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Degradation of Atrazine by White Rot and Soil Fungi

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Abstract

The widespread use of atrazine in agriculture has lead to an abundance of this toxic chemical in the environment. Fungi that have the ability to degrade atrazine into less toxic products have been identified and used in the remediation of atrazine. In this study atrazine degradation in a defined liquid media was characterized in a diverse group of white rot basidiomycete and deuteromycete soil fungi. Atrazine did not have an effect on fungal growth although each species produced a different amount of biomass in culture. Statistical analysis showed that biomass production was an important factor in determining the amount of atrazine removed. Two of the twelve fungal species tested, *Armillaria gallica* and *Aspergillus niger*, removed amounts of atrazine from culture. Analysis of high pressure liquid chromatography chromatograms did not show the production of atrazine degradation products in fungal cultures that could be differentiated from control chromatograms. Gas chromatography-mass spectrometry analysis of organic extracts of fungal cultures also indicated that no chlorinated atrazine metabolites were produced in any of the cultures although unidentified compounds were detected in *Mycena leaiana*, *Aspergillus flavus*, and *Aspergillus niger* cultures that may be hydroxylated atrazine metabolites. These data indicate that atrazine degradation did not occur in most of the fungal cultures and although it may have occurred in *Mycena leaiana*, *Aspergillus flavus*, and/or *Aspergillus niger*, albeit at appreciable levels only in *Aspergillus niger* and *Armillaria gallica*. Removal of atrazine from *Aspergillus niger* and *Armillaria gallica* cultures might have been due to atrazine sequestration in fungal biomass although the culture conditions might not have been conducive to atrazine degradation.

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Introduction

Atrazine is a triazine herbicide used to control weed pests in agriculture, primarily in the cultivation of corn. Atrazine is used in the treatment of 75% of all corn fields in the United States which accounts for the majority of the approximately 34.5 million kg consumed every year (USEPA 2011). Atrazine kills non-resistant plants by binding to photosystem II (PSII) in chloroplasts and preventing electron transfer, leading to an accumulation of reactive oxygen species that damage lipid membranes in plant cells and lead to cell death (Steinback et al 1981; Fedtke 1982). Resistant plants such, as corn and sorghum, are not affected by atrazine because they are able to either metabolize atrazine before it binds to PSII or they possess a variant protein, natural or engineered, involved in PSII that does not allow atrazine to bind and inhibit electron transfer (Fedtke 1982; Shimabukuro and Swanson1969).

The widespread use of atrazine has raised concerns because atrazine has been shown to act as an endocrine disruptor in several animals. Atrazine exposure, even at levels at or below 3 parts per billion (ppb), the safe recommended limits for drinking water outlined by the United States Environmental Protection Agency, has been shown to alter sexual development, tissue maturation, and reduce survival rates in amphibians (Hayes et al 2006; Lenkowski et al 2008; Storrs and Kiesecker 2004; "Atrazine Background" 2011). In addition, rainbow trout treated with atrazine show altered liver metabolism (Iurgi et al. 2009). These studies suggest that atrazine is a direct threat to the survival of wildlife and that the widespread application of atrazine has the potential to disrupt natural ecosystems. Mammalian systems have also shown detrimental responses to atrazine exposure. Rats exposed to atrazine exhibit delayed sexual development,

hastened reproductive aging, and increased incidence of mammary tumors caused by an altered pattern of female hormone secretion (Cooper et al. 2007; Ashby et al. 2002). These studies indicate that atrazine acts as an endocrine disruptor in model animal systems and may act as a human endocrine disruptor.

The potential health risks of atrazine exposure, coupled with its persistence in the environment, make it a very real public health concern. Atrazine is a recalcitrant chemical that is relatively mobile in soils and is able to leach into surface and groundwater (Periera and Rostad 1990; Ma and Selim 1996). Atrazine has an average half life in soils of 60 days which can vary depending on environmental conditions (USDA ARS, 2008) and this allows it to readily make its way into human drinking water supplies. The United States Department of Agriculture reported in its 2009 Pesticide Data Program Summary that 88% of drinking water sampled from across the United States contained detectable levels of atrazine, making chronic human exposure at low levels unavoidable.

The half-life of atrazine depends on a number of factors including soil pH, chemical composition, and microbial communities present in contaminated soil (Krutz et al. 2008). Microbial communities in particular have a strong effect on the removal of atrazine from soils. Soils that have a history of treatment with atrazine tend to harbor microbes that are able to degrade atrazine more efficiently than soils untreated with atrazine (Krutz et al. 2008). In addition, fungi acclimatized to atrazine containing environments degraded atrazine more quickly in synthetic media than non-acclimatized fungal isolates (Singh et al. 2008). This suggests that exposure to atrazine induces the production of atrazine degrading genes in soil microbes.

Several bacterial species have been identified that have at least one or more atrazine degrading genes present in their genome and demonstrate the ability to degrade atrazine. The bacterial strain *Pseudomonas* ADP was isolated from atrazine contaminated soil and shown to mineralize atrazine. Atrazine can be used as the sole source of nitrogen for *Pseudonmonas* (Mandelbaum et al. 1995). Since then, *Psudomonas* ADP has been used as a model organism for bacterial atrazine mineralization and metabolism. Genes involved in atrazine mineralization in *Pseudomonas* may have evolved specifically for the purpose of deriving a source of nitrogen from atrazine and may have offered a selective advantage to *Pseudomonas* in agricultural fields rich in atrazine.

Although bacteria capable of mineralizing atrazine were first isolated only two decades ago (Wackett et al. 2002), atrazine metabolizing genes are now widespread among soil microbes. Atrazine metabolizing genes may have arisen in different bacteria due to the widespread presence of atrazine in the environment. In addition, because atrazine metabolizing genes are carried on a single plasmid in some species, they could have easily been spread by horizontal gene transfer (Wackett et al. 2002).

The genes involved in atrazine degradation have been described in *Pseudomonas* ADP. All of the genes involved in atrazine metabolism are present on a single plasmid, pADP-1 which contains 104 putative genes including genes necessary for translocation, transcriptional regulation, and membrane transport proteins (Martinez et al. 2001). Atrazine is degraded by the hydrolysis of its chlorine atom by the atzA protein. This is followed by two successive N-dealkylations, the hydrolysis of the N-ethyl group carried out by the atzB protein, and the hydrolysis of the N-isopropyl group by the atzC protein to yield cyanuric acid. The triazine ring in cyanuric acid is then cleaved and further

degraded to CO_2 and urea by proteins atzD, atzE, and atzF (Sene et al. 2010). Urea can then be used as a nitrogen source by shuttling it through the urea cycle. The triazine ring is used solely as a source of nitrogen by *Pseudomonas*, but the hydrolyzed alkyl groups yield ethanol and isopropanol which can be utilized as a source of carbon.

