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Isolation of anthrax spores from the environment and ecological investigation of *Bacillus anthracis* in endemic regions of northern Canada

by

Daniel Christopher Dragon

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Department of Medical Microbiology and Immunology

Edmonton, Alberta

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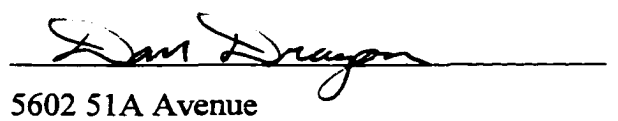

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Isolation Of Anthrax Spores From The Environment And Ecological Investigation Of *Bacillus anthracis* In Endemic Regions Of Northern Canada submitted by Daniel Christopher Dragon in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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Abstract

Bacteriological studies of anthrax endemic regions in northern Canada were initiated in order to determine the extent of anthrax spore contamination and to examine the ecology of *Bacillus anthracis* spores. Efforts to develop a field immunoassay to detect viable anthrax spores proved unsuccessful and experiments were undertaken to investigate ways to improve the sensitivity of selective polymyxin, lysozyme, ethylenediaminetetraacetic acid, thallium acetate (PLET) medium. In laboratory tests with seeded sterile soil, solutions of high specific gravity sucrose plus nonionic detergent extracted significantly more spores than the standard water extraction used with PLET medium.

PLET medium was evaluated for recovery of spores from a number of environmental and clinical strains of *Bacillus* species. While inhibitory to most non-*anthracis Bacillus*, the medium supported the growth of *B. anthracis* ATCC 4229, *B. mycoides* MU 711/84, *B. thuringiensis* QC 12093, *B. subtilis* 1A289, and environmental strains of *B. pumilus* and *B. circulans*. Not all *B. anthracis* ATCC 4229 spores germinated when applied to PLET indicating it is not an optimal medium for anthrax spore recovery.

Using a 1.22 g/ml sucrose plus 0.5% (v/v) Triton X-100 extraction solution, ethanol purification, PLET medium and confirmatory assays, *B. anthracis* spores were detected in 11 of 588 (1.9%) environmental specimens collected from anthrax endemic regions in northern Canada. All positive samples were associated with disposal sites of bison carcasses from previous anthrax epizootics. Viable anthrax spores were found at three of

six carcass burial sites surveyed in the Parson's Lake Road region and at four of eight carcass cremation sites examined in the Falaise Lake region. Anthrax spore concentrations of > 500 spores/g were observed in a red fox scat and specimens collected from a distinct bone bed found within cremation sites. The positive fox scat is the first bacteriological evidence that carnivorous mammals are capable of disseminating anthrax spores in northern Canada. Within the cremation bone beds, mats of bison hair and maggot casings were also found with anthrax spores, indicating the bed had been protected from the heat of the blaze by the bulk of the carcass.

Acknowledgments

Compared with other graduate students, I started this project backwards, developing a thesis proposal and then fielding it out to various University faculties searching for a supervisor. Dr. Bob Rennie took up the challenge of a presumptuous student and a proposal strong in enthusiasm if a little weak on execution. Throughout the resultant, unexpectedly long, program Dr. Rennie has been unflagging in his faith and support, being both a mentor and friend, and for this I thank him.

I also thank Dr. Brett Elkin of the Northwest Territories (NT) Department of Resources, Wildlife and Economic Development (RWED) and Dr. Cormack Gates now of the University of Calgary for their commitment and support without which this project would never have started or finished. It was Drs Elkin and Gates who first suggested I look into graduate work on anthrax and bison in the NT and have been patiently guiding the project ever since. Without hesitation, they have provided financial and technical field support whenever needed, and words of encouragement during some frustrating and difficult times.

I am grateful to Dr. John Ezzell of the United States Army Research Institute of Infectious Diseases, Fort Detrick, Maryland for his donation of samples of the FDF 1B9 and EAII-6G6-2-3 monoclonal antibodies. I also thank Dr. Pam Coker of Louisiana State University, Baton Rouge for extracting DNA from my environmental isolates and Dr. Paul Keim's laboratory at Northern Arizona University, Flagstaff for analyzing this DNA and confirming my isolates were indeed *B. anthracis*.

This graduate project is notable for the long commutes involved; my regular laboratory was located in Edmonton, the field areas were in remote regions of the NT and the level III biocontainment laboratory necessary to work with virulent *Bacillus anthracis* was in Lethbridge. While the distance traveled was substantial, the help and amity I encountered everywhere more than compensated for it and facilitated the completion of the project.

I am grateful to Troy Ellsworth of RWED, Dean Robertson of Yellowknife and Daniel Gates formerly of Fort Smith for their assistance and woodsman skills during the sometimes grueling and adventurous sample collection process at Hook Lake, Parson's Lake Road and Falaise Lake. I thank George Mercer and Wood Buffalo National Park for permission to sample carcass disposal sites along Parson's Lake Road. I also thank Jan Stirling and Kate Capshaw, NT Public Health nurses in Yellowknife, for hunting down and providing me with anthrax vaccine throughout this project.

I thank Thea Hefner for her contribution to this project. As a technician in our laboratory in Edmonton, Thea prepared and performed several spore isolations from soil to verify my initial findings thereby freeing up my time to prepare for screening of environmental specimens in Lethbridge. I am grateful to Karen Emde of the University of Alberta Civil Engineering Environmental Laboratory, Dr. W. Wenman of the University of Alberta Hospital Department of Pediatrics and the Animal Diseases Research Institute (ADRI) in Lethbridge for their donations of *Bacillus* strains. I thank Marie Kaplan, technician for Dr. Randy Irvin at the Department of Medical Microbiology and Immunology, for her insight and expertise during antibody and hybridoma production. During antibody production, I had the opportunity to work with Eugene Ambrosie and Tony Panter of the Provincial Laboratory of Northern Alberta Vivarium who helped me with animal immunizations and took care of the animals.

I thank Dr. Vic Gannon, Dr. Stacey Tessaro and ADRI, Lethbridge, for the use of their level III diagnostic facility. I had thought I was a patient, attentive worker at the bench but former ADRI technician Jack Burchak revealed a whole new level of caution and preparedness; the only rational way to work in biocontainment facilities; when he introduced me to *B. anthracis* and for this I am eternally grateful. Greg Tiffin, Jennifer Whorley, Shirley Stolk and Robin King also provided endless technical support while I was at ADRI for which I am grateful.

During this project I had the good fortune to be part of the Provincial Laboratory for Northern Alberta research group headed by Dr. Rennie, Dr. Jim Talbot and Dr. Greg Tyrrell. Technician Lillian Cook provided excellent laboratory maintenance and attention to the endless minutiae in the facility which insured experiments ran smoothly and without delay. Fellow graduate students Claire Lukey, Laura Sly and Robert Liao called me on the worst of my northern tall tales and I can think of no better peers to unwind with. The group was a pleasure to work along side and an excellent source of fresh ideas.

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Finally, though far from least, I thank my parents, brothers and extended family for their unending support; financially, emotionally and spiritually. They may not have always understood what I was doing or why it was taking so long but their faith in my abilities never wavered. They were convinced that all would work out in the end and they were absolutely right.

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List of Abbreviations

AAAUS	Amino acid, adenosine, uracil, salt
AB	Alberta
ABTS	2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
ADRI	Animal Diseases Research Institute
ANOVA	Analysis of variance
APC	Antigen presenting cell
ASA	American Standards Association
ATCC	American Type Culture Collection
ATCCSB	ATCC sporulation broth
BSA	Bovine serum albumin
CMB	Cooked meat broth
CO_2	Carbon dioxide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DPA	Dipicolinic acid
DSSM	Double strength skim milk
EDTA	Ethylenediaminetetraacetate acid
EF	*B. anthracis* edema factor
ELISA	Enzyme-linked immunosorbent assay
ENP	Etosha National Park, Namibia
Envir.	Environmental isolate
GPS	Global positioning system
HEPA	High efficiency particle arresting
Ig	Immunoglobulin
im	intramuscularly
ip	intraperitoneally
kbp	Kilobase pairs
kDa	Kilodaltons
KNP	Kruger National Park, South Africa
LF	*B. anthracis* lethal factor
MBS	Mackenzie Bison Sanctuary, Northwest Territories

MHC	Major histocompatibility complex
MLVA	Multiple-locus variable-number tandem repeat analysis
MPHL	Microbiology and Public Health Laboratory
MTSA	Modified Tarr's sporulation agar
NAP	Nutrient agar plate
NAPL	Northern Alberta Provincial Laboratories
ND	Not done
NS	No surveillance
nm	Nanometer
NT	Northwest Territories
ONPG	o-Nitrophenyl-β-d-galactophyranoside
PA	*B. anthracis* protective antigen
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Potential hydrogen
PLET	Polymyxin, lysozyme, ethylenediaminetetraacetic acid, thallium acetate
PLR	Parson's Lake Road, Wood Buffalo National Park, Northwest Territories
ppi	post primary injection
ppm	Parts per million
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SASP	Small, acid-soluble proteins
sc	subcutaneously
SBA	Sheep blood agar
SRL	Slave River Lowlands, Northwest Territories
TES	N-tris(Hydroxymethyl)methyl-2-aminoethanesulfonic acid
T_h	Helper T cells
TSA	Trypticase soy agar
TSB	Trypticase soy broth
UAHS	University of Alberta Health Sciences
UV	Ultraviolet radiation
WBNP	Wood Buffalo National Park

Chapter I. Introduction

I.1. *Bacillus anthracis*

I.1.a. The organism

Bacillus anthracis is a straight or slightly curved rod with boxed ends, 1.0 - 1.2 μm wide and 3.0 - 5.0 μm long. The microbe is non-motile, although one motile, flagellated strain has been reported (Brown and Cherry, 1955). It can form endospores that are highly resistant to many adverse conditions. The spores are ellipsoidal in shape, between 1.0 - 2.0 μm in size and are formed in the center of the vegetative cell. During sporulation, the vegetative mother cell does not elongate or swell but retains its regular dimensions.

The bacterium stains Gram positive. It is an aerobe but can tolerate added CO_2. It produces a poly-γ-D-glutamic acid capsule *in vivo* or on serum plates in a 5% CO_2 atmosphere (Meynell and Meynell, 1964). This is the basis of the McFadyean reaction which involves staining with polychrome methylene blue to give blue rods surrounded by purple- or pink-stained serrated capsular material in a positive test.

Several DNA studies have demonstrated a very high homology among *B. anthracis*, *B. cereus*, *B. thuringiensis* and *B. mycoides* suggesting they be considered a single species (Sommerville and Jones, 1972; Kaneko et al., 1978; Roloff et al., 1996). This is particularly true of *B. anthracis* and *B. cereus* which have been shown to possess identical 16S rRNA sequences and only two differences in their 23S rRNA sequences (Ash et al., 1991; Ash and Collins, 1992). Currently the four species are referred to as the *B. cereus*-group with *anthracis*, *thuringiensis* and *mycoides* considered subspecies of *cereus*.

I.1.b. Endospores

All species of *Bacillus* form metabolically dormant life stages, called endospores, in response to nutrient-poor conditions or dehydration; which effectively limits the diffusion of nutrients to the microbe and isolates it in a depleted microenvironment. Sporulation of *Bacillus* has been induced in the laboratory by culturing vegetative cells in a medium with limited metabolizable carbon, nitrogen, phosphate or sulfate (Grelet, 1957; Schaeffer, 1969; Lee and Brown, 1975). Each vegetative cell is capable of forming one spore, hence, sporulation is not a form of replication, but a protective cryptobiotic state. When favorable nutrient levels return to the local environment, the spores germinate and new vegetative cells outgrow to exploit the new resources.

Endospore survival mechanisms are primarily cellular rather than molecular (Marquis et al., 1985). Resistance to deleterious environmental factors is not the result of the manufacture of new, more resistant metabolic enzymes but rather is achieved through the overall structure and composition of the spore which provide a stabilizing environment for the internal macromolecules (e.g., enzymes, DNA and RNA). Many of these adaptations protect against more than one kind of deleterious agent and often work together redundantly and synergistically to protect the spore contents against specific environmental factors.

The *Bacillus* spore is comprised of several layers, each conferring a cumulative protection on the inner layers and spore core (Figure I-1). The spore core stores the macromolecules necessary for regular vegetative cell growth and metabolism upon germination. The macromolecules are immobilized within the core by an extensive salt lattice of calcium and dipicolinic acid (DPA; Gould, 1984). The immobilization protects the macromolecules from denaturation and disruption due to heat, freezing and pressure (Bender and Marquis, 1982; Belliveau et al., 1992). DNA is further protected by α/β-type small, acid-soluble proteins (SASP) which bind non-specifically to the entire spore chromosome and coil it

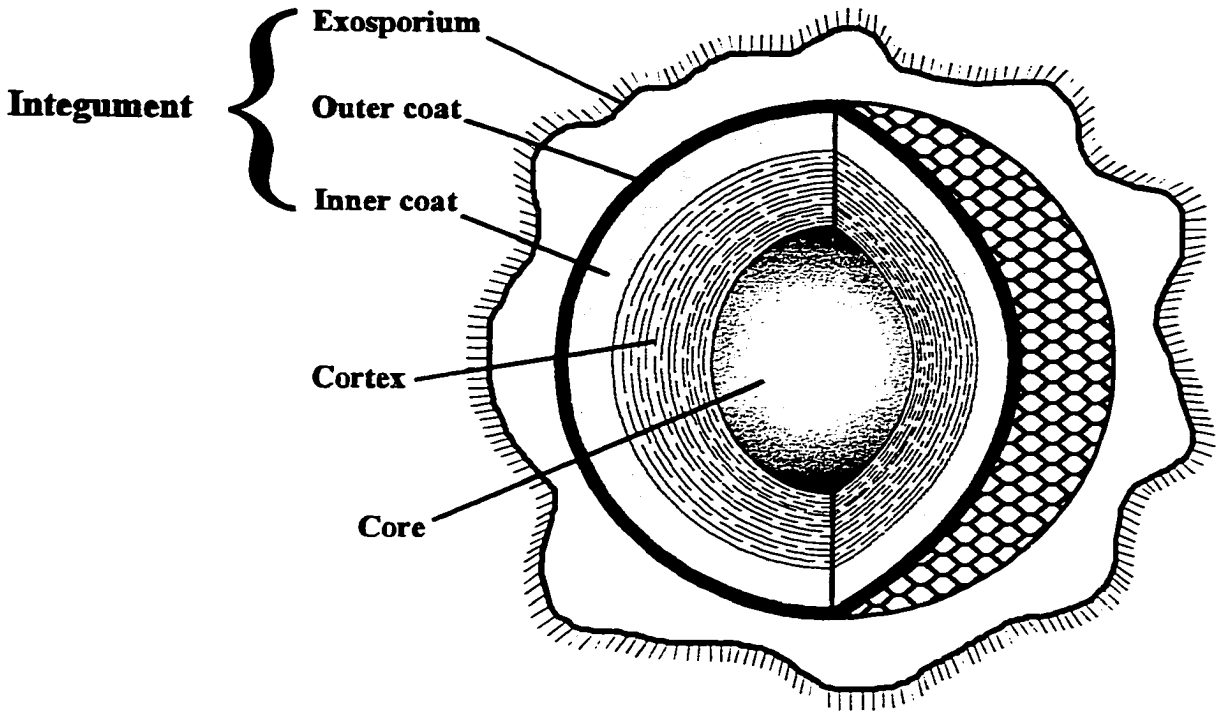

Figure I-1. Generalized structure of a *B. cereus*-group endospore (Foster and Johnstone, 1990).

and coil it into negative supertwists (Setlow, 1988). Alpha/beta-type SASP have been shown to protect DNA against cleavage by heat, hydrogen peroxide and freeze-drying (Fairhead et al., 1993, 1994; Setlow and Setlow, 1993, 1994). The α/β-type SASP are also the major, if not sole, factor in spore resistance to UV and ionizing radiation (Tanooka, 1968; Setlow, 1992).

Surrounding the core is an extensive layer of minimally cross-linked peptidoglycan called the cortex (Popham and Setlow, 1993). The cortex exerts osmotic and hydrostatic pressure on the core conferring a low water activity (Gould, 1977; Popham and Setlow, 1993). The low water activity of the core acts in synergy with core mineralization to protect macromolecules, membranes and ribosomes from disruption due to heat, freezing, dessication and pressure (Belliveau et al., 1992). The core is not completely dry but remains fully permeable to water (Marshall and Murrell, 1970). The low water activity conferred by the cortex, however, greatly limits diffusion of environmental molecules into the core. Even molecules as small as methylamine require many hours to penetrate endospores via passive diffusion (Gerhardt and Black, 1961; Setlow and Setlow, 1980), compared with an equilibrium time of minutes for vegetative cells (Marquis et al., 1985). The decreased penetration into the dehydrated core acts to protect its contents against deleterious chemicals.

The exosporium and spore coat; known collectively as the integument; comprise the surface of the spore. They have a low permeability to environmental reagents and further increase spore core equilibrium time (Gerhardt and Marquis, 1989). The integument is impermeable and resistant to a number of harmful enzymes, chemicals and surfactants, and protect the cortex from chemical disruption (Gould and Hitchins, 1963; Foster and Johnstone, 1990). The exosporium is composed primarily of protein with carbohydrate and lipid also present (Warth, 1978). It is divided into an ordered lattice basal layer, an intermediate layer, and an outer layer of hair-like projections (Gerhardt and Ribi, 1964; Moberly et al., 1966). The exosporium of *B. cereus*-group species is loosely associated

with the spore proper (Figure I-1); other *Bacillus* species have tight fitting exosporia and still others have no apparent exosporium (Matz et al., 1970). It has been postulated that all *Bacillus* species possess exosporium but some are so closely associated with the spore coat as to be indistinguishable (Sousa et al., 1976).

The coats of *Bacillus* endospores are comprised primarily of protein. The spore coat of *B. cereus*-group species consists of two morphological layers, an inner or under coat and a thinner lamellar outer coat (Horn et al., 1973). There is evidence that there is only one protein monomer in the coats and that the two distinct layers are due to secondary modification of this protein (Aronson and Fitz-James, 1971, 1976; Aronson and Horn, 1972). The inner coat is preferentially solubilized by sulfhydryl reducing agents indicating that the layer forms through intermolecular disulfide bond formation. At the time of outer coat formation, there is a disulfide interchange between cysteine and protein cystine bonds resulting in the disruption of one or two cystine bonds in each outer coat monomer (Aronson and Fitz-James, 1973). The disruption may permit portions of the polypeptide chains to assume a new tertiary conformation where hydrophobic interactions are relatively more important and this may account for the need of reagents that disrupt both disulfide bonds and hydrophobic interactions in order to solubilize the outer coat (Aronson and Fitz-James, 1976). The hydrophobicity of the outer coat of *B. cereus*-group spores affects both permeability to soluble reagents (Gerhardt and Black, 1961) and spore surface adhesion (Doyle et al., 1984; Wiencek et al., 1990; Singh and Doyle, 1993).

I.1.c. The disease

Several forms of anthrax are recognized in humans depending on the route of entry. Cutaneous anthrax is by far the most common and accounts for 95 - 99% of human cases worldwide (Whitford and Hugh-Jones, 1994). The spores gain entry via small cuts, abrasions, or insect bites. Two to five days after exposure, the affected site begins to itch and a red papule appears. The papule develops into a fluid filled vesicle and extensive

edema may evolve in adjacent tissues. Satellite vesicles may appear near the initial lesion and the vesicular fluid, initially clear, becomes dark bluish-black. When the vesicle ruptures, necrosis occurs at its center and it eventually develops into a typical black eschar.

Mild systemic symptoms may accompany the developing eschar. Malaise and fever are experienced along with occasional tenderness of lymph nodes near the affected site. Cutaneous anthrax on the face or neck may be complicated with obstructive airway disease due to the associated edema. Seven to ten days after onset, 80% of cases progress to healing. The eschar loosens and eventually falls off leaving a distinctive scar. In the remaining 20% of untreated cases there is progressive extension of the infection to the regional lymph nodes and circulatory system that rapidly develops into bacteremia and toxemia terminating in death (Whitford and Hugh-Jones, 1994).

Intestinal anthrax results from ingestion of spores in contaminated meat and is most commonly reported in poor, undeveloped countries and regions suffering from low availability of protein. After ingestion, the spores penetrate the intestinal mucosa and germinate, and the resulting vegetative cells multiply in the submucosal tissue. Ulcers develop at the site of infection, usually in the terminal ileum or cecum. Ulceration may be so extensive as to cause hemorrhage. Symptoms manifest 2 - 5 days after ingestion of the contaminated meal. Nausea, vomiting, anorexia and fever develop and are followed by abdominal pain, hematemesis and occasionally bloody diarrhea. The disease may then progress to a generalized toxemia, shock, cyanosis and death in 25 - 50% of cases (Brachman, 1972).

Inhalation anthrax occurs when viable spores gain access to the alveoli of the lungs. The spores are phagocytized by macrophages and transported to the mediastinal lymph nodes where they germinate. The bacteria then colonize the circulation through the efferent lymphatics and produce a rapidly fatal toxemia and bacteremia. At the onset of illness, the

symptomology is mild and resembles that of a common upper respiratory tract infection including low-grade fever, malaise, myalgia and non-productive cough. The disease is often misdiagnosed at this stage resulting in improper treatment and increased mortality. After several days of mild symptoms, the patient exhibits a sudden onset of acute dyspnea, diapheresis and cyanosis with death usually occurring within 24 h (Whitford and Hugh-Jones, 1994).

Herbivores are much more susceptible to the disease than carnivores; humans are relatively resistant and fall on the carnivore side of the spectrum. Herbivores that die of anthrax are often discovered in otherwise good physical condition owing to the rapidly acute nature of the disease. The route of entry of spores into animals is often unknown and as a result animal cases of the disease are classified by the severity of symptoms.

The disease may occur in a peracute or fulminant form with the animal developing a rapid septicemia. The animal dies within 48 h of exhibiting signs of an apoplectic seizure or often may not exhibit any signs of illness at all. The peracute form of anthrax has been observed in Asian elephants (*Elephas maximus*), and kudu (*Tragelaphus strepsiceros*) in South Africa (Choquette, 1970).

Anthrax may also occur in an acute form, running a course of 2 - 3 days or more and ending in death. This is the form most commonly observed in bison (*Bison bison*; Novakowski et al., 1963; Gates et al., 1995). Morbid bison appear gaunt and depressed and are inordinately indifferent to activity around them. They may lie down and completely ignore the noise of nearby clean-up crews and surveillance aircraft. Most morbid animals walk with apparent difficulty, staggering at times, and exhibit a stiff-legged gait when forced to run. Many exhibit edematous swellings usually in, but not limited to, the preputial and umbilical regions. Death appears sudden with the animals literally falling over in their tracks. No signs of struggle or flailing are observed in the soil immediately surrounding the carcasses.

The disease may also run a nonlethal course lasting several days, with the animals displaying symptoms of the previous two forms but recovering. This form has been observed in ungulates in South Africa, livestock and bison although its prevalence within an affected herd has never been quantitated (Pienaar, 1967; Novakowski et al., 1963; Choquette et al., 1972).

I.1.d. Virulence factors

The virulence of *B. anthracis* is due to the presence of the glutamic acid capsule and production of a tripartite protein exotoxin comprised of edema factor (EF), lethal factor (LF) and protective antigen (PA) (Sterne, 1937; Keppie et al., 1955). The genes for the capsule and exotoxin are located on two plasmids; pX01 (184.5 kbp) encodes for the production of the exotoxin components and pX02 (95.3 kbp) contains genes for the synthesis of the capsule. Both plasmids are required for full virulence; the loss of either results in an attenuated strain.

Within the host, synthesis of the capsule begins immediately after spore germination and the capsule is fully formed before outgrowth, ensuring that the vegetative form is completely surrounded by the capsule before exposure to the immune system (Ezzell and Abshire, 1996). The capsule is weakly antigenic and acts to prevent phagocytosis of the cells by the immune system. Polyglutamic acid also blebs off the capsule and is itself a powerful immunosuppressor (Ezzell and Abshire, 1996).

Edema factor is an adenyl cyclase that produces edema when injected with PA subcutaneously (sc; Leppla, 1982). Lethal factor is so named because it causes death when injected intravenously with PA into susceptible animals. Lethal factor is a zinc-metalloprotease which causes host macrophages to release proinflammatory cytokines which result in sudden and fatal shock of the host (Hanna et al., 1993; Klimpel et al., 1994; Duesbery et al., 1998; Hammond and Hanna, 1998).

Both LF and EF work intracellularly and if purified and injected individually into a host have no effect. To work, the two factors require PA which binds to the surface of susceptible host cells, is cleaved into an active form by the host protease furin, binds LF or EF and forms a transmembrane porin through which the factors may pass (Milne et al., 1994). Because of its central role in the delivery and action of anthrax toxin, PA is an ideal target for the immune response, hence its name. By binding and eliminating extracellular PA with antibodies before it forms porins, a primed immune system can prevent large scale tissue damage and disruption of the immune system.

I.1.e. Life cycle

The dormant endospore is the infectious agent of anthrax (Hanna and Ireland, 1999). Infections occur only when spores enter the body from the external environment. There are no well-documented cases of vegetative anthrax bacilli in a natural system and no natural examples of live-animal-to-live-animal transmission of the disease. The resistance of the spore allows the microbe to survive phagocytosis and passage through the stomach of the host. In inhalation anthrax, and probably other forms of the disease, once inside macrophages the microbe germinates and begins producing capsule and toxin so that it is one step ahead of the immune response of the host when it lyses the macrophage (Ezzell and Abshire, 1996; Guidi-Rotani et al., 1999).

In an animal with terminal anthrax, the microbe enters the circulatory system and a bacteremia rapidly arises with massive invasion of all body tissues. There is a rapid buildup of bacteria in the blood in the last few hours before death with terminal blood concentrations of $> 10^7$ cfu/ml (Lindeque and Turnbull, 1994). After the death of the host, some *B. anthracis* cells escape in exudates to the external environment where they can sporulate but the vast majority are unable to replicate further because of a lack of oxygen and can not sporulate in the nutrient-rich viscera. If the carcass is left unopened, vegetative *B. anthracis* are rapidly destroyed by putrefactive anaerobes growing out from

the intestinal tract (Stein, 1947; Tolstova, 1960). However, animal carcasses are rarely left undisturbed. Avian and terrestrial scavengers quickly arrive to utilize the resource, borrowing into and dismembering the carcass, and allowing *B. anthracis* access to the proper conditions for sporulation. *Bacillus anthracis* spores released into the environment may remain viable and infective for decades, awaiting the opportunity to infect a new host and initiate a new cycle of disease.

I.2. Distribution and Epidemiology

I.2.a. Global anthrax

Anthrax is global in its distribution and is endemic to regions of all continents except Antarctica. In 1981 (the last year data was tabulated) the World Health Organization reported 3200 human cases of anthrax in 43 countries (Brachman, 1991). The incidence of the disease has decreased in developed countries but it remains a considerable medical and agricultural problem in developing countries. The disease is under-reported in domestic livestock and wild animal populations in both developed and developing countries due to a lack of surveillance and reporting (Hugh-Jones, 1996).

In the United States, there are two main endemic areas; northwestern Mississippi and adjacent southeastern Arkansas where outbreaks occur primarily in cattle (*Bos taurus*), and western Texas and adjacent Mexico where outbreaks are reported sporadically in sheep (*Ovis aries*) and white-tailed deer (*Odocoileus virginianus*). In Mexico, reports of anthrax in cattle and sheep are widespread. The epidemiological pattern in North American livestock suggests that contamination of feed with anthrax infected carcasses is primarily responsible for the continuing occurrence of outbreaks (Hugh-Jones, 1996).

Anthrax is a serious problem in Central America, with the apparent exception of Belize. The disease appears absent from the Carribean, with the exception of Haiti where it is a serious zoonosis and the human case rate may exceed 2000/y. Anthrax is endemic in many South American countries including Chile, Brazil, Uruguay and Paraguay.

In Europe, the major affected regions are Turkey, Greece, Albania, southern Italy, Romania and central Spain. Norway, which had not reported a case of the disease in years, recently reported several cases in cattle (Hugh-Jones, 1996). War and civil unrest in the Balkan region and Russia have led to a breakdown in veterinary services and an increase in the incidence of the disease. Southern Russia experiences the greatest frequency of outbreaks in that nation, however, sporadic outbreaks also occur in northern Russia in the domestic reindeer (*Rangifer tarandus*) herds (Cherkasskiy, 1999). From southern Europe sporadic outbreaks of anthrax continue along a belt extending into areas of India and Pakistan.

The disease is endemic in southeast Asia where it frequently affects water buffalo (*Bubalus bubalis*) and pigs (*Sus scrofa*). Although no cases had been reported in Korea since 1978, two confirmed cattle and 24 human cases were reported in 1994 (Hugh-Jones, 1996). In China, vast areas are contaminated with anthrax spores and the disease is endemic in ten provinces. During 1990 - 1993, there were 8122 human cases of anthrax in China, of which 324 (4.0%) were fatal. The main source of human infection was reported to be sheep in the north and cattle in the south (Xudong et al., 1996).

In Africa, extended outbreaks involving humans, livestock and wildlife species occur especially in West Africa. South Africa suffers from sporadic epizootics with large numbers of mortalities in affected herds. Anthrax cases are diagnosed every year in the wild herbivores of Etosha National Park (ENP) in Namibia, however, the incidence has been low in recent years (Lindeque et al., 1996). In the Park, the disease primarily affects Burchell's zebra (*Equus burchelli*), blue wildebeest (*Connochaetes taurinus*) and

elephants (*Loxodonta africana*). The disease occurs sporadically in the wild herbivore population of Kruger National Park (KNP) in South Africa with major epizootics occurring in a cyclic pattern with a periodicity of 10 y or multiples thereof. The principal animal species affected in the Park is the kudu, although during major outbreaks dozens of species may be affected (de Vos and Bryden, 1996).

Australia has traditionally had a small area affected by the disease in New South Wales but has recently seen cases in Western Australia as well. In 1997, an explosive outbreak occurred in north-central Victoria killing 202 cattle and 4 sheep over a 45-day period (Turner et al., 1999).

I.2.b. Anthrax in southern Canada

Prior to the 1960's, 90% of reported anthrax outbreaks in Canada occurred in Ontario and Quebec (Moynihan 1963). The majority of these outbreaks were confined to the southern portions of the two provinces and were often associated with pastures contaminated with effluent from tanneries and other textile industries dealing with raw animal materials. In 1952, introduction of Federal regulations requiring disinfection of imported untanned hides, raw wool and hair greatly reduced the incidence of disease in eastern Canada. Except for one lone outbreak on Prince Edward Island, the remaining epidemics in the first half of the twentieth century occurred in western Canada with the majority occurring in Saskatchewan. In all areas, cattle were by far the most affected species although outbreaks were also reported in horse (*Equus caballus*) and sheep herds, mink (*Mustela vison*) farms and exotic species in zoological gardens. The origin of disease in mink and zoological gardens was inevitably traced to infected livestock carcasses bought as cheap feed from local ranchers.

Since 1962, domestic outbreaks of the disease have been reported almost exclusively in cattle in the western prairie provinces (Gainer and Saunders, 1989; MacDonald et al.,

1992; Coupland and Henderson, 1996) with the exception of one outbreak in eastern Ontario (Lusis and Smart, 1996). A small outbreak occurred at the Winnipeg Zoo in Manitoba in July 1998 where three bison and a white-tailed deer housed together in the same enclosure succumbed to the disease.

In June 1999, a prolonged and expanding epizootic occurred in the Rocky Mountain House area of eastern Alberta with at least 31 cattle dying of the disease at seven ranches. With this exception, cattle outbreaks in the last four decades have been small; limited to one ranch and few mortalities. A number of outbreaks were recorded on farms which had previous experience with the disease and where recent excavation was believed to have cycled anthrax spores back to the surface (Lusis and Smart, 1996) or, in Saskatchewan, on waterways where outbreaks had been recorded years previously on farms upstream of the affected site (Heath and Brewitt, 1982). For the majority of these outbreaks, however, no indigenous source of spores was identified.

Anthrax is a rare disease in humans in Canada. Between 1931 and 1961, Statistics Canada reported a total of 23 cases of human anthrax (Varughese, 1977). However, lack of familiarity with the disease and its rapid convalescence with antibiotic treatment have probably prevented the diagnosis of cases and led to an under-reporting of the disease. Two additional cases of anthrax were retrospectively identified in employees of Wood Buffalo National Park (WBNP) in 1952, a full decade before the first recorded outbreak of the disease in the bison (see next section; Gates et al., 1995). Both men developed eschar-like lesions after handling a bison carcass. The men's illness resolved quickly with penicillin treatment but *B. anthracis* was not isolated.

Since 1961, there have been only four reported cases of human anthrax in Canada. Two cases occurred in 1962 during the anthrax outbreak in wild bison at Hook Lake, Northwest Territories (NT). One man developed cutaneous anthrax after performing an unprotected necropsy on a bison carcass. A second man developed the pulmonary form of

the disease while disposing of carcasses. He had repeatedly cleaned the blade of his backhoe of infected blood and offal. Both men survived thanks to prompt medical treatment (Pyper and Willoughby, 1964). A human cutaneous case was reported in Alberta during bovine outbreaks in 1991 and involved a rancher handling an infected carcass (MacDonald et al., 1992). In 1990, a woman in British Columbia developed cutaneous anthrax following a vacation in the Bahamas. She denied any agricultural contact at home or in the Bahamas and the source of her infection was never identified (Wong and Ng, 1991).

I.2.c. Anthrax in the bison herds of the Northwest Territories and northern Alberta

In summer 1962, a large anthrax epizootic occurred in the free-ranging bison herds of the NT (Novakowski et al. 1963). The epizootic occurred in the Hook Lake region of the Slave River Lowlands (SRL) with most of the carcasses concentrated on two large open fields of prime bison habitat (Figure I-2). Out of a herd estimated at 1300 head, 281 carcasses were found and buried. The total carcass count was an underestimation of the actual bison losses to the disease as tall grass, tree foliage and dismemberment by scavengers undoubtedly hid many carcasses from visual detection by aerial surveillance and clean-up crews. During the next two summers, outbreaks of the disease continued in the Hook Lake region and spread; first across the Slave River to the Grand Detour and Park Central regions and then across the Peace River to the Lake One region. Over the next three decades, sporadic summer outbreaks of anthrax continued in the bison herds of these four areas and they are now considered endemic for the disease.

In the summer of 1993, anthrax appeared in the bison herd of the Mackenzie Bison Sanctuary (MBS), across the Great Slave Lake from the previously affected areas (Gates et al. 1995). Bison carcasses were found in five localized areas; Falaise Lake, Boulogne Lake, Slave Point, Calais Lake and Mink Lake. One hundred sixty-nine bison from a herd estimated to be 2000 strong were found dead (Figure I-2). Because of the ability of

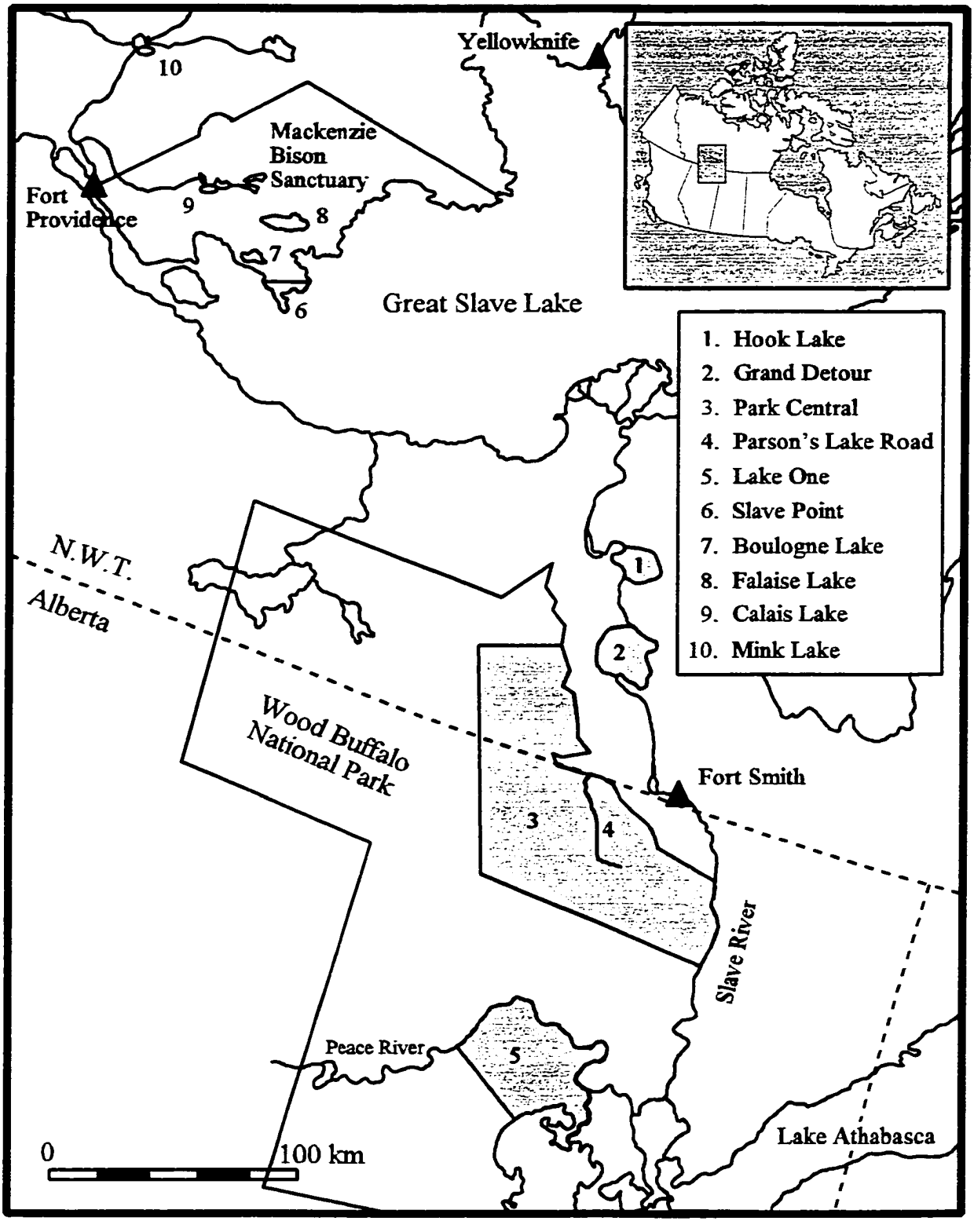

Figure I-2. Endemic anthrax regions in northern Canada.

anthrax spores to survive for long periods in the environment and because of the previous history of the disease in other northern bison herds, the MBS must also be considered endemic for anthrax.

Between 1962 and 1993, nine anthrax epizootics have been recorded in northern Canada resulting in the death of over 1300 bison (Table I-1). Several moose (*Alces alces*) carcasses were also found and diagnosed with anthrax during the largest epizootics. The epizootics have varied in magnitude in both area and number of animals affected from the 299 carcasses found over all four southern regions in 1964 to the single case detected in the Lake One region in 1968. Again numbers reflect only the total carcasses discovered by clean-up crews and are at best minimal estimates of true mortality. This past summer, a tenth epizootic occurred in WBNP with the wardens reporting over 100 bison carcasses found in the Lake One region and along the Peace River watershed in the south-western portion of the Park (Adam Moreland, WBNP, personal communication).

I.2.d. Vectors and disseminators

Infected herbivores may shed anthrax spores in their feces and urine but the main release of spores occurs after death when scavengers rend the body contaminating the local environment (Choquette, 1970). Direct transmission between herbivore hosts is considered to be of minor importance in anthrax epizootics. Furniss and Hahn (1981) refer to one instance where direct ingestion of spores from a carcass may have occurred in a kudu that was observed to lick the face of a recently dead member of the herd. Clark (1938) speculated that osteophagia (bone-eating) in phosphate deficient areas may be an important route of anthrax infection in cattle and wild herbivores though natural infection by this route has yet to be demonstrated. It should be noted, however, that several outbreaks in developed countries have been traced to the production of bone meal and other nutritional supplements with anthrax-infected carcasses (Green and Jamieson, 1958; Hugh-Jones, 1996).

Table I-1. Number of bison carcasses found in anthrax epizootics in northern Canada (Novakowski et al., 1963; Cousineau and McClenaghan, 1965; Choquette, 1970; Choquette et al., 1972; Broughton, 1992; Carbyn et al., 1993; Gates et al., 1995, Dragon and Elkin, 2001).

	SLAVE RIVER LOWLANDS		WOOD BUFFALO NATIONAL PARK		MACKENZIE BISON SANCTUARY				
YEAR	Hook Lake	Grand Detour	Park Central	Lake One	Falaise Lake	Boulogne Lake	Slave Point	Calais Lake	Mink Lake
1962	281[a]	NS[b]	NS	NS	NS	NS	NS	NS	NS
1963[c]	15	242	47	NS	NS	NS	NS	NS	NS
1964[c]	44	259	49	11	NS	NS	NS	NS	NS
1967	0	0	0	120	NS	NS	NS	NS	NS
1968	0	0	0	1	NS	NS	NS	NS	NS
1971	33	0	0	0	NS	NS	NS	NS	NS
1978	12	27	40	0	NS	NS	NS	NS	NS
1991	0	0	32	0	NS	NS	NS	NS	NS
1993	0	0	0	0	110[d]	26	7[e]	23	3

[a] One black bear and one moose carcass were also found in the affected region but because of advanced decomposition *B. anthracis* was not isolated from either carcass.

[b] NS = No surveillance.

[c] Anthrax was also diagnosed in moose carcasses in the same general area. The number of moose mortalities was not specified.

[d] One moose dead of anthrax was also found and disposed of following anthrax clean-up procedures.

[e] Two moose dead of anthrax were also found and disposed of following anthrax clean-up procedures. One black bear carcass was also found but no specimens were collected for bacteriological investigation.

Scavengers, with their high innate resistance to the disease, may also act as carriers transporting and dispersing the spores over large areas. Anthrax spores have been isolated from scavenger feces collected around carcasses during epizootics in ENP (Lindeque and Turnbull, 1994). Similarly, herring gulls (*Larus argentatus*) and ravens (*Corvus corax*) collected during epizootics in bison were found to harbor spores in their gastrointestinal tracts (Choquette, 1970; Gates et al., 1995). Besides intestinal carriage, scavengers may also transport anthrax spores in viscera adhering to their fur or feathers. Not all carriers of anthrax are scavengers; *B. anthracis* was isolated from the digestive tracts of two house sparrows (*Passer domesticus*) in Great Britain (Strewsbury and Barson, 1952). There were no reported cases of the disease in the area at the time of the isolation but the birds were captured near a veterinary barn where numerous necropsies including some of anthrax infected carcasses had been performed. In ENP, the microbe was recovered from the bill and gizzard of an Egyptian goose (*Alophochen aegyptiacus*) shot on a water pan contaminated with anthrax spores (Ebedes, 1976).

In Africa, necrophilic insects, such as blowflies (*Chysomya* spp.), have been shown to carry spores to surrounding forage and contaminate the vegetation while cleaning themselves or regurgitating infected viscera (de Vos, 1973, 1990). Mechanical transmission of anthrax spores by biting insects, including mosquitoes and tabanids, has been documented under laboratory and field conditions (Mitzmain, 1914; Olsuf'ev and Lelep, 1938; Krisna Rao and Mohiyadeen, 1958; Turell and Knudson, 1987), however, the contribution of biting insects to the spread of disease remains unknown. Most studies demonstrated successful transmission only if the insect was interrupted while feeding on the infected animal a few hours before or just after death when *B. anthracis* blood concentrations were at their highest and before the putrefactive process began. In the terminal stages of the disease, most morbid animals would be indifferent to insect harassment allowing the insects to have a full, uninterrupted meal and therefore have little reason to visit a second herbivore.

Anthrax outbreaks have often been associated with low-lying areas such as river valleys and flood plains (van Ness, 1971). Water has been implicated in the dispersion of anthrax spores in the environment but the phenomenon has rarely been conclusively demonstrated. Flooding may precede an outbreak and it has been hypothesized that water may uncover anthrax spores buried after a previous outbreak and concentrate them into low lying areas to levels that are infectious to the local herbivores (Ebedes, 1976; Dragon et al., 1996). Natural and artificial waterholes in Africa have been found contaminated with anthrax spores but the spores could have been transported to the water by scavengers or contaminated by infected herbivore carcasses. A bacteriological survey of Fever Tree Depression, in the region of highest frequency of anthrax outbreaks in KNP, demonstrated the poorly drained Depression acted as a catchment and concentrator area for anthrax spores (de Vos, 1990).

Wind has also been implicated as a possible disseminator of anthrax spores from carcasses and contaminated soil (Ebedes, 1976). Turnbull et al. (1998) recently studied the airborne movement of spores from three heavily contaminated carcass sites in ENP. They detected anthrax spores downwind of the sites only during the highest winds or when the sites were mechanically disturbed. Calculations demonstrated that winds quickly dispersed the aerosolized spores to non-infectious concentrations.

I.2.e. Meteorology

Anthrax outbreaks in the northern bison herds have occurred in late summer usually prior to the rut or breeding season. For many of the northern epizootics, the regions affected experienced heavy precipitation or flooding during the spring or early summer and a prolonged hot, dry spell immediately prior to the outbreak. Heavy precipitation was noted in the Hook Lake region in the early summer months preceding the 1962 outbreak and in WBNP in the spring of 1991 prior to the Park Central outbreak (Novakowski et al., 1963;

Broughton, 1992). In both cases the excess precipitation resulted in flooding of bison habitat within the affected regions. Bison habitat in the MBS was flooded for several summers before a summer-long drought the year of its epizootic (Gates et al., 1995).

Anthrax outbreaks in the Canadian bison herds also terminate with the arrival of cooler weather and precipitation (Choquette et al., 1972; Broughton, 1992; Gates et al., 1995; Dragon and Elkin, 2001). Following three straight summers of anthrax in northern Canada in which the disease progressively spread across the SLR and WBNP, the entire region experienced 3 months of rain in the fall of 1964 and anthrax mortalities for the year promptly ended (Dragon and Elkin, 2001). The rain resulted in complete flooding of bison habitat in the areas. Interestingly, anthrax did not occur in the SRL and WBNP endemic areas the following two summers even though conditions appeared favorable.

I.2.f. Initiation of epizootics

Several hypotheses have been put forward to explain the initiation of anthrax epizootics not associated with imported contaminated feed. The one that has received the most attention is the "incubator area" hypothesis which states that under conditions of alkaline pH, high soil moisture and the presence of organic matter *B. anthracis* may undergo cycles of germination, vegetative outgrowth and resporulation in the summer heat that can cause an overall increase in spore numbers and lead to new outbreaks of the disease (van Ness, 1971). Such soil conditions have been observed in anthrax endemic areas in the United States, Great Britain and Asia (Hugh-Jones and Hussaini, 1975; Kasianenko et al., 1984).

In all recorded epizootics among the northern bison herds, significantly more sexually mature bulls succumbed to the disease than cows, juveniles and calves (Choquette et al., 1972; Broughton, 1992; Gates et al., 1995). It is not uncommon during an outbreak for > 80% of the carcasses to be mature bison bulls. Two hypotheses have been developed to

explain the meteorology and sex bias associated with anthrax in northern Canada. The "modified host resistance" hypothesis proposes that the bison are chronically exposed to low levels of *B. anthracis* spores which establish subclinical infections prior to the outbreaks proper and that outbreaks occur when the animals become immunocompromised due to accumulated transient stresses (Gainer and Saunders, 1989). The meteorological conditions associated with anthrax result in diminished food and water resources, crowding around the remaining resources, sweltering weather and high concentrations of biting insects, all of which could contribute to an overall immunosuppression of the animals. At the same time, fighting and other physical displays during the rut take a heavy toll on the energy reserves of the bulls which, compounded with the other factors, could preferentially leave the bulls susceptible to disease.

The "wallow concentrator" hypothesis proposes that during the anthrax season, spores in the environment are carried by water action and concentrated into low lying areas, specifically bison wallows (Dragon et al., 1996). Wallows are circular depressions formed by bulls pawing and rolling upon the ground. Wallows are revisited year after year and can form substantial depressions in otherwise level pastures. Wallowing can be a territorial display and its frequency increases as the rut approaches, therefore anthrax spores concentrated in wallows would be preferentially encountered by mature bulls. The wallowing bulls could aerosolize spore-laden dust clouds which could then be inhaled thereby establishing disease.

I.3. Detection of anthrax spores

I.3.a. Isolation of *Bacillus* spores

Bacillus spores are commonly isolated from environmental samples by agitation of the specimen in deionized water or saline. Non-sporulating contaminants in a sample are not generally a problem because the resistant properties of the spores can be used as a

prescreen to remove the vegetative microbes. The most common pretreatment is to subject the sample to wet heat at a level which will kill vegetative cells; except for extreme thermophiles; but will allow the spores to survive. It should be kept in mind, however, that heat resistance varies among the spores of *Bacillus* species and some have little more heat resistance than vegetative cells (Norris et al., 1981). Furthermore, spores formed in natural environments may possess different heat sensitivities than those which have been produced under optimal conditions in culture (Claus and Berkeley, 1986). A heat pretreatment developed in the laboratory with cultured spores may be too stringent for natural spores to survive.

Heat treatment of bacterial endospores can lead to mutations (Zamenhof, 1960; Nisida et al., 1969; Hayase et al., 1974; Kadota et al., 1978). To minimize the possibility of mutations yet still take advantage of heat prescreening, isolation protocols for anthrax spores utilize low pasteurization temperatures for short periods of time (Manchee et al., 1981; Carman et al., 1985; Turnbull et al., 1986). Once samples have been pretreated to kill vegetative contaminants, *B. anthracis* must be isolated and differentiated from other spore-forming species present.

An alternative method of prescreening which has not been commonly used and which does not cause mutation is ethanol treatment. Bond et al. (1970) successfully used ethanol as a spore-selective agent. In a comparison of heat (80°C for 15 min) and ethanol (50% (v/v) for 1 h) treatments, the latter consistently resulted in higher recoveries of *Clostridium* and *Bacillus* species from mixed cultures and intestinal samples (Koransky et al., 1978). A similar study demonstrated that spores of several *Bacillus* species survived exposure to 50% ethanol for up to 1 h whereas vegetative cells of *Bacillus* and other species were killed (Claus and Berkeley, 1986).

I.3.b. Immunodetection

Bacillus anthracis may be detected in environmental samples either directly with antibodies against spore surface epitopes or following incubation in germination medium with antibodies against vegetative cell antigens. However, both routes have proven problematic as antibodies raised against vegetative cells or spores of *B. anthracis* cross-react extensively with preparations from other *Bacillus* species, especially with other members of the *B. cereus*-group (Fluck et al., 1977; Phillips and Martin, 1983; Phillips et al., 1983; Phillips and Ezzell, 1989). It is apparent that *B. anthracis* vegetative cells and spores possess some unique antigens. Pre-adsorption of polyclonal serum raised against *B. anthracis* spores or cells with like preparations of *B. cereus* often results in sera specific for *B. anthracis* (Phillips et al., 1983; Phillips and Ezzell, 1989; Niederwöhrmeier and Böhm, 1990).

Monoclonal antibodies specific for *B. anthracis* cells and spores have also demonstrated the presence of epitopes unique to the species and their use may eliminate the need for preadsorption of sera prior to immunoassays. Monoclonal antibodies have been developed against the glutamic acid capsule and a cell wall galactose-N-acetylglucosamine polysaccharide of the microbe; both of which are considered rare in other *Bacillus* species (Ezzell et al., 1990; Ezzell and Abshire, 1996). Monoclonal antibodies have also been produced against *B. anthracis* Ames strain spores that have variable specificity for other *B. anthracis* strains and minimal cross-reaction with *B. cereus* spores (Phillips et al., 1988).

Enzyme immunoassays specific for *B. anthracis* vegetative cells, spores and virulence factors have been developed for the identification of the microbe and for environmental detection. Niederwöhrmeier and Böhm (1990) developed an enzyme immunoassay using pre-absorbed polyclonal sera linked to polystyrene beads that could detect as low as 10^4 vegetative cells of *B. anthracis* with minimal cross-reaction to non-*anthracis* strains.

Ezzell and Abshire (1996) combined monoclonal antibodies against anthrax capsule and the galactose-N-acetylglucosamine polysaccharide in a direct immunofluorescent assay that could specifically identify *B. anthracis* cultures. An enzyme immunoassay using *B. anthracis* spore specific polyclonal serum linked to paramagnetic beads was recently developed and could detect as few as 10^4 anthrax spores in spiked soil samples (Bruno and Yu, 1996).

I.3.c. Selective media

Several selective media have been developed for the isolation of *B. anthracis* from the environment (Pearce and Powell, 1951; Morris, 1955; Shapovalova, 1966; Tomov and Todocov, 1966; Turnbull et al., 1986). Of these, the most satisfactory is heart infusion agar medium supplemented with polymyxin, lysozyme, ethylenediaminetetraacetic acid (EDTA) and thallium acetate (PLET medium; Knisely, 1966). PLET medium has been successfully used to detect anthrax spores at herbivore carcass sites in outbreak areas in Africa (Turnbull et al., 1986, 1989, 1991, 1998; Lindeque and Turnbull, 1994), to delineate areas contaminated with anthrax spores during World War II biological weapons testing on Gruinard Island, Scotland (Manchee et al., 1981, 1983), and to diagnosis anthrax during epizootics (Carman et al., 1985).

