

ABSTRACT

PRICE, MICHAEL SCOTT. Characterization of *Aspergillus niger* for Removal of Copper and Zinc from Swine Wastewater. (Under the direction of Gary A. Payne.)

The United States has experienced a recent boom in pork production. Associated with this growth has been a shift from traditional small family farm units to large confined housing operations. North Carolina, with 9.5 million swine, has been the leader in the development of large, efficient swine operations and is second only to Iowa in pork production. This change has resulted in production of more swine on less land and an increase in animal waste application to adjoining farm land. The repeated application of swine waste may result in increased accumulation of copper and zinc in soils. There is concern that these two metals, which are added to swine feed, will accumulate to phytotoxic levels in agricultural soils. The objective of the research described in this thesis was to investigate the ability of fungi to remove copper and zinc from swine wastewater. The imperfect fungus *Aspergillus niger* was found to be the most resistant (of six fungi examined) to copper, and the one best able to remove copper from culture media and swine wastewater. *A. niger* was able to remove as much as 91% of the copper and 70% of the zinc from hog wastewater collected from an aerobic/anaerobic swine waste treatment facility. Interestingly, the majority of the copper and zinc removed by the fungus was by absorption. Absorption of metal by fungi has not been reported as a useful method for bioremediation. These studies show that *A. niger* is a promising candidate for the removal of copper and zinc from swine wastewater.

**CHARACTERIZATION OF *ASPERGILLUS NIGER*
FOR REMOVAL OF COPPER AND ZINC FROM
SWINE WASTEWATER**

by

Michael Scott Price

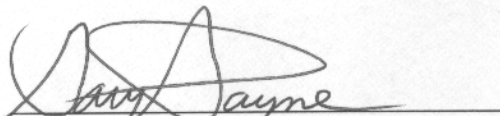
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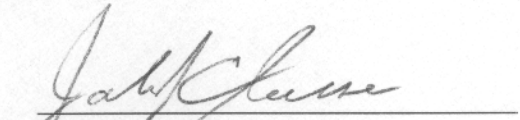
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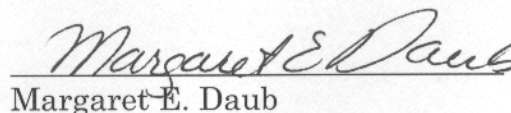
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DEDICATION

The work contained in this thesis is dedicated to my wife, Jennifer. She always persevered through late night lab and study sessions, and her understanding and love means the world to me. She understands my goals, and helps me every step of the way. Thank you honey, I love you.

BIOGRAPHY

Michael Price was born in Wilmington, Delaware on December 22, 1973, at 5:52 PM. He attended New Castle Baptist Academy from kindergarten until graduating high school in 1991. Upon graduation he entered the University of Delaware Honors Program, majoring in Animal Science with a minor in Biology. While at UD, he also entered the Degree with Distinction program, and conducted thesis research under the direction of Dr. John Dohms. His thesis work was completed in May, 1995, and he graduated from UD with a B.S. in Agriculture with Distinction.

After graduating from the University of Delaware, Michael entered the Microbiology Department at Colorado State University. After realizing this program was not for him, he left and returned home to take a year off before entering the Plant Pathology Department at North Carolina State University. Currently, he is a Master's Degree candidate under the direction of Dr. Gary Payne. Future plans include pursuing a Ph.D. in Plant Pathology at NCSU, also under the direction of Dr. Gary Payne.

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COPPER AND ZINC IN SWINE PRODUCTION

Swine production in North Carolina has become a lucrative business in which large confinement operations are replacing many smaller family farms. Currently, North Carolina is second in the nation in hog production with 9.5 million swine (USDA, 1999). Intensive hog production leads to vast quantities of waste that must be treated, stored and used on adjacent farmland. Current dietary recommendations for elevated copper (250 ppm) and zinc (3000 ppm) in feed for growing swine (Carlson and Pettigrew, 1999) results in hog waste that is high in both metals. The concentration of these metals in hog waste poses a serious environmental threat to arable land receiving repeated applications of swine waste.

Copper

Copper is an essential mineral found in almost all living systems. In animals copper is necessary for hemoglobin (Hb) production and required for the uptake and utilization of iron. It is one constituent of the metalloenzyme superoxide dismutase, which eliminates harmful oxygen radicals from the body. Copper is also a component of cytochrome oxidases, enzymes that play many key roles in organisms, including oxidative phosphorylation. A cytochrome oxidase is also indispensable in the proper deposition of myelin around nerve tissue, and copper deficiency in sheep is known to lead to

neonatal ataxia and death. Another copper containing enzyme, ceruloplasmin, is required for the oxidation of a number of substrates and necessary for normal bone formation. This enzyme is required for the conversion of iron from the ferrous (+2) to the ferric (+3) valence state, and is also the main copper transport protein synthesized in the liver. Copper homeostasis is achieved mainly through biliary excretion in non-ruminant animals, such as swine. Ruminants, on the other hand, rely more on urinary excretion for copper regulation. The excreted copper is bound to metallothionein (MT), a short, cysteine-rich protein which is very efficient at binding metals such as copper and zinc (Richards, 1989). Metal binding by this protein takes place mainly in either the intestinal lumen (non-ruminants) or the kidney (ruminants) and is unavailable for re-absorption.

Given the diverse and important roles of copper in organisms, it is surprising that the biological significance of this metal was still largely unknown just 50 years ago. Copper was known to be a component of plants as early as 1817, but was not accepted as being ubiquitous in flora until 1920 (Elvehjem, 1935). Although high concentrations of copper were known to occur in marine organisms such as gastropods as early as 1847, its presence in higher animals was considered a rarity. In the gastropods, copper is a component of the oxygen-carrying molecule hemocyanin, while in higher animals it is required for the production of the iron-containing oxygen

transport molecule hemoglobin. Until the early 1900's copper was known to play some role in both cellular metabolism and anemia, but the specifics eluded researchers. Copper was not verified as a component of human tissue until 1921 (Elvehjem, 1935).

Church was the first to report the presence of copper in a non-marine animal (Church, 1869). He described the red pigment turacin in certain species of Touracou, an African bird related to the cuckoo (Church, 1869). Turacin is only slightly water soluble, but extremely soluble in alkali. Under alkaline conditions turacin is blue. Church characterized the properties of turacin and found it to resemble hemoglobin. After analysis of the ashed protein, however, he was surprised to find not iron, as would be expected of a heme protein. Instead, he found a large amount of copper. Turacin was also evenly distributed in the feathers, unlike hemoglobin, which is usually found in aggregates, such as inside red blood cells. The results of Church showed that turacin was unlikely a hemoglobin protein, and his paper is considered the first report of copper as a constituent of a major animal pigment.

The next major report of copper in animals was made in 1928, when researchers found copper necessary for hemoglobin production in the rat (Hart *et al.*, 1928). When the researchers fed rats a diet of whole cow's milk, known to be low in both iron and copper, the rats became anemic. Previous studies by these researchers (Waddell *et al.*, 1928) had shown that feeding

purified iron salts to anemic rats failed to prevent or correct anemia associated with a diet of cow's milk. In an attempt to identify a compound to prevent anemia, the researchers examined an ashed liver preparation from Eli Lilly and Co. The ash was digested with HCl, separated with hydrogen sulfide, filtered and the filtrate treated with ammonia and ammonium sulfide. The H₂S precipitate was found to be capable of reversing the anemia. This fraction contained nearly all of the copper from the liver preparation. As a result of these studies they fed one rat CuSO₄ directly. Although realizing this trial was statistically inadequate, the overwhelmingly positive response observed by the authors compelled them to include the results of this assay in the manuscript. Based on the results obtained from one rat and the presence of copper in the active fraction, the authors concluded that copper was required for hemoglobin synthesis. This was the first such report.

The biological significance of copper in swine was elucidated by Teague and Carpenter (1951). The objective of their study was to determine the level of dietary copper necessary for normal hematopoiesis, but during the study they also discovered previously unseen effects of copper deficiency on the joints and bones of pigs. To address the level of dietary copper necessary for normal hematopoiesis, the investigators fed the pigs a milk diet with differing levels of iron and copper over time. In the first eighty days, Hb levels in the blood fell dramatically. However, after ten days of consuming

iron and copper supplemented feed, Hb levels more than doubled. Further, pigs fed a diet deficient in copper exhibited signs of rickets with swollen joints, crookedness in the forelimbs, and general unthriftiness. This condition was reversed by the addition of copper to the diet, and the pigs appeared almost normal (Teague & Carpenter, 1951).

In 1955, Barber *et al.* wrote a short communication to the journal *Chemistry and Industry* on a feed formulation containing high levels of copper (Barber *et al.*, 1955). A craving for copper by pigs had been described previously (Braude, 1945). Pigs in the Barber *et al.* study were fed a barley/wheat offal/white fish meal diet with or without mineral mix containing copper as CuSO_4 . Pigs receiving the copper supplement exhibited better feed conversion and feed intake than the control pigs. However, the differences were not statistically significant. The pigs used in this study were found to be part of a herd suffering from viral pneumonia. This was an unfortunate complication, for it obscured the ability of copper to increase feed conversion (kg feed per kg gain) and efficiency in pigs.

A period of inactivity followed the inconclusive report of Barber *et al.* After almost 20 years, Gipp and colleagues (Gipp *et al.*, 1973) performed three studies to determine the response of swine to high levels of dietary copper. In one study, eight pigs were fed a corn-soybean meal diet supplemented with or without 250 ppm copper. For the initial period of

development (10-40 lb. body weight), there was a statistically significant increase in gain for those pigs fed supplemental copper versus those without the extra copper. In other periods, the gain was not statistically different. In the second experiment, however, the researchers found that supplemental copper decreased weight gain, whether administered orally (250 ppm) or subcutaneously (300 ppm). It is interesting to note, however, that overall feed conversion rates were highest in those pigs given copper orally. Unfortunately, the authors did not analyze that data for statistical significance. In the third experiment, the pigs were fed either a corn-soybean meal diet with or without supplemental copper or a dry skim milk diet with copper. Severe anemia and low plasma and liver iron levels were noted in those pigs on the skim milk diet with copper. This was not surprising given the nature of the diet, as milk is naturally low in iron. The only differences seen between treatments were shorter carcass length and smaller cross-sectional area in the pigs fed the skim-milk diet with copper. No real advantage could be ascribed to copper supplementation of pig rations.

Despite the findings made by Gipp *et al.* described above, it became generally accepted that the addition of copper in swine feed would enhance growth in these animals. Next the focus shifted to how to get better absorption of the copper in the pigs diet. Zoubek *et al.* (1975) looked at the effects of different copper sources on absorption. Basically two sources of

copper were used: CuSO_4 and copper chelated to ethylene diamine tetraacetic acid (EDTA). These sources of copper were either fed alone, or in combination with the basal diet.

The researchers found no difference in final copper concentration in the blood of pigs after eight hours. There was a difference, however, in the profile of plasma copper over the entire time-course. The changes in plasma copper for each treatment were statistically different depending on when the samples were taken. This cast light on why previous studies involving copper uptake measured by plasma copper concentration showed no differences between treatments.

Another aspect of pig health was addressed in a 1979 study, in which the effects of copper and zinc on performance and cholesterol level of pigs were assessed (Eisemann *et al.*, 1979). In the study, 6-8 week old pigs were fed either 8 or 125 ppm copper and 100 or 500 ppm zinc as a supplement to a corn-soybean meal diet. At four weeks, the plasma cholesterol levels decreased in the pigs fed the higher copper supplement, regardless of the amount of zinc fed. However, this effect diminished over time, and neither zinc nor copper, at these dietary levels, had any long term effect on the cholesterol status of swine.

At this time it was accepted that high levels of copper caused an increase in feed consumption, and therefore an increase in weight gain in

swine. To test the validity of the hypothesis, researchers at Virginia Tech conducted paired-feeding experiments (Zhou *et al.*, 1994). They showed that while copper may have an impact on feed consumption, it also has intrinsic growth promoting properties. Interestingly, increases in serum mitogenic activity and improvements in the weight gain to feed ratio were observed.

These researchers also examined the response of pigs fed two different sources of copper, CuSO₄ and Cu-lysine. Two copper levels for each source were used (15 and 200 ppm). In addition, possible differences between ad libitum and restricted feeding were assessed. Average daily gain was higher for those pigs fed copper as Cu-lysine as opposed to CuSO₄. Further, restricted feeding of 15 ppm Cu-lysine was as effective as restricted feeding of 200 ppm CuSO₄. The gain-to-feed ratios between the two sources were not different for a given concentration. It was noted that at 200 ppm copper, both average daily gain and feed intake were higher for pigs fed the Cu-lysine diet. This implies a performance advantage for pigs fed elevated levels of copper, and that organically complexed copper is a better feed supplement.

Recent studies have failed to confirm the findings of Zhou *et al.* (1994). Other researchers at Virginia Tech fed pigs a basal diet (containing 15 ppm copper) supplemented with 0, 100, 150 and 200 ppm Cu-lysine or CuSO₄ (Apgar *et al.*, 1995). They found no conclusive evidence that one source of copper had any more impact on growth and feed intake than the other.

Similarly, Cromwell *et al.* (1998) did not find tribasic copper chloride to support greater average daily gain, feed intake or gain-to-feed ratio than CuSO_4 . From these studies it appears that copper is well absorbed in most forms.

Dove looked at the effect of copper concentration on animal fat utilization by swine (Dove, 1995). Animal fat is a cheap energy source used in pig feed. However, weanling pigs are unable to utilize this energy source for up to 14 days post-weaning. Two copper concentrations (15 and 250 ppm) and two fat contents (0 and 5%) were examined. Pigs fed the 5% fat diet containing 250 ppm copper grew faster and consumed more feed than did pigs on the other diets. In contrast, pigs fed the 5% fat diet containing 15 ppm copper had the lowest average daily gain and feed intake values. Regardless of the fat content of the diet, pigs fed diets supplemented with 250 ppm copper consumed more feed than pigs fed diets with 15 ppm copper.