Fungi can also degrade atrazine, but with very different mechanisms than seen in bacteria. Many of the fungi that have been shown to degrade atrazine are white rot wood-degrading fungi (Bending et al. 2002). White rot fungi are characterized by their ability to completely remove lignin, a polyphenolic biopolymer, from woody substrates, leaving the wood with a white appearance due to the remaining cellulosic material. This is accomplished this by using a unique set of enzymes including lignin peroxidases (LiPs) and manganese peroxidases (MnPs). These enzymes oxidize low molecular weight substrates to free radicals that act as reaction mediators and oxidize the β-O-4 aryl ether bonds between lignin monomers (Hammel and Cullen 2008). White rot fungi have been extensively studied because of their potential use in commercial delignification and bioremediation. The non-specific free radical mechanism by which white rot fungi de-polymerize lignin allows them to oxidize a variety of xenobiotic compounds such as polyaromatic hydrocarbons and polychlorinated biphenyls (Novotny et al. 2004; Chupungars et al. 2009). Several widely studied white rot species have been shown to degrade atrazine including *Phanerochaete chrysosporium* Burds, *Pleurotus pulmonarius* (Fr.) Quel., and *Trametes versicolor* (L.)Lloyd (Bastos and Magan 2009; Mougin et al. 1997; Masaphy et al. 1993). A larger diversity of common soil fungi, including many deuteromycetes, are also able to degrade atrazine and some were isolated from atrazine

contaminated soils (Singh et al. 2008; Kaufman and Blake 1970; Khromonygina et al. 2003).

Unlike bacteria, fungi do not produce proteins specifically for the purpose of atrazine degradation. Non-substrate specific enzymes produced for other purposes, such as lignin degradation, degrade many xenobiotics, one of which may be atrazine, although a role of ligninolytic enzymes in atrazine degradation has not been demonstrated. In addition, no fungi have been shown to mineralize the triazine ring; they only modify atrazine by N-dealkylation, hydroxylation, and/or de-hydrogenation of the parent structure. N-dealkylated atrazine metabolites, more specifically deethyl atrazine, deisopropyl atrazine, and deethyl deisopropyl atrazine are the most commonly produced compound by white rot and imperfect fungi (Kaufman and Blake 1970; Sene et al 2010). De-chlorinated metabolites such as hydroxyatrazine and its de-alkylated analogs are also produced in imperfect fungi and white rot fungi (Singh et al. 2008; Masaphy et al. 1996).

Because deuteromycete fungi do not produce the LiPs and MnPs found in white rot fungi, they must employ some other mechanism to degrade atrazine. Cellular detoxification enzymes, such as fungal cytochrome P450s, may also play a role in the degradation of atrazine by fungi (Masaphy et al. 1996; Mougin et al. 1997). The utilization of these widely distributed enzymes in atrazine degradation would help explain why such a diverse group of fungi have the ability to degrade atrazine.

The de-alkylated metabolites of atrazine have a reduced but significant phytotoxicity which are thought to contribute to the persistence of herbicidal effects of atrazine in agricultural fields (Fedke 1982). Few studies on the toxicity of atrazine

metabolites in animal systems have been completed, but existing work shows that they are generally less toxic than atrazine. Aquatic invertebrates and algae chronically exposed to atrazine showed lower survival rates than those exposed to its de-alkylated metabolites (Ralston-Hooper et al. 2009). In addition, hormonal secretion from mouse pituitary glands was altered in mice that were fed atrazine, while mice that were fed the de-isopropyl-de-ethyl atrazine metabolite did not show altered hormonal secretions (Fraites et al. 2009).

The ability to degrade atrazine and other xenobiotics into less toxic or non-toxic metabolites makes fungi good candidates for use in mycoremediation, specifically the use of fungi in bioremediation. Fungi may be used in the mycoremediation of atrazine in heavily contaminated areas or as atrazine degrading biofilters in soils frequently exposed to atrazine spills. In order to test the hypothesis of mycoremediation of atrazine by fungi, fungi with the ability to modify atrazine and reduce its toxicity must be identified. This can be accomplished by screening fungi for the ability to degrade atrazine and subsequently characterize degradation products. Previous research that has identified atrazine degrading fungi can be used to target fungi with similar capabilities. Because wood decaying white rot fungi and soil inhabiting deuteromycetes are known to degrade atrazine, fungi with similar ecological roles and metabolic properties may also be able to degrade atrazine. Targeted screening of fungi and characterization of atrazine degradation products will provide the information needed to identify fungal species that are well suited for use in the mycoremediation of atrazine.

In this study, a diverse group of soil inhabiting deuteromycete and white rot wood decay fungi were surveyed to identify atrazine detoxifying species that could be used in

mycoremediation of atrazine. The degree of atrazine removal was measured, the effects of atrazine on fungal growth were assessed, and atrazine metabolites produced were identified for each species. Fungi tested were expected to produce N-dealkylated atrazine metabolites, much like metabolically and ecologically similar fungi in which atrazine has been previously characterized. This work will help identify species of fungi that are best suited for use in the mycoremediation of atrazine and have the proper metabolic capabilities to detoxify contaminated substrates.

Methods

Isolation of Fungi

Basidiomata of white rot wood decay fungi were collected and identified to species. Voucher specimens were dried and deposited in the cryptogamic herbarium at Eastern Illinois University. Wedges of fresh pileus tissue containing lamellae were suspended over malt extract agar (MEA) until basidiospores were deposited on the agar surface. Germinated basidiospores were transferred to potato dextrose agar (PDA) until axenic fungal cultures were isolated. Fungal cultures were maintained on PDA for use in bioassays.