Despite its wide-scale use in epidemiological studies and diagnostic laboratories, there have been few in-depth studies of PLET medium. Manchee et al. (1981) suggested a sensitivity limit of 3 anthrax spores/g for environmental isolation with PLET but the value was a theoretical limit based on the dilutions of the extraction protocol and assumed that water was able to extract all spores present in a sample and that all anthrax spores would germinate equally on the medium.

I.3.d. Confirmatory assays

One advantage of using selective medium to detect *B. anthracis* is that further tests may be run on suspect colonies to confirm their identity. Most confirmatory assays are geared towards differentiating *B. anthracis* from other *B. cereus*-group species. *Bacillus anthracis* is normally non-motile, non-hemolytic on blood agar and susceptible to penicillin whereas most other *B. cereus*-group members are motile, β-hemolytic and resistant to penicillin (Carman et al., 1985). Virulent strains of *B. anthracis* produce capsular material and form mucoid colonies on bicarbonate medium when incubated in an atmosphere containing 10% CO_2. Capsule formation can be further confirmed with McFadyean's reaction mentioned previously. *Bacillus anthracis* is also susceptible to gamma phage (McCloy, 1951, 1956). When the phage is added to a confluent lawn of vegetative *B. anthracis* the bacillus lyse forming plaques; other *B. cereus*-group species are generally resistant to the phage (Brown and Cherry, 1955; Brown et al., 1958).

Several genetic assays have also been developed to differentiate *B. anthracis* from contaminating *B. cereus*-group species. Current tests are based on polymerase chain reaction (PCR) and most amplify genetic sequences of the anthrax virulence genes (Carl et al., 1992; Makino et al., 1993; Reif et al., 1994). Recent genetic analysis of *B. anthracis* has identified several amplified fragment length polymorphism DNA markers in both virulence plasmid and genomic DNA that are able to differentiate the microbe from other *B. cereus*-group members and to discriminate strains within the species (Keim et al., 1997). Analysis of an isolate at the genetic level is the most accurate method of conclusively identifying the microbe.

I.4. Objectives of this study

In-depth epidemiological studies have not been undertaken in the anthrax endemic areas of northern Canada. Research in the region has been limited to field observations during epizootics and cataloguing of the carcasses found. The source and movement of *B. anthracis* outside of the bison host are almost totally unknown. Bacteriological studies of environmental specimens from several of the northern endemic regions were initiated in order to determine the extent of anthrax spore contamination, to evaluate carcass disposal methods used in past outbreaks, to examine the ecology of *B. anthracis* spores and to investigate hypotheses about the initiation of epizootics.

The study was initially designed to develop a portable field assay system for the detection of viable *B. anthracis* spores. Development centered on the production of monoclonal or polyclonal antibodies specific for *B. anthracis* spores to be used in an indirect plate enzyme-linked immunosorbent assay (ELISA) system. Attempts were also made to adapt the limited germination immunofluorescent laboratory assay of Ezzell and Abshire (1996) to a plate ELISA format.

Later experiments focussed on the use of PLET medium to recover *B. anthracis* spores from environmental samples. The medium was investigated for its ability to germinate and recover a variety of *Bacillus* species. The standard procedure used with the medium was tested for ways to improve its sensitivity at either the extraction, purification or culturing steps. The modified procedure was then evaluated against the standard PLET procedure in its ability to isolate anthrax spores from spiked sterile soil and used to isolate *B. anthracis* spores from environmental specimens collected from around carcass disposal sites and bison habitat in the northern endemic areas.

Chapter II. Methods and Materials

II.1. Safety protocols

II.1.a. Field study regions

All personnel collecting environmental samples from areas potentially contaminated with *B. anthracis* spores were educated in the symptomology of anthrax and either vaccinated with the US anthrax vaccine preparation (Michigan Department of Public Health, Lansing, Michigan) or given prophylactic erythromycin tablets to take while in the field at a dosage of 500 mg/day. Public Health Nurses in the nearby communities of Fort Providence, Fort Smith and Yellowknife were informed of the nature of the study and who was involved.

Personnel collecting samples wore protective clothing comprised of rubber boots, disposable coveralls with hood and elastic cuffs, double-layered latex gloves, and a full-face HEPA-filtered M-95 respirator. After each collection, rubber boots were wiped down with 5% (v/v) formaldehyde and the outside of the respirator was washed with 5% (v/v) bleach. The rest of the protective clothing was then removed and stored in plastic garbage bags and personnel moved to the next collection site. At the end of each day, the coveralls and gloves were incinerated. All other protective clothing was stored in sealed garbage bags away from sleeping and eating areas.

II.1.b. Level III laboratory

All handling of live virulent *B. anthracis* strains was performed in Level III biocontainment facilities at the Animal Diseases Research Institute (ADRI), Canadian Food Inspection Agency, Lethbridge, Alberta. The facilities were also used during bacterial endospore extraction and identification of *B. anthracis* in environmental specimens collected from anthrax endemic regions of northern Canada. While at ADRI,

research personnel abided by the Level III safety protocols and standard operating procedures of the Institute. All liquid and solid waste was autoclaved in small, loosely-packed batches at 121°C for 90 min. Solid waste was then repackaged and incinerated. Surfaces and equipment in contact with cultures were wiped down with 5% (v/v) formaldehyde allowing a contact time of at least 10 min before removal. At the completion of experiments, high use areas of the laboratory were swipe tested, and the swabs were heat treated and used to inoculate sheep blood agar (SBA) and PLET plates to insure there was no anthrax spore contamination of the laboratory.

Prior to entry into the facility, research personnel changed into hospital greens, dedicated rubber boots, surgical masks and latex gloves. Inside the laboratory proper, personnel donned a disposable smock with sleeve cuffs and a second pair of gloves, taping the outer pair of gloves over the smock sleeves. When leaving the laboratory researchers removed the smock and discarded the gloves and mask. In an antechamber outside the laboratory, personnel soaked their boots in a 6% (v/v) bleach bath for 30 s before removing the boots and leaving them in the antechamber.

II.1.c. Research laboratory

Bacillus anthracis ATCC 4229 is an avirulent, toxin-negative, capsule-positive strain that was used in immunoassay and soil isolation experiments outside a Level III facility. Because of the tenacity of bacterial endospores, even the spores of non-virulent *Bacillus* and *Clostridium* species can create long-term contamination problems in a research laboratory. There was also the possibility of viable bacterial endospores spreading from the laboratory to contaminate the adjacent University of Alberta Hospital. Therefore, all *Bacillus* and Clostridial strains used in experiments, including the inactivated *B. anthracis* preparations, were handled with the same level of precaution and stringent clean-up protocol.

All direct manipulation of the microbes was performed in a biological safety cabinet with personnel wearing yellow smocks and latex gloves. Barrie et al. (1994) reported an outbreak of *B. cereus* meningitis caused by spore contamination of hospital linen in hampers and the subsequent failure of the hospital laundry to disinfect the linen. To prevent such an occurrence, smocks used during manipulation of the sporeformers were bagged at the end of the day and autoclaved at 121°C for 90 min before being allowed to enter the laundry system. When possible, all sporulation cultures were grown in sealed containers; tissue culture flasks for solid medium and screw-capped test tubes for liquid medium. All culture of *Bacillus* and *Clostridium* species was performed in a small, table-top incubator dedicated to this purpose. After completion of culture work, the inside of the incubator was washed down with 5% (v/v) formaldehyde with a contact time of 15 min for all interior surfaces. All surfaces and equipment to come into contact with the micro-organisms were washed down with 5% (v/v) formaldehyde with a contact time of at least 10 min. All solid waste was collected and autoclaved at 121°C for 90 min before being repackaged and sent out for incineration.

All liquid waste was collected and chemically treated prior to autoclaving. Even liquid waste from 96-well ELISA plates was aspirated out of the wells and pooled for treatment. Originally liquid waste was to be treated with formaldehyde at a final concentration of 5% (v/v), however, concern was expressed about the generation of toxic formaldehyde fumes upon subsequent autoclaving. Instead, Presept tablets (sodium dichloroisocyanurate, Johnson and Johnson) were be added to the liquid waste to give a final concentration of 5000 ppm. Presept-treated waste was left in labeled screw-capped bottles in the safety cabinet overnight at ambient temperature and then autoclaved at 121°C for 90 min.

All *Bacillus* and *Clostridium* species stocks were clearly labeled and stored together in a dedicated compartment in the 4°C cold room. For long-term storage, all live *Bacillus* and *Clostridium* preparations; except for *B. anthracis* ATCC 4229 spore stock which was left

at 4°C; were placed in a -20°C freezer. A single nutrient agar slant of *B. anthracis* ATCC 4229 was stored double-bagged in biohazard zip-lock bags in a sealed drawer at room temperature as an alternative culture inoculum.

II.2. Bacterial strains

II.2.a. Sources

Virulent *B. anthracis* strains were recovered from old, often dehydrated, trypticase soy agar (TSA) slants stored at room temperature in the Level III facility at ADRI. The *B. anthracis* strains used and their origin are listed in Table II-1; all were kindly provided by ADRI. *Bacillus anthracis* strains were recovered in nutrient broth. After overnight incubation at 37°C, portions of the culture were streaked on SBA, bicarbonate and nutrient agar plates to check for purity. The plates were incubated overnight at 37°C; the bicarbonate plates in a candle jar. Isolated colonies of the correct phenotype from the blood agar and bicarbonate plates were smeared on microscope slides, and inactivated and fixed onto the glass with 10% (v/v) formalin for 30 min. The blood slide was Gram stained and the bicarbonate slide was treated with Geimsa stain to confirm the identity of the culture (Collins et al., 1995).

Pseudomonas aeruginosa S169-90, *Clostridium* and non-*anthracis Bacillus* strains used in this study came from several sources (Table II-2). The *P. aeruginosa* strain was used to test the bactericidal effects of high specific gravity sucrose plus nonionic detergent extraction, and the heat and ethanol spore purification treatments against Gram negative bacteria. Isolates obtained from the Department of Pediatrics were supplied by Dr. W. Wenman. Isolates from the Civil Engineering Environmental Laboratory were kindly donated by Karen Emde. Non-*anthracis Bacillus* species and *P. aeuriginosa* were recovered from long-term storage in either skim milk broths held at -70°C, dried onto filter paper stored at 4°C or lyophilized in sealed vials held at 4°C. The strains were recovered

Table II-1. Origin of *B. anthracis* strains.

Strain	Origin
ATCC 4229	Toxin-negative, avirulent laboratory strain
93-189C	Bison blood isolate from 1993 Mackenzie Bison Sanctuary epizootic
93-196C	Bison ear isolate from 1993 Mackenzie Bison Sanctuary epizootic
9604	Bovine spleen isolate from a 1996 domestic outbreak near High Level in northern Alberta

Table II-2. Source and culture of *P. aeruginosa* S169-90 and Clostridial and non-*anthracis Bacillus* strains.

Species	Strain[a]	Source[b]	Storage medium[c]	Recovery medium[d]	Sporulation medium[e]
B. brevis	ATCC 8246	Pediatrics	Filter paper	TSB	MTSA
	Envir.	Civil	DSSM	TSB	MTSA
B. cereus	ATCC 14579	MPHL	DSSM	NAP	MTSA
	CPS 85-03A	MPHL	Lyophilized	TSA	ATCCSB
	Envir.	Civil	DSSM	TSB	MTSA
	F382	MPHL	DSSM	NAP	MTSA
	F447-90	MPHL	DSSM	NAP	MTSA
	R593	MPHL	DSSM	NAP	MTSA
	R618	MPHL	DSSM	NAP	MTSA
	R2281	MPHL	DSSM	NAP	MTSA
	R2483	MPHL	DSSM	NAP	MTSA
	RC-9-90	MPHL	DSSM	NAP	MTSA
B. circulans	Envir.	Civil	DSSM	TSB	MTSA
	LCDC 88-2	MPHL	Lyophilized	TSB	ATCCSB
	R1080	MPHL	DSSM	NAP	MTSA
B. licheniformis	Envir.	Civil	DSSM	TSB	MTSA
	R1984	MPHL	DSSM	NAP	MTSA
	R2138	MPHL	DSSM	NAP	ATCCSB
B. mycoides	MU711/84	MPHL	Lyophilized	TSA	MTSA
B. pumilus	Envir.	Civil	DSSM	TSB	MTSA
B. sphaericus	13A3	Pediatrics	DSSM	NAP	MTSA
	13A4	Pediatrics	Filter paper	TSB	MTSA
B. subtilis	1A289	Pediatrics	Filter paper	TSB	MTSA
	ATCC 6633	MPHL	DSSM	NAP	MTSA
	ATCC 23059	Civil	DSSM	TSB	ATCCSB
	CPS 85-03B	MPHL	Lyophilized	TSA	ATCCSB
	Envir.	Civil	DSSM	TSB	MTSA
	R417	MPHL	DSSM	NAP	MTSA
B. thuringiensis	QC12093	MPHL	DSSM	NAP	MTSA
C. bifermentans	13124	MPHL	CMB	CMB	-

C. butyricum	19398	MPHL	CMB	CMB	-
C. difficile	ATCC 9689	MPHL	CMB	CMB	-
C. novyi	F28	MPHL	CMB	CMB	-
C. paraputrificum	25780	MPHL	CMB	CMB	-
C. perfringens	13124	MPHL	CMB	CMB	-
C. ramosum	25582	MPHL	CMB	CMB	-
C. septicum	12464	MPHL	CMB	CMB	-
C. sordellii	9714	MPHL	CMB	CMB	-
C. sporogenes	3585	MPHL	CMB	CMB	-
C. tertium	14573	MPHL	CMB	CMB	-
C. tetani	19406	MPHL	CMB	CMB	-
P. aeruginosa	S169-90	MPHL	DSSM	TSA	-

[a] Envir. = Environmental isolate

[b] Civil = Civil Engineering Environmental Laboratory, University of Alberta
MPHL = Microbiology & Public Health Laboratory, University of Alberta Hospitals
Pediatrics = Department of Pediatrics, University of Alberta Hospitals

[c] CMB = Cooked meat broth
DSSM = Double strength skimmed milk

[d] NAP = Nutrient agar plate
TSA = Trypticase soy agar
TSB = Trypticase soy broth

[e] ATCCSB = ATTC sporulation broth
MTSA = Modified Tarr's sporulation agar

either on nutrient or trypticase soy agar plates, or in trypticase soy broth; all culturing was at 37°C for approximately 24 h. Non-*anthracis Bacillus* species and *P. aeruginosa* S169-90 were plated on SBA plates; overnight at 37°C; and Gram stained to check for purity. Clostridial species were obtained from old cooked meat broths stored at ambient temperature for at least 2 months. The purity of the *Clostridium* strains was checked via Gram stain.

II.2.b. Production of bacterial endospores

For the majority of *Bacillus* strains, including all *B. anthracis* strains, bacterial spore stocks were produced on modified Tarr's sporulation medium (Table II-2). In order to minimize the possibility of spore contamination of the laboratory or cross contamination of sporing cultures, sporulation was performed in sealed 225 cm^2 tissue culture flasks. To aid in dispersal of the inoculum and harvesting of the spores, 30 ml of sterilized glass beads (1 mm diameter) were added aseptically to each flask.

Sporulation flasks were seeded with a large loopful of a confirmed SBA culture of the *Bacillus* and 100 µl of 0.85% (w/v) physiological saline. The flask was sealed and the glass beads were swirled across the agar surface for a full minute to disperse the inoculum and then left in place on the surface of the medium for another minute before the flasks were inverted and the beads allowed to fall away. The cultures were incubated at 37°C for 14 days.

Sporulation flasks were harvested with 10 ml of either phosphate buffered saline (PBS) or inactivation solution (3% (v/v) formaldehyde or 5% (v/v) alkaline glutaraldehyde). The liquid was added to the flask which was then tightly sealed. The glass beads and liquid were carefully swirled across the agar surface for 2 min. The flask was set standing upright and tilted so that the liquid and beads all collected in one corner. The flask was

left in this position for 5 min in order for aerosols to settle and then the liquid was removed with a 25-ml pipette and pooled to a disposable centrifuge tube. After spore harvest, modified Tarr's sporulation flasks were sealed and incinerated.

Some *Bacillus* strains failed to produce high yields of spores on the modified Tarr's sporulation agar. Spore stocks for these strains were obtained through culture in ATCC sporulation broth. Each broth was inoculated with a large loopful of confirmed SBA culture of the *Bacillus* and the culture tube was loosely sealed. The broths were incubated at 37°C for 20 days. To harvest, the broth was gently finger vortexed to resuspend the culture and the suspension was transferred to a suitably sized centrifuge tube for washing. Spores of *Clostridium* species were collected directly from the aged cooked meat broth storage medium. Seven millilitre portions of each *Clostridium* culture were removed to a suitable disposable centrifuge tube for washing.

All spore preparations were washed twice with 20 ml of physiological saline; centrifuged at 5100 x g for 10 min at 4°C and vortexing for 30 s to resuspend. Portions of each stock were Gram and spore stained to confirm the presence of spores and to determine the level of vegetative contamination. Purity of the stocks was deemed satisfactory if there was < 10 clumps of vegetative debris per 10 microscope fields on both slides when viewed at 400x magnification. Stocks contaminated with vegetative matter were washed twice more with saline as described above and retested. Some strains, especially among *Clostridium* species, had to be sealed in screw-capped, o-ring Eppendorf tubes and submersed in a sonicator bath for 30 min at room temperature then washed twice with saline in order to sufficiently reduce the level of vegetative contamination. Despite repeated sonication, it was not possible to improve the purity of *C. novyi* F28 and *C. sordellii* 9714 stocks to below 100 vegetative clumps per 10 microscope fields. After washing was completed, the spore concentration of each stock was determined by phase contrast microscopy. The stocks were stored at 4°C until required.

II.2.c. Inactivation of spores

Spore samples of virulent *B. anthracis* strains, *B. anthracis* ATCC 4229, *B. cereus* ATCC 14579 and *B. subtilis* ATCC 6633 were inactivated with either 3% (v/v) formaldehyde in PBS, pH 7.2 or an aqueous solution of 5% (v/v) glutaraldehyde and 0.3% (w/v) sodium bicarbonate, pH 7.9 (alkaline glutaraldehyde). Spores were harvested directly from sporulation flasks with 10 ml of either inactivation solution as described above. Disposable centrifuge tubes containing the harvested spores were tightly sealed with parafilm and secured to a platform shaker in a horizontal position. The tubes were shaken overnight at 150 rpm at room temperature.

The next day the tubes were opened and portions from each were diluted 1 in 10 in PBS and tested in triplicate on SBA, TSA and nutrient agar plates. Neat aliquots were added in triplicate to 10 ml trypticase soy and nutrient broths (overall dilution 1/200). From his many years of experience with the organism, Jack Burchak of ADRI found that recovery of *B. anthracis* was enhanced in nutrient broth by constant aeration with magnetic stirring. Therefore, a portion from each tube was seeded to a sealed 75-ml Erlenmeyer flask containing 20 ml of broth (overall dilution 1/400) and a stir bar. The cultures were placed on magnetic stirrers set at 100 rpm in the incubator. Dilution of samples prior to spreading on agar plates and the application of samples to broths many times their volume was used to dilute out any inactivation reagent present and prevent its killing of any viable *Bacillus* upon germination. Safety considerations required that the *B. anthracis* spores were 100% inactivated and not held in a sporostatic state. All cultures were incubated at 37°C for 48 h to see if any growth occurred. While cultures were incubated, the spore tubes were stored at 4°C still under their inactivation solution. If growth occurred in any culture, the corresponding tube was returned to the platform shaker and agitated for another night at 150 rpm before retesting for complete inactivation.

Only after complete inactivation had been demonstrated were the spore stocks centrifuged at 10,000 x g for 15 min at 4°C and the inactivation solution removed. The stocks were then washed as described above and stored in saline containing 1% (v/v) of the aldehyde used to inactivate the stock. The stocks were stored at 4°C until required.

Spore stocks of virulent *B. anthracis* strains were not removed from the Level III biocontainment facility at ADRI until complete inactivation had been confirmed. To remove the stocks from the laboratory, the tubes were wrapped in paper towels soaked with 40% (v/v) formalin and placed in a zip-lock plastic bag for 20 min. The tubes were then removed from the bag and towels at the laboratory door. Outside the laboratory, the stocks were washed and stored as described above.

II.3. Immunodetection of *B. anthracis* spores

II.3.a. Production of serum titres in mice against *B. anthracis* spores

Inbred BALB/c and outbred Swiss strain mice were immunized with combinations of formaldehyde- or glutaraldehyde-inactivated anthrax spores, or isolated spore coat proteins and various adjuvants in an effort to obtain animals with high anti-anthrax spore serum titres (Table II-3). For all protocols, only female mice were employed and all were approximately 6 weeks old at the time of primary inoculation.

Prior to the primary injection, each mouse was pre-bled via jugular bleeds and the sera, pooled for each protocol, was saved as a negative control (pre-bleed serum). In general, injections were spaced 2 - 3 weeks apart depending on the adjuvant used. Beginning after the second booster, jugular test bleeds were taken from each mouse to monitor the developing immune response. Test bleeds were taken 10 - 14 days after the last booster and at least 3 days was allowed between the bleed and next booster.

Table II-3. Mouse immunization schedules with inactivated *B. anthracis* spore preparations.

Mouse strain	Number of mice	Spore preparation	Concentration	Adjuvant	Boosters (days ppi)	Test bleeds (days ppi)
BALB/c	5	93-189C spore coat proteins	0.13 mg/ml	Aluminum hydroxide	None	None
	4	93-196C spore coat proteins	0.11 mg/ml	Aluminum hydroxide	23, 43, 57, 74	51, 67, 86
	5	Formaldehyde-inactivated 93-196C whole spores	4.64×10^6 spores/ml	None	23, 43, 64, 78, 111	74, 90, 122
	4	Glutaraldehyde-inactivated 93-196C whole spores	8.72×10^6 spores/ml	None	21, 42, 63, 96	59, 75, 107
	3	Formaldehyde-inactivated 93-196C whole spores	2.32×10^6 spores/ml	Freund's	15, 30, 46, 61	57, 71
	3	Glutaraldehyde-inactivated 93-196C whole spores	4.35×10^6 spores/ml	Freund's	15, 30, 46, 61	57, 71
	3	Formaldehyde-inactivated 93-196C whole spores	2.33×10^6 spores/ml	TitreMax	25, 40	36, 50
	3	Glutaraldehyde-inactivated 93-196C whole spores	4.35×10^6 spores/ml	TitreMax	25, 40	36, 50
	3	Formaldehyde-inactivated 93-196C whole spores	2.33×10^6 spores/ml	Adjuvax	27, 43, 58	54, 68
	3	Glutaraldehyde-inactivated 93-196C whole spores	3.48×10^6 spores/ml	Adjuvax	27, 43, 58	54, 68
Swiss	3	93-196C spore coat protein	1.0 mg/ml	Freund's	14, 30, 48, 63, 85, 106	58, 73, 97, 120
	3	Extracted formaldehyde-inactivated 93-196C whole spores	2.21×10^5 spores/ml	Freund's	14, 30, 48, 63, 85, 106	58, 73, 97, 120
	3	Extracted glutaraldehyde-inactivated 93-196C whole spores	2.49×10^5 spores/ml	Freund's	14, 30, 48, 63, 85, 106	58, 73, 97, 120
	3	Glutaraldehyde-inactivated 93-196C whole spores	2.00×10^5 spores/ml	Freund's	14, 30, 48, 63, 85, 106	58, 73, 97, 120

At the termination of each protocol, mice were euthanized by cervical dislocation. The anti-anthrax spore response of the pre-bleed and test sera were determined using an indirect serum titre ELISA (see section A.6.).

Spores of *B. anthracis* strains 93-189C and 93-196C were produced in modified Tarr's sporulation flasks, harvested with either 3% (v/v) formaldehyde or 5% (v/v) alkaline glutaraldehyde, and stored in 1% (v/v) aldehyde (see section II.2.c.). Prior to protein extraction or mouse immunization with whole spores, the required amount of spores was washed twice with saline, as described previously, in order to remove unreacted aldehyde. After washing, the spore concentration of the preparation was calculated via phase contrast microscopy.

The outer coat proteins of formaldehyde-inactivated spores of the two strains were selectively isolated following a procedure described by Aronson and Fitz-James (1971; see section A.4.). However, instead of precipitating the solubilized proteins around spore protoplasts to regenerate whole spores, the proteins were particulated in the presence of aluminum hydroxide particles in an effort to reconstitute the native outer spore coat structures within an aluminum hydroxide matrix. Spore coat protein and aluminum hydroxide were mixed in a 1:2 ratio by weight (protein concentrations of 0.13 mg/ml and 0.11 mg/ml for strain 93-189C and 93-196C, respectively). All injections of the coat protein plus aluminum hydroxide preparation in BALB/c mice were given intraperitoneally (ip) at a dosage of 250 µl.

Bacillus anthracis 93-196C whole spore preparations were injected into groups of BALB/c mice alone or with Freund's adjuvant, Adjuvax (Alpha-Beta Technology, Worcester, MA) or TitreMax (CytRx Corporation, Norcross, GA) following the manufacturer's directions. With Freund's adjuvant, the primary inoculation was done in complete adjuvant and all boosters were with incomplete adjuvant. Spore suspensions were mixed 1:1 with the appropriate Freund's adjuvant in Eppendorf tubes and shaken on

a tube mixer at 4°C for 90 min prior to injection ip into mice at a dosage of 130 µl. TitreMax was mixed 1:1 with the spore suspensions in tubes and shaken as above prior to sc injection of 60 µl at the base of the animal's tail. One milligram of Adjuvax was mixed with the appropriate number of spores in 1 ml of saline and shaken as above before sc injection into both hind thighs of the animal at a dosage of 100 µl/thigh (200 µl total). All immunizing preparations were made fresh on the day of injection. Boosters with TitreMax and Adjuvax were given at the same volume but with one-tenth the concentration of the primary injection as per the directions of the manufacturers.

Swiss strain mice were tested for their immune response to inactivated whole spore and spore coat protein preparations. One group of three mice received a preparation of 93-196C spore coat proteins dialyzed in reconstitution buffer in the absence of aluminum hydroxide particles. Two groups of three mice each received preparations of formaldehyde- or glutaraldehyde-inactivated 93-196C spores pretreated with one wash of total spore coat extraction solution (see section A.4.). A final group received glutaraldehyde-inactivated whole 93-196C spores. All preparations were mixed with Freund's adjuvant as described above. All inoculations were ip with 150 µl of spore preparation.

II.3.b. Production of polyclonal serum against *B. cereus*-group spores in rabbits

Two New Zealand white rabbits were immunized with repeated injections of formaldehyde-inactivated *B. anthracis* strain 9604 whole spores in Freund's adjuvant. Strain 9604 was isolated from a bovine carcass sampled during a small outbreak in northern Alberta in 1996 while the other wild *B. anthracis* isolates used in this study were obtained from bison carcasses found during the 1993 MBS epizootic (Table II-1). *Bacillus anthracis* is one of the most molecularly monomorphic bacteria known (Keim et al., 1997). The spore epitopes of the bison and cattle isolates should be the same even though multiple-locus variable-number tandem repeat analysis (MLVA) has demonstrated

that the cattle and bison isolates belong to two separate but closely related genotypes (Keim et al., 2000). The differences in short polymorphic genetic sequences identified by MLVA between the two genotypes should not translate into structural differences in the spore surfaces of the isolates.

As with mouse inoculations, the spores were harvested from the flasks directly with the inactivating formaldehyde and stored in 1% (v/v) aldehyde until required. Prior to injection, portions of the spore stock were washed twice with saline and the spore concentration of the preparation was calculated with phase contrast microscopy. The spore preparation was mixed 1:1 with Freund's adjuvant as described in the previous section.

Three days prior to the primary injection the rabbits were pre-bleed via the marginal ear vein and the resultant sera was pooled as a negative control. Injections were spaced 2 - 3 weeks apart depending on the apparent health of the animals. Beginning after the third booster, ear bleeds were taken from each rabbit to monitor the developing immune response. Test bleeds were taken 10 - 14 days after the last booster and at least 3 days elapsed after a bleed before the next booster was administered. At the termination of the protocol, the rabbits were exsanguinated via cardiac punctures and euthanized by pentobarbital overdose.

At each injection, rabbits were inoculated sc at four sites along the upper backbone between the shoulder blades with 250 μl at each site and once intramuscularly (im) in the upper thigh (alternating thighs every injection) with 500 μl (Harlow and Lane, 1988). The immunizing preparation had a concentration of approximately 1.79×10^6 inactivated spores/ml for a total dose of approximately 2.69×10^6 spores per injection. All injections were at the same strength. Boosters were given on days 15, 29, 43, 57, 72, 93, 115, 136,

158, 178, 206 and 227 post primary injection (ppi). Test bleeds were taken on days 52, 66, 84, 107, 129, 150, 171, 192 and 220 and the rabbits were terminally bled and euthanized on day 241.

The anti-anthrax spore response of the test sera was tested using the serum titre ELISA described in section A.6. Sera from day 66 onwards was frozen at -20°C for at least 2 days before testing the anti-anthrax spore response (see section III.1.b.). Sera collected from day 107 onwards was pooled together for subsequent purification as polyclonal serum. The serum was purified by ammonium sulfate precipitation followed by low salt protein A chromatography (section A.7.). At each step of the purification, the protein concentration was determined by UV spectroscopy at 280 nm (section A.7.) and the serum titre against formaldehyde-inactivated *B. anthracis* 9604 spores was determined by the serum titre ELISA (section A.6.).

The sensitivity of the purified polyclonal serum for inactivated *B. anthracis* 9604 spores, and live and inactivated spores of avirulent *B. anthracis* ATCC 4229 and other selected *Bacillus* species was determined for specific serum dilutions with the spore titre ELISA (section A.6.). The specificity of the purified serum against approximately 10^5 spores/well of numerous *Bacillus* and *Clostridium* species was tested via the serum titre ELISA.

II.3.c. Experimentation with monoclonal antibodies FDF 1B9 and EAII-6G6-2-3

Raw ascites samples of monoclonal antibodies FDF 1B9 and EAII-6G6-2-3 were kindly donated by Dr. J.W. Ezzell Jr. of the United States Army Research Institute of Infectious Diseases, Fort Detrick, Maryland. Immunoglobulins were purified from the ascites fluid by 50% (w/v) ammonium sulfate precipitation preceded by a 33% (w/v) precipitation to remove high molecular weight contaminants (Harlow and Lane, 1988). After precipitation, monoclonal FDF 1B9, which was designated an IgG, was further purified using Protein A chromatography under low salt conditions; EAII-6G6-2-3, designated an

IgM, was purified via size exclusion chromatography using Sephadex G150 beads. Following their respective column purifications, the monoclonals were concentrated with a second 50% (w/v) ammonium sulfate precipitation, this time omitting the 33% (w/v) pre-precipitation (section A.7.).

Purification of the antibodies during the procedures was monitored by protein absorbance at 280 nm and the serum titre ELISA (sections A.6 and A.7) using approximately 10^5 cells/well of *B. anthracis* ATCC 4229 from overnight blood agar cultures as the antigen for EAII-6G6-2-3 and from overnight candle jar cultures on bicarbonate plates as the antigen for FDF 1B9. The *B. anthracis* ATCC 4229 grown on SBA did not produce any capsular material to mask the cell wall polysaccharide target of EAII-6G6-2-3 whereas the microbe cultured on bicarbonate was fully encapsulated and reactive with FDF 1B9. The immunofluorescent assay for viable *B. anthracis* spores described in Ezzell and Abshire, 1996 was adapted to an indirect plate ELISA format. With this viable spore ELISA, the sensitivity and specificity of the purified monoclonal antibodies were determined against *B. anthracis* ATCC 4229, *B. cereus* ATCC 14579 and *B. subtilis* ATCC 6633 spores.

II.4. Selective PLET medium

PLET medium was formulated according to Knisely (1966) and Carman et al. (1985), except 300 U/ml of lysozyme was used instead of 40 μg/ml. Fifty microlitres of each *Bacillus* spore stock were inoculated to PLET and SBA plates. Samples of several *Bacillus* strains were also inoculated to plates of PLET supplemented with either 5% (v/v) horse blood or 1.0 μg/ml nitrocefin. The cultures were incubated at 37°C and observed 24 and 48 h post-inoculation. Plates of SBA and PLET were compared for their ability to recover *B. anthracis* ATCC 4229 spores from a standard stock suspension. In triplicate, 100 μl portions of the stock were spread on SBA and PLET plates. The plates were incubated at 37°C and the colonies were counted after 24 and 48 h incubation.

II.5. *Bacillus anthracis* spore isolation

II.5.a. Recovery of anthrax spores from seeded sterile soil

Viable, avirulent *B. anthracis* ATCC 4229 spores were added to autoclaved samples of potting, wallow and field soil types and used to test the efficacy of several wash solutions for recovery of spores. Sucrose and detergent solutions used for spore extraction were autoclaved at 121°C for 20 min to sterilize. Hydrometer measurements indicated that autoclave treatment did not effect the specific gravity of the sucrose solutions.

Potting soil used in the experiments was a commercially available, acidic (pH 3.82; see Table A-1), general-use soil comprised of 50% peat and 50% loam. The wallow and field soil types were collected from an open glacial lake bed surrounding Falaise Lake in the MBS, NT (Gates et al., 1995). Both soil types were alkaline (pH 7.45 to 8.22; see Table A-1), clay based and calcareous (> 6000 ppm) due to a high content of crushed mollusk shells. The field soil type was comprised of a mixture of loam and clay and supported a diverse array of sedges and grasses. The wallow soil came from a bison wallow where the rolling and pawing of the animals had denuded the area of vegetation. There was very little loam associated with the wallow soil. The chemical composition of the soil types are listed in Table A-1 of the Appendix.

Soils were dispensed in 2.5 g portions to Universal bottles and autoclaved at 121°C for 25 min. The soil samples were transferred to appropriately sized disposable centrifuge tubes. Between 2 - 8 x 10^5 *B. anthracis* ATCC 4229 spores were seeded to the soil in a liquid volume of 0.5 ml and the samples were shaken vigorously by hand to mix. The seeded soil specimens were stored in a darkened drawer at room temperature for 3 days prior to extraction experiments.

To extract spores, the appropriate wash solution was added to a seeded sample, the tube was shaken vigorously for 1 min and then secured to a platform shaker and agitated for 15 min at 75 rpm. The samples were placed upright in a rack for 10 min to allow large soil particles and aerosols to settle. The samples were centrifuged at 2000 x g for 45 s to settle minute soil particles and surface foam. With extraction solutions of high specific gravity, the top 3 ml of supernatant were transferred to a new tube containing 6.0 ml of filtered 1% (w/v) bovine serum albumin in PBS (BSA/PBS) in order to reduce the specific gravity and allow collection of spores via centrifugation. The new tube was gently agitated to mix the contents and centrifuged at 5100 x g for 10 min. The supernatant was discarded and the pellet was resuspended in 1 ml of 1% (w/v) BSA/PBS. For water and detergent solutions, the dilution step was unnecessary and the top 3 ml of supernatant were transferred to an empty tube. The sample was centrifuged as before and the pellet was resuspended in 1% (w/v) BSA/PBS.

The secondary tubes in which the spores were pelleted were all pre-blocked with 3% (w/v) BSA/PBS for 2.5 h at room temperature before addition of the extracted sample. Use of BSA/PBS as a blocking agent and dilutant minimized spore clumping and spore adherence to the inner tube walls, and greatly reduced the variance in spore concentration calculations.

In experiments with seeded sterilized soil samples, no heat or ethanol purification was performed and the samples were plated on SBA rather than PLET in order to maximize germination of the spores recovered. Ten-fold serial dilutions of the isolated samples were made in 1% (w/v) BSA/PBS and 100 µl portions of the neat and diluted samples were spread on SBA plates and incubated overnight at 37°C. The next day colonies on the plates were counted and used to determine the percentage of spores recovered.

The sensitivity limits of PLET medium with the standard water or 1.22 g/ml sucrose plus 0.5% Triton X-100 (sucrose plus Triton) extraction were compared. Spore isolation tests

were performed on sterilized 2.5 g wallow and field soil samples seeded in a dilution series with 10^4 - 10^1 ATCC 4229 spores. Extraction was as described above except the samples were plated on PLET medium.

II.5.b. Bactericidal and sporicidal effects of ethanol- and heat-purification and sucrose plus Triton X-100 extraction

Stocks of *B. anthracis* ATCC 4229 spores and overnight vegetative cultures of *B. cereus* ATCC 14579 and *P. aeruginosa* S169-90 were prepared in 1% (w/v) BSA/PBS. The microbe concentration of each stock was determined by serial dilution and overnight incubation on SBA plates. Multiple 1 ml samples of each stock were placed in a 63°C water bath for 20 min. A second set of 1 ml samples were centrifuged at 5100 x g for 10 min and the supernatant was replaced with 1 ml of filtered 50% (v/v) ethanol. The microbes were resuspended in the ethanol and gently agitated for 60 min at room temperature. The samples were then centrifuged as before and the ethanol was replaced with 1 ml of 1% (w/v) BSA/PBS. A third set of 1 ml samples were centrifuged and the pellets were resuspended in 350 µl of 1.22 g/ml sucrose plus Triton. The samples were gently agitated for 15 min at room temperature and then 700 µl of 1% (w/v) BSA/PBS was added to each tube. The samples were centrifuged and the pellets were resuspended in 1 ml of 1% (w/v) BSA/PBS. Samples from all three treatments were serially diluted and plated on SBA. The blood plates were incubated overnight at 37°C.

II.5.c. Comparison of spore extraction and purification methods in non-sterile soil

Deionized water and 1.22 g/ml sucrose plus Triton extraction solutions, and heat and ethanol spore purification were compared for their ability to detect *B. anthracis* spores in environmental specimens collected from anthrax carcass disposal sites in northern Canada. The samples had been collected following anthrax epizootics in free-roaming bison and stored up to 5 y prior to testing. Some of the specimens had been previously shown to

contain *B. anthracis* spores via 1.22 g/ml sucrose plus Triton extraction, ethanol purification and PLET culture (see section III.5.a). Because some of the soil samples contained virulent *B. anthracis* spores, the experiments were performed under full Level III biosafety precautions in the anthrax diagnostic laboratory at ADRI. Any colonies isolated in these experiments with a morphology similar to *B. anthracis* on the PLET plates were further tested to confirm their identity (see section II.6.b).

Eighteen environmental specimens were divided into duplicate 3.0 g samples and extracted with 12 ml of 1.22 g/ml sucrose plus Triton or deionized water. The extraction was as described above except the final isolated pellet was resuspended in 1 ml of 50% (v/v) ethanol and shaken at 75 rpm for 1 h at room temperature. The samples were then centrifuged at 5100 x g for 10 min and the ethanol was replaced with 1 ml of 1% (w/v) BSA/PBS. One hundred microlitres of each sample were spread on PLET plates which were incubated at 37°C for 48 h.

A second set of 18 environmental specimens was divided into duplicate 3.0 g samples and extracted with 12 ml of 1.22 g/ml sucrose plus Triton. One set of samples was treated with 50% (v/v) ethanol for 1 h as described above. The other set was treated with heat to kill vegetative cells present. The extracted pellet was resuspended in 1.0 ml of 1% (w/v) BSA/PBS and placed in a 63°C water bath for 20 min. Afterwards, 100 µl portions of each sample were spread on PLET plates and incubated at 37°C for 48 h.

II.6. Field collections in endemic regions of northern Canada

II.6.a. Environmental sampling and specimen storage

Between 1992 and 1997, several field trips were undertaken to anthrax endemic regions in northern Canada to collect environmental specimens from carcass disposal sites and bison habitat (Figure I-2). In August 1992 and July 1993, specimens were collected from

anthrax burial mounds in the Hook Lake region and from burial sites along Parson's Lake Road (PLR) in central WBNP. Samples collected from the Park were obtained under research permit 92-13 (WBNP, Environment Canada; Environmental assessment registry number WB 92-32). In July of 1994 and 1997, trips were made into the Falaise Lake region of the MBS to collect specimens from carcass disposal sites under the authority of NT wildlife research permits WL001018 and WL001547. A limited number of soil specimens from bison wallows and open meadows not associated with known anthrax carcass sites were also collected during the Falaise trips. The wallow and meadow samples were collected during differential transect surveying of a pie-shaped section of Falaise field north of the lake.

Although an anthrax epizootic occurred in the MBS during the time period of this study, no environmental specimens were collected during the outbreak. Because of the large scale of the outbreak and limited resources, all efforts went towards carcass location and disposal. As a result, all specimens were collected at least a year after an outbreak and all carcass sites sampled had experienced some form of disposal treatment.

During field observations the location of disposal sites was determined using a portable Global Positioning System (GPS) unit. Each carcass site was visually surveyed to detect elevations and depressions, roughly mapping the details by hand. At each site, a central wooden peg was hammered into the ground. Environmental specimens were collected from around the site recording the distance and bearing of each relative to the wooden peg using a tape measure and compass. Similarly, the position of nearby wallows, elevations, vegetation and other phenomena of interest were recorded relative to the peg so that the entire site could be remapped in detail.

Surveyed disposal sites in the Hook Lake and PLR regions were identified by a two digit number; the first digit referring to the year sampled (1 = 1992; 2 = 1993) and the second to the order encountered. During clean-up operations of the 1993 MBS anthrax

epizootic, each carcass disposal site was given a key identification number. Surveyed carcass disposal sites in the Falaise Lake region were matched to their key identification number by comparing their coordinates with those listed in clean-up records.

During the July 1994 trip to Falaise Lake, a wooden peg was sunk in the field at 61° 21' 0" N, 116° 12' 50" W. Moving directly south from the peg towards the lake proper, shallow soil samples were collected every 3 m, starting right beside the peg and ending 72 m away on the mud flat shore of the lake. The final sample was taken from the lake and contained water as well as mud.

During July 1997, a pie-section of Falaise Lake field from the shore of the lake proper to the escarpment surrounding the field was surveyed using differential transecting. A permanent iron pole was sunk in the lake approximately 5 m from the shoreline and the water level was marked on the pole with a length of aluminum wire. A fixed length of nylon rope, 25.8 m long, was used to sink a second pole on shore at that distance from the first. The elevation of the second pole relative to the water mark on the first pole was recorded with a transect and 3-m stick. Using the rope to keep a constant distance and the transect to determine the bearing from the last point, transect points were leap-frogged across the field measuring their elevation relative to the last transect point. At each transect point a wooden peg was sunk and, using a 25-m tape measure and the transect, the immediate surrounding area was surveyed relative to the transect point. During these survey arcs special attention was paid to nearby bison wallows and trails. Environmental samples were collected from the surrounding field and several of the wallows recording their location relative to the nearest transect point.

Environmental specimens collected were comprised of a heterogeneous mixture which included soil, charred bone, bison hair, animal feces, maggot casings, vegetation and ash. Samples were collected using disposable plastic spoons and were taken only to a maximal depth of 5 cm as there was no core sampler available in the field to accurately collect

specimens from greater depths. The samples were carefully transferred to labeled Universal bottles; approximately 15 ml (3 - 12 g) of sample was collected in each bottle. The lids of the bottles were ringed with parafilm in an effort to form an airtight seal. The sample bottles were stored at ambient temperature in a secure facility until they could be screened for viable anthrax spores.

II.6.b. Spore extraction and *B. anthracis* identification

Bacterial spore extraction and identification of *B. anthracis* in the environmental specimens was done under full Level III biosafety precautions in the anthrax diagnostic laboratory at ADRI. Approximately 3 g of each specimen were transferred to a 30-ml centrifuge tube. Twelve millilitres of a 1.22 g/ml sucrose plus 0.5% (v/v) Triton X-100 solution were added to each sample and the tubes were briefly shaken by hand to suspend the specimen. The tubes were secured to a platform shaker and agitated at 150 rpm for 10 min. The samples were left in an upright position for 10 min then spun at 850 x g for 45 s to settle large particles and surface foam. The top 3 ml of supernatant were transferred to a 15-ml centrifuge tube containing 6 ml of filtered 1% (w/v) BSA/PBS in order to reduce the specific gravity and allow the collection of any spores present by centrifugation. The 15-ml tube was gently shaken to mix the contents and then centrifuged at 5000 x g for 10 min. The supernatant was discarded and the pellet was resuspended in 1 ml of filtered 50% (v/v) ethanol. The samples were secured to the platform shaker and agitated at 150 rpm for 1 h. The tubes were centrifuged as before and the ethanol was discarded. The pellets were resuspended in 1 ml of 1% (w/v) BSA/PBS and a 200 µl aliquot was spread onto a PLET plate. The cultures were incubated at 37°C for 48 h.

After 24 and 48 h incubation, PLET plates were observed for growth and any colonies present were counted. Colonies isolated with a morphology resembling *B. anthracis* on the PLET plates were further tested to confirm their identity. One in five of the suspect colonies were picked from the plate, spread over a quarter section of a SBA plate and overlaid with a 10 unit disc of penicillin G sulfate. The blood plates were incubated at 37°C overnight. Colonies with any zone of inhibition around the penicillin disc were for

the purposes of this investigation designated "penicillin-sensitive" even though this inhibition does not conform to established criteria for determining microbial antibiotic susceptibility in most species of Gram-positive bacteria (National Committee for Clinical Laboratory Standards, 1997) Any non-hemolytic, penicillin-sensitive isolates were picked off the blood plates and spread on bicarbonate plates which were incubated overnight at 37°C in 10% CO_2. Isolates forming mucoid colonies on bicarbonate were smeared to microscope slides, formalin fixed to the slide and treated with Geimsa stain. The slides were observed under 1000x magnification with an oil emersion lens to detect the presence of encapsulated bacilli (Carman et al., 1985).

Any PLET isolates that were non-hemolytic, penicillin-sensitive, mucoid-positive, capsule-positive bacilli were subcultured to TSA slants and sent to the Louisiana State University, Baton Rouge for DNA extraction which was performed by Dr. Pam Corker. The genetic material was then forwarded to the laboratory of Dr. Paul Keim at the Department of Biology, Northern Arizona University, Flagstaff where final identification was achieved via MLVA (Keim et al., 2000).

II.7. Statistical analyses

All statistical analyses were performed using SigmaStat 1.0 (Jandel Scientific, 1992) with the alpha value of each test set at 0.05. All analyses were tested for their power to resolve a statistical difference in the data set if indeed there was one present (type II error). If a test failed to find a significant difference yet had a power < 0.800, its negative results were suspect. In experiments where a significant difference between treatment groups was detected via a one-way ANOVA, multiple pairwise comparisons were performed using the Student-Newmann-Keuls method. In most of the experiments performed in this study the sample sizes were < 10. Because variance is inversely proportional to sample size and affected by small values, it was represented by standard error, which accounts for sample size, rather than standard deviation (Norman and Streiner, 1994). All logarithms in figures and text are to the base of ten.

Chapter III. Results

III.1. Immunodetection of anthrax spores

III.1.a. Immune response of mice to *B. anthracis* spores

Fifteen days after primary injection with isolated *B. anthracis* strain 93-189C spore coat proteins plus aluminum hydroxide, three of the five BALB/c mice were found dead in their cage. The two remaining mice had red swellings approximately 8 mm in diameter at the site of injection. The next day the peritoneal swelling on the two remaining mice had approximately doubled in size and the animals appeared in obvious discomfort. The mice were euthanized by cervical dislocation. The bodies along with two Eppendorf tubes of the 93-189C spore coat plus aluminum hydroxide preparation, including the tube used for the primary injections, were packed on ice and sent down to ADRI for bacteriological investigation.

Gram and Geimsa staining of the 93-189C immunizing preparation found no whole vegetative cells or spores present. No micro-organism grew out of samples of the preparation seeded to SBA or TSA plates, bicarbonate agar plates incubated in a candle jar, nutrient broths, or SBA plates after a 24-h pre-incubation in nutrient broth; all media were incubated at 37°C for 72 h. Sheep blood agar cultures of saline washes of the spleen, heart and peritoneal cavity of the carcasses and a subcutaneous nodule found at the inoculation site of one of the mice failed to grow any microbes after 72 h at 37°C. Four Swiss strain mice (no BALB/c mice were available for injection at ADRI at the time) were injected ip with a dosage of the preparation identical to that given to the first group of mice in an effort to duplicate the morbidity and mortality. No deaths or illness occurred in the mice up to 5 weeks after the primary injection when the test was discontinued.

With no living agent isolated from the dead mice or immunizing preparation, it was hypothesized that the mortalities were probably due to residual dithiothreitol in the spore coat extraction. The remaining 93-189C preparation was pooled and dialyzed overnight at 4°C against two changes of 6.0 l of PBS (molecular weight cut-off of the dialysis tubing was 12 - 14 kDa). The preparation was redistributed into Eppendorf tubes for storage as before and 50 µl samples were injected ip into five new BALB/c mice. No deaths occurred and the mice were boostered ip on day 15 ppi with another 50 µl of the preparation. Two days later, two of the mice were found dead. On day 31, two more animals were found dead. The remaining mouse was euthanized and the protocol was discontinued.

Although not associated with any mouse mortalities, spore coat proteins of *B. anthracis* strain 93-196C reconstituted in the presence of aluminum hydroxide proved poorly immunogenic in BALB/c mice (Figure III-1). Even after repeated boosters, the anti-anthrax spore serum titre of the mice did not rise above 100. Formaldehyde- and glutaraldehyde-inactivated whole 93-196C spores proved more immunogenic than the isolated spore coat protein eliciting higher serum titres against their respective inactivated spores, however, the resultant titres were still low. Coinjection of the inactivated spores with adjuvant did little to improve the resultant immune response in the mice. Serum titres of mice inoculated with spore preparations containing Freund's, Adjuvax or TitreMax were of the same magnitude as those obtained in mice injected with roughly the same amount of spores and no adjuvant.

Due to the poor immune response of BALB/c mice to the spore preparations, the immunization protocols were continued with Swiss strain mice. Groups of Swiss mice were immunized with isolated spore coat protein, glutaraldhyde-inactivated whole spores, or formaldehyde- or glutaraldehyde-inactivated spores pretreated with total spore coat extraction solution; all in Freund's adjuvant. All four protocols elicited approximately equal serum titres against the inactivated test spores that, although higher than serum titres

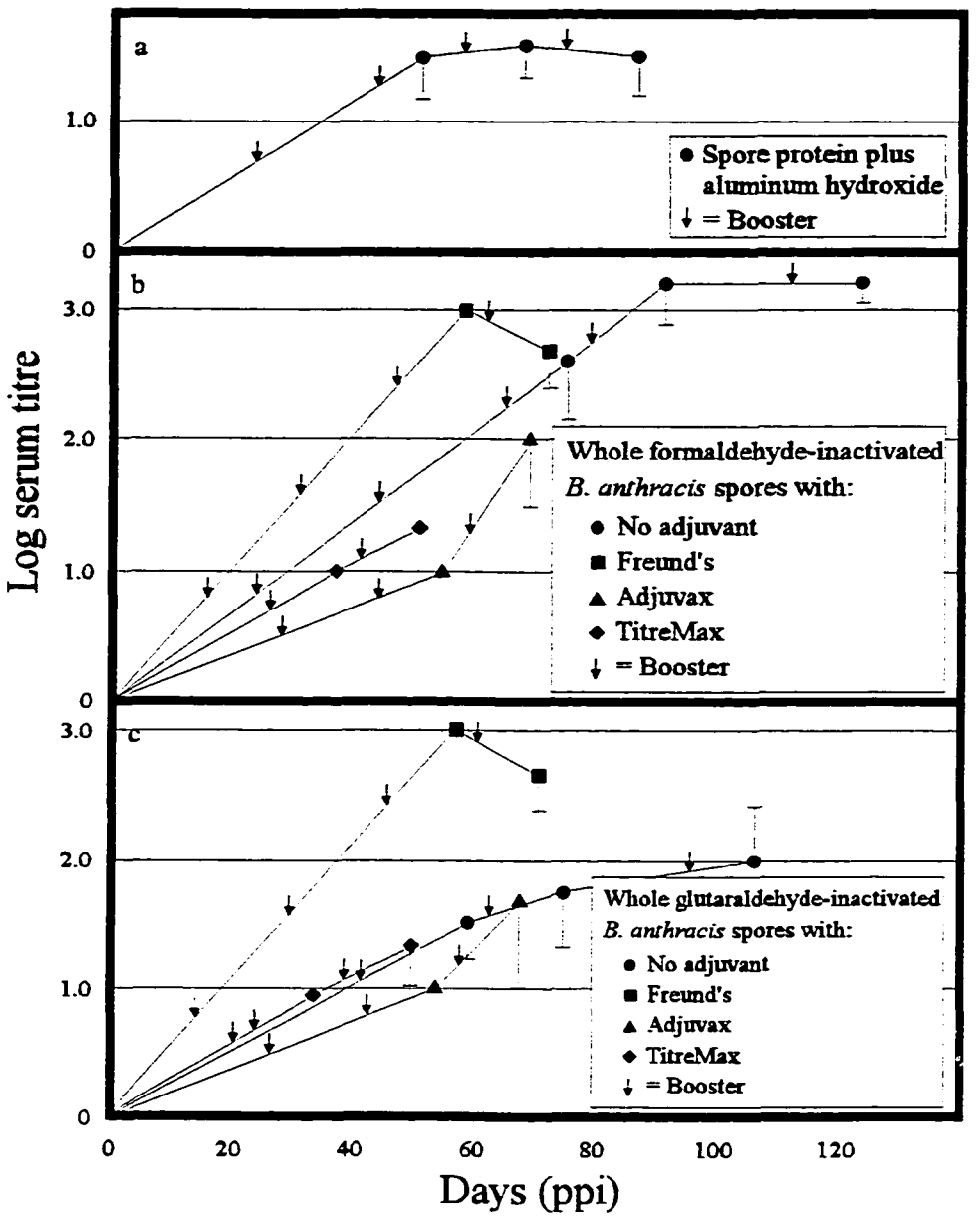

Figure III-1. Immune response of BALB/c mice to *B. anthracis* 93-196C spore protein with aluminum hydroxide, and formaldehyde- and glutaraldehyde-fixed whole spores with various adjuvants. The sera of mice receiving spore protein (panel a) or formaldehyde-fixed spores (panel b) was assayed against formaldehyde-fixed spores while the sera of mice receiving glutaraldehyde-fixed spores (panel c) was tested against glutaraldehyde-fixed spores.

obtained in BALB/c mice, were still not sufficient for monoclonal antibody production (Figure III-2). A hybridoma fusion was attempted with the best responding mice; those receiving the whole glutaraldehyde-inactivated spores. Fusion of splenocytes from these mice and mouse myeloma NS-1 cells was successful and 122 hybridomas were produced. However, none of the hybridomas produced monoclonal antibodies that were reactive against whole glutaraldehyde-inactivated *B. anthracis* 93-196C spores (data not shown).

