AN ABSTRACT OF THE THESIS OF

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Rivers, lakes, ponds, marshes, wells, taps, drinking fountains, distilled and bottled waters in eastern Kansas were examined for the presence of Mycobacteria. Several experiments were conducted. Cetylpyridinium chloride (CPC) and formaldehyde (HCHO) were used as decontaminating agents. Samples were passed through millipore filters, the filters were then rinsed and placed on Middlebrook 7H10 or Lowenstein-Jensen agar. Plates were incubated at 25 C, 37 C or at ambient temperature either in air or in 5 % CO_2 depending on the experiment. When CPC was used as the decontaminating agent only M. chelonei and M. fortuitum were isolated. M. chelonei was recovered from 1 of 3 distilled water samples, 2 of 22 tap samples, 1 of 6 pond samples, 1 of 10 well samples, 1 of 2 river samples, and 2 of 3 bottled water samples. M. fortuitum was recovered from 2 of 10 well samples and 1 of 10 lake samples. No mycobacteria was recovered from any of 5 marsh samples. When HCHO was used as the decontaminating agent M. gordonae, M. fortuitum, M. chelonei, M. szulgai, M. avium complex, M. scrofulaceum, and an unidentified Group III and 3 unidentified Group IV organisms were isolated. M. gordonae was isolated from 4 of 24 tap and 5 of 23 drinking fountain samples. M. fortuitum was isolated from 3 of 24 tap, 1 of 10 well, 1 of 6 bottled and 1 of 23 drinking fountain

samples. M. chelonei was isolated from 2 of 24 tap, 2 of 6 bottled and 1 of 2 distilled water samples. M. szulgai was isolated from 3 of 24 tap and 2 of 23 drinking fountain samples. M. avium complex was isolated from 1 of 24 tap and 1 of 23 drinking fountain samples. M. scrofulaceum was isolated from 1 of 24 tap samples. Three unidentified Group IV organisms were isolated from 3 of 24 tap samples. One unidentified Group III organism was isolated from 1 of 24 tap samples. One home aquarium and one outdoor decorative fountain failed to yield any acid-fast organisms. Samples containing excessive foreign material were pelleted resuspended in Middlebrook 7H9. The use of acetic acid as a decontaminating agent for these samples was investigated. A review of the literature is also given.

MYCOBACTERIA OTHER THAN TUBERCULOSIS IN EASTERN KANSAS WATER SUPPLIES

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INTRODUCTION

A. THE MYCOBACTERIA

Mycobacteria (Manual of Clinical Microbiology 1985) are aerobic, nonmotile, asporogenic, acid-fast and alcohol-fast bacilli. For growth, rapid growing Mycobacteria require 2 to 3 days at temperatures ranging from 20 C to 40 C. Most other species of Mycobacteria require 2 to 6 weeks on complex media at very specific temperatures for growth to occur.

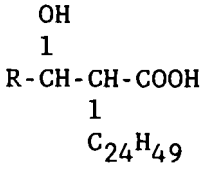
Mycobacteria have an unusually high lipid content. Smith (1982) states that more than 25 % of the mycobacterial cell wall is made up of lipids, while only 3 % of the cell wall of gram-negative bacteria and 0.5 % of the cell wall of gram-positive bacteria consists of lipids. Boyd and Marr (1980) claim that up to 60 % of the total weight of mycobacteria may be lipids, while the cell walls of gram-negative bacteria contain approximately 20 % lipid and the cell walls of gram-positive organisms contain 1 % to 4 % lipid. This high lipid content (Smith 1982) is responsible for the acid-fastness and alcohol-fastness of Mycobacteria, as well as for their resistance to drying, alkali and certain germicides. It also makes the Gram stain useless for characterizing the Mycobacteria.

Only Mycobacteria and a few species of Nocardia (Smith 1982) are acid-fast. This makes the acid-fast stain an extremely useful diagnostic tool.

Some of the unusual lipids found in Mycobacteria (Smith 1982) include mycolic acid, mycosides, waxes D, trehalose-6,6'-dimycolate and

sulfolipid. Mycolic acid is found in many of the complex lipids in Mycobacteria. Mycolic acid has the general formula of $C_{88}H_{176}O_4$ +or- $5CH_2$. The general structure of mycolic acid is shown in Figure 1.

Figure 1. Structure of mycolic acid in Mycobacterium tuberculosis. The R group contains about 60 carbon atoms and an undetermined oxygen function. It occurs in three chains. (Smith, 1982)


OH
R-CH-CH-COOH
$C_{24}H_{49}$

Mycosides (Smith 1982) are glycolipids or glycolipid peptides which contain mycolic acid. According to Randall and Smith (1964) a chemically distinct mycoside is associated with each species of Mycobacteria. However, the procedure used to identify the mycoside present in an isolate is too complex for routine use as a diagnostic tool. Some of the mycosides occur on the outer surface of the cell and act as mycobacteriophage receptors.

The formation of long, serpentine cords (Boyd and Marr 1980) is associated with virulent strains of mycobacteria. Cording is due to a mycoside known as 6,6'-dimycolyltrehalose, but commonly referred to as cording factor. The structure of cording factor is detailed in Boyd and Marr (1980). When this surface lipid is extracted the cells lose their virulence and tend to disperse in aqueous solution. Purified cording factor (Smith 1982) has been shown to have several properties such as (1) lethality in mice, (2) inhibition of migration of polymorphonuclear leukocytes, (3) induction of protection against virulent infection, and (4) induction of granuloma formation. Mycobacteria obtained from animals (Boyd and Marr 1980) have a higher cording factor content than organisms grown on artificial media for a long period of time. Other lipids that are toxic to macrophages are also present in higher concentration in virulent organisms. The role of cording factor and other toxic lipids in the pathogenesis of mycobacteria is currently unknown.

Virulent strains of M. tuberculosis (Burnett and Schuster 1973) bind the dye neutral red, while avirulent strains do not. This is due (Smith 1982) to a sulfonated glycolipid characterized as a tetraester

of trehalose. Its role in pathogenesis is also unknown.

Waxes D (Smith 1982) contain mycolic acid, peptides and polysaccharides. Those extracted from M. tuberculosis (1) enhance antibody production against a protein antigen incorporated in a wax D oil emulsion and (2) induce a cell-mediated immune response against the protein. Due to these adjuvant properties, wax D may contribute to the pathogenicity of M. tuberculosis. Also because of its enhancing properties (Boyd and Marr 1980) wax D is often used as an emulsion known as Freund's adjuvant. It produces a delayed-hypersensitivity reaction to almost any antigen incorporated in it. Since tuberculoprotein alone is poorly immunogenic, wax D is probably responsible for inducing delayed-hypersensitivity to tuberculoprotein. Despite its name, wax D is not a true wax.

The type species of Mycobacterium is M. tuberculosis which, as the name implies, causes tuberculosis in man. M. bovis causes tuberculosis in cattle and other animals. It occurs (Manual of Clinical Microbiology 1985) sporadically in cattle, but today rarely causes human disease in the United States. It is, however, isolated occasionally from immigrants.

Bacillus Calmette-Guerin (BCG) was derived from M. bovis and may be isolated from some patients receiving cancer immunotherapy. In some countries immunization with BCG is used to reduce the number of tuberculosis cases. Hart et al. (1967) showed that vaccination with BCG lowers the incidence of tuberculosis by 70 % to 80 % in the immunized population. Benenson (1985) claims that more than 90 % of uninfected individuals who are immunized are protected. He points out that

protection has varied greatly among field trials and that perhaps these differences are related to some special characteristic of the population or to the quality of the vaccine. Some controlled trials have shown protection to last up to 20 years in areas of high incidence, while other trials have shown no protection. Since the incidence of tuberculosis in the United States is low, BCG is not routinely used. Its use invalidates the tuberculin skin test, thus limiting surveillance of tuberculosis. BCG may be used in the United States in persons who are at high risk of becoming infected such as health care providers in areas of high incidence where periodic tuberculin testing and treatment of reactors is not feasible.

Stanford et al. (1981) reported that exposure to environmental mycobacteria can enhance the efficacy of BCG vaccinations. According to them exposure to environmental mycobacteria elicits one of two types of immune responses referred to as the Listeria-type and the Koch-type responses. The Koch response results when cells containing mycobacteria or having mycobacterial products on their surface are destroyed. This leads to a hypersensitivity reaction. The Listeria-type response is due to macrophages which are activated by specifically primed thymus derived lymphocytes. These macrophages are bacteriocidal, and thus the Listeria-type response is more protective than the Koch phenomenon. Some mycobacterial species can induce only one type of response, while others can induce either type. According to Stanford et al. (1981) if an individual with a Listeria-type response is vaccinated with BCG that response is enhanced and the individuals ability to recognize other mycobacterial species is

increased. If an individual with a Koch-type response to an environmental mycobacteria is vaccinated with BCG the hypersensitivity is reinforced and the level of protective immunity may be reduced.

Another mycobacterial disease (Benenson 1985) that is rare in the United States is leprosy, caused by M. leprae. This organism cannot be grown in artificial media or tissue cultures. It can only be cultivated in mouse foot pads and in the nine-banded armadillo. However, some (Lyons and Naafs 1987) believe that prior exposure to environmental mycobacteria may influence the type of leprosy an individual develops. Lyons and Naafs (1987) compared antibody levels to 16 environmental mycobacteria in leprosy patients and healthy controls. They compared the responses between patients in Zimbabwe where lepromatous leprosy is predominant and patients from an area where more tuberculoid leprosy cases exist, and found significant differences in responses to mycobacteria other than tuberculosis (MOTT) antigens. They believe this suggests that exposure to some MOTT may influence the type of leprosy a patient develops.

Abrahams (1970) has also considered the immunological effect of exposure to environmental mycobacteria. In Australia routine testing is done using both human and avian PPD. A larger reaction to the human PPD in considered indicative of true tuberculosis. Many school children react more to the avian type. To determine if this was due to BCG vaccination or to prior exposure, children who were vaccinated as newborns while still in the maternity ward were tested with both strains. Such early immunization is done for children born into a family where a member has an active case of tuberculosis. Sixty-six

children ranging from less than one year to 16 years of age were tested. Children in this group reacted more strongly to the human antigen than to the avian strain. This contrasted sharply with all other groups tested. Abrahams (1970) suggests that the first mycobacterial infection may set the antigenic reaction pattern, and subsequent exposure to a different mycobacterial antigen will lead to the production of antibodies that react better with the primary antigen than with the one eliciting the response. This is similar to the "original antigenic sin" phenomena seen with influenza.

For many years if a mycobacteria was isolated in a clinical laboratory and was not either M. tuberculosis or M. bovis it was not identified any further, but was simply labelled as anonymous or atypical mycobacteria. These organisms (Manual of Clinical Microbiology 1985) are not atypical, but rather are characteristic of their particular species. Today the phrases "mycobacteria other than tubercle bacilli" (MOTT) and "nontuberculous mycobacteria" are preferred.

Runyon (1959) proposed a classification system for MOTT that placed these organisms in one of four groups. Group I consists of photochromogens. These organisms form a yellow pigment only after being exposed to light for 1 hour from a 300-watt bulb. Growth is visible in 3 to 4 weeks at 20 to 25 C, and these organisms can grow slightly faster at 37 C. Group II are the scotochromogens which produce a yellow or orange pigment if grown in the dark and a more reddish pigment if grown in continuous light. Group II organisms are also slow growers, producing visible growth at about the same rate as

M. tuberculosis at 37 C. Growth occurs more slowly from 20 to 25 C and usually fails to occur at 45 C. Group III organisms are nonphotochromogens which usually have no pigment or one that develops slowly. Growth is similar to that of Groups I and II, except growth at 45 C may indicate avian strains. Group IV are rapid growers which produce growth in 48 hours at 20 to 25 C.

Nontubercle mycobacteria are ubiquitous. However, Chadwick (Chadwick 1982) stated that they have been found almost everywhere except Alaska.

Kubica (1978) shows forty (40) named species. These are listed in Table I. Over the years many names have been used for mycobacterial species. Some of these names are legitimate while others are not. Some species were referred to by several different names by different investigators. Kubica's update on nomenclature assisted both workers in the field and those attempting to determine which species were referred to in the literature.

Table I. Approved Mycobacteria Species (Kubica, 1978)

M. africanum	M. gordonae	M. scrofulaceum
M. asiaticum	M. haemophilum	M. senegalense
M. aurum	M. intracellulare	M. simiae
M. avium	M. kansasii	M. smegmatis
M. bovis	M. leprae	M. szulgai
M. chelonei	M. lepraemurium	M. terrae
M. chitae	M. malmoense	M. thermoresistible
M. duvalii	M. marinum	M. triviale
M. farcinogenes	M. microti	M. tuberculosis
M. flavescens	M. nonchromogenicum	M. ulcerans
M. fortuitum	M. neoaurum	M. vaccae
M. gadium	M. parafortuitum	M. xenopi
M. gastri	M. paratuberculosis	
M. gilvum	M. phlei	

B. DISEASES CAUSED BY MOTT

As previously mentioned, MOTT were not identified in the clinical laboratory until recently. Several investigators have found that as the incidence of tuberculosis declines the incidence of MOTT infections increases. Marks (1969) noted that MOTT infections in Wales doubled from 1953-1957 and doubled again between 1963-1967. This increase was not believed to be due to better laboratory diagnosis. During this same period the incidence of tuberculosis in Wales decreased by two-thirds. Paull (1973) showed similar increases in Wales from 1953 to 1970. Grange (1987) remarked that although the prevalence of MOTT infections is still relatively low compared to that of tuberculosis and leprosy, the incidence of disease due to these organisms appears to be on the increase in the Western world. Ahn et al. (1979) reported an increase in the number of infections due to M. kansasii and M. avium-intracellulare in Texas from 1967 to 1976. During that same period the incidence of tuberculosis decreased. In British Columbia between 1972 and 1981 (Isaac-Renton et al. 1985) MOTT isolates increased in both relative and absolute numbers while the incidence of tuberculosis decreased. MAIS (M. avium-intracellulare-scrofulaceum complex) organisms accounted for 73.3 % of the MOTT isolates.

Modern medical science has inadvertently increased the population that is susceptible to MOTT infections (Du Moulin and Stottmeier 1986). Complicated and prolonged surgeries such as organ transplants and open heart surgery (Centers for Disease Control 1977) leave patients open to attack by these organisms. Patients in chemotherapy or on cytotoxic drugs (Wolinsky 1979), those on hemodialysis (Bolan et al. 1985; Katz

and Hull 1971) and those who are immunosuppressed (Gold 1986; Woods and Washington II 1987) are also highly susceptible to these organisms. Certain disease such as some retrovirus infections (Du Moulin and Stottmeier 1986), leukemia (Winter et al. 1985) and AIDS (Good 1985) also increase a patients vunerability to MOTT infection. Prince et al. (1989) found that pulmonary disease due to M. avium can affect persons, particularly elderly women, without predisposing conditions. There are four main types of diseases (Grange 1986) caused by MOTT infections. These are (1) local lesions after traumatic injury, (2) localized lymph node involvement, (3) pulmonary infections similar to tuberculosis and (4) disseminated disease.

Several species of mycobacteria can cause skin and subcutaneous disease. These include M. scrofulaceum, M. kansasii, M. avium-intracellulare, M. haemophilum, M. terrae complex (Wallace 1987), M. fortuitum-chelonei, M. marinum and M. ulcerans (Wolinsky 1979). A traumatic injury (Wallace 1987) generally precedes an infection in an immunocompetent host by four to six weeks. No history of trauma is usually presented in cases in immunocompromised patients.

Localized abscesses may occur at the site of injection. These have occurred in an insulin dependent diabetic (Kelley 1987) and following injections of penicillin, iron dextran and BCG (Wolinsky 1979). Epidemics of 50 cases following injection of diphtheria-pertussis-tetanus-polio vaccine and 12 cases following histamine injections (Wolinsky 1979) have also occurred.

A case of lymphadenitis due to M. haemophilum was reported (Thibert 1990) in a 3-year-old female after trauma due to repair of

dental caries. M. gordonae has been reported to cause chronic cutaneous granuloma (Shelley and Folkens 1984) that was possibly connected to wounds sustained while working with rosebushes. Following a puncture wound by a century plant thorn, M. fortuitum caused a localized lesion (Wallace 1987) in an otherwise healthy female.

Deeper infections may follow injury. Minkin et al. (1987) reported on a case of osteomyelitis of the scaphoid after a puncture wound of the thumb from a hook used in a slaughter house. M. aquae (now known as M. gordonae) was reported (Gonzales et al. 1971) to be the etiologic agent of a case of meningitis in a child with hydrocephalus and multiple shunt implants. A case of septicemia possibly caused by M. fortuitum (Katz and Hull 1971) was reported in a nurse on home dialysis. To save time in the procedure the patient had rinsed out the formalin compartment with tap-water rather than sterile saline. Gran et al. (1987) reported a case of monoarthritis that resembled "fish fanciers finger", a disease caused by M. marinum, in a female farmer with no history of injury. The causative agent was M. chelonei.

