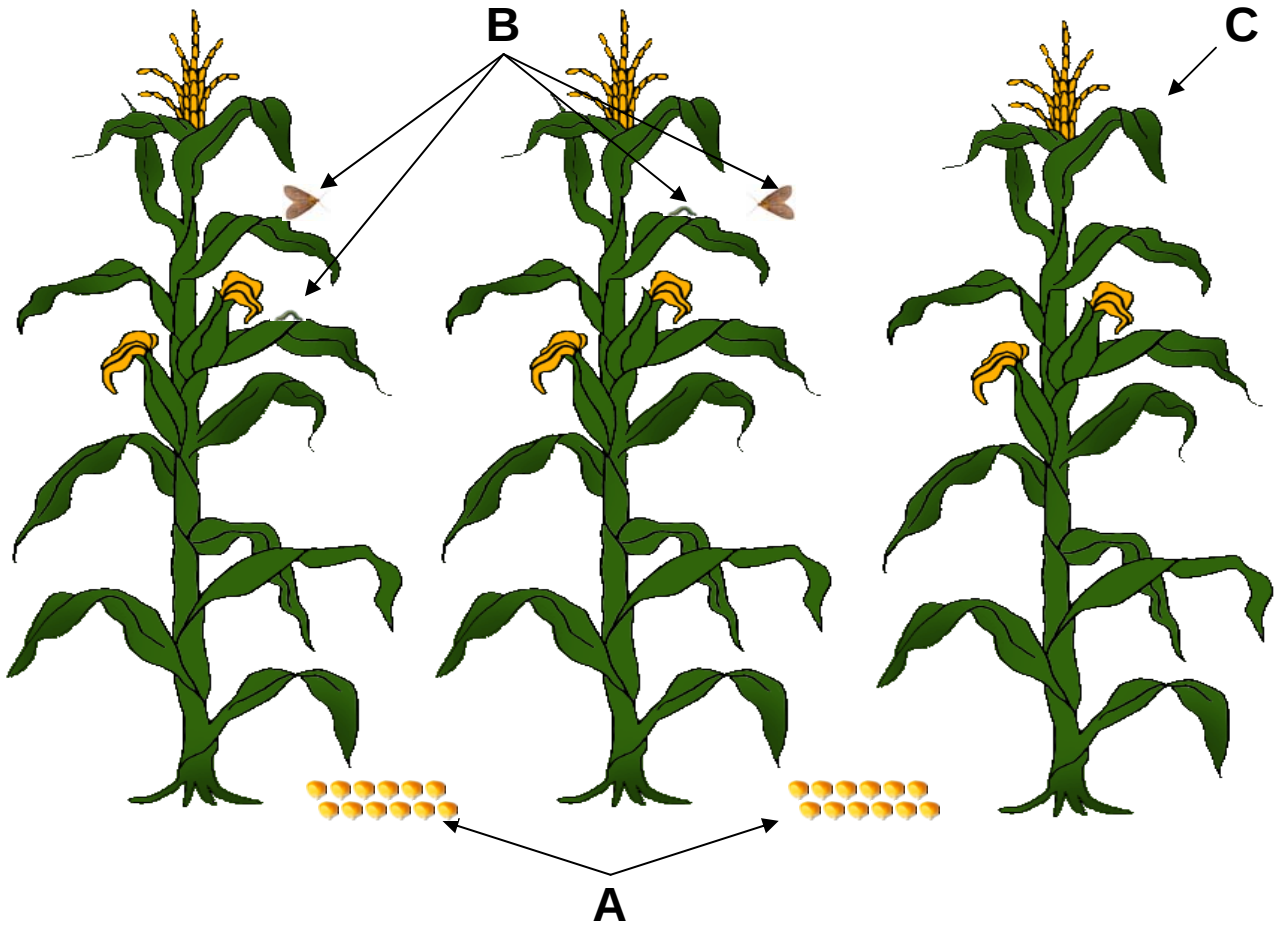


Figure 5. Factors that contribute to *Fusarium* contamination of maize. Insect damage from *Ostrinia nubilalis* (**B**) is directly correlated to contamination of maize by *Furarium verticillioides/ proliferatum*. Fallen maize or other plants can act as a host for *Fusarium* spp. innoculum (**A**). Maize hybrids with the *Bacillus therogenesis* gene have been effective in reducing contamination from *Fusarium* spp (**C**).

successful in that niche (Miller, 2001). The success or failure of competing fungi is dependant on temperature and water activity which influence growth and production of toxins. The energy and material commitment required for *Fusarium* spp. to produce fumonisin is significant. It is plausible that resource expenditure for the production of these toxins by *Fusarium spp*. hinges on two functions: energy storage and protection from other organisms, although reviews of current literature have been unable to confirm or refute this hypothesis.

1.4 Fumonisin B_1 production by *Fusarium* spp.

Fumonisin B_1 contamination of cereal grains is an endemic problem (Shephard et al., 1996; Marin et al., 2004). It was first identified in 1988 by Gelderblom et al. as a novel toxin produced by *Fusarium moniliforme* that had cancer promoting properties (Gelderblom et al., 1988) . Since then, FB_1 has been shown to be a secondary metabolite produced by multiple species of the fungal Genus *Fusarium*, but is predominantly produced by *F. verticillioides* and *F. proliferatum*. As previously mentioned, the conditions that lead to growth and proliferation of *Fusarium spp*. capable of producing FB_1 may not necessarily lead to the production of the mycotoxin.

The structural components of the A and B fumonisins were first described by Bezuidenhout in 1988 (Bezuidenhout et al. 1988). Advances in technology have allowed further characterization of the components of FB_1. Stable isotope labeling of fumonisin has shown that the backbone of FB_1 is of polyketide origin;

predominantly due to the condensation of acetate. Studies feeding radio labeled amino acids to *F. verticillioides* corroborate the hypothesis of polykeitde synthesis versus a lipid biosynthetic method in ideal conditions for production of FB1 (ApSimon, 2001). The methyl groups at C12 and C16 are derived from methionine; the tricarballylic acid side chains are derived from glutamic acid; and the amino group is derived from serine (Marin et al., 2004). Oxygen moieties found in the FB_1 molecule are derived from 2 sources: molecular oxygen is utilized for the oxygen containing moieties along the carbon backbone and water is used for the tricarballylic acid groups esterified at C14 and C15 (ApSimon, 2001). Fumonisin production under stress conditions has yet to be analyzed as far as substrate utilization is concerned.

The stereochemical structure of FB_1 was delineated from 1024 possible stereoisomers using reactive chemical analysis, x-ray crystallography, and NMR (ApSimon, 2001). It was found to be structurally similar to *Alternaria alternata lycopersic*i (AAL) toxin, the only naturally occurring non-fumonisin inhibitor of sphinganine N-acetyl transferase activity (Abbas and Riley, 1996). Although the lewis structure and skeletal formula for FB_1 depict a molecule that is spread out, molecular modeling and energy minimization studies by Beier and Stanker indicate that the fumonisin molecule is most likely folded on its self to form a cage-like structure. Intermolecular hydrogen bonding has been identified as the most likely explanation of this phenomenon. The implications of this structural orientation on membrane interactions and intercellular transport have yet to be

determined (Beier and Stanker, 1997). Structurally, fumonisins are very similar to endogenous sphingolipids and it is this resemblance that is believed to be the basis of their toxicity (Merrill et al., 1996).

1.5 Fumonisin B_1 toxicity and mechanism of action

The mechanism of fumonisin B_1 toxicity is complex and not fully understood. FB_1 is known to produce a number of intracellular and structural changes in target cells due to its enzyme specific activity. These changes are more marked in some species than others and are most likely the source of species specific pathology following exposure to fumonisin toxins. Because of the diversity of these effects, an all encompassing mechanism of toxicity is currently unknown.

1.5.1 Cellular effects of fumonisin B_1 exposure

Although the details of fumonisin transport have not been fully elucidated, these toxins are thought to gain entrance into hepatic and kidney cells through organic anion/cation transporters, which lead to their untoward effects. Although no structural differences have been reported between organic transporters in human and animal species (suggesting toxin mechanistic similarity), variations in the concentration of these cell surface transporters may account for the differences in species sensitivity (Tachampa et al., 2008). These variations may be significant contributors in the development of species specific

diseases such as equine leukoencephalomalacia and porcine pulmonary edema after ingestion of FB_1. Once inside target cells, FB_1 has been reported to cause a number effects including: moderation of apoptosis, disruption of cell signaling pathways, increased production of reactive oxygen species, activation of caspase 3, reduction of glutathione, increases in tumor necrosis factor – α, and moderation of interferon and interleukin production. (Stockmann-Juvala and Savolainen, 2008; Sweeney et al., 1998, Merrill et al, 2001; Panjarian et al., 2008). These effects are most likely a down stream result of the disruption of sphingolipid metabolism through the inhibition of ceramide synthase (Merrill et al., 1996; Smith and Merrill, 1995; Hannun et al., 1986).

1.5.2 Inhibition of ceramide synthase by fumonisin B_1 and derivative molecules

Ceramide synthase (CerS) is the cornerstone enzyme involved in sphingolipid metabolism. CerS is responsible for the production of ceramide and complex sphingolipids from the simple sphingolipids sphinganine/sphingosine and fatty acyl-coAs of varying length (C16-C24) (Pewzner-Jung et al, 2006) (Figure 6). Ceramide is involved in the intracellular and extracellular functions critical to the survival and activity of a cell including differentiation, growth promotion, proliferation, senescence, apoptosis, and necrosis (Kolestnick, 2002; Okazaki et al. 1989; Venable et al., 1995; Hetz et al., 2002; Adam et al., 2002;

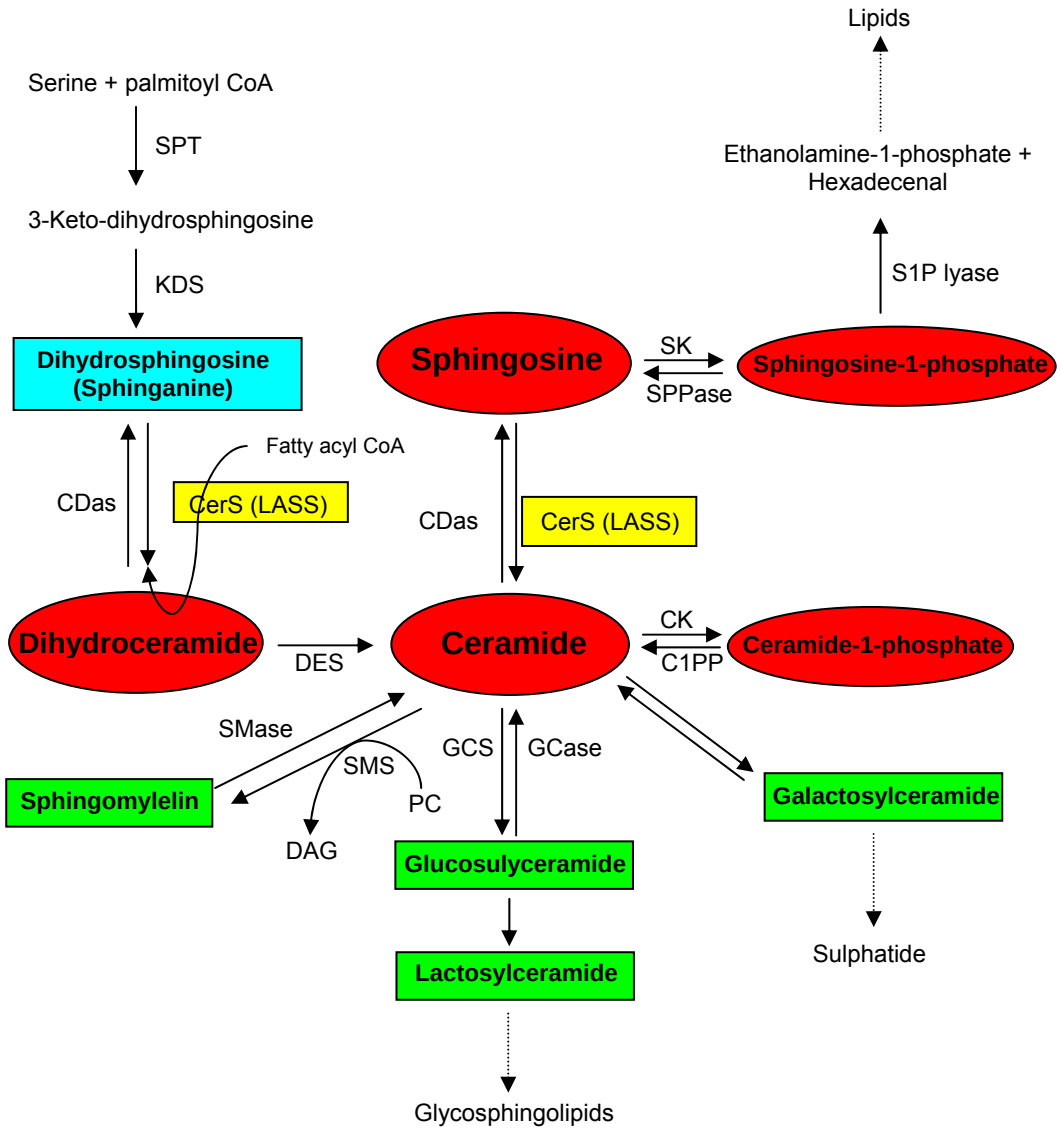


Figure 6. Scheme of sphingolipid metabolism. Bioactive SL metabolites are highlighted in red. Sites of fumonisin B_1 inhibition are highlighted in yellow which causes an increase in sphinganine highlighted blue. Cell membrane components and complex sphingolipids are highlighted in green. SPT, serine palmitoyl transferase; KDS, 3-keto-dihydrosphingosine reductase; DES, dihydroceramide desaturase; SPPase, Sphingosine phosphate phosphatase; CK, Cer kinase; C1PP, C1P phosphatase; SMS, Sphingomyleline synthase; PC, phosphatidylcholine; DAG, diacylglycerol; GCS, glucosylceramide synthase; GCase, glucosyl CDase. CDas, Ceramidase. Adapted from Bartke and Hannun, 2009.

Obeid et al, 1993; Wang et al., 2005; Blazquez et al. 2000; Ruvolo, 2003; Heinrich et al, 1999).

In addition to its activities as a moderator of cellular function, ceramide also serves as a transient precursor molecule in the production of complex sphingolipids. These complex sphingolipids are critical for the maintenance of membrane structure (especially microdomains such as caveolae), and serve as binding sites for extracellular matrix proteins as well as for some microorganisms, microbial toxins, and viruses. These sphingolipids are also found in lipoproteins and other lipid-rich structures used in transport and signaling (Merill et al, 2001).

Fumonisin B_1 is known to exert its toxic effects through the inhibition of ceramide synthase (sphinganine/ sphingosine N- acetyl transferase) (Merrill, et al., 1993;Merrill et al, 1996; Desai et al, 2002; Riley et al., 1996). Fumonisins are structural analogs of the sphingoid bases sphinganine (SA) and sphingosine (SO), posessing a similar, but not identical, aminopentol backbone structure (fumonisin B_1 possesses a 20- C aminopentol while sphinganine/ sphingosine contain an 18-C aminopentol). Fumonisins are esterfied at the C14 and C15 carbons with tricarballylic acid side chains, whereas SA and SO are not. Additionally, the C1 carbon is hydroxylated in sphinagnine/ sphingosine and not fumonisin B_1. Despite these differences, there is enough similarity between fumonisins and sphingolipids to bind to ceramide synthase, but not enough for FB_1 to be used as a substrate in natural form (Figure 7). For this reason, direct

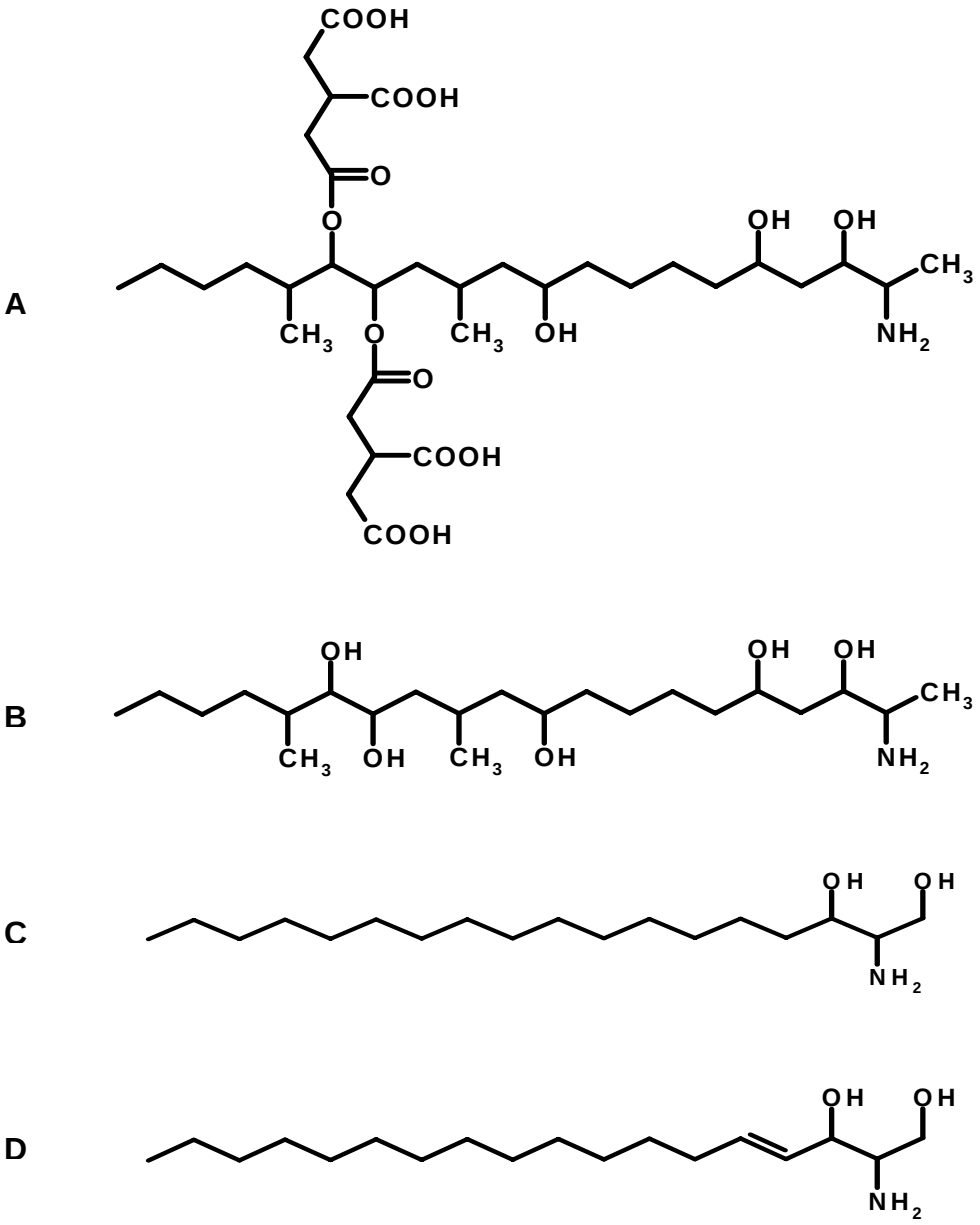


Figure 7. Two dimensional structures of fumonisin B_1, aminopentol, sphinganine, and sphingosine. (fumonisin B_1 (**A**), aminopentol (**B**), sphinganine (**C**), and sphingosine (**D**)) Note the similarity in backbone structure. All of the above can be used as substrates by ceramide synthase except for FB_1. Aminopentol (**B**) is the product of base catalyzed hydrolysis of the FB_1 tricarballylic acid esters due to the conditions in the maize nixtamalization process.

competitive inhibition of enzymatic activity is thought to be the major route of toxicity. In the current model of how fumonisin B_1 inhibits ceramide synthase, the aminopentol backbone competes for binding of the sphingoid base substrate, whereas the anionic tricarballylic acids interfere with binding of the fatty acyl-CoA (Desai et al, 2002; Merrill et al., 2001) (Figures 8 and 9).

Merrill et al. demonstrated that FB_1 most likely interacts with the binding sites for sphinganine and fatty acyl–coenzyme A (CoA) because its potency is influenced by the concentrations of both substrates (Merrill et al, 2001). Merrill also noted that removal of the tricarballylic acid side-chains decreased the initial effectiveness of ceramide synthase inhibition in vitro by approximately 10-fold. Moreover, FB_1 was not found to be acylated by ceramide synthase, but removal of the tricarballylic acid sidechains (producing the aminopentol form of fumonisin B_1 or AP_1) converted this inhibitor into a substrate. This was only possible if AP_1 occupies the sphingoid-base binding site and leaves the fatty acyl–CoA binding site accessible. The product of the reaction involving fully hydrolyzed fumonisins (AP-1) and palmitoyl co-A is N-palmitoyl-AP_1 (PAP_1) which has interesting properties. It is has been shown to be an inhibitor of ceramide synthase in vitro and causes a greater increase in sphinganine in HT-29 cells than FB1 or AP1 cause at comparable concentrations (Humpf et al., 1998; Desai et al, 2002, Seiferlein et al. 2007).

Although the mechanism for the inhibition of ceramide synthase by N-palmitoyl-AP_1 (PAP_1) is not known, the simplest explanation is that PAP_1

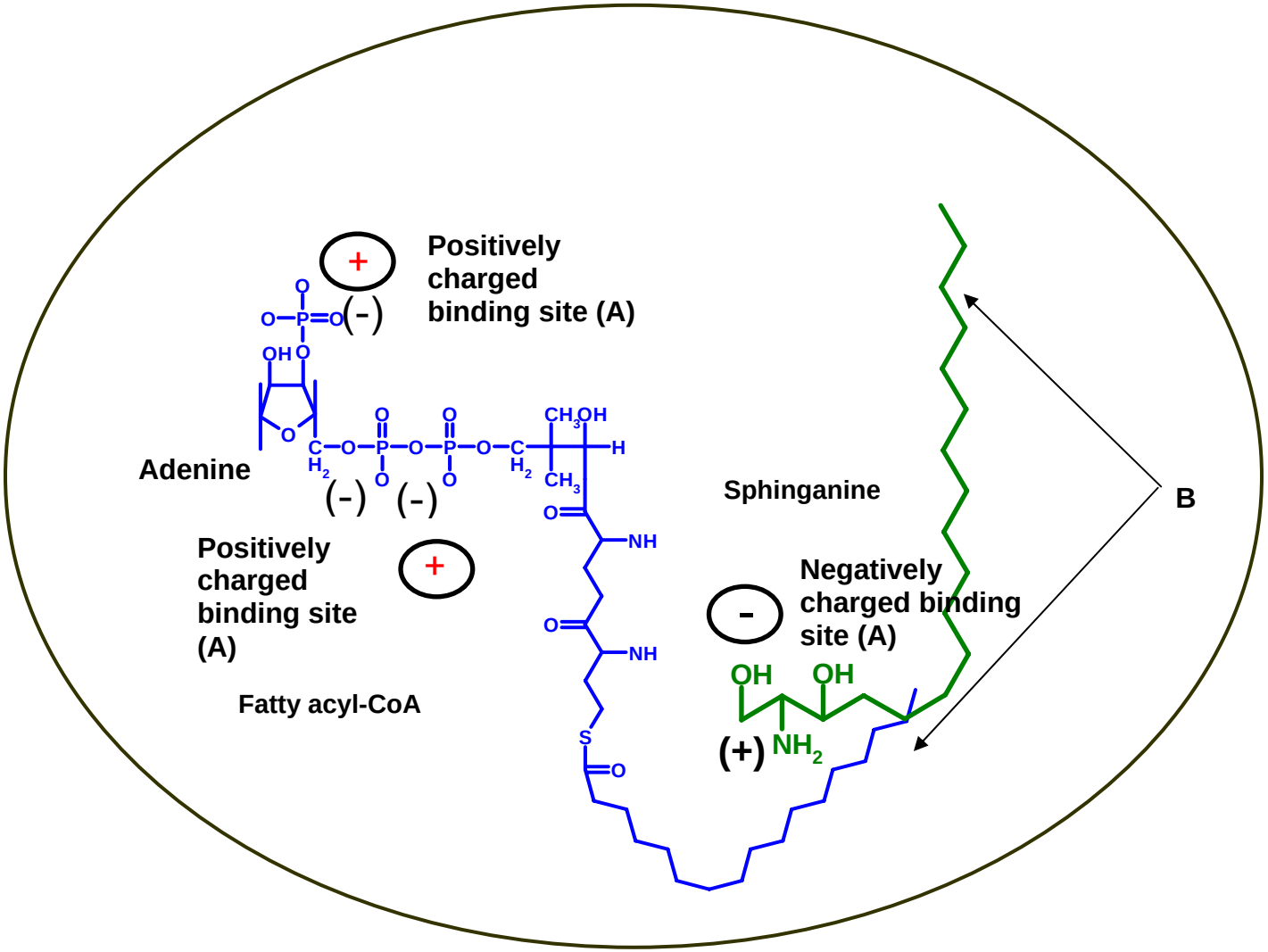


Figure 8. A model for the active site of ceramide cynthase and how fatty acyl-CoA and sphinganine interact electrostatically at sites to activate enzyme activity (**A**). Hydrophobic interactions may also play a role n the production of (dihydro)ceramide (**B**). Adapted from Merrill et al., 2001.