The addition of copper also increased the digestibility of the dry matter in the diet and increased nitrogen retention (Dove, 1995). Copper did not, however, effect the retention of calcium, phosphorus and magnesium. Copper, iron and zinc retentions were lowest in those pigs fed the 5% fat diet containing 15 ppm copper. It was hypothesized by the author that the fat in the diet was the interfering factor leading to decreased retention. However, this effect was not observed in the pigs fed the 250 ppm copper. This

suggests the higher copper concentration was able to overcome the adverse effect of high dietary fat on retention of copper, iron and manganese.

This research shows conclusively that copper plays a major role in overall metabolism in swine, and that the addition of 250 ppm copper enhances desirable growth traits in weanling pigs. A subsequent study (Luo & Dove, 1996) confirmed these results by examining nutrient balance in weanling barrows. These researchers confirmed the previous data regarding fat digestibility and nitrogen retention, and also found that increased copper led to an increase in the activity of lipase and phospholipase A in the small intestine. These new data could explain the enhanced ability of pigs fed a copper enriched diet to metabolize fat.

Zinc

Zinc, like copper, is found in almost all living systems. It is present throughout the body of man and animals, including all the major organs, muscle, bone and plasma. Zinc is an integral component of numerous enzymes including carbonic anhydrase (important in maintaining acid/base balance of the blood), collagenase (important in bone formation), and retinal reductase (important in vitamin A metabolism). Further, zinc has been implicated in maintaining chromatin structure. Zn-metallothionein produced by the intestinal mucosa is chiefly responsible for maintaining zinc

homeostasis (Richards, 1989). Interestingly, this protein is induced by zinc, but has a higher affinity for copper (Blalock *et al.*, 1988).

In 1934, researchers at the University of Wisconsin designed experiments aimed at elucidating the role, if any, of zinc in growth of rats (Todd *et al.*, 1934). A possible role of zinc had been reported by others (McHargue, 1926; Hubbell & Mendel, 1927), but unfortunately the diets used in these studies were deficient in one or more vital nutrients, and thus conclusions could not be drawn. In the Wisconsin study (Todd, *et al.*, 1934) rats were furnished with rations that were complete for all the nutrients known to be essential at the time except for zinc. The addition of zinc to the diet supported increased growth in the rats. They also showed that zinc increased the body weight of both males and females.

Zinc deficiency has also been associated with the cause of parakeratosis, or “swine dermatitis”. Tucker and Salmon (1955) ruled out parasitic organisms as the cause for the condition, and showed that the dermatitis was exacerbated by the addition of calcium and phosphate to the diet. They further showed the curative effects of $ZnCO_3$. However, zinc at concentrations between 34 and 44 ppm could not prevent dermatitis when calcium and phosphorus concentrations were high in the ration. Thus it was shown that parakeratosis was caused by a zinc deficiency, and that the level of zinc required by the animal to prevent parakeratosis is greatly dependent

on feed calcium and phosphorus levels. Liptrap *et al.* (1970) further showed that the concentration of zinc required to prevent parakeratosis was higher for gilts and boars than for barrows (castrated male pigs). Barrows, for example, failed to exhibit symptoms of parakeratosis when fed less than 30 ppm zinc. In contrast, approximately one-third of boars and gilts fed the same diet suffered from parakeratosis.

As was found for copper, the requirement of swine for zinc is related to the composition of the diet (Babatunde, 1972). For example, the addition of lard as a cheap energy source to the diet results in an increased requirement for zinc. The relationship between zinc and lard is further influenced by calcium. Babatunde (1972) found that a 0.2% increase in dietary calcium above the National Research Council guidelines led to a two fold increase in the requirement for zinc.

The maximum tolerated level of zinc as a food supplement to gilts was determined in 1983 (Hill & Miller, 1983a). Gilts fed a diet containing 0, 50, 500 or 5000 ppm zinc as ZnO showed no obvious effects for 12 weeks. By 20 weeks, however, gilts fed the highest levels of zinc had the lowest body weights. Clinical examination of the gilts in this study revealed that by the end of four weeks gilts fed the highest level of zinc had significantly lower serum copper levels than the other pigs in the study. A good hypothesis for this drop in serum copper is the production of metallothionein. A study from

1960 suggested that the reason for the lower serum copper levels was increased copper excretion, brought about by increased zinc in the diet (Magee & Matrone, 1960). Another study pointed to zinc interfering with copper absorption by inducing the synthesis of a copper binding entity (Fischer *et al.*, 1981). Metallothionein binds copper and is known to occur in the small intestine, liver and kidney in animals.

There has been some controversy in the literature on which form(s) of zinc are most available and produce the most favorable effects in swine (Hill *et al.*, 1986; Schell & Kornegay, 1996; Katouli *et al.*, 1999). Hill *et al.* (1986) looked at the difference between inorganic ($ZnSO_4$) and organic (Zn-methionine) forms of zinc on performance and growth of adult pigs. No difference was found among the forms of zinc on the performance of pigs. They also showed that the ligand picolinic acid did not aid in zinc absorption from Zn-methionine. Later studies provided evidence both for and against the hypothesis that zinc improved performance in weanling pigs (Fryer *et al.*, 1992; Kavanagh, 1992; Poulsen, 1992; Tokach *et al.*, 1992; Hahn & Baker, 1993). Schell and Kornegay (1996) reported no increase in growth performance for zinc fed at 1000, 2000 or 3000 ppm. However, the authors did point out that none of the pigs fed the supplemented zinc suffered from scouring, which is a common problem in the swine industry. A recent study showed that feeding ZnO to pigs for the first 2 weeks post-weaning promoted

microbial diversity in the intestine, which lead to decreased incidence of scouring in those animals (Katouli, *et al.*, 1999).

Both copper and zinc are required for maximum performance in swine and both metals are added to feed. Little research, however, has focused on the possible interaction between these two metals. Hill *et al.* (1983b) demonstrated that gilts fed pharmacological levels of zinc (5000 ppm) contained up to an order of magnitude less copper in their milk than gilts fed lower levels of zinc. Zinc levels in these gilts were expectedly higher, but only by approximately 4 µg/ml. In a related study, piglets born to and nursing from dams fed 5000 ppm ZnO were severely copper deficient (Hill *et al.*, 1983c). Attempts to confirm an interaction between zinc and copper have given puzzling results (Smith *et al.*, 1997). Four experiments were performed in which pigs of weaning age were fed different concentrations of ZnO and CuSO₄. In the first two experiments, a diet with copper fixed at 250 ppm was combined with either 110 (control) or 3110 ppm zinc. No differences in any of the growth parameters were seen between the control and assayed diets. In the third experiment both copper (16.5 or 250 ppm) and zinc (165 or 3000 ppm) were varied in the diet. In the diets containing 3000 ppm zinc, there was a statistically significant increase in average daily gain, contrary to previous reports (Fryer, *et al.*, 1992; Tokach, *et al.*, 1992; Schell and Kornegay, 1996). Lastly, Smith and colleagues looked at possible carryover

effects of feeding 3000 ppm zinc from 0-2 weeks post-weaning, followed by a diet containing varying levels of zinc (165 or 3000 ppm) and copper (16.5 or 250 ppm). The authors found that feeding 3000 ppm zinc in this fashion led to increases in average daily gain and feed intake when the level of zinc was held at 3000 ppm for the entire 4 weeks of the study. The authors concluded that further research needs to be done to look at the interaction with copper, since their results with copper did not meet those from other labs. This sentiment was echoed in a 1998 review of copper and zinc (Poulsen, 1998).

BIOREMEDIATION AND METAL UPTAKE BY FUNGI

Microbes have been used for millennia to aid mankind in the production of food and enzymes. However only recently have microbes gained importance in another important area, remediation of environmental pollution. In the early 1970's, researchers proposed releasing a genetically modified bacterium for remediation of an oil spill (Keeler, 1991). Since that time, the use of microbes to remediate waste spills and other toxic sites has greatly increased.

Bioremediation is a term that encompasses removal of contaminants from groundwaters and soils (biostimulation), sludges and highly contaminated soils (bioslurry) and lightly contaminated soils with less than 1% organics (biofarming). There are two processes by which bioremediation occurs, biodegradation or bioaccumulation. Biodegradation is the process employed in the clean up of petroleum spills or other complex organic pollutants. In this procedure, microbes seeded onto the contaminated area break down the complex hydrocarbons into simpler organic compounds. Bioaccumulation is used when the contaminants are simple compounds such as metal that cannot be further degraded. In this latter case, the microbes used usually accumulate the polluting substance by cell wall binding or internalization of the compound.

In North Carolina, the preferred waste management system for hog waste has been anaerobic lagoons for treatment and spray fields for disposal. While this system is effective if managed properly, concerns have been raised in recent years as to its environmental safety. This is due mainly to past failures of such systems during periods of high rainfall. In addition to the potential negative environmental impact should the lagoons rupture and spill hog waste into streams, this system of treating waste has further environmental concerns. This system relies on the repeated application of wastewater and accumulated biosolids to adjacent farm land. This practice can result in accumulation of phytotoxic levels of copper and zinc, and the contamination of soils and groundwater with nitrogen and phosphorous.

Sutton *et al.* (1983) reported that the application of waste from swine that had been fed elevated levels of copper to soil had no negative effect on corn yield over a 4 year period. They also reported no significant accumulation of copper in soils receiving waste from animals fed a diet containing either 125 or 250 ppm copper. Upon closer examination of the data, however, it should be noted that when manure from pigs fed 250 ppm copper was spread on fields, the soil concentrations of copper did increase numerically from year 2 through year 5.

A more extensive study was performed by Anderson *et al.* (1991). They examined the effect of continuous amendment of soils with swine manure for

11 years on corn yield and plant copper levels. The amount of copper in the manure was amended at a rate of 45 kg/ha, which was higher than United States Environmental Protection Agency guidelines (USEPA, 1983). The treatment did not result in significant differences in yield and crop copper content. The authors did note that soil pH was kept above 6.5 and that there was a high amount of organic matter associated with the manure, both of which could dramatically lower the availability of the copper in the manure. This, in turn, would alleviate any toxicity to plants.

The need for a more thorough study to address the impact of copper in waste applied to soil soon became evident. Kerr & McGavin (1991) found chronic copper toxicity in sheep grazing on pastures that had been fertilized the previous 7 years with swine manure. The level of copper in the pasture soil was about 26.35 mg/kg, far above the 7.25 mg copper/kg soil found in surrounding soils. This study shows that the repeated application of swine waste for only 7 years is sufficient to cause chronic toxicity to sheep.

Because of the environmental concerns, several new treatment procedures are being tested. One system that holds promise is used at the Center for Environmental Farming Systems (CEFS) in Goldsboro, NC. This system uses an alternating sequence of aerobic and anoxic tanks to remove nitrogen through nitrification and denitrification reactions. The resulting wastewater is approximately 0.5% solids, and possesses copper and zinc at

concentrations of 0.03 and 0.18 mM, respectively. While this system addresses the concern of nitrogen contamination found with lagoon systems, it still does not address the concern of metal accumulation in lands receiving the waste.

This type of facility is well adapted for additional bioremediation because of its sequential treatment design; an additional metal removal step can be added easily. Bacteria are not likely candidates for this type of system because of the difficulty of removing unicellular organisms from an aqueous environment after treatment. Fungi, due to their mycelial nature and well-documented ability to accumulate metals of all families (Gadd, 1993), are good candidates for bioremediation of metals from swine waste.

There are three ways in which fungi can be used to detoxify metals in the environment. First, fungi can be used to leach metals from solid substrates. Because swine wastewater is a liquid, this method is not applicable to the current treatment systems. Second, fungi have been shown capable of removing metals from aqueous solution by adsorption. This process takes advantage of the metal binding properties of the fungal cell wall. Lastly, fungi can absorb metals from aqueous solutions and internalize the metal into their mycelia. This last process has not been exploited for bioremediation as of yet, but may be the best way to employ certain fungi for

bioremediation. Intuitively, the best solution is one that results in near total metal removal and is adaptable to industry.

Adsorption of Metals by Fungi

There are numerous papers and reviews on biosorption (adsorption) of metals by fungi. This property was first described in the late 70's (Stokes & Lindsey, 1979; Siegel *et al.*, 1990) and received some attention in the 1980's (de Rome & Gadd, 1987). Most of the research in this area, however, was done in the 1990's in response to increased pressure to find economically sound treatment systems for industrial waste (Wales & Sagar, 1990; Junghans & Straube, 1991; Luef *et al.*, 1991; Wnorowski, 1991; Mullen *et al.*, 1992; Volesky, 1994; Akthar & Mohan, 1995; Morley & Gadd, 1995; Volesky & May-Phillips, 1995; Kapoor & Viraraghavan, 1997; Kapoor & Viraraghavan, 1998; Kapoor *et al.*, 1999; Zhou, 1999). *Saccharomyces cerevisiae* and other yeasts have been proposed as potential bioremediation agents (Junghans and Straube, 1991; Brady & Duncan, 1994; Volesky and May-Phillips, 1995). In 1991, German researchers performed a comparative study of yeasts from different genera, looking at their ability to adsorb copper from aqueous solution (Junghans and Straube, 1991). All cultures used were viable but the brevity of the experiments precluded any significant internal absorption taking place. The performance of *S. cerevisiae* was comparable to the other yeasts. At a cell density of 3 grams fungal dry weight per liter, *S.*

cerevisiae was able to remove up to 61% of the copper from a 0.2 mM copper solution. The highest removal rate achieved was 71% by *Candida famata* and *Rhodotorula* sp. M2, at the same cell density. However, no yeast did well at the higher metal concentration (2.0 mM), suggesting that these species may not be suited for industrial use. In fact, *S. cerevisiae* growth is inhibited by 1.0 mM copper (Fogel & Welch, 1982).

The practical reason for using *S. cerevisiae* for bioremediation is its abundance as a byproduct from the alcohol and baking industries. Both industries have problems with disposing of spent yeast cultures. A Canadian group looked at the adsorptive properties of baker's, brewer's and active dry yeast in 1995 (Volesky and May-Phillips, 1995). The yeast cultures were killed and examined for their ability to remove copper, zinc and uranium from solution. The three strains differed in their ability to remove metals. The baker's yeast was best at removing copper, with greater than 0.3 mmoles copper per gram cells removed from a solution of 3.0 mM copper. Unfortunately, the authors chose to focus on uranium, and little more investigation of copper removal was done.