Culture Conditions

Fungi were grown in synthetic liquid media modified from Kirk et al. (1978) supplemented with 1% glucose as a carbon source. The media contained per liter of autoclaved water: 0.2 g KH_2PO_4, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.0132 g $CaCl_2 \cdot 2H_2O$, 0.096 g NH_4NO_3, 1ml of mineral salts solution, and 0.5 ml of vitamin solution. The mineral salts solution contained per 50 ml of autoclaved water: 0.15 g $MgSO_4 \cdot 7H_2O$, 0.075 g

nitrilotriacetate, 0.025 g $MnSO_4 \cdot H_2O$, 0.05 g NaCl, 0.005 g $FeSO_4 \cdot 7H_2O$, 0.0077 g $CoCl_2 \cdot 2H_2O$, 0.0055 g $CaCl_2 \cdot 2H_2O$, 0.0089 g $ZnSO_4 \cdot 7H_2O$, 0.0026 g $CuSO_4$, 0.0009 g $AlK(SO_4)_2 \cdot 12H_2O$, 0.0005 g H_3BO_3, and $NaMoO_4 \cdot 2H_2O$. The vitamin solution was made with de-ionized water filter sterilized after it was made and stored at -20 °C. The vitamin solution contained per 200 ml: 0.0004 g biotin, 0.001 g thiamine HCl, 0.0004 g folic acid, 0.001 g riboflavin, 0.001 g nicotinic acid, 0.001 g calcium panothenate, 0.001 g *para*-amino benzoic acid, and 0.002 g pyroxidine. Some of the vitamins included in the media described in Kirk et al. (1978) were not included because they were not available due to lack of funding.

100 ml cultures were made with and without atrazine supplemented with 1% glucose (1 g per culture). Atrazine containing cultures were made using a 30 ppm stock solution of atrazine and atrazine-free cultures were made using de-ionized water. A total of 95 ml of water or atrazine solution per culture was autoclaved. After cooling, 5 ml of the 20x mineral salt solution described above was added to each culture to bring each to a final volume of 100 ml. The final concentration of atrazine in cultures was 28.5 ppm. Cultures were inoculated with agar plugs 4 mm in diameter covered in mycelium taken from the actively growing margin of fungal cultures. Uninoculated atrazine-containing controls were made and incubated alongside each group of fungal cultures. All cultures were replicated four times. Cultures were incubated for 21 days at 25 °C without shaking in regular cycles of 12 h of light and dark.

Biomass Production and Atrazine Removal

After 21 days of incubation fungal cultures were filtered using a pre-weighed Whatman No. 1 paper filter to remove biomass. The biomass was placed pre-weighed, dried glass jars and dried for two days at 60 °C. The final dry biomass was measured after drying.

The extracellular media was analyzed using high pressure liquid chromatography (HPLC), and a Hitachi HPLC. Each run was 15 minutes long with a flow rate of 1ml/minute. The solvent system used was 50:50 water:acetonitrile and 10 µl of sample was injected per run. Solutes were detected at 222 nm. The integration of peaks corresponding to atrazine was used to determine the amount of atrazine removed from solution relative to un-inoculated controls. Any media that was not immediately analyzed was stored at -20 °C.

Statistical Analysis

Fungi tested were grouped in four different groups each with its own control culture. SAS was used to analyze the data using a nested ANOVA to determine if atrazine removal was significant within each control group. Each group of fungi with a separate control was analyzed as a batch. A contrast statement was performed on the batches to determine if they were different from one another. Because the effect of batch was not significant, the controls were pooled from each of the groups and compared to experimental cultures to determine if atrazine removal by each species was significant. The univariate procedure was run to identify any outliers in the data set.

An ANCOVA was performed using dry fungal biomass as a covariate was used to measure the effect of mass production on overall atrazine removal. The effect of species on atrazine removal was tested using ANOVA and a contrast statement was run between each species and the control group.

Identification of Atrazine Metabolites

The culture medium from atrazine cultures (~400 ml) was split into 200 ml fractions and each was extracted separately with 50 ml followed by 30 ml of dichloromethane. The organic fractions for each culture were pooled and evaporated using a rotovapor. The solid was dissolved in a minimal portion of dichloromethane and diluted to 100-200 ppm for analysis with GC-MS.

Results

Total fungal biomass for atrazine-containing and atrazine-free cultures varied for each species at the end of 21 days (Table 1). An ANOVA detected no overall effect of atrazine on total dry biomass production (Table 2). Different species produced different amounts of biomass in culture ($p < 0.0001$). Atrazine affected the growth different fungal species to varying degrees ($p = 0.0009$). However, atrazine did not have a significant effect on the production of fungal biomass.

Analysis of the culture medium with HPLC did not identify any atrazine metabolites (Figure 1). Atrazine-containing cultures showed several small peaks eluting before atrazine in all of the species tested. However, un-inoculated controls and atrazine-free controls also contained several peaks that eluted earlier than atrazine's retention time (around 8.5 min) and no unique peaks could be identified. *Aspergillus flavus* cultures

displayed several large peaks of eluting before atrazine, but atrazine-free cultures showed the same peaks, indicating that these were normal fungal metabolites and not atrazine metabolites (Figure 1, D1 and D2). HPLC analysis was not able to identify any difference between atrazine-containing and atrazine-free controls other than differences in the concentration of atrazine.

Two outliers were identified, one in *Aspergillus niger* Tiegh. treatments and one in *Armillaria gallica* Marxm. and Romagn. treatments. Only two species removed atrazine from solution, *Aspergillus niger* ($p=0.0337$) and *Armillaria gallica* ($p=0.0486$). Another analysis was run without the outliers and the p values changed to 0.0494 for *Aspergillus niger* and 0.0710 for *Armilliaria gallica* (Table 2). The separation of the variation due to mass in the ANCOVA increased the p values for *Aspergillus niger* and *Armillaria gallica* made their responses similar to other species tested (Table 2).

Organic extracts of the extracellular medium of atrazine-containing cultures were analyzed with GC-MS. No chlorinated atrazine metabolites were identified in any of the gas chromatograms and atrazine was the only chlorinated compound found in the mass spectra. Metabolites of unknown identity were detected in *Mycena leaiana* (Berk.)Sacc.(3), *Aspergillus flavus* Link.(11), and *Aspergillus niger* (1) cultures (Figure 2. A, B, and C). Only two metabolites in *Mycena leaiana* cultures, biphenyl and benzene propanal, were identified and these had no apparent structural relationship to atrazine. Analysis with GC-MS did not support the production of chlorinated atrazine metabolites by any of the fungi tested and it was not clear if the unidentified metabolites detected were de-chlorinated metabolites from atrazine metabolism.