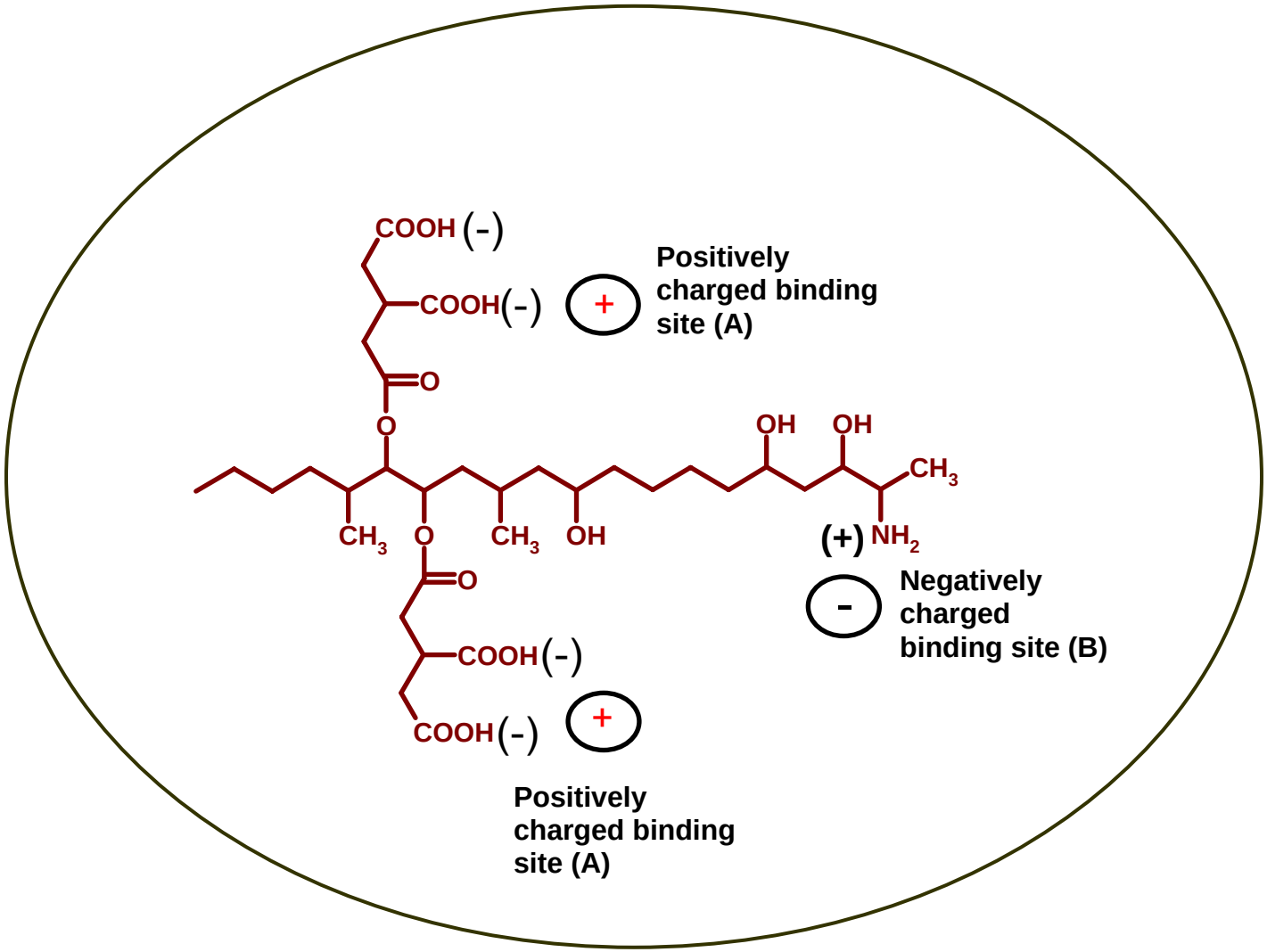


Figure 9. A model for the active site of ceramide synthase and how FB_1 mimics regions of both the sphingoid base and the fatty acyl-CoA substrates to interact electrostatically with ceramide synthase(**A and B**). FB_1 can not be acylated and simply acts as a competitive inhibitor. Adapted from Merrill et al., 2001.

most likely interacts with the hydrophobic binding domains for the substrates and product. As would be predicted from the increase in sphinganine, PAP_1 is more toxic than FB_1 or AP_1 for HT- 29 cells. Together, these findings suggest that when AP1 is taken up by cells, it can be converted to a more potent (toxic) inhibitor of ceramide synthase (Merrill et al., 2001; Humpf et al., 1998; Desai et al, 2002, Seiferlein et al. 2007). This may account for the observation that ceramide synthase is inhibited much less by AP_1 than FB_1, yet toxic effects still occur. During in vivo toxicity experiments, Voss et al. found that feeding nixtamalized Fusarium moniliforme culture material (which contains AP_1) to rats causes lesions in liver and kidney that are indistinguishable from those caused by consumption of a diet prepared from untreated (FB1-containing) culture material (Voss et al, 1996).

Although the focus of our study was non-hydrolized FB_1, the toxicity of AP1 warrants further investigation. The processing and preparation of food that results in the loss of tricarballylic side chains is a common practice in many regions of the world where maize is a staple, such as Central and South America where as much as 185 μg AP_1 has been found per gram of tortillas (Merrill et al., 2001). The nixtamalization of maize common in latin american food preparation results in a base catalyzed hydrolysis of the tricarballylic acids from fumonisin. An acid catalyzed hydrolysis of fumonisin may occur during the preparation of indigenous foods (kenkey) due to the fermentation process common in many African countries (Halm et al., 1996). Ultimately whether

through acid or base catalyzed hydrolysis, amounts of AP1 are formed, and are subject to metabolic activity of ceramide synthase leading to the formation of N-palmitoyl-AP_1 and inhibition of cerS. This is critical because risk assessment of fumonisin exposure currently focuses on parent FB_1 in food and biomarkers of exposure (urinary FB_1 and changes in sphingolipid ratio). Little to no attention is given to the potential presence of AP_1 in food and its FB_1 mimicking effects in the absence of detectable FB_1.

Increases in free sphinganine following cerS inhibition is due to the enzyme serine palmitoyl transferase (SPT-1) possessing no mechanistic feedback tied to ceramide synthase or intracellular sphinganine increases. SPT-1 is known as the rate limiting enzyme in de novo ceramide synthesis. SPT-1 continues to produce sphinganine as long as the precursor molecules are available regardless of the activity of downstream enzymes in sphingolipid metabolism. This leads to the initial change in the intracellular sphingolipid ratio that normally favors sphingosine concentration over sphinganine. This change is transient, as enzymes with complex sphingolipid catabolic abilities (ie, sphingmyelinases) are activated to break down complex sphingolipids into precursor molecules, notably ceramide and sphingosine. The increase in sphingosine helps to restore the “proper” intracellular sphingolipid ratio (He et al.,2006).

1.5.3 Effects of ceramide synthase disruption

Disruption of the de novo sphingolipid biosynthesis pathway due to inhibition from ingested fumonisin B_1 can lead to a number of untoward effects. In humans, the most notable and widely recognized effect associated with the consumption of FB_1 contaminated corn is the development of esophageal squamous cell carcinoma (Gong et al., 2009; Marasas et al., 1988; Syndenham et al., 1990; Hendricks and Parker, 2002). Ceramide a product of de novo sphingolipid biosynthesis is intimately involved in the apoptotic pathway mediated by caspase 3. The relationship between ceramide and caspase 3 has been demonstrated in vitro using human keratinocytes, In these studies there was a lack of apoptotic activity following irradiation with ultraviolet light in cell dosed with FB_1 or myriocin (a serine-palmitoyl transferase inhibitor). The inhibition of apoptosis seemed to occur in a caspase 3- independent fashion (Uchida et al, 2003). Fumonisin B_1 has also been known to indirectly interact (most likely through changes in the sphingolipid ratio) with other mediators of cellular mitogenic and apoptotic activity including protein kinase C, NF-κB, tumor necrosis factor –α. These cellular affectors possess both cell growth and apoptosis responsibility and it is suspected that in this manner FB_1 possesses both mitogenic and apoptotic capabilities. FB_1 related cellular changes have also been shown to interfere with mediators of the cell cycle including cyclin D1 (Bondy and Pestka, 2000; Buchner, 2000; Soriano et al., 2005). Early studies

with fumonisin B_1 failed to identify any genotoxic activity, and for this reason FB_1 has not been classified as a complete or initiating carcinogen in humans.

Following administration of a known genotoxin (diethylnitrosamine), however, FB_1 was able to promote the induction of hyperplastic foci in rat liver. Based on this and similar work, FB_1 is widely considered to be a promoter of neoplastic formation and as such has been classified in class 2B by IARC (IARC monograph, 2003). This classification is also due to in vivo studies in which rats fed FB_1 developed neoplasms in their kidneys and liver in the absence of another initiator. Fumonisins have not been shown to have a significant effect on P53 or peroxisome proliferation, however fumonisin consumption has caused accumulation of fatty acids in the liver which are subject to lipid peroxidation, free radical formation and damage leading to neoplasm formation (Gelderblom et al, 1996).

1.5.4 Hepatocellular carcinoma and the consequence of co-exposure to aflatoxin B1 and fumonisin B_1

Hepatocellular carcinoma (HCC) is the most common form of malignant liver cancer with an elevated incidence focused in developing regions of the world. In 2002, it was estimated that there were 600,000 new cases of liver cancer, 82% of which occurred in developing countries. Regardless of geographic region, diagnosis of HCC has an extremely poor prognosis due to its propensity to metastasize and form secondary tumor sites. The 5 year (1988-

2001) survival rate in the US was 8%, 9% in Europe (1995-1999), and only 5% when developing regions of Africa and Asia were compared. Of the numerous factors that contribute to the high incidence (diagnosed and un-diagnosed) of HCC, hepatitis infection was the most serious contributor. It was estimated the carriers of the hepatitis B surface antigen are 100 times more likely to develop HCC than their sero-negative counterparts (Chuang et al., 2009).

Mycotoxins, specifically aflatoxin B1 (AFB1), are also identified as significant risk factors for the development of HCC. Aflatoxin B1 is known to be a potent liver carcinogen; acting as an initiator by forming adducts at the N7 guanine of liver DNA and serving as a promoter, triggering inflammation in the organ. This N7 guanine adduct can lead to the formation of a more stable aflatoxin formamidopyrimidine (AFB1-FAPY) adduct which is often found as a persistent mutation in populations chronically exposed to aflatoxins (Wild and Gong, 2010). The synergism between HBV and aflatoxin exposure is demonstrated in populations that have an increased odds ratio of HCC compared to populations with hepatitis sans aflatoxin exposure (Kumagia et al, 2009). This is most likely due to AFB1's activity as a potent, persistent initiator, while HBV acts as a potent promoter of carcinogenesis due to inflammation.

There have been a number of studies describing the exposure to AFB1 in Africa, specifically the liver status and immune effects of AFB1 exposure in Ghanaians (Jolly et al, 2006). AFB1 appears to be ubiquitous throughout West Africa with >95% of sampled populations possessing the AFB1-albumin

biomarker of exposure (Wild and Gong, 2010). Combined with the high incidence of HBV in Ghana, HCC is the most common cause of cancer related death in adult men and third highest in adult women (Wiredu and Armah, 2006).

Notwithstanding the contributions of AFB1 and HBV to the incidence of HCC in Ghana, there is evidence to suggest that there may be another significant contributor to the high levels of liver cancer. In a study by Ankrah et al., although a large number of the participants demonstrated markers of liver inflammation, a significant difference between AFB1 exposed and non-exposed participants did not exist. One notable result from this study was 29% of the AFB1 positive and 62% of the AFB1 negative participants with markers of liver inflammation also demonstrated indicators of bile duct hyperplasia/obstruction. (Ankrah et al., 1994). This ductular hyperplasia phenomenon has been observed in rodent studies in which participants were exposed to fumonisin B_1, a common food contaminant (Gelderblom et al, 1996). While it is plausible, all be it unlikely, that AFB1 could be involved in a mechanism that protects the liver from inflammation, it's more likely that another toxin (such as FB_1) contributed to the observed inflammation.

In a study by Kpodo et al., 53% of maize samples intended for maize consumption from Ghanaian markets were found to be co-contaminated with AFB1 and FB_1 (Kpodo et al., 2000). Co-exposure to multiple mycotoxins is a cause of concern because so many have been shown to be potent toxic agents with diverse effects and a synergetic nature. In a recent study, McKean et al.

examined the effects of co- exposure to AFB1 and FB_1 in both in vitro and in vivo models. A significant synergistic effect was observed when the LD50 of the individual toxins were compared to their combined potency (Mc Kean et al., 2005). For this reason, co-exposure to AFB1 and FB_1 should be investigated as a significant contributor to the prevalence of HCC in West Africa (Kumagia et al, 2009).

1.5.5 Species specific effects of fumonisin B_1 exposure

As evidenced by the complexity of fumonisin B_1's mechanism of toxicity, the species on which FB_1 can exert its effects are no less varied. Ingestion of as little as 5-10 ppm fumonisin B_1 in horses has been shown to induce development of equine leukoencephalomalacia (ELEM) (Marasas et al., 1988; Kellerman et al., 1990; Uhlinger, 1997). Signs of ELEM include nervousness, wide-based stance, trembling, ataxia, paresis of the lower lip and tongue, anorexia, and hypodypsia. Although the exact mechanism responsible for the white mater necrosis of is unknown, it should be clear that a significant portion of neural tissue is composed of complex sphingolipids (i.e sphingomyelin). Catabolism of these complex sphingolipids in an effort to restore sphingolipid ratios could be a critical process in etiopathogenesis of ELEM. Additionally, there is evidence that suggests that ELEM may be due to species specific permeability of the blood brain barrier. In mice, intracerebroventricular infusion of FB_1 lead to a focal upregulation of inflammatory mediators and changes in the

sphingolipid ratios similar to those seen in leukoencephalomalacia (Osuchowski et al, 2005). Studies that investigate the involvement of intestinal microflora metabolism of FB_1 in equid toxicity are warranted but lacking.

Swine, while seeming unrelated to the equids are also susceptible to FB_1 toxicity. With the exception of horses, swine are significantly less resistant to the effects of FB_1 ingestion than other foodstock animals. Levels > 92ppm lead to development of porcine pulmonary edema. In mammals, pulmonary edema is generally caused by either: left ventricular failure or increased pulmonary capillary permeability subsequent to injury of the alveolar endothelium and epithelium. In studies evaluating the cardiovascular toxicity of FB_1 in swine, left ventricular hypertrophy and pulmonary arterial hypertrophy were observed indicating pulmonary hypertension of some etiology.

The exact mechanism by which pulmonary edema in swine develops is unknown (Hasachek et al., 2001; Fodor et al., 2008). There is evidence that suggests that fumonisin B1 acts as a cardiotoxin through the increased production of TNF-α (He et al., 2001; Soriano et al., 2005). TNF-α has been shown to have negative inotropic influences on cardiac tissue (Duncan et al., 2007). Additionally, the disruption of sphingolipid metabolism by FB_1 causes collapse of cell to cell junctions leading to disruptions in the endothelial barrier, which can cause “leaky” vessels (Ramasamy et al., 1995; Soriano et al., 2005). The increased permeability of the lung vessels coupled with the congestive heart failure induced by FB_1 would lead to pulmonary edema. Again, species specific

sensitivity most likely plays a significant role. Other effects of fumonisin B_1 exposure in swine include decreased weight gain, changes in serum biochemical analytes, immunologic response suppression, and histopathologic lesions in liver and kidney (Harvey et al. 1995; Harvey et al. 1996).

Despite theses species specific signs of fumonisin toxicity, changes in the kidney and liver are almost always seen as FB_1 pathognomonically targets these organs regardless of the species evaluated. Biopsy of liver and kidney tissue following administration of fumonisin B_1 in horses and swine, demonstrate low level storage of the toxin and histopathological changes prior to any clinical changes (Hasachek et al., 2001; Fodor et al., 2008). Experimentally, male Fisher 344 rats developed renal adenomas and carcinomas when consuming FB_1 contaminated feed at 50ppm and higher. Paradoxically, only female M6C3F1 mice developed hepatic adenomas and carcinomas (NTP, 2001).

In addition to its carcinogenic potential, FB_1 has been implicated as the causative agent responsible for increased neural tube defects along the US/ Mexico border (Missmer et al, 2006; Graham et al., 2010). Neural tube defects are birth defects of the brain and spinal cord and are classified into anterior and posterior (Barber et al., 1999). The most common neural tube defects are spina bifida, encepahlocele, and anencephaly. In spina bifida, the fetal spinal column fails to close completely during the first month of pregnancy. There is usually nerve damage that causes at least some paralysis of the legs. Encephalocele is characterized by sac-like protrusions of the brain and meninges through

openings in the skull. In anencephaly, much of the brain does not develop. Infants with anencephaly are either stillborn or die shortly after birth. Although there are many proposed explanations for these events, the common element between them is the involvement of folic acid and the folate receptor (Marasas et al., 2004; Solomons, 2007). FB_1 has demonstrated the ability to mediate folate receptor/transport disruption (Cabrera et al., 2004). Strategies to reduce the incidence along the Texas/ Mexico border have included monitoring for FB_1 contaminated food sources and folate supplementation (Graham et al., 2010).

NTD incidence is not a problem that affects only developed countries. Populations in developing countries experience a high number of NTD's caused by unavoidable consumption of FB_1 contaminated foods. Some regions have strikingly high frequencies (compared to that for the general U.S. population of 3/10,000 live births), such as 106 NTD/10,000 live births in Quetzaltenango, Guatamala, which has a mostly indigenous population that consumes high amounts of maize as their staple food (Nguyen et al., 2008; Marasas et al., 2004). The most frequent defect noted from this population was myelomeningocele (a type of spina bifida). In South Africa, high NTD incidences have been found in a rural Transkei district in the Eastern Cape Province (61/ 10,000) and in rural areas in the Limpopo Province (35/10,000); in contrast, far lower incidence figures are reported in urban regions such as Cape Town (1.06/10,000), Pretoria (0.99/10,000), and Johannesburg (1.18/ 10,000). High NTD incidence rates (57 to 73/10,000) have been reported in rural areas in the

northern provinces of China. The inhabitants of both the Transkei region and the northern provinces of China are likely to be exposed periodically to high fumonisin levels as a result of the consumption of fungal contaminated maize (Marasas et al., 2004). Reduction of FB_1 consumption and enhanced prenatal care/ nutrition would most likely cause a significant reduction in NTDs worldwide.

A lesser known and studied effect of FB_1 exposure is mycotoxin mediated immune suppression. In vitro and in vivo studies demonstrate that exposure to FB_1 suppresses immune response in animals (Odhav et al., 2008; Bondy and Pestka, 2000). Much of the early work concerning fumonisin effects on the immune system were performed in poultry. Poultry are relatively insensitive to fumonisin B_1 and few effects of toxicity were observed below 75mg FB_1 /kg feed during in vivo studies. Ingestion of diets containing 25% or greater F. moniliforme culture content (by weight) caused depressed thymus weights and, depressed antibody responses to sheep red blood cells (SRBC) and Brucella abortus in chickens (Bondy and Pestka, 2000; Sharma et al., 2008). It should be noted in these early studies that fungal culture materials was used instead of FB_1 isolate and the associated effects of administration could be influenced by the presence of more than one toxin in the isolate material. Turkey poults ingesting feed contaminated with F. moniliforme culture material containing fumonisins at levels of 100 or 200 mg/kg feed displayed lesions indicative of immunotoxicity, including diffuse thymic cortical thinning, mild bursal follicular atrophy, and mild splenic lymphocyte depletion (Li et al., 2000).

Cattle and swine have been evaluated as ruminant and non-ruminant animal models of immune suppression, respectively. In calves, ingestion of feed experimentally adulterated with fumonisin contaminated corn screenings containing up to 148 µg/g total fumonisins inhibited neutrophil migration but not phagocytosis or antibody-dependent cytotoxicity (Bondy and Pestka, 2000). Lymphocyte blastogenesis following phytohemagglutinin induction was suppressed in pigs given fumonisin-contaminated culture material for 28 days at levels as high as 100 mg FB_1/kg feed (Harvey et al., 1996).

Only one study to date addresses the potential immunotoxicity of purified FB_1 following ingestion in rodents. Tryphonas et al. administered FB_1 by gavage at doses from 1 to 25 mg/kg body weight/d for 14 d to Sprague- Dawley rats. This treatment caused depressed responses to SRBC at the 25-mg/kg dose level, and a significant trend toward increased numbers of colony-forming bacteria in the spleens of rats experimentally infected with Listeria monocytogenes. There were no marked effects on spleen lymphocyte blastogenesis, calcium mobilization in lymphocytes, natural killer (NK) cell activity, or peripheral blood monocyte phagocytic activity (Tryphonas et al,, 1997). Conversely, in a study of the effects in isolated rodent splenic immune cells, exposure to FB_1 caused an increased production of nitric oxide and increased proliferation in macrophages and T lymphocytes, respectively (Dombrink-Kurtzman et al., 2000). The implications of these immune effects have yet to be fully explored.

In humans, immune suppression has been demonstrated in isolated macrophages and lymphocytes (Odhav et al., 2008). Currently, there is no available literature directly connecting immune suppression to FB_1 exposure in human populations.

1.6 Regulation of fumonisin B_1 exposure

Because of the myriad of different diseases fumonisin exposure can cause following ingestion, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has recommended a provisional maximum tolerable daily intake (PMTDI) of 2 µg /kg b.w. for fumonisins B_1, B_2 and B_3, alone or in combination. This is based on an experimentally derived NOEL for fumonisin B1 exposure and a safety/ uncertainty factor of 100. The US FDA has recommended maximum levels for fumonisins in human foods and in animal feeds that they consider achievable and sustainable with the use of good agricultural and good manufacturing practices (Tables 5 and 6). At 2 – 4 mg/ kg maize for humans and 5 mg / kg maize for horses, these levels are significantly greater than the level recommended by JECFA (IARC, 2003). Although these recommendations are in place, socioeconomic conditions often force the citizens of less developed countries to consume whatever is grown regardless of the condition. Economically sensitive, yet culturally acceptable solutions for reducing exposure to mycotoxins like fumonisin B_1 are in high demand.

TABLE 5

FDA maximum levels of fumonisins in human foods and animal feeds in the USA

Product	Total fumonisins (B1+B2+B3) ppm (mg/kg)
Human foods	
Degermed dry milled corn products (e.g., flaking grits, corn products (e.g. flaking grits, corn grits, corn meal, corn flour with fat content <2.25%, dry weight basis)	2
Whole or partially degermed dry milled corn products (e.g., flaking grits, corn products (e.g. flaking grits, corn grits, corn meal, corn flour with fat content <2.25%, dry weight basis)	4
Dry milled corn bran	4
Cleaned corn intended for masa production	4
Cleaned corn intended for popcorn	3
Animal feeds	
Corn and corn by-products intended for:	
Equids and rabbits	5
Swine and catfish	20
Breeding ruminants, breeding poultry and breeding mink	30
Ruminants > 3 months old being raised for slaughter and mink being raised for pelt production	60
Poultry being raised for slaughter	100
All other species or classes of live stock and pet animals	10

Adapted from FDA, 2001a

TABLE 6

Levels of total fumonisins (B_1+B_2+B_3) in corn, corn by-products and the total ration for various animal species recommended by the FDA in the US

Animal or Class	Recommended maximum levels of total fumonisins in corn and corn by-products (ppm; mg/kg)	Feed factor	Recommended maximum level of total fumonisins in the total ration (ppm)
Equids	5	0.2	1
Rabbit	5	0.2	1
Catfish	20	0.5	10
Swine	20	0.5	10
Ruminants	60	0.5	30
Mink	60	0.5	30
Poultry	100	0.5	50
Ruminant, poultry and mink breeding stock	30	0.5	15
All others	10	0.5	5

Feed factor is contribution of ingredient in feed 1.0 = 100%. Adapted from FDA, 2001c

1.7 Methods for reducing fumonisin exposure

There are 2 types of strategies for reducing fumonisin exposure: pre-harvest and post harvest. Pre-harvest strategies encompass all of the methods used to reduce fumonisin contamination of maize while still in the field. Post harvest strategies cover all methods of remediation once the toxin has been harvested. Generally speaking, pre-harvest strategies tend to be less expensive to implement and easier to adapt to a specific region. Examples of these strategies include the use of genetically modified (GM) crops to reduce insect damage and fungal contamination, implementation of more effective crop rotation practices, the development of environmentally responsible fungicides that target metabolism specific to mycotoxic fungi (Duvick, 2001; Brown et al., 2005). Implementation of GM corn plants has yielded success in controlled conditions. The introduction of the Bacillus therogenesis (Bt) gene into corn, has produced a crop that is more resistant to insect damage and less likely to be infected by Fusarium mold (Bucchini and Goldmann, 2002). Unfortunately, a crop that has been modified to produce its own pesticide presents another set of problems. There are numerous questions about what affects will be seen in the general population from consuming this corn/ pesticide combination, will target pest species eventually become resistant, and concerns about whether resistance will be passed on to other organisms, and how will introducing a foreign gene into a crop affect its yield/quality? It is these and other societal concerns that have prevented the introduction of these GM crops in the market

place in developing or developed countries (Parrott, 2010). Enhanced crop rotation practices that reduce the ability of the soil based Fusarium primary innoculum might be of use, however in developing countries, the availability of fertile soils needed for proper rotation is most likely a limiting factor (Munkvold, 2003). Phenolic compounds have gained notoriety both for their anti-oxidant properties as well as endocrine disruption activity. Beekrum et al. described the use of naturally phenolic compounds such as chlorophorin, iroko, maakianin, vanillic acid, and caffeic acid in reducing fumonisin production by Fusarium by as much as 94% (Beekrum et al., 2003). These compounds are produced by a variety of plants and could serve as a sustainable method of fumonisin control. Currently, further testing on the toxicokinetics of compounds intended for use and methods of environmentally friendly production and isolation of these compounds are barriers to implementation.