Several authors have reported corneal ulcers due to mycobacteria other than tuberculosis. Lazar et al. (1974) reported on four patients who were referred by the same physician in a two year period, and he suspected that contaminated solutions or instruments may have been the source of infection. Newman et al. (1984) also reported three cases of corneal infection due to M. chelonei who were treated in a common ophthalmological office after eye surgery. Again it was believed that the source was in the doctor's office, but no organisms could be

cultured. This could be due to the passage of nine to 17 months between exposure and the collection of specimens. Meisler et al. (1982) and Knapp et al. (1987) both reported on cases of corneal ulcers due to MOTT after accidental injury to the eye. Aylward et al. (1987) reported on an infection of a corneal graft due to M. chelonei which was associated with the use of an extended wear contact lens. Gangadharam et al. (1978) reported a case of ulcerative keratitis due to M. chelonei in which the mode of entry of the organism was unknown. Turner (1970) published a review on atypical infections in ophthalmology.

M. marinum (Johnston and Izumi 1987) was discovered in 1926 in saltwater fish in the Philadelphia aquarium. In 1954 it was found to be the cause of "swimming pool granuloma". In 1959 (Mollahan and Romer 1961; Schaefer and Davis 1961) the organism caused an epidemic of tuberculoid lesions in as many as 290 persons who used a public swimming pool in Colorado. A study was done in Tennessee (Park and Brewer 1976) on indoor swimming pools to detect the presence of M. marinum and determine its resistance to chlorine. It was found that the organism was resistant to concentrations of 1.5 ppm free chlorine. Cases of "swimming pool granuloma" have also been reported in Britain (Morgan and Blowers 1964), as well as other countries, including Sweden and Canada. Several cases have been reported in patients who had been swimming and/or fishing in the Chesapeake Bay or its tributaries (Zeligman 1972). Aubrey and Fam (1987) reported a case in a patient who had been swimming and fishing off the gulf coast of Florida. Adams et al. (1970) reported on six cases of M. marinum infection in patients

who cleaned an aquarium with a minor abrasion on their hand which allowed the organism entry. Several other reports (Barrow and Hewitt 1971; Heineman et al. 1972; Littlejohn and Dixon 1984) of "fish fanciers finger" can be found in the literature. These infections can be extremely difficult to treat (Ljungberg et al. 1987) and therapy may require multiple antibiotics, surgery and/or a long recovery period. Although M. marinum is usually associated with superficial cutaneous infections it can also involve the deep structures of the hand and wrist (Chow et al. 1987). This organism is also well suited for the environments of whirlpools, saunas, physical therapy pools and water slides (Bercherer 1987).

M. ulcerans causes (David 1976) a chronic, indolent ulceration of the skin which spreads extensively and becomes crippling. Infection commonly occurs between five and 15 years of age (Wolinsky 1979), but adults can also be infected (Igo and Murthy 1988). Disease due to this organism is mainly found in the tropics of Africa and in Australia (Wolinsky 1979). The natural reservoir of M. ulcerans and its mode of transmission to man have not been determined. However, some evidence points to an association with certain vegetation in swampy areas (Wolinsky 1979). Hayman (1987) reported that the distribution of disease due to M. ulcerans is similar to the discontinuous distribution of some plants. For instance, it has been proposed that M. ulcerans normally exists in a symbiotic relationship with the root systems of certain rainforest plants and their fungi. The distribution of these plants is related to plate tetonics, that is, they were once associated with the great land mass Gondwana and when it broke up to

form the southern continents the plates and their symbiotic fungi also moved. Endemic foci of disease due to M. ulcerans can be explained, according to Hayman (1980), if it is assumed that the bacteria also moved with the plants.

Atypical mycobacterial infections can be varied. A common type of MOTT infection is cervical lymphadenitis in children (Escajadillo et al. 1987). Spark et al. (1988) reported an increased incidence of adenitis in children at the Tucson Medical Center between 1979 and 1983. All cases during that period were due to M. avium-intracellulare complex.

Pulmonary diseases (Good 1985) caused by MOTT often cannot be clinically, radiologically or histologically distinguished from tuberculosis. Chronic pulmonary disease resembling tuberculosis (Wolinsky 1979) is the most important clinical disease caused by MOTT. The organism must be isolated repeatedly (Good 1985) and identified and tested for its sensitivity to antimycobacterial drugs because many of the MOTT are highly resistant to them. M. kansasii is a frequent cause of pulmonary disease in the United States (Youmans 1980) as well as in Europe (Jones 1969). Pulmonary disease may also be caused by M. avium-intracellulare, M. scrofulaceum, M. fortuitum, M. chelonei (Wolinsky 1979), M. xenopi, M. szulgai, M. simiae and M. malmoense (Woods and Washington II 1987). Generally some preexisting chronic pulmonary disease (Wolinsky 1979) is present before a MOTT infection occurs. Some of the most common predisposing conditions (Wolinsky 1979) for MOTT infection are pneumoconiosis, chronic bronchitis, chronic obstructive lung disease, bronchiectasis, chronic aspiration from

esophageal disease and previous tuberculosis and malignancy. Pulmonary disease due to MOTT (Wolinsky 1979) can occur in patients without apparent lung disease or immunodeficiency. A retrospective study (Prince et al. 1989) covering a 10-year period in two Philadelphia hospitals found that 18 % of 119 patients with pulmonary infection due to M. avium had no predisposing conditions. Prince (1989) also noted that the disease without any predisposing condition was more common in elderly females than in males, with 17 females and only 4 males being diagnosed between 1978 and 1987.

In AIDS patients M. avium-intracellulare often causes severe disease (Gold 1986), which is usually disseminated (Good 1985). This may include involvement of the gastrointestinal tract, liver, spleen and lymph nodes (Gold 1986). Similar disease occurs in patients with hairy-cell leukemia (Winter et al. 1985). M. avium-intracellulare infections are extremely difficult to treat because the organism is highly resistant to antimycobacterial therapy (Gold 1986). Hirasuna (1987) also reported disseminated disease due to M. kansasii in an AIDS patient. Martin-Scapa et al. (1987) also reported a case of disseminated disease due to M. kansasii in a previously healthy person. He pointed out the need to avoid classifying a patient with disseminated mycobacterial disease as an AIDS case without evidence of HIV (Human Immunodeficiency Virus) infection. Rogers et al. (1988) reported on two AIDS patients with disseminated disease due to M. haemophilum. Although there are few reports of M. haemophilum infections, they believe the incidence is probably higher and that the organism is not identified due to its fastidious growth requirements.

Thilbert et al (1990) also reported a case of M. haemophilum infection in an AIDS patient. A case of disseminated disease due to M. gordonae was reported (Kurnik et al. 1983) in an alcoholic who may have been further predisposed to disease by a prior malignancy. A survey of 9,760 AIDS patients in the United States (Du Moulin and Stottmeier 1986) showed that 4 % of the patients had disseminated mycobacterial infection, but the incidence is most probably higher. Disseminated mycobacterial disease (Du Moulin and Stottmeier 1986) is the third most common opportunistic terminal infection in AIDS patients, with only Pneumocystis carinii pneumonia and disseminated cytomegalovirus infection occurring more frequently.

MOTT may be present in sarcoidosis, which is a chronic generalized granulomatous increase in reticuloendothelial cells that can involve any organ (Dorland's Medical Dictionary 1980). These are suspected of being mycobacterial L-forms (Cantwell 1981; Cantwell 1982a; Cantwell 1982b).

Cantwell et al. (1982c) also reported finding cell wall deficient mycobacteria, along with other organisms, in 7 patients with cutaneous lupus erythematous. He believes that these mycobacterial L-forms may even be the etiologic agent or agents of lupus erythematosus.

M. paratuberculosis is known to be the etiologic agent of paratuberculosis or Johne's disease in ruminants. The disease (Chiodini et al. 1984a) occurs worldwide, including the United States, and is one of the most serious infectious diseases in the cattle industry. The primary lesions are limited to the gastrointestinal tract and regional lymph nodes. It is believed that the organism is

transmitted by ingestion and possibly by sperm and from mother to fetus. Crohn's disease is a granulomatous enteritis of humans that resembles tuberculosis. Chiodini et al. (1984b) isolated two mycobacteria belonging to Runyon Group III from Crohn's disease patients. These organisms appear to be most closely related to M. paratuberculosis. Thayer et al. (1984) fed these organisms to infant goats and a granulomatous ileitis was produced. The organism was later found (Graham, D. et al. 1988) to be M. paratuberculosis strain linda. However, it was reported (Van Kruiningen 1988) that the goats had a tuberculoid response to the organism rather that the classical Johne's disease reaction. Van Kruiningen (1988) questioned if Crohn's disease might not be a tuberculoid reaction to intestinal mycobacteria. In one study (Colemont et al. 1988) acid-fast bacilli were found in smears of biopsy specimens from 11 of 32 Crohn's disease patients. Two strains of M. chelonei were isolated from two Crohn's disease patients. Colemont et al. (1988) points out that convincing evidence that Mycobacteria are the etiologic agent of Crohn's disease is not yet available, but further investigations are needed.

No evidence (Chapman 1982) of person-to-person transmission of mycobacteria other than tuberculosis infections exists, but the evidence suggests an environmental source for human infections. The natural reservoirs of some environmental mycobacteria, such as M. kansasii, (Chapman 1977) remain unknown. Disease due to these organisms is becoming a growing concern (Grange 1987), especially in developed countries. Exposure to these organisms may also affect the response to BCG (Stanford et al. 1981) and the type of leprosy (Lyons

and Naafs 1987) that may occur. For these reasons the search for the natural reservoirs and methods of transmission of these organisms continues.

Grange (1987) suggests that three factors determine the prevalence and nature of disease due to environmental mycobacteria. The first factor is the conditions such as pH, hydration, temperature, mineral content, vegetation and animal reservoirs that determine the number and species of Mycobacteria in the environment. Secondly, the opportunity for the organisms to move from the environment to man must exist. The third factor is the susceptibility of the human population to these organisms.

C. MYCOBACTERIA IN THE ENVIRONMENT

Myobacteria other than tuberculosis have been isolated from many environmental sources. Beerwerth (1967) isolated 677 atypical or non-classifiable strains from animal droppings. Mycobacteria were isolated in fecal specimens from 86 % of cattle, 31 % of sheep, 56 % of horses and 8 % of pigs tested. Of the organisms isolated 81 % belonged to Group II, 7.5 % to Group III and 11.4 % to Group IV. Beerwerth et al. (1979) has also isolated MOTT from arthropods. Arthropods collected on pasture ground carried mainly Group II organisms. Those collected from arable land, stables and saw mills carried primarily Group III organisms. It was noted that strains of M. avium-intracellulare-scrofulaceum were frequently found in arthropods from saw mills (20.5 % of strains), but rarely isolated (4.4 % of strains) in other biotypes. MOTT have also been isolated (Nassal et al. 1974)

from fruits and vegetables. It was learned that mycobacteria could be found only in plants which had edible parts growing in or near the soil such as radishes, potatoes, carrots, strawberries, lettuce, chicory and cucumber. No MOTT were found in cereals or in fruit growing on trees or bushes. Washing the vegetables decreased the number of Mycobacteria present, but did not eliminate them. MOTT were found inside fruit which suggested that the organisms are picked up by the roots and distributed through the plant by the sap. All organisms isolated by Nassal et al. (1974) were members of Group III. Kadza (1973b) watered lettuce plants with a suspension of M. intracellulare serotype Davis (0.1 mg/ml) and found that the organism lodged in crevices of the plant despite frequent washings. However, he was unable to demonstrate the organism inside the plant.

Many investigators have examined soil for the presence of Mycobacteria. Frey and Hagan (1931) isolated Mycobacteria from 100 soil samples collected from around the United States. They found that all of these "saprophytic" organisms were capable of growth at 47.5 C. However, since most of the currently recognized species were not identified until the 1950's or later (Chapman 1977), it is difficult or perhaps impossible to ascertain which species Frey and Hagan isolated. Gordon and Hagan (1937) recognized that other saprophytes existed that were not capable of growth at 47.5 C and examined soil samples using a nitrogen-free medium and incubation at room temperature. They isolated 60 cultures, 27 of which were not capable of growth at 47.5 C. Singer and Rodda (1961) examined soil and other materials in Queensland, Australia. They succeeded in isolating MOTT from 12 of 25

(45 %) of the soil samples studied. Kubica et al. (1961) isolated Mycobacteria in 204 of 452 (45 %) soil samples from Georgia. Kubica et al. (1963) pointed out that there are differences in the physical and biochemical properties of Mycobacteria found in soil and in humans, but he went on to speculate that changes could occur in an organism and its progeny once they are in a host animal.

In Texas a study (Jones and Jenkins 1965) was conducted on soil samples from the home environment of school children who had positive skin tests to PPD-B (Group III antigen). They isolated 101 strains of Mycobacteria form 77 of 92 soil samples. Ninety-three of the organisms isolated were studied and five belonged to Group II, three to Group III and 85 to Group IV. However, one must question the usefulness of this information since no soil samples were taken from the home environments of children who had negative skin tests to PPD-B.

Wolinsky and Rynearson (1968) examined 72 soil samples from four different states. These included clay, loam and sand. Eighty-six per cent of the samples yielded Mycobacteria. Sixty-four per cent of the samples contained M. fortuitum, 54 % contained scotochromogens and 42 % yielded Group III organisms. Clay tended to have a higher yield of M. fortuitum, while mud tended to be higher in scotochromogens. When biochemical reactions of the soil isolates were compared to those of disease-associated organisms they were found to differ. Wolinsky and Rynearson (1968) believed these results to indicate that soil was not the usual reservoir for human infections unless it could be shown that the biochemical reactions could be altered by the organism living in soil or in human tissue.

Another study of soil samples from Queensland (Reznikov and Leggo 1974) found M. avium-intracellulare-scrofulaceum complex (MAIS) in 36 of 74 samples. Twenty-seven of the MAIS isolates proved to be serologically similar to disease-associated strains. Of the typable strains from soil samples, strains similar to Schaefer's serotype Boone were predominant. It is interesting to note that serologically similar strains were the predominate type from both clinical material of patients and housedusts in southeastern Queensland. In one study in Queensland Dawson (1971) examined house dust for the presence of MOTT. Over half the strains isolated were human pathogens. M. avium-intracellulare-scrofulaceum complex made up 56 % of the isolates. Since dust could be inhaled the investigators believed this is an excellent possible source of human infection. Singer and Rodda (1961) had previously examined dusts and found 40 % of the samples yielded Mycobacteria. Dawson (1971) suggested that the differences in types of organisms found in Queensland and by the various investigators in the United States might be due to the method of decontamination each investigating group used.

Kleeberg and Nel (1973) examined soil and dust in South Africa for the presence of MOTT. Thirty-one of 54 (57.4 %) of the dust samples and 15 of 37 (40.5 %) of the soil samples yielded Mycobacteria. All of the soil and dust samples had low colony counts except for dust taken from pigsties.

In Japan (Ichiyama et al. 1988) MOTT have also been isolated from soil, mud, housedusts and river water. They found Mycobacteria in all samples of soil and mud examined, but only five and four samples

respectively were collected. Seventeen of 22 samples of housedust also yielded Mycobacteria.

Another source examined by several investigators is milk. Singer and Rodda (1961) found Mycobacteria in 14 of 44 (32 %) milk samples in Australia. In a study of raw milk in the United States (Chapman et al. 1965) it was noted that seasonal variations occurred in the number of positive samples. Of 351 winter samples 49.5 % were positive, while only 24.6 % of 207 spring samples and 16.5 % of 212 summer samples yielded MOTT. These differences were found to be significant. More pigmented and photochromogenic strains were isolated in winter than during the other two seasons. Isolates belonging to Groups II, III and IV were identified. A few of the isolates resembled M. kansasii, but further identification was not possible.

Another study (Jones et al. 1966) isolated 83 strains from 77 of 123 (62.6 %) raw milk samples. M. phlei, M. fortuitum and M. smegmatis were isolated along with 23 slow growers. A different study (Holt and McDurmount 1975; cited by Wolinsky 1979) recovered M. avium serotypes 9, 13 and 19 from raw milk.

Chapman and Speight (1968) also examined 458 cartons of refrigerated homogenized pasteurized milk. Thirteen (2.8 %) yielded MOTT. The organisms found were one isolate of M. fortuitum, nine organisms belonging to Group III and three unclassified organisms. Chapman suggested three possible sources for the Mycobacteria in pasteurized milk: (1) the milk itself, (2) piping in the pasteurization plant and (3) the cartons. These studies indicate that both raw and pasteurized milk can be a possible mode of transmission for

Mycobacteria into the human gastrointestinal tract.

An excellent source of Mycobacteria other than tuberculosis is water. After a study (Kubica et al. 1961) of soil and water samples in Georgia, Kubica remarked that the data indicated that many of the MOTT may reside in soil and be carried from there to water supplies where it can then reach animals or man by inhalation or ingestion. Mycobacteria are notoriously resistant to disinfectants. M. chelonei was isolated from patients with peritonitis (Bolan et al. 1985) in a hospital peritoneal dialysis center. The organism was also recovered from the dialysate and other fluids of the dialysis machine. This strain could survive 2 % formaldehyde (HCHO). An earlier study (Carson et al. 1978) of M. chelonei isolated from peritoneal fluid of patients and peritoneal dialysis machines found that some organisms this strain survived 2 % HCHO for up to 24 hours, and was decreased by only two log units in 8 % HCHO after two hours. Reference strains of M. chelonei and M. fortuitum succumbed to 2 % HCHO in two hours and to 8 % HCHO in 15 minutes. The disease associated strain survived one hour in 2 % alkaline glutaraldehyde, whereas the reference strains had no survivors after two minutes exposure. All strains survived 1 hour of exposure to 0.3 micrograms of free chlorine per ml at pH 7. Four of five strains survived 30 minutes exposure to 0.7 micrograms per ml free chlorine at pH 7. All Mycobacteria were allowed to acclimate to sterile distilled water (SDW) for five days.