Discussion

Atrazine had no effect on fungal growth under the culture conditions tested. The methods utilized tested fungal growth in cultures containing 28.5 ppm atrazine, four orders of magnitude greater than concentrations that have been shown to produce an endocrine disrupting effect in amphibians and the highest level of atrazine that is legally allowed in drinking water in the United States (Hayes et al. 2006). It is not clear if atrazine had any effects at the cellular level in these fungi because this study only measured the total dry biomass produced (Table 1). The levels of atrazine tested in this study may have been too high to have an effect on fungal growth. If atrazine only affects fungal growth at lower concentrations, (Hayes et al. 2006) then any negative impacts of atrazine might not be seen with this experimental design. Therefore, the results of this study cannot be used to make predictions about the effect of atrazine on fungi at field concentrations.

The different fungal species produced different amounts of dry biomass in the same time period. This is not unexpected considering the diversity of organisms tested. For example, the deuteromycetes tested grew much faster than the white rot basidiomycetes (Table 1).

Removal of atrazine was observed relative to controls in fungal cultures of *Armillaria gallica* and *Aspergillus niger*, two of the twelve fungi tested. *Armillaria gallica* is a white rot wood-degrading basidiomycete and *Aspergillus niger* is a soil dwelling, saprobic deuteromycete that is commonly isolated from air and soil samples. Similar kinds of fungi have been shown to degrade atrazine previously (Singh et al. 2008;

Masaphy et al. 1993; Bending et al. 2002). After analyzing the data set without the outliers, p values generated from contrasts between each species and its control did not change drastically. The p value for *Armillaria gallica* increased from 0.0486 to 0.0710 (Table 2), not significant at 95% confidence interval, but low enough to say that *Armillaria gallica* did have an effect on the level of atrazine in solution. For *Aspergillus niger* p increased from 0.0337 to 0.0494, still significant with a 95% confidence interval (Table 2).Either way both species were able to remove atrazine from solution.

Separation of the variation due to mass from the analysis made the previously significantly different levels of atrazine removal non-significant, indicating. The amount of mass produced by the fungus was important in determining how much atrazine was removed from cultures. This suggests that the fungi may be bioaccumulating atrazine in their hyphae rather than degrading it in this experiment. Benoit et al. (1998) showed that atrazine was adsorbed onto the walls of the fungal hyphae of two species of fungi isolated from straw compost grown in potato dextrose broth. The concentration of atrazine in solution dropped quickly in the first few days of incubation. Biosorbtion was responsible for the removal of all of the atrazine that was removed from solution and no degradation took place. Biosorbtion might have been responsible for the removal of atrazine from fungal cultures in this experiment because mass production had an effect on the removal of atrazine. If biosorbtion was the route of atrazine removal, a greater biomass production would have created a greater surface area on the fungal hyphae for atrazine to adsorb and allowed cultures with more biomass to remove more atrazine.

There was no correlation however between atrazine removal and biomass production. For example, *Trichoderma* spp. did not significantly remove atrazine from

culture, but it produced a greater amount of dry fungal biomass than *Aspergillus niger* or *Armillaria gallica*, the only two species that significantly removed atrazine from culture (Table 1).

If atrazine was removed via biosorption to fungal hyphae, and the total mass of hyphae produced was the only factor involved in determining the amount of atrazine removed, then species that produced a greater mass of hyphae should remove the most atrazine. This is not the case and thus other factors may play a role. For example, different species of fungi could secrete variable polysaccharide coatings which may vary in their ability to bind atrazine. Alternatively, the difference in atrazine removal from culture may have been a randomly generated difference among the different species tested and is not dependent on any of the species' characteristics.

Although HPLC analysis of the culture medium showed the production of metabolites that eluted earlier and later than atrazine, without direct examination using liquid chromatography-mass spectrometry (LCMS) there was no way to determine their identity. Peaks with similar retention times were present in atrazine-free controls and uninoculated controls, although it is not possible to determine if they represented the same compounds. The detection method used in this experiment was designed to detect the triazine ring in atrazine and metabolites visible in HPLC chromatograms (Figure 1). The absence of clearly unique signals in atrazine cultures suggests that no atrazine metabolites were produced. Without a method to directly detect each compound eluting from the column, an identity cannot be assigned to the compounds detected in HPLC chromatograms and they might be normal fungal metabolites.

GC-MS analysis of organic extracts of fungal cultures also showed that no chlorinated atrazine metabolites were present in the fungal cultures. Atrazine was the only chlorinated compound detected (Figure 3. E). Chlorinated compounds display a unique M+2 peak two units heavier and about one-third the intensity of the molecular ion peak for that compound. No patterns indicative of chlorination were detected in mass spectra indicating that no chlorinated metabolites were produced. Two of the metabolites produced in *Mycena leaiana* were identified, one was biphenyl, an aromatic compound consisting of two phenyl rings separated by a covalent bond, and the other was identified as benzene propanal, a compound consisting of benzene with a single propanal functional group. These metabolites are structurally unrelated to atrazine metabolism and are probably a normal fungal metabolite. The other metabolite could not be identified beyond the fact that it did not contain chlorine.

Cultures of *Mycena leaiana* turned a dull orange color after the incubation period indicating the presence of some kind of pigmented compound in the media. This orange pigment was transferred to organic extracts and analyzed with GC-MS. Benzene propanal is a fragrant, clear to slightly yellow liquid at room temperature and may have imparted some of the pigmentation in *Mycena leaiana* cultures. Another unknown compound detected in gas chromatograms of *Mycena leaiana* culture extracts may have also been involved in the pigmentation of the culture medium. The unknown compound was in low abundance relative to atrazine. Because atrazine was not removed from *Mycena leaiana* cultures, any atrazine metabolite produced would have to be produced in very small quantities. The unknown compound may have been a de-chlorinated atrazine metabolite

produced in trace amounts that did not lead to statistically significant removal of atrazine in *Mycena leaiana* cultures.