Post harvest strategies to control fumonisin contamination have not been successful to date. Controlling the conditions in which maize is stored is a way to reduce fumonisin production that occurs post harvest; however the facilities needed for such an undertaking are usually not economically feasible in the regions that would benefit most from their application. Ozonation of mycotoxins has been proposed as an effective means of detoxifying contaminated foods. Application of ozone was successful in detoxifying aflatoxin contaminated material, however, it demonstrated little to no effect on material containing FB_1 (McKeneziee et al., 1997). Other strategies have targeted the C-2 amine, as it is

critical to fumonisin B_1's potency as a toxin. Lemke et al. demonstrated that reacting the fumonisin B_1 with sodium nitrite in acidic conditions caused the formation of a diazonium salt which lead to the formation of an FB_1 diol (Lemke et al., 2001). This product was found to be less toxic than unreacted FB_1. In a method also targeting the primary amine of FB_1, Castelo et al. demonstrated that cooking fumonisin contaminated corn at high temps in the presence of glucose reduced the toxicity (Castelo et al. 2001). This was most likely due to the conjugation of the primary amine to glucose through the Maillard reaction described by Louis-Camile Maillard in the 1910's (Rindernecht and Jurd, 1958). This glucose fumonisin reaction has been shown to partially decrease the toxicity of FB_1 in swine (Fernandez-Surumay et al., 2004). While novel in application, the infrastructure required for implementation in developing countries would be the limiting barrier of the above strategies.

The use of food additives is an accepted practice in animal production. The application of materials generally regarded as safe for use as "food additives" in the reduction of aflatoxin bioavailability has garnered a lot of attention over the years. Phillips et al. first described the use of hydrated sodium calcium aluminosilicate (HSCAS) in protecting chickens from the effects of aflatoxin exposure (Phillips et al., 1988). Subsequent studies from Grant and Phillips verified the hypothesis that HSCAS was a high affinity sorbent for aflatoxin requiring an interlayer for sorption (Grant and Phillips, 1998). These studies helped lay the foundation for the testing and application of dioctahedral

smectite materials as sorbents for other mycotoxins. A survey study by Avantaggiato et al. demonstrated that bentonite, a dioctahedral smectite, displayed a pH dependent reduction in bioavailability of fumonisin B_1 in a simulated gastrointestinal model (Avantaggiato et al, 2005). Aly et al. reported the ability of HSCAS to reduce the concentration of FB1 in malt extract by almost 92% (Aly et al., 2004). Although these studies demonstrate promise, further studies focusing on the efficacy of smectites in binding fumonisins are lacking.

1.8 Research objectives

It has been established that exposure to fumonisins can result in a number of untoward effects in a number of different species. Despite extensive research into the mechanisms of toxicity and hazard assessment of FB_1 exposure, a practical method of assessing individual exposure for the purposes of risk assessment has yet to be identified and fully accepted. Without individual exposure assessment, it is difficult if not impossible to assess the efficacy of any remediation strategy utilized in a fumonisin exposed population.

On a related note, the efficacy of Novasil (NS), a smectite clay, in reducing the biomarkers of aflatoxin exposure in humans and animals is well documented. The success of NS clay in preventing the symptoms of aflatoxicosis has led to the examination of the potential for fumonisin

sequestration through similar target-sorbent interactions. Therefore the principle goals of this research were to:

1) Identify sorbent materials that would be compatible with the chemical characteristics of fumonisin B_1 and evaluate their ability to sequester the toxin using established in vitro techniques. Isothermal analysis and an alternative animal model were examined and compared to previously published results to determine the sorbent toxin interaction activity in vitro as a predictor of in vivo efficacy.

2) Evaluate urinary FB_1 as a biomarker of exposure from a fumonisin contaminated diet. An HPLC method for detection and quantitation of urinary FB_1 was developed based on methods previously adapted for primary amine and biomarker analysis. Urinary FB_1 was evaluated as an HPLC detectable biomarker using a rodent model.

3) Utilize urinary FB_1 as a diagnostic tool to evaluate the efficacy of NS in reducing biomarkers of FB_1 bioavailability in a Ghanaian population suspected to be co-exposed to aflatoxins and fumonisins. Previously established methods were utilized to evaluate the fumonisin exposure status of a population known to be at high risk for mycotoxicosis.

CHAPTER II

EVALUATION OF SORBENT MATERIALS FOR FUMONISIN B_1 REMEDIATION

Fumonisin B_1, B_2, and B_3 isoforms have been reported in corn based food and commodities worldwide with FB1 identified as the most prevalent and toxic congener (Shephard et al., 1996; Marin et al., 2004; IARC 2003). The primary source of exposure to FB_1 is the consumption of contaminated food and feedstuffs by humans and animals. Acute and chronic exposures to FB_1 are major contributors in the eitopathogenesis of equine leukoencephalomalacia and porcine pulmonary edema (Marasas et al., 1988; Kellerman et al., 1990; Uhlinger, 1997; Hasachek et al., 2001; Fodor et al., 2008). Chronic FB_1 exposures have been implicated in the formation of liver and kidney neoplasms in rats and mice as well as increased incidences of esophageal cancer in humans Gelderblom et al., 1996; NTP, 2001; Gong et al., 2009; Marasas et al., 1988; Syndenham et al., 1990; Hendricks and Parker, 2002). Consumption of fumonisin contaminated products has also been associated with increased incidences of neural tube defects (NTD's) and other teratogenic anomalies in populations that consume maize products as a staple of their diet (Nguyen et al., 2008; Marasas et al., 2004). The complete mechanism of FB_1 toxicity is not fully understood; however, inhibitions of ceramide synthetase and folate transport are

well-defined (Merrill, et al., 1993; Merrill et al, 1996; Desai et al, 2002; Riley et al., 1996; Marasas et al., 2004).

Common food production methods, such as nixtimalization and heat processing, do not significantly reduce the toxicity of fumonisins in corn and can lead to the production of more potent toxins (Bullerman et al., 2002; Humpf and Voss, 2004). Thus, alternative strategies have been proposed for the remediation of contaminated foodstuffs. One strategy that has enjoyed a measure of success has focused on the prevention of Fusarium growth on corn plants through genetic resistance mechanisms (Bucchini and Goldmann, 2002; Munkvold et al., 1999). Alternatively, novel methods of fumonisin detoxification using heat, ozone, or oxidative and non-oxidative deamination have also been reported. These methods have yielded limited success (Lemke et al., 2001; McKenezie et al., 1997). Recent efforts have focused on decreasing the bioavailability of mycotoxins in the gastrointestinal (GI) tract (i.e., enterosorption), however, sorption and reduction of bioavailability of FB_1 to blood and target organs has not been previously demonstrated. This may be attributed to the zwitterionic properties of the fumonisins, which lead to varying charge characteristics at different pH values. With ingestion being the primary route of exposure, it would be desirable to have a safe enterosorbent material to effectively bind the fumonisins despite changes in pH throughout the GI tract.

Due to the zwitterionic charge characteristics of FB_1, we investigated two representative classes of sorbent material to determine which charge found on

fumonisin B_1 (positive or negative) could be exploited most effectively for sequestration. 2-Line ferrihydrite (FH) was selected as a potential sorbent based on the hypothesis that the positive surface charge of the sorbent would interact with the negatively charged “free” carboxylic acid moieties of the fumonisin B_1 molecule. Ferrihydrite is a naturally occurring meta-stable iron oxide that is produced when an Fe^{2+} containing soil is rapidly oxidized in the presence of crystallization inhibitors (i.e. organic matter) (Figure 10). Experimentally, FH can be produced by the rapid hydrolysis of an Fe III containing solution. FH is characterized based on the number of X-ray diffraction peaks (2-6) that appear during analysis which are directly related to its level of crystallization. Due to both its small particle size and poor crystallinity, FH typically has a surface area greater than $200 m^2/ g$ (Shwertmann and Cornell, 2000). Ferrihydrite also has a high density of local or point defects, such as dangling bonds and vacancies. These defects coupled with the pH of the environment directly influence the polarity of FH surface charge; below pH 8, FH exhibits a positive charge (Bigham et al., 2002).

Montmorillonite was selected as the negatively charged sorbent based on the hypothesis that its negative interlayer would interact with the protonated C-2 amine group (at acidic pH) and sequester the toxin. Montmorillonite clays are dioctahedral smectite clays in which a portion of the Al in the octahedral layer has been isomorphically substituted with Mg (Figure 11). Due to this elemental substitution, there is a reduction in positive charge which is no longer sufficient

to balance the negative charge of the structural oxygen molecules. This charge imbalance is a major contributor to the characteristic net negative interlayer surface charge found in montmorillonite clays. This charge is balanced in nature by exchangeable cations; the amount of positive charge needed for equilibrium is referred to as cation exchange capacity. Conditions conductive to the formation and stability of smectites in soil environments include high Si and Mg activity, basic pH and poor drainage which allow precipitation of the soil constituent. Small particle size and the ability of the interlayer to expand promoting ion exchange result in a large surface area of 600-800 m^2/ g in montmorillonite clays (Bigham et al., 2002). Due to their polarity, large surface areas, and zwitterionic characteristics, FH and montmorillonite were targeted as potential sorbent materials for fumonisins.

Thus, the primary objective of this study was to evaluate the ability of FH and montmorillonite to sequester and sorb FB_1 from aqueous solutions in vitro and in vivo. This objective was investigated using analytical methods well-established in our laboratory to estimate the capacity of sorption and to predict safety and efficacy of selected materials.

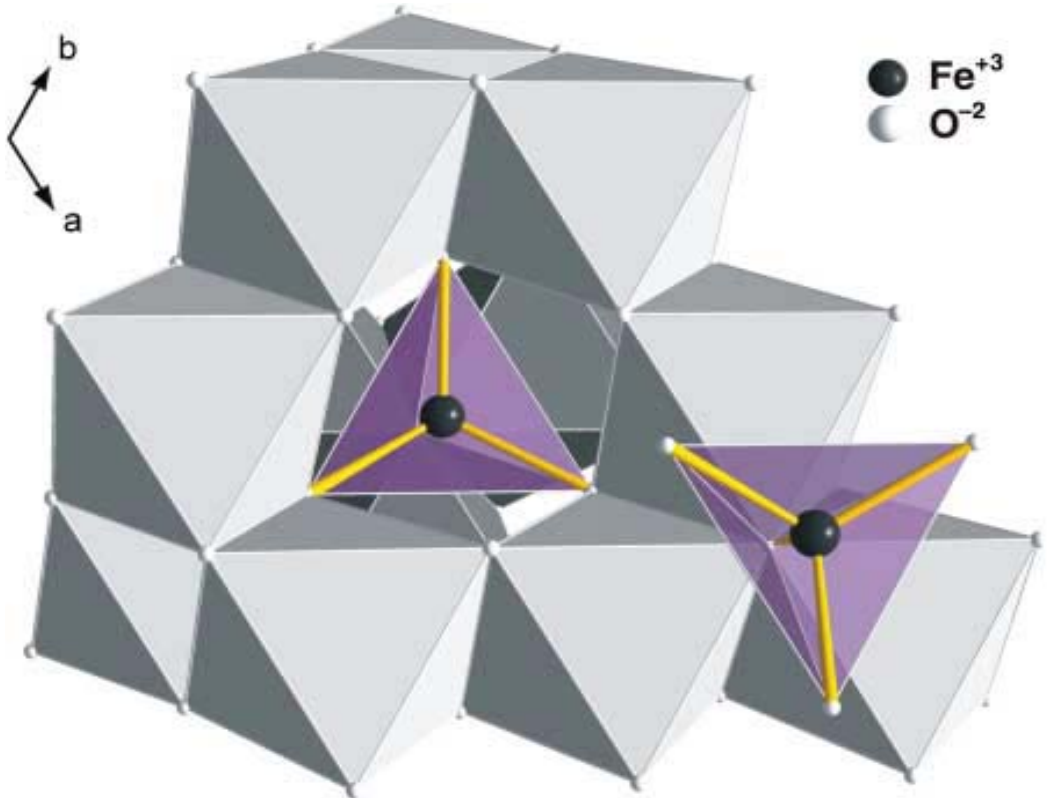


Figure 10. Three dimensional representation of ferrihydrite. Ferrihydrite is a poorly ordered iron oxide with a pH dependant ability to sequester anions.

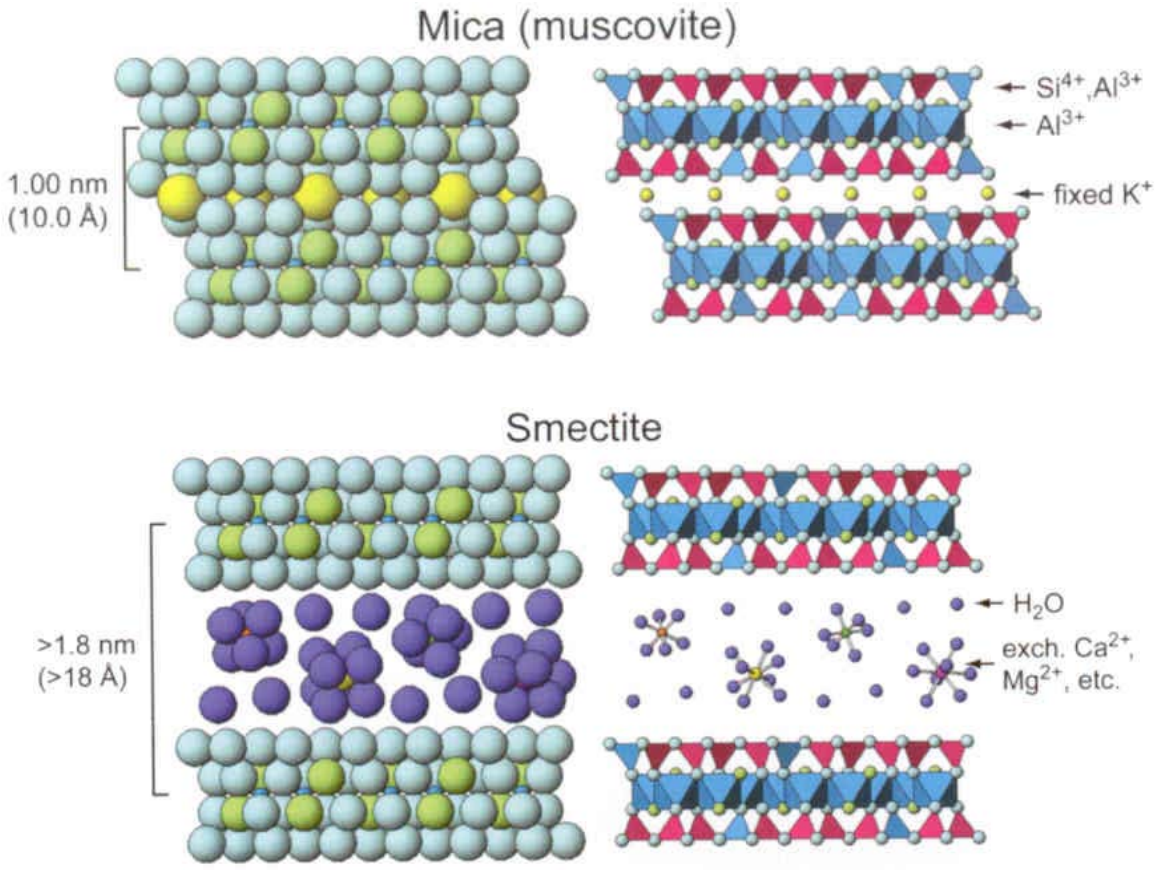


Figure 11. Three dimensional representations of two 2:1 dioctahedral clays. Smectite clays possess an expanded negatively charged interlayer which is critical to their binding ability.

2.1 Materials and methods

2.1.1 Chemicals and reagents

Fumonisin B_1, o-phthalaldehyde (OPA), methanol, 2- mercaptoethanol, sodium phosphate dibasic, and phosphoric acid were purchased from Sigma-Aldrich Corporation. Acetonitrile and sodium hydroxide were purchased from Fisher Scientific. Hydrochloric acid and boric acid were purchased from TJ Baker. Clay samples including sodium montmorillonite (SWY-2) and calcium montmorillonite (NovaSil) were obtained from the Clay Repository (University of Missouri, Columbia) or Engelhard Chemical Corporation, Cleveland, Ohio. Ferrihydrite (FH) was prepared in the laboratory according to methods described by Schwertmann and Cornell in 2000. Heat collapsed clays were prepared by heating 100 mg samples to 200°C for 30 minutes, followed by 800°C for 60 minutes. These were transferred and maintained in air tight containers prior to analysis. Fumonitest wide bore immunoaffinity columns were purchased from VICAM. Test tubes were from VWR international (16 x 125 mm borosilicate glass) and used in conjunction with an IKA- VIBRAX- test-tube shaker. HPLC sample vials were obtained from Supelco. Visualization of Hydra attenuata was performed using a Nikon microscope and Nikon MKII optic light. Centrifugation of samples was performed using an International centrifuge Model UV from International equipment company; a Corning Pinnacle 530 pH meter and Corning “3 in 1” probe was utilized to determine pH. Distilled, deionized water was used in all experiments (18mΩosm).

2.1.2 Fumonisin analysis using HPLC

FB_1 analysis was performed using a Waters HPLC System with auto-injection (Model 717A) and fluorescence detection (Model 2475). Separation of FB_1 was achieved using a C-18 column (Supelcosil LC-ABZ, 15 x 4.6 cm, 5 um particle size from Supelco, Bellefonte, PA) proceeded by a pre-column filter (RP – 185 u 7.5 x 4.6 mm). FB_1 was detected using the wavelengths 335nm excitation and 440nm emission.

2.1.3 Preparation of mobile phase

The mobile phase was prepared by combining 230 ml of 0.1M sodium phosphate dibasic solution with 770 ml of methanol. The pH of the 0.1M sodium phosphate solution was adjusted to 3.35 with phosphoric acid prior to mixing with methanol to equilibrium. The mobile phase solution was then filtered through Whatman nylon membrane filters (0.45um) and cavitated using sonication to remove gas from the solution. Mobile phase was transferred to a 1 liter HPLC solvent bottle and sealed.

2.1.4 Preparation of o-phthalaldehyde solution

O-phthalaldehyde reagent solution was prepared according to Riley et al. with minor modifications (Riley et al., 1994). A 3% borate solution was prepared and the pH was adjusted to 10.5 using sodium hydroxide solution (1M). The solution was stirred until the pH was stable. OPA (5 mg) was dissolved in 100 ul

of methanol followed by the addition of 5 ul of 2-mercaptoethanol and 10ml of 3% borate buffer.

2.1.5 Calibration curve for fumonisin B_1 detection

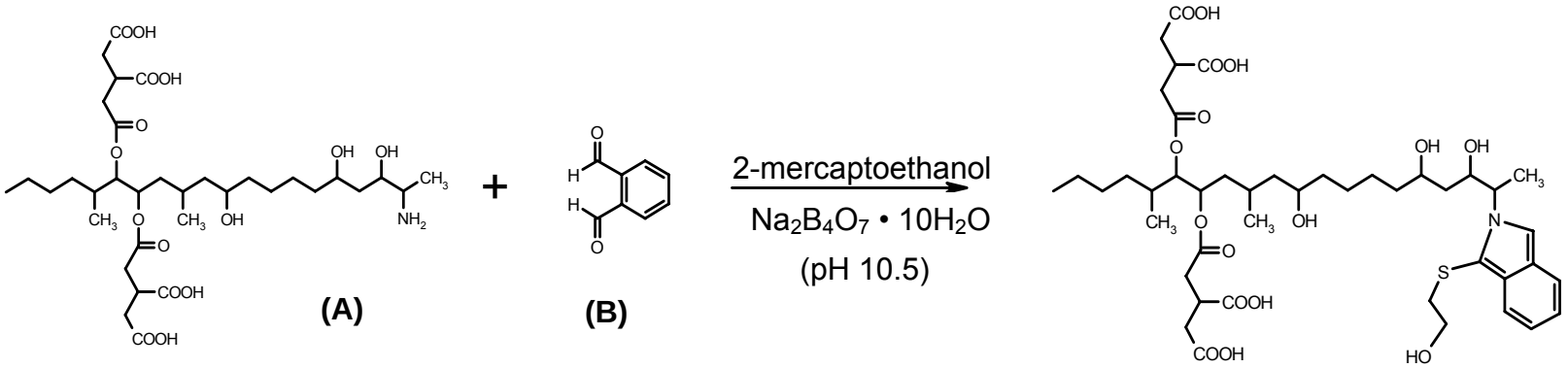
Fumonisin standards were prepared for high and low concentration calibration curves (A and B, respectively). All calibration curve samples were prepared with a 2 ml volume in the following concentrations: 100 ppm, 50 ppm, 10 ppm, and 0 ppm FB_1 for calibration curve A; 0 ppm, 10 ppm, 5 ppm, and 2.5 ppm for calibration curve B. Prior to injection, all samples were prepared in the following manner: an HPLC injection vial containing 100 µl of the FB_1 sample was derivatized with 200 µl of freshly prepared OPA solution (Figure 12), sealed, and vortexed for approximately 20 sec. The HPLC was programmed to inject 100 ul of the sample with a run time of 10 min. Data from these injections were recorded in a Microsoft Excel spreadsheet and XY scatter graphs were prepared. Linearity of the calibration curve was tested by observing the r^2 value of the line (close to 1.00) formed by the points from each calibration curve. Because of the similarity of the low concentration sample set to the analytical concentrations in our experiments, the low concentration calibration curve was utilized as the standard for subsequent isothermal analyses.

2.1.6 Fumonisin B_1 sorption by FH and SWY-2 at different pH values

A 100 ml stock solution of 4 ppm FB_1 was prepared by dissolving 400 ug of FB1 in 100 ml ddH_2O. This solution was divided into two 50 ml aliquots, A and B. Aliquot A was adjusted to pH 2 with HCl and labeled A. Aliquot B was adjusted to pH 7 with NaOH and labeled B. Borosilicate tubes were filled with 2.5 ml of the 4 ppm FB1 solution (A or B) and combined with sorbent (i.e., FH, SWY-2 and 50:50 wt/wt FH/SWY-2) in the following concentrations: 0.01%, 0.02%, 0.05%, 0.1%, 0.15%, and 0.2%. FB_1 samples were prepared without sorbent to serve as non-treated pH controls. Samples for analysis were prepared in triplicate. Tubes were agitated at 1000 rpm for 18 h at 25°C for equilibration. After equilibration, tubes were centrifuged at 700 x g for 20 min. After centrifugation, all samples were prepared and analyzed using HPLC as previously described. Standard FB_1 peaks were identified manually with a retention time of 4.5 to 5.5 min. Peaks were integrated by Waters Breeze software. Peak areas of treated samples were compared to standards with similar pH. The amount remaining in solution was calculated based on the peak areas and sample injection volume. Results were reported as mass FB_1 toxin bound vs. mass of initial sorbent in the sample.

2.1.7 Isothermal analyses of fumonisin sorption

Aqueous sorbent suspensions (1 mg/ml) of Ferrihydrite, NOVASIL, and SWY-2 were prepared. Two 12.5 ppm stock solutions of FB_1 were prepared and



Fluorescent Adduct (ex 335nm, em 440nm)

Figure 12. The reaction of the primary amine of FB_1 with o-phthaladehyde. (FB_1 (A) and o-phthalaldehyde (B)). The reaction occurs in the presence of β- mercaptoethanol, forms the 1-thiol-2 alkyl isoindol fluorescent product that is detected using high performance liquid chromatography separation and fluorescence detection.

individually adjusted with HCl or NaOH to pH 2 or 7, respectively. Triplicate samples (2.5 ml total vol.) were prepared in the following concentrations: 1, 2.5, 4, 5.5, 7.0, 8.5, and 10 ppm by combining 12.5ppm FB_1 stock solution, ddH2O, and 0.5 ml of sorbent suspension. Samples were agitated and allowed to equilibrate at 4°C, 25°C and 37°C for 18 h. A 10 ppm FB_1 sample without sorbent was also prepared for calibration curve and linearity analysis. Following equilibration and centrifugation, analysis was performed as stated previously for all samples.

2.1.8 FB_1 MEC determination in hydra

Hydra attenuata (HA) were cultured and maintained in our laboratory as previously described by Mayura et al. (Mayura et al., 1991). The minimum effective concentration of FB_1 or (MEC) resulting in a toxic endpoint (tulip stage or disintegration) was determined by placing 3 adult hydra in small Petri dishes containing 4 ml of hydra media combined with logarithmic concentrations of FB_1 from 0.1 ppm to 1000 ppm. The minimum effective dose (MEC) was then titrated based on the initial logarithmic findings. The hydra response to FB_1 toxin was recorded at time points 0, 4, 20, 28, 44, 68, and 92 h.

