EVALUATION OF THE POTENTIAL ENVIRONMENTAL TOXIC EFFECTS OF A NYLON FIBERS ADDITIVE

by

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(ABSTRACT)

New chemical substances being considered for use today are required by law to be evaluated for potential toxic effects upon disposal to the environment. A thorough evaluation, however, is complex, time-consuming, expensive, and impossible to perform on each new substance. In this study the potential toxic effects of a new carpet additive with antimicrobial properties and the associated process waste stream from a textile facility were considered. The wastewater from the rest of the plant was currently being treated in a land application disposal system. An assessment of the toxicity of the antimicrobial additive was made using conventional greenhouse studies. This assessment was compared to the results obtained from three short-term toxicity tests performed on the same set of solutions. The short-term tests used were a corn seedling bioassay, adenosine triphosphate measurements and bacterial bioluminescence. These short-term tests were evaluated as to their utility as screening tools and as monitoring devices for toxic substances.

The greenhouse studies indicated that at the anticipated level of the antimicrobial additive in the wastewater no adverse effects would be noted in the land application system. The corn seedling bioassay root test was reliable in predicting the greenhouse responses and was considered to be a good candidate for a screening tool. However, the procedure was too time consuming to be considered as a monitoring tool in most situations. The adenosine triphosphate measurements were difficult to reproduce, but average values compared well to the greenhouse results. An appropriate number of replicates might increase the reliability of the method and its usefulness as a screening tool, but, of course, would decrease the utility of the test as a monitoring tool. The bacterial bioluminescence test was rapid to perform and provided reproducible results; however, the test was very sensitive and yielded what appeared to be overly conservative estimates of toxicity.

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I. INTRODUCTION

The Toxic Substances Control Act specifically states in Section 2 that

> "...adequate data should be developed with respect to the effect of chemical substances and mixtures on health and the environment..."

In accordance with the Act, then, adequate testing of a chemical's effect upon disposal to the intended receiving system is imperative. Tests that most accurately reflect or mimic the true conditions of the receiving system are generally the most accurate in predicting any effects. This type of testing, however, is complicated, time consuming and expensive. In this project, for example, where the receiving system was soil with a crop cover, a significant time lag occurred between initiating the first greenhouse study, performing additional studies, and analyzing the final results. Costs for personnel would be high over that time period in addition to the cost of supplies. A shorter-term bioassay that correlates well with the more elaborate tests therefore would be useful as a screening tool. The short-term bioassay would help eliminate false starts by providing data that would allow the more expensive test to be better designed. In this way more information would be gained per dollar invested.

Short-term bioassays that correlate well with the more elaborate, accurate tests and that can be performed with a minimum amount of laboratory space, equipment, and expertise would also be useful for monitoring potentially toxic wastewaters for changes in toxicity. A short-term bioassay would be especially useful when a wastewater is

composed of a matrix of compounds. The effect of the matrix of compounds and any associated antagonistic or synergistic reactions might be monitored rather than simply measuring the level of individual components.

In this project, which was performed for the Allied Corporation, both the disposal of a potentially toxic effluent and monitoring of the effluent for changes in toxicity were considered. Effluent samples were collected at Allied's textile plant in Chesterfield County, Virginia, where the bulk of the industrial wastewater generated was being land applied. The overall wastewater disposal system consisted of three holding ponds, spray irrigation equipment, and about 34 acres of spray irrigation fields with fescue grass. At the time of this project a new process had been recently introduced in the production of carpet fiber at the Allied plant. An anti-microbial agent was being added to the finish applied to the nylon fibers to retard bacterial growth and aid in controlling carpet odors. Due to confidentiality agreements with Allied Corporation the antimicrobial agent cannot herein be described in detail and will henceforth be referred to as the AM agent.

The Allied Corporation's goal was to be able to mix the wastewater from the new AM process with the main industrial waste stream and spray the combined effluent on the existing land application system. There was a question, however, as to whether it would be safe to land-dispose the wastewater containing the AM component. In addition, there was concern about the possible fluctuation of AM additive levels in the wastewater due to increased carpet production or accidental spills which

might result in damage to the land application system before the fluctuation could be detected.

Given these concerns, the objectives of this project were to:

1. Investigate the potential environmental toxic effects to a soil environment of the AM agent alone, in combination with other compounds, and in the matrix of Allied's wastewater;
2. Define an appropriate land application rate for the AM wastewater on fescue (Festucia arudinaceae) grass using conventional greenhouse studies and secondly, investigate the effect to the plants of a rinse cycle after wastewater application;
3. Determine if any correlation existed between the results of a traditional greenhouse study and the data generated by means of three, short-term, toxicity-assessment techniques: corn-seedling bioassay, bacterial bioluminescence, and adenosine triphosphate (ATP) measurements; and,
4. Evaluate the effectiveness of the short-term assessment techniques as screening tools and as monitoring techniques.

II. LITERATURE REVIEW

Land Application of Wastewaters

Historical Aspects of Land Application Systems

Land application systems for the disposal of wastewater have successfully been used for centuries. The first land application system designed specifically for wastewater disposal was begun in 1559 in Prussia and operated more than 300 years (78). Land disposal was the preferred method of disposal until early in the twentieth century when advances were made in wastewater treatment including Imhoff tanks, trickling filters, and activated sludge. In 1908, the Great Britain Second Royal Commission on Sewage Disposal conceded to technology and stated "we are satisfied that it is practicable to purify the sewage of towns to any degree required by either land treatment or by artificial means" (31).

Today, although the majority of wastewater is treated by conventional processing, there are a substantial number of systems that still land apply wastes. A survey of the United States in 1965 listed 947 sanitary facilities and 1300 industries that land applied wastes (74). Neither figure includes the over 50 million people that are served by septic tank systems. The sum of these figures was still a small percentage of the total number of wastewater facilities.

The passage of PL 92-500 in 1972 restored interest in land application systems. The Clean Water Act which sought "to restore and maintain the chemical, physical, and biological integrity of the

Nation's waters" (Section 101), encouraged the use of wastewater treatment processes that provided for "the recycling of potential sewage pollutants through the production of agriculture, silviculture, or aquaculture products, or any combination thereof." It also withheld funding of any project unless alternative waste treatment techniques had been studied, evaluated, and the best practicable technology utilized. Land application was identified as one of the alternative treatment methods.

Under the 1972 Act land bought for land application systems was eligible for federal grant funds (57). Later amendments in 1977 allowed for up to 85% funding for construction of alternative waste treatment systems (57). Three hundred and fifty new land application projects were funded as a direct result of the 1972 Act. As of 1982, it had been approximated that over 1000 new systems had been scheduled to begin in the near future (57).

Land application remains a controversial topic, however. To its proponents, land application is an excellent alternative to conventional in-plant treatment and has a special appeal in water short areas, in areas where a suitable receiving stream is unavailable and in areas with adequate land supplies. Given that the soil geology, hydrology, and climate are favorable, land disposal has the potential to provide for the equivalent of aerobic tertiary treatment and for at least a 90% reduction in oxidizable carbon (36).

Nitrogen, phosphorus, and other nutrients in the wastewater are utilized by the crops when present. Constituents of the wastewater not

adsorbed by the crops are acted upon by biological, chemical, and physical processes as the water moves through the soil column. Ultimately an effluent of better quality than the waste originally applied is produced and with less pretreatment than needed for disposal to water or air (14). The effluent also recharges groundwater supplies instead of sending needed water supplies downstream (67).

Use of the soil-plant receiver system rather than water or air also immobilizes polluting constituents within the application area, reducing the possibility of widespread contamination (37). The exception to this is if groundwater supplies become contaminated.

The opponents to land application point out the need for large expanses of suitable land (14). In an urban area it would be impractical, expensive, if not impossible to obtain the needed acreage. Odor and dike leakage associated with holding lagoons have also been cited as negative aspects (74).

The possibility of groundwater contamination is a very valid concern in land application systems. This is especially critical where aquifer materials are fractured or are covered only by a thin layer of soil (14). Soluble contaminants such as nitrate can easily be leached to the groundwater.

If managed properly a land application system should last at least as long as its concrete counterpart. However, improper waste loadings and poor management can severely damage the soil-plant receiver system. Unlike water or air, once a soil system is overloaded it has little natural powers of recovery (36).

Land application, like any other waste treatment process, is suitable for some situations and not for others. Proper site selection, waste loading, and management is necessary to produce a high quality effluent.

Types of Land Application Systems

There are three basic types of systems used in land application; slow rate irrigation, overland flow, and high rate infiltration. The following descriptions are based on Overman (57).

Slow Rate Irrigation. Hydraulic loadings of 25 to 100 mm/week are typical for this type of system and are applied either at the surface or with sprinkler systems. The main goal of this type of system is usually crop production and percolation of the effluent with nutrient removal through crop uptake.

Overland Flow. This system is useful in areas of low permeability and high runoff potential. Application rates are typically 50-400 mm/week. The wastewater flows over the soil where a crop cover has been planted to stabilize the soil and engage in some nutrient removal. The system is managed to promote biological reduction of carbon and nitrogen. There is still a discharge to surface water occurring and prevailing discharge standards must be met.

High Rate Infiltration. A basin not planted in crops is inundated with wastewater. The water is allowed to percolate through the soil. Hydraulic loadings of 25-250 mm/day are common. Soil clogging is common and a drying period is usually

necessary to restore the infiltration capacity. Two or more basins are usually used for this reason. Groundwater quality criteria must be met since complete percolation takes place.

Soil Processes

There are numerous processes occurring in the soil that influence the effectiveness of land application. They include (67,68):

1. physical retention
2. ion exchange
3. chemical precipitation
4. biological transformation
5. biological adsorption

Together these processes act on the components of the wastewater to neutralize or remove them as the water passes through the soil.

Physical Retention

The filtering action that occurs as wastewater moves through the soil removes virtually all of the suspended solids and most of the organic material (62,68). Microorganisms attack the organic matter, utilizing it as a carbon source. The microbial degradation is encouraged by aerobic conditions, favorable moisture, and a soil pH of 5-9 (49).

About 92-97 percent of bacteria and viruses present in the wastewater are removed within the first centimeter of soil (68). The survival rate for human pathogens in soil is dependent on the organic content, moisture and temperature, of the soil as well as competition from soil microbes. Salmonella typhoid bacillus has survived in soil

for periods of six months to one year, but only for one week in sandy soil (68). Coliforms are reported to survive up to four years in soil.

The movement of water through the soil is governed by the type of flow condition that prevails, either saturated or unsaturated. Under saturated flow all of the soil pores are filled with water while during unsaturated flow conditions soil micropores contain water and the macropores are air filled (16).

The volume of water moving through the soil in both types of flow is dependent on the hydraulic conductivity, or the ease with which water passes through the soil pores, and also a driving force or pressure. The hydraulic conductivity is related to the intrinsic properties of the soil's geometry and chemistry and the liquid's density and viscosity (16). The driving force for each flow type is quite different and aids in defining the flow condition. In saturated flow water is forced through the soil macropores due to positive pressure. Water flow in the micropores is minimal resulting in reduced physical, biological, and chemical treatment of the wastewater in the same soil volume. Unsaturated flow is characterized by a negative pressure (matric potential) resulting in a reduced flow rate through soil micropores. Consequently the volume of water flowing through the soil during unsaturated conditions is much smaller than during saturated flow, but maximum treatment of the wastewater results.

Depending on the wastewater volume and composition for disposal by a land application system, one flow condition or the other may be encouraged and in some cases a combination of the two flow conditions

may be desirable or produce alternating anaerobic and aerobic conditions.

With time, soil being continually used for disposal of wastewater may experience a disintegration of the soil structure with a concurrent increase in biological slimes and deposits of ferrous sulfide which effectively clog the soil pores. This soil clogging is the result of (68):

1. various biochemical reactions,
2. excessive inorganic and organic loading rates,
3. excessive hydraulic loading,
4. the geometry of the soil surface and profile,
5. perpetuation of anaerobic conditions, and/or
6. inadequate soil microbial populations.

Remedial measures involve restoring an aerobic environment by allowing for a drying period after wastewater application. Incorporating materials into the soil to add to the surface area of the soil-water interface would also be beneficial.

Ion Exchange

Cation Exchange. Soil colloids have the ability to hold cations in an exchangeable form according to the following lyotropic series (16).

$$Al > Ca > Mg > K > Na$$

A cation added (i.e. applied) to the soil solution can affect the exchangeable form as the mass action of a cation can force it to replace another on the exchange complex. This ability of soils to 'hold'

cations allows time for the cations to be acted upon by the microbial and plant populations and reduces the possibility of leaching of the cations to the groundwater.

Cation exchange in soil results from two main processes; isomorphic substitution (the permanent charge) in crystalline clay minerals and the disassociation of the H^+ ions at a high pH from both clays and organic matter (pH dependent charge).

Amorphous materials such as humus and some of the aluminosilicates, hydroxyoxides, and silica are extremely small with large surface areas. These exhibit high pH-dependent cation exchange capacities (CEC). Humus has a higher CEC than any of the clay fractions, however, up to 200 meq/100 g (33).

The crystalline clay minerals vary in the size of the particles and the source of the charge. For example. kaolinite which is a large sized (0.2 - 2.0 μm), low surface area, non-expanding clay mineral, exhibits little isomorphic substitution. Most of the CEC for Kaolinite is associated with the disassociation of OH groups at the edges of broken clay crystals. As a result the CEC of Kaolinite is largely pH dependent and relatively low at 3-15 meq/100 g (16). In contrast, montmorillonite, a small (0.01 - 1.0 μm), expanding crystalline clay with a higher surface area than Kaolinite has a much higher CEC of 80-100 meq/100 g. Montmorillonite undergoes a high degree of isomorphic substitution so that it has a permanent charge associated with it.

Soil clays are not as well ordered as the clay minerals and are often smaller in size than the pure mineral. The charge of these particles, however, is not directly related to size as they are often coated with iron and aluminum oxides and organic matter which can greatly affect the clay's physical and chemical properties.

In general, the CEC of a soil varies with the humus content and the amount and type of clay fraction involved. A high CEC is not necessarily an advantage to a land application system depending on the other properties of the soil. A soil with a large montmorillonite fraction would have a high CEC but the expanding and contracting nature of the clay may invoke concerns over water movement through the soil.

Particular attention has been given to systems where Na predominates as the exchangeable ion resulting in potentially severe problems with the soil structure, infiltration rate, and permeability rate. In comparison to calcium, sodium causes a strong shift in the osmotic pressure which draws water between the clay units. The problem is enhanced in soils that contain clays like montmorillonite that tend to swell easily. This swelling restricts water movement through the soil column and restricts plant growth due to the dispersed conditions (70).

The amount of sodium adsorbed by a soil can be expressed as a function of the proportion of sodium or potassium ions to the calcium and magnesium ions (70).

$$\text{Sodium Absorption Ratio (SAR)} = Na^{+}/[(Ca^{++} + Mg^{++})/2]^{1/2}$$

An SAR greater than 15 is generally considered unacceptable, but depending on the clay, up to 20 may be acceptable. Those soils predominated by swelling clays would have serious problems at an SAR of 8 or 10 (33). Treatment of the soil profile with calcium sulfate may be able to leach the excess sodium or potassium and replace it with calcium (15).

Anion Exchange. Anion exchange usually occurs at lower pH's due to the disassociation of an OH^- group or addition of an H^+ to a clay mineral resulting in a net positive charge. Anions such as $H_2PO_4^-$, SO_4^{--}, NO_3^-, and Cl^- are attracted to these sites and are held in an exchangeable form. For some of these ions, such as NO_3^-, anion exchange is the only mechanism available to remove them from the soil water except for microbial and plant uptake or action. Unfortunately, anion exchange is limited in most soils and anions such as NO_3^- may actually be concentrated in the bulk soil solution (negative absorption). The affinity of soils for phosphate greatly reduces the probability of significant NO_3^- absorption particularly in the upper part of the soil profile.

Chemical Precipitation

Phosphorus is one of the more important elements removed from the soil by chemical precipitation/adsorption as an insoluble compounds. At pH's less than 6 phosphorus binds with iron and aluminum to form strengite, $Fe(H_2PO_4)(OH)_2$, and variscite, $Al(H_2PO_4)(OH)_2$, respectively (33). At pH's 6.5 - 7 phosphorus reacts with calcium to form

octocalcium phosphate, $Ca_4H(PO_4)_3$ - 3 H_2O (33). At pH 6 - 7 phosphorus tends to remain in a more soluble form.

Sorption reactions occur over a wide pH range in soils containing amorphous clays, such as allophane or hydrous oxide clays of iron and aluminum, resulting in hydroxyphosphate compounds. In moderately acid conditions fixation of phosphates also occurs with silicate clays. The aluminum is dissolved from the crystalline structure and may form insoluble compounds such as variscite. In the presence of calcium above a pH of 7, phosphate is fixed as calcium phosphate.

Phosphate can also be held in an exchangeable form at lower pH's as a result of anion exchange reactions, especially in amorphous clays. See Anion Exchange.

Studies have shown that up to 95% of added phosphorus can be immobilized given an adequate retention time in the soil column for precipitation/adsorption and fixation to occur (10). The major portion of the added phosphate, up to 90%, will generally be found in the upper 40 centimeters of the soil column.

It is estimated that disposal of phosphorus could be continued for years without an increase in the discharge of phosphorus in the effluent. The precipitation of insoluble phosphate complexes exposing new reaction sites and the continual formation of iron and aluminum oxides as a result of weathering constantly add new reaction sites for the removal of phosphates.

Biological Transformation

The soil environment is composed of a number of different inhabitants including bacteria, actinomycetes, fungi, myomycetes, yeasts, algae, lichen, viruses, microplasmas, protozoans, nematodes and earthworms (49). Although all of these play a part in the degradation of wastes, bacteria are the most numerous inhabitants and are generally considered to have the more important roles in degrading wastes.

The role of bacteria in waste degradation is best exemplified by the fate of nitrogen once it is applied to the soil. Most nitrogen in wastewater is in the form of nitrate (NO_3^-), ammonium (NH_4^+), or bound organic matter (33). Depending on the initial form and soil conditions nitrogen can undergo a number of fates.

Nitrogen in organic matter is released when microorganisms utilize the molecule as a carbon source. Some nitrogen is used by the organism to synthesize needed compounds. The rest is released as NH_4^+. When an organism converts nitrogen to an organic compound, such as in the synthesis of amino acids, the process is referred to as immobilization. The release of nitrogen from an organic form to a mineral form is termed mineralization.

There are two other processes that can affect the mineralized forms of nitrogen, nitrification and denitrification. These will be discussed in detail.

Nitrification. Nitrification is the conversion of ammonium nitrogen to nitrite and nitrate. This conversion can be stimulated directly by light or occur during the metabolism of some heterotrophic

microorganisms (38). The bulk of nitrification is attributed to two autotrophic bacteria, of the Nitrosomonas and Nitrobacter genera. These bacteria are able to utilize the energy obtained from the oxidation of ammonium (NH_4^+) or nitrite (NO_2) to drive the reduction of carbon to carbohydrates for cellular synthesis. The following formulaes describe the reactions and subsequent energy evolved (36).

$$NH_4^+ + 1\text{-}1/2\ O_2 \rightarrow NO_2^- + 2\ H^+ + H_2O + 66\ Kcal$$

$$NO_2^- + 1\text{-}1/2\ O_2 \rightarrow NO_3^- + 17.5\ Kcal$$

Nitrosomonas is associated with the oxidation of NH_4^+ to NO_2^- and Nitrobacter completes the oxidation of NO_2^- to NO_3^-.

Since the reduction of carbon is coupled with the oxidation of ammonia, an adequate carbon source is needed for the process to continue, not just for the nitrifying organisms but more importantly for the organisms that breakdown organic compounds for their carbon. If the carbon to nitrogen ratio is less than 22:1, adequate nitrogen is available with respect to the amount of organic carbon so ammonium is released as a waste product from the breakdown of the organic material. The ammonium, in turn, fuels the nitrification reaction. At ratios higher than 22:1 the soil microbes that degrade the organics are using all of the available nitrogen for their own needs. The breakdown of organic matter and subsequent release of ammonium, under proper conditions, is generally considered the rate controlling step for nitrification (12).

Optimum pH for nitrification is 7.8 to 8.8 in pure culture (49). Due to the H^+ ions produced by the reaction, the pH may tend to drop. Often, however, the wastewater applied is usually sufficiently buffered to offset any acid production (36).

The optimal temperature lies between 30°C and 35°C (12). The overall range is believed to be 4° to 50°C with much lower nitrification rates above and below the optimum (7).

Nitrification is generally most effective in aerobic well-drained soils since oxygen acts as the terminal electron acceptor (49). Tilstra et al. (76) demonstrated the differences in nitrification rates in a Detroit lake peat soil in a saturated state as opposed to a well-drained state. The percolate from the well-drained soil contained approximately three times as much nitrate nitrogen as in the saturated soils' percolate. Pilot and Patrick (60) varied the moisture tension in soil cores and recorded the production of nitrate nitrogen at each moisture tension. They concluded that as the moisture tension reached zero so that no oxygen was completely diffusing through the soil, production of nitrate nitrogen stopped. The levels of nitrate nitrogen then began decreasing as the denitrification process began.

