RECOVERY OF MAIS ORGANISMS FROM COASTAL SWAMPS AND KEY PHYSIOCHEMICAL VARIABLES INFLUENCING THEIR GROWTH

by

Richard A. Kirschner, Jr.

Thesis submitted to the Faculty of the

Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

Master of Science

in

Biology (Environmental Microbiology)

APPROVED:

Bruce C. Parker, Chairman

Joseph O. Falkinham, III

John L. Neal

April, 1991

Blacksburg, Virginia

RECOVERY OF MAIS ORGANISMS FROM COASTAL SWAMPS AND KEY PHYSIOCHEMICAL VARIABLES INFLUENCING THEIR GROWTH

by

Richard A. Kirschner, Jr.

Bruce C. Parker, Committee Chairman

(ABSTRACT)

Mycobacterium avium, *M. intracellulare*, and *M. scrofulaceum* (MAIS) organisms were isolated and identified from waters, soils, aerosols, and droplets ejected from water during several seasons in four geographically separate aquatic environments (Okefenokee Swamp, GA; Dismal Swamp, VA; Claytor Lake, VA; and Cranberry Glades, WV). Recovery of MAIS was significantly higher from waters, soils, and aerosols collected from the first two, which were acid, brown-water swamps located in the southeastern coastal plain. High MAIS numbers correlated with (1) warmer temperature, (2) low pH, (3) high humic acid, (4) high fulvic acid, (5) low dissolved oxygen, and (6) high soluble zinc, which characteristized these two swamp waters and soils.

Addressing especially variables 1-4, I sought to determine the factors favoring the growth and survival of MAIS organisms that might explain their relative abundance in the waters, soils, and aerosols of the swamps of the southeastern United States coastal plain. Growth of a majority of MAIS strains was stimulated by humic or fulvic acids at concentrations found in the swamps. Most MAIS also grew significantly better at 37°C compared to 16°C, and exhibited a broad pH tolerance, though most strains tested grew better at low pH (i.e., 4.0 or 5.5).

Most additions of humic or fulvic acids did not stimulate respiratory uptake of oxygen in MAIS strains tested, suggesting that rapid uptake and breakdown of these natural substances is not the primary cause of their growth stimulation.

This research, in relation to previous findings for the geographic distribution and physiological ecology of MAIS, supports the conclusion that waters, soils, and aerosols of the acid, brown-water swamps of the southeastern United States coastal plain represent major environmental sources likely connected with the higher incidence of human infection in this region.

ACKNOWLEDGEMENTS

I wish to express my deepest graditude to Dr. Bruce Parker for his unlimited encouragement, advice, and friendship. I also thank Dr. Joseph Falkinham for his insight into the "world of mycobacteria" and for allowing me to be a part of this world. Dr. John Neal deserves much thanks for his helpful advice and service on my advisory committee.

Many thanks go to all the people whom I have had the pleasure of working with or just getting to know: Myron Beaty, Lori Brookman, Glenn Carlisle, Jamie Daucher, Markus Jucker, Dr. Noel Krieg, Mark Schweitzer, Joe "Toast" Sucic, Tad Syler, Ujwala Warek, Lori Wenkert, Laura Via, Andrew Yurochko, and the "B-ball" crew. Their friendship and fond memories of our experiences will be cherished for years to come. I would also like to thank Han for his help with the oxygen uptake studies and Stuart Fossceco and Sungsue Rheem for their help in the statistical analysis of the data.

Words cannot begin to describe the appreciation and respect I have for my wife, Cindy, and my parents, Richard and Terri, who have provided nothing but support and love during my studies. Thank you with all my heart.

Table of Contents

List of Tables

Chapter 1

List of Figures

INTRODUCTION AND REVIEW OF LITERATURE

MAIS CHARACTERIZATION

Members of the genus *Mycobacterium* are usually straight to slightly curved, rarely branched rods that are typically 1 to 4 um in length. Mycobacterial cells are structurally Gram-positive, but staining is poor and irregular because of failure of the dye to penetrate the cell wall's outer layers, rich in lipids and mycolic acids (Laskin and Lechevalier, 1973). Waxy lipids and mycolic acids account for approximately 60% of the dry weight of the cell wall and when stained with carbol-fuchsin following the Ziel-Neelsen procedure, give mycobacteria their characteristic acid-fast staining property.

The genus *Mycobacterium* includes the human pathogens *M. tuberculosis* and *M. bovis* (*M. tuberculosis* complex) and the non-cultivatable pathogen *M. leprae*. Other mycobacteria (i.e., nontuberculous) include the photochromogenic pathogens (i.e., *M. kansasii* and *M. marinum*) and slow and rapidly growing pathogens. Members of the *Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum* (MAIS) organisms are distinguished from other nontuberculous mycobacteria on the basis of their slow growth (i.e., visible growth requiring at least 7 days incubation on Lowenstein-Jensen slants at 37°C; Runyon, 1974; Wolinsky, 1979) and their inability to hydrolyze polyoxyethylene-sorbitan monooleate detergent (Tween 80; Wayne, et al., 1964; HEW, 1969). *M. scrofulaceum* is scotochromongenic (i.e., pigmented when grown in light or dark), whereas *M. avium* and *M. intracellulare* are scotochromogenic or nonphotochromogenic (i.e., nonpigmented). Representatives of the three species can be distinguished on the basis of pigmentation and urease and catalase activities (Reznikov and Dawson, 1973; Hawkins, 1977; Portaels, 1978; Wolinsky,

1979). *Mycobacterium avium* and *M. intracellulare* are unpigmented and lack urease and catalase activities. By contrast, *M. scrofulaceum* are pigmented and have urease and catalase activities. However, there are representatives of intermediate types designated as MAIS intermediate biovars (Hawkins, 1977; Portaels, 1978). Recently, it was shown that *M. avium* can be distinguished from *M. intracellulare* on the basis of the latter's higher arylsulfatase activity (Tomioka, et al., 1990). In addition, serotyping (Schafer, 1979) and specific radioactively labeled DNA probes (Drake, et al., 1987; Kiehn and Edwards, 1987) offer alternative, though more expensive, ways of identifying MAIS organisms.

EPIDEMIOLOGY

Members of the *Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum* (MAIS) group are opportunistic pathogens of environmental origin that cause pulmonary infections in humans (Wolinsky, 1979). An estimated 2,000 United States residents are infected annually (Good, 1980; Good and Snider, 1982). Recently it has been shown that a substantial proportion of patients with acquired immunodeficiency syndrome (AIDS) have disseminated MAIS infections (Blaser and Cohn, 1986; Horsburgh and Selik, 1989). The highest frequencies of isolates from infected patients (Good, 1980; Good and Snider, 1982) and of persons reacting to either PPD-B (purified protein derivative of *M. intracellulare* Battey strain, L. Edwards, et al., 1969) or PPD-G (*M. scrofulaceum* Gause strain, P. Edwards, 1970) are found in the southeastern United States. That correlates well with the higher numbers of MAIS recovered from water samples (Falkinham, et al., 1980), soils (Brooks, et al., 1984), and aerosols (Wendt, et al., 1980) collected in the southeastern coastal plain. In fact, most aerosol and 25% of MAIS water isolates share characteristics with clinical isolates (Meissner and Falkinham, 1986; Fry et al., 1986). The well established absence of any evidence for transmission from person-to-person (Wolinsky, 1979) implies that one or more environmental source(s) of these bacteria must exist.

In 1987, Martin, et al., showed that MAIS organisms were absent from groundwater, while Falkinham, et al. (1989), showed that MAIS were not found in chicken litter. These findings eliminated these two environments as possible sources. MAIS organisms have been recovered more frequently and in higher numbers from acidic soils, high in organic matter (Brooks, et al., 1984). A majority of clinical and environmental isolates grow optimally at acid pH (George and Falkinham, 1986). Further, many MAIS strains grow at relatively high temperatures up to 43°C (Fry, et al., 1986) and grow under microaerobic conditions (Falkinham, unpublished). Experimental evidence also suggests that MAIS are readily absorbed on gas bubbles, which on bursting, eject concentrated MAIS aerosols from water into the air (Parker, et al., 1983).

Thus, based on the skin sensitivity data, the clinical case data, and the recovery of higher numbers of MAIS in southeastern environments, it was hypothesized that one major source of MAIS strains are the warm, low oxygen content, acidic, brown swamp waters and soils rich in humic and fulvic acids which are widely distributed within the southeastern coastal plain. This hypothesis was tested by recovering, enumerating, and identifying MAIS from waters, soils, dusts, and aerosols collected from coastal swamps (Okefenokee Swamp, GA and Great Dismal Swamp, VA) in regions of high frequency of PPD-B and PPD-G (Edwards, et al., 1969; Edwards, 1970) reactors (as an index of MAIS infection). By contrast, and as a potentially negative control MAIS habitat with low frequency of PPD-B and PPD-G reactors, Clatyor Lake, VA and Cranberry Glades, WV were selected. In parallel with the enumeration of MAIS from these four habitats, data were also collected for physiochemical variables (e.g., pH, temperature, dissolved oxygen, zinc, humic and fulvic acid contents).

Complete identification of an environmental source and an understanding of its pathway to human infection are critical for describing the epidemiology of MAIS. Such knowledge is of value, because MAIS-infections are difficult to cure due to the drug-resistance of these pathogens (Wolinsky, 1979; Iseman, et al., 1985). Note that a single source is not proposed, but rather

many sources, some of which are more significant in terms of infection by virtue of: (1) number of infective units, (2) higher survival or growth rates, (3) greater human exposure, or (4) greater human susceptibility. Thus, MAIS are probably ubiquitous, but their population size differences and specific habitats may explain the epidemiology of this disease. The recovery of MAIS organisms from the four locations and key physiochemical variables influencing their growth is presented in Chapter 1.

Based on initial findings of higher recovery of MAIS organisms from the two coastal swamps compared to the Appalachian region, a second phase of research was initiated to test certain physiochemical variables and their influence on MAIS growth in laboratory media. The abundance of MAIS organisms in the coastal swamp regions implies that their growth and survival are favored by one or a combination of several physiochemical variables: high temperature, low oxygen content, low pH, high zinc, and high humic and fulvic acid concentrations. This hypothesis was tested through laboratory experiments with both environmental and clinical isolates, addressing especially the variables of temperature, pH, and humic and fulvic acid content. These experiments attempted to further describe the present understanding of the epidemiology of MAIS in human infection and disease. Chapter 2 presents these findings.

Much of the work in Chapters 1 and 2 represents submitted manuscripts that had not yet been published at the time of acceptance of this thesis. The style of writing for each chapter fits the format outlined of the two journals. Chapter 1, "*Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum* in Acid, Brown-Water Swamps of the Southeastern United States and Their Association With Environmental Variables", was submitted to American Review of Respiratory Diseases; Chapter 2, "Humic and Fulvic Acid Growth Stimulation of *Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum*", was submitted to Applied and Environmental Microbiology. Additional data and discussion is presented in this thesis that represents work not included or only briefly mentioned in the two manuscripts.

RESEARCH OBJECTIVES

In accordance with the problems and potential solutions to the epidemiology of MAIS in the environment, the following objectives were undertaken:

(1) Determine the number of MAIS organisms in water, soil, dust, and aerosolized water droplet samples collected from areas of high and low frequency of PPD-B and PPD-G reactors.

(2) Determine if the recovery of MAIS organisms was statistically more significant from the two coastal swamps compared to the two Appalachian locations.

(3) Measure physiochemical and biological variables of samples which might influence MAIS numbers (e.g., pH, temperature, dissolved oxygen content, humic acid, fulvic acid).

(4) Determine if statistical correlations existed between MAIS numbers and values for physiochemical variables.

(5) Determine the influence on clinical and environmental MAIS growth of certain physio-chemical variables (i.e., humic and fulvic acids, pH, temperature) in the laboratory.

(6) Determine if humic and fulvic acids were utilized as substrates by MAIS organisms, by measuring respiratory oxygen uptake.

LITERATURE CITED

Blaser, M. J., and D. L. Cohn. 1986. Opportunistic infections in patients with AIDS: Clues to the epidemiology of AIDS and the relative virulence of pathogens. Rev. Infect. Dis. **8**:21-30.

Brooks, R. W., B. C. Parker, H. Gruft, and J. O. Falkinham, III. 1984. Epidemiology of infection of nontuberculous mycobacteria. V. Numbers in eastern United States soils and correlation with soil characteristics. Am. Rev. Respir. Dis. **130**:630-633.

Drake, T. A., J. A. Hindler, O. G. W. Berlin, and D. A. Bruckner. 1987. Rapid identification of *Mycobacterium avium* complex in culture using DNA probes. J. Clin. Microbiol. **25**:1442- 1445.

Edwards, L. B., F. A. Aquaviva, V. T. Livesay, R. W. Cross, and C. E. Palmer. 1969. An atlas of sensitivity to tuberculin, PPD-B, and histoplasmin in the United States. Am. Rev. Respir. Dis. **99**:1-131.

Edwards, P. Q. 1970. Interpretation and significance of the tuberculin test in typical and atypical mycobacterial infections, p. 43-55. *In* J. E. Johnson, III (ed.), Symposium on rationale therapy and control of tuberculosis. University of Florida Press, Gainesville.

Falkinham, J. O., III, B. C. Parker, and H. Gruft. 1980. Epidemiology of infection by nontuberculous mycobacteria. I. Geographic distribution in the eastern United States. Am. Rev. Respir. Dis. **121**:931-937.

Falkinham, J. O. , III, K. L. George, and B. C. Parker. 1989. Epidemiology of infection by nontuberculous mycobacteria. VIII. Absence of mycobacteria in chicken litter. Am. Rev. Respir. Dis. **139**:1347-1349.

Fry, K. L., P. S. Meissner, and J. O. Falkinham, III. 1986. Epidemiology of infection by nontuberculous mycobacteria. VI. Identification and use of epidemiologic markers for studies of *Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum*. Am. Rev. Respir. Dis. **34**:39-43.

George, K. L., and J. O. Falkinham, III. 1986. Selective medium for the isolation and enumeration of *Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum*. Canad. J. Microbiol. **32**:10-14.

Good, R. C. 1980. Isolation of nontuberculous mycobacteria in the United States, 1979. J. Infect. Dis. **142**:779-783.

Good, R. C., and D. E. Snider, Jr. 1982. Isolation of nontuberculous mycobacteria in the United States, 1980. J. Infect. Dis. **146**:829-33.

Hawkins, J. E. 1977. Scotochromogenic mycobacteria which appear intermediate between *Mycobacterium avium-intracellulare* and *Mycobacterium scrofulaceum*. Am. Rev. Respir. Dis. **116**:963-964.

Health, Education, and Welfare (U. S. Dept. of). 1969. Procedures for the isolation and identification of mycobacteria, 118 pp. HEW, PHS Publ. No. 19995.

Horsburgh, C. R., Jr., and R. M. Selik. 1989. The epidemiology of disseminated nontuberculous mycobacterial infection in the acquired immunodeficiency syndrome (AIDS). Am. Rev. Respir. Dis. **139**:4-7.

Iseman, M. D., R. F. Corpe, R. J. O'Brien, D. Y. Rosenzweig, and E. Wolinsky. 1985. Disease due to *Mycobacterium avium-intracellulare*. Chest **87**:139S-149S (Supplement).

Kiehn, T. E., and F. F. Edwards. 1987. Rapid identification using specific DNA probe of *Mycobacterium avium* complex from patients with acquired immunodeficiency syndrome. J. Clin. Microbiol. **25**:1551-1552.

Laskin, A. I., and H. A. Lechevalier (eds.). 1973. Handbook of Microbiology. II. Microbial Composition, 1926 pp. CRC Press, Cleveland, OH.

Martin, E. C., B. C. Parker, and J. O. Falkinham, III. 1987. Epidemiology of infection by nontuberculous mycobacteria. VII. Absence of mycobacteria in southeastern groundwaters. Am. Rev. Respir. Dis. **136**:344-348.

Meissner, P. S., and J. O. Falkinham, III. 1986. Plasmid DNA profiles as epidemiologic markers for clinical and environmental isolates of *Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum*. J. Infect. Dis. **152**:325-331.

Parker, B. C., M. A. Ford, H. Gruft, J. O. Falkinham, III. 1988. Epidemiology of infection by nontuberculous mycobacteria. IV. Preferential aerosolization of *Mycobacterium intracellulare* from natural waters. Am. Rev. Respir. Dis. **128**:652-656.

Portaels, F. 1978. Difficulties encountered in identification of *M. avium-intracellulare*, *M. scrofulaceum* and related strains. Am. Rev. Respir. Dis. **118**:969.

Reznikov, M., and D. Dawson. 1973. Serological examination of some strains that are in the Mycobacterium *avium-intracellulare-scrofulaceum* complex but do not belong to Schaefer's serotypes. Appl. Microbiol. **26**:470-473.

Runyon, E. H. 1974. Ten mycobacterial pathogens. Tubercle **55**:235-240.

Schaefer, W. B. 1979. Serological identification of atypical mycobacteria. Methods Microbiol. **13**:323-344.

Tomioka, H., H. Saito, K. Sato, and D. J. Dawson. 1990. Arysulfatase activity for differentiating *Mycobacterium avium* and *Mycobacterium intracellulare*. J. Clin. Microbiol. **28**:2104-2106.

Wayne, L. G., J. R. Doubek, and R. L. Russell. 1964. Classification and identification of mycobacteria. I. Tests employing Tween 80 as substrate. Am. Rev. Respir. Dis. **90**:588-597.

Wendt, S. L., K. L. George, B. C. Parker, H. Gruft, and J.O. Falkinham, III. 1980. Epidemiology of infection by nontuberculous mycobacteria. III. Isolation of potentially pathogenic mycobacteria from aerosols. Am. Rev. Respir. Dis. **122**:259-263.

Wolinsky, E. 1979. Nontuberculous mycobacteria and associated diseases. Am. Rev. Respir. Dis. **119**:107-159.

Chapter 1

Mycobacterium avium, *M. intracellulare*, and *M. scrofulaceum* in Acid, Brown-Water Swamps of the Southeastern United States and Their Association With Environmental Variables

Abstract

Mycobacterium avium, *M. intracellulare*, and *M. scrofulaceum* (MAIS) organisms were isolated and identified from waters, soils, aerosols, and droplets ejected from water collected during several seasons from four geographically separate aquatic environments (Okefenokee Swamp, GA; Dismal Swamp, VA; Claytor Lake, VA; and Cranberry Glades, WV). Recovery of MAIS was significantly higher from waters, soils, and aerosols collected from the two acid, brown-water swamps located in the southeastern coastal plain. High MAIS numbers correlated with warmer temperature, low pH, low dissolved oxygen, high soluble zinc, high humic acid, and high fulvic acid. This research, in relation to previous findings for the geographic distribution and physiological ecology of MAIS, supports the conclusion that waters, soils, and aerosols of the acid, brown-water swamps of the southeastern United States coastal plain represent major environmental sources likely connected with the higher incidence of human infection in this region.

Introduction

Members of the *Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum* (MAIS) group are opportunistic pathogens that cause pulmonary infections in humans (1). An estimated 2,000 or more United States residents are infected annually by these organisms (2, 3), and recently it has been shown that a substantial proportion of patients with AIDS have disseminated MAIS infections (4, 5). In the absence of any evidence for a human-to-human transfer of MAIS organisms (1), searches for one or more environmental sources for MAIS have ensued.

Higher frequencies of persons reacting to either PPD-B (6) or PPD-G (7) and of MAIS isolates from patient samples (2, 3) correlated with the higher numbers of MAIS recovered from water (8) and soil (9) samples collected in the southeastern United States coastal plain. Further, the fact that a high proportion of MAIS aerosol isolates from this region (10) shared characteristics in common with clinical isolates (11, 12) supports the hypothesis that at least one source of MAIS organisms is in the southeastern United States environment.

Based on the observation that MAIS organisms were recovered more frequently and in higher numbers from acidic soils and those high in organic matter (9), that they grow optimally at pH 5.0 to 5.5 (13), and that most clinical MAIS isolates grow at 43° C (12) but die or grow slowly below 15.5° C (14), we hypothesized that the warm, acid, brown, low oxygen-containing swamp waters and associated soils would have high MAIS numbers. We sought to test this hypothesis by recovering, enumerating, and identifying MAIS from waters, soils, dusts, and aerosols collected from coastal swamps (Okefenokee Swamp, GA and Great Dismal Swamp, VA) in regions of high frequency of PPD-B and PPD-G (6, 7) reactors (as an index of MAIS infection). By

contrast, and as potentially negative control MAIS habitats with low frequency of PPD-B and PPD-G reactors, we selected Claytor Lake, VA, and Cranberry Glades, WV. In parallel with the enumeration of MAIS from these four habitats, we sought to identify physiochemical variables (e.g., pH, temperature, dissolved oxygen, zinc, humic and fulvic acid content) which correlated with MAIS numbers.

Methods

Sampling Locations

Four sampling locations (Okefenokee Swamp in coastal Georgia, Great Dismal Swamp in coastal Virginia, Claytor Lake in the Appalachian mountains of Virginia, and Cranberry Glades in the Appalachian mountains of West Virginia) were chosen because of differences in frequency of PPD-B and PPD-G reactors (6, 7). Figures 1A-E show the four sampling locations and the specific sites within these locations where aerosols, dusts, ejected water droplets, waters, and soils were collected. Both coastal locations represent two of the larger acidic swamps of high organic matter surrounded by forest and agricultural farmland (15). These and numerous smaller swamps also occur in this region of Quaternary and Tertiary geological formations, which are composed mostly of sands and gravels lacking limestones or other high pH minerals, and which contribute significantly to the acidic nature of the swamps (16). The characteristic vegetation of these environments leads to the accumulation of humins, and the degradative by-products of humic and fulvic acids (16, 17). Claytor Lake is a man-made reservoir located in a mountainous region (elevation 2,000 ft/615 m), and its waters, which frequently drain through limestone, are contrastingly high in alkalinity and hardness, but low in humic substances. Cranberry Glades is in a mountainous region (elevation 3,400 ft/1050 m) lacking limestone geology and has acid, brown-waters in its bogs. The mean annual temperatures of the four sites are also different and reflect the higher and lower temperature ranges; the Okefenokee Swamp has the warmest mean (20.3° C), the Dismal Swamp is next (15.1° C), then Claytor Lake (11.7° C), and Cranberry Glades (8.4° C) (18).