III.1.b. Polyclonal rabbit serum against *B. cereus*-group spores

During the course of the immunization protocol, the rabbits developed hemorrhagic nodules at the sc injection sites along the upper vertebrae. The vivarium staff determined that the lesions were a common response to Freund's adjuvant. At first, the rabbits appeared unbothered by the nodules but as the protocol continued and more nodules formed, the animals weakened visibly and became arthritic. The protocol was halted, the animals were euthanized and their blood was collected via a terminal cardiac bleed.

Sera collected during test and final bleeds of the rabbits was serially diluted and tested in an indirect immunoassay against formaldehyde-inactivated *B. anthracis* 9604 spores in order to determine the anti-spore titre. It was noted upon repeated testing of the sera that the titre dropped up to 1000-fold after short-term (2 - 7 days) storage at -20°C. Limited subsequent freezing and thawing did not result in a further decrease in the titre. As long-term storage of the polyclonal serum at -20°C would be required if it were to be used in a diagnostic field ELISA system, the higher serum titre prior to freezing was disregarded. All titres quoted for rabbit serum and the purified polyclonal serum are for samples of sera that had been frozen at least 48 h at -20°C prior to the immunoassay.

The immune response of both rabbits to the immunogen preparation is depicted in Figure III-3. Whole formaldehyde-inactivated *B. anthracis* 9604 spores in the presence of Freund's adjuvant elicited a much higher immune response than the immunization

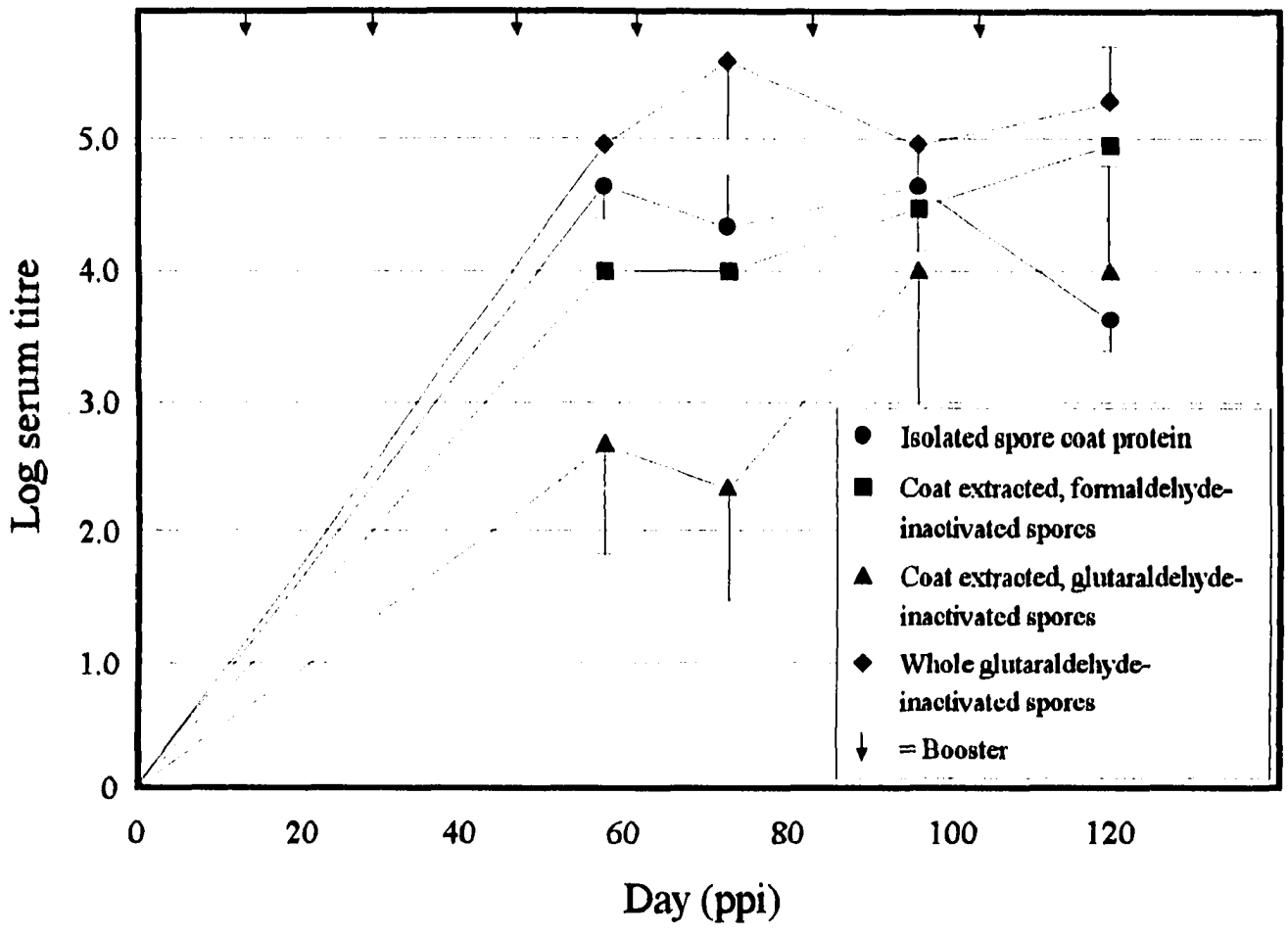

Figure III-2. Immune response of Swiss mice to *B. anthracis* 93-196C spore protein, glutaraldehyde-fixed whole spores, and formaldehyde- and glutaraldehyde-fixed, coat extracted spores. All spore preparations were combined with Freund's adjuvant. The sera of mice receiving spore protein or formaldehyde-fixed spores was assayed against formaldehyde-fixed spores while the sera of mice receiving glutaraldehyde-fixed spores was tested against glutaraldehyde-fixed spores.

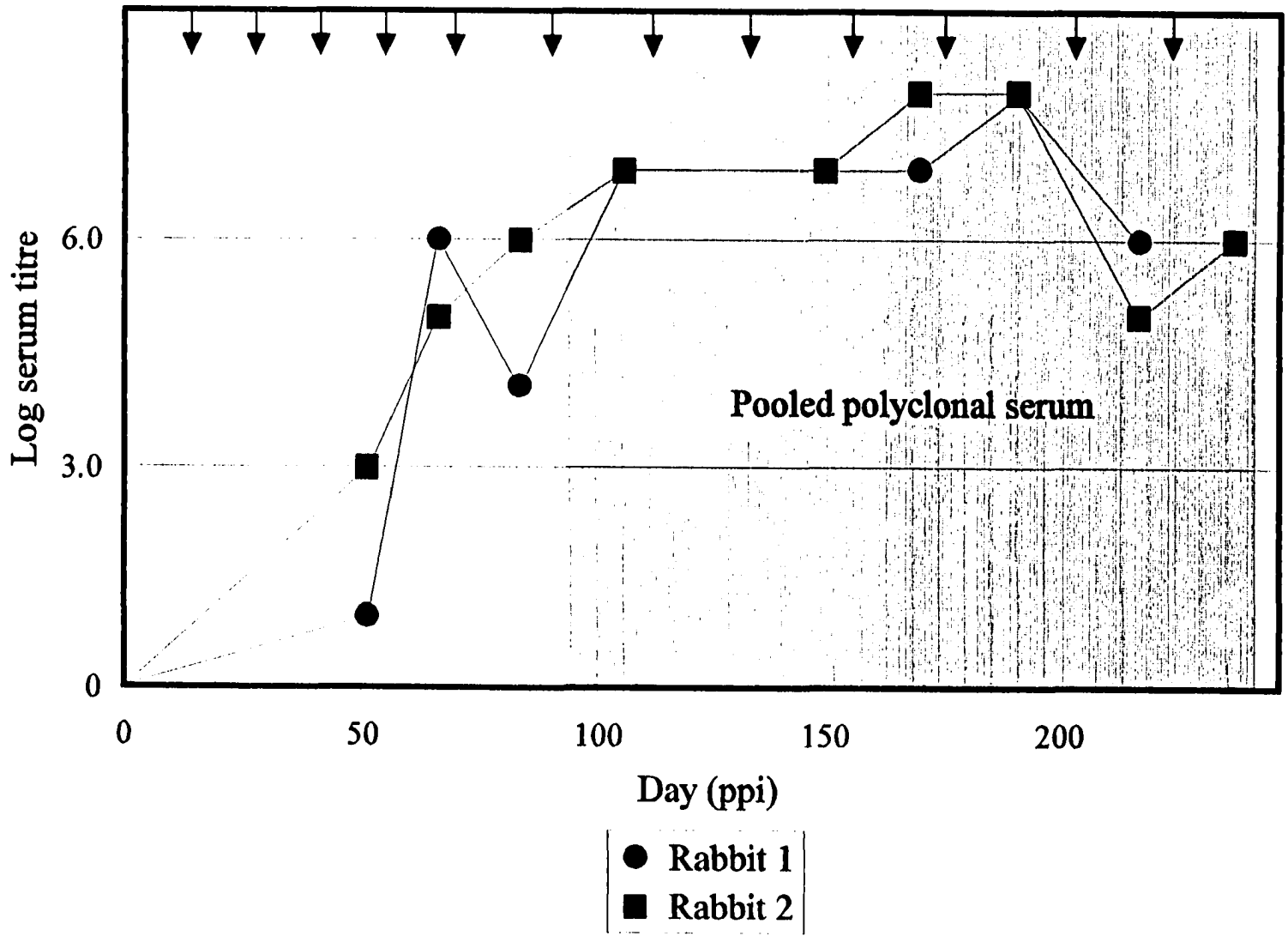

Figure III-3. Immune response of New Zealand white rabbits inoculated with formaldehyde-fixed *B. anthracis* 9604 spores to formaldehyde-fixed anthrax spores. Each sera was tested in triplicate after short- term storage at -20°C for 48 h.

protocols used with BALB/c and Swiss mice. The serum titre of both rabbits peaked at 10^8 after the ninth booster on day 158 ppi and then quickly deteriorated as the health of the animals declined. All sera collected from the rabbits after the sixth booster on day 93 ppi was pooled together as raw polyclonal serum. At each test bleed, approximately 5 ml of purified serum was collected from each rabbit and at the terminal bleed approximately 30 ml of serum/rabbit was obtained for a total raw polyclonal serum volume of 120 ml.

The immunoglobulins in the pooled raw serum were purified through 50% (w/v) ammonium sulfate precipitation; with a 33% (w/v) pre-precipitation step to remove any high molecular weight contaminants; followed by low salt Protein A chromatography (see Appendix A.7.). Immunoglobulins reactive against formaldehyde-inactivated *B. anthracis* 9604 spores eluted from the Protein A column in a large single protein peak (Figure III-4). No other protein peaks were observed in the elution profile of the column up to the fiftieth fraction (25 ml or five column volumes of elutent). Overall, the ammonium precipitation and Protein A procedure purified the polyclonal serum 156.5-fold by weight and improved the serum titre against formaldehyde-inactivated *B. anthracis* 9604 spores 1000-fold (Table III-1).

After purification, serial dilutions of the polyclonal serum were tested in an indirect ELISA against approximately 10^5 spores of various strains of *Bacillus* and *Clostridium* species (Figures III-5 and III-6). The polyclonal serum bound most successfully to the formaldehyde-inactivated spores of *B. anthracis* 9604 and *B. anthracis* 93-196C (titre 10^9). High serum titres (10^6 to 10^5) were also obtained against *B. anthracis* ATCC 4229 spores and most other strains of the *B. cereus*-group tested except *B. cereus* CPS 85-03A, the environmental *B. cereus* isolate and *B. mycoides* MU711/84 (titres 10^4 to 10^3). Titres against the remaining *Bacillus* species tested were low (10^2 to 10^1).

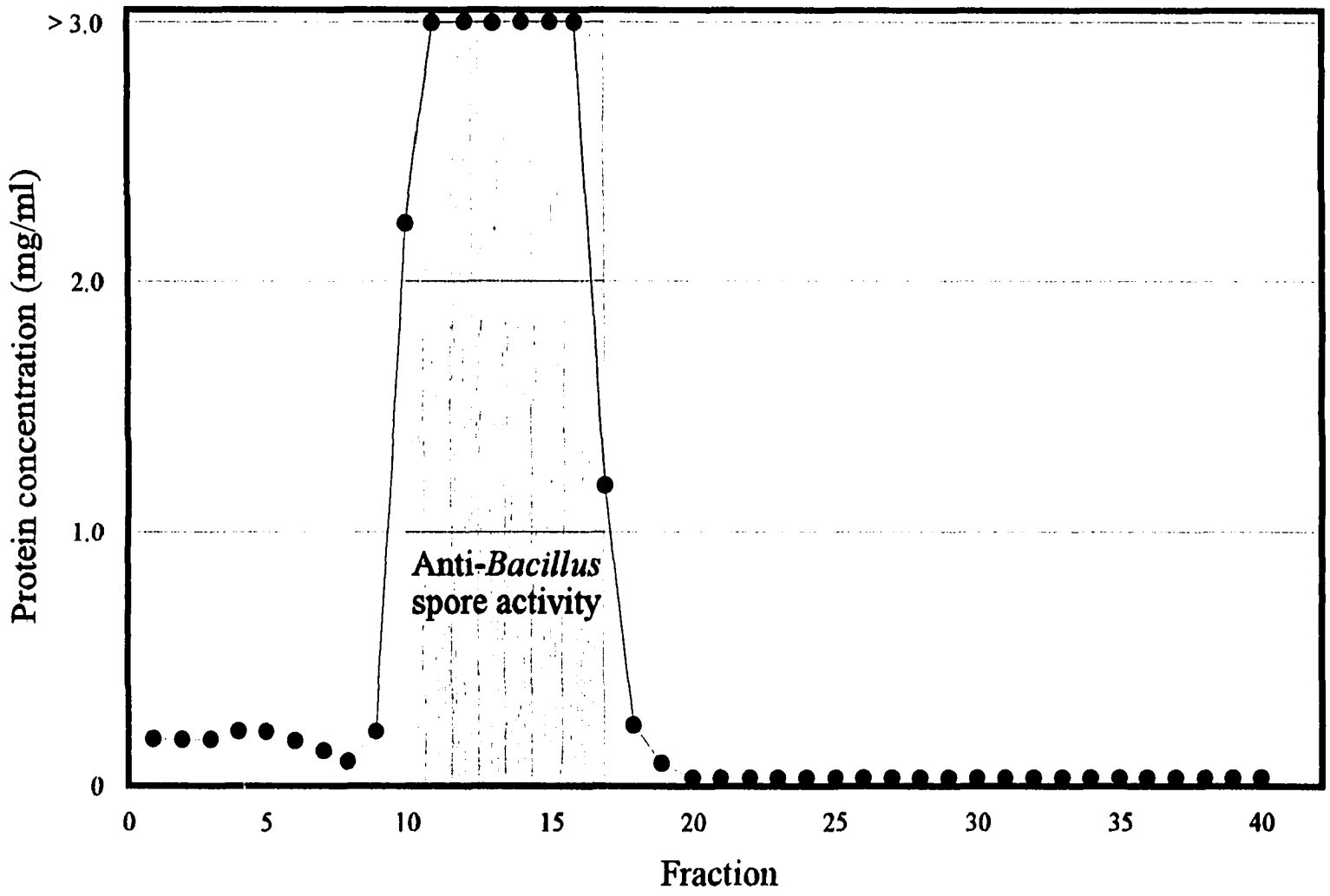

Figure III-4. Elution profile of rabbit polyclonal serum from low salt Protein A column. The protein concentration of each fraction was determined by UV absorbance at 280 nm and the activity against formaldehyde-inactivated *B. anthracis* 9604 spores was determined via the serum titre ELISA.

Table III-1. Step purification of anti-*Bacillus* spore rabbit polyclonal serum.

Sample	Protein concentration[a] (mg/ml)	Sample volume (ml)	Purification by mass[b]	Log sample titre[c]
Serum	75.3	120	-	6
33/50% Ammonium sulfate precipitation	20.2	40	11.2	8
Protein A chromatography	8.13	7.1	156.5	9

[a] Protein concentration determined by solution absorbance at 280 nm (Appendix A.7.).

[b] Purification by mass = $\frac{\text{Protein concentration of sample x Sample volume}}{\text{Protein concentration of serum x serum volume}}$

[c] Log sample titre determined by serum titre ELISA (Appendix A.6).

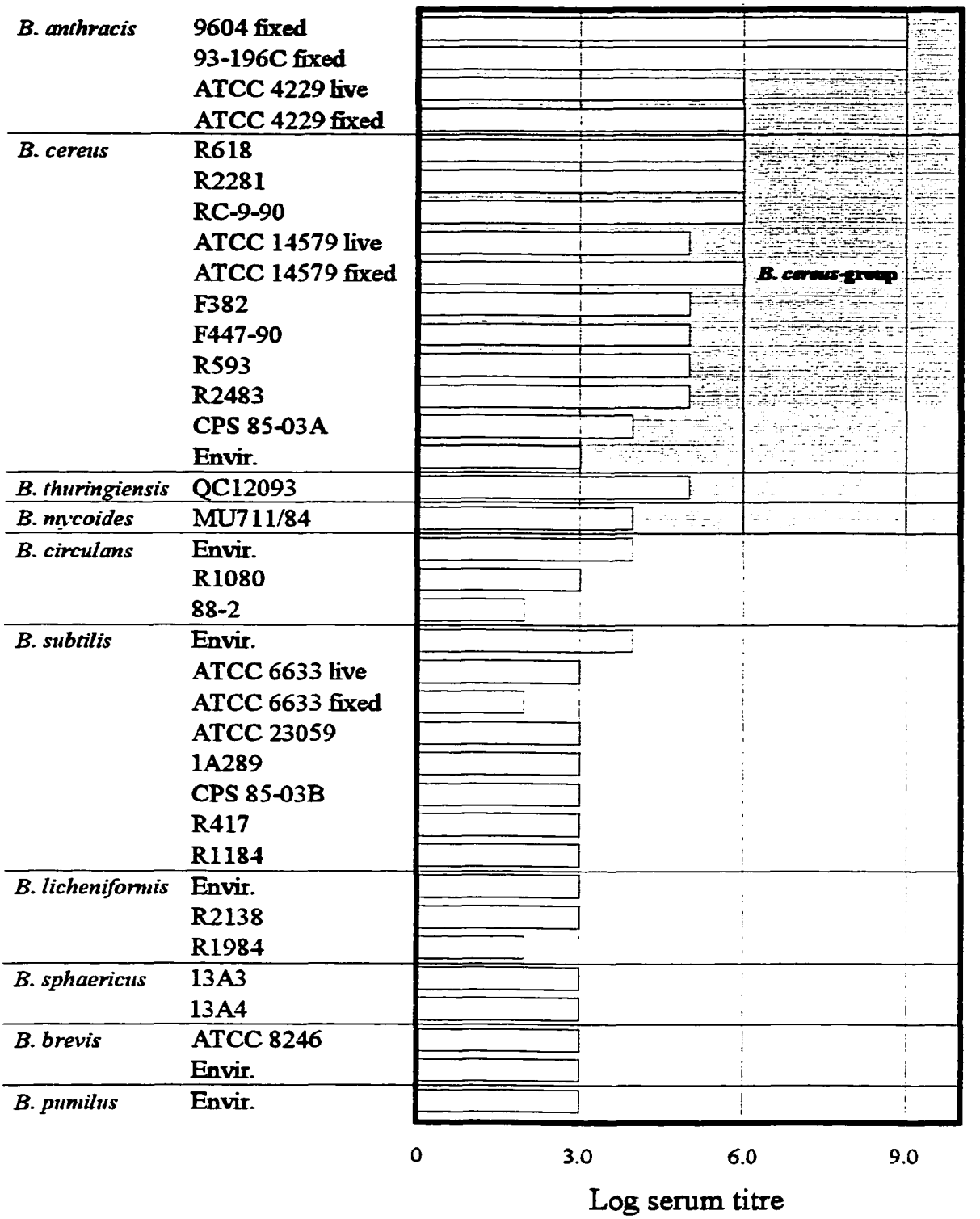

Figure III-5. Titres of the purified rabbit polyclonal serum tested against spores of *Bacillus* species.

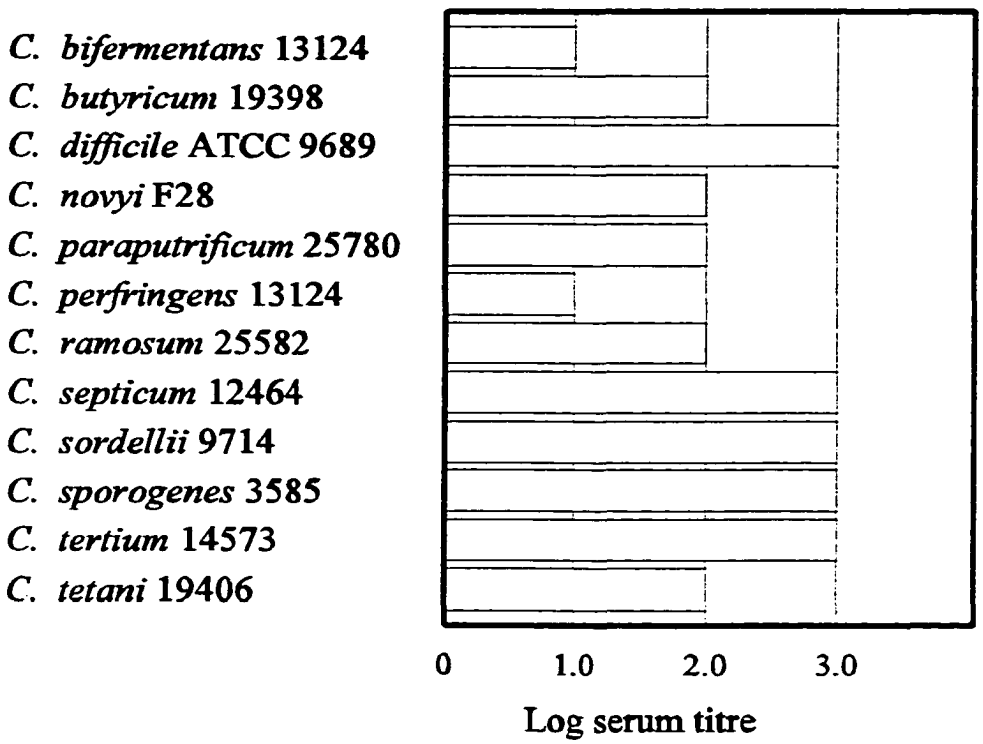

Figure III-6. Titres of the purified rabbit polyclonal serum tested against spores of *Clostridium* species.

Formaldehyde fixation did not appear to appreciably alter the native epitopes on the surface of *Bacillus* spores. There was no difference in the serum titre against live and fixed preparations of *B. anthracis* ATCC 4229 spores and only a single log change in titres against *B. cereus* ATCC 14579 and *B. subtilis* ATCC 6633 spores (Figure III-5).

Against the Clostridial strains tested, the polyclonal serum gave only low titres (Figure III-6). The polyclonal serum was also tested against vegetative cells of *B. anthracis* ATCC 4229, *B. cereus* ATCC 14579, *B. subtilis* ATCC 6633 and *C. difficile* ATCC 9689 from liquid cultures undergoing logarithmic growth (data not shown). Against vegetative *Bacillus* cells, only neat polyclonal serum was reactive (serum titre of 1). The polyclonal serum was not reactive to *C. difficile* vegetative cells at any dilution.

To verify the ELISA results were due to the sensitivity of the polyclonal serum and not to differential adhesion of the spores of the different strains to the plate wells, a plate was loaded with the various spore stocks and blocked with 3% (w/v) BSA/PBS as per the serum titre ELISA (section A.6). After blocking, the wells were rinsed repeatedly with PBS to simulate the regular immunoassay. Each plate well, containing a different bacterial endospore stock, was then viewed under an inverted light microscope at 400x magnification. Just prior to viewing, the well was loaded with 50 µl of 0.2% (w/v) Trypan blue dye. Diffusion of the dye into the metabolically dormant spores was delayed and allowed visualization of spores present in the wells via negative staining. Large numbers of spores were observed adhering to the bottoms of the wells loaded with *Bacillus* strains and in most of the wells loaded with Clostridial strains. The spores tended to clump together forming large aggregates and amassed at a higher density in the center of the well than near the side walls. *Clostridium perfringens* 13124 and *C. ranosum* 25582 were the exceptions with much fewer spores observed in their wells than the other stocks. This may have contributed to the low titre obtained against *C. perfringens* 13125 in the serum ELISA (Figure III-6).

A second series of indirect immunoassays was performed testing a range of dilutions of the purified polyclonal serum against dilutions of live and fixed spores of *B. anthracis*, *B. cereus* and *B. subtilis* (Figure III-7). At 1/50 and 1/100 dilutions, the polyclonal serum detected roughly the same number of live or fixed spores of the three *Bacillus* species. Differences in reactivity of the serum to the spore preparations were not statistically significant as measured by a one-way ANOVA test. If the number of spores in the ELISA wells was $< 5 \times 10^5$ spores, the serum was unable to detect them. Again, formaldehyde fixation did not appear to alter the reactive surface epitopes of the spores. Similar spore titres were obtained with polyclonal serum at dilutions of 1/5, 1/10, 1/20, 1/67, 1/80, 1/500 and 1/1000 (data not shown).

III.1.c. Adaptibility of monoclonal antibodies FDF 1B9 and EAII-6G6-2-3

The overall purification by mass and serum titre of each step of the purification protocols of monoclonal antibodies FDF 1B9 and EAII-6G6-2-3 are listed in Tables III-2 and III-3, respectively. Overall, the combined procedures purified FDF 1B9 and EAII-6G6-2-3 by weight 18- and 6.5-fold, respectively, and improved the titre of the antibody solutions 10- and 1000-fold against their respective target cells. During the purification procedures neither antibody preparation was tested against the target cells of the other.

Monoclonal EAII-6G6-2-3 eluted rapidly as a single peak from the Sephadex G150 column in the first 50 ml (void volume) of the elution buffer (Figure III-8). A smaller peak directly behind the first peak contained no anti-*B. anthracis* vegetative cell activity and was not pooled with the first peak. No other protein peaks were eluted after 250 ml (five column volumes) of elution buffer. Two closely spaced protein peaks were also observed in the elution profile of monoclonal antibody FDF 1B9 from a low salt Protein A column (Figure III-9). Both peaks displayed activity against encapsulated *B. anthracis* vegetative cells and were pooled together. No other protein peaks were observed in the elution profile after 25 ml (five column volumes).

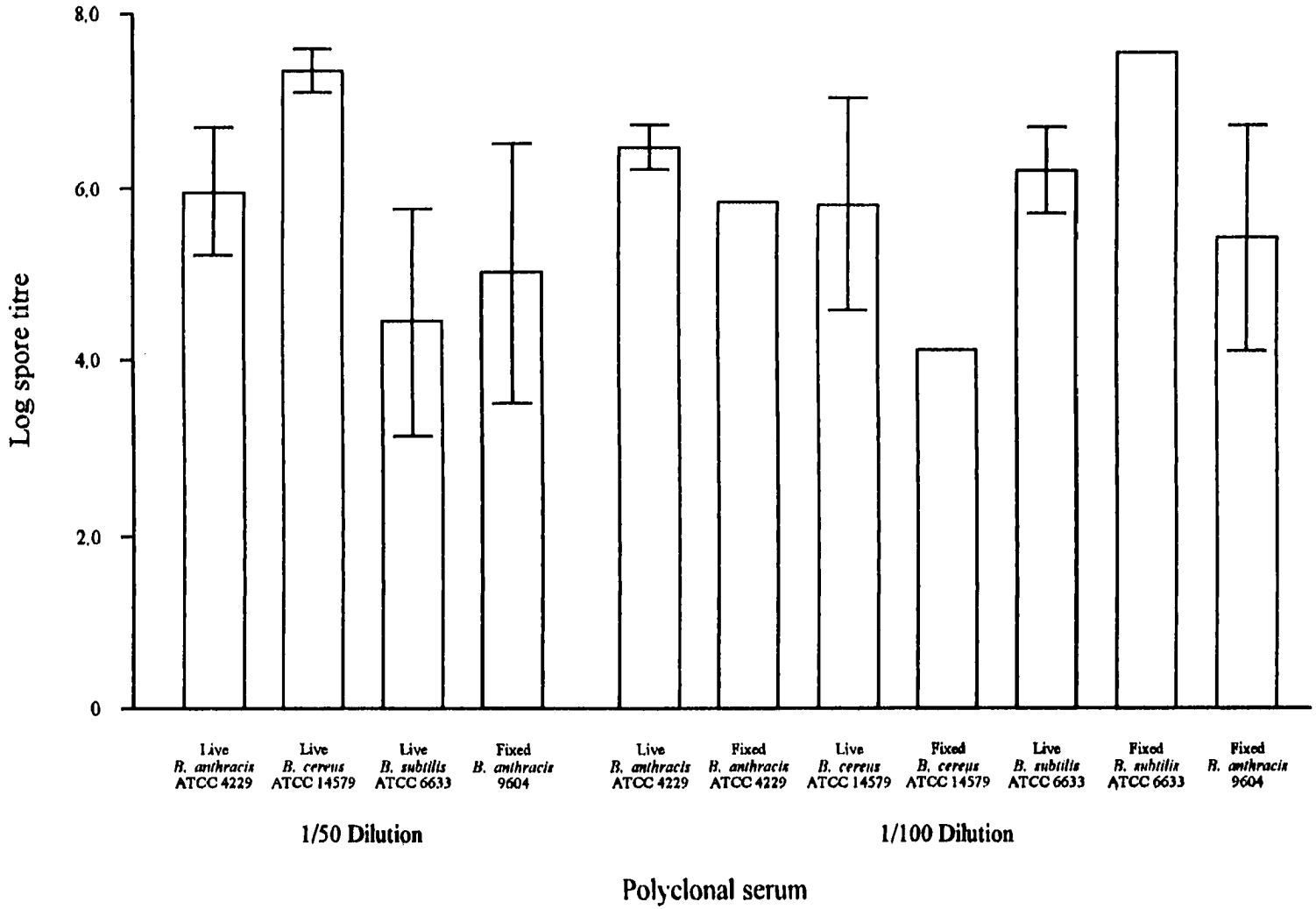

Figure III-7. Titres of the purified rabbit polyclonal serum at 1/50 and 1/100 dilution tested against live and formaldehyde-fixed spores of *B. anthracis* ATCC 4229, *B. cereus* ATCC 14579 and *B. subtilis* ATCC 6633, and formaldeyde-fixed spores of *B. anthracis* 9604.

Table III-2. Step purification of monoclonal antibody FDF 1B9.

Sample	Protein concentration[a] (mg/ml)	Sample volume (ml)	Purification by mass[b]	Log sample titre[c]
Raw ascites	31.5	10	-	2
33/50% Ammonium sulfate precipitation	18.1	5	3.5	2
Protein A chromatography	1.85	9.9	17.2	3
50% Ammonium sulfate precipitation	2.33	7.5	18	3

[a] Protein concentration determined by solution absorbance at 280 nm (Appendix A.7).

[b] Purification by mass = $\frac{\text{Protein concentration of sample x Sample volume}}{\text{Protein concentration of raw ascites x Raw ascites volume}}$

[c] Log sample titre determined by serum titre ELISA (Appendix A.6).

Table III-3. Step purification of monoclonal antibody EAII-6G6-2-3.

Sample	Protein concentration[a] (mg/ml)	Sample volume (ml)	Purification by mass[b]	Log sample titre[c]
Raw ascites	27.2	10	-	1
33/50% Ammonium sulfate precipitation	16.9	5	3.2	2
Sephadex G150 chromatography	0.73	80	4.7	4
50% Ammonium sulfate precipitation	3	14	6.5	4

[a] Protein concentration determined by solution absorbance at 280 nm (Appendix A.7).

[b] Purification by mass = $\frac{\text{Protein concentration of sample x Sample volume}}{\text{Protein concentration of raw ascites x Raw ascites volume}}$

[c] Log sample titre determined by serum titre ELISA (Appendix A.6).

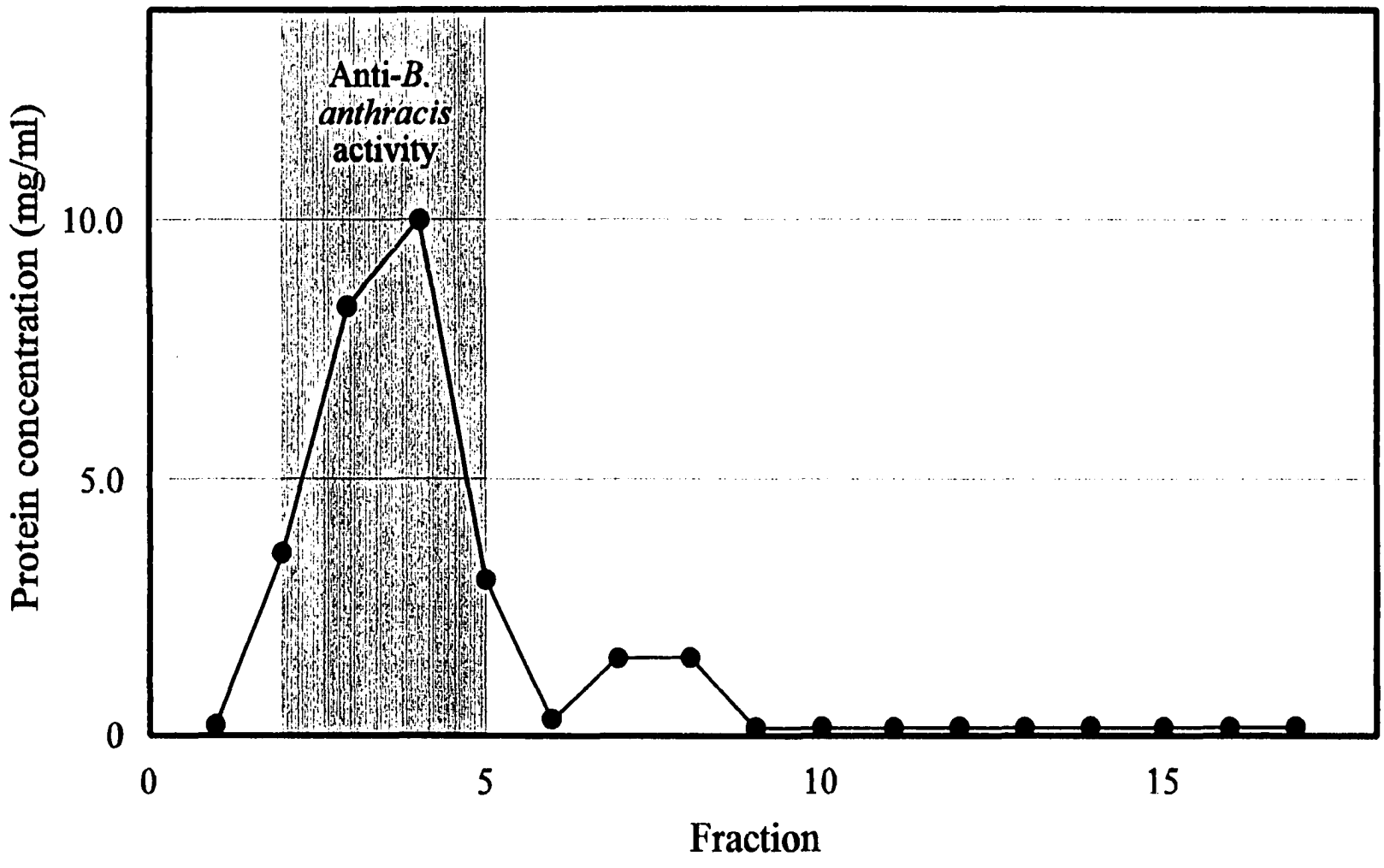

Figure III-8. Elution profile of monoclonal antibody EAII-6G6-2-3 from Sephadex G150 column. The protein concentration of each fraction was determined by UV absorbance at 280 nm and the activity against an overnight SBA culture of *B. anthracis* ATCC 4229 was determined via the serum titre ELISA.

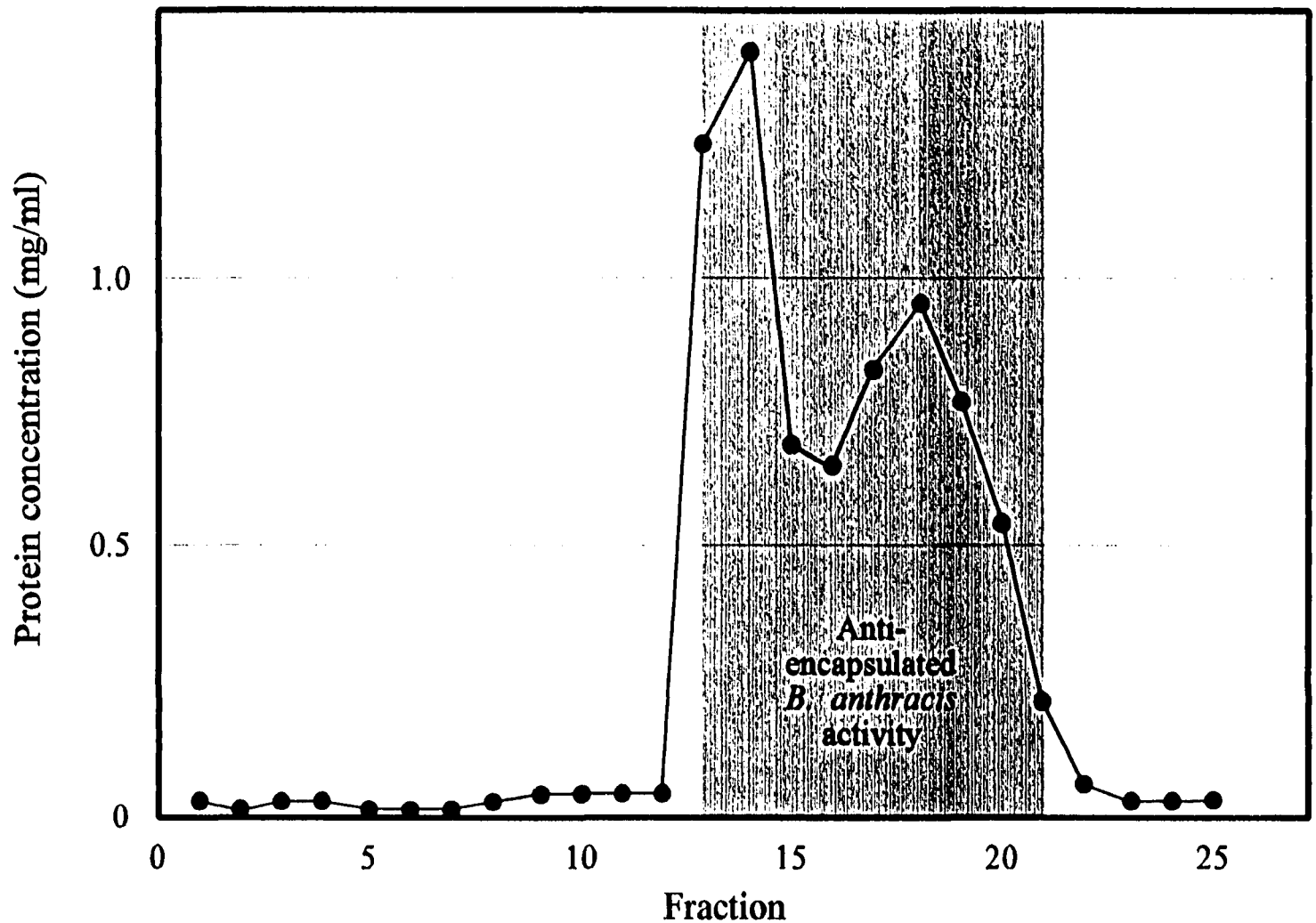

Figure III-9. Elution profile of monoclonal antibody FDF 1B9 from low salt Protein A column. The protein concentration of each fraction was determined by UV absorbance at 280 nm and the activity against an overnight bicarbonate culture of *B. anthracis* ATCC 4229 was determined via the serum titre ELISA.

Adaptation of the direct immunofluorescent staining assay of Ezzell and Abshire (1996) for viable *B. anthracis* spores to an indirect plate format proved disappointing. In the immunofluorescent assay, the monoclonal antibodies were used with broths that maximized spore germination and production of each antigen; FDF 1B9 was used with capsule broth and EAII-6G6-2-3 with polysaccharide broth. Plate well absorbances with either monoclonal antibody in the indirect plate format (see section A.6) did not rise above background levels against spores incubated in the appropriate germination broth at 37°C for 45, 90 and 180 min (data not shown). The monoclonal antibodies were only successful in the plate format against *B. anthracis* spores which had been left in the broth cultures overnight (Table III-4). Even then, monoclonal EAII-6G6-2-3 had poor sensitivity against *B. anthracis* spores (> 2.50×10^6 spores/well) in its germination medium (polysaccharide broth). Purified monoclonal FDF 1B9 was much more sensitive to anthrax spores germinated in capsule broth and was able to detect approximately 2000 spores/well. The lengthy incubation in the germination broths, however, also resulted in the production of cross-reactive antigens in cultures of *B. cereus* and *B. subtilis* incubated in the appropriate broth for each monoclonal and cultures of *B. anthracis* incubated in the other germination broth. Cross-reactivity of EAII-6G6-2-3 with *B. cereus* and *B. subtilis* incubated in polysaccharide broth and encapsulated *B. anthracis* from capsule broth was roughly equal to the sensitivity for unencapsulated *B. anthracis*. The sensitivity of FDF 1B9 to overnight cultures of *B. subtilis* and *B. cereus* in capsule broth and *B. anthracis* in polysaccharide broth was roughly equal but the monoclonal was approximately 1000-fold more sensitive against its target antigen; encapsulated *B. anthracis* from capsule broth.

III.2. Investigation of PLET medium

When functioning properly, PLET medium allowed few non-*anthracis Bacillus* strains to grow (Table III-5). *Bacillus anthracis* ATCC 4229 formed white, circular colonies 2 - 3 mm in diameter after overnight incubation. By 48 h, the colonies had domed up on the medium and were 5 - 8 mm in diameter. None of seven *B. cereus* strains tested on the

Table III-4. Purified monoclonal antibodies FDF 1B9 and EAII-6G6-2-3 against overnight capsule or polysaccharide broth cultures of *B. anthracis* ATCC 4229, *B. cereus* ATCC 14579 and *B. subtilis* ATCC 6633.

Bacillus species	Broth medium	1/50 dilution of monoclonal antibody	
		FDF 1B9	EAII-6G6-2-3
B. anthracis ATCC 4229	Capsule	1965	1.97×10^5
	Polysaccharide	$> 2.50 \times 10^5$	$> 2.50 \times 10^6$
B. cereus ATCC 14579	Capsule	$> 2.50 \times 10^6$	ND[a]
	Polysaccharide	ND	$> 2.50 \times 10^6$
B. subtilis ATCC 6633	Capsule	$> 1.31 \times 10^6$	ND
	Polysaccharide	ND	1.63×10^6

[a] ND = Not done.

Table III-5. *Bacillus* species growth on sheep blood agar, PLET, and PLET supplemented with 5% (v/v) horse blood or 1 μg/ml nitrocefin.

Bacillus species	Strain	Sheep blood agar hemolysis	Growth on						
			PLET		Blood + PLET			Nitrocefin + PLET	
			24 h	48 h	24 h	48 h	Hemolysis	24 h	48 h
B. anthracis	ATCC 4229[a]	non	Y	Y	Y	Y	non	N	N
B. brevis	ATCC 8246[a]	non	N	N	N	N	-	N	N
	Envir.[b]	β	Y	Y	Y	Y	β	N	N
B. cereus	ATCC 14579[a]	β	N	N	N	N	-	N	N
	Envir.	β	N	N	N	Y	β	N	N
	F382[c]	β	N	N	N	N	-	N	N
	F447-90[c]	β	N	N	Y	Y	non	N	N
	R2281[c]	β	N	N	ND[d]	ND	-	ND	ND
	R2483[c]	β	N	N	Y	Y	β	N	N
	RC-9-90[c]	β	N	N	N	N	-	N	N
B. circulans	Envir.	β	N	Y	N	Y	β	N	N
	R1080[c]	non	N	N	ND	ND	-	ND	ND

B. licheniformis	Envir.	non	Y	Y	Y	Y	non	N	N
B. mycoides	MU 711/84[a]	β	Y	Y	Y	Y	β	Y	Y
B. pumilis	Envir.	non	N	Y	N	Y	non	N	N
B. sphaericus	13A3[a]	non	N	N	N	N	-	N	N
B. subtilis	1A289[a]	non	Y	Y	N	Y	non	N	N
	ATCC 6633[a]	non	N	N	N	N	-	N	N
	Envir.	β	N	N	N	N	-	N	N
	R417[c]	α	N	N	N	N	-	N	N
B. thuringiensis	QC 12093[a]	β	Y	Y	Y	Y	β	N	N

[a] Designates a laboratory strain.

[b] Envir. = Environmental isolate.

[c] Designates a clinical isolate.

[d] ND = Not Done

medium formed colonies after 48 h of incubation, however, strains tested from two other species of the *B. cereus*-group, *B. mycoides* MU711/84 and *B. thuringiensis* QC12093, both formed colonies after 24 h that were indistinguishable from those of ATCC 4229. One of four *B. subtilis* strains tested grew within 24 h into colonies morphologically identical to ATCC 4229. Environmental isolates of *B. brevis* and *B. licheniformis* grew within 24 h but remained as microcolonies that were easily distinguished from ATCC 4229. Environmental isolates of *B. circulans* and *B. pumilis* formed colonies resembling ATCC 4229 on PLET medium but their formation was delayed; colonies were not observed until after 48 h incubation. Thus, if growth on the medium after 24 h incubation at 37°C was used in the selection criteria, PLET medium could differentiate *B. anthracis* ATCC 4229 from all test strains except *B. mycoides* MU 711/84, *B. thuringiensis* QC 12093 and *B. subtilis* 1A289. If incubation time was not included in the criteria, then the environmental isolates of *B. pumilus* and *B. circulans* would also be indistinguishable from *B. anthracis* ATCC 4229 on the medium.

On PLET medium supplemented with 5% (v/v) horse blood, *B. anthracis* ATCC 4229 formed non-hemolytic, slightly irregular, cream colonies 2 - 3 mm in diameter after 24 h incubation. Three strains of *B. cereus* formed colonies on the medium after 48 h incubation, however, the strains were readily distinguishable from *B. anthracis*; two formed β-hemolytic colonies and the third remained a microcolony. *Bacillus mycoides* and *B. thuringiensis* also grew on the medium but their colonies were β-hemolytic. Blood supplemented PLET grew more non-*anthracis* test strains (10 of 18; 56%) versus those tested on regular PLET (7 of 20; 35%). However, 24 h incubation on supplemented medium allowed for the selective differentiation of *B. anthracis* ATCC 4229 from all other test strains. When the incubation was extended to 48 h, differentiation between *B. anthracis*, *B. subtilis* 1A289 and the *B. pumilus* environmental strain was lost.

Bacillus anthracis ATCC 4229 failed to grow on the PLET medium supplemented with 1 μg/ml nitrocefin. In fact, of the 20 *Bacillus* strains tested on the medium, only *B. mycoides* MU711/84 grew, forming off-white, irregular colonies 4 - 5 mm in diameter after 24 h of incubation.

Sheep blood agar recovered 3 - 4 times more *B. anthracis* ATCC 4229 spores than PLET medium (Figure III-10). A one-way ANOVA test with multiple pairwise comparison demonstrated that SBA recovered significantly more *B. anthracis* spores after 24 h than PLET medium after 24 and 48 h ($P<0.001$). After 24 h incubation, PLET recovered 25.5% of the colony-forming units observed on blood agar. Prolonged incubation reduced the discrepancy somewhat as colonies continued to form on PLET but no new colonies were observed on SBA. However, even after 48 h PLET medium had still only recovered 37.6% of the colonies observed on blood agar.

III.3. Recovery of *B. anthracis* spores from soil

III.3.a. Extraction of anthrax spores from seeded sterile soil

A range of sucrose solutions of increasing specific gravity with and without nonionic detergent were tested against samples of sterile field soil seeded with *B. anthracis* ATCC 4229 spores (Figure III-11). Neither detergent nor sucrose alone recovered significantly more spores than deionized water. However, the combination of detergent and high gravity sucrose recovered significantly greater numbers of spores than water (one way ANOVA, $P<0.001$). Increasing the specific gravity resulted in even higher recoveries. Multiple pairwise comparison of the treatment groups demonstrated the 1.22 g/ml sucrose plus Triton X-100 or Nonidet P-40 recovered significantly more spores than the other treatments but not each other. Similarly, there was no difference in the percentage of spores recovered by 1.18 g/ml sucrose plus Triton or Nonidet but their recoveries were significantly different from the number of spores isolated by the other treatments.

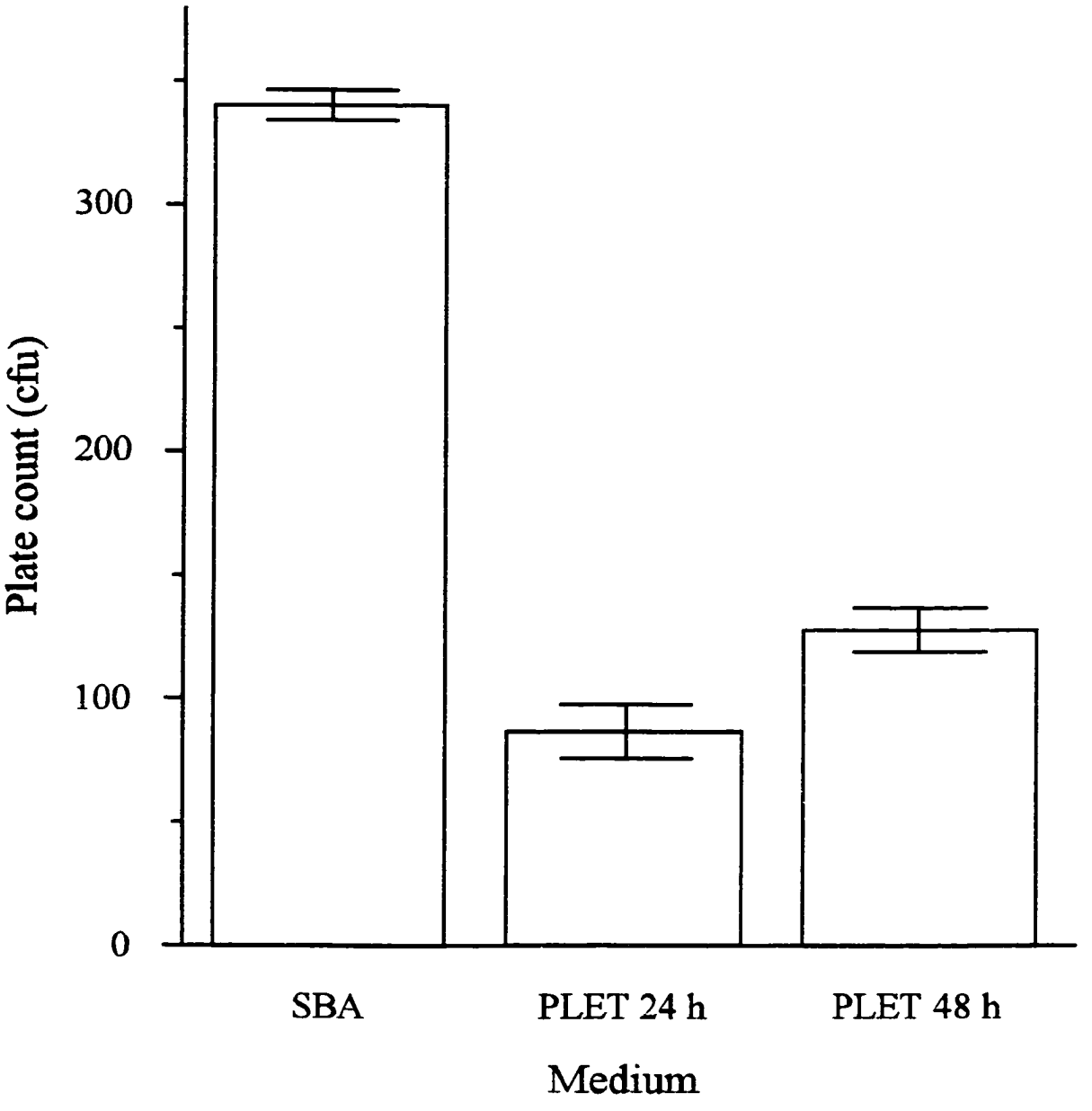

Figure III-10. Recovery of *B. anthracis* ATCC 4229 spores with PLET medium after 24 and 48 h at 37°C and SBA after 24 h at 37°C. The number of spores inoculated was 372 ± 21 as determined by hemocytometer counts of the spore stock using a phase contrast microscope.