Joynson (1979) inoculated soil and water samples with M. kansasii to determine its survival rates in hopes of identifying its natural habitat. All four soil samples yielded M. kansasii immediately after

inoculation, but only three survived for one month. None could be isolated during the next five months with repeated sampling. Water seeded with M. kansasii, however, yielded three or more strains each month for the next 12 months. These data suggested that the natural habitat of M. kansasii is water since the organism does not seem to be capable of survival in soil for more than one month.

Kazda (1973a) isolated M. intracellulare serotype Davis from moorland water. This water had a pH of 4.0 to 4.5 and proved to be suitable for the multiplication of this organism. Using water models, he Kazda, (1973b) also demonstrated that M. intracellulare serotype Davis and M. avium serotype 2 could multiply in water with a pH of 4.2 to 4.7, but did not multiply in waters of pH 7.3 to 8.3 or 6.9 to 9.1. However, the moorland water models reached a maximum concentration of 10^4 to 10^5/ml and then decreased to 10^3/ml. The other water models remained constant at concentrations of 10^6 or decreased only slightly.

It has been demonstrated the M. avium-intracellulare-scrofulaceum complex (MAIS) can be grown in the laboratory (George et al. 1980) in natural waters from northeastern and southeastern states. Growth occurred without addition of any substrates. Growth rates and maximum yields were similar for all waters tested and were not dependent on the area from which the waters were collected nor whether or not MAIS isolates were recovered from the natural source. The data suggested that temperature is an influencing factor in the differences of isolation rates of these organisms between northeastern and southeastern states. The organisms grew poorly at 15.5 C and may have

slightly decreased in number when held at <9.4 C for 30 days. The northeastern part of the United States thus supplies a shorter growing period for MOTT than that found in the southeastern region. Another investigation (Falkinham III et al. 1980) found that 33 % of the water samples from the southeastern United States yielded MAIS while only 20 % of the water samples from the northeastern United States contained these organisms.

It was further shown (Falkinham III et al. 1980) that MAIS organisms were isolated in fewer numbers from marine waters than from fresh waters. Another study (George et al. 1980) found that salt concentration had an effect on all Mycobacteria isolated from environmental samples. They found that waters with 0.1 %-1.9 % salt had the highest percentage of MAIS isolates.

A study conducted along the eastern seaboard of the United States (Gruft et al. 1981) found that M. intracellulare was predominant in the North while M. scrofulaceum was predominant in the South. However, regional differences of M. intracellulare did not prove to be statistically significant. Other MOTT isolated included M. terrae, M. gordonae and M. fortuitum-chelonei.

Gruft et al. (1975) conducted a study to aid in explaining the geographic distribution of skin sensitivity to M. intracellulare. Using sterilized sea water at temperatures normally found off the southeastern coast of the United States he demonstrated that M. intracellulare can survive or can remain in a state similar to the stationary growth phase for long periods of time. When bubbles were passed through a saline suspension of M. intracellulare the organisms

were concentrated on the bubbles. When the bubbles burst at the surface droplets were ejected into the atmosphere that contained much greater concentrations of Mycobacteria than the suspension. Gruft et al. (1975) pointed out the possibility that such droplets could be carried by wind and air currents and be transported for long distances inland.

In a separate study Gruft et al. (1979) collected 38 subsurface and surface microlayer water samples from estuaries and ocean waters from Virginia to Florida. A total of 30 isolates were cultured from 16 positive samples. Nineteen of the 30 isolates were identified as M. avium-intracellulare-scrofulaceum (MAIS), but only four of these were typable. Only four strains of M. gordonae were isolated. Several samples were overgrown with non-acid-fast bacilli. This isolation of MOTT from ocean and estuary waters is consistent with the hypothesis that aerosols from these sources present a possible source of infection for man and animals.

Another group of investigators (Wendt et al. 1980) explored the question of whether naturally occurring droplets and aerosols contained mycobacteria. They used a site in the James River at Richmond, Virginia, from which Mycobacteria had previously been isolated. They found that potentially pathogenic M. avium-intracellulare-scrofulaceum complex organisms were present in the freshwater subsurface and microlayer, Andersen and water-to-air aerosols and rainwater in that area. This work established that a water-to-air pathway for transmission of MOTT resembling those isolated from humans does exist. An interesting finding of the study was that 13 isolates of M.

intracellulare were recovered from aerosols while no M. scrofulaceum was found in aerosols, although M. scrofulaceum is generally in higher concentrations in the waters of the area than M. intracellulare. No explanation for this phenomenon was available. It was found (Parker et al. 1983), however, that M. intracellulare was preferentially concentrated in aerosols over M. scrofulaceum. The enrichment factor was defined as the concentration of cells per droplet volume divided by the concentration of cells in the bulk suspension per equivalent volume. The enrichment factor for M. intracellulare ranged from 68 to 15,000 with an average of 2922; and for M. scrofulaceum ranged from 35 to 550 with an average of 177. Even after vortexing to decrease the aggregation of M. intracellulare the organism had a higher aerosolization than M. scrofulaceum. Increasing salt concentration also increased the aerosolization of both organisms. Other pathogens including Legionella were also concentrated in droplets and transferred from water to air. This suggested that aerosolization of organisms from natural waters may be a significant mode of transmission of respiratory diseases for humans and animals.

Collins and Yates (1984) suggested that aerosols created by shower heads and when tap water splashes against basins, baths and sinks could lead to the inhalation of M. xenopi or M. kansasii which have been found in tap waters.

In England (Rao et al. 1980) it has been shown that M. tuberculosis can apparently be spread by minimal exposure to droplet nuclei. A swimming pool attendant was found to have an active case of tuberculosis. He supervised swimmers from a six foot high platform at

the center of one side of an indoor heated pool. Although contact was minimal with the swimmers, 3764 children age eight to 11 years were tested and 108 (2.9 %) were found to have evidence of disease. There were 16 cases of tuberculosis and the other 92 children had tine tests of grade three or four with or without radiological or clinical signs. Above one end of the pool there is an intake fan and above the other end of the pool extractor fans are housed. This causes an airflow from the deep end to the shallow end of the pool. This airflow may have caused droplet nuclei from the index case to move toward the shallow end of the pool where the children were swimming. The children were in the pool for 20-30 minutes on 10-15 occasions. There might also have been brief contact between the children and the index case in a locker room. Considering the results of Wendt et al. (1980) and Gruft et al. (1975) one must question whether the water and aerosols of the swimming pool aided in concentrating the tuberculosis organism and thus increased its spread.

In Japan (Saito and Tsukamura 1976) 90 % of the pulmonary infections due to MOTT are caused by M. intracellulare. A study of public bath water in Hiroshima yielded 14 strains of slow growing, nonphotochromogenic mycobacteria. Five of the strains were further identified and all were found to be M. intracellulare.

In 1973 a sudden increase in positive acid-fast smears in the Bacteriology Laboratory of Long Island Medical College (Dizon et al. 1976) was found to be due to the laboratory tap water. The tap water contained M. gordonae. The organism colonized the deionizer and thus contaminated reagents used in the preparation of clinical specimens.

Another pseudoepidemic (Gangadharam et al. 1976) which involved M. gordonae occurred in Texas. The organism was isolated from aerosol-induced sputum from patients without any mycobacterial disease. It was learned that the patients gargled and rinsed their mouths with water from a tap near the aerosol induction machines. This tap harbored M. gordonae and an oral rinse of only a few seconds duration was enough to contaminate specimens.

Two subgroups of M. kansasii (Manual of Clinical Microbiology 1985) based on their catalase production are recognized. The strains with high catalase production, those which generate >45mm of bubbles, are generally associated with disease. In 1967 (Bailey et al. 1970) it was found that a high catalase producing strain of M. kansasii was in the city water supply of Martinez, California. Taps tested in the County Hospital and the Contra Costa County Health Department yielded a scotochromogen and M. kansasii. Water taken from a hospital on a different water system and from the Martinez Treatment plant where the treated water entered the system did not contain acid-fast bacilli. No seasonal variations occurred.

In Texas (Steadham 1980) high catalse strains of M. kansasii were isolated from eight of 19 taps. All but one of the positive sites were dead-end mains where debris can build up and protect organisms from chlorination. Using a different procedure in a later study (Powell and Steadham 1981) nine isolates of high catalase M. kansasii were cultured from 233 samples. The high catalase producing organism was also isolated from a river in central Texas which was the first isolation of this organism in a natural source.

In 1973 a pseudoepidemic due to M. kansasii (Maniar and Vanbuckenhout 1976) occurred when six isolates were obtained from sputa, bronchial washings or gastric lavages of patients at Brandon Mental Hospital in Manitoba. M. kansasii was found to be present in the tap water. It was assumed that measuring cylinders used in the preparation of reagents for pretreatment of clinical specimens were contaminated when rinsed with tap water. This led to contamination of clinical specimens. All M. kansasii strains isolated in this study had low catalase activity and did not appear to be associated with any disease process. It is interesting to note that the hospital water supply is from an on-site reservoir which was deepened by digging out the soil prior to the isolation of M. kansasii from the tap water.

After sputum samples from 10 patients on one ward of a hospital in England yielded M. xenopi (Bullin et al. 1970) an environmental source of contamination was suspected. Pairs of hot and cold water taps in three hospitals and 10 private homes were tested. In the hospital where contamination was first suspected 111 pairs of taps were tested. Sixty-five (55 %) yielded M. xenopi, 11 (10 %) yielded scotochromogens and one yielded M. kansasii. In the second hospital 27 % of the taps tested yielded M. xenopi and 50 % yielded scotochromogens. In the third hospital M. xenopi was found in only five of the taps tested. No scotochromogens were isolated. In 10 private homes 34 pairs of taps were examined and only two (6 %) yielded M. xenopi. M. xenopi is thermophilic (42-44 C). Because of this high temperature range and the high incidence in coastal areas of England and Europe, sea birds are a possible source. Bullin et al. (1970) tested pigeon droppings from the

hospitals in this study since two of the buildings had water towers on the roof which were not entirely protected from birds. No acid-fast bacteria were found in these droppings, nor in reagents used in the laboratory. How the organism gained entrance to the water system is unknown.

Between 1969 and 1975 M. xenopi was isolated at least once from 683 patients (Gross et al. 1976) at West Haven Veterans Administration Hospital. Pulmonary disease due to M. xenopi was diagnosed in 11 patients. Prior to this only three cases of pulmonary disease due to M. xenopi had been reported in the literature and a pseudoepidemic was suspected. The hospital water supply was sampled where city water enters the hospital, as well as from pumps, storage tanks, cold water tanks and hot water generators. Heavy growth of M. xenopi was isolated from all of the hot water generators. One and two colonies were isolated from two of the cold water tanks. State law requires that hot water tanks be kept at 43 C, which is an optimum temperature for growth of the thermophilic M. xenopi. Other MOTT isolated from the hospital water supply included M. avium, M. kansasii, M. gastri and M. gordonae. No outside source of contamination of the water system could be determined.

In London (McSwiggan and Collins 1974) a similar case of suspected environmental contamination of sputum samples due to M. xenopi occurred. A total of 97 samples were taken from 65 taps in the hospital. Mycobacteria was isolated from 37 (57 %) of the taps. M. xenopi was found to constitute only 14 % of the isolates, while M. kansasii accounted for 47 %. The remaining isolates were

scotochromogens (30 %) and nonchromogens (9 %).

In a more recent pseudoepidemic in Denver, Colorado, (Grahman, Jr. et al. 1988) M. avium complex organisms were isolated from urine samples of several patients. An investigation revealed M. avium in the phenol red solution used to process urine samples in the mycobacteriology laboratory. The organism was subsequently isolated from the deionized tap water used to make the reagent, the laboratory tap water and taps from four hospital wards. A microbiological filter was installed for the deionized tap water. Only two urine isolates of M. avium were recovered in the following year compared to 26 isolates during the year before installation of the filter.

In Australia, Singer and Rodda (1965) looked at several water sources. A total of 102 cultures of acid-fast bacteria (including four cultures of Nocardia) were isolated from 165 swimming pools. The majority of these isolates were slow-growing chromogens, and only two strains of M. marinum were found. Twelve rainwater tanks were tested and 13 isolates were recovered. Two of the tanks had more than one isolate and one strain of M. marinum was isolated. A total of 19 isolates were recovered from 34 samples of the Brisbane city water supply. All isolates came from filters, mains and reservoirs, and none were found in tap samples.

In Bergen, Norway, a swimming pool (Eilertsen 1969) was contaminated with M. marinum, a Group I organism, for several months. The organism was found to be resistant to chlorine and at least 10 mg per liter of chlorine was needed to inhibit growth. It was necessary every two or three months to over-chlorinate the pool with

concentrations as high as 15 mg per liter. Such high concentrations are not compatible with everyday use of the pool. The organism was also resistant to several antimycobacterial drugs. Of 3,000 children 13 % had positive reactions to M. marinum, while only 1.5 % of the same age group had virulent tuberculosis. Eilertsen (1969) pointed out that exposure to M. marinum creates problems for the use of skin tests of large groups for tuberculosis. Part of the water in the pool was sea water collected from Bergen harbor. The sea water contained Group IV mycobacteria.

Cationic exchange resins used in water-softener units (Stamm et al. 1969) were examined for microorganisms in one investigation. Forty-four different genera of bacteria and fungi were recovered. Bacillus, Candida, Clostridium and Staphylococcus were the most common genera isolated. Mycobacterium was the ninth most frequently isolated organism. Most Mycobacterium isolates came from private homes, but a hospital and a dairy also yielded the organisms.

In Ohio (Showalter and Wolinsky 1974) several water sources were cultured. These included taps from the city water supply, wells, cisterns, drinking fountains, ice machines, reservoirs, Lake Erie, small lakes and rivers, swimming pools, lakeside and oceanside sand, snow, aquarium tanks and zoo ponds. From 255 samples 201 isolates were recovered. Fifty-two of the isolates were rapid growers and 43 of these were identified as M. fortuitum. Four of the isolates had an intermediate growth rate. The remaining 145 isolates were slow growers which tentatively included M. terrae-triviale, M. gastri, M. avium-intracellulare, M. scrofulaceum and M. gordonae. The disease-

associated strains that were found constituted only a small portion of the isolates. It was noted (Goslee and Wolinsky 1976) that the city aquarium contain the greatest abundance of Mycobacteria and that the highest yield of positive cultures resulted from waters in contact with zoo animals or fish.

Rotterdam (Engel and Berwald 1980) has a high isolation rate of M. kansasii. The strain found in Rotterdam differs from other isolates in its nitrate activity and phage susceptibility. Bimonthly samples were taken of 78 water taps in Rotterdam in an attempt to locate the source of infection. The reservoirs at the water plant, 10 distribution mains and water after slow sand filtration were also sampled. M. kansasii was isolated at least once from 49 % of the taps. Other MOTT were isolated from 78 % of the taps. The highest number of positive taps and the highest mean density scores were obtained in November and December, and the lowest of both parameters were obtained in May. Some taps had high numbers of the organism while others, even in the same building, had none. This suggests that M. kansasii colonizes different areas of the distribution system. Phage typing indicated that the isolates in tapwater were the same type as those found in clinical specimens. Only one of 112 samples from distribution mains yielded M. kansasii. None of the reservoir samples or samples taken after slow sand filtration yielded M. kansasii. However, one of three samples taken from raw water basins yielded the organism.

M. kansasii (Steadham 1980) was isolated from eight of 19 taps in Texas. All taps tested yielded M. gordonae and two contained M. fortuitum.

Kaustova et al. (1981) examined 1079 water samples and scrapings from the coal mine water system in Czechoslovakia. M. kansasii was present in 20 samples collected from four different mines. Thirteen of these samples came exclusively from shower heads in one district. A total of 233 samples (21.6 %) were positive for acid-fast bacteria, most of which were scotochromogens and/or rapid growers. These or similar organisms, but no M. kansasii were isolated from water treatment plants in the area. The area where the highest number of M. kansasii were isolated is an area of high incidence of infection by this organism. It was proposed that aerosols created from the showerheads may be an important factor in the transmission of pulmonary disease.

In 1976 an increase in M. avium-intracellulare-scrofulaceum complex (MAIS) was noticed at Beth Israel Hospital (Du Moulin and Stottmeier 1986) in Boston. Other area hospitals were also isolating these organisms, but none of them were associated with disease. An environmental study was performed in which samples of water were taken from heated nebulizer reservoirs, ice machines, hot and cold drinking-water faucets, sprays from toilets and utility room sinks, bedside carafes and water fountains. A bedside carafe and ice yielded the highest numbers of isolates, providing 50 CFU/ 100 ml and 42 CFU/ 100 ml, respectively. One of five connections of the municpal water system also grew MAIS. MAIS were revealed in the water systems of five of 12 hospitals sampled in the greater Boston area. The isolates from water sources and clinical specimens were serologically identical which suggested contamination of specimens from the environment.