While yeast is a readily available biomass from industry, its unicellular growth makes it difficult to adapt it for use in wastewater treatment systems. One way to circumvent this problem is to encapsulate the microbe in a polymer matrix. Brady and Duncan examined the ability of

S. cerevisiae fixed in a polyacrylamide matrix to remove copper, cobalt, and cadmium from solution (Brady and Duncan, 1994). Here, a column of polyacrylamide beads impregnated with the yeast was constructed, and chloride salt solutions of each metal were passed through it. The fungus removed greater than 70% of the copper from 800 ml of effluent containing 0.2 mM copper. Less cobalt and cadmium were removed.

Filamentous fungi have the advantage over yeast and bacteria in that recovery from aqueous systems is easier due to the nature of these fungi to grow as mycelial mats. Researchers at the University of Dundee studied the ability of *Rhizopus arrhizus*, *Cladosporium resinae*, and *Penicillium italicum* to remove copper from aqueous solution (de Rome and Gadd, 1987). The main purpose of this work was to determine standards that could be used to express the metal binding capacity of biological material, and experiment with alternative methods of determining the amount of metal bound. Atomic absorption spectrometry is the gold standard for elucidating metal concentration in a substance, but is time consuming. The authors found that using a copper specific electrode to determine copper content was much faster and of acceptable accuracy. They also studied the binding of copper to cell walls, and described the kinetics of copper binding by different isotherm models. Two of the three fungi examined exhibited single layer adsorption characteristics whereas *R. arrhizus* yielded a more complex adsorption

pattern. *R. arrhizus* was able to remove over 300 nmoles copper per gram dry weight, more than the other two fungi tested.

Zhou (1999) compared cell walls of *R. arrhizus* and six other fungi for their ability to remove zinc from solution. *A. nidulans* and *A. niger* were next to last in zinc removal, only proving better than *Schizosaccharomyces pombe*. In contrast to the study by de Rome and Gadd (1987) above, *R. arrhizus* was able to remove approximately 130 μ moles zinc per gram fungal dry weight.

Rhizopus nigricans has also been examined for removal of lead from aqueous solutions (Zhang *et al.*, 1998). Lead adsorption by a killed fungal biomass was highly pH dependent, with adsorption increasing as the pH increased. This phenomenon is supported by others who found that alkaline treatment of fungi increased adsorptive capacity (Feofilova *et al.*, 1994; Akthar and Mohan, 1995; Akthar *et al.*, 1995).

The ability of a fungus to adsorb metals is a function of the constituents of its cell wall, but there is evidence that adsorption can be enhanced by chemical treatment (Muzzarelli *et al.*, 1980), or through the addition of clay or other materials (Morley and Gadd, 1995). Muzzarelli (1980) showed that NaOH treatment of *Aspergillus niger* biomass leads to increased metal adsorption. This enhancement may be due to the exposure of amine or carboxyl groups in the cell wall (Kapoor and Viraraghavan, 1997). Morley and Gadd (1995) reported that clay soil had higher adsorptive

tendencies for copper, cadmium, and zinc than either *R. arrhizus* or *Trichoderma viride*. Fungal uptake capacity increased with increasing clay concentration. This suggests that a formulation of industrial waste fungi and clay can be effective in removing metals from an aqueous system.

One of the first papers to describe the feasibility of using pre-treated fungi to remove metals from aqueous solutions in a commercial system was presented at a meeting held by the Society of Chemical Industry in London in 1990 (Wales and Sagar, 1990). Here the authors described a process of using NaOH to activate the amine groups of the chitin/chitosan in the cell walls of six fungi. Crude columns were constructed using wet-laid paper made from mycelia mixed with either manilla-hemp or ashless clippings. These were used to test the ability of six fungi to remove copper from solutions at commercially acceptable flow rates. *Mucor mucedo* and *Rhizomucor miehei* proved to be better than the other fungi tested (*Aspergillus oryzae*, *Neurospora crassa*, *Penicillium chrysogenum*, and *Trichoderma viride*) at removing copper from aqueous solution. These fungi removed greater than 80% of the metal, even after 6 cycles of washing and regeneration. Results from the study argue for the utility of using fungi to remove metals from waste streams. The authors also provided evidence that cell density is important in the mechanics of adsorption as they found the capacity of metal uptake to decrease as cell density increased.

Aspergillus niger is widely used in industry for such things as the production of citrate and gluconate (Berka *et al.*, 1992; Shankaranand & Lonsane, 1994). *A. niger* grows well in aqueous solutions and can be readily removed from culture solutions. This trait plus its ability to produce a number of enzymes and metabolites has led to its widespread use in the fermentation industry.

Because of the traits mentioned above, *A. niger* has been studied for its ability to adsorb metals from solution. In a comparative study, *A. niger* was found to be superior to *P. chrysogenum* and *Claviceps paspali* in its ability to remove zinc from solution (Luef, *et al.*, 1991). The fungus was able to remove zinc when suspended in shake culture or when the solution was passed through a column containing the fungus. The authors found, as have others (Muzzarelli, *et al.*, 1980), that NaOH treatment of the mycelia prior to assaying enhances the metal uptake by *A. niger*.

Researchers in India found that alkali treated *A. niger* was able to remove more copper and zinc from polluted lake water than Dowex-50, an ion exchange resin (Akthar and Mohan, 1995). Here, either the fungus or the resin was bagged in cheese cloth and submerged in the polluted water for 60 minutes. The fungus was able to remove 38 times more copper and 41 times more zinc than the resin. Kapoor and Viraraghavan (1997) used different chemical treatments designed to block or modify certain functional groups in

the cell wall of *A. niger*. They reported severe decreases in biosorption of copper, cadmium, and lead when either the carboxyl groups were esterified or the amine groups were methylated. This suggests these groups are important for metal binding by *A. niger*.

These researchers took their analysis a step further in 1998 and examined the effects of different pre-treatments on metal uptake in *A. niger* (Kapoor and Viraraghavan, 1998). They reported increased accumulation of lead, cadmium, and copper after pre-treatment with either NaOH, dimethyl sulfoxide, formaldehyde, or commercial laundry detergent. Nickel adsorption was decreased by all the treatments performed. Treatment of the fungus with certain chemicals prior to use seems to increase its ability to remove certain metals which can pose an environmental threat.

Absorption of Metals by Fungi

Internal absorption of metals by fungi has received less attention than cell wall adsorption. One reason is that by absorbing the metal, the fungus cannot be readily re-used. Second, not much is known about metal uptake in filamentous fungi. Most of the information on the uptake of metals by fungi was obtained from studies using *S. cerevisiae*, which is not very resistant to metal toxicity. Metal absorption and resistance to toxicity are related phenomenon. This point was made in 1982, when it was shown that multiple

copies of copper metallothionein in *S. cerevisiae* enhanced its resistance to copper (Fogel and Welch, 1982).

There are basically three mechanisms for absorption of metals: 1) vacuolar compartmentalization, 2) binding with phytochelatin, and 3) production of metallothionein (Gadd, 1993). Vacuolar compartmentalization of metals has been described in yeast, but mainly involving metals such as strontium and lithium. This mechanism of metal removal has not been described in filamentous fungi. Filamentous fungi have been shown to contain phytochelatin (Kneer *et al.*, 1992). These γ -glutamyl peptides were first characterized in plants as a means to defend against cadmium toxicity (cited in Zenk, 1996). They have since been described in a few fungi (Mehra & Winge, 1991; Kneer, *et al.*, 1992). In *S. pombe*, as well as plants, it has been demonstrated that a sub-unit of phytochelatin, glutathione (γ ECG tripeptides), can be transported inside vacuoles of the fungus (Zenk, 1996; Li *et al.*, 1997). Since neither vacuolar compartmentalization of metals or phytochelatin have been reported in *A. niger*, it seems unlikely these two mechanisms would be responsible for metal uptake in this fungus.

The most likely mechanism for removal of metals from solution involves metallothioneins (MTs). These short, cysteine rich proteins bind many different transition IIb metals. MTs are named for the metal that induces their transcription. Currently the two filamentous fungi known to

possess MT are *A. niger* and *N. crassa* (Lerch, 1980; Kermasha *et al.*, 1993). Both of the fungi produce copper MTs, however, neither the *A. niger* MT gene nor protein has been cloned or sequenced. Unfortunately, little is known about the regulation of metallothionein genes in these two fungi. *S. cerevisiae* also possesses CuMT, and much is known about this protein and its genetic regulation.

The MT gene in *S. cerevisiae* is known as *cup1*. This gene was first described as a resistance mapping locus in 1955 (Brenes-Pomales *et al.*, 1955). The gene was sequenced by two independent groups almost 30 years later (Fogel and Welch, 1982; Butt *et al.*, 1984a). The sequence of the gene revealed a structure similar to other known MTs, and was shown to be important in copper resistance of yeast by this same group (Fogel and Welch, 1982; Welch *et al.*, 1983; Karin *et al.*, 1984; Jeyaprakash *et al.*, 1991).

The regulation of *cup1* does not appear to be complex. Copper crosses the plasma membrane and undergoes a valence change from +2 to +1. The copper ion then interacts with a transcriptional regulator, *ace1* (Thiele, 1988). This Cu-ACE1 complex then binds the upstream activating sequence (UAS) of *cup1* and transcription commences. Because of this scheme, the gene is positively regulated. As intracellular copper levels wane, transcription of the gene shuts down. But due to the simplicity of the system, the fungus is able

to rapidly respond to increased copper concentrations. It is reasonable to assume that this system is similar in other species, such as *A. niger*.

The data reviewed here show copper and zinc are important dietary requirements of animals. Their inclusion in swine feed at pharmacological concentrations leads to increased copper and zinc excretion. The use of hog manure and wastewater to fertilize soils has been shown to increase soil concentrations of copper and lead to copper toxicity in sheep. The goal of this thesis research project was two-fold. First, fungi were evaluated for their ability to remove copper from semi-defined media. Of the fungi tested, *A. niger* proved best at removing copper from solution. The second goal was to determine how *A. niger* removes copper and zinc from aqueous solutions and swine wastewater. The report contained in this thesis demonstrates that *A. niger* removes copper and zinc by an active process leading to internalization of these metals.

***Aspergillus niger* absorbs copper and zinc from swine wastewater.¹**

Michael S. Price, John J. Classen, and Gary A. Payne.

Wastewater from swine confined-housing operations contains elevated levels of copper and zinc due to their abundance in feed. These metals may accumulate to phytotoxic levels in some agricultural soils of North Carolina due to land application of treated swine effluent. We evaluated fungi for their ability to remove these metals from wastewater and found *Aspergillus niger* best suited for this purpose. *A. niger* was able to grow on plates amended with copper at a level five times that inhibitory to the growth of *Saccharomyces cerevisiae*. We also found evidence for internal absorption as the mechanism used by *A. niger* to detoxify its environment of copper, a property of the fungus that has not been previously exploited for metal bioremediation. In this report we show that *A. niger* is capable of removing 91% of the copper and 70% of the zinc from treated swine effluent.

¹ Submitted to Bioresource Technology

Introduction

Fungi are used widely in biotechnology for many processes, including the production of antibiotics, enzymes, food products, industrial acids, and alcohol. Fungi also have been utilized for removal of eutrophication agents and bioremediation of metal contaminated waste streams (Thanh & Simard, 1973; Akthar & Ghaffar, 1986; Akthar and Mohan, 1995; Bosshard *et al.*, 1996). There is current interest in the use of microorganisms for the removal of nitrogen, phosphorus, and metals from commercial and municipal waste (Cassidy *et al.*, 1996).

North Carolina is currently the second largest pork producing state in the U. S., with an on-farm inventory of 9.5 million swine (USDA, 1999). Nationwide, increased pork production has been accompanied by changes to modern farming methods, including confinement housing and higher population densities. These changes have led to larger volumes of waste being generated on smaller areas of land. While other research is targeting carbon, nitrogen, and phosphorus removal from wastewater, copper and zinc removal has been neglected. Copper and zinc salts are liberally included in swine feed as additives. These metals promote immune system function and growth in swine (Omole, 1980; O'Dell, 1998), are inexpensive, and are

relatively non-toxic to hogs at doses greater than that required for maintenance due to their lower bioavailability (O'Dell, 1998).

The unabsorbed copper and zinc in swine feed are excreted. Over time, these metals could accumulate to phytotoxic levels in soil receiving swine manure or wastewater. Primary swine waste treatment systems target carbon stabilization. Current land application disposal systems are based on a balance of nitrogen application and uptake by plants. New systems under investigation attempt to enhance nitrogen and phosphorous removal from liquid effluent in order to reduce the amount of land required for final disposal. These enhanced nutrient removal systems would therefore raise the amount of regulated metals applied to a given area of land. Industrial methods to remove these metals, such as ion exchange resins, are inappropriate for use in animal agriculture due to the high cost of implementing these systems and their non-specific metal binding properties. An ideal system for the removal of regulated metals, such as copper and zinc, would be one that is economical and safe for the environment, as well as efficient in removing target metals.

One fungus shown capable of removing metals from a number of substrates is *Aspergillus niger*. This filamentous fungus is widely used in the fermentation industry because of its ease of culturing and lack of pathogenicity to humans and animals (Berka, *et al.*, 1992). *A. niger* has been

used to remove metals from the environment by either adsorption of the metals to fungal cell wall components, or complexation of the metals with organic acids produced by the fungus (Akthar and Mohan, 1995; Bosshard, *et al.*, 1996). Other fungi also have been used for metal removal with varying degrees of success (de Rome and Gadd, 1987; Zhou & Kiff, 1991; Mullen, *et al.*, 1992; Brady and Duncan, 1994; Al-Asheh & Duvnjak, 1995; Zhang, *et al.*, 1998). Specifically, *Fusarium* species have been used as biosorbents, and *Penicillium simplicissimum* has been used to precipitate metals from solution (Burgstaller & Schinner, 1993; Gadd, 1993; White *et al.*, 1997). However, *A. niger* has been consistently listed among the top metal biosorbents (Luef, *et al.*, 1991; Mullen, *et al.*, 1992; Gadd, 1993; Akthar and Mohan, 1995; Bosshard, *et al.*, 1996).