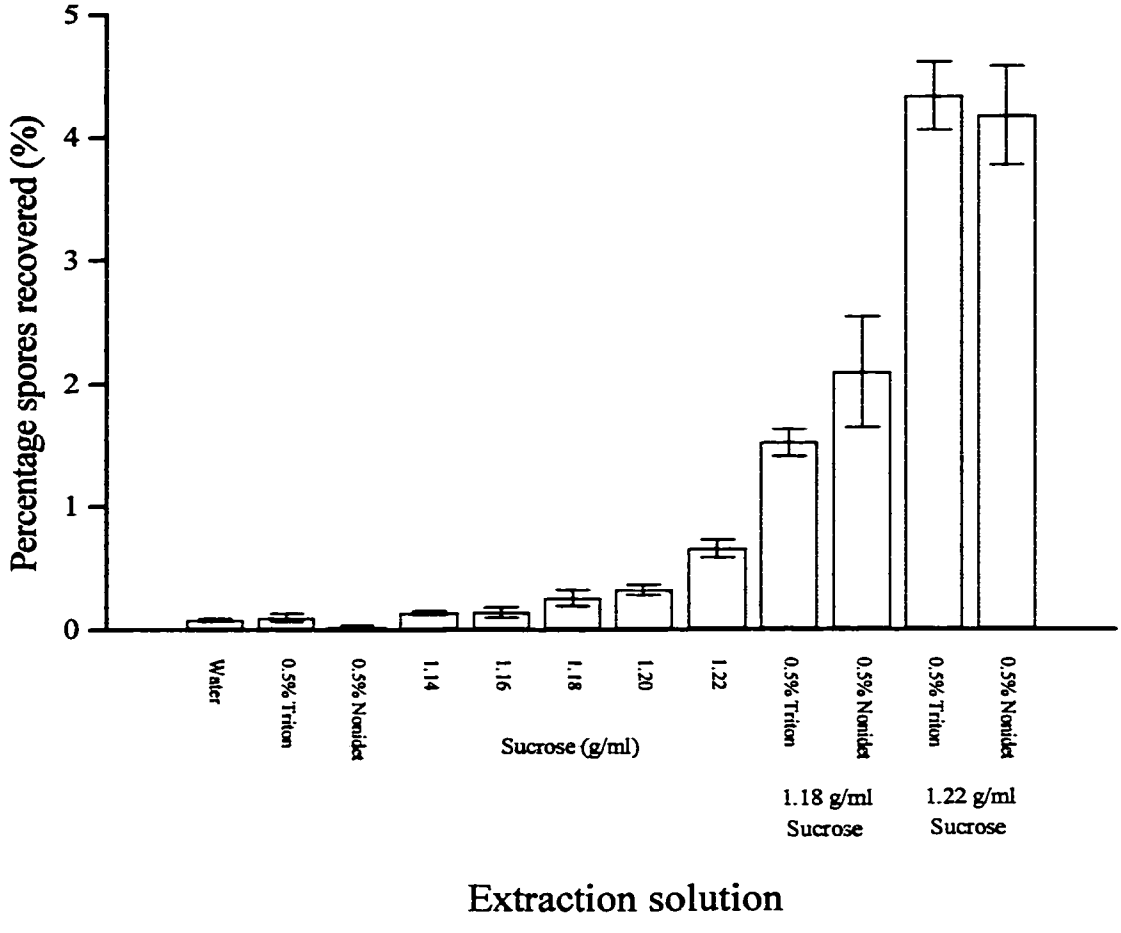

Figure III-11. Percentage recovery of seeded *B. anthracis* ATCC 4229 spores from sterile field soil with deionized water and sucrose solutions of increasing specific gravity with or without nonionic detergents 3 days post-seeding. Soil samples were sterilized prior to seeding with *B. anthracis* spores by autoclaving at 121°C for 25 min.

Anionic N-lauroylsarcosine (0.5% w/v) alone or in combination with 1.18 g/ml and 1.22 g/ml sucrose was also used to try and extract anthrax spores from sterile field soil. However, unlike with solutions containing nonionic Triton X-100 or Nonidet P-40, in those containing N-lauroylsarcosine the detergent began to precipitate upon addition to the soil. The precipitate formed a thick, white pellicle at the surface of the suspension after centrifugation that made supernatant and subsequent spore recovery impossible.

Various sucrose and nonionic detergent solutions were used to recover *B. anthracis* spores from sterile potting soil (Figure III-12). Compared to the extraction experiments performed with field soil, there was less variance between the treatments used against potting soil. Still, one-way ANOVA analysis demonstrated a statistically significant difference between the treatment groups ($P<0.001$). Multiple pairwise comparison identified that the 1.18 g/ml sucrose plus Triton solution recovered significantly more spores than all the other groups. The pairwise comparison also indicated the 1.18 g/ml sucrose plus Nonidet, 1.22 g/ml sucrose, 1.22 g/ml sucrose plus Triton and 1.22 g/ml sucrose plus Nonidet solutions all recovered significantly more spores than water.

Water and 1.22 g/ml sucrose both with and without Triton X-100 or Nonidet P-40 were tested for their ability to recover seeded *B. anthracis* spores from sterile wallow soil (Figure III-13). According to a one-way ANOVA analysis of the data, there was no significant difference among the percentage of spores recovered by the six extraction solutions ($P = 0.1831$). However, the power of the analysis was calculated at only 0.1968 indicating the possibility of a type II error; i.e., there was a significant difference but there was not enough resolving power in the data set to detect the difference.

Extraction with 1.22 g/ml sucrose plus Triton was compared against standard water extraction in sterile field and wallow soil samples seeded with decreasing amounts of *B. anthracis* ATCC 4229 spores (Figure III-14). The extraction solutions were equally sensitive and consistently detected down to 40 spores/g in both soil types.

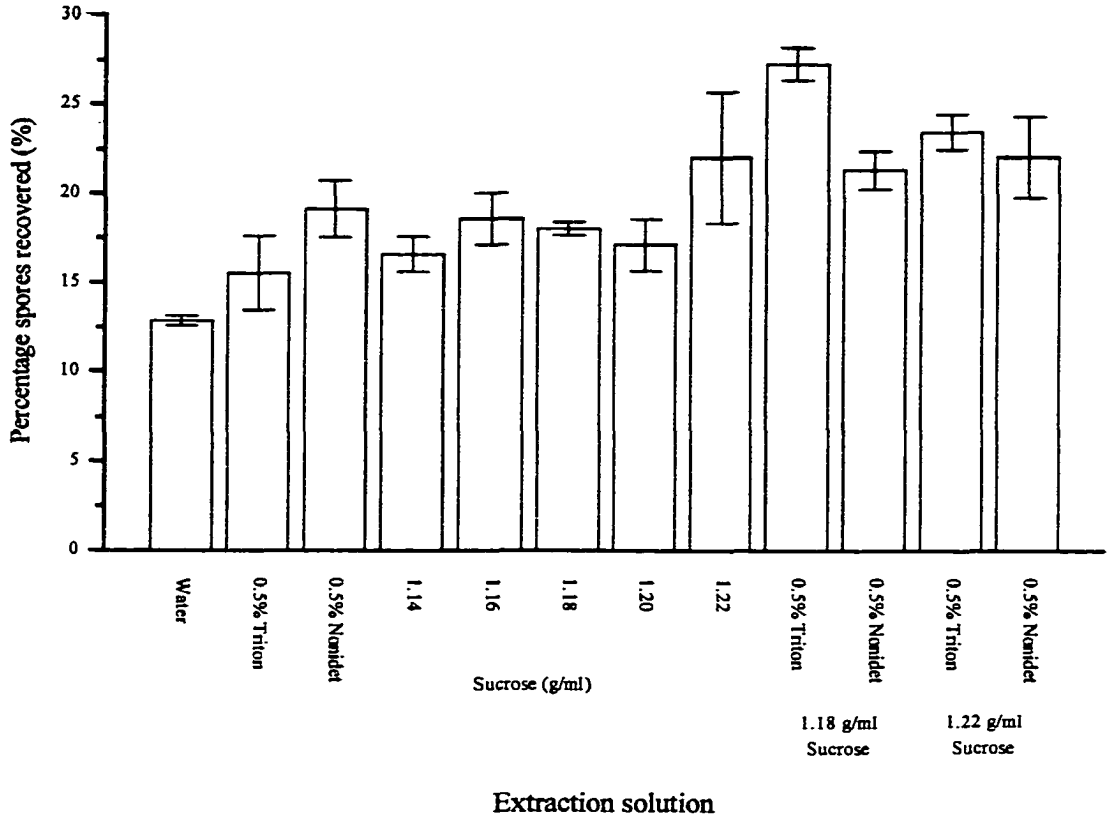

Figure III-12. Percentage recovery of seeded *B. anthracis* ATCC 4229 spores from sterile potting soil with deionized water and sucrose solutions of increasing specific gravity with or without nonionic detergents 3 days post-seeding. Soil samples were sterilized prior to seeding with *B. anthracis* spores by autoclaving at 121°C for 25 min.

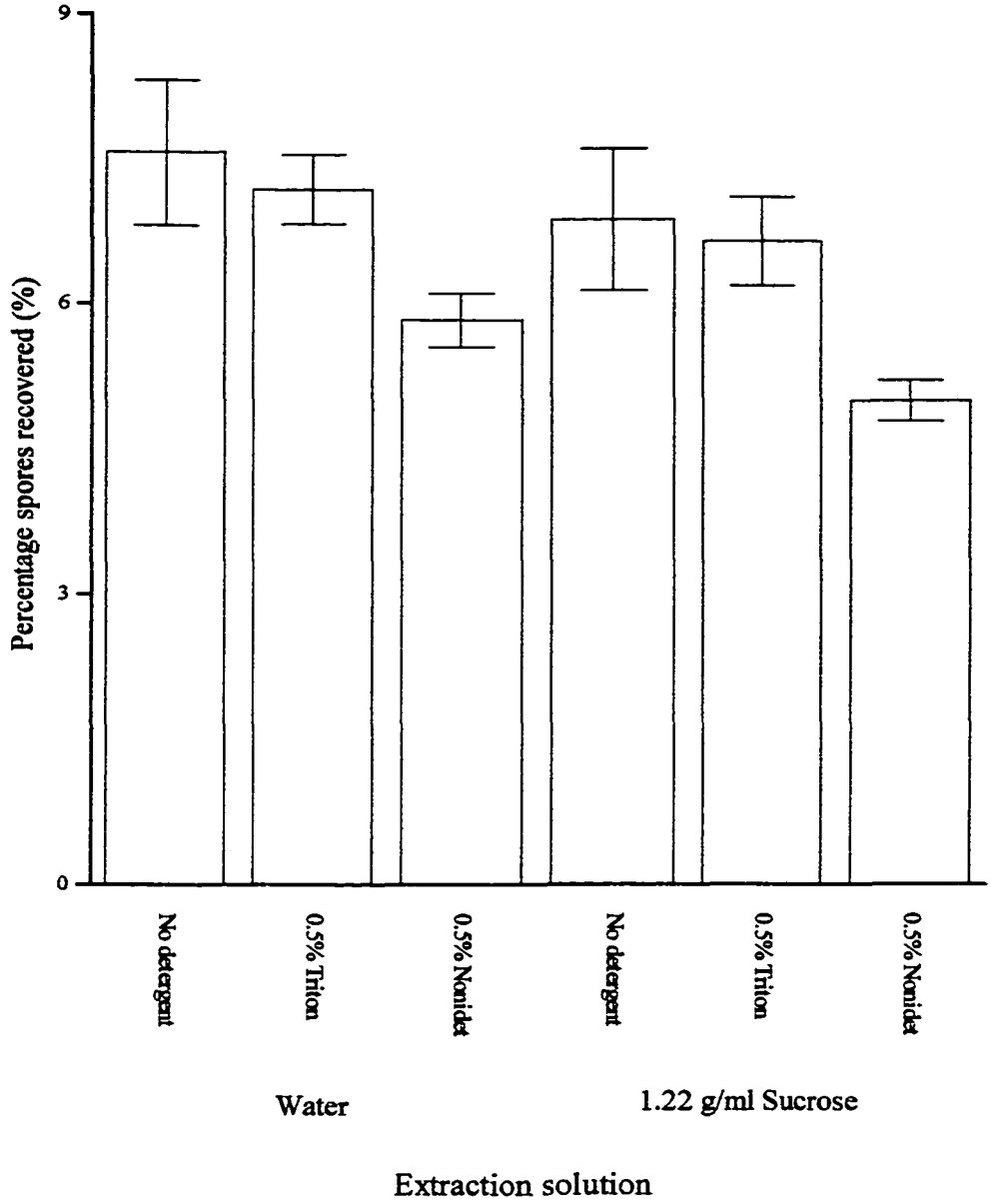

Figure III-13. Percentage recovery of seeded *B. anthracis* ATCC 4229 spores from sterile wallow soil with deionized water and 1.22 g/ml sucrose with or without nonionic detergents 3 days post-seeding. Soil samples were sterilized prior to seeding with *B. anthracis* spores by autoclaving at 121°C for 25 min.

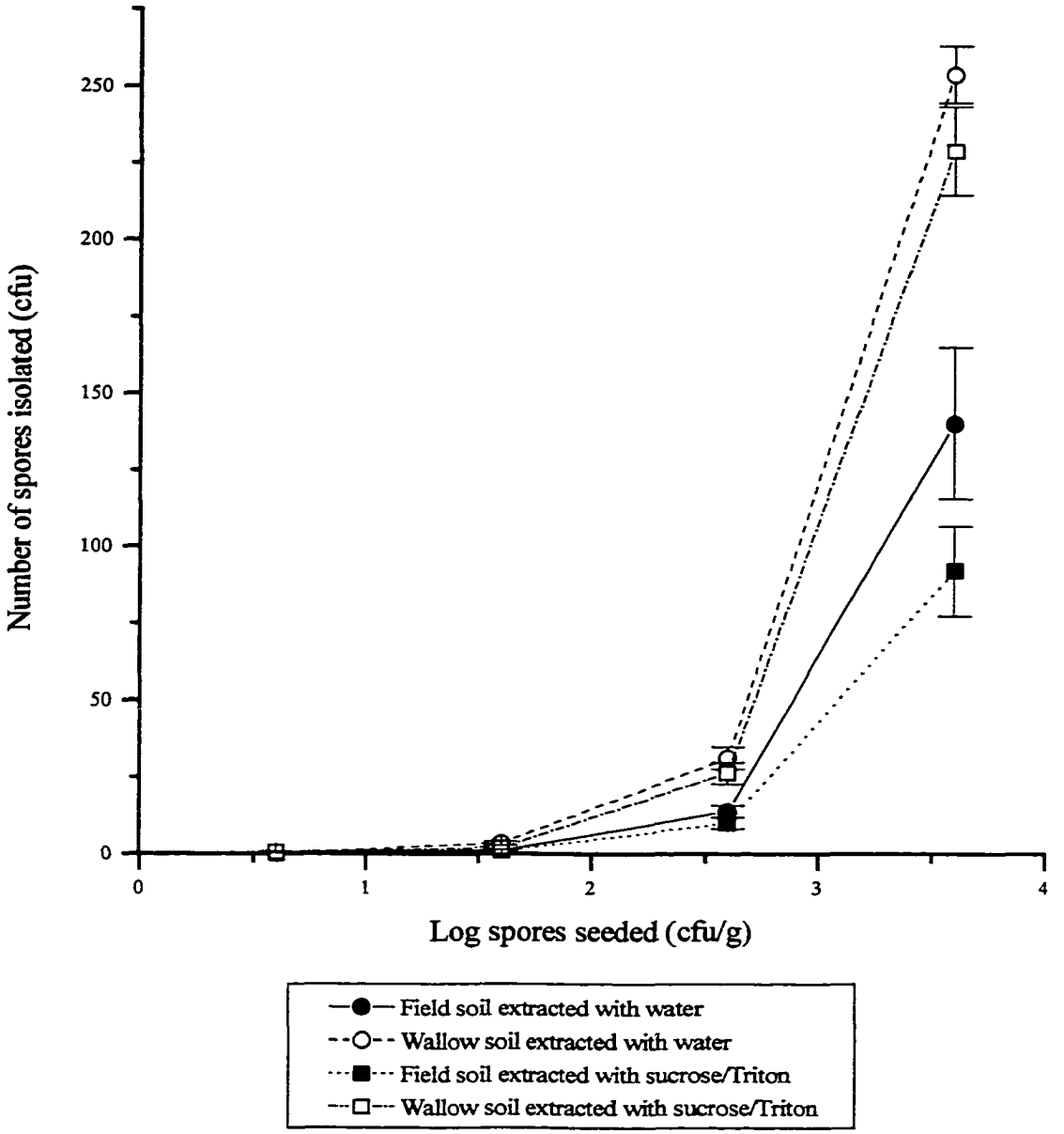

Figure III-14. Sensitivity limits of PLET medium with standard water wash or 1.22 g/ml sucrose plus 0.5% (v/v) Triton X-100 extraction against seeded *B. anthracis* ATCC 4229 spores in sterile field and wallow soil 3 days post-seeding. Soil samples were sterilized prior to seeding with *B. anthracis* spores by autoclaving at 121°C for 25 min.

III.3.b. Bactericidal and sporicidal effects of sucrose plus detergent, and heat and ethanol purification methods

The 1.22 g/ml sucrose plus Triton extraction solution was tested against representative Gram positive (*B. cereus* ATCC 14579) and negative (*P. aeruginosa* S169-90) vegetative cells and *B. anthracis* ATCC 4229 spores to see if high osmolarity sucrose and detergent had a detrimental effect on the microbes (Figure III-15). At the same time the microbial suspensions were treated with heat and ethanol to determine their bactericidal and sporicidal effects. Exposure to sucrose plus detergent resulted in a 100-fold reduction in the concentration of the *B. cereus* and *P. aeruginosa* suspensions. Treatment with heat and ethanol completely killed the *P. aeruginosa* suspension and resulted in an approximately 100-fold reduction in the *B. cereus* vegetative cells. The *B. cereus* vegetative cells were obtained from an overnight broth culture and it was thought the microbe would still be in a logarithmic growth phase with few spores present. However, subsequent spore staining of the *B. cereus* suspension revealed that approximately 1% of the vegetative cells contained fully formed spores. It therefore appears that the three treatments killed the vegetative cells present in the *B. cereus* stock but not the spores. Exposure to sucrose plus Triton, ethanol or heat did not result in a significant change in *B. anthracis* spore viability.

III.3.c. Comparison of deionized water and sucrose plus Triton extraction, and heat and ethanol spore purification in non-sterile soil

Water and 1.22 g/ml sucrose plus Triton were compared for their ability to isolate *B. anthracis* spores from environmental samples collected from carcass disposal sites from northern anthrax endemic regions. The extraction liquids were tested against 18 duplicate soil samples; four of which had been shown to be positive for viable anthrax spores (see section III.5.a). Neither solution identified any new *B. anthracis* containing samples

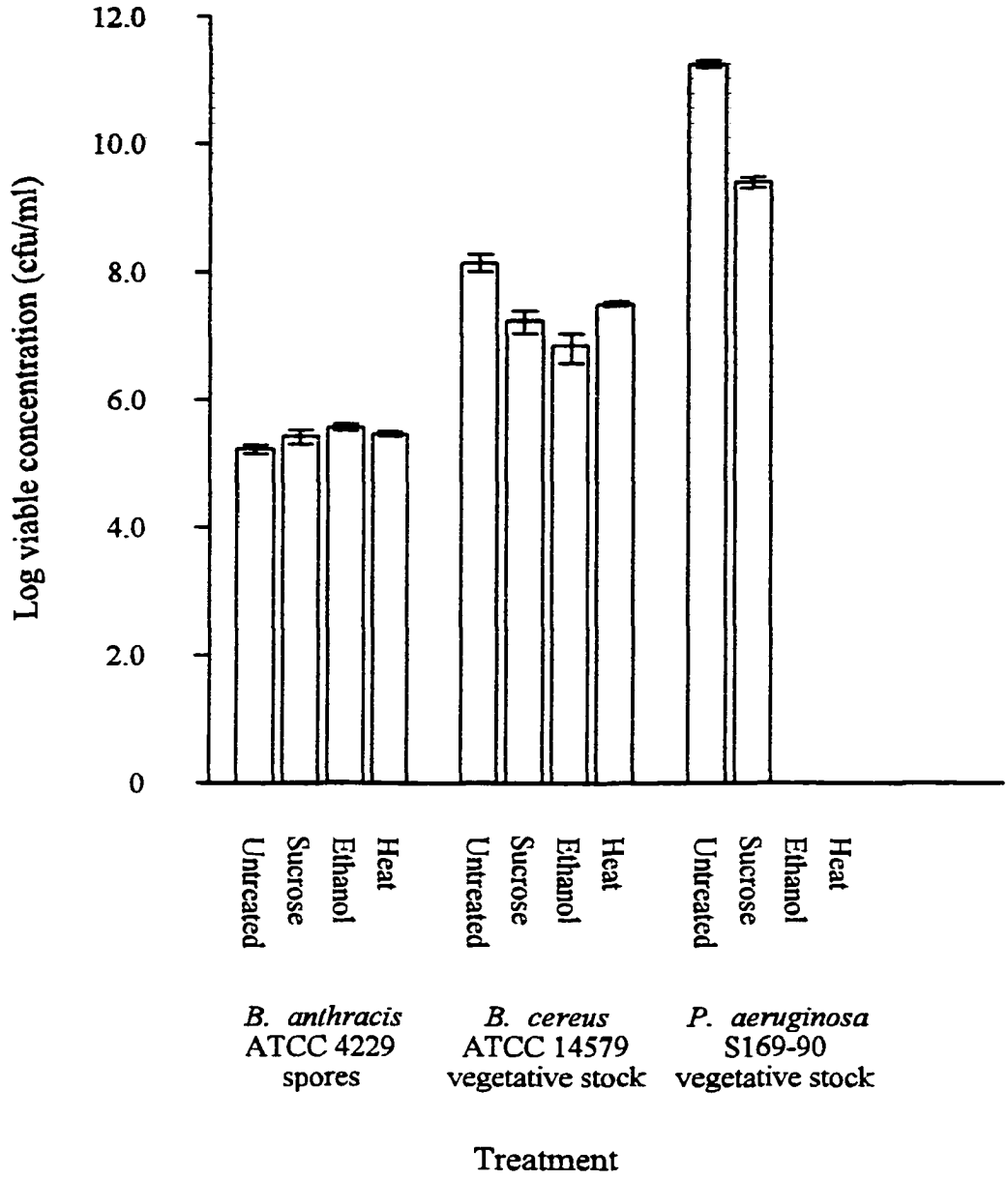

Figure III-15. Effect of 1.22 g/ml sucrose plus 0.5% (v/v) Triton X-100, ethanol and heat treatment on viability of *B. anthracis* ATCC 4229 spores, *B. cereus* ATCC 14579 vegetative cells and *P. aeruginosa* S169-90. Microbes were treated with 1.22 g/ml sucrose plus 0.5% Triton X-100 for 15 min, with 50% (v/v) ethanol for 1 h, and at 63°C for 20 min. No colonies developed on *P. aeruginosa* plates after ethanol and heat treatment.

amongst the negative specimens and neither managed to isolate anthrax spores from all four positive samples; the microbe was detected in three samples with sucrose plus Triton and in three samples with water (Table III-6). A Fisher exact test indicated there was no significant difference in the proportion of positive specimens identified by the two liquids ($P = 0.6582$), however, the small sample size of positive specimens tested greatly reduced the resolving power of the test.

There was no statistically significant difference in the number of anthrax spores recovered from positive samples with either extraction (Mann-Whitney rank sum test, $P = 0.6099$). According to a t-test, there was no significant difference in the number of non-*anthracis* contaminants carried over with either method ($P = 0.9846$). An average of 141 ± 29 contaminant colonies were found on water extraction PLET plates while 142 ± 31 contaminating colonies developed on sucrose plus Triton extraction PLET plates.

In a similar experiment, heat and ethanol treatments were compared for their ability to isolate anthrax spores from carcass disposal site samples. The treatments were tested against 18 duplicate soil samples; three of which had been shown to be positive for anthrax spores. No new positives were detected with either purification method in the negative specimens. All three positive samples were detected using the ethanol treatment but only two were detected with the heat treatment (Table III-7). A Fisher exact test indicated there was no significant difference in the proportion of positive samples identified with either treatment ($P = 0.6581$) but the small sample size of positive specimens tested reduced the resolving power of the test. There was no difference in the number of anthrax spores recovered from positive samples (rank sum test, $P = 0.9619$). There was also no difference between the number of contaminants surviving either treatment (t-test, $P = 0.4184$). An average of 161 ± 31 and 199 ± 35 contaminants developed on the PLET plates after heat and ethanol treatment, respectively.

Table III-6. Comparison of water and sucrose plus Triton X-100 extraction of *B. anthracis* spores from environmental specimens from northern Canada.

Environmental specimen	*B. anthracis* colonies isolated with	
	Water	1.22 g/ml sucrose plus 0.5% Triton X-100
S22[a]	0	0
41-4	0	0
41-6	5	7
41-17	0	0
89-2	509	337
89-3	0	0
110-23	0	0
141-14	0	0
142-1	0	0
142-6	0	0
142-7	0	0
142-10	0	0
142-15	0	0
142-16	0	0
142-19	0	1
142-23	1	0
142-24	0	0
142-25	0	0

[a] In the specimen designation, the first number is the carcass identification number assigned during the 1993 MBS clean-up operations and the second number refers the order samples were collected at the disposal site. The S in the first specimen designation indicates the specimen was collected along the 1994 Falaise field transect (section II.6.a).

Table III-7. Comparison of ethanol- and heat-purification of *B. anthracis* spores from environmental specimens from northern Canada. Samples were treated with 50% (v/v) ethanol for 60 min and at 63°C for 20 min.

Environmental specimen	*B. anthracis* colonies isolated with	
	Ethanol	Heat
S10[a]	0	0
41-2	1	1
41-6	5	0
41-7	0	0
41-19	0	0
41-20	0	0
41-21	0	0
89-3	0	0
142-2	0	0
142-3	0	0
142-4	0	0
142-5	0	0
142-8	0	0
142-11	0	0
142-13	0	0
142-19	1	1
142-20	0	0
142-21	0	0

[a] In the specimen designation, the first number is the carcass identification number assigned during the 1993 MBS clean-up operations and the second number refers the order samples were collected at the disposal site. The S in the first specimen designation indicates the specimen was collected along the 1994 Falaise field transect (section II.6.a).

Even single colonies of *B. anthracis* stood out for easy identification amongst roughly 150 contaminating colonies after 48 h of incubation on PLET. Prior to 48 h, however, it was impossible to differentiate colonies, even when plates were viewed under a dissecting microscope. *Bacillus anthracis* formed raised, circular white colonies from 4 - 8 mm in diameter after 48 h incubation while most contaminants remained a brown-grey. The two most common contaminants were a circular, mucoid colony-type that remained flush with the medium and a large, multi-lobed spreading colony which pitted the surface of the medium. The identity of either contaminant was not established.

III.4. Characterization of anthrax endemic areas in northern Canada

III.4.a. Carcass disposal sites and surveyed northern endemic areas

The Hook Lake region consists of large, open alluvial fields broken by stands of trembling aspen (*Populus tremuloides*) and willow (*Salix spp.*). Sedge and grass species dominate the cover of the open fields and provide excellent grazing for bison. The first recorded anthrax epizootic in northern bison herds occurred in the region in 1962 and over the next 16 y the area was involved in four more outbreaks (Carbyn et al., 1993). During the outbreaks, the majority of carcasses were found and disposed of on the open fields. In these earlier outbreaks, the preferred method of carcass disposal was deep burial under a mound of earth (Choquette et al., 1972).

Decades after their formation, the anthrax mounds remain clearly distinguishable even at a distance of up to a km across the fields (Figure III-16). Mounds observed in the region rose 0.3 - 1.0 m above the surrounding flat meadows. Yellow foxtail barley (*Hordeum jubatum*) appeared to grow preferentially on the mounds causing them to stand out even further against the green fields. In general, the mounds were elliptical with gentle slopes and a flat crown.

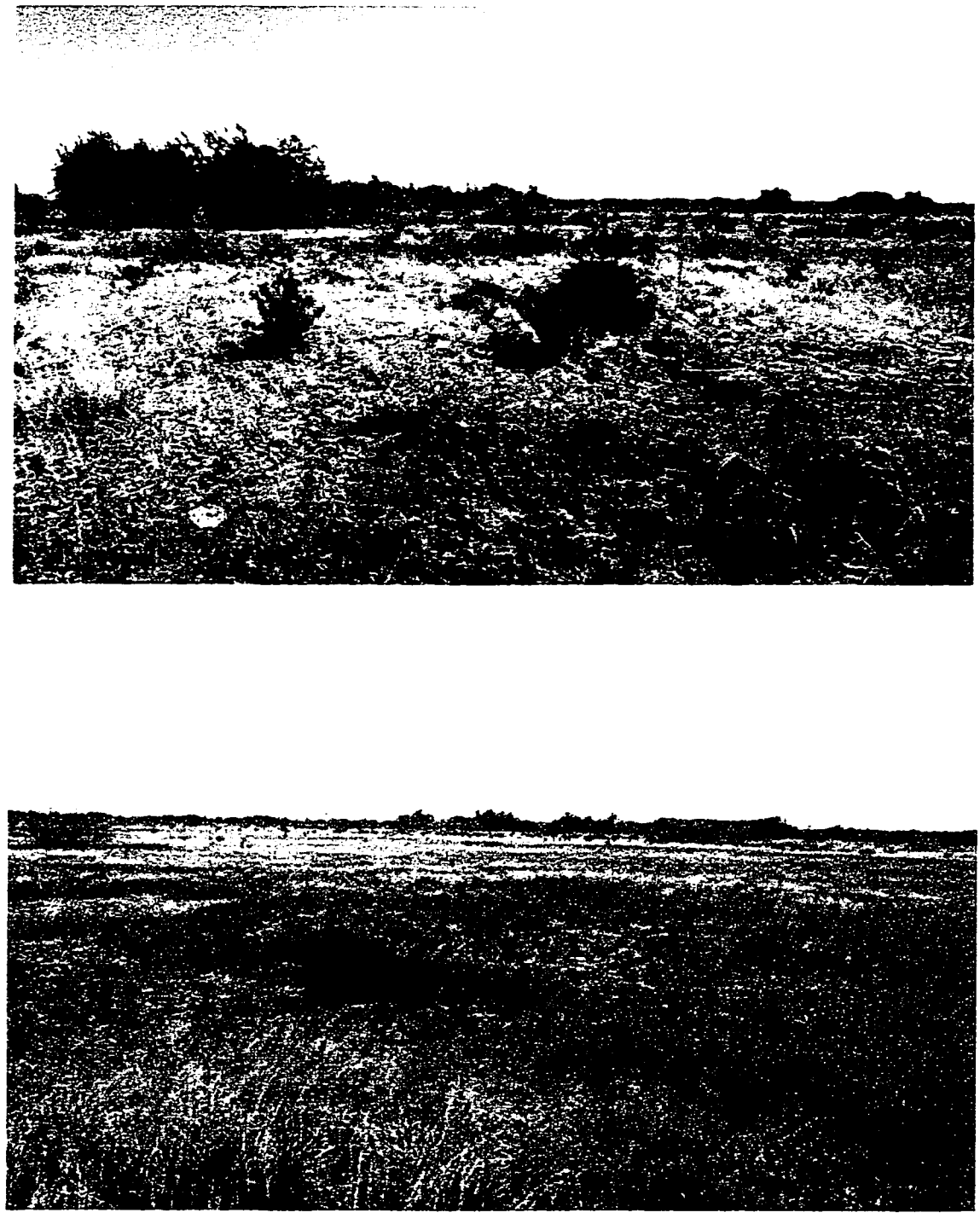

Figure III-16. Anthrax burial mounds at Hook Lake, NT, July 1993.

The anthrax mounds observed in the region appeared predominantly intact. One small mound had three sinkholes measuring 40 - 70 cm across and 20 - 40 cm deep in its crown and another had a single circular depression 1.2 m across and 45 cm deep at its apex. A third mound had a fresh borrow at its base leading into the mound proper but no evidence of bone excavation. All eight of the observed mounds had multiple bison wallows about their bases. The wallow depressions allowed for the collection of standing water around the mounds; one mound with five wallows about its base appeared surrounded by a moat. The 1992 field trip to Hook Lake coincided with a spell of hot weather and, while the rest of the field appeared dry, water remained in several of the mound wallows.

Parson's Lake Road is a one-lane gravel track that cuts through an old growth forest of predominantly spruce (*Picea* spp.) and jack pine (*Pinus banksiana*) in the north-central part of WBNP. There are few open glens and the trees grow right up to the road side. The soil is sand-based and would be expected to drain more quickly than the clay-based soils of the Hook and Falaise Lake regions. During the 1991 epizootic, several carcasses were found and burned or buried along the road (Broughton, 1992).

Disposal sites along the road were surveyed during the summers of 1992 and 1993. Despite thick forest overgrowth and the lack of a definite burial mound, the sites were easily detected due to the path of downed trees caused by the heavy machinery used to excavate the burial pits and the thick white covering of lime spread over the site (Figure III-17). Six sites were surveyed; all within 30 m of the road. From the close crowding of live trees and the lack of charred wood around the sites, it was unlikely the carcasses were cremated before burial. The burial sites were roughly square in shape. All were in areas of loose-packed sand and except for a few small sinkholes appeared intact and undisturbed. Although some bison wallows were observed along the road side, there were no wallows in the immediate vicinity of the surveyed sites.

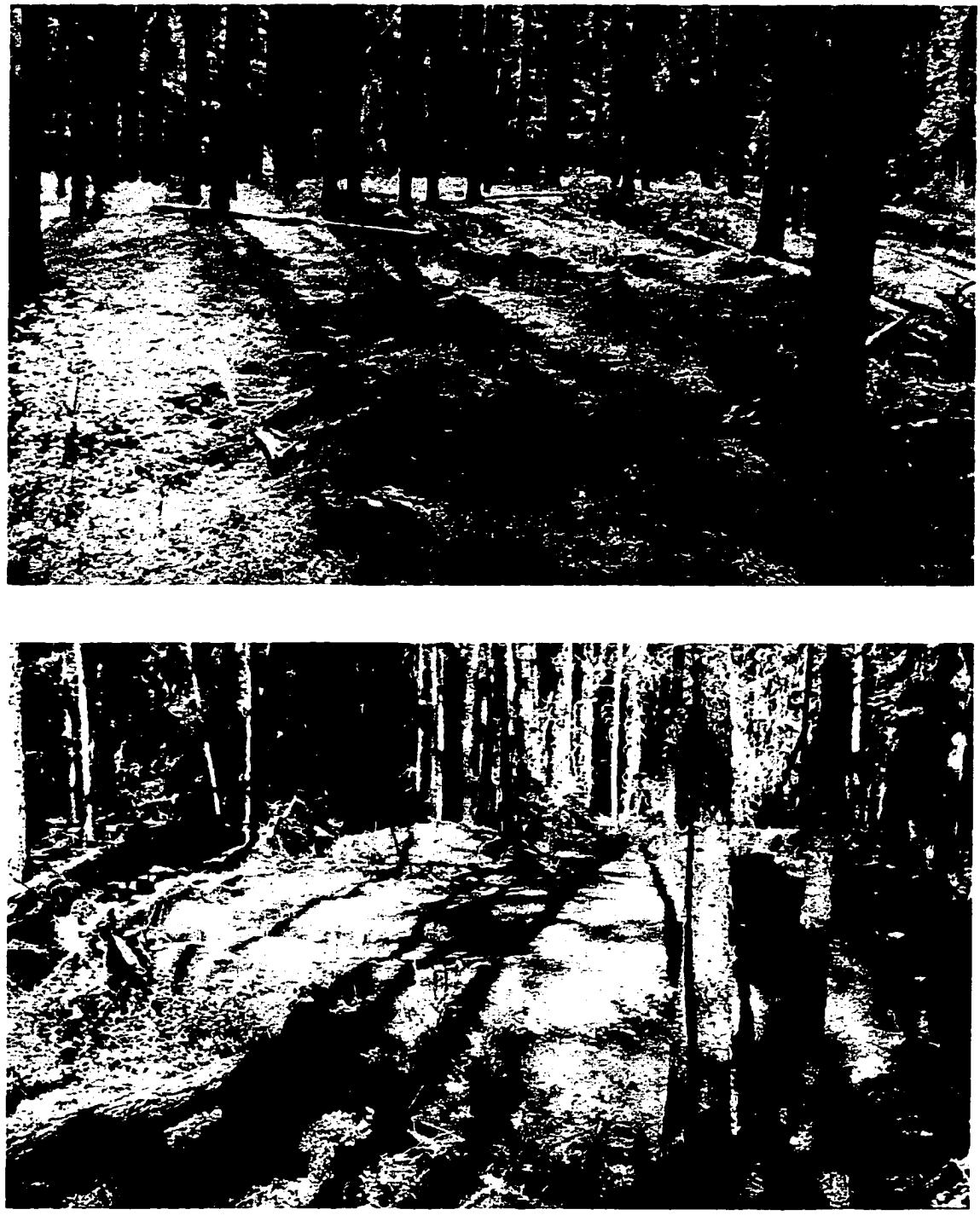

Figure III-17. Typical anthrax burial sites along Parson's Lake Road in WBNP, NT, July 1993.

The Falaise Lake region in the MBS is the ancient bed of a large glacial lake. A shallow lake occupies the center of the bed and is surrounded by alluvial fields dominated by sedge and grass species. At approximately 150 m from the lake edge, irregular bands of poplar and willow running parallel to the shore begin and are inter-spaced with smaller meadows. The rim of the lake bed is delineated by an abrupt escarpment roughly 2 m in height. While the soil of the bed is alkaline and calcium-rich due to a high concentration of fragmented mollusc shells, above the escarpment the soil is acidic and supports muskeg and coniferous forest habitats (Gates et al., 1995).

During the 1993 epizootic, carcasses were disposed of by incineration with either wood or coal and each site was treated either before or after cremation with 400 l of 3 - 5% formaldehyde. One year after the epizootic, the cremation sites were covered in ash with the charred remnants of logs about their perimeter (Figure III-18). Trees and shrubs immediately beside the sites had often been killed by the heat of the fire but usually remained unburned. Grass and sedge species grew right up to the edge of the burn site and appeared unaffected by the formaldehyde treatment. On some sites, foxtail had already begun to recolonize the charred area. At each site, slightly offset from the center was a bed of charred bones comprised of fragments of ribs, vertebrae and some long bones. At six of the eight sites surveyed, there were thick mats of bison cape hair and large concentrations of maggot casings underneath the bone bed (Figure III-19). Although sometimes singed, the hair and casings were intact and undamaged. Four years after the epizootic, the cremation sites at Falaise Lake were revisited. In the intervening years, deciduous herbs and shrubs, including lamb's-quarters (*Chenopodium album*), yarrow (*Achillea millefolium*) and wild mint (*Mentha arvensis*) had overgrown the sites (Figure III-20). No new wallows had been formed near the sites and none of the vegetation showed signs of grazing or trampling.

Figure III-18. Cremation sites on Falaise Lake field in the MBS, NT 1 y following the 1993 epizootic.

Figure III-19. Mats of bison hair found in bone bed of Falaise Lake cremation site.

Figure III-20. The author at a typical Falaise Lake cremation site in July 1997, 4 y after the MBS epizootic.

III.4.b. Topographical analysis of Falaise Lake field

Topographical surveying of a subsection of Falaise Lake field via differential transecting revealed a gentle rise from the lake shore to the escarpment (Figure III-21). The majority of bison wallows observed in the area were located in the first large open meadow surrounding the lake proper where they often formed substantial clusters (Figure III-22). The wallows were roughly circular with a mean diameter of 2.9 ± 0.3 m and a mean depth of 6 ± 1 cm, and were able to hold a volume of approximately 400 l. The wallows were often connected via worn bison trails running parallel to the lake shore. The majority of bison carcasses found during the 1993 epizootic were discovered amongst the bands of trees and smaller meadows above the central field.

III.5. Recovery of *B. anthracis* from environmental specimens from endemic regions of northern Canada

III.5.a. Screening of environmental specimens

During field studies of the endemic regions, 169 environmental specimens were collected from five anthrax mounds in the Hook Lake area, 157 specimens were collected from six burial sites along PLR and 195 specimens were collected from eight cremation sites in the Falaise Lake area (Table III-8). In addition, 23 samples from bison wallows and 44 soil samples were collected from areas of meadow not associated with carcass sites.

After spore extraction with a high specific gravity sucrose plus Triton solution and purification with 50% (v/v) ethanol, samples were spread on PLET plates. Although colonies were observed on the plates after overnight incubation, it was impossible to differentiate colonies morphologically until 48 h. Of the 588 specimens screened with PLET medium, 174 (29.6%) exhibited domed, circular, white colonies 4 - 8 mm in diameter that were morphologically similar to *B. anthracis* (Table III-8). The highest

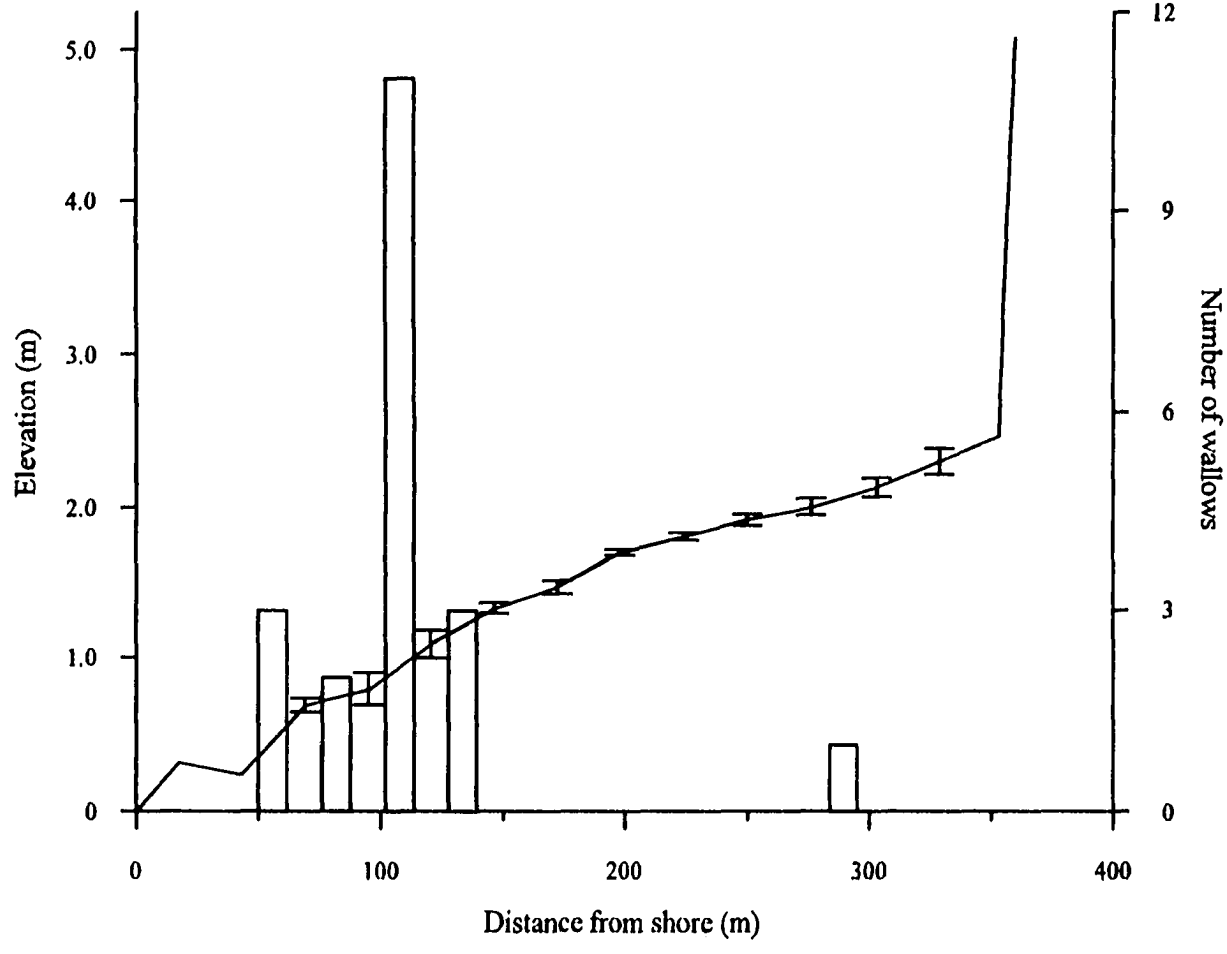

Figure III-21. Mean rise relative to the lake water level from the shore to the surrounding escarpment of Falaise Lake and the distribution of bison wallows.

Figure III-22. Aerial photograph of bison wallows dotting Falaise Lake field. The wallows are the circular areas of bare earth.

Table III-8. Number of *B. anthracis* isolates and contaminants with *B. anthracis*-like properties recovered from environmental specimens from anthrax endemic regions in northern Canada.

Specimens	Hook Lake	Parson's Lake Road	Falaise Lake		Total
			Disposal sites	Open field[a]	
Collected	169	157	195	67	588
B. anthracis-like colonies on PLET	19 (11.2%)	32 (20.4%)	96 (49.2%)	27 (40.3%)	174 (29.6%)
Non-hemolytic, penicillin sensitive	0	3 (1.9%)	19 (9.7%)	3 (4.5%)	25 (4.3%)
Mucoid on bicarbonate	0	3 (1.9%)	11 (5.6%)	0	14 (2.3%)
Encapsulated bacilli	0	3 (1.9%)	8 (4.1%)	0	11 (1.9%)
PCR positive	0	3 (1.9%)	8 (4.1%)	0	11 (1.9%)

[a] Comprised of specimens collected from bison wallows and meadows not associated with known carcasses.

percentage of specimens with *anthracis*-like colonies were from Falaise Lake cremation sites and open field samples while the lowest percentage was recovered from Hook Lake anthrax mounds.

Despite the selectivity of PLET medium, contamination on the plates was observed. The mean number of contaminants/plate, including *B. anthracis*-like colonies that were later discounted through other tests, was 191 ± 132 (error represented by standard deviation), 13 ± 50, 160 ± 134 and 158 ± 119 for specimens from Hook Lake, Parson's Lake, and Falaise Lake cremation sites and open field samples, respectively. A one-way ANOVA test demonstrated that significantly fewer contaminants were isolated with PLET medium from Parson's Lake specimens than from samples from the other regions ($P < 0.001$). PLET cultures of Parson's Lake specimens were also less likely to show any microbial growth after 48 h of incubation. Eighty-six (54.8%) Parson's Lake samples failed to yield any colonies compared to 3 (1.8%) of Hook Lake samples, 11 (5.6%) of Falaise Lake carcass site specimens and none of the open field samples.

Bacillus anthracis-like colonies from the PLET were picked and tested with SBA plates and penicillin discs. Only 25 of the specimens yielded isolates that, like *B. anthracis*, were non-hemolytic and penicillin-sensitive (Table III-8). None of the Hook Lake samples yielded non-hemolytic, penicillin-sensitive isolates, whereas only three specimens each from PLR and Falaise open field and 19 specimens from the Falaise cremation sites exhibited colonies of this phenotype. Isolates were considered susceptible to penicillin if any amount of growth inhibition was observed around the penicillin disc. The mean diameter of the zone of inhibition for isolates that were later confirmed to be *B. anthracis* by MLVA was 25 ± 7 mm with a range of 13 - 40 mm. In contrast, β- and non-hemolytic contaminants that were susceptible had a mean zone of inhibition of 16 ± 8 mm (range 7 - 34 mm). A t-test demonstrated there was a significant difference between *B. anthracis* and contaminant zones of inhibition ($P = 0.0141$).

None of the isolates from the three remaining Falaise open field specimens formed mucoid colonies on bicarbonate plates after overnight incubation in 10% CO_2 (Table III-8). Isolates from the three remaining Parson's Lake specimens and 11 of the 19 remaining Falaise cremation samples developed heavily mucoid colonies on the bicarbonate plates that were consistent with *B. anthracis*. Geimsa stained smears prepared from the bicarbonate cultures of 11 of the mucoid isolates displayed rods with thick enveloping purple capsules compatible with *B. anthracis* and often referred to as "fuzzy caterpillars" (Figure III-23). These 11 isolates were later confirmed to be *B. anthracis* of the bison genotype by MLVA.

III.5.b. Location of *B. anthracis* positive specimens

The 11 *B. anthracis* positive specimens came from seven carcass disposal sites; three burial sites along PLR (50% of the sites in the region surveyed) and four (50%) cremation sites around Falaise Lake (Table III-9). No positive samples were isolated from Hook Lake specimens. In the Falaise Lake region viable *B. anthracis* were limited to the carcass disposal sites; no anthrax spores were recovered from the open field specimens. However, the sample size of open field specimens was small and they were collected roughly a km away from the nearest positive disposal site.

In six (54.5%) of the 11 positive specimens, *B. anthracis* was detected at the sensitivity limit of the extraction and PLET culture procedure; 2 spores/g; represented by a single colony on the PLET medium (Table III-9). At five (71.4%) of the seven positive carcass disposal sites, *B. anthracis* was isolated from a single environmental specimen. High levels of anthrax spores were associated with a red fox (*Vulpes vulpes*) scat and soil specimens collected from within the bone bed of cremation sites. Low anthrax spore concentrations were found dispersed across the disturbed area of the disposal sites.

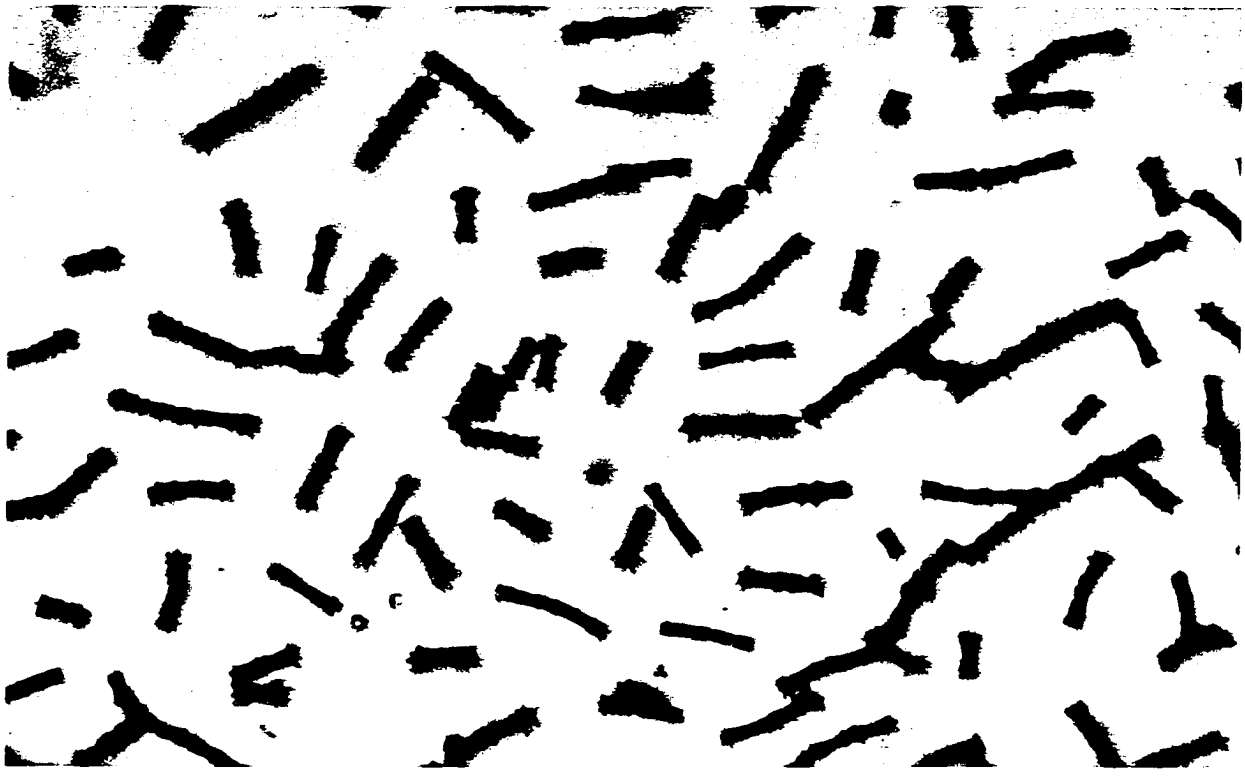

Figure III-23. Geimsa stained virulent *B. anthracis* isolate from environmental specimen 89-2 following overnight culture on bicarbonate. Isolate is displaying the "fuzzy caterpillar" morphology of a positive McFadyean reaction.

