CONTRIBUTION OF MICROORGANISMS TO ZINC IMMOBILIZATION IN SOIL

By

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ABSTRACT

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A soil perfusion system was used to determine Zn immobilization by soil microorganisms in Rubicon sand (pH 5.9). A 17 $mgkg^{-1}$ (.26 mM) Zn solution (320 mL) was perfused through 12.5 g of gamma-irradiated (sterilized) or biologically active soil. Approximately 75% of the perfusate Zn was inactivated by chemical and physical mechanisms. The introduction of biologically active soil microorganisms and sterile nutrient broth into a sterile soil perfusion system resulted in an additional significant reduction in Zn concentration of the soil perfusate. The level of Zn in the perfusate of the sterile perfusion system remained constant (3.9 mgL^{-1} of Zn) during the same 72 hour perfusion period where the Zn level in the perfusate of the biologically active (inoculated) system decreased to 0.7 mgL^{-1} of Zn. The enhanced immobilization represented over 90% of the Zn from the perfusion solution indicating that microorganisms immobilized a fraction of Zn (15%) in addition to that activated by chemical and physical mechanisms.

A soil sample was obtained from the soil perfusion column after maximum fixation had been attained. This soil sample was diluted in sterile water and surface plated on soil extract agar containing ^{65}Zn. After colonies of microorganisms developed, the agar plate was placed on Kodak film X-OMAR-AR or NO-SCREEN (NS-2T) for autoradiography. The colonies that accumulated sufficient levels of radioactive ^{65}Zn to

expose the X-ray film were identified by comparison with the developed film. The colonies were isolated, grown in pure culture, and reconfirmed as "zinc accumulators" by the autoradiographic plating technique on ^{65}Zn enriched agar.

Most of the isolated Zn immobilizing organisms were fungi, identified as predominantly Penicillium species. Other Zn-immobilizing fungi were Fusarium, Paecilomyces, Cladosporium, Cephalosporium, Mucor, and Aspergillus spp. The most abundant Zn-immobilizing bacteria were spore-forming Bacillus spp., and another unidentified gram-positive rod.

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INTRODUCTION

The role of soil microorganisms in the inactivation of micronutrients such as Zn by microbial immobilization in soil has been considered to be insignificant when compared to chemical and physical mechanisms of fixation. A significant microbial impact upon the solubility of soil micronutrients that can readily undergo oxidation and reduction, such as Fe or Mn, has been reported. However, the influence on Fe and Mn solubility was probably due to chemical and physical changes which were initiated by the change in the oxidation state of micronutrients rather than by immobilization by soil microorganisms. Likewise, the importance of soluble soil organic matter upon micronutrient solubility in soil through complexation and chelation has been reported by several investigators (Hodgson, 1963; Randhawa and Broadbent, 1965; Stevenson and Ardakani, 1972; Stevenson, 1976, 1977 and 1982). According to the National Research Council (1979) newly formed organic substances which are mobile (especially fulvic acids and biochemical intermediates), can solubilize Zn and increase the metal's availability to plants and other biological systems. A direct link with microbial immobilization has not been established in these reactions and the primary mechanisms are reported to be chemical and physical in nature. Generally, data that define the magnitude of micronutrient immobilization in soils are very limited, and data which describe the relative importance of different microorganisms of the soil microbial population upon micronutrient immobilization are extremely limited.

Zinc is a logical micronutrient choice for model investigations involving microbial immobilization in soil because: it is an essential micronutrient; it is not subject to oxidation and reduction under usual soil conditions; it is relatively nontoxic (according to Brown et al., 1964, its contamination may cause illness with food poisoning); it has been shown to be accumulated by and cause growth response in Aspergillus; and there is considerable experience and background data on soil fixation (Krauskopf, 1956; Foy et al., 1978; Reddy and Perkins, 1974). In addition, there is considerable current interest in the environmental impact of Zn-contaminated waste materials such as municipal sewage sludge and industrial wastes. Human health minimum daily requirements and associated benefits of Zn supplementation of the human diet as well as potential toxic influences on human and animal systems have been the subject of many recent popular and scientific literature sources. There is a growing recognition of the importance of Zn in health and certain areas of potential research such as the influence of Zn and other trace elements upon microbial activity in digestive systems of livestock has not really been investigated.

A model approach to demonstrate the principles of the concept of significant Zn immobilization by soil microorganisms was decided upon and a system was needed that would include a soil in which usual Zn chemical and physical fixation reactions were minimal and the development of a population of soil microorganisms could be achieved very rapidly under controlled environmental conditions. The combination of Rubicon sand soil (which has a low cation exchange capacity, low organic matter content and a pH of 5.9) with a soil perfusion system and

a Zn spiked perfusate provided an opportunity to test the hypothesis that soil microorganisms can immobilize a fraction of Zn from soil solution beyond that which would be fixed by chemical and physical reactions on a short term basis.

Accumulated colonies which were isolated in "pure culture" on agar slants were used as the source population to confirm that they were Zn accumulators. Finally, preliminary attempts were made to identify the genus of some major Zn accumulator colonies.

The opportunity to identify, select and develop populations of soil microorganisms that can selectively immobilize soluble Zn in soils can have important practical benefits for environmental cleanup of Zn contaminated soils. The processes of microbial immobilization could be used to concentrate low levels of metals such as Cd by bacterial cells (Kurek et al., 1982), organic compounds as well as radioactive elements from contaminated solution. Strandberg et al. (1981) used microbial cells as biosorbents to accumulate uranium. The uranium was accumulated extracellularly on the surfaces of Saccharomyces cerevisiae and Pseudomonas aeruginosa and could be removed chemically. The cells could then be reused as a biosorbent.

This study was undertaken to test the hypothesis that Zn could be immobilized by soil microorganisms in quantities great enough to lower the Zn levels in soil solution--especially when soils of low cation exchange capacity (CEC) were amended with material high in Zn and available energy and nutrient supply (carbon, nitrogen, phosphorus, etc.)--such as when Zn enriched sewage sludge is added to sandy soils.

LITERATURE REVIEW

General Characteristics of Zinc

Zinc is a silvery metal that quickly tarnishes to a blue-gray appearance. This color is due to an adherent coating of carbonate, $Zn_2(OH)_2CO_3$, which protects the underlying metal from further corrosion. The metal is hard and brittle at ordinary temperatures but ductile and malleable at 100-150°C (Nebergall et al., 1972). Zinc has 18 electrons in the underlying shells but only two in its outer shell therefore the divalent oxidation state prevails. Coordination numbers of both 4 and 6 are quite common in the mineral structures and complexes of this element despite its large size (0.74 A). Taylor (1964) stated the average amount of Zn in the earth's crust was 70 $mgkg^{-1}$ and in igneous rocks was 40 to 100 $mgkg^{-1}$. Turekian and Wedepohl (1961) reported the Zn content of sedimentary rocks (sandstone, limestone and shale) as being 16, 20 and 95 $mgkg^{-1}$, respectively. Allaway (1968) reported the total Zn content of the soils in the range of 10 to 300 $mgkg^{-1}$, including the majority of soils but certainly not the extremes (Zn content of soils near sulfide deposits). Quartz is low in Zn, so sandy soils often contain only 10-30 $mgkg^{-1}$ total Zn (Lindsay, 1972a). Soil near highways may be contaminated appreciably; the source of the Zn may be from the wearing of tires containing ZnO and from emissions of motor oil to which Zn-dithiophosphite has been added; these sources of Zn are in addition to industrial emanations (Lagerwerff and Specht, 1970). Klein (1972) found that soil from an industrial area near Grand Rapids, Michigan, had a mean content of 57 $mgkg^{-1}$ Zn, whereas nearby agricultural and

residential areas had respective means of 22 and 21 $mgkg^{-1}$ Zn. The fact that Zn occurs chiefly as the single sulfide mineral sphalerite (ZnS) indicates that the metal is largely chalcophile in its behavior at the earth surface. The transportation of Zn in solution is not a problem except in solutions containing sulfide ion. Even in fairly alkaline non-sulfide solutions, appreciable Zn can remain dissolved in the presence of common anions, and the solubility is not affected by changes in redox potential (Mortvedt, et al., 1972). Hodgson (1963) stated that a wide range of biochemical compounds and also organic substances like humic and fulvic acids form stable combinations with Zn. The soluble organic complexes have a profound effect on the mobility and availability of Zn. Some of the Zn-organic complexes are so stable that Zn is essentially unavailable to living systems, but these are rare.

One main group of organic compounds that form stable compounds with Zn in the soil are direct biological products which originated in living organisms and contain various organic acids, peptides, proteins and polysaccharides. A second group includes organic polymers such as humic and fulvic acid formed by secondary organic synthesis reactions and result in more complex organic compounds (National Research Council, 1979). These two groups cannot always be clearly separated because Zn can function as a linkage in binding these organic compounds and it can be chelated by them (Stevenson and Ardakani, 1972). Lucas and Knezek (1972) concluded that climatic and soil conditions were partly responsible for Zn deficiency in crops. Cool, wet spring weather often aggravates or induces Zn deficiency in field crops. Zinc deficiency occurs in calcareous soils with pH of 7.4 or higher because the

solubility of Zn decreases as soil pH increases. In calcareous soil, adsorption of Zn by carbonates contributes to low Zn availability (Lindsay, 1972b; and Lucas and Knezek, 1972). Elgabaly and Jenne (1943) and Elgabaly (1950) reported that some of the Zn^{2+} and $Zn(OH)^{+}$ adsorbed on montmorillonite was not replaceable and had entered the octahedral layers of clay. Lindsay (1972a) noticed severe Zn deficiency in corn planted after beets (high in carbohydrates for microbial growth and activity) rather than following corn or other crops. DeRemer and Smith (1964) observed reduction of available Zn to the succeeding bean crop when sugar beet tops were plowed down. Zinc deficiencies have been reported in areas where the topsoil containing organic matter was removed. Lindsay (1972a) concluded that Zn deficiencies increased because the exposed subsoil was lower in organic matter and higher in pH and carbonate than the surface soil.

Zinc Metalloenzymes and Effect of the Heavy Metals on Enzyme Activity in Soil

The study of soil enzymatic activity is a promising approach to the study of biological effects of heavy metal ions on the decomposition process. These effects may be elucidated in more detail than is possible with simple measurement of soil respiration. More than 10 different Zn metalloenzymes including proteases, transcarboxylases, carboxylases, phosphatases and dehydrogenases occur in microorganisms. The metal Zn may have catalytic, functional or both catalytic-functional importance in these enzymes. They contain fixed quantities of metal which are firmly bound as an integral part of the protein molecule (Tyler, 1980). Zinc plays an important role in protein synthesis; it is

essential for normal activity of both DNA and RNA polymerase (National Research Council, 1979). Removing Zn from DNA polymerase inhibits its activity; with Zn the complete functionality will resume. Riordan and Vallee (1974) and Holmquist and Vallee (1976) reported that both Bacillus subtilis and Bacillus thermoproteolyticus produce enzyme proteases in which the atom Zn (1 Zn atom/mole) serves as a component of an active site and directly participates in the catalytic process (hydrolysis of the peptides). The enzyme transcarboxylase produced by Propionibacterium shermanii contains both Zn and Co (2 Co atoms, 4 Zn atoms/mole) and its catalytic reaction is the carboxylation of pyruvate (Northrop and Wood, 1969). Zinc may maintain the structure of the protein enzyme and act as a structural constituent as it does in the enzyme aspartate transcarbamylase which is produced by Escherichia coli and converts carbamyl phosphate to carbamyl-aspartate (Jacobson and Stark, 1973 and Riordan, 1977). However, in some enzymes (alcohol dehydrogenase and alkaline phosphatase) produced by Escherichia coli (Li and Vallee, 1973) and Saccharomyces spp. (Simpson and Vallee, 1968; Anderson et al., 1976), the metal Zn appears to have both catalytic and structural importance. Alkaline phosphatase has 4 Zn atoms per mole with 2 catalytically active and 2 are structure stabilizers. The specificity of a metal in an enzyme may be so exact that substitution by some other metal species usually results in an almost complete loss of enzymatic activity. At the same time the metal ion may activate the enzymes but metal specificity may be lower and similar ions may substitute with no loss or only partial loss of activity.

Knowledge of the relative effects of trace elements on urease activity is important because its substrate, urea, is added to soils as a synthetic fertilizer and in animal excreta. Tabatabai (1977) and Juma and Tabatabai (1976) showed that 20 trace elements including Zn and Cd that are commonly found in sludge samples inhibited the activity of urease and phosphatase in soils and that their order of effectiveness as inhibitors depends on the soil. Cadmium is known to exert its toxic effects by replacing Zn in carboxypeptidase enzyme systems which catalyze peptide hydrolysis (Mahler and Cordes, 1966). Consequently, the indirect effect of Cd on glucose metabolism may not have been noticed until large quantities of Cd were present. A copper ion reacts more readily with sulfhydryl groups than Zn and so it is a considerably more powerful inhibitor of urease activity than the Zn ion (Hughes et al., 1969). This was also found by Tyler (1976) to be the case with phosphatase enzyme where Cu and Zn pollution reduces the decomposition rate, phosphatase activity, and phosphorus mineralization rate in the mor horizon of coniferous forest soil surrounding a brass mill in Sweden. Price (1970) reported that several dehydrogenases were sensitive to Zn deficiency in Euglena gracilis, therefore the metabolism of aquatic plants can be influenced markedly by changes in the availability of Zn. In plants, the main function of Zn is as a metalloenzyme. Several Zn-requiring dehydrogenases, proteinases and peptidases have been identified in various organisms, including aquatic plants such as Euglena gracilus (Price et al., 1972).