Extracts of *Aspergillus flavus* cultures contained eleven unknown metabolites (Figure 2. A). Five of these showed a high abundance relative to atrazine and were likely not atrazine degradation products since atrazine was not removed from these cultures and production of atrazine metabolites would have reduced the amount of atrazine in solution. The other six metabolites in lower abundance may have been de-chlorinated metabolites such as hydroxyatrazine, but this cannot be confirmed because molecular standards for these compounds were not available. None of the compounds found in *Aspergillus flavus* cultures had a molecular weight that was identical to any of the possible hydroxylated/de-alkylated atrazine metabolites that may be produced in fungal atrazine metabolism. HPLC analysis of *Aspergillus flavus* cultures show the presence of several high abundance compounds eluting before atrazine in atrazine-containing and atrazine-free controls (Figure 1. D1 and D2). These compounds must be normal fungal metabolites not related to atrazine because they are produced in atrazine-free control cultures as well. They likely account for some, if not all, of the metabolites seen in GC-MS.

Cultures of *Aspergillus niger* contained one unidentified metabolite in high abundance relative to atrazine (Figure 2.B). Atrazine was removed from *Aspergillus niger* cultures so this unknown metabolite may represent some de-chlorinated degradation product. On the other hand it might not associated with atrazine. The molecular ion peak for this metabolite was not clearly identifiable. It could have been at m/z= 196, 178, or 168. A possible de-chlorinated atrazine metabolite, deethyl hydroxyatrazine, has a molecular weight of 169, close to one of the possible molecular

ion peaks for this compound. Without molecular standards, the identity of this metabolite cannot be confirmed. GC-MS analysis of *Armillaria gallica* cultures showed no metabolites. In this case, the removal of atrazine seen in *Armillaria gallica* cultures could be attributed to the previous biosorbtion explanation; none of the atrazine was degraded and instead it was adsorbed onto the fungal hyphae.

Curiously, atrazine removal or the production of atrazine metabolites was not detected in *Trametes versicolor* cultures even though previous studies have shown its ability to degrade atrazine (Bastos and Magan 2009). Protein synthesis is a tightly regulated process influenced by many exogenous and endogenous signals. Both endogenous and exogenous signals allow cells to regulate protein expression in order to prevent futile production of proteins. Production of proteins that may be involved in the degradation of atrazine, such as LiPs and MnPs, are tightly regulated in white rot fungi by nutrient availability. The culture conditions tested in this study may not have promoted the production of atrazine degrading enzymes in the fungi tested. This could be why no degradation of atrazine by *Trametes versicolor* was observed and why other fungi tested may be able to degrade atrazine under the proper conditions.

Lignin metabolism in white rot fungi is mediated by LiPs and MnPs and lignin metabolism in *Phanerochaete chrysosporium* is regulated by a number of factors the most important of which is nitrogen limitation. Lignin degradation was increased in "low" concentration (2.4 mM) nitrogen cultures of *P. chrysosporium* versus "high" concentration (24 mM) nitrogen cultures (Kirk et al. 1978). Thiamine was the only vitamin that was necessary for lignin metabolism in *P. chrysosporium* and it was included in the salts medium utilized in this study. Fungal cultures in this experiment used the

lower concentration of nitrogen, 2.4 mM nitrogen (NH_4NO_3), found by Kirk et al. (1993) to maximize LiP and MnP production. Despite the efforts to stimulate LiP and MnP production, no atrazine metabolism was detected. It is possible that a further reduction in nitrogen concentration would have aided atrazine metabolism by stimulating further production of lignin degrading enzymes.

Nitrogen limitation is an important factor in determining the rates of atrazine biodegradation in the environment. Soil microbial communities respond to nitrogen limitation with an increased level of atrazine biodegradation. Sims et al. (2006) found that soils that had been farmed without nitrogen fertilization for 130 years harbored microbial communities that were able to mineralize a higher percentage of atrazine than microbial communities from soil that was regularly treated with nitrogen supplements. Soil fungi contributing to the degradation of atrazine in these microbial communities must have been conditioned by nitrogen limitation to more efficiently degrade atrazine.

Mn concentration was also shown to affect the transformation of atrazine by *Pleurotus pulmonarius*, another white rot fungus. The production of N-dealkylated atrazine metabolites was maximized in cultures containing 300 μM of Mn, showing a 265% increase in the production of N-dealkylated metabolites relative to the un-supplemented control containing 0.8 μM Mn (Masaphy et al. 1996). The media used in this study contained 3.31μM Mn, two orders of magnitude lower than the concentration that yielded the maximum production of N-dealkylated atrazine metabolites by *P.pulmonarius*. This may have contributed to the failure to reduce atrazine concentrations in fungal cultures.

The stimulation of lignin degrading enzymes is important for the decomposition of many xenobiotics. However, the same enzymes may not be responsible for the degradation of atrazine by fungi. The degradation of atrazine by soil deuteromycetes cannot involve the action of LiPs and MnPs because they are only produced by white rot wood degraders. Several deuteromycetes have been shown to degrade atrazine into N-dealkylated and hydroxylated metabolites which suggests that different pathways are used in these fungi to degrade atrazine (Singh et al. 2008; Kaufman and Blake 1970). Cytochrome p450s (p450s) are detoxification enzymes that catalyze the oxidation of a wide variety of substrates that are conserved in all classifications of living organisms (Van den Brink et al. 1998). Fungal p450s help deuteromycetes adapt to and degrade different substrates encountered in their environment. Some evidence suggests that fungi such as *Pleurotus pulmonarius* and *Phanerochaete chrysosporium* may metabolize atrazine using p450s (Mougin et al. 1997; Masaphy et al. 1996).

The use of p450s in atrazine degradation would explain why techniques used to increase lignin degradation, such as nitrogen reduction, did not result in the degradation of atrazine. The expression of p450s in *Phanerochaete chrysosporium* is not drastically affected by nitrogen levels or the type of carbon source that is given. The primary inductive agent of p450s appears to be xenobiotic substrates of the enzyme (Doddapenei et al. 2005). Therefore, nutrient limitation would not affect the amount of atrazine removed from solution if p450s catalyze its degradation. This would also explain why deuteromycetes are able to degrade atrazine without the production of lignin degrading enzymes.

Atrazine degradation was not observed in most of the fungal cultures utilized in this study including *Trametes versicolor*, a species that had previously been shown to degrade atrazine. The detection methods used in this study might have been inadequate to identify low concentration atrazine metabolites. While HPLC allows for the detection atrazine in the part per billion range and low abundance metabolites would have been detectable, there was no way to identify peaks separated from atrazine directly. Direct identification of metabolite peaks using LCMS would have assigned identities to each of the peaks seen in HPLC chromatograms. GC-MS allowed for the identification compounds from organic extracts of fungal cultures and no chlorinated atrazine metabolites were observed, but the identity of other metabolites detected in GC-MS could not be confirmed. As such, it is unlikely that the compounds were below detection limits.