2.1.9 In vivo sorbent efficacy assay

Hydra attenuata (HA) were cultured and maintained in our laboratory as previously described. Hydra were fasted 24 h prior to testing and during 96-h

acute toxicity tests. Triplicate hydra were placed in 11 Petri dishes each containing 4 ml of hydra media. Hydra media was prepared with FB_1 at 400 ppm in 7 of the dishes to produce the toxic endpoint as determined by previous experiments. The FB_1 contaminated dishes were divided into 2 groups (3 per group) and used to evaluate the ability of the sorbents to protect the hydra from the effects of FB_1 exposure. One hydra dish was prepared without sorbent as a positive control for FB_1 toxicity. In group 1, sorbent (FH, NS, or SWY-2) was allowed to equilibrate with toxin prior to being exposed to hydra. In group 2, sorbent was not allowed to equilibrate with toxin prior to being exposed to the hydra. Sorbents were added to the hydra dishes based on a theoretical maximum FB_1 binding capacity calculated from the Q_{max} of the respective sorbents. In three of the remaining FB_1 free petri-dishes, hydra were exposed to sorbent without FB_1 in the media to evaluate potential sorbent toxicity. One dish was maintained toxin and sorbent free as an absolute control (Figure 13). Hydra were monitored and toxicity was recorded as previously described.

2.2 Results

2.2.1 Fumonisin B_1 sorption by FH and SWY-2 at different pH values

Fumonisin B_1 (FB_1) is zwitterionic in nature. FB_1 At acidic pH is protonated (and positively charged) at the amino group on C2 of the molecule. In this form, it is electrostatically attracted to clays such as SWY-2 and NovaSil that contain a net negative charge on their interlayer surfaces. FB_1 tends to interact

less with these surfaces at neutral to basic pH due to less positive charge on the molecule. Identifying sorbent materials capable of accommodating the pH change of the gastrointestinal tract in mammalian organisms from acidic in the stomach to neutral/slightly basic in the intestine and the subsequent change in the polarity of FB_1 was the major objective of this study. An initial sorption study was conducted to explore the potential of montmorillonite (SWY-2) and ferrihydrite (FH) as FB_1 sorption materials. In this study, 0.02% SWY-2 was shown to bind 94% of 4 ppm FB_1 at pH 2. At pH 7, however, SWY-2 was less effective and sorption of FB_1 (8.8%) was only shown at sorbent levels of 0.05% or higher (Figure 14).

At neutral pH, FB_1 was significantly sorbed by FH and this interaction was probably due to interface of deprotonated carboxyl groups in the tricarboxyllic acid moieties with the positive surface charge of the sorbent. FH sequestered greater than 92% of the total FB_1 at 0.05% sorbent levels at pH 2 and 7. (Figure 2) This sorption was comparable with SWY-2 binding at pH 2, but better than SWY-2 at pH 7. From these results, SWY-2 was found to be the most effective sorbent for FB_1 at pH 2. Conversely, FH was an effective sorbent for FB_1 at pH 7 (Figure 14). Further investigation was warranted to determine the binding capacity of these sorbents.

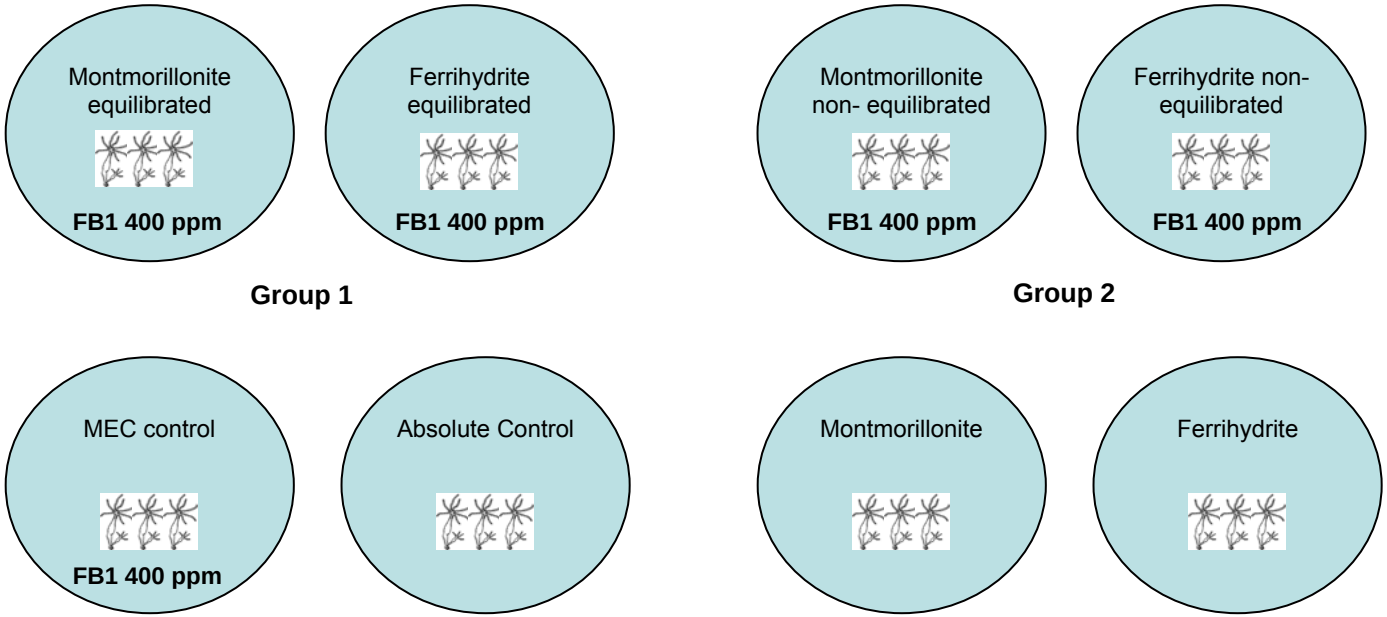


Figure 13. Study design of the *Hydra attenuata* FB_1 toxicity/sorbent protection assay. The hydra depicted are representative of timepoint 0, prior to any effects from toxin or sorbent. Dishes depicted with montmorillonite represent both SWY-2 and NS containing dishes. SWY-2 and NovaSil are trade names for our representative sodium and calcium montmorillonites, respectively.

2.2.2 Isothermal analyses of ferrihydrite and montmorillonite

Isothermal analyses were performed at 25°C to determine the maximal binding capacity of SWY-2 and FH. Isotherm equations have previously been used to model the adsorption of compounds in aqueous solutions to solid surfaces and to provide estimates of their Q_{max} or maximal binding capacity (Figure 15). These modified equations are based on the original Langmuir equations that related the coverage or adsorption of molecules on a solid surface to gas pressure or concentration of a medium above the solid surface at a fixed temperature (Grant et al., 1998). Initially, both sorbents were added at 0.02% but the sorbent concentration was too high to produce a saturation curve. Therefore, concentrations of SWY-2 and FH were titrated down to 0.004% for use in the isothermal analyses to derive accurate measures of capacity (Q_{max}). The Q_{max} values for SWY-2 at pH 2 and 7 were 0.13 and 0.03 mol FB_1/kg sorbent, respectively (Figure 16). The Q_{max} values for FH at pH 2 and pH 7 were 0.02 and 0.09, respectively (Figure 17).

SWY-2 and FH isothermal analyses were also performed at 4°C and 37°C. SWY-2 was evaluated at pH2 while FH was evaluated at pH7. Q_{max} values for SWY-2 were 0.22 and 0.09 at 4°C and 37°C, respectively (Figure 18). The Qmax values for FH were 0.08 and 0.21 at 4°C and 37°C, respectively (Figure 19). Based on these results, sorption of FB_1 by montmorillonites appeared to be more thermodynamically favored when compared to binding of

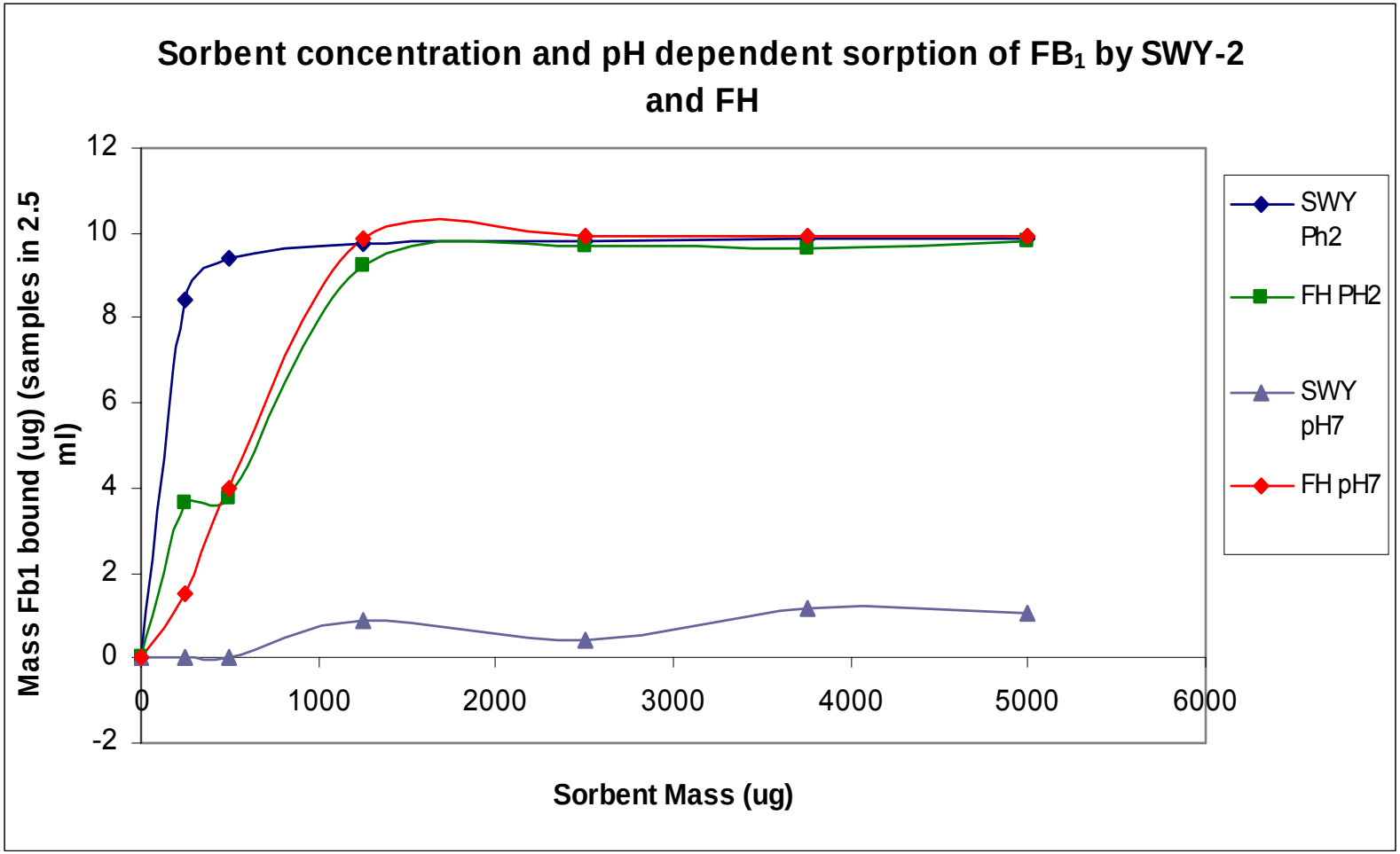
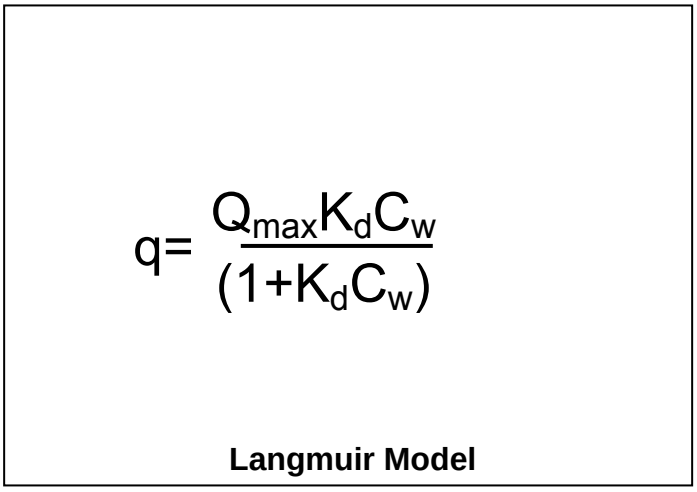


Figure 14. Results from the preliminary evaluation of ferrihydrite (FH) and montmorillonite (SWY-2) sorption of FB_1 at physiologically relevant pH.



q = sorbate adsorbed (mol/ kg), Q_{max} = maximum capacity (mol/kg), K_d = distribution constant, C_w = equilibrium concentration of sorbate

Figure 15. Langmuir equation commonly used to fit isothermal adsorption data.

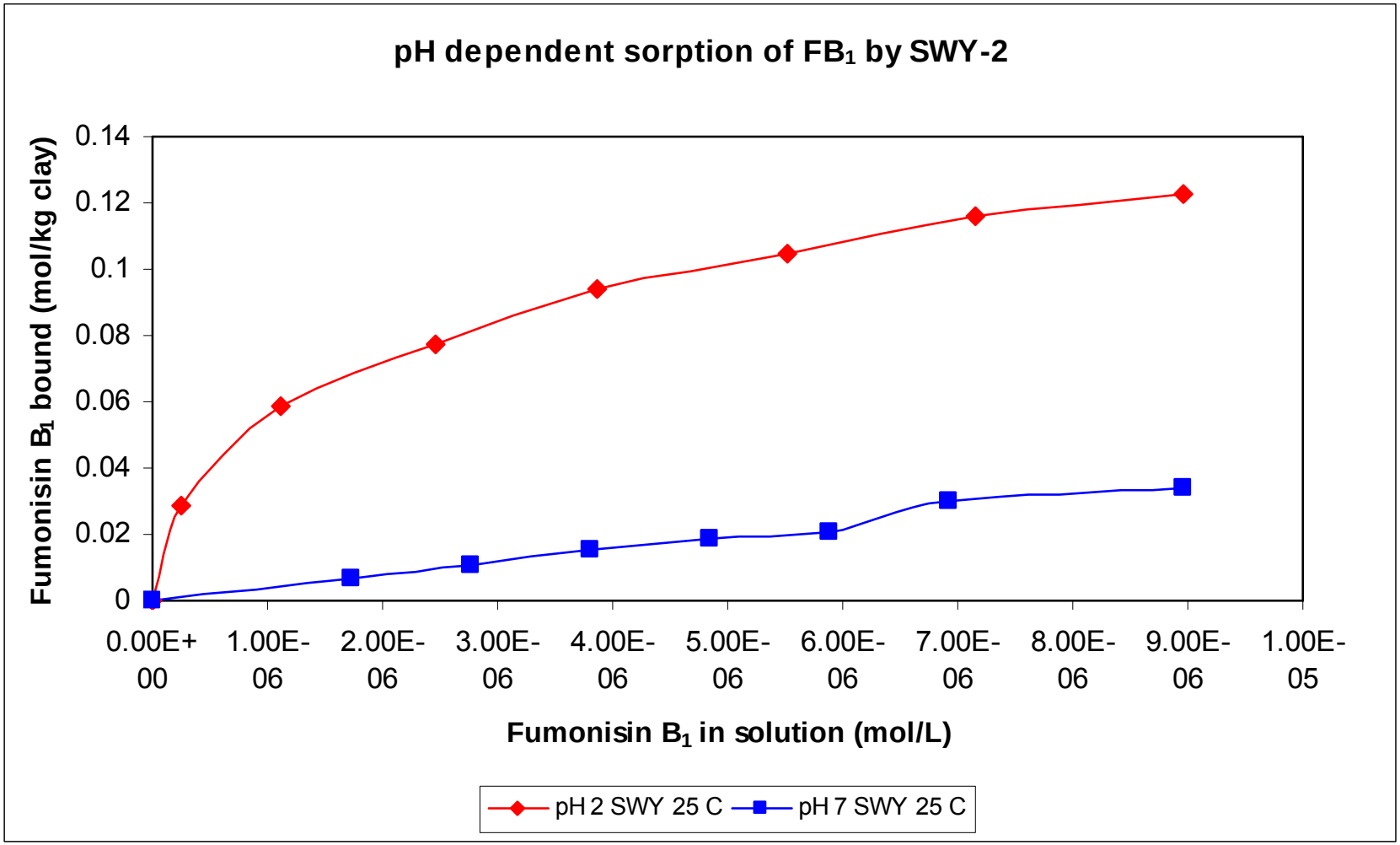


Figure 16. pH dependent sorption of FB_1 by SWY-2. This representative montmorillonite clay demonstrates pH dependent sorption of FB_1.

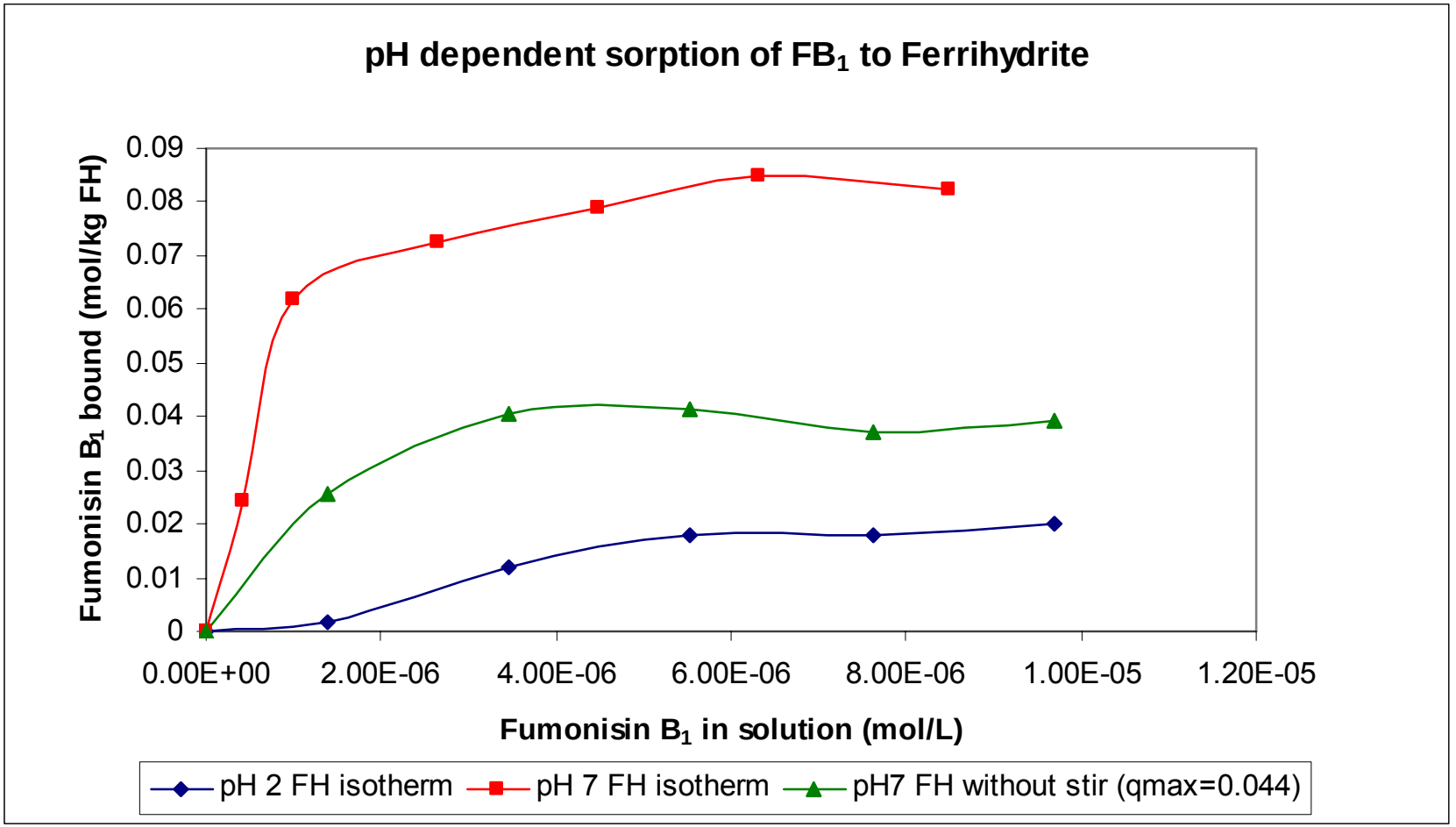


Figure 17. pH dependent sorption of FB_1 by FH. This representative iron oxide demonstrates pH dependent sorption of FB_1.

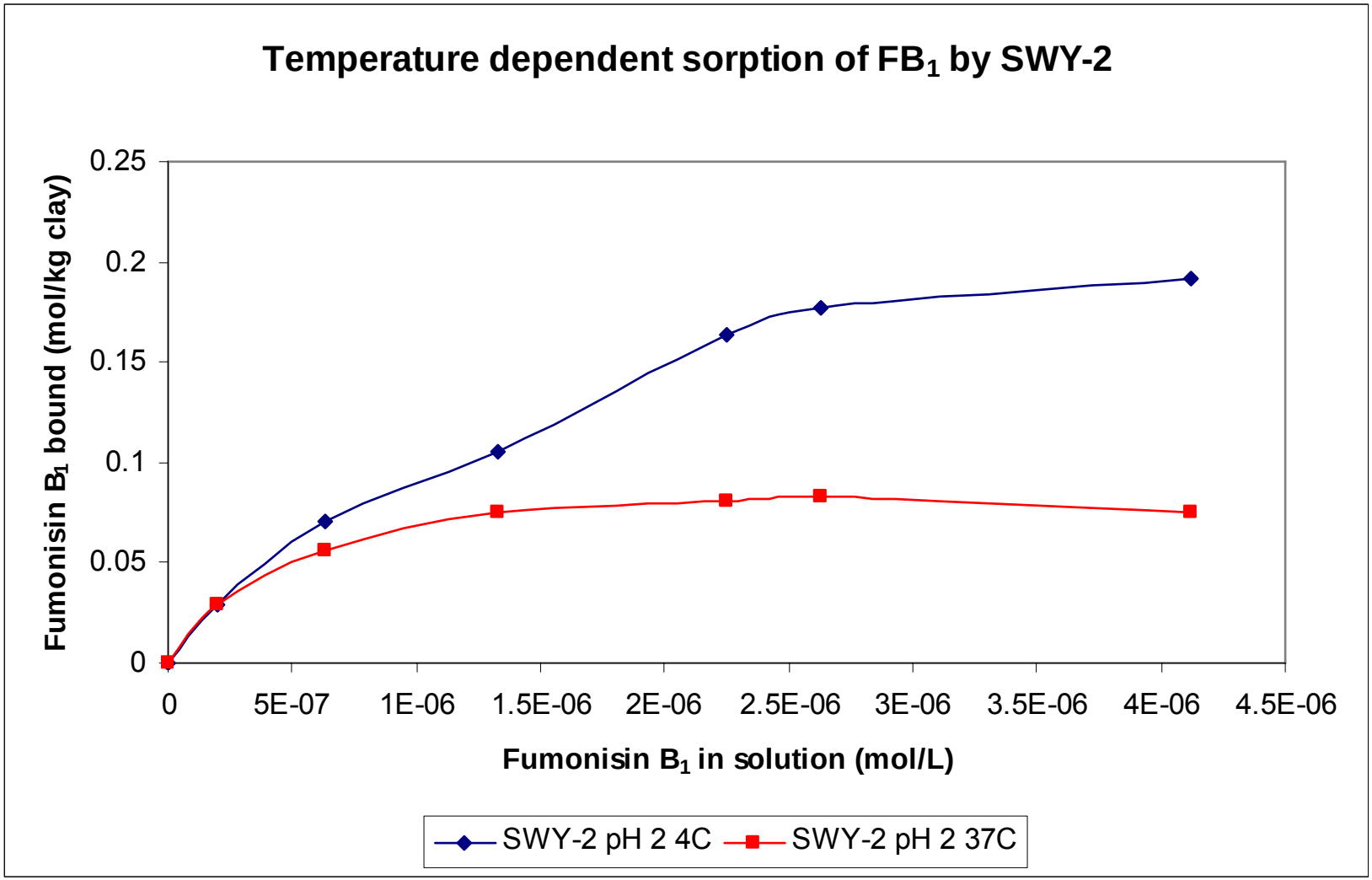


Figure 18. Temperature dependent sorption of FB_1 by SWY-2. Isothermal analysis of SWY-2 sorption of FB_1 at 4°C (**blue**) and 37°C (**red**).