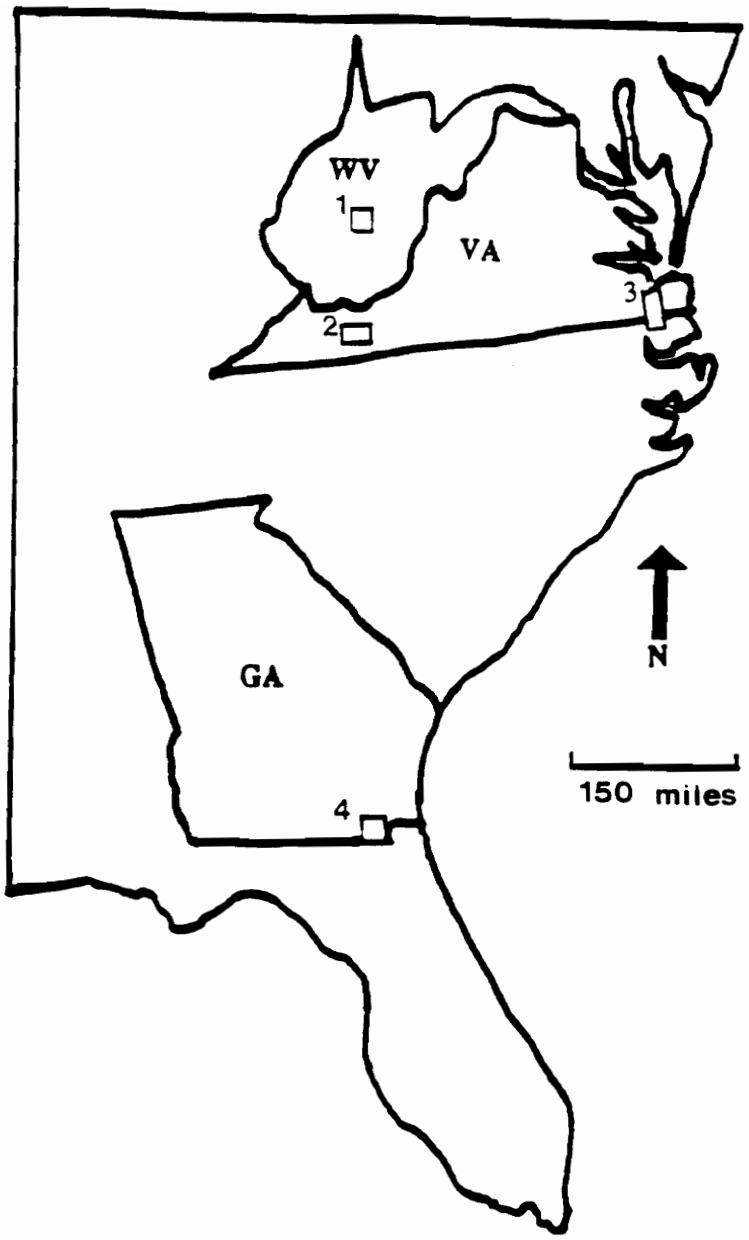

Fig. 1. Composite map of West Virginia and the southeastern United States, showing the four sampling locations. Key to the locations: (1) Cranberry Glades Botanical Area, Monongahela National Forest, West Virginia, (2) Claytor Lake State Park, Virginia, (3) Great Dismal Swamp National Wildlife Refuge, Virginia, and (4) Okefenokee Swamp National Wildlife Refuge, Georgia.

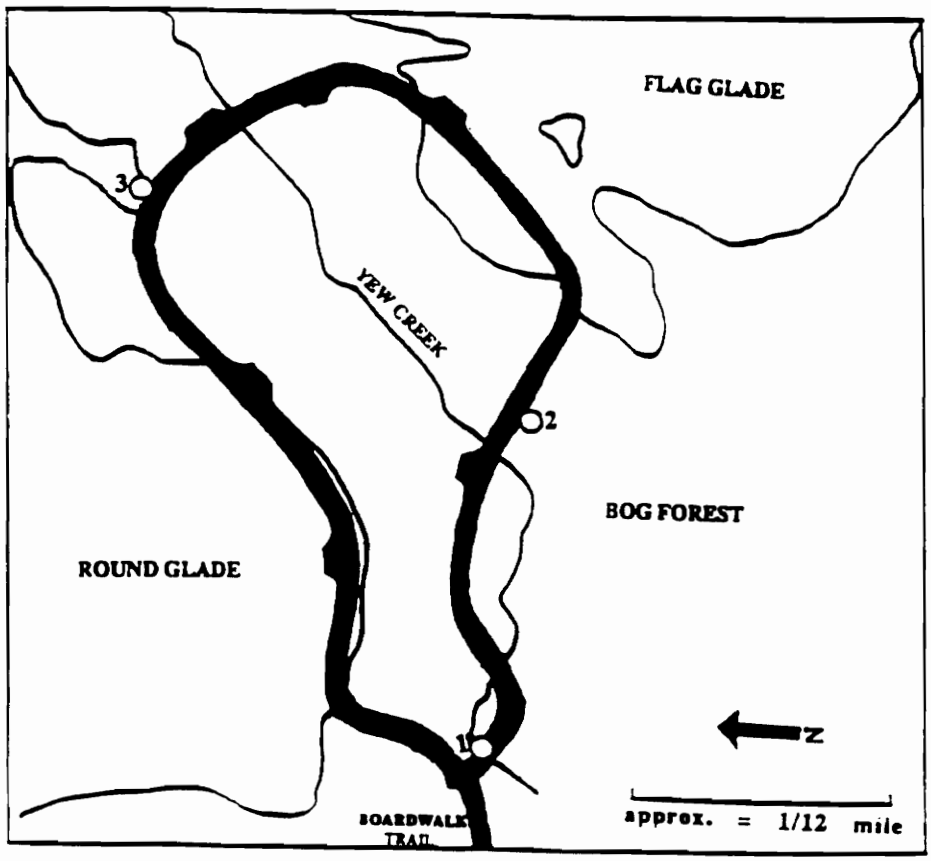

Fig. 1B (Location 1). Cranberry Glades Botanical Area, Monongahela National Forest, Pocathontas County, West Virginia: (1) aerosols, ejected droplets, water, and soil, (2) water and soil, and (3) water and soil.

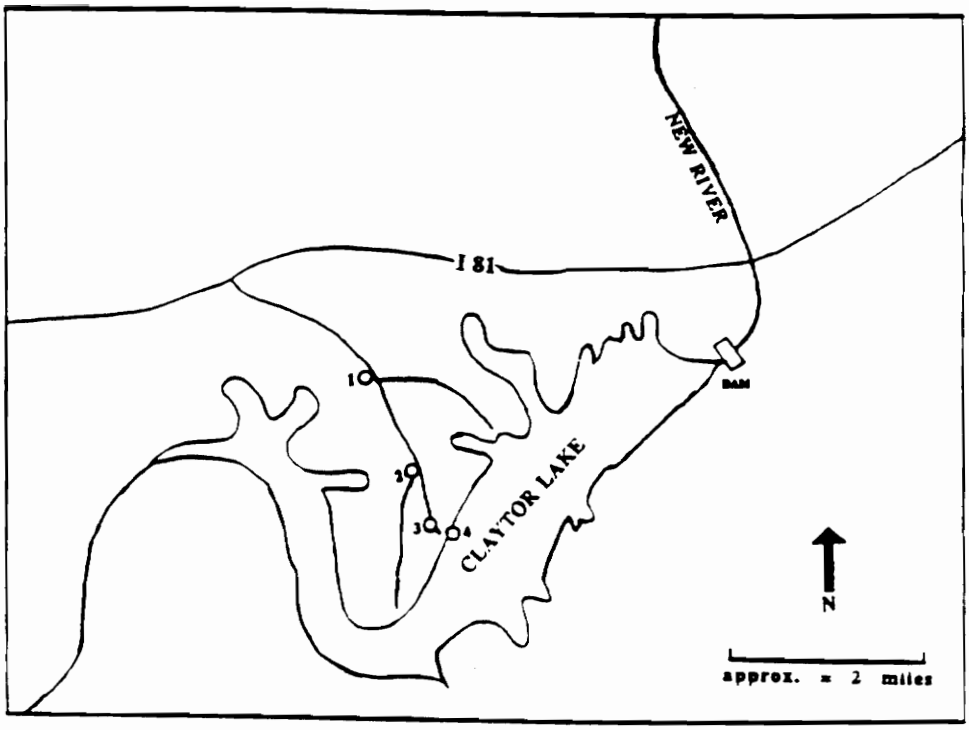

Fig. 1C (Location 2). Claytor Lake State Park, Pulaski County, Virginia: (1) soil, (2) soil, (3) soil, and (4) aerosols, ejected droplets, water, and soil.

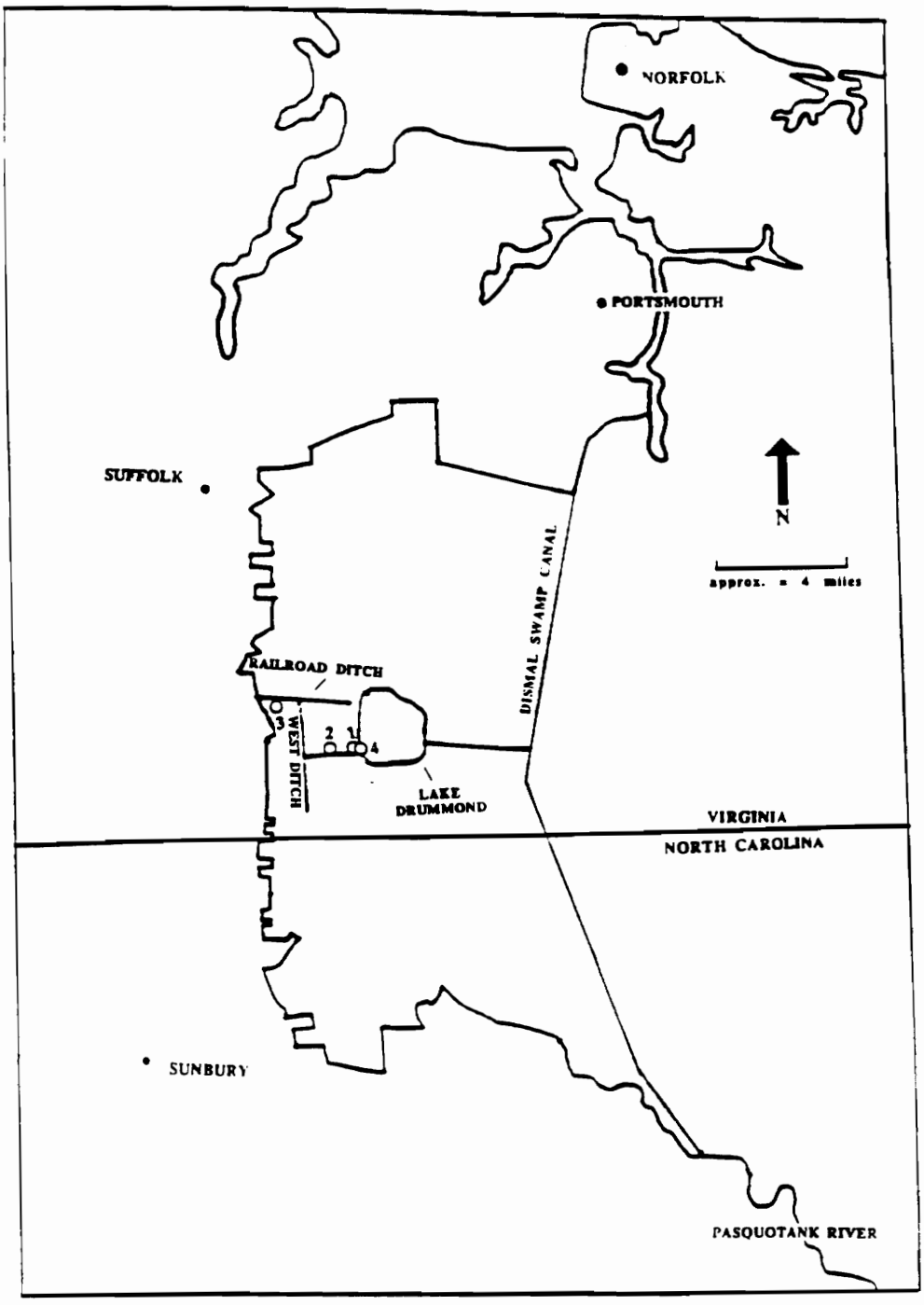

Fig. 1D (Location 3). Great Dismal Swamp National Wildlife Refuge, Virginia: (1) water, (2) water and soil, (3) water and soil, and (4) aerosols, ejected droplets, water, and soil.

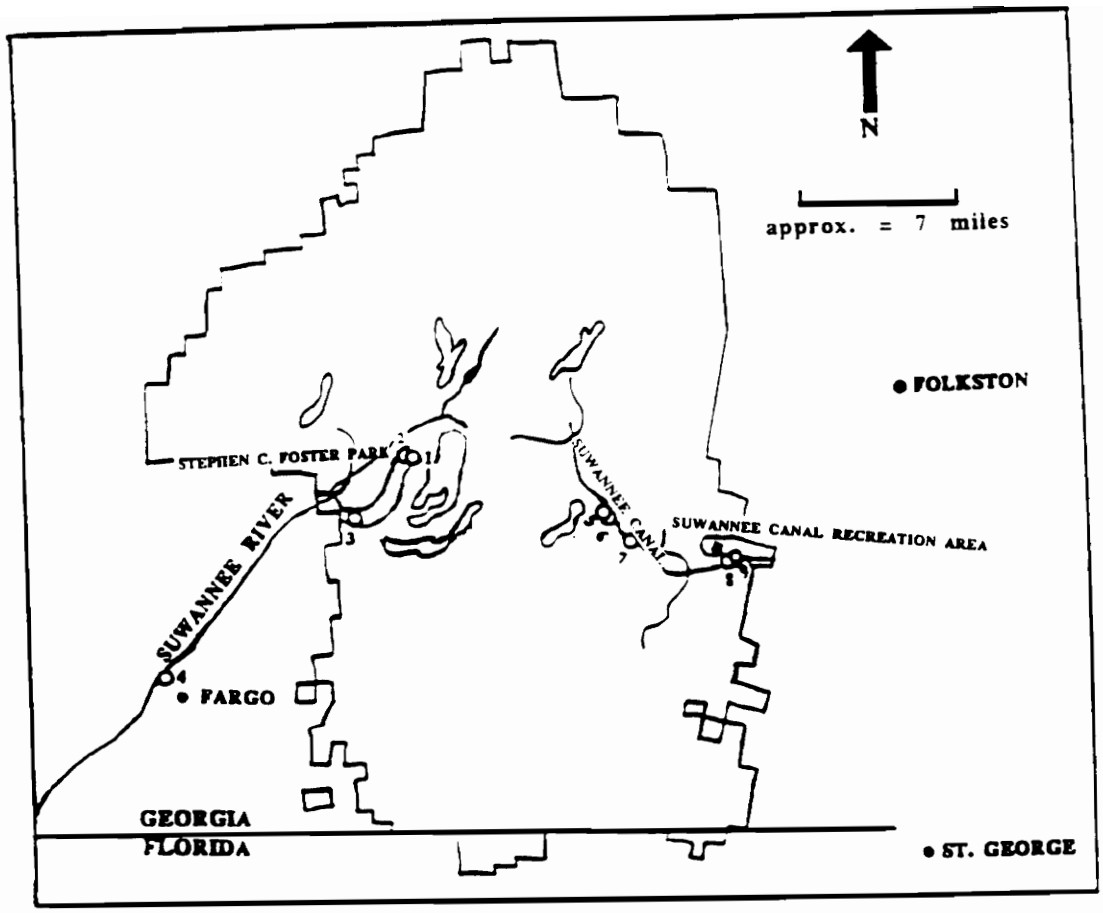

Fig. 1E (Location 4). Okefenokee Swamp National Wildlife Refuge, Georgia: (1) aerosols, ejected droplets, water, and soil, (2) water, (3) water and soil, (4) water and soil, (5) aerosols, ejected droplets, water, and soil, (6) soil, (7) soil, (8) water, (9) soil.

Samples, Sample Collection, and MAIS Isolation

Water Samples. Triplicate surface water samples were collected after rinsing and submerging ethanol-sterilized, one liter polypropylene bottles. Bottles were returned to the laboratory in coolers held near 5° C and processed within 24 h. Isolation of MAIS from water involved spreading 0.1 ml onto each of at least 5 replicate plates of TTC selective agar medium (13). MAIS were also isolated on Middlebrook and Cohn 7H10 medium (M7H10; BBL Microbiology Systems, Cockeysville, MD) containing 0.5% (vol/vol) glycerol, following sodium hydroxide-decontamination treatment and HCL neutralization (19).

Soil Samples. Soil samples were collected using a clean trowel after removing any surface leaf litter from approximately 0.1 m^2. Up to 1,000 grams of soil (to 4 cm depth) were transferred into 710-ml ethylene oxide-sterilized Whirl-Pak bags (Nasco Industries, Inc., Fort Atkinson, WI). These samples were returned to the laboratory in coolers held near 5° C and processed within 24 h. After vortexing 1 g (undried) of soil in 40 ml of one-tenth strength Nutrient Broth (BBL Microbiology Systems, Cockeysville, MD) for 5 min, each soil suspension was shaken 5 min, and sonicated 5 min. Following centrifugation at 1,000 x *g* for 10 min at 4° C to pellet the soil particles, 0.1 ml samples of the supernatant suspension were spread onto TTC agar medium. In addition, 20 ml of the supernatant was centrifuged for 20 min at 8,000 x *g* at 4° C and the resulting pellet treated with sodium hydroxide, neutralized with HCl, and 0.1 ml samples spread onto M7H10 medium (19).

Aerosol Samples. Dust and airborne water droplet samples were collected using a pair of six-stage Andersen Aerosol samplers (Research Appliance Co., Gibsonia, PA) as described by Wendt, et al. (10). Dust samples were collected at sites upwind of any bodies of water, whereas sampl-

es more likely enriched with airborne water droplets were collected adjacent to and downwind from bodies of water.

Ejected Water Droplet Samples. Ejected water droplets were collected by bubbling air below the water surface. Pumped air passing through tubing to aquarium bubblers suspended at 20 to 30 cm created a cloud of tiny bubbles, which on rising to the surface, burst and ejected smaller droplets of water beneath a floating platform. The platform, 5 to 10 cm above the water surface, held 10 Petri plates with the agar medium facing down so ejected droplets impacted on the surface. Ejected droplet sizes (150 um to 450 um) of waters from the four locations overlapped the size range reported by Falkinham, et al. (20) for natural aquatic aerosols. TTC agar medium was used for collection of the aerosol and ejected water droplet samples. Following collection, plates were sealed in parafilm, returned to the laboratory in coolers held near 5° C, and spread within 24 h.

After all sample types were processed, plates were sealed with parafilm and incubated at 37° C in candle jars. Checks for colonies were done at 7-day intervals, and plates were removed from the incubator after sufficient incubation time to allow colony development (28 to 42 days).

Mycobacterium Identification

Acid-fast (Ziehl-Neelsen stain) colonies were picked and transferred to Lowenstein-Jensen (LJ) slants (BBL Microbiology Systems, Cockeysville, MD). Slowly growing isolates, (>7 days for visible colony formation) were picked and tested for Tween 80 hydrolysis, as described (8). Tween 80 negative strains were classified as MAIS, while Tween 80 positive strains were classified as non-MAIS. Those strains showing visible colony formation on LJ slants <7 days were classified as rapidly-growing mycobacteria.

Acid-fast isolates failing to grow on LJ slants were transferred to M7H10 plates or slants containing 0.5% (vol/vol) glycerol and 10% (vol/vol) oleic acid-albumin enrichment (OAA) (21) and incubated at 37° C. If visible colonies still did not form, the isolates were transferred again to 2 ml of Middlebrook and Cohn 7H9 broth medium (M7H9; BBL Microbiology Systems, Cockeysville, MD) containing 0.5% (vol/vol) glycerol and 10% (vol/vol) OAA and incubated on a rotator at 37° C. Isolates that grew in the broth media, were transferred to LJ slants for identification. Any acid-fast isolates which failed to grow under all conditions were not counted in the study.

Measurement of Physiochemical Variables

During dust and aerosol collections, air temperature, wind velocity and direction, relative humidity, and light intensity were measured. Water pH, temperature, and transparency (Secchi disc depth) were measured in the field and modified-Winkler determinations of dissolved oxygen (22) were completed in the laboratory. Soil sample pH, temperature, organic matter content, and moisture content were measured as described by Brooks, et al. (9). Various cationic elements and heavy metals, including zinc, were determined in filtered waters or soil extracts using Inductively Coupled Plasma spectral analysis (ICAP-9000 Plasma Spectrometer; Jarrell-Ash, Inc., Waltham, MA) (22).

Isolation and Measurement of Humic and Fulvic Acids

Humic and fulvic acids were extracted from soils following a modification of the methods outlined by Schnitzer (23). Duplicate 10 g (dry weight basis) soil samples were suspended in 100 ml of 1 N NH_4OH in methanol in a 250-ml polypropylene centrifuge bottle and shaken vigorously for 5 min. Following 24 h storage under a N_2 atmosphere, samples were centrifuged at 10,000 x g for 20 min and the supernatant containing the soluble humic and fulvic acid components saved. The soil pellet was resuspended and the extraction procedure repeated. Supernatant solutions were

combined and concentrated to 20 to 35 ml in a flash evaporator (Buchler Instruments, Fort Lee, NJ) at 40° C under vacuum. Concentrates transferred to 40-ml polypropylene centrifuge tubes were acidified to pH 2.0 using 1 N HCl, precipitating humic acid from the fulvic acid fraction. Samples were refrigerated 48 h; then tubes were centrifuged at 10,000 x g for 20 min. The fulvic acid (supernatant) component was transferred to a tared glass Petri dish, dried at 80° C for 24 h, and washed with 25 ml of 0.01N HCl and 25 ml of distilled water. Samples were redried at 80° C for 24 h, placed in a desiccator, and weighed when cooled. Traces of hymatomelanic acid were removed from the humic acid precipitate by suspending the pellet in 10 ml of ethanol, manually shaking for 5 min, and centrifuging at 10,000 x g for 20 min. The supernatant was discarded and the humic acid pellet resuspended in 20 to 50 ml of distilled water, transferred to a tared glass Petri dish, dried at 80° C for 24 h, and washed with 25 ml of 0.01 N HCl and 25 ml of distilled water. Samples were redried at 80° C for 24 h, placed into a desiccator, and weighed when cool.

Humic and fulvic acid fractions were isolated from water samples and concentrations measured using a modification of the procedure of Carder, et al. (24). Duplicate 500 ml water samples were concentrated to 25 to 50 ml by evaporating samples at 80° C over a period of several days. The solutions were acidified to pH 2.0 using 1 N HCl and applied to 40 x 60 mm Amberlite XAD-2 resin columns (Rohm and Haas Company, Philadelphia, PA) pretreated with 50 ml of 0.01 N HCl. Salts were removed with 200 ml of 0.01 N HCl. Humic and fulvic acids were eluted with 200 ml of 1 N NH_4OH in methanol (4 h), and 100 ml of methanol. Eluents were combined and the humic and fulvic acid fractions were recovered, purified, and weighed as described above for soils.

Determination of the Microaerobic Nature of MAIS

A 0.2 ml inoculum for each strain was mixed slowly for 1 min with semi-solid M7H9 medium, which was prepared by autoclaving (15 min; 15 psi) M7H9 broth medium containing 0.5% (vol/vol)

glycerol, and 0.1% (wt/vol) agar, cooling to 60°C and adding 10% (vol/vol) OAA, as described by Collins and coworkers (25). Cultures were incubated at 37° C for 3 to 4 wk, whereupon growth was measured as mm from the medium surface and the microaerobic nature determined.