Characteristics of Sewage Sludge Containing Zn and Other Heavy Metals

Application of sewage sludge on cultivated land surrounding a population center is economical and solves the disposal problem in a beneficial way. But recently a concern has been expressed regarding the introduction of heavy metals into natural biological systems in conjunction with its application on land. Heavy metals pose a hazard to the food chain since they can be readily transferred from soil to plants and up the food chain to animals or humans. The chemical composition of sewage sludge that results from an industrial or domestic activity is dependent upon numerous factors including the type of chemical digestion performed, the extent and nature of industrialization in the sanitary district, and the seasonal variability of sewage entering the treatment facility. Initial studies on the composition of sewage sludge were primarily concerned with total amounts of N, P, K and trace elements essential for plant growth. More recently, the metal content of sewage sludge has received emphasis because of the toxicity of some metals like Zn, Cd and Ni to microorganisms, plants, animals and even humans.

Bradford et al. (1975) characterized the metropolitan sewage sludges and effluents in relation to their total and water extractable trace elements. They found that the total concentration of trace elements in sludges and effluents were highly variable depending upon the source. Concentrations of Zn, Cu, Ni, Co and Cd were consistently greater in the saturation extracts obtained from sludges than those obtained from a large sampling of California soils. They attributed the increased solubility of trace elements in sludge extracts to the formation of soluble metal chelates.

Sommers et al. (1976) collected sewage sludge samples over a 2 year period from eight Indiana cities and analyzed them for C, N, P, K, Ca, Mg, Fe, Cd, Zn, Cu, Ni and Pb. The dried sludge contained approximately 50% organic matter and 1-4% inorganic C. Inorganic N, organic P, K and all metals were found to be quite variable over time for sewage sludge produced at a given city. Page (1974) mentioned that Zn concentrations in sewage sludge ranged from 700 to 49 000 $mgkg^{-1}$, whereas Ni ranged from 20 to 5 300 $mgkg^{-1}$, Cu from 200 to 8 000 $mgkg^{-1}$ and Cd from 1 to 1 500 $mgkg^{-1}$. The rationale commonly used to recommend the rate of sludge application to agricultural land is based upon limiting available N to that which a crop can utilize and limiting metal additions to an amount such that excessive metals will not accumulate in or be toxic to the crop. The variable nature of inorganic N and metal contents of sludges indicates that a comprehensive sampling and analysis program is essential prior to formulating recommendations for rates of sewage sludge application on soils used for crop production. There is an inherent heterogeneity in sewage sludges. If one wishes to study the effect of a particular heavy metal like Zn or Cd in sewage sludge on microorganisms or soil accumulation and plant response, it is desirable to have only one kind of sewage sludge and then amend it to get various levels of Zn rather than having different sludges with varying concentrations of this metal. Kirkham (1975) added 14 or 140 $mgkg^{-1}$ Cd as $CdSO_4$ to an anaerobically digested sludge containing 65 $mgkg^{-1}$ Cd to study the uptake of Cd and Zn from sludge by barley plants grown under four different irrigation regimes. However, metals supplied as inorganic salts are usually more biologically active than those forms

associated with sludge (Cunningham et al., 1975). Dijkshoorn and Lampe (1975) found that an application of sewage sludge rich in metals resulted in about half as much Zn and Cd in potted perennial ryegrass as when the same amount of the metal was added in a sulfate form. This variation in uptake may be attributed to the differences in the chemical form of Zn and Cd in the sewage sludge. Interestingly enough, Giordano and Mortvedt (1976) found that mobility of heavy metals, including Cd, from inorganic sources was slightly greater than from sewage sludge. Sludge organisms can become adapted to toxic substances when these are continuously present in the waste (Tomlinson et al., 1966). The concentration of metals necessary to inhibit nitrification in activated sludge is much higher than that required to give the same effect in pure cultures. Tomlinson et al. (1966) found that as much as 150 mgL^{-1} Cu, added as cupric sulphate, were required to produce 75% inhibition in unadapted activated sludge plus sewage compared with only 4 mgL^{-1} Cu in the pure culture. When sewage was replaced by an ammonium salt solution, nitrification was inhibited at a much lower concentration of metals suggesting that organic matter removed the toxic metals by chelation or precipitation. Premi and Cornfield (1969) reported that nitrification was unaffected, partially inhibited, and totally inhibited by 100, 1 000, and 10 000 $mgkg^{-1}$ Zn respectively. Wilson (1977) confirmed that nitrification in soil is temporarily inhibited by 100 $mgkg^{-1}$ Zn and totally by 1 000 $mgkg^{-1}$ Zn.

In addition to the chemical and biological changes which would take place in the land as the result of the sludge application, there would be changes in the physical structure of the soil as well.

Microbial Activity as Affected by Environmental and Heavy Metal Contamination

Microorganisms are key components in the biochemical cycling of various chemical elements, in the incorporation of energy (photo and chemosynthesis), and in those processes necessary for the maintenance of the fertility of aquatic and terrestrial habitats and for waste reduction. Consequently, the elimination, diminution, or enhancement of any specific microbial population may influence the overall ecology of the biosphere. In addition, pollutants may affect complex microbial interactions, such as parasitism and mutualism (Babich and Stotzky, 1980). Abiotic factors such as pH, Eh, moisture, clay minerals, hydrous metal oxides, organic matter, ion exchange capacity, solar radiation, hydrostatic pressure, and inorganic, anionic, and cationic composition all influence the chemical reaction and hence, availability and toxicity of the contaminants to microorganisms. Biotic factors like slime production, physiological age, morphological state, nutritional state, physiological and genetic adaptation influence the response of the indigenous microbial populations to pollutants in the environment (Babich and Stotzky, 1980). The activities, ecologies and population dynamics of microorganisms in many habitats are influenced, usually adversely, by the increased deposition of contaminants from both industrial and domestic sources.

Pollutants are seldom, if ever, present in the environment as single constituents. Usually, industrial and domestic activities simultaneously emit numerous pollutants that may be deposited into a common microbial habitat. Interaction between pollutants, whether

synergistic or antagonistic, may evoke responses from the indigenous microbiota that are different from those evoked by exposure to individual pollutants.

Most studies of environmental toxicology dealing with microorganisms have been concerned only with determining the concentration of a pollutant that causes an inhibitory and/or lethal response in the target microbe under artificial laboratory conditions. However, there is little information on antagonistic mechanisms whereby one heavy metal increases or decreases the toxicity of a second heavy metal. Antagonistic interactions may result from competition between metals for common sites on the cell surface with the more efficient competitor preventing the uptake of another metal. For example, increasing the concentration of Mg^{2+} reduced the toxicity of Zn and Cd to the growth of Aspergillus niger (MacLeod and Snell, 1950 and Laborey and Lavollay, 1973). The uptake of Cd by Euglena gracilis was greater in Zn-deficient than in a Zn-sufficient medium (Nakano et al., 1978). However, other studies have shown that although Fe reduced the amount of inhibition of growth of Aspergillus niger by Cu, Ni or Zn, it did not suppress uptake of these metals (Adiga et al., 1962). Contradictory results have been reported by many researchers regarding synergistic or antagonistic effects of metals and microbial response. Upitis et al. (1973) stated that the toxicity of Cd to growth of a Chlorella sp. was reduced by the addition of Zn, Mn or Fe while Hart and Scaife (1977) reported that Zn addition did not influence the uptake of Cd by Chlorella pyrenoidosa. No synergistic or antagonistic effect between Cu and Zn was noted in the growth of Selanastrum capricornutum (Bartlett

et al., 1974). The generation time of a Zn-deficient medium containing 20 $mgkg^{-1}$Cd was 56 hours, whereas after the addition of 2 $mgkg^{-1}$ Zn, the generation time was reduced to 27 hours (Nakano et al., 1978).

The protective effect of one cation on the toxicity of a second heavy metal cation probably is a reflection of competition for uptake with a reduction in the subsequent accumulation of the heavy metal inside the cell. In natural environments, heavy metal pollutants and the natural inorganic cations each with different chemical affinities for functional ionogenic groups on the surface of indigenous cells probably compete in sorption to the cells (Stotzky and Marshall, 1976). Pre-exposure of Esherichia coli or Saccharomyces cerevisiae to Zn permitted the cells to accommodate Cd to toxic levels (Mitra et al., 1975 and Macara, 1978). Conversely, low levels of Zn increased the lethal action of Cd to Klebsiella pneumonia (Pickett and Dean, 1976).

Living organic matter (viable cells) sorb heavy metals. They bind differentially to specific ionogenic groups on the surfaces of these cells. The cell walls of Saccharomyces cerevisiae have been shown to sorb Cd (Itoh et al., 1975) and those of Salmonella enteritidis sorb Zn, Mn and Fe by cation exchange (Chipley and Edwards, 1972). Zwarum and Thomas (1973) reported that cells of Pseudomonas stutzeri have a CEC of 3400 $Mmol(+).kg^{-1}$ of dry weight and are capable of sorbing substantial quantities of Al to their cell walls. In the environment, both dead and living organic matter probably compete for heavy metal ions. For example, both river sediment and Pseudomonas fluorescens sorbed Hg with the bacterium being the more efficient adsorber (Ramamoorthy et al., 1977). However, sediments may sequester sufficient quantities of heavy

metals to reduce the effective concentration (availability to the biota) of the pollutant in that environment. The protective effect of the sediment was related to its high organic matter content which apparently removed the heavy metal from solution and thus reduced its availability to the bacteria (Hamdy and Wheeler, 1978). Soluble organic matter reacts with heavy metal by either covalent or coordination (chelation) bonding, and such complexes are, in general, less toxic to the microbiota than are the free metal ions (Pan et al., 1961). Another mechanism of antagonism between heavy metals may involve the sorption of one heavy metal to the amorphous complex of the other heavy metal. For example, Steeman and Kamp-Nielson (1970) explained that at alkaline pH levels, addition of Fe suppressed the inhibition of growth of Chlorella pyrenoidosa caused by Cu, presumably because of the cupric cations sorbed to the negatively charged sites on the micelles of amorphous $Fe(OH)_3$, rendering Cu unavailable for uptake by the alga. Synergistic interactions between heavy metals on microbial cells may result from the adsorption of both metals to the surface of the cell, with the adsorption of one metal increasing the permeability to the second. Although the majority of the responses of microbes to pollutants is negative, low concentrations of pollutants may, in time, exert a stimulatory effect on the responding microbiota. According to Henriksson and daSilva (1978), concentrations of Zn, Cd and Pb ranging from 0.005 to 0.025 $mgkg^{-1}$ stimulated nitrogen-fixation of Nostoc sp., whereas concentration of these metals between 0.025 to 0.1-5 $mgkg^{-1}$ was inhibitory.

Stimulation of the growth and metabolic activities of microorganisms by low concentrations of a toxicant may reflect an Arndt-Schulz effect, where the accumulation of a nonlethal concentration of a poison at the surface of a cell induces, among other changes, an alteration in the permeability that permits a freer flow of nutrients across the plasma membrane and, thereby, an increase in cellular metabolic activity (Babich and Stotzky, 1980). When the concentration of a contaminant is increased above tolerable levels, the initial response is an inhibition of microbial activity and, as the concentration increases, cell death results. Adverse responses of microbes to pollutants include: prolongation of the generation time of bacteria; decreased spore production by fungi; decreased nitrogen fixation by blue-green alga and lichens; decreased photosynthetic activity of green alga and lichens; and inactivation of viral infectivity (Babich and Stotzky, 1980).

Babich and Stotzky (1977b) tested a variety of microorganisms for sensitivity to Cd and noted a wide range. The toxicity of Cd to eubacteria, actinomycetes and fungi appeared to be pH dependent as it was generally potentiated at pH 8 or 9. So, it appears that not all microorganisms are equally susceptible to the toxic effects of metals in the environment. According to Novick and Roth (1968), strains of Staphylococcus aureus carry plasma conferring resistance to Zn, Cd, Hg, Pb and As. Kondo et al., (1974) also found that the penicillinase plasmid in Staphylococcus aureus contains gene(s) giving resistance to Cd ions. Resistant cells allow Ca but not Cd to enter the cell. Metal resistant plasmids are now thought to be common among bacteria, and this

could explain the differences found among the species. It is possible that the cell wall played an important role in protecting the organism. Tornabene and Edwards (1974) found that when their test bacteria were exposed to 2 500 mgL^{-1} Pb, over 99% of the Pb associated with the cells was on the cell walls and outer membranes of the bacteria. Less than 1% of the Pb had penetrated into the cytoplasm which may explain why this extremely high Pb concentration had no effect on the bacteria. Cadmium would behave in a similar manner to Pb since both cations have many similar properties.

Fixation and Availability of Heavy Metal by a Soil Component

The quantity and availability of the ions in soil are a function of chemical and biochemical processes alone or in conjunction with other factors. For example, the pH of the environment into which a pollutant is deposited may affect the chemical form, mobility, solubility and toxicity of heavy metal pollutants. According to Lucas and Knezek (1972), Zn may be rapidly leached under acid conditions from a soil where rainfall is high. Wear (1956) suggested that as pH of the soil increases, extractable Zn decreases and there is a reduction in Zn uptake by the plant. Peech (1947) stated that at a pH of 4.0 virtually all Zn applied to soil could be extracted, but as the pH increased the extractable Zn would be diminished. In alkaline environments such as sea water, Zn exists as a precipitated dibasic compound and Pb as $PbOH^{+}$ (Hahne and Kroonje, 1973). Cadmium, like Zn, is most mobile in soil at a pH of 5 or lower (e.g., in acid peat), whereas in soils of high pH (alkaline soils rich in $CaCO_3$), Cd is immobile presumably due to formation of insoluble $Cd(OH)_2$ and $CdCO_3$ (Hutchinson and Collins, 1978).