In this study, we evaluated six fungi for their ability to grow on and remove copper and zinc from culture media and swine wastewater. The objective of this study was to evaluate potential fungi for use in bioremediation of copper and zinc from swine wastewater. Further, we wanted to determine if current bioremediation protocols for wastewater metal removal utilize fungi in the most efficient manner. Of the fungi tested in an initial screen, *A. niger* was the most effective at removing copper from liquid media. Its ability to remove copper and zinc from treated swine wastewater was further studied.

Methods

Microorganisms used. *Aspergillus niger* (NRRL 326) and *Penicillium simplicissimum* (NRRL 1075) were obtained from the USDA/ARS National Center for Agricultural Utilization Research, Peoria, IL. *Fusarium verticillioides* (*Gibberella fujikuroi* strain A00149) was obtained from the Fungal Genetics Stock Center, Kansas State University. *Rhizoctonia solani* and *Aquathanatephorus pendulus* isolates were obtained from Dr. Marc Cubeta, North Carolina State University (NCSU). The *Geotrichum* isolate was collected from soil adjacent to the Lake Wheeler Road Field Laboratory Swine Educational Unit lagoon, Wake County, NC. All fungi were cultured on potato dextrose agar for production of conidia, or in case of *R. solani* and *A. pendulus*, for mycelium. All liquid cultures in the metal uptake and detoxication mechanism studies were carried out in duplicate.

Fungal growth screen. The fungi were grown for 7 days at 28°C on either yeast extract/sucrose (YES) agar (yeast extract, 2%; sucrose, 5-10%; Bacto® agar, 15 g/l), YES + 5mM CuSO₄·5H₂O, or a minimal medium of the following composition (g/l): L-asparagine, 0.5; K₂HPO₄, 0.5; MgSO₄·7H₂O, 0.2; FeSO₄·7H₂O, 0.01; D-glucose, 2.0; Bacto® agar, 15. The center of plates containing the respective media was inoculated with 5000 spores of either *A. niger*, *F. verticillioides*, *P. simplicissimum*, or the *Geotrichum* sp. isolate. A

3.5 mm plug from the margin of growing colonies was used to inoculate plates with *R. solani* and *A. pendulus*. Three plates of each fungus were used for each treatment. The mean of perpendicular diameter measurements was recorded for each plate on days 4 and 7.

Copper uptake screen. To determine the relative ability of the fungi tested to remove copper from liquid media, the fungi were cultured in 100 ml YES broth amended with 0.1 mM CuSO₄. The medium was inoculated with 10⁶ conidia/ml for all fungi except *R. solani* and *A. pendulus*. For these fungi, ten 3.5 mm plugs were added to each flask. All cultures were incubated at 28°C for 4 days. The fungal mats were then washed for 30 min in 0.1 N HCl. Due to the yeast-like growth of *F. verticillioides* and *Geotrichum* sp., the washes were separated from these fungi by centrifugation at 10,000 x g for 30 min. The mats of all other cultures were separated by filtration through P5 Whatman paper. All fungal mat samples were lyophilized. The spent broths, wash filtrates, and lyophilized mats were analyzed for copper content.

Determination of metal removal mechanism(s) in *A. niger*. *A. niger* was further assayed for its ability to remove metal from YES broth amended with 0.0, 0.01, 0.1 or 1.0 mM CuSO₄·5H₂O or ZnSO₄·7H₂O. The +2 valence form of both metals have been used in other studies (Junghans and Straube, 1991; Luef, *et al.*, 1991; Kermasha, *et al.*, 1993; Al-Asheh and Duvnjak, 1995; Morley and Gadd, 1995; Sanchez *et al.*, 1999). In further

studies to assess possible interaction between copper and zinc uptake, both metals were present at equal concentrations at the levels listed above. Also, titration experiments were designed with each metal titrated against the other. For example, copper was fixed at 30 μM (the concentration of copper in fresh waste), while zinc was varied over a range from 50 to 500 μM (a range including the concentration of zinc in fresh waste). The fungus was grown at 28°C, harvested, and the mats and filtrates prepared for analysis as described above.

To determine if uptake was due to an active or passive process, the removal of copper from a 0.1 mM solution of CuSO_4 by live and killed cultures of *A. niger* was compared. *A. niger* spores (10^5 spores/ml) were inoculated into two flasks containing 1 l each of a modified Watson and Smith medium (Kermasha, *et al.*, 1993) and incubated as shake cultures (125 rpm) for 4 days at 28°C. The two cultures were then harvested by filtration through P5 Whatman paper and the mycelial mats of each flask were divided into halves. Each half was further cut into squares approximately 1 cm x 1 cm. Squares from half of each culture were then killed by exposure to chloroform vapor for 20 min. All of the diced halves were then weighed and inoculated into flasks (three replicates per treatment) containing 100 ml each of the Watson and Smith broth with 0.1 mM CuSO_4 and incubated at 28°C for 24 h as shake

cultures (125 rpm). The cultures were then harvested, and the mats and filtrates were prepared for analysis as described above.

Metal removal from swine wastewater. For the evaluation of metal removal from treated swine wastewater, *A. niger* was grown in waste liquid obtained from the Center for Environmental Farming Systems, Goldsboro, NC, operated by the North Carolina Department of Agriculture. This type of system is likely to be implemented in swine waste treatment. Waste samples were obtained from the house flush tank containing effluent from an alternative treatment system. This system uses aerobic and anoxic tanks in alternating sequence to remove nitrogen by nitrification and denitrification. Seed cultures were grown to approximately 1.0 g dry weight in 100 ml YES broth for 48 h at 28°C, and resuspended in 100 ml of treated effluent. One seed culture was used for each effluent culture. All effluent cultures were incubated at 28°C in a rotary shaker incubator (150 rpm) for 24 h. At the end of the incubation, the mats were removed by filtration as described above and the liquids were centrifuged at 10,000 x g for 30 min to remove any organic debris. The mycelium retained on the filters was washed with sterile 0.05% Tween-20 to remove any microbes associated with the fungal cell wall. The wash filtrates were collected by filtration through P5 Whatman paper and the fungal mats were lyophilized. The supernatants,

pellets, wash filtrates, and fungal mats were all analyzed for copper and zinc content.

Sample analysis. All samples were analyzed by atomic absorption spectroscopy (Clesceri *et al.*, 1990) at the Biological and Agricultural Engineering Department's Environmental Analysis Laboratory, NCSU. Filtered fungal mats and centrifuged pellets were lyophilized and weighed prior to analysis. All glassware used in these experiments was rinsed with a solution of 25% ACS grade nitric acid to remove any metal contaminants. Statistical analysis was performed on data using the Microsoft Excel 97/98 software package (Microsoft Inc., Redmond, WA).

Results

Fungal growth screen. All of the fungi tested except *R. solani* were able to grow in the presence of 5.0 mM CuSO₄ (Figure 1). *A. niger*, however, grew considerably better on copper-amended medium than any other fungus and obtained a colony diameter of 84.5 mm in 7 days (Figure 1). Further, the morphology of *A. niger* grown on copper-amended medium did not appear different from that observed on control YES media. The fungus grew luxuriantly on the copper-containing medium and produced abundant conidia. In contrast, the presence of 5.0 mM copper in the medium caused observable changes in the morphology of the other fungal species tested. For

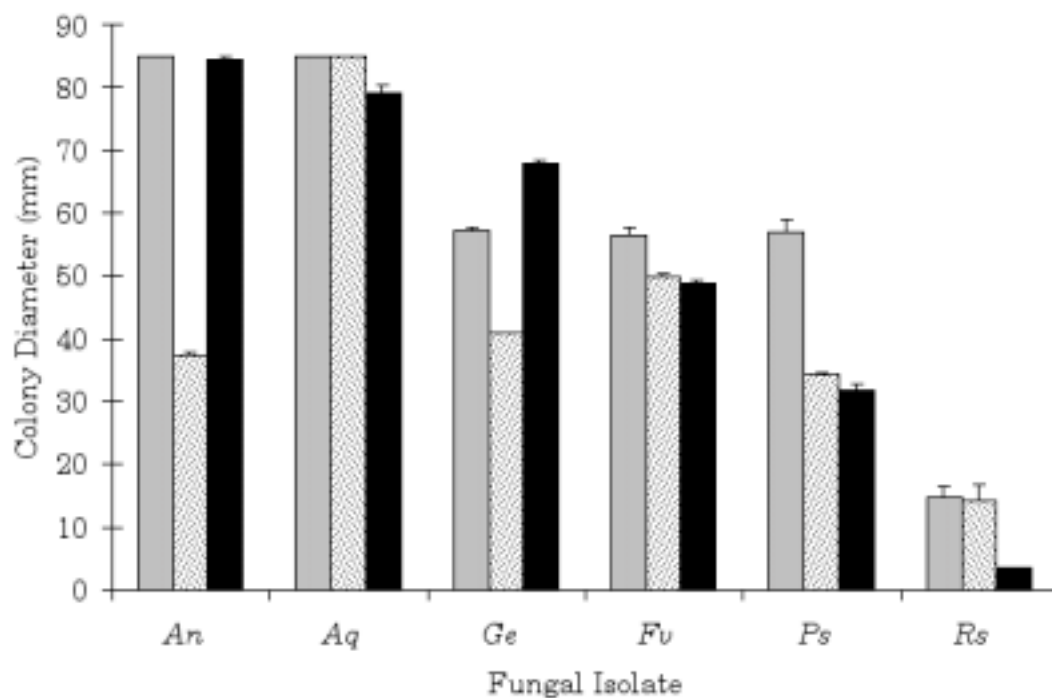


Figure 1. Growth of six fungi on solid media with or without 5.0 mM CuSO₄. Fungi were grown at 28°C for seven days on YES , YES + 5.0 mM CuSO₄ or minimal media (MM) . Bars represent standard error. *An* = *Aspergillus niger*, *Aq* = *Aquathanatephorus pendulus*, *Ge* = *Geotrichum* sp., *Fv* = *Fusarium verticillioides*, *Ps* = *Penicillium simplicissimum*, *Rs* = *Rhizoctonia solani*.

example, *A. pendulus*, which obtained a colony diameter nearly as large as *A. niger* on copper-containing media, produced only sparse mycelial growth.

Copper uptake screen. Five of the six fungal species were further tested for their ability to remove copper from broth solution amended with 0.1 mM CuSO₄. The amount removed varied from 0.14 (*R. solani*) to 0.52 (*A. niger*) mg copper/g fungal dry weight. *A. niger* not only grew better than the other fungi (3.1 g dry weight), but it also removed more copper per gram fungal dry weight than did the other fungi (Figure 2). *Geotrichum* and *F. verticillioides* removed 0.41 and 0.29 mg copper/g dry weight, respectively, and grew to 1.1 g and 1.0 g dry weight, respectively. In the solution, both *Geotrichum* and *F. verticillioides* grew as conidia or short mycelial strands, whereas the other fungi grew as mycelium.

Mechanism of copper and zinc removal by *A. niger*. We chose *A. niger* for further study because of its ability to efficiently remove copper from solution. As our initial interest was to use a fungus to remediate swine lagoon waste water, we evaluated *A. niger* for its ability to grow in and to remove copper and zinc from swine lagoon wastewater. Swine waste samples from a representative lagoon contained 0.11 mM copper as well as 0.037 mM zinc. To begin our comparative studies of copper and zinc uptake by *A. niger*, we examined the profile of copper and zinc accumulation by the fungus. *A. niger* was grown in shake culture (125 rpm) at 28°C for one week and the

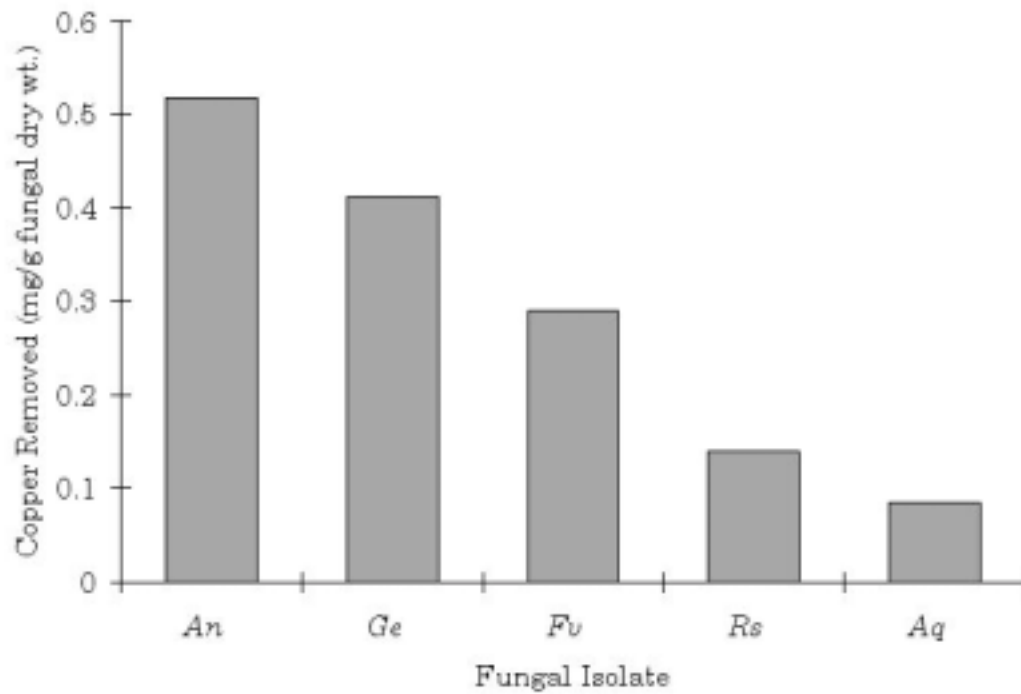


Figure 2. Amount of copper removed from a 0.1 mM solution of CuSO₄ by various fungal species. Fungi were grown at 28°C for four days in YES broth supplemented with 0.1 mM CuSO₄. Abbreviations same as used in Figure 1. Values represent the means from two replicated experiments.

mats and filtrates were assayed daily for metal content. When *A. niger* was grown in the presence of 0.1 mM copper, approximately the copper concentration found in swine lagoons, removal of copper increased from 58% after 1 day to 99% removal after 7 days (Figure 3A). Copper removal by the fungus was near maximal by day four (Figure 3A). Interestingly, the amount of copper removed per gram fungal dry weight decreased over time (Figure 3B). Thus the increase in copper removal from solution over time was due to increased mycelial growth, not more efficient copper absorption.