Effect of Betaine on MAIS Aerosolization and Growth

For the last sampling trips to the different locations (Spring, 1990), TTC selective medium containing 2 mM betaine (Sigma Chemical Co., St. Louis, MO) was used for potential isolation of MAIS organisms during aerosol collection to compare MAIS recoveries with TTC medium with no betaine addition, since Marthi and Lighthart (26) demonstrated higher recoveries of airborne bacteria other than mycobacteria when betaine was added to water. In addition, MAIS strains at concentrations of 10^5 to 10^6 cells/ml were plated (in duplicate) onto (1) TTC, (2) TTC with 2 mM betaine, and (3) M7H10 with 0.5% (vol/vol) glycerol and 10% (vol/vol) OAA and incubated at 37° C for 2 wk. Qualitative comparisons were made on the basis of growth on the three media types.

Statistical Analysis

Due to the high percentage of samples that yielded no MAIS, nonparametric one-way analysis procedure employing the Kruskal-Wallis test (chi-square approximation) of the Statistical Analysis Systems (SAS) program (27) was used to compare the numbers of MAIS between the four locations. The General Linear Model (GLM) procedure of SAS (27) was performed to compare MAIS recovery at each sampling site in the four locations with the location's water, soil, and aerosol physiochemical characteristics. The influence of both individual characteristics and multifactorial interactions between the characteristics were compared. For significance, a confidence interval of 95% was selected for both procedures.

Results

Mycobacterium Recovery

Tables 1-4 summarize the number of mycobacteria recovered for the 4 different sample types collected at each site within the 4 sampling locations. Values for water (Table 1) or soil (Table 2) samples reflect the average number of MAIS organisms recovered from both M7H10 medium following NaOH decontamination and TTC medium. MAIS were recovered in greater frequency and higher numbers compared to rapidly-growing and non-MAIS mycobacteria species from waters collected at the two coastal swamp locations, while no mycobacteria were recovered from Appalachian waters (Table 1). MAIS, rapidly-growing mycobacteria, and non-MAIS species were recovered from Okefenokee and Dismal Swamp soils, though MAIS organisms were recovered at higher frequencies (Table 2). Soils from Cranberry Glades yielded both MAIS and rapidly-growing mycobacteria, but only rapid-growers were recovered from Claytor Lake soils (Table 2). Most aerosol samples yielded no mycobacteria, but some MAIS isolates were collected on samples taken from the Okefenokee and Dismal swamps (Table 3). In contrast, no aerosol samples collected from Claytor Lake or Cranberry Glades yielded any mycobacteria species. Ejected droplets collected only at the two coastal swamps yielded MAIS organisms, while mycobacteria collected from the Appalachian locations were rapidly-growing (Table 4). A listing of the identity and source of the mycobacteria strains isolated from the different sample types collected from the 4 sampling locations appears in Table 5. The rest of this chapter deals solely with MAIS recovery and identification of some physiochemical variables influencing their growth in the environment.

TABLE 1. Recovery* of mycobacteria from waters collected from the four sampling locations

Location (Date)†	Site‡	Total Mycobacteria	MAIS	Rapid-Growers	Non-MAIS
Okefenokee Swamp					
(12/17/88)	1	0.2	0.2(100)	<0.1§	<0.1
	2	0.7	0.7(100)	<0.1	<0.1
	3	2.1	2.1(100)	<0.1	<0.1
	4	<0.1	<0.1	<0.1	<0.1
(6/22/89)	5	0.5	<0.1	0.5(100)	<0.1
	8	0.1	<0.1	0.1	<0.1
(5/16/90)	5	16.9	16.9(100)	<0.1	<0.1
	8	<0.1	<0.1	<0.1	<0.1
Dismal Swamp					
(11/20/88)	1	23.9	20.9(87)	3.0(13)	<0.1
	2	11.7	11.7(100)	<0.1	<0.1
	3	0.1	0.1(100)	<0.1	<0.1
	4	5.6	0.8(14)	4.8(86)	<0.1
(9/10/89)	1	20.0	20.0(100)	<0.1	<0.1
	2	3.1	2.6(84)	<0.1	0.5(16)
	3	4.5	2.6(58)	1.9(42)	<0.1
	4	<0.1	<0.1	<0.1	<0.1
(5/25/90)	1	35.0	33.0(94)	<0.1	2.0(6)
	2	<0.1	<0.1	<0.1	<0.1
	3	15.0	15.0(100)	<0.1	<0.1
	4	<0.1	<0.1	<0.1	<0.1
Claytor Lake					
(3/4/89)	4	<0.1	<0.1	<0.1	<0.1
(6/17/89)	4	<0.1	<0.1	<0.1	<0.1
(6/9/90)	4	<0.1	<0.1	<0.1	<0.1
Cranberry Glades					
(10/4/89)	1	<0.1	<0.1	<0.1	<0.1
	2	<0.1	<0.1	<0.1	<0.1
	3	<0.1	<0.1	<0.1	<0.1
(5/7/90)	1	<0.1	<0.1	<0.1	<0.1
	2	<0.1	<0.1	<0.1	<0.1
	3	<0.1	<0.1	<0.1	<0.1

*Values represent average number of colony-forming units (CFU) of mycobacteria/ml water recovered on either M7H10 medium following NaOH decontamination or TTC medium. Numbers in parenthesis represent percentages of total mycobacteria.

†Date of collection.

‡Refer to Figure 1A-E for the location.

§Represents the limit of detection.

TABLE 2. Recovery* of mycobacteria from soils collected from the four sampling locations

Location (Date)†	Sample Site‡	Total Mycobacteria	MAIS	Rapid-Growers	Non-MAIS
Okefenokee Swamp					
(12/17/88)	1	17.9	0.3(2)	17.6(98)	<0.1§
	3	0.7	<0.1	0.7	<0.1
	4	7.5	<0.1	4.5(60)	3.0(40)
(6/22/89)	5	1.7	1.2(71)	0.5(29)	<0.1
	6	2.1	2.1(100)	<0.1	<0.1
	7	1.8	1.6(89)	0.2(11)	<0.1
	9	2.7	1.6(59)	<0.1	1.1(41)
(5/16/90)	5	2.0	2.0(100)	<0.1	<0.1
	6	10.9	10.8(99)	0.1(1)	<0.1
	7	0.1	0.1(100)	<0.1	<0.1
	9	33.5	25.0(75)	8.5(25)	<0.1
Dismal Swamp					
(11/20/88)	2	1.0	0.2(20)	<0.1	0.8(80)
	3	<0.1	<0.1	<0.1	<0.1
	4	<0.1	<0.1	<0.1	<0.1
(9/10/89)	2	<0.1	<0.1	<0.1	<0.1
	3	9.0	8.6(96)	0.3(3)	0.1(1)
	4	<0.1	<0.1	<0.1	<0.1
(5/25/90)	2	32.2	<0.1	32.2(100)	<0.1
	3	7.5	<0.1	7.5(100)	<0.1
	4	5.3	0.3(6)	5.0(94)	<0.1
Claytor Lake					
(3/4/89)	1	<0.1	<0.1	<0.1	<0.1
	2	397.0	<0.1	397.0(100)	<0.1
	3	31.6	<0.1	31.6(100)	<0.1
	4	8.3	<0.1	8.3(100)	<0.1
(6/17/89)	1	0.2	<0.1	0.2(100)	<0.1
	2	0.1	<0.1	0.1(100)	<0.1
	3	<0.1	<0.1	<0.1	<0.1
	4	0.2	<0.1	0.2(100)	<0.1
(6/9/90)	1	<0.1	<0.1	<0.1	<0.1
	2	<0.1	<0.1	<0.1	<0.1
	3	<0.1	<0.1	<0.1	<0.1
	4	<0.1	<0.1	<0.1	<0.1
Cranberry Glades					
(10/4/89)	1	<0.1	<0.1	<0.1	<0.1
	2	<0.1	<0.1	<0.1	<0.1
	3	<0.1	<0.1	<0.1	<0.1
(5/7/90)	1	0.5	0.5(100)	<0.1	<0.1
	2	2.0	0.1(5)	1.9(95)	<0.1
	3	<0.1	<0.1	<0.1	<0.1

*Values represent average number of colony-forming units (CFU) of mycobacteria/g dried soil recovered on either M7H10 medium following NaOH decontamination or TTC medium. Numbers in parenthesis represent percentages of total mycobacteria.

†Date of collection.

‡Refer to Figure 1A-E for the location.

§Represents the limit of detection.

TABLE 3. Recovery* of mycobacteria from aerosols† collected from the four sampling locations

Location (Date)‡	Sample	Total Mycobacteria	MAIS	Rapid-Growers	Non-MAIS
Okefenokee Swamp					
(12/17/88)	1	<0.6§	<0.6	<0.6	<0.6
	2	<0.6	<0.6	<0.6	<0.6
	3	<0.6	<0.6	<0.6	<0.6
	4	<0.6	<0.6	<0.6	<0.6
(6/22/89)	1	0.6	<0.6	<0.6	0.6(100)
	2	43.4	43.4(100)	<0.6	<0.6
	3	<0.6	<0.6	<0.6	<0.6
	4	1.2	1.2(100)	<0.6	<0.6
(5/16/90)	1	13.7	13.7(100)	<0.6	<0.6
	2	<0.6	<0.6	<0.6	<0.6
	3	<0.6	<0.6	<0.6	<0.6
	4	<0.6	<0.6	<0.6	<0.6
	5‖	<0.6	<0.6	<0.6	<0.6
	6‖	<0.6	<0.6	<0.6	<0.6
Dismal Swamp					
(11/20/88)	1	66.1	66.1(100)	<0.6	<0.6
	2	<0.6	<0.6	<0.6	<0.6
	3	<0.6	<0.6	<0.6	<0.6
	4	<0.6	<0.6	<0.6	<0.6
(9/10/89)	1	<0.6	<0.6	<0.6	<0.6
	2	<0.6	<0.6	<0.6	<0.6
	3	<0.6	<0.6	<0.6	<0.6
	4	<0.6	<0.6	<0.6	<0.6
(5/25/90)	1	12.5	7.7(62)	4.8(38)	<0.6
	2	14.3	14.3(100)	<0.6	<0.6
	3	<0.6	<0.6	<0.6	<0.6
	4	108.3	0.6(1)	107.7(99)	<0.6
	5‖	<0.6	<0.6	<0.6	<0.6
	6‖	<0.6	<0.6	<0.6	<0.6
Claytor Lake					
(3/4/89)	1	<0.6	<0.6	<0.6	<0.6
	2	<0.6	<0.6	<0.6	<0.6
	3	<0.6	<0.6	<0.6	<0.6
	4	<0.6	<0.6	<0.6	<0.6
(6/17/89)	1	<0.6	<0.6	<0.6	<0.6
	2	<0.6	<0.6	<0.6	<0.6
	3	<0.6	<0.6	<0.6	<0.6
	4	<0.6	<0.6	<0.6	<0.6
(6/9/90)	1	<0.6	<0.6	<0.6	<0.6
	2	<0.6	<0.6	<0.6	<0.6
	3	<0.6	<0.6	<0.6	<0.6
	4	<0.6	<0.6	<0.6	<0.6
Cranberry Glades					
(10/4/89)	1	<0.6	<0.6	<0.6	<0.6
	2	<0.6	<0.6	<0.6	<0.6
	3	<0.6	<0.6	<0.6	<0.6
	4	<0.6	<0.6	<0.6	<0.6
(5/7/90)	1	<0.6	<0.6	<0.6	<0.6
	2	<0.6	<0.6	<0.6	<0.6
	3	<0.6	<0.6	<0.6	<0.6
	4	<0.6	<0.6	<0.6	<0.6
	5‖	<0.6	<0.6	<0.6	<0.6
	6‖	<0.6	<0.6	<0.6	<0.6

*Values represent colony-forming units (CFU) of mycobacteria/m³ · h⁻¹. Numbers in parenthesis represent percentages of total mycobacteria.

†Total mycobacteria isolated on 6 plates of TTC medium using the 6-stage Andersen Aerosol sampler.

‡Date of collection.

§Represents the limit of detection.

‖TTC medium with the addition of 2 mM betaine.

TABLE 4. Recovery* of mycobacteria from ejected water droplets collected from the four sampling locations

Location (Date)†	Sample	Total Mycobacteria	MAIS	Rapid-Growers	Non-MAIS
Okefenokee Swamp					
(12/17/88)	1	<0.01‡	<0.01	<0.01	<0.01
	2	0.32	0.32(100)	<0.01	<0.01
(6/22/89)	1	0.13	<0.01	<0.01	0.13(100)
	2	<0.01	<0.01	<0.01	<0.01
(5/16/90)	1	1.26	1.26(100)	<0.01	<0.01
	2	0.28	0.28(100)	<0.01	<0.01
Dismal Swamp					
(11/20/88)	1	1.64	1.64(100)	<0.01	<0.01
	2	0.01	0.01(100)	<0.01	<0.01
(9/10/89)	1	ns§			
	2	ns			
(5/25/90)	1	4.57	0.61(13)	<0.01	3.96(87)
	2	0.14	<0.01	0.14(100)	<0.01
Claytor Lake					
(3/4/89)	1	<0.01	<0.01	<0.01	<0.01
	2	<0.01	<0.01	<0.01	<0.01
(6/17/89)	1	18.50	<0.01	18.50(100)	<0.01
	2	<0.01	<0.01	<0.01	<0.01
(6/9/90)	1	<0.01	<0.01	<0.01	<0.01
	2	<0.01	<0.01	<0.01	<0.01
Cranberry Glades					
(10/4/89)	1	<0.01	<0.01	<0.01	<0.01
	2	<0.01	<0.01	<0.01	<0.01
(5/7/90)	1	<0.01	<0.01	<0.01	<0.01
	2	1.59	<0.01	1.59(100)	<0.01

*Values represent colony-forming units (CFU) of mycobacteria/$cm^2 \cdot h^{-1}$. Numbers in parenthesis represent percentages of total mycobacteria.

†Date of collection.

‡Represents the limit of detection.

§ns = not sampled.

Table 5. Reference list of the Mycobacteria strains isolated from the different locations

STRAIN*	IDENTIFICATION	BIOVAR†	DATE‡	SOURCE§
DSA4	*M. avium* complex	---	11/88	e.d.
DSA10	*M. scrofulaceum*	++-	11/88	e.d.
DSA16	M. *avium* complex	+--	11/88	aerosol
DSA19	M. *avium* complex	---	11/88	aerosol
DSA24	*M. scrofulaceum*	++-	11/88	aerosol
DSW2	M. *avium* complex	---	11/88	water
DSW16	*M. scrofulaceum*	+++	11/88	water
DSW23	*M. scrofulaceum*	++-	11/88	water
DSW25	Rapid (Group IV)		11/88	water
DSW43	M. *avium* complex	+--	11/88	water
DSS1	Non-MAIS		11/88	soil
DSS2	*M. scrofulaceum*	+-+	11/88	soil
OKA16	M. *avium* complex	---	12/88	aerosol
OKA19	M. *avium* complex	---	12/88	e.d.
OKA23	M. *avium* complex	+--	12/88	e.d.
OKW73	M. *avium* complex	+--	12/88	water
OKW75	*M. scrofulaceum*	++-	12/88	water
OKW77	M. *avium* complex	+--	12/88	water
OKS28	Rapid (Group IV)		12/88	soil
OKS30	Rapid (Group IV)		12/88	soil
OKS41	M. *avium* complex	+--	12/88	soil
OKS47	Rapid (Group IV)		12/88	soil
OKS48	Rapid (Group IV)		12/88	soil
OKS55	Non-MAIS		12/88	soil
OKS56	Rapid (Group IV)		12/88	soil
CLS9	Rapid (Group IV)		03/89	soil
CLS10	Rapid (Group IV)		03/89	soil
CLS11	Rapid (Group IV)		03/89	soil
CLS107	Rapid (Group IV)		06/89	soil
CLS116	Rapid (Group IV)		06/89	soil
CLS120	Rapid (Group IV)		06/89	soil
CLS121	Rapid (Group IV)		06/89	soil
OKA102	Non-MAIS		06/89	aerosol
OKA106	M. *avium* complex	---	06/89	aerosol
OKA107	M. *avium* complex	+--	06/89	aerosol
OKA111	*M. scrofulaceum*	+++	06/89	aerosol
OKA112	M. *avium* complex	---	06/89	aerosol
OKA114	*M. scrofulaceum*	+-+	06/89	aerosol
OKA121	Non-MAIS		06/89	e.d.
OKA122	M. *avium* complex	---	06/89	e.d.

*Key for the strains collected from the various locations:
DS=Dismal Swamp, OK=Okefenokee Swamp, CL=Claytor Lake,
CG=Cranberry Glades

†Presence (+) or absence (-) of pigmentation, urease, and catalase activity.

‡Date of collection.

§Type of sample from which the strain was isolated. (ejected droplet = e.d.)

Table 5. cont...

STRAIN*	IDENTIFICATION	BIOVAR†	DATE‡	SOURCE§
OKA126	*M. scrofulaceum*	++-	06/89	e.d.
OKS147	*M. scrofulaceum*	++-	06/89	soil
OKS160	M. *avium* complex	+--	06/89	soil
OKS167	M. *avium* complex	+--	06/89	soil
OKS175	Rapid (Group IV)		06/89	soil
OKS176	Rapid (Group IV)		06/89	soil
OKS177	Rapid (Group IV)		06/89	soil
OKS178	*M. scrofulaceum*	++-	06/89	soil
OKS182	Rapid (Group IV)		06/89	soil
OKS183	Rapid (Group IV)		06/89	soil
OKS186	*M. scrofulaceum*	+++	06/89	soil
OKS188	M. *avium* complex	-+-	06/89	soil
DSW123	Rapid (Group IV)		06/89	water
DSW127	M. *avium* complex	-+-	06/89	water
DSW138	M. *avium* complex	-+-	06/89	water
DSS141	M. *avium* complex	+--	06/89	soil
DSS148	M. *avium* complex	---	06/89	soil
DSS151	Non-MAIS		06/89	soil
DSS153	Non-MAIS		06/89	soil
DSS154	Rapid (group IV)		06/89	soil
DSW1	Non-MAIS		09/89	water
DSW2	M. *avium* complex	---	09/89	water
DSW51	M. *avium* complex	---	09/89	water
DSW69	M. *avium* complex	+--	09/89	water
DSW70	Rapid (Group IV)		09/89	water
DSW73	M. *avium* complex	---	09/89	water
DSW74	M. *avium* complex	---	09/89	water
DSW75	Rapid (Group IV)		09/89	water
DSS7	M. *avium* complex	---	09/89	soil
DSS8	Rapid (Group IV)		09/89	soil
DSS9	Non-MAIS		09/89	soil
CGS8	Rapid (Group IV)		10/89	soil
CGS9	Rapid (Group IV)		10/89	soil
CGS212	M. *avium* complex	---	05/89	soil
CGS215	M. *avium* complex	---	05/90	soil
CGS219	Rapid (Group IV)		05/90	soil
CGA228	Rapid (Group IV)		05/90	e.d.
OKA202	M. *avium* complex	---	05/90	aerosol
OKA219	M. *avium* complex	---	05/90	e.d.
OKA220	M. *avium* complex	---	05/90	e.d.

*Key for the strains collected from the various locations:
DS=Dismal Swamp, OK=Okefenokee Swamp, CL=Claytor Lake,
CG=Cranberry Glades

†Presence (+) or absence (-) of pigmentation, urease, and catalase activity.

‡Date of collection.

§Type of sample from which the strain was isolated. (ejected droplet = e.d.)

Table 5. cont...

STRAIN*	IDENTIFICATION	BIOVAR†	DATE‡	SOURCE§
OKA224	M. *avium* complex	---	05/90	e.d.
OKA227	M. *avium* complex	+--	05/90	e.d.
OKA235	M. *avium* complex	---	05/90	e.d.
OKW236	M. *avium* complex	---	05/90	water
OKW237	M. *avium* complex	---	05/90	water
OKW243	M. *avium* complex	+--	05/90	water
OKS246	M. *avium* complex	---	05/90	soil
OKS247	M. *avium* complex	---	05/90	soil
OKS248	M. *avium* complex	---	05/90	soil
OKS249	*M. scrofulaceum*	+-+	05/90	soil
OKS250	M. *avium* complex	---	05/90	soil
OKS251	Rapid (Group IV)		05/90	soil
OKS254	M. *avium* complex	---	05/90	soil
OKS258	*M. scrofulaceum*	+-+	05/90	soil
OKS260	Rapid (Group IV)		05/90	soil
OKS261	M. *avium* complex	--+	05/90	soil
OKS262	M. *avium* complex	---	05/90	soil
DSA201	M. *avium* complex	---	05/90	aerosol
DSA203	M. *avium* complex	---	05/90	aerosol
DSA207	Rapid (Group IV)		05/90	aerosol
DSA209	M. *avium* complex	---	05/90	aerosol
DSA211	M. *avium* complex	+--	05/90	aerosol
DSA213	Rapid (Group IV)		05/90	aerosol
DSA214	M. *avium* complex	---	05/90	e.d.
DSA216	Non-MAIS		05/90	e.d.
DSA218	M. *avium* complex	---	05/90	e.d.
DSA219	M. *avium* complex	+--	05/90	e.d.
DSA221	Rapid (Group IV)		05/90	e.d.
DSA222	Rapid (Group IV)		05/90	e.d.
DSS229	Rapid (Group IV)		05/90	soil
DSS246	Rapid (Group IV)		05/90	soil
DSS251	M. *avium* complex	---	05/90	soil
DSS252	Rapid (Group IV)		05/90	soil
DSS253	Rapid (Group IV)		05/90	soil
DSS255	Rapid (Group IV)		05/90	soil
DSW256	M. *avium* complex		05/90	soil
DSW257	Non-MAIS		05/90	soil
DSW264	M. *avium* complex	---	05/90	soil
DSW265	M. *avium* complex	---	05/90	soil
DSW266	Rapid (Group IV)		05/90	soil

*Location key for the various isolated strains.
DS=Dismal Swamp, OK=Okefenokee Swamp, CL=Claytor Lake,
CG=Cranberry Glades

†Presence (+) or absence (-) of pigmentation, urease, and catalase activity.

‡Date of collection.

§Type of sample from which the strain was isolated. (ejected droplet = e.d.)