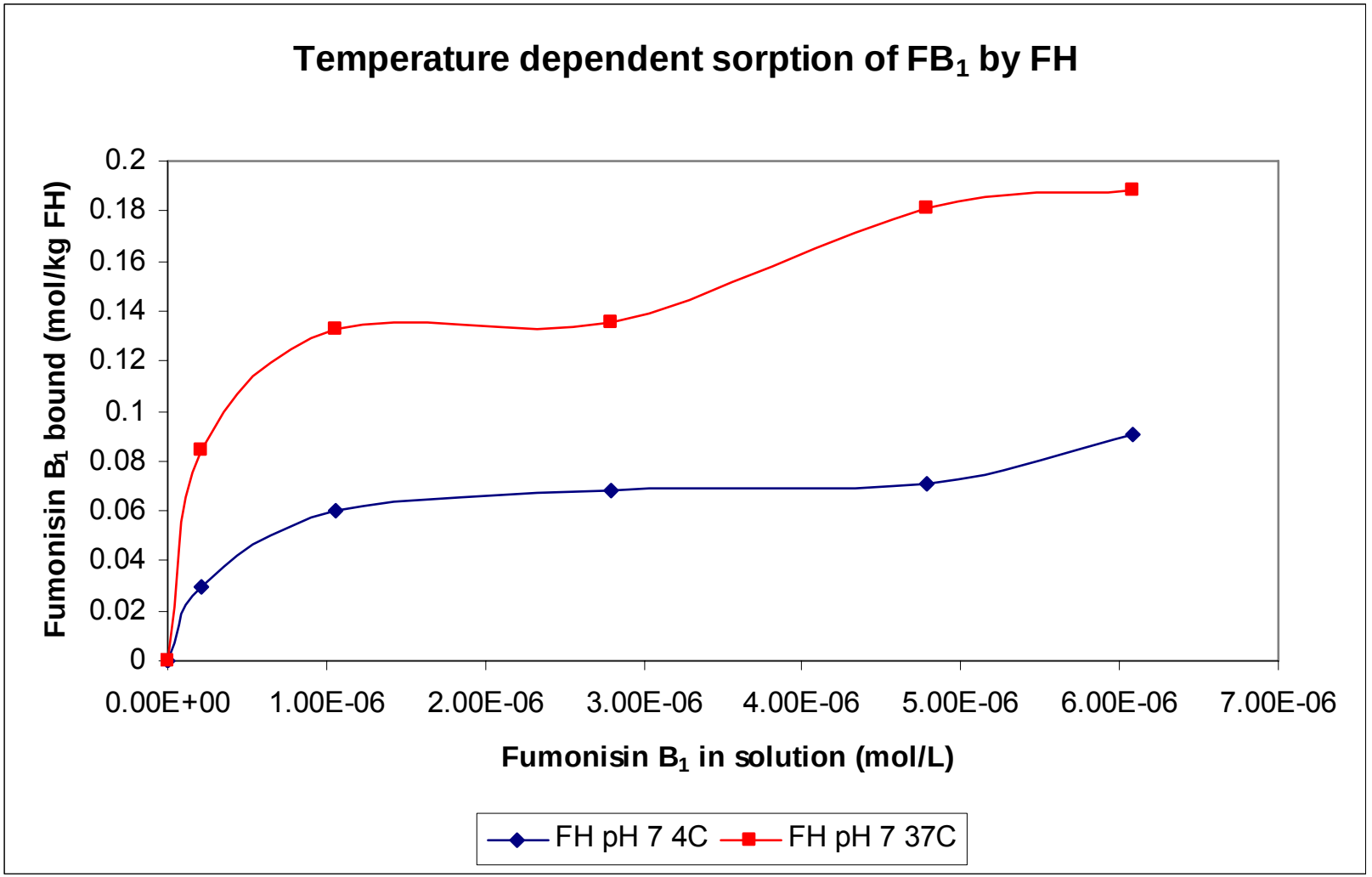


Figure 19. Temperature dependent sorption of FB_1 by FH. isothermal analysis of ferrihydrite (FH) sorption of FB_1 at 4°C (**blue**) and 37°C (**red**).

FB_1 to FH. Interestingly, increasing the time that FH was allowed to mix in the suspension prior to toxin addition increased its binding capacity (Figure 16). An increased mixing time of 12 hours vs. 30-60 minutes prior to the addition of FB_1 toxin yielded a significantly increased Qmax value of 0.09 vs. 0.044 at pH 7 and 25°C. This phenomenon may be due to the dissolution of FH aggregates leading to an increased surface area for sorption of FB_1. No differences were observed in binding capacity when suspension mixing times for SWY-2 were altered.

In order to determine whether there was a difference in binding capacity for sodium montmorillonite vs. calcium montmorillonite, an isothermal analysis of NovaSil (NS) was performed at pH 2 and pH 7 and 25°C (Figure 20). NS displayed a Qmax of 0.130 at pH2 which is comparable to the SWY-2 Qmax of 0.126. NS also showed a Qmax of 0.04 at pH 7. These Qmax values suggested similar preference to sorption of FB_1 by calcium montmorillonite when compared with sodium montmorillonite at identical pH values. Isothermal analyses were also performed on heat collapsed samples of NS and SWY-2 to determine if FB_1 sorption is dependent on the inner layer of the clays.

The isothermal analysis of the heated clays at pH 2 and 25°C revealed no measurable Qmax, suggesting that the collapsed interlayer of montmorillonite clay prevents FB_1 from binding. Based on these findings, it is probable that the interlayer of montmorillonite plays a significant role in the ability of montmorillonite clay to act as a sorbent for FB_1.

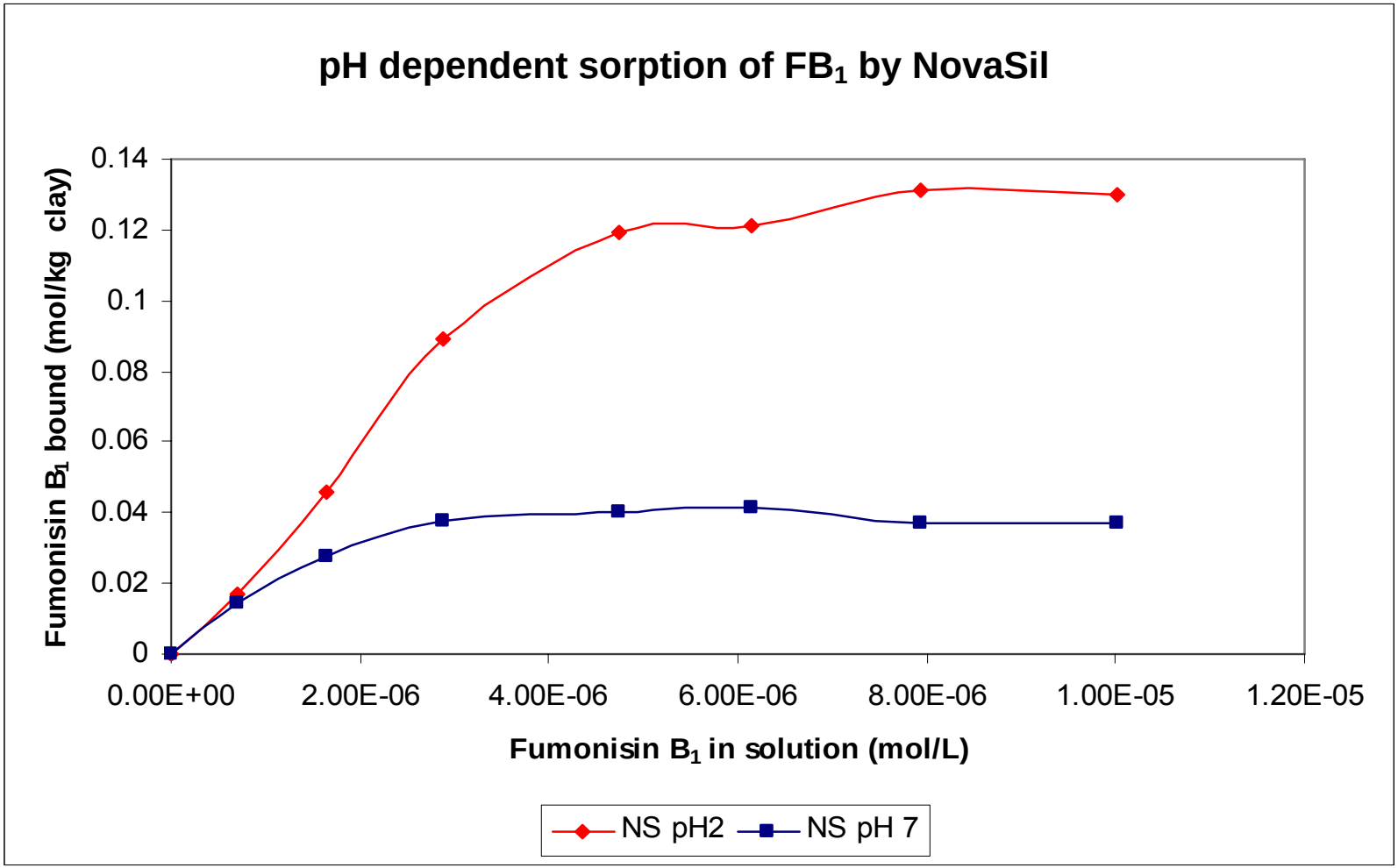


Figure 20. pH dependent sorption of FB1 by NovaSil. This calcium montmorillonite clay demonstrates pH dependent sorption of FB_1 similar to that of the sodium montmorillonite clay SWY-2.

2.2.3 *Hydra attenutta* toxicity and sorbent protection assay

FB_1 toxicity was confirmed through the use of the Hydra toxicity assay. Based on the toxin dilution results, the MEC needed to induce the toxic endpoint was 300 ppm. In our hydra assay, 400 ppm was used as the standard to produce a toxic endpoint. Montmorillonite (SWY-2 and NS) at 0.35% and FH at 1.25% were added to the individual sample dishes based on the calculated Q_{max} of the respective sorbents. In this assay, only the hydra exposed to 400 ppm FB_1 displayed effects of toxin insult and reached the tulip stage in 28 h with disintegration occurring by 44 h. FB_1 exposed hydra treated with either FH or montmorillonite were protected from the toxic effects and remained viable throughout the 96 h duration of the study. These results suggest that the clay provided protection to the hydra by sequestering the FB_1 from surrounding hydra media, thus lowering the bioavailability of FB_1 below the level that was toxic to the hydra (Figures 21 and 22).

2.3 Discussion and conclusions

In summary, in vitro studies in our laboratory have demonstrated that both sodium and calcium montmorillonite clays interact with FB_1 at low pH. The mechanism for this interaction is likely due to the negatively charged surfaces within the interlayer of these clays reacting with the protonated (and positively charged) amino group of the FB_1 molecule. Sorption studies also demonstrated that FH is an effective sorbent material for FB_1.

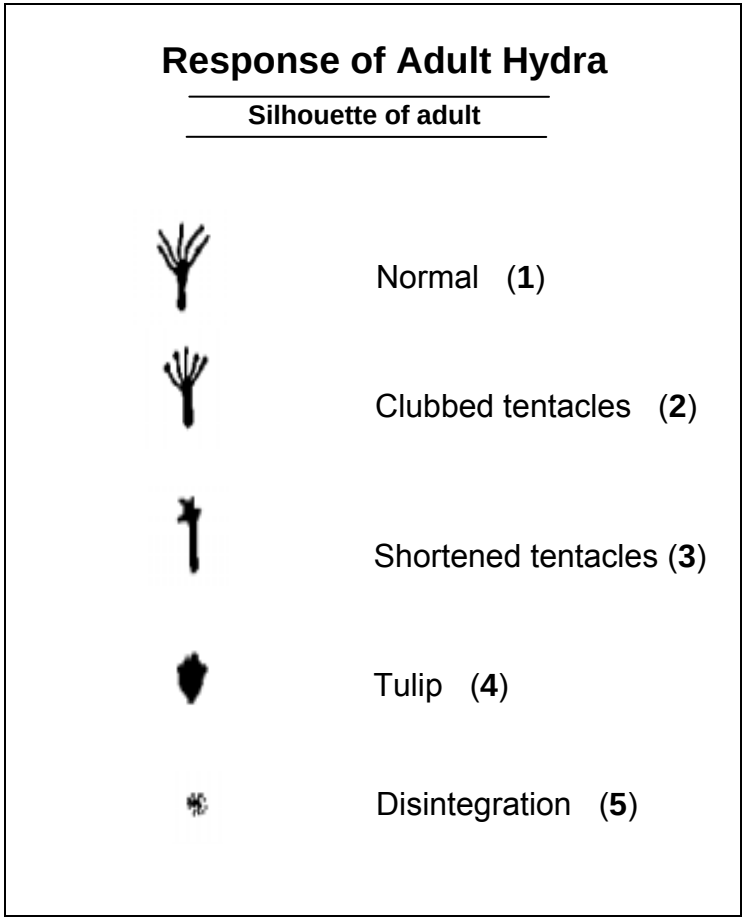


Figure 21. Schematic illustrating the adult hydra response to a toxin and the grading system used to plot the results.

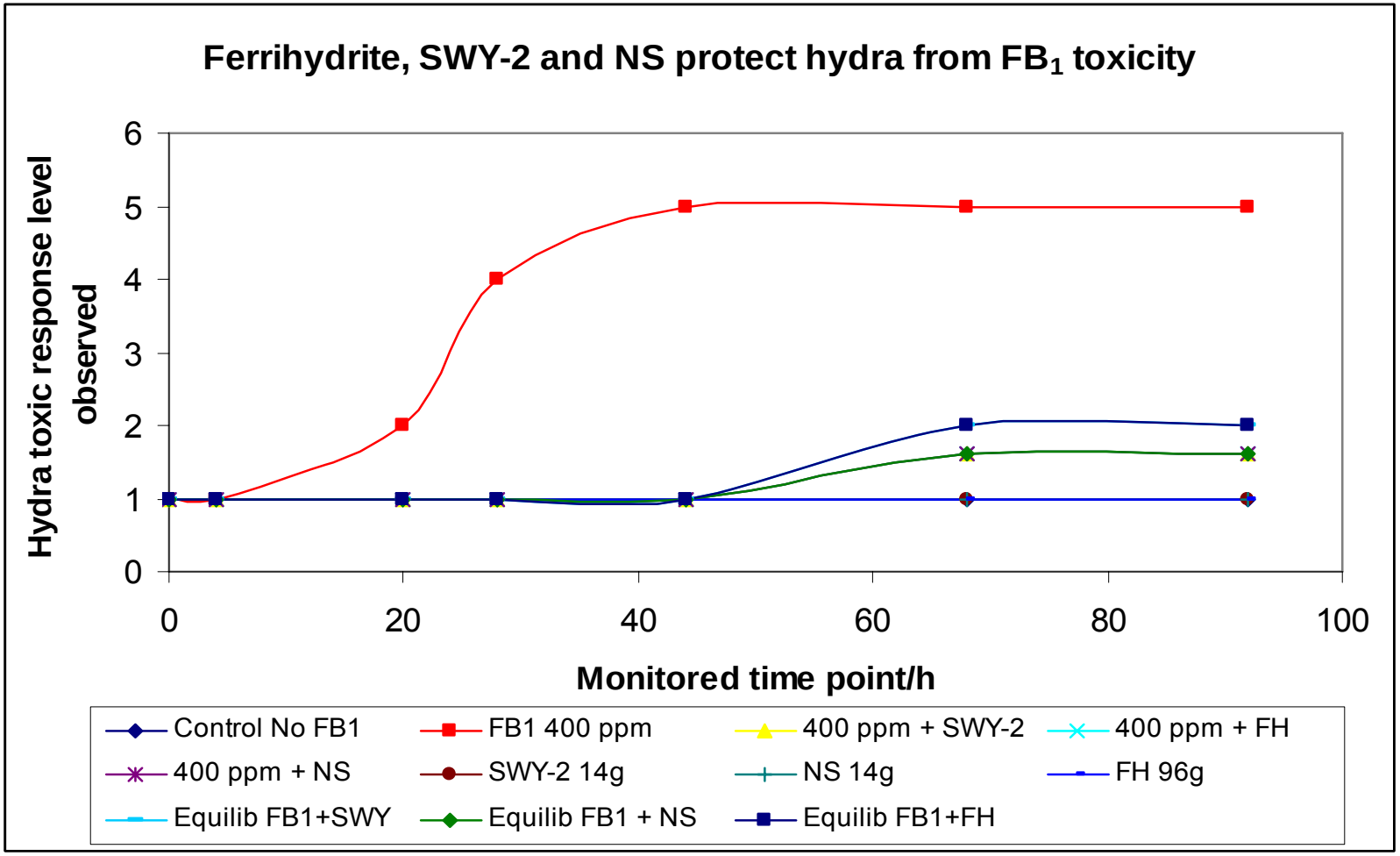


Figure 22. Ferrihydrite, SWY-2, and NS protect hydra from FB_1 toxicity. Montmorillonite clay (NS and SWY-2) and ferrihydrite (FH) protected the hydra from the exposure to FB_1. Hydra that were not treated with montmorrilonite or ferrihydrite reached the toxic endpoint by the 28h.

This interaction is likely due to the interface of positively charged sorbent material with negatively charged tricarballylic acids on the FB_1 molecule at pH 7. SWY-2, NS, and FH were protective against the effects of FB_1 in the hydra assay. Moreover, none of the tested sorbents produced untoward effects in the hydra during the assay at neutral pH.

Fumonisin B_1 is a toxin that causes a variety of pathologic conditions in many different species. It has been identified as a potential carcinogen and has been linked to the prevalence of esophageal cancer in South Africa and China. Exposure to FB_1 has also been shown to cause liver and kidney carcinomas in rats and mice. In addition to its carcinogenic effects, FB_1 poses a significant threat as a teratogen via interaction with the folate receptor. With so many potential effects attributed to FB_1 exposure, the need for a novel intervention strategy for FB_1 is highly warranted.

The ability of selected materials to sequester FB_1 and lower its bioavailability is influenced by the relative pH and temperature at which the FB_1/sorbent interaction occurs. Preliminary studies in our laboratory showed that montmorillonite clays interact with FB_1 at low pH. The mechanism for this interaction is likely due to the negatively charged surfaces within the interlayer of these clays reacting with the protonated (and positively charged) amino group of the FB_1 molecule. Sorption studies also demonstrated that FH is an effective FB_1 sorbent material at neutral pH. This interaction is possibly due to the sorbent

material attraction to the (deprotonated) and negatively charged tricarballylic acids on the FB_1 molecule at pH 7.

Montmorillonite clays (SWY-2 and NovaSil) and ferrihydrite (FH) were capable of sequestering free FB_1 from an aqueous solution at pH 2 and pH 7, respectively, which was verified by HPLC analysis. Based on isothermal analysis, SWY-2 and NovaSil exhibited similar sorption capacity of FB_1 at pH 2 and pH 7. Hydra were protected from the lethal effects of 400 ppm FB_1 when sodium/calcium montmorillonites and ferrihydrite were added to the test dishes at concentrations of 0.35% and 1.25%, respectively. Importantly, exposure to montmorillonite or FH produced no discernable toxic effects in hydra. Further work is warranted to confirm the protection conveyed by montmorillonite and ferrihydrite against FB_1 exposure and the potential application of these materials as enterosorbents of fumonisins in the diet.

CHAPTER III

EVALUATION OF URINARY FB_1 AS A BIOMARKER OF FUMONISIN B_1 EXPOSURE AND NOVASIL EFFICACY IN A RODENT MODEL

Assessing the risk of fumonisin B_1 (FB_1) exposure has been a goal of fumonisin research since the toxin was first described in 1988 (Gelderblom et al., 1988). Quality risk assessment has three major components: hazard identification, dose response assessment, and exposure assessment; without these, no realistic estimation of risk can be made (Faustman and Omenn, 2008). There are numerous studies that identify FB_1 exposure as a global hazard. One of the most comprehensive of these studies was performed by Shepherd et al. as part of a global risk assessment of fumonisin exposure. This survey included a literature review encompassing reports of suspected exposure hazard from 1985 to early 1995. Thirty-two laboratories from 32 countries submitted data for this review which utilized the most accepted methodology for fumonisin detection to date. From this study, it was confirmed that fumonisin B_1 was a common contaminant of corn in developed and developing countries with levels of maize contamination ranging from 0-330 ppm in corn. It is interesting that the highest level of FB_1 contamination in this study was found in corn from the United States (Shephard et al., 1996). Since 1996, there have been other studies confirming the hazard of FB1 exposure in Brazil, Cote d' Ivoire, China,

Guatemala, Ghana, and Iran (Caldas, E.D. and Sliva, 2007; Sangare-Tigori et al., 2006; Xu et al., 2010; Torres et al., 2007, Kpodo et al., 2000; Ghiasian et al., 2006). These hazard assessments are important as they serve as the foundation linking potential fumonisin B_1 exposure to its myriad of health effects in humans and animals.

Fortunately, the presence of a hazard does not translate to danger unless an exposure occurs. This principle has been the caveat of many reported health effects from suspected fumonisin B_1 exposures worldwide. For example, elevated incidence rates of esophageal cancer (EC) in regions of South Africa and China have been correlated to potential chronic exposures to fumonisin B_1 (Shephard et al., 2007; Sun et al., 2007). These studies have examined the elevated morbidity and mortality data and the elevated EC rates have been strongly correlated with FB_1 contamination of maize at levels as high as 155 ppm in foods intended for human consumption (Chu and Li, 1994; Marasas, 1995; Rheeder et al., 1992; Syndenham et al., 1990). While these data were compelling, to date there are no in vivo studies in which any form of esophageal hyperplasia was a consequence of FB_1 exposure. Additionally, evidence of individual exposure to FB_1 was assumed and not definitive. Although common sense suggests that the presence of FB_1 in food and cancer development would be sufficient for the causal relationship, by definition, without evidence of individual exposure and a dose response assessment with esophageal cancer

as the end point, risk does not exist. Thus the relationship between FB_1 and esophageal cancer remains circumstantial and correlated at best.

Aside from excretion patterns following oral and parenteral administration, relatively little is known about the toxicokinetics and toxicodynamics of fumonisin B_1. FB_1 is not significantly metabolized in vivo and >95% is excreted in the feces, with a considerable portion of absorbed FB_1 being excreted in the bile. Significant efforts have been directed at establishing a physiologic marker or biomarker of FB_1 exposure. Previously, exposures to fumonisins were estimated by measuring concentrations of the agent or fungal producer in food, water, or other media with which a population was in contact. However, this external exposure was only a rough estimate for the internal exposure (agent dose or its metabolite at the critical target in the organism). Factors that influence this internal exposure or dose are bioavailability of the agent, variations in concentrations and routes of exposure, and individual variation in rates of metabolism, distribution, and excretion. All of these affect the concentration of the toxic agent at the target organ, which is the most important information for risk assessment. Thus, internal exposure is best measured by determining the concentration of the toxicant or its ultimate metabolite at the critical site in the target organ or by determining adducts with cellular macromolecules such as proteins, amino acids, DNA, or its bases. These markers are often readily available in experimental toxicology from animal experiments, but only occasionally from humans as they often require invasive

procedures such as biopsies. Therefore, more accessible body fluids or tissues are used, such as blood, urine, or adipose tissue, or adducts with macromolecules such as albumin or hemoglobin in the blood (Greim et al., 1995).

Unfortunately, most of these efforts, while academically fulfilling, have left researchers with more questions than answers. What is known about the mechanism of FB_1 toxicity centers around its inhibitory effect on ceramide synthase. This inhibition of de novo ceramide production leads to a significant disruption in sphingolipid metabolism often causing a disruption in the sphinganine:sphingosine ratio which has been identified as a potential biomarker of fumonisin exposure (Cai et al., 2007). While diagnostic in laboratory animals chronically exposed to fumonisin B_1, changes in the sphingolipid ratio do not seem to be an effective means by which fumonisin exposure can be monitored in humans (Wild and Gong, 2010). Species differences in toxicokenetics and toxicodynamics are most likely responsible for this incongruency. Thus the need for sensitive, reliable, non species specific biomarkers of FB_1 exposure still exists.

Previously, montmorillonite clay was shown to reduce the amount of free FB_1 in an aqueous environment and provide protection against the toxin's effects in a hydra toxicity assay. Although ferrihydrite (FH) was also able to sequester FB_1, the long term effects of feeding an iron based material as an enterosorbent are not known. Conversely, NovaSil is a calcium montmorillonite that has

previously been shown to be a safe and effective mycotoxin enterosorbent in animals and humans (Phillips et al., 2008). While in vitro results from both sorbents were encouraging, this preliminary in vivo study focused only on smectite materials to verify previous findings and assess the potential of montmorillonite clay as an enterosorbent for FB_1.

The Fischer 344 rat has been well established as an appropriate animal model for fumonisin toxicity studies (NTP, 2001; Cai et al., 2007). The use of an animal model provides the appropriate bridge for translation of in vitro success into in vivo solutions. The use of urinary FB_1 as a biomarker of exposure has been previously demonstrated in Fischer 344 rats, and we hypothesized that this biomarker would be sufficiently sensitive to detect any reduction of FB_1 bioavailability due to NS treatment (Cai et al., 2007).

O-phthalaldehyde (OPA) derivatization is currently considered the standard detection method for quantitative and qualitative measurement of FB_1 using HPLC. While this method of derivatization offers many advantages, its disadvantages led us to consider the use of naphthalene 2,3 dicarboxaldehyde (NDA) as the derivatization agent in our analysis (Williams et al., 2004b; Cho et al., 2002; He et al., 2005). NDA derivatization has previously demonstrated greater sensitivity in sphingolipid detection compared to OPA, and due to its stability, provided the opportunity for automation of our analysis procedure, which increased sample throughput without jeopardizing precision or accuracy.

Therefore, the main objectives of this study were to evaluate the use of NDA derivatization versus OPA derivatization for detecting low concentrations of FB_1, to adapt these analytical methods in the development of a sensitive and reliable method for the detection of urinary FB_1, and to assess FB_1 exposure and evaluate the ability of NS to reduce FB_1 bioavailability in a Fischer 344 rodent model.

3.1 Materials and methods

3.1.1 Chemicals and reagents

Fumonisin B_1, o-phthalaldehyde (OPA), methanol, 2- mercaptoethanol, sodium phosphate dibasic and phosphoric acid were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Naphthalene 2, 3-dicarboxaldehyde (NDA), potassium cyanide (KCN), sodium tetraborate (STB), phosphate buffered saline (PBS) and potassium hydroxide (KOH) were also obtained from Sigma. Fumonitest WB columns were purchased from VICAM (Watertown, MA, USA). All of the experiments were performed using filtered and deionized water (18.2 MΩ·cm) (Millipore, Milford, MA), and all other chemicals and reagents were purchased commercially at the highest degree of purity available.

3.1.2 Preparation of fumonisin B_1 standard solution

A 10 ppm FB_1 standard was prepared in 50:50 methanol: water. FB_1 standards were prepared daily.