The removal of zinc over time showed a similar profile to that for copper, except that the absolute amount removed was an order of magnitude less than for copper (Figure 4A, B). Only 10% of the zinc from a 0.05 mM solution (a concentration comparable to that found in swine lagoon wastewater) was removed in the first day of culture. The maximum amount of zinc removed, 37%, occurred after 7 days. The amount of zinc removed per gram fungal dry weight was also less than that for copper and ranged from 0.1 to 0.2 mg Zn/g fungal dry weight.

To determine if the presence of copper and zinc together in solution altered the uptake of either metal, *A. niger* was cultured in a solution containing 0.1 mM copper and 0.05 mM zinc. The profile of copper accumulation was nearly identical to that found when copper was present alone (Figure 5A, B). Similarly, the profile of zinc removal in the copper/zinc

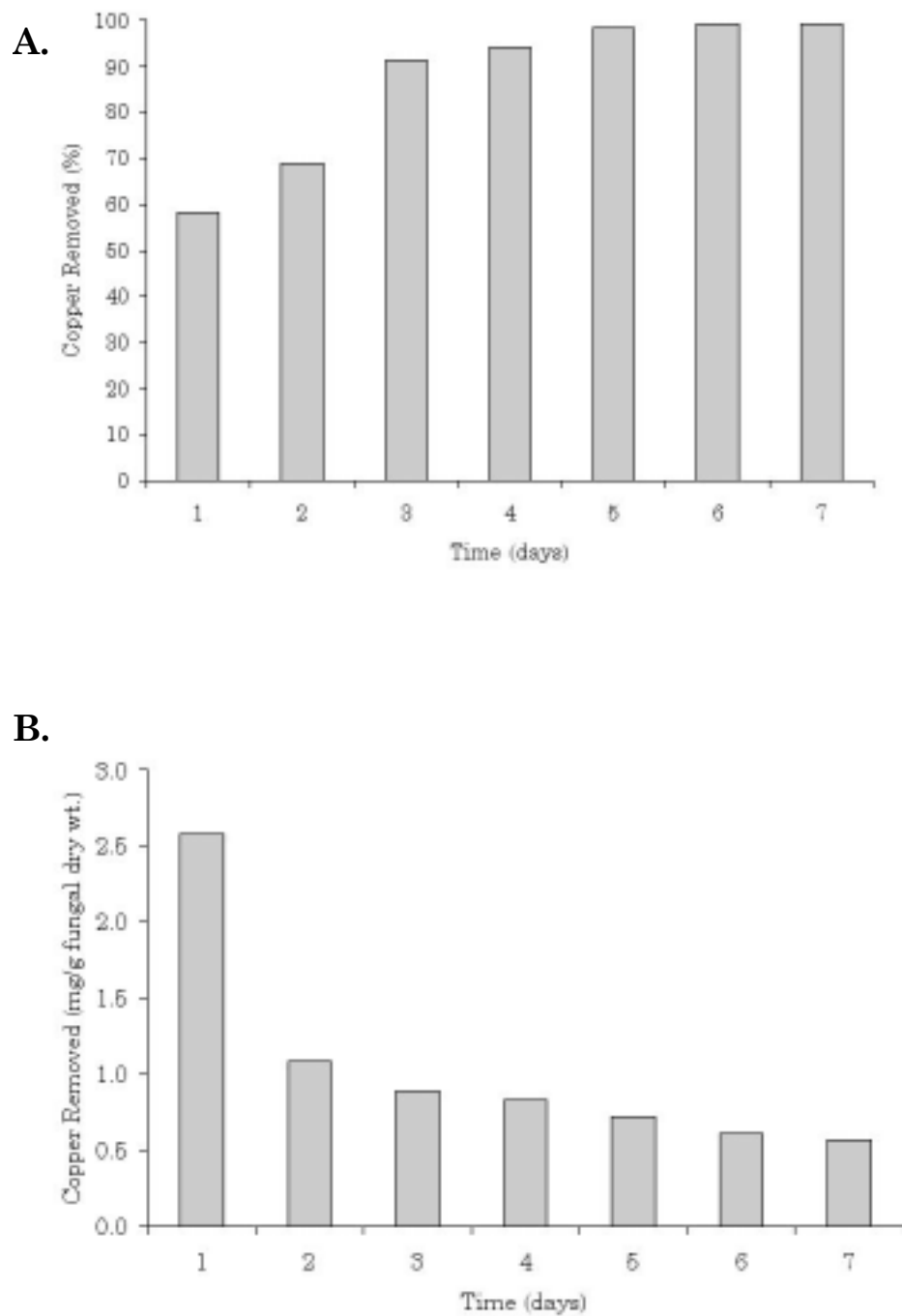
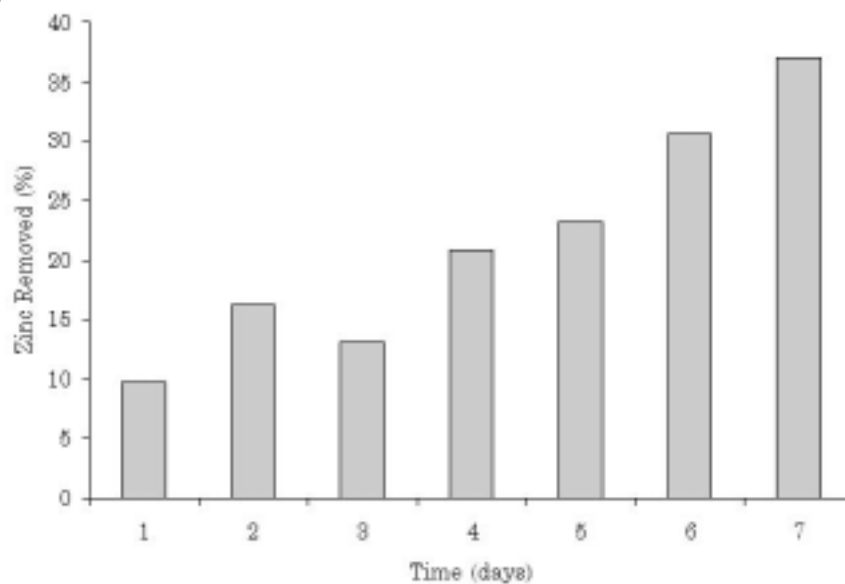


Figure 3. Accumulation of copper over time by *Aspergillus niger*. *A. niger* was grown at 28°C for seven days in YES broth supplemented with 0.1 mM CuSO₄. Copper removed expressed as percent of total copper present (A) or as a mass ratio of copper removed (B). Values represent the means from two replicated experiments.

A.



B.

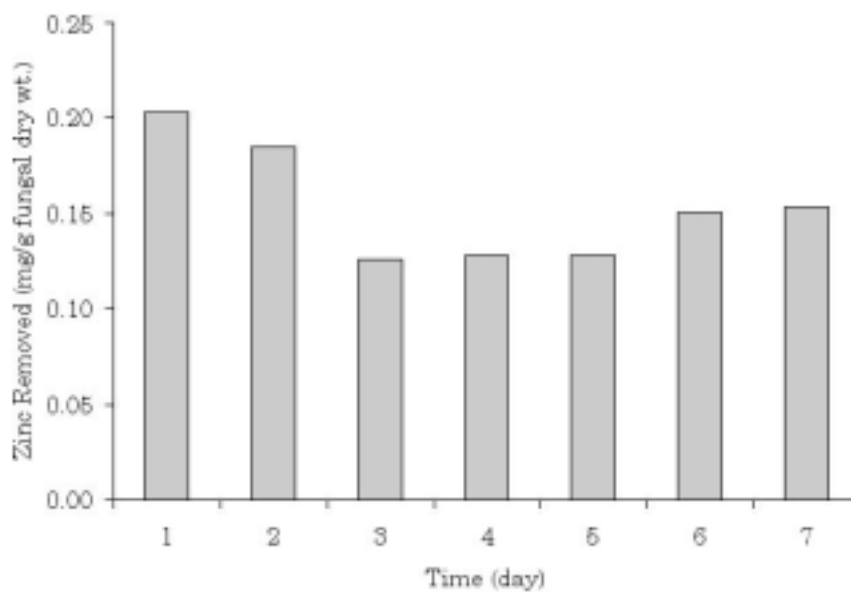


Figure 4. Accumulation of zinc over time by *Aspergillus niger*. *A. niger* was grown at 28°C for seven days in YES broth supplemented with 0.05 mM ZnSO₄. Zinc removed expressed as percent of total zinc present (A) or as a mass ratio of zinc removed (B). Values represent the means from two replicated experiments.

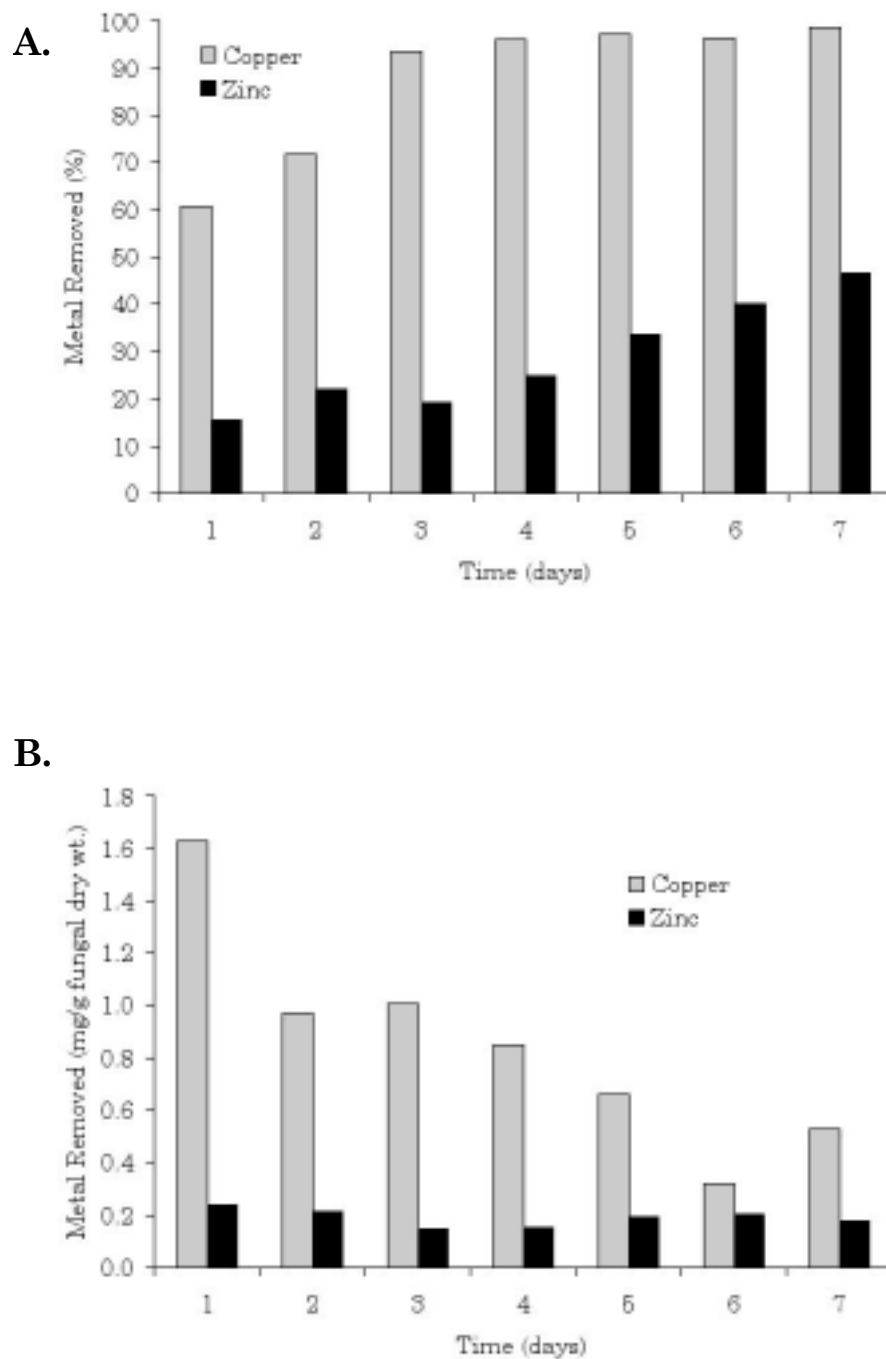


Figure 5. Copper and zinc accumulation by *Aspergillus niger*. *A. niger* was grown at 28°C for seven days in YES broth amended with 0.1 mM CuSO₄ and 0.05 mM ZnSO₄. Metal accumulation expressed as percent of total metal present (A) or as a mass ratio of metal removed (B). Values represent the means from two replicated experiments.

solution mimicked that found when zinc was present alone, except that slightly less zinc was removed from the solution also containing copper.

In order to determine if the uptake of copper and zinc was concentration dependent, *A. niger* was further tested for its ability to remove these metals from medium containing 0.01, 0.1 or 1.0 mM CuSO₄ or ZnSO₄. In these studies the mycelial mats were treated with 0.1 N HCl after harvest and the concentration of copper or zinc determined in the medium, in the HCl wash, and in the mycelial mat. Treatment of the fungal mycelium with 0.1 N HCl has been reported to remove any copper adsorbed to the mycelium (Gadd, 1993; Akthar and Mohan, 1995). In our studies, all copper and zinc remaining with the mat after this treatment was assumed to be absorbed internally.