MAIS Recovery

Table 6 summarizes the frequency of recovery and number of MAIS from the 4 sampling locations. Values for water or soil samples reflect the average number of MAIS organisms recovered from both M7H10 medium following NaOH decontamination and TTC medium. Higher numbers/ml water were recovered on TTC compared to M7H10 following NaOH decontamination, while higher numbers of MAIS/g soil were recovered on M7H10 following NaOH decontamination than on TTC (Table 7). MAIS were recovered from 13 of 20 (65%) water samples and 12 of 20 (60%) soil samples collected over the two year period from the two coastal swamps. By contrast, none of 9 (<11%) water samples and only 2 of 18 (11%) soil samples collected from Claytor Lake and Cranberry Glades yielded MAIS. MAIS numbers were different in those soil samples collected at different times of the years at the same site. Higher numbers of MAIS were typically recovered during warmer seasons. MAIS were also recovered from aerosols [7/24 (29%)] and ejected droplets [6/10 (60%)] collected at the Okefenokee and Dismal Swamps, while no Claytor Lake or Cranberry Glades aerosol [0/20 (<5%)] or ejected droplet [0/10 (<10%)] samples yielded MAIS. When MAIS were recovered from the two swamp locations, their numbers were significantly higher ($p \leq 0.05$) for all four of the sample types compared to the two Appalachian regions (Table 6). MAIS were recovered more frequently from soils from the Okefenokee Swamp, while waters from the Dismal Swamp yielded more MAIS than any of the other locations.

MAIS Recovery and Site Characteristics

The physiochemical characteristics for waters, soils, and those characteristics measured during aerosol collections appear in Tables 8, 9, and 10, respectively. Tables 11 and 12 list the elemental analyses of waters and soils, respectively. The swamp waters had relatively lower pH, lower dissolved oxygen, and higher humic and fulvic acid contents compared to those waters from Claytor Lake and Cranberry Glades (Table 8). Table 9 shows that soils from the Okefenokee and Dismal

TABLE 6

SUMMARY OF TOTAL MAIS RECOVERED* FROM AEROSOLS, EJECTED WATER DROPLETS, WATERS, AND SOILS COLLECTED FROM THE FOUR SAMPLING LOCATIONS

Location (Collection Date)	Sample Type: Aerosols (CFU/m³/h)	Ejected Droplets (CFU/cm²/h)	Water (CFU/ml)	Soil (CFU/g dried)
Okefenokee Swamp				
(Winter, 1988)	<0.6 (0/4)	0.32 (1/2)	3.0 (3/4)	0.3 (1/3)
(Summer, 1989)	44.6 (2/4)	<0.01 (0/2)	<0.1 (0/2)	6.4 (4/4)
(Spring, 1990)	13.7 (1/4)	1.54 (2/2)	16.9 (1/2)	37.9 (4/4)
Dismal Swamp				
(Winter, 1988)	66.1 (1/4)	1.65 (2/2)	33.3 (4/4)	0.2 (1/3)
(Fall, 1989)	<0.6 (0/4)	NS+	25.2 (3/4)	8.6 (1/3)
(Spring, 1990)	22.6 (3/4)	0.61 (1/2)	48.0 (2/4)	0.3 (1/3)
Claytor Lake				
(Winter, 1988)	<0.6 (0/4)	<0.01 (0/2)	<0.1 (0/1)	<0.1 (0/4)
(Summer, 1989)	<0.6 (0/4)	<0.01 (0/2)	<0.1 (0/1)	<0.1 (0/4)
(Spring, 1990)	<0.6 (0/4)	<0.01 (0/2)	<0.1 (0/1)	<0.1 (0/4)
Cranberry Glades				
(Fall, 1989)	<0.6 (0/4)	<0.01 (0/2)	<0.1 (0/3)	<0.1 (0.3)
(Spring, 1990)	<0.6 (0/4)	<0.01 (0/2)	<0.1 (0/3)	1.2 (2/3)

*Frequency of samples that yielded MAIS are shown in parentheses following the total MAIS recovery numbers (CFU = colony-forming units).

+NS = Not Sampled.

TABLE 7. Comparison of MAIS recovery on M7H10 medium following NaOH decontamination or TTC medium from waters and soils collected from the four sampling locations

Location (Date)‡	Site§	Water* Medium M7H10	Water* Medium TTC	Water* Total	Soil† Medium M7H10	Soil† Medium TTC	Soil† Total
Okefenokee Swamp							
(12/17/88)	1	0.4	<1.7	0.4	0.6	<0.1	0.6
	2	1.3	<1.7	1.3			
	3	4.2	<1.7	4.2	<0.1	<0.1	<0.1
	4	<0.1	<1.7	<0.1	<0.1	<0.1	<0.1
(6/22/89)	5	<0.1	<1.7	<0.1	<0.1	2.3	2.3
	6				4.2	<0.1	4.2
	7				<0.1	3.2	3.2
	8	<0.1	<1.7	<0.1			
	9				0.2	2.9	3.1
(5/16/90)	5	1.8	32.0	35.8	4.0	<0.1	4.0
	6				21.6	<0.1	21.6
	7				0.2	<0.1	0.2
	8	<0.1	<1.7	<0.1			
	9				50.0	<0.1	50.0
Dismal Swamp							
(11/20/88)	1	<0.1	41.7	41.7			
	2	<0.1	23.3	23.3	<0.1	0.4	0.4
	3	0.1	<1.7	0.1	<0.1	<0.1	<0.1
	4	1.5	<1.7	1.5	<0.1	<0.1	<0.1
(9/10/89)	1	<0.1	40.0	40.0			
	2	1.1	5.0	6.1	<0.1	0.4	0.4
	3	<0.1	5.1	5.1	<0.1	<0.1	<0.1
	4	<0.1	<1.3	<0.1	<0.1	<0.1	<0.1
(5/25/90)	1	<0.1	66.0	66.0			
	2	<0.1	30.0	30.0	<0.1	<0.1	<0.1
	3	<0.1	<2.0	<0.1	<0.1	<0.1	<0.1
	4	<0.1	<2.0	<0.1	<0.1	0.6	0.6
Claytor Lake							
(3/4/89)	1				<0.1	<0.1	<0.1
	2				<0.1	<0.1	<0.1
	3				<0.1	<0.1	<0.1
	4	<0.1	<1.7	<0.1	<0.1	<0.1	<0.1
(6/17/89)	1				<0.1	<0.1	<0.1
	2				<0.1	<0.1	<0.1
	3				<0.1	<0.1	<0.1
	4	<0.1	<1.7	<0.1	<0.1	<0.1	<0.1
(6/9/90)	1				<0.1	<0.1	<0.1
	2				<0.1	<0.1	<0.1
	3				<0.1	<0.1	<0.1
	4	<0.1	<1.7	<0.1	<0.1	<0.1	<0.1
Cranberry Glades							
(10/4/89)	1	<0.1	<1.1	<0.1	<0.1	<0.1	<0.1
	2	<0.1	<1.1	<0.1	<0.1	<0.1	<0.1
	3	<0.1	<1.1	<0.1	<0.1	<0.1	<0.1
(5/7/90)	1	<0.1	<2.0	<0.1	<0.1	1.0	1.0
	2	<0.1	<2.0	<0.1	<0.1	0.2	0.2
	3	<0.1	<2.0	<0.1	<0.1	<0.1	<0.1

*Values represent colony-forming units (CFU) of MAIS/ml water.
†Values represent CFU of MAIS/g soil (dry weight basis).
§Date of collection.
‡Refer to Figure 1A-E for the location.

TABLE 8. Physiochemical characteristics of waters collected from the four sampling locations

Location (Date)*	Sample Site†	Temp. (°C)	pH	Dissolved oxygen (mg/L)	% O_2 saturation	Humic acid (mg/L)	Fulvic acid (mg/L)
Okefenokee Swamp							
(12/12/88)	1	3.0	3.3	nd‡	nd	15.3	117.0
	2	3.0	3.3	nd	nd	16.2	148.3
	3	1.0	3.6	nd	nd	33.4	127.3
	4	5.0	3.3	nd	nd	12.3	93.6
(6/22/89)	5	28.0	3.7	1.83	23	15.6	92.0
	8	28.0	3.8	nd	nd	12.3	61.2
(5/16/90)	5	22.5	3.9	0.89	10	20.2	121.4
	8	23.0	3.7	3.13	37	25.4	104.3
Dismal Swamp							
(11/20/88)	1	18.0	4.5	nd	nd	28.9	268.2
	2	18.0	4.4	nd	nd	12.6	342.6
	3	17.0	5.6	nd	nd	9.8	127.7
	4	19.0	4.1	nd	nd	10.0	144.3
(9/10/89)	1	32.0	3.6	<0.26	<3	53.4	371.1
	2	31.0	3.7	0.44	6	36.7	356.1
	3	27.0	5.7	0.26	3	8.6	86.6
	4	29.5	3.9	2.94	35	30.2	203.3
(5/25/90)	1	12.0	3.8	0.79	7	39.6	421.8
	2	13.0	3.8	0.53	5	44.6	254.2
	3	12.0	5.8	0.49	5	<0.1	2.0
	4	18.0	4.2	4.60	49	27.1	180.4
Claytor Lake							
(3/4/89)	4	8.0	6.5	nd	nd	<0.1	1.0
(6/17/89)	4	24.0	6.6	7.20	90	<0.1	0.7
(6/9/90)	4	20.0	7.9	9.23	112	<0.1	0.4
Cranberry Glades							
(10/4/89)	1	8.0	5.9	9.19	77	<0.1	0.4
	2	8.0	5.7	0.10	12	5.3	79.1
	3	8.0	5.5	2.85	24	7.6	66.4
(5/7/90)	1	7.0	6.1	9.17	85	<0.1	1.2
	2	6.5	5.8	5.07	46	11.6	75.2
	3	6.0	6.2	6.24	59	24.1	40.4

*Date of collection.
†Refer to Figure 1A-E for the location.
‡nd = not determined.

TABLE 9. Physiochemical characteristics of soils collected from the four sampling locations

Location (Date)*	Sample Site†	pH	% Moisture	% Organic matter	Humic acid (mg/10g)‡	Fulvic acid (mg/10g)‡
Okefenokee Swamp						
(12/17/88)	1	4.0	62.0	5.7	36.9	22.3
	3	5.5	16.0	1.2	0.9	1.0
	4	5.1	9.0	0.5	3.5	42.0
(6/22/89)	5	4.0	21.0	3.0	17.3	11.2
	6	3.2	72.0	48.5	76.7	216.6
	7	5.1	6.0	3.8	1.9	12.1
	9§	4.8	94.0	49.0	161.2	122.0
(5/16/90)	5	5.4	20.5	6.0	9.8	55.0
	6	3.7	44.5	43.5	161.9	314.7
	7	5.3	4.0	3.7	9.0	23.3
	9§	4.5	93.0	50.5	262.5	189.2
Dismal Swamp						
(11/20/88)	2	5.1	18.0	1.0	2.5	52.8
	3	3.8	34.0	15.0	96.9	35.0
	4	4.2	18.0	3.8	12.9	25.1
(9/10/89)	2	3.8	74.8	50.0	247.0	266.3
	3	3.9	58.0	23.5	317.0	212.2
	4	4.3	78.5	4.8	21.7	32.1
(5/25/90)	2	3.7	72.3	55.5	350.5	313.8
	3	3.9	65.0	52.5	253.7	204.0
	4	4.3	19.8	4.1	13.0	40.9
Claytor Lake						
(3/4/89)	1	5.1	22.0	0.8	<0.1	3.8
	2	5.7	53.0	13.0	268.1	224.5
	3	5.3	19.0	1.6	11.1	12.9
	4	6.2	19.0	3.4	5.1	12.8
(6/17/89)	1	5.4	23.0	0.8	<0.1	0.5
	2	5.7	31.0	12.4	26.8	50.5
	3	5.4	21.0	1.1	11.1	12.9
	4	6.0	22.0	5.0	5.1	12.8
(6/9/90)	1	4.8	18.0	0.9	1.7	4.6
	2	4.8	32.5	13.0	47.6	98.6
	3	5.3	24.3	1.1	2.0	6.6
	4	4.6	7.5	4.4	9.4	27.3
Cranberry Glades						
(10/4/89)	1	5.0	60.0	11.0	65.0	68.5
	2	5.6	50.0	5.5	14.2	33.1
	3	4.3	57.0	13.6	37.7	48.1
(5/7/90)	1	4.5	42.5	12.2	25.6	91.7
	2	5.5	45.0	10.2	19.9	45.0
	3	5.1	39.5	8.3	17.2	17.2

*Date of collection.
†Refer to Figure 1A-E for the location.
‡Soil dry weight basis.
§Represents peat sample floating on water.

TABLE 10. Physiochemical characteristics measured during aerosol collections from the four sampling locations

Location (Date)*	Samples†	Air Temp. (°C)	Relative Humidity (%)	Light (lux)	Wind (mph/dir)
Okefenokee Swamp					
(12/17/88)	1,2	5.0	63	10,000	1 SW
	3,4	5.0	75	40,000	0-2 SW
(6/22/89)	1,2	27.0	88	40,000	0-0.5 N
	3,4	28.0	80	40,000	0-0.5 N
(5/16/90)	1,2	22.5	94	82,000	0-0.5 SW
	3,4	23.0	94	100,000	0-0.5 SW
	5,6‡	25.0	94	100,000	5 W
Dismal Swamp					
(11/20/88)	1,2	21.0	98	5,000	0-2 SE/NE
	3,4	21.0	98	20,000	0-2 SE/NE
(9/10/89)	1,2	28.0	95	40,000	<0.5 SW
	3,4	28.0	98	80,000	<0.5 SW
(5/25/90)	1,2	11.0	87	80,000	0.5 SW
	3,4	13.0	85	80,000	0.5 SW
	5,6‡	15.0	84	80,000	0.5 SW
Claytor Lake					
(3/4/89)	1,2	7.5	100	1,200	1-4 NE
	3,4	7.5	100	1,200	1-4 NE
(6/17/89)	1,2	22.0	80	80,000	5-8 SW
	3,4	21.5	80	100,000	5-8 SW
(6/9/90)	1,2	24.5	71	120,000	0-3 SW
	3,4	25.0	60	100,000	0-3 SW
Cranberry Glades					
(10/4/89)	1,2	12.0	70	12,000	1 NE
	3,4	15.0	70	12,000	1 NE
(5/7/90)	1,2	8.0	67	42,000	0-5 SE
	3,4	10.0	67	42,000	0-5 SE
	5,6‡	10.0	67	42,000	0-5 SE

*Date of collection.
†Two Andersen aerosol samplers ran simultaneously.
‡TTC medium with the addition of 2 mM betaine.

TABLE II. Selected elemental composition* of waters collected from the four sampling locations

Location (Date)†	Sample Site‡	P	K	Ca	Mg	Mn	Zn	Fe	Al	Cu	B	S	Ni
Okefenokee Swamp													
(12/17/88)	1	0.0683	0.3000	1.400	0.5614	0.0065	0.0288	0.3607	0.1884	0.0130	0.0324	0.9601	5.2280
	2	0.2809	0.4838	2.094	0.7395	0.0114	0.0438	0.8747	0.4637	0.0163	0.0324	1.1170	5.6730
	3	0.0728	0.3586	2.642	0.8658	0.0537	0.1035	0.8604	1.0430	0.0212	0.0324	1.2640	5.8750
	4	0.0600	0.3000	1.559	0.7557	0.0130	0.0260	0.3433	0.3333	0.0114	0.0324	1.0170	5.1340
(6/22/89)	5	0.6460	0.4189	1.379	0.6117	0.0090	0.0155	0.6036	0.1250	0.0140	0.0290	1.1180	6.3030
	8	0.9260	0.4677	1.215	0.4075	0.0525	0.0214	0.4711	0.3000	0.0194	0.0261	1.3640	4.9760
(5/16/90)	5	0.0600	0.9345	2.390	1.1970	0.0227	0.1428	0.7717	0.1236	0.0063	0.0184	1.4560	8.3000
	8	0.0600	1.0210	3.179	1.6340	0.0361	0.0621	0.5040	0.3183	0.0047	0.0461	3.1950	24.1300
Dismal Swamp													
(11/20/88)	1	0.0922	5.6950	16.240	4.7110	0.2654	0.0748	2.1740	0.3681	0.0237	0.0738	5.1860	12.0000
	2	0.1556	3.8290	19.500	4.9560	0.2006	0.0748	1.3140	0.2582	0.0126	0.0463	11.8000	9.0910
	3	0.1724	4.2080	6.257	1.1890	0.0679	0.0833	4.6500	0.1345	0.0316	0.0875	30.1900	10.2600
	4	0.0618	1.6160	9.064	2.0080	0.0582	0.0562	1.6540	0.7527	0.0205	0.0446	7.2990	9.7150
(9/10/89)	1	0.0600	1.0690	14.050	2.5580	0.1033	0.1015	4.3900	1.8840	0.0149	0.0272	5.8830	11.2900
	2	0.0600	0.9845	12.810	1.9410	0.0654	0.0728	4.4100	1.8710	0.0149	0.0272	5.4260	7.4890
	3	0.0600	2.1210	5.283	1.2960	0.0052	0.0247	0.4270	0.6060	0.0066	0.0272	1.4850	6.1700
	4	0.0600	1.6140	8.207	1.5850	0.0549	0.0675	3.6640	1.3130	0.1320	0.0255	4.0240	6.4590
(5/25/90)	1	0.5196	2.8160	8.271	1.2510	0.0321	0.1385	2.0900	1.2500	0.0095	0.0258	4.7400	43.0600
	2	0.0600	0.9382	10.830	1.6250	0.0428	0.1592	2.7030	1.5840	0.0079	0.0221	4.8290	8.6110
	3	0.0600	1.6030	3.780	1.5590	0.0267	0.0632	0.7852	0.0250	0.0020	0.0165	2.4720	11.1900
	4	0.0600	1.0870	5.349	1.1540	0.0321	0.1243	1.9120	0.8608	0.0020	0.0165	2.2170	4.8320
Claytor Lake													
(3/4/89)	4	0.0600	2.3290	15.470	6.3500	0.0010	0.0186	0.0364	0.0250	0.0034	0.0435	4.7140	9.1940
(6/17/89)	4	0.0684	1.4240	7.373	2.9650	0.0010	0.0103	0.0218	0.0250	0.0059	0.0305	2.1730	4.3910
(6/9/90)	4	0.0600	1.3270	8.660	3.5240	0.0010	0.0294	0.0123	0.0250	0.0020	0.0202	2.2980	10.4600
Cranberry Glades													
(10/4/89)	1	0.0600	0.4043	2.616	0.6670	0.0157	0.0223	0.0050	0.0250	0.0066	0.0102	1.6410	1.8370
	2	0.0600	1.1890	6.871	1.4810	0.9579	0.0524	14.0400	0.1695	0.0149	0.0272	1.0660	3.4760
	3	0.0600	0.6402	3.728	0.8887	0.0444	0.0378	0.4135	0.1014	0.0116	0.0119	1.5050	1.9320
(5/1/90)	1	0.0600	0.3894	3.325	0.8195	0.0107	0.0196	0.0190	0.0250	0.0020	0.0092	1.8060	1.7160
	2	0.0600	0.5929	3.186	0.7319	0.4440	0.0937	2.5060	0.1097	0.0020	0.0092	2.1980	1.7480
	3	0.0600	0.4948	3.092	0.7351	0.0722	0.0632	0.6659	0.0401	0.0020	0.0165	1.5980	1.7840

*All elements are given as ppm (mg/L water).
†Date of collection.
‡Refer to Figure 1A-E for the location.

TABLE 12. Selected elemental composition* of soils collected from the four sampling locations

Location (Date)†	Sample Site‡	P	K	Ca	Mg	Zn	Mn	Soluble Salts	NO_3-N
Okefenokee Swamp									
(12/17/88)	1	45	17	108	20	2.3	2.4	115	5
	3	3	4	108	13	1.9	1.3	1	3
	4	1	3	36	6	2.4	0.9	1	3
(6/22/89)	5	2	15	84	14	1.3	1.5	128	3
	6	4	44	216	55	1.9	1.2	768	5
	7	7	26	420	45	5.4	4.2	128	8
	9	20	19	158	57	5.9	0.6	409	3
(5/16/90)	5	3	20	360	38	4.6	2.4	115	3
	6	4	37	240	101	1.5	1.5	435	3
	7	11	34	576	84	6.1	5.4	1	3
	9	25	23	168	57	6.1	0.9	371	3
Dismal Swamp									
(11/20/88)	2	25	61	840	80	1.1	14.3	51	3
	3	1	22	240	31	1.6	1.9	102	3
	4	1	17	72	18	1.2	0.7	1	3
(9/10/89)	2	5	20	588	56	2.3	3.1	243	35
	3	3	18	732	59	2.6	8.1	205	20
	4	1	18	120	19	1.6	0.8	1	3
(5/25/90)	2	2	25	552	48	6.1	1.8	179	8
	3	3	23	792	66	3.6	8.2	102	10
	4	2	31	168	32	1.6	1.5	1	3
Claytor Lake									
(3/4/89)	1	1	23	252	39	0.3	1.5	154	3
	2	30	112	1200	120	6.1	16.1	243	3
	3	3	63	264	81	0.5	3.0	1	3
	4	60	150	312	48	0.9	2.4	1	3
(6/17/89)	1	1	37	420	62	0.4	1.6	64	3
	2	47	107	1200	120	6.1	16.1	294	3
	3	1	20	444	114	1.4	5.5	51	3
	4	29	157	1200	120	6.1	16.1	179	5
(6/9/90)	1	2	37	300	67	0.4	2.1	1	3
	2	6	53	1176	120	4.1	16.1	38	3
	3	0	36	324	81	0.4	3.7	1	3
	4	53	66	204	26	2.9	4.8	1	3
Cranberry Glades									
(10/4/89)	1	4	53	720	66	6.1	16.1	1	3
	2	3	26	636	68	3.9	16.1	1	3
	3	1	25	288	37	3.4	11.5	26	3
(5/7/90)	1	3	47	360	45	3.0	16.1	102	13
	2	3	80	840	107	3.8	16.1	102	20
	3	3	67	360	59	1.5	16.1	154	38

*All data given as ppm (mg/kg soil).
†Date of collection.
‡Refer to Figure 1A-E for the location.

Swamps had somewhat higher organic matter content, higher humic and fulvic acid contents, and lower pH compared to soils from the two Appalachian locations. Physiochemical measurements taken during aerosol collection were variable with no trend observed when comparing the different locations (Table 10).

MAIS colony-forming units (CFU)/ml water and some selected physiochemical characteristics and MAIS CFU/g dried soil and selected physiochemical variables were correlated (Figure 2, Table 13). Water samples in the Okefenokee and Dismal Swamps that yielded high MAIS numbers had lower pH and dissolved oxygen and higher zinc, humic acid, and fulvic acid contents, as compared to the Appalachian waters (Figure 2). More MAIS were recovered from relatively warmer waters (Figure 2). Similarly, soil samples that were lower in pH and higher in zinc, organic matter, humic, and fulvic acid contents (abundant within the Okefenokee and Dismal Swamps) yielded more MAIS than soils of Claytor Lake and Cranberry Glades (Figure 2). Water samples had higher concentrations of fulvic than humic acid, since some humic acid may have been insoluble and precipitated at the low pH's of the waters (17, 23). However, soils had about equal concentrations of both humic and fulvic acids (Figure 2, Table 9).