As pH and Eh are related, the ionic form of heavy metal pollution is influenced jointly by the availability of H^+ and electrons in the environment (Gadd and Griffiths, 1978). Krauskopf (1956) and Foy et al., (1978) reported that under reducing conditions, like those encountered in anaerobic environments, the microbial conversion of SO_4^{2-} to sulfide (S^{2-}), may lead to the precipitation of heavy metals such as ZnS, CdS, HgS and FeS. Adsorption of heavy metal ions may occur on the surface of $Si(OH)_4$ and $Al(OH)_2^-$ cation exchange sites, and on amorphous Fe hydroxide below the isoelectric pH. Clay minerals may fix appreciable quantities of metals by precipitation, by physical entrapment in clay lattices of metals, and by exchange adsorption (Reddy and Perkins, 1974). The metal toxicants introduced into the environment may be adsorbed or exchanged for the cations on the exchange sites of clay minerals and thereby removed from solution, at least temporarily, and be unavailable for uptake by the microorganisms. Montmorillonite is an effective adsorbent of Zn, Cu, Co, Ni, Hg, Pb and Cd, whereas kaolinite is a comparatively poor exchanger for heavy metals due to its lower CEC (Babich and Stotzky, 1980).

Increasing the concentration of the clay minerals increased their protection and this can be related to the CEC, since at equivalent concentrations, the sequence of protection is in the order of the CEC magnitudes. However, the protection provided by kaolinite and montmorillonite for bacteria and fungi in soil against the toxicity of heavy metals like Cd^{2+} may not be operable in sea water as Cd exists there primarily as $CdCl_2$ and $CdCl_3^-$ which may not bind as effectively to the negatively charged clay minerals (Pan et al., 1961). The sorbtion

of some heavy metals by clay minerals appears to be dependent on the pH of the environment. For example, the sorption of Zn and Pb by montmorillonite, illite and kaolinite increased when the pH was increased from 3 to 7; at higher pH values, they were precipitated as sparingly OH^- species. This increase was attributed to the adsorption of OH^- groups to the clay, which presumably acted as bridges to metal ions or through abstraction of a proton from functional groups. The ability of the clay minerals to exchange heavy metals is apparently also influenced by the concentration and type of soluble ligands present in the various environments. For example, in sea water, both OH^- and Cl^- anions compete for the same metals: with Zn and Pb, $PbOH^+$ and $Zn(OH)_2$ (which is sparingly soluble) species predominate over Cl^- complexes. The form and chemical activity of many heavy metal pollutants is not only influenced by the concentration and type of the anionic ligand present in the environment, but these different coordination complexes exert different toxicities to microorganisms. Microorganisms produce biochemicals such as amino acids, sugar acids, phenols, simple aliphatics and other compounds in soil which chelate metal ions. Although these constituents have only a transitory existence, significant amounts may be present in the soil during periods of intense biological activity which occur near decomposing residues, in the rhizosphere and in the soils amended with organic wastes (Stevenson, 1982). Biochemicals produced in this way as well as other chelating compounds occurring naturally in the soil (humic and fulvic acids) are all rich in functional groups (metal binding sites) and have the capability of transforming the solid phase forms of metals into soluble

and/or stable complexes. Even though simple biochemical compounds are of little significance in soil because of their rapid destruction by microorganisms, in many agricultural soils with high organic matter content (OM) the combined total of potential chelate formers in the aqueous phase is probably sufficient to account for the minute quantities of metal ions normally present (Stevenson, 1982). Zinc, like other multivalent cations (Cu^{+2}, Mn^{+2}), has the potential for complex formation or linkage with humic acid. Randhawa and Broadbent (1965) reported that in the retention of Zn by humic acids, three types of sites are involved. The less stable complexes were believed to be associated with phenolic OH groups and weakly acidic COOH; the more stable complex was believed to involve strongly acidic COOH. Although the strongly bound Zn represented less than 1% of the total retained, the sites responsible were believed to be of great importance because small quantities of Zn would be bound preferentially as the most stable complex. Humic and fulvic acids form both soluble and insoluble complexes with polyvalent cations depending upon degree of saturation. However, metal complexes of fulvic acids are more soluble than those of humic acids because of their high acidities and relatively low molecular weights. According to Stevenson (1976, 1977) the metal complexes are soluble at low metal-humic acid ratios, but precipitation occurs as the chainlike structure grows and COOH groups become neutralized through salt bridges.

Stevenson (1982) reported that in the natural soil a balance exists between the metal ions that occur in the solution phase (free ions and/or soluble chelate complexes) and in insoluble mineral and

organic forms.

The quantity and availability of micronutrients at any one time are affected by the synthesis and destruction of biochemical chelating substances as well as by transformations carried out by microorganisms. The requirement of micronutrients for bacteria, actinomycetes, and fungi are the same as higher plants, and they immobilize available nutrients when levels are suboptimum for growth. The relationship is analogous to N immobilization when crop residues with wide C/N ratios undergo decomposition in soil. According to Tisdale and Nelson (1975) when a C/N ratio in OM is greater than 30, there is immobilization of soil N during the initial decomposition process. For ratios between 20 and 30 there may be neither immobilization nor release of mineral N. And if the C/N ratio is less than 20, then there is a release of mineral N early in the decomposition. Besides the C/N ratio, many other factors influence the decomposition of organic materials and the release or immobilization of N.

Effects of Sterilization on Soil and Microbial Response

Sterilization is the process of killing or removing all living cells from a medium. It can be accomplished by exposure to lethal physical or chemical agents or, in special cases, by filtration of solutions. The maintenance of cellular equilibria is a function of various enzymes which in turn are regulated by genes on the cell DNA-chromosome. Many essential enzymes are associated with the cytoplasmic membrane which must be intact in order for the enzyme activities to contribute to cell maintenance (Carpenter, 1972). The vital activities of any organism or cell helps to maintain a dynamic

state or equilibrium condition. The disturbance of any cellular equilibrium by altering the factors controlling it may lead to cellular death. Radiation can induce in the medium reactive chemical radicals, of which the most important is the hydroxyl free radical (OH). It is the most reactive and potent oxidizing agent of various oxygen intermediates known and is capable of attacking many of the organic substances present in cells (Brock, 1979). Ionizing radiation can act on all cellular constituents, but death usually results from effects on DNA. The only valid criterion of death in the case of microorganisms is irreversible loss of its ability to reproduce. This is usually determined by quantitative plating methods with survivors being detected by colony formation. Cell membranes may be altered in permeability, be dissolved, be removed or be ruptured by sterilization and therefore cause destruction of the equilibria that maintained constant composition and osmotic pressure. Increased membrane permeability would permit toxic substances to enter or vital components to be lost from the cell and decreased permeability would retard the entrance of nutrients or excretion of toxic wastes. In studies of the chemistry and microbiology of soil, it is often desirable to work with a soil which is sterile, but use of heat or chemicals for sterilization changes the chemical and nutritional properties of soils rather markedly (Russell and Russell, 1950). The use of X and γ-radiation or an electron beam provides a useful means for partial or complete heatless sterilization of soil with the possibility of leaving the soil and its enzymes largely unchanged (Paul and McLaren, 1981). Radioisotopes in general, and especially those which emit γ-rays, provide high energy radiation which far exceeds

many chemical bond energies and can induce chemical reactions and/or disrupt structures in biological systems. Ionizing radiation occurs as a result of spontaneous disintegrations or transformation of atomic nuclei. These nuclear changes can give rise to several types of radiation; one being γ-rays which have an electromagnetic nature (photons) (Wolf, 1969). Photons are electrically neutral particles with zero mass when the particle is at rest and, consequently, they can penetrate matter with little interaction. This characteristic allows γ-rays to have a greater effective range in matter than that of alpha or beta particles of comparable energy (Wang et al., 1975). Gamma radiation causes ionization, and brings about sudden and drastic changes in the microbial population of soils. McLaren et al., (1957) sterilized the soil by an electron beam of sufficient intensity and energy and showed that doses of 1 and 2 million roentgen (roetgen(r) = 98 ergs g^{-1} of tissue) will kill all fungi; and eliminate all bacteria and actinomycetes respectively in soil. At a dose of 2.4×10^5 r the number of viable organisms are reduced to less than 1%. They also reported that soil urease activity was not retained in a similar soil where autoclaving was used as a means of sterilization. Doses of 2, 8, 32, 64, 128 and 250 kiloroentgens (kr) of gamma rays from a Co^{60} source were used by Stotzky and Mortenson (1959) to irradiate samples of Rifle peat. No significant effect was observed 54 days after an incubation period on the development of the bacterial population, pH, mineralization of phosphorus and evolution of CO_2. Growth of the fungal population and ammonia utilization was increasingly inhibited by doses of 8 to 250 kr. Since gross changes did not take place in the metabolism of the soil

microflora, apparently higher dose levels are required than those used for the sterilization of organic soils. Arredondo fine sand was exposed to gamma radiation from a Co^{60} source at doses of 1, 4, 16, 32, 64, 256, 512, 1 024 and 2 048 kr. Popenoe and Eno (1962) reported that the percentage survival of fungi and bacteria decreased with each increase in radiation dose to 4% at 1 024 kr. Algae were not as drastically reduced as bacteria and fungi. Some survived at 2 048 kr but greatest reduction occurred above 64 kr. With 1 024 kr, a few nematodes remained in the soil 2 days after irradiation, but at a dose rate of 256 kr, after 14 and 28 days none were recoverable. Two populations, one more sensitive to radiation than the other, were probably involved in this reaction. Stotzky and Mortensen (1959) reported that the number of viable cells diminished as a function of radiation dose and that fungi were more susceptible to radiation damage than bacteria in the pure culture. McLaren and et al. (1957) stated that bacteria and bacterial spores are more sensitive to electron beam radiation than viruses and enzymes. For example, bacteria and their spores require from $2x10^5$ to $2x10^6$ r for 100% sterilization in broth, whereas urease in soy flour was only inactivated 9.4% by $2x10^6$ r. These results also indicate that comparable doses are required for killing microorganisms in soil. Comar (1955) reported examples of physiological response in X or γ-radiation of many organisms. He indicated that dosages of 200 000 r, 500 000 r, and 1 000 000 r destroyed bacterial colonies, sterilized spores of bacteria and inactivated tobacco mosiac viruses, respectively. According to Carpenter (1972), the ionizing radiation can produce mutants in the surviving population besides the death which it causes by

rupturing the cell surface. These genetically altered forms differ from the parent population usually in only one characteristic, such as resistance to bacteriophage or the ability to synthesize a substance necessary for metabolic activity.

Bacteria vary markedly in sensitivity to ionizing radiation (Brock, 1979). Typical sensitive organisms are species of Pseudomonas, and resistant organisms are species of Micrococcus and Streptococcus. Probably the main reason that spores are resistant is their low water content, which reduces the efficiency of ionization events. Another factor affecting radiation resistance is the presence of chemical protective agents. These are mainly organic sulphydryl compounds that react with radiation-induced ionized substances converting them to normal substances. Such protective compounds may be present in high amounts in organisms that are unusually radiation resistant.

There is little information concerning the influence of soil organic matter on the lethality of radiation toward the microflora, although work of Dertinger and Jung (1970) with organisms in pure culture indicated that some organic compounds of high molecular weight have a protective effect that may be partly explained by their reactivity with free radicals.

Use of Soil Perfusion Apparatus in Studying Microbial Processes in the Soil

Lees and Audus first developed the soil perfusion apparatus in 1946. Temple (1951) described a modified design of the Lees's device as being a useful tool for the study of microbial processes and investigation of soil reactions. This modified and compact unit has the

advantages of seldom requiring mechanical adjustment, having fewer glass to rubber joints and being easier to maintain in aseptic or uncontaminated conditions. The perfusion technique is applicable to many bacterial systems utilizing a water-insoluble substrate and is superior for static studies of many processes in the soil such as chemical and biochemical metal fixation, nitrification and pesticide degradation where a solid is immersed in a liquid medium. The apparatus was especially well adapted to the studying of bacteria on sulfur compounds in coal and associated rocks. Substances other than soil, coal or rock also may be used in the unit. For example, Thiobacillus thiooxidans grows well in this unit on Waksman's sulfur medium when the sulfur is placed in the inner perfusion tube. Wildung et al., (1969) reported that perfusion units were useful in investigations designed to establish rate and mechanisms of pesticide degradation, in isolating organisms responsible for this process and evaluating the role of environmental variables on rates and pathways of degradation. The precise control of soil moisture and soil atmosphere composition during perfusion was accomplished by regulation of pressure and composition of gases surrounding the soil column. However, due to a lack of uniformity of moisture and aeration regimes within the soil column, the relationship between degradation rates in perfusion units and field condition was difficult to evaluate. Stewart et al. (1975) used soil perfusion techniques to study the transformations of N in fractions of anaerobically digested sewage sludge. The soil perfusion experiments indicated that only the ammonium-N of the sludge solids was oxidized to NO_3-N and the rates of nitrification of sludge were 37 ug No-3^--N/g soil

day $^{-1}$ for 2.5 cm hr^{-1}. Zamani et al. (1980) utilized a soil perfusion system and extensively studied static Zn immobilization by microorganisms as well as organic matter in different soils. The system was structured to resemble the natural soil system where the solid phase (soil body) was immersed in a liquid medium (application of liquid sewage on the land). Contamination of underground water (medium in filter flask of the soil perfusion apparatus) was possible if the soil and its enhanced microbial populations did not act as an effective "living filter".

The ability to directly sample at any specified time interval and analyze the solution which percolated through the soil was a major advantage of the soil perfusion system over other methods.

MATERIALS

Soils

Surface samples of Rubicon sand (Muskegon County, MI) Brookston clay loam, (Ingham County, MI) and Houghton muck (Clinton County, MI) were used to compare the relative amounts of Zn inactivation in soil due to microbial activity and to physical and chemical factors. The Rubicon sand and Brookston clay loam were air dried, passed through a 2 mm sieve and stored in the air dry state at room temperature (25°C) until required for experimental work. Houghton muck was passed through a 2 mm stainless steel sieve and stored in a moist state to avoid rewetting problems.