As shown in figure 6, the amount of copper removed from solution (absorbed and adsorbed) increased linearly (on a log-log plot) in response to increased copper concentrations. *A. niger* was capable of removing up to 3.65 mg copper/g fungal dry weight from a 1.0 mM solution of CuSO₄. The fungus absorbed 88.5% (1.43 mg) and adsorbed 9% (0.15 mg) of the copper from a 0.1 mM CuSO₄ solution, which corresponded to 0.518 mg copper/g fungal dry weight. In 0.01 mM copper, the fungus removed approximately 79% of the copper present (0.044 mg copper/g fungal dry weight), with 73% by absorption and 6% by adsorption. Thus *A. niger* was able to remove 97% of the copper

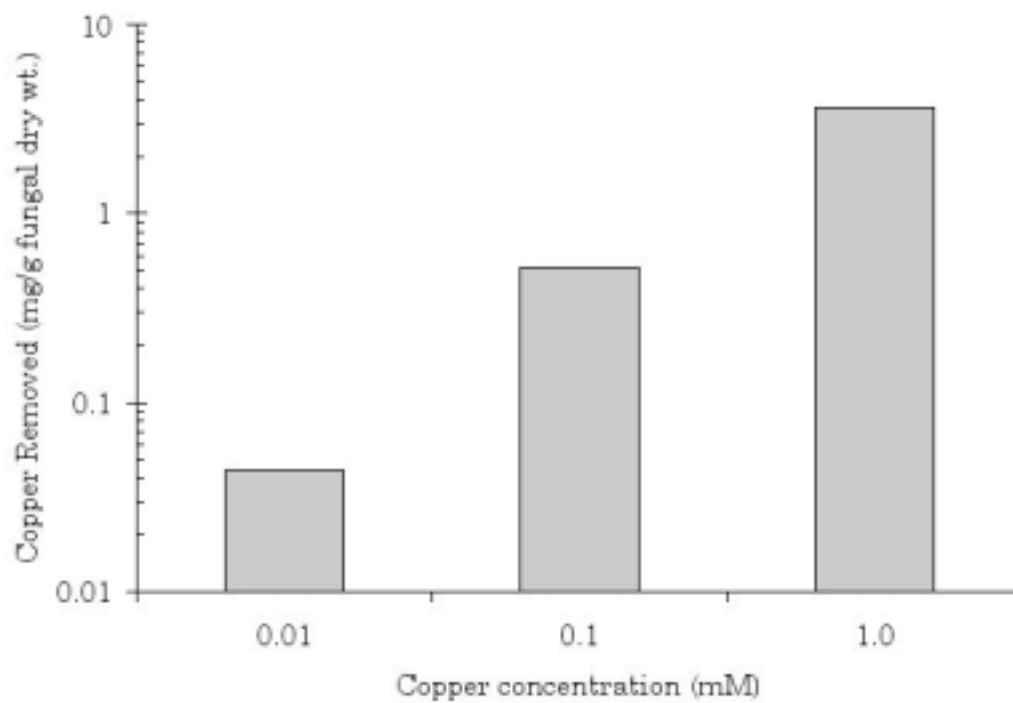


Figure 6. Copper accumulation by *Aspergillus niger* grown in different concentrations of CuSO_4 . *A. niger* was grown in YES broth amended with CuSO_4 at the concentrations shown, and was grown in shake flasks (150 rpm) at 28°C for 7 days. Values represent the means from two replicated experiments.

from a solution containing approximately the same concentration of copper found in swine lagoon wastewater (0.11 mM).

To determine if copper removal was accomplished by an active or passive process (i.e. binding fungal cell walls), copper removal by living and chloroform-killed mats was compared. As shown in figure 7, the living fungal tissue absorbed five times more copper (on a mass basis) than a metabolically inactive mat. The living fungal mat removed 82% of the total copper present in the medium while the killed material removed only 7%.

A. niger was grown in solutions containing $ZnSO_4$ at the same concentrations used for the copper study to determine if it could also remove zinc from solution. As shown in figure 8, *A. niger* removed 0.533 mg zinc/g fungal dry weight from a 1.0 mM solution of $ZnSO_4$. Zinc, like copper, was both absorbed and adsorbed by the fungus. In a 0.01 mM solution of $ZnSO_4$, *A. niger* removed 0.164 mg zinc/g fungal dry weight, which corresponded to 66% absorption of the total zinc present. At 0.1 mM zinc, the concentration nearest that found in swine wastewater (0.194 mM in treated swine effluent), *A. niger* was able to remove 0.37 mg zinc/g fungal dry weight (Figure 8). The 40.1% of zinc removed was almost evenly divided between absorption (20.4%) and adsorption (19.7%).

Copper and zinc did not affect the removal of one-another from a solution containing 0.1 mM copper and 0.05 mM zinc (Figures 3, 4 and 5). A

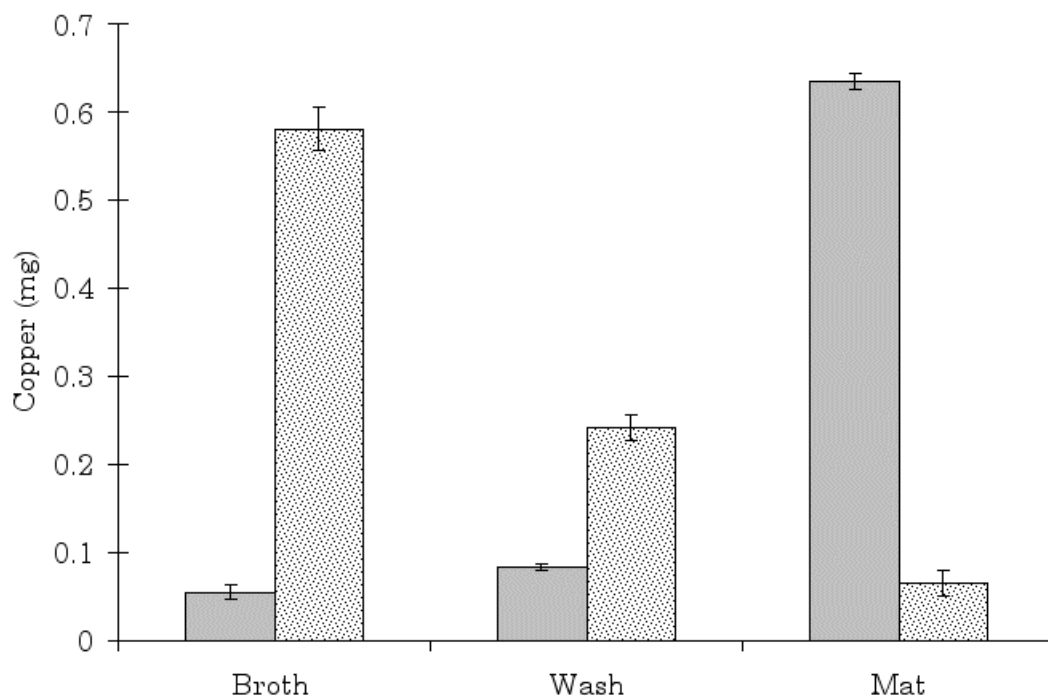




Figure 7. Effect of metabolic status on copper removal by *Aspergillus niger*. *A.* *niger* was grown at 28°C for four days in a modified Watson and Smith medium in shake flasks at 125 rpm. At this time half the biomass was killed with chloroform vapor, and the live  and dead  biomass were challenged separately with 0.1 mM CuSO₄ in shake culture for 24 h. Bars represent standard error.

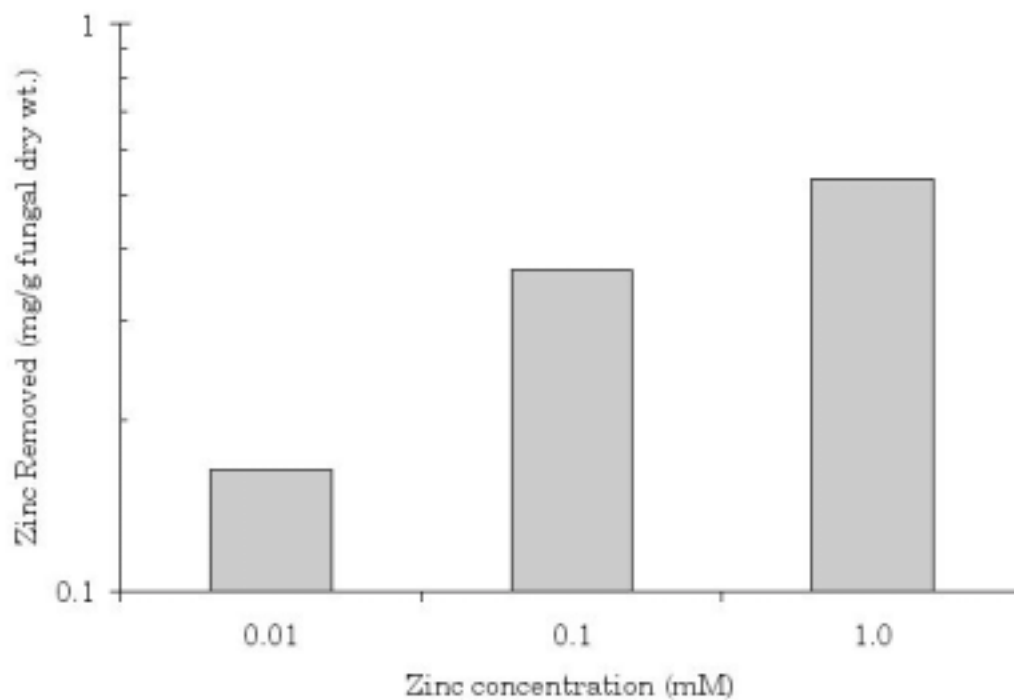


Figure 8. Zinc accumulation by *Aspergillus niger* grown in different concentrations of ZnSO₄. *A. niger* was grown in YES broth amended with ZnSO₄ at the concentrations shown, and was grown in shake flasks (150 rpm) at 28°C for 7 days. Values represent the means from two replicated experiments.

possible interaction between the two metals was further examined by measuring the uptake of each metal from a solution containing equal molar concentrations of copper and zinc in a range from 0.01 to 1.0 mM. The weight of each metal removed (mg/g fungal dry weight) when present together at either 0.01 or 1.0 mM was approximately the same as when each metal was present alone (Figures 6, 8 and 9). The amount of copper removed at 0.1 mM (when present with zinc) decreased compared to copper alone, from 0.52 to 0.39 mg copper/g fungal dry weight (Figures 6 and 9). Likewise, zinc removal (when present with copper) decreased compared to zinc alone (0.1 mM), from 0.37 to 0.13 mg zinc/g fungal dry weight (Figures 8 and 9).

Metal removal from swine wastewater. The observation that *A. niger* can remove both copper and zinc from defined media encouraged us to study its ability to remove the two metals from swine effluent. We assayed treated swine effluent by atomic absorption spectroscopy and found copper present at 0.029 mM and zinc at 0.194 mM. As shown in figure 10, the fungus was able to remove 91% of the copper and 70% of the zinc from treated swine wastewater after 24 h incubation of the fungal mat. These percentages correspond to 0.05 and 0.96 mg metal/g fungal dry weight of copper and zinc, respectively, being removed by the fungus.

Effect of varying the metal concentration on the uptake of the other metal. The amount of zinc removed from swine wastewater was

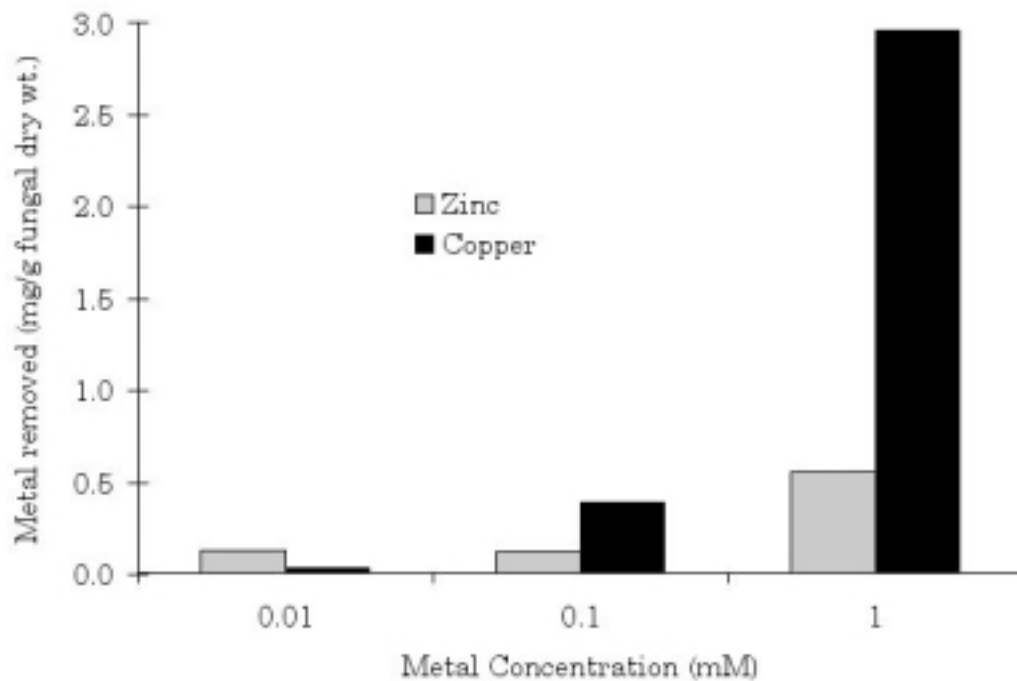


Figure 9. Accumulation of copper and zinc by *Aspergillus niger* grown in a medium containing both metals. *A. niger* was grown at 28°C for seven days in YES broth amended with 0.01, 0.1, or 1.0 mM each of CuSO₄ and ZnSO₄. Spent broth, 0.1 N HCl wash filtrate, and fungal mat samples were analyzed for metal content by atomic absorption spectroscopy. Removed metal is defined as the metal associated with the wash and mat. Values represent the means from two replicated experiments.