Analyses indicate that total biomass production was important in determining atrazine removal. If p450s catalyze the degradation of atrazine, then nitrogen limitation may have actually worked against the degradation of atrazine by limiting fungal growth and decreasing the amount of detoxifying biomass in culture. It is possible that the incubation period might have been too short to produce enough fungal growth to remove atrazine from solution using the culture conditions tested and the fungal strains isolated. Although none of the twelve species tested in this study were proven to degrade atrazine, further testing using different culture conditions may yield different results and identify fungal species with the ability to degrade atrazine.

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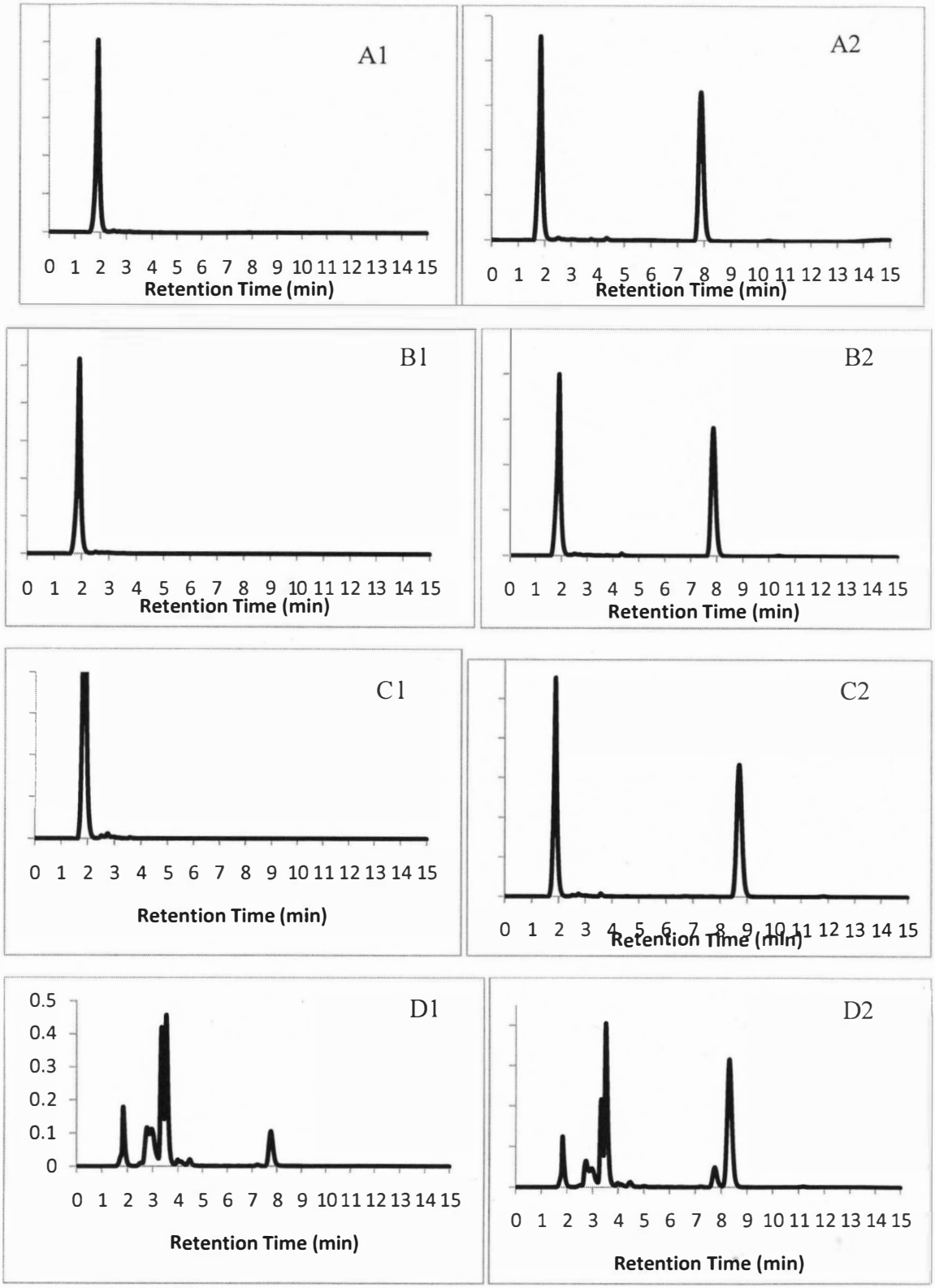
Tables and Figures