Denitrification. Denitrification is the subsequent reduction of NO_3^- or NO_2^- to a gaseous form of nitrogen, either nitrous dioxide (NO_2), nitrous oxide (N_2O), or elemental nitrogen (N), by soil bacteria. The bacteria utilize the oxygen available in nitrate and nitrite as a terminal electron acceptor instead of elemental oxygen (12).

In a strict sense, nitrification and denitrification are exclusive of each other. As the oxygen level drops below 3 micromoles of oxygen per liter of water nitrification processes shut down and denitrification begins (12). In reality, denitrification occurs frequently in well-aerated soils where nitrification processes are proceeding. This is possible due to anoxic pockets or zones that are created in the soil environment as respiration rates exceed the oxygen diffusion rates (38,49).

Optimal temperatures for denitrification are 65° to 75°C. However, warm soils in general encourage denitrification (61). A pH of 8 - 8.6 is optimal for the process (61). As the pH increases the relative amounts of N_2O and NO evolved decrease relative to the elemental nitrogen fraction formed (49).

Stoichiometrically the minimal carbon/nitrogen ratio to support denitrification is 0.7, but the most favorable to the process is 2:1 - 3:1 (43,49). It varies depending on the type of carbon and its practical availability to the soil microorganisms.

Denitrification is the only process available that removes nitrogen completely from a wastewater and creates no unwanted by-products. By encouraging this process through proper management of C/N, soil oxygen content, and application rates, removals of 60 - 90% of nitrogen may be obtained (68).

Biological Adsorption

Biological adsorption refers to the uptake of carbon, nitrogen, and other elements by microbial and plant populations. The majority of

actual uptake, however, occurs with plants. Plants actively and selectively take up potentially toxic and non-toxic compounds and elements, storing them within various parts of the plant and effectively removing them from the water and soil (45). Overcash and Pal (56) noted that pathways for uptake and subsequent neutralization by plant metabolism have been demonstrated for 23 complex and toxic organic compounds, metals excluded. For elements like nitrogen, plants are, in general, more effective than denitrification for removing nitrogen from the soil. Most existing land application systems receiving domestic waste have been unable to remove more than 30% of added nitrogen by denitrification (45) and rely on plant uptake and crop management for greater removal. Exceptions to this include some high rate infiltration systems that report removal of up to 80% of nitrogen (14).

An early successional stage ecosystem, such as grass, which is accumulating biomass is generally more efficient at removing nutrients and toxics from wastewater than a late stage stable ecosystem such as a hardwood forest. A system in which biomass is growing rapidly will have a net accumulation of nutrients. As the system matures nutrient incorporation will be less and less. Eventually an equilibrium is reached where the net input equals the net output (77). A study that compared losses of nitrate from an early successional forest to an old-age forest found that streams draining the younger forests were much lower in nitrates than the streams draining the older forest (77). In selecting a crop for wastewater treatment, then, it would be beneficial to use a crop with a high growth rate that could be harvested

periodically to permanently remove nutrients and toxics and also to encourage new growth. Depending on the composition of the wastewater it may be possible to not only renovate the wastewater, but also to produce a cash crop that could offset the expense of the system.

There are of course other considerations that should be examined in selecting a crop. Loehr et al. (46) suggest the following factors.

1. Water requirement and tolerance
2. Nutrient requirement and tolerance
3. Nutrient utilization and renovation efficiency
4. Sensitivity to potentially toxic elements and salts
5. Insect and disease problems
6. Season of growth and dormancy requirements
7. Natural range
8. Ecosystem stability
9. Demand or market for the product

A number of different types of crops have been used. They include forage crops such as hay, alfalfa, and other grasses; field crops like corn and sorghum; turf to be sold as sod or irrigated as in golf courses; and trees (57). The forage crops generally require some sort of storage facility for silage. With field crops, like corn, there is down time for drying and harvesting when the fields can't be sprayed.

Also, uptake of nutrients is generally for only a short time. Corn accumulates 90% of its nitrogen during a one month period (29). Irrigation of turf is most often used where the hydraulic loading is large and nutrient removal is of secondary concern. Several species of

trees have been used favorably for wastewater renovation although insect and odor problems can occur.

If concern over bioaccumulation of toxics or toxic metabolites exists then a fiber, ornamental, or other non-food crop could be raised instead such as cotton, jute, or hemp, or corn for fuel (56). Sod has also been used since it can be irrigated and then physically removed from the site (46). Removal would not only eliminate the toxics problem but would also extend the longevity of the site.

For many systems perennial grasses appear to be the best suited for wastewater disposal (54). Excess nitrogen (N) is common in wastewaters, including the wastewater obtained from Allied Corporation. Too much N can be damaging to a number of crops such as small grains, tubers, sugar beets, melons, squash and tomatoes (46). Lush vegetative growth results at the expense of tubers. Sugar content is decreased in beets. Increased fruit rot and decreased vitamin C due to shading occurs in melons, squash, and tomatoes.

Secondary effluent was applied to corn and grasses in an experiment that compared N removal rates of the plants. The concentration of inorganic N in the soil water was consistently lower under the grass croppings than the corn cropping (29). Another study considered ryegrass, ryegrass and corn, and corn for renovation treatments. Again the ryegrass was the best and corn and ryegrass intercropping second, for removal of nitrogen. Corn, by itself, had the lowest N removal rate (32).

Table 1 from Overcash and Pal (56) shows clearly that the grasses are most efficient at removing nitrogen from wastewater. The various tree combinations have the next highest removals along with the forage crops. Note that alfalfa, although a legume, has been shown to utilize the N in the applied wastewater rather than fix it from the air (31).

Perennial grasses have several advantages over annual crops. Their fibrous root system and sod forming characteristics aid in erosion control and promote high uptake rates. They are tolerant of a wide range of environmental conditions and have long growing periods (54). Another consideration is low maintenance. Once the grass is established it is essentially maintenance-free except for occasional cuttings.

In most land application systems, plants are the primary absorbers of nutrients and other substances. Removal, or harvesting, of the plant removes the nutrients, toxics, etc. from the system. Perennial grasses appear to be best suited to land application system due to their low maintenance, high uptake rates, and erosion control properties.

Production of Nylon-6

Nylon has been defined as "a manufactured fiber in which fiber forming substances are any long-chain synthetic polyamide having recurring amide groups (-CONH-) as an integral part of the polymer" (11). Nylon-6 is so named because the repeating unit is composed of the 6-carbon cycloamide caprolactam (Figure 1a). Commercial production of nylon-6 began in 1955 with the large scale availability of caprolactam when the Allied Corporation began producing it and nylon-6.

Table 1. Anticipated nitrogen removal by crops.

Crop	Nitrogen Removal (kg/ha/yr)
Coastal Bermudagrass with rye overseed	570 + 205 = 775
Coastal Bermudagrass	480 to 600
Reed Canary Grass	226 to 359
Fescue	275
Alfalfa	155 to 220
Sweet Clover	158
Red Clover	77 to 126
Lespedeza Hay	130
Johnson Grass	890
Peanuts	140
Corn	155
Soybeans	94 to 113
Irish Potatoes	108
Sugar Beets	73
Cotton	66 to 100
Milo Maize	50 to 76
Sweet Potatoes	75
Barley	63
Oats	53
Tobacco	85
Mixed Hardwood Trees	200
Red Pine	160
White Spruce	250
Pioneer Succession Vegetation	250

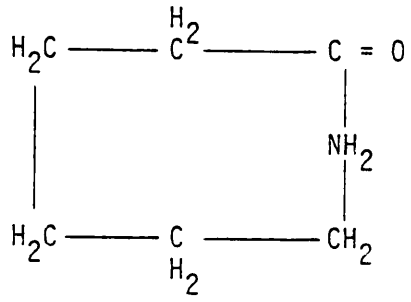


Figure 1a. Structure of ε-caprolactam

(1) $HN(CH_2)_5C = O + H_2O \rightleftharpoons H_2N(CH_2)_5COOH$

aminocaproic acid

(2) $H_2N(CH_2)_5COOH + (n-1)HN(CH_2)_5C = O \rightleftharpoons H[HN(CH_2)_5CO]_nOH$

(3) $xH[HN(CH_2)_5CO]_nOH \rightleftharpoons H(HN(CH_2)_5CO]_{x\ n}OH + xH_2O$

low molecular weight high molecular weight

Figure 1b. Polymerization of ε-caprolactam to Nylon-6.

Polyamide fibers are noted for their strength, dyeability, fatigue and abrasion resistance, wearability, resistance to rot, insect resistance, generally low shrinkage, and silk-like appearance. These characteristics have made polyamide fibers, and, especially nylon-6, popular for a variety of products including fabric, ropes, carpet, clothes, and kitchen appliances (11).

A hydrolytic polymerization technique is used to form over 95% of all polyamide fibers (11) (Figure 1b). The first step is termed initiation and addition. Caprolactam is able to add successively to aminocaproic acid once one molecule has been formed by hydrolysis with water under pressure. This results in short chains of 8,000 to 14,000 molecular weight (MW).

The second step, condensation and polymerization, produces molecules with a molecular weight of 18,000-33,000 and is primarily a joining of the short chains from the first step. The third and final step is an equilibrium step which involves a chain stopping element. A molecular weight of 20,000 to 36,000 is achieved.

Once the polymerization reaction occurs the molten nylon is spread onto a belt and cooled by a water spray. The cooled nylon is chipped and stored as a solid which is later blended on a batch basis to ensure product uniformity.

The molten nylon is extruded through a spinneret and a finish oil applied which protects the fiber as it passes draw pins, guides, and other fibers. The finish provides for surface lubricity, plasticizing action, and static protection (11). These finishes applied during the

spinning are called "spin finishes." Special purpose finishes can also be added which may extend the life of a product, or, as in the case of the AM finish used in this project, one that protects the fibers from microbial degradation.

The finish is circulated through the finishing system from a holding tank. Any one of a number of methods can be used to apply it, such as, spraying the finish directly onto fibers, passing the fibers through the finish, or passing the fibers over a constantly revolving roller. The finished fiber can then be spooled or sent on for further processing into finished products.

Due to the high cost of finish, efforts are usually made to reduce the amount of finish lost to wastewater. The largest contribution of finish to the wastewater results from washing out the holding tanks.

Components of the AM Finish Wastewater

The Allied wastewater from this new finishing process was segregated from the main process flow, stored in 208 L (55 gallon) drums and, then transferred to an outside storage tank. The components of interest to the project include caprolactam, the main process component; the antimicrobial additive; the finish containing the additive; sodium lauryl sulfate, reported to be a neutralizing agent for the AM agent; and metasol, a fungicide. Each of these components are discussed in detail in the following sections.

Caprolactam

Caprolactam is a synthetic six carbon cyloamide which has never been found in nature (34). Nonetheless, a number of bacterial strains are able to utilize the compound and some use caprolactam as their sole source of carbon and nitrogen (5,34). It is believed that the bacteria acquire an enzyme that allows them to split the molecule. That enzyme, loosely termed the "lactam splitting enzyme" has not been isolated (35). The following is the presumed pathway for degradation of caprolactam (34).

ε-caprolactam → 6-aminocaproic acid →
adipaldehydic acid → adipic acid

Brown (18) considered degradation of caprolactam in an activated sludge system using wastewater from the main process flow at the Allied Corporation plant in Chesterfield County, Virginia. Lab-scale continuous-flow, conventional activated sludge reactors were operated at Θ_c of 5, 10, and 15 days. Percent reductions of caprolactam were reported as 65, 96 and 100%, respectively, with an influent strength of 1000 mg/L caprolactam. This would tend to verify the literature reported biodegradability of ε-caprolactam.

Earlier work with Allied's wastewater indicated that caprolactam produced a phytotoxic effect in grass. Donley (28) utilized increasing concentrations of caprolactam, up to 10,000 mg/L, in greenhouse and seedling bioassays. Leaf tip burn was evident in the greenhouse studies

with fescue and bermuda grass although bermuda-grass was found to be more tolerant. Root uptake and translocation were believed to be the dominant mechanisms involved. Regrowth was evident in a few days, however, indicating a possible loss in toxicity of the compound with time.

Caprolactam is therefore subject to biological degradation by certain bacteria that naturally occur in the environment. It is also amenable to degradation within an activated sludge system. Land application of caprolactam, however, may result in a phytotoxic response by the plant receiving system. Use of a more tolerant soil-plant receiver system such as bermuda grass would improve the likelihood of a successful land application system.

Antimicrobial Agent

The antimicrobial (AM) agent is an organosilane consisting of a long chain alkyl group and a quaternary ammonium salt (23). Since it polymerizes rapidly with water, the agent is packaged as 42% active in a methanol solvent. The AM's physical properties and mode of action are listed in Table 2.

It is the rapid polymerization/bonding action around the silane group that makes this agent well-suited to its purpose. In the presence of water it creates a strong silicon-oxygen bond that binds the molecule to surfaces. In this way the active quaternary ammonium group is held firmly in place resulting in long-term effectiveness (Figure 2).

Table 2. Physical properties and mode of action of the antimicrobial agent.

42% active organo silane in methanol solvent	
pH	7.5
boiling point	150°F/65°C
specific gravity	0.88
vapor pressure	100 mm
vapor density	> 1.0
% volatile by volume (%)	> 40%
evaporation rate (ether = 1)	< 1.0
solubility in water	> 90%
stable to 125°C	
mode of action: disrupts microbial cell membrane	

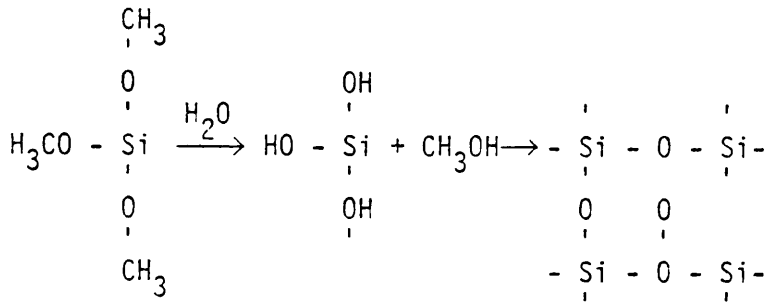


Figure 2. Bonding of antimicrobial agent through silicon-oxygen bonding.

The silicon-oxygen bonding appears to be a better fixing method than other attempts such as the procedure described by Blazej et al. (13) where fibers were coated with a fine dispersion of inorganic particles followed by the application of a quaternary ammonium salt. With the AM agent the quaternary ammonium salt is already chemically linked to the bonding agent.

Although it is an effective bacteriostat, the AM agent does not appear to be highly toxic to higher forms of life. In numerous tests reported by Dow Corning (23), the AM agent caused severe problems only in exposure to eyes and caused only slight skin irritation. Mutagenic and teratogenic tests were negative. Tests using fabric treated with the agent resulted in no observable skin or vaginal irritation. Static bioassays (96 hr) with fingerling rainbow trout and blue gills resulted in a TL_{50} of 0.56 mL/L and 0.51 mL/L, respectively. The AM agent has a high affinity for microbial cells which results in the agent being extremely toxic to activated sludge in a wastewater treatment system at concentrations greater than 1 mL/L.

By standard biological oxygen demand (BOD) and chemical oxygen demand (COD) analyses, the agent does not appear to be biodegradable (23). Fortunately, durable bonding to surfaces should result in a minimal amount of the AM agent being released to the environment.

The AM agent can reportedly be neutralized with nonionic Triton X-100 at 10,000 mL/L 1.0% (vol/vol) or greater to bath solutions up to 1.0% (vol.vol) of AM agent. Anionic detergents, such as sodium lauryl

sulfate, may be used to block the functional group and deactivate it. Neutralization can also be achieved by heating a solution of the AM agent to 320°F (160°C) for 30 minutes (23).

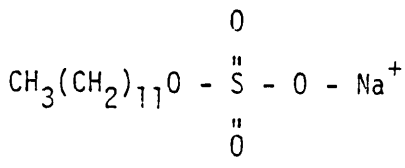
AM Finish

The finish which contains the antimicrobial agent is applied to the fibers at 6% wet pickup. Water constitutes the major portion of the finish (≅ 80%) while the lubricating system composed of emulsifying, wetting, and antistatic agents, is the next largest fraction at 10 to 15%. The remainder of the finish includes the AM agent and additional wetting agents. Due to the confidential nature of the finish composition specific components cannot be provided. The finish without the new additive has been used before at the Allied plant. Previous tests on the wastewater have not identified any of the components of the finish as being present at toxic levels (28).

Sodium Lauryl Sulfate

Sodium lauryl sulfate (SLS) is an anionic synthetic detergent whose chemical structure is given in Figure 3 (8). Allied selected SLS as the neutralizing agent for the AM finish, based on reports prepared by Dow Corning (23). In general, an anionic and cationic agent together should result in a precipitation reaction, neutralizing the bacteriostatic properties of the AM agent.

Sodium lauryl sulfate has been used as a pre-operative skin cleanser and in medicated shampoos due to its bacteriostatic action



Sodium lauryl sulfate

Figure 3. Chemical structure of sodium lauryl sulfate.

against gram positive bacteria (4). Large doses of ionic surfactants lead to structural damage of cells while small doses reduce the efficiency of mucousal cells (4).

In the aquatic environment, surfactants exert both physical and chemical effects on their surroundings. For example, foam formation interferes with reaeration rates and has a negative effect on flocculation and sedimentation. These effects could increase the toxicity of other compounds and/or decrease the photosynthetic capacity of submerged green plants and algae (48). Toxicity values of SLS, reported as lethal concentrations, for various species of fish to SLS range from 6 to 8.5 mg/L but have been reported up to 25 mg/L (48). Investigators in this field tend to agree that death is the result of interference with the respiratory system. A thickening of the respiratory epithelium and lesions have been noted in fish exposed to SLS and other surfactants (48).

In terrestrial plants the coating action of the surfactant may be sufficient to block the exchange of gases and/or light absorption. Due to its coating action, SLS may also increase the contact time of toxic compounds on the stems and leaves which would normally be rinsed or drained off.

Metasol TK-100

Metasol TK-100 is a fungicide which was under consideration by Allied as an additive in its finish to improve the antimicrobial properties of nylon. It was included only in the initial phases of this project as a side study.

Metasol has a three-ring structure, as presented in Figure 4 (22). Its solubility in water is 25 µg/mL at 23°C and appears to be stable under normal conditions (30). Metasol has been used for the treatment of post harvest diseases in concentrations ranging up to 6000 mg/L in water suspensions or wax emulsions (30). It has also been under study for the treatment of various fungal infections of the eye (64).

This fungicide appears to act primarily as an inhibitor of nuclear division (47). Inhibition of respiration also has been described as a mode of action, but this may be a side effect rather than a main effect (47). Not all fungi are susceptible to Metasol and a few resistant strains have been identified (47).

Metasol appears to be very stable in the soil. Sijpesteijn et al. (65) reported that the fungicidal activity of 50 mg/L in soil disappeared in 12 to 18 weeks. Some photodecomposition to benzimidazole and benzimidazole-2-carboxamide has been identified (65). In fate studies using C_{14} and cotton plants, 60% of the Metasol was recovered after three days as the parent compound. The remainder was bound as higher weight compounds (65). The potential for breakdown to a more toxic compound appears to be minimal.

Greenhouse Studies

Greenhouse studies have been used for years to research a number of topics in fields such as agronomy, horticulture, plant pathology, and genetics. The utility of the greenhouse tests in assessing the suitability of effluents for land application has been well-accepted.

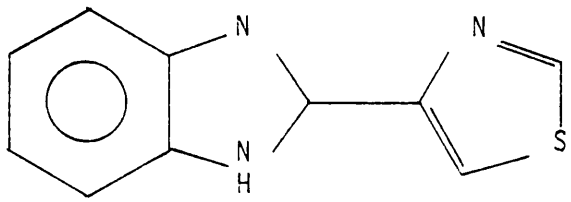


Figure 4. Chemical Structure of Metasol TK-100.

The advantages of using greenhouse studies over field studies are obvious. The greenhouse represents a closed environment where the researcher can control year-round a number of environmental and biological factors which reduces variation and increases the reliability (6). Being confined also reduces the possibility of harm to the environment which is of special concern where potentially toxic wastewaters are being considered.

Greenhouse studies are cheaper to perform than field studies in that the cost of a singular experiment is reduced (6). The number of tests can then be multiplied to obtain information over a larger experimental space of variables and to reduce the error associated with the experiment. Greenhouse studies are time-saving as well and are fairly accurate representations of field conditions. Results from the greenhouse studies can be used to predict the results under field conditions and to better design field scale experiments.

Greenhouse studies, however, are not ideal and certain drawbacks are evident. A greenhouse provides for an optimum of light and temperature, so the degree of correlation between greenhouse results and actual field experience may be questionable. Evapotranspiration, the sum of evaporation from the soil and plant surfaces (12), would be enhanced in a greenhouse. The use of polyethylene liners in the pots, reduces evaporation from the soil, but allows for the passage of oxygen and carbon dioxide (12). The liners, however, could lead to a saturated soil, producing anoxic conditions whereas in the field excess liquid would more readily leach past the root zone. An argument could be made

that excess water would be the result of poor experimental design. However, if an effluent application should inhibit plant growth then the moisture requirements for that system are also depressed thereby resulting in excess moisture.

Greenhouse testing, although much faster than field scale testing due to year-round availability, is still slow. Depending on the plant used the lag time between time of planting and maturity can be a matter of days, months, or years. With grass studies, 4 to 8 weeks are needed to achieve a full stand of grass. As a routine monitoring device, greenhouse studies are far too slow to catch any plug flows of toxics in an effluent stream.