A 12 year study exposed a five-fold increase in MAIS isolation (Du Moulin et al. 1985) in Massachusetts. The statewide isolation rate of 0.87 per 100.000 persons in 1972 rose to 4.6 per 100,000 persons in 1983.

A study in which 1454 patients (Du Moulin and Stottmeier 1986) were monitored over a ten year period revealed that MAIS isolation was highest in densely populated communities. No two cases occurred in the same family.

In continuing research (Du Moulin and Stottmeier 1986) 43 strains of MOTT have been isolated. These were recovered from drinking water supplies in seven coastal communities with groundwater supplies, two inland communities with surface supplies, two inland reservoirs, and hot and cold hospital taps.

From February to June, 1987, water samples were taken from two vacant floors of Beth Israel Hospital (Du Moulin et al. 1988) in Boston. A total of 34 sites, including shower heads were cultured. Eleven of 16 hot water taps and three of 18 cold water taps yielded M. avium. Shower heads provided the highest number of isolates. Of the 14 M. avium isolates 11 were shown by seroagglutination to be serovar four which is the predominate serovar isolated from clinical specimens of AIDS patients. The National Institutes of Health has recovered M. avium from 40 % of the AIDS patients autopsied there. Water is a suspected mode of transmission.

Prolonged time in the water distribution system, resistance to chlorine, optimal growth temperatures, availability of nutrients, and stagnant or interrupted water flow (Du Moulin and Stottmeier 1986) all

aid in the waterborne dissemination of MOTT. Pitting and incrustations inside water pipes (Geldrich 1977; cited by Du Moulin and Stottmeier 1986) protect bacteria from chlorine. The bacteria can be periodically swept through the water system by vibrations caused by construction, the use of fire hydrants and storm runoff. This movement allows the bacteria to colonize new areas of the water distribution system.

D. PURPOSE OF STUDY

Most studies of MOTT in water in the United States have been conducted along the eastern seaboard. A few studies have been performed in California and Texas. The purpose of this study was to determine whether or not Mycobacteria other than tubercle bacilli are present in the water supplies of northeast Kansas, and if so, which species are residing in these waters.

MATERIALS AND METHODS

A. SAMPLE COLLECTION BOTTLES

Two liter plastic bottles that originally contained a carbonated beverage were used for the collection of samples. These bottles could not withstand autoclaving. The bottles were sterilized by adding approximately 5 % sodium hypochlorite (household bleach) in sterile water and allowing to stand for a minimum of 24 hours. An average total content taken from several bottles was 2120 ml. A volume of 120 ml sodium hypochlorite was added and the bottle was filled with sterilized distilled water. Due to the volume of water required and a limited supply of distilled water this was later changed to sterilized tap water. The cap was loosely applied and some of the bleach solution was allowed to rinse the cap as it leaked from the bottle. Before use the sterilizing solution was discarded. Each bottle was rinsed three times with a total volume of one liter of sterile distilled water. Caps were also rinsed with the same rinses.

B. SAMPLE COLLECTION

Tap water and drinking fountains were sampled by catching the stream directly in the collection bottle. Wells were sampled either at taps or at hand pumps, and the stream was collected directly in the collection bottle. Taps were allowed to run for one to three minutes before a sample was taken. Aseptic technique was used in that nothing other than the sample water was allowed to touch the neck of the bottle nor the inside of the cap. The cap was removed only when collection of

a sample was imminent to avoid possible contamination of the bottle by airborne organisms.

Rivers, lakes, ponds and marshes were sampled by submerging the collection bottle in the water to be sampled. Usually a full sample could be collected, however, if the water at the collection point was shallow only a partial sample might be collected. This method of sampling collected not only the water, but also organic matter and debris in and floating on the water.

As soon as possible after collection all samples were stored in the cold until they were to be decontaminated.

C. DECONTAMINATION OF SAMPLES

1. TAP AND WELL SAMPLES

In the first study the method of Du Moulin and Stottmeier (1978) using cetylpyridinium chloride (CPC) was used with modification. A final concentration of 0.05 %, instead of 0.04 %, was added to the sample. A stock solution of 4.0 % CPC was made. After thorough shaking, approximately 50 ml of the sample was discarded and 47.25 ml of the CPC stock solution was added to the sample bottle. The sample was shaken vigorously by hand and allowed to stand for 24 hours before filtering.

In the second study formaldehyde (HCHO) was used in accordance with the research of Carson et al. (1988). A small portion of the sample was removed and the pH was tested. Enough of the sample was discarded so that approximately 1946 ml remained in the collection bottle. This was approximated by comparing to a line drawn on another

bottle at the known volume of 1946 ml. To this volume 54 ml of 37 % stock solution formaldehyde was added. This gave a final concentration of 1 % HCHO. The sample was vigorously shaken by hand and allowed to stand for 5 minutes before filtering.

2. RIVER, LAKE, POND AND MARSH SAMPLES

River, lake, pond and marsh samples were thoroughly mixed by vigorously shaking by hand. CPC was added, the sample was again shaken by hand, and then allowed to sstand at ambient temperature for 24 hours. The sample was again mixed by shaking vigorously and 200 ml of the sample was removed. This was divided among four polypropylene centrifuge tubes. The tubes were centrifuged for five minutes at 4000 rpm. The supernatant was discarded and the pellet was washed three times with sterile distilled water. After the final wash the pellet was resuspended in Middlebrook 7H9 broth and incubated. The remainder of the sample, or the largest portion possible until the filter clogged with foreign matter, was filtered as other samples.

After incubation some of the samples contained both acid-fast and non-acid-fast organisms. In an attempt to decontaminate the broths the samples were vortexed and a portion was removed. This was treated with 4 % NaOH (Vestal 1981) at room temperature for 10 minutes and neutralized with 2N HCl. When this did not destroy the contaminating organisms or resulted in death of the acid-fast organism the procedure was reversed (Vestal 1981) adding the 2N HCl for 10 minutes at room temperature and neutralizing with 4 % NaOH. No success was achieved with this method.

In another attempt to decontaminate some of these heavily contaminated specimens acetic acid was used. This method was adapted from Corper and Uyei (1930). All of the samples had been stored in the cold for several months to over a year after their original 8 weeks of incubation. Middlebrook 7H9 broth was added to any tubes that had dried out or had little liquid left, to a total volume of approximately 5 ml. All tubes were vortexed and allowed to incubate at 37 C for 24 hours to allow any spores to germinate An equal volume of 3 % acetic acid was added to the volume of broth in each tube. Tubes were then incubated at 37 C for 30 minutes. The tubes were centrifuged for 5 minutes at 4000 rpm. The pellets were washed three times with sterile distilled water. The remaining pellet was resuspended in the smallest possible volume of sterile distilled water. The samples were vortexed and the entire contents of each tube was aseptically poured onto a 60 x 15 mm plate of Middlebrook 7H10 without added antibiotics. The plates were left at ambient temperature in a safety cabinet overnight to dry. The plates were bagged and incubated at 25 C in 5 % CO_2.

D. CONCENTRATION OF SAMPLE

After the decontamination procedure was performed one liter of a sample was passed through a 0.45 micron millipore filter. The filter was washed three times with a total volume of one liter of sterile distilled water. The aluminum foil cover used during autoclaving was kept over the opening of the filter apparatus as much as possible during filtering and rinsing to prevent the introduction of airborne contaminates. The filter was aseptically placed on a plate of the

selected media. The plates were placed in Gladlock (a registered trademark) reclosable plastic bags and incubated under the selected conditions. At least two filterings of each sample were done to allow plates to be incubated under different conditions. Those conditions varied in different experiments. When the Lowenstein-Jensen media was used in conjunction with Middlebrook 7H10, four filterings of one liter each were performed on each sample. In those cases two collection bottles were filled with each sample.

E. MEDIA

Several media were used in the experiments. At first Middlebrook 7H10 agar was used. In an attempt to inhibit the growth of contaminating organisms, a total of 2.5 microliters per ml Amphotercin B and 1000 Units per ml Penicillin G was added to the medium. In the early experiments the media was made in our lab. Throughout most of the experiments, however, the media was supplied by the Kansas Department of Health and Environment.

In the literature (Manual of Clinical Microbiology 1985, p. 228) it was noted that at least two types of media should be used when attempting to isolate mycobacteria. It was stated that a primary, nonselective, inspissated egg medium and a nonselective agar medium should be employed. To accommodate this both Lowenstein-Jensen and Middlebrook 7H10 media were used in some experiments. Due to the heavy amount of foreign matter on many of the filters it was not practical to attempt to cut the filters and place them in tubes, so filters were put on plates of Middlebrook 7H10 or plates of Lowenstein-Jensen media.

The only broth used was Middlebrook 7H9. No antibiotics were added to this media or to slants of Middlebrook 7H10.

F. INCUBATION

All plates and tubes were incubated for a minimum of 8 weeks unless severe contamination prevented it. Specific incubations will be given with each experiment. Some were incubated at 37 C in a normal atmosphere, others at 25 C in a normal atmosphere. Still others were incubated in 5 % CO_2 at ambient temperature. Ambient temperature varied from 22 C to 33 C, but was generally at 24 C to 27 C.

In early experiments plates were incubated uncovered in the incubator. Many plates dried out, however, so thin plastic bags were used to prevent this. The bags had to be taped and often tore whenever opened. To avoid having to replace a bag after each opening the bags were changed to one quart size Gladlock reclosable storage bags. Each bag measured 7 inches x 8 inches x 1.75 mm according to the manufacturer. Each bag could easily hold four to five 100 x 60 mm plates and could be opened and closed repeatedly. The use of these bags also made it easy to tell when a good seal was achieved because the sealing strips are yellow and blue and when successfully sealed together the line becomes green. Plates incubated in a normal atmosphere were sealed as soon as filtering was accomplished. Those plates put in the CO_2 incubator were placed in a bag and the bag was left open for a minimum of one hour in the 5 % CO_2 atmosphere before sealing.

G. ACID-FAST STAINS

To determine which isolates were possibly Mycobacteria and needed to be isolated, all dissimilar colony types were acid-fast stained. Since the researcher lacked experience in working with Mycobacteria at the beginning of these studies the use of colonial morphology was not feasible.

The first acid-fast stain used in these experiments was the Ziehl-Neelsen method (Manual of Clinical Microbiology 1985, p. 225). After experience was gained with the procedure it was believed that many acid-fast organisms were being missed. It was learned through experimentation that limiting the decolorization step to 10 seconds instead of two minutes (Vestal 1985) allowed more of the environmental isolates to be identified. In the first study this resulted in six of 21 isolates sent for identification being false positives. As more experience was gained no false positives occurred among 45 isolates sent for identification.

Since it was apparent that some of the environmental forms were variably or only slightly acid-fast, the Alexander triple stain procedure (Alexander-Jackson 1944) was attempted. The procedure was more time consuming, allowed for a greater chance of error on the part of the performer, and failed to identify any organisms not identified by simply shortening the decolorization time in the Ziehl-Neelsen stain. Therefore, the procedure was abandoned.

For the last study the Kinyon "cold" stain was used. Again the procedure was adapted in that the decolorization with acid alcohol was limited to 10 seconds.

Reagents for all stains were made in our laboratory, except the carbol-fuchsin for the Kinyon stain which was manufactured by Becton Dickinson for BBL Microbiology Systems in Cockeysville, MD. When carbol-fuchsin was made in our laboratory sterile distilled water was used to avoid contamination of the reagent.

False positives were found when the organisms were sent for identification. However, there was no method available to identify any false negatives that might have occurred. It has been found (Marraro et al. 1975; Boyd and Marr 1975) that the acid-fast stain is unreliable. In a 12 month study (Marraro et al. 1975) of paired cultured and acid-fast stained clinical specimens only 58 % were true positives. Forty-two per cent showed false-positives and 76 % were false-negative results. False negatives occurred in 81 % of the cases in which cultures were later identified as M. tuberculosis, while only 43 % were true positive results. When used for the detection of MOTT 57 % gave true positive results, while 69 % were false negative. This study was done using the auramine-rhodamine fluorescent technique which does differ from the procedures used in the current experiments. Boyd and Marr (1975) found that for every true positive result a false positive result also occurred, that is, the ratio of true positive to false positive results was one to one. However, since the false positive results after shortening the decolorization step in this lab were much smaller it is assumed that false negatives were also less likely to occur particularly since shortening the decolorization step is more likely to cause false positive results. Another difference is that the studies by Marraro et al. and Boyd and Marr were using the

acid-fast smear as a screening technique before culture, while in the current experiments generally colonies were smeared and stained. The only time specific colonies were not stained was with river, lake, pond and marsh samples when smears would be made from the incubated broths after vortexing. A third difference is that only environmental samples were cultured and/or stained in the current experiments while the other studies were concerned only with clinical specimens.

H. SPECIES IDENTIFICATION

After a colony was determined to be acid-fast it was cultured on a slant of Middlebrook 7H10 agar and sent to the Mycology and Mycobacteriology Unit of the Kansas Department of Health and Environment where Mr. Dwight Huntley and Ms. Beverly Spring identified the organisms to species.

I. CONTROL EXPERIMENTS

Several sets of control experiments were conducted during the investigation. All were performed in basically the same manner.

In the first controls M. gordonae, M. phlei, or M. chelonei were used. The organism was grown in Middlebrook 7H9 broth, either in test tubes or in flasks incubated at ambient temperature on a shaker. The culture was pelleted and washed with SDW three times. It was then resuspended in 10 ml SDW. The contents of one tube was added to one liter of SDW in a sterile collection bottle and mixed. A second tube was added and the appropriate amount of SDW was added. The volume of SDW varied because the total volume was to be 2 liters. If no CPC was

to be added to the bottle, 980 ml SDW would be added. If CPC was to be added, only 953.5 ml SDW was added. This allowed 26.5 ml of CPC to be added to bring the total volume to 2 liters. In each control experiment a bottle containing an acid-fast organism in only SDW and a bottle containing an acid-fast organism in SDW plus CPC were set up and filtered for each dilution used. Plate counts were also performed to determine the actual number of organisms added to each bottle. CPC was added immediately after the bottle was set up, or the bottle was stored in the cold to prevent multiplication of the organisms.

In the control experiments using formaldehyde there was a slight alteration in method. The organisms used included M. chelonei, M. fortuitum and M. gordonae. The organisms were grown in 7H9 broth at ambient temperature on a shaker to allow for thorough aeration and mixing of the organisms. Once sufficient growth was achieved the organisms were washed 3 times in 30 ml SDW. The M. chelonei and M. fortuitum achieved turbid growth in 3 days, while the M. gordonae required 12 days on the shaker for turbid growth to occur. The organisms were then resuspended in 20 ml SDW and allowed to set for a minimum of 1 week and a maximum of 11 months to acclimate to the water. This change was made after reading the methods used by in a study at the Centers for Disease Control (Carson et al. 1988).

RESULTS

A. Cetylpyridinium Chloride As The Agent For Decontamination.

Cetylpyridinium chloride was used as a decontaminating agent in several sets of experiments. In one set of experiments using Middlebrook 7H10 (7H10), the plates were incubated at 37 C and 25 C. In a smaller set of experiments the same temperatures were used, but the samples were put on both Lowenstein-Jensen (L-J) agar and Middlebrook 7H10 (7H10) agar and one plate of each was incubated at each temperature. In another set of experiments the plates were incubated only at 25 C, but each sample was placed on both L-J agar and 7H10 agar and one plate of each medium was incubated in a normal atmosphere and one plate of each medium was incubated in 5 % CO_2. In the following tables the medium used will be specified. Those samples that contained excessive amounts of foreign matter were pelleted and resuspended in Middlebrook 7H9 broth (7H9) in all sets of experiments.

A total of 61 samples contained 11 positive samples. No Mycobacteria was isolated from any of the samples using both L-J and 7H10. Mycobacteria was isolated from only one 7H9 sample. Table two shows that the number of positive samples varied from one to three depending on sample type, and that positive samples varied from 0 to 50 % of the total samples of a specific type. However, Mycobacteria was isolated from 18 % of the overall samples. All samples used in these experiments are identified in Appendix A.

A total of three M. fortuitum and eight M. chelonei were isolated from the 61 samples. The M. fortuitum took from 12 to 28 days for growth on initial isolation. The M. chelonei required five to 37 days

for initial growth. The results shown in Table three tend to suggest that M. chelonei is more widespread in the waters tested than M. fortuitum. Table four shows the species isolated from specific samples.

Table 2. Cetylpyridinium Chloride as Decontaminating Agent

SAMPLE TYPE	NO. SAMPLES	NO. POSITIVE*	% POSITIVE
Distilled	3	1	33.33
Tap	22	2	9.09
Pond	6	1	16.67
Well	10	3	30.00
River	2	1	50.00
Lake	10	1	10.00
Marsh	5	0	0
Bottled	3	2	66.67
Total	61	11	18.03

* All positive samples used Middlebrook 7H10 medium.

Table 3. Isolates by Sample Type and Temperature

CPC Experiments
Middlebrook 7H10

Mycobacterium fortuitum

SAMPLE TYPE	TEMPERATURE	DAYS TO INITIAL GROWTH
Well	37 C	28
	25 C	15
Lake	37 C	12

Mycobacterium chelonei

SAMPLE TYPE	TEMPERATURE	DAYS TO INITIAL GROWTH
Distilled*	37 C	5
Tap	25 C	18 and 29
Pond	25 C	15
Well	25 C	15
River	25 C	**
Bottled	25 C	27 and 37

* No decontamination was performed. No mycobacteria was isolated from a later sample from the same source decontaminated with CPC.