Soil Perfusion System

Soil perfusion units of a modified design of the Lees soil percolation apparatus (Temple, 1951), as shown in Figure 1, were used in the soil perfusion experiments. Soil perfusion units were connected to a vacuum pump in series with the particular experiment being conducted determining the number of soil perfusion units in use (Figure 2).

The soil perfusion unit is a vacuum driven device which can be used to move perfusate from a soil perfusion reservoir through a soil column under a variety of experimental conditions such as aseptic environment, type of soil column, composition of perfusate, and aerobic or anaerobic conditions. Soil perfusion units are well suited for experiments which require accelerated impacts of particular soil microorganisms upon the biology and chemistry of soils under specific environmental and experimental conditions. The perfusion cell or unit

Figure 1. Soil perfusion apparatus used in these studies. Note the indicated column of packed soil.

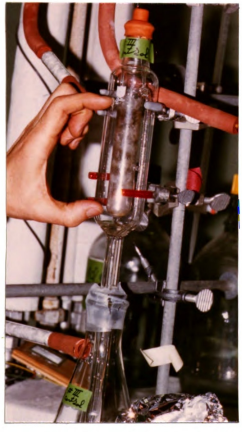
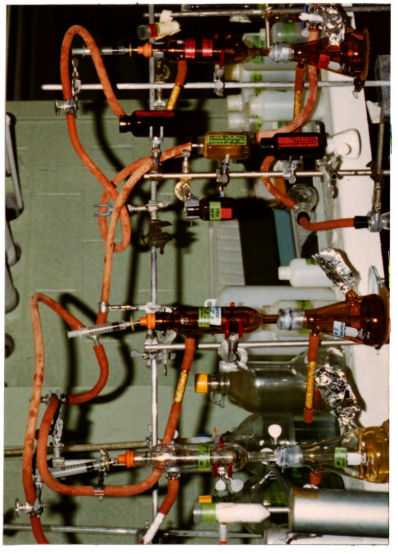


Figure 2. Three soil perfusion units connected in series. Glass became tinted brown as a result of gamma radiation.



is composed of two pieces of pyrex glassware. One part is a 500 mL reservoir or filtering flask with a stoppered cap and a built-in needle for sample removal. Air entry ports are part of the reservoir design so that a constant supply of fresh filtered air is available to the cell in experiments under aerobic conditions. The second part of the soil perfusion cell is the sample percolation unit constructed of Pyrex tubing of an appropriate size to allow a vacuum driven device to function.

Soil Perfusion Column and Perfusate

The soil perfusion soil column was prepared by combining 12.5 g of soil with 12.5 g of silica sand and 1 g of glass wool. A plug of glass wool was inserted into the bottom of the soil perfusion column, and the mixture was inserted into the soil column tube (Figure 1) and packed in such a manner that a uniform rate of percolation and distribution of perfusate was obtained regardless of the soil being used or experimental treatment being applied. The column and all other openings of the soil perfusion system were sealed and the soil perfusion unit was wrapped with aluminum foil to reduce recontamination of the unit after sterilization by either autoclaving or by gamma irradiation using a variable flux gamma irradiator with a ^{60}Co gamma source. Filter flasks containing 320 mL of deionized water to be used as perfusate were capped with stoppers containing needle-entry ports, covered with aluminum foil and subjected to the same sterilization procedure. The Zn solution and microbial energy and nutrient additions were subjected to similar sterilization procedures.

Energy and Nutrient Enrichment of Perfusate

Denatured Difco bacto nutrient broth solution was selected as the energy and nutrient supplement used to enhance microbial activity in the soil perfusion system. The five mL solution added 389 mg of C and 140 mg of N (C:N ratio of 2.8:1) to the 320 mL of purfusate in the reservoir. A 32 hour growth period was required after an energy and nutrient addition and microbial inoculation before maximum microbial activity was achieved and experimentation with Zn addition could begin.

Agar Plate Preparation for Dilution and Counting

A mixture of 1 kg soil, 1L H_2O and 10 g $CaCO_3$ were autoclaved for one hour, cooled and filtered through Whatman #2 filter paper. The soil extract was adjusted to pH 7 and sterilized. A subsample of 200 mL was mixed with 800 mL H_2O, 1 g glucose, 15 g dipotassium phosphate and 15 g bacto-agar. The pH was again adjusted to 7 and the mixture autoclaved for 15-20 minutes and then either mixed with labeled Zn, unlabeled Zn source, or unaltered and poured into dispo petri dishes and allowed to solidify.

METHODS

Soil Characteristics and Textural Analysis

Rubicon sand soil (Muskegon County, MI) was used in all the soil perfusion experiments. Brookston clay loam (Ingham County, MI) and Houghton muck (Clinton County, MI) were also used in selected experiments. The methods and procedures used in the chemical and physical characterization of the soils are those employed in the Michigan State University Soil Testing and Plant Analysis Laboratory (Dahnke, 1980). The chemical and physical properties of these soils are presented in Tables 1 and 2.

Table 1. Textural analysis of Rubicon sand and Brookston clay loam.

Soil series	Particle size		
	sand	silt	clay
	---------- % ----------		
Rubicon sand	88.5	4.4	7.1
Brookston clay loam	22.5	32.4	35.1

Inorganic Zn Analysis

A one mL sample of the soil perfusate was removed from the perfusion reservoir at periodic intervals during the operation of the soil perfusion system and brought up to a 10 mL volume with deionized water. Analysis was conducted using a Perkin Elmer-303 atomic

Table 2. Chemical characteristics of Rubicon sand, Brookston clay loam and Houghton muck.[+]

Soil series	pH	Extractable nutrients				0.1 N HCL Extractable				Soluble salts	Exchangeable bases			CEC
		P	K	Ca	Mg	Zn	Mn	Cu	Fe		K	Ca	Mg	
		mg kg^{-1}				mg kg^{-1}				dS m^{-1}	%			mmol(+)$\cdot kg^{-1}$
Rubicon sand	5.9	11	16	320	32	4	5	1	20	.15	2	84	14	32
Brookston clay loam	7.5	138	448	2240	435	11	69	14	60	.20	7	70	23	175
Houghton muck	6.0	76	100	3894	365	33	6	27	32	2.10	1	86	13	704

[+]All values are the average of three replications.

absorption spectrophotometer.

Inhibitory (static) and Lethal (cidal) Effect of Zn

Duplicate samples of 0, 10, 20, 30, 40, 50, 100, 150, 200, 250 and 300 $mgkg^{-1}$ Zn in a 40% volume of thioglycollate medium were autoclaved for 20 minutes, cooled, aseptically inoculated with 0.1 mL of soil suspension (1:1 soil:water) and incubated for 1 to 2 weeks at room temperature.

Following the incubation period, those samples showing no microbial growth, as indicated by turbidity, were subsampled into fresh thioglycollate medium, set aside for 1 or 2 weeks at room temperature and then checked for microbial growth. If microbial growth was nil, a cidal effect from Zn on microorganisms was assumed.

Sterilization and Microbial Growth Inhibitors

Autoclaving the intact perfusion unit (with packed soil column in place) for one hour per day on 2 consecutive days and for 17 hours on the third day at 120°C at 1.1 kg cm^{-2} was found in several trials to be the most effective means of steam sterilization and was used in all subsequent autoclaving experiments. Similarly, the most effective sterilization using gamma irradiation was accomplished by ^{60}Co irradiation for 24 hours with a total radiation dosage of 4.9 megarads of the entire soil perfusion unit with the packed soil column in place.

To insure uniformity of sample treatment, all treatments were autoclaved or irradiated, and then those samples scheduled to contain microbial activity were reinoculated with a fresh soil suspension (1 g unsterilized soil:10 mL deionized water) made up from the soil being studied. A similar soil suspension, which had been sterilized, was

added aseptically to the treatments scheduled to have an inactive microbial population so that a complete check could be maintained.

A microbial viability test was performed before and after each sterilization and the sterilized perfusate was monitored throughout the experiment as regular aseptic perfusate withdrawal raised the risk of contamination. To determine the effectiveness of the sterilization and subsequent aseptic manipulations, one mL of soil perfusate was added to 10 to 15 mL of sterile bacto-fluid thioglycollate medium and was incubated for one to two weeks at room temperature. After preparation, the thioglycollate medium separated into two colored layers, the pink or aerobic zone on the top (containing resazuring as an Eh indicator) and the yellow or anaerobic zone at the bottom. If the incubated solution appeared turbid in either zone, microbial activity was present and complete sterilization had not been achieved.

Uniformity of Perfusate Solution Mixing

The soil perfusion units were packed with identical mixtures of soil, sand, and glass wool and perfused with 320 mL of solution containing 20 $mgkg^{-1}$ Zn (as $ZnCl_2$). Addition of a few drops of a dye mixture of bromo-cresol and methyred (an indicator for N determination) to the soil perfusion system while it was in operation indicated a complete mixing action while the Zn contents of drawn samples from different units were compared to verify uniformity between units.

Soil Perfusion System Operation in Experimental Mode

The sterilized soil perfusion units were assembled in a series and hooked to a vacuum pump as shown in Figure 2. Either 5 mL of soil suspension and 5 mL of sterile deionized H_2O or 5 mL of sterile soil

suspension plus 5 mL of nutrient broth were injected into a column and incubated for 32 hours to allow for microbial growth. Following incubation, the soil perfusion units were started and regulated. A baseline soil perfusate sample was drawn and five mL of 1 000 $mgkg^{-1}$ Zn (5 000 ug Zn/320 mL perfusate) solution was added to the soil perfusion units by aseptic injection onto the soil column. Eleven serial samples were drawn over a period of 72 hours and analyzed for microbial activity and Zn content. The total volume of the system was kept constant by timely additions of deionized H_2O.

Statistical Procedures

Facilities of the university computer center and STAT routines system (version 4.4) of the Agricultural Experiment Station were used for statistical analysis. The perfusate Zn concentration data were subjected to an analysis of variance. The experiment was arranged in a split plot design with 3 replications. The main plot was treatment which consisted of four combinations of inoculation with microorganisms and amendment with nutrient broth:

sterile and unamended

sterile and amended

inoculated and unamended

iooculated and amended

The sub-plot was time of sampling which occurred at 12 time intervals: 0, 0.5, 1, 2, 4, 8, 12, 24, 36, 48, 60 and 72 hours. Simple effects of treatment and time of sampling and the interaction between the two were carefully analyzed.

Dilution and Counting of Soil Microorganisms

The Rubicon sand soil perfusion experiment ran 72 hours during which the maximum number of Zn immobilizing-microorganisms developed. One g of soil from the soil perfusion column was mixed with 10 mL of sterile deionized water. Dilutions of $1:10^n$ (where n varied from 1 to 8) were prepared in a sequential manner by transferring 1 mL of a previous dilution into a 10 mL volumetric flask and filling it to volume with sterile deionized water.

In order to determine which dilution gave the desired number of colonies, replicate plates were prepared by inoculating 0.1 mL from each of several dilutions in the anticipated critical range. The diluted soil suspension was placed on the agar plate medium and spread in a manner that resulted in a good distribution of organisms. The plates were then incubated at room temperature until the colonies became visible. The assumption is that each viable microorganism trapped in or on a nutrient agar medium would multiply and produce a visible colony. The number of colonies would therefore be the same as the number of viable microorganisms inoculated on the agar in the petri dish. Plates with yields between 30 and 300 colonies could be counted easily and accurately.

Isolation of Zn Immobilizing Soil Microorganisms

A technique combining standard microbial plating techniques in a ^{65}Zn impregnated agar and autoradiography was devised to determine what percentage of the microorganisms in the soil population were immobilizing Zn. The technique was utilized to isolate pure cultures of the Zn immobilizing soil microorganisms.

Tracer Zn solutions of 0, 11 000, 22 000, 33 000, 44 000, 66 000, 88 000, and 176 000 disintegrations per second, equivalent to 0.0, 0.024, 0.048, 0.072, 0.096, 0.144, 0.192 and 0.384 $mgkg^{-1}$ ^{65}Zn, were mixed with soil agar and the extract was transferred to petri dishes and allowed to solidify. A separate set of agar plates with non-labeled Zn in identical concentrations was also prepared to determine the toxicity of ionizing radiation. Both sets were spread with diluted soil suspension and then incubated for 4 to 7 days. Response of x-ray film to the time of exposure was determined for each concentration of radioactive Zn by selecting the plates with a specific concentration which had a desired number of colonies (30 to 80), setting petri dishes above the film and exposing KODAK NO-SCREEN X-RAY film (NS-2T) or X-OMAR-AR film for various time periods. A good response consisted of distinct black spots on the processed film relating directly to ^{65}Zn concentration and length of exposure time to the film.

Isolation of Pure Culture of Microorganisms

The microbial biomass (colony) uhich accumulated radioactive ^{65}Zn on the agar plate was marked carefully by using a glow box (source of light) to match with the distinct exposure spots on the films. Overlapping colonies and contamination of different species of microorganisms on plates as the result of overgrowth during lengthy periods of x-ray film exposure time occurred so doses of ^{65}Zn were adjusted to speed up the autoradiograph. The use of the streak plate technique with subsequent transfer to a tube culture after colonies were formed provided a simple procedure for isolation purposes. A transfer needle was sterilized and inserted into a specified colony and a loopful

of inoculum was streaked rapidly and lightly back and forth across the new medium. Under sterile conditions, the plate contained a single group of Zn immobilizing microorganisms.

After colonies developed, a loopful of colony was transferred to a new culture tube and stored in a cold room until time for identification.