Thus, greenhouse studies are excellent for anticipating the effect that a given effluent would have on a soil/plant system. They are cheaper and faster to conduct than field-scale experiments. Greenhouse studies also give a more realistic result than if each component of the system, i.e., soil, fungi, plant, etc., were assayed separately. However, greenhouse studies are not appropriate for detecting sudden changes in a given effluent and such testing might not accurately duplicate field results.

Short-Term Bioassays

There are many bioassay techniques in use today that evaluate the impact of a substance or substances on a variety of receiving systems. The techniques range in complexity, length, and cost, and each is designed to enlarge the data base upon which a more complex, realistic, and expensive test can be designed.

For example, a short-term bioassay could be used to screen effluents and provide information to set up a better greenhouse study. It could save time and money by eliminating unnecessary factors from the greenhouse design.

A short-term bioassay could also be used to monitor changes in a wastewater's toxic characteristics. A bioassay is preferable over a chemical determination because of the complexity of most wastewaters. Even with a comprehensive list of potential components of a water, it would be difficult for a technician to complete all the necessary chemical tests on a daily or even weekly basis. Identification of all by-products of those components due to photodegradation, microbial action, or chemical interaction would also be difficult, if not impossible, to determine. In addition, chemical tests may not be sensitive enough to detect toxic concentrations of a substance. A bioassay, however, is able to test the toxicity of a waste stream without having to identify individual components. The bioassay chosen should reflect the sensitivity of the receiving system and be completed within a short time period.

Three short-term tests were considered in this research. All three could potentially be used to screen compounds for a more complex bioassay. One test, however, the corn seedling bioassay, takes 3 to 5 days to perform, so it is not useful as an effluent monitoring procedure, unless effluent was released on a batch basis. The other two tests, Microtox and Lumac, can be performed within an hour, making them good candidates for use as monitoring tools. Each of these bioassays are

evaluated to determine their utility for the Allied Corporation as either screening or monitoring tools based on their correlation with the greenhouse study.

Corn Seedling Bioassay

The corn seedling bioassay (CSB) evaluates the effect of a substance on root and/or shoot growth. Root tests can be completed in three days. Shoot tests take five days to perform. The method used in this study was adopted from procedures proposed by Parker (58) and Donley (28). (See Materials and Methods for a detailed description.)

Anderson (3) was the first to use a corn seedling bioassay to evaluate the toxicity of Allied's wastewater. His method differed from Donley's in that the corn seeds were germinated on filter paper, rather than on sand, soaked with a dilution of the wastewater. His results indicated a near linear relationship between root growth inhibition and increasing wastewater concentration. Donley (28) investigated both root and shoot growth using Allied's wastewater and caprolactam. The study, which identified caprolactam as the primary toxicant in the wastewater, was supported by greenhouse studies with fescue and bermuda grass. The seedling bioassay was also instrumental in defining root uptake and translocation as the mechanisms for producing leaf tip burn in the grasses.

These previous tests with Allied's wastewater by Anderson (3) and Donley (28) indicated that the corn seedling bioassay may have utility

as a screening device for more elaborate testing. Since the seedlings are a plant system, the seedlings are more likely to reflect the true response of another plant system to a toxicant than, for instance, fish bioassays. However, what this bioassay gains in realism is offset by time. As with the greenhouse studies, the CSB is best suited as a screening tool to be used before a compound is used in full-scale operations. It would be a poor choice as a monitoring system of a wastewater flow that fluctuates daily in its composition and/or strength.

Microtox and Lumac

These two systems, Microtox and Lumac, utilize bioluminescence to assess a given substance. The Microtox Test challenges a known bioluminescent bacterial population with a suspected toxicant and measures any subsequent change in light output. The Lumac system uses firefly luminescence to measure the change in adenosine triphosphate (ATP) in a biological system after the system has been challenged by a toxicant.

There are a number of organisms that have the ability to emit light besides certain bacteria and the firefly. They include fungi, worms, sponges, corals, fish, and crustaceans (51). A number of these organisms, like the luminescent bacteria and firefly, utilize an enzymatic luciferin-luciferase to produce light. Although the actual composition of the luciferin-luciferase system varies, the general reaction is the same. The luciferin is catalyzed by luciferase into an oxidized state and light is emitted. Cofactors have been identified in

some of the systems as being essential to the reaction. In the firefly system ATP is the cofactor. In the bacterial system a flavin mononucleotide ($FMNH_2$) is the cofactor (41).

The general reaction for the bacterial system is given in Figure 5 (52). It is this type of system that is utilized by Microtox. The Microtox Toxicity Analyzer by Beckman (Model 2055) uses a marine photobacterium resembling Photobacterium phosphoreum to assess the toxicity of a sample. In this test a series of dilutions of an agent are made and then used to dose prepared samples of the reconstituted, luminescent bacteria. The change in light output is recorded over time and the agent concentration effective in reducing the light emitted by 50% is commonly reported (EC_{50}).

The theory behind the Microtox system rests on the intrinsic tie between the metabolic processes of the cell and the luminescent reaction. Damage to the metabolic processes result in a change in light output. Consequently the Microtox can be used to evaluate the toxicity of a broad spectrum of chemicals having diverse modes of action (19).

Comparisons of bacterial bioluminescence results with fish and invertebrates bioassay results show a fairly good correlation for most compounds tested. In tests with fossil fuel process wastewater, rainbow trout (96 hr flow through tests) were slightly more sensitive than the bacteria but the two tests were significantly correlated ($r = 0.82$, $p < 0.05$)(44). Fathead minnows using the same wastewater did not correlate well with the bacteria using 96 hours, flow-through tests. Twenty-four hour static tests, however, showed a very significant ($r = 0.97$)

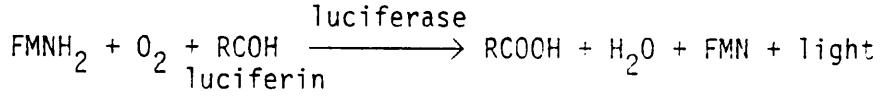


Figure 5. Bacterial bioluminescence reaction.

correlation (44). In this same study phenolic compounds were also considered, but no significant correlation between rainbow trout and the bacterial response was found. The response of fathead minnows, had a weak correlation with that of the bacteria ($r = 0.67$), but the correlation was still higher than the correlation between the two fish bioassays ($r = 0.65$).

Bulich et al. (21) investigated a number of pure compounds using the Microtox analyzer and compared the results to literature reported values. The comparison is presented in Table 3 (21). Again a good agreement can be seen in most cases, however, no attempts were made to correct for changes in water quality, i.e., hardness, pH, temperature, etc., which may have positively or negatively affected the results.

Bulich (20) compared the results of 235 fish bioassays to Microtox bioassays and 155 Daphnia bioassays to Microtox bioassays using a variety of complex effluents. In these tests 97.5% of the fish and Microtox results and 96.1% of the Daphnia and Microtox results were within a 1.5 order of magnitude. Kenaga (42) in his review of bioassays noted that for bluegill bioassays compared to other blue gill bioassays, 95% of the results were within 2 orders of magnitude. Fathead minnow and Daphnia results were with 2 to 3 orders magnitude. For freshwater fish in general, 92% of the results were within 2 orders of magnitude and 97% within 3.

Based on just these data, the Microtox Analyzer would appear to be at least as accurate as the most common bioassays in use today.

Table 3. Comparison of Microtox EC_{50} and fish LC_{50} data using pure compounds.

Toxicant	Microtox, 5-min, EC_{50} mg/L	Fish Assay 24 to 96h LC_{50}, mg/L
Mercury II	0.065	0.01 to 0.9
Pentachlorophenate	0.5	0.21 to 0.6
Aroclor 1242	0.7	0.3 to 1.0
p-Cresol	1.5	3.5 to 19
Sodium lauryl SO_4	1.6	5 to 46
Ammonia (free)	2.0	0.068 to 8.2
Benzene	2.0	17 to 32
Zinc II	2.5	0.24 to 7.2
Malathion	3.0	0.07 to 19.5
Formaldehyde	3.0	18 to 185
Copper II	8.0	0.1 to 10.7
Cyanide (HCN)	8.5	0.1 to 0.44
Trinitrotoluene	20	26
Phenol	25	9 to 66
Chromium VI	70	29 to 133
Nitrite	420	19 to 230
1-Butanol	3,300	1,940
Isopropanol	42,000	4200 to 11,130
Urea	24,000	12,000
Ethanol	31,000	13,500

Microtox results can be obtained within 30 minutes, as compared to 24 hour or 96 fish bioassays.

The Lumac system has not been widely used, but the basic premise of its operation, firefly luminescence, has been in use for a number of years. The firefly luminescent reaction is dependent on ATP (50)(see Figure 6). Although the light emission can be influenced by other ribonucleotide-5'-triphosphates, they each derive their terminal phosphate from ATP, except for guanosine triphosphate, (GTP) which derives its phosphate from the citric acid cycle. If one considers, however, that each represents an equivalent ATP molecule, then the distinction is not important (59).

Analyses of ATP have a 2% relative standard deviation (17). It is because of this accuracy, plus the fact that ATP is a good indicator of cell viability, that ATP analysis has been studied for years as a possible measure of microbial biomass in a number of systems, including activated sludge and soil systems.

Activated sludge systems, under steady state conditions, possess a relatively constant ATP pool. Any sudden changes in that ATP pool implies an environmental change (17). While changes in temperature up to 37°C do not affect ATP content, decreasing oxygen concentration produces a decline in ATP (17). With the reintroduction of oxygen, ATP pools increase to near previous levels. Toxic agents produce a variety of response levels (17). Nickel produces a linear decrease in ATP with increasing concentration, while cyanide produces a hyperbolic plot. Copper appears to have a threshhold concentration of 2.5 mg/L, above which ATP pools decreased.

$$\text{Luciferin} + \text{Luciferase} + \text{ATP} \xrightarrow{Mg^{++}} \text{Luciferin - Luciferase - AMP} + \text{PP}$$

$$\text{Luciferin - Luciferase - AMP} \xrightarrow{O_2} \text{D-carboxyluciferin} + \text{luciferase} + \text{AMP} + CO_2 + \text{light}$$

Figure 6. Firefly bioluminescence reaction.

Chiu et al. (24) reported that at constant biomass levels one might still see changes in ATP due to different ATP content between microbial species. Another problem in assessing ATP is the ionic composition of the medium. Patterson et al. (59) found ions to affect light emission in the following order:

$$Ca^{++} > K^{+} > Na^{+} > Rb^{+} > Li^{+}$$

In spite of the problems, however, it has been found that ATP concentration responds rapidly to cell viability, population shifts, and growth phase (24,59).

Results with soil microbial biomass have had mixed results. Nannipieri et al. (53) compared CO_2 evolution, phosphatase, urease activity, and ATP to microscopic determination of bacterial and fungal biomass. The ATP concentration varied with phosphorus and soil type with a standard error of 5 to 10%. The authors concluded that no one measurement of microbial biomass or activity is sufficient to interpret microbial growth in a soil system.

After adjusting their ATP determination to reflect incomplete ATP extraction, Jenkinson and Oades (40) obtained a close correlation between ATP content and carbon (C) and nitrogen (N) in soils of $r = 0.86$ and $r = 0.89$, respectively. They were also able to determine that extracellular ATP was rapidly broken down and did not contribute much to the overall ATP pool. Another study by Oades and Jenkinson (55) also found a strong correlation between C and ATP ($r = 0.98$) using a

heterogenous group of 11 soils. The C to ATP ratios can change based on incubation and storage techniques (1).

Tate and Jenkinson (71) used a luminometer designed specifically for measuring bioluminescence. They reported that the Picolite Model 6160 by Packard Instruments, Co. was more convenient to use than the conventional scintillation counter and produced very comparable results.

The luminescent meter used in this study is referred to as Cell Tester Model 1030 by Lumac Systems. The technique proposed by Lumac (3M) is a simpler method of ATP extraction and subsequent luminescent measurement than previously available. In all of the studies cited here the samples for ATP analysis underwent a boiling extraction process, lasting usually 5 to 10 minutes, before the luciferin-luciferase agents were introduced. The Lumac system includes a nucleotide releasing agent that releases ATP from most microbial cells in 15 seconds without boiling. The luciferin-luciferase extract is reconstituted with a bottled buffer containing Mg^{++}, as well as a chelating agent to offset any interferences from Ca^{++} and other ions.

In summary, the Lumac system makes the ATP analysis a much faster and easier method than was previously available. The system can be adapted to a variety of microbial systems and the theory is well-documented in activated sludge and soil systems.

III. METHODS AND MATERIAL

A series of three greenhouse studies, hereafter referred to as Phases I, II, and III studies, were performed to meet the first two objectives of the study which were: (1) to investigate the potential environmental toxic effects of the AM agent alone and in a wastewater matrix; and (2) to define an appropriate loading rate for the AM wastewater on fescue grass. The first phase was conducted to approximate appropriate loading limits to maintain a healthy grass population, while the next two phases were designed to refine and expand the information gained from Phase I. The third objective of testing for correlations between the greenhouse study results and short-term bioassay results was achieved by using a Corn Seedling Bioassay, a Lumac ATP analyzer, and a Microtox bioluminescent procedure.

All solutions used in the tests were supplied by Allied except for Metasol which were obtained commercially. Wastewater from the new finishing process was segregated from the major wastewater flow and stored in 208 L (55 gallon) drums. The wastewater sample used in the Phase I and II greenhouse studies was a composite of waters from four drums. The wastewater contained 132 mg/L of the AM agent and 284 mg/L total Kjeldahl nitrogen (TKN). The third phase and all short term tests were conducted with a second wastewater sample supplied by Allied. This composite sample was much weaker than the first, having only 48 mg/L TKN and 22 mg/L of the AM agent. Distilled, deionized water (hereinafter

simply referred to as distilled water) was used as a diluent in all experiments.

Greenhouse Studies

Phase I

The first greenhouse study was conducted with cores of fescue grass obtained from the Virginia Tech Turf Center. The cores were placed into 15 cm (six-inch) diameter plastic pots that were lined with plastic bags to eliminate moisture losses due to leaching. Sand was used to fill in along the sides and bottoms of the pots surrounding the cores, to allow for drainage. A small amount of bentonite clay was placed on top of the sand around the upper edge of the pot, to reduce evaporation.

Because the cores were obtained during the winter, they were maintained in a greenhouse for 68 days to allow for regrowth before treatments were administered. The pots were fertilized frequently with a standard, commercial-grade fertilizer, Miracle-Gro, and watered to 80% of field capacity with distilled water daily. Over the 68 days the pots received a total of 0.193 g nitrogen (N), 0.193 g K_2O, and 0.386 g of P_2O_5. The grass was clipped to a uniform height twice during that period to encourage uniform root and tissue growth. The clippings from the second harvest were dried overnight in a 70°C oven, and the dried weights were compared. Based on the dry weights and visual observations, the healthiest grasses were selected for the Phase I study.

The treatments used for the first greenhouse study were designed on the basis of information supplied by Allied about their finishing

process and the nature of the AM agent. The most relevant facts included the following:

1) The AM agent is used in the finish at about a 2% concentration. The wastewater from this finishing process contained the finish plus wash water from rinsing the machinery. The highest concentration of the AM agent in the wastewater was then estimated to be less than or equal to 1%.
2) The antimicrobial effect of the AM agent was reported to be neutralized by 60 parts of a 30% sodium lauryl sulfate solution for every part of a 42% solution of the AM agent.
3) A fungicide, Metasol, was being considered as an additive to a proposed finish. The estimated concentration of the fungicide in the wastewater was 1 mg/L.

Based on this information, nine treatments were developed for this study and are presented in Table 4.

Treatments were based on nitrogen content. The semi micro-Kjeldahl (420.B.) and macro-Kjeldahl (420.A.) techniques described in Standard Methods (69) were used to determine the nitrogen content of the AM wastewater and the AM agent solution. The first five treatments listed in Table 4 were applied at three different loading rates based on a 200-day growing season: 308 Kg N/ha/year(275 lbs N/acre/year), 616 Kg N/ha/year (550 lbs N/acre/year), and 1232 Kg N/ha/year (1100 lbs N/acre /year). These three rates were selected because they define a range

Table 4. Treatments applied to fescue grass in Phase I.

Treatment	Description
1	1% AM agent solution made with distilled water
2	1% AM agent with SLS added at a 60:1 ratio (60 pts. SLS: 1 pt. AM agent) in distilled water
3	Wastewater from the new finishing process (AM wastewater)
4	AM wastewater neutralized with 30% SLS in a 60:1 ratio to an assumed 1% active concentration of the AM agent
5	AM wastewater with 1% concentration of AM agent; assumed no AM agent was initially present in the wastewater
6	30% SLS added to distilled water in same quantity as Treatment 2
7	AM wastewater plus 1% AM agent added, AM agent neutralized with SLS (60:1 ratio), 1 mg/L Metasol
8	AM wastewater with 1 mg/L Metasol added
9	1 mg/L Metasol in distilled water

of loading rates extending somewhat below and above rates which might actually be used in a land application system. The assumed 200-day growing season approximates Allied's schedule of wastewater applications. The last four treatments, six through nine in Table 4 were applied at the 616 kg N/ha/year, only, because the main emphasis of the study was concerned with the effects of the first five treatments. Treatments six and nine, the sodium lauryl sulfate (SLS) and Metasol in water treatments, respectively, did not contain a N contributor, so the solutions were added on a volume basis equal to an AM wastewater treatment at 616 Kg N/ha/year. A N solution of ammonium nitrate was added to treatments six and nine, as well as to the controls at the 616 Kg N/ha/year rate to avoid a N deficiency. Each treatment and loading rate was repeated in triplicate.

Plants were dosed Monday through Friday, corresponding to a five-day work week, with the appropriate treatment. The doses were applied to the vegetative matter and the soil of each pot. Ten mL of distilled water were then applied in the same fashion to partially rinse the leaves. The supplemental N was added to the soil where appropriate. The pots were then watered daily to 80% of field capacity, the water being applied to the soil surface. On Saturday and Sunday all plants received 25 mL of a N-free nutrient solution and then were watered to 80% of field capacity. (See Table 5 for the composition of the nutrient solution.) The weekend treatments were necessary to eliminate the possibility of nutrient deficiency and moisture stress.

Table 5. Nitrogen-free nutrient solution*.

0.002 M	$CaSO_4$ $2H_2O$
0.002 M	$MgSO_4$
0.005 M	$Ca(H_2PO_4)_2$ H_2O
0.0025 M	K_2SO_4

* made with distilled water

The experiment was discontinued after 21 days for most of the treatments. A four-member panel rated the effects of treatments on plant growth based on percent of viable, green foliage remaining in the pots. The pots receiving the AM agent in water, metasol in water, (treatments 1 and 9, respectively) and the controls were continued for a total of 79 days and were analyzed together with the Phase II pots. Only a visual assessment was deemed necessary for Phase I treatments because the objective was only to roughly characterize the response of fescue grass to the various agents.

Phase II

Cores for the second greenhouse study were obtained and set-up, as in Phase I. The plants, obtained in warm weather, were maintained for 21 days in the greenhouse prior to dosing. During that time the plants were given 25 mL of the N-free nutrient solution described in Table 5, and were watered to 80% of field capacity daily.

As stated earlier, the principal objectives of this phase were to further refine an appropriate application rate of AM wastewater and the AM agent on fescue grass and also to investigate potential environmental toxic effects. Secondary objectives of these Phase II studies were to determine if rinsing the plants after dosing, as Allied does in its operation, would affect plant responses, and to determine if soil microbial populations were affected by the treatments. The treatments used are presented in Table 6.

The 100% AM wastewater treatment was applied at 616 Kg N/ha/year based on the TKN values determined in Phase I. The other wastewater

Table 6. Treatments applied to fescue grass in Phase II.

Treatment	Sample Concentration (%)	Rinsing
AM Wastewater	100	YES
	75	NO
	50	NO
	50	YES
	25	NO
Sodium Lauryl Sulfate	1.76	YES
	1.76	NO
	0.18	YES
	0.18	NO
	0.02	YES
	0.02	NO
AM agent	0.10	NO
	0.01	NO
	0.001	NO

loadings were, in effect, dilutions of this loading level. Sodium lauryl sulfate and the AM agent solutions were added on a volume basis equal to the 100% AM wastewater application rate. The concentrations of SLS listed in Table 6 are the levels resulting from making 1:10, 1:100, and 1:1,000 dilutions of the amount of the 60:1 solutions of SLS applied in Phase I. The levels, therefore, would correspond to 6:1, 0.6:1, and 0.06:1 ratios of SLS to the AM agent, if the AM agent was present at a 1% level. Rinsing the plants receiving the AM agent was not considered because the effects noted with 1% solutions of the agent were not severe.

The plants were dosed Monday through Friday (to simulate a five-day workweek) with the treatments being applied to both the leaves and the soil. A N supplement was added to all but the 100% AM wastewater treatment to provide for 308 Kg N/ha/year. The plants were then watered to 80% of field capacity. To "rinse" the plants water was allowed to drain over the leaves, flushing the major portion of the applied solution into the soil. In the no-rinse pots care was taken to add the water to the soil surface only. On Saturday and Sunday 25 mL of the N-free solution (Table 5) were applied to all pots which were then watered to 80% of field capacity to avoid any extraneous nutrient or moisture deficiencies.