These physiochemical variables and interactions between them were then tested for correlation with MAIS CFU in both water ($n = 29$) and soil ($n = 38$) samples. The statistical significance of these correlations for the samples collected at the four locations is shown in Table 13. Noteworthy are the highly significant correlations between high zinc and fulvic acid content and the interaction between these two variables for the water samples. Thus, any multivariant analysis involving zinc and/or fulvic acid with any other variables results in decreased p values, indicating a greater degree of significance. MAIS CFU/g soil was most significantly influenced by humic or fulvic acid concentrations or total organic matter contents of the soils and any interactions with other variables involving those three. There was no correlation for the aerosol samples between MAIS CFU/m^3/h and light intensity, relative humidity, or air temperature, nor was there any

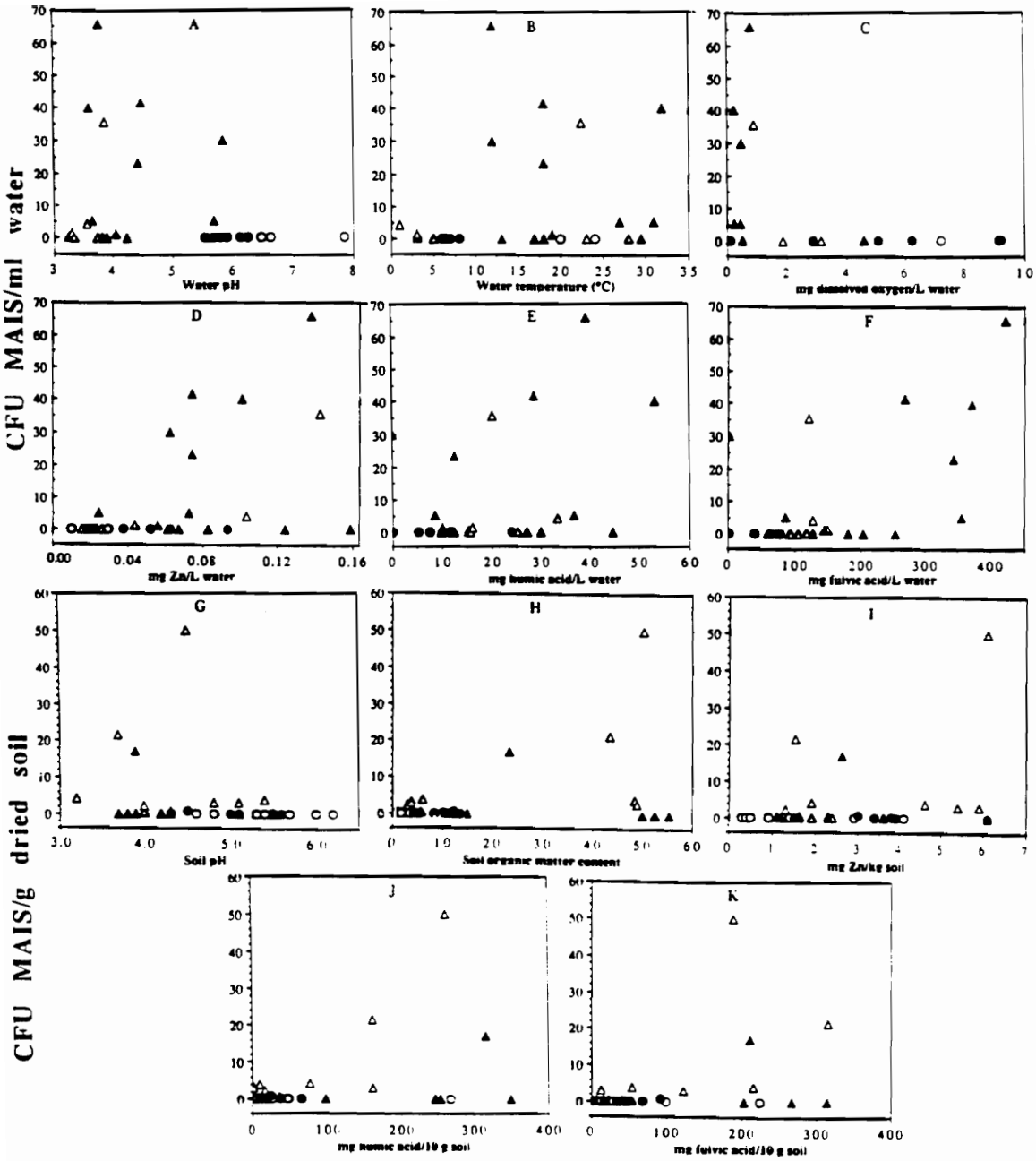

Fig. 2. Relationship between MAIS colony-forming units (CFU)/ml water and (A) pH, (B) temperature, (C) dissolved oxygen, (D) zinc content, (E) humic acid content, and (F) fulvic acid content, and between MAIS CFU/g soil (dry weight) and (G) pH, (H) organic matter content, (I) zinc content, (J) humic acid content, and (K) fulvic acid content. Key to the sampling sites: △= Okefenokee Swamp, ▲= Dismal Swamp, ○= Claytor Lake, and ●= Cranberry Glades.

TABLE 13

LEVELS OF SIGNIFICANCE (p VALUES) FOR CORRELATIONS OF SELECTED PHYSIOCHEMICAL VARIABLES WITH HIGHER NUMBERS OF MAIS FROM WATER AND SOIL SAMPLES COLLECTED FROM ALL FOUR LOCATIONS

Variable	Water	Soil
pH (<5.5)	0.22	0.15
Dissolved oxygen (<2 mg/L water)	0.65	--
Zinc (>0.75 mg/L water; >4 mg/kg soil)	0.03	0.23
Temperature (>15°C)	0.92	--
Organic matter (>15%)	--	0.004
Humic acid (>20 mg/L water; >15 mg/g soil)	0.13	0.004
Fulvic acid (>200 mg/L water; >15 mg/g soil)	0.008	0.006
pH and dissolved oxygen	0.45	--
pH and zinc	0.09	0.12
pH and organic matter	--	0.02
pH and humic acid	0.33	0.02
pH and fulvic acid	0.03	0.02
Zinc and dissolved oxygen	0.09	--
Zinc and organic matter	--	0.02
Zinc and humic acid	0.09	0.02
Zinc and fulvic acid	0.03	0.02
Organic matter and humic acid	--	0.01
Organic matter and fulvic acid	--	0.01
Humic acid and dissolved oxygen	0.33	--
Humic acid and fulvic acid	0.01	0.01
Fulvic acid and dissolved oxygen	0.03	--
Organic matter, humic acid, and fulvic acid	--	0.03
All variables measured	0.08	0.19

correlation for ejected water droplet samples between MAIS CFU/cm²/h and water temperature. Likewise, there were no correlations between MAIS CFU/ml water and concentrations of Mn, Mg, Ca, P, K, Cu, Al, Fe, B, Na, and S or MAIS CFU/g soil and concentrations of Mn, Mg, Ca, P, K, NO_3-N, and soluble salt. Correlations between MAIS CFU and some heavy metals (e.g., Cd, Cr, Hg, Pb, Ni, and Tl) in water could not be determined because concentrations were below the limit of detection for all samples.

Oxygen Tolerance

Table 14 shows oxygen tolerance classifications determined for a variety of MAIS strains. Of 33 MAIS isolates tested, 23 (70%) isolates were classified as microaerobic, exhibiting growth both on the medium surface and >5 mm below, while 5 (15%) isolates exhibited growth solely >5 mm below the medium surface and were classified as obligately microaerobic and growth of the 5 (15%) remaining strains (aerobic) occurred on the medium surface but not >5 mm below (Table 14). Four of 5 MAIS strains that exhibited only aerobic growth were aerosol isolates. Isolates collected from ejected droplets, water, or soils usually exhibited microaerobic growth.

Effects of Betaine on MAIS Aerosolization and Growth

Comparison of recovery of aerosolized MAIS organisms using TTC medium with and without the addition of betaine are shown in Table 3. MAIS were not recovered on TTC + betaine for any of the aerosol collections, but some MAIS were recovered on TTC with no additions. All 5 MAIS strains tested grew best on M7H10 medium with OAA, while only 1 of 5 (20%) strains grew on TTC, either with or without betaine addition (Table 15). Strain 2812U grew slightly better on TTC compared to TTC + betaine.

TABLE 14. MAIS strain oxygen tolerance

Strain†	Classification*		
	Aerobic (<5mm)	Micro-aerobic (0->5mm)	Micro-aerophillic (>5mm)
Aerosol:			
DSA16	X		
DSA24	X		
DSA211	X		
OKA16			X
OKA23			X
OKA106	X		
OKA112		X	
OKA114		X	
Ejected Droplets:			
DSA10		X	
OKA126		X	
Water:			
DSW23		X	
DSW43		X	
DSW127		X	
DSW1			X
DSW2		X	
DSW69		X	
DSW73		X	
DSW74		X	
OKW75		X	
OKW135			X
OKW143		X	
Soil:			
CGS212	X		
DSS141		X	
DSS148		X	
DSS151		X	
DSS153		X	
OKS41		X	
OKS178		X	
OKS160		X	
OKS167		X	
OKS186		X	
OKS258			X
OKS261		X	
Totals:	5/33 (15%)	23/33 (70%)	5/33 (15%)

*Growth distribution (mm from medium surface) in semi-solid M7H9+ medium.
†Refer to Chapter 1, Table 5 for strain listings.

TABLE 15. Qualitative comparison of MAIS growth on different media types

Strain	Media Type*		
	TTC	TTC+2 mM betaine	M7H10+OAA
13S	NG, NG	NG, NG	+++, +++
Va2	NG, NG	NG, NG	+++, +++
Va14(O)	NG, NG	NG, NG	+++, +++
2812U	++, ++	++, +	+++, +++
OKS41	NG, NG	NG, NG	+++, +++

*Refer to Methods for media preparations. Results are for duplicate platings, in which growth ranged from + (slight growth) to +++ (excellent growth). NG = No Growth.

Discussion

MAIS organisms were recovered more frequently from waters, soils, aerosols, and ejected droplets compared to recovery of rapidly-growing or non-MAIS mycobacteria (Tables 1-4). The data on recovery of MAIS from the four sampling locations show that significantly higher numbers are recovered from water, soil, and aerosol samples of the Okefenokee and Dismal Swamps compared to Claytor Lake and Cranberry Glades (Table 6). "Recovery" by our methods represents only a percentage of the total MAIS numbers present. The different numbers recovered by these methods from similar environmental compartments at different locations should therefore reflect the comparative sizes of the resident MAIS communities. Therefore, our finding of higher MAIS recovery in the two coastal swamps agrees well with the geographic distribution of MAIS in water (8) and soil (9) samples, and with the geographic incidence of skin sensitivity to PPD-B (6) and PPD-G (7) and clinical isolates from infected patients (2, 3).

Lower recovery of MAIS organisms from Okefenokee waters compared to Dismal Swamp waters (Table 7) could be due to the fact that Okefenokee waters were of higher dissolved oxygen and lower humic and fulvic acid concentrations (Figure 2). Okefenokee soils yielded more MAIS than Dismal Swamp soils (Table 7). Possibly this source of MAIS results in the higher frequency of PPD-B (6) and PPD-G (7) reactors in the Okefenokee Swamp compared to the Dismal Swamp. Values for soil pH, organic matter, zinc, humic acid, and fulvic acid were similar (Figure 2). Comparisons of these recovery data using our methods cannot be made reliably with those of Falkinham, et al. (8) for water and Brooks, et al. for soil (9), because different media were used.

However, numbers of MAIS recovered on TTC medium per ml water in the Dismal Swamp (Table 7) were higher than those from non-swamp waters in Virginia (20).

A highly significant correlation for the data was apparent between high numbers of MAIS and high zinc and fulvic acid levels of water samples. In soils, significant correlations between high MAIS numbers and humic and fulvic acids and organic matter contents was also observed. Zinc probably was not limiting nor toxic for most organisms in any of the water samples whose zinc concentration ranged from 10.3 to 159.2 ug/L (Table 11), a range typical for many fresh-waters in the United States (28). It has been established that some mycobacteria have a high zinc requirement (29), that zinc is a common metal in many metalloenzymes (28), and that humic and fulvic acids complex with and chelate zinc (28). The high correlation between the recovery of MAIS and high zinc concentrations, therefore, may be related to MAIS abundance in the environment. This finding also may explain high recoveries of MAIS from hospital water systems with galvanized pipes consisting of zinc alloys (30).

Higher recovery of MAIS from samples rich in humic and fulvic acids agrees with earlier work by Kazda (31), demonstrating *M. intracellulare* growth stimulation inside dead storage cells of *Sphagnum*, a moss genus long recognized as a primary constituent of acid bogs and important in acid peat and humic acid production (32). Results also agree with the findings of Brooks, et al., (9) who demonstrated higher MAIS recoveries from acidic soils, high in organic matter. Humic and fulvic acids compose a significant portion of water and soils' organic fractions (17, 23).

Most MAIS have also been shown to be microaerobic (Table 14), thereby agreeing with our data suggesting higher recoveries of MAIS in less oxygenated waters. Higher frequencies of isolation of MAIS from warmer waters also agrees with the work of George, et al. (14) suggesting greater recoveries of MAIS in temperatures above 15.5° C. Additions of betaine to TTC medium did not enhance MAIS recoveries. Physiochemical characteristics differed little (Table 10) or showed no major correlations with the results of aerosol sampling, thereby eliminating those

as possible explanations regarding the lack of MAIS recovery when TTC medium + betaine was employed. Also, failure of most MAIS organisms to grow on TTC medium suggests the need to develop other MAIS selective media.

Our data suggests that MAIS organisms probably are much more abundant in the acid, brown swamp waters and associated soils of the southeastern coastal plain than those waters and soils found elsewhere. On a weight basis, somewhat higher MAIS numbers were recovered from water compared to soil. However, strong binding of MAIS to soil particles (19), suggests that swamp soils may harbor higher numbers and be as important or more so than swamp water as a source of infection. In fact, if one corrects for the loss of MAIS following the decontamination procedure and subsequent plating onto M7H10, our recovery numbers would be about 100-fold higher for water and 1,000-fold higher for soil (19). On a volume basis, the numbers in aerosols are still smaller, but the volume of air regularly inhaled by humans makes this third potential source of infection also important.

Probably the abundance of MAIS organisms in these swamps results from several interacting variables. The combination of higher temperatures, low oxygenated waters, and lower pH soils and waters higher in zinc and humic and fulvic acids, most likely favor growth and survival of MAIS organisms in the environment. A single environmental (soil, water, or aerosols) source for members of the *M. avium*, *M. intracellulare*, and *M. scrofulaceum* group is neither necessary nor likely. The swamp soils, waters, and aerosols containing MAIS may all play a role in the epidemiology of MAIS infections in humans within this geographic region. Sources having higher numbers of infective units or sources with greater frequencies of human exposure can be more significant for human infection. Whereas MAIS complex organisms may be ubiquitous, but their population size and proximity to routes of transfer to humans may be of key importance for explaining the epidemiology of infection. Apparently the acid, brown-water swamps of the coastal plain of the southeastern United States aptly fits the epidemiologic model.

References

1. Wolinsky E. Nontuberculous mycobacteria and associated diseases. Am Rev Respir Dis 1979; 119:107-59.

2. Good RC. Isolation of nontuberculous mycobacteria in the United States, 1979. J Infect Dis 1980; 142:779-83.

3. Good RC, Snider DE Jr. Isolation of nontuberculous mycobacteria in the United States, 1980. J Infect Dis 1982; 146:829-33.

4. Young LS, Inderlied CB, Berlin OG, Gottlieb MS. Mycobacterial infections in AIDS patients, with an emphasis on the *Mycobacterium avium* complex. Rev Infect Dis 1986; 8:1024-33.

5. Horsburgh CR Jr, Selik RM. The epidemiology of disseminated nontuberculous mycobacterial infection in the acquired immunodeficiency syndrome (AIDS). Am Rev Res Dis 1989; 139:4-7.

6. Edwards LB, Aquaviva FA, Livesay VT, Cross RW, Palmer CE. An atlas of sensitivity to tuberculin, PPD-B, and histoplasmin in the United States. Am Rev Respir Dis 1969; 99:1-131.

7. Edwards PQ. Interpretation and significance of the tuberculin test in typical and atypical mycobacterial infections. In: Johnson JE III, ed, Symposium on rationale therapy and control of tuberculosis. Gainesville: University of Florida Press, 1970; 43-55.

8. Falkinham JO III, Parker BC, Gruft H. Epidemiology of infection by nontuberculous mycobacteria. I. Geographic distribution in the eastern United States. Am Rev Respir Dis 1980; 121:931-7.

9. Brooks RW, Parker BC, Gruft H, Falkinham JO III. Epidemiology of infection of nontuberculous mycobacteria. V. Numbers in eastern United States soils and correlation with soil characteristics. Am Rev Respir Dis 1984; 130:630-3.

10. Wendt SL, George KL, Parker BC, Gruft H, Falkinham JO III. Epidemiology of infection by nontuberculous mycobacteria. III. Isolation of potentially pathogenic mycobacteria from aerosols. Am Rev Respir Dis 1980; 122:259-63.

11. Meissner PS, Falkinham JO III. Plasmid DNA profiles as epidemiologic markers for clinical and environmental isolates of *Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum*. J Infect Dis 1986; 152:325-31.

12. Fry KL, Meissner PS, Falkinham JO III. Epidemiology of infection by nontuberculous mycobacteria. VI. Identification and use of epidemiologic markers for studies of *Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum*. Am Rev Respir Dis 1986; 34:39-43.

13. George KL, Falkinham JO III. Selective medium for the isolation and enumeration of *Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum*. Canad J Microbiol 1986; 32:10-4.

14. George KL, Parker BC, Gruft H, Falkinham JO III. Epidemiology of infection by nontuberculous mycobacteria. II. Growth and survival in natural waters. Am Rev Respir Dis 1980; 122:89-94.

15. Murray RE, Hodson RE. Microbial biomass and utilization of dissolved organic matter in the Okefenokee Swamp ecosystem. Appl Environ Microbiol 1984; 47:685-92.

16. Oaks RQ Jr, Whitehead DR. Geologic setting and origin of the Dismal Swamp, southeastern Virginia and northeastern North Carolina. In: Kirk PW Jr, ed, The Great Dismal Swamp. Charlottesville: University Press of Virginia, 1979; 1-24.

17. Flaig W, Beatelspacher H, Rietz E. Chemical composition and physical properties of humic substances. In: Gieseking JE, ed, Soil components, Vol 1. New York: Springer-Verlag, New York, Inc, 1975; 1-211.

18. National Climatic Data Center. Surface, land daily cooperative summary of the day. Asheville: NOAH, 1990. TD-3200.

19. Brooks RW, George KL, Parker BC, Falkinham JO III, Gruft H. Recovery and survival of nontuberculous mycobacteria under various growth and decontamination conditions. Canad J Microbiol 1984; 30:1112-7.

20. Falkinham JO III, George KL, Ford MA, Parker BC. Collection and characteristics of mycobacteria in aerosols. In: Morey PR, Feeley JC Sr, Otten JA, eds. Biological contaminants in indoor environments, ASTM STP 1071. Philadelphia: American Society for Testing and Materials, 1990; 71-81.

21. Pethel ML, Falkinham JO III. Plasmid-influenced changes in *Mycobacterium avium* catalase activity. Infect Immun 1989; 57:1714-8.

22. American Public Health Association, American Water Works Association, and Water Pollution Control Federation (APHA-AWWA-WPCF). Standard Methods for the Examination of Waste Water. 16th ed. Washington: American Public Health Association, 1985; p. 1268.

23. Schnitzer M. Organic Matter Characterization. In: Page AL, Miller RH, Keeney DR, eds, Methods of soil analysis, Part 2. 2nd ed. Madison, WI: American Society of Agronomy, Inc, 1982; 581-94.

24. Carder KL, Steward RG, Harvey GR, Ortner PB. Marine humic and fulvic acids: Their effect on remote sensing of ocean chlorophyll. Limnol Oceanogr 1989; 34:68-81.

25. Collins CH, Yates MD, Grange JM. Subdivision of *Mycobacterium tuberculosis* into five variants for epidemiologic purposes: methods and nomenclature. J Hyg Camb 1982; 89:235-42.

26. Marthi B, Lighthart B. Effects of betaine on enumeration of airborne bacteria. Appl Environ Microbiol 1990; 56:1286-9.

27. SAS Institute, Inc. SAS User's Guide: Statistics, Version 5.0. Cary, NC: 1985; p. 956.

28. Committee on Metabolic and Biologic Effects of Environmental Pollutants, Subcommittee on Zinc. Zinc. Baltimore: University Park Press, 1979; p. 471.

29. Winder FG, O'Hara C. Effects of iron deficiency and of zinc deficiency on the composition of *Mycobacterium smegmatis*. Biochem J 1962; 82:98-108.

30. duMoulin GC, Stottmeier KD, Pelletier PA, Tsang AY, Hedleywhyte J. Concentration of *Mycobacterium avium* by hospital water systems. J Am Med Assoc 1988; 260:1599-601.

31. Kazda J. Vermehrung von Mykobakterien in der grauen Schicht der Sphagnum-Vegetation. Zbl Bakt Hyg I Abt Orig B 1978; 166:463-9.

32. Scagel RF, Bandoni RJ, Maze JR, Rouse GE, Schofield WB, Stein JR. Nonvascular plants. An evolutionary survey. Belmont, CA: Wadsworth Publishing Co., 1983; 477.

Chapter 2

Humic and Fulvic Acid Growth Stimulation of *Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum*

ABSTRACT

We sought to determine factors favoring the growth and survival of clinical and environmental strains of *Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum* (MAIS group) that might explain their relative abundance in the waters, soils, and aerosols of the swamps of the southeastern United States coastal plain. Characteristics of these swamp waters and soils include relatively (1) warm temperature, (2) low pH, (3) high humic and (4) high fulvic acids, (5) low dissolved oxygen, and (6) high zinc concentrations. We show that the growth of a majority of MAIS strains was stimulated by humic or fulvic acids at concentrations found in acid, brown-water swamps, such as the Okefenokee (GA) or Great Dismal (VA) Swamps. Most additions of humic or fulvic acids did not stimulate oxygen uptake in MAIS strains tested, suggesting that rapid uptake and breakdown of these natural substances is not the primary cause of their growth stimulation. Other possibilities include the incorporation as carbon skeletons not requiring oxygen and chelation of metals, for example, zinc. These results at least partially explain the relative abundance of MAIS in the coastal plain swamps of the southeastern United States, a region long-established for its higher rate of human infection by these mycobacteria.

INTRODUCTION

Members of the *Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum* (MAIS) group are slow-growing, opportunistic pathogens of environmental origin that cause pulmonary infections in humans (34). An estimated 2,000 United States residents are infected annually (14, 15). Recently it has been shown that a substantial proportion of patients with acquired immunodeficiency syndrome (AIDS) have disseminated MAIS infections (1, 17). The highest frequencies of isolates from patient specimens (14, 15) and the highest percentage of persons reacting to either PPD-B (purified protein derivative of *M. intracellulare* Battey strain) (7) or PPD-G (*M. scrofulaceum* Gause strain) (8) occur in the southeastern United States. This correlates well with the higher numbers of MAIS recovered from water samples (9), soils (2), and aerosols (32) collected in the southeastern coastal plain. In fact, most aerosol and 25% of MAIS water isolates share many characteristics with clinical isolates (11, 21). The well established absence of any evidence for transmission from person-to-person (34) implies that one or more environmental source(s) of these bacteria may be important in the epidemiology of infection.