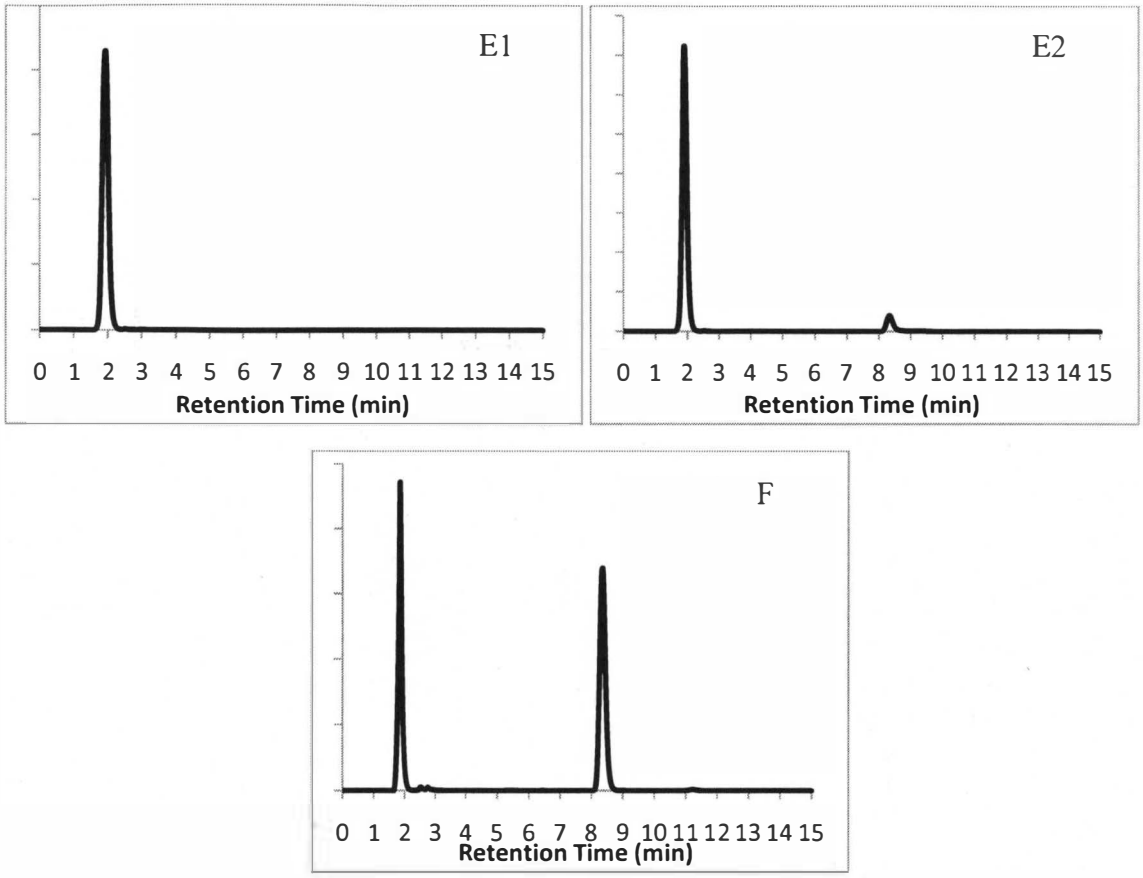


Figure 1. HPLC chromatograms from culture media of fungal cultures. *Armillaria gallica* atrazine-free (A1) and atrazine-containing (A2)*Trametes versicolor* atrazine-free (B1) and atrazine-containing (B2) *Mycena leaiana* atrazine-free (C1) and atrazine-containing (C2) *Aspergillus flavus* atrazine-free (D1) and atrazine-containing (D2) *Aspergillus niger* atrazine-free (E1) and atrazine-containing cultures (E2) and an atrazine-containing, uninoculated control (F).

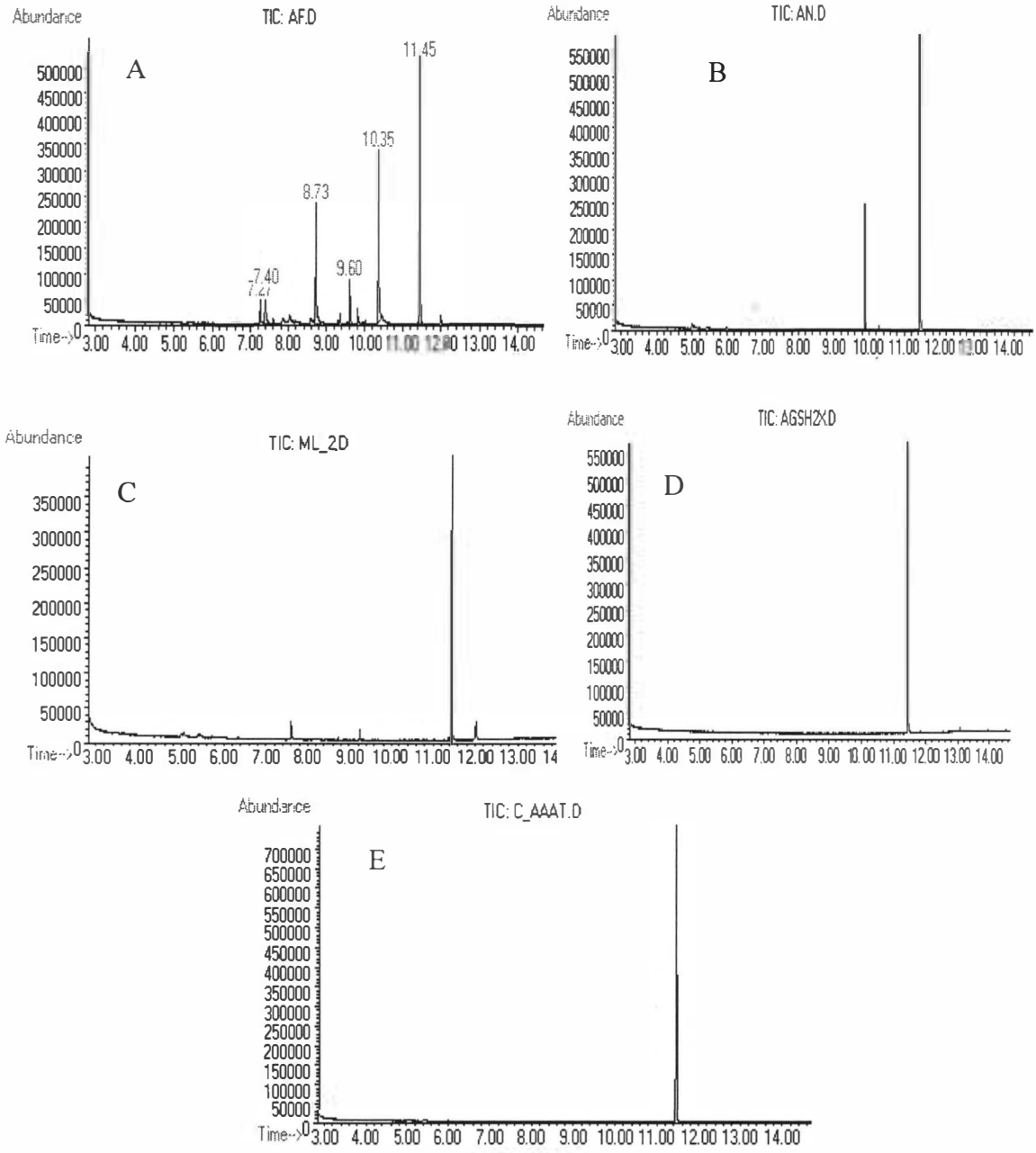


Figure 2. GC chromatograms for dichloromethane extracts. *Aspergillus flavus* (A) *Aspergillus niger* (B) *Mycena leaiana* (C) *Armillaria gallica* (D) and atrazine-containing un-inoculated control cultures (E).