3.1.3 Preparation of mobile phase

23:77 0.1M sodium phosphate: methanol mobile phase was prepared as described in the previous study.

3.1.4 Preparation of o-phthalaldehyde solution

O-phthaladehyde solution was prepared daily as described in the previous study.

3.1.5 Preparation of naphthalene 2,3 dicarboxaldehyde solution

NDA solution was prepared as described by He et al. (He et al., 2005). Briefly, 3 ml of a 4% aqueous sodium tetraborate dibasic solution was added to 17 ml of HPLC grade methanol and vortexed for 10 s. Next, 200 µl of a 0.83 mM aqueous KCN solution was added to the methanol/tetraborate solution. Then, 3.6 mg of naphthalene 2,3 dicarboxaldehyde was added to the solution and vortexed until dissolved (approx 3 m). The prepared NDA solution was wrapped in foil and refrigerated until use. The NDA derivatization agent was prepared daily.

3.1.6 Comparison of OPA versus NDA derivatization sensitivity

Detection of both OPA and NDA derivitized FB_1 was linear, with NDA having a lower limit of detection equal to 0.242 ng. The detected fluorescent intensity of NDA derivatized FB_1 was two to five times the intensity of OPA

derivatized FB_1 (Figures 23 and 24).

3.1.7 Urinary FB_1 rodent model experimental design

Eighteen five -week-old male (110–130 g) Fisher 344 rats were purchased from Harlan (Houston, TX) and maintained on nutritionally complete powdered feed (Teklad rodent diet 8604, Harlan, Madison, WI) and water ad libitum. In this study, rats were randomly placed in 3 equal groups (A, B, C). The rats in group A and C were fed untreated basal rodent feed; the rats in group B received basal rodent feed containing 2% NS (w/w). After a brief acclimation period (1week), the rats were placed in metabolism cages (1 rat/cage) for 8 days and urine was collected. Rodents in groups A and B were administered FB_1 at 25 mg/kg b.w. or 3.75 mg of toxin (based on 150g body weight) via aqueous gavage. Fischer rats from group C served as the absolute control group and were not exposed to FB_1 or clay. The dietary NS clay concentration was based on the highest level previously determined to be safe in a chronic rodent study (Afriyie-Gyawu et al., 2005). All rats were housed in a climate-controlled environment (temperature 22– 25 C°) that was artificially illuminated (12 hr dark/12 hr light) and free from chemical contamination. Rats were inspected daily for general appearance, behavioral changes and signs of morbidity and mortality. Body weights were measured initially then twice per week throughout the course of the study. Upon termination, organs were not collected because the short duration of the study and the relatively low FB_1 dose selected were

unlikely to produce remarkable pathology.

3.1.8 Preparation of urine samples and urinary FB_1 analysis

Urine samples were stored at -80^{o}C until analysis. Samples were thawed at room temperature and centrifuged at 500 g for 5 min. Samples (10 ml) were passed through Fumonitest WB columns (VICAM, Watertown, MA) at a flow rate of approximately 1 ml/min. Columns were washed with 10 ml phosphate buffered saline before elution with 2 ml 100% methanol. Samples were dried at 50^{o}C under nitrogen gas and reconstituted in 300 µl of 50:50 methanol:water. NDA solution (300 µl) was added to samples which were vortexed and heated (50^{o}C) for 10 min (Cho et al. 2002). Analyte extracts were injected (150 µl) onto a 250 x 4.6 mm C-18 LUNA column, 5 µm particle size (Phenomenex, Torrance, CA) with mobile phase consisting of 77:23 methanol:sodium phosphate dibasic (0.1 M , pH 3.35) at a flow rate of 1 ml/min. FB_1 analysis was performed using a Waters HPLC System equipped with an auto-injector (model 717A) and fluorescence detector (model 2475). Excitation and emission parameters were set at 335 and 440 nm, respectively, for detection of OPA-derivatized material, and at 252 and 483 nm, respectively, for detection of NDA-derivatized material. Creatinine concentrations in urine samples were measured at the Texas Veterinary Medical Diagnostic Lab in College Station, TX.

3.1.9 Statistical analysis

Data generated from HPLC analyses were transferred into an Excel database for management. Mean, median, confidence interval (CI) and range were calculated for concentrations of FB_1. To show the consequence of NS ingestion on FB_1 levels, statistical evaluation focused on the comparisons among different treatment groups at three different time points. Non-detectable samples = LOD/2 for statistical analysis. One way ANOVA and Fishers LSD were used to compare the differences among and between treatment groups. A p-value $\leq$ 0.05 (two-tailed) was considered significant. Statistical analyses were done using SPSS software version 15.0 (SPSS Headquarters., Chicago, IL).

3.2 Results

3.2.1 Comparison of OPA versus NDA derivatization sensitivity

Detection of both OPA and NDA derivitized FB_1 was linear, with NDA having a lower limit of detection equal to 0.242 ng. The detected fluorescent intensity of NDA derivatized FB_1 was two to five times the intensity of OPA derivatized FB_1 (Figures 23 and 24).

3.2.2 Measuring FB_1 exposure and NovaSil efficacy in a Fischer 344 rodent model

Urine samples were collected from 3 groups (A,B,C) of Fisher 344 rats for a duration of 8 days. Only urine from days 1-4 were used in this analysis as the

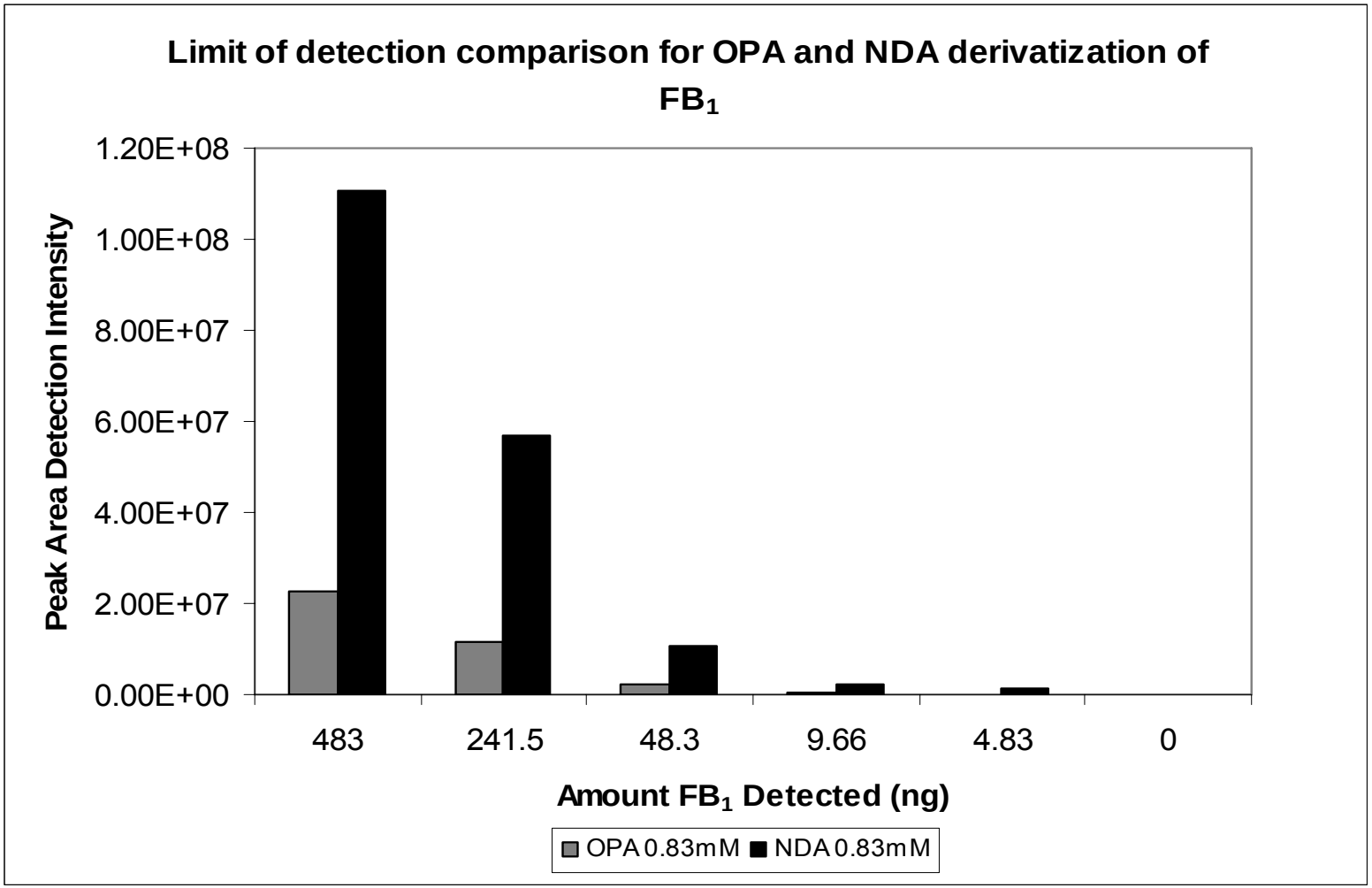


Figure 23. Limit of detection comparison for OPA and NDA derivatization of FB_1 (high concentration). Detection intensity comparison of OPA derivatized FB_1 vs. NDA derivatized FB_1 at higher concentrations.

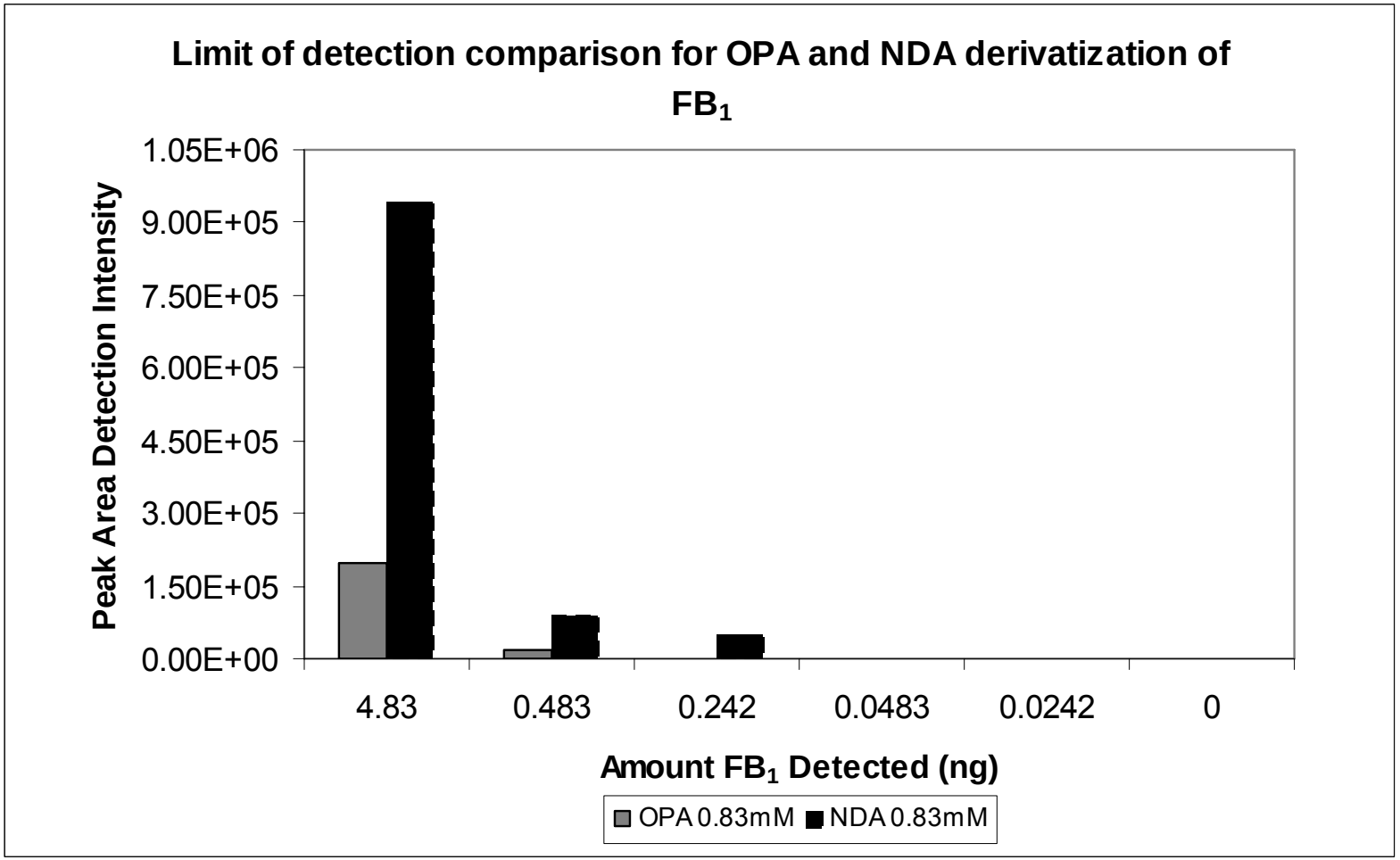


Figure 24. Limit of detection comparison for OPA and NDA derivatization of FB_1 (low concentration). Detection intensity comparison of OPA derivatized FB_1 vs. NDA derivatized FB_1 at low concentrations.

urinary FB_1 biomarker was undetectable 3 days after mycotoxin ingestion. Urine was collected at day 1 prior to gavage in groups A and B. Rats in group A received 25 mg FB_1/kg b.w. in a one time aqueous gavage at the end of day 1. Mean total urinary FB_1 excretion for rats in group A was 86.5 ng FB_1/ml urine on Day 2; on day 3, excretion was reduced to 18.3 ng FB_1/ml urine. Median values were similar at 89.2 and 18.4 ng FB_1/ml urine on days 2 and 3, respectively. Creatinine standardized urinary FB_1 for group A had a mean value of 194.1 ng FB_1/mg creatinine on day 2 and 9.4 ng FB_1/mg creatinine on day 3. Median values were similar at 192.0 and 9.9 ng FB_1/mg creatinine on day 2 and day 3, respectively.

Rats in group B received 25mg FB_1/kg b.w. in a one time aqueous gavage while being maintained on control rodent feed supplemented with 2% NovaSil. Mean total urinary excretion for rats in group B was 62.6 ng FB1/ml urine on day 2; on day 3 excretion was reduced to 8.3 ng FB_1/ml urine. Median values were similar at 59.0 ng FB_1/ml urine on day 2 and 9.2 ng FB_1/ml urine on day 3. Creatinine standardized urinary FB_1 for this group had a mean value of 158.2 FB_1/mg creatinine on day 2 and on day 3 excretion was only 4.4 ng FB_1/mg creatinine.

Median values were similar at 154.3 ng FB_1/mg creatinine for day 2 and 5.0 ng FB_1/mg creatinine on day 3. Rats in group C were the absolute control group and were only fed control feed. Urinary FB_1 was not detected in this group (Figures 25 and 26; Table 7). There was a statistically significant

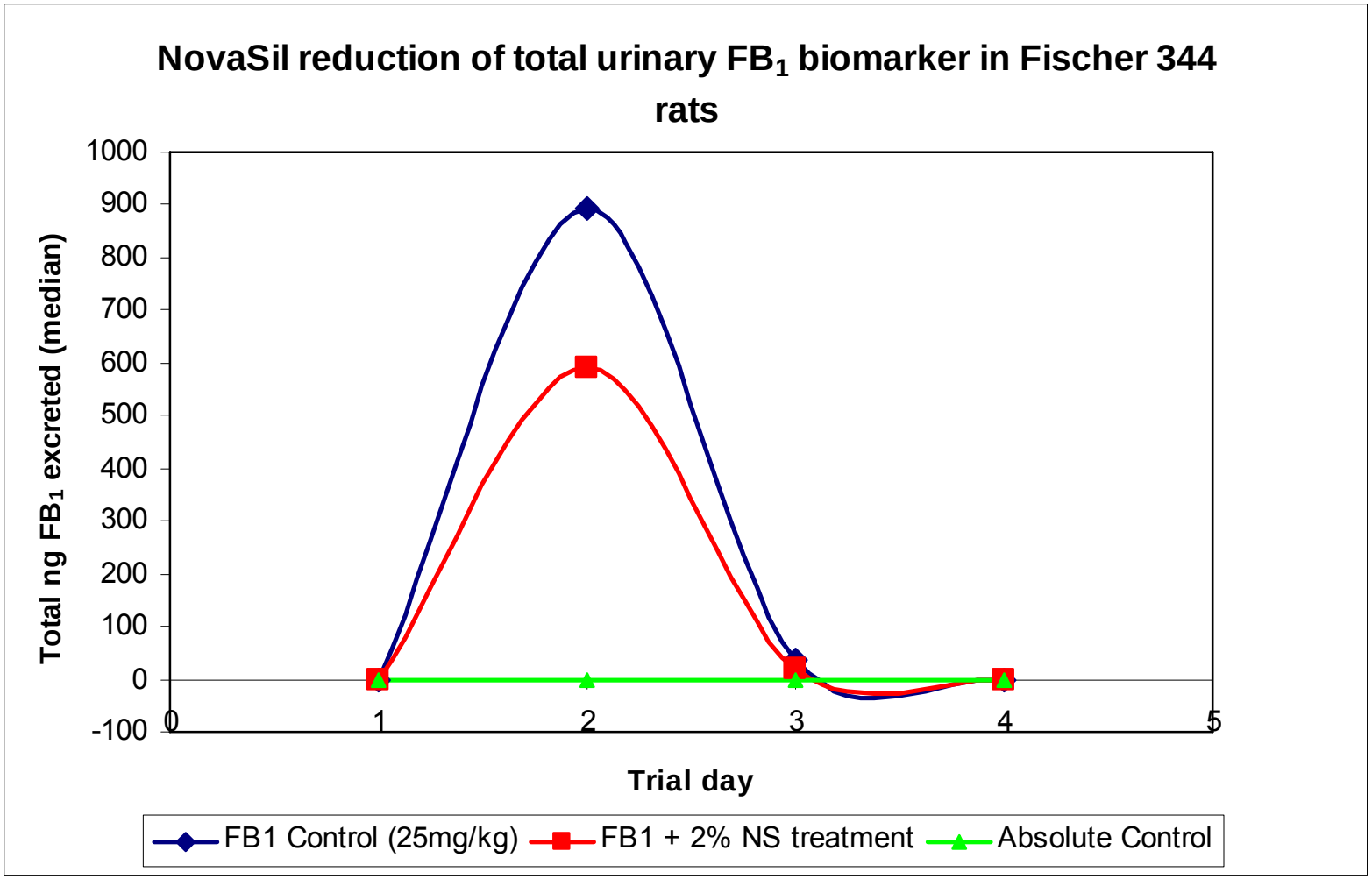


Figure 25. Novasil reduction of total excreted urinary FB_1 in Fischer 344 rats.

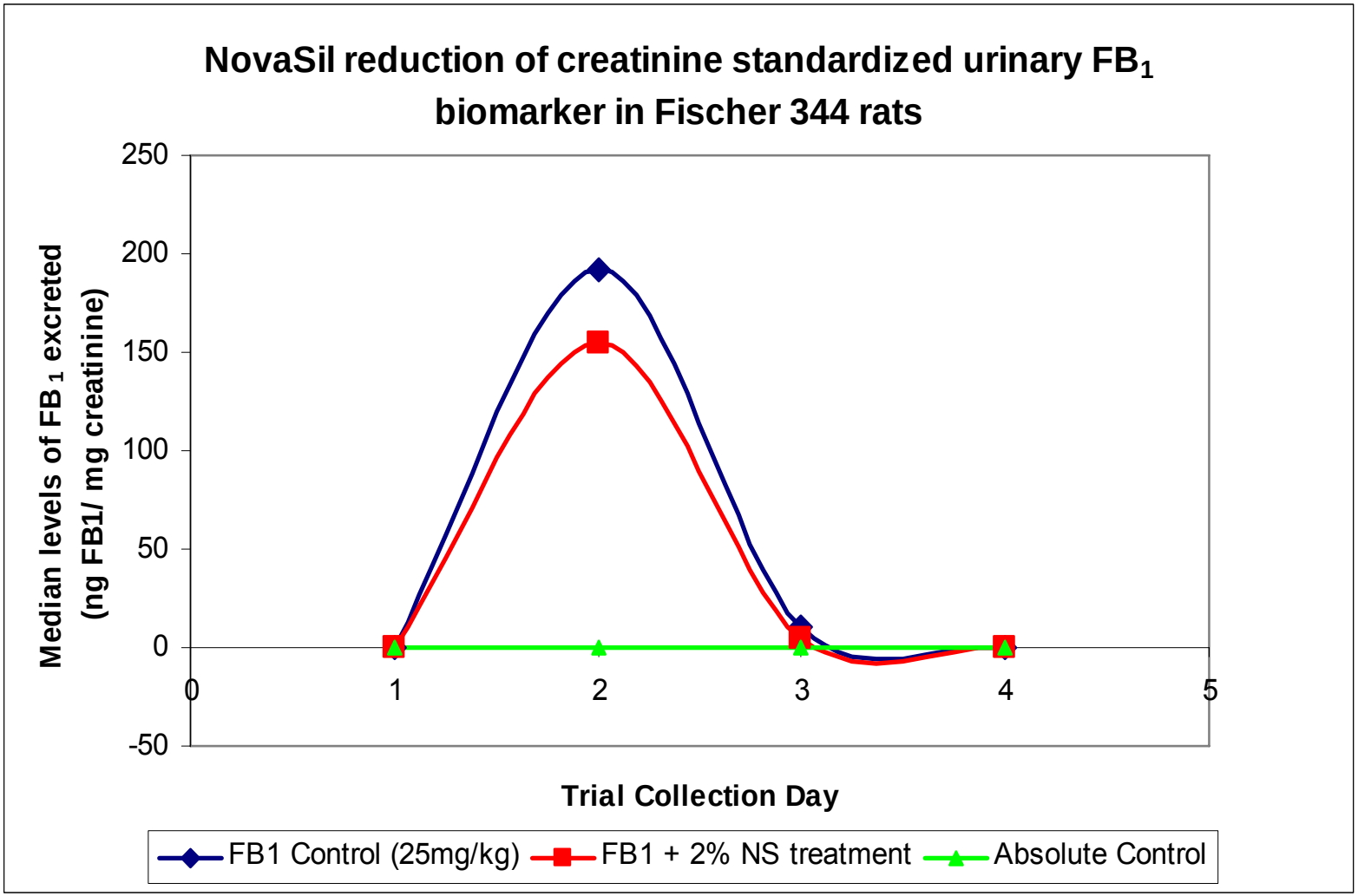


Figure 26. Novasil reduction of creatinine standardized urinary FB_1 in Fischer 344 rats.

TABLE 7

NovaSil reduction of urinary FB_1 biomarker in Fischer 344 rats

	Absolute Control	FB_1 control	FB_1 + 2% NS treatment
Urine Collection Day 2			
% detectable FB_1 samples	0 (n=6)	100 (n=6)	100 (n=6)
Mean FB_1 (95% CI)	N.D.	864.68 (719-1010.14)	*625.77 (554.17-696.83
Median FB_1 (ng/ ml urine)	N.D.	892.35	590.41
Mean FB_1 (95% CI)	N.D.	194.07 (161.55-226.59)	*158.17 (138-21-178.14)
Median FB_1 (ng/ mg creatinine)	N.D.	191.95	154.32
Urine Collection Day 3			
% detectable FB_1 samples	0 (n=6)	100 (n=6)	100 (n=6)
Mean FB_1 (95% CI)	N.D.	183.07 (110.16-255.98)	*83.29 (18.40-148.19)
Median FB_1 (ng/ ml urine)	N.D.	183.65	91.92
Mean FB_1 (95% CI)	N.D.	9.38 (6.03-12.72)	*4.42 (1.00-7.85)
Median FB_1 (ng/ mg creatinine)	N.D.	9.91	4.95

Data represent the mean, median, confidence interval (CI) and median from each treatment group

* Indicates $p \leq 0.05$ compared to FB_1 control group

difference in the means between all three treatment groups. Total urinary FB_1 was significantly reduced by 27.6% in the NS treatment group compared to the control group on day 2. When standardized with creatinine, urinary FB_1 was reduced by 19%. By day 3 however, total urinary FB_1 was significantly reduced by 54.5% in the NS treatment group compared to the control group. When standardized with creatinine, urinary FB_1 was reduced by 53%.

3.3 Discussion and conclusions

Exposure to fumonisins through the diet is a problem for animals and humans. Development of strategies to combat this problem requires sensitive and reliable methods to assess their efficacy before they can be used to aid affected populations. In this study, we determined that naphthalene 2,3 dicarboxaldehyde derivatization of FB_1 was better suited to our project goals than OPA derivatization and this method was used to evaluate urinary FB_1 as a biomarker of exposure. Furthermore we utilized this analytical method and biomarker to evaluate the efficacy of NS in decreasing FB_1 bioavailability in a rodent model.