All treatments were performed in triplicate. The pots were randomly dedicated to certain treatments. In order to minimize variations in light due to position in the greenhouse, the pots were rotated to different positions on a weekly basis.

The experiment was conducted for 44 days, 12 of which were weekends when the plants were not treated. A visual assessment was made by a three member panel to rank the condition of the plants. All of the soil from Phase II, the soil remaining from Phase I (1% AM agent and 1 mg/L metasol), and the controls, were analyzed for pH, P, K, Ca, Mg, soluble salts, NH_4^+, NO_2^-, total microbial population, and the NH_4^+ and NO_2^- oxidizing microbial populations.

The cores were prepared for analysis by first removing the vegetative matter and then sieving the soil through a No. 10 screen. Soil samples for the microbial studies and the nitrogen determinations were placed in sterile bags and stored at 4°C until processed. Samples for the remaining chemical analyses were placed in cardboard containers supplied by the Virginia Tech Soils Testing Laboratory. The Testing Lab analyzed the soil for pH, P, K, Ca, Mg, and soluble salts.

Ammonium and Nitrate Determinations

Ammonium (NH_4^+) and nitrate (NO_3^-) were determined from the refrigerated soil samples. The moisture content of each soil sample was first determined. Approximately 5 g of soil was transferred to a pre-weighed pan and the exact weight of the soil was recorded. The soil was then dried overnight at 105°C and reweighed, so that the dry weight could be recorded. Percent soil is defined as follows:

$$\frac{\text{wt. (g) of dry soil}}{\text{wt. (g) of wet soil}} (100) = \% \text{ soil}$$

Another 5 g sample was transferred to a 50 mL centrifuge tube which contained 50 mL of 2 N potassium chloride (KCl). The tubes were capped and placed on a reciprocating shaker for one hour. The neutral potassium salt solution replaces the NH_4^+ and NO_3^- in the soil so that these ions are released into solution. The samples were then filtered and colorimetrically analyzed on a Scientific CFA200 Auto-Analyzer, using an indophenol method for NH_4^+ and a cadmium reduction method for NO_3^-.

Microbial Studies

Soil samples for the microbial studies were stored at 4°C for 10 to 11 days before being processed. Samples may be stored in this manner for 7 to 14 days without significant alterations of their biological properties (79). The three replicates for each treatment were composited to form one sample for analysis.

Samples for total microbial population assays were prepared according to the methods outlined in "Cultural Methods for Soil Microorganisms" by Wollum (79) for non-rhizosphere samples. For each composited sample, 10 g of soil were placed in 95 mL of a sterile, peptone diluent along with 15 to 20, glass beads (2 mm diameter). Once capped, the bottle was shaken using a windmilling motion approximately 200 times. Just before use, the bottle was shaken vigorously and 10 mL were aseptically transferred to another 90 mL of the sterile peptone solution. This dilution process was continued until a dilution of $1:10^{-6}$ was reached.

Standard plate count agar was prepared, sterilized, and poured into 15 mm by 100 mm Petri plates. Aliquots of 0.1 mL from each of the four highest dilutions were transferred to three of the petri plates and spread with a sterile glass spreader. Three plates for each of the four dilutions were prepared. The plates were inverted and incubated for seven days in the dark at 25° to 28°C. Colonies were counted at the end of the seven day period.

Because the Allied waste had a high organic N content, a healthy population of nitrifying bacteria would be needed to facilitate the transformation of organic-N to NO_3^-, which could then be utilized by the plants. Cultures of nitrifying bacteria, however, are slow growing, sparse in numbers and susceptible to contamination. In an attempt to determine if the nitrifying bacteria were affected by the treatments the density of NH_4^+ and NO_2^- oxidizing bacteria in the soils was measured by means of a most probable number method described by Schmidt and Belser (63).

Ten g of composited soil were added to a blender along with 95 mL of sterile, 1 mM phosphate buffer and blended for 30 to 60 seconds. The blended sample was transferred to a sterile bottle and shaken vigorously. Ten mL were then aseptically transferred to a sterile dilution bottle with 90 mL of phosphate buffer. This dilution procedure was repeated until a dilution of $1:10^{-5}$ was reached. Ammonium and NO_2^- oxidizers were enumerated by transferring one mL aliquots of each dilution to five tubes containing an appropriate medium for NH_4^+ and

NO_2^- oxidizers (Table 7)(63). Once inoculated, the tubes were incubated for a period of 10 weeks.

Weekly spot checks were made of each tube during the last three weeks to check for the appearance of NO_2^- in the NH_4^+-oxidizer tubes (a positive result) and the disappearance of NO_2^- in the NO_2^--oxidizer tubes (a positive result) using a diazotizing reagent and a coupling reagent. The titer of bacteria in the samples was then estimated using a most probable number (MPN) table (2).

Phase III

Phase III studies were designed based on the following concepts which were derived from Phases I and II:

1. Wastewater with the AM agent appeared to be more toxic than the AM agent alone.
2. Sodium lauryl sulfate appeared to be quite toxic to the plants.
3. Metasol did not appear to adversely affect the plants. Work with Metasol therefore, was discontinued.

Another consideration in the design of the experiments was that if the decision was made to land-apply the wastewater from the new finishing operations, it would not be directly applied, but rather would first be mixed in the holding ponds with the wastewater from the rest of the plant and then be applied through the main irrigation system. Allied projected that a realistic ratio of existing wastewater flow to the flow from the new finishing process would be 99.5 to 0.5. There was

Table 7. Medium for nitrifier determinations.

Chemical Constituent	Concentration of stock solution, g/100 mL	Stock Solution required (mL) per liter of media	
		NH_4^+ oxidizer	NO_2^- oxidizer*
$(NH_4)_2SO_4$	5.0	10.0	--
KNO_2	0.85	--	1.0
$CaCl_2 \cdot 2H_2O$	1.34	1.0	1.0
$MgSO_4 \cdot 7H_2O$	4.0	1.0	5.0
Bromothymol blue	0.04	5.0	--
K_2HPO_4 (0.2M)	3.48	--	4.0
KH_2PO_4 (0.2M)	2.72	7.5	1.0
Chelated iron		1.0	1.0
$FeSO_4 \cdot 7H_2O$	0.246		
EDTA disodium	0.331		
Trace Elements		1.0	1.0
$NaMoO_4$	0.01		
MCl_2	0.02		
$CoCl_2 \cdot 6H_2O$	0.0002		
$ZnSO_4 \cdot 7H_2O$	0.01		
$CuSO_4 \cdot 5H_2O$	0.002		

*$CaCl_2 \cdot 1H_2O$ and $MgSO_4 \cdot 7H_2O$ were combined, autoclaved separately, and added aseptically to a sterile solution of remaining ingredients.

also some concern that certain degreasers might have contaminated wastewater from the new finishing operations. Based on this and the information from the previous greenhouse studies, the treatments listed in Table 8 were used.

For this greenhouse study the grass plants were started from seed using soil from Allied's irrigation plots. Soil (1025 g) was added to plastic pots, which were approximately 15 cm (6 inches) deep and 13 cm (5 inches) in diameter. Twenty-five mL of the nutrient solution used in Phases I and II were added to each pot, and then the pots were watered to 80% of field capacity with distilled water. Fescue grass (Festucia arudinaceae) seeds (approximately 60-65) were distributed over the surface of each pot, covered with 20 g of soil and then moistened with water applied as a mist. The pots were sprayed twice daily until the seeds germinated. Once germinated, the plants were given a dose of the nutrient solution and watered to 80% of field capacity on a daily basis. The pots were maintained for 89 days prior to dosing and received a total of 0.223 g of N, 0.445 g of P_2O_5, and 0.223 g of K_2O.

The treatments were applied at a rate of 616 Kg N/ha/yearwhich was based on the assumption of a 200-day growing season per year. Total Kjeldahl nitrogen concentrations were determined for the AM wastewater, the Pond water, the Finish with and without the AM agent, but they were estimated for the AM agent (see Table 9).

A note should be made of the weak nature of the AM wastewater used in this phase as compared to the sample used in Phases I and II. The Phase III wastewater was less than one-fifth the strength of the first

Table 8. Treatments applied to fescue grass in Phase III

Treatment	Concentration (%) or Ratio
AM Wastewater	100
	75
	50
	25
Pond Wastewater (supplied by Allied) (no AM agent or finish)	100
	75
	50
	25
Pond Wastewater:AM Wastewater	99.5:0.5
	90:10
	80:20
	60:40
Finish with AM agent	100
	75
	50
	25
Finish without AM agent	100
	75
	50
	25
AM agent	1.00
	0.75
	0.50
	0.25

Table 9. TKN values of Phase III treatments.

Treatment	TKN (mg/L)
AM wastewater	47
Pond water	615
Finish with AM agent	941
Finish without AM agent	483
AM agent (1%)	229

wastewater sample. However, by basing the application rate on TKN and not applying on a volume basis alone, it was believed that the effect of the different wastewater strengths was minimized.

The pots for the 100% treatments (the Pond, AM wastewater mixtures, and 1% AM agent) received the 616 Kg N/ha/year loading rate on the basis of N in the samples. The diluted treatments were applied on a volume basis equal to the 100% rate, and nitrogen levels were supplemented by the addition of the N solution used in Phases I and II, in quantities that equaled 616 Kg N/ha/year.

The plants were dosed Monday through Friday with the treatments, and the N solution was added when necessary as in the previous studies. The treatments were added to both the vegetation and the soil, followed by a rinse of distilled water to provide for 80% of field capacity. On Saturday and Sunday the plants received 25 mL of the no-N nutrient solution (Table 5) and were watered to 80% of field capacity.

The pots were dosed on 15 days over a period of 19 days. At the end of that period the pots were examined and then processed for tissue and soil analyses. The tissue was analyzed for TKN, P, Ca, Mg, and K levels. The soil analyses included pH, P, K, Ca, Mg, soluble salts, NH_4^+, NO_3^-, and TKN. A portion of the soil sample was also used for a short-term, Lumac soil microbial study. (See later section.)

Tissue Analysis

The dry weight of the tissue was determined by cutting the vegetative matter even with the top of the pot, placing it in paper bags, and drying the sample bags in a 70°C oven overnight. The dried

tissues were then ground with a Thomas Wiley Mill (Model ED-5, Philadelphia, PA) to ensure the production of a more uniform sample for the chemical analysis.

A 0.2 g sample of the ground tissue from each pot was analyzed for TKN. The sample was transferred to a 50 mL digestion tube, along with a mercury catalyst. After mixing, 3.0 mL of concentrated H_2SO_4 were added to each tube. The tubes were mixed and placed into a digestion block and heated to 400°C. When the block temperature reached 200°C, it was assumed that all of the water had evaporated, and small funnels were placed in the tubes to facilitate a refluxing action. The samples were refluxed for 1.5 hours. After removing the tubes from the block, water was carefully added to the tubes to bring the volume to 50 mL. The tubes were capped, mixed, and allowed to settle for several hours. The supernatant in the tubes was then analyzed colorimetrically for NH_4^+ with an autoanalyzer as described in the Phase II section.

A dry-ashing procedure was used in the preliminary preparation of the tissues for the remaining chemical analyses. A 0.5 g sample of the ground tissue was transferred to a 30 mL glass beaker and then placed in a muffle furnace at 450°C for 5 hours. Once cool, 5 mL concentrated HCl were added to each beaker and allowed to stand for 30 minutes. Ten mL of water were then added to each beaker and allowed to stand an additional 20 minutes. At the end of this period, each sample was transferred to a graduated test tube and diluted to 50 mL. The tubes were capped, centrifuged for 10 minutes at 30 rpm, and the sample was

allowed to settle overnight. The supernatant was used in the actual testing.

For the analysis of P a 0.5 mL sample of the supernatant was mixed with 49.5 mL of water in a graduated test tube. Standard solutions were prepared by adding appropriate amounts of a 5.0 mg/L KH_2PO_4 standard to the test tube and diluting the solution to 50 mL. A sulfur-molybdate color reagent was added to each tube, mixed well, and the sample was allowed to stand for 15 minutes. Absorbance at 880 nm was determined for each sample using an Hitachi Spetrophotomer.

The tissue samples were analyzed for K by diluting 0.5 mL samples of the supernatant and 5 mL of a 10,000 mg/L NaCl solution to 50 mL with water. The NaCl solution was added to overcome interferences related to ionization that can occur with K. The samples were analyzed with a Model 503 Perkin-Elmer atomic absorption spectrophotometer (AAS) with an air-acetylene flame.

Calcium and Mg were determined from the same sample. A one mL sample was placed in a 50 mL graduated test tube along with 5 mL of lanthanum chloride ($LaCl_3$). The $LaCl_3$ was prepared by dissolving 58.0 g of La_2O_3 in 500 mL of concentrated hydrochloric acid (HCl) and diluting the mixture to 1 L with water. The La controls interferences from silicon (Si), aluminum (Al), phosphate (PO_4), and sulfur (S). Each of these agents depresses the sensitivity of the test for Ca, whereas Si and Al create interferences during the analysis of Mg. The samples were then diluted to 50 mL and analyzed by means of AAS using an air-acetylene flame.

Soil Analysis

The soil was prepared for analysis by first passing the soil through a No. 10 screen to remove any remaining vegetation and to create a more uniform sample. Part of the soil was placed in a container supplied by the Virginia Tech Soils Testing Lab. A second part was placed in a sterile, plastic bag and refrigerated at 4°C. A third portion was also placed in sterile bags, but was frozen.

The Virginia Tech Soils Testing Lab analyzed the soils for pH, P, K, Ca, Mg, and soluble salts. The frozen samples were used for N analyses, specifically TKN, NO_3^-, and NH_4^+. The refrigerated sample was held for short term bioassays with a Lumac instrument (to be described).

Ammonium and NO_3^- levels were determined as in Phase II. The TKN procedure for soils is similar to the tissue method. The samples were first allowed to air dry to remove as much moisture as possible prior to digestion. A 0.5 g sample was placed in a digestion tube. From this point, the method was exactly the same as the tissue TKN determination.

Short-Term Toxicity Studies

The main objective of these studies was to determine if a short term bioassay could be used to predict the plant toxicity of the various substances. These studies were prompted by the fact that greenhouse studies are quite time consuming and expensive, and could not be used by Allied to monitor sudden waste stream changes.

Lumac Microbial Studies

The Lumac instrument, manufactured by the 3M Corporation, measures the fluorescence of a luciferin-luciferase extract in the presence of adenosine triphosphate (ATP), the high-energy compound present in living cells. By measuring changes in ATP, one might have a good index of the toxic effects of a treatment. The versatility of the Lumac technique lies in the fact that it can be adapted to any type of living cell and any luminescent reaction that produces light between 300 and 900 nm. Two sources of ATP were used for this study: the first was the refrigerated soil samples from the Phase III greenhouse study, and the second was activated sludge from Virginia Tech's pilot plant on Allied's property.

The soil samples were held for 10 days at 4°C. Samples for each dilution considered were composited by mixing equal parts (volume basis) of soil from each of three replicate pots. Nine mL of tris-EDTA buffer (3M product) were added to a 1 g sample of the composite, and the suspension was blended for one minute. One hundred µL of the blended mixture was then added to a cuvette with 100 µL of a nucleotide releasing agent for bacterial cells obtained from 3M. The sample was mixed and after 20 seconds was placed into the counting chamber of the Lumac. One hundred µL of the luciferin-luciferase extract was automatically injected into the cuvette. The instrument integrated the signal over a ten second interval and the final reading was taken from the display as relative light units (RLU's). This process was repeated three times for each composite sample.

The reading was corrected for variations in the weight and moisture content of the mixed sample. Percent soil was determined as mentioned earlier. The final values reported were calculated by using the following expression:

$$\frac{\text{relative light units}}{(\text{sample weight} \times \% \text{ soil})} = \text{relative light units/g soil}$$

The activated sludge from the Allied plant was chosen over a municipal sludge because the microorganisms at the pilot plant were already acclimated to the existing wastewater. Therefore, the pilot plant would better mimic the conditions that the AM wastewater would create in a biological system being used to treat Allied's industrial waste.

Samples were taken directly from the aeration basin. One hundred mL of mixed liquor were placed directly into 250 mL flasks and aerated continuously. Five flasks, a control and four dilutions of a given solution, were used in each trial (Table 10). One mL of a given dilution for each sample was added to flasks, except in the case of the AM wastewater where two mL of sample were needed to obtain a response.

After dosing, a 0.1 mL sample was taken from each flask at 5, 20, 40, and 60 minute intervals. Each 0.1 mL sample was diluted with 4.9 mL of tris-EDTA buffer (3M product) and then sonicated using a Biosonik III sonicator (Bronwill) for 15 seconds. One hundred µL of the sonicated sample was pipetted into a cuvet and 100 µL of the nucleotide releasing agent was added. The sample was mixed and after 15 seconds, the cuvette

Table 10. Treatments applied to activated sludge.

Treatment	Concentration % or ratio
AM Wastewater	100
	75
	50
	25
Pond Wastewater	100
	75
	50
	25
AM agent (1% solution)	100
	75
	50
	25
AM Finish with the AM agent	100
	75
	50
	25
AM Finish without the AM agent	100
	75
	50
	25
Pond Wastewater:AM Wastewater (on a volume basis)	99.5: 0.5
	90 :10
	80 :20
	60 :40

was placed into the counting chamber of the Lumac instrument. The machine injected the luciferin-luciferase extract into the cuvette. The ten second integrated signal was recorded as relative light units (RLU's).

Microtox Toxicity Analysis

As described previously, the Microtox Toxicity Analyzer assesses the toxicity of a sample based on the change in light emission by a marine photobacterium resembling Photobacterium phosphoreum. The instrument has a precooling well for the reconstituted bacteria, fifteen wells for sample preparation, and a turret well where the actual light reading is taken. The arrangement is shown in Figure 7 (9).

The treatments used in this analysis are listed in Table 11. Samples were prepared by first adjusting the salt concentration to 2% NaCl to maintain the osmotic balance of the luminescent bacteria. Temperature of the wells was adjusted to 15°C. Microtox diluent (1.5 mL) was added to cuvettes in wells A1 to A4 and 0.5 mL of the diluent was added to cuvettes in wells B1 to B5 and C1 to C5. A 1.5 mL sample was added to cuvettes A4 and A5. Cuvette A4 was mixed and then 1.5 mL of A4 was transferred to A3. A3 was mixed and 1.5 mL of A3 was then transferred to A2. After mixing, 1 mL of A2 was discarded. A1 contained the control.

The reconstituted bacteria (10 µL) were added to cuvettes B1 to B5 and C1 to C5 and mixed. After a fifteen minute stabilization period, readings were obtained for cuvettes B1 to C5. The bacteria were then challenged with the diluted sample by transferring 500 µL from A1 to B1

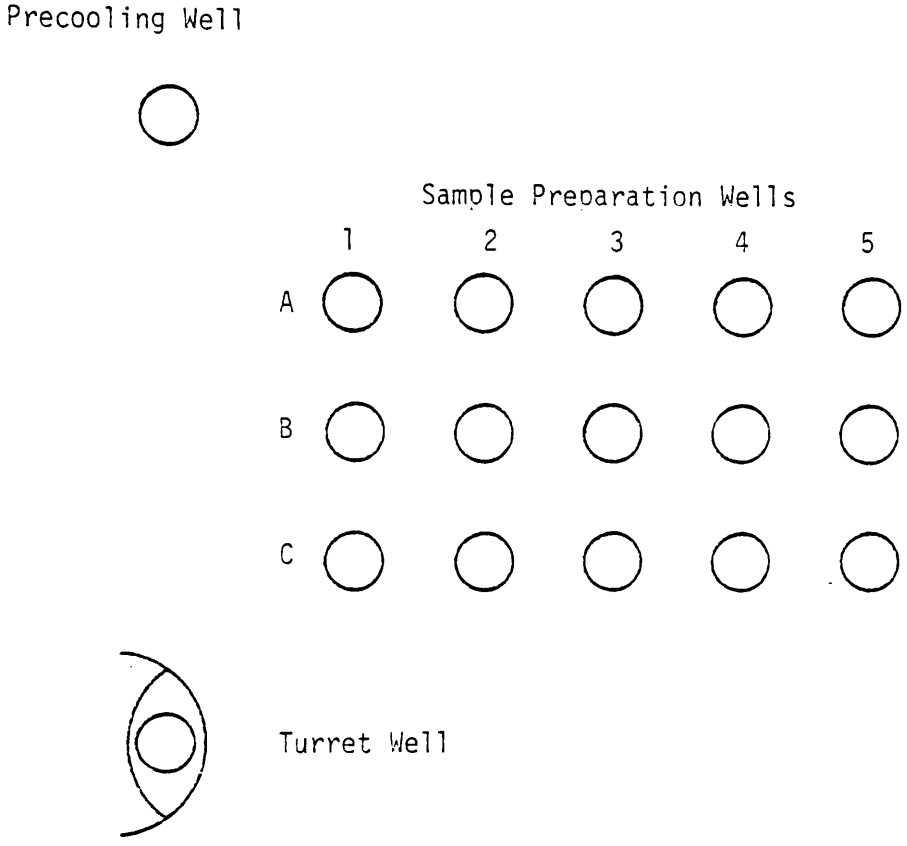


Figure 7. Well arrangement for microtoxicity analyzer.