Identification of Zn Immobilizing Microorganisms

Staining techniques and microscopic examination were used to identify the Zn immobilizing microorganisms. Inoculant was removed from the refrigerated culture tubes with a sterile loop and placed on the plates containing special growth media. Potato dextrose agar (3.9 g 100 mL^{-1} of deionized H_2O) was used for fungal growth and tryptophan glucose extract agar (2.4 g 100 mL^{-1} of deionized H_2O) was used for bacteria and actinomycetes. New colonies developed on the respective media after a short incubation period of 2 to 4 days and were stained for microscopic study. Staining of cell samples consisted of spreading a drop of an aqueous cell suspension on a glass slide, allowing it to dry, applying gentle heat to fix the cells, followed by the application of the staining solutions. The cells were stained by the application of a single staining solution (such as lactophenol cotton blue for fungi). Differential staining procedures were used to study the bacteria and actinomycetes as follows: Using a sterile transfer loop, two loopfuls of water were placed on a clean, dry slide. A portion of a colony was spread to cover an area of 1.5 cm. It was air dried and then fixed by flame. After slide preparation, selected stains were applied to the smear: Crystal violet, one minute; the smear rinsed with Grams iodine

and then sustained for an additional period of one minute; rinsed with 95% ethanol and then restained with a few drops of safranin. After 30 seconds excess stain was removed by gentle running water, and then blotted dry with absorbent paper. A slide stained and prepared in this way was studied under the light microscope using the oil-immersion objective.

Identification of an organism is the process of determining its species. As many as possible of its characteristics are ascertained by appropriate observations and tests, and the accumulated information was then compared with published descriptions of the various species. The organism was considered properly identified when a species description was found that was identical with the observed characteristics.

RESULTS AND DISCUSSION

The information presented in Tables 1 and 2 show that Rubicon sand is a relatively infertile sandy soil with a low cation exchange capacity (32 mmol(+)$\cdot kg^{-1}$) and a pH of 5.9 while Brookston clay loam is a very fertile soil with a moderate cation exchange capacity (175 mmol(+)$\cdot kg^{-1}$) and a pH of 7.5. Houghton muck, by contrast, is an organic soil of moderate fertility, a higher cation exchange capacity (705 mmol(+)$\cdot kg^{-1}$) and a pH of 6.0. The data suggest that Rubicon sand should be an ideal soil for microbial immobilization of excessive Zn addition provided nutrients and energy are supplied to the soil to enhance the microbial population.

Verification of consistent performance between the three soil perfusion units used in the experiments is given in Figure 3. Performance consistency between different soil perfusion units was determined visually by rate of mixing of a dye as well as by analyzing the Zn concentration in the soil perfusion reservoir of each unit with time. Uniform distribution of the dye solution after introduction into each unit indicated a complete mixing action. Similarity of Zn concentration in the samples drawn from separate units at specified time intervals verified the operational consistency of multiple units connected in series (Figure 3). The Zn in the perfusate reached an equilibrium concentration of about 4 mgkg^{-1} prior to five hours of operation.

Effective soil sterilization should reduce Zn removal from the perfusion solution if microorganisms are involved in the removal. The

Figure 3. The performance consistancy of three different soil perfusion units. A solution of Zn (5 mL containing 5 000 ug Zn) was added to each soil column at time equal zero, and the Zn concentration in the reservoir was determined at intermittant intervals.

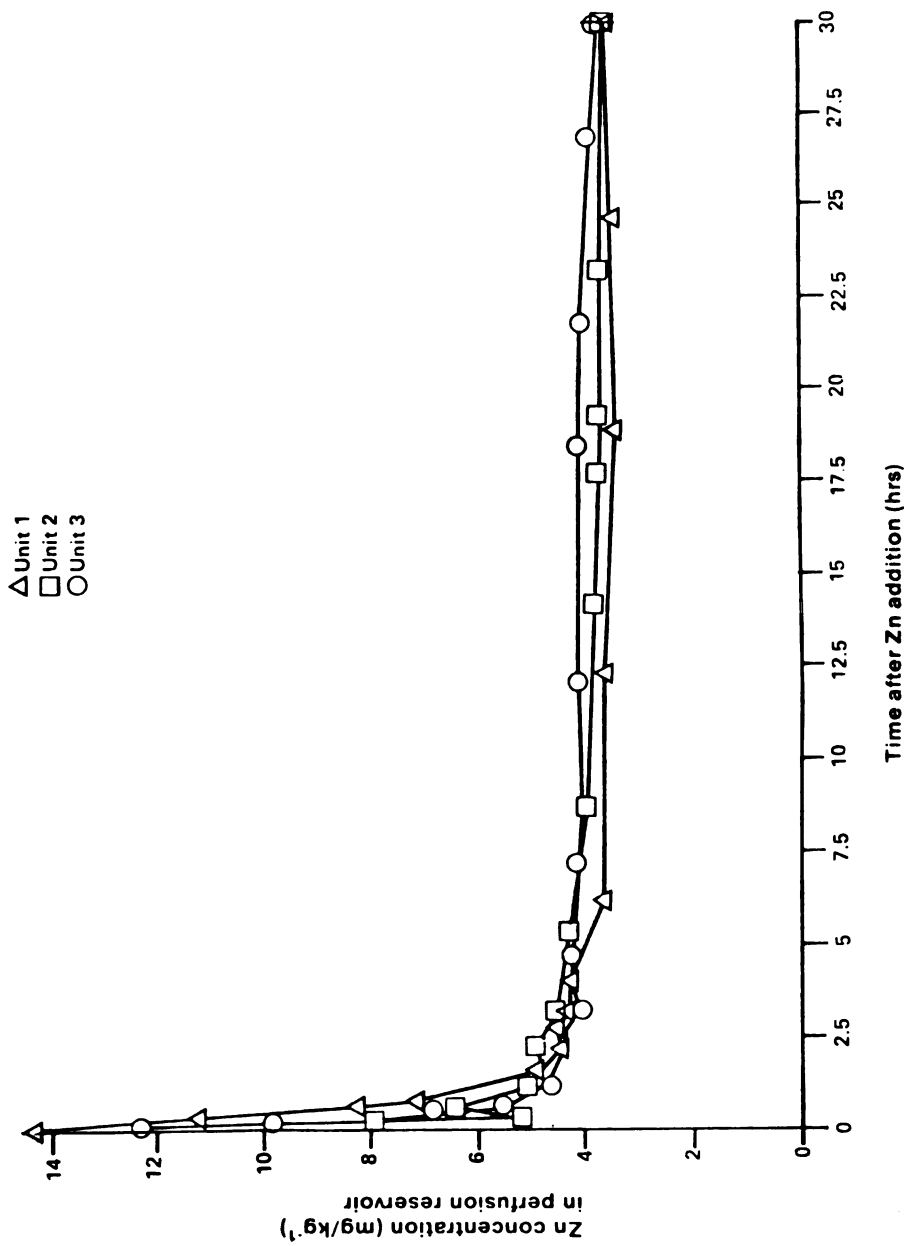

Unit 1
Unit 2
Unit 3
Zn concentration (mg/kg⁻¹)
in perfusion reservoir
14
12
10
8
6
4
2
0
0
2.5
5
7.5
10
12.5
15
17.5
20
22.5
25
27.5
30
Time after Zn addition (hrs)

Figure 3.

data in Figures 4 and 5, respectively, show that autoclaving and gamma-irradiation suppressed Zn removal from the perfusion solution. The removal of Zn from Rubicon sand soil perfusion solution after sterilization, addition of nutrient broth and inoculation with a fresh microbial population was similar in both methods. However, the equilibrium level of Zn in the soil perfusion reservoir sterilized by gamma radiation (Figure 5) was only half that with autoclaving (Figure 4). This observation indicates that hydrolysis of organic matter may have occurred during autoclaving and this left more soluble Zn-organic complexes in the reservoir. Therefore, the Zn level was higher in the autoclaved perfusion solution than in the gamma-irradiated solution after sterilization. Based on this information, the relatively nondestructive gamma-irradiation technique was used for microbial suppression.

Data in Figure 5 also show that inoculation of a sterile and amended perfusion treatment after 68 hours of incubation will induce Zn removal from the perfusion solution. These results are evidence that the Zn removal pattern observed is indeed due to the presence of soil microorganisms from the inoculate.

Additional evidence for soil microorganisms being involved was through filtration of the perfusate. Results of unfiltered and corresponding filtered samples from soil treated with increasing amounts of a food and energy source (1, 2 and 3 mL soybean meal extract) as represented in Table 3 indicate that the Zn concentration was reduced noticeably by as much as 2.8 $mgkg^{-1}Zn$ as the result of filtration or almost 53%. Since the pore size of Gelman Acrodisc disposable filter

assembly (0.2 u) is smaller than the general size range of microorganisms (0.5 to 20-50 u), it would therefore eliminate the microorganisms which presumably immobilize Zn. Beside microorganisms, this screening action would also eliminate large sized Zn-adsorbing particles (>0.2u) such as colloidal clay, organic matter, and microbial products or by-products. The filtration method was only indirect evidence that microorganisms participate in Zn-immobilization in the soil.

Table 3. Filtration of the soil perfusate over time to determine if Zn in the reservoir is present in a particulate form.

Organic matter level	Filtered (0.2 u)	Zinc concentration (hrs after addition) 4	15	32	39
		mg kg^{-1}			
1	no	5.0	2.3	2.3	1.8
	yes	4.7	1.7	1.8	1.2
2	no	6.9	2.8	5.5	4.5
	yes	6.5	2.2	3.5	2.4
3	no	6.0	3.3	5.1	5.3
	yes	5.4	2.3	3.7	2.5

Zinc removal data for replicated treatments consisting of sterile and unamended, sterile and amended, inoculated and unamended, and inoculated and amended soil perfusion systems with Rubicon sand, Brookston clay loam and Houghton muck soil columns are given in Figures 6, 7 and 8, respectively.

Figure 4. The influence of microorganisms and denatured nutrient broth upon Zn immobilization in a soil perfusion apparatus which was sterilized by autoclaving. Percolate from the reservoir was sampled periodically and analyzed for Zn by atomic absorption spectrophotometry.

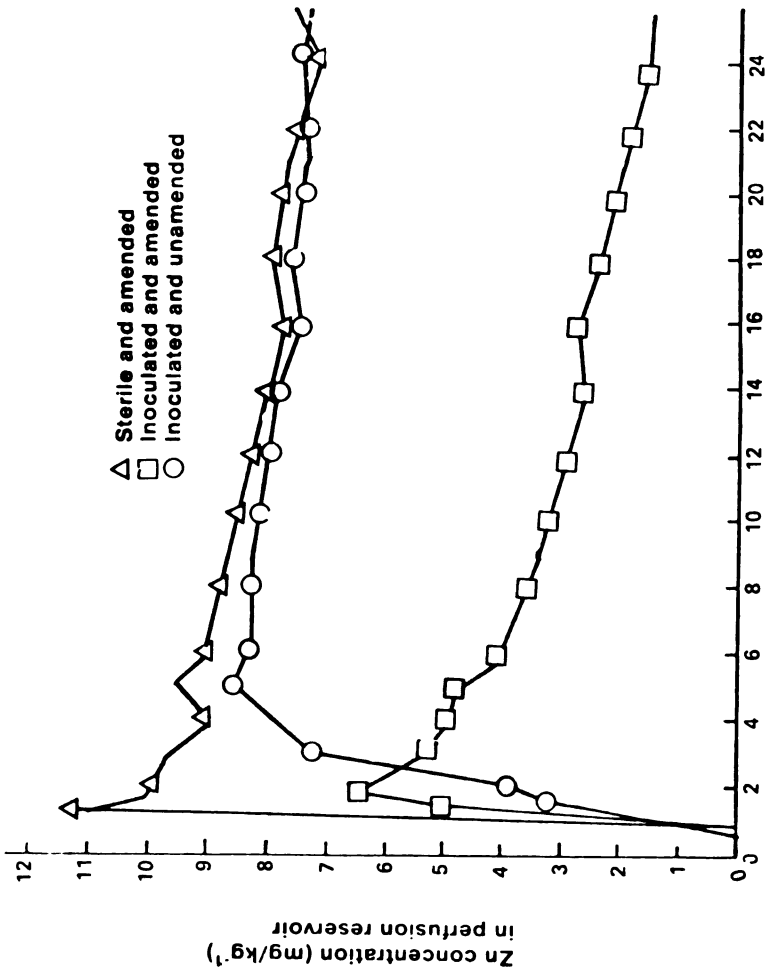

Sterile and amended
Inoculated and amended
Inoculated and unamended
Zn concentration (mg/kg⁻¹)
in perfusion reservoir
Time after Zn addition (hrs)
0
1
2
3
4
5
6
7
8
9
10
11
12
0
2
4
6
8
10
12
14
16
18
20
22
24

Figure 4.

Figure 5. The influence of microorganisms and denatured nutrient broth upon Zn immobilization in a soil perfusion apparatus which was sterilized by gamma radiation. Microorganisms were added to one sterile treatment at the 68th hour with a subsequent increase in Zn immobilization. Percolate from the reservoir was sampled periodically and analyzed for Zn by atomic absorption spectrophotometry.