** Isolated in 7H9 only after eight weeks incubation, but time required for growth is unknown.

Table 4. Mycobacteria Isolated By Sample - CPC Experiments
Middlebrook 7H10

SAMPLE	ORGANISM ISOLATED	GROWTH CONDITIONS	DAYS TO INITIAL GROWTH
Dr	M. chelonei	25 C	5
T7	M. chelonei	25 C	18
T8	M. chelonei	25 C	29
P4	M. chelonei	25 C	15 and *
W1	M. fortuitum	37 C	28
W1B	M. fortuitum	25 C	15
W2B	M. chelonei	25 C	15
R2x	M. chelonei	25 C	*
L1	M. fortuitum	37 C	12
B1	M. chelonei	25 C	27 and 37
B2	M. chelonei	25 C	27

* Grown in Middlebrook 7H9 broth which was incubated for eight weeks before attempts were made to recover any organisms.

It is interesting to note that one well sample provided two colony types of M. chelonei after 15 days incubation. However, one tap sample had two colony types which were both identified as M. chelonei, but one was isolated after 18 days of incubation while the other required 29 days for growth. One sample of commercially bottled water also contained two colony types of M. chelonei, but one was isolated after 27 days and one after 37 days incubation. These observations point out the importance of weekly inspection of plates and for noting the colonies present at each inspection when evaluating water for the presence of these organisms.

False positives were detected in four samples. A pond sample and

a marsh sample each contained two false positives. A well sample and a lake sample contained one false positive each. This is shown in Table five.

Table 5. Samples Showing False Positives - CPC Experiments

SAMPLE	GROWTH CONDITIONS
P1B*	25 C in 7H9 broth
L2x	25 C in 7H9 broth
M1x*	25 C in 7H9 broth
W3	37 C on 7H10 plate

* Each of these samples yielded two false positive organisms.

One reason that some isolates may have been missed is that many plates were lost to mold and/or bacterial contamination. Table six shows that 15 to 50 % of the plates were lost to mold or bacteria depending on the sample type. Overall almost one quarter of the plates were lost to contamination before eight weeks incubation could be completed. Table seven shows that the average number of days the various sample types were incubated before being lost to contamination varied from 13 to 43 days. Table eight demonstrates that incubation at 37 C allowed for longer incubation times before loss due to contamination than did incubation at 25 C. In fact, incubation at 37 C allowed for 44.6 % to 73.2 % of the intended eight weeks of incubation to occur, while 25 C incubation allowed only 14.2 % to 44.6 % of the intended eight week incubation period to pass before contamination occurred. Table nine shows that little difference occurred in the

length of time before contamination prevented further incubation in respect to the type of atmosphere and/or the medium used. Table 10 shows that in experiments where only 7H10 was used that some Mycobacteria were isolated from plates that were later lost to contamination. The plates from which isolates were recovered were incubated from 13 to 43 days.

Table 6. Mold and Bacterial Contamination - CPC Experiments

SAMPLE TYPE	NO. PLATES	NO. PLATES LOST	% PLATES LOST
Distilled	6	1	16.67
Tap	52	8	15.38
Pond	12	6	50.00
Well	22	4	18.18
River	4	1	25.00
Marsh	12	3	25.00
Bottled	6	3	50.00
Total	138	32	23.19

Table 7. Average Incubation Time in days of Samples Lost to Mold and Bacterial Contamination - CPC Experiments

SAMPLE TYPE	37 C	25 C
Distilled	13	*
Tap	36	32.86
Pond	33	28.50
Lake	32	17.00
Well	34	16.00
River	*	18.00
Marsh	25	19.67
Bottled	*	43.00

* No plates were lost.

Table 8. Incubation time in Days of Samples Lost to Mold or Bacteria on L-J and 7H10 at Different Temperatures

CPC Experiments

Sample	37 C		25 C	
	L-J	7H10	L-J	7H10
Tap 17				25
Lake 8	41		11	11
Well 7			8	
Marsh 2			17	
Marsh 3			17	
Marsh 4	25		19	25
Average	33		13.25	20.33

No Mycobacteria was isolated from any plates in this series of experiments.

Table 9. Incubation time in Days of Samples Lost to Contamination on L-J and 7H10 in Normal Atmosphere and 5 % CO_2 at 25 C - CPC Experiments

SAMPLE	NORMAL		CO_2	
	L-J	7H10	L-J	7H10
Tap 19	10		10	
Tap 20	15	15	15	15
Tap 21	45	53		15
Tap 22	7		47	
Lake 9	10	10	10	10
Lake 10	14	14	14	14
Well 8	8	8	8	8
Marsh 5	8	8	8	8

No Mycobacteria was isolated from any plate in this series of experiments.

Table 10. Incubation Time in Days of Samples Lost to Contamination at 37 C and 25 C - CPC Experiments

SAMPLE	37 C	25 C	MYCOBACTERIA ISOLATED
Distilled	13		M. chelonei at 25 C
Tap 2		32	No
Tap 3	36		No
Tap 4		22	No
Tap 5		50	No
Tap 8		38	M. chelonei at 25 C
Tap 12		28	No
Tap 14		35	No
Pond 1	39	33	No
Pond 2		27	No
Pond 4	27	27	M. chelonei at 25 C
Pond 5		27	No
Lake 1		23	M. fortuitum at 37 C
Lake 2		23	No
Lake 4	23		No
Well 1B		34	M. fortuitum at 25 C
Well 2B	34		M. chelonei at 25 C
Well 5		6	No
River 2		18	M. chelonei at 25 C *
Bottled 1		43	M. chelonei at 25 C
Bottled 2		43	M. chelonei at 25 C
Bottled 3		43	No

* Isolated from 7H9 broth, not from filters on plates.

It should also be noted that some acid-fast organisms had been found upon staining that were lost to mold, contaminating bacteria or drying of the medium as isolates. Some could not be isolated. These included two isolates from commercially bottled water samples lost to molds, and one from the same type of source that dried out. An isolate from one sample of distilled water was lost because of mold

contamination.

In the experiments where 7H10 plates were inoculated with portions of the 7H9 broth cultures several were lost due to mold, contaminating bacteria or drying of the medium. One such sample each of a pond, river and marsh were lost to mold. These plates were incubated for 13 days, four days and eight days, respectively. Two lake samples showed acid-fast organisms upon staining, but no such organism could be isolated.

B. Formaldehyde (HCHO) as the Agent For Decontamination.

In the experiments using formaldehyde as the decontaminating agent all sample filters were placed on 7H10 agar with added penicillin G and amphotercin B. One filter of each sample was incubated in a 25 C incubator with a normal atmosphere. The other filter of each sample was incubated in 5 % CO_2 at ambient temperature. The room temperature varied from 22 C to 33 C, but was generally between 24 C and 27 C.

A total of 67 samples contained 20 positive samples. Eight of the 20 samples contained two or more species of Mycobacteria. Table 11 shows that Mycobacteria were isolated from 10 % to 38 % of the samples depending on sample type, and that almost 30 % of the total samples yielded acid-fast organisms. It is tempting to compare this 30 % of the total samples to the 18 % of the samples in the experiments using CPC as a decontaminating agent. However, since different kinds of water sources were evaluated in the earlier experiments those results must be adapted to compare with those where HCHO was the decontaminating agent. By considering only the distilled, tap, well and bottled samples tested in the procedure using CPC as the decontaminating agent the percent of

the total samples positive increases to 21 %. This is still significantly lower than the percent positive achieved by using HCHO as the decontaminating agent.

Table 11. Formaldehyde as Decontaminating Agent

SAMPLE TYPE	NO. SAMPLES	NO. POSITIVE	% POSITIVE
Taps	24	9	37.5
Drinking fountains	23	7	30.4
Bottled	6	2	33.3
Wells	10	1	10.0
Other	4	1	25.0
	67	20	29.85

C. Isolates Recovered With HCHO as the Decontaminating Agent.

A total of 36 isolates were recovered. These included nine M. gordonae, seven M. fortuitum, five each of M. chelonei and M. szulgai, three chromogenic Group IV unidentified species, two M. avium complex, one M. scrofulaceum and an unidentifed Group III species. Occasionally more than one colony type of a given species was present, but these were considered as a single isolate when identified as the same species. Table 12 shows that although M. gordonae was present in the greatest number of samples, M. fortuitum was present in the greatest number of sample types. The isolations of M. chelonei and M. szulgai were quite similar to each other in both the number of isolations and the number of sample types. Other Mycobacteria were occasionally isolated. Table 13 shows species isolated by specific sample and type of atmosphere, and the days required to obtain initial

Table 12. Mycobacteria Isolated by Sample Type and Atmosphere

HCHO Experiments

SAMPLE TYPE	ATMOSPHERE	DAYS TO INITIAL GROWTH
Mycobacterium fortuitum		
Tap	Normal	42, 42 and 43
Drinking fountain	Normal	39 and 42
Bottled	Normal	53
Well	5 % CO_2	23
Mycobacterium chelonei		
Tap	Normal	42 and 43
	5 % CO_2	10
Bottled	Normal	23
	5 % CO_2	23
Mycobacterium gordonae		
Tap	Normal	42 and 43
	5 % CO_2	36, 39 and 42
Drinking fountains	Normal	43
	5 % CO_2	39, 40, 43, 57

Table 12. continued

Mycobacterium szulgai

SAMPLE TYPE	ATMOSPHERE	DAYS TO INITIAL GROWTH
Taps	Normal	14 and 56
	5 % CO_2	39 and 42
Drinking fountains	Normal	44
	5 % CO_2	57

Mycobacterium scrofulaceum

SAMPLE TYPE	ATMOSPHERE	DAYS TO INITIAL GROWTH
Taps	5 % CO_2	36

Mycobacterium avium complex

SAMPLE TYPE	ATMOSPHERE	DAYS TO INITIAL GROWTH
Taps	5 % CO_2	36
Drinking fountains	5 % CO_2	43

Chromogenic Group IV other

SAMPLE TYPE	ATMOSPHERE	DAYS TO INITIAIL GROWTH
Taps	Normal	36, 43 and 56

Group III other

Drinking fountains	5 % CO_2	39

Table 13. Mycobacteria Isolated by Sample - HCHO Experiments

SAMPLE	ORGANISM ISOLATED	GROWTH CONDITIONS*	DAYS TO INITIAL GROWTH
T1	M. fortuitum	N	43
T4	M. gordonae	N	43
		+	39
	M. chelonei	N	43
		+	10
	Group IV other	N	43
	M. szulgai	+	39
T6	M. fortuitum	N	42
	M. chelonei	N	42
	M. gordonae	N	42
T11	M. scrofulaceum	+	36
	M. avium complex	+	36
T12	M. gordonae	+	36
	Group IV other	N	36
T13	M. szulgai	N	14**
	Group IV other	N	56
T20	M. gordonae	+	42
T21	M. fortuitum	N	42
T23	M. szulgai	N	56
		+	42
U3	M. chelonei***	+	15
W10	M. fortuitum	+	23
B3	M. chelonei	N	23
B5	M. chelonei	+	23
	M. fortuitum	N	53

* N = Normal atmosphere; + = 5 % CO_2

** Five colony types of M. szulgai were isolated from this sample.

*** Identified as M. chelonei subspecies abscessus.

Table 13. continued

SAMPLE	ORGANISM ISOLATED	GROWTH CONDITIONS*	DAYS TO INITIAL GROWTH
F1	M. fortuitum	N	42
F4	M. fortuitum	N	39
	M. szulgai	N	44
	M. gordonae	+	40
	Group III other	+	39
F5	M. gordonae	N	43
		+	39
F6	M. gordonae	N	43
F18	M. gordonae	+	57
	M. szulgai	+	57
F21	M. avium complex	+	43
F23	M. gordonae	+	43

* N = Normal atmosphere; + = CO_2

growth. Incubation necessary for initial growth varied from 14 to 57 days. This points out that although many of the rapid growers can be isolated in shorter incubations, some do require eight weeks of incubation for initial isolation from environmental sources. Table 14 shows that 15 isolates were recovered only by incubating in a normal atmosphere, 14 isolates were recovered only in 5 % CO_2, and only 4 isolates were recovered from both atmospheric types. If a single species was isolated in either one or both types of atmospheres from a single sample it was considered to be a single isolate.

Table 14. Numbers of Each Species Isolated by Atmosphere HCHO Experiments

	ATMOSPHERE			
ORGANISM ISOLATED	N	+	B	TOTAL
M. gordonae	2	5	2	9
M. fortuitum	6	1	0	7
M. chelonei	2	2	1	5
M. szulgai	2	?	1	5
Group IV other	3	0	0	3
M. avium complex	0	2	0	2
M. scrofulaceum	0	1	0	1
Group III other	0	1	0	1
Totals	15	14	4	33

* N = Normal air, + = 5 % CO_2, B = Both Normal and 5 % CO_2

Table 15. HCHO Isolates by Sample and Atmosphere* Types

M. gordonae

SAMPLE TYPE	ATMOSPHERE			TOTAL
	N	+	B	
Tap	1	2	1	4
Fountains	1	3	1	5
Totals	2	5	2	9

M. fortuitum

SAMPLE TYPE	ATMOSPHERE			TOTAL
	N	+	B	
Taps	3	0	0	3
Wells	0	1	0	1
Bottled	1	0	0	1
Fountains	2	0	0	2
Totals	6	1	0	7

M. chelonei

SAMPLE TYPE	ATMOSPHERE			TOTAL
	N	+	B	
Taps	1	0	1	2
Bottled	1	1	0	2
Other (distilled)	0	1	0	1
Totals	2	2	1	5

M. szulgai

SAMPLE TYPE	ATMOSPHERE			TOTAL
	N	+	B	
Taps	1	1	1	3
Fountains	1	1	0	2
Totals	2	2	1	5

* N = Normal atmosphere, + = 5 % CO_2, B = Both Normal and 5 % CO_2.

Table 15. Continued

Group IV Other

SAMPLE TYPE	ATMOSPHERE*			TOTAL
	N	+	B	
Taps	3	0	0	3

M. avium complex

SAMPLE TYPE	ATMOSPHERE*			TOTAL
	N	+	B	
Taps	0	1	0	1
Fountains	0	1	0	1
Totals	0	2	0	2

M. scrofulaceum

SAMPLE TYPE	ATMOSPHERE*			TOTAL
	N	+	B	
Taps	0	1	0	1

Group III Other

SAMPLE TYPE	ATMOSPHERE*			TOTAL
	N	+	B	
Fountains	0	1	0	1

* N = Normal atmosphere, + = 5 % CO_2, B = Both Normal and 5 % CO_2.

As in the CPC experiments, many plates in the HCHO experiments were lost to mold and overgrowth by sporeforming bacteria. Table 16 shows that over half of the plates originally inoculated were lost due to contamination. Table 17 shows that plates incubated in a normal atmosphere could be incubated almost one week longer than those incubated in 5 % CO_2. Table 18 indicates that only five plates lost to

contamination yielded Mycobacteria. One plate of the samples T1, T11 and F18 were lost, but Mycobacteria were recovered from the uncontaminated plate of those samples. The contaminated plates were incubated for 43, 11 and 22 days, respectively. However, it is unknown whether Mycobacteria would have grown on the contaminated plates if they could have been incubated longer because as shown in Table 14 most isolates were recovered in only one type of atmosphere.

Table 16. Mold And Bacterial Contamination - HCHO Experiments

SAMPLE TYPE	NO. PLATES	NO. PLATES LOST	% PLATES LOST
Taps	48	23	47.92
Fountains	46	18	39.13
Wells	20	20	100.00
Other	8	6	75.00
Bottled	12	2	16.67
Total	134	69	51.49

Table 17. Average Incubation Time In Days of Samples Lost to Contamination - HCHO Experiments

SAMPLE TYPE	ATMOSPHERE	
	NORMAL	CO_2
Taps	25.3	13.7
Fountains	26.3	13.9
Wells	16.3	16.6
Other	19.0	26.0
Bottled	15.0	*
Total	22.05	15.87

* No plates were lost.

Table 18. Incubation time in Days of Samples Lost to Contamination.