Table III-9. Source and relative anthrax spore concentration of *B. anthracis* positive environmental specimens.

Specimen	Region	Carcass disposal method	Location	Concentration (cfu/g)
16-03[a]	Parson's Lake	Burial	Fox scat	800
22-24	Parson's Lake	Burial	Sand	2
23-16	Parson's Lake	Burial	Sand	2
37-19	Falaise Lake	Cremation	Bone bed	70
41-2	Falaise Lake	Cremation	Bone bed	2
41-6	Falaise Lake	Cremation	Bone bed	20
41-13	Falaise Lake	Cremation	Bone bed	600
41-14	Falaise Lake	Cremation	Bone bed	2
89-2	Falaise Lake	Cremation	Bone bed	1200
142-19	Falaise Lake	Cremation	Soil	2
142-23	Falaise Lake	Cremation	Soil	2

[a] In the specimen designations for Parson's Lake samples, the first digit refers to the year sampled (1 = 1992; 2 = 1993) and the second digit to the order the burial sites were encountered. In the designations for Falaise Lake samples, the number before the dash is the carcass identification number assigned during the 1993 MBS clean-up operations. In all cases, the number after the dash refers to the order specimens were collected at the disposal site.

Site 16 was right along side PLR and was sampled in both 1992 and 1993 for a total of 47 specimens (Figure III-24). The site has a small mound at its center approximately 60 cm high. The sole positive sample from this site, containing roughly 800 anthrax spores/g, was a red fox scat found on top of the mound in 1992. The site was intact and no bison or other animal remains were observed in its immediate vicinity.

Sites 22 and 23 were both located away from PLR under the forest canopy. From 22 and 25 specimens collected from sites 22 and 23, respectively, only a single *B. anthracis* colony was isolated from each site (Figures III-25 and III-26). The positive specimen from site 22 was collected at the very edge of the disturbed sand while the positive specimen from site 23 was obtained from near the center of the disturbed area.

At three of the four positive cremation sites at Falaise Lake, anthrax spores were found closely associated with the charred bone bed. Twenty-five specimens were collected from each of these sites in 1994 but none were resampled in 1997. Sites 37 and 89 each yielded one positive sample with approximately 70 and 1200 viable anthrax spores/g, respectively, from within their bone beds (Figures III-27 and III-28). Site 41 yielded the most positive specimens with 4, all of which were associated with the bone bed. As depicted in Figure III-29, the highest concentration of anthrax spores was found near the center of the bone bed and the concentration of viable anthrax spores appeared to decrease toward the bed's periphery. Two positive samples representing 2 spores/g each were found at site 142 (Figure III-30). Both were located on the south side of the site well away from the bone bed. Maps of surveyed carcass disposal sites that did not yield *B. anthracis* isolates are displayed in section A.10 of the Appendix.

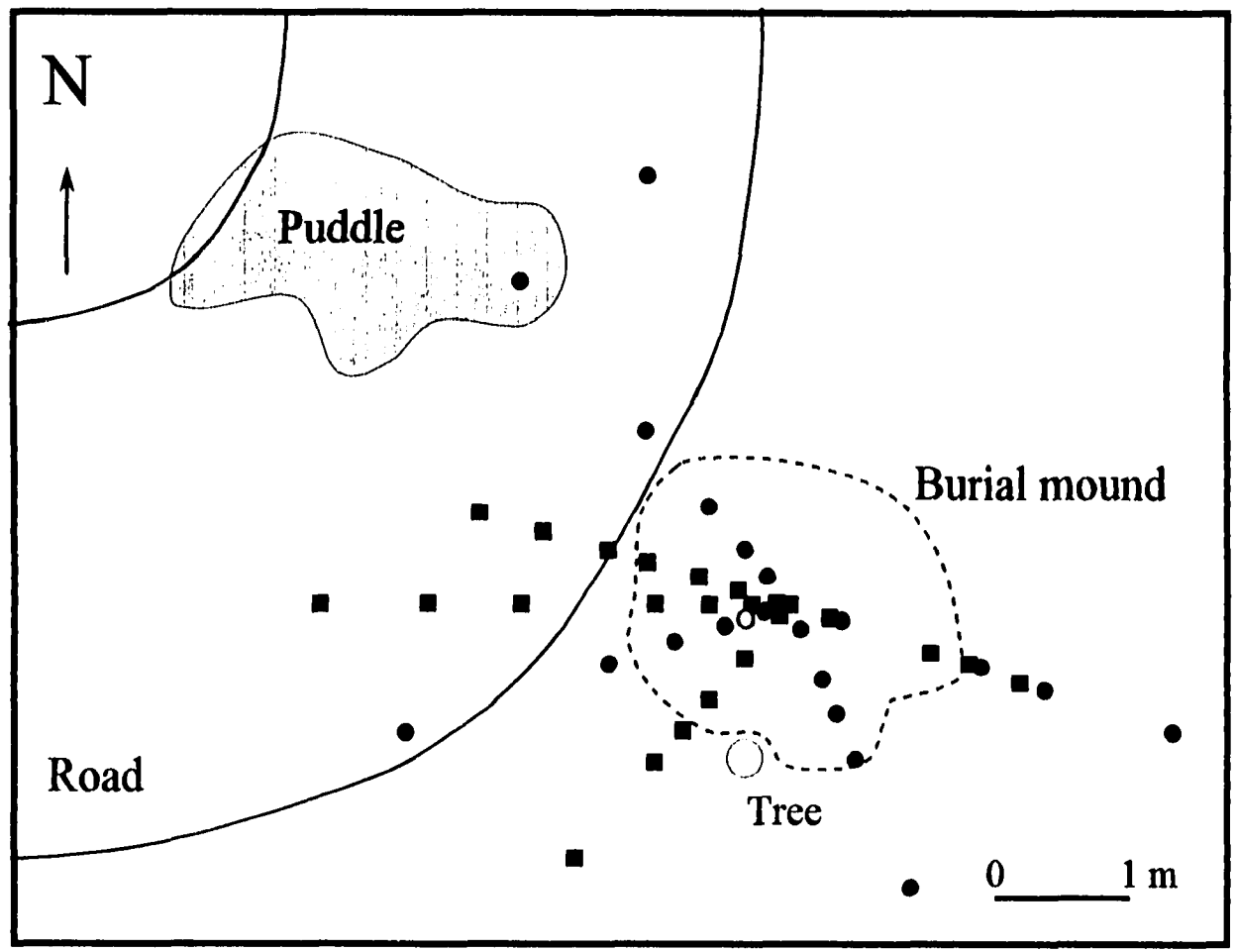

Figure III-24. Specimen collection around burial site 16 at 59° 45' 50" N, 112° 17' 0" W, Parson's Lake Road, WBNP, AB. Negative samples collected in 1992 and 1993 are represented by solid circles and solid squares, respectively. The open circle denotes a sample that was positive for *B. anthracis* spores and contained approximately 800 cfu/g.

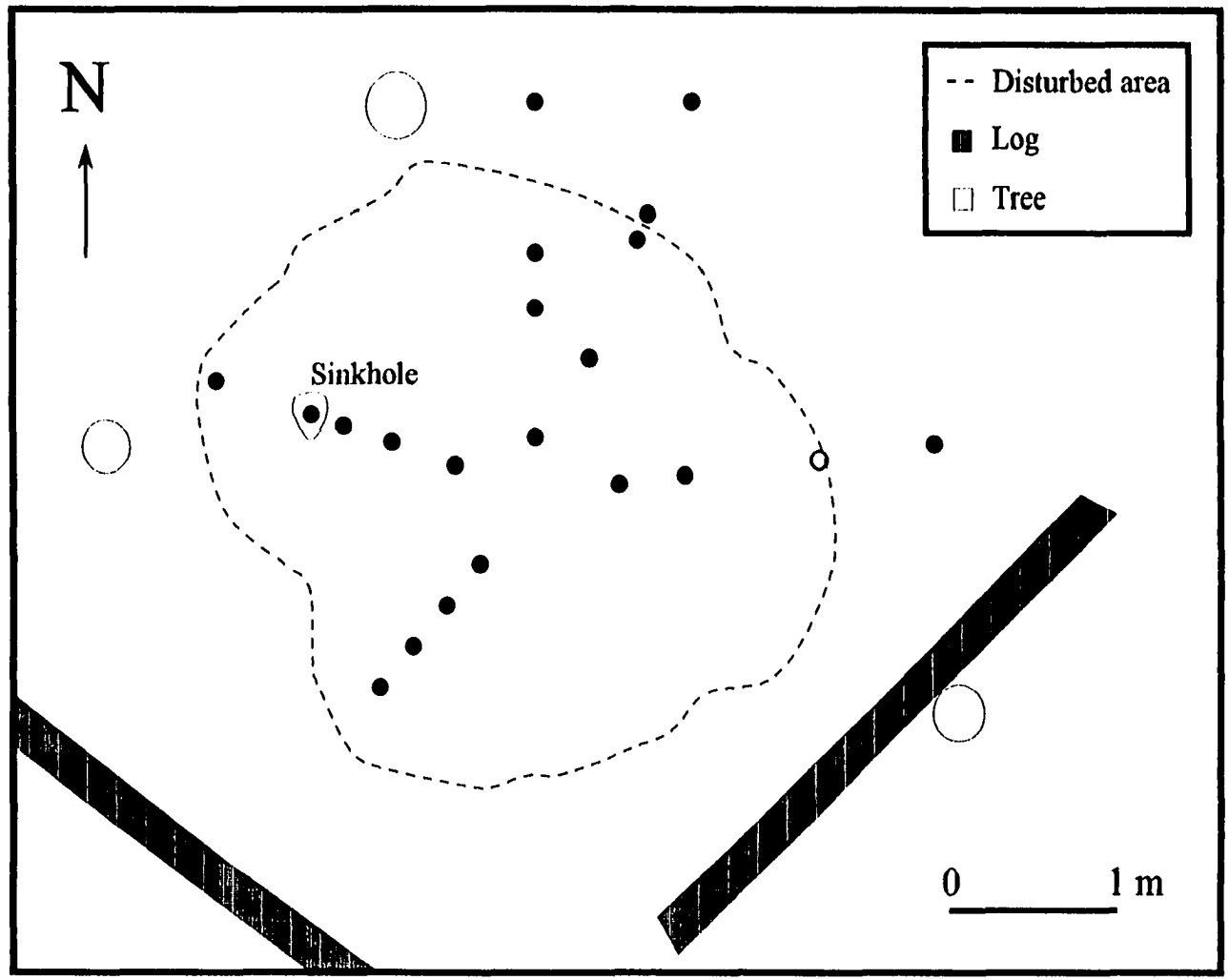

Figure III-25. Specimen collection around burial site 22 at 59° 45' 10" N, 112° 16' 20" W, Parson's Lake Road, WBNP, AB. Sampling locations are represented by circles with negative samples shown as solid circles. The open circle denotes a sample that was positive for *B. anthracis* spores and contained approximately 2 cfu/g.

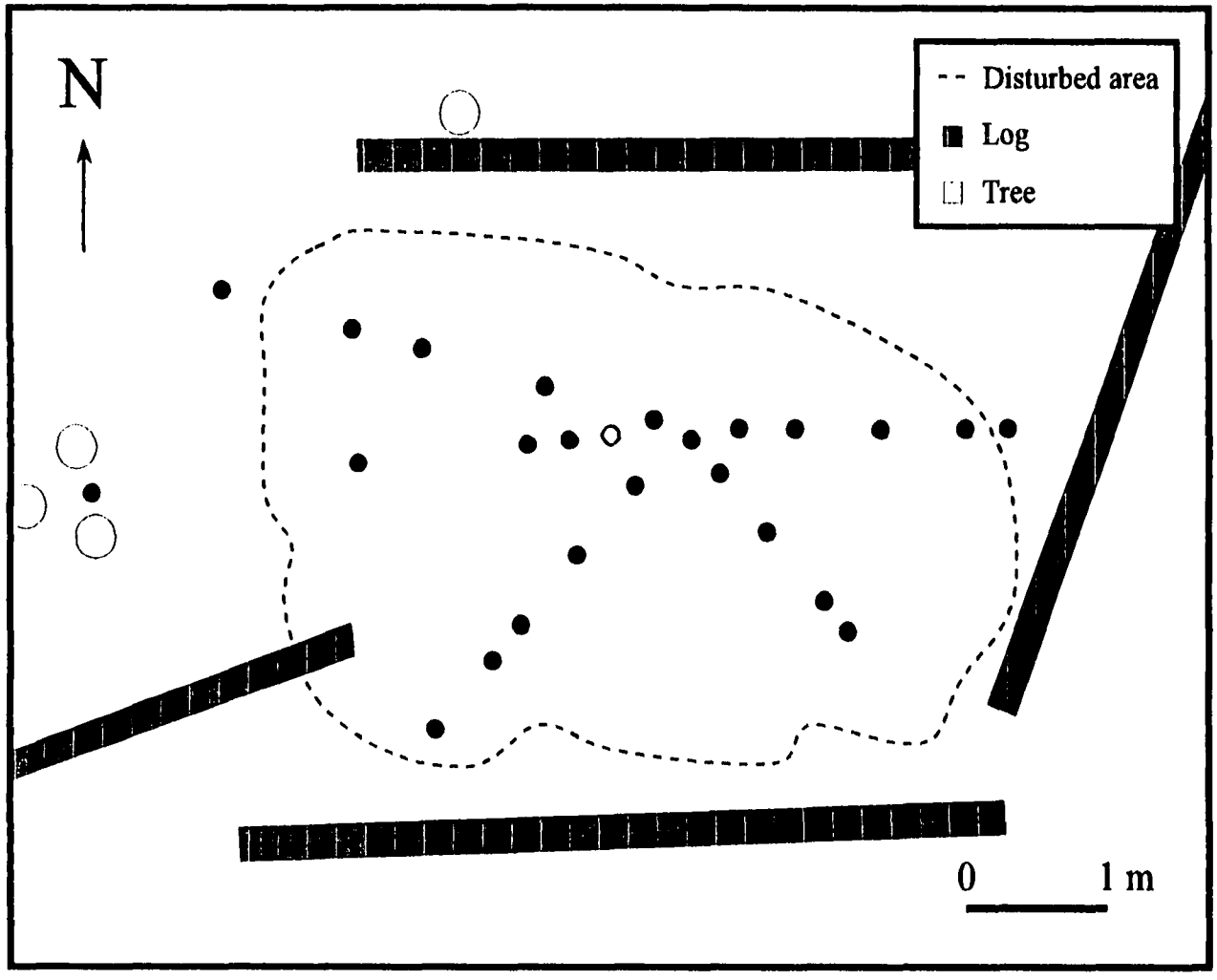

Figure III-26. Specimen collection around burial site 23 at 59° 45' 10" N, 112° 16' 40" W, Parson's Lake Road, WBNP, AB. Sampling locations are represented by circles with negative samples shown as solid circles. The open circle denotes a sample that was positive for *B. anthracis* spores and contained approximately 2 cfu/g.

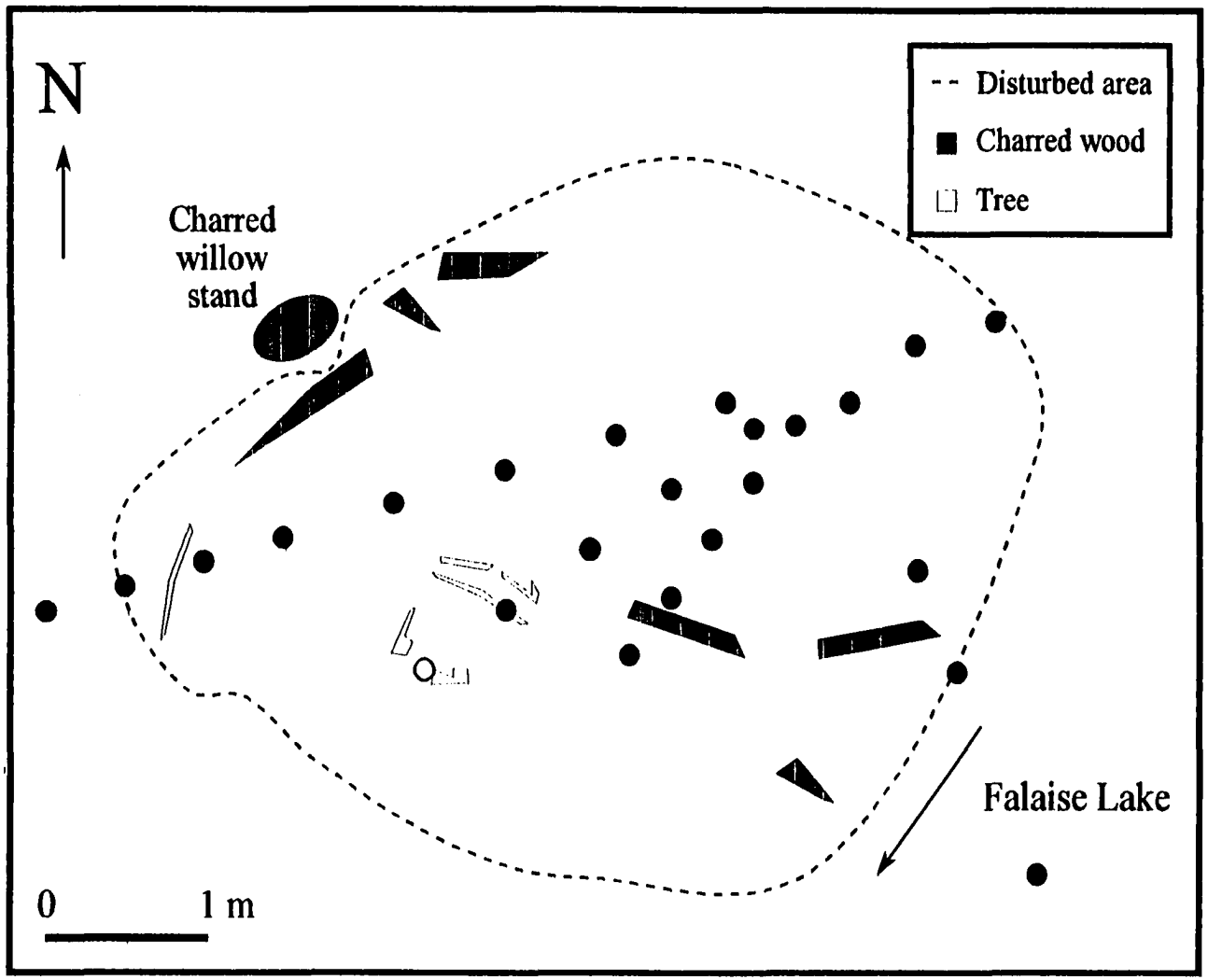

Figure III-27. Specimen collection around cremation site 37 at 61° 28' 0" N, 116° 19' 30" W, Falaise Lake, MBS, NT. Sampling locations are represented by circles with negative samples shown as solid circles. The open circle denotes a sample that was positive for *B. anthracis* spores and contained approximately 70 cfu/g.

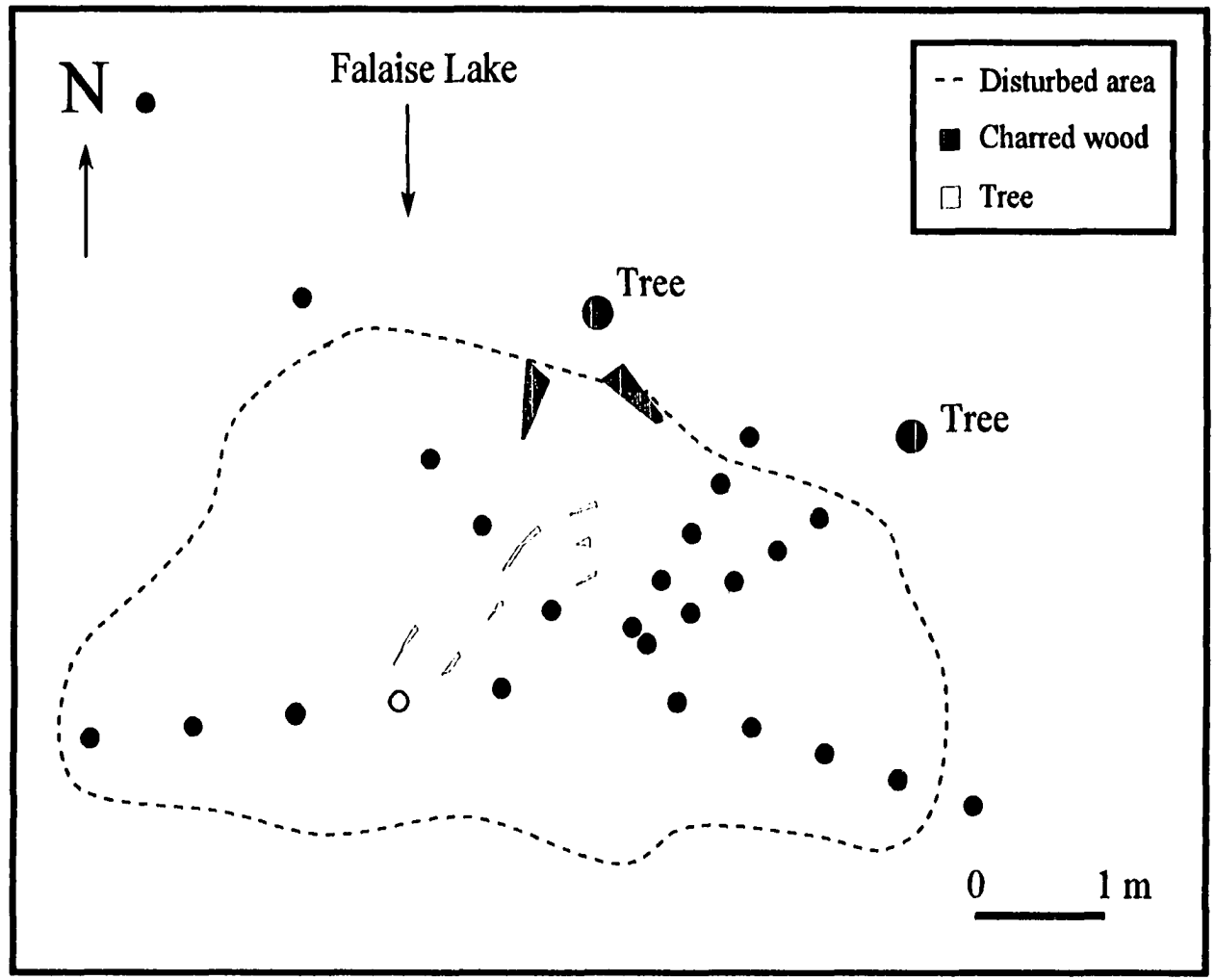

Figure III-28. Specimen collection around cremation site 89 at 61° 29' 10" N, 116° 13' 30" W, Falaise Lake, MBS, NT. Sampling locations are represented by circles with negative samples shown as solid circles. The open circle denotes a sample that was positive for *B. anthracis* spores and contained approximately 1200 cfu/g.

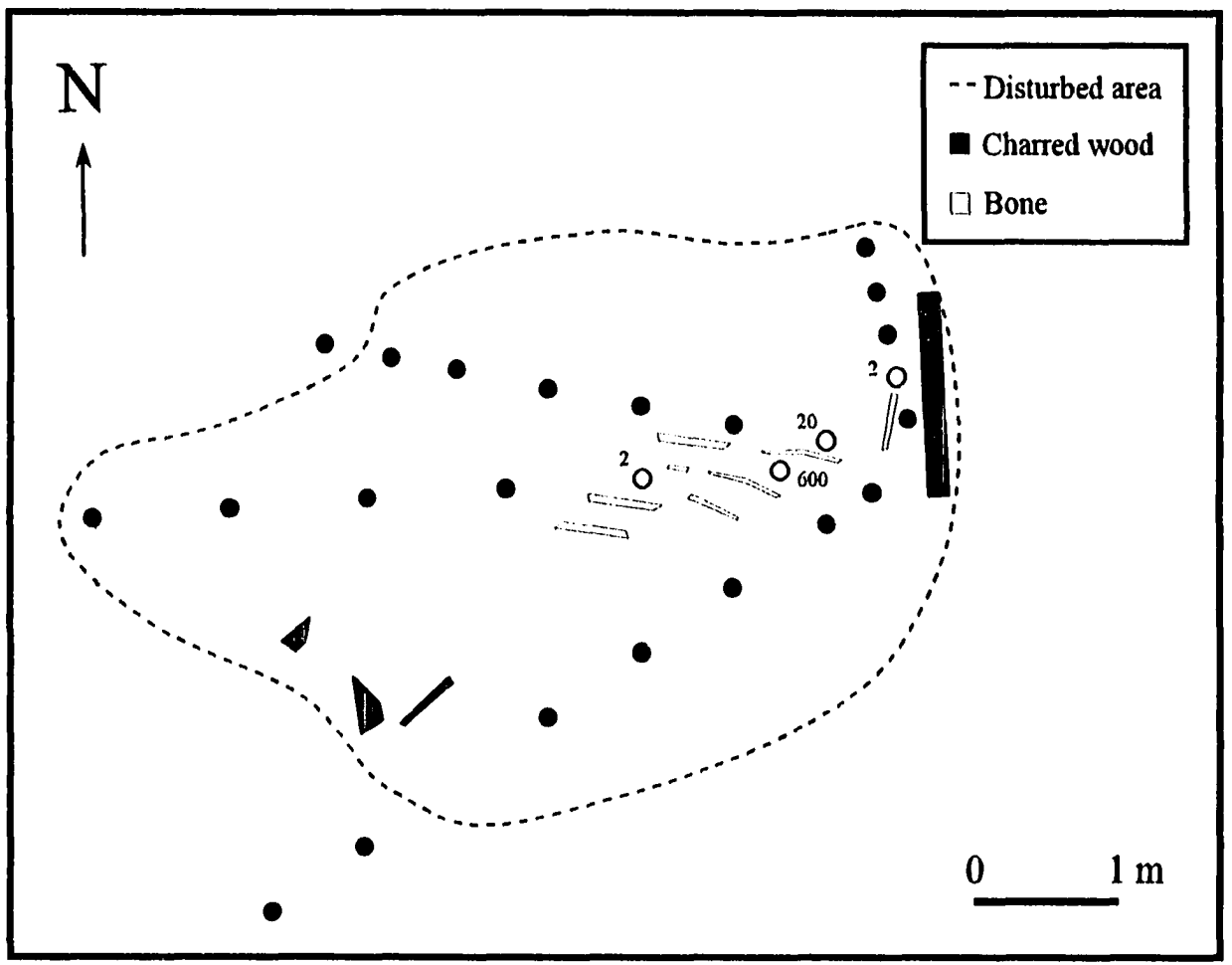

Figure III-29. Specimen collection around cremation site 41 at 61° 28' 50" N, 116° 15' 50" W, Falaise Lake, MBS, NT. Sampling locations are represented by circles. Negative samples are shown as solid circles and positive samples are shown as open circles. The numbers off to the side of the positive samples represent the concentration in cfu/g of anthrax spores recovered.

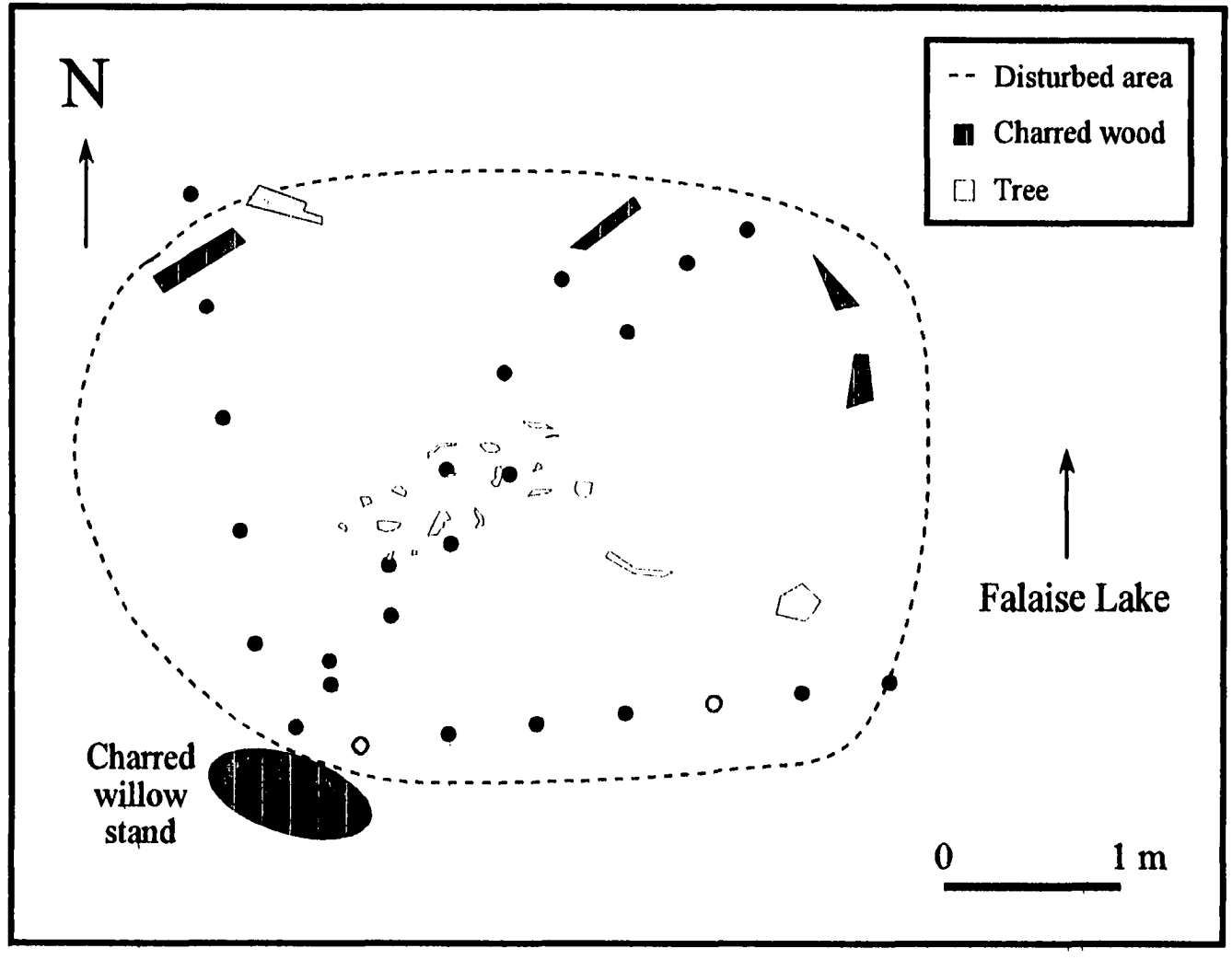

Figure III-30. Specimen collection around cremation site 142 at 61° 27' 40" N, 116° 18' 20" W, Falaise Lake, MBS, NT. Sampling locations are represented by circles with negative samples shown as solid circles. The open circles denote samples that were positive for *B. anthracis* spores and each contained approximately 2 cfu/g.

Chapter IV. Discussion

IV.1. Antigenicity of *B. anthracis* spores

The first observations on the antigenicity of *B. anthracis* and other *Bacillus* spores were performed by Defalle (1902). He injected live preparations of *Bacillus* spores and vegetative cells into rabbits and demonstrated the two life forms possessed unique antigens. Antiserum from rabbits injected with vegetative cells did not agglutinate homologous spores although antibodies which agglutinated the bacilli were present. Spore antisera showed some ability to agglutinate cells, a fact which Defalle (1902) attributed to vegetative contamination of the spore preparations but was more likely due to germination of the live spores upon inoculation. Agglutination experiments with the spore antisera demonstrated variations in the antigens of the species tested suggesting spore antiserum might be used to differentiate species, however, a high level of cross-reactivity was noted. *Bacillus mycoides* spore antiserum gave high titre agglutinations against the spores of *B. mycoides*, *B. anthracis* and *B. subtilis* and low titre reactions against *B. mesentericus vulgatus* and *B. alvei* spores. Norris (1962) stated the *B. mesentericus vulgatus* strain Defalle used was probably a *B. licheniformis*, *B. pumilus* or *B. subtilis*, and contented that the *B. subtilis* strain used was one of the "large-celled" or Michigan strains later shown by Conn (1930) to be *B. cereus*. Thus, Defalle's antiserum could discriminate *B. cereus*-group spores from those of non-group species but not from each other.

Subsequent taxonomic investigation using *Bacillus* spore agglutinins demonstrated clear serological groups within the genus but had minimal success distinguishing *B. cereus*-group species (Lamanna, 1940a, b). H.P. Chu achieved some success producing diagnostic antisera that could differentiate *B. cereus* and *B. anthracis* spores after cross-absorption of the sera with the heterologous spores (Norris, 1962). Lamanna and Eisler (1960) failed to distinguish between spores of several strains of *B. anthracis* and *B. cereus*

with agglutinogens and precipitinogens, however, they did not carry out absorption experiments. A follow-up study to attempt to differentiate strains of *B. cereus*, *B. thuringiensis* and *B. anthracis* with spore agglutinogens found extensive cross-agglutination and the species could not be distinguished serologically even with pre-absorption of the antisera with heterologous spores (Lamanna and Jones, 1961).

The application of more sensitive immunofluorescent analysis, where the reaction of the antibody with the spore surface could be visualized directly with a fluorescent microscope, helped to clarify the serology of the *B. cereus*-group and identify antigenic differences between *B. anthracis* and *B. cereus* spores. Kim and Goepfert (1972) examined 116 strains of *Bacillus* with fluorescently-labeled polyclonal serum prepared against spores of *B. cereus* T. Except for a single strain of *B. pumilus*, none of the non-*B. cereus*-group spores demonstrated any sign of staining. Conversely, the spores of 58 of 59 *B. cereus*, eight of eight *B. anthracis*, 12 of 12 *B. thuringiensis* and four of four *B. mycoides* strains reacted with the serum. Absorption of the serum with *B. anthracis* or *B. mycoides* spores resulted in the elimination of staining of these organisms. Absorption with *B. thuringiensis* ATCC 10792 removed antibodies reacting with six of the 12 *thuringiensis* strains, however, absorption with *B. thuringiensis* var. *galleriae* spores removed antibodies against *B. cereus* to such a degree that the serum was unusable.

Fluorescein-conjugated rabbit IgG polyclonal serum raised against spores of the Vollum strain of *B. anthracis* reacted in immunofluorescent assays with spores of 11 out of 20 *B. cereus* strains but these cross-reactions were removed by absorption of the serum with spores of two *B. cereus* strains (Phillips et al., 1983). The absorbed polyclonal serum reacted with a range of anthrax strains including Vollum and Sterne but a second polyclonal serum raised against Sterne spores and absorbed with *B. cereus* spores as before reacted with the spores of all test anthrax strains save Vollum, suggesting that at least two unique anthrax spore antigens exist (Phillips and Martin, 1983, 1988).

The existence of antigens unique to *B. anthracis* spores was confirmed through the production of a specific monoclonal antibody E12 (Phillips et al., 1988). The antibody was generated from BALB/c mice with a single inoculation of 10^9 *B. anthracis* Ames spores inactivated by heating at 90°C for 1 h. The monoclonal antibody did not stain *B. anthracis* vegetative cells in immunofluorescent assays nor did it react with spores of non-*anthracis Bacillus* including six strains of *B. cereus* and two of *B. thuringiensis*. The monoclonal antibody reacted with spores of all 24 *B. anthracis* strains tested regardless of whether they were live or inactivated with heat or formaldehyde.

IV.1.a. Generation of anti-anthrax spore response in mice

Immunizations of laboratory mice with anthrax spore preparations in combination with various adjuvants were performed in an effort to produce high anti-anthrax spore serum titres in the animals so that they could be used as a source of splenocytes for hybridoma fusion and monoclonal antibody production (Kohler and Milstein, 1975). All spore preparations used with the mice were inactivated with formaldehyde or alkaline glutaraldehyde. The aldehydes react with proteins, peptidoglycan, DNA and RNA producing intramolecular cross-linkages which inactivate the biomolecules (Walker, 1964; Russell and Hopwood, 1976; Power and Russell, 1990). The aldehydes react with proteins on the surface of the spore but also slowly penetrate through the integument to disrupt the peptidoglycan of the cortex and the macromolecules of the core (Sykes, 1970; Power and Russell, 1990). While it is possible that aldehyde cross-linkages may alter the surface antigenicity of the spore, monoclonal antibody E12 reacted equally with live and formaldehyde-fixed anthrax spores (Phillip et al., 1988). Aldehyde-inactivation of spores was chosen over the heat-inactivation because chemical treatment was easier to control and monitor.

The spore coat of *B. cereus*-group species consists of two distinct morphological layers which are apparently comprised of a single protein monomer which differs only in

secondary modifications between the layers. Aronson and Fitz-James (1971) selectively extracted the coats from *B. cereus* T spores and, depending on the absence or presence of cysteine, reconstituted the protein into configurations morphologically and chemically similar to inner or outer coats around coatless spore protoplasts.

For use in immunizing BALB/c mice, coat protein was extracted from *B. anthracis* spores following the Aronson and Fitz-James (1971) protocol and an attempt was made to reconstitute the protein around aluminum hydroxide particles in the outer coat configuration. Isolated coat protein was used in the initial animal immunizations in order to minimize the possibility of animals being inoculated with viable, virulent anthrax spores. Also, because inner spore coat protein could apparently be reconfigured to outer coat, the isolation process helped to maximize the amount of antigen available for immunizations.

The exosprium of *B. cereus*-group spores is a loose covering easily sheared off by mild mechanical forces (Moberly et al., 1966; Tipper and Gauthier, 1972). Morphological studies have demonstrated large holes and tears in the exosporium and solute uptake studies have not demonstrated that the exosporium limits permeability (Gerhardt and Black, 1961; Beaman et al., 1972; Gerhardt et al., 1972). Even though the exosporium is the outermost layer of a spore, its apparent fragility made it an unsuitable target for monoclonal antibodies and production efforts were focussed on more resilient spore coat antigens. However, the spore coat protein extraction protocol used also selectively isolated exosporium components (Aronson and Fitz-James, 1971). Thus, exosporium antigens were present in the aluminium hydroxide preparation and could have contributed to an immune response in the mice.

Despite formaldehyde-treatment to inactivate spores prior to protein extraction and repeated filtration of the extract, a high mortality rate was observed in BALB/c mice receiving immunizations of the spore protein plus aluminium hydroxide preparation produced with *B. anthracis* 93-189C spores. However, extensive bacteriological

investigation of the preparation and mouse cadavers failed to isolate *B. anthracis* or any other virulent microbial agent and it is unlikely the animals died of anthrax or other bacterial infection. Repeated dialysis of the 93-189C preparation to remove toxic reagents used in the protein extraction and reconstitution process failed to decrease the mortality rate and protocols with the preparation were discontinued. Because of the edema associated with the injection site on the mice, the most probable cause of death was the co-isolation of anthrax toxin components with the spore coat protein. The subunits of the anthrax toxin are large enough that they would not have diffused out of the dialysis tubing during the purification procedure.

Although mice were boostered repeatedly with the 93-196C spore protein plus aluminium hydroxide preparation, they failed to develop high serum titres against formaldehyde-inactivated anthrax spores (Figure III-1). The immunodominant epitopes on the spore proteins were either not expressed on the surface of whole spores or did not assume confirmations identical to native spore surface epitopes upon reconstitution. Phillips et al. (1988) obtained similar results during monoclonal antibody production with extracted spore protein. The resultant antibody, A9, did not bind to intact anthrax spores but did react with the protein used to inoculate the mice. The serum titres of the mice in our experiments were not determined against extracted 93-196C spore protein.

BALB/c mice were also immunized with whole spore preparations of *B. anthracis* 93-196C inactivated with either formaldehyde or alkaline glutaraldehyde by themselves or in the presence of three commercial adjuvants. Despite proven efficacy improving the immune response to other antigens (Bennett et al., 1992), none of the three adjuvants significantly enhanced the immune response of the BALB/c mice to the inactivated *B. anthracis* spores relative to inoculations of homologous spores without adjuvant (Figure III-1). Although boostered repeatedly, the immune response of the BALB/c mice was unsatisfactory. In producing monoclonal antibody E12, Phillips et al. (1988) relied on a primary response to their whole spore immunogen and only administered a single dosage

of spores before harvesting the mouse splenocytes five days later for hybridoma fusion. A primary immune response to a novel antigen results in small amounts or low titres of antibody of the desired specificity being formed, most of which is of the IgM subclass as was the case of monoclonal antibody E12. Although Phillips et al. (1988) were successful with a single inoculation, most monoclonal antibody protocols call for multiple boosters of the mice to allow for the generation of a memory response where each subsequent response to the inoculum is quicker and stronger and converts from IgM to IgG via class switching (Harlow and Lane, 1988; Barrett, 1994). Although IgM antibodies have a higher avidity for antigens due to their pentamer configuration, singular IgG antibodies generally have a higher overall affinity to the antigen because of affinity maturation which takes place during memory responses. Most monoclonal protocols strongly suggest waiting until the serum titre of the mice is $\geq 10^6$ against the antigen of interest before harvesting the splenocytes and carrying out hybridoma fusion, in order to maximize the chances of isolating high affinity monoclonal antibodies with high selectivity for the antigen. None of the whole spore immunization schedules used with the BALB/c mice generated serum titres of sufficient magnitude to allow for subsequent hybridoma fusion.

The failure of the BALB/c mouse immune response to mature against the whole spore preparations was most likely due to poor processing or presentation of the antigen by cells of the immune system. Normally after injection, antigens are phagocytosed by APCs and specific B cells and partially degraded (Harlow and Lane, 1988). The antigen fragments are then displayed on the surface of the cells bound with major histocompatibility complex (MHC) class II molecules. In total, any one animal can produce between 6 and 40 different class II molecules all differing in their amino acid sequence in the cleft which holds the antigen fragments, thus allowing a wide range of fragments to be displayed.

BALB/c mice are an inbred strain and as a result have a limited set of MHC class II proteins. If none of the class II proteins present are compatible to the fragments produced from the antigen or the antigen fragments are altered due to chemical treatment, no

fragment-class II protein complexes will be displayed on the surface of APC and B cells and the immune system will not be activated. Outbred strains, because of the greater heterozygosity of their MHC genes, have a much larger repertoire of class II molecules and are therefore able to display a greater variety of antigen fragments. To investigate if class II presentation of spore antigens was a problem in the BALB/c mouse immune response, subsequent immunization schedules were performed using outbred Swiss mice.

The coats of *B. cereus*-group spores are quite resistant to enzymatic degradation and the APC and B cells of the BALB/c mice may not have been able to fully process the spores so that fragments could be displayed in class II proteins on the surface of the cells. To compensate for this possibility, extracted spore protein and inactivated whole anthrax spores pretreated with total spore coat extraction solution were used in subsequent immunization schedules. The proteins were extracted as before from *B. anthracis* 93-196C spores according to Aronson and Fitz-James (1971) but were reconstituted without spore protoplasts or aluminium hydroxide. For comparison, glutaraldehyde-inactivated whole 93-196C spores untreated with coat extraction solution were also injected into Swiss mice.

The serum titres in Swiss mice were higher with all four immunogen preparations than any titres obtained in BALB/c mice (Figure III-2). The improved immune response in Swiss mice may have been due to improved presentation of the antigen by immune cells. Although high serum titres were obtained with both preparations containing spores pretreated with coat extraction solution, the highest serum titres were obtained in mice that received untreated whole spores indicating the immune cells of the Swiss mice had little problem processing and presenting anthrax spores. The extracted protein preparation elicited a higher immune response in the Swiss mice than the BALB/c mice suggesting that at least some of the proteins in the preparation assumed native configurations that were present on the surface of anthrax spores.

Despite the improved immune response in the Swiss mice, their titres were still below the 10^6 minimum suggested by monoclonal antibody production protocols (Harlow and Lane, 1988). In light of the success of Phillips et al. (1988) in producing an anti-anthrax spore monoclonal antibody with only a primary response, a hybridoma fusion was attempted with the highest responding Swiss mouse group; those receiving the whole glutaraldehyde-inactivated spores (Figure III-2). Although hybridomas were produced, none of them secreted antibodies that were reactive against gutaraldehyde-inactivated spores. The fusion results are consistent with the recommendation to obtain as high a serum titre response as possible against the immunizing agent in order to maximize the probability that a mouse splenocyte secreting the desired antibody will meet and fuse with a myeloma cell to produce the desired hybridoma.

IV.1.b. Evaluation of polyclonal rabbit serum against *B. cereus*-group spores

In comparison to mouse immunizations with anthrax spore preparations, immune responses were much stronger and easier to obtain with rabbits (Figure III-3). Like Swiss mice, New Zealand white rabbits are an outbred population and the strong immune response compared to BALB/c mice again coincided with a larger variety of class II proteins due to a higher degree of heterogeneity in the rabbit MHC genes. Despite following the manufacturer's instructions and the University's animal safety guidelines, and restricting the use of Freund's complete adjuvant to the primary inoculation, the rabbits developed weeping granulomas at the injection sites along with the vertebrae and progressive arthritis. As soon as sufficient amounts of high anti-anthrax spore titre serum was collected from the animals, they were humanely euthanized.

The anti-anthrax spore titres of the test and terminal bleed sera were observed to decrease by 100- to 1000-fold after freezing at -20°C for storage. An additional cycle of freezing and thawing did not result in any further titre decreases. The decrease was attributed to degradation of the fragile j-chain holding rabbit IgM pentamers together (Harlow and

Lane, 1988). With the destruction of the j-chain, the avidity of the IgM molecules, and therefore their overall affinity, would greatly decrease as their number of antigen-binding sites went from ten to two.

After pooling and purification of high titre polyclonal serum, the serum was serially diluted and tested against spores and vegetative cells of a wide variety of *Bacillus* and *Clostridium* species in an indirect plate ELISA. The highest serum titres were obtained against formaldehyde-inactivated spores of *B. anthracis* 9604; the same spore preparation used to immunize the rabbits; and *B. anthracis* 93-196C (Figure III-5). The equal titres of the serum against both strains verifies that although they belong to two separate but closely related genotypes based on MVLA (Keim et al., 2000) they share similar spore surface epitopes. The serum titres against formaldehyde-inactivated and live *B. anthracis* ATCC 4229 spores were equal to each other but 1000-fold less than against the other two strains. This suggests that *B. anthracis* ATCC 4229 possesses spore surface epitopes that are different from those of *B. anthracis* 9604 and 93-196C and that the use of formaldehyde to inactivate the spores did not appreciably modify the spore surface epitopes. *Bacillus anthracis* ATCC 4229 is a laboratory conditioned strain which has undergone decades of culture since its original isolation and, although it has not yet been analyzed with MVLA, probably belongs to a genotype well separated from the other two strains. The genetic drift between the strains could have allowed for alterations between their spore surface epitopes. Previous work with absorbed polyclonal serum against Sterne and Vollum laboratory strains (which belong to widely separate MLVA genotypes) have also suggested anthrax strains can carry differing spore surface epitopes (Phillips and Martin, 1983, 1988).

Like previous studies of anti-*Bacillus* spore polyclonal sera, the serum displayed various degrees of cross-reactivity to the spores of non-*anthracis* species (Figure III-5). As expected, the highest cross-reactive titres were obtained against spores of the closely related *B. cereus*-group species although some variability was noted. Spores of three

strains of *B. cereus* reacted as strongly with the serum as *B. anthracis* ATCC 4229 spores, whereas two other strains had only moderate titres (10^4 - 10^3) against the serum. The serum reacted strongly with spores of the *B. thuringiensis* strain tested but only moderately with spores of the *B. mycoides* test strain. All non-*B. cereus*-group strains tested gave only moderate or low (10^2 - 10^1) titres against the serum. The serum also gave moderate to low titres against the spores of several Clostridial species indicating the two genus share common surface epitopes (Figure III-6). Like other polyclonal sera raised against *Bacillus* spores, the serum displayed minimal cross-reactions with *Bacillus* vegetative cells.

Serial dilutions of the polyclonal serum were tested against a fixed number of bacterial endospores; approximately 10^5 spores/ELISA plate well; in order to determine the serum titre against *Bacillus* and Clostridial spores. When the complementary ELISA was performed; testing fixed dilutions of the serum against serial dilutions of endospores; reactivity against the spores was quickly lost (Figure III-7). The purified serum could not detect live or fixed spores of *B. anthracis* ATCC 4229, *B. cereus* ATCC 14579 and *B. subtilis* ATCC 6633 and fixed spores of *B. anthracis* 9604 below concentrations of roughly 10^5 - 10^4 spores/well. The abrupt disappearance of reactivity after spore dilution suggested the target antigens on spores of all reactive strains were only present at low concentrations on the surface of the spores. While the purified serum was useful for identifying *B. cereus*-group spore stocks, its limited sensitivity and selectivity made it unsuitable as a capture or detection agent in an ELISA system for detecting low levels of anthrax spores in the environment.

IV.1.c. Utility of monoclonal antibodies FDF 1B9 and EAII-6G6-2-3 in environmental assays for *B. anthracis* spores

Monoclonal antibodies FDF 1B9 and EAII-6G6-2-3 were developed by Dr. J.W. Ezzell Jr. for the identification of *B. anthracis* (Ezzell et al., 1990; McGee et al., 1994) and for the

study of *B. anthracis* germination (Ezzell and Abshire, 1996). FDF 1B9 is an IgG antibody which recognizes the negatively-charged glutamic acid capsule of *B. anthracis*. EAII-6G6-2-3 is of the IgM class and binds to a galactose-N-acetyl-D-glucasamine polysaccharide component of the cell wall that is apparently universal in *B. anthracis* vegetative cells but rare in non-*anthracis Bacillus* species (Ezzell et al., 1990). Although other *Bacillus* species, including strains of *B. subtilis*, *B. licheniformis* and *B. megaterium*, are known to produce glutamic acid capsules (Claus and Berkeley, 1986) and two *B. cereus* strains produce the cell wall polysaccharide, the combination of both traits is strongly indicative of *B. anthracis* (Ezzell et al., 1990).

Immunostaining studies with fluorescently labeled monoclonals revealed that *B. anthracis* strains display both antigens upon germination before outgrowth (Ezzell and Abshire, 1996). A *B. anthracis* spore identification system was developed using the monoclonals in which the spores are briefly incubated in one of two broths to maximize germination and elaboration of the target antigens before immunostaining with the labeled antibodies and viewing under a fluorescent microscope. Incubation in the germination broths was limited to 30 - 45 min allowing for spore germination but not vegetative outgrowth. The germination broths utilize a rich heart infusion medium base fortified with a variety of amino acids, adenosine, uracil and salts that allow > 90% of the *Bacillus* spores present in a sample to germinate (Ezzell and Abshire, 1996). The basic medium, designated polysaccharide broth, is used in combination with monoclonal antibody EAII-6G6-2-3 as the germinating *B. anthracis* produce the cell wall antigen but no capsule. Germination medium supplemented with 50% (v/v) heat-inactivated horse serum and 0.8% (w/v) sodium bicarbonate (capsule broth) is used in combination with antibody FDF 1B9 and *B. anthracis* germinated in this medium form thick capsules which bleb out through the spore coats (Ezzell and Abshire, 1996). The assay system allows for the rapid identification of viable *B. anthracis* spores in the laboratory without the outgrowth and exponential multiplication of the pathogenic micro-organism required with selective and differential media.

An attempt was made in the laboratory to adapt the identification system to an indirect plate ELISA format more amenable to field study and the processing of large numbers of environmental samples. *Bacillus anthracis* spores were bound into wells of polystyrene 96-well plates and then treated with the germination broths. As the spore core elongates into a vegetative cell, the spore coat hemispheres remain attached to both terminals (Ezzell et al., 1990). Therefore, germination of the spores should not disrupt their adhesion to the solid matrix of the plates wells. A blocking agent was unnecessary in the assay as proteins in the germination broth blocked the remaining adhesion sites in the plate wells.

Neither of the monoclonal and broth combinations detected *B. anthracis* ATCC 4229 spores after short term incubations. Both were able to detect spores only after overnight incubation in the germination broths (Table III-4). As spore germination takes about 1 h for completion (Moberly et al., 1966), the results indicate that the assay required exponential growth of the vegetative organism in order to amplify the signal; germination alone did not generate sufficient antigen to be detected in the plate ELISA format.

Against overnight polysaccharide broth cultures of *Bacillus* species, monoclonal antibody EAII-6G6-2-3 lacked specificity for *B. anthracis* and was equally sensitive to *B. cereus* and *B. subtilis* strains, detecting starting concentrations of roughly 10^6 spores/well. The antibody was also cross-reactive, but to a lesser extent, to *B. anthracis* cultured in capsule broth. Production of capsule in the enriched capsule broth may have slowed the growth of microbe compared to equal length incubation in polysaccharide broth resulting in less cells/well or the capsule itself may have restricted access of the antibody to the cell wall of the microbe and was thus responsible for the decreased sensitivity. Previous experiments with fluorescently labeled EAII-6G6-2-3 failed to immunostain encapsulated *B. anthracis* cells isolated from infected guinea pigs (Ezzell et al., 1990).