Table 11. Treatments used in the Microtox analysis

Treatments	Concentration % or ratio
AM Wastewater	100
Pond Wastewater	100
AM Agent	1
AM Finish with the AM agent	100
AM Finish without the AM agent	100
Pond Wastewater:Am Wastewater	99.5:0.5

and 500 µL from A1 to C1, repeating the transfer procedure for the other replicates (A2 to B2, A2 to C2, etc.) After the transfer, light readings were taken at five minutes and fifteen minutes.

Five minute, blank ratios were calculated for cuvettes B1 and C1 (the controls) using the following formula (76):

$$R(t) = \frac{I(t)b}{I(0)b}$$

where, $R(t)$ = blank ratio at time t

$I(0)b$ = initial light reading for the blank cuvette

$I(t)b$ = final light reading at time t;

If R(t) for each cuvette, B1 and C1, differed by less than 0.02, then it was assumed that no spurious blanks occurred, and the test was considered valid. Otherwise, the test was discontinued. If the blank ratios were acceptable, the test was continued and the results calculated for each light reading and dilution using the following formulae (76):

$$\bar{R}(t) = \frac{R(t)B_1 + R(t)C_1}{R(0)B_1 + R(0)C_1}$$

$$\Gamma(t,T) = \frac{\bar{R}(t) * I(0)}{I(t)}$$

where:

$\bar{R}(t)$ = mean blank ratio at time t

$R(t)B_1$, $R(t)C_1$ = blank readings at time t

$R(0)B_1$, $R(t)C_1$ = blank readings at time 0

$I(0)$ = the initial light reading for any given test cuvette at zero time

$I(t)$ = the final reading for the corresponding test cuvette at time (t)

$\Gamma(t,T)$ = the Γ effect calculated for exposure time t at temperature T

The Γ value was plotted against concentration on log-log paper. The EC_{50} is defined as the concentration causing a 50% reduction in light at exposure time t and temperature T. A 50% light loss corresponds to $\Gamma = 1.00$.

Corn Seedling Bioassay

Table 12 lists the types and concentrations of agents considered in this portion of the project.

The corn seedling bioassay was performed based on a method proposed by Parker (58). Iowa Chief corn seeds (Zea mays) were germinated by placing the seeds between two sheets of filter paper in a Petri dish. Fifteen mL of distilled deionized water were added to the dish to saturate the paper. The plates were then placed in an incubator for 24 hours at 25°C in the dark. Silica sand was sifted through a number 10 mesh sieve to remove any extraneous debris and then dried at 110°C for 24 hours to remove excess moisture.

The dried sand was used to fill several Petri plates (15 mm by 100 mm). Twenty mL of a given solution were added to each plate, nine plates per dilution. Seven of the pregerminated seeds were placed in a row on the surface of the sand. All of the seeds were oriented, so that the embryos faced the upper plate and the radicles faced in the same

Table 12. Treatments used in Corn Seedling Bioassay

Treatment	Concentration (%) or Ratio
AM wastewater	100
	75
	50
	25
Pond water	100
	75
	50
	25
Pond: AM wastewater	99.5:0.5
	90:10
	80:20
	60:40
Finish with AM agent	15
	10
	5
	1
Finish without AM agent	15
	10
	5
	1

direction, perpendicular to the row. The plates were taped shut to hold the seeds in place and reduce evaporative losses.

The plates were divided into three test groups, three plates per test for the evaluation of shoot growth in the dark, shoot growth in the light, and root growth in the dark. All plates were initially placed in a dark, 24°C incubator. The root test group was incubated at a 15° angle from the vertical with the radicles facing downward to force the roots to grow along the surface of the plate. After 16 to 18 hours, the root tip positions were marked on the surface of the plate. After an additional 24 hours in the dark incubator, the tip positions were marked again. The difference between these two marks was recorded.

The shoot growth tests were performed with the plates tilted in the opposite direction, 15° from the vertical, to force the shoots to grow along the surface of the plate. Six plates per dilution were incubated at 24°C in the dark for 40 hours and then the position of the shoot tips were marked on the surface of the plate. Three of the plates were moved into the light and three remained in the incubator. The position of the shoot tips of the corn seeds in the dark incubator were marked again, after an additional 24 hours. The position of the shoot tips of the corn seeds in the light were marked again, after an additional 48 hours. The difference between the first and second set of marks for each seed was recorded.

IV. RESULTS AND DISCUSSION

Greenhouse Studies

Phase I

By the third day, all plants dosed with sodium lauryl sulfate (SLS) were showing signs of bleaching on the leaves. The effect was especially evident on the plants for treatments 4, 6, and 7. By the 10th day, the SLS and AM wastewater treatments had to be discontinued due to the poor condition of the plants.

When the experiment was discontinued after 21 days, a visual assessment of the percent of viable, green foliage remaining in the pots was made. The results are reported in Table 13. The treatments were ranked from worst to best based on this visual evaluation (see Table 14).

The Metasol in water appeared to stimulate growth, at least for the duration of this experiment. The low loading of AM agent produced some leaf tip burn and yellowing, but the effect was not extensive and was considered acceptable based on the visual analysis. For all of the rest of the treatments, the effects noted were considered unacceptable. All the other plants exhibited yellowing of the leaves followed by varying degrees of necrosis.

The SLS at the suggested neutralization rate for 1% AM agent (60 pts. SLS:1 pt. AM agent) caused severe bleaching and deterioration of the plants. The Metasol did not appear to negatively affect the plants.

Table 13. Percent of viable foliage remaining after 21 days as compared to control plants in Phase I.

Treatment	Loading Rate Kg N/ha/year	Percent Viable			
		Rep A	Rep B	Rep C	Avg*
1. 1% AM in H_2O	308	90	75	80	82
	616	75	65	65	68
	1232	50-55	45	40	46
2. 1% AM in H_2O + SLS (SLS:AM = 60:1)	308	20-25	5	5-10	12
	616	15	3	3	7
	1232	1	1	1	1
3. AM Wastewater	308	40	45-50	20-25	37
	616	20	40	20-25	28
	1232	20	30	5	18
4. AM Wastewater and SLS	308	15	15	7	12
	616	10	6	2	6
	1232	3	1	1	2
5. AM Wastewater 1% AM	308	35-40	45-50	30	38
	616	30	30	10	23
	1232	20	10	5	12
6. SLS	616	1	2	3	2
7. AM Wastewater, 1% AM , SLS,and 1 mg/L Metasol	616	5	1	3	3
8. AM Wastewater and 1 mg/L Metasol	616	15	40-50	12	19
9. 1 mg/L Metasol and H_2O	616	100	100	100	100

* in the case of ranges, midpoints were used

Table 14. Ranking of treatments from Phase I based on visual analysis.

WORST	AM Wastewater + SLS
	Metasol + AM Wastewater + AM agent + SLS
	SLS + H_2O
	H_2O + AM agent + SLS
	AM Wastewater + Metasol
	AM Wastewater
	AM Wastewater + AM agent
	AM agent + H_2O
BEST	Metasol + H_2O

Phase II

A visual assessment of the condition of plants in the Phase II trials was made after 44 days. The results of this evaluation are provided in Table 15. The treatments continued from Phase I were not included in this visual assessment as they were unchanged in appearance from the first assessment.

Plants dosed with AM agent and at the two lower levels of SLS, 0.02% and 0.18%, were not significantly affected. The rest of the treatments, however, resulted in unacceptable damage to the plants.

At the highest SLS level considered (1.76%) rinsing appeared to help, but the treatment still produced an unacceptable effect. All of the AM wastewater treatments were unacceptable, but rinsing did improve the appearance of the plants. It should be noted that application of a 50% concentration of the AM wastewater with a rinse produced essentially the same result as applying a 25% concentration of the AM wastewater without a rinse.

The Virginia Tech Soils Testing Laboratory analyzed the soil samples for pH, P, K, Ca, Mg, and soluble salts. Those results are presented in Table 16.

The pH levels of the soils were substantially increased by the addition of the AM agent, the AM wastewater and the SLS (no rinse) treatments. The SLS rinse treatments resulted in increased soil pH values, but the increases were not as great as those in the other treatments. In the Phase I studies, where the control had a pH of 7.1, the AM agent, and Metasol treatments slightly decreased soil pH.

Table 15. Visual assessment of plant conditions after Phase II treatments.

Plant Treatments		Extent of Damage[a]					
		Rep. A		Rep. B		Rep. C	
Substance	Level, %	Rinse[b]	No Rinse	Rinse	No Rinse	Rinse	No Rinse
AM Agent	0.001		-[c]		-		-
	0.01		-		-		-
	0.1		-		-		-
Sodium laury1 sulfate (SLS)	0.02	-	-	-	-	-	-
	0.18	-	-	-	-	-	+
	1.76	+	++	+	+	+	++
AM Wastewater	100	+++++		+++++		+++++	
	75		++++		++++		++++
	50	++	+++	++	+++	++	+++
	25		++		++		++

[a]Harmful effects were ranked over a range of being slight with one plus sign (+) to being severe with five plus signs (+++++).

[b]Plants rinsed with distilled water after test compound applied.

[c]A minus sign (-) indicates no harmful effects were observed.

Table 16. Chemical analysis of various soil cores from Phase II. (All values expressed in mg/kg except for pH values.)

Treatment	pH		P		K		Ca		Mg		Sol. Salts	
	*Rep	Avg	*Rep	Avg	*Rep	Avg	*Rep	Avg	*Rep	Avg	*Rep	Avg
	Phase II Samples											
AM agent, 0.001%	6.6		37		26		1020		108		179	
	6.3	6.23	43	40	37	27	708	800	68	78	166	162
	6.0		40		17		672		57		141	
AM agent, 0.01%	6.5		44		37		840		120		154	
	6.5	6.5	54	49	44	33	1008	944	120	120	141	150
	6.5		49		18		984		120		154	
AM agent, 0.1%	6.7		55		44		1200		120		141	
	6.5	6.65	51	51	26	37	900	1040	120	120	154	150
	6.8		48		40		1020		120		154	
AM Wastewater 25%, no rinse	7.1		49		82		1056		120		192	
	7.1	6.92	53	51	75	62	996	936	120	108	282	226
	6.7		50		28		756		83		205	
AM Wastewater 50%, no rinse	7.5		60		99		1200		120		333	
	7.3	7.16	60	59	91	89	1080	1052	120	109	333	312
	6.9		57		77		876		87		269	
AM Wastewater 50%, rinse	7.3		60		83		1068		120		358	
	7.3	7.3	51	56	96	87	996	1048	120	120	320	328
	7.3		58		82		1080		120		307	
AM Wastewater 75%, no rinse	7.4		52		85		960		120		384	
	7.3	7.36	49	51	77	84	816	896	113	118	397	371
	7.4		51		91		912		120		333	

(Continued)

Table 16. (Continued)

Treatment	pH *Rep	pH Avg	P *Rep	P Avg	K *Rep	K Avg	Ca *Rep	Ca Avg	Mg *Rep	Mg Avg	Sol. Salts *Rep	Sol. Salts Avg
	Phase II Samples											
AM Wastewater	7.4		60		90		924		120		486	
100%, rinse	7.3	7.39	43	52	78	88	660	832	73	104	448	461
	7.5		53		95		912		120		448	
SLS, 0.02%,	7.1		60		48		1200		120		192	
no rinse	6.4	6.49	54	54	28	36	948	1012	120	105	166	166
	6.3		49		33		888		74		141	
SLS, 0.18%,	6.6		59		75		1068		120		256	
no rinse	6.8	6.79	59	58	56	58	1140	1136	120	120	256	256
	7.1		55		42		1200		120		256	
SLS, 1.76%,	4.8		57		112		816		84		38	
no rinse	5.0	5.06	59	58	88	95	1044	964	120	108	1	94
	7.2		57		85		1032		120		243	
SLS, 0.02%,	4.7		52		66		756		81		1	
rinse	4.8	4.92	45	52	20	42	816	924	83	95	1	176
	8.4		60		39		1200		120		525	
SLS, 0.18%,	7.8		59		74		1140		120		858	
rinse	5.1	5.28	44	54	61	64	984	1108	120	120	422	649
	5.1		60		56		1200		120		666	
SLS, 1.76	5.0		47		48		636		61		154	
rinse	6.7	5.12	39	45	80	60	840	732	120	86	794	337
	4.9		48		51		720		78		64	

Table 16. (Continued)

Treatment	pH *Rep	pH Avg	P *Rep	P Avg	K *Rep	K Avg	Ca *Rep	Ca Avg	Mg *Rep	Mg Avg	Sol. Salts *Rep	Sol. Salts Avg
	Phase II Samples											
Control	3.8		60		55		1200		120		179	
	3.1	3.50	53	56	59	70	792	1024	75	105	474	265
	5.7		56		96		1080		120		141	
	Phase I Samples											
AM agent, 308	4.8		60		55		840		110		179	
kg N/ha/yr	7.9	5.24	60	60	53	52	900	804	117	105	205	188
	5.8		60		47		672		89		179	
AM agent, 616	5.8		60		44		780		96		678	
kg N/ha/yr	6.6	6.04	60	60	83	64	948	864	115	106	192	435
AM agent, 1232	6.4		60		26		888		120		102	
kg N/ha/yr	6.3	6.25	60	60	39	44	1008	988	120	120	64	77
	6.1		60		66		1068		120		64	
Metasol, %	6.7		60		59		1164		120		1	
1 mg/L	6.2	5.95	60	60	80	72	1104	1108	120	120	1	43
	5.6		60		78		1056		120		128	
Control	7.1		60		53		1200		120		154	
	7.1	7.1	60	60	59	56	1032	1116	120	120	179	166

*Replicate

All of the cores would be considered to have high P values according to Thomas and Peaslee (73). Except for a slight depression in P content at the 0.001% level AM treatment level and at the 1.76% SLS rinse treatment from 56 mg/kg P in the control soil to 40 mg/kg and 45 mg/kg, respectively, P remained stable. Soil Ca levels increased slightly with increasing AM agent concentration from 800 mg/kg at the 0.001% level to 1,040 mg/kg at the 0.1% level, and decreased slightly less than 100 mg/kg, with increasing wastewater concentration. All Ca levels, however, were within the 250 to 5000 mg/kg range reported to provide for no apparent deficiency or excess in plants (27). Magnesium levels were variable, but again an adequate supply, greater than 50 mg/kg, was available in all of the soils tested (27).

The soluble salt levels were all low in the Phase II soils according to accepted soluble salt limits (70). Salinity effects could be considered to be negligible. Increases in soluble salts could be seen, however, with increasing wastewater concentration from 226 mg/kg at 25% to 461 mg/kg at 100%. Increases in soluble salts with increasing SLS dosages was expected, but the variation between replications was too great to allow for a definitive statement about the response.

All soils also had sufficient K, but K appeared to vary the most of all parameters measured (27). Plants treated with the AM agent had somewhat depressed values of K, 27 to 37 mg/kg, as compared to the controls (70 mg/kg), but these plants exhibited the least amount of negative effects. Plant growth in the pots receiving wastewater applications was poor, but soil K levels were high and increased with

increasing wastewater doses. This level of K in the soil could be related to the extent of tissue growth. Most available K in the soil for the AM agent treatments was probably absorbed by the plants. The other treatments retarded plant growth, so K concentrated in the soil. However, this theory does not account for the high K levels found in the Phase II control pots and the pots treated with Metasol, a treatment continued from Phase I. These plants were healthy with no apparent signs of any deficiencies or toxic effects.

The Phase I plants, however, had been receiving the nutrient solution containing K twice as long as the Phase II plants, since the start of Phase I. It may be that K was applied in excess of the plant's requirement with the excess accumulating in the soil.

The NH_4^+ and NO_3^- concentration in the soil cores are presented in Table 17. Note that in all samples, the NH_4^+ concentration was higher than the NO_3^- concentration. The ratio of NH_4^+:NO_3^- is perhaps an easier form to use in evaluating the results. In the AM agent and AM wastewater treatments, a trend toward higher ratios was observed. The NH_4^+:NO_3^- ratios for controls and treatments for Phase I pots were very similar. Under normal conditions, the conversion of organic-N to NH_4^+ is the rate controlling step (12,25). The conversion of NO_2^- to NO_3^- is generally considered the fastest step (25), so NH_4^+ and NO_2^- are usually converted quickly to NO_3^- and do not accumulate in the soil (12). The accumulation of NH_4^+ present in the Phase II soils could indicate that 1) NH_4^+ was forming more rapidly than the nitrifying population could metabolize it; 2) the nitrifying population was inhibited and was not

Table 17. Soil nitrate and ammonia levels from treatments in Phases I and II. (All values reported in terms of mg/kg)

Treatment	NO_3^-	Avg	NH_4^+	Avg	NH_4^+/NO_3^-
	Phase II Samples				
AM agent, 0.001% in	2.1 2.5 2.8	2.5	7.9 10.5 10.7	9.7	3.95
AM agent, 0.01% in	2.7 2.4 2.2	2.4	15.7 16.3 21.6	17.9	7.37
AM agent, 0.1% in	2.5 2.9 2.7	2.7	16.5 20.4 11.4	16.1	6.00
AM Wastewater, 25%, no rinse	2.2 2.6 2.2	2.3	12.7 9.9 11.1	11.2	4.83
AM Wastewater, 50%, no rinse	3.5 2.8 2.0	2.8	10.7 14.2 6.5	10.5	3.80
AM Wastewater, 50%, rinse	2.2 1.9 1.6	1.9	14.4 15.6 13.2	14.4	7.70
AM Wastewater, 75%, no rinse	1.9 1.6 2.4	2.0	11.6 13.2 7.7	10.8	5.53
AM Wastewater,100%, rinse	2.0 1.4 2.4	1.9	8.3 7.2 2.7	6.0	3.14
SLS, 0.02%, no rinse	2.8 2.5 2.0	2.4	17.0 10.7 4.8	10.9	4.45
SLS, 0.18%, no rinse	3.9 3.9 3.1	3.6	4.9 4.8 5.1	4.9	1.36

(Continued)

Table 17. (Continued)

Treatment	NO_3^-	Avg	NH_4^+	Avg	NH_4^+/NO_3^-
			Phase II Samples		
SLS, 1.76%, no rinse	1.8 3.3 3.2	2.8	6.9 3.5 2.3	4.2	1.51
SLS, 0.02%, rinse	1.7 1.4 2.7	1.9	5.9 5.2 5.4	5.5	2.86
SLS, 0.18%, rinse	3.0 3.5 4.9	3.8	7.6 2.9 3.9	4.8	1.25
SLS, 1.76%, rinse	1.3 2.1 1.7	1.7	6.1 1.0	4.3 (w/o 1.0, avg 5.8 = 6.0)	3.53
Control	2.9 2.1 3.0	2.7	8.1 8.0 8.2	8.1	3.01
			Phase I Samples		
AM agent, 308 kg N/ha/yr	1.4 1.5 1.0	1.3	7.7 6.7 4.2	6.2	4.76
AM agent, 616 kg N/ha/yr	1.2 1.1	1.1	7.5 5.5	6.5	5.69
AM agent, 1232 kg N/ha/yr	1.5 1.4 1.2	1.4	6.4 7.5 7.9	7.3	5.34
Metasol (1 mg/L)	1.3 0.9 1.3	1.2	8.8 6.2 10.1	8.2	6.94
Control	1.1 1.5	1.3	6.4 9.5	8.0	5.98

metabolizing at normal rates; or, 3) the NO_3^- produced was being taken up by the plant, so that lower residuals were left in the soil.

The fact that NH_4^+ levels in the the controls were elevated suggests that perhaps some environmental factor was affecting nitrification negatively and/or the conversion of organic-N to NH_4^+ positively. The average temperature in the Phase II greenhouse studies was approximately 24°C. Optimum temperature for ammonium oxidation is 30 to 35°C (12). The lower temperature, 24°C, may have reduced the overall ammonium oxidation rate.

Another possible explanation concerns oxygen tensions in the soil. Excessive hydraulic loading could result in low oxygen tensions in the soil which would encourage denitrification (12). An hydraulic overload may have occurred due to a reduction in evapotranspiration caused by plant die-back or overcast days. If denitrification was occurring a decrease in soil NO_3^- levels would be expected. There was still a sufficient difference in the NH_4^+ to NO_3^- ratios between treatments, however, to suspect some other factors besides the environmental factors, such as a treatment effect, were involved.

The total numbers of microorganisms found in the soils of the Phase II treatments are provided in Table 18. The growth of microorganisms appeared to be stimulated somewhat by most of the treatments. Based on these data, however, it is not possible to state whether or not the treatments harmed the soil systems. If microbial numbers had been reduced to low levels, the effect would, of course, have been negative. Because the results obtained do not reflect the types and distribution

Table 18. Gross microbial numbers in the soil cores of Phases I and II.

Treatment	Concentration %	Population of Organisms (x 10^6 per gm)
	Phase II Samples	
AM agent	0.001	9.1
	0.01	23.2
	0.1	36.4
AM Wastewater, no rinse	25.0	39.1
	50.0	60.9
	75.0	75.7
AM Wastewater, rinse	50.0	54.5
	100.0	64.1
SLS, no rinse	0.02	23.9
	0.18	12.4
	1.76	21.2
SLS, rinse	0.02	3.1
	0.18	4.6
	1.76	6.2
Control		6.8
	Phase I Samples	
AM agent, 308 kg N/ha/yr	1.0	5.7
Control		5.6

of microbial populations, it is difficult to make judgements about the health of the soil environments. Many of the plates were also overgrown with a filamentous population (apparently fungi) making some of the counts questionable.