HCHO Experiments

SAMPLE	ATMOSPHERE[1]	REASON[2]	DAYS INCUBATION	ISOLATES
T 1	N	B	43	*
T 2	N	M	43	
	+	M	11	
T 3	N,+	M	12	
T 4	N	B,M	39	M. chelonei
				M. gordonae
		Group IV other		
T 5	N	M	42	
	+	M	10	
T 6	N	B	42	M. chelonei
				M. fortuitum
				M. gordonae
	+	M	10	
T 7	N	M,B	15	
	+	M,B	11	
T 8	N	M,B	15	
	+	M,B	11	
T 9	+	M,B	11	
T10	N	M,B	11	
T11	N	M	10	**
T15	N,+	M	12	
T16	+	M	24	
T17	N,+	M	25	
T18	N	M	20	
W 1	N,+	M	9	
W 2	N,+	M	9	
W 3	N,+	M	9	
W 4	N	M	15	
	+	M	7	
W 5	N,+	M	25	
W 6	N,+	M	25	
W 7	N,+	M	25	
W 8	N,+	B,M	17	
W 9	N,+	B,M	17	
W10	N	M	12	
	+	B,M	23	M. fortuitum
U 1	N,+	M	25	
U 2	N	B	10	
	+	M	10	
U 3	+	B,M	43	
U 4	N	M	22	

Table 18. Continued

SAMPLE	ATMOSPHERE[1]	REASON[2]	DAYS INCUBATION	ISOLATES
F 1	N	M	44	M. fortuitum
	+	M	12	
F 3	N	M	44	
	+	M	12	
F 6	N	B	44	M. gordonae
	+	M	11	
F 7	N	B,M	15	
	+	B	11	
F 8	N,+	B	11	
F 9	+	M	23	
F10	N	M	16	
	+	M	9	
F13	N	M	43	
F14	N	M	15	
F16	N	M	9	
F18	N	M	22	***
F19	+	M	22	
B 1	N	M	15	
B 6	N	M	15	

1. N = Normal atmosphere; + = 5 % CO_2

2. B = Contaminating Bacteria; M = Mold

* M. fortuitum was isolated from the CO_2 plate of sample T1.

** M. scrofulaceum and M. avium complex were isolated from the CO_2 plate of sample T11.

*** M. gordonae and M. szulgai were isolated from the CO_2 plate of sample F18.

D. Samples Decontaminated with Both CPC and HCHO.

Seven sources that were sampled during the CPC experiments were again sampled in the HCHO experiments. It can be seen in Table 19 that one tap sample that yielded no Mycobacteria in the CPC experiments yielded M. szulgai and a chromogenic Group IV species when decontaminated with HCHO. Two repeated tap samples failed to yield any Mycobacteria with either decontaminating agent.

The repeated well sample yielded M. chelonei with CPC decontamination (LC-W2), but M. fortuitum was isolated with the HCHO decontamination (PW10). However, M. fortuitum was found in a sample of the same well taken upstream the home filter and decontaminated with CPC (LC-W1). Therefore, it may have also been present in the sample taken downstream the filter (LC-W2), but missed being isolated because of the similar colonial morphology of the the two species. It is also feasible that M. chelonei was present on the PW10 filter and was not isolated for the same reason.

One bottled water sample (LC-B1) yielded M. chelonei with CPC decontamination, but the same source (PB2) yielded no acid-fast organisms when decontaminated with HCHO (PB2). Another bottled water sample (LC-B2) yielded M. chelonei when decontaminated with CPC, but both M. chelonei and M. fortuitum when HCHO was the decontaminating agent. Again, both organisms could have been present on the LC-B2 filter and one was missed because of the similarities in colonial morphology. Another bottled water sample yielded no Mycobacteria when either CPC (LC-B3) or HCHO (PB1) was used. One source of distilled water yielded M. chelonei when either CPC (LC-D2) or HCHO (PU3) was used.

Table 19. Samples Repeated in CPC and HCHO Experiments and the Mycobacteria Species Isolated

CPC	HCHO
LC-T2	PT13
NONE	M. szulgai
	Chromogenic group IV other
LC-T6	PT10
NONE	NONE
LC-T11	PT16
NONE	NONE
LC-W2	PW10
M. chelonei	M. fortuitum
LC-B1	PB2
M. chelonei	NONE
LC-B2	PB5
M. chelonei	M. chelonei
	M. fortuitum
LC-B3	PB1
NONE	NONE
LC-D2	PU3
M. chelonei	M. chelonei

E. Acetic Acid Experiment.

Organisms isolated with the acetic acid decontamination were not identified except as being acid-fast with the modified Kinyon stain procedure. Because no identification was attempted false positives possibly occurred. The samples used for this experiment are listed in Appendix C. All samples were originally taken during the CPC experiments. Appendix A contains further information on each of the samples used.

A total of 4 samples out of 48 samples tested yielded acid-fast bacteria. A total of nine acid-fast colony types were isolated. Sample R1 yielded one colony type from the 25 C tube and two colony types from the 37 C tube. These may have actually been one species or they may have been different species. Sample R2 yielded 4 colony types from the 37 C tube, but no acid-fast organisms were found in the 25 C tube. It should be noted that in the CPC experiments the 25 C Middlebrook 7H9 tube of sample R2 yielded M. chelonei. Again all isolates could have been the same species or different species.

None of the samples that produced false positives in the CPC experiments yielded any acid-fast organisms after the acetic acid procedure. This suggests that acetic acid may eliminate at least some false positives.

Sample T17 from the 25 C tube and sample L6 from the 37 C tube yielded mixed results upon staining. However, further attempts at isolation did not produce more than one colony type. This could be due to the fact that some environmental isolates of Mycobacteria are weakly acid-fast. Some isolates in previous studies that were later identified as Mycobacteria demonstrated similar staining appearances.

Tubes of known Mycobacteria that had been stored in the cold for varying lengths of time were used as controls. These are listed in Appendix C. Growth of acid-fast organisms occurred on all plates from these tubes. No plate counts were done since the original viable populations of the tubes were unknown.

The results of this experiment suggest that the use of acetic acid as a decontaminating agent, at least in samples with excessive amounts

of foreign material, may be a useful technique. Further studies are necessary to determine if acetic acid would also be useful with cleaner samples such as tap water, drinking fountains, etc. It is also unknown what species of Mycobacteria can survive exposure to acetic acid and what exposure times produce the best results.

Table 20. Acid-fast Organisms Isolated Using Acetic Acid as the Decontaminating Agent

SAMPLE	ORIGINAL TEMPERATURE	NOTES
T17	25 C	Mixed stain results
L 6	37 C	Mixed stain results
R 1A	25 C	Rough *
R 1B	37 C	Rough
R 1C	37 C	Smooth
R 2A1	37 C	Pinpoint colony, medium sized rods **
R 2A2	37 C	Small colony, large rods ***
R 2B1	37 C	Pinpoint colony, medium rods
R 2B2	37 C	Small colony, medium rods

* Sample R1 may contain only one species with different colony types present.

** Sample R2 at first yielded a small, smooth colony (R2A) and a medium sized smooth colony (R2B). When streaked for isolation each colony produced two colony types. Judging by microscopic appearances R2A1, R2B1 and R2B2 may be the same organism.

*** R2A2 consists of much larger cells than the other isolates and may be a false positive.

F. Control Experiments

1. CPC Control Experiments

In the control experiment in which M. phlei was used, the organisms were passed through an 18 gauge needle in an attempt to put them into suspension and prevent the organsims from adhering to each other. No acid-fast organisms were recovered from the bottles containing M. phlei in SDW plus CPC. Filtrations were done of 50 ml and 900 ml and filters of each volume were incubated at 25 C and 37 C. All 25 C plates were lost to mold. The 900 ml plate of M. phlei in SDW only yielded 5 acid fast colonies and one mold colony. It was estimated that the entire 2 liter volume contained 100 organisms, or 0.05 organisms per ml. This sample was expected to yield 45 colonies from 900 ml.

Another control experiment using M. chelonei was performed. Again the bottles containing CPC failed to yield any acid-fast colonies. The plate count showed that 1.6×10^4 bacteria were added to each bottle. The bottle without CPC was TNTC. The total count was estimated by averaging the number of colonies on 10 randomly selected squares on the filter and multiplying by 166 squares. This gave a count of 4150 CFU/filter or 4.15 CFU/ml. This was a recovery of only 0.023% of the seeded organisms.

Another control experiment was performed using organisms that had been acclimated to water. Bottles were seeded with 36 CFU/ml and with 8 CFU/ml. The results are shown in Table 21. The addition of CPC apparently prevented the recovery of many organisms. Table 22 shows the percentage of organisms recovered. The filters having the highest

initial input (36 CFU/ml) yielded recoveries from 100 % to a low of 93.3 %, while lower inputs (8 CFU/ml) yielded recoveries ranging from 42 % to 52 %.

Table 21. CPC Control Experiment

SEEDED TO CONTAIN 36 CFU/ml			
AMOUNT FILTERED	EXPECTED COUNT	CPC COUNT*	NO CPC COUNT**
500 ml	1.8×10^4	1	TNTC***
250 ml	9.0×10^3	3	8.8×10^3
125 ml	4.5×10^3	0	4.2×10^3
50 ml	1.8×10^3	1	1.8×10^3
SEEDED TO CONTAIN 8 CFU/ml			
AMOUNT FILTERED	EXPECTED COUNT	CPC COUNT	NO CPC COUNT
500 ml	4.0×10^3	0	2.08×10^3
250 ml	2.0×10^3	0	8.87×10^2
125 ml	1.0×10^3	0	4.20×10^2
50 ml	4.0×10^2	0	1.92×10^2

* Average of plates filtered from bottle to which CPC was added.

** Average of plates filtered from bottle to which CPC was not added.

*** TNTC = Too numerous to count.

Table 22. Per Centage of Organisms Recovered - CPC Control
No CPC Added

AMOUNT FILTERED	% SEEDED ORGANISMS RECOVERED	
	8 CFU/ml	36 CFU/ml
500 ml	52.0	*
250 ml	44.4	97.8
125 ml	42.0	94.2
50 ml	48.0	100.0

* The colonies on this plate were too numerous to count and an accurate estimation of the colonies could not be made due to solid growth in some areas of the plate.

A final control experiment was performed using CPC. The organisms used in this experiment had been acclimating at ambient temperature in SDW for over 11 months. The following volumes were filtered without decontamination: 10 ml, 50 ml, 100 ml and 250 ml. Four filtrations were made of each dilution at 10 ml and 50 ml volumes, and two filtrations were made at 100 ml and 250 ml of each dilution. Two filtrations were made of each of the following volumes when CPC was used as the decontaminating agent: 50 ml, 100 ml, 250 ml and 500 ml. All plates that were filtered without decontamination were too numerous to count and the population could not be estimated. From the bottle seeded to contain 2.6×10^3/ml without decontamination the following contamination occurred: one 50 ml filter had two red bacterial colonies, one 100 ml filter had a fungal colony and one 250 ml filter was lost due to mold contamination. From the bottle seeded to contain 2.6×10^2/ml without decontamination the following contamination

occurred: one 50 ml and one 100 ml filters were lost due to mold. The only contaminate to occur in the bottles decontaminated with CPC occurred on a 50 ml filter from the bottle seeded to contain 2.6 x 10^3/ml. The filter was countable, but did have a mold colony present. Table 23 shows that generally less than one percent of the expected counts were recovered. The 50 ml filters seeded made from bottles seeded to contain 2.6 x 10^2 yielded 68 CFU and 929 CFU, thus ranging from .52 % to 7.1 % of the expected count. Due to this high discrepancy in the number of CFU recovered these plates were considered to have little validity.

Table 23. Control Experiment - CPC Used as Decontaminating Agent

SEEDED TO CONTAIN 2.6×10^3			
AMOUNT FILTERED	EXPECTED COUNT	AVERAGE COUNT	% RECOVERED
500 ml	1.3×10^6	5.14×10^2	0.04
250 ml	6.5×10^5	2.64×10^2	0.04
100 ml	2.6×10^5	1.13×10^2	0.04
50 ml	1.3×10^5	6.20×10^1	0.05
SEEDED TO CONTAIN 2.6×10^2			
AMOUNT FILTERED	EXPECTED COUNT	AVERAGE COUNT	% RECOVERED
500 ml	1.3×10^5	2.5×10^1	0.02
250 ml	6.5×10^4	1.7×10^1	0.03
100 ml	2.6×10^4	4.0×10^0	0.02
50 ml	1.3×10^4	$5.0 \times 10^{2*}$	3.84*

* These figures may be misleading. One plate contained 68 colonies, while the other contain 929 colonies.

2. HCHO Control Experiments

The first control experiment using M. chelonei that was acclimated to SDW and HCHO yielded the confusing results shown in Table 24. As shown in Table 24 almost the same number of organisms were recovered from 550 ml and from 31.25 ml with the former yielding 623 CFU and the latter yielding 601. The issue was clouded further in that 250 ml, 125 ml and 62.5 ml filters yielded counts of 1320, 1190, and 1000 CFU respectively. Why the percentage of organisms recovered decreased as

the volume increased is unknown. However, the smaller volumes were filtered first, thus leaving the organisms in the larger volumes exposed to the HCHO for a longer period of time.

Plate counts of acclimated M. chelonei were performed and another set of experiments were conducted. Bottles were seeded to contain 66.8 CFU/ml and 8.4 CFU/ml. The results of this experiment are shown in Tables 25, 26 and 27. It is shown in Table 25 that the percentage of organisms recovered decreased as the volume increased, as had occurred in the previous experiment. Table 26 indicates that the percentage of organisms recovered increased as the volume increased when no decontamination was used. This lends support to the hypothesis that exposure time to the HCHO has an effect on the number of organisms recovered. Table 27 compares the number of CFU recovered with and without decontamination. Only the 50 ml filter from the bottle seeded with 66.8 CFU/ml yielded a higher count with decontamination than without. The 50 ml filter from the bottle seeded with 8.4 CFU/ml yielded 96.21 % of the number of CFU recovered without decontamination. However, with decontamination 127 CFU were recovered while 132 were recovered without decontamination. A difference of only 5 CFU in 50 ml is probably not significant. In all other cases the lack of decontamination greatly improved the recovery of Mycobacteria.

Table 24. First HCHO Control Experiment

AMOUNT FILTERED	EXPECTED COUNT	ACTUAL COUNT	% RECOVERED
1000 ml	3.77×10^5	TNTC*	unknown
500 ml	1.89×10^5	6.23×10^2	0.32
250 ml	9.43×10^4	1.32×10^3	1.40
125 ml	4.71×10^4	1.19×10^3	2.53
62.5 ml	2.36×10^4	1.00×10^3	4.24
31.25 ml**	1.18×10^4	6.01×10^2	5.09

* TNTC = Too numerous to count.

** This was an approximate measurement in that the hundredths were estimated.

Table 25. Second HCHO Control Experiment - HCHO Used

SEEDED TO CONTAIN 66.8 CFU/ml

AMOUNT FILTERED	EXPECTED COUNT	AVERAGE COUNT	% RECOVERED
1000 ml	6.68×10^4	7.01×10^2	1.05
500 ml	3.34×10^4	1.57×10^3	4.70
250 ml	1.67×10^4	4.07×10^3	24.37
50 ml	3.34×10^3	2.02×10^3	60.48

SEEDED TO CONTAIN 8.4 CFU/ml

AMOUNT FILTERED	EXPECTED COUNT	AVERAGE COUNT	% RECOVERED
1000 ml	8.4×10^3	2.68×10^2	3.19
500 ml	4.2×10^3	7.68×10^2	18.29
250 ml	2.1×10^3	5.10×10^2	24.29
50 ml	4.2×10^2	1.27×10^2	30.24

Table 26. Second HCHO Control Experiment - No Decontamination

SEED TO CONTAIN 66.8 CFU/ml

AMOUNT FILTERED	EXPECTED COUNT	AVERAGE COUNT	% RECOVERED
1000 ml	6.68×10^4	TNTC	unknown
500 ml	3.34×10^4	TNTC	unknown
250 ml	1.67×10^4	9.43×10^3	56.44
50 ml	3.34×10^3	1.51×10^3	45.33

SEEDED TO CONTAIN 8.4 CFU/ml

AMOUNT FILTERED	EXPECTED COUNT	AVERAGE COUNT	% RECOVERED
1000 ml	8.4×10^3	3.88×10^3	46.13
500 ml	4.2×10^3	1.45×10^3	34.57
250 ml	2.1×10^3	***	unknown
50 ml	4.2×10^2	1.32×10^2	31.43

*** These plates were contaminated and no count could be taken.

Table 27. Second HCHO Control Experiment - Comparison of Organisms Recovered With and Without Decontamination With HCHO

SEEDED TO CONTAIN 66.8 CFU/ml

AMOUNT FILTERED	HCHO COUNT	NO HCHO COUNT	HCHO/WITHOUT HCHO
1000 ml	7.01×10^2	TNTC	unknown
500 ml	1.57×10^3	TNTC	unknown
250 ml	4.07×10^3	9.43×10^3	43.18 %
50 ml	2.02×10^3	1.51×10^3	133.42 %

SEEDED TO CONTAIN 8.4 CFU/ml

AMOUNT FILTERED	HCHO COUNT	NO HCHO COUNT	HCHO/WITHOUT HCHO
1000 ml	2.68×10^2	3.88×10^3	6.92 %
500 ml	7.68×10^2	1.45×10^3	52.89 %
250 ml	5.10×10^2	**	unknown
50 ml	1.27×10^2	1.32×10^2	96.21 %

** Plates lost to contamination, no counts could be made.

DISCUSSION

There are several discrepancies among the results of the current experiments and those of previous works. Du Moulin and Stottmeier (1978) used 5 day old cultures grown in Middlebrook 7H9 to seed one liter samples of sterile water. The samples were then treated with 0.04 % CPC, allowed to stand at ambient temperature for 24 hours and then filtered. When this lab used the same procedure no Mycobacteria (M. chelonei, M. fortuitum, M. gordonae) were recovered. Carson et al (1988) grew their organisms on agar, and then harvested, washed and suspended them in autoclaved water. The organisms were diluted after one week and left to adapt to the water for four to five weeks before being used as inocula. This method was modified by this lab and met with some success. The organisms were grown in Middlebrook 7H9. The organisms were pelleted, washed and resuspended in sterile distilled water (SDW). The organisms were left for several weeks at ambient temperature to acclimate to the water before being used to seed samples of SDW. This allowed for the recovery of some of the seeded organisms.