Monoclonal antibody FDF 1B9 was highly sensitive against overnight capsule broth cultures of *B. anthracis* and was able to detect starting concentrations down to

approximately 2000 spores/well. However, the antibody was also cross-reactive to overnight capsule broth cultures of *B. cereus* and *B. subtilis* and to unencapsulated *B. anthracis* cells from overnight polysaccharide broth cultures, although the sensitivity to these other cells was roughly 1000-fold less than against encapsulated *B. anthracis*. Monoclonal FDF 1B9 binds to highly charged poly-D-glutamic acid moeities in the capsule of *B. anthracis*. The cross-reactivity to cultures of nonencapsulated *Bacillus* species was probably due to non-specific interactions with negatively-charged components on the cell walls of these species.

Although after overnight incubation the FDF 1B9 and capsule broth combination was very sensitive to *B. anthracis*, it lacked the selectivity required for an environmental detection system; false-positive results from overgrowth by ubiquitous non-*anthracis Bacillus* species would be highly likely. As well the plate system requires overnight culture and exponential outgrowth of pathogenic *B. anthracis* in order to detect the micro-organism, negating the speed and safety of the immunofluorescent format.

IV.2. Detection of *B. anthracis* spores with PLET medium

IV.2.a. Selectivity and sensitivity of PLET medium

PLET medium was developed by Knisely (1966) as a selective medium for *B. anthracis* and with slight modifications has become the standard for the isolation and quantitation of viable anthrax spores in environmental samples. Polymyxin is present in the medium as a general inhibitor of Gram negative bacteria. The antibiotic functions as a cationic surface-active detergent creating holes in the cell membrane of bacteria (Hoeprich, 1970). Gram positive micro-organisms are generally more resistant to the antibiotic, especially at low concentrations like that used in PLET medium, because their thick outer cell wall prevents the drug from reaching their cell membrane (LaPorte et al., 1977). Lysozyme also acts as a general inhibitor of Gram negative bacteria although it does effect Gram positive

microbes including some *Bacillus* species. In a review of published literature, Claus and Berkeley (1986) reported > 90% resistance to lysozyme in strains of only ten *Bacillus* species; *B. alvei*, *B. anthracis*, *B. cereus*, *B. larvae*, *B. laterosporus*, *B. lentimorbus*, *B. mycoides*, *B. popilliae*, *B. schlegelii* and *B. thuringiensis*.

The main selective mechanism in the medium is the combination of EDTA and thallium acetate which allows the recovery of *B. anthracis* strains but generally inhibits strains of other *Bacillus* species. Knisely (1966) hypothesized that the effect was due to EDTA acting as a chelating agent which combined with an essential cation required by *Bacillus* species for growth. Thallium cations could be effectively utilized as a substitute for this cation by *B. anthracis* but not by other species. Bowen et al. (1996) recently demonstrated that while EDTA was more essential than thallium when the reagents were used singularly in the medium, the best selective growth of *B. anthracis* occurred when the two reagents were used in combination. They also found evidence in support of the chelating hypothesis; the addition of >2% (w/v) soil to PLET medium removed its selectivity for *B. anthracis*. The inhibition of the medium was demonstrated to be largely due to cations present in the soil chelating with EDTA and preventing its synergistic action with thallium cations.

The importance of EDTA in the selectivity of the medium was demonstrated in preliminary experiments with PLET performed in this study. The first several batches of PLET produced were not selective and allowed the majority of *Bacillus* species tested to germinate and grow. Failure of the medium was traced to the EDTA used in its formulation which was over 10 y old. The chelating ability of the old batch of EDTA was apparently reduced as production of the medium with a new bottle of EDTA resulted in medium that was selective for *B. anthracis*, inhibitory for *B. cereus* and semi-selective against the other *Bacillus* species tested (Table III-5).

Published literature on the selectivity of PLET medium against non-*anthracis Bacillus* species is limited. Knisely (1966) reported that the medium successfully recovered 21 of 22 *B. anthracis* strains after 24 h incubation at 37°C with the remaining strain recovered after 48 h incubation. The medium inhibited growth of ten of 11 strains of *B. cereus* and the strain that did grow lagged behind *B. anthracis* in morphological development. Of four strains of *B. megaterium* tested, one strain, described as "lysozyme-resistant", was able to grow within 24 h of incubation. Although *B. cereus* has received the most attention as a contaminator of PLET medium, multiple strains of *B. subtilis* var. *niger* were able to grow on the medium; 60% (three out of five strains) of the var. *niger* strains compared to only 9% of the *B. cereus* strains tested. Knisely (1966) did not state whether the colonies of *B. subtilis* var. *niger* could be distinguished from colonies of *B. anthracis* on the medium. All other *Bacillus* strains tested by Knisely; *B. mycoides* (5 strains tested), *B. polymyxa* (1), *B. pumilius* (2), *B. sphaericus* (3), *B. sphaericus* var. *fusiformis* (1), *B. subtilis* (5) and *B. thuringiensis* (2); were inhibited on the medium. It is noteworthy that few isolates of each species were included in the study by Knisely and a broad range of species was not tested.

Because only one strain of *B. anthracis* was tested against PLET medium, it was the inhibitory rather than the selective abilities of the medium that were actually being studied in our experiments. The ability of *B. anthracis* to grow on PLET has already been well established by Knisely (1966) and in environmental studies of the microbe (Manchee et al., 1981; Turnbull et al., 1986; 1989; 1991; Lindeque and Turnbull, 1994). Strain ATCC 4229 was used in this study as a positive control to ensure that the medium was functioning properly and for comparison of colony morphology with other successfully growing *Bacillus* strains. Functional PLET in this study allowed the growth of *B. anthracis* ATCC 4229 and inhibited all seven strains of *B. cereus* tested (Table III-5). However, the medium failed to inhibit strains from two other *B. cereus*-group species; *B.*

mycoides and *B. thuringiensis*; whose colonies were indistinguishable from those of ATCC 4229. Colonies of *B. subtilis* 1A289 were also indistinguishable from ATCC 4229 colonies on the medium.

Although only six environmental *Bacillus* isolates were tested, two; *B. pumilus* and *B. circulans*; were indistinguishable from *B. anthracis* ATCC 4229 on PLET and raise concerns about differentiating *B. anthracis* from indigenous *Bacillus* species in environmental studies. Knisely (1966) only tested the medium against laboratory strains of non-*anthracis* species. Strains adapted to enriched media culture in the laboratory may have lost an ability to scavenge cations or utilize thallium cations that was inherent to the wild parent strain. More laboratory studies of PLET should be performed against environmental isolates of *Bacillus* species that have received minimal culture in order to properly address this issue.

As mentioned previously, one of the assumptions of the often quoted sensitivity limit of 3 *B. anthracis* spores/g of soil for PLET (Manchee et al., 1981) is that all the anthrax spores present in a population germinate equally on the medium. This is clearly not the case. Even after 48 h incubation, *B. anthracis* ATCC 4229 spore recovery on PLET plates was only 38% of the number recovered with nutrient-rich SBA from the same stock (Figure III-10). McGetrick et al. (1982) also observed various degrees of recovery of *B. anthracis* spores on PLET medium. For five of 12 *B. anthracis* strains tested, PLET recovery of spore stocks was < 40% that recovered with enriched, non-selective medium. The inhibition appeared to effect either the ability of the microbe to germinate or outgrow as, for most of the 12 strains, PLET recovery of vegetative stocks was comparable with recovery by the enriched medium. Thus, although PLET is selective for *B. anthracis*, it is not an optimal recovery medium and may miss a significant proportion of an anthrax spore population.

IV.2.b. Modification of PLET medium

In an effort to improve the ability of PLET to differentiate between *B. anthracis* and other *Bacillus* species, especially other members of the *B. cereus*-group, the medium was supplemented with either horse blood or nitrocefin. Nitrocefin is a penicillin or β-lactam analog that when cleaved by penicillinases produces a colored product. *Bacillus anthracis* strains are, in general, non-hemolytic and lack penicillinase whereas other *B. cereus*-group members are β-hemolytic and produce penicillinase (Carman et al., 1985). It was hypothesized that clear haloes of lysis around colonies on blood supplemented PLET or yellow colonies on nitrocefin supplemented PLET plates would help to differentiate *B. cereus*-group contaminants from *B. anthracis* colonies.

The addition of horse blood to PLET allowed for the differentiation of *B. anthracis* ATCC 4229 from all test strains except *B. subtilis* 1A289 and the environmental strain of *B. pumilus* (Table III-5). However, the blood also decreased the selectivity of the medium presumably by providing cations that could chelate and inactivate EDTA in the medium or act as alternatives to thallium cations. The decreased inhibitory ability of the supplemented medium increased the likelihood of *B. anthracis* colonies being overgrown and masked by non-*anthracis* contaminants and made the supplemented medium unsuitable for preliminary screening of specimens in epidemiological and environmental investigations.

Of the *Bacillus* strains tested against nitrocefin supplemented PLET, only *B. mycoides* MU 711/84 grew and its off-white color indicated that either the strain lacked penicillinase or insufficient levels of nitrocefin had been used in the medium to result in a visibly colored product if the enzyme was present (Table III-5). It is unknown whether the nitrocefin itself or in combination with the regular components of PLET resulted in the inhibition of *B. anthracis* ATCC 4229, *B. thuringiensis* QC 12093 and the other strains that had successfully grown on regular PLET. Alternately, before addition to the medium,

the nitrocefin was solubilized in a reconstitution solution containing dimethylsulfoxide (DMSO; final concentration in PLET medium of 0.017% w/v) and it is possible that this reagent was responsible for the inhibition of the previously successful strains. However, no experiments were performed with PLET medium supplemented with only DMSO in order to confirm if the reagent was responsible for the inhibition.

IV.2.c. *Bacillus anthracis* spore purification by heat and ethanol

Because of uncertainty about thermophilic contaminants and the thermoresistance of natural spore stocks, spore purification with heat has varied from study to study (Manchee et al., 1981; Turnbull et al., 1986). In recent years, a standard treatment of 62.5°C for 15 min has arisen (Turnbull et al., 1991, 1998; Lindeque and Turnbull, 1994), however, care must be taken when adapting this treatment because the samples must be pre-incubated to reach the desired temperature. The length of pre-incubation varies with the volume of sample and the composition of the sample container. In our experiments, it was necessary to pre-incubate the 1 ml samples in their polyethylene centrifuge tubes an extra 5 min before they came up to temperature. On the other hand, the same volume of sample reached 63°C in pyrex glass tubes within 1 min.

Although heat treatment of bacterial endospores can successfully purify them from vegetative contaminants, it can also lead to mutations. An alternative method of prescreening endospores from environmental samples which has not been commonly used, yet is non-mutagenic, is ethanol. One hour incubation in 50% (v/v) ethanol was just as effective a bactericidal agent against Gram positive and negative species as heating for 20 min at 63°C (Figure III-15). The treatments might have reduced the *B. cereus* stock further but staining revealed a substantial population of spores within the stock that were resistant to the treatments. Neither treatment resulted in a reduction in the viability of *B. anthracis* ATCC 4229 spores.

In comparisons of the two treatments with non-sterile specimens, ethanol was just as effective as heat at isolating anthrax spores and there was no significant difference in the number of spores isolated or the number of positive specimens identified (Table III-7). Both treatments failed to kill an equal number of morphologically similar contaminants. Ethanol treatment proved less sensitive to incubation time than heat treatment; a 50% increase in incubation time did not significantly alter the outcome of the treatment (data not shown). Extended storage of bacterial spore crops in ethanol has been shown to have no effect on viability or heat resistance (Favero, 1967). Ethanol treatment also did not require a variable pre-incubation step and treatment began as soon as the reagent was added.

IV.3. Selective isolation of *B. anthracis* spores from sterile soil

The 3 spores/g sensitivity limit of PLET medium suggested by Manchee et al. (1981) assumed that water is able to extract all spores present in an environmental specimen. However, in complex specimens, such as soil, there is a heterogeneous mixture of matrices and microenvironments, some of which may have a high affinity for bacterial endospores. It has been well established that *Bacillus* spores, especially those of *B. cereus*-group species are hydrophobic (Doyle et al., 1984; Koshikawa et al., 1989; Rönner et al., 1990; Wiencek et al., 1990). Husmark and Rönner (1990) demonstrated that hydrophobic interactions were the predominant attractive force for spore adhesion to a variety of solid matrices. Water, by itself, may not be able to disrupt these interactions and extract the spores for inoculation to the medium. Any extraction solution that is able to free and concentrate more spores in the supernatant than water alone would increase the sensitivity of the PLET detection system.

Because of their partially dehydrated core, bacterial endospores, like many other resistant cryptobiotic microbial life stages (i.e., parasitic cysts, fungal teliospores), have a high buoyant density. Bakken (1985) reported a buoyant density of up to 1.29 g/ml for

bacterial endospores. Due to a combination of their buoyancy and hydrophobicity, *Bacillus* spores have been demonstrated to selectively accumulate in surface foam (Boyles and Lincoln, 1958) and in the upper hydrophobic phase of a polyethylene glycol-aqueous system (Sacks and Alderton, 1961). Flotation in solutions of high specific gravity is a common method of parasitic cyst isolation from fecal matter in the diagnostic laboratory and high specific gravity solutions of sucrose were tested against standard water extraction for recovery of anthrax spores. Although most cyst isolation procedures use high specific gravity ionic solutions, the sucrose solutions used here allowed the use of nonionic detergents to disrupt hydrophobic interactions between spores and their substrates. Hydrophobic interactions between spores and solid mattrices would have been enhanced in an ionic extraction solution (Shaltiel, 1974; Lindahl et al., 1981; Doyle et al., 1984).

Experiments with seeded sterile soil samples confirmed that both a reagent to disrupt hydrophobic interactions and a high specific gravity solution to float the spores were necessary to recover maximal numbers of spores from field and potting soils (Figures III-11 and III-12). There was no significant difference between the percentage of spores recovered by water and high gravity sucrose plus Triton in wallow soil samples but, as noted, the resolving power of the statistical analysis was hindered by the small sample size (Figure III-13).

Higher percentage yields of anthrax spores were obtained from potting soil than from the other two soil types using identical extraction solutions. The difference in spore yields could be due to structural differences between the peat-based potting soil and the clay-based Falaise soils. Alternately, electrostatic interactions have been shown to play a pH-dependent role in the binding of *B. cereus*-group spores (Husmark and Rönner, 1990) and the acidic pH of the potting soil compared to the alkaline pH of the Falaise soils may have reduced the adhesive properties of the spores by altering their surface charge (Table A-1). However, the most probable cause for the discrepancy is the water content of the soil

types. Wallow and field soil had a water content of roughly 10% of their dry weight while the water content of the potting soil was > 50% (Table A-1). The higher water content of the potting soil may have resulted in a hydration barrier around the soil particles and prevented the hydrophobic spores from adhering optimally to the particles.

Although sucrose plus nonionic detergent did not appear to improve spore recoveries with wallow soil, this soil did not contain any loam (i.e., decaying plant matter) and its calcareous clay content may have contained few hydrophobic moieties to bind spores. Similar results were obtained by Bowen et al. (1996) who adapted the two phase aqueous polymer system developed by Sacks and Alderton (1961) to the selective recovery of *B. anthracis* spores from seeded samples of several soil types. The glycol formed a buoyant hydrophobic layer into which free spores concentrated. Spore recoveries with the biphasic system were greater with sandy soil than with loamy clay soil (similar to the field soil used here). The biphasic system recovered significantly more spores than straight water extraction with soil samples that had been freshly seeded, however, if the spores were left in the samples for longer periods before extraction and had a chance to dry onto soil particles, extraction efficiency with glycol was greatly reduced (Bowen et al., 1996). No experiments were performed to test the effect of spore contact time with soil prior to extraction in our studies. However sucrose plus nonionic detergent extraction should be less sensitive to spore-soil binding than glycol extraction because of the ability of the detergents to disrupt hydrophobic interactions and wash spores free of solid matrices. Further experiments with older seeded soil samples should be performed to confirm this hypothesis.

The 1.22 g/ml sucrose plus Triton solution was chosen for further comparisons with water. Although 1.22 g/ml sucrose plus 0.5% (v/v) Triton X-100 had consistently isolated higher yields of anthrax spores than water from seeded sterile potting and field soil samples, in sensitivity tests against decreasing numbers of spores in sterile soil samples, the two extraction solutions were equally sensitive, consistently detecting down to 40

spores/g of wallow and field soil (Figure III-14). Similarly, in comparisons of the two extraction solutions with non-sterile environmental specimens from northern Canada, there was no significant difference in the number of positive samples identified or the number of *B. anthracis* spores isolated (Table III-6). There was also no difference in the number of contaminating microbes carried over from the samples by the extraction solutions to the PLET plates. However, the small number of samples that contained anthrax spores and the low concentration of spores in these samples may have reduced the resolving power of the statistical analysis to detect a difference between the two solutions. Further comparisons should be undertaken with a greater variety of anthrax spore containing environmental samples.

IV.4. Detection of *B. anthracis* spores in environmental samples from endemic regions of northern Canada

IV. 4.a. Isolation and confirmation of *B. anthracis* spores

It is a commonly held notion that PLET medium has a short shelf life. During screening of the environmental samples from northern Canada, the stock of PLET plates used was checked weekly against a positive control (*B. anthracis* ATCC 4229 spores) and negative controls (spores of *B. cereus* ATCC 14579 and *B. subtilis* ATCC 6633) in order to insure it remained selective. Throughout the screening, the medium remained fully functional and selective up to eight weeks after its manufacture when the screening was completed. There was no decrease in the number of colony forming units isolated from standardized spore suspensions inoculated on the medium during this period.

Despite the isolation of contaminants from the environmental specimens on PLET, the medium was still able to differentiate *B. anthracis* morphologically from the majority of contaminants. Even on plates containing over 300 contaminant colonies, colonies displaying the milk-white, domed morphology of *B. anthracis* stood out and were readily

isolated for further testing. Although it is possible that some of the hardier contaminants may have overgrown and masked *B. anthracis* isolates, on several plates *anthracis*-like colonies were observed overgrowing contaminant colonies.

Although PLET medium was able to detect and differentiate *B. anthracis* ATCC 4229 spores from pure stocks in the laboratory after 24 h incubation, it was necessary to incubate wild anthrax strains recovered from the northern Canada field samples for 48 h before they could be counted and assessed. Thus, incubation time should not be used as a selective criterion for identification of *B. anthracis* on PLET in epidemiological studies.

In subsequent penicillin sensitivity tests, isolates displaying any degree of sensitivity were considered presumptive *B. anthracis* (Table III-8). Statistical comparison of confirmed *B. anthracis* and penicillin-sensitive, non-*anthracis* isolates demonstrated the zone of inhibition was significantly greater with *B. anthracis* and one may be tempted to assign a minimal diameter to the zone of inhibition before accepting an isolate as *B. anthracis*. However, the wide range of zones for both populations and the high level of variance within each population, suggest such a strategy would result in a number of false negative samples while still misidentifying a number of isolates as *B. anthracis*. Accepting any sensitive isolate as *B. anthracis* would minimize the number of false-negative samples and any false-positive isolates could be identified later in follow-up confirmatory tests.

The anthrax spore concentration of each positive sample was calculated from the number of *B. anthracis*-like colonies formed on PLET plates taking into account the percentage of isolates screened in confirmatory tests that proved to be *B. anthracis* (Table III-9). The theoretical sensitivity limit of the extraction and PLET culture procedure based on sample dilution was 2 spores/g, however, the calculation is based on the same assumptions criticized previously, namely that the extraction procedure was able to isolate every anthrax spore in a given sample and the spores were completely germinable on the PLET medium. As mentioned previously, both assumptions are highly unlikely. Therefore, the

anthrax spore concentrations listed in Table III-9 for positive specimens can only be considered rough estimates of the true concentrations. However, relative to each other, the concentrations can still be used to identify high and low levels of anthrax spores in the environment.

IV.4.b. Level of environmental anthrax spore contamination

Of the 588 environmental specimens collected, 11 (1.9%) were shown to contain viable *B. anthracis* spores (Table III-8). All 11 were directly associated with the disturbed area of carcass disposal sites. Three were associated with burial sites along PLR and the remainder were found at cremation sites around Falaise Lake; no positive samples were found at anthrax mounds sampled in the Hook Lake region. The variance in anthrax recovery rates among the regions probably has more to do with differences in soil type and the amount of time elapsed between spore deposition and sample collection than with differences in carcass disposal methods. Hook Lake mounds were between 14 - 30 y old when sampled while PLR burial and Falaise cremation sites were < 2 y old when samples were collected. The discrepancy in age allows a much greater opportunity for spores at the Hook Lake sites to have been either inactivated or dispersed from the mound by environmental forces. Although the PLR and Falaise sites were approximately the same age when sampled, the sandy soil of the PLR region may be poorly suited for the maintenance of spores (see below).

At all three of the positive carcass disposal sites along PLR, *B. anthracis* spores were found in one collected environmental specimen. At one site, high levels of anthrax spores were recovered from a fox scat whereas at the other two sites, barely detectable levels of spores were found in specimens of sand. It is difficult to interpret the low levels of anthrax spores found in the sand specimens. It is possible that burial of the carcasses and surrounding earth effectively removed all anthrax spores present from the immediate environment. It is, however, equally possible that the acidic, sandy soil of the region was

not conducive to the maintenance of spores that escaped burial. In several parts of the world, recurrent anthrax outbreaks have been associated with low-lying, alkaline soils rich in organic matter (van Ness, 1971; Hugh-Jones and Hussaini, 1975). It was originally believed that these soil conditions influenced vegetative anthrax bacilli and allowed for cycles of germination, vegetative growth and resporulation resulting in an overall increase in spore concentration (van Ness, 1971). However, vegetative *B. anthracis* have very specific nutrient and physiological requirements and are unlikely to survive outside of a host. Instead, the specific soil factors linked to endemic areas may reflect environmental conditions that aid in maintaining anthrax spores at the site and prolonging their viability (see section IV.5.c).

It is difficult to consider the spores of *Bacillus* species in terms of ecosystems (Claus and Berkeley, 1986). Although there have been many reports on the presence and numbers of spores of *Bacillus* species in certain habitats, the habitat approach has its pitfalls in that the metabolically inactive spores could have been passively transported to the site or could have been formed at the site when it was experiencing a different set of environmental parameters and was, for all intents, a different habitat. Parson's Lake Road burial site specimens contained significantly fewer spores from *Bacillus* species able to grow on PLET medium than the other two regions surveyed ($P < 0.001$). It is possible that the sandy soil was unsuitable for holding spores and any present are quickly removed via water action. The acidity of the soil may have reduced the long-term viability of spores formed at the site (Dragon and Rennie, 1995). It is also possible the PLR habitat supported a large number of *Bacillus* species whose spores were unable to germinate and grow on PLET medium and the perceived difference between the regions is an artifact introduced by the isolation protocol.

In bacteriological studies of *Bacillus* species in an English pine forest soil, Siala et al. (1974) observed that the majority of the *Bacillus* population in the acidic surface horizon was present as active vegetative cells. Underneath the acidic surface soil was an alkaline

horizon where the *Bacillus* population was comprised mostly of spores. Environmental specimens from PLR were only collected to a maximal depth of 5 cm and it is unknown if the acidic surface sand was underlaid by an alkaline soil. It is possible that a large population of *Bacillus* spores, including *B. anthracis*, existed at the PLR burial sites at a depth below that surveyed.

The majority of *B. anthracis* spore isolates from Falaise Lake were associated with the bone beds of cremation sites. The beds were comprised of charred shards of vertebrae and ribs indicating the bulk of the carcass had rested above the bed prior to burning. Along with viable anthrax spores, the beds often contained thick mats of bison cape hair and masses of maggot casings under the bones, demonstrating the area had been shielded from the fire. Cord wood and coal used to burn the carcasses was piled on top and beside the body, doused with aviation fuel and lit. Although on average the blazes are reported to have burned for 7.3 h, the hottest and most intense period was at the beginning and was focussed on the top of the carcass. The bulk of the body insulated the ground underneath from the intense heat and flame of the fire. By the time the fire had consumed the body, its temperature was much reduced and the final layer of hide above the ground was probably consumed by a smoldering flame, like the wick of a candle, rather than by a raging blaze. While the higher concentration of anthrax spores in the center of the bone bed at site 41 compared to the edges may have been due to dispersion of an originally localized spore deposit, it is more likely the result of decreased protection of spores from the destructive effects of fire nearer the edges of the zone (Figure III-29).

Overall, the level of anthrax spore contamination in the three endemic regions surveyed appeared low. All positive samples were obtained in the immediate vicinity of carcass disposal sites within the area disturbed by clean-up operations. A study in the Falaise Lake region of wallow and meadow samples not associated with known carcass locations failed to detect any *B. anthracis* spores, however these areas were not surveyed as intensively as the disposal sites and the results are not conclusive. The wallow and

meadow samples were also collected well away from any cremation sites that subsequently proved positive for anthrax spores. The low recovery of anthrax spores from carcass disposal sites; locations where maximal anthrax spore contamination of the environment should have occurred; illustrates the need to thoroughly survey a site before declaring it anthrax free. Based on this study, high concentrations of anthrax spores in the environment of northern Canada appear limited to scavenger fecal matter and anthrax carcass sites.

Ecological studies of anthrax spores with PLET medium in African endemic regions have also demonstrated an association of high spore concentrations with scavenger feces and soil around carcass sites and spores were only rarely found in environmental specimens not associated with carcasses (Turnbull et al., 1989, 1991; Lindeque and Turnbull, 1994). The majority of African isolates not found around carcasses were transient low spore concentrations in water and mud associated with watering sources around which wildlife would congregate. The spores were probably deposited during previous epizootics by herbivores dying in the water, infected animals and carnivorous carriers defecating at the site or scavengers washing in the water. No such areas of large-scale wildlife congregation are known in the endemic regions of northern Canada.

The African studies with PLET medium detected anthrax spores at a greater frequency and at higher concentrations around animal carcass sites than found in northern Canada during this study (Turnbull et al., 1989, 1991; Lindeque and Turnbull, 1994). Anthrax spores were isolated from all 106 soil samples collected from around anthrax carcass sites in ENP (Lindeque and Turnbull, 1994). In many of the specimens the anthrax spore concentrations were in excess of 10^5 spores/g. However, in the areas surveyed in the African studies anthrax is much more common and occurs almost yearly in the abundant herbivore herds. More importantly, due to a lack of equipment and fuel, most carcasses in the African regions are neither burned nor buried but left to be rendered and consumed by

scavengers allowing for the increased dispersion of anthrax spores. Under these conditions, it is expected that much higher concentrations of anthrax spores would survive.

IV.4.c. Implications for carcass disposal in future anthrax outbreaks

There has been some debate over whether burial or burning is more appropriate for disposal of carcasses infected with *B. anthracis*. Historically, neither method was meant to decontaminate an area of spores but rather to remove the carcass as a source of infection from the local environment. While bacteriological comparison of the two methods is not possible because of the confounders mentioned previously, burning and burial appeared equally effective methods of disposal, although empirical observations suggest both methods could be improved.

Burning is often considered the more effective method of disposal as the carcass is permanently removed from the environment and there is much less chance for later dissemination of spores by scavenging or entry into the water table. The fire consumes vegetative anthrax in the carcass as well as any spores it touches. The main problem with burning carcasses is to insure that the fire reaches and consumes all of the carcass. At three of the cremation sites surveyed on Falaise Lake field, burning was incomplete and viable anthrax spores remained in the bone bed. A simple solution would be to revisit the sites after the initial burn and treat the bone bed with formaldehyde or spray with aviation fuel and burn a second time. After burning, bison interaction with the cremation sites was minimal. There was no evidence of wallowing at the sites and the nutrients and ash released by the blaze promoted the growth of leafy herbs and shrubs that were not prime bison forage species.

At two of the four positive cremation sites, including site 41 which yielded multiple positive soil specimens from its bone bed, wood was used in the cremation of the carcass.

At the other two positive sites, coal was used. A comparison of burning times with the two fuel sources for all carcasses treated in the 1993 MBS epizootic demonstrated no significant difference and both fuelled cremations for an average of roughly 7.3 h (data not shown). Although no hard data were collected, clean-up crews felt that coal provided a much hotter fire and resulted in a more complete cremation of the carcass than wood. The sample size of positive cremation sites is too small to determine if the type of fuel used or burn time of the carcass favored the maintenance of anthrax spores at the site.

During the first anthrax epizootics in northern Canada, burial was the method of choice for the disposal of carcasses. Burial places the carcass in an anaerobic environment where further sporulation of *B. anthracis* is halted and, if buried long enough, vegetative anthrax organisms are destroyed by bacterial antagonism and the natural putrefactive process. Burial does not destroy spores that were formed at the site before disposal and these spores may be buried with the carcass, spread over the surface, or dispersed in the intervening soil. It is, therefore, important that the site be left undisturbed and to bury the carcass above the local water table so that any viable spores are not later returned to the surface.

Rather than minimizing interaction with the environment, building a mound on top of a burial site appears to focus animal attention on the site. Even decades after their construction, anthrax mounds in the Hook Lake region attract mammalian carnivores as observation and denning sites. The slope of the mounds have also made them attractive sites for bison to wallow. Not only could this increase the likelihood of contact between bison and anthrax spores, the depressions caused by wallowing result in standing pools of water around the mound which may help to percolate viable spores to the surface.

Burial sites along PLR were left unmounded and as a result appear to have less interaction with the local fauna. It is interesting to note that the presence of a small mound at one site resulted in a scavenger depositing viable anthrax spores from another location. Pooling of

run-off from the gravel road was observed at two burial sites abutting the road and illustrate the importance of not only determining the water table depth in an area but observing physical features in the local environment that could funnel water onto the sites at a later date.

The use of formaldehyde at the Falaise Lake carcass sites was the first attempt at thorough decontamination in northern Canada. Previously, application of the aldehyde allowed for the complete decontamination of Gruinard Island, Scotland, site of World War II biological weapons testing with anthrax spores (Manchee et al., 1990, 1994). Detonation of several anthrax bombs had resulted in the contamination of 4.7 ha in the southwest corner of the island with spores that remained viable and infectious for over 40 yr. Although the aldehyde is extremely reactive and toxic, results from Gruinard Island indicated treatment resulted in local, though severe, damage to the ecosystem but the effects were short term and the ecosystem quickly recovered. Formaldehyde reacts rapidly with organic matter and leaves no lingering toxic byproducts (Miles et al., 1988; Manchee et al., 1994).

Most carcass disposal sites in the MBS 1993 epizootic were treated with approximately 400 l of 3 - 5% formaldehyde. Two of the four positive cremation sites are known to have received formaldehyde treatment, however, the sample size of positive cremation sites is too small to determine if formaldehyde treatment had an appreciable effect on the survival of anthrax spores at the sites. Furthermore, evaluation of its true effectiveness is not possible because of the confounding influence of cremation. Like cremation, the effectiveness of formaldehyde depends on its ability to reach all the spores present at a site. In most cases, the carcass was treated with formaldehyde before cremation as the treatment helped to prevent scavenging. The formaldehyde was dropped on carcasses from a fire bucket held 7 m above the ground by a helicopter. The solution hit the carcass

and splashed over the surrounding ground. Although the top of the carcass and the immediate surrounding area would have been soaked, the solution may not have been able to diffuse under the body in an appreciable concentration to inactivate spores.

Formaldehyde treatment appeared to have little effect on microbial and plant succession at the cremation sites. There was no significant difference between the number of contaminants recovered from cremation sites and regular open field specimens collected within a year of the epizootic (Table III-8). However, the isolation and purification processes employed in our study selected for spores which instead of forming at the site may have been passively carried to the cremation sites during spring thaw. There was little plant growth at the sites 1 y after cremation but when the region was revisited in 1997 the sites all supported thick growths of herbs and shrubs (Figure III-20). In hindsight, this is not surprising as formaldehyde denatures organic matter into components with fertilizer-like properties which would encourage the rapid recolonization of the treated sites (Manchee et al., 1990).

IV.5. Ecology of anthrax spores in northern Canada

IV.5.a. Role of scavengers

Bacillus anthracis spores have been recovered from the feces of black-backed jackals (*Canis mesomelas*) and spotted hyenas (*Crocuta crocuta*) collected from around carcass sites during active anthrax epizootics in ENP and Luangwa Valley, Zambia (Turnbull et al., 1989, 1991; Lindeque and Turnbull, 1994). While carnivorous mammals have been suspected of intestinal carriage and dissemination of anthrax spores in northern Canada, the recovery of anthrax spores from fox fecal matter in our study is the first time it has been bacteriologically demonstrated. Like the fecal isolates from Africa, the positive fox scat was probably deposited during the outbreak in the year before collection. The location of the scat on top of the intact burial site suggests that the spores were carried by

the fox from another location. The burial site is right along side PLR and the carcass would have been one of the first disposed of by clean-up crews allowing the fox ample time to feed on nearby untreated carcasses and deposit the scat.

The anthrax spore concentration in the red fox scat was comparable to the mean spore count found in positive jackal fecal matter but was much less than the mean spore count of hyena feces (Lindeque and Turnbull, 1994). It has been suggested that the difference in fecal spore counts between the two African species, though not statistically significant, may reflect specific feeding habits. The jackal prefers to feed on soft tissue and viscera from fresh carcasses while the stronger jawed hyena can dismember and digest more of the body including scraps of hide and bone from older remains. The older the carcass, the greater the opportunity for sporulation to have occurred and, therefore, the higher the chance of the hyena ingesting larger numbers of spores. North American foxes are similar to jackals in their scavenging preferences and focus on the soft underbelly and viscera of bison carcasses.

IV.5.b. Role of water

Falaise Lake transect data support the possibility of water run-off carrying and concentrating anthrax spores from carcasses to wallows. The field surrounding the lake has a gentle incline from the escarpment to the lake shore (Figure III-21). The majority of carcasses in the 1993 outbreak were found among the stands of poplar and willow and the small meadows in between. Most of the wallows were found below the trees on the open field surrounding the lake. Run-off from the carcasses would encounter and could be collected by the wallows on its way to the lake. Depressed bison trails running parallel to the lake shore and connecting the wallows could also help to funnel run-off into the wallows which can hold sizable amounts of water. Unfortunately, none of the cremation sites or wallows tested near the transects were positive for anthrax spores and the hypothesis remains speculative.

Site 16 along PLR was the only *B. anthracis* positive disposal site from which specimens were collected during multiple summers. The fox scat specimen was the only positive sample collected from the site and no dissemination of spores from the scat was observed. All other positive sites were sampled only once and it was, therefore, not possible to investigate movement of anthrax spores in the environment over time. At the positive cremation sites around Falaise Lake there was no correlation between the location of the spores and the slope of the ground towards the lake which could have supported water transport of the spores. In-depth study of the role of water in the movement of anthrax spores in northern Canada will have to await the identification of sites with high concentrations of anthrax spores whose dispersal may be tracked over time and correlated with the topography.

IV.5.c. Initiation of outbreaks

Van Ness (1971) put forward his incubator area hypothesis in order to explain outbreak-associated conditions of alkaline soil, high organic soil content, high soil moisture and ambient temperatures > 15.5°C (van Ness, 1961). These factors, he believed, would have little influence on static spores, therefore they must be influencing the more sensitive vegetative cells cycling in the environment. Experimental evidence however indicates that the vegetative cells of *B. anthracis* have very specific nutrient and physiological requirements, and survive poorly outside a host or complex artificial medium (Sterne, 1959; Manchee et al., 1981; Turnbull et al., 1989). Experimental germination of *B. anthracis* in the environment has only been successful in soil or water that has been artificially enriched with animal blood or viscera (Minett and Dhanda, 1941; Manchee et al., 1981; Turnbull et al., 1989, 1991; Lindeque and Turnbull, 1994). Even when adequate nutrients are provided to trigger germination and outgrowth of *B. anthracis* spores, the vegetative cells are very susceptible to antagonism from other bacterial species present (Minett and Dhanda, 1941; Sterne, 1959; Vasil'eva, 1960; Zarubkinskii, 1960; Turnbull et al., 1989). This antagonism, though poorly characterized, leads to an overall

decline in the number of anthrax spores, even when all other conditions favor growth. It has also been noted that growth of *B. anthracis* outside a host leads to a rapid loss of virulence. This is the basis for the successful development of many anthrax vaccines (Gainer and Saunders, 1989). The Sterne strain, for example, was made avirulent after incubation under 30% CO_2 on horse serum nutrient agar for 24 h (Turnbull, 1991). Outside the laboratory setting, it is difficult to imagine a microenvironment better suited for successful germination and multiplication of *B. anthracis* than within a mammalian host.

The modified host resistance hypothesis was based primarily on the apparent absence of *B. anthracis* spores in a limited number of soil samples collected from anthrax mounds around Hook Lake years after the last recorded outbreak in the region (Gainer and Saunders, 1989). The 75 samples collected were analysed using culture on non-selective sheep blood and nutrient agar plates. From these tentative findings, Gainer and Saunders (1989) concluded anthrax outbreaks in bison were much like epizootics of clostridal diseases in cattle, such as blackleg, malignant edema and bacillary hemoglobinuria, where chronic exposure to low levels of spores in the environment resulted in subclinical infections that later became virulent after accumulated transient stresses immunosuppressed the host.

The more in-depth bacteriological survey of the anthrax endemic regions in northern Canada performed here with selective PLET medium has demonstrated that, though rare, substantial concentrations of anthrax spores exist at treated carcass disposal sites. Although stored in glass bottles within 2 y of their formation at the carcass sites, the spores were still subject to northern temperatures in the unheated storage facility yet remained viable in the environmental specimens for up to 7 y before screening. The results of our study indicate that concentrations of anthrax spores sufficient to cause lethal infection in bison probably exist and remain viable for years at carcass sites missed by

clean-up crews and in carnivore fecal matter disseminated across the endemic regions. Thus, prior establishment of subclinical infections in the animals is unnecessary for the establishment of an outbreak.

The modified host resistance hypothesis attributes the meteorological pattern preceding anthrax outbreaks in northern Canada with causing a number of accumulating stresses on the bison which result in an overall immunosuppression of the animals leaving them more susceptible to disease (Gainer and Saunders, 1989). The hypothesis suggests that sexually mature bulls are further stressed and predisposed to develop anthrax by the rut where the bulls vie with each other for the right to breed. However, the majority of anthrax outbreaks in northern Canada have occurred prior to or during the early stages of the rut when the males are in peak physical condition (Figure IV-1). Stress from the rut should be greatest on the bulls at its peak and terminal stages when the animals have spent most of their energy.

Rather than stress, bulls may be selectively immunosuppressed by a build up of testosterone prior to the rut. Testosterone has been shown to be immunosuppressive in a wide variety of mammalian species (Levine and Madin, 1962; Rifkind et al., 1973; Franks, 1975; Blazkovec and Orsini, 1976; Cohn, 1979; Weinstein et al., 1984; Grossman and Roselle, 1986). In circumpolar regions, the warble fly, *Hypoderma tarandi*, is a common parasite of reindeer. The female flies lay their eggs on the reindeer and upon hatching the first instar larva burrow into the flesh of the reindeer and migrate to the vertebrae where they encyst and develop into second and third instars. Sexually mature male reindeer carry a higher abundance of second and third instars than females which has been attributed to a higher survival rate of migrating first instars in males (Breyev, 1961). The larval migration coincides with increased testosterone levels in male reindeer prior to their rut and a positive correlation has been demonstrated between testosterone levels and number of surviving larvae (Folstad et al., 1989). It has been hypothesized that sexually mature males have a reduced ability to elicit an effective immune response due to high

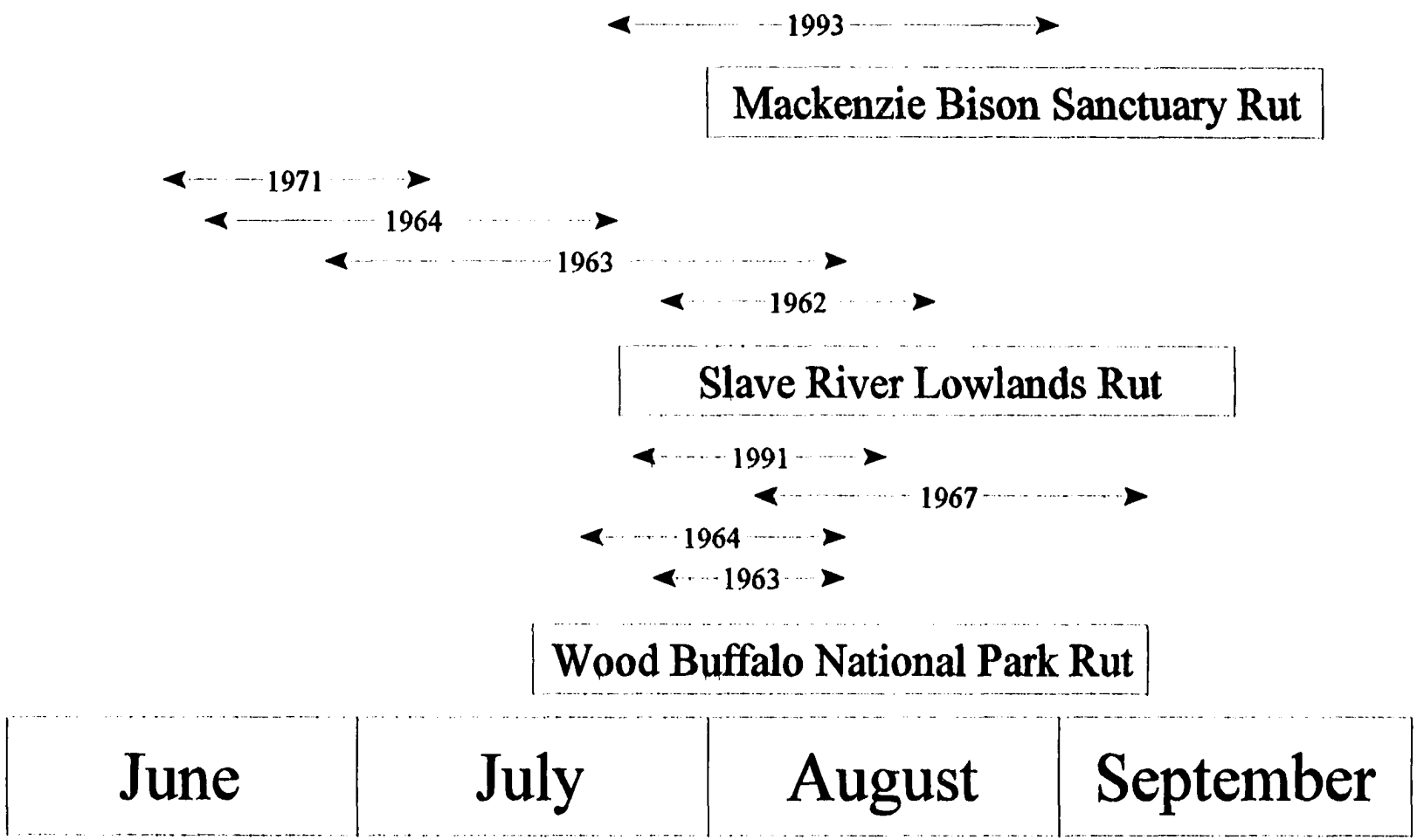

Figure IV-1. Occurrence of anthrax epizootics in northern Canada in relation to the bison rut of the area involved.

testosterone levels in the blood. Castrated reindeer which do not experience raising testosterone levels have warble larval densities similar to those of females. A similar window of immunosuppression may occur in bison bulls rendering them susceptible to anthrax in the period preceding their rut. However, such hormonal experiments have not been undertaken with bison and testosterone suppression of the bison immune system remains speculative.

The wallow concentrator hypothesis contends that the conditions van Ness (1971) found associated with anthrax outbreaks act on the spores which, though more resistant than vegetative cells, are still subject to their environment especially over prolonged periods. Calcium may be one spore component that is affected by extended interactions with the environment. Calcium cations have been shown to be important participants in both germination and the maintenance of dormancy (Foerster and Foster, 1966; Rode and Foster, 1966a, 1966b; Rowley and Levinson, 1967; Grecs and Tang, 1970; Stastna and Vinter, 1970; Sacks, 1972; Kamat et al., 1985; Shibata et al., 1992). In his studies, the alkaline soil pH van Ness (1971) correlated with anthrax outbreaks was often a direct result of calcareous soils and it may be the high calcium levels of these soils that is the actual predisposing condition. High levels of calcium in the soil may buffer the calcium levels in the spore and help to maintain viable spores in the environment for longer periods of time, thereby increasing their chance of coming into contact with and successfully infecting a new host. No experiments were conducted in this study on the ability of calcium to improve anthrax spore longevity in the environment. This would require testing periods of 10 - 50 y.

The other conditions and low-lying areas that van Ness (1971) correlated with anthrax outbreaks may implicate the movement of spores by water action and concentration of spores through evaporation (Dragon et al., 1996). The wallow concentrator hypothesis contends water run-off during flooding in the spring preceding an outbreak carries spores dispersed in the environment from previous outbreaks into low-lying wallows where they

are subsequently concentrated by evaporation during the drought conditions normally leading up to an outbreak. Unfortunately, while the lay of the land and locations of carcass sites and wallows around Falaise Lake supports the movement of anthrax spores by water, the process could not be demonstrated in this study and the hypothesis remains speculative.

It is highly unlikely anthrax spores undergo replicative cycling and build up in the environment of northern Canada. Nor is it likely the microbe establishes a subclinical infection in the bison prior to an outbreak. Anthrax outbreaks in northern Canada are probably initiated by the exposure of bison to doses of spores high enough to cause fulminant disease. The observations of this investigation suggest that acquisition may occur as a result of inhalation or ingestion of environmental spore deposits left by a previously infected herbivore or disseminated from an infected carcass by scavengers. Whether the spores are transported and concentrated from initial deposit sites by water or other environmental forces remains unknown. It is also unknown whether bison are predisposed to infection by transient stresses, hormonal immunosuppression or behavioral patterns. None of these factors is mutually exclusive and all three may act to varying degrees to make bison more susceptible to the disease and initiate epizootics.

IV.5.d. Status of endemic regions

While sporadic outbreaks in bison have proven concentrations of anthrax spores high enough to cause disease are obviously present somewhere in the endemic regions, it is unknown if the levels of spores found in this study represent a sufficient dosage to cause disease. Neither the infectious dosage of anthrax spores for bison nor how the animal contracts the disease is known. Bison may acquire the disease through inhalation of aerosolized spores during wallowing, ingestion of lethal levels of spores while grazing or may be infected with the disease through the bite of contaminated insects. Further

complicating the matter is the possibility of seasonal transient stresses that result in an immunocompromised state and reduce the infectious dosage required (Gainer and Saunders, 1989).

Anthrax was last detected in the Hook Lake region in 1978 and no *B. anthracis* isolates were recovered from the region during this study. It may be tempting to assume the region is no longer endemic for the disease. However, the region is very remote with little human activity during the anthrax season and regular anthrax surveillance flights have not been flown over the area since 1987. It is possible that anthrax epizootics have occurred in the intervening years and went unnoticed. Secondly, since the early 1970's there has been a steady decline in the bison population of the Hook Lake region and the rest of the SRL due to high rates of predation and diseases other than anthrax; herd infection rates with brucellosis and tuberculosis are upwards of 30% each (Van Camp and Calef, 1987; Broughton, 1987). Presently there are approximately 500 bison in the entire SRL region (John Nishi, NT Department of Resources Wildlife and Economic Development, personal communication) and their density may be insufficient to sustain a large noticeable outbreak of anthrax. The Hook Lake region has already demonstrated its ability to harbor viable *B. anthracis* spores for long periods of time between epizootics and it very likely continues to do so.

IV.6. Summary

Efforts to develop a field ELISA system to detect viable *B. anthracis* spores in the environment proved unsuccessful. Immunizations of BALB/c and Swiss mice with anthrax spore preparations and various adjuvants failed to develop strong enough immune responses in the animals to allow for hybridoma fusion and the production of anti-anthrax spore monoclonal antibodies. Polyclonal antiserum was produced in rabbits against formaldehyde-fixed *B. anthracis* 9604 spores. The serum proved selective for spores from species of the *B. cereus*-group, however, it had a low sensitivity making it unsuitable

for use in a field ELISA. Attempts to adapt monoclonal antibodies FDF 1B9 (specific for *B. anthracis* capsule) and EAII-6G6-2-3 (specific for a *B. anthracis* vegetative cell wall component) from an immunofluorescent format to an indirect plate ELISA proved unsuccessful as, again, the antibodies lacked the sensitivity and selectivity necessary for a field ELISA.

PLET medium was evaluated for recovery of spores of a number of environmental and clinical *Bacillus* isolates. While inhibitory to most non-*anthracis Bacillus*, the medium failed to distinguish *B. anthracis* ATCC 4229 from *B. mycoides* MU 711/84, *B. thuringiensis* QC 12093, *B. subtilis* 1A289, and environmental strains of *B. pumilus* and *B. circulans*. Not all *B. anthracis* ATCC 4229 spores germinated when applied to PLET indicating it is not an optimal medium for spore recovery. The addition of 5% (v/v) horse blood to the medium improved its ability to differentiate *B. anthracis* from other strains but decreased its selective ability making the modified medium unsuitable for environmental screening. The addition of nitrocefin to the medium proved inhibitory to all *Bacilus* species tested save *B. mycoides*.

Alternative spore extraction and purification procedures to use with PLET medium were investigated as ways of improving the sensitivity of the procedure. Ethanol purification of spores was less sensitive to incubation time than regular heat purification and in tests against non-sterile soil specimens from northern anthrax endemic areas proved as effective at removing vegetative contaminants. In laboratory tests with seeded sterile soil samples, solutions of high specific gravity sucrose plus nonionic detergent extracted significantly more spores than water alone, though neither extraction solution was able to isolate all the spores present in a sample. However, in further comparisons both extraction solutions proved equally sensitive with spiked soil samples; detecting down to 40 spores/g; and non-sterile environmental specimens from northern anthrax endemic areas.

Using a 1.22 g/ml sucrose plus 0.5% (v/v) Triton X-100 extraction solution, ethanol purification, PLET medium and a number of confirmatory assays, *B. anthracis* spores were detected in 11 of 588 (1.9%) environmental specimens collected from anthrax endemic regions in northern Canada. All positive samples were associated with disposal sites of bison carcasses found during previous anthrax epizootics. Viable anthrax spores were found at three of six carcass burial sites surveyed in the PLR region of WBNP and at four of eight carcass cremation sites examined in the Falaise Lake region of the MBS; no anthrax spores were recovered from decades-old anthrax mounds in the Hook Lake region. Anthrax spores were not recovered from 67 specimens not associated with known carcass sites collected from bison wallows and meadows around Falaise Lake. Anthrax spore concentrations of > 500 spores/g were associated with a red fox scat and soil specimens collected from a distinct bone bed found within cremation sites. The positive fox scat is the first bacteriological evidence that carnivorous mammals are capable of disseminating anthrax spores in northern Canada.

Within the cremation bone beds, mats of bison hair and maggot casings were also found with anthrax spores, indicating the bed had been protected from the heat of the blaze by the bulk of the carcass. At three of the four positive cremation sites, positive specimens were obtained from within the bone bed and demonstrate the need to revisit disposal sites after cremation to reburn the bed. Empirical observations at carcass burial sites in the Hook Lake and PLR regions suggested that mounded sites still had substantial interactions with wildlife and did not seclude the carcass from the environment.

Although topographical data in the Falaise Lake region supported the wallow concentrator hypothesis and the possibility of water transport of anthrax spores from carcass sites to bison wallows, direct movement of spores by water could not be demonstrated. The recovery of substantial concentrations of viable anthrax spores from treated carcass disposal sites indicate that infectious levels of anthrax spores are probably released from infected carcasses and preclude the need for spore cycling in the environment or subclinical infections of hosts to establish an epizootic.

Chapter V. Future Studies

Although ELISAs have proven helpful in the diagnosis of anthrax and the laboratory confirmation of *B. anthracis*, this study demonstrated that the development of monoclonal antibodies of sufficient selectivity and sensitivity to detect low concentrations of anthrax spores in diverse environmental specimens will be extremely difficult. Alternatively, selective culture with PLET medium continues to be a proven simple and sensitive method for *B. anthracis* isolation from the environment. Further investigations of PLET medium against a wider variety of *Bacillus* strains should be undertaken in order to determine the extent of the selectivity of the medium. Special attention should be given to environmental isolates of *Bacillus* species which have received minimal culture on artificial media as strains adapted to laboratory culture may have lost an ability to scavenge cations or utilize thallium cations that was inherent to the wild parental strain. Attempts to modify PLET medium not only to improve its selectivity but also its ability to germinate anthrax spores should continue. Tyrosine, L-alanine, adenosine and O-carbamyl-D-serine have been shown to induce germination of *B. anthracis* spores *in vitro* (Hills, 1950; Titball and Manchee, 1987) and their inclusion in PLET medium may help to improve the germination efficiency of the medium. In future environmental screening studies with PLET, the identity of non-*anthracis* contaminants should be determined in order to better assess the selectivity of the medium.