Based on our initial work, NDA derivatization of fumonisin B_1 was found to have a lower limit of detection when compared to the OPA derivatized product (Figure 27). This was a significant finding since a lower limit of detection (LOD) allowed for a more reliable quantitation of FB_1 exposure. This lower LOD is possibly attributed to the difference in aromatic components of OPA and NDA,

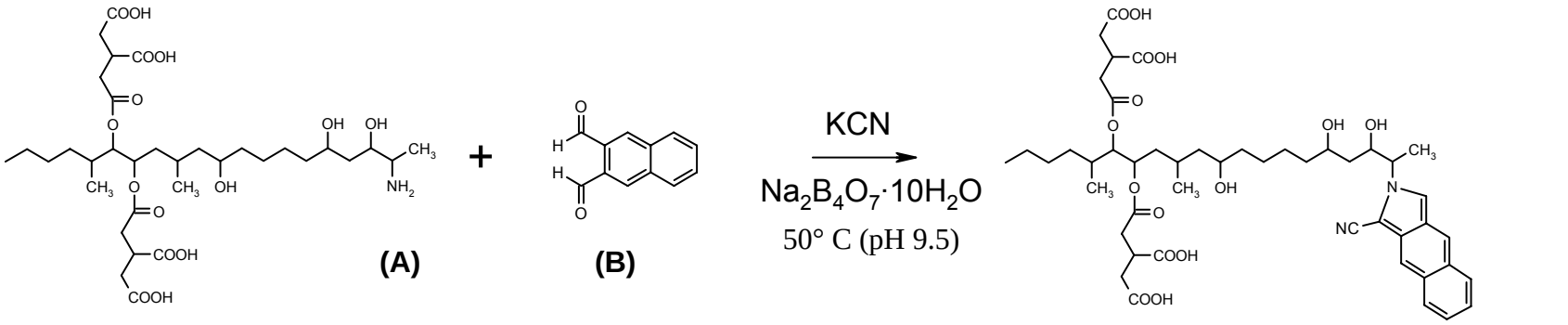


Figure 27. The reaction of the primary amine of FB_1 with naphthalene 2,3 –dicarboxaldehyde. (FB1 (**A**) and naphthalene 2,3 –dicarboxaldehyde (**B**)) The reaction occurs in the presence of potassium cyanide, forms the 1-cyano-2 alkyl isoindol fluorescent product that is detected using high performance liquid chromatography and fluorescence detection.

benzene and naphthalene, respectively. Also, NDA has more delocalized electrons than OPA which produce a stable isoindole product compared to the OPA derivative. Additionally, the increased electron count can be correlated to the amount of fluorescence produced by the derivatized product, or its quantum yield. Assuming a set wavelength of light, excitation energy is spread over more electrons in NDA when compared to OPA. This transfer of energy moves the delocalized electrons to an excited state, and in an effort to return to their natural lower energy state, they produce light and heat. As energy can only be transferred, the energy levels of individual electrons in OPA will be greater when compared to the energy levels of individual electrons in NDA after excitation from a finite source of energy. Assuming that the amount of energy converted to heat is directly related to the excited energy level of an electron (higher energy level = more vibration), the larger number of electrons in NDA resonating at a lower energy level will transfer less energy to heat than the smaller number of electrons in OPA resonating at a higher energy level; more energy will be given off as photons at a lower wavelength. This explanation is further substantiated when comparing the quantum yield of benzene (0.053) to naphthalene (0.23) (Du et al., 1998).

OPA derivatization has been previously utilized as the standard detection method for FB_1 in foodstuffs and urine (Williams et al., 2004b; Cai et al., 2007; Shephard et al., 2007; Wild and Gong, 2010). While OPA derivatization offers advantages, including low cost, ease of use, relatively low toxicity, reproducible

results at ppm concentrations, and relatively short run times, disadvantages are evident. It has been reported that OPA derivatization forms unstable FB_1-OPA derivatives that must be analyzed in an especially short time frame for maximal results (~2min) (Williams et al., 2004b; Cho et al., 2002; He et al., 2005). In addition, OPA derivatization tends to lose its ability to resolve low concentrations of FB_1 as the concentrations decrease to the low ppb-ppt range (figure 24). Since OPA derivatization must take place immediately before HPLC analysis, this methodology eliminates the possibility of automation of the procedure, limiting the sample throughput per day. Use of NDA was favored over OPA due to its increased stability and its increased fluorescent properties (Cho et al., 2002; He et al., 2005).

Shetty and Bhat first proposed a sensitive method for detecting FB_1 in urine in 1998 (Shetty and Bhat, 1998). Recently a method for the detection of free FB_1 in urine was developed for humans using LC/MS. Gong et al. developed and utilized this method to see if a there was a correlation between the consumption of tortillas suspected to contain FB1 and the levels of FB_1 in the urine (Gong et al., 2008). This study suggested a dosimetric relationship between tortilla consumption and urinary FB_1 levels. Cai et al. had previously demonstrated dose dependent excretion of FB_1 in urine by rats exposed to FB_1 (Cai et al., 2007). One caveat of the Gong study was the lack of direct measurement of FB_1 in the tortillas which translated to a lack of exposure assessment at the individual level. These omissions lead to an inability to

definitively prove a causal relationship between the excreted levels of urinary FB_1 and contaminated tortilla consumption. For this reason urinary FB_1 must be further validated for dosimetry before it will be fully accepted as a viable biomarker. Urinary FB_1, however, showed significant promise as a biomarker because of positive correlations to FB_1 exposure in more than one species of interest.

Detection and quantitation of fumonisin B_1 from a matrix is most often accomplished by using chromatographic methods. Analyses requiring high levels of sensitivity typically utilize high performance liquid chromatography (HPLC) coupled with absorbance or fluorescence detection or liquid chromatography mass spectrometry (LC-MS). Because of the ability to verify the identity of an analyte based on mass/charge ratio, LC-MS (and variations of this configuration) are considered the gold standard. LC-MS systems are expensive, however, often prohibitively so to the regions and populations that might most benefit from the technology. For this reason, HPLC is often substituted for LC-MS offering similar sensitivity without the expensive start-up and maintenance costs. HPLC offers an “affordable” option promoting sustainability in intervention situations with the ultimate goal of empowering affected populations to maintain progress.

Once a sufficient limit of detection was obtained (ppb/ppt), we used our HPLC method to measure the exposure to ingested FB_1 in a rodent model using urinary FB_1 as a biomarker. Urinary FB_1 was detected in both groups that were

exposed to FB_1 in this study. Similar to previous studies, less than 1% of our FB_1 dose was excreted in the urine. FB_1 is poorly absorbed from the GI tract and the majority of FB_1 elimination occurs through the feces, either through non-absorption or biliary excretion (Shephard et al., 1992a; Shephard et al., 1992b). All animals dosed with fumonisin B_1 displayed the urinary FB_1 biomarker within 24 h. In urine analyzed 48 h after gavage, the biomarker was reduced by almost 80% and by 72 h, urinary FB_1 was not detectable. This 48 h window is similar to other urinary biomarkers of mycotoxin exposure (Wang et al, 2008).

A major objective of this study was the verification of in vitro sorption of FB_1 by NovaSil, and evaluation of its ability to reduce bioavailability in vivo. The ultimate goal of this evaluation was to decide whether NS, a safe and effective enterosorbent for aflatoxins, could confer protection on populations exposed to fumonisins through their diet. NS is a calcium montmorillonite that has previously been shown to be safe and effective in reducing the bioavailability of ingested mycotoxins in animals and humans (Wang et al, 2008, Phillips et al.,2008). Additionally, montmorillonites have been shown in previous studies to sequester FB_1 from aqueous solutions and protect hydra from the toxic effects of FB_1 exposure. In this study, NovaSil was able to significantly reduce total urinary FB1 by 27.6% in the NS treatment group compared to the control group on day 2. When standardized with creatinine, urinary FB_1 was reduced by almost 20%. The reduction of FB_1 bioavailability was even greater on day 3 as total urinary FB_1 was significantly reduced by 54.5% in the NS treatment group

compared to the control group. When standardized with creatinine, urinary FB_1 was reduced by 53%. This enhanced reduction was probably a function of concentration dependent urinary FB_1 excretion on day 3 due to concentration dependent metabolism and release from storage organs, plasma, and bile that could potentially act as multipliers of urinary FB_1 excretion in the control group. This reduction could also be an artifact of the larger variation at the lower biomarker levels. Further study is warranted to further examine these effects and the efficacy of montmorillonite as a fumonisin enterosorbent. Based on the results of this study, urinary FB_1 was found to be a sensitive biomarker of fumonisin exposure that can be analyzed using NDA derivatization, HPLC separation and fluorescence detection methods. Additionally, NS has demonstrated an ability to reduce FB_1 bioavailability in an animal model. This effect is significant as literature suggests that populations are co-exposed to aflatoxins and fumonisins from their diet. A multifunctional enterosorbent that could sorb both mycotoxins simultaneously would be of great benefit to public health.

CHAPTER IV

SURVEY OF FB_1 EXPOSURE AND EFFECTS OF NOVASIL IN A GHANAIAN POPULATION

Fumonisin B_1 (FB_1) is the most abundant of the naturally occurring fumonisins, a group of mycotoxins produced by *Fusarium* fungi. FB_1 has been shown to be hepatotoxic, nephrotoxic, and carcinogenic in a number of species (Voss et al., 2002). Epidemiological studies have correlated *Fusarium spp*. and fumonisin contamination of food sources with increased incidences of esophageal cancer in regions of China and South Africa, neural tube defects along the Texas-Mexico border, and primary liver cancer in China (Marasas et al., 2004; Shephard et al., 2007a; Shephard et al., 2007b; Chu et al., 1994; Ueno et al., 1997). Currently, there are no reports describing the metabolism or kinetics of FB_1 in humans that definitively demonstrate a causative relationship (Stockmann-Juvala et al., 2008). While methods assessing the hazard of fumonisins in foodstuffs exist, few are capable of determining the actual exposure of populations considered to be “at risk” (Shephard et al., 1996; Shephard et al., 2007b).

Changes in sphingolipid ratio due to the inhibitory effect of FB_1 on the de novo ceramide synthase activity are commonly utilized as biomarkers for FB_1 exposure (Voss et al., 2002; Sadbourdy et al., 2008; He et al., 2006). Alterations in the sphinganine:sphingosine ratio have accurately reflected fumonisin

exposure in some lab animal species, but have not been shown to be consistent indicators of exposure in human populations consuming fumonisin-contaminated foods (Solfrizzo et al., 2004; Abnet et al., 2001). Metabolic studies in non-human primates and swine have shown that excretion of FB1 is mainly through the feces with <1% urinary excretion (Shephard et al., 1994; Fodor et al., 2008). Due to high dietary levels of FB_1 in developing countries, urinary biomarkers have been successfully used to characterize exposure in human populations (Gong et al., 2008). These same biomarkers can be applied to evaluate intervention strategies that could reduce exposure to fumonisins. (Shetty and Bhat 1998;Turner et al.; 1999; Gong et al.; 2008)

Our laboratory has previously reported that a Ghanaian population is highly exposed to aflatoxins (AFs) due to the frequent consumption of AF-contaminated foods (Jolly et al. 2006, Wang et al. 2008, Phillips et al. 2008, Williams et al. 2004a). It has been well-documented that aflatoxins cause immunosuppression, malnutrition, and hepatocellular carcinoma (Williams et al. 2004, Wogan 1992). Among a variety of strategies that have been reported to reduce AF exposure, the use of NovaSil (NS), a dioctahedral smectite clay, as a primary intervention for the enterosorption of toxin has been shown to be safe and effective in humans (Phillips et al. 2008).

In vitro and in viv*o* studies have demonstrated that FB_1 can potentiate the effects of AFs (McKean et al. 2005). Kpodo et al. verified the co-occurrence of *Fusarium* spp. and fumonisins with AFs in maize samples from Ghanaian

markets (Kpodo et al. 2000). It was postulated that participants from our previous study in Ghana, shown to be at high risk for aflatoxicosis, may be co-exposed to fumonisins. Thus, the main objectives of this study were to apply a sensitive, cost-effective, and reliable method for the detection of urinary FB_1, to assess exposure to fumonisins in a Ghanaian population highly exposed to AFs using the FB_1 urinary biomarker; and assess the efficacy of NS clay to reduce fumonisin exposure. Importantly, strategies that reduce co-exposures to AFs and fumonisins are highly desirable for populations at risk for both agents.

4.1 Materials and methods

4.1.1 Chemicals and reagents

Fumonisin B_1, methanol, 2- mercaptoethanol (cas # 60-24-2), sodium phosphate dibasic and phosphoric acid were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Naphthalene 2, 3-dicarboxaldehyde (NDA), potassium cyanide (KCN), sodium tetraborate (STB), phosphate buffered saline (PBS) and potassium hydroxide (KOH) were also obtained from Sigma. Fumonitest WB columns were purchased from VICAM (Watertown, MA, USA). All of the experiments were done using filtered and deionized water (18.2 $M\Omega \cdot cm$) (Millipore, Milford, MA), and all other chemicals and reagents were purchased commercially at the highest degree of purity available.

4.1.2 Preparation of fumonisin B_1 standard solution

A 10 ppm FB_1 standard was prepared in 50:50 methanol:water. Naphthalene 2,3-dicarboxaldehyde (NDA) solution was prepared daily using methods previously described.

4.1.3 Preparation of mobile phase

23:77 0.1M sodium phosphate: methanol mobile phase was prepared as described in the previous study.

4.1.4 Preparation of naphthalene 2,3 dicarboxaldehyde solution

NDA solution was prepared daily as described in the previous study.

4.1.5 Preparation of urine samples and Ghanaian urinary FB_1 analysis

Urine samples were stored at -80^{o}C until analysis. Samples were thawed at room temperature and centrifuged at 500 g for 5 min. Samples (10 ml) were passed through Fumonitest WB columns (VICAM, Watertown, MA) at a flow rate of approximately 1 ml/min. Columns were washed with 6 ml phosphate buffered saline and 6ml water before elution with 2 ml 80% methanol. Samples were dried at 50^{o}C under nitrogen gas and reconstituted in 150 µl of 50:50 methanol:water. Aliquots of NDA solution (300 µl) were added to samples which were vortexed and heated (50^{o}C) for 10 min (Cho et al. 2002). Analyte extracts were injected (200 µl) onto a 250 x 4.6 mm C-18 LUNA column, 5 µm particle

size (Phenomenex, Torrance, CA) with mobile phase consisting of 77:23 methanol: sodium phosphate dibasic (0.1 M , pH 3.35) at a flow rate of 1 ml/min. FB_1 analysis was performed using a Waters HPLC System equipped with an auto-injector (model 717A) and fluorescence detector (model 2475). Excitation and emission parameters were set at 252 and 483 nm for detection of NDA-derivatized material. Creatinine concentrations in urine samples were measured at St. Joseph's Regional Health Center Laboratory in Bryan, TX.

4.1.6 Urinary FB_1 MALDI-TOF mass spectrometry

The identity of the derivatized urinary FB_1 metabolite was confirmed via MALDI-TOF on a Kratos Kompact Probe MALDI-MS instrument (Kratos Analytical). A saturated solution of alpha-cyano-4-hydroxycinnamic acid (in methanol) was used to facilitate ionization of the sample as previously described by Neupert et al. (Neupert et al., 2009).

4.1.7 Study site and population

Stored urine samples were available from our previous Phase IIa clinical trial assessing the safety and efficacy of NovaSil for reducing AF exposure in a Ghanaian population (Phillips et al., 2008). Volunteers were recruited from six communities within the Ejura-Sekyedumase district in the Ashanti Region of Ghana. Biomarker data regarding exposure to fumonisins was unknown for this area; however, maize samples had been shown to contain fumonisins (Kpodo et

al., 2000). To our knowledge, this was the first study to measure FB_1 in a human population in Ghana. A total of 180 volunteers (male and female) were selected to participate in the NS intervention study based on predetermined inclusion criteria. Participants met the following conditions: signed consent form, healthy status based on physical examination, age 18-58 years, intake of corn and/or groundnut-based foods at least four times per week, blood AFB_1-albumin adduct levels > 0.5 pmol AFB_1/mg albumin, no history of chronic disease(s), no use of prescribed medications, non-pregnant and non-breastfeeding females, normal ranges of hematological parameters, normal liver and renal function indicators (blood and urine parameters).

4.1.8 Study design and protocol

The study protocol was approved by the Institutional Review Boards at Texas A&M University and Noguchi Memorial Institute for Medical Research in Ghana for Ethical Clearance. All participants were provided written informed consent, as well as an oral explanation prior to beginning the study. The overall study design adhered to guidelines set for a randomized, double-blind, placebo controlled Phase IIa clinical trial (Sangare-Tigori et al., 2006). Participants were randomly divided into one of the three groups: High dose (NS 3.0 g/day), low dose (NS 1.5 g/day) or placebo control (microcrystalline cellulose) for a period of 3 months. Trained study monitors collected blood and urine samples from each participant at multiple time points. Aliquots of the first urines were stored

separately in polypropylene tubes and shipped frozen to Texas A&M University where they were stored at -80°C prior to biomarker analysis. Samples collected at week eight and ten from all three treatment groups (186 samples) were used in this preliminary study to assess fumonisin exposure and intervention efficacy.

4.1.9 Statistical analysis

Data generated from HPLC analyses were transferred into an Excel database for management. Mean, median, confidence interval (CI) and range were calculated from analysis of FB_1. To show the consequence of NS ingestion on FB_1 levels, statistical evaluation focused on the comparisons among different treatment groups at two different time points. Non-detectable samples = LOD/2 for statistical analysis. Since the parameters were not normally distributed, the Kruskal-Wallis test and Wilcoxon rank sum test was used to compare the differences among and between treatment groups. A p-value $\leq$ 0.05 (two-tailed) was considered significant. Statistical analyses were done using SPSS software version 15.0 (SPSS Headquarters., Chicago, IL).

4.2 Results

4.2.1 Ghanaian exposure assessment using urinary FB_1

Urine samples (186) from study participants at week 8 and week 10 were analyzed for the presence of FB_1 using immunoaffinity clean-up and HPLC analysis. Chromatograms of NDA derivatized FB_1 standard, spiked human urine

and a representative urine sample from a study participant were compared for continuity (Figure 28). Mean and median levels of FB_1 are presented in Table 8. In the week 8 placebo treatment group (n = 23), 14 samples (60.9 %) were found to be positive for the presence of FB_1 with a mean urinary FB_1 concentration of 7.12 ng/mg creatinine. In the week 10 placebo group (n = 43), the percentage of samples with detectable FB_1 was higher (72.1%), but the mean urinary concentration of 6.29 ng FB_1/mg creatinine was similar to week 8. Analysis of the week 8 high dose (n = 23) and low dose (n = 22) NS treatment groups revealed a lower percentage of participants with detectable urinary FB_1 (39.1 and 45.5%, respectively). Week 10 high (n = 35) and low dose (n = 40) treatment groups also displayed a reduction in the percentage (54.3 and 52.5 %, respectively) of urinary samples with detectable FB_1 along with decreased FB_1 levels of 1.02 and 1.69 ng/mg creatinine, respectively. A similar trend was observed in the treatment groups from both week 8 and 10 when comparing non-creatinine adjusted urinary FB_1 levels. Because the FB_1 data was not normally distributed, non-parametric analysis was applied for all statistical evaluations.

Median FB_1 levels for the high dose treatment group were significantly lower ($p \leq 0.05$) than the placebo group at week 8. FB_1 levels from both treatment groups, high and low dose, were significantly reduced compared to placebo at week 10 (Figure 29, Table 8).

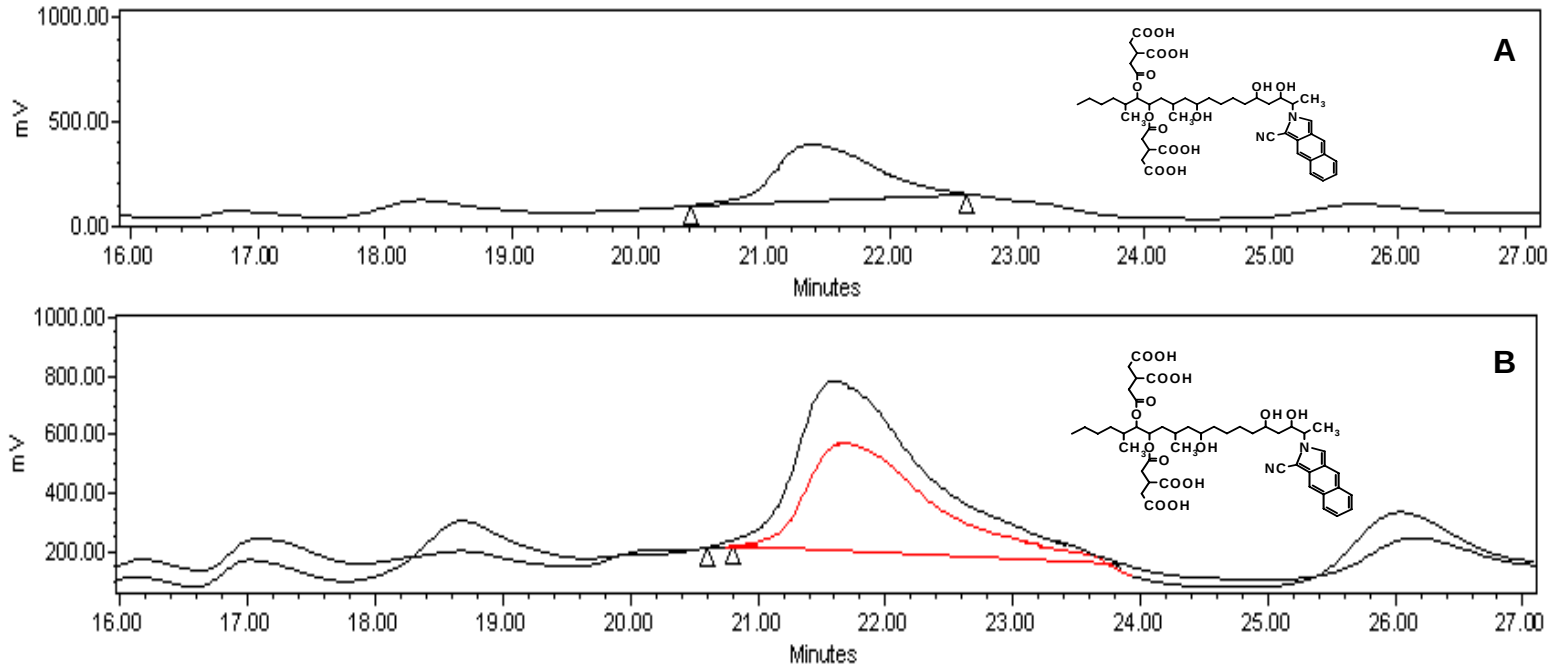


Figure 28. **A.** HPLC chromatogram of NDA derivatized FB_1 standard (retention time ~21.5 min). **B.** HPLC chromatogram of spiked human urine (retention time ~21.5 min) and a representative urine sample from a study participant containing NDA derivatized FB_1 (**red**) (retention time ~21.5 min).

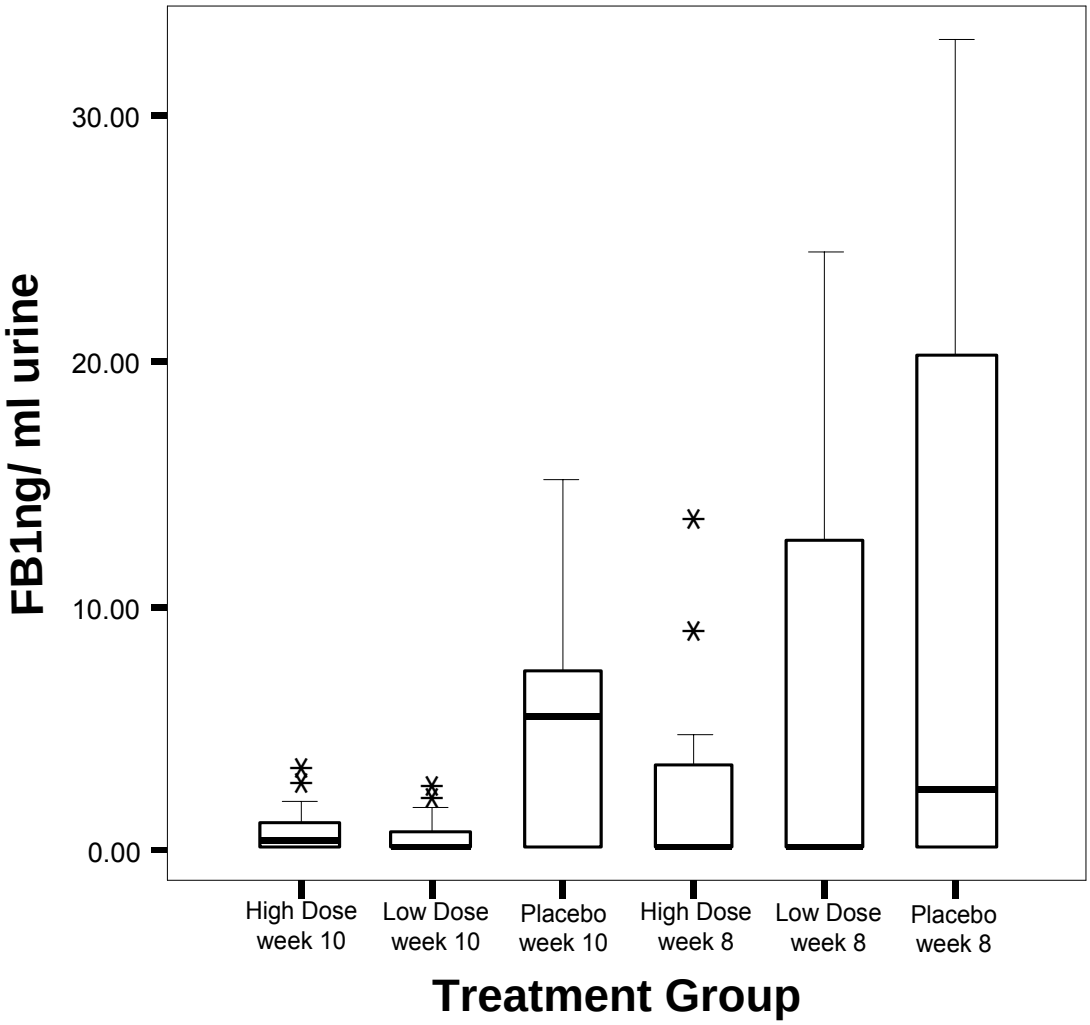


Figure 29. Distribution of total urinary FB_1 between treatment groups at weeks 8 and 10 (ng FB_1/ml urine). Data represents the distribution of FB_1 in the various treatment groups based on a NovaSil intervention in Ghana. Extreme outliers are not depicted. There was a statistically significant difference among treatments ($p<0.05$). There was a significant difference between the median FB_1 levels in the NS treated groups versus the placebo group ($p<0.05$). There was not a significant difference between median FB_1 levels in the high and low dose groups ($p = 0.566$).