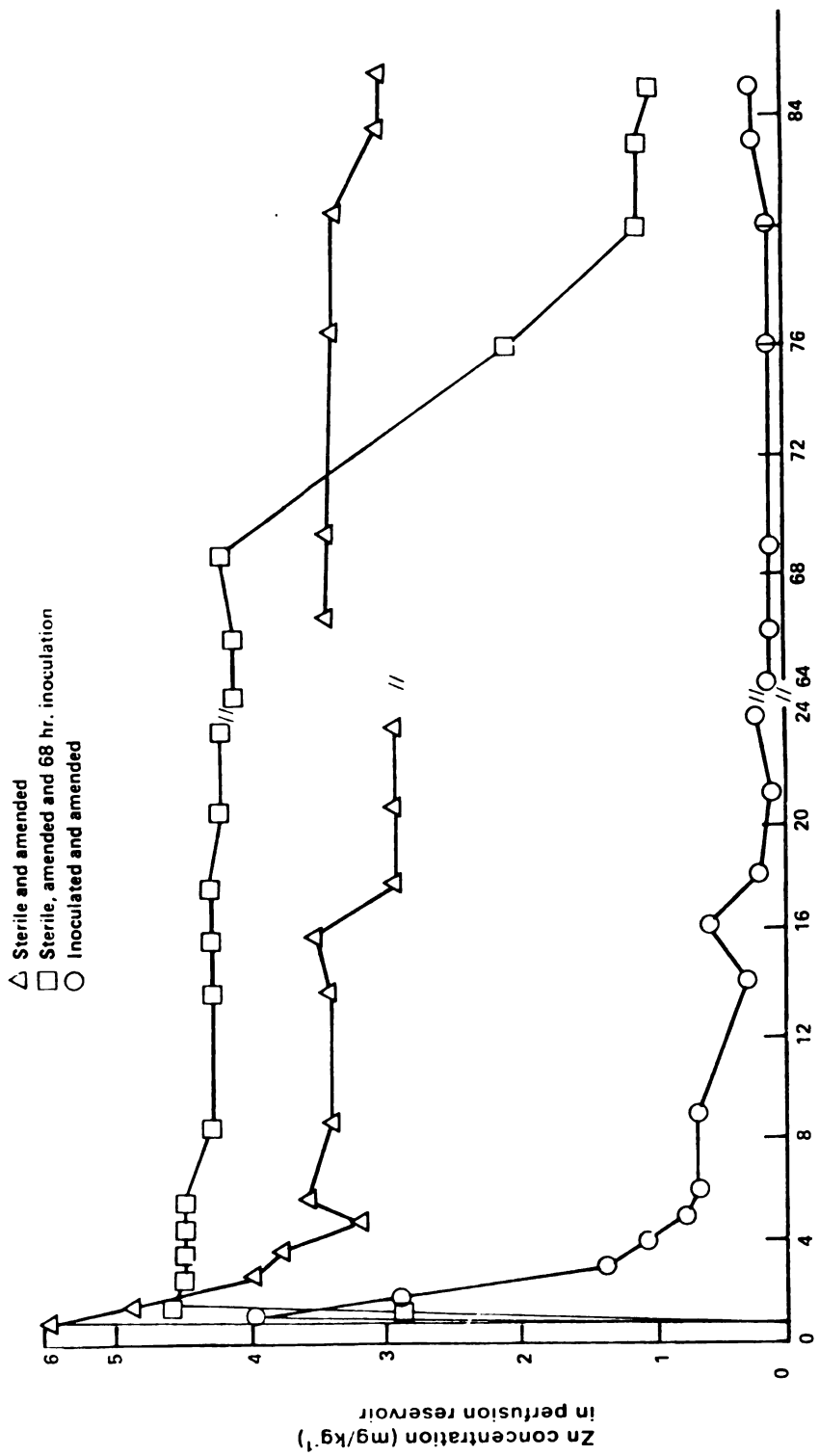

Sterile and amended
Sterile, amended and 68 hr. inoculation
Inoculated and amended
Zn concentration (mg/kg⁻¹)
in perfusion reservoir
Time after Zn addition (hrs)
0
1
2
3
4
5
6
0
4
8
12
16
20
24
64
68
72
76
84

Figure 5.

Data in Figure 6 for Rubicon sand show that the inoculated and amended treatment resulted in a significant greater removal of Zn from the soil perfusion solution with time than did the other treatments. These results imply that inoculation with viable soil microorganisms and amendment with nutrient broth stimulated microbial growth and subsequent Zn immobilization. The increase in Zn removal was significantly greater than the amount removed from solution by the control treatment (sterile and amended). While not significant, there tended to be a greater Zn removal with the inoculated and unamended as compared to either sterile treatments. Therefore, the Rubicon sand appears to need supplemental nutrients and an energy source to produce enough of a microbial population to achieve maximum immobilization of Zn. The Zn levels in the perfusion solution generally reached an equilibrium state within 12 hours even though the inoculated treatments tended to immobilize additional Zn with time.

Data in Figure 7 for Brookston clay loam show a rapid inactivation of Zn from the soil perfusion soil to a level less than one $mgkg^{-1}$ with no significant difference between treatments. These results indicate that chemical and physical factors dominated the control of the level of Zn in the soil perfusion system while microorganisms were relatively unimportant in the inactivation. Such results were expected since Brookston clay loam has a pH of 7.5 and a high clay content and cation exchange capacity to provide surfaces and sites for Zn adsorption.

The information shown on Figure 8 for Zn removed from the soil perfusion solution with Houghton muck shows no significant difference between treatments. Presumably, the large number of active organic

Figure 6. The influence of microorganisms and denatured nutrient broth upon Zn immobilization in a Rubicon sand soil column contained in a soil perfusion apparatus which was sterilized by gamma radiation. Percolate from the reservoir was sampled periodically and analyzed for Zn by atomic absorption spectrophotometry.

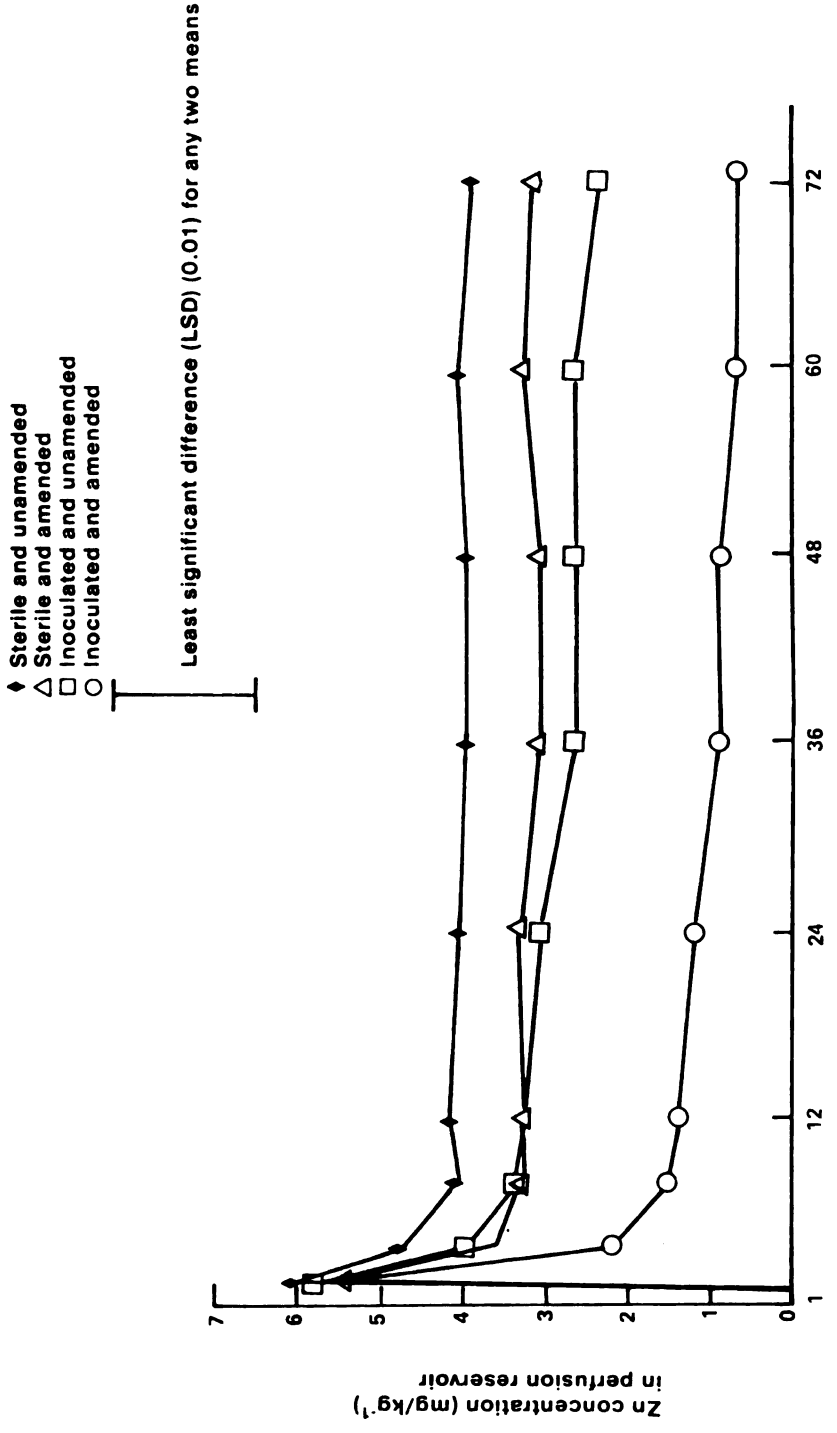

♦ Sterile and unamended
△ Sterile and amended
□ Inoculated and unamended
○ Inoculated and amended
Least significant difference (LSD) (0.01) for any two means
Zn concentration (mg/kg⁻¹) in perfusion reservoir
0
1
2
3
4
5
6
7
1
12
24
36
48
60
72
Time after Zn addition (hrs)

Figure 6.

Figure 7. The influence of microorganisms and denatured nutrient broth upon Zn immobilization in a Brookston clay loam soil column contained in a soil perfusion apparatus which was sterilized by gamma radiation. Percolate from the reservoir was sampled periodically and analyzed for Zn by atomic absorption spectrophotometry.

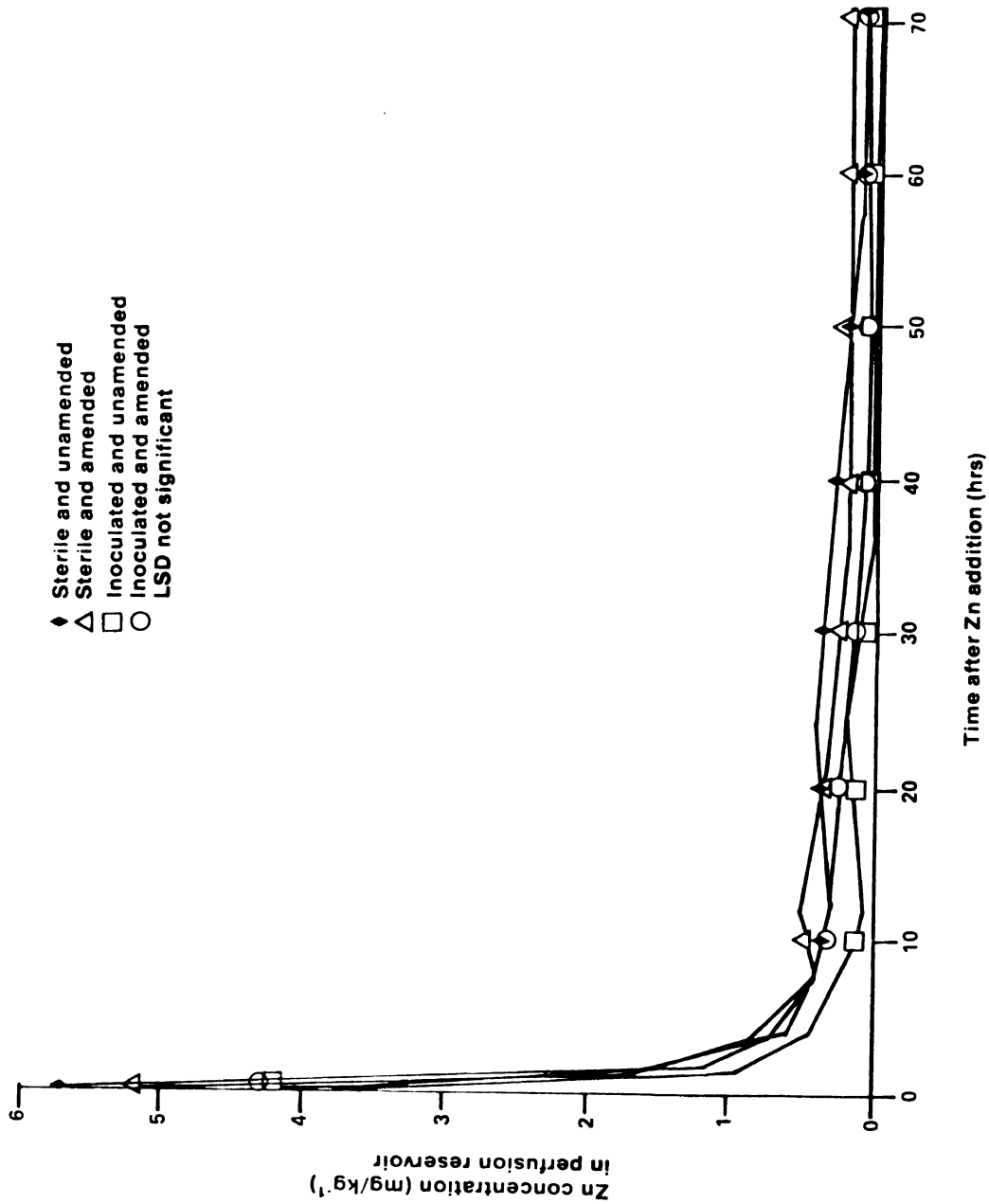

Sterile and unamended
Sterile and amended
Inoculated and unamended
Inoculated and amended
LSD not significant
Zn concentration (mg/kg⁻¹)
in perfusion reservoir
Time after Zn addition (hrs)
0
1
2
3
4
5
6
0
10
20
30
40
50
60
70

Figure 7.

Figure 8. The influence of microorganisms and denatured nutrient broth upon Zn immobilization in a Houghton muck soil column contained in a soil perfusion apparatus which was sterilized by gamma radiation. Percolate from the reservoir was sampled periodically and analyzed for Zn by atomic absorption spectrophotometry.