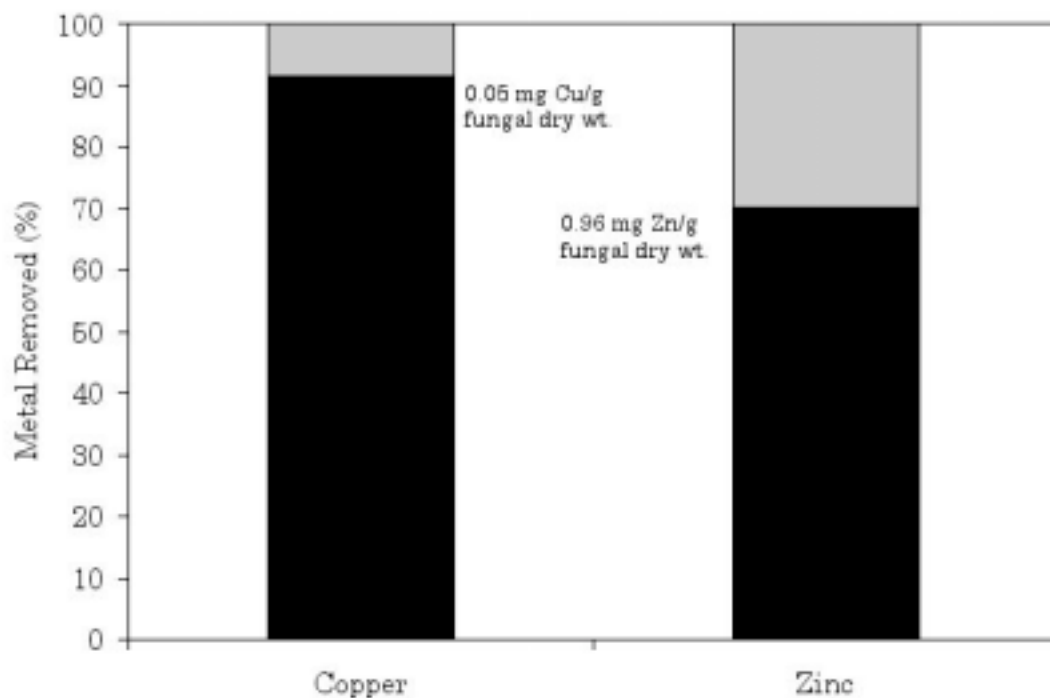


Figure 10. Removal of copper and zinc in cultures of *Aspergillus niger* grown in treated swine effluent. Fungal mats were grown up from spores in YES broth at 28°C for 48 h and resuspended in treated swine effluent for 24 h. The fungus was separated by filtration, and the effluent and fungus were analyzed for metal content. Values represent the means from two replicated experiments.

higher than observed when the fungus was grown in culture media amended with zinc alone (Figure 4A) or with copper (Figure 5A). One explanation for this is that copper facilitates zinc uptake at the zinc concentration present in wastewater. To test this hypothesis, solutions were made in which zinc concentration was held constant at 180 μM and the concentration of copper varied from 1-50 μM . As can be seen in figure 11, copper concentration in the range of that found in swine wastewater did not affect zinc removal from solution. Increasing the concentration of copper did result in increased copper uptake as would be expected. The effect of differing zinc concentrations on copper uptake was also examined. *A. niger* was grown in solutions in which zinc was increased from 10-500 μM with a constant copper concentration of 30 μM . Increasing zinc concentration in this range increased zinc removal by the fungus but did not affect copper removal from the media (Figure 12).

Discussion

Filamentous fungi are used widely in industrial fermentation and bioremediation (Friedrich *et al.*, 1983; Friedrich *et al.*, 1986; Luef, *et al.*, 1991; Berka, *et al.*, 1992; Mullen, *et al.*, 1992; Burgstaller and Schinner, 1993; Feofilova, *et al.*, 1994; Akthar and Mohan, 1995; Bosshard, *et al.*, 1996; Plaza *et al.*, 1996). They are preferred over other organisms for

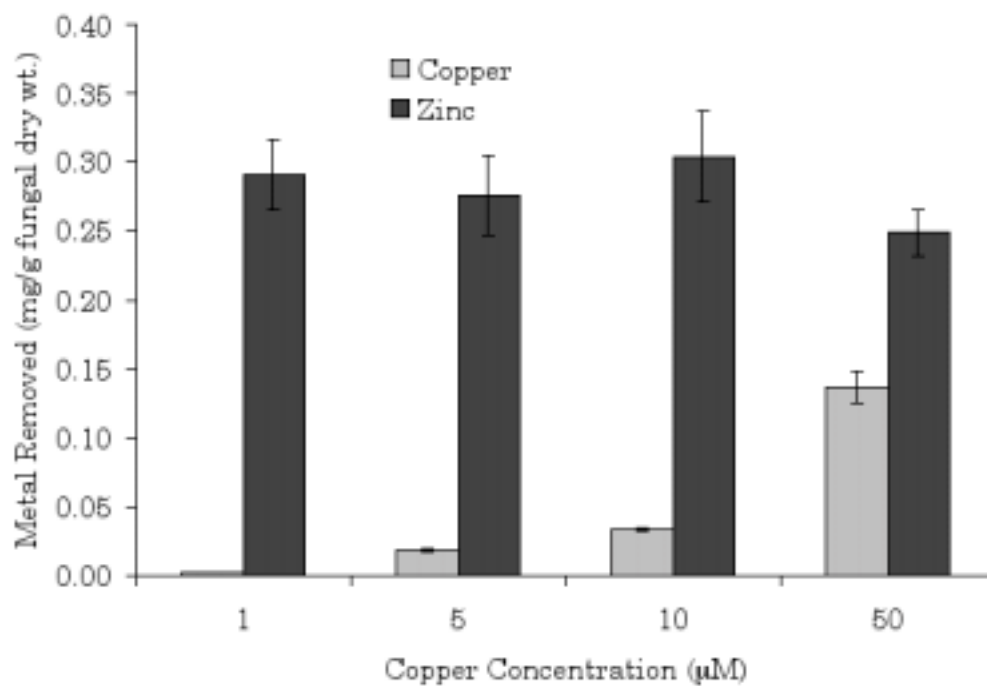


Figure 11. Titration of copper against a constant level of zinc and its effect on metal uptake by *Aspergillus niger*. *A. niger* was grown at 28°C for four days in a modified Watson and Smith medium in shake flasks at 150 rpm. Zinc concentration held constant at 180 μM as ZnSO_4 , and copper concentration varied at either 1, 5, 10 or 50 μM as CuSO_4 . Bars represent standard error.

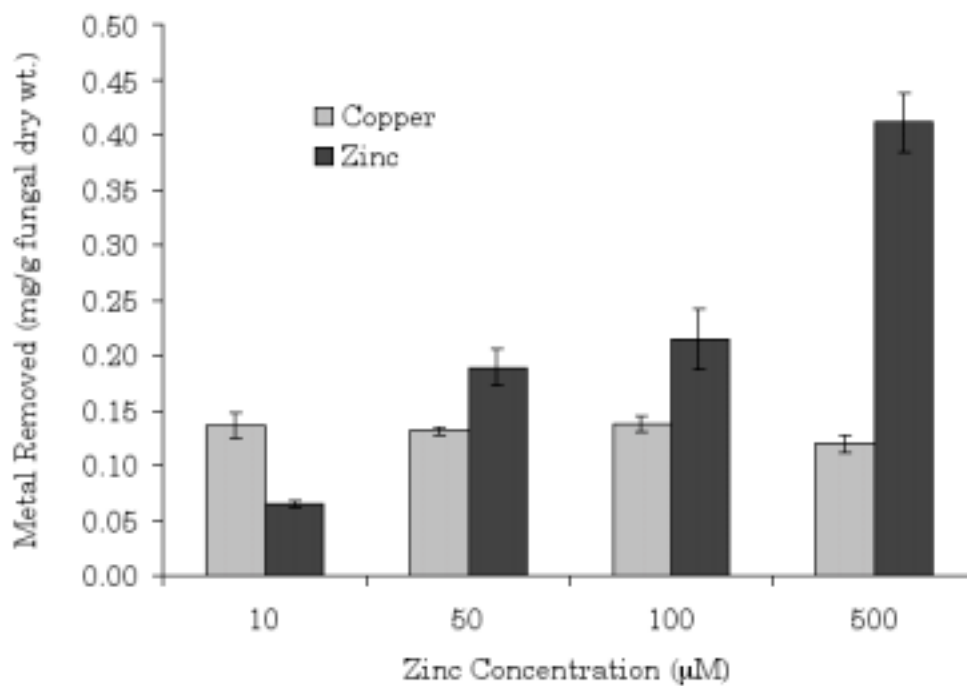


Figure 12. Titration of zinc against a constant level of copper and its effect on metal uptake by *Aspergillus niger*. *A. niger* was grown at 28°C for four days in a modified Watson and Smith medium in shake flasks at 150 rpm. Copper concentration held constant at 30 µM as CuSO₄, and zinc concentration varied at either 10, 50, 100 or 500 µM as ZnSO₄. Bars represent standard error.

bioremediation because they are easier to remove from liquid substrates. Metal contamination of swine waste is a good example of a system in which bioremediation is ecologically sound. Swine wastewater does not contain high concentrations of metals *per se*, but the contaminating metals may accumulate to phytotoxic levels in soils receiving repeated applications of swine effluent, as is the practice in North Carolina.

Our studies indicate that *A. niger* is capable of thriving on substrates containing elevated levels of copper. It grew better in the presence of copper than all the other fungi tested, to a maximum of 3.1 g dry weight after 4 days in liquid culture containing 0.1 mM copper. Further, this fungus is able to grow normally on plates amended with a level of copper five times greater than that inhibitory to the growth of *S. cerevisiae* (Fogel and Welch, 1982). Our data suggest a copper concentration greater than 20 mM is required for inhibition (data not shown). We further show that *A. niger* can accumulate copper and zinc from semi-defined media as well as swine wastewater. This fungus is capable of removing up to 0.533 mg Zn and 3.65 mg Cu/g fungal dry weight from semi-defined media (Figures 6 and 8). When assayed in semi-defined media with copper and zinc present at the same concentrations found in treated swine effluent, *A. niger* was able to remove only 13% of the zinc and 48% of the copper present (data not shown). In treated swine effluent,

however, this fungus was able to remove 70% of the zinc and 91% of the copper present (Figure 10).

There was no clear interaction between copper and zinc that would affect the uptake of these metals from solution by *A. niger*. When the copper concentration in solution was held constant at 30 μM and zinc concentrations were adjusted from 10-500 μM , neither copper nor zinc uptake differed compared to each present in solution alone (Figures 8 and 11). A similar phenomenon was observed for copper when zinc was held constant at 180 μM and copper concentrations were adjusted from 1-50 μM (Figures 6 and 12). Further, in experiments in which copper and zinc were in solution in equal concentrations of 0.01 and 1.0 mM, no effect was observed (Figures 6, 8, 9). An exception to this occurred when copper and zinc were present together at 0.1 mM (Figure 9), in which removal of zinc was less than when zinc was assayed separately (Figure 8). When present with an equal molar concentration of copper, removal of zinc slipped from 0.367 mg/g fungal dry weight (zinc alone) to 0.126 mg/g fungal dry weight (zinc with copper). One possible explanation for this reduction could be the optimization of the system for copper trafficking, as removal of copper as a percentage of total copper present (with zinc) is near the level of removal observed for copper alone.

Other researchers have examined *A. niger* for its ability to remove contaminating metals (Akthar and Mohan, 1995; Bosshard, *et al.*, 1996). Akthar and Mohan (1995) used killed mycelium of *A. niger* to remove copper and zinc from contaminated lake waters. The levels of copper and zinc in these lake waters was comparable to those found in treated swine effluent. The killed biosorbent was able to remove 17.02 μg Cu and 912.5 μg Zn/g fungal dry weight from the contaminated lake water. Bosshard, *et al.* (1996) reported the use of *A. niger* to remove copper, zinc and other metals from incinerated municipal (fly) ash by organic acid leaching. The fungus is known to produce large quantities of citrate and gluconate, both of which are capable of leaching or precipitating metals out of materials. While this method works well with substrates possessing extremely high metal content (0.11% Cu, 3.1% Zn in fly ash), it may not be suitable for use in the swine wastewater system (0.000003% Cu and 0.000018% Zn). Indeed, we found that using similar growth conditions as were used in the fly ash study, *A. niger* preferred to absorb copper rather than precipitate it. However, this does not discount the potential to utilize organic acid production by this fungus to remediate solid swine waste.

Our data support an active process as being responsible, at least in part, for the fungus' ability to remove such large amounts of copper and zinc from solution. A live fungal mat is able to remove more than twice the

amount of copper from solution than a dead mat (Figure 7). We found that *A. niger* removes metals, especially copper, from its environment mainly by absorption. This fungus was able to remove 51.1 µg Cu and 963 µg Zn/g fungal dry weight from treated swine effluent (Figure 10), which is superior to the killed-fungus biosorption method used by Akthar and Mohan (1995). The absorptive properties of *A. niger* have not been previously reported for use in bioremediation.

The actual mechanism(s) which *A. niger* uses to detoxify metals present in its environment is not known. Fungi have been shown to possess two classes of metal binding molecules: metallothionein (MT) and phytochelatin (Butt & Ecker, 1987; Mehra and Winge, 1991; Zhou & Thiele, 1993; Cervantes & Gutierrez-Corona, 1994; Kosman, 1994; Joho *et al.*, 1995; Perego & Howell, 1997). Phytochelatins have been shown to provide protection against cadmium in certain fungal species, while metallothioneins have been shown responsible for copper detoxication by others (Mehra and Winge, 1991; Gadd, 1993; Zenk, 1996). MTs are postulated as responsible for detoxication of a variety of class IIb metals such as Cu, Zn, Cd, and Ag, in many different species (Lerch, 1980; Jeyaprakash, *et al.*, 1991; Mehra and Winge, 1991; Gadd, 1993; Cervantes and Gutierrez-Corona, 1994; Kosman, 1994; Cizewski-Culotta *et al.*, 1995; Joho, *et al.*, 1995; Kelly *et al.*, 1996;

Ramirez-Salgado *et al.*, 1996; Perego and Howell, 1997), and are usually named by the metal which induces transcription of the protein.