TABLE 8

Reduction of FB_1 with NovaSil Treatment in a Ghanaian population

	NovaSil Treatment		Control
	High Dose (3.0 g/Day)	**Low Dose** (1.5 g/Day)	**Placebo** (1.5 g/Day)
Week 8 Urinary FB_1 analysis			
% detectable FB_1 samples	39.1 (9/23)	45.5 (10/22)	60.9 (14/23)
Mean FB_1 (95% CI) Median FB_1 (ng/ml urine)	*4.05 (0.99 – 7.10) 0.19	5.61 (1.74 – 9.48) 0.19	9.36 (4.51 – 14.22) 5.45
Mean FB_1 (95% CI) Median FB_1 (ng/mg creatinine)	*1.07 (0.22 – 1.91) 0.20	5.61 (0.81 – 10.42) 0.20	7.12 (1.69– 12.52) 2.00
Week 10 Urinary FB_1 analysis			
% detectable FB_1 samples	54.3 (19/35)	52.5 (21/40)	72.1 (31/43)
Mean FB_1 (95% CI) Median FB_1 (ng/ml urine)	*0.81 (0.53 – 1.09) 0.44	*0.73 (0.37 – 1.08) 0.21	5.03 (3.76 – 6.30) 5.45
Mean FB_1 (95% CI) Median FB_1 (ng/mg creatinine)	*1.02 (0.45 – 1.59) 0.28	*1.69 (0.51 – 2.87) 0.20	6.29 (3.75– 8.84) 2.91

Data represent the mean, confidence interval (CI) and median from each treatment group
Placebo = microcrystalline cellulose
*Indicates $p < 0.05$ compared to placebo group

Mean urinary FB_1 was reduced >40% in the NS low and high dose treatment groups at week 8 and > 80% at week 10. Similar results were seen after creatinine standardization for urine volume (Figure 30, Table 8). Mean urinary FB_1 was reduced >80% in the high dose treatment groups at both week 8 and 10. There was also a 73% reduction in mean urinary FB_1 in the low dose treatment group at week 10. Although dose-dependent differences in FB_1 median levels between the high and low dose treatment groups were not found to be statistically significant, these results suggest a high affinity of the NS clay for FB_1.

4.2.2 MALDI-TOF analysis of urinary FB_1

MALDI-TOF mass spectrometry (MTMS) analysis of parent ($M+H^+$), NDA derivatized, and a NDA derivatized + sodium ion ($M+Na^+$) confirmed a mass/charge of 723.0, 906.5, and 928.3, respectively (Figure 31A-C). MTMS analysis of FB_1 peaks collected from HPLC analysis of randomly selected positive urine samples verified the presence and identity of FB_1, with peaks at 907.4 ($M+H^+$) and 928.5 ($M+Na^+$) (Figure 31C). Worth noting, is the lack of parent peak (~722.8) in MTMS analyzed samples which may provide an indirect indicator of the efficiency of the NDA derivatization reaction.

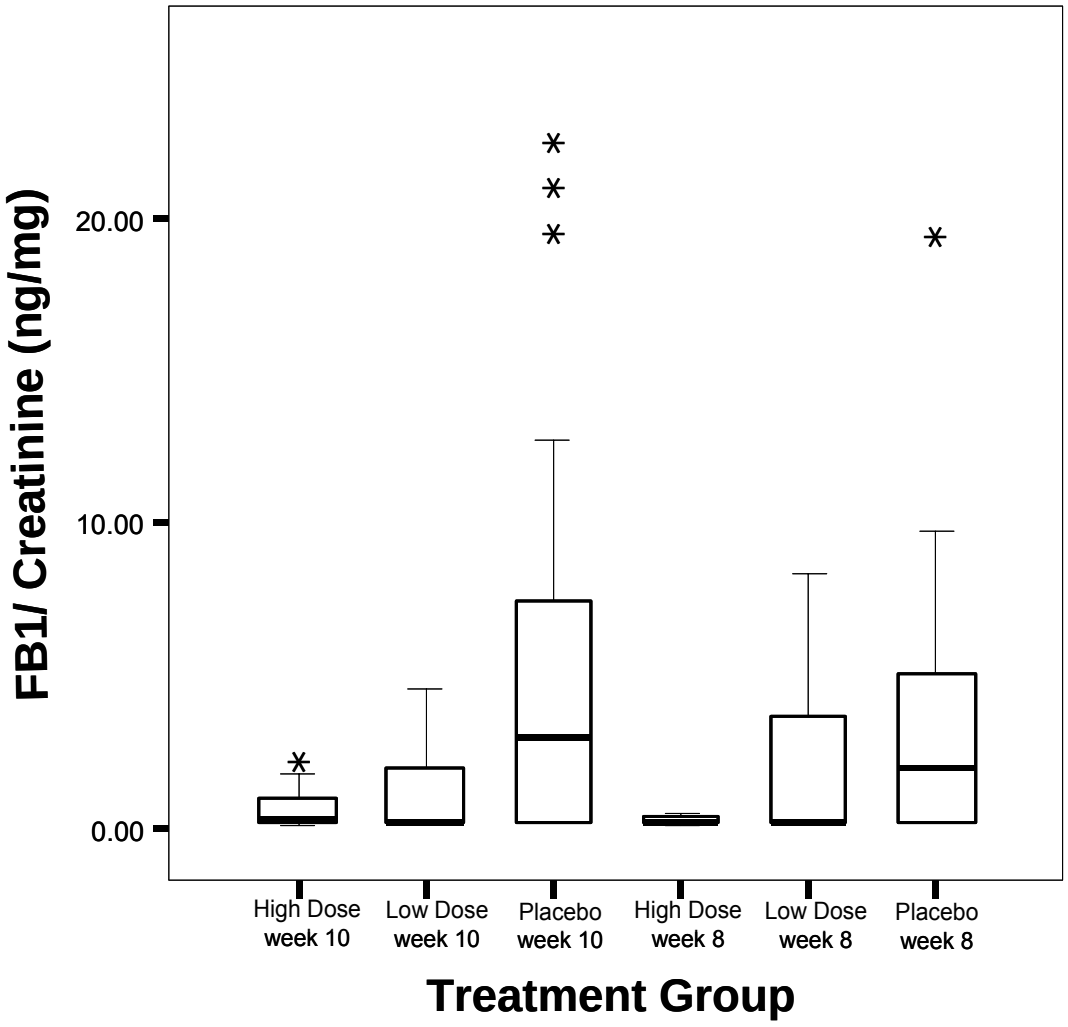


Figure 30. Distribution of creatinine standardized urinary FB_1 between treatment groups at weeks 8 and 10 (ng FB_1/mg creatinine). Data represents the distribution of FB_1 in the various treatment groups based on a NovaSil intervention in Ghana. Extreme outliers are not depicted. There was a statistically significant difference among treatments ($p<0.05$). There was a significant difference between the median FB_1 levels in the NS treated groups versus the placebo group ($p<0.05$). There was not a significant difference between median FB_1 levels in the high and low dose groups ($p = 0.955$).

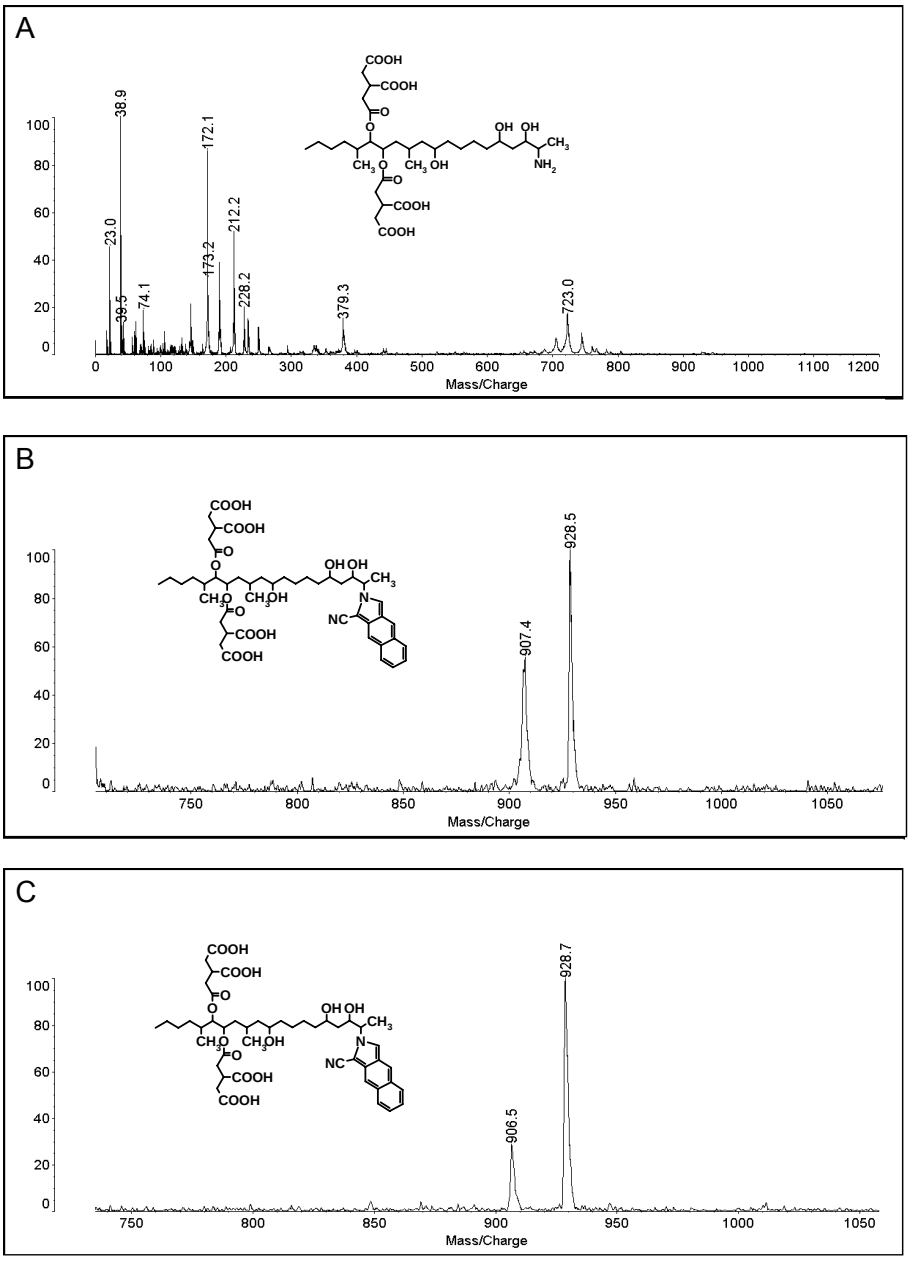


Figure 31. MALDI-TOF mass spectra of parent and derivatized FB_1(MS). MS are based on relative intensity (y-axis) of depicted peaks (highest peak = 100%). **A**. MS of underivatized standard FB_1 in water (723 m/z). **B**. MS of NDA derivatized FB_1 from spiked urine sample (~906.1 m/z) and NDA derivative + Na ion (~928 m/z). **C**. MS of a representative FB_1 positive participant urine sample verifying presence of FB_1.

4.3 Discussion and conclusions

Human exposure to multiple mycotoxins has received increased attention, and recent studies in West Africa have confirmed the co-occurrence of AFs and fumonisins in foodstuffs found in Cote d'Ivoire and Ghana (Kpodo et al. 2000, Sangare-Tigori et al. 2006). Surveys have demonstrated that maize-based products are often co-contaminated with diverse mycotoxins; however, there is a paucity of published data measuring human exposure to fumonisins and AFs. Use of urinary FB_1 as a biomarker of exposure in humans was first proposed by Shetty and Bhat in 1998 (Shetty and Bhat 1998). In 1999, Turner et al. reported that analysis of urinary FB_1 held significant promise as there was a need for a reliable biomarker of exposure to fumonisins (Turner et al. 1999). A recent study by Gong et al. demonstrated a dose-response relationship between consumption of tortillas containing fumonisins and human urinary FB_1 (Gong et al. 2008). In our study, biomarker data in humans from Ghana support the work by Gong et al. in Mexico. Moreover, these same data shows that NS clay is able to mitigate FB_1 exposure from the diet possibly by diminishing the bioavailability of this mycotoxin in the gastrointestinal tract.

The week 8 and 10 mean FB_1 levels measured in our study population (9.36 and 5.03 ng FB_1/ml urine, respectively) were higher than mean urinary FB_1 concentration reported previously in Mexico (0.147 ng FB_1/ml urine) (Gong et al. 2008).

Based on ~1% urinary excretion, an estimated metabolic rate of 1500 ml urine/day and the average size of an adult (70kg), it can be estimated that the exposure to FB_1 at week 8 was 20.06 µg/kg b.w./day and 10.78 µg/kg b.w./day at week 10 in our placebo groups (Shephard et al. 1994, Fodor et al. 2008). These values are higher than the FB_1 levels reported from Mexico (0.368 µg/kg b.w./ day) (Gong et al. 2008). A number of possible explanations may account for this difference in the levels of FB_1 observed in humans in Ghana versus Mexico. These include variations in: (1) corn production techniques, (2) climate, (3) analytical methodologies, (4) maize preparation, processing, and amount consumed, (5) age, health, nutritional status, gender and (6) fumonisin metabolism due to population genetic polymorphisms. Furthermore, a market survey in Ghana detected levels of FB_1 between 70 and 2621 (mean 608.8) µg/kg in maize intended for human consumption (Jolly et al. 2006). Based on an average maize intake of 369.5 g/day (MacIntyre et al. 2002), a 1% carryover of FB_1 from the diet, and an average urinary excretion (1500 ml), Ghanaians from our site could potentially excrete urinary FB_1 levels between 0.172 to 6.46 ng/ml urine. Mean Biomarker levels measured in this preliminary study are similar to this range.

In the previous clinical trial in Ghana, significant decreases in median urinary aflatoxin M_1 levels were observed at 12 weeks after NS intervention ($p = 0.0445$) (Wang et al. 2008). Since samples that were collected at 12 weeks were depleted for aflatoxin biomarker analysis, we utilized the remaining samples at

the closest time points during the intervention (week 8 and 10), in order to assess the effect of NS treatment on urinary FB_1 levels. Following treatment with NS, the week 8 samples had mean values of 4.05 and 5.61 ng/ml urine in the high and low dose treatment groups respectively. At week 10 samples had further reduced mean values of 0.81 and 0.72 ng FB_1/ml urine in the high and low dose treatment groups, respectively, versus 5.03 ng FB_1/ml urine in the placebo group. Further studies are warranted to establish whether a temporal relationship exists allowing NS treatment to exert more effect at later time points. Based on these 10 week mean urine values, exposure could be estimated at 1.72 and 1.54 µg/kg b.w./day for the high and low dose participants, respectively. Following consumption with NS, participant exposure to FB_1 was reduced below the JECFA provisional maximum tolerable daily intake for FB_1 of 2 µg/kg b.w./day (WHO 2002).

Based on our previous research, NS clay has displayed: 1) favorable thermodynamic characteristics of aflatoxin sorption, 2) tolerable levels of priority metals, dioxins/furans and other hazardous contaminants, 3) safety and efficacy in multiple animal species and humans, 4) safety and efficacy in long-term animal studies, and 5) negligible interactions with vitamins, iron and zinc and other micronutrients. Studies in vitro with NS clay have suggested that it sorbs aflatoxins and similar congeners with stereo- and regiospecificity. A potential chemical reaction that may explain the high capacity and affinity of aflatoxins for clay surfaces is an electron donor-acceptor mechanism. This

mechanism involves sharing electrons from the negative surface of the clay with partially positive atoms in the β-dicarbonyl system in the aflatoxins. Moreover, animal studies have confirmed the preference of NS clay for aflatoxins, in that NS in the diet did not protect animals from the effects of other structurally diverse mycotoxins, including zearalenone, deoxynivalenol, T-2 toxin, ochratoxin A, cyclopiazonic acid, and ergotamine (Phillips et al., 2008).

Dioctahedral smectite clays, including NS, have been evaluated in vitro for FB_1 sorption and found to interact with this mycotoxin at interlayer surfaces (Lemke 2000). As previously stated, the potential mechanism for FB_1 sorption by NS is protonation of the amino group at C2 on the molecule. The charged FB_1 may facilitate a cationic exchange reaction at negatively charged surfaces of the clay similar to the interaction(s) of cationic surfactants or quaternary amines at interlayer surfaces in common dioctahedral smectite clays. Further work is warranted to delineate the molecular mechanism and surface chemistry involved in the sorption process of FB_1 onto NS and to confirm the efficacy and safety of NS clay as a multifunctional intervention for aflatoxins and fumonisins in animals and humans.

Mycotoxins have been linked to death and disease, with an estimated 4.5 billion people highly exposed in developing countries (Williams et al. 2004a). The mycotoxin problem to public health is longstanding, unavoidable, and seemingly inextricable. Frequent and concurrent exposure to aflatoxins and fumonisins may enhance/synergize the toxic, carcinogenic, and teratogenic

effects of these food-borne contaminants. This study is the first to demonstrate the efficacy of enterosorbent intervention for FB_1 exposure using HPLC with NDA derivitization to detect fumonisin biomarkers in human urine. Importantly, it also illustrates that a population in Ghana co-exposed to aflatoxins and fumonisins may be protected from the adverse effects of these mycotoxins using NS clay. As a primary intervention, capsules or other dose forms of NS could be used in clinical practice for both “acute emergencies” and “chronic exposures.” Additional studies are planned to confirm these findings in long-term clinical intervention trials in Ghana. Clay-based enterosorbent strategies represent a novel approach to the problem in populations that are at high risk for mycotoxicosis.

CHAPTER V

SUMMARY AND CONCLUSIONS

Fumonisins contaminate the diet of a large proportion of the world's population with as many as 4.5 billion people at risk for mycotoxicosis. Fumonisin toxicosis is a problem in developed regions but more so in developing regions where these mycotoxins affect staple foods, such as maize (corn) and other cereals. Exposures to fumonisins are responsible for the development of equine leukoencephalomalacia, porcine pulmonary edema, and neoplasm formation in the liver and kidneys of rodents. Exposure to fumonisin toxins are also highly correlated with increased incidences of esophageal and hepatic carcinomas in humans and are thought to be a major contributor to the global incidence of neural tube defects. Fumonisin B_1 exerts its effects through the competitive inhibition of de novo ceramide production which results in disruption of most, if not all, cellular processes.

Global public health organizations have tried to limit exposure to fumonisins and other mycotoxins to levels where the public health risk was minimal. These recommendations are based on in vitro and in vivo data from mycotoxin studies, epidemiological studies in populations perceived to be exposed, and socioeconomic pressures. It is this last variable that leads to large discrepancies in regulatory levels; one organization is focused on the elimination of all risk while another regulatory agency proposes levels that are deemed achievable and sustainable with the use of good agricultural and good manufacturing practices. There have been difficulties in establishing guidelines

for public exposure to mycotoxins in which co-exposure is the driving force for regulation. The scientific basis for previous mycotoxin regulations has been the study of these toxins and their effects in isolation, unknowingly, but likely to the detriment of public health.

Adding to this epidemic is the fact that the exposures and co-exposures to mycotoxins have largely been ignored as a public health concern. Despite occasional high profile incidents such as acute poisoning outbreaks or the presence of mycotoxins in nutritional supplements, mycotoxins have not been widely prioritized from a public health perspective in low-income countries. Where attention has been paid, it is perceived to have been largely driven by the need to meet stringent import regulations on mycotoxin contamination in the richer nations of the world rather than to protect the population producing and consuming the contaminated crops locally.

Recently there has been a shift in research focus from identification and study of mycotoxins such as fumonisin B_1 in isolation to studies where their co-existence with other mycotoxins such aflatoxin B_1 are targeted. Mycotoxins rarely exist in isolation in nature and the implications of co-exposure in “at risk” populations are of significant concern. Fumonisin B_1 and aflatoxin B_1 have been shown to be synergistic in the formation of hepatic neoplasms. While a few studies have examined the hazard of co-exposure to these potent mycotoxins, to date, there are no studies that have assessed the actual co-exposure to these toxins in “at risk” populations and their resulting effects on public health. This is due largely to the lack of reliable methods to assess the concurrent individual exposure to these toxins. In an effort to reduce the

potential for exposure, intervention strategies have been proposed at the pre and post harvest level. However, much like previous exposure assessments, these remediation strategies disregard the co-existence and co-exposure of populations at high risk for multiple mycotoxins.

As previously stated, regulatory mandates and recommendations exist for animal and human exposure to fumonisins in developed and developing parts of the world. The impetus for compliance with these regulations is more likely a function of socioeconomic status than concern for well-being when poorer countries are compared to more developed nations. Often the regions that would benefit most from concerted efforts of regulation and remediation of mycotoxin exposure are the regions least adapted to do so. Socioeconomic conditions often force the citizens of less developed countries to consume whatever is grown regardless of the condition. Economically sensitive, yet culturally acceptable solutions for reducing exposure to mycotoxins like fumonisin B_1 are in high demand.

Our studies were designed to adapt existing technology for the purpose of identification, quantitation, and remediation of fumonisin exposure in populations frequently exposed to mycotoxins. Montmorillonite clays such as NovaSil are safe, inexpensive and represent a remediation strategy that is culturally accepted in many parts of the world. Isothermal analyses were used to evaluate the binding capacity of a negatively charged sorbent (montmorillonite) and a positively charged sorbent (ferrihydrite). The results from these in vitro studies demonstrate that montmorillonite clays and ferrihydrite were able sequester fumonisin B_1 from an aqueous environment and

protect lower organisms from the effects of toxicity. Montmorillonite had a greater capacity for sorption of fumonisin B_1 based on Q_{max} values. In this case, the basis of this efficacy is probably the interaction of the positively charged amine and negatively charged interlayer that results from the 2:1 dioctahedral structure of the clay. This was indirectly confirmed by the lack of binding by a montmorillonite in which the interlayer had been collapsed using heat. In ferrihydrite, it is possible that the negatively charged acid groups on deprotonated fumonisin interacted with the positive surface charge of the sorbent. Questions about the bioavailability of iron from chronic ferrihydrite treatment and the potential development of hemosiderosis and hemochromatosis were of concern and warrant further study. Previous studies have shown, however, that montmorillonite clays such as NovaSil, are non-toxic at levels up to 2% w/w and were effective in aflatoxin sorption in rodent assays. For this reason, NovaSil was selected for in vivo studies to assess its potential as an enterosorbent for fumonisin exposure.

A critical barrier to the assessment of NovaSil as an enterosorbent was the lack of a sensitive yet reliable method of assessing levels of fumonisin B_1 bioavailability. Fumonisins require conjugation to a fluorescent molecule in a process known as derivatization before they can be analyzed using HPLC. O-phthalaldehyde (OPA) and naphthalene 2, 3 dicarboxaldehyde (NDA) were compared as potential derivatization agents for FB_1. Derivatization of FB_1 with NDA was found to produce an isoindole product that was stable and displayed a higher fluorescent intensity than the OPA derivatized product. This could be due to increased numbers of delocalized electrons in NDA allowing for greater

molecular stability and increased quantum yield. Through the use of naphthalene 2,3 dicarboxaldehyde derivatization, an HPLC method was adapted to the task of assessing fumonisin B_1 exposure. This method was critical as the validity of previous methods in assessing fumonisin B_1 exposure were heavily dependent on the species studied (ie. sphinganine: sphingosine ratio in rodents). In this work, urinary FB_1 was shown to be a reliable biomarker of fumonisin B_1 exposure in a Fischer 344 rodent model, confirming previous reports in the literature. This biomarker reached it peak intensity within 24 hours of ingestion and was not detectable 72 hours after exposure. These results suggest that urinary FB_1 is a short term biomarker offering a window of exposure assessment of only 48 hours.

This short term biomarker was the cornerstone of our efforts to assess the efficacy of NovaSil as a fumonisin enterosorbent. There was a significant reduction in fumonisin B_1 bioavailability in the animals treated with NovaSil, confirming the results of our in vitro studies. Despite our success, further studies are warranted to further assess potential species differences in the dosimetry of this biomarker.

Utilizing urinary FB_1 as a short term biomarker, a sub-set of a Ghanaian population known to be exposed to aflatoxin B_1 was found to be co-exposed to fumonisin B_1. This was noteworthy due to the known synergistic effects of co-exposure to these mycotoxins. In participants treated with NovaSil clay, urinary FB_1 was reduced in a dose dependent fashion similar to the reduction seen in aflatoxin M1 biomarker. No adverse effects from the ingestion of NovaSil were noted. To our knowledge, this is the first report of co-exposure to fumonisin B_1

and aflatoxin B_1 and successful application of a multifunctional remediation strategy. Techniques described in this document are currently being adapted in Ghana in hopes that the progress gained will be sustained and used to enhance public health in the Region.

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