The results of this study indicated that the sensitivity of screening procedures with PLET medium can be improved by the use of high density sucrose solutions containing nonionic detergents to increase spore recoveries from specimens. Spore extraction with high gravity sucrose plus Triton X-100 should be performed on a larger number and variety of spiked soil samples in order to verify the results of this study. The extraction procedure should also be tested against other environmental matrices such as vegetation, animal hair

and bones. Seeded spores should be left in the samples for extended periods of time prior to extraction in order to determine if spore adhesion to environmental substrates increases over time.

Although high specific gravity sucrose plus nonionic detergent extracted significantly more anthrax spores than water from spiked soil samples, spore recovery rates with sucrose plus detergent remained below 30%. Further investigations to improve bacterial endospore extraction from environmental specimens should be undertaken in order to improve the sensitivity of PLET and other anthrax spore detection systems. Hydrophilic siliconized extraction vessels may help to minimize spore losses due to adhesion to vessel surfaces. While hydrophobic interactions have been demonstrated as the predominant adhesive force for *B. cereus*-group spores, electrostatic interactions are also involved to various degrees (Husmark and Rönner, 1990). At neutral and alkaline pH, *Bacillus* spores are negatively-charged (Husmark and Rönner, 1990) and the addition of EDTA to the extraction solution may help to disrupt electrostatic interactions and improve spore recoveries. The addition of EDTA to the extraction solution would also help to minimize the contamination of PLET medium with calcium and other divalent cations which could decrease the selectivity of the medium.

Positive carcass disposal sites in the Falaise Lake region, especially those that yielded multiple positive specimens, should be resampled in greater detail to see if movement or inactivation of anthrax spores has occurred in the intervening years. One problem with the environmental survey performed here was that all the carcass sites sampled had received some form of treatment to minimize anthrax spore contamination; the very phenomenon we were investigating. Because of the remote area, and monetary and logistic constraints, the carcasses of bison dying during the 2000 WBNP anthrax epizootic were inventoried (with GPS coordinates collected) but not treated in any way. The anthrax spore detection

system described herein could be used to survey these sites to better assess the sensitivity and selectivity of the system, and to study the movement and viability of anthrax spores in the environment of northern Canada.

In the event of another anthrax epizootic in the bison herds of northern Canada, the detection system described in this study may be used to initiate a full-scale epidemiological study of anthrax spores. The system could be used to correlate the level of spore contamination around a carcass with the severity of scavenging. Samples of scavenger feces and biting insects could be collected for testing to determine the role played by these animals in the dispersal of anthrax spores during an outbreak. Wallows and vegetation in the area of the first carcasses could be surveyed in an effort to determine how the outbreak was initiated. The system could also be used to determine spore contamination levels at a carcass site before and after disposal in order to evaluate disposal procedures. Because of the sporadic nature of anthrax outbreaks in northern Canada, sampling protocols and equipment should be prepared and stored in advance so that when an outbreak does occur field work can begin without delay.

Chapter VI. References

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Appendix

A.1. Buffers

Phosphate buffered saline (PBS), pH 7.2

48.0	g	Sodium chloride
1.2	g	Potassium chloride
8.64	g	Sodium phosphate, dibasic
1.44	g	Potassium phosphate, monobasic
6.0	l	Deionized water, filtered

Combined reagents together and dissolved. Adjusted pH of buffer to 7.2. If sterilization was required, buffer was either passed through a 0.22 μ filter or dispensed into 500-ml screw-capped bottles and autoclaved at 121°C for 20 min (Harlow and Lane, 1988). Solution was stable at ambient temperature for 6 months.

0.85% Physiological saline

17.0	g	Sodium chloride
2.0	l	Deionized water, filtered

Dissolved sodium chloride in water. Dispensed in 400- and 100-ml volumes and autoclaved at 121°C for 15 min. Saline was stable for up to 6 months at ambient temperature.

Double strength skim milk

200	g	Skim milk powder, generic store brand
1.0	l	Deionized water, filtered

Dissolved milk powder in water. Dispensed in 50-ml volumes and autoclaved at 116°C for 18 min. Solution was stable for up to 2 months at 4°C.

A.2. Bacteriological media

A.2.a. Culture media

Bicarbonate agar plates

7.00	g	Bovine albumin, fraction V
20.0	g	Nutrient agar
7.00	g	Sodium bicarbonate
1.0	l	Deionized water, filtered

Measured 90% of the water into a flask. Added nutrient agar and heated to dissolve. Autoclaved at 121°C for 15 min. Cooled to 50°C. To the remaining 10% of the water added bicarbonate and albumin and dissolved. Passed the bicarbonate-albumin solution through a 0.22 μ filter and added to the cooled agar solution. Swirled medium to mix and dispensed in 20 ml portions to 100-mm Petri plates. Allowed to solidify and stored at 4°C for up to 2 weeks.

Cooked meat broth

125.0	g	Cooked meat medium
1.0	l	Deionized water, filtered

Dissolved medium in water. Allowed to stand for 10 min at ambient room temperature. Dispensed into 10 ml volumes and autoclaved at 121°C for 15 min. Broth was stable for up to 1 month at 4°C.

Nutrient agar plates

34.0	g	Tryptose blood agar base
1.0	l	Deionized water, filtered

Dissolved base in water. Autoclaved at 121°C for 15 min. Allowed the medium to cool to below 60°C then dispensed in 20 ml portions to 100-mm Petri plates and allowed to solidify. Stored plates for up to 2 months at 4°C.

Nutrient broth

34.0	g	Tryptose blood base
1.0	l	Deionized water, filtered

Dissolved base in water. Dispensed medium in 10 ml portions to 15-ml snap-top glass test tubes. Autoclaved broth at 121°C for 15 min. Stored broth for up to 2 months at 4°C.

Sheep blood agar plates, citrated

50.0	ml	Sheep blood, citrated
40.0	g	Tryptic soy agar
1.0	l	Deionized water, filtered

Warmed the blood to 37°C. Combined water and tryptic soy agar together in a flask and heated to dissolve. Autoclaved agar solution at 121°C for 15 min then cooled to 50°C. Aseptically added the warmed blood to the agar solution and swirled to mix. Dispensed medium in 20 ml portions to 100-mm Petri plates and allowed to set. Stored plates at 4°C for up to 3 months.

Trypticase soy agar plates

40.0	g	Tryptic soy agar
1.0	l	Water, deionized

Combined agar and water and heated to dissolve. Autoclaved solution at 121°C for 35 min to sterilize. Allowed medium to cool to 50°C before dispensing in 20 ml portions to 100-mm Petri plates and allowing to set. Stored plates at 4°C for up to 1 month.

Trypticase soy broth

30.0	g	Trypticase soy broth base
1.0	l	Deionized water, filtered

Dissolved base in water. Dispensed 6 ml portions into 10-ml snap-top glass test tubes. Autoclaved at 121°C for 15 min to sterilize. Broth was stable at 4°C for up to 1 month.

A.2.b. Sporulation media

ATCC sporulation broth

0.33	g	Yeast extract
0.33	g	Beef extract
0.67	g	Tryptose
trace		Ferric chloride hexahydrate
3.33	g	Glucose
1.0	l	Deionized water, filtered

Dissolved reagents and adjusted the pH to 7.2. Dispensed 15 ml portions of the broth to 20-ml screw-capped test tubes. Autoclaved test tubes at 121°C for 15 min (Cote et al., 1984). Shelf life of ATCC sporulation broth was 2 months at 4°C.

Modified Tarr's sporulation agar

1.5	g	Sucrose
1.5	g	Ammonium phosphate, dibasic
1.5	g	Sodium chloride
0.75	g	Magnesium sulfate heptahydrate
7.5	g	Potassium phosphate, monobasic
75	mg	Manganese sulfate monohydrate
150	mg	Calcium chloride dihydrate
trace		Ferric chloride hexahydrate
1.5	l	Deionized water, filtered
11.25	g	Bacto-agar

Mixed all the reagents except agar together and dissolved. Adjusted the pH of the solution to 7.4 and added agar. Autoclaved the medium at 121°C for 20 min. Cooled medium to 50°C and readjusted the pH to 7.4. Dispensed 100 ml portions of the medium into 225-cm^2 tissue culture flasks. Poured the medium slowly down the side of the flask in order to minimize the generation of bubbles. The flasks were sealed with 0.22 μ filtered caps and laid down in their natural position for tissue culture, and the medium was allowed to solidify. Medium was stable at 4°C for 4 months (Tarr, 1933; Claus and Berkeley, 1986). Just prior to use, 30 ml of sterilized glass beads (1 mm diameter) were added to each flask to aid in inoculation and harvesting.

A.2.c. Germination broths

Amino acid, adenosine, uracil, salt (AAAUS) stock, 40x

0.140	g	L-Tryptophan
0.260	g	Glycine
0.100	g	L-Cystine
1.149	g	L-Lysine, hydrochloride
0.692	g	L-Valine
0.920	g	L-Leucine
0.680	g	L-Isoleucine
0.480	g	L-Threonine
0.292	g	L-Methionine
0.736	g	L-Aspartic acid
2.709	g	L-Sodium glutamate monohydrate
0.172	g	L-Proline
0.220	g	L-Histidine, hydrochloride
0.500	g	L-Arginine, hydrochloride
0.500	g	L-Phenylalanine
0.940	g	L-Serine
0.004	g	L-Thiamine, hydrochloride
0.026	g	Calcium chloride
0.071	g	Magnesium sulfate heptahydrate
0.004	g	Manganese sulfate monohydrate
0.006	g	Uracil
0.007	g	Adenine, hemisulfate salt
100.0	ml	Deionized water, filtered

Mixed all reagents together and dissolved. Dispensed in 5 ml portions to Bijou bottles. Solution is stable for 1 month at 4°C and for 1 y at -20°C (Ristroph and Ivins, 1983).

Heat-inactivated horse serum

500	ml	Horse serum, filter sterilized

Incubated serum in a 56°C water bath for 30 min. Centrifuged serum at 10,000 x g for 12 min at 4°C. Sterilized the supernatant with a 0.22 μ filter. Heat inactivated horse serum may be stored for up to 2 months at 4°C and for up to 1 y at -20°C.

Capsule broth

50	ml	Difco heart infusion broth, 10% (w/v)
5	ml	AAAUS stock, 40x
100	ml	Heat-inactivated horse serum
25	ml	Deionized water, filtered

Mixed reagents together to dissolve and passed through a 0.22 μ filter. Dispensed into adequate volumes. The medium is stable at 4°C for 3 months and at -20°C for 1 y. Just prior to use, mixed 9 parts medium with 1 part fresh 8% (w/v) sodium bicarbonate in order to activate the broth. Activated medium is stable for up to 3 weeks if stored in an airtight container at 4°C (Ezzell and Abshire, 1996).

Polysaccharide broth

50	ml	Difco heart infusion broth, 10% (w/v)
5	ml	AAAUS stock, 40x
145	ml	Deionized water, filtered

Mixed reagents together to dissolve and passed through a 0.22 μ filter. Dispensed into adequate volumes. Medium is stable at 4°C for 3 months and at -20°C for 1 y (Ezzell and Abshire, 1996).

A.2.d. *B. anthracis* selective media

Polymyxin, lysozyme, EDTA, thallium acetate (PLET) agar medium

40.0	g	Difco heart infusion agar
0.300	g	Ethylenediaminetetraacetate (EDTA), disodium salt
0.04	g	Thallium acetate
1.0	l	Deionized water, filtered
3.8	mg	Polymyxin B sulfate (30,000 U)
4.3	mg	Lysozyme from chicken egg white (300,000 U)

Dissolved agar, EDTA and thallium acetate in 990 ml of the water. Autoclaved solution at 121°C for 20 min. Meanwhile dissolved polymyxin and lysozyme in the remaining 10 ml of water and passed through a 0.22 μ filter. Placed agar solution in a warm water bath

and allowed to cool to 48°C. Added polymyxin-lysozyme solution and swirled to mix (Knisely, 1966; Carman et al., 1985). Dispensed the medium in approximately 20 ml portions to 100-mm Petri plates and allowed to solidify. Stored at 4°C.

Blood PLET agar medium

24.0	g	Difco heart infusion agar
0.180	g	Ethylenediaminetetraacetate (EDTA), disodium salt
0.024	g	Thallium acetate
570	ml	Deionized water, filtered
2.29	mg	Polymyxin B sulfate (30,000 U)
2.57	mg	Lysozyme from chicken egg white (300,000 U)
30	ml	Sheep blood, sterile (5% w/v)

Dissolved agar, EDTA and thallium acetate in 560 ml of the water. Autoclaved solution at 121°C for 20 min. Meanwhile dissolved polymyxin and lysozyme in remaining 10 ml of water and sterilized through a 0.22 μ filter. Placed agar solution in a warm water bath and allowed to cool to 48°C. Added polymyxin-lysozyme solution and swirled to mix. Added sheep blood and swirled to mix. Dispensed medium in approximately 20 ml portions to 100-mm Petri plates. Stored the medium at 4°C.

Nitrocefin PLET agar medium

24.0	g	Difco heart infusion agar
0.180	g	Ethylenediaminetetraacetate (EDTA), disodium salt
0.024	g	Thallium acetate
598	ml	Deionized water, filtered
2.29	mg	Polymyxin B sulfate (30,000 U)
2.57	mg	Lysozyme from chicken egg white (300,000 U)
1	mg	Nitrocefin, lyophilized
2	ml	Nitrocefin resolubilization solution (5.0% v/v dimethylsulfoxide in 0.1 M phosphate buffer, pH 7.0)

Dissolved agar, EDTA and thallium acetate in 590 ml of the water. Autoclaved solution at 121°C for 20 min. Meanwhile resolubilized nitrocefin. Dissolved polymyxin and lysozyme in remaining 8 ml of water, combined with the nitrocefin solution and sterilized through a 0.22 μ filter. Placed agar solution in a warm water bath and allowed to cool to

48°C. Added polymyxin-lysozyme-nitrocefin solution and swirled to mix. Dispensed medium in approximately 20 ml portions to 100-mm Petri plates. Stored the medium at 4°C.

A.3. Staining procedures

Geimsa stain

The stain was used to detect the presence of a capsule around vegetative *B. anthracis* grown on bicarbonate plates in the presence of 10% CO_2. An isolated colony from the plate was smeared with a loop into a drop of physiological saline on a microscope slide. The slide was left for 10 - 15 min to dry and then immersed in 30% formalin for 30 min to fix the bacterial film. The slide was removed from the formalin, placed on a staining rack and flooded with Geimsa stain. The stain was left in place until almost dry, then washed off with tap water followed by phosphate buffer (0.001 M, pH 7.0). The slide was blotted dry and viewed under a light microscope with oil emersion at 1000x magnification. Vegetative cells appeared blue while capsule, if present, stained purple (Collins et al., 1995).

Gram stain

An isolated colony or drop of culture in liquid medium was smeared onto a microscope slide. The slide was left 10 - 15 min to dry. The smear was heat-fixed to the slide. The slide was placed on a staining rack and allowed to cool. The smear was flooded with crystal violet stain. After 30 s, the stain was washed off with tap water. The slide was then covered with Gram's iodine for 45 s before being washed a second time with tap water. The smear was decolorized with acetone-alcohol (3 parts ethanol to 1 part acetone). The slide was washed with acetone-alcohol just until no more blue leached from the smear. At that precise moment the slide was washed with tap water. The slide was

flooded with safranin stain for 30 s to counterstain the smear. The excess safranin was washed off the slide with tap water and the slide was blotted dry. The slide was viewed under a light microscope with oil emersion at 1000x magnification. With the stain, Gram-positive bacteria appeared dark blue or purple and Gram-negative bacteria appeared red or pink (Collins et al., 1995).

Spore stain

The stain was used to confirm the presence of spores and to check for vegetative cell contamination. One drop of spore stock was spread across a microscope slide and allowed to dry for 10 - 15 min. The sample was heat-fixed to the slide. The slide was allowed to cool to ambient temperature. The slide was placed over a rapidly boiling container of water and flooded with aqueous 5% malachite green. The slide was left over the water to steam for 6 min, before it was removed and the excess stain was rinsed off with tap water. The slide was counterstained by flooding with safranin. After 30 s the excess safranin was rinsed off with tap water. The slide was then blotted dry with filter paper. Viewed the slide under a light microscope with oil emersion at 1000x magnification. Spores were seen as green spheres while vegetative debris, if present, stained red (Hendrickson et al., 1985).

Phase contrast counts

Because of their dehydrated core, bacterial endospores are highly refractive when viewed under a phase contrast microscope. One in ten dilution series of sample were made up in Eppendorf tubes pre-blocked for 3 h with filtered 1% (w/v) BSA/PBS to prevent spore adherence to the inner walls. Dilutions were made in 1% (w/v) BSA/PBS to minimize spore clumping. Ten microlitres of each dilution was loaded onto a hemocytometer and the bright spheres were counted under a phase contrast microscope set at 400x magnification. Because not all the spores were on the same plane within the

hemocytometer, it was necessary to continually adjust the fine focus while scanning the hemocytometer grid in order to observe all the spores present. For a statistically accurate determination of the spore concentration, between 100 and 200 spores had to be counted over at least one large square. The concentration of viable spores in the original sample was calculated using the equation,

$$\text{Spores/ml} = \frac{\text{Number of spheres counted x Dilution factor x } 10^4}{\text{Number of large squares covered}} \qquad (1)$$

(Harlow and Lane, 1988).

A.4. *Bacillus* spore coat extraction

Inner spore coat extraction solution

0.901	g	Dithiothreitol (50 mmol/ml)
100	ml	Deionized water, filtered

Mixed solution with a metal stir bar and magnetic stirrer to dissolve the dithiothreitol. Adjusted pH to 10.5 with 4.0 M sodium hydroxide solution. Passed solution through a 0.22 μ filter to sterilize and stored at 4°C (Aronson and Fitz-James, 1971). The solution was stable at 4°C for 2 days.

Total spore coat extraction solution

0.901	g	Dithiothreitol (50 mmol/ml)
24.02	g	Urea (4.0 M)
100	ml	Deionized water, filtered

Mixed solution with a metal stir bar and magnetic stirrer to dissolve the solids. Adjusted pH to 10.5 with 4.0 M sodium hydroxide solution. Passed solution through a 0.22 μ filter to sterilize and stored at 4°C (Aronson and Fitz-James, 1971). The solution was stable at 4°C for 2 days.

Saturated ammonium sulfate

270.81	g	Ammonium sulfate (4.1 M)
500	ml	Deionized water, filtered

Mixed to dissolve with a stir bar and magnetic stirrer. Adjusted pH to neutral with 1.0 N HCl. Filtered solution through a 0.22 μ filter and stored at ambient temperature. The solution was stable at ambient temperature for 4 months (Harlow and Lane, 1988).

Resolubilization solution

0.360	g	Dithiothreitol (20 mmol/ml)
14.41	g	Urea (2.4 M)
100	ml	Deionized water, filtered

Mixed solution with a metal stir bar and magnetic stirrer to dissolve the solids. Adjusted pH to 10.2 with 4.0 M sodium hydroxide. Passed solution through a 0.22 μ filter to sterilize and stored at 4°C (Aronson and Fitz-James, 1971). The solution was stable at 4°C for 2 days.

Aluminum hydroxide salt

1.0	g	Aluminum potassium sulfate
10	ml	Deionized water, filtered
22.8	ml	Sodium hydroxide, 0.25 N, filtered

Dissolved aluminum potassium sulfate in water. Passed solution through a 0.22 μ filter to sterilize. While vortexing aluminum potassium sulfate solution in a test tube, slowly added the 0.25 N sodium hydroxide, dropwise. Sealed tube and incubated at room temperature for 10 min. Centrifuged at 1000 x g for 10 min. Removed and discarded supernatant. Added 50 ml of filtered, deionized water to the pellet and resuspended. Centrifuged at 1000 x g for 10 min. Removed and discarded supernatant (Harlow and Lane, 1988). Resuspended pellet in 10 ml of resolubilization solution and used immediately.

Reconstitution buffer

1.375	g	N-tris(Hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)
1.428	g	Magnesium chloride

0.210	g	Cystine
3.0	l	Deionized water, filtered

Mixed solution with a metal stir bar and magnetic stirrer to dissolve the solids and adjusted pH to 7.0. Passed through a 0.22 μ filter and used immediately (Aronson and Fitz-James, 1971).

Spore coat isolation

Approximately 10^8 formaldehyde-inactivated anthrax spores stored in 1% (v/v) formaldehyde/PBS were washed twice with filtered PBS; centrifuging the suspension at 10,000 x g for 10 min at 4°C to isolate the spore pellet. The pellet was resuspended in 7 ml of inner coat extraction solution and gently agitated at room temperature for 60 min. The solution was centrifuged as before, and the supernatant was removed and saved as "inner coat wash". The pellet was resuspended in 7 ml of total coat extraction solution and agitated slowly at 37°C for 90 min. The suspension was pelleted and the supernatant was saved as "total coat wash". The pellet was suspended in a second volume of total coat extraction solution and incubated as before. The suspension was pelleted and the supernatant was pooled with the first total coat wash. The total coat wash was passed through a 0.22 μ filter that had been pretreated with the inner coat wash.

A 1/3 volume of saturated ammonium sulfate was slowly added dropwise to the filtered total coat wash to give a final ammonium sulfate concentration of 25%. During the addition, the 30-ml centrifuge tube containing the total coat wash was swirled to completely disperse the ammonium sulfate. The resulting suspension was incubated overnight at 4°C on a rotary test tube platform shaker set at 50 rpm. The next day, the suspension was centrifuged at 10,000 x g for 16 min at 4°C and the supernatant was decanted. The pellet was suspended in 7 ml of resolubilizing solution and incubated with gentle agitation on the rotary platform shaker at 37°C for 3 h (Aronson and Fitz-James, 1971).

Reconstitution of spore coat protein with aluminum hydroxide

The protein concentration of the resolubilized spore coat protein was calculated with the UV spectrometer against a resolubilization solution blank (see section A.7.). The spore coat protein was combined with fresh aluminum hydroxide particles in a 1:2 ratio (by weight) in a suitable length of prepared dialysis tubing (molecular cut-off weight 12 - 14 kDa). The tubing was sealed with double clips at each end and placed overnight in 3.0 L of reconstitution buffer at 4°C. After dialysis, the suspension was collected from the tubing and centrifuged at 15,000 x g for 20 min at 4°C. The supernatant was decanted and the pellet was washed once with 25 ml of PBS and centrifuged as before. The pellet was resuspended in 0.85% saline to give a final protein concentration of approximately 0.10 mg/ml and a aluminum hydroxide concentration of roughly 0.20 mg/ml. The solutions were dispensed in 1.25 ml portions to Eppendorf tubes and stored at -20°C.

A.5. Animal handling procedures

A.5.a. Mouse

BALB/c mice were obtained from the University of Alberta Health Sciences (UAHS) vivarium colony. Swiss strain mice were procured from the Northern Alberta Provincial Laboratories (NAPL) vivarium colony. All mice used were housed in an isolated, dedicated room of the NAPL vivarium. BALB/c mice brought over from the UAHS vivarium were acclimatized for at least 1 week in the NAPL vivarium prior to the commencement of the immunization schedules.

The mice for each monoclonal immunizing schedule were housed together in a single cage. Neither ear nor toe clipping were used to identify individual mice, instead at test bleeds animals were colored on the back of their head, neck and shoulders with permanent

marker; a different color for each mouse in the cage. The colored ink was long-lasting and was often still visible on the mice 3 weeks later at the next test bleed. The ink marking did not mutilate or harm the mice and allowed later identification of high-titre responders.

Immunizing inoculation

Prepared immunizing suspension in Eppendorf tubes as detailed in section II.3.a. Loaded suspension into a 3.0-ml syringe with a 26 gauge needle and flicked the side of the syringe to remove any air bubbles present. An assistant held the mouse tightly with the scruff of the neck between his thumb and side of his index finger and the tail tucked between his pinkie and palm. With the mouse thus immobilized, it was injected sc or ip with the immunizing suspension depending on the type of adjuvant used. Mice in the same protocol were inoculated from the same needle.

Jugular bleed

Jugular bleeds were used to draw pre-bleed and test sera from the mice during immunization schedules; approximately 0.1 to 0.2 ml of blood was collected at each bleed. In a fumehood, the mouse was anesthetized with methoxyflurane. The mouse was monitored constantly throughout the procedure using the toe pinch (pedal) reflex, mucous membrane color and respiratory rate to ensure that surgical plane anesthesia was maintained. The mouse was secured to the fume hood bench in a dorsal recumbency position. The hair above the left side of the chest was wetted with a mixture of hibitane and warm water. Holding the skin taut in the neck region and using a #22 scalpel blade the area was shorn of hair using short strokes.

The jugular vein was visualized in the shaved area. A 26 gauge x 3/8" needle attached to a 1.0-ml syringe was inserted 1 - 2 mm caudal to the cranial edge of the pectoral muscle at a 30° angle. A minute vacuum was created in the syringe barrel by pulling back slightly on

the plunger and the needle was slowly advanced. Once the needle was in the trunk of the vein, a small drop of blood appeared in the needle hub. A milking action was used to draw blood from the vein; drawing slightly on the plunger and releasing to allow the syringe to fill slowly. Once the desired volume of blood had been obtained, slight digital pressure was applied to the injection site before withdrawing the needle in order to prevent a hemotoma from forming at the site. The needle was removed from the syringe and the blood was ejected to a labeled Eppendorf tube. The mouse was released from its bindings and placed by itself in a cage to recover (UAHS Laboratory Animal Services Technical Standard Operating Procedures #018).

Preparation of serum

After collection, the mouse blood was allowed to clot for 60 min at 37°C. The clot was separated from the sides of the container using a flame-sterilized straightened paper clip. The collection vessel was placed at 4°C overnight for the clot to contract. The serum was removed from the clot and placed in a new tube. The tube was centrifuged at 10,000 x g for 10 min at 4°C to pellet any remaining insoluble material and the serum was transferred to a second labeled tube. The serum was stored at -20°C (Harlow and Lane, 1988).

Cervical dislocation

Cervical dislocation was used to euthanize mice at the termination of the immunization schedules. The mouse was anesthetized with methoxyflurane as described previously in the non-invasive jugular bleed protocol and maintained in surgical plane anesthesia. The animal was placed on its belly on the bench top and a rod or plastic pen was pressed across the base of the skull. With the other hand, the base of the tail was quickly and firmly pulled causing separation of the cervical vertebrae from the skull (Andrews et al., 1993). After dislocation, the carcass was properly bagged for autoclaving and incineration.

A.5.b. Rabbit

New Zealand white strain laboratory rabbits were obtained from the NAPL vivarium colony. The rabbits were housed in individual cages in an isolated, dedicated room of the NAPL vivarium and were identified by permanent ear tattoos.

Immunizing inoculation

Prepared immunizing suspension in Eppendorf tubes as described in section II.3.b. Loaded suspension into a 3.0-ml syringe with a 26 gauge needle and flicked the side of the syringe to remove any air bubbles present. An assistant held the rabbit tightly on the benchtop with hands holding either side of the rabbit and the head tucked under the arm. With the rabbit thus immobilized, it was injected sc at four sites along the upper back and once im in the hind thigh with the immunizing suspension. A different needle and syringe was used for each rabbit.

Ear Vein Puncture

This procedure was used to collect pre-bleed and test serum from each rabbit during the immunization schedule. The rabbit was secured in a restraining box. The ventral edge of the outer surface of the ear was wet with soapy water and shaved using a #10 scalpel blade. The ear was dried and a thin film of vaseline was applied on the skin over the ear vein. A drop of xylene was applied to the tip of the ear to dilate the blood vessel. Pressure was applied to the vein just ahead of the point of entry and the vein was punctured with a 22 gauge butterfly needle attached to a vacutainer holder. The needle was held in place with the butterfly and approximately 30 ml of blood was collected in Corvac tubes from each rabbit. The needle was removed and direct pressure was applied

to the puncture site to stop any bleeding. The ear was washed thoroughly with 70% ethanol then soapy water, paying special attention to the tip of the ear where the xylene had been applied (NAPL Vivarium Blood Standard Operating Procedure #1, 1997).

Cardiac Puncture

Cardiac punctures were used for final serum collection upon termination of the immunization protocol. The rabbits were anesthetized with a xylazine 5 mg/kg, ketamine 35 mg/kg, and acepromazine 0.5 mg/kg mixture administered im at multiple injection sites. The anesthetized rabbit was placed on a V-board . A 20 guage needle with attached vacutainer holder and Corvac tube was inserted at an approximately 30° angle immediately posterior to the xyphoid process to puncture the heart cavity. Approximately 100 ml of blood was collected from each rabbit in Corvac tubes, exchanging to a fresh tube when filled. After the rabbit had been exsanguinated, it was euthanized with a pentobarbital overdose; 3 ml of euthanyl (240 mg/ml of sodium pentobarbital; NAPL Vivarium Blood Standard Operating Procedure #3, 1997).

Preparation of serum

The Corvac tubes containing blood were left at room temperature for 1 h to clot then centrifuged at 1000 x g for 10 min to isolate the serum. The serum was carefully removed and placed in labeled Universal bottles. Approximately 15 ml of serum was collected from each rabbit at each test bleed and 50 ml at the terminal bleeds. The serum was stored at -20°C.

A.6. Enzyme-linked immunosorbent assay formats

ELISA controls and calculations

All tubes used for endospore dilution were pre-blocked with 3% (w/v) BSA/PBS for 2.5 h at room temperature in order to prevent spore adherence to the inner surface of the tube. One percent BSA/PBS was used as a dilutant to interfere with spore clumping within a sample. The use of BSA as a blocker and dilutant greatly reduced the variance in titre calculations.

As a positive control to insure that the reporter serum and substrate were working, 10 µl of diluted reporter serum was added to 5 ml of its substrate. If both reagents were functioning properly, the solution changed color within 5 min.

Each sample dilution was run in triplicate in the ELISA. The corrected absorbance at 405 nm was calculated as the mean dilution absorbance against spores minus the mean absorbance of the serum against wells not containing spores (blocked with 3% (w/v) BSA/PBS) ± the cumulative standard error of the dilution and negative control wells. A threshold value equal to three times the standard error of the absorbance of pre-bleed serum against spore-free wells (negative control) was calculated. Dilutions were considered positive if their corrected mean absorbance minus their cumulative standard error was greater than the threshold value. The titre for a given sample was the inverse of the last dilution in the series that gave a positive colorimetric reaction.

2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate

0.486	g	Sodium phosphate, dibasic
0.311	g	Citric acid
100	ml	Deionized water, filtered
1		2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), diammonium salt tablet (Sigma A9941)
50	µl	Hydrogen peroxide, 30%

Dissolved sodium phosphate and citric acid in water. Adjusted the pH to 5.0. Dissolved the ABTS tablet in the phosphate-citrate buffer. In this form, the substrate solution was stable for up to 3 months at 4°C. Just prior to use, dispensed the volume of solution required and added the hydrogen peroxide to activate. Mixed well.

o-Nitrophenyl-β-d-galactophyranoside (ONPG) substrate

0.245	g	Bicine
0.027	g	o-Nitrophenyl-β-d-galactophyranoside (ONPG)
0.030	g	Sodium azide
30.0	ml	Deionized water, filtered

Dissolved reagents together and adjusted pH to 8.2. The solution was stable for up to 4 months in a darkened container at 4°C (Craven et al., 1965).

Serum titre ELISA

Samples of the endospore stock of interest were diluted to 2.00 x 10^6 spores/ml in filtered 1% (w/v) BSA/PBS. Fifty microlitre aliquots of the spore dilution were added to appropriate wells of 96-well plates to give a total of approximately 1.00 x 10^5 spores/well. The plates were covered and incubated at either ambient temperature for 6 h or at 4°C overnight. Spore preparations were removed from the plate wells and the remaining protein binding sites in the wells were blocked by adding 320 µl of 3% (w/v) BSA/PBS and incubating covered plates at ambient temperature for 2.5 h. The BSA/PBS was removed and each well was washed three times with PBS. A 1:10 dilution series of each test serum was prepared in filtered 1% (w/v) BSA/PBS and 60 µl aliquots of the dilutions were added to wells of the coated ELISA plates. The plates were covered and incubated at ambient temperature for 90 min on a platform shaker set at 130 rpm. After the incubation, the serum was removed and each well washed twice with PBS. Sixty microlitres of a 1/1000-dilution of anti-mouse or anti-rabbit immunoglobulin goat polyclonal serum conjugated to horseradish peroxidase in 1% (w/v) BSA/PBS (reporter serum) was added to the test wells depending on the source of the test serum and the

plates were incubated at ambient temperature for 60 min on the platform shaker set at 130 rpm. The reporter serum was removed from the plate and the wells were washed twice with PBS. One hundred microlitres of activated ABTS substrate (see above) was added to the wells and the plates were incubated at ambient temperature for 45 min, during which time positive wells containing spore-Ig complexes turned from colorless to dark green. The absorbance of the wells were measured at 405 nm on a plate reader in order to quantify the assay.

Endospore titre ELISA

One-in-ten dilution series from 10^{-1} to 10^{-6} in 1% (w/v) BSA/PBS were made up of the spore stocks of interest. Fifty microlitre aliquots of each dilution were added to appropriate wells of 96-well plates. The plates were covered and incubated at either ambient temperature for 6 h or at 4°C overnight. Spore preparations were removed from the plate wells and the remaining protein binding sites in the wells were blocked by adding 320 µl of 3% (w/v) BSA/PBS and incubating plates at ambient temperature for 2.5 h. The BSA/PBS was removed and each well was washed three times with PBS. Each test serum was prepared in filtered 1% (w/v) BSA/PBS and 60 µl portions of test serum dilutions were added to wells of the coated ELISA plates. The plates were covered and incubated at ambient temperature for 90 min on a platform shaker set at 130 rpm. After the incubation, the serum was removed and each well washed twice with PBS. Sixty microlitres of a 1/1000-dilution of anti-rabbit immunoglobulin goat polyclonal serum conjugated to horseradish peroxidase in 1% (w/v) BSA/PBS (reporter serum) was added to the test wells and the plates were incubated at ambient temperature for 60 min on the platform shaker set at 130 rpm. The reporter serum was removed from the plate and the wells were washed twice with PBS. One hundred microlitres of activated ABTS substrate (see above) was added to the wells and the plates were incubated at ambient temperature for 45 min. The absorbance of the wells were measured at 405 nm on a plate reader in order to quantify the assay.

Viable spore ELISA

One-in-ten dilution series from 10^{-1} to 10^{-6} in 1% (w/v) BSA/PBS were made up of the spore stocks of interest. Fifty microlitre aliquots of each dilution were added to appropriate wells of 96-well plates. The plates were covered and incubated at either ambient temperature for 6 h or overnight at 4°C. Spore preparations were removed from the wells and 250 µl of either polysaccharide or activated capsule broth were added to the coated wells, and the plate was incubated at 37°C for various lengths of time. The medium was removed from the wells and each well was washed three times with PBS. Samples of purified monoclonal antibody prepared in filtered 1% (w/v) BSA/PBS were added in 60 µl portions to wells of the coated ELISA plates. The plates were covered and incubated at ambient temperature for 90 min on a platform shaker set at 130 rpm. After the incubation, the serum was removed and each well washed twice with PBS. Sixty microlitres of a 1/1000-dilution of anti-mouse immunoglobulin goat polyclonal serum conjugated to β-galactosidase in 1% (w/v) BSA/PBS (reporter serum) were added to the test wells and the plates were incubated at ambient temperature for 60 min on the platform shaker set at 130 rpm. The reporter serum was removed from the plate and the wells were washed twice with PBS. One hundred microlitres of ONPG substrate was added to the wells and the plates were incubated at ambient temperature for 45 min, during which time positive wells containing spore-mouse immunoglobulin complexes turned from colorless to bright yellow. The absorbance of the wells were measured at 405 nm on a plate reader in order to quantify the assay.

A.7. Antibody purification

Protein concentration

The absorbance of antibody preparations was measured at 280 nm before and after purification steps in order to determine the protein concentration of the preparation and

the percentage purification. A 1 in 10 dilution series from 10^{-1} to 10^{-4} of the antibody preparation was prepared in filtered PBS. The absorbance of the dilutions was read against a PBS blank at 280 nm with a UV spectrometer. The relationship between protein concentration and absorbance is most linear between absorbance readings of 1 and 0; at an absorbance of greater than 1 the curve begins to plateau . The protein concentration of the neat solution was calculated from the dilution that gave an absorbance reading between 0 and 1 according to the equation,

$$\text{Concentration (mg/ml)} = (A_{280}\ \text{dilution} - A_{280}\ \text{PBS blank}) \times bcd \qquad (2)$$

where d was the dilution factor, c was the width of the sample cuvet (1 cm) and b was the absorbance constant for 1 mg/ml of protein. The absorbance constant was assumed to be 1.0 for a heterogeneous protein sample, 1.35 for a sample enriched with IgG and 1.2 for a sample enriched with IgM (Harlow and Lane, 1988). All sample dilutions were measured in triplicate to give a mean value and standard error.

Ammonium sulfate precipitation

The procedure was used to purify anti-*Bacillus* spore polyclonal serum and EAII-6G6-2-3 and FDF 1B9 monoclonal antibodies. The antibody sample to be purified was centrifuged at 3000 x g for 30 min at 4°C. The volume of the supernatant was determined and the solution was transferred to an appropriately sized beaker. A sterile stir bar was added and the beaker was placed on a magnetic stirrer. While the antibody solution was gently stirring, a 0.5 volume of saturated ammonium sulfate was added slowly dropwise to give a final concentration of 33%. The beaker was moved to a magnetic stirrer at 4°C and stirred gently overnight.

The solution was centrifuged at 3000 x g for 30 min at 4°C. The supernatant was carefully removed to a clean beaker, a stir bar was added and the beaker was placed on a

magnetic stirrer at room temperature. While the solution was stirring gently, a second 0.5 volume of ammonium sulfate (based on the original starting volume of the antibody sample) was slowly added dropwise to bring the final ammonium sulfate content to 50% saturation. The solution was gently stirred at 4°C overnight.

The solution was centrifuged at 3000 x g for 30 min at 4°C. The supernatant was carefully decanted and the precipitate was resuspended in 0.3 volumes of the starting sample volume in filter-sterilized PBS and transferred to an appropriate length of prepared dialysis tubing. Both ends of the tubing were double clipped and it was dialyzed against three changes of 4.0 l PBS overnight at 4°C with magnetic stirring of the PBS. The antibody solution was then removed from the tubing to a suitable sterile container. For short-term storage, the purified antibodies were kept at 4°C; for long-term storage the antibody solution was frozen at -20°C (Harlow and Lane, 1988).

Gel filtration chromatography

This procedure was used to purify monoclonal antibody EAII-6G6-2-3 from raw ascites taking advantage of the larger size of IgM molecules compared to other immunoglobulin classes. Ten grams of Sephadex G150 beads (Sigma, medium-sized beads 150 μm in diameter with an exclusion limit of 5 - 300 kDa globular proteins) were prepared following the instructions of the manufacturer. The beads were added to 150 ml of filtered PBS with 0.02% sodium azide and incubated at room temperature for 48 h to hydrate. The final volume of the hydrated beads was approximately 50 ml.

The prepared beads were transferred to an appropriately sized column equipped with a stopcock. The beads were prewashed with 1.0 l (20 column volumes) of filter sterilized PBS to which sodium azide had been added to a final concentration of 0.02%. The PBS was allowed to run just into the top of the column bed and then the flow was stopped. Two and a half millilitres of antibody sample was carefully added to the top of the column

and the stopcock was released. Just as the last of the antibody solution ran into the column, the column was carefully topped off with filtered PBS (no sodium azide). While the column was running the upper reservoir of the column was periodically filled with PBS to keep it from running dry (Harlow and Lane, 1988).

After the antibody sample had been loaded, 5 ml fractions of the column effluent were collected in separate test tubes. After 50 fractions had been collected, the flow of the column was stopped. Each fraction was tested for the presence of protein by measuring its absorbance at 280 nm (see above). The fraction number was plotted against the absorbance at 280 nm and fractions comprising protein peaks were pooled together. Dilutions of each pooled peak were tested in a serum titre ELISA (see section A.6.) to identify peaks containing the antibody of interest.

Low salt Protein A chromatography

This procedure was used to purify anti-*Bacillus* spore polyclonal rabbit sera and the FDF 1B9 monoclonal antibody. The pH of the crude antibody preparation was adjusted to 8.0 by adding 1/10 volume of filter-sterilized 1.0 M Tris (pH 8.0). Five millilitres of the antibody preparation was added to the top reservoir of a 5-ml prepacked Protein A column (Sigma) connected to a peristalic pump. The pump was turned on and the preparation was allowed to flow into the column. Just after the antibody preparation had entered the column, the flow was stopped and 50 ml (10 column volumes) of filter-sterilized 100 mM Tris (pH 8.0) was carefully added to the upper reservoir. The flow through the column was restarted. After the 100 mM Tris had run through, the column was washed with 50 ml of filter-sterilized 10 mM Tris (pH 8.0).

The column was eluted with filter-sterilized 100 mM glycine (pH 3.0). Beginning with the addition of the glycine, 500 µl fractions were collected in Eppendorf tubes containing 50 µl of filtered 1 M Tris (pH 8.0). After each fraction was collected, it was mixed via finger

vortexing to bring the pH back to neutral. Fifty fractions were collected and then the column was stopped. Each fraction was tested for the presence of protein by measuring its absorbance at 280 nm against a 100 mM glycine blank. The fraction number was plotted against absorbance at 280 nm and fractions comprising protein peaks were pooled together. Dilutions of each pooled peak were tested in a serum titre ELISA (section A.6.) to identify peaks containing the antibody of interest. The Protein A column was regenerated by washing sequentially with 50 ml each of filtered 2 M urea and 100 mM glycine (pH 2.5) (Harlow and Lane, 1988). The column was stored at 4°C under filter-sterilized 100 mM Tris (pH 8.0).

A.8. Soil characteristics

Samples of Falaise field soil and soil used in spore extraction experiments were tested with the LaMotte combination soil kit, model STH-14 (LaMotte Chestertown, Maryland; Table A-1). All chemical tests were carried out as detailed in the instruction manual of the kit, however, pH was measured with a pH meter rather than colorimetrically. The water content of the soil types was determined by drying a sample of known initial weight in a 85°C oven until its weight became constant. The water content was the weight lost divided by the initial weight and expressed as a percentage.

Table A-1. Some chemical characteristics of soils seeded with *B. anthracis* ATCC 4229 spores.

Test	Milli-Q water	Autoclaved potting soil	Autoclaved wallow soil	Autoclaved field grass soil	Field grass soil
Drainage[a]	-	Excellent	Excellent	Good	Good
pH	6.3	3.8 ± 0.3	8.2 ± 0.1	8.0 ± 0.1	7.5 ± 0.5
Aluminum[b]	< 5.0	5.0 ± 0	5.0 ± 0	8.3 ± 1.7	5.0 ± 0
Calcium[b]	< 150	1400 ± 200	6300 ± 700	8000 ± 1000	7000 ± 0
Chloride[b]	< 25	58 ± 22	50 ± 0	46 ± 27	38 ± 13
Ferric iron[b]	< 2.5	< 2.5	4.2 ± 1.7	5.8 ± 1.7	< 2.5
Magnesium[b]	< 5.0	5.0 ± 0	4.2 ± 0.8	5.0 ± 0	4.2 ± 0.8
Manganese[b]	< 4.0	4.0 ± 0	7.7 ± 2.3	12.0 ± 0	12.0 ± 0
Nitrogen, ammonia[b]	< 5.0	5.0 ± 0	5.0 ± 0	7.5 ± 2.5	5.0 ± 0
Nitrogen, nitrate[b]	< 5	88 ± 0	< 5	5 ± 0	13 ± 3
Nitrogen, nitrite[b]	< 1.0	1.0 ± 0	< 1.0	1.0 ± 0	< 1.0
Phosphorus[b]	< 5	100 ± 0	58 ± 8	5 ± 0	29 ± 4
Potassium[b]	< 25	88 ± 17	172 ± 6	417 ± 33	200 ± 12
Sulfate[b]	< 50	33 ± 8	42 ± 8	< 50	42 ± 8

a As measured by the time required for 14 ml of Universal extraction solution to drain through the soil.

b Values in ppm ± standard error.

A.9. Soil wash solutions

Sucrose solutions

500	g	Sucrose
1.0	l	Deionized water, filtered

Added sucrose and a stir bar to the water. Placed on a magnetic stirrer set on high and dissolved. Poured a 200 ml portion of the solution into a 200-ml graduated cylinder with a stir bar in the bottom and measured the specific gravity with a hydrometer, reading the gravity at the bottom of the meniscus. By adding more water or sucrose, mixing the solution on a magnetic stirrer and remeasuring with the hydrometer, the solution was brought to the desired specific gravity. The sucrose solutions were autoclaved on liquid cycle at 121°C for 15 min to sterilize because the solutions were too viscose to filter-sterilize. Autoclaving did not affect the specific gravity of the sucrose solutions. The solutions were stored at room temperature for up to 3 months.

Sucrose + 0.5% (v/v) Nonidet P-40

199	ml	Sucrose solution of the desired specific gravity, non-sterilized
1.0	ml	Nonidet P-40

Combined the reagents, sealed bottle and shook vigorously to mix. Autoclaved the solution on liquid cycle at 121°C for 15 min to sterilize. Stored the solution for up to 3 months at room temperature.

Sucrose + 0.5% (w/v) N-lauroylsarcosine

200	ml	Sucrose solution of the desired specific gravity, non-sterilized
1.0	g	N-lauroylsarcosine, sodium salt

Combined the reagents, sealed bottle and shook vigorously to mix. Autoclaved the solution on liquid cycle at 121°C for 15 min to sterilize. Stored the solution for up to 3 months at room temperature.

Sucrose + 0.5% (v/v) Triton X-100

199	ml	Sucrose solution of the desired specific gravity, non-sterilized
1.0	ml	Triton X-100

Combined the reagents, sealed bottle and shook vigorously to mix. Autoclaved the solution on liquid cycle at 121°C for 15 min to sterilize. Stored the solution for up to 3 months at room temperature.

A.10. Site maps

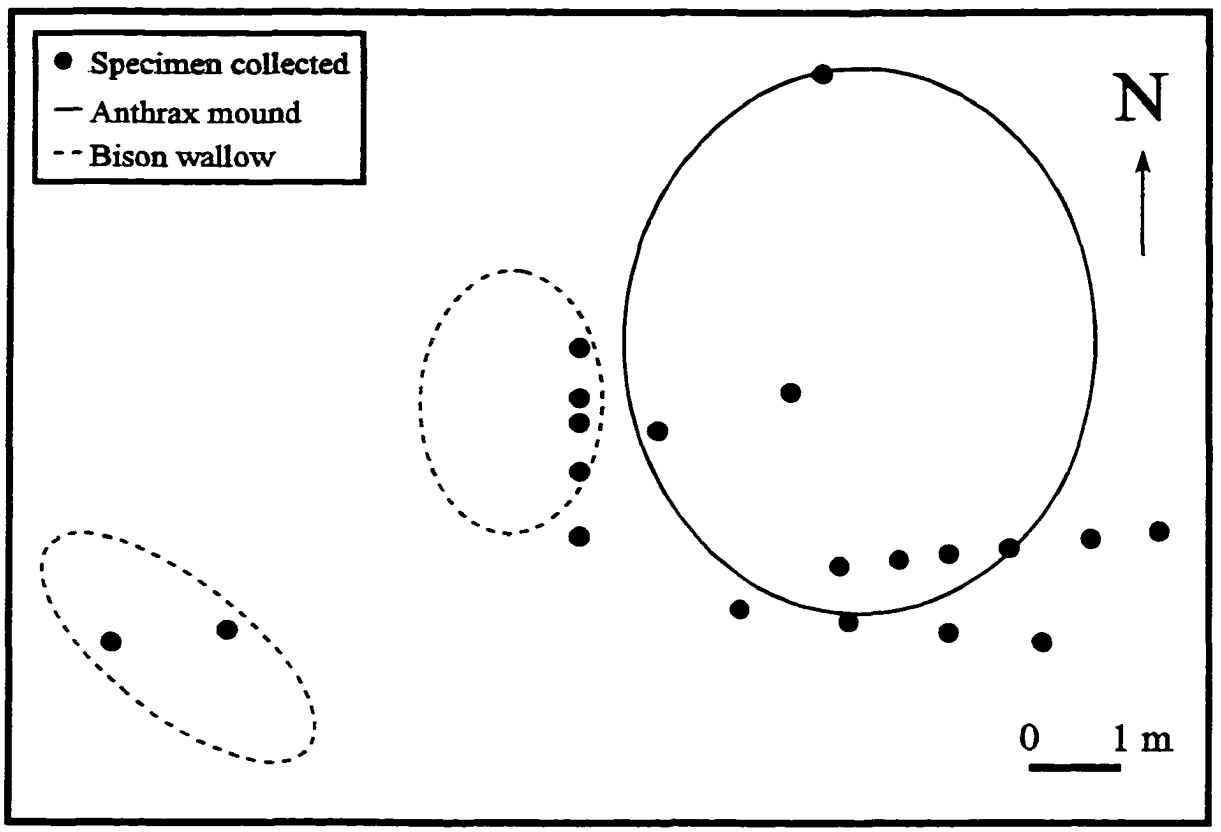

Figure A-1. Specimen collection around anthrax mound 11 at 60° 52' 30" N, 112° 45' 40" W, Hook Lake, NT.

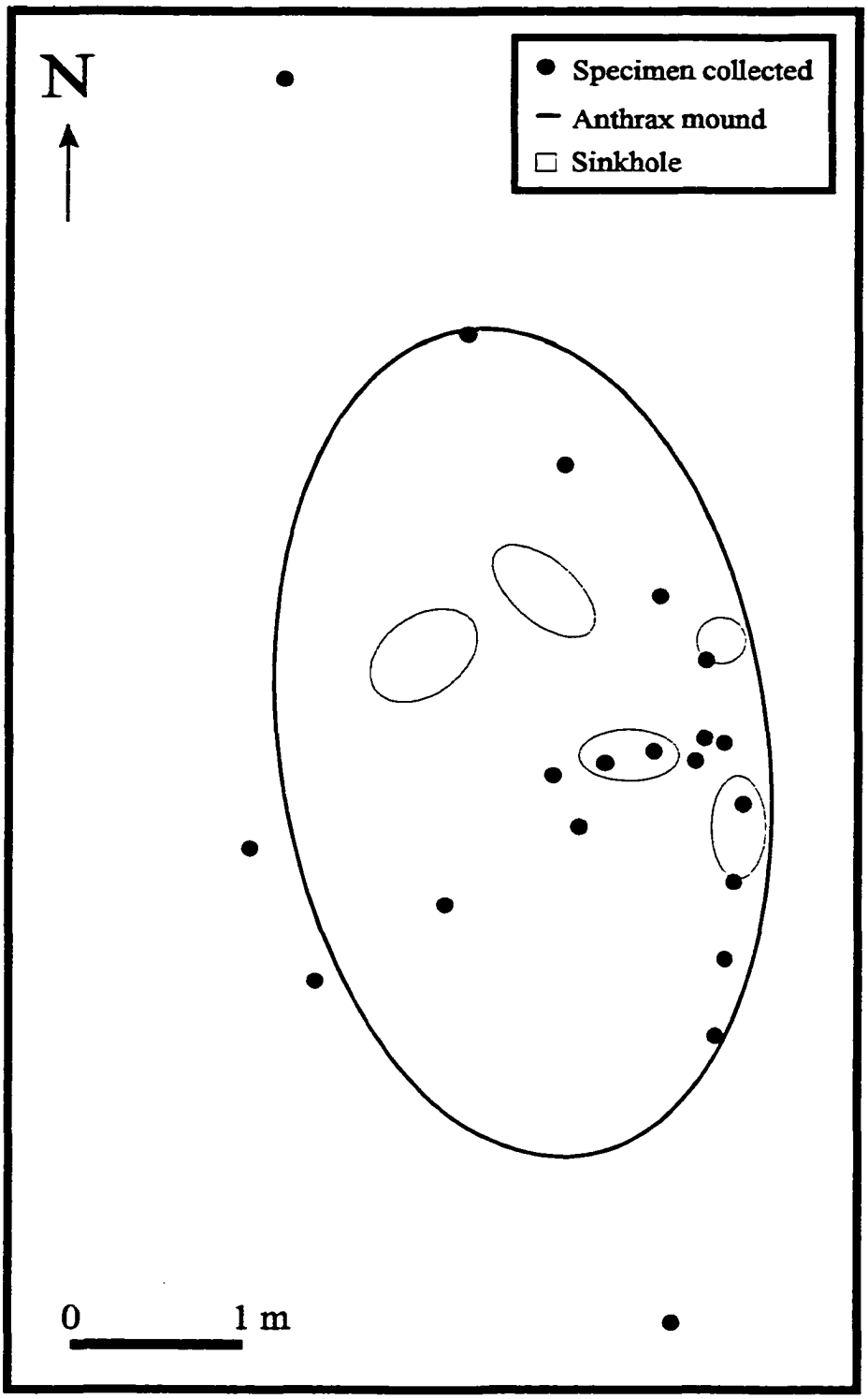

Figure A-2. Specimen collection around anthrax mound 12 at 60° 52' 40" N, 112° 45' 10" W, Hook Lake, NT.