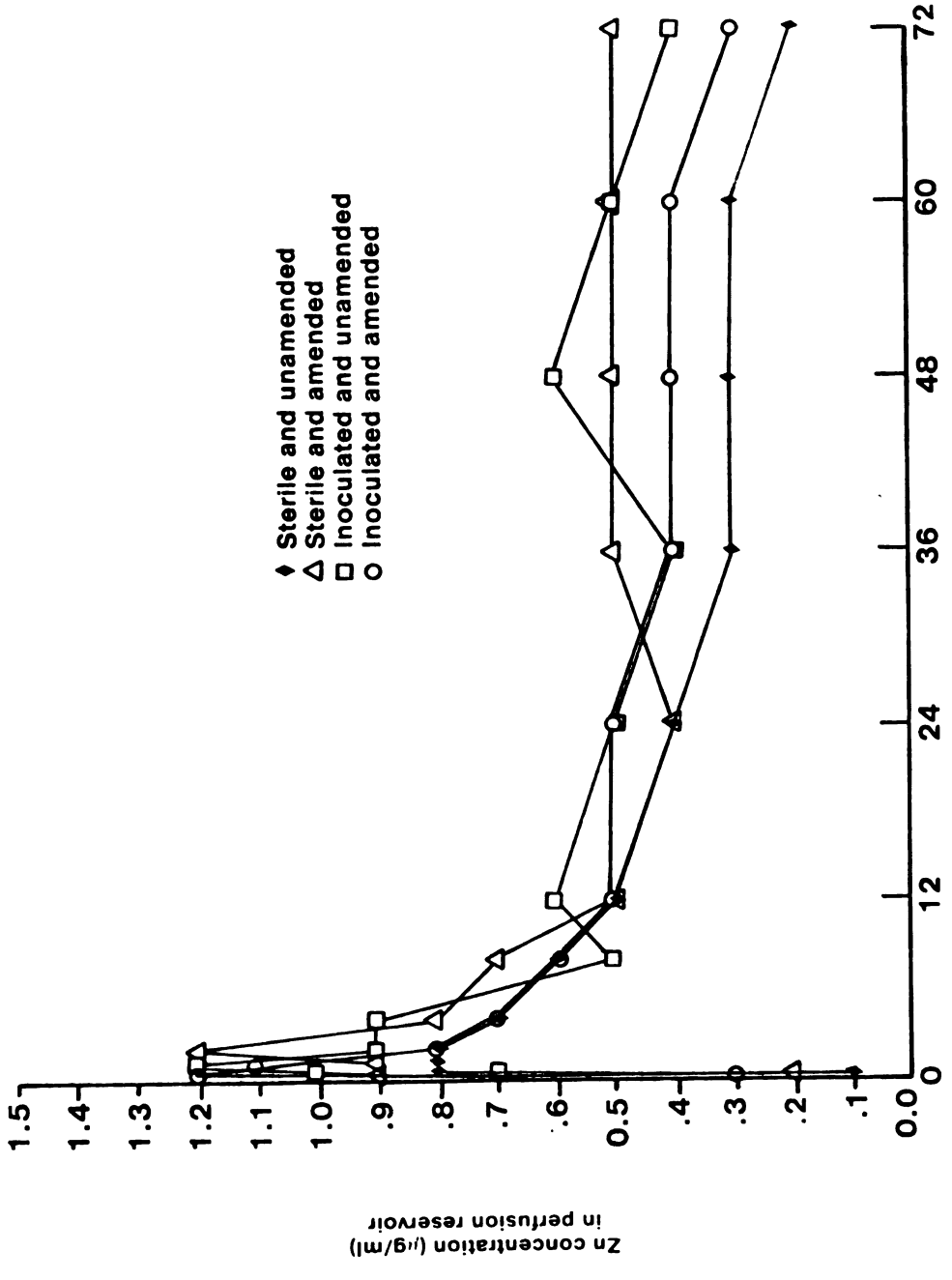

Sterile and unamended
Sterile and amended
Inoculated and unamended
Inoculated and amended
Zn concentration (μg/ml)
in perfusion reservoir
Time after Zn addition (hrs)
0.0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
1.1
1.2
1.3
1.4
1.5
0
12
24
36
48
60
72

Figure 8.

binding sites enabled the Zn level to be buffered at a one $mgkg^{-1}$ level regardless of treatment. While the difference in Zn levels in solution were not statistically significant, there did tend to be a greater inactivation with the inoculated and amended treatment.

Data in Figure 9 show the results of Mn immobilization in Rubicon sand. The procedures are the same as those used in obtaining the data for Zn shown in Figure 6 except that Mn, as $MnSO_4$, rather than Zn was applied. Results given in Figure 9 showed that microbial activity and organic amendment significantly reduced the level of Mn in the soil perfusion solution and the patterns of Mn immobilization with time were similar to that of Zn. The phenomenon of relatively rapid inactivation of Mn in this experiment was attributed to either a complexation or adsorption phenomenon in the soil organic fraction or to enriched microbial fixation as reported by numerous investigators. The higher equilibrium values of Mn than Zn in the perfusion solution at the end of the experiment was probably due to less precipitation and adsorption of Mn than Zn under these experimental conditions.

The dark areas in Figure 10 represent autoradiographic exposure of x-ray film by isolated colonies of ^{65}Zn-immobilizing soil microorganisms isolated from a perfused Rubicon sand column and which were grown on an agar plate impregnated with ^{65}Zn. Larger exposure areas represent a greater accumulation of the radioactive isotope. The greater ^{65}Zn accumulation could be due to either larger colony mass or to a differential amount of uptake by different microorganisms. Since only 20% of the colonies formed accumulated ^{65}Zn in amounts discernable from background levels by autoradiography, there is good circumstantial

Figure 9. The influence of microorganisms and denatured nutrient broth upon the Mn immobilization in a Rubicon sand soil column contained in a soil perfusion apparatus which was sterilized by gamma radiation. Percolate from the reservoir was sampled periodically for Mn by atomic absorption spectrophotometry.

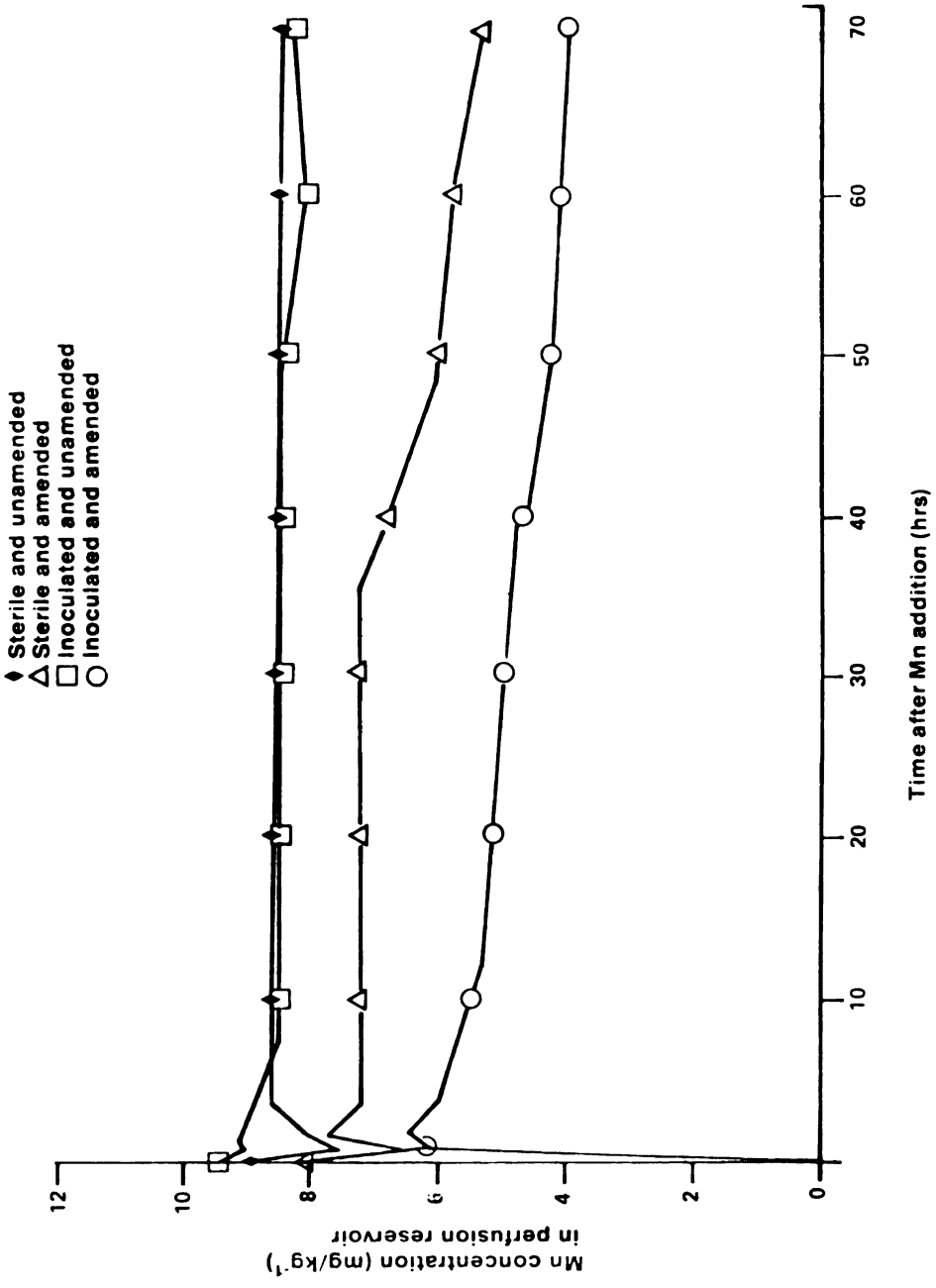

Sterile and unamended
Sterile and amended
Inoculated and unamended
Inoculated and amended
Mn concentration (mg/kg⁻¹)
in perfusion reservoir
Time after Mn addition (hrs)
0
2
4
6
8
10
12
10
20
30
40
50
60
70

Figure 9.

evidence for differential uptake between even efficient ^{65}Zn accumulator species. But, no actual mass measurements were recorded. Figure 11 serves as a control for Figure 10 to show the uniformity of ^{65}Zn distribution in the agar plate. Figure 11 is an autoradiographic exposure of x-ray film by a sterile control plate which represents the background exposure of x-ray film by the ^{65}Zn in the agar medium for a longer film exposure time than that used in Figure 10.

The uniformity of exposure shown in Figure 11 is contrasted with an apparent zone of ^{65}Zn depletion from the agar medium near the site of colony accumulation shown in Figure 10. The zone of ^{65}Zn depletion from the agar medium is shown very clearly in Figure 12 which represents reconfirmation of ^{65}Zn immobilization by an isolated pure culture of Penicillium using the autoradiography plate technique. The single large ^{65}Zn immobilizing colony represented by the dark area in the center of autoradiograph shown in Figure 12 was the result of isolating and culturing a single ^{65}Zn immobilizing colony similar to one of those shown on Figure 10. Similar colony isolation and reconfirmation of ^{65}Zn immobilization was accomplished for a number of individual colonies.

Pure tube cultures were established from the agar plates on which ^{65}Zn immobilization had been reconfirmed.

Once the pure tube cultures were well established, phase contrast microscopy was combined with standard microbiological staining and related identification techniques to tentatively identify as many of the species of microorganisms as possible. Photomicrographs were taken of representative slide material and the results are species of the following: Penicillium, (Figure 13), Paecilomyces (Figure 14), Fusarium

(Figure 15), Cladosporium (Figure 16), Aspergillus (Figure 17), Mucor (Figure 18), Rhizopus (Figure 19), Bacillus (Figure 20), and a culture of an unidentified gram-positive rod-shaped bacteria (Figure 21). Isolates of Penicillium (Figure 13) were the most abundant even though a number of fungi tended to be represented as ^{65}Zn immobilizers. The most abundant ^{65}Zn-immobilizing bacteria developing in these plates were spore-forming Bacillus sp., and another unidentified gram-positive rod (Figures 20, 21).

Figure 10. Autoradiographic exposure of X-ray film by isolated colonies of ^{65}Zn-immobilizing soil microorganisms.

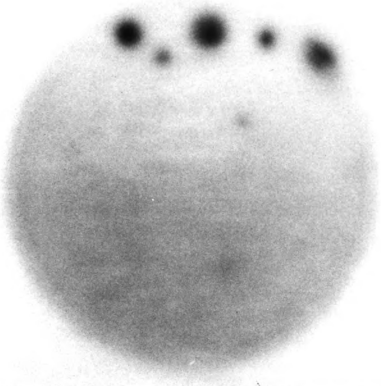


Figure 11. Autoradiographic exposure of X-ray film by a sterile control plate, which represents the background exposure of X-ray film by the ^{65}Zn in the culture medium.

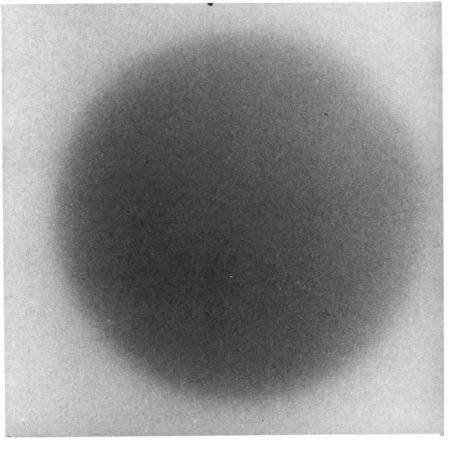


Figure 12. Reconfirmation of ^{65}Zn immobilization by a pure culture of Penicillium using the autoradiography plate technique.