The test used to enumerate nitrifying populations (see Table 19) was a most probable number method which has a high amount of inherent statistical error. The results, however, can be used to evaluate relative changes in organism populations but not as an exacting measure of population changes.

The majority of the Phase II applications produced only moderate reductions in the NH_4^+ oxidizer populations. Only the higher wastewater applications produced NH_4^+ oxidizer values noticeably below the control level. The Phase I AM agent sample, however, produced a significant deterioration of that population. This may indicate that, over time, the AM agent disrupts the normal populations of NH_4^+ oxidizers. The NO_2^- oxidizing population, on the other hand, appeared to be adversely affected by all treatments. These low NO_2^--oxidizer levels would explain the low NO_3^- levels present in the soil samples. The transition from NO_2^- to NO_3^- was apparently impaired.

The results of the Phase II efforts indicated that:

1) With respect to soil extractable nutrients, pH, and soluble salts, the levels measured were not large enough or small enough to be considered detrimental to plant growth.
2) When applied at reasonable rates, the AM agent and Metasol were not toxic to the plants. With time, however, the AM agent may

Table 19. Nitrifying microbial populations in ten grams of soil from treatments in Phases I and II.

Treatment	Concentration, %	Nitrite Oxidizers	Ammonium Oxidizers
		Phase II Samples	
AM agent	0.001	2,700	17,000
	0.01	17,000	17,000
	0.1	7,900	14,000
AM Wastewater, no rinse	25.0	1,100	17,000
	50.0	1,700	79,000
	75.0	7,900	2,300
AM Wastewater, rinse	50.0	13,000	3,300
	100.0	2,100	7,000
SLS, no rinse	0.02	17,000	4,900
	0.18	340	4,900
	1.76	7,900	26,000
SLS, rinse	0.02	7,900	11,000
	0.18	5,600	33,000
	1.76	22,000	130,000
Control		33,000	26,000
		Phase I Samples	
AM agent, 308 kg N/ha/yr	1.0	7,900	3,300
Control		79,000	13,000

adversely affect ammonium and nitrite oxidizing microbial populations.

3) Toxicity due to SLS was observed at concentrations equal to or greater than 1.76%.

4) Wastewater retarded growth at all levels. It should be remembered, however, that toxicity effects may be more severe in greenhouse studies than under field conditions due to the extreme root proliferation and elevated transpiration rates encountered under greenhouse conditions.

5) A rinse cycle using uncontaminated water after a treatment application seemed to improve the condition of the plant.

Phase III

The results derived from visually examining the pots used in Phase III are provided in Table 20. As mentioned earlier, treatments were applied over a period of 19 days in this experiment. The most severe damage was seen with the Finish applications, both with and without the AM agent. These plants showed signs of bleaching, drying, and leaf tip burn.

The 50% AM wastewater applications and higher proved to be unacceptable. Plant leaves, as a result of the applications, were folded, browning, and either dead or dying. Plants treated with a 25% dilution of AM wastewater showed some of these signs, but the effects were considered acceptable.

All of the Pond water and the AM agent applications considered were acceptable. Some leaf-tip burn was evident on the 100 and 75% Pond

Table 20. Visual assessment of plant conditions after Phase III greenhouse treatments.

Treatment	Concentration (%) or Ratio	Result[a]
Pond	100	+
	75	+
	50	-
	25	-
AM Wastewater	100	+++++
	75	+++++
	50	+++
	25	++
Pond: AM Wastewater	99.5:0.5	+
	90:10	+
	80:20	+++
	60:40	++
Finish with AM agent	100	+++++
	75	+++++
	50	++++
	25	+++
Finish without AM agent	100	+++++
	75	+++++
	50	++++
	25	+++
AM agent	1.00	++
	0.75	+
	0.50	+
	0.25	-

[a]Effects ranked from minus(-) meaning no visible effect to five pluses (+++++) which indicates a severe effect. Visual effects of ++ or less were acceptable.

water treatments, possibly due to the presence of caprolactam, but the effect was not severe. The Pond to AM wastewater mixes were acceptable at the 99.5:0.5 and 90:10 rates. Leaf-tip burn was evident in both cases, however, and probably was also caused by the Pond water. Interestingly, the 80:20 mix was more harmful than the 60:40 mix, possibly suggesting that there was interaction among agents in the two media.

The tissue was analyzed for dry weight, N, P, Ca, Mg, and K (Table 21). A non-parametric regression analysis was performed by the Virginia Tech Statistics Department to identify significant differences in the results ($p \leq 0.05$). (See Appendix A for statistical results.) A significant decrease in dry weight was observed only with increasing AM wastewater concentrations. Tissue Ca and Mg were stable for all treatments except for a slight decrease in tissue Mg as a result of the Pond and AM wastewater mixture treatment ($p = 0.05$).

The AM wastewater treatments, the Pond and AM wastewater mixtures, and the Finish with and without the AM agent all reduced N in the plant tissue to a significant degree. Tissue P was reduced only by the AM wastewater treatment and the Finish with the AM agent.

Decreases in tissue K were seen as a result of the Pond:AM wastewater, the Finish with AM agent and the Finish without the AM agent treatments. The seemingly lower tissue K values, as a result of the AM wastewater treatments, were not statistically significant at the 0.05 level. The apparent increase in tissue K in the AM agent treatments also were not statistically significant.

Table 21. Tissue analysis of plants treated in Phase III (Average values are listed).

Treatment	Tissue wt,g	N%	P%	Ca%	Mg%	K%
Controls	2.18	3.07	0.583	0.46	0.633	3.53
Pond 25%	2.17	2.93	0.576	0.463	0.620	3.40
50%	2.13	3.27	0.598	0.456	0.623	3.70
75%	2.19	3.06	0.573	0.441	0.638	3.56
100%	2.06	3.26	0.592	0.442	0.593	3.53
AM Wastewater						
25%	2.13	2.97	0.570	0.382	0.580	3.37
50%	2.15	2.90	0.557	0.380	0.568	3.07
75%	1.95	2.73	0.549	0.367	0.560	3.03
100%	1.78	2.67	0.536	0.380	0.602	3.10
Pond:Wastewater						
99.5:0.5	2.21	3.00	0.584	0.425	0.628	3.60
90:10	2.16	3.10	0.586	0.422	0.615	3.53
80:20	2.30	2.90	0.587	0.428	0.623	3.43
60:40	2.15	2.80	0.585	0.405	0.580	3.40
Finish with AM agent						
25%	1.93	3.10	0.584	0.440	0.622	3.53
50%	1.95	2.90	0.587	0.523	0.647	3.50
75%	1.90	2.90	0.583	0.457	0.635	3.30
100%	1.96	2.83	0.563	0.427	0.610	3.30
Finish without AM agent						
25%	1.97	2.97	0.602	0.452	0.614	3.57
50%	1.81	3.03	0.580	0.437	0.617	3.23
75%	1.79	2.93	0.580	0.442	0.629	3.23
100%	1.87	2.73	0.573	0.408	0.600	3.03
1% AM agent						
0.25%	2.36	2.83	0.555	0.433	0.615	3.47
0.50%	2.23	2.80	0.549	0.433	0.637	3.63
0.75%	2.10	2.80	0.552	0.423	0.590	3.67
1.00%	2.22	2.73	0.547	0.443	0.612	3.67

In general, none of the nutrients in the tissue was low. The concentration of the nutrients in the tissue indicate that for most of the nutrients, the transport system through the roots was unaffected by the treatments. However, the stunted growth of tissues may have resulted in a concentration of nutrients in what little tissue was left.

The Virginia Tech Soils Laboratory analyzed the soil samples for pH, P, K, Ca, Mg, and soluble salts (SS). Additional tests were performed to determine TKN, NO_3^-, and NH_4^+ (Table 22).

All parameters measured by the Soils Lab were within acceptable limits (12,27,73). A non-parametric regression analysis, performed by the Virginia Tech Statistics Department, pointed to some significant correlations at the 0.05 level (See Appendix A). Soil pH increased as a result of increasing AM wastewater concentration and Pond water concentration. In all other treatments no significant pH change was noted. Soil Ca levels were increased by the Pond water application, but were decreased by the AM wastewater application. In the soils receiving the Pond:AM wastewater mixture, however, no significant increase or decrease was detected suggesting that each of these treatments neutralized the effect of the other on soil Ca. Significant increases in soluble salts were seen with increasing concentrations of AM wastewater, AM agent, and Finish with and without the AM agent, but the soluble salts were still extremely low for all treatments and should not have adversely affected the plants. No significant effects to the soil P and Mg were noted with any of the treatments.

Table 22. Chemical analysis of soil cores from Phase III (Average values expressed in mg/kg except for pH values).

Treatment	pH	P	K	Ca	Mg	SS	TKN	NO_3^-	NH_4^+
Control	6.04	51	38	547	120	181	1257	1.2	3.3
Pond 25%	6.19	52	36	508	120	192	1263	1.2	3.2
50%	6.42	51	38	548	120	145	1297	1.2	3.0
75%	6.36	52	32	556	120	175	1277	1.0	2.6
100%	6.45	52	35	568	120	154	1283	1.0	2.6
AM Wastewater									
25%	6.73	54	61	576	120	294	1273	1.1	2.9
50%	7.10	58	66	564	120	371	1317	0.8	3.2
75%	7.23	55	64	548	120	512	1260	0.8	3.0
100%	7.50	58	94	532	120	520	1263	0.8	3.5
Pond:AM Wastewater									
99.5:0.5	6.52	51	37	516	120	209	1220	0.5	1.7
90:10	6.40	51	39	219	120	154	1270	0.5	1.5
80:20	6.46	53	41	536	120	205	1300	0.7	2.4
60:40	6.45	52	41	548	120	183	1287	0.6	2.5

(Continued)

Table 22 (Continued)

Treatment	pH	P	K	Ca	Mg	SS	TKN	NO_3^-	NH_4^+
Control	6.04	51	38	547	120	181	1257	1.2	3.3
Finish with AM agent									
25%	6.43	53	38	548	120	286	1243	0.8	1.9
50%	6.32	46	43	564	120	205	1297	1.0	2.3
75%	6.28	47	52	552	120	290	1230	0.7	2.3
100%	6.16	53	52	520	120	568	1317	0.6	2.6
Finish without AM agent									
25%	6.22	49	38	528	120	183	1360	0.6	2.1
50%	5.79	52	48	560	120	273	1260	0.7	2.1
75%	5.69	51	49	516	120	256	1320	0.7	2.0
100%	5.99	51	57	520	120	315	1297	0.8	2.1
1% AM agent									
0.25%	6.20	52	36	580	120	158	1240	1.6	3.5
0.50%	6.23	55	41	580	120	222	1253	2.2	3.8
0.75%	6.19	50	37	556	120	188	1210	1.4	1.9
1.00%	6.29	52	37	556	120	256	1173	1.1	2.3

As in the Phase II studies, soil K was significantly elevated with increasing concentrations of AM wastewater, the Pond:AM wastewater mixtures, Finish with the AM agent and Finish without the AM agent. The AM agent and Pond water treatments did not significantly affect soil K levels.

The elevated K levels in some of the soils represent a reverse of what was found in the tissue. Figures 8 through 10 illustrate the decrease in tissue K with a concomittant increase in soil K for the AM wastewater and the two Finish treatments. Figure 11 depicts the increase in tissue K for the AM agent treatment, but shows a relatively constant soil K. This would seem to substantiate the observation made in Phase II that due to poor tissue growth K concentrated in the soils of the AM wastewater and Finish treatments. The AM agent treatments exhibited better plant growth and had lower soil K levels because the K was being translocated to the plant tissues.

The soil nitrogen analyses were also subjected to a non-parametric regression analysis. (See Appendix A.) Nitrate levels were reduced significantly by the Pond water, AM wastewater, the Finish with the AM agent, and the AM agent alone. The Pond and AM wastewater mixtures reduced NO_3^- when compared to the controls but produced a significant increase in NO_3^- from the 99.5:0.5 application to the 60:40 application. Overall, the soil NO_3^- values were low, as in the Phase II studies.

Ammonium levels were higher than the NO_3^- levels in the soils, but were low in comparison to the Phase II soil NH_4^+. Ammonium levels were reduced significantly by the Pond water and the AM agent applications.

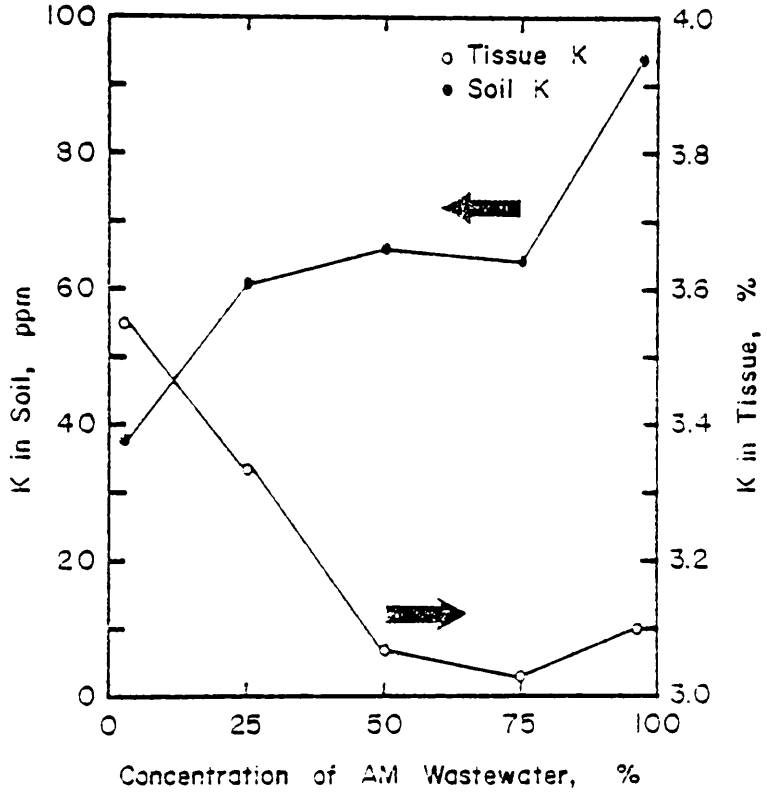


Figure 8. Potassium levels in soil and plant tissue as a function of the AM wastewater.

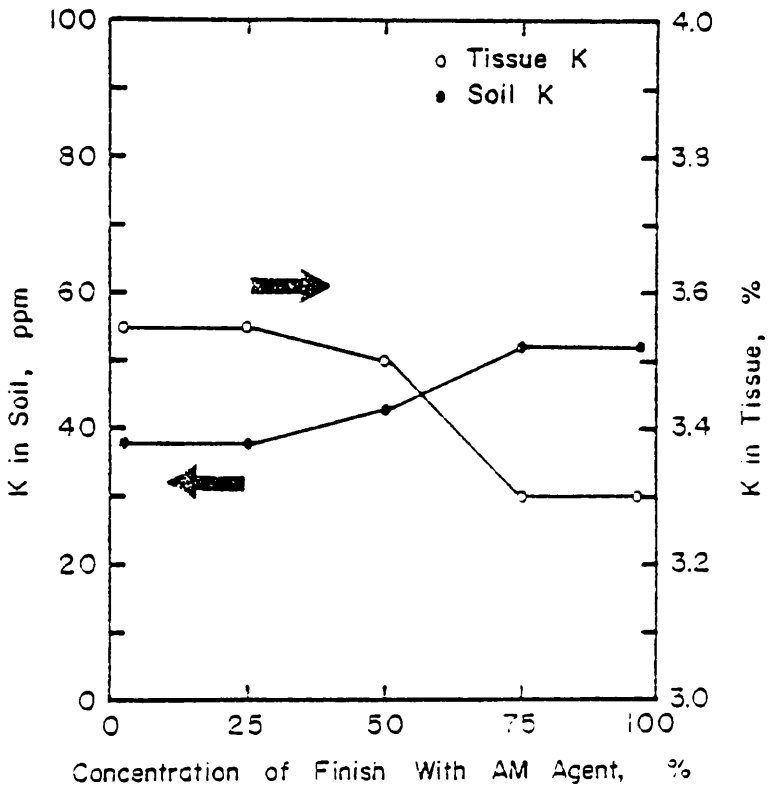


Figure 9. Potassium levels in soil and plant tissue as a function of the Finish with the AM agent.

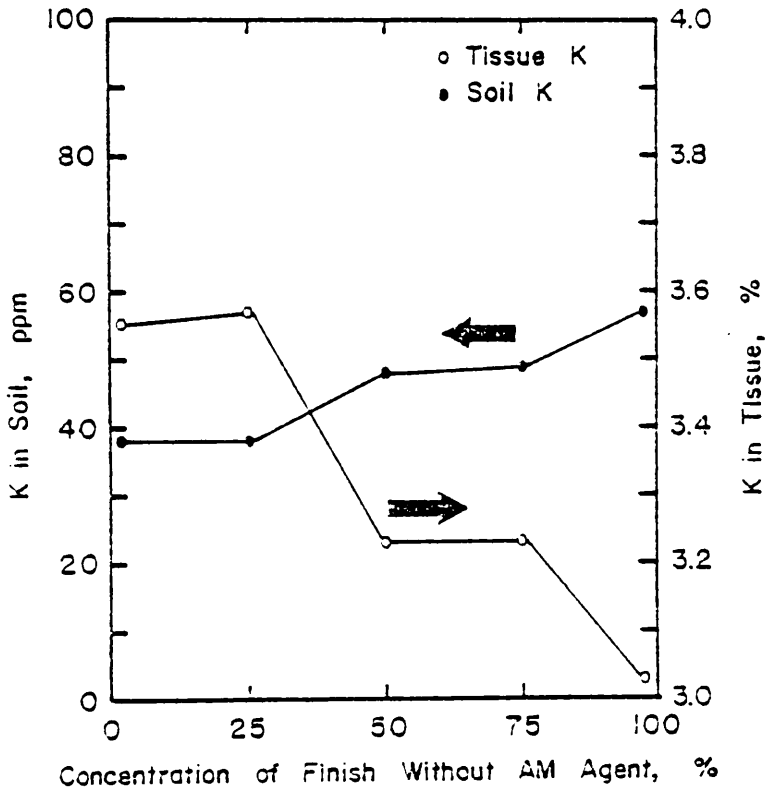


Figure 10. Potassium levels in soil and plant tissue as a function of the Finish without the AM agent.

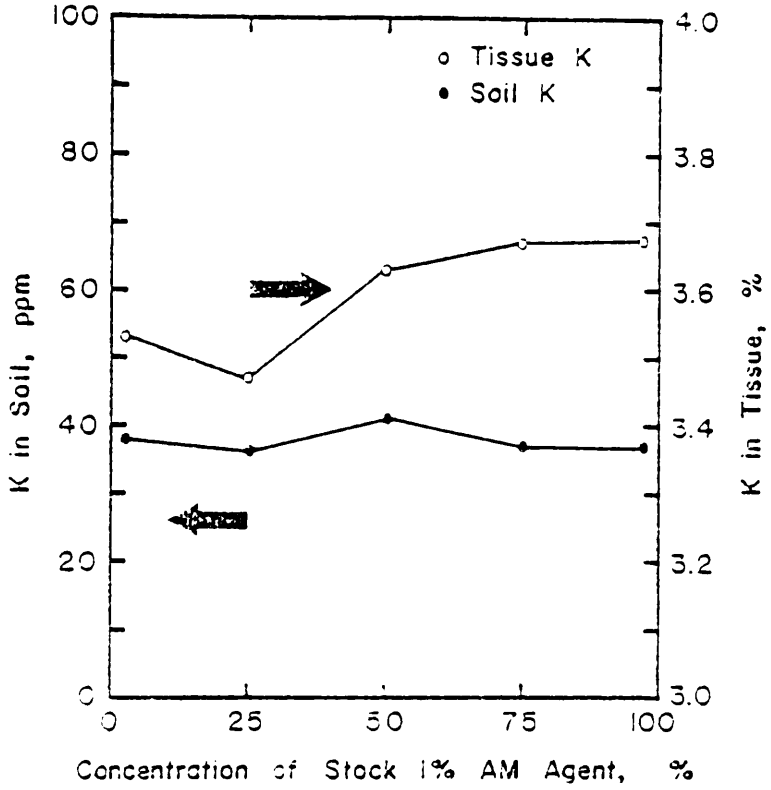


Figure 11. Potassium levels in soil and plant tissue as a function of the AM agent.

Soils treated with Finish containing the AM agent had increased NH_4^+ values as did the soils treated with Pond and AM wastewater mixtures. The Finish without the AM agent produced no significant variation in NH_4^+ between application rates, but consistently lowered the NH_4^+ levels by 1.3 mg/kg from the control values.

In general, the NO_3^- levels were lower than the NH_4^+ levels which suggests that the nitrifying population was inhibited to some degree, as was indicated by the Phase II studies. The possibility of denitrification occurring due to low oxygen tensions in the soil may also explain the low NO_3^- values.

Soil TKN data were mostly statistically insignificant. Increases in soil TKN were noticeable with all treatments but the AM agent, which decreased soil TKN. This decrease in soil TKN by the AM agent was the only significant change in TKN produced by the Phase III treatments.