A. niger is known to possess a copper metallothionein (CuMT), but neither the protein nor the gene has been sequenced (Kermasha, *et al.*, 1993). The CuMT from *A. niger* exhibits strong biochemical similarity to other CuMTs. Its transcription is induced in the presence of copper, and its size and moles copper per mole protein are similar to other known CuMTs (Kermasha, *et al.*, 1993). Thus, the *A. niger* CuMT could be responsible for the high tolerance of the fungus to copper and zinc. However, other possibilities, such as phytochelatin and superoxide dismutase, cannot be overlooked. Our results suggest that phytochelatin binding is not the mechanism involved in metal uptake by *A. niger* due to the lack of enhanced uptake of zinc, which is known to activate this system in other species (Mehra and Winge, 1991; Zenk, 1996). Metallothionein binding, which is a more discriminating system, is more likely responsible due to the apparent specificity of copper uptake in *A. niger* (Butt *et al.*, 1984b; Winge *et al.*, 1985). In any case, copper resistance in this organism is due to an active process, and not simply due to passive metal binding on the cell wall.

The potential for copper removal in swine wastewater was predicted in our semi-defined media studies (Figures 3A and 5A). The level of zinc removal, however, was not predicted by any of our other data. Surprisingly,

A. niger's ability to absorb zinc was enhanced in the swine wastewater environment. *A. niger* accumulated 1.68 mg Zn/g fungal dry weight (highest for a given experiment) from the wastewater. The mechanism for increased zinc accumulation is not known. One hypothesis is that copper and zinc uptake is mediated by a CuMT. If this is true, the copper present in the waste may act to induce the production of CuMT. Once produced, the CuMT then removes zinc first due to its relative abundance. However, this hypothesis is not supported by the titration studies where zinc concentration was held constant and copper concentration were varied (Figure 11). If the hypothesis were true, one would expect zinc uptake to increase as copper concentration increased.

In conclusion, we have shown that the fungus *A. niger* is capable of removing both copper and zinc from treated swine effluent, and that the mechanism of removal appears to be mainly due to an active metabolic process leading to internal absorption of the metal. Bioremediation by internal absorption has not been previously reported in the literature. If the fungus were to be used in a treatment regime, the spent fungus could be replaced with fresh biomass and possibly fed to swine as a dietary supplement for copper and zinc.

Acknowledgments

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APPENDIX

Lagoon and Fresh Wastewater Assays

In order to determine the utility of *A. niger* in the remediation of other types of swine wastewater, experiments were conducted using lagoon and freshly flushed wastewater. Lagoon wastewater was collected from the Lake Wheeler Road Field Laboratory Swine Educational Unit for use in this assay, and was found to be near neutral in pH (pH = 7.19). *A. niger* was incubated in YES overnight at 28°C as a shake culture (175 rpm) to obtain a mass of hyphae. The hyphae was then resuspended into either septic lagoon wastewater, or lagoon wastewater which had been sterilized by autoclaving at 121°C for 20 minutes, and incubated for 24 hours at 28°C, 175 rpm.

The fungus was able to sequester copper and zinc from lagoon wastewater, whether or not it was in the presence of other microorganisms (Figure 13). However, *A. niger* was unable to survive in the lagoon wastewater, and lost more than half its biomass in 24 hours whether or not the wastewater was sterilized (data not shown). This suggests there is a substance in lagoon wastewater that survives autoclaving and is detrimental to the growth of the fungus.

Due to public outcry demanding an end to lagoon waste treatment systems, it seemed necessary to address metal removal in an alternate waste

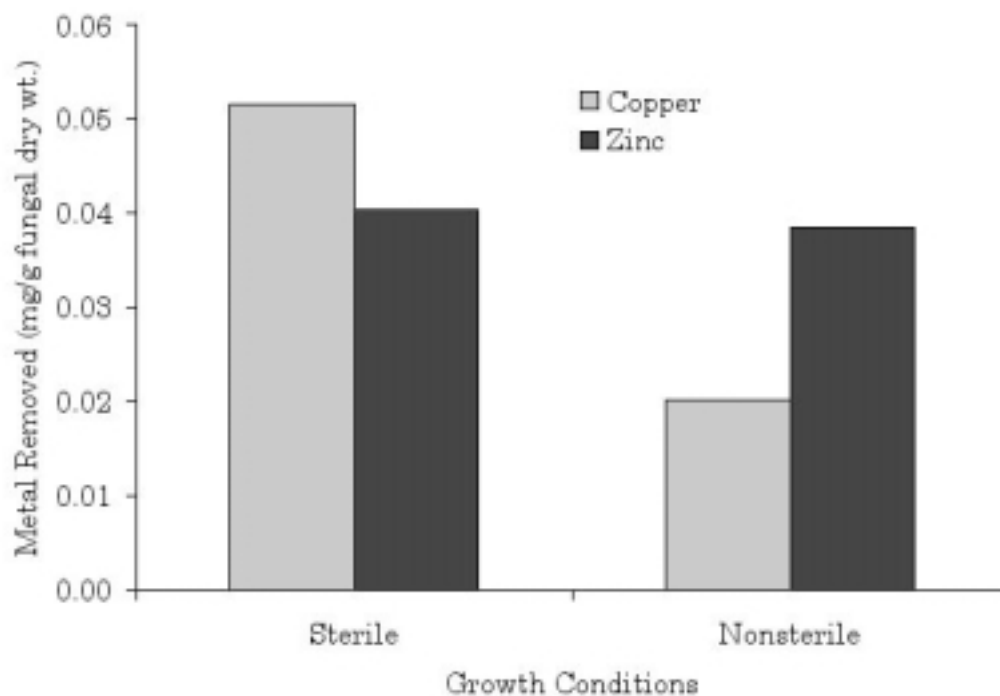


Figure 13. Removal of copper and zinc in cultures of *Aspergillus niger* grown in septic or sterilized swine lagoon liquid. Fungal mats were grown up from spores in YES broth at 28°C overnight in shake culture at 175 rpm. The hyphal mats were then resuspended in either septic or sterilized swine lagoon liquid and incubated in shake culture at 175 rpm for 24 h at 28°C. Values represent the means from two replicated experiments.

treatment system. The Center for Environmental Farming Systems in Goldsboro, NC, uses a semi-closed waste treatment system consisting of alternating aerobic and anoxic tanks for nitrogen removal of wastewater that has been screen-filtered to remove large particles. This treated wastewater then is moved to flush tanks, to be re-used in flushing the hog-house every three hours.

Wastewater was collected from the first collection tank, which contained the freshly flushed wastewater prior to screen-filtering. This wastewater was used in experiments to determine if *A. niger* could survive in hog waste, and remove copper and zinc. A fungal seed culture was grown for 48 hours at 28°C, 150 rpm and resuspended in the wastewater. In half of the flasks yeast extract was added. As a further sub-treatment, half of the flasks were autoclaved at 121°C for 25 minutes. The fungi were then treated the same as in previous experiments, with the exception that no HCl wash was performed. The broths and mats were then analyzed for copper and zinc content.

The fungus grew better in the waste from the alternative system than in lagoon liquid. The wastewater cultures without the addition of YE did not grow, but no loss of biomass was observed. The cultures with the addition of YE did show a doubling in biomass (data not shown). The fungus was more efficient at removing copper and zinc from the septic cultures without YE and

was able to remove 0.11 mg Cu and 0.86 mg zinc/g fungal dry weight (Figure 14).

Swine lagoon wastewater is viscous. To determine if the poor growth of *A. niger* in the wastewater was due to poor aeration, the cultures were aerated with compressed air. A manifold was fashioned using vacuum tubing and syringes and attached to an air compressor. All tubing was sterilized by washing with 100% isopropanol. This system was used to aerate fresh wastewater inoculated with *A. niger*. The aeration system appeared to work well as bubbles of air were constantly produced in the flask. As seen in the previous assay, conditions providing the most efficient removal of the metals was the sterile wastewater without YE, where 0.28 mg copper and 1.93 mg zinc/g dry weight were removed (Figure 15). The addition of YE seemed to hinder uptake, as was seen in the previous assay. Interestingly, all treatments that were aerated with the compressed air exhibited better copper and zinc uptake than those from the previous assay (Figures 14 and 15). *A. niger* was able to remove 98% of the copper and 99% of the zinc from the sterile swine wastewater when aerated with compressed air.

The fresh wastewater results are most encouraging, as *A. niger* was able to remove at least as much copper and zinc as in the treated waste system described in the manuscript. Indeed, improving the aeration of the untreated wastewater boosted copper and zinc removal to 98% and 99%,

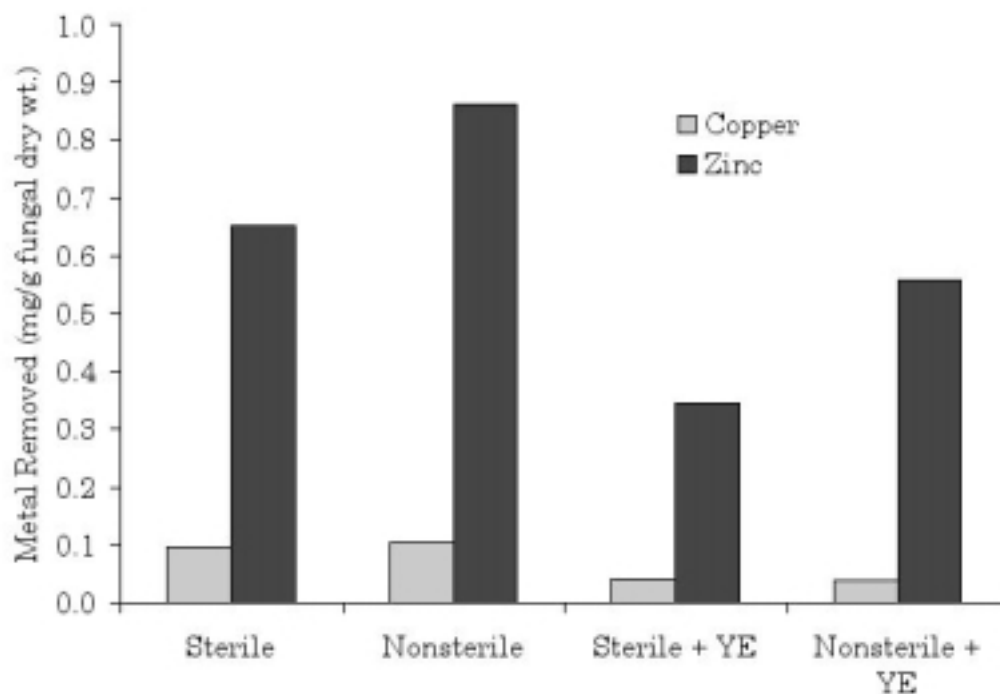


Figure 14. Removal of copper and zinc in cultures of *Aspergillus niger* grown in fresh swine wastewater. Fungal mats were grown up from spores in YES broth at 28°C for 48 h in shake culture at 150 rpm. The hyphal mats were then resuspended in either septic or sterilized wastewater with or without added yeast extract to aid growth. These cultures were then incubated in shake flasks at 150 rpm for 24 h at 28°C. Values represent the means from two replicated experiments.

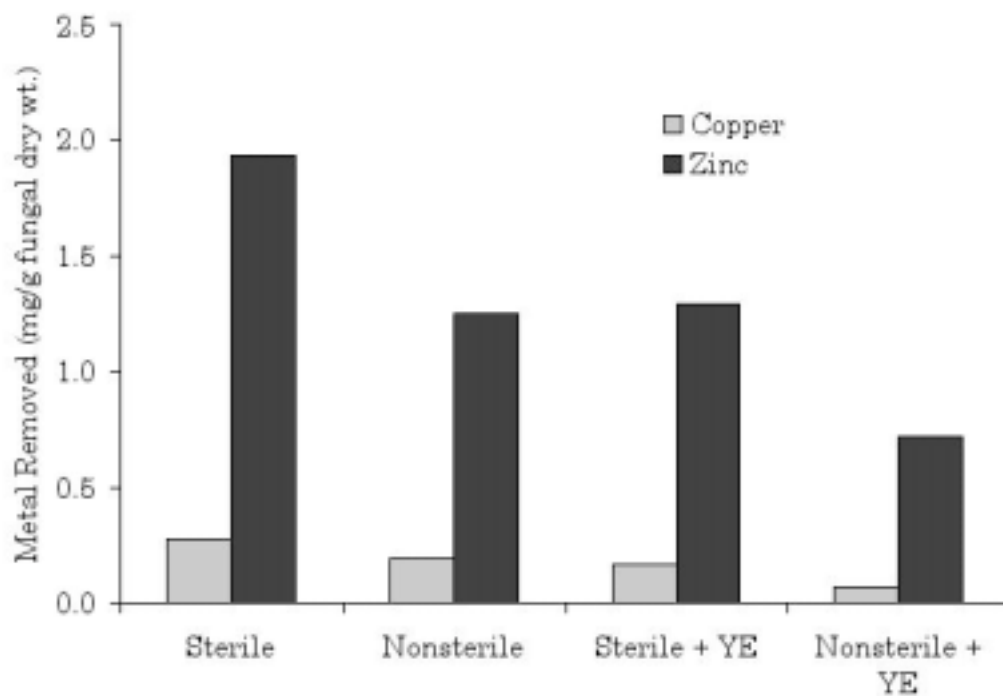


Figure 15. Effect of aeration on removal of copper and zinc from swine wastewater by *Aspergillus niger*. *A. niger* treated same as in Figure 14. Swine wastewater cultures aerated using an air compressor linked to a manifold. Values represent the means from two replicated experiments.

respectively. This is an improvement over the observations made with treated waste, where only 91% of the copper and 70% of the zinc were removed. The work contained in this thesis shows that *A. niger* is a good choice for removing copper and zinc from swine effluent.

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