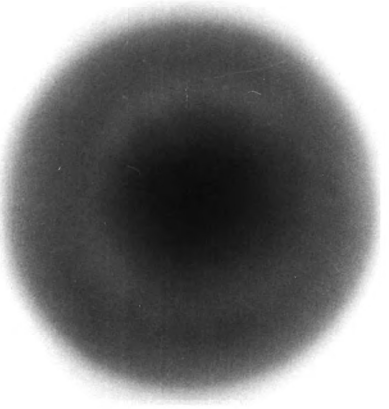


Figure 13. Phase contrast photomicrograph of a culture of Zn-immobilizing *Penicillium sp.* (x2652).

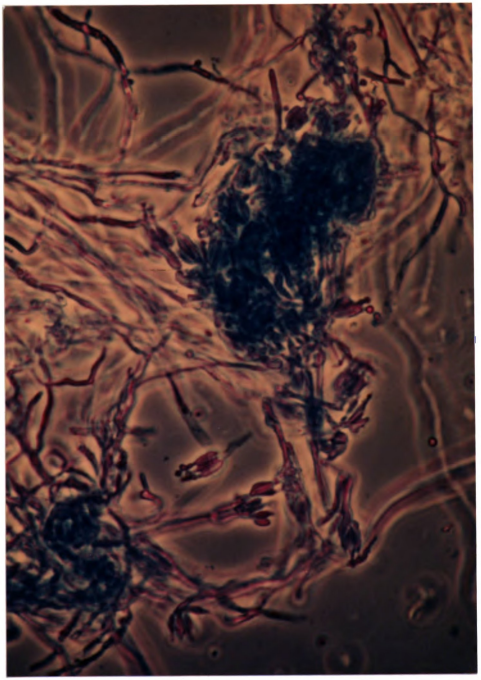


Figure 14. Phase contrast photomicrograph of a culture of Zn-immobilizing *Paecilomyces sp.* (x2652).

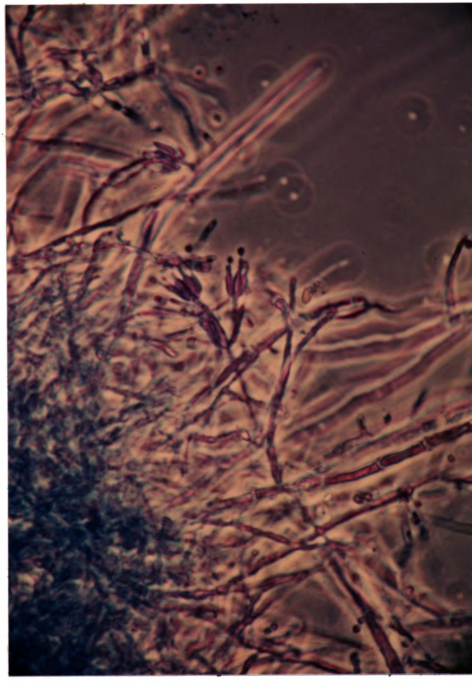


Figure 15. Phase contrast photomicrograph of a culture of Zn-immobilizing Fusarium sp. (x2652).

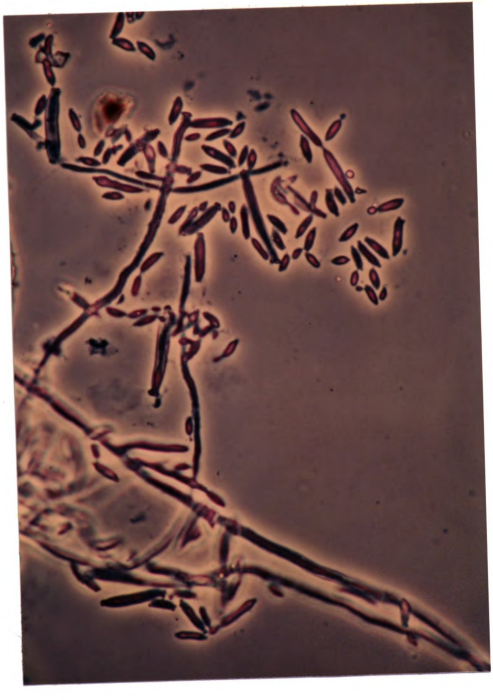


Figure 16. Phase contrast photomicrograph of a culture of Zn-immobilizing *Cladosporium sp.* (x2652).

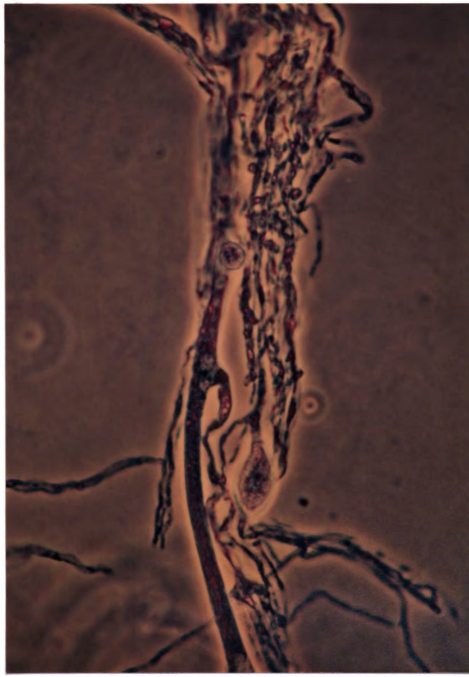


Figure 17. Phase contrast photomicrograph of a culture of Zn-immobilizing *Aspergillus sp.* (x2652).

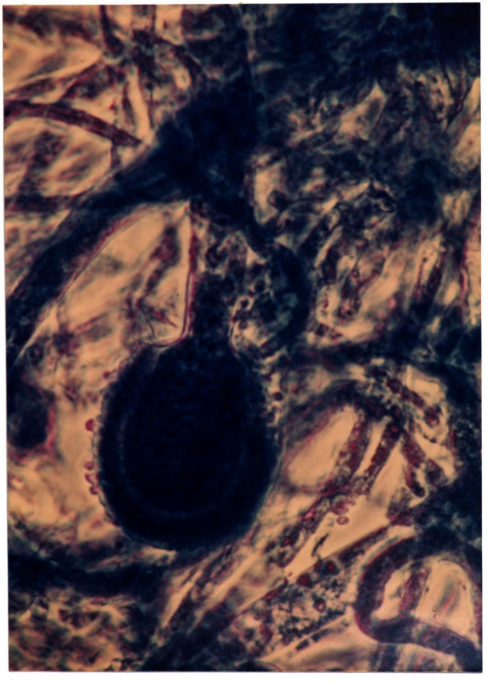


Figure 18. Phase contrast photomicrograph of a culture of Zn-immobilizing Mucor sp. (x2652).

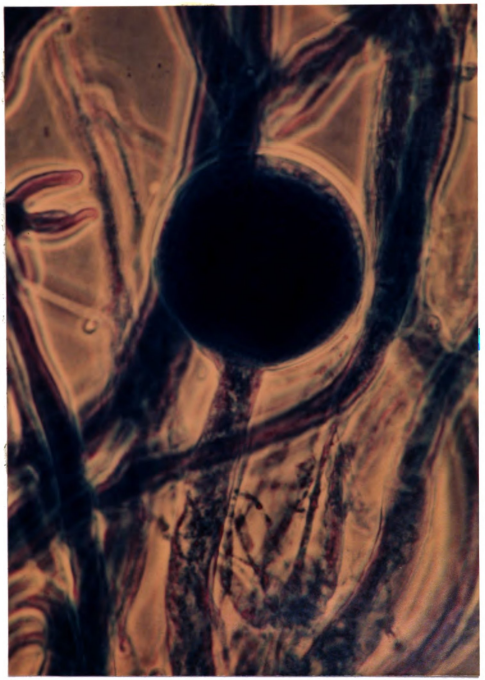


Figure 19. Phase contrast photomicrograph of a culture of Zn-immobilizing Rhizopus sp. (x2652).

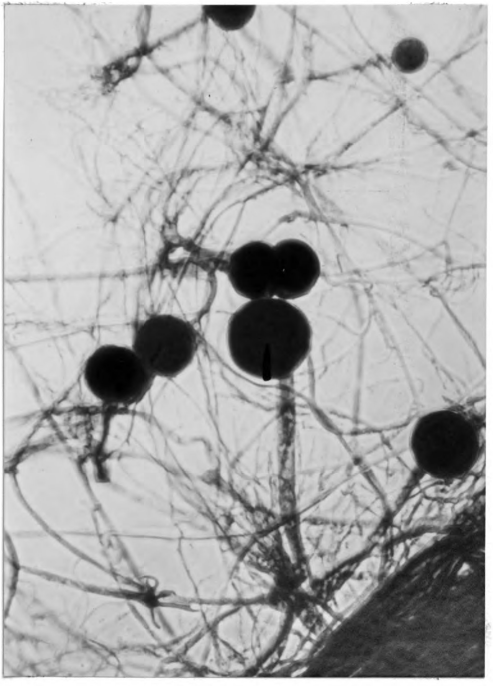


Figure 20. Phase contrast photomicrograph of a culture of gram-positive Bacillus sp. which immobilized Zn. (x6637).

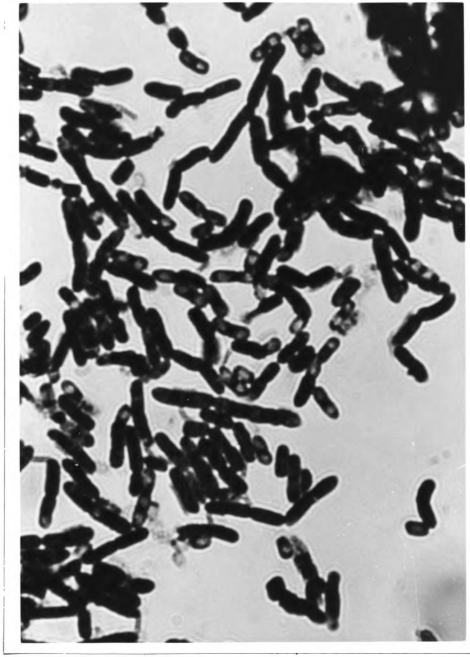
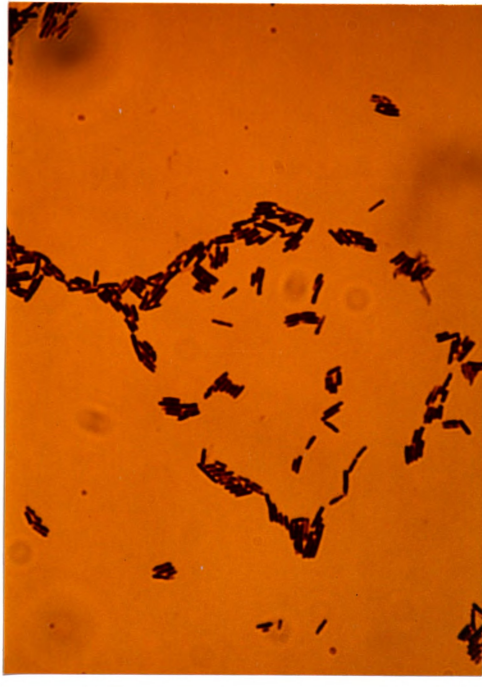


Figure 21. Phase contrast photomicrograph of a culture of unidentified, gram-positive rod which immobilized Zn (x6637).



SUMMARY

A soil perfusion system was used to produce a population of soil microorganisms that could significantly reduce the level of Zn in soil solution to a level lower than that attainable by soil chemical and physical factors.

Soil was sterilized in a nondestructive manner by using ^{60}Co gamma radiation. The investigation produced data on soil and microbial inactivation as a result of time of perfusion and level of microbial activity where differing levels of microbial activity were obtained by stimulating microbial populations. Microbial populations were increased by adding increasing amounts of nutrient broth for energy and nutrient sources.

The microbial populations did respond to energy and nutrient additions and the amount of Zn which could be inactivated by a soil with a high level of microbial activity was significantly greater than that which could be inactivated by soil without measurable biological activity and the additional inactivation was due to microbial immobilization. The enhanced Zn inactivation by soil generally reached a new plateau (Zn level in perfusion solution) within 48 to 72 hours after the addition of an energy and nutrient source to the perfusion system.

Subsequent tests using radioactive Zn (^{65}Zn) and autoradiography along with standard microbial plating techniques demonstrated that only certain microorganisms were involved in the elemental uptake. These could be isolated as colonies in pure culture, plated out again on

^{65}Zn-containing nutrient medium and be reconfirmed as Zn immobilizers. These reconfirmed isolates were tentatively identified as species of Penicillium, Paecilomyces, Fusarium, Mucor, Cladosporium, Aspergillus, Cephalosporium, and Rhizopus. Isolates of Penicillium were the most abundant. The most abundant Zn-immobilizing bacteria developing in these plates were spore-forming Bacillus sp., and another unidentified gram-positive rod.

CONCLUSIONS

From the results presented in this study the following conclusions can be drawn:

1. The soil perfusion apparatus is a useful tool for static, dynamic studies and in evaluating the role of environmental variables on biological and nonbiological processes in the soil.
2. Addition of nutrient and energy sources for microbial growth and activity, especially in sandy soil, increases the Zn immobilization capacity by increasing the activity of the microorganisms.
3. Removal or immobilization of Zn by direct microbial uptake appears to be relatively unimportant in organic soil. Similarly, in Brookston clay loam the presence of microbes and energy and nutrients is of minor importance in Zn uptake because soil chemical and physical factors control the level of Zn in the soil perfusion solution.
4. Radioactive Zn and autoradiography along with standard microbial plating techniques can be used to isolate and confirm colonies of microorganisms that immobilize Zn.
5. The colonies of Zn-immobilizers isolated in pure culture were identified as species of Penicillium (most abundant), spore-forming Bacillus sp., and another unidentified gram-positive rod.

6. Only 21% of the platable microorganisms in the soil tested (Rubicon sand) were Zn immobilizers.
7. Variations in the capability of Zn immobilization exist between different and even the same species of microorganisms.

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