Du Moulin and Stottmeier (1978) used a 24 hour exposure to 0.04 % CPC and recovered only 8.4 % of the seeded M. gordonae. Carson et al (1988) exposed the sample to 25 micrograms/ml (0.00025 %) CPC for 10 minutes and retrieved 96.3 % of the seeded M. gordonae. Using the above techniques respectively, Du Moulin and Stottmeier (1978) retrieved only 1.1 % of seeded M. fortuitum while Carson et al (1988) recovered 94.8% of that organism. The current work tends to agree with the findings of Du Moulin and Stottmeier (1978) since no M. gordonae was recovered during the experiments using CPC as the decontaminating agent and only

18 % of the samples tested in those experiments were positive for Mycobacterium.

The control experiments performed in this thesis differ greatly from the results claimed by Du Moulin and Stottmeier (1978). Organisms that were seeded into untreated SDW were recovered at rates ranging from 42 % to 102 %. However, the same organisms failed to be recovered when they were seeded into SDW and treated with CPC. Even experiments using acclimated organisms generally yielded less than 1 % of the seeded organisms.

The control experiments using HCHO yielded much better results. However, these experiments were not as efficient as those reported by Carson et al. (1988). One apparent reason for at least some of the decrepancies between the findings of Carson et al. (1988) and this thesis was filtering time. The samples filtered first from a sample bottle generally provided a much better recovery rate than those filtered later. In the experiments by Carson et al. (1988) one bottle was apparently used for each sample filtered. In this thesis a two liter bottle of sample provided two to eight filters of that sample. Filtering was begun after five minutes exposure to HCHO, however, the last of the sample may have been exposed to the decontaminant for 20 to 30 minutes. This was due to the time it took to filter the water, rinse the filter three times, place the filter on a plate, aseptically prepare a new filter, and to repeat this procedure as many times as necessary. If the water filtered rapidly and larger volumes were filtered so that less time was required for handling the filters and setting up the equipment, a higher recovery rate was noted than if a

large number of small volume filtrations were performed. The results of control experiments in this thesis suggest that there is a rapid kill rate of some Mycobacteria after prolonged exposure to HCHO. This had a definite effect on the outcome of control experiments, and undoubtedly also affected other experimental samples. In theory the recovery of Mycobacteria from drinking water samples could be increased by using separate collection bottles for each filtration, and thereby decreasing the exposure time of the organisms to HCHO.

When one first examines the results of the second HCHO control experiment the organisms recovered varied from 1.05 % to 60.48 % of the expected counts. However, when the number of organisms recovered with decontamination with HCHO is compared to the number of organisms recovered with no decontamination the results are much more efficient. The bottles seeded with 66.8 CFU/ml yielded an average recovery rate of 22.65 % of the seeded organisms when HCHO was used. When no decontamination was used the countable plates showed a recovery rate of 51 %. However, when the number of organisms recovered with HCHO is compared to those recovered without formaldehyde the decontaminated samples yielded 43 % to 133 % of the organisms recovered without decontamination. In the bottles seeded with 8.4 CFU/ml the decontaminated bottles yielded an average of 19 % of the organisms recovered, while the undecontaminated samples provided an average recovery rate of 37.4 %. When the number of recovered organisms from decontaminated and undecontaminated samples are compared, the use of HCHO still allowed recovery rates varying from 7 % to 96 %. The large discrepancy in recovery rates is most likely due to the length of time

the samples were actually exposed to the HCHO.

Another difficulty with methods used in this thesis surfaced during the control experiments. In a CPC control experiment two 50 ml filters from the same sample bottle yielded 68 and 929 colonies. This was most likely due to a failure to mix the organisms efficiently before each filtration. The bottles were routinely shaken during filtration, but with a quantity as large as two liters that amount of mixing was probably not very efficient. One would expect for the mixing to improve as the amount left in the bottle to be filtered decreased. However, all samples would be affected by all prior mixing. For instance, if an extra large number of acid-fast organisms were allowed to be filtered after the first shaking, fewer organisms would be available for later filtrations despite any improvement that might occur in the mixing procedure. The opposite could also occur, in that too many organisms might be available for the later filtrations. This would have a profound effect on all plate counts. Since the mixing of each bottle could vary there was no way to adjust for this. The only method that could improve this possible error would be to use several small sample bottles rather than one large bottle.

It is also well known that Mycobacteria tend to adhere to one another. Clumps of organisms formed in this way would also decrease the number of colonies recovered. Therefore, not only do the organisms need to be thoroughly mixed in the SDW, but also with a substance such as Tween 20 to prevent clumping. No experiments were done using Tween, but one would expect better recovery rates, at least when no decontamination is used. The clumping probably protects some organisms

from the CPC or HCHO, so recovery rates may drop due to more organisms being exposed to the decontaminant, while the recovery rate increases due to the separation of aggregations of Mycobacteria into single organisms. Experiments would be necessary to determine the overall effect of the presence of a surfactant. The choice of surfactant might also have an effect. For instance, some species of Mycobacteria, such as M. gordonae, M. malmoense and most nonphotochromogens (Manual of Clinical Microbiology 1985, p. 236), are capable of hydrolyzing Tween 80.

Another possible error that was not tested for in the control experiments is that some of the Mycobacteria probably adhered to the inside of the plastic bottle. It is not known if this was a significant number of organisms, but this researcher believes it most likely did have some effect on yields. The number of organisms that might adhere could also vary due to species. In further experiments this area should be investigated.

One more factor that could greatly affect recovery rates is a difference between laboratory and wild strains of Mycobacteria. The strains of M. gordonae and M. phlei used in control experiments were obtained from the Kansas Department of Health and Environment. The M. chelonei and M. fortuitum used in control experiments were originally recovered from water samples, but had been repeatedly subcultured both in this lab and at the Kansas Department of Health and Environment. Mutations or adaptations may have occurred during laboratory growth so that the cultures were no longer identical to those originally recovered. Wild strains may be adapted to survive in water better than

laboratory strains, even after the domesticated strains have been acclimated to water.

Du Moulin and Stottmeier (1978) claimed that 24 hour exposure to 0.04 % CPC resulted in no survivors among the gram negative species tested (Pseudomonas aeruginosa, Pseudomonas cepacia, Flavobacterium sp., Acinetobacter calcoaceticus). Carson et al (1988) used a mixture of 15 strains of gram negative bacteria commonly found in water distribution systems in hemodialysis centers (Acinetobacter anitratus, Aeromonas hydrophila, Alcaligenes faecalis, Achromobacter xylosoxidans, Bordetella bronchiseptica, Pseudomonas aeruginosa, P. cepacia, P. diminuta, P. maltophilia, P. palleronii, P. paucimobilis, P. pickettii, P. putida, P. stutzeri, and P. thomasii). They found that 99.5 % of these organisms were recovered after a 10 minute exposure to 0.00025 % CPC. Carson et al (1988) also found that a 30 minute exposure to 500 micrograms/ml (0.5 %) or a 15 minute exposure to 1000 micrograms per milliliter (1.0 %) CPC was necessary to achieve 100-fold reductions in gram negative bacteria, but that these concentrations also led to up to 100-fold reductions in some M. chelonei and in M. chelonei-like organisms. In the current experiments filters were infrequently, if at all, contaminated with gram negative bacteria. Gram stains were not performed routinely. However, most, if not all, bacterial contaminants were sporeforming rods and were therefore assumed to be gram positive organisms.

Du Moulin and Stottmeier (1978) stated that in previous work Stottmeier and others found that Pseudomonas aeruginosa and Proteus vulgaris as well as gram positive species were destroyed by 5 hour

exposure to 0.004 % CPC. The original paper (Stottmeier, Woodley and Beam, 1970) was not available, so the gram positive species tested are not known. Since the current work had such gross contamination with sporeformers one is left to wonder if sporeforming rods were tested and/or if young cultures with no spores present were used. Carson et al. (1988) did not test the survival of any gram positive organisms.

In testing hospital water supplies Du Moulin and Stottmeier (1978) reported that only 5 of 54 (9.3 %) of the filters were lost to mold contamination and none were lost to bacterial contamination. In the current work over 23 % (32 of 138) of the plates were lost to mold and bacterial contaminants. Even if only the tap water samples are considered for comparison over 15 per cent (8 of 52) were lost to contamination. Although a few of the plates in our lab may have been contaminated by airborne organisms the majority of the contamination appeared to come from the samples themselves. It is difficult to believe that the waters of Kansas carry that many more species of bacteria and molds than the waters of Massachusetts, or that the species found in Kansas would be more resistant to the detergent than the species found in the northeastern part of the country. Perhaps some work should be done to determine if contaminating bacteria in different parts of the U.S. differ greatly in their resistance to decontamination. If that is the case techniques would have to be modified in various parts of the country. However, these discrepancies are probably due to differences in reporting or simply due to unseen and uncontrollable differences between laboratories.

Some of the calculations by Du Moulin and Stottmeier (1978) are

questionable. They show that an untreated sample of M. intracellulare contained 4.4 x 10^2 CFU/1000 ml and that the sample treated with CPC contained 7.4 x 10^3 CFU/1000 ml of that organism. They call this a 100 % survival rate. If these figures are correct it is actually a 1,682 % survival rate! One must assume that both the untreated and the treated samples were seeded with the same number of organisms. The remainder of their calculations verify this assumption allowing for slight discrepancies due to rounding. If the numbers of CFU were turned around (4.4 x 10^2 for treated and 7.4 x 10^3 for untreated) only 6 % of the organisms survived. If the treated sample CFU should have read 7.4 x 10^2 instead of 7.4 x 10^3 there would still have been a 168 % survival rate. One is left to wonder why M. intracellulare proliferated during exposure to CPC while other organisms apparently did not. Carson et al. (1988) did not test this organism, so no comparison of results can be made.

Although their methods and descriptions seem primitive and perhaps almost unscientific, it is unfortunate that the older scientific literature is often ignored. In this day of computerized indices, the literature prior to the 1960's has slipped into oblivion. Few take the necessary time to seek out older papers in their area of research. Although they are obviously out of step with today's methods and some of their theories have been out-dated by modern developments, many of the older papers contain gems of discovery and insight that could be the impetus of tomorrow's research. For instance, the paper by Corper and Uyei (1930) was obtained for its discussion of the use of different media with "nonpathogenic" Mycobacteria. However, a small segment of

the paper described the use of different chemical reagents with "nonpathogenic acid-fast bacilli". It was shown that some species that were destroyed by HCL and NaOH were resistant to acetic acid. This led to the present experiments using acetic acid as a decontaminating agent. In the current work 4% NaOH and 2N HCl had been used in attempts to decontaminate broth cultures that showed acid-fast organisms upon staining. However, no acid-fast organisms could be recovered. As suggested by Vestal (1975) the procedure was reversed by using the 2N HCl and neutralizing with the 4% NaOH, but the contaminants still remained and/or the acid-fast organism was destroyed. Thus an antiquated paper led to what may prove to be a useful technique for the decontamination of specimens containing Mycobacterium other than tuberculosis. An unexpected advantage of the acetic acid procedure is that it also appears to cut down on false positives.

The current work used broth samples that had been stored in the cold for several months to over a year. Further work needs to be done using young cultures of known concentrations of MOTT to determine the best concentration of acetic acid, the best exposure time and the temperature during exposure for the optimum recovery of MOTT. The susceptibility of different species and strains of Mycobacterium also needs to be tested. The survival rates of gram negative and gram positive species as well as molds should also be evaluated. Also Corper and Uyei (1930) noted that acetic acid was toxic to M. tuberculosis and M. bovis, but the rapid growing species known at that time and a virulent strain of "avian tubercle bacilli" were resistant.

They suggested that acetic acid might be used to differentiate between the avian organism and the human and bovine organisms. Today there are obviously biochemical tests that provide such identifications, but perhaps acetic acid could be used for a more rapid tentative identification.

The results of these experiments make it obvious that at least some species of Mycobacteria are present in a significant percentage of the water supplies of eastern Kansas. Some of the species isolated in these experiments are potential pathogens. However, it remains unknown whether or not water is a vehicle for human mycobacterial infections. To determine if the presence of these organisms is a danger to those persons susceptibile to nontuberculous mycobacterial infections an epidemiologically based study would be necessary. Such a study could be conducted in several ways.

First, a retrospective study could be conducted. That is, the number of infections due to the mycobacterial species isolated in specific areas could be determined. Although the Kansas Department of Health and Environment identifies many mycobacterial isolates, they do not know which species actually caused disease nor do they generally have the patient's address. For these reasons such a study was not feasible.

A second way to perform an epidemiological study would be to ask physicians to report all infections due to nontuberculous Mycobacteria. These reports would be followed up by sampling water supplies to which the patient had been exposed. A potential problem with such a study would be that the exposure would have occurred weeks

original source of the infection the organism might no longer be present, so absence of the organism would not rule out water as a source.

A third method of conducting such a study would be to repeatedly sample water supplies in a specific area over a period of time and ask physicians in that area to report any infections due to nontuberculous Mycobacteria. This method has several limitations. Mycobacterial species may be isolated in only some taps in the same building or in some buildings and not others (Bullin and Tanner, 1970; Du Moulin and Stottmeier, 1978 and 1986). It would be impossible to examine all water taps in an area of any size. Since these diseases have a low incidence rate it is not probable that infections would necessarily occur in persons exposed to the tested water supplies. Therefore only generalized statements could be made assuming that the same species found in some water supplies in the area also caused disease among the exposed population. Other sources of exposure to the organisms would not be ruled out.

Because the incidence of infection is low amd because these organisms are opportunistic pathogens the best way to attempt to determine if water is a vehicle of these organisms might be to repeatedly sample water supplies to which susceptible persons are exposed. It can be assumed that persons such as AIDS patients, chemotherapy patients and organ transplant patients are often limited in their movements and are exposed to a more limited number of water

supplies than healthy persons. It might, therefore, be feasible to repeatedly sample most, if not all, water supplies to which a reasonably sized group of such persons are exposed. These samples would have to be taken at regular intervals over a long period of time, such as six months to one year. Any infections which occurred in these patients could then be compared to the mycobacterial species isolated from their individual water supplies. However, even if the species in the water supply and the infection matched, other sources of infection would not be ruled out. For a thorough study water and other environmental sources would have to be tested regularly.

Ethical problems could arise while conducting such a study. For instance, assume that the taps in a home occupied by an AIDS patient have been tested regularly. The first samples contain no MOTT, but later in the study two taps yield M. avium. Knowing that disease due to M. avium is potentially fatal in AIDS patients it would seem prudent (in this investigator's opinion) to notify the patient that those particular taps should be avoided. This should not adversely affect the study because allowing for initial growth, isolation and identification the patient would possibly have been exposed to this source for several weeks. All taps could still be tested regularly. The only adaptation of the study would be to note the date when the patient was no longer exposed to those taps. However, is it fair to cause the patient added anxiety? What if it is found that the only tub/shower available to the AIDS patient yields M. avium? Knowing that the patient is unable to change his residence, should the investigator inform him that with every shower he takes he exposes himself to a

potentially fatal organism? The investigator would have to be prepared to handle such situations.

Since no isolates of M. gordonae were found in the CPC experiments it seemed probable that at least that species and probably other species were being missed. Changing to the method of using HCHO as the decontaminating agent proved this assumption to be true. However, control experiments showed that exposure time to HCHO killed or inhibited at least some of the organisms. One reason more isolates were found on plates grown in a 5 % CO_2 atmosphere may be technique. These filters were generally done first so that the opened bags could be placed in the modified atmosphere for at least one hour before sealing. Therefore the samples that were filtered and placed in a normal atmosphere were exposed longer to the decontaminating agent. Control experiments suggested that exposure time to the decontaminating agents affected the recovery rate of at least some species of Mycobacteria.

Throughout both the CPC and the HCHO studies sporeforming bacteria and molds destroyed many plates. The addition of penicillin G and amphotercin B greatly diminished the number of contaminating bacteria, and the number was even less when HCHO was used as the decontaminating agent. Some plates with mold colonies or contaminating bacteria present in small quantities were left in the incubators. However, it is unknown whether the presence of these contaminating organisms might have inhibited the growth of nontuberculous Mycobacteria. Neither is it known if the presence of certain species of nontuberculous Mycobacteria could have inhibited the growth of other

species in the genus.

Although these experiments have shown that some nontuberculous Mycobacteria reside in some waters of eastern Kansas more work needs to be done. If the contamination problems can be brought under control perhaps even more species will be found. Better control of contamination would also allow a study in which a quantitative approach was used, i.e. determining the number of mycobacteria per liter of each source. More work is needed on decontaminating agents, particularly those that can help control gram positive organisms and molds. From the current work the use of HCHO appears to be the best decontaminating agent, but further work needs to be done with different concentrations and exposure times using more organisms than Carson et al. (1988). One technique that might enhance the recovery of MOTT would be to collect several smaller samples from each source. By collecting four 500 ml samples rather than one 2 liter sample the exposure time of all portions filtered could be more standardized. As previously mentioned, the use of acetic acid as a decontaminating agent also needs to be investigated.

Further studies should also include other pertinent information on water samples. Such factors as the date collected, the outside temperature, the water temperature, chlorine content, pH, and perhaps even the presence of other substances such as metals might be determined.