Based on the results of this study several points can be made:

1) The difference in plant effects caused by the finish with and without AM agent was minimal. AM agent at the 1% application level did not appear to adversely affect the plants.
2) Potassium levels in the soil and plant tissues and NO_3^- in the soil appeared to be the chemical factors most influenced by the treatments.
3) The Pond water appeared to have minimal impact on the grass. The 99.5:0.5 projected flow ratio of Pond to AM wastewater should be acceptable, assuming the wastewater used in the Phase III work

was a representative sample. These statements are obviously contingent upon the samples being applied at the rates considered herein, 616 kg N/ha/yr with 308 kg N/ha/yr being recommended to allow for a safety factor.

Short-Term Toxicity Studies

Lumac Microbial Studies

The results of the Lumac studies performed on the soil microbial population from the Phase III greenhouse study are presented in Table 23. Pond water appeared to stimulate the growth of the soil microbes judging by the apparent increase in relative light units (RLU) with increasing Pond water concentration. This stimulatory response may have been due to a biological population within the Pond water acting as acclimated seed organisms when applied to the soil. This theory is supported by the fact that biological degradation of caprolactam, the main component of the Pond water, is well-documented (5,34,35).

All other treatments exhibited depressed RLU's as compared to the controls. Increasing AM wastewater concentrations increasingly depressed the RLU's to a statistically significant degree ($p < 0.05$) (See Appendix A).The 75% AM wastewater concentration produced an RLU = 0.0. The Pond and wastewater mixtures produced an unexpected result. The 99.5:0.5 mix was highly toxic to the soil microbes (reduced RLU's by 0.153 from the control) whereas Pond water, the major component of the mixture, stimulated microbial growth by up to 0.244 RLU's above the control level when applied separately. The AM wastewater, alone,

Table 23. Microbial analysis of soil from the Phase III greenhouse study using the Lumac device.

Treatment	Concentration (%) or ratio	Adjusted Relative Light Units
Pond	25	0.323
	50	0.273
	75	0.262
	100	0.411
AM Wastewater	25	0.244
	50	0.076
	75	0.000
	100	0.041
Pond:AM Wastewater	99.5:0.5	0.014
	90:10	0.032
	80:20	0.039
	60:40	0.018
Finish with AM agent	25	0.037
	50	0.071
	75	0.134
	100	0.122
Finish without AM agent	25	0.141
	50	0.038
	75	0.169
	100	0.110
AM agent	0.25	0.119
	0.50	0.130
	0.75	0.108
	1.00	0.116
Controls (4)		0.167

depressed RLU's at concentrations greater than 25% which is a much higher concentration than the 0.5% AM wastewater found in the 99.5:0.5 Pond water to AM wastewater mixture. A synergistic reaction may have occurred between the Pond water and the AM wastewater.

The AM agent depressed RLU values randomly. The Finish with the AM agent produced statistically significant ($p < 0.05$) increasing RLU's with increasing concentration, although the overall RLU (less than 0.135 RLU) were depressed when compared to the control value of 0.167. The AM Finish without the AM agent depressed RLU levels from 0.141 RLU at a 25% concentration to 0.110 RLU when applied at full strength, but the difference was not significant at the 0.05 level.

The results obtained from the Lumac studies with activated sludge were more consistent than the data obtained from the soil Lumac study (see Table 24). The AM wastewater, the AM agent, and the Pond and AM wastewater mixtures produced no adverse effects on the luminescent reaction. The Pond water significantly increased the RLU's at the 20 minute reading, but by the 40 minute reading a significant decrease in the RLU was detected.

The Finish without the AN agent did not significantly affect the sludge biological population to any discernible extent after 20 minutes. At the 40 and 60 minute readings, however, lower RLU's were evident especially after 60 minutes. The Finish with the AM agent was clearly more toxic than the Finish without the AM agent and produced statistically significant decreases at each reading. For example, at the 5 minute reading the 100% Finish with AM agent treatment reduced the

Table 24. Microbial analysis of activated sludge using the Lumac device.

Treatment	Concentration (%) or Ratio	Relative Light Units After Contact Period			
		5 min	20 min	40 min	60 min
Pond	100	1.89	1.66	1.44	1.44
	75	1.48	1.61	1.45	1.51
	50	1.76	1.55	1.54	1.40
	25	1.39	1.37	1.55	1.43
	0	1.49	1.49	1.84	1.59
AM Wastewater	100	1.88	1.56	1.24	1.45
	75	1.65	1.77	1.53	1.36
	50	1.57	1.45	1.72	1.43
	25	1.78	1.89	1.67	1.41
	0	1.75	1.64	1.46	1.34
Pond: AM Wastewater	99.5:0.5	1.72	1.24	1.31	1.32
	90:10	1.96	1.37	1.31	1.11
	80:20	1.44	1.61	1.27	1.17
	60:40	1.20	1.37	1.37	1.14
	0	1.25	1.62	2.16	1.34
Finish with AM agent	100	0.80	0.52	0.35	0.37
	75	0.97	0.55	0.58	0.51
	50	1.06	0.60	0.60	0.52
	25	1.82	1.02	0.87	0.82
	0	1.75	1.83	1.53	1.69
Finish without AM agent	100	1.16	0.75	0.68	0.63
	75	1.16	0.74	0.71	0.72
	50	1.81	1.15	0.87	1.12
	25	1.66	1.78	1.70	1.61
	0	1.49	1.61	1.67	1.51
AM agent, 1%	100	1.64	1.70	2.11	1.68
	75	1.93	1.80	1.64	1.49
	50	1.80	1.70	1.70	1.72
	25	1.90	1.64	1.66	1.78
	0	1.84	1.73	1.58	1.51

RLU's by 0.95 while the 100% Finish without AM agent treatment reduced RLU's only 0.33 units from the 0% value. This tends to support the fact that the AM agent has antimicrobial properties.

Microtox Toxicity Analysis

The solutions used in the Phase III greenhouse study were examined using the Microtox System (see Table 25). The AM agent was the most toxic solution used, as would be expected with an EC_{50} for Photobacterium phosphoreum equal to 8.50 mg/L. The Finish was toxic, but the Finish with the AM agent was less toxic (42 mg/L) than the Finish without the AM agent (34 mg/L). This result seems contradictory, but perhaps there was some deterioration of the AM agent in the Finish or there may have been some sort of antagonistic reaction between the Finish and AM agent. In assessing the effect of the AM agent, solutions had to be tested immediately because the AM agent polymerized in water, thereby resulting in a decrease in toxicity over time. This same reaction may have occurred with the Finish.

If these results are compared to those obtained from a Microtox analysis performed six months earlier on the same samples (Table 26), the EC_{50} for the Finish without the AM agent seemed to remain relatively constant (32 mg/L vs 534 mg/L). The EC_{50} for the Finish with the AM agent, however, changed considerably from 14 mg/L in 8/83 to 42 mg/L in 2/84. This change was verified in six different trials suggesting that a loss of toxicity over time occurred with the AM agent, possibly due to a polymerization reaction. Note that evidence of a possible

Table 25. Microtox analysis of treatments used in Phase II (February, 1984).

Treatment	EC_{50}* at 15°C After Contact Period			
	5 min		15 min	
	mg/L	%	mg/L	%
Pond	18,350	1.835	16,150	1.615
AM Wastewater	315	0.0315	290	0.029
Pond:AM Wastewater(99.5:0.5)	26,600	2.66	24,200	2.420
Finish with AM agent	42	0.0042	32	0.0032
Finish without AM agent	34	0.0034	25	0.0025
AM agent	8.5	0.00085	-	0.0000

* EC_{50} - concentration of sample required to decrease light output of Photobacterium phosphoreum by 50%. Lower values indicate greater toxicity.

Table 26. Microtox analyses performed in August, 1983.

Treatment	Sample Collection Date	EC_{50}* in mg/L after 5 min at 15°C
Pond Wastewater	8/83	10,500
AM Wastewater (from large tank)	8/83	140
Finish with AM agent	8/83	14
Finish without AM agent	8/83	32
AM agent (old sample)	12/82	8
AM agent (new sample)	8/83	9

*EC_{50} = concentration of sample required to decrease light output of Photobacterium phosphoreum by 50%. Lower values indicate greater toxicity.

antagonistic effect alluded to previously was not apparent in the 1983 series of experiments (i.e., the possible interaction between AM agent and the Finish). Results obtained with AM agent compared favorably in the two experiments.

The Pond water (Table 25) EC_{50} was high (18,350 mg/L, but those of the AM wastewater and the AM wastewater plus degreasers, were 315 mg/L and 155 mg/L, respectively. It may be of importance to note that the toxicity of the Pond:AM wastewater combination (EC_{50} = 26600 mg/L)was less than either the Pond or AM wastewater, alone. Some type of antagonistic reaction might have occurred.

Corn Seedling Bioassay

The Pond water produced no statistically significant response in the corn seedling bioassays ($p < 0.05$)(Table 27). (See Appendix A for statistical significarce.) The AM wastewater produced significant decreases in growth of at least 4.36 mm, as compared to the controls, for the roots and shoots in the dark. Shoots in the light showed no effect until after the 50% AM wastewater level which reduced shoot growth by 2.76 mm, but not to a statistically significant degree. The Pond and AM wastewater mixtures decreased growth significantly in both the root and the shoot bioassays with the roots exhibiting the more sensitive response ($p = .002$).

The AM agent significantly inhibited root growth but only marginally affected the shoots. The Finish containing the AM agent was clearly more toxic than the Finish without the AM agent when root response is considered. The Finish without the AM agent produced 29,

Table 27. Results of corn seedling bioassay tests.

Treatment	Avg. Length of Roots mm	Avg. Length Shoots in Dark, mm	Avg. Length Shoots in Light, mm
Control	16.38	8.48	10.55
Pond			
25%	14.83	8.81	11.31
50%	12.92	7.07	12.83
75%	13.29	8.24	11.57
100%	7.74	8.29	9.38
AM Wastewater			
25%	11.32	8.14	10.97
50%	5.10	4.36	12.67
75%	5.21	6.67	7.79
100%	3.36	2.21	5.71
Pond:AM Wastewater			
99.5:0.5	7.83	7.55	8.47
90:10	3.00	8.00	10.54
80:20	2.60	5.50	11.24
60:40	2.78	5.21	5.50
AM Finish with AM Agent			
1%	5.23	9.70	11.81
5%	0.74	5.45	10.17
10%	0	1.71	1.21
15%	0	0.36	0.38
AM Finish without AM Agent			
1%	11.65	10.05	9.83
5%	2.02	4.28	9.02
10%	1.02	1.93	7.59
15%	0.14	1.17	2.71
1% AM Agent			
25%	8.26	8.23	13.33
50%	3.62	8.48	10.67
75%	2.91	5.28	12.56
100%	2.09	6.62	9.61

88, 94, and 99% reductions in root growth at 1, 5, 10, and 15% concentrations, respectively. The Finish with the agent was more toxic and depressed root growth by 68, 95, 100 and 100% at 1, 5, 10, and 15% concentrations of Finish, respectively. The two finishes, however, produced the same statistical response in the roots ($p = .0001$). Shoot growth in the light also produced evidence that the Finish with the AM agent was more toxic than the Finish without the agent with $p = .0001$ and $p = .0341$, respectively. At the 10% concentration Finish with the agent reduced shoot growth 86%, while the Finish without the agent reduced shoot growth by only 28% at the 10% concentration. On the basis of shoot growth in the dark, however, the toxicity of the two treatments appeared to be about the same. Both depressed shoot growth by about 78% at the 10% concentration.

For all of the solutions, the root growth test appeared to be the most sensitive of the three seedling bioassays. The increased sensitivity of the root tests, as compared to the shoot tests, may be seen in Figures 12 through 17.

Evaluation of Short-Term Tests

The Lumac tests were a bit awkward to perform and required some special equipment, such as blender for small fluid volumes (less than 10 mL) and a sonicator. Both the soil and sludge Lumac data exhibited variability between replications. If a 50% decrease in the adjusted RLU is used, the averaged values, however, were results consistent with the greenhouse results (Table 28) with a few exceptions. The soil Lumac test was more sensitive to the Pond and AM wastewater mixtures than the

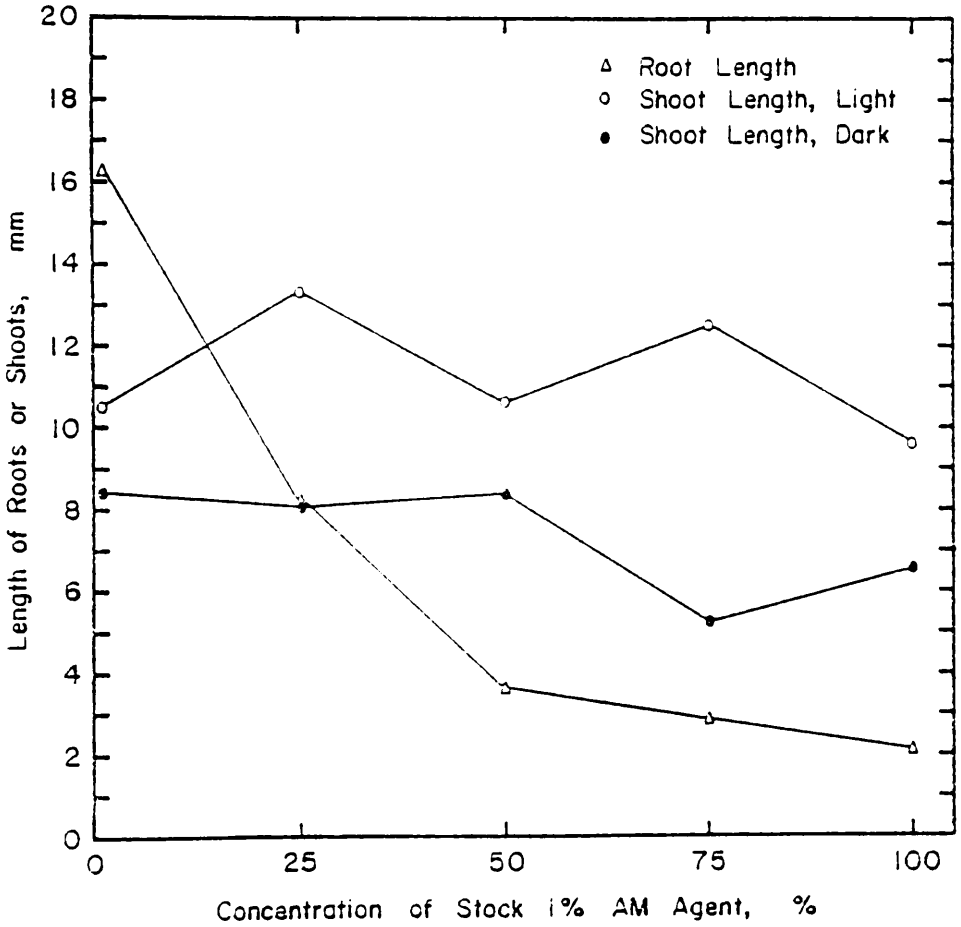


Figure 12. Corn seedling root length, shoot length in the light, and shoot length in the dark as a function of AM agent concentration.

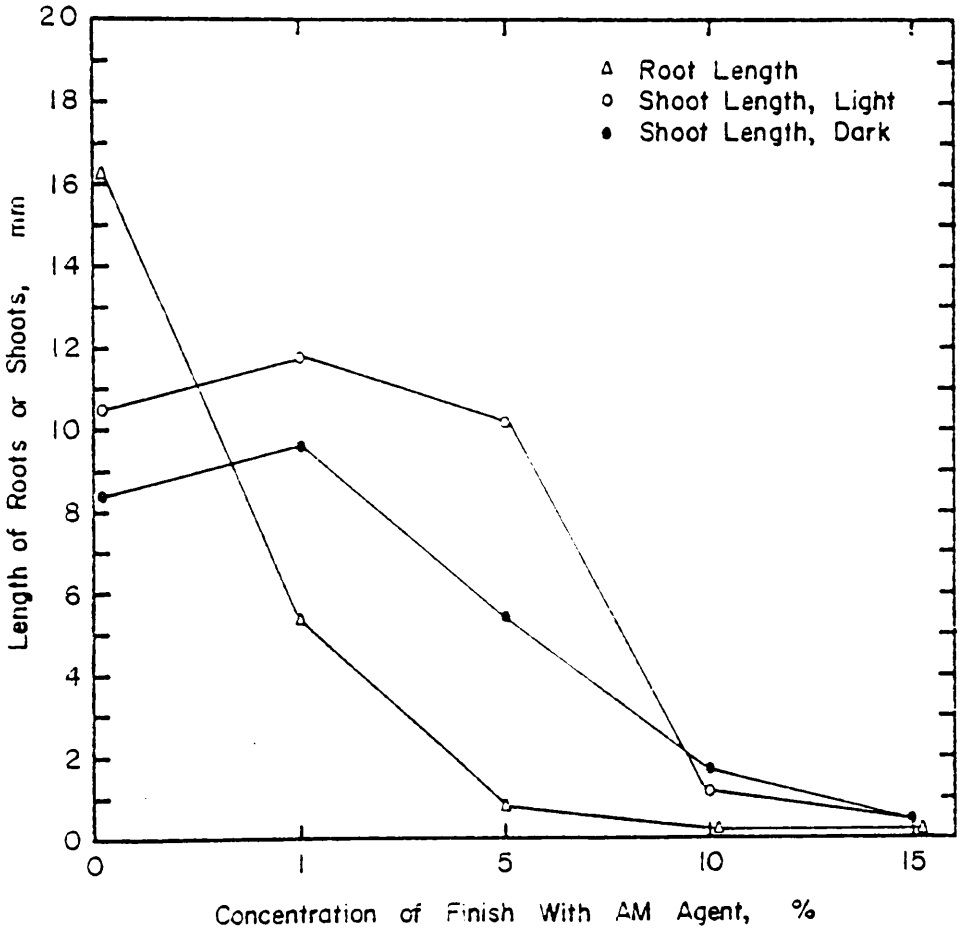


Figure 13. Corn seedling root length, shoot length in the light, and shoot length in the dark as a function of Finish with AM agent concentration.

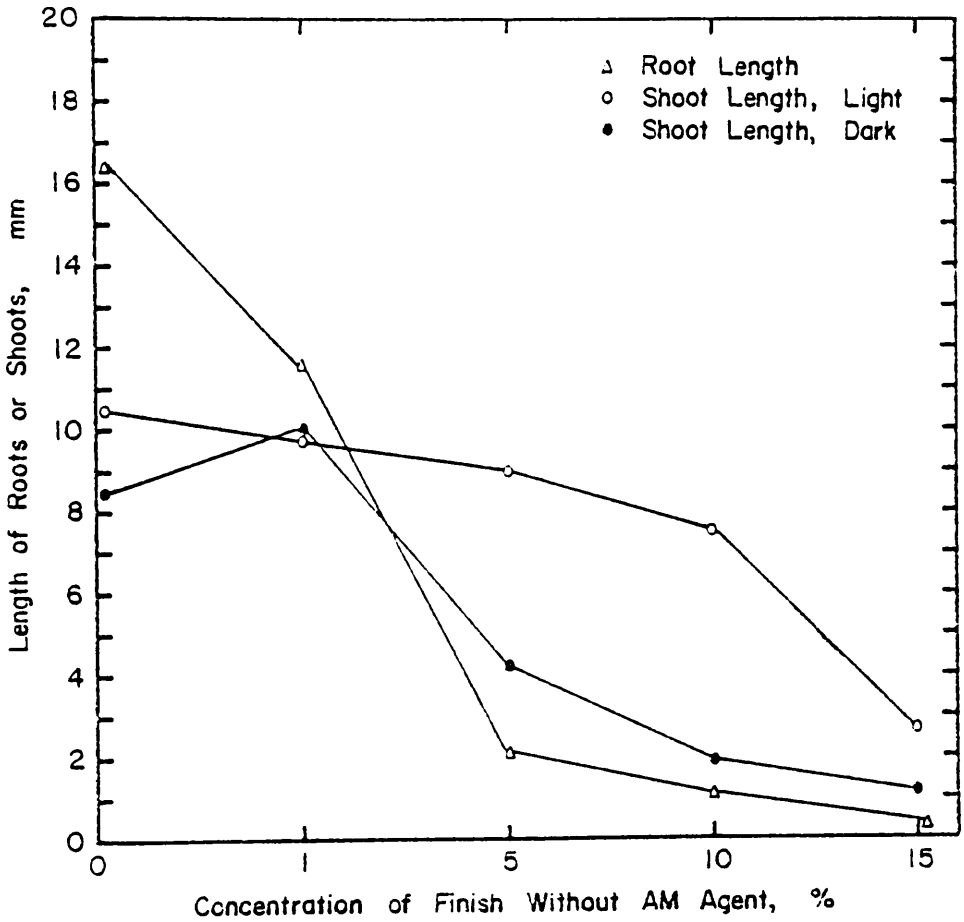


Figure 14. Corn seedling root length, shoot length in the light, and shoot length in the dark as a function of Finish without AM agent concentration.