Recently higher numbers of MAIS have been recovered from two southeastern coastal plain swamps (Great Dismal Swamp, VA and Okefenokee Swamp, GA) compared to two colder aquatic habitats in the Appalachian region (Claytor Lake, VA and Cranberry Glades, WV) (Chapter 1). It was shown that the higher MAIS numbers correlated well with the higher humic and fulvic acid concentrations, lower pH, and warmer temperature of the swamps' soils and waters. The abundance of MAIS organisms in the coastal swamp regions implies that their growth and survival may be favored one or a combination of these physiochemical variables. Higher tempera-

ture, lower pH, higher humic, and higher fulvic acid concentrations may explain MAIS abundance in those swamp environments. Therefore, we sought to test this hypothesis through laboratory experiments with both environmental and clinical isolates. These experiments attempt to further describe the present understanding of the epidemiology of MAIS in human infection and disease.

MATERIALS AND METHODS

Bacterial strains. Table 1 lists the 18 MAIS isolates used in this study with information on their species and habitat source. *M. avium* complex includes both *M. avium* and *M. intracellulare*.

Growth media and growth of the bacterial strains. Log phase cultures of MAIS strains were established by growing strains for 1 to 3 wk at 37°C in 16 x 125 mm screw-capped tubes containing 6 to 10 ml of Middlebrook and Cohn 7H9 medium (M7H9; BBL Microbiology Systems, Cockeysville, MD) with 0.5% (vol/vol) glycerol and 10% (vol/vol) oleic acid-albumin enrichment (OAA) (23). Cultures were stored at 4°C after reaching 10^6 - 10^8 cells/ml. Growth media consisted of filter-sterilized (0.2 um pore size) natural water collected from the four sampling locations: Claytor Lake, VA (pH 7.85, 0.03 ug zinc/ml, <0.001 mg humic acid/ml, and <0.001 mg fulvic acid/ml), Cranberry Glades, WV (pH 5.78, 0.09 ug zinc/ml, 0.01 mg humic acid/ml, and 0.08 mg fulvic acid/ml), Great Dismal Swamp, VA (pH 4.24, 0.12 ug zinc/ml, 0.03 mg humic acid/ml, and 0.18 mg fulvic acid/ml), and Okefenokee Swamp, GA (pH 3.85, 0.14 ug zinc/ml, 0.02 mg humic acid/ml, and 0.12 mg fulvic acid/ml). Other experiments used the basal (BS) medium of Ratledge and Hall (24), containing glycerol, L-asparagine, and KH_2PO_4 adjusted to pH 5.5. Filter-sterilized (0.2 um pore size) solutions of humic and fulvic acids also adjusted to pH 5.5 and extracted and purified (29) from soils collected from the Dismal Swamp were added to both Claytor Lake water and BS medium to measure the effects of each on MAIS growth. MAIS strains were inoculated into 5 ml of medium in 16 x 150 mm screw-capped tubes containing different volumes of stock humic or fulvic acids. Cultures were incubated at 37°C. Growth was

Table 1. Mycobacterial strains and sources

Strain	Identification	Origin[a]	Reference
CLINICAL			
2812P	*M. avium* complex	Human (AIDS)	31
2812U	*M. avium* complex	Human (AIDS)	31
Val4(O)	*M. avium* complex	Human (Non-AIDS)	11
Val4(T)	*M. avium* complex	Human (Non-AIDS)	11
Va2	*M. avium* complex	Human (Non-AIDS)	11
2279	*M. avium*	Chicken	16
ENVIRONMENTAL			
13S	*M. avium*	ejected droplet (JR)	32
DSA16	*M. avium* complex	aerosol (DS)	This study
DSW2	*M. avium* complex	water (DS)	This study
DSW69	*M. avium* complex	water (DS)	This study
DSW73	*M. avium* complex	water (DS)	This study
DSW74	*M. avium* complex	water (DS)	This study
DSW127	*M. avium* complex	water (DS)	This study
OKA23	*M. avium* complex	ejected droplet (OK)	This study
OKS41	*M. avium* complex	soil (OK)	This study
OKW75	*M. scrofulaceum*	water (OK)	This study
OKA126	*M. scrofulaceum*	ejected droplet (OK)	This study
OKS147	*M. scrofulaceum*	soil (OK)	This study

[a]Key for the origin of the strains: JR = James River, Richmond (VA), DS = Dismal Swamp (VA), and OK = Okefenokee Swamp (GA).

measured as increases in turbidity at 580 nm to reduce interference from the yellow pigment of some MAIS strains.

Growth studies involving pH, temperature, and metals. Growth stimulation by humic and fulvic acids could be due to a decrease in pH after adding the acidic compounds (pH 5.5) to Claytor Lake water, since MAIS grow best at pH 5-5.5 (13). To test for a potential pH stimulatory effect, 6 selected MAIS strains were grown in Claytor Lake adjusted with 0.5 N HCl or 0.5 N NaOH to four different pH's (4.0, 5.5, 7.0, and 8.5). As a comparison, the strains were also grown in Claytor Lake water containing 0.05 mg humic acid/ml or 0.4 mg fulvic acid/ml and adjusted to the same pH values. Growth (turbidity changes) was measured (in triplicate) as described above.

Seven MAIS strains were grown in natural waters collected from the four sampling locations to compare growth at two temperatures (37° and 16°C). In addition to turbidity changes (done in triplicate), growth was also measured as viable cell counts (CFU/ml) were determined after being diluted in sterile, distilled water and spread in duplicate onto Middlebrook and Cohn 7H10 medium (M7H10; BBL Microbiology Systems, Cockeysville, MD) with 0.5% (vol/vol) glycerol and 10% (vol/vol) OAA.

Growth comparisons (in duplicate) were also done using 7 MAIS strains in BS medium containing the following metals: iron ($FeSO_4$; 40 uM final concentration), magnesium ($MgSO_4$; 2 uM), manganese ($MnSO_4$; 5 uM), and zinc ($ZnSO_4$; 7 uM), in the presence or absence of growth-stimulatory concentrations (0.1 mg/ml) of humic acid (Aldrich Chemical Company, Inc., Milwaukee, WI). Each of the metal additions consisted of filter-sterilized (0.2 um pore size) stock solutions prepared accordingly to achieve the desired final concentrations.

Measurement of oxygen uptake. MAIS strains used in the oxygen uptake studies were grown at 37°C with continuous shaking at 100 rpm (Orbital Shaker Model 3590, Lab Line Instruments, Inc., Melrose Park, IL) in six 1,000 ml screw-capped flasks containing 100 ml of BS medium and 10% (vol/vol) OAA for 5 to 7 days until mid-log phase was reached. Cells were harvested by centrifugation at 10,000 x g for 10 min at 4°C. The cells were washed in 50 ml 0.05 M KH_2PO_4 buffer (pH 5.5) by gently shaking for 5 min and collected by centrifugation at 7,500 x g for 10 min at 4°C. The washing process was repeated, and the cell pellet suspended in 25 ml of buffer and clumps disrupted by passage through a tissue homogenizer (Arthur H. Thomas Co., Philadelphia, PA), then stored at 4°C.

Oxygen consumption was measured using a YSI Model 5331 Oxygen Probe covered with a FEP Teflon membrane, a YSI Model 53 Biological Monitor (Yellow Springs Instrument Co., Inc., Yellow Springs, OH), and a water-jacketed, 1.7 ml Clark cell (Gilson Medical Electronics, Inc., Middleton, WI) maintained at 37°C with a circulating water bath. The system was calibrated for dissolved O_2 using the method of Robinson and Cooper (25). After establishing an endogenous oxygen consumption rate (6 to 7 min), the effects on oxygen uptake following the addition of (i) OAA [final concentration 0.0018% (vol/vol) oleic acid and 0.15% (wt/vol) bovine serum albumin], (ii) 0.005, 0.05, and 0.5 mg humic acid/ml, (iii) 0.04, 0.4, and 4.0 mg fulvic acid/ml, and (iv) combinations of the above were measured. Following substrate addition and establishment of a constant oxygen uptake rate (6 to 7 min), the effects of adding KCN (final concentration 5.9 mM) were measured.

The oxygen consumption rates due to endogenous respiration, substrate addition, and KCN addition were measured sequentially for each assay. All experiments were performed in duplicate at 37°C. The rate of change between the fourth and sixth minutes after obtaining a constant rate was used for all oxygen uptake calculations. Dry cell weight (mg/ml) for each cell suspension was determined by dispensing (in duplicate) 1.0 ml of each culture, in a dried, tared, aluminum pan.

The culture was allowed to dry at 100°C in an oven for 24 h, then weighed. The weight of 1.0 ml buffer was subtracted from the dried cell suspension values.

Statistical analysis. Duncan's Multiple Range test or Student's T test of the Statistical Analysis Systems (SAS) program (30) were performed to compare differences in MAIS growth in media and waters subjected to the variety of treatments. For significance, a confidence interval of 95% was selected for both procedures.

RESULTS

Growth stimulation by humic and fulvic acids. Fig. 1 illustrates growth of *M. avium* complex strain OKS41 in Claytor Lake water. The relatively slow growth is a characteristic of all MAIS organisms. Humic and fulvic acids both stimulated growth (Fig. 1). Fourteen of 15 (93%) strains tested showed similar stimulatory responses in Claytor Lake water. The concentrations of humic and fulvic acids chosen were similar to those in the natural swamp waters. Further, we sought to determine if humic and fulvic acids could stimulate MAIS growth in an organically enriched medium (i.e., BS medium). Because BS medium contains sufficient carbon (glycerol) and nitrogen (L-asparagine), stimulation by humic or fulvic acids might suggest that the substances were stimulating MAIS growth through mechanisms other than nutrient supplementation. Growth of 8 of 18 (44%) strains tested were stimulated in BS medium containing either humic or fulvic acids, as illustrated for *M. scrofulaceum* strain OKA126 (Fig. 2). The remaining 10 of 18 (56%) strains tested grew relatively well in BS medium and did not show marked humic or fulvic acid stimulation, as illustrated for *M. avium* strain 13S (Fig. 3). Thus, humic and fulvic acid growth stimulation in BS medium was only observed in those 8 strains which grew poorly in BS (Fig. 2). Table 2 summarizes the stimulatory responses that were observed for each MAIS strain.

The growth response of 3 selected MAIS strains in either BS or Claytor Lake water, containing or not containing different types of humic or fulvic acids from soils from the Okefenokee Swamp, Dismal Swamp, and Claytor Lake, indicated that none of the 3 humic and fulvic acid types clearly enhanced MAIS growth better than the others (Table 3). Therefore, since humic and fulvic acids purified from soils collected from the Dismal Swamp were equivalent to others, these were used in the remaining growth studies.

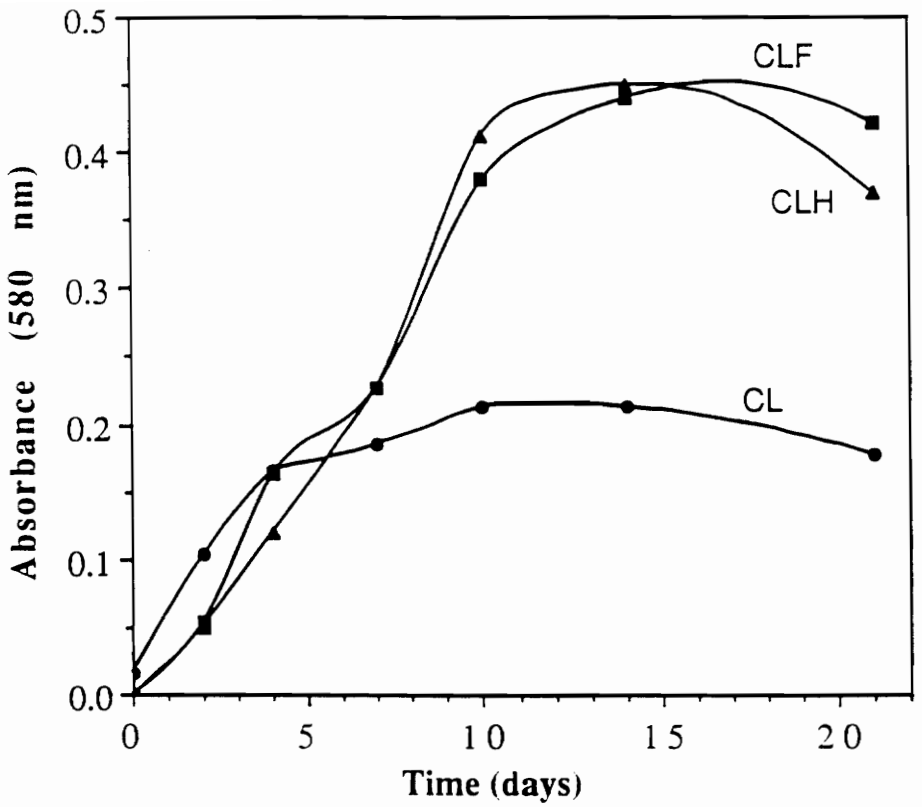

Figure 1. Growth (average of triplicate measurements) at 37°C of strain OKS41 in Claytor Lake (CL) water and Claytor Lake water containing 0.05 mg humic acid/ml (CLH) or 0.4 mg fulvic acid/ml (CLF).

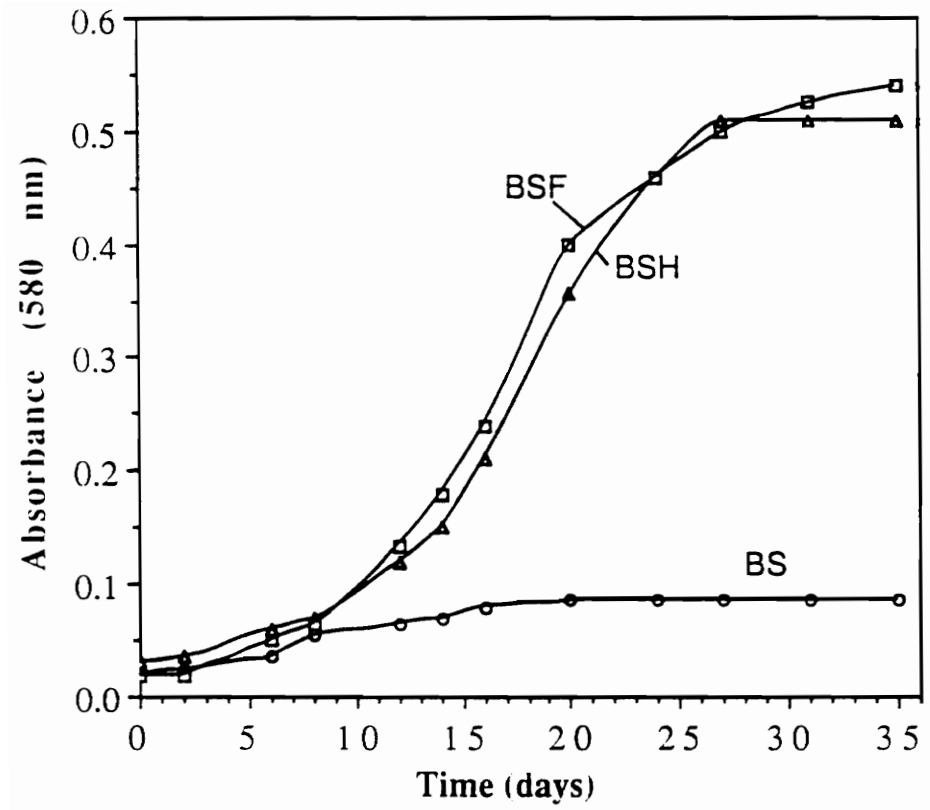

Figure 2. Growth at 37°C of strain OKA126 in basal medium (BS) and basal medium containing 0.1 mg humic acid/ml (BSH) or 0.5 mg fulvic acid/ml (BSF).

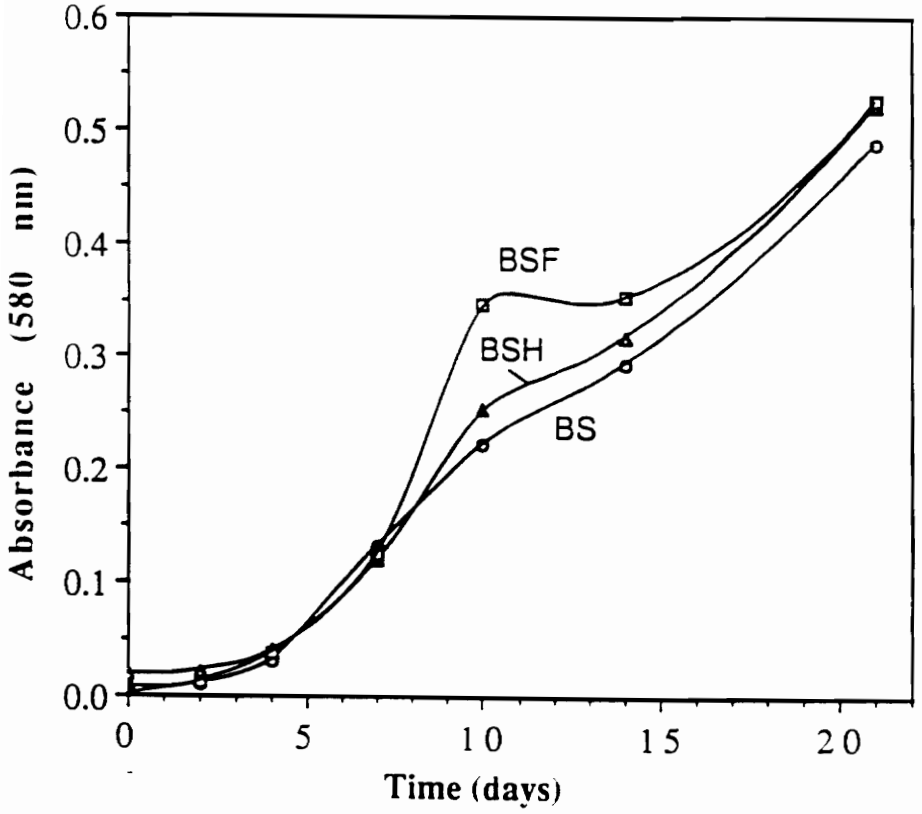

Figure 3. Growth (average of triplicate measurements) at 37°C of strain 13S in basal medium (BS) and basal medium containing 0.05 mg humic acid/ml (BSH) or 0.4 mg fulvic acid/ml (BSF).

Table 2. Observation of humic and fulvic acid stimulation[a] of MAIS growth

Strain[b]	Identification	Claytor Lake water	Basal medium
CLINICAL			
2812P	*M. avium* complex	YES	NO
2812U	*M. avium* complex	YES	NO
Va14(O)	*M. avium* complex	ND[c]	NO
Va14(T)	*M. avium* complex	ND	YES
Va2	*M. avium* complex	ND	NO
2279	*M. avium*	YES	NO
ENVIRONMENTAL			
13S	*M. avium*	YES	NO
DSA16	*M. avium* complex	YES	YES
DSW2	*M. avium* complex	YES	NO
DSW69	*M. avium* complex	YES	NO
DSW73	*M. avium* complex	YES	YES
DSW74	*M. avium* complex	YES	NO
DSW127	*M. avium* complex	NO	NO
OKA23	*M. avium* complex	YES	YES
OKS41	*M. avium* complex	YES	YES
OKW75	*M. scrofulaceum*	YES	YES
OKA126	*M. scrofulaceum*	YES	YES
OKS147	*M. scrofulaceum*	YES	YES

[a]Responses refer to the observation of marked humic and fulvic acid stimulation (YES) or no observed humic and fulvic acid stimulation (NO) of the MAIS strains grown in Claytor Lake water or basal medium, with and without humic or fulvic acids.

[b]Key for the origin of the strains: JR = James River, Richmond (VA), DS = Dismal Swamp (VA), and OK = Okefenokee Swamp (GA).

[c]ND = Not Determined.

Table 3. Comparison of different humic (H) or fulvic (F) acids[a] influence on MAIS growth[b]

Medium[c]	Strain		
	OK-S41	OK-A23	DS-W69
Claytor Lake water			
CL	0.042	0.000	<0.000
CL+H(OK)	0.035	0.003	0.000
CL+H(DS)	0.087	0.009	0.001
CL+H(CL)	0.051	0.006	0.003
CL+F(OK)	0.061	0.021	0.012
CL+F(DS)	0.111	0.049	0.008
CL+F(CL)	0.093	0.016	0.008
Basal medium			
BS	0.007	0.074	0.005
BS+H(OK)	0.030	0.065	0.007
BS+H(DS)	0.017	0.060	0.009
BS+H(CL)	0.014	0.065	0.012
BS+F(OK)	ND[d]	0.074	0.009
BS+F(DS)	0.044	0.081	0.011
BS+F(CL)	0.079	0.084	0.018

[a]Humic and fulvic acids were added to achieve final concentrations of 0.1 mg/ml and 0.5 mg/ml, respectively.

[b]Growth represents changes in OD over the first 10 days.

[c]Key to the humic and fulvic acid source locations: OK = Okefenokee Swamp (GA), DS = Dismal Swamp (VA), and CL = Claytor Lake (VA).

[d]ND = not determined due to contamination.

Dose-response effect of humic and fulvic acids on growth. Table 4 shows the dose-response effect (triplicate measurements) of humic and fulvic acid on growth rates (0-4 days) and yield (defined here as change in turbidity over the first 10 days) of 4 selected MAIS strains in BS medium, with no additions, and with additions of 0.1, 1.0, and 10 times the concentrations of humic and fulvic acids found in natural swamp waters. Strains 13S (Fig. 3) and DSW69 were selected because they previously showed only slight stimulation of growth in BS medium containing humic and fulvic acids compared to BS medium alone, while strains OKS41 and OKW75 exhibited substantial increases in growth in BS medium containing the two organic acids compared to BS medium alone (Table 2). Generally strains grew faster and had higher yields as the concentrations of humic or fulvic acids increased (Table 4). Statistically significant differences (T test, $p \leq 0.05$) were observed for strains OKS41 and OKW75 in 0.05 and 0.5 mg humic acid/ml, and strains OKS41, OKW75, and DSW69 in all three concentrations of fulvic acid added to the medium compared to growth rates in BS medium alone. All four of the strains tested had faster growth rates and higher yields in media with the highest concentration of humic acid, while 2 of 4 grew fastest and had higher yields in media with the highest concentration of fulvic acid (Table 4). Even at the highest concentrations, though not necessarily yielding most rapid or extensive growth, neither humic nor fulvic acid inhibited growth.

Effect of pH on growth. As noted above (Table 2), strains grew significantly better (Duncan's Multiple Range test, $p \leq 0.05$) in Claytor Lake water containing humic or fulvic acids, than in lake water alone regardless of the pH (Table 5). For 11 of the 18 (61%) treatments (i.e., pH, strain, and water ± humic or fulvic acids combinations), strains grew better at pH 4.0 or 5.5 compared to pH 7.0 or 8.5, although growth was observed in each water type at every pH, suggesting a broad pH tolerance.