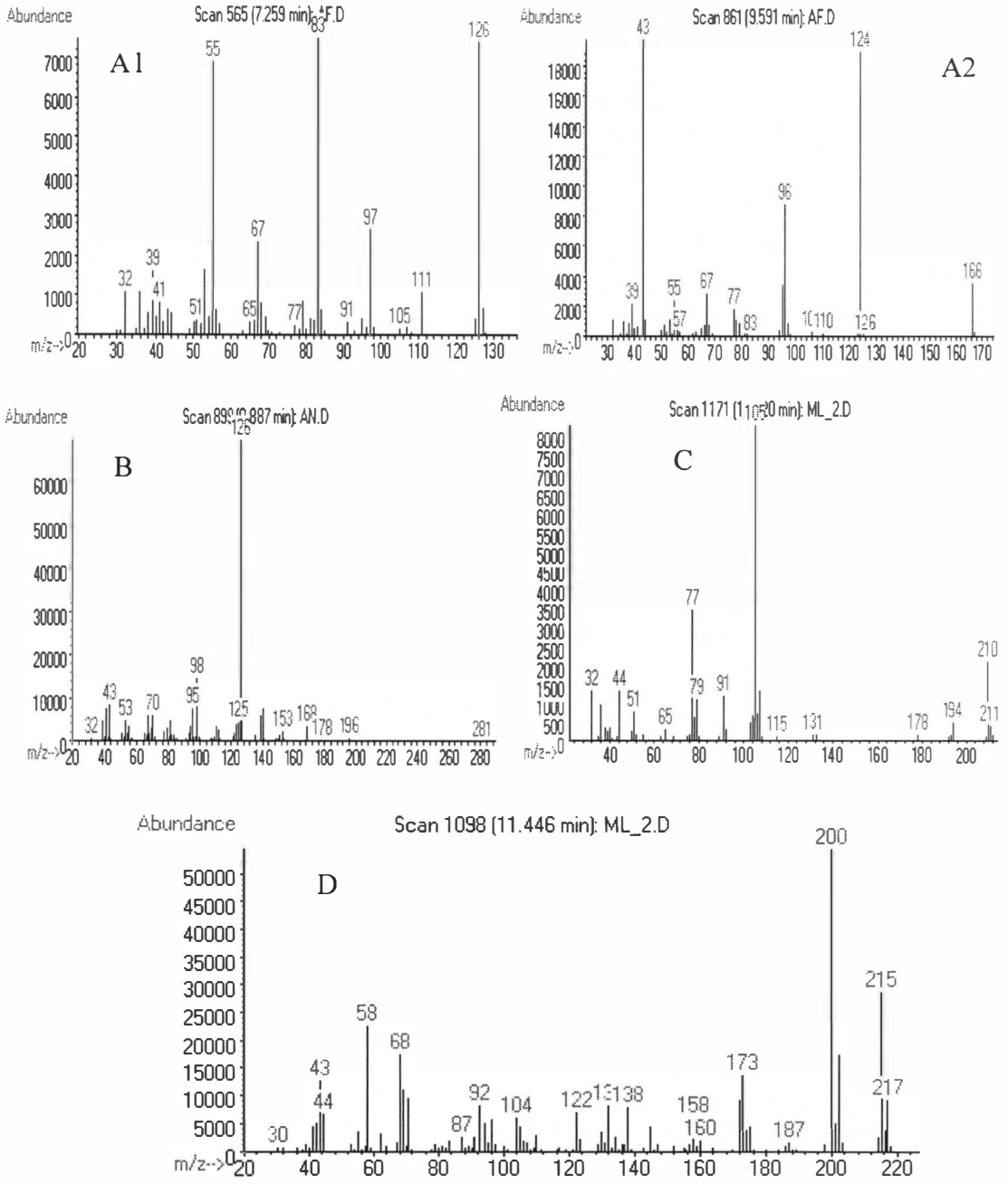


Figure 3. Mass spectra from compounds found in dichloromethane extracts. *Aspergillus flavus* (A1 and A2) *Aspergillus niger* (B) and *Mycena leaiana* (C) with a mass spectrum of atrazine showing a prominent M+2 peak at m/z=217 and, two mass units higher than the molecular ion peak at m/z=215 (D).

Table 1. Average dry fungal biomass produced in liquid culture after 21 days of incubation.

Species	Atrazine	Average Mass (g)
Armillaria gallica	Y	0.0447
Armillaria gallica	N	0.0368
Megacollybia rodmani	Y	0.0167
Megacollybia rodmani	N	0.0181
Trametes versicolor	Y	0.0576
Trametes versicolor	N	0.0221
Cyathus striatus	Y	0.0111
Cyathus striatus	N	0.0128
Mycena leaiana	Y	0.0145
Mycena leaiana	N	0.0332
Xeromphalina kauffmanii	Y	0.0299
Xeromphalina kauffmanii	N	0.0328
Auricularia auricular	Y	0.0043
Auricularia auricular	N	0.0055
Crucibulum laeva	Y	0.0201
Crucibulum laeva	N	0.0247
Alternaria alternate	Y	0.1050
Alternaria alternate	N	0.1250
Aspergillus flavus	Y	0.0835
Aspergillus flavus	N	0.0807
Aspergillus niger	Y	0.1054
Aspergillus niger	N	0.1097
Trichoderma sp.	Y	0.1212
Trichoderma sp.	N	0.1431

Table 2. P values from ANOVA and ANCOVA analyses. Contrasts between control group and different species determined if atrazine removal was significant.

	ANOVA	No Outliers	ANCOVA
Control v *Aspergillus flavus*	0.5988	0.4866	0.9432
Control v *Armillaria gallica*	0.0486	0.071	0.1814
Control v *Aspergillus niger*	0.0337	0.0494	0.5366
Control v *Alternaria alternate*	0.6905	0.5978	0.8536
Control v *Auricularia auricula*	0.8992	0.8664	0.8535
Control v *Crucibulum laeva*	0.7668	0.694	0.8706
Control v *Cyathus striatus*	0.9896	0.9862	0.9096
Control v *Mycena leaiana*	0.8164	0.7579	0.7058
Control v *Megacollybia rodmani*	0.3902	0.2592	0.4371
Control v *Trichoderma* sp.	0.7919	0.7262	0.7951
Control v *Trametes versicolor*	0.2096	0.1036	0.5804
Control v *Xeromphalina kauffmanii*	0.7202	0.6348	0.9118