Nontuberculous Mycobacteria are an increasing cause of infection mainly because the susceptible population is increasing in size (Du Moulin and Stottmeier, 1986). Since many of these infections prove

fatal to patients, particularly those with AIDS, it may become necessary to find the common sources of these infections and attempt to limit the exposure of susceptible persons. These organisms are ubiquitous, but the presence of an organism is not enough to cause disease. The organism must be somehow disseminated in a sufficient dose to a portal of entry of a susceptible host. Perhaps this is done by wind-blown dust, or it may occur through aerosols created while taking a bath or shower. Our knowledge of how most species of nontuberculous Mycobacteria are disseminated leading to infection and disease is limited. Further studies in this area are needed to help prevent unnecessary infections in susceptible persons.

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APPENDIX

APPENDIX A

SAMPLES USED IN EXPERIMENTS WITH CETYLPYRIDINIUM CHLORIDE

TAPS

LC-T1 cold water tap, left sink in SH 59 at ESU. No CPC used.

LC-T2 cold water tap, left sink in SH 59 at ESU. Added CPC.

LC-T3 hot water tap, left sink in SH 59 at ESU.

LC-T4 cold water bathtub tap in Apartment G-73 of Married Student Housing complex, Emporia,KS. Collected by Milt Demory.

LC-T5 cold water tap, kitchen sink at 556 Hackberry, Americus, KS.

LC-T6 kitchen sink tap, Route 2, Box 126, Chanute, KS.

LC-T7 city water, Goodwell, OK

LC-T8 campus tap water, Panhandle State University, Goodwell, OK.

LC-T9 outdoor tap at Hartford Ramp area along Neosho River.

LC-T10 outside tap at Stop 2 Shop in Lyndon, KS.

LC-T11 hot and cold taps of laundromat sink in Lebo, KS.

LC-T12 outside tap with garden hose attached, BETO Junction Truck Stop at I-35 and Highway 75.

LC-T13 Outdoor tap at Lincoln Park, Osage City, KS.

LC-T14 outdoor tap at Greenwood Cemetery in Council Grove, KS.

LC-T15 outside tap at Stop 2 Shop in Council Grove, KS.

LC-T16 outdoor drinking fountain at Jones Youth Park in Allen, KS.

LC-T17 outdoor tap in Emporia Cemetery near 18th St., Emporia, KS.

LC-T18 laundromat sink tap, Chase County WashHouse in Strong City, KS.

WELLS

LC-W1 Dr. Sobieski's well before home filter, Rt. 1, Emporia, KS. Collected by Dr. Sobieski.

LC-W2 Dr. Sobieski's well after home filter, Rt 1, Emporia, KS. Collected by Dr. Sobieski.

LC-W3 chlorinated well water, Burnrnhyde, Rt 1, Emporia, KS. Collected by Dr. Sobieski.

LC-W4 well water, Rt 1, Emporia, KS. Collected by Dr. Sobieski.

LC-W5 well water, Rt 1, Emporia, KS. Collected by Dr. Sobieski.

LC-W6 well water, 816 Main, Americus, KS.

LC-W7 hand pump at Cottonwood Falls Cemetery, rural Cottonwood Falls, KS.

LC-W8 hand pump at Chase County Lake marked "not fit for human consumption". Chase County, KS.

LC-W1B repeated sample of LC-W1. Collected by Dr. Sobieski.

LC-W2B repeated sample of LC-W2. Collected by Dr. Sobieski.

PONDS

LC-P1 farm pond, Rt. 1, Emporia, KS. Collected by Dr. Sobieski.

LC-P2 little pond on Ross Reservation, Lyon County, KS.

LC-P3 pond, Ross Reservation, Lyon County, KS.

LC-P4 Gladfelter pond, Ross Reservation, Lyon County, KS.

LC-P5 Peter Pan Park, Emporia, KS.

LC-P1B repeated sample of LC-P1. Collected by Dr. Sobieski.

LAKES

LC-L1 Lyon County Lake, collected on south side of lake near shelter house area. Lyon County, KS.

LC-L2 Lyon County Lake, collected as runoff at low water bridge near dam and swimming area. Lyon County, KS.

LC-L3 east outlet, Council Grove Lake, Council Grove, KS.

LC-L4 Canning Cove area, Council Grove Lake, Council Grove, KS.

LC-L5 boat ramp at damsite, Council Grove Lake, Council Grove, KS.

LC-L6 Santa Fe Lake, collected on south edge of lake near the road and before the playground area, Chanute, KS.

LC-L7 Tulakes, collected at southwest corner of lake, Wilson County, KS.

LC-L8 Chase County Lake. Collected when lake level was above normal and high winds were present. Chase County, KS.

MARSHES

LC-M1 stagnant pool, Ross Reservation, Lyon County, KS.

LC-M2 salt marsh, Barton County, KS. Collected by Dr. Spencer.

LC-M3 salt marsh, Barton County, KS. Collected by Dr. Spencer.

LC-M4 salt marsh, Barton County, KS. Collected By Dr. Spencer.

LC-M5 Cheyenne Bottoms, Barton County, KS. Collected by Dr. Spencer.

RIVERS

LC-R1 Neosho River, Dittmer's ford, east of Chanute, KS.

LC-R2 Neosho River, Hartford ramp, near Hartford, KS.

BOTTLED

LC-B1 Hinckley and Schmitt Natural Spring Water. Purchased at Dillons in Emporia, KS.

LC-B2 Dillons Drinking Water. Purchased at Dillons in Emporia, KS.

LC-B3 Hinckley and Schmitt Nursery Drinking Water. Purchased at Dillons in Emporia, KS. Statement on label "Distilled" water with added minerals, calcium chloride, potassium bicarbonate, and magnesium chloride scientifically controlled to improve flavor. Contains 0 mg. sodium per eight fluid ounce serving".

DISTILLED

LC-D1 distilled water, Chemistry Department, Panhandle University, Goodwell, OK.

LC-D2 distilled water SH 66 at Emporia State University, Emporia, KS.

APPENDIX B

SAMPLES USED IN EXPERIMENTS WITH FORMALDEHYDE

TAPS

PT1 outside tap with garden hose attached, Kerr McGee station on west Highway 50, Emporia, KS. Collected 08/05/88.

PT2 outside tap on restroom building at Jones Park, Emporia, KS. Collected 08/05/88.

PT3 tap near front gate of park, Soden's Grove, Emporia, KS. Collected 08/05/88.

PT4 outside tap with garden hose attached, C-Mart station on east 12th St., Emporia, KS. Collected 08/05/88.

PT5 outside tap, Americus City Park on Main St., Americus, KS. Collected 08/05/88.

PT6 outside tap, Memorial Lawn Cemetery, Emporia, KS. Collected 08/05/88.

PT7 outside tap near 14th St. gate, Elmwood Cemetery Memorial Lawn section, Chanute, KS. Collected 08/10/88.

PT8 outside tap near northwest corner, Catholic Cemetery, Chanute, KS. Collected 08/10/88.

PT9 outside tap in Elmwood Cemetery on first road north of mausoleum, Chanute, KS. Collected 08/10/88.

PT10 kitchen tap, Rt. 2, Box 126, Chanute, KS. Collected 08/10/88.

PT11 bathtub tap, 1017 N. Santa Fe, Chanute, KS. Collected 08/10/88.

PT12 kitchen tap, 1017 N. Santa Fe, Chanute, KS. Collected 08/10/88.

PT13 cold water tap, left sink, SH 59 at Emporia State University, Emporia, KS. Collected 07/15/88.

PT14 tap near ball diamond, Jones Youth Recreation Park, Emporia, KS. Collected 09/25/88.

PT15 tap near shelter house, Lyndon City Park, Lyndon, KS. Collected 09/25/88.

PT16 cold water tap, laundromat sink, Lebo, KS. Collected 09/25/88.

PT17 outside tap with garden hose attached, Rt. 2, Box 126, Chanute, KS. Collected 08/10/88.

PT18 cold water tap, Chemistry storeroom, SH 209 at Emporia State University, Emporia, KS. Collected 11/07/88.

PT19 cold water tap from sink in SH 21 at Emporia State University, Emporia, KS. Collected 11/07/88.

PT20 cold water tap, animal room, Science Hall at Emporia State University, Emporia, KS. Collected 11/15/88.

PT21 cold water tap from handicapped equipped sink with hose attached in first floor restroom near Rm 155 in Morse Hall, Emporia State University, Emporia, KS. Collected 11/15/88.

PT22 cold water tap from sink along north wall in SH 65 at Emporia State University, Emporia, KS. Collected 11/22/88.

PT23 cold water tap from sink in front bech of SH 169 at Emporia State University, Emporia, KS. Collected 11/22/88.

PT24 mixed hot and cold taps, Biology Office sink, SH 174A at Emporia State University, Emporia, KS. Collected 11/22/88.

DRINKING FOUNTAINS

PF1 playground area of Jones Park, Emporia, KS. Collected 08/05/88.

PF2 near Johnson Shelter House in Jones Park, Emporia, KS. Collected 08/05/88.

PF3 near Kiwanis Shelter House in Peter Pan Park, Emporia, KS. Collected 08/05/88.

PF4 behind ball diamond at Peter Pan Park, Emporia, KS. Collected 08/05/88.

PF5 beside bandstand in Casitas Park, Emporia, KS. Collected 08/05/88.

PF6 outside restroom building in Hammond Park, Emporia, KS. Collected 08/05/88.

PF7 near main shelter house and tennis courts in Highland Park, Chanute, KS. Collected 08/10/88.

PF8 Kustom Electronics, Chanute, KS. Collected 08/10/88.

PF9 BETO Junction Truck Stop at I-35 and Highway 75. Collected 08/10/88.

PF10 near ball diamond next to swimming area at Melvern Lake, Osage County, KS. Collectdd 09/25/88.

PF11 parking area below spillway at Melvern Lake, Osage County, KS. Collected 09/25/88.

PF12 across from SH 177 at Emporia State University, Emporia, KS. Collected 10/19/88.

PF13 across from SH 150 at Emporia State University, Emporia, KS. Collected 10/19/88.

PF14 near SH 170 across from stairwell at Emporia State University, Emporia, KS. Collected 10/19/88.

PF15 across from SH 46 at Emporia State University, Emporia, KS. Collected 10/25/88.

PF16 between exit and animal room on ground floor of Science Hall at Emporia State University, Emporia, KS. Collected 10/25/88.

PF17 by SH 216 at Emporia State University, Emporia, KS. Collected 11/07/88.

PF18 by SH 30 and planetarium at Emporia State University, Emporia, KS. Collected 11/07/88.

PF19 by SH 230 at Emporia State University, Emporia, KS. Collected 11/07/88.

PF20 Emporia State University Memorial Union concourse between bulletin board and vending machines. Emporia, KS. Collected 11/15/88.

PF21 Emporia State University Memorial Union across from Post Office. Emporia, KS. Collected 11/15/88.

PF22 Emporia State University Student Health Center waiting area. Emporia, KS. Collected 11/15/88.

PF23 across from Rm 155 in Morse Hall at Emporia State University, Emporia, KS. Collected 11/15/88.

WELLS

PW1 untreated well water at 2840 Edwards, Manhattan, KS. Collected by Milt Demory on 08/08/88.

PW2 outside faucet, well water after home softener treatment, 2840 Edwards, Manhattan, KS. Collected by Milt Demory on 08/08/88.

PW3 tub faucet, well water after softener treatment, 2840 Edwards, Manhattan, KS. Collected by Milt Demory, 08/08/88.

PW4 kitchen tap, well water after softener treatment, 2840 Edwards, Manhattan, KS. Collected by Milt Demory, 08/08/88.

PW5 Warren farm, Rt. 1, Emporia, KS. Collected by Dr. Sobieski on 09/15/88.

PW6 Wellnitz farm, Rt. 1, Emporia, KS. Collected by Dr. Sobieski on 09/15/88.

PW7 Burnenhyde farm, Rt. 1, Emporia, KS. Collected by Dr. Sobieski on 09/15/88.

PW8 Royal farm, Rt. 1, Emporia, KS. Collected by Dr. Sobieski on 09/15/88.

PW9 McQuenn farm, Rt. 1, Emporia, KS. Collected by Dr. Sobieski on 09/15/88.

PW10 Sobieski farm, Rt. 1, Emporia, KS. Collected by Dr. Sobieski on 09/15/88.

OTHER

PU1 55 gallon fish tank housing a large African knifefish, Rt. 2, Box 126, Chanute, KS. Collected 08/10/88.

PU2 decorative fountain at Highland Park, Chanute, KS. Collected 08/10/88.

PU3 distilled water from prep room still, SH 66F at Emporia State University, Emporia, KS. Collected 10/19/88.

PU4 distilled water from Chemistry storeroom still, SH 209 at Emporia State University, Emporia, KS. Collected 11/07/88.

BOTTLED

PB1 Hinckley and Schmitt Nursery Drinking Water. Distilled water with added minerals, calcium chloride, potassium bicarbonate and magnesium chloride scientifically controlled to improve flavor. Contains 0 mg per eight ounce serving. H & S - Hinckley and Schmitt Chicago IL 60638. One gallon.

PB2 Hinckley and Schmitt Natural Spring Water. Exceeds all federal, state and local bottled water quality standards. H & S - Hinckley and Schmitt Chicago IL 60638. One gallon.

PB3 Hinkley and Schmitt Hi-Pure Sodium Free* Drinking Water. Purified Water. Purified by deionization with added minerals, calcium chloride, potassium bicarbonate and magnesium chloride scientifically controlled to improve flavor. *Contains 0 mg sodium per eight ounce serving. H & S - Hinckley and Schmitt Chicago IL 60638. One gallon.

PB4 Hinkley and Schmitt Distilled Water. Salt Free Purified Water. Purified by distillation to meet USP standards. Contains 0 mg sodium per eight ounce serving. H & S - Hinkley and Schmitt Chicago IL 60638. One gallon.

PB5 Dillons Crystal C;ear Drinking Water. Dist. by Dillons Stores Box 1608 Hutchinson, Ks. 67504-1608 PLT. 20-283. One gallon.

PB6 Evian natural spring water from the French Alps. Imported. Non carbonated. 1.5 liters - 50.7 fl. oz. Evian water: Evian, France, the source of Evian Water, is a spa town situated in the region of Mont-Blanc in the French Alps. Evian is a pure, natural spring water and under French law, can only be bottled at its source, the Evian Cachat Spring. The French Ministry of Health, under the recommendation of the French Academy of Medicine, gave its approval for the bottling of Evian at the spring in 1878. Since then they have maintained regulatory controls. Source: Bottled at Evian's Cachat Spring. S. A. Evian Co. 74503. Evian-Les-Bains, France. Store in a clean, dry and cool place.

Composition in mg/liter

bicarbonates	357	sodium	5*
calcium	78	nitrates	4
magnesium	24	chlorides	2
silica	14	potassium	1
sulphates	10		

*Salt Free (Only 1.2 mg per eight ounce serving).
Dissolved solids - 309 p.p.m. Neutrally balanced pH = 7.2.
Product of France. For further information write U.S.A. office: Evian Waters of France, Inc., 600 West Putnam Ave., Greenwich, CT 06830. Any questions? Please call toll free, 1-800-633-3363.

APPENDIX C

MIDDLEBROOK 7H9 SAMPLES USED IN ACETIC ACID EXPERIMENTS

Appendix C

Middlebrook 7H9 Samples Used in Acetic Acid Experiments

SAMPLE	ORIGINAL DATE	ATMOSPHERE OR TEMPERATURE
T11	08-18-82	25 C, 37 C
T17	04-08-88	25 C, 37 C
R 1	05-27-87	25 C, 37 C
R 2	10-10-87	25 C
W 8	07-02-88	+, N
P 1	01-21-87	25 C, 37 C
P 1B	06-09-87	25 C, 37 C
P 2	01-26-87	25 C, 37 C
P 3	01-26-87	25 C, 37 C
P 4	01-26-87	25 C, 37 C
P 5	05-18-87	25 C, 37 C
M 1	01-26-87	25 C, 37 C
M 2	05-10-88	25 C
M 3	05-16-88	25 C
M 4	05-10-88	25 C
M 5	07-22-88	+, N
L 1	01-30-87	25 C, 37 C
L 2	01-30-87	25 C, 37 C
L 3	05-22-87	25 C, 37 C
L 4	05-22-87	25 C, 37 C
L 5	05-22-87	25 C, 37 C
L 6	05-25-87	25 C, 37 C
L 7	05-25-87	25 C, 37 C
L 8	04-08-88	25 C, 37 C
L 9	07-09-88	+, N
L10	07-05-88	+, N
M. chelonei	02-26-88	37 C
M. chelonei	02-28-88	25 C
M. chelonei	03-01-88	Unknown
M. chelonei	04-17-88	25 C
M. gordonae	02-09-88	Unknown
M. gordonae	11-17-88	25 C
M. phlei	01-17-87	25 C
M. smegmatis	01-17-87	25 C
M. fortuitum	01-17-87	25 C

Linda K. Cox
Signature of Graduate Student

Signature of Major Advisor

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5 3 90
Date

Mycobacteria Other Than Tuberculosis in Eastern Kansas Water Supplies
Title of Thesis/Research Report

Signature of Graduate Office Staff Member

Date Received