Table 4. Comparison of early log-phase growth rates and yields (average of triplicate measurements) at different humic and fulvic acid concentrations added to basal medium of four MAIS strains

	Strain							
	OKS41		OKW75		DSW69		13S	
Concentration (mg/ml)	Rate[a] (gen/day)	Yield[b] (OD)	Rate[a] (gen/day)	Yield[b] (OD)	Rate[a] (gen/day)	Yield[b] (OD)	Rate[a] (gen/day)	Yield[b] (OD)
Humic acid:								
0	0.53	0.030	0.17	0.003	0.56	0.158	0.50	0.107
0.005	0.48	0.078	0.09	0.004	0.58	0.155	0.31	0.124
0.05	0.77	0.108*	0.31	0.017*	0.42	0.143	0.26	0.120
0.5	1.3	0.130*	1.2	0.107*	1.1	0.205	0.58	0.148
Fulvic acid:								
0	0.53	0.030	0.11	0.003	0.56	0.158	0.50	0.107
0.04	0.83	0.085*	0.12	0.033*	0.83	0.317*	0.42	0.138
0.4	1.7	0.200*	0.95	0.076*	1.3	0.341*	1.0	0.184
4.0	1.3	0.150*	1.5	0.110*	1.4	0.408*	0.32	0.155

[a]Rate = early log-phase growth rate over the first 4 days, expressed as generations/day.

[b]Yield = change in OD over the first 10 days of growth. Values that were statistically different (Duncan's Multiple Range Test; $p \leq 0.05$) than those for basal media alone are marked with an *.

TABLE 5. Influence of pH on MAIS growth[a] in Claytor Lake (CL) water

		Treatment[b]											
		CL				CL+H				CL+F			
Strain	pH	8.5	7.0	5.5	4.0	8.5	7.0	5.5	4.0	8.5	7.0	5.5	4.0
2812P		0.061 (0.005)	0.062 (0.013)	0.066 (0.005)	0.053 (0.007)	0.154* (0.019)	0.138* (0.026)	0.151* (0.017)	0.170* (0.017)	0.123* (0.013)	0.126* (0.010)	0.143* (0.004)	0.119* (0.004)
2812U		0.004 (0.006)	0.006 (0.003)	0.008 (0.002)	0.003 (0.001)	0.025* (0.007)	0.023* (0.005)	0.020* (0.005)	0.017* (0.007)	0.034* (0.007)	0.036* (0.004)	0.047*† (0.014)	0.014* (0.006)
13S		0.086 (0.005)	0.130 (0.016)	0.123 (0.004)	0.133 (0.011)	0.143* (0.012)	0.142 (0.016)	0.164*† (0.004)	0.121 (0.016)	0.095 (0.001)	0.101 (0.003)	0.125† (0.011)	0.148† (0.037)
OKS41		0.143 (0.033)	0.103 (0.010)	0.117 (0.005)	0.093 (0.006)	0.199* (0.012)	0.225* (0.010)	0.240*† (0.012)	0.286*† (0.023)	0.189* (0.004)	0.206* (0.003)	0.243*† (0.008)	0.259*† (0.033)
OKW75		0.133 (0.021)	0.129 (0.011)	0.129 (0.024)	0.092 (0.013)	0.157*† (0.007)	0.157*† (0.009)	0.117 (0.009)	0.099 (0.013)	0.156*† (0.003)	0.156*† (0.001)	0.125 (0.004)	0.107 (0.005)
DSW69		0.219 (0.064)	0.193 (0.061)	0.193 (0.018)	0.317† (0.020)	0.316* (0.022)	0.361*† (0.028)	0.272* (0.016)	0.182 (0.039)	0.311* (0.006)	0.353* (0.015)	0.305* (0.006)	0.220 (0.004)
Optimum growth no.[c]		1 (17)	1 (17)	2 (33)	2 (33)	1 (17)	2 (33)	1 (17)	2 (33)	1 (17)	1 (17)	3 (50)	1 (17)

[a]Growth represents change (average of triplicate measurements) in OD (± SD) over the first 10 days. When growth of the strain in Claytor Lake water with humic or fulvic acid was significantly different (Duncan's Multiple Range test; $p \leq 0.05$) from growth in Claytor Lake water alone adjusted to the same pH, values show an *.

[b]Humic (H) and fulvic (F) acids were added to achieve final concentrations of 0.05 mg/ml and 0.4 mg/ml, respectively.

[c]pH at which strain growth was optimal for each of the three treatments. Percentages are given in the parentheses. When growth at the same pH for each treatment (i.e., CL, CL+H, and CL+F) was significantly different (Duncan's Multiple Range test; $p \leq 0.05$) from growth in the other pH's, values show an †.

Effect of temperature on growth. Growth, as both turbidity changes and viable colony counts, indicated that most strains (6 of 7; 86%) grew statistically (Duncan's Multiple Range test, $p \leq 0.05$) better (≈2 - 10X higher) at 37°C compared to growth at 16°C (Table 6). OKW75 grew fairly well in cooler temperature waters, as indicated by change in turbidity, however viable colony counts showed that warmer waters produced higher viable cell numbers over a two week period compared to viable counts for cooler temperature water. Overall, pH or humic and fulvic acid content of the natural waters had less of an influence on growth and survival of MAIS than temperature, because the cultures grew well in all of the waters at 37°C.

Effect of metals on growth. Data in Table 7 show that a majority [4/7; (57%)] MAIS strains tested grew better in BS medium containing both metals and humic acid, compared to growth in BS medium alone or BS medium containing either metals or humic acid alone. Two of 7 (29%) strains grew best in BS medium containing only humic acid, although this growth was not statistically (T test, $p \leq 0.05$) different than growth in BS medium with metals and humic acid. The remaining strain (OKS41) grew best in BS medium containing only metals. None (<14%) grew optimally in BS medium alone.

Oxygen uptake. Oxygen uptake was significantly (T test, $p \leq 0.05$) stimulated above the endogenous rate for only 1 of 5 strains when OAA, humic acid (0.5 mg/ml only), or fulvic acid (4.0 mg/ml only) were provided as substrates (Table 8). Interestingly, growth of that strain (13S) was not significantly stimulated by either humic or fulvic acid in BS medium (Fig. 3). Oxygen uptake was stimulated, though not significantly, for more strains when OAA was added with humic (3 of 5 strains) or fulvic (4 of 5 strains) acids. The stimulation of O_2 uptake also showed a dose response relationship under those circumstances (Table 8). All endogenous and substrate-stimulated rates were inhibited (>90%) by 5.9 mM KCN of the strains.

TABLE 6. Influence of temperature on MAIS growth in natural waters

	Natural Water															
	Okefenokee Swamp, GA				Cranberry Glades, WV				Dismal Swamp, VA				Claytor Lake, VA			
	OD[a]		CFU/ml[b]		OD[a]		CFU/ml[b]		OD[a]		CFU/ml[b]		OD[a]		CFU/ml[b]	
Strain	37°C	16°C	37°C	16°C	37°C	16°C	37°C	16°C	37°C	16°C	37°C	16°C	37°C	16°C	37°C	16°C
2812P	0.044* (0.004)	0.019 (0.001)	14.2	4.5	0.025* (0.004)	0.013 (0.006)	12.9	3.4	0.185* (0.005)	0.063 (0.008)	50.6	8.1	0.236* (0.048)	0.062 (0.004)	64.7	9.2
2812U	0.041* (0.003)	0.009 (0.004)	23.8	5.0	0.025* (0.002)	0.013 (0.004)	15.9	5.0	0.038* (0.005)	0.018 (0.009)	25.0	7.4	0.053* (0.002)	0.029 (0.004)	4.7	0.3
13S	0.044* (0.017)	0.014 (0.004)	87.9	6.4	0.021* (0.004)	0.008 (0.001)	15.5	3.6	0.030* (0.006)	0.017 (0.002)	19.7	16.3	0.126* (0.013)	0.076 (0.008)	7.6	4.5
OK-S41	0.065* (0.004)	0.039 (0.007)	21.6	6.8	0.123* (0.012)	0.031 (0.008)	43.2	8.2	0.164* (0.020)	0.025 (0.006)	46.2	23.3	0.159* (0.016)	0.053 (0.004)	18.6	6.2
OK-W75	0.056 (0.009)	0.058 (0.006)	70.3	26.4	0.063* (0.019)	0.039 (0.001)	92.3	31.9	0.036 (0.006)	0.049 (0.008)	73.5	48.1	0.101 (0.003)	0.168* (0.014)	39.2	30.0
DS-W2	0.053 (0.008)	0.040 (0.002)	10.0	5.0	0.037* (0.006)	0.015 (0.005)	6.4	3.0	0.047* (0.006)	0.018 (0.007)	7.0	4.7	0.118* (0.013)	0.081 (0.015)	32.3	17.0
DS-W69	0.083* (0.005)	0.008 (0.001)	41.2	18.8	0.070* (0.008)	0.000 (0.004)	12.9	3.6	0.123* (0.002)	0.075 (0.015)	35.8	23.5	0.187* (0.014)	0.125 (0.006)	71.8	56.3

[a]Growth represents change (average of triplicate measurements) in OD (± SD) over the first 14 days. When growth at 37°C was significantly different (Student's T test; $p \leq 0.05$) from growth at 16°C, values show an *.

[b]Growth is also expressed as the number of viable cells/ml after 14 days divided by initial number of viable cells/ml.

Table 7. Comparison of MAIS growth[a] in basal (BS) medium ± metals[b] (M) and/or humic acid[c] (HA)

Strain	Medium			
	BS	BS+M	BS+M+HA	BS+HA
2812P	0.19 ± 0.00	0.17 ± 0.00	**0.23** ± 0.00	0.22 ± 0.02
2812U	0.12 ± 0.01	0.11 ± 0.01	**0.15** ± 0.02	0.14 ± 0.01
Va14(O)	0.33 ± 0.04	0.31 ± 0.04	0.36 ± 0.07	**0.44** ± 0.02
Va14(T)	0.01 ± 0.01	0.04 ± 0.01	**0.09** ± 0.03	0.03 ± 0.01
Va2	0.06 ± 0.03	0.05 ± 0.01	**0.09** ± 0.02	0.06 ± 0.00
13S	0.18 ± 0.01	0.24 ± 0.02	0.27 ± 0.04	**0.29** ± 0.06
OKS41	0.01 ± 0.01	**0.79** ± 0.16	0.51 ± 0.04	0.21 ± 0.13

[a]Growth represents the average change (duplicate samples) in OD ± SD over the first 10 days for the following strains: 2812P, 2812U, Va14(O), Va14(T), and Va2; the first 7 days for strains: 13S and OKS41. The medium that yielded the highest growth is indicated in bold.

[b]Refer to Materials and Methods for the type and final concentration of each metal that was added.

[c]Humic acid (Aldrich Chemical Company) was added to achieve a final concentration of 0.1 mg/ml.

Table 8. Oxygen uptake[a] of log-phase cell suspensions of five MAIS strains

Addition	Strain OKW75	OKS41	13S	2812P	DSW69
Endogenous	0.46 ± 0.08	0.42 ± 0.12	0.32 ± 0.09	0.73 ± 0.10	0.14 ± 0.03
OAA	0.57 ± 0.17	NS[b]	0.55 ± 0.18*	NS	NS
Humic acid:					
0.005 mg/ml	NS	NS	NS	NS	NS
0.05 mg/ml	NS	NS	NS	NS	NS
0.5 mg/ml	NS	NS	0.58 ± 0.00*	NS	NS
Fulvic acid:					
0.04 mg/ml	NS	NS	NS	NS	NS
0.4 mg/ml	NS	NS	0.38 ± 0.08	NS	NS
4.0 mg/ml	0.49 ± 0.04	NS	0.57 ± 0.04*	NS	NS
OAA+ Humic acid:					
0.05 mg/ml	0.48 ± 0.19	NS	0.47 ± 0.09	NS	NS
0.5 mg/ml	0.50 ± 0.06	NS	0.57 ± 0.08*	0.80 ± 0.07	NS
OAA+ Fulvic acid:					
0.4 mg/ml	NS	NS	0.35 ± 0.15	NS	NS
4.0 mg/ml	0.57 ± 0.07	0.48 ±0.01	0.66 ± 0.01*	0.81 ± 0.10	NS

[a]μL oxygen uptake/mg dry cell weight · min^{-1} ± SD (average of duplicate measurements). Numbers shown are values which exceeded the endogenous rate. Those values that were significantly different (T test; $p \leq 0.05$) than the endogenous rate are marked with an *.

[b]NS = O_2 uptake not stimulated above endogenous rate.

Tracings of amperometric measurements of respiration are also given for the 5 MAIS stains (Figures 4 - 8) illustrating the results summarized above. Although 3 concentrations of humic and fulvic acid were tested, only the 10X concentrations are shown in order to avoid making the figures too detailed, thereby adding confusion. The 10X concentration of each acid always exhibited the largest effect on O_2 uptake compared to the lower concentrations that were added, so respiration trends shown in the figures represent the best O_2 uptake following humic and fulvic acid additions. Oxygen uptake (endogenous rate and subsequent rates following addition of substrates) was inhibited >90% by KCN (5.9 mM) in all strains tested, though only KCN inhibition of endogenous rates are illustrated (Figures 4 - 8), once again avoiding excessive detail.

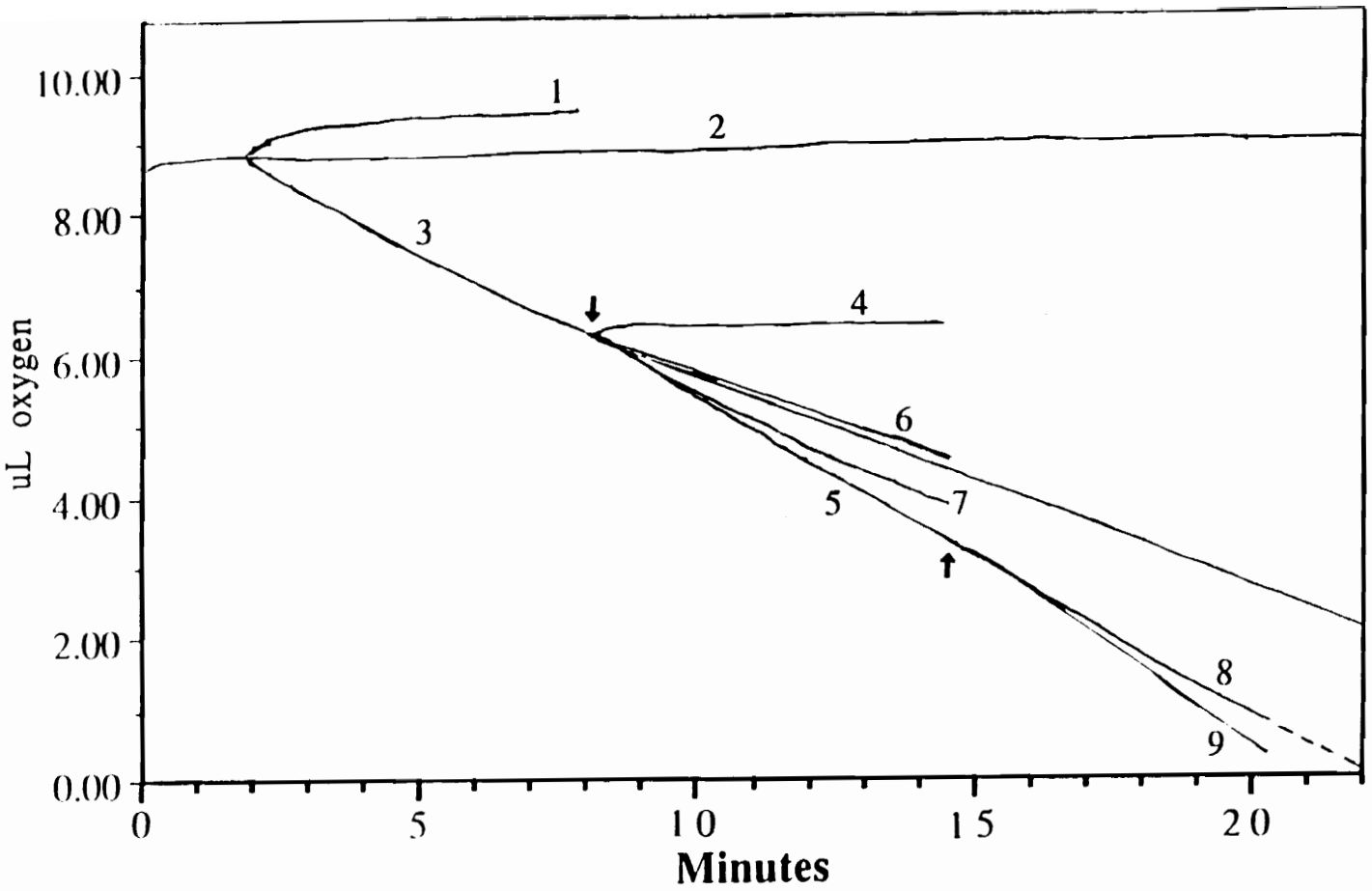

Figure 4. Tracings of amperometric measurements of respiration following substrate addition to 0.85 mg dry cell weight of MAIS strain OKW75. Key to the tracings: (1) boiled cell suspension, (2) sterile KH_2PO_4 buffer (pH 5.5), (3) endogenous rate, (4) 5.9 mM KCN, (5) 0.06% (v/v) oleic acid and 5.0% (w/v) albumin (OAA), (6) 0.5 mg humic acid/ml, (7) 4.0 mg fulvic acid/ml, (8) combination of 0.5 mg humic acid/ml and OAA, and (9) combination of 4.0 mg fulvic acid/ml and OAA. Arrows indicate times when the different substrates were added to the cell suspension.

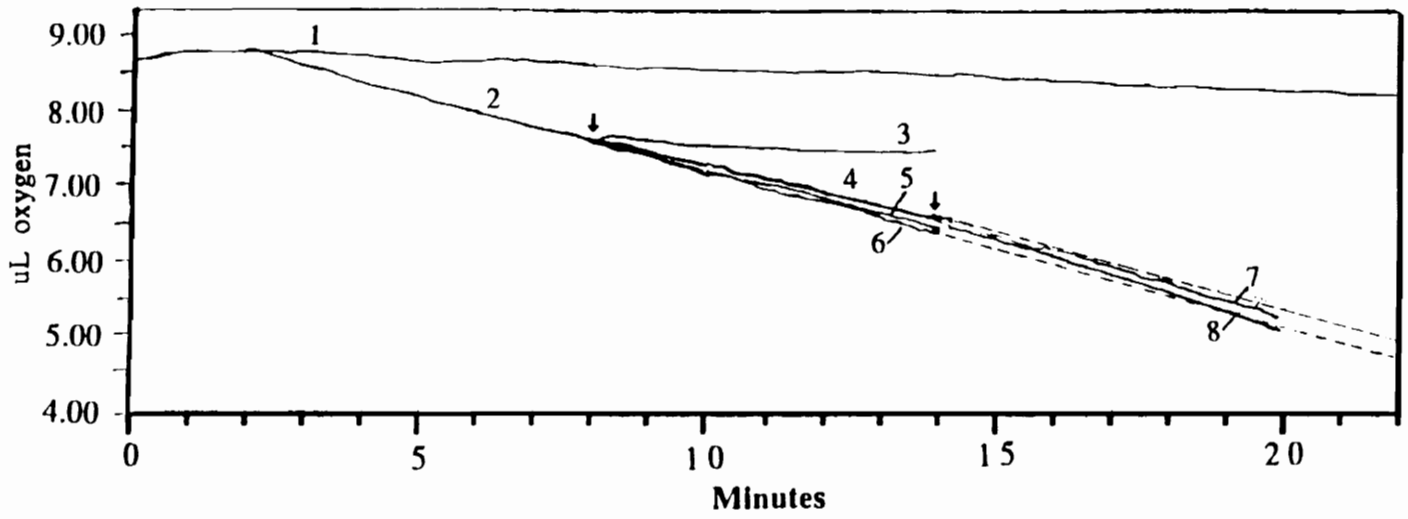

Figure 5. Tracings of amperometric measurements of respiration following substrate addition to 1.60 mg dry cell weight of MAIS strain OKS41. Key to the tracings: (1) sterile KH_2PO_4 buffer (pH 5.5), (2) endogenous rate, (3) 5.9 mM KCN, (4) 0.06% (v/v) oleic acid and 5.0% (w/v) albumin (OAA), (5) 0.5 mg humic acid/ml, (6) 4.0 mg fulvic acid/ml, (7) combination of 0.5 mg humic acid/ml and OAA, and (8) combination of 4.0 mg fulvic acid/ml and OAA. Arrows indicate times when the different substrates were added to the cell suspension.

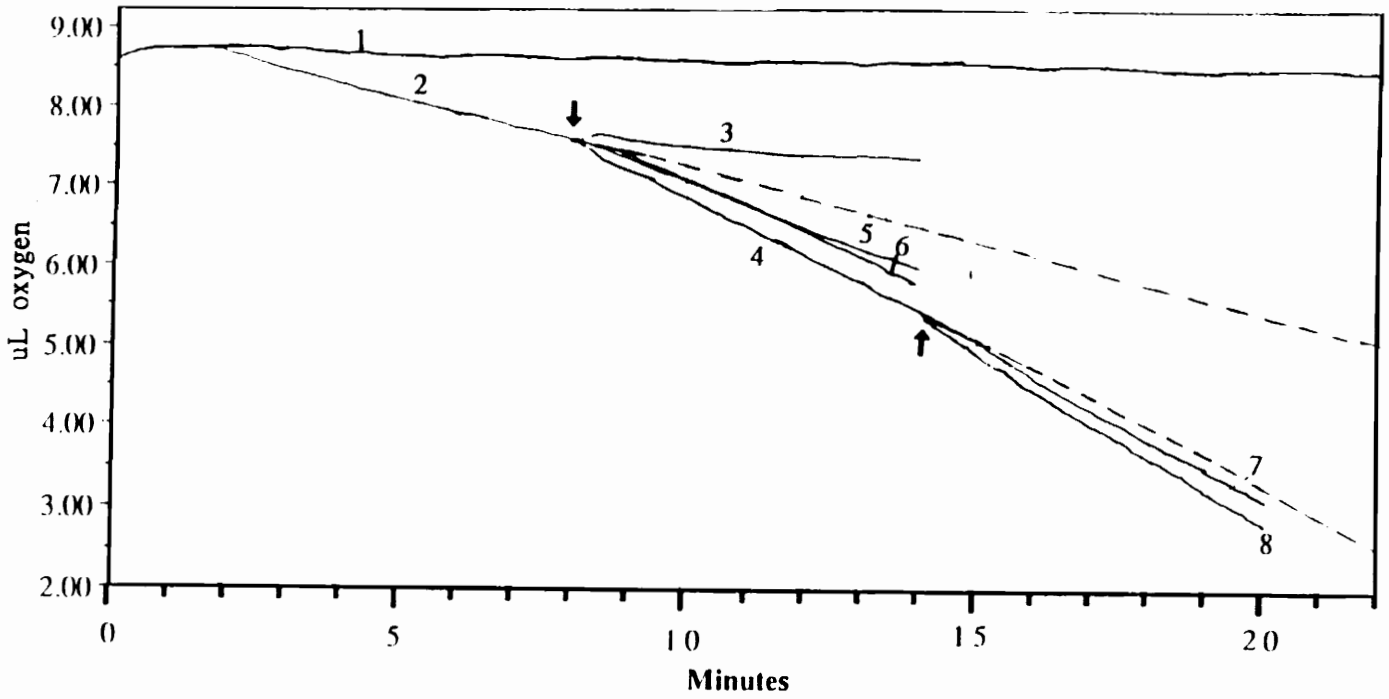

Figure 6. Tracings of amperometric measurements of respiration following substrate addition to 0.65 mg dry cell weight of MAIS strain 13S. Key to the tracings: (1) sterile KH_2PO_4 buffer (pH 5.5), (2) endogenous rate, (3) 5.9 mM KCN, (4) 0.06% (v/v) oleic acid and 5.0% (w/v) albumin (OAA), (5) 0.5 mg humic acid/ml, (6) 4.0 mg fulvic acid/ml, (7) combination of 0.5 mg humic acid/ml and OAA, and (8) combination of 4.0 mg fulvic acid/ml and OAA. Arrows indicate times when the different substrates were added to the cell suspension.