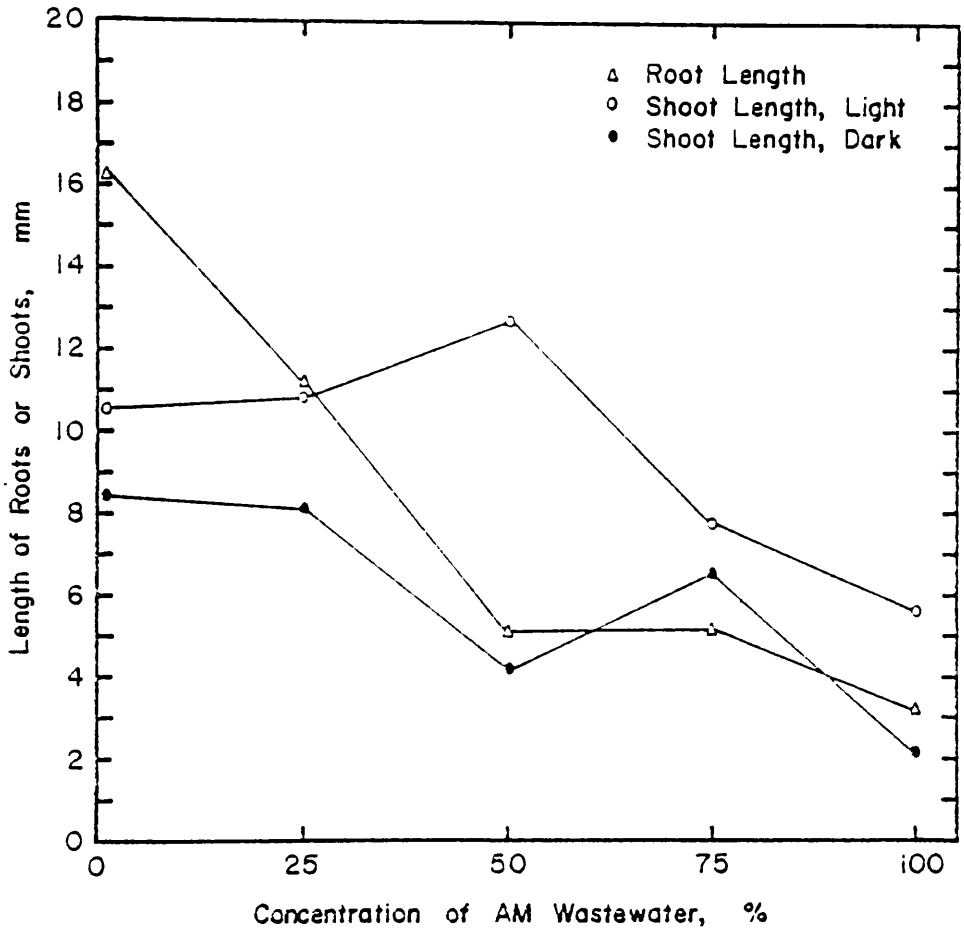


Figure 15. Corn seedling root length, shoot length in the light, and shoot length in the dark as a function of AM wastewater concentration.

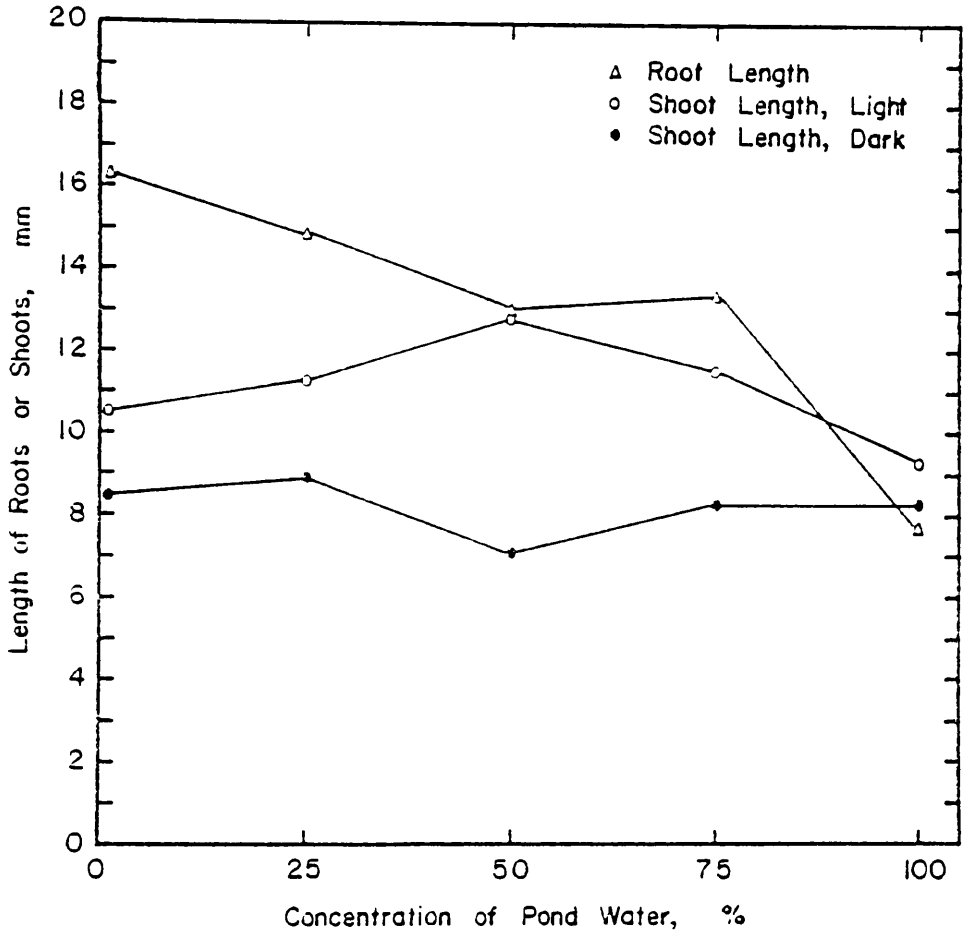


Figure 16. Corn seedling root length, shoot length in the light, and shoot length in the dark as a function of Pond water concentration.

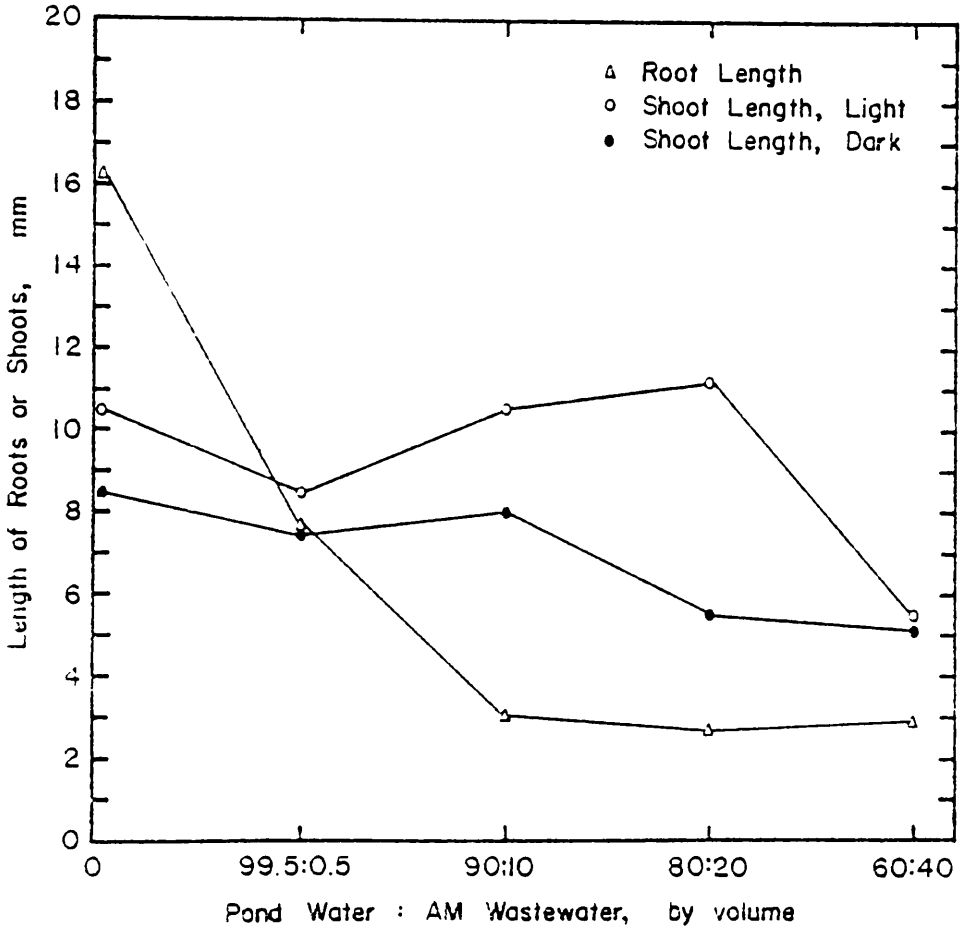


Figure 17. Corn seedling root length, shoot length in the light, and shoot length in the dark as a function of the Pond water and AM wastewater mixture.

Table 28. Levels at which a 50% decrease in the parameter measured was detected; Concentration expressed in terms of percent unless otherwise noted.

	Bioassay						
Treatment	Greenhouse[a] Phase III	Lumac[b] Soil	Lumac Sludge[c]	Microtox[b]	Corn Seedling Roots	Shoots-Dark	Shoots-Light
Pond Water	N.E.[d]	N.E.	N.E.	1.835	100	N.E.	N.E.
AM Wastewater	50	50	N.E.	0.0315	50	100	N.E.
Pond:AM Wastewater (in ratios)	80:20	at all levels	N.E.	2.66% of 99.5:0.5	at all levels	N.E.	N.E.
Finish with the AM Agent	25	<50	25	0.0042	1	10	10
Finish without the AM Agent	25	50 only	75	0.0034	15	15	15
AM Agent	N.E.	N.E.	N.E.	0.0085	0.5	N.E.	N.E.

[a] based on Table 20
[b] 5 minute readings used
[c] 60 minute readings used
[d] no effect

greenhouse studies. Both Finish treatments produced erroneous results in the soil Lumac study. The Finish with the AM agent produced a 50% reduction in RLU at the 25 and 50% treatment level, but not at the 75 or 100T. The Finish without the AM agent lowered the RLU by at least 50% at the 50% concentration only. The Lumac activated sludge test was less sensitive than the greenhouse study and reported no discernible effect from AM wastewater, the Pond water, the Pond and AM wastewater mixtures, and the AM agent.

The basic problem with the Lumac soil and activated sludge test is the lack of a uniform microbial sample population which probably produced the variability in the data. Well-mixed soil samples from a confined structure, such as a greenhouse pot, or a well-mixed activated sludge sample might be assumed to be uniform. Day-to-day changes in biomass and the ability of microbes to form aggregate clusters tend to negate this assumption. The use of a large number of replicates would appear to be the only method available at present to offset variations between sample populations. The number of replications required would decrease the utility of the Lumac procedures as a monitoring device, however, these procedures may be useful for screening tools.

The Microtox studies were relatively simple to perform. This test has the advantage that the test bacterial population is of a known size and type, so the test is more reproducible than the Lumac methods discussed earlier. The $EC_{50's}$, if expressed as percent concentrations (Table 25), appear to be overly conservative in predicting the toxicity of the treatments considered. For example, Pond water produced no

discernible adverse effects in the plants at any concentration but the Microtox test results indicated that a 1.84% solution of Pond water would kill 50% of the Microtox bacterial population. There appeared to be magnitudes of difference in the results of the greenhouse study and the Microtox analysis. The appropriateness of the Microtox test for predicting the response of plants is a concern that could only be addressed on a case-by-case basis. The results of this study would have to conclude that the Microtox is not appropriate for predicting plant response to the treatments considered. The Microtox procedure would be a good screening or monitoring tool if and only if a method could be found to correlate the Microtox results with the results of a more realistic assay such as greenhouse studies.

The corn seedling bioassay was easy to perform. No special equipment was needed, except an incubator. It was expected that the corn seeds would mimic the plant responses best, since both corn and the grasses used are monocots. The corn seedlings, however, did appear to be more sensitive to the Finish treatments than the Fescue plants. A preliminary test using a 25% Finish concentration, both with and without the AM agent, resulted in zero growth of the corn seedlings. As a result additional corn seedling tests were performed with Finish concentrations less than 25%. The fescue plants were negatively affected at 25% Finish but were not killed completely. Since the concentrations of the Finishes used in the two tests do not overlap, it is hard to compare the results.

The seeds followed the same general response pattern as the plants, especially in the shoot growth tests. The root test, was consistently and statistically more sensitive than the shoot test, however. Because the root test was the quickest and most sensitive of the three seedling bioassays, Allied should consider using such a test for screening purposes in the future. The time lag of several days in performing the test would render the corn seedling bioassay an inappropriate monitoring tool.

Tables 28 through 29 summarize the above information.

Table 29. Relative ranking of toxicity of treatments where zero equals no effect at the levels considered as indicated in Table 28.

Treatment	Bioassay						
	Greenhouse Phase III	Lumac Soil	Lumac Sludge	Microtox	Roots	Corn Seedling Shoots-Dark	Shoots-Light
Pond Water	0	0	0	5	1	0	0
AM Wastewater	3	3	0	5	3	1	0
Pond:AM Wastewater	3	5	0	5	5	0	0
Finish with the AM Agent	4	*	4	5	5	5	5
Finish without the AM Agent	4	*	2	5	5	5	5
AM Agent	0	0	0	5	3	0	0

*erroneous results

V. SUMMARY AND CONCLUSIONS

Allied Corporation introduced a new antimicrobial (AM) finish for their carpet fibers. Concern over the effect of releasing this AM agent to the environment via a land disposal wastewater treatment system prompted this study. Greenhouse studies were used to evaluate possible environmental effects of the agent, alone, and in combination with other compounds, and to determine an appropriate land application rate for the agent. Three short-term bioassays were also conducted in order to evaluate their usefulness as screening tools and monitoring devices for potentially toxic mixtures, using the greenhouse studies as a reference for accuracy.

The greenhouse studies produced evidence to suggest that the AM agent, alone, and in combination with other compounds may disrupt the normal activity of nitrifying bacterial populations. This was indicated by the low nitrate values, as compared to the ammonium levels in greenhouse soils, as well as the depressed numbers of nitrite oxidizing bacteria in the same soils. There are four possible explanations for these results: 1) Ammonium-N was forming more rapidly than the nitrifying population could metabolize it; 2) the nitrifying population was inhibited; 3) the nitrate produced was being removed from the soil through plant uptake, and/or 4) an environmental effect such as low oxygen tensions in the soil due to hydraulic overloading caused a shift to denitrification which would result in low nitrate levels in the soil.

The AM wastewater and the Finish, both with and without the AM agent, produced toxic effects in all plants, as evidenced by necrosis of

plant tissues and subsequent concentration of nutrients in the soil. A 1% solution of the AM agent alone, which was the maximum concentration anticipated in the AM wastewater, did not adversely affect the plants based on a chemical analysis of the soil and plant tissue. In addition no adverse effects to the plants were noted at a ratio of 99.5% Pond water to 0.5% AM wastewater by volume, which is the composition of the effluent Allied would like to land apply.

The short-term bioassays produced varied results. A high variability was noted in the Lumac test performed with soil and activated sludge. The average response, of the soil Lumac test, was similar to that seen in the greenhouse studies except for erroneous responses to the Finish treatments. The sludge Lumac tests were generally less sensitive to the applied treatments than the greenhouse study. It may be that given enough replications, the Lumac technique might be useful as a screening tool, but the results of simply a few tests could be misleading.

In performing the Microtox test a known and constant biological population was utilized, and, as a result, the test exhibited good reproducibility. The test can be performed quickly and easily, making it a likely candidate for either a monitoring or a screening tool. However, the results of this study indicated that the Microtox method is very sensitive and may give overly conservative estimates of the toxicity of a compound in soil systems. Additional work should be performed to investigate the possibility of utilizing a correction factor with the Microtox results.

The Corn Seedling Bioassay (CSB) is a plant system and, as such, represents a logical means of assessing plant system resporses. In this study the results indicated that the root test was more sensitive than the shoot tests and is a good method for screening potentially toxic agents. Since it takes a few days to perform a CSB, its utility as a monitoring tool would be limited unless effluent discharges were made on a batch basis.

Based on these observations, the following conclusions were derived:

1) The AM agent alone and in combination with the other compounds considered may reduce nitrifying populations or produce conditions that favor denitrification. A chemical analysis of the plant tissue and the soil, however, did not indicate that the plants were under any nutrient stress as a result of the AM agent treatments.

2) Based on Allied's anticipated application ratio of 99.5% Pond water to 0.5% AM wastewater (by volume), a 200 day growing period, and a 5 day per week application schedule, a land application rate of 308 Kg N/ha/yr is recommended with a rinse cycle of uncontaminated water following each application.

3) Of the short term toxicity tests considered, the CSB root test provided results which were most consistent with those of the greenhouse study.

4) Although the Microtox produced fast (<1 hour) reproducible results which would make it a good monitoring and screening

tool, additional work would have to be performed to determine its applicability to soil-plant systems. The Lumac procedures considered in this study exhibited high variability and would therefore not be acceptable as screening or monitoring tools unless multiple replications are performed. The root test of the CSB was easy to perform and had good reproducibility, making it a good candidate as a screening device. The time lag (days) involved in performing the CSB analysis, however, makes it unsuitable for most wastewater monitoring applications.

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APPENDIX A

Appendix Table 1. Statistical significance (p) of Phase III soil chemical analyses*

Treatment	Parameters								
	pH	P	K	Ca	Mg	SS	TKN	NO_3^-	NH_4^+
Pond Water	0.043	0.527	0.175 (-)**	0.027	0.527	0.369 (-)	0.293	0.004 (-)	0.016 (-)
AM Wastewater	0.0	0.23	0.0085	0.036 (-)	0.527	0.003	0.369 (-)	0.031 (-)	0.125
Pond: AM Wastewater	0.369	0.076	0.031	0.175	0.527	0.293	0.098	0.004	0.007
Finish with AM Agent	0.076 (-)	0.30	0.0085	0.076 (-)	0.527	0.007	0.175	0.022 (-)	0.043
Finish without AM Agent	0.19 (-)	0.110	0.0	0.245 (-)	0.527	0.004	0.175 (-)	0.058	0.473 (-)
AM Agent	0.155	0.273 (-)	0.33 (-)	0.245 (-)	0.527	0.004	0.036 (-)	0.043 (-)	0.031 (-)

*Significant at $p \leq 0.05$.
**(-) indicates a negative correlation.

Appendix Table 2. Statistical significance (p) of Phase III plant tissue analyses*

Treatments	Dry wt.	N	P	Ca	Mg	K
Pond Water	0.25 (-)*	0.076	0.319	0.125 (-)	0.098 (-)	0.34
AM Wastewater	0.0035 (-)	0.0015 (-)	0.007 (-)	0.34 (-)	0.34	0.08 (-)
Pond: AM Wastewater	0.42 (-)	0.043 (-)	0.5	0.319 (-)	0.05 (-)	0.031 (-)
Finish with AM Agent	0.50 (-)	0.031 (-)	0.027 (-)	0.23 (-)	0.21 (-)	0.027 (-)
Finish without AM Agent	0.125 (-)	0.019 (-)	0.076 (-)	0.098 (-)	0.25 (-)	0.0005 (-)
AM Agent	0.058 (-)	0.250 (-)	0.276 (-)	0.42	0.29 (-)	0.06

*Significant at $p \leq 0.05$
**(-) indicates a negative correlation

Appendix Table 3. Statistical significance (p) of short-term toxicity test*

Treatments	Short-Term Tests							
		Lumac Sludge				Corn Seeds		
	Lumac Soil	minutes 5	15	40	60	Shoots Dark	Shoots Light	Roots
Pond Water	0.125	0.242	0.042	0.008 (-)**	0.408 (-)	0.8859	0.1322	0.4012
AM Wastewater	0.010 (-)	0.408	0.408 (-)	0.408 (-)	0.117	0.0109 (-)	0.1331 (-)	0.0367 (-)
Pond: AM Wastewater	0.420	0.117	0.08 (-)	0.18 (-)	0.408 (-)	0.0117 (-)	0.0059 (-)	0.0002 (-)
Finish with AM Agent	0.007	0.042 (-)	0.008 (-)	0.008 (-)	0.008 (-)	0.0001 (-)	0.0001 (-)	0.0001 (-)
Finish without AM Agent	0.319 (-)	0.30 (-)	0.117 (-)	0.042 (-)	0.042 (-)	0.0001 (-)	0.0341 (-)	0.0001 (-)
AM Agent	0.50 (-)	0.408 (-)	0.50	0.117	0.408 (-)	0.1265	0.1428	0.0011 (-)

*Significant at $p<0.05$
**Indicates a negative correlation

VITA

Marcia J. Degen was born December 22, 1957, in Baltimore, Maryland. She completed her secondary education at St. Maria Goretti High School in 1975, after moving to Hagerstown, Maryland, in 1968. Ms. Degen began her college career at Hagerstown Junior College receiving an Associate in Arts degree in Biology in 1978. She continued her education at Virginia Polytechnic Institute and State University and graduated in 1980 with a Bachelor of Science degree in Biology. After working for two years as a Research Associate with an environmental consulting firm in Springfield, Virginia, GKY & Associates, Inc., Ms. Degen returned to VPI&SU to pursue a Master's degree in Environmental Sciences and Engineering through the Civil Engineering Department. She is presently employed as a Research Associate in the Department of Agronomy at Virginia Tech.