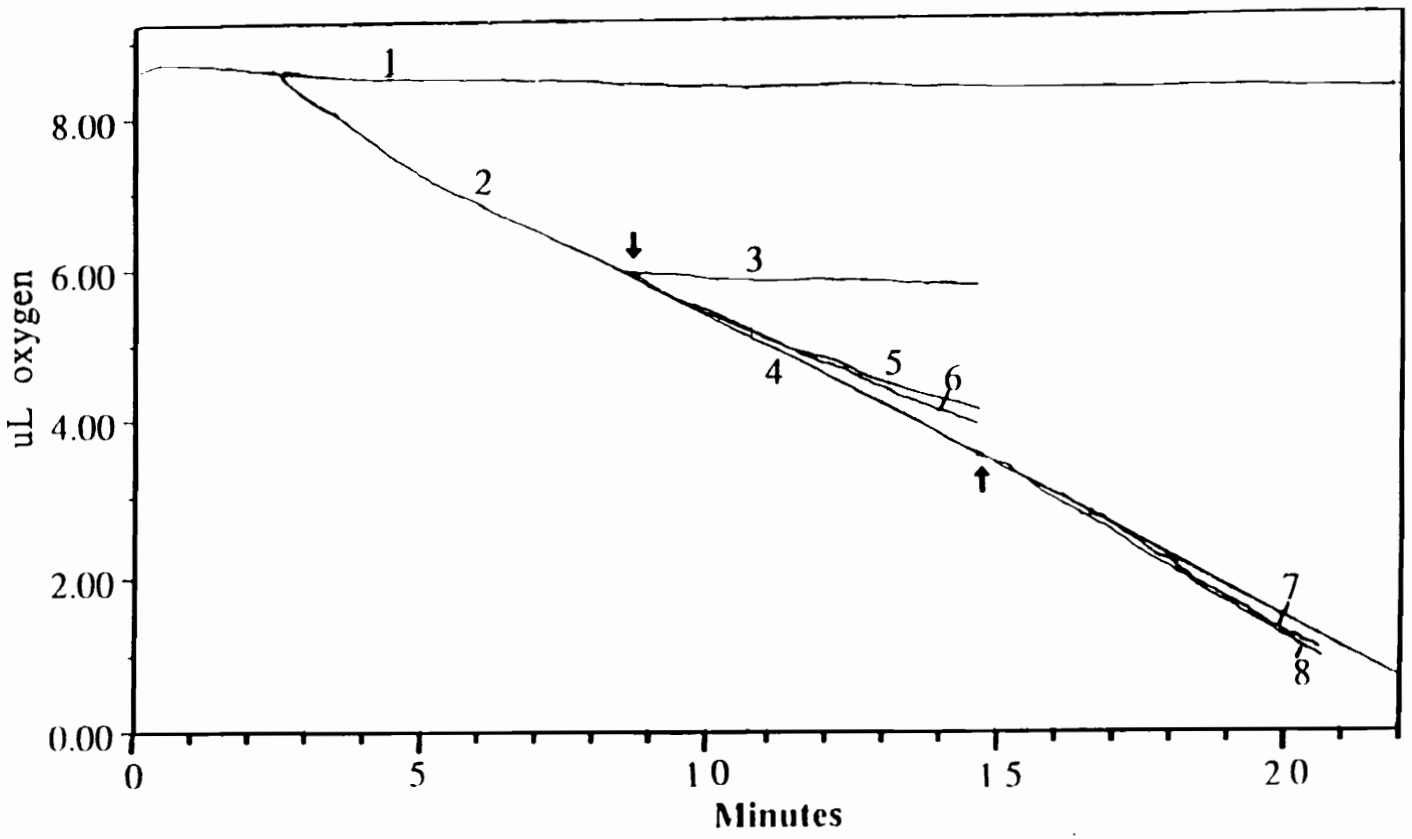

Figure 7. Tracings of amperometric measurements of respiration following substrate addition to 0.40 mg dry cell weight of MAIS strain 2812P. Key to the tracings: (1) sterile KH_2PO_4 buffer (pH 5.5), (2) endogenous rate, (3) 5.9 mM KCN, (4) 0.06% (v/v) oleic acid and 5.0% (w/v) albumin (OAA), (5) 0.5 mg humic acid/ml, (6) 4.0 mg fulvic acid/ml, (7) combination of 0.5 mg humic acid/ml and OAA, and (8) combination of 4.0 mg fulvic acid/ml and OAA. Arrows indicate times when the different substrates were added to the cell suspension.

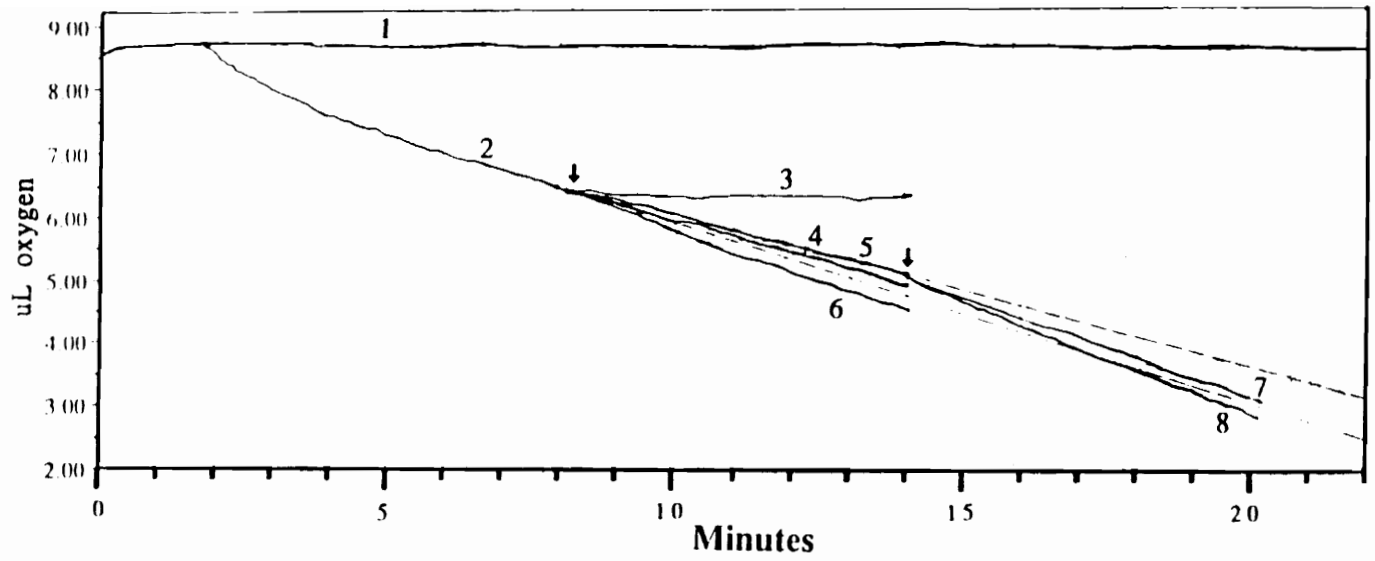

Figure 8. Tracings of amperometric measurements of respiration following substrate addition to 1.95 mg dry cell weight of MAIS strain DSW69. Key to the tracings: (1) sterile KH_2PO_4 buffer (pH 5.5), (2) endogenous rate, (3) 5.9 mM KCN, (4) 0.06% (v/v) oleic acid and 5.0% (w/v) albumin (OAA), (5) 0.5 mg humic acid/ml, (6) 4.0 mg fulvic acid/ml, (7) combination of 0.5 mg humic acid/ml and OAA, and (8) combination of 4.0 mg fulvic acid/ml and OAA. Arrows indicate times when the different substrates were added to the cell suspension.

DISCUSSION

Our investigations have aimed at understanding more completely the basis for occurrence of higher numbers of MAIS in the warmer, low oxygen, humic and fulvic acid-rich, higher zinc, brown swamp waters and associated soils of the southeastern United States. The data show that MAIS growth often can be stimulated by additions of humic or fulvic acids to either natural water or sometimes basal salts medium (Fig. 1 and 2; Tables 2 - 5 and 7). Failure of humic or fulvic acids to stimulate growth for 10 of 18 strains in BS medium was due to the fact that those strains grew well in the organically enriched BS medium. This finding of growth stimultion agrees with earlier work by Kazda (20), demonstrating *M. intracellulare* growth stimulation inside dead storage cells of *Sphagnum*, a moss genus long recognized as a primary constituent of acid bogs and important in peat and humic acid production (27). Results also agree with the findings of Brooks, et al. (2), who demonstrated higher MAIS recoveries from acidic soils, high in organic matter. The fact that samples rich in humic and fulvic acids yielded higher MAIS numbers (Chapter 1), fits well our findings that these substancess, which can compose a significant portion of the organic fraction of some waters and soils (10, 29), also stimulate growth.

Little difference occurred in MAIS growth stimulation by humic and fulvic acids isolated and purified from soils from the different sample sites (Table 3). A dose-response relationship was apparent between the concentration of humic and fulvic acid and growth rates and yields of 4 MAIS isolates (Table 4). Growth stimulation by humic and fulvic acids was not due to their affect on lowering pH (Table 5). Also, most MAIS strains grew better in natural waters held at 37°C compared to growth in waters at 16°C (Table 6), agreeing with earlier work showing that

MAIS grew well at higher temperatures up to 43°C (11) and that MAIS organisms grew slowly at temperatures below 15.5°C (12).

Results from the respiration studies suggest that humic and fulvic acids do not act as energy substrates for MAIS (Table 3). This is expected, since humic substances have been described as possessing a complex, high molecular weight refractory core (3) surrounded by numerous chemical substances, ranging from inorganic ions to amino acids and pesticides (10, 28). Therefore, oxygen uptake stimulation might have been due to the utilization of any labile substates possibly still attached to the humic or fulvic acid core. In addition, humic substances have also been shown to be cometabolized during bacterial utilization of more labile substates (5, 6), which would explain our observed increase of oxygen uptake when both OAA and humic or fulvic acids were added. This conclusion also may be drawn for the relatively small utilization of humic complexes prepared from Okefenokee waters by the entire microbial community (21), but not axenic mycobacterial strains as tested.

Although humic and fulvic acids are not utilized as substrates, these compounds probably enhance growth by other means. Instead of rapid respiration, MAIS organisms also might use these compounds as carbon skeletons, which would not necessarily stimulate oxygen uptake, but over several days might stimulate MAIS growth. Such stimulation could be due to the surfactant activity of humic and fulvic acids or their ability to chelate metals (26). High MAIS numbers in the swamps correlated with high zinc (but not other metals) (Chapter 1), and it has been observed that the metal mixture containing zinc in BS medium significantly stimulated the growth of strains 13S and OKS41 (Table 7). The report of a high zinc requirement for the rapidly-growing *M. smegmatis* (33), coupled with the fact that humic and fulvic acids chelate metals (26), suggest that the growth stimulation reported here could be due to metals associated with humic and fulvic acids. The function of zinc in many metalloenzymes found in mycobacteria has been firmly established (4).

The physiological similarities between clinical and environmental MAIS suggest a close relationship. We have not proved that environmental MAIS are pathogenic, but we have demonstrated that the acid, brown swamp waters and soils widely distributed in the Southeast provide humic and fulvic acid rich environments ideal for growth and survival of MAIS organisms whose physiology resembles closely those of pathogenic isolates. Therefore, we suggest that these swamp waters, soils, and aerosols provide compatible habitats for the year-round survival and growth of MAIS. Because Kazda (18, 19) also demonstrated recovery of MAIS from moorland waters in Europe, implicating acid, brown swamps as primary sources, the epidemiologic model for MAIS infection may well be similar the world over.

LITERATURE CITED

1. **Blaser, M. J., and D. L. Cohn.** 1986. Opportunistic infections in patients with AIDS: Clues to the epidemiology of AIDS and the relative virulence of pathogens. Rev. Infect. Dis. **8**:21-30.

2. **Brooks, R. W., B. C. Parker, H. Gruft, and J. O. Falkinham, III.** 1984. Epidemiology of infection of nontuberculous mycobacteria. V. Numbers in eastern United States soils and correlation with soil characteristics. Am. Rev. Respir. Dis. **130**:630-633.

3. **Campbell, C. A., E. A. Paul, D. A. Rennie, and K. J. McCallum.** 1967. Applicability of the carbon-dating method of analysis to soil humus studies. Soil Sci. **104**:217-224.

4. **Committee on Metabolic and Biologic Effects of Environmental Pollutants, Subcommittee on Zinc.** 1979. Zinc, p. 471. University Park Press, Baltimore.

5. **De Haan, H.** 1974. Effect of fulvic acid fraction on the growth of *Psuedomonas* from Tjeukemeer (the Netherlands). Freshwater Biol. **4**:301-310.

6. **De Haan, H.** 1977. Effect of benzoate on microbial decompostion of fulvic acids in Tjeukemeer (the Netherlands). Limnol. Oceanogr. **22**:38-44.

7. **Edwards, L. B., F. A. Aquaviva, V. T. Livesay, R. W. Cross, and C. E. Palmer.** 1969. An atlas of sensitivity to tuberculin, PPD-B, and histoplasmin in the United States. Am. Rev. Respir. Dis. **99**:1-131.

8. **Edwards, P. Q.** 1970. Interpretation and significance of the tuberculin test in typical and atypical mycobacterial infections, p. 43-55. *In* J. E. Johnson, III (ed.), Symposium on rationale therapy and control of tuberculosis. University of Florida Press, Gainesville.

9. **Falkinham, J. O., III, B. C. Parker, and H. Gruft.** 1980. Epidemiology of infection by nontuberculous mycobacteria. I. Geographic distribution in the eastern United States. Am. Rev. Respir. Dis. **121**:931-937.

10. **Flaig, W., H. Beatelspacher, and E. Rietz.** 1975. Chemical composition and physical properties of humic substances, p. 1-211. *In* J. E. Gieseking (ed.), Soil components, Vol 1, Springer-Verlag, New York, Inc., New York.

11. **Fry, K. L., P. S. Meissner, and J. O. Falkinham, III.** 1986. Epidemiology of infection by nontuberculous mycobacteria. VI. Identification and use of epidemiologic markers for studies of *Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum*. Am. Rev. Respir. Dis. **34**:39-43.

12. **George, K. L., B. C. Parker, H. Gruft, and J. O. Falkinham, III.** 1980. Epidemiology of infection of nontuberculous mycobacteria. II. Growth and survival in natural waters. Am. Rev. Respir. Dis. **122**:89-94.

13. **George, K. L., and J. O. Falkinham, III.** 1986. Selective medium for the isolation and enumeration of *Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum*. Canad. J. Microbiol. **32**:10-14.

14. **Good, R. C.** 1980. Isolation of nontuberculous mycobacteria in the United States, 1979. J. Infect. Dis. **142**:779-783.

15. **Good, R. C., and D. E. Snider, Jr.** 1982. Isolation of nontuberculous mycobacteria in the United States, 1980. J. Infect. Dis. **146**:829-33.

16. **Gross, W. B., J. O. Falkinham, III, and J. B. Payeur.** 1989. Effect of environmental-genetic interactions on *Mycobacterium avium* challenge infection. Avian Dis. **33**:411-415.

17. **Horsburgh, C. R., Jr., and R. M. Selik.** 1989. The epidemiology of disseminated nontuberculous mycobacterial infection in the acquired immunodeficiency syndrome (AIDS). Am. Rev. Respir. Dis. **139**:4-7.

18. **Kazda, J.** 1973a. Die Bedeutung von Wasser fur die Verbreitung von potentiell pathogenen Mykobakterien. I. Moglichkeiten fur eine Vermehrung von Mykobakterien. Zbl. Bakt. Hyg., I Abt. Orig. B **158**:161-169.

19. **Kazda, J.** 1973b. Die Bedeutung von Wasser fur die Verbreitung von potentiell pathogenen Mykobakterien. II. Vermehrung der Mykobakterien in Gewassermodellen. Zbl. Bakt. Hyg., I Abt. Orig. B **158**:170-176.

20. **Kazda, J.** 1978. Vermehrung von Mykobakterien in der grauen Schicht der Sphagnum-Vegetation. Zbl. Bakt. Hyg., I Abt. Orig. B **166**:463-469.

21. **Meissner, P. S., and J. O. Falkinham, III.** 1986. Plasmid DNA profiles as epidemiologic markers for clinical and environmental isolates of *Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum*. J. Infect. Dis. **152**:325-331.

22. **Moran, M. A., and Hodson, R. E.** 1990. Bacterial production on humic and nonhumic components of dissolved organic carbon. Limnol. Oceanogr. **35**:1744-1756.

23. **Pethel, M. L., and J. O. Falkinham, III.** 1989. Plasmid-influenced changes in *Mycobacterium avium* catalase activity. Infect. Immun. **57**:1714-1718.

24. **Ratledge, C., and M. J. Hall.** 1971. Influence of metal ions on the formation of mycobactin and salicylic acid in *Mycobacterium smegmatis* grown in static culture. J. Bacteriol. **108**:314-319.

25. **Robinson, J., and J. M. Cooper.** 1970. Method of determining oxygen concentrations in biological media, suitable for calibration of the oxygen electrode. Anal. Biochem. **33**:390-399.

26. **Saar, R. A., and J. H. Weber.** 1982. Fulvic acid: modifier of metal-ion chemistry. Environ. Sci. Technol. **16**:510-517.

27. **Scagel, R. F., R. J. Bandoni, J. R. Maze, G. E. Rouse, W. B. Schofield, and J. R. Stein.** 1983. Nonvascular plants, an evolutionary survey, p. 477. Wadsworth Publishing Co., Belmont, CA.

28. **Schnitzer, M., and S. U. Khan.** 1972. Humic substances in the environment, p. 327. Marcel Dekker, Inc., New York.

29. **Schnitzer, M.** 1982. Organic matter characterization, p. 581-594. *In* A. L. Page, R. H. Miller, and D. R. Keeney (ed), Methods of soil analysis, part 2, 2nd ed. American Society of Agronomy, Inc, Madison, WI.

30. **SAS Institute, Inc.** SAS user's guide: statistics, version 5.0, p. 956. SAS Institute, Inc., Cary, NC.

31. **Stormer, R. S., and J. O. Falkinham, III.** 1989. Differences in antimicrobial susceptibility of pigmented and unpigmented colonial variants of *Mycobacterium avium*. J. Clin. Microbiol. **27**:2459-2465.

32. **Wendt, S. L., K. L. George, B. C. Parker, H. Gruft, and J.O. Falkinham, III.** 1980. Epidemiology of infection by nontuberculous mycobacteria. III. Isolation of potentially pathogenic mycobacteria from aerosols. Am. Rev. Respir. Dis. **122**:259-263.

33. **Winder, F. G., and C. O'Hara.** 1962. Effects of iron deficiency and of zinc deficiency on the composition of *Mycobacterium smegmatis*. Biochem. J. **82**:98-108.

34. **Wolinsky, E.** 1979. Nontuberculous mycobacteria and associated diseases. Am. Rev. Respir. Dis. **119**:107-159.

SUMMARY

The data on recovery of MAIS from the four sampling locations show that significantly higher numbers are recovered from water, soil, and aerosol samples of the Okefenokee and Dismal Swamps compared to Claytor Lake and Cranberry Glades. These finding of higher MAIS recovery in the two coastal swamps agrees well with previous studies regarding the geographic distribution of MAIS in water and soil samples, and with the geographic incidence of skin sensitivity to PPD-B and PPD-G and clinical isolates from infected patients.

A highly significant correlation for the data was apparent between high numbers of MAIS and high zinc and fulvic acid levels of water samples. In soils, significant correlations between high MAIS numbers and humic and fulvic acids and organic matter contents was also observed. Most MAIS were shown to be microaerobic, thereby agreeing with the data suggesting higher recoveries of MAIS in less oxygenated waters.

The data showed that most environmental and clinical MAIS strains grew better or were stimulated by warm temperature and by humic and fulvic acids, while low pH is less of an influencing variable. The fact that samples rich in humic and fulvic acids yielded higher MAIS recoveries, fits well our findings that these substances also stimulate growth. Little difference occurred in MAIS growth stimulation by humic and fulvic acids isolated and purified from soils from the different sample sites. A dose-response relationship was apparent between the concentration of humic and fulvic acid and growth rates and yields of 4 MAIS isolates. Growth stimulation by humic and fulvic acids was not due to their affect on lowering pH.

Results from the respiration studies suggest that humic and fulvic acids do not act as energy substrates for MAIS, but probably enhance growth by other means. Growth stimulation

could be due to the surfactant activity of humic and fulvic acids or their ability to chelate metals. Humic and fulvic acids might also be used as carbon skeletons.

MAIS organisms probably are much more abundant in the acid, brown swamp waters and associated soils of the southeastern coastal plain than those waters and soils found elsewhere. On a weight basis, somewhat higher MAIS numbers were recovered from water compared to soil. However, strong binding of MAIS to soil particles suggest that swamp soils may harbor higher numbers and be as important or more so than swamp water as a source of infection. On a volume basis, the numbers in aerosols are still smaller, but the volume of air regularly inhaled by humans makes this third potential source of infection also important, especially for pulmonary diseases. Probably the abundance of MAIS organisms in these swamps results from several interacting variables. The combination of higher temperatures, low oxygenated waters, and lower pH soils and waters higher in zinc and humic and fulvic acids, most likely favor growth and survival of MAIS organisms in the environment. Thus, these studies have demonstrated that the acid, brown swamp waters and soils widely distributed in the southeastern United States provide environments ideal for the year-round growth and survival of MAIS organisms whose physiology resembles closely those of pathogenic isolates, and that apparently these coastal swamps aptly fit the epidemiologic model.

The vita has been removed from the scanned document