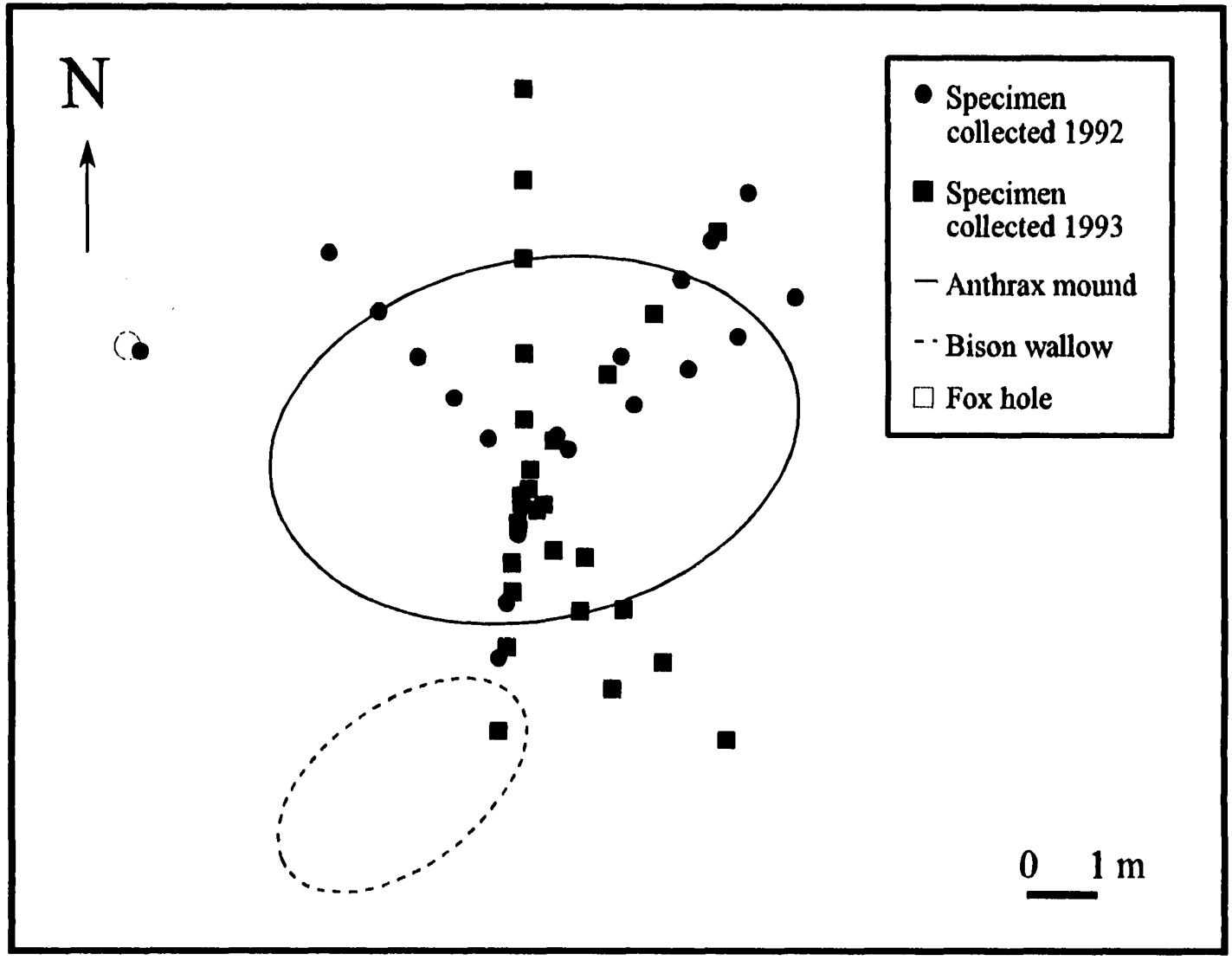

Figure A-3. Specimen collection around anthrax mound 13 at 60° 53' 10" N, 112° 47' 30" W, Hook Lake, NT.

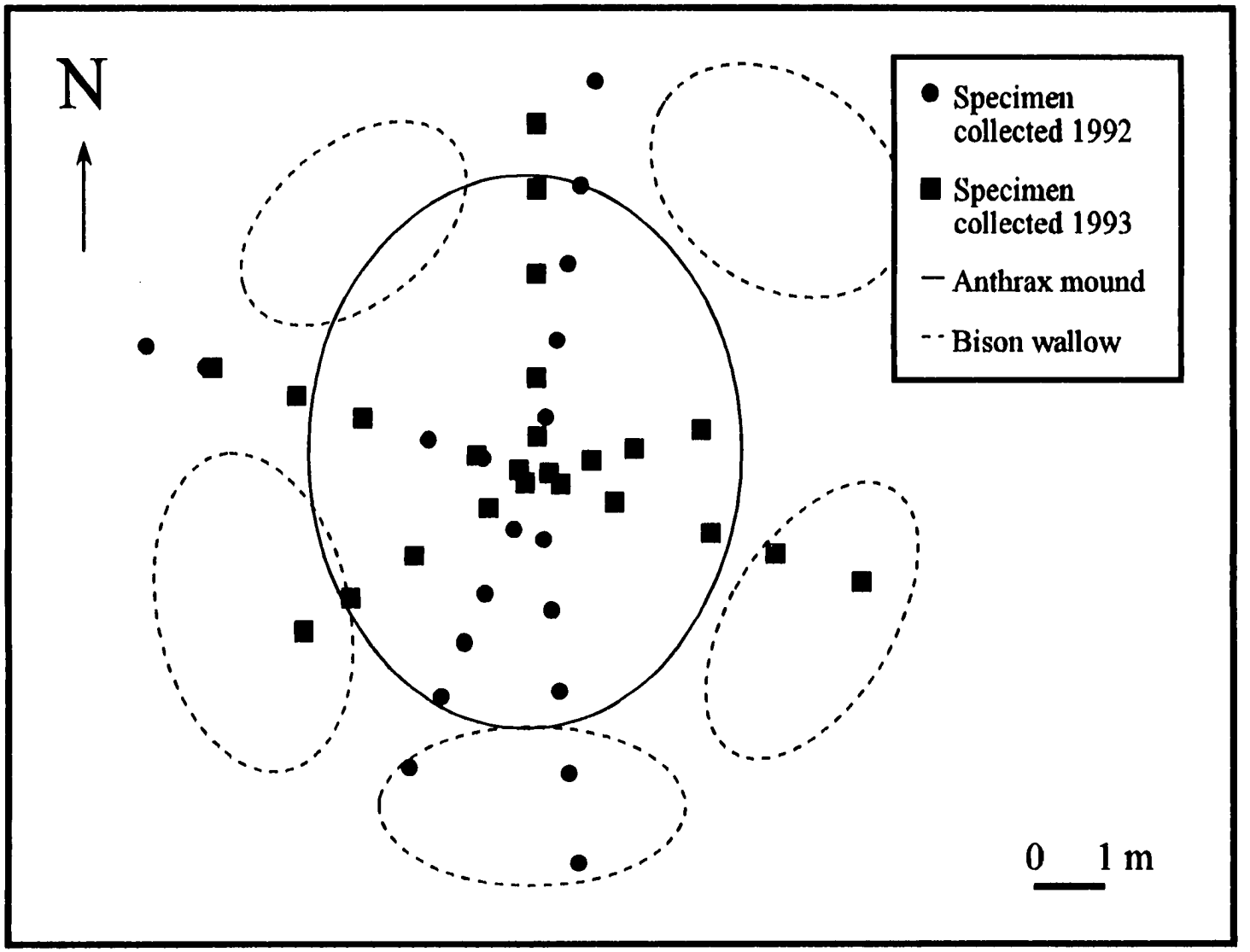

Figure A-4. Specimen collection around anthrax mound 14 at 60° 52' 50" N, 112° 48' 20" W, Hook Lake, NT.

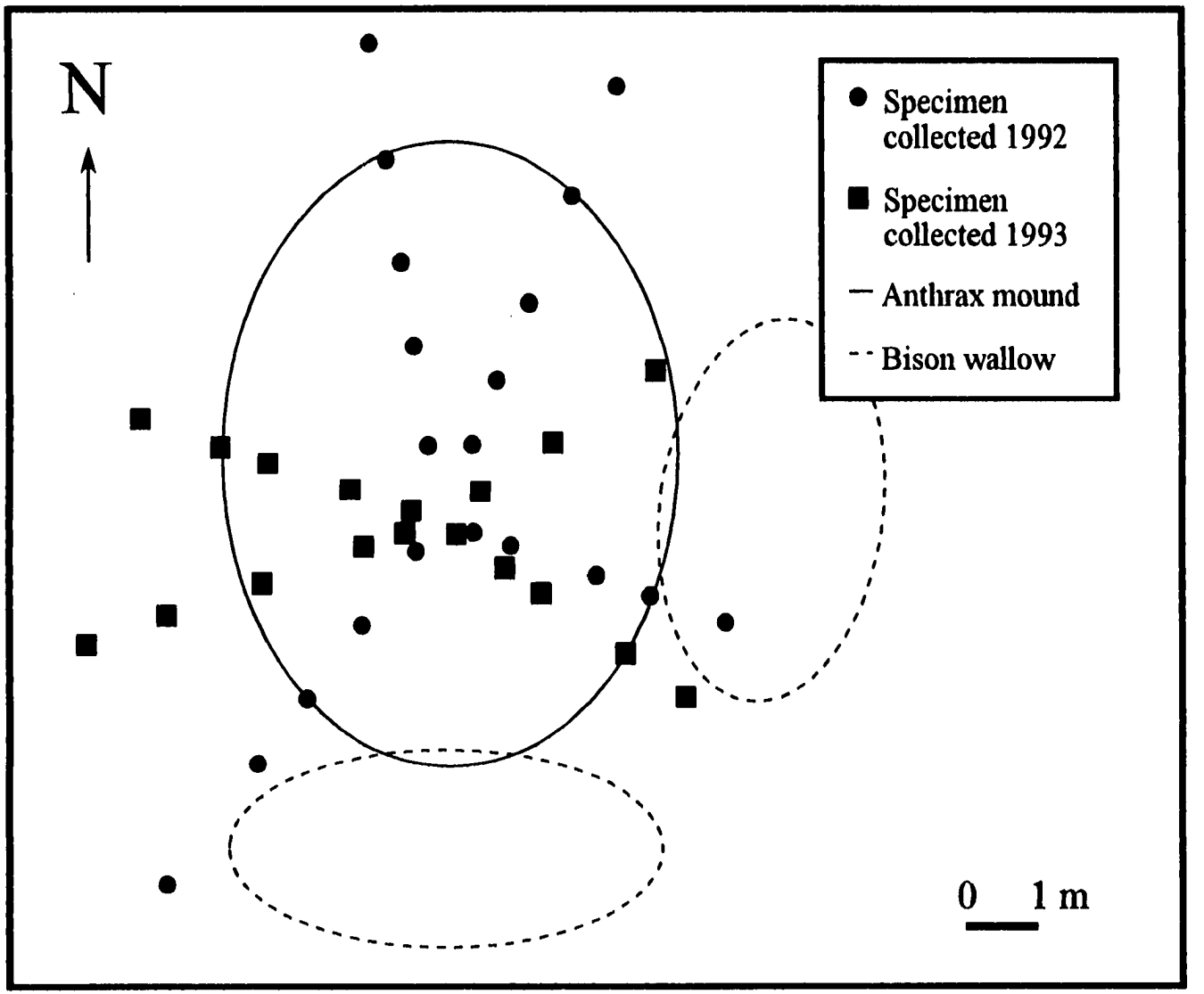

Figure A-5. Specimen collection around anthrax mound 15 at 60° 52' 40" N, 112° 47' 50" W, Hook Lake, NT.

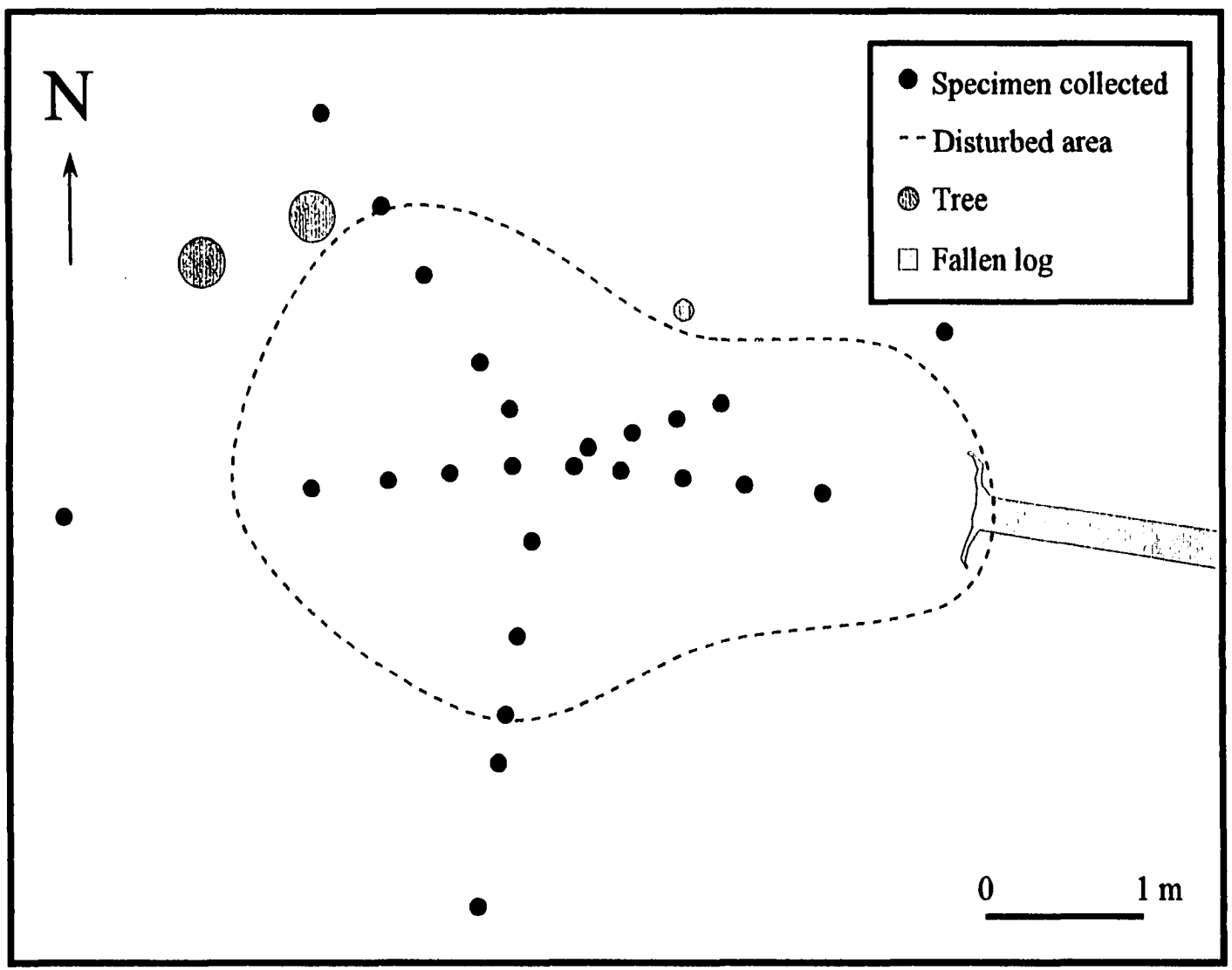

Figure A-6. Specimen collection around burial site 21 at 59° 44' 0" N, 112° 16' 10" W, Parson's Lake Road, WBNP, AB.

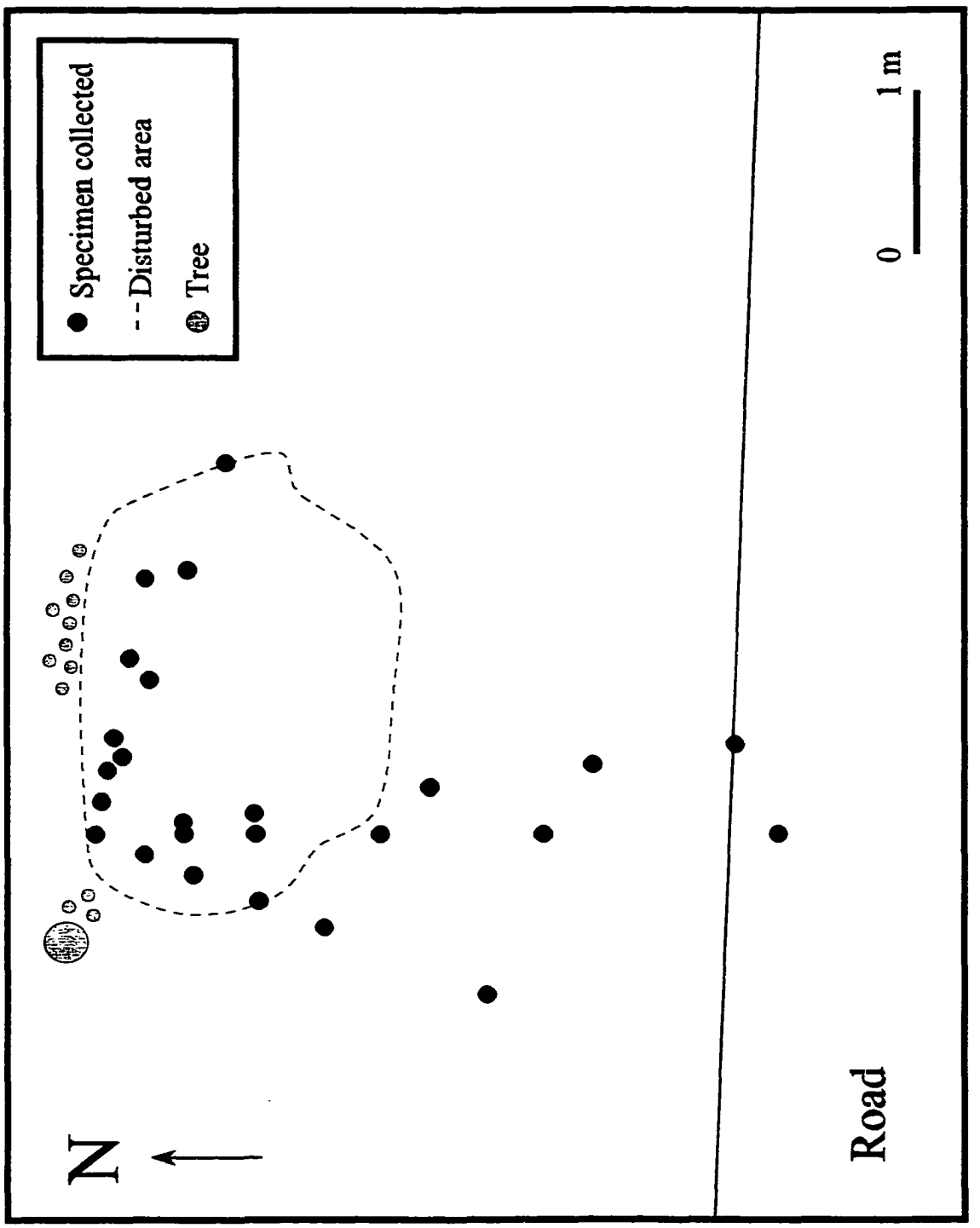

Figure A-7. Specimen collection around burial site 24 at 59° 45' 50" N, 112° 16' 40" W, Parson's Lake Road, WBNP, AB.

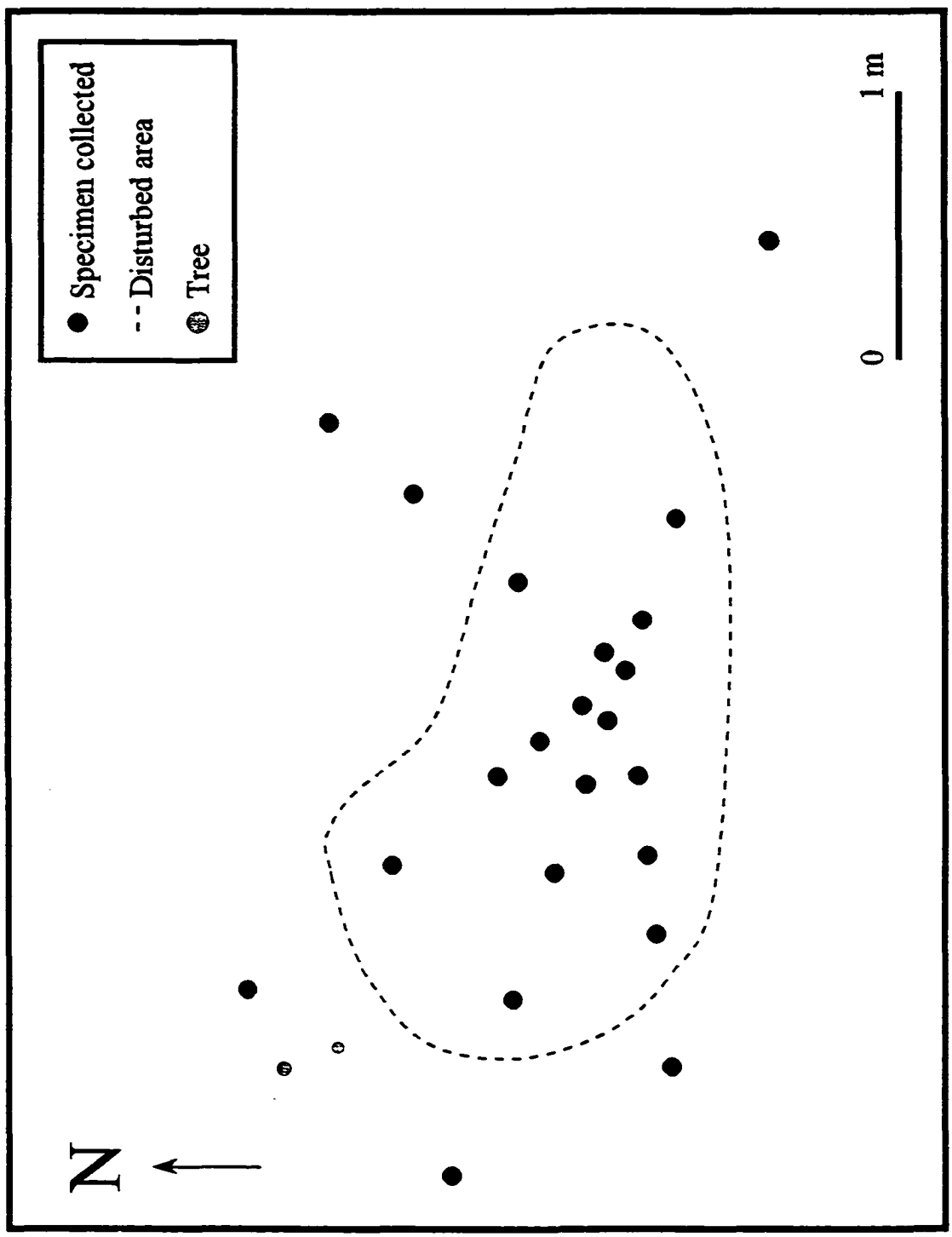

Figure A-8. Specimen collection around burial site 25 at 59° 46' 50" N, 112° 19' 40" W, Parson's Lake Road, WBNP, AB.

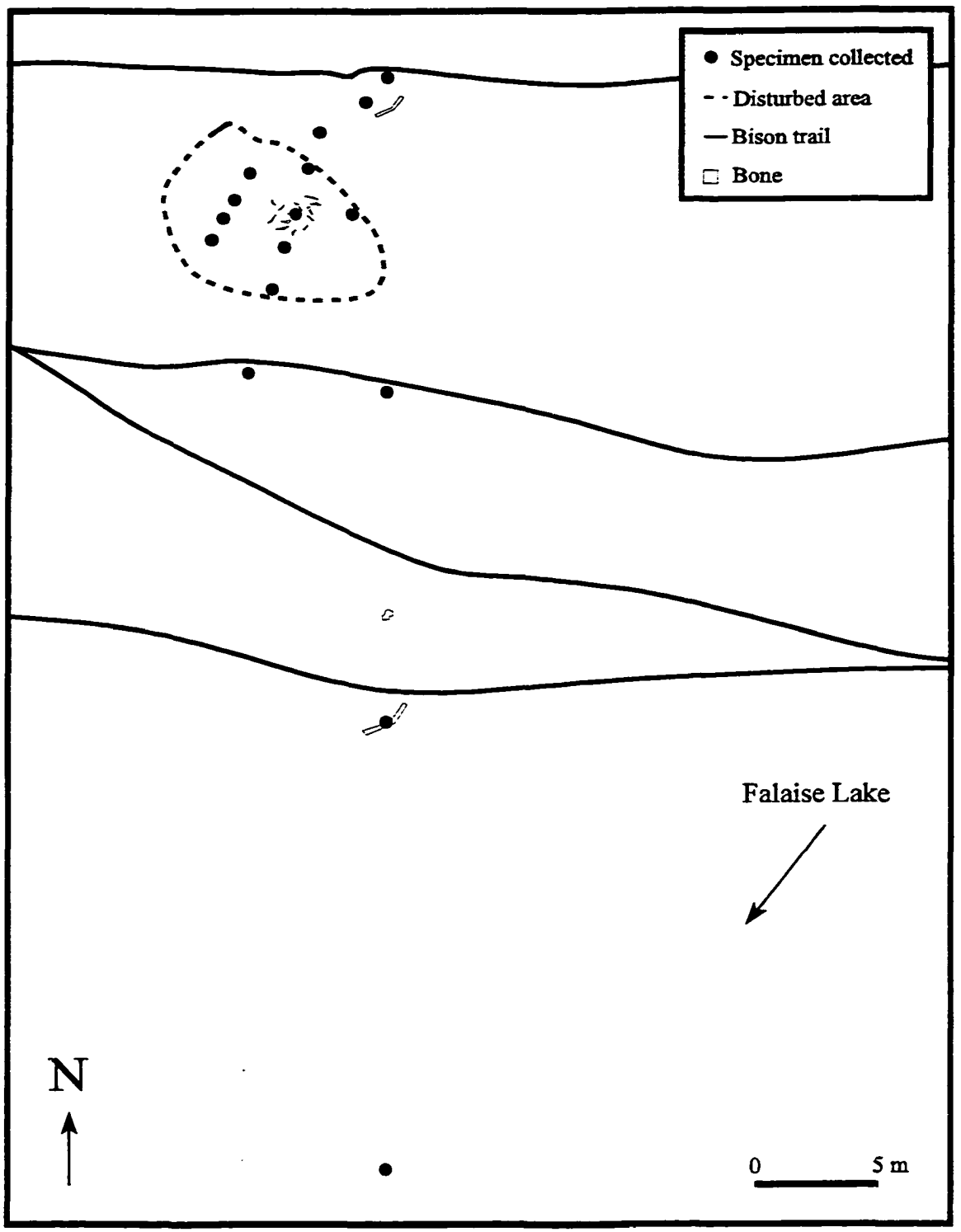

Figure A-9. Specimen collection around cremation site 87 at 61° 29' 10" N, 116° 11' 40" W, Falaise Lake, MBS, NT.

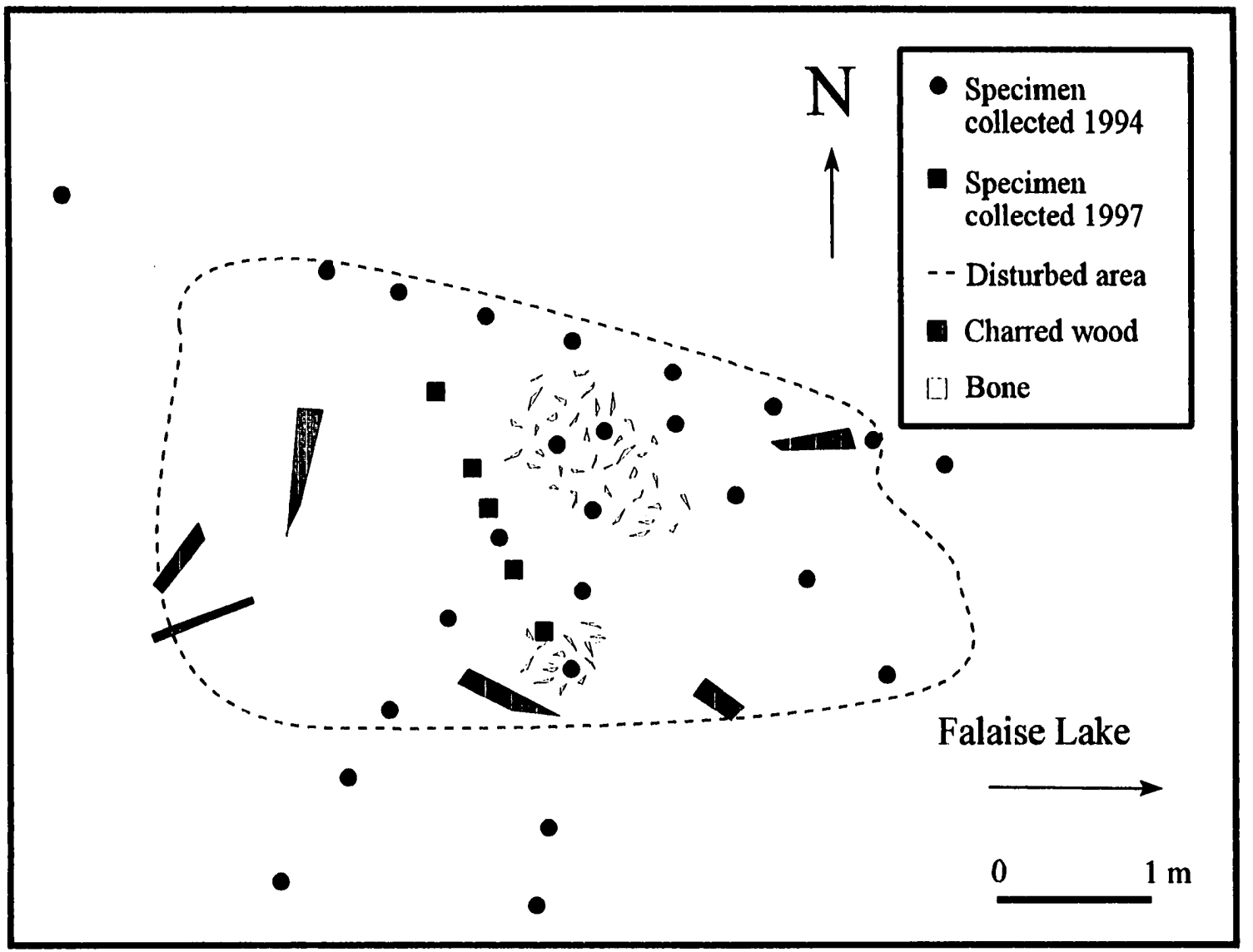

Figure A-10. Specimen collection around cremation site 90 at 61° 29' 0" N, 116° 13' 40" W, Falaise Lake, MBS, NT.

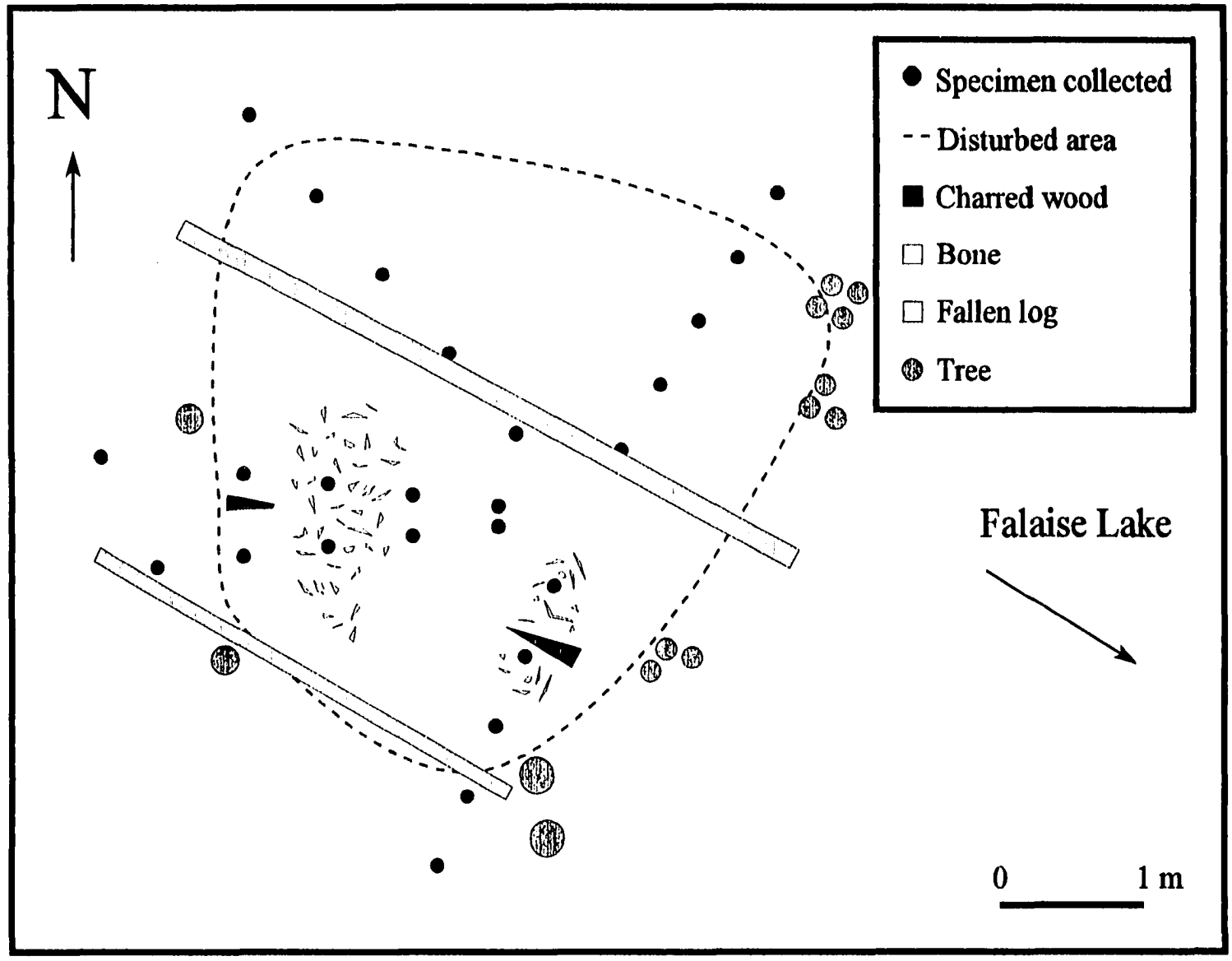

Figure A-11. Specimen collection around cremation site 110 at 61° 29' 0" N, 116° 13' 20" W, Falaise Lake, MBS, NT.

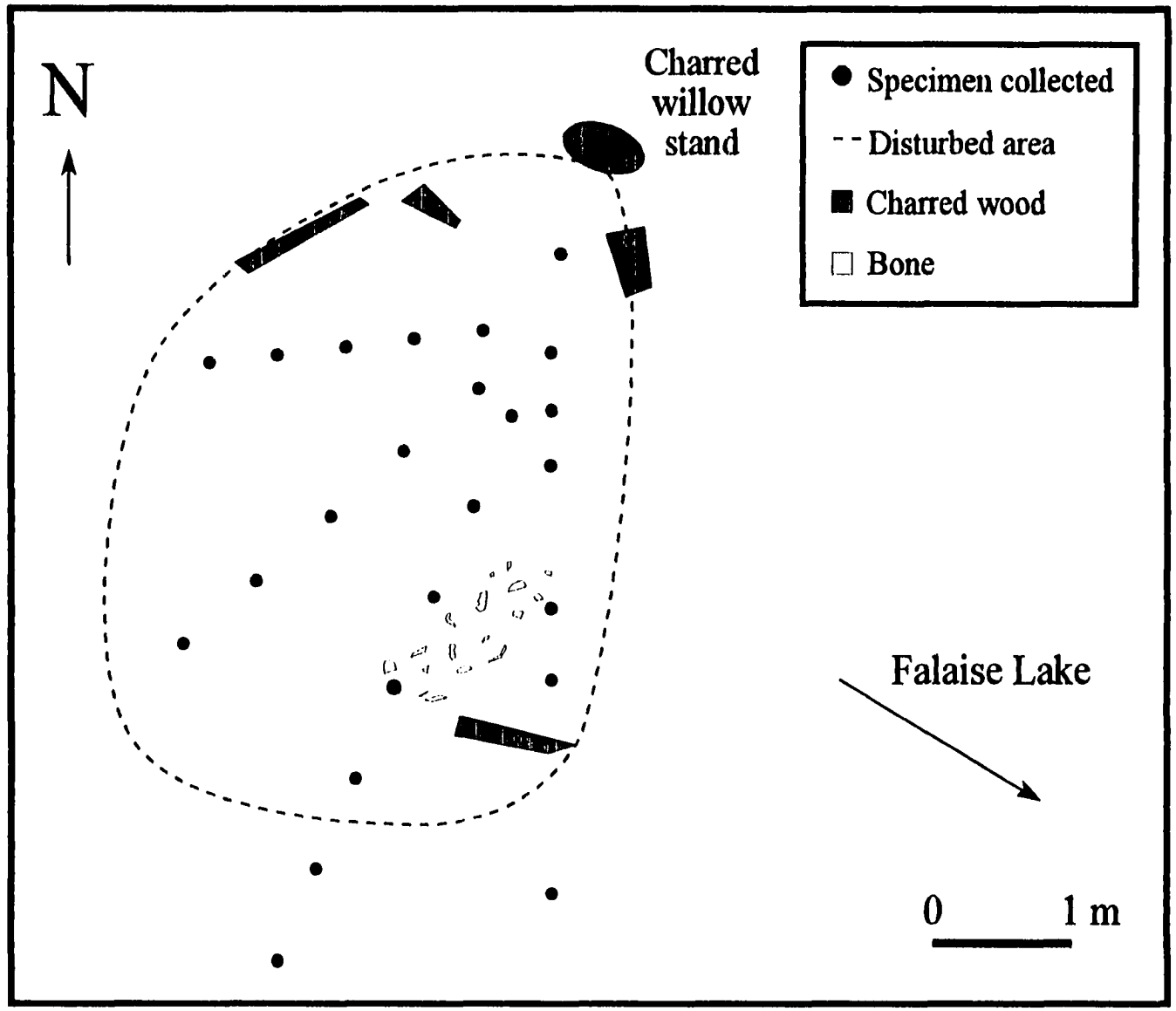

Figure A-12. Specimen collection around cremation site 141 at 61° 27' 30" N, 116° 18' 20" W, Falaise Lake, MBS, NT.

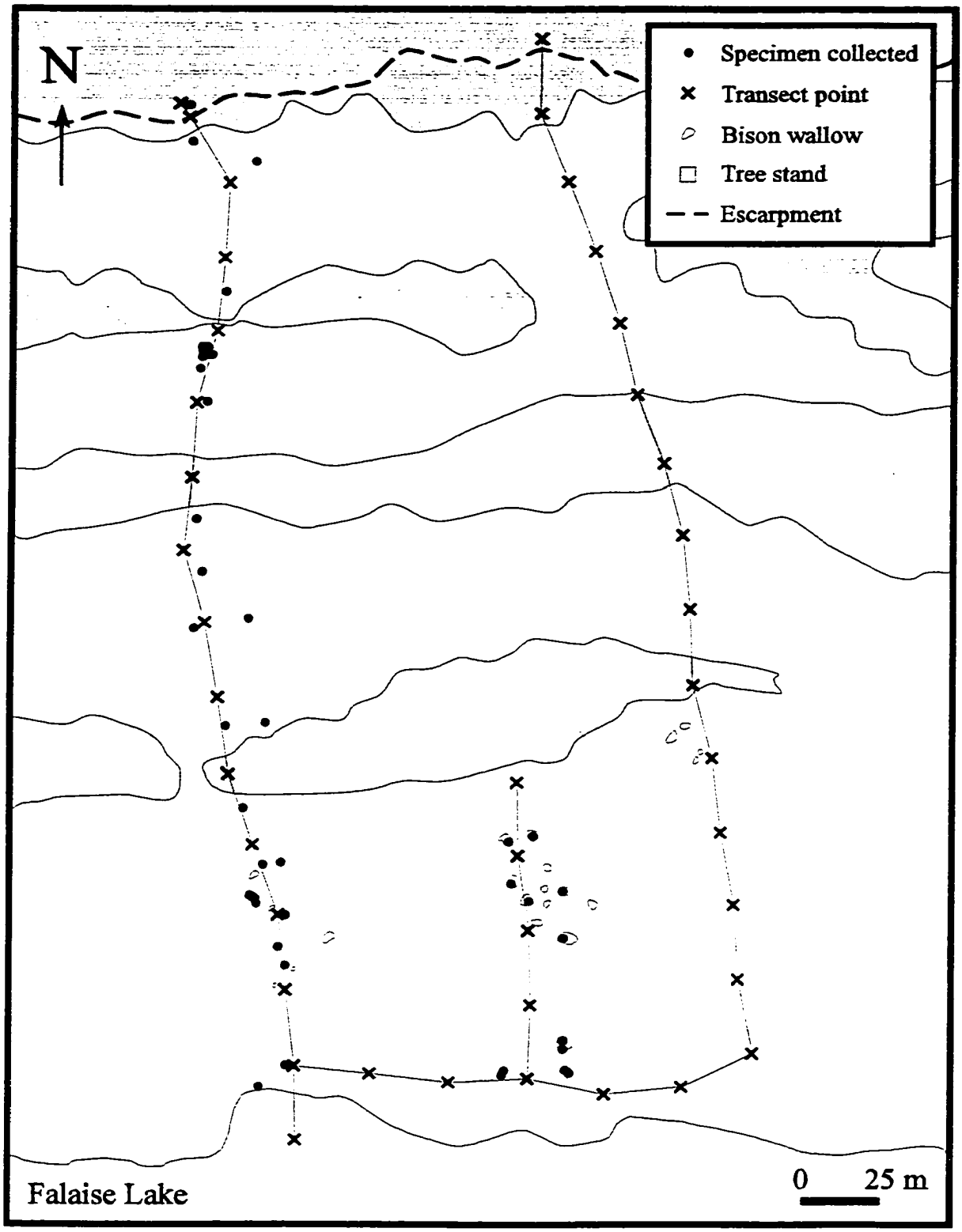

Figure A-13. Transect lines run across Falaise Lake field, July 1997.

A.11. Published papers

Dragon, D.C. and R.P. Rennie. 1995. The ecology of anthrax spores: Tough but not invincible. Canadian Veterinary Journal 36:295-301.

Gates, C.C., B.T. Elkin and D.C. Dragon. 1995. Investigation, control and epizootiology of anthrax in a geographically isolated, free-roaming bison population in northern Canada. Canadian Journal of Veterinary Research 59:256-264.

Dragon, D.C., R.P. Rennie and C.C. Gates. 1996. Bison and anthrax in northern Canada. In: Proceedings of the Second International Workshop on Anthrax, Winchester, England, September 19-21, 1995. (P.C.B. Turnbull, Ed.). Salisbury Medical Bulletin 87 (Special Supplement):22-23.

Dragon, D.C., B.T. Elkin, J.S. Nishi and T.R. Ellsworth. 1999. A review of anthrax in Canada and implications for research on the disease in northern bison. In: Proceedings of the Third International Workshop on Anthrax, Plymouth, England, September 7-10, 1998. (D.E.S. Stewart-Tull, I.M. Feavers and G.R. Gibson, Eds.). Journal of Applied Microbiology 87:208-213.

A.12. Submitted manuscripts

An overview of early anthrax outbreaks in northern Canada: Field reports of the Health of Animals Branch, Agriculture Canada, 1962-1971.

D.C. Dragon and B.T. Elkin

ABSTRACT

Between 1962 and 1971, six outbreaks of anthrax occurred in the bison herds of the Northwest Territories and northern Alberta. In response the Federal Health of Animals Branch dispatched staff veterinarians to oversee carcass disposal operations and later to take part in bison depopulation and vaccination programs. Recently a collection of documents from the agency concerning the outbreaks was discovered in the Federal archives. Included in the collection were field reports from the veterinarians which provide valuable, detailed first-person accounts of the outbreaks and later programs that have generally been lacking in the published literature. The reports identify at least 1102 bison died of anthrax during the six outbreaks; dozens more than reported previously; and indicate the disease had spread into Wood Buffalo National Park in 1963 rather than 1964 as had been previously reported. A minimum of 598 healthy bison were also killed in depopulation programs aimed at preventing the spread of anthrax into the Park even though the targeted regions were repopulated within weeks and anthrax carcasses had already been discovered within the Park. Coverage and revaccination rates were low throughout the vaccination program and a further 828 bison died during the vaccine roundups.

Key words: anthrax, bison, disease, Northwest Territories, Wood Buffalo National Park

INTRODUCTION

In Canada, a large outbreak of anthrax occurred in the free-roaming bison (*Bison bison*) herds of the Hook Lake region of the Northwest Territories during the summer of 1962 (Figure I-2) (Novakowski et al., 1963). Over the next two summers, anthrax

outbreaks continued in the region and spread, first across the Slave River to the Grand Detour region and later into the Park Central and Lake One regions of Wood Buffalo National Park (WBNP) (Choquette et al., 1972). Between 1962 and 1991, there were eight sporadic anthrax outbreaks of varying size in the bison of these four regions which resulted in the deaths of over a thousand animals. As recently as 1993, a large-scale outbreak of the disease occurred in the bison herds of the Mackenzie Bison Sanctuary (MBS) across Great Slave Lake from the previously affected areas (Gates et al., 1995).

Because of the threat the outbreaks posed to recovery efforts for the endangered bison and the possibility of the disease spreading to inhabited regions and domestic livestock, large scale clean-up operations were initiated. The primary focus of the operations was to locate and dispose of carcasses as quickly as possible and this is reflected in the published literature which generally contains only cursory epidemiological details. Recently a large collection of files from the Health of Animals Branch (HAB) concerning the department's response to the early anthrax outbreaks in northern Canada was discovered in the archives of the Canadian Food Inspection Agency. The files provide a wealth of previously unpublished observations on the early outbreaks from 1962 to 1971. The novel data is herein summarized and placed within the framework of previously published literature in order to augment the known scientific literature on the disease and to provide greater detail on this period of history in northern Canada.

HISTORICAL SOURCES

Anthrax is a reportable disease under the Federal Health of Animals Act. During the period of the early northern anthrax outbreaks the diagnosis and containment of the disease was the responsibility of Agriculture Canada (today the control of anthrax outbreaks falls under the jurisdiction of the Canadian Food Inspection Agency). When the northern outbreaks occurred the federal Department sent a veterinarian from its Health of Animals Branch to collect samples for bacteriological confirmation of the disease and to supervise carcass disposal operations. Later, veterinarians were also sent north to help in other anthrax-related operations, including large-scale anthrax vaccination campaigns, and

inspection of bison carcasses during a depopulation/harvest program in the Grand Detour region.

While in the field the HAB veterinarians wrote daily reports on the progress of the operations along with empirical observations on the weather, wildlife and surrounding country. Copies of these reports along with correspondence pertaining to the anthrax operations were maintained in an anthrax file at the HAB regional office in Calgary, Alberta. Although most federal files are destroyed after 20 years, the anthrax documents were either misfiled or spared by an unknown party who realized their value and were recently rediscovered allowing present day anthrax researchers to benefit from the first hand accounts of the early outbreaks. Fittingly, the HAB anthrax file has now been archived in the Glenbow Museum in Calgary.

CHRONOLOGY AND MORTALITY

Original outbreak at Hook Lake - 1962. The first anthrax outbreak at Hook Lake was discovered by chance. On July 28, 1962, a Canadian Wildlife Service (CWS) biologist, Dr. G. Kolensky, was conducting a helicopter survey of bison habitat over the remote region and discovered 32 dead bison on two meadows (Novakowski et al., 1963). The animals had been dead only a few days, and samples were collected from several of the carcasses in an attempt to determine the cause of death. Over the next few days Dr. Kolensky continued to survey the region and observed more bison carcasses whose locations he began to map. Between July 28 and August 7, eight lethargic, morbid bison were shot in the region by CWS personnel and more samples were collected. On August 14 anthrax was confirmed as the cause of death by the federal Health of Animals Laboratory by direct culture of *Bacillus anthracis* from the field samples collected. Plans were immediately developed for a full-scale clean-up of infected carcasses in the region (MacEwan, 1995).

To better assess the situation, Dr. A.E. Lewis, HAB Alberta District Veterinarian, travelled to Fort Smith and surveyed the region by helicopter with Dr. Kolensky on August 15. In a letter to his superiors, Dr. Lewis reported seeing nearly 100 carcasses

spread over approximately 1500 km^2, although most were concentrated on the two original meadows. Dr. Lewis also observed anthrax eschars on Dr. Kolensky's forearm. With no previous history of anthrax in the area to arouse suspicion, Dr. Kolensky had collected specimens from the first carcasses without the benefit of gloves or other protective gear. A week later he developed a general malaise, and carbuncles appeared on his forearm. Fortunately, the local physician in Fort Smith was originally from England and apparently had experience with the disease and recognized the condition. Treatment was started immediately with the antibiotic tetracycline, which rapidly cleared his malaise although the black eschars typical of cutaneous anthrax were slower to resolve.

Due to the logistical difficulties involved in barging supplies and equipment down the Slave River during the summer and setting up a quarantined base camp to insure the living quarters remained uncontaminated by the hardy and infectious anthrax spores, the initiation of disposal operations was delayed (Pyper and Willoughby, 1964). At its height, the operation employed 40 men, five caterpillar tractors, two bombardier-type vehicles and one muskeg tractor to dispose of the carcasses, all of which had to be barged into the area (MacEwan, 1995). Dr. Lewis did not remain at Hook Lake to supervise the operations but instead assigned the duty to Dr. W.M. Norton. According to his field reports, Dr. Norton arrived at the base camp, a hastily converted abandoned sawmill, on August 21 but disposals did not begin until August 23 as they still had to wait the arrival of two tons of lime.

According to Novakowski et al. (1963) approximately 120 carcasses were located between July 28 and August 4. Despite continued surveillance, no new deaths were observed from August 4 to August 15. A second wave of mortality was then reported, and 161 more carcasses were found between August 15-21 (Table 1). However, Dr. Norton's field report described a somewhat different sequence of events. Norton reported Dr. Kolensky had located only 156 carcasses by August 21 and that aerial surveillance was not completed until August 28 at which time 253 carcasses had been located and flagged. Included with the report was a copy of Dr. Kolensky's map with all 253 carcass locations. One can assume that Dr. Kolensky numbered the carcasses sequentially as he found them.

Carcasses 1 to 156 were all located on the two meadows where the original 32 carcasses were found. Carcasses assigned numbers higher than 156 were generally found at a distance from these two main meadows. Instead of representing a second wave of bison mortalities, the delayed discovery of the last 97 carcasses may have been due to their being in previously unsearched areas. These 97 likely died at the same time as the rest of the infected bison as Dr. Norton repeatedly reported that no fresh bison carcasses were observed after August 19 indicating that the epizootic had already ended by this date. The remaining carcasses noted by Novakowski et al. (1963) were later located by clean-up crews travelling through areas of thick vegetation which had likely obscured aerial detection. In the 1993 MBS outbreak, 55% of the carcasses were located in forested or shrub-covered sites and were not visible from aircraft used to search for carcasses (Gates et al. 1995).

Major outbreak west of the Slave River - 1963. Anthrax was detected in the Hook Lake region again the following summer and this time Dr. J.F. Gallivan of the HAB was dispatched to supervise clean-up operations. In his field report, Dr. Gallivan stated a single carcass had been discovered in the region on June 27 by CWS biologists conducting an aerial survey of sandhill cranes. The biologists had been involved in clean-up operations the previous summer and immediately suspected anthrax. They were also familiar with Dr. Kolensky's encounter with the disease and upon landing took the appropriate safety precautions before collecting biological specimens. These precautions were justified as *B. anthracis* was later isolated from the specimens.

Despite the possibility of another large anthrax outbreak, it was impossible to resurvey the Hook Lake area until July 3 as all available aircraft were involved in fighting a large forest fire in WBNP. The surveillance flight on July 3 only observed five more carcasses which were buried that same day along with the original carcass. Although subsequent surveys were flown over the area daily, by July 17 only twelve carcasses had been detected. Surveillance flights over the area continued for several more weeks yet only three more carcasses were found with the last one located around July 23.

If the second outbreak appeared anticlimactic compared to the first, it did not

remain that way for long. Sometime between July 18 and July 29 a much larger outbreak was detected on the west side of the Slave River. In the minutes of an August 29 meeting of the anthrax response committee in Edmonton it was reported that 62 carcasses had been located in the Grand Detour region and another 24 had been discovered in the northeast corner of WBNP within the Park Central region. This last point is interesting as previous accounts of the outbreak stated carcasses were limited to the Hook Lake and Grand Detour areas (Choquette et al., 1972; Broughton, 1987). However, the discovery of carcasses within the Park boundaries is verified repeatedly in Dr. Gallivan's report and other correspondence. On August 7 Dr. Gallivan stated 117 and 37 carcasses had been found in the Grand Detour and Park Central regions, respectively. Disposal operations were completed on August 15 with a total of 217 carcasses buried west of the Slave River. In his August 15 entry, Dr. Gallivan stated,

> "The current anthrax outbreak [to the west of the Slave River] would appear to be terminated. I would estimate that all carcasses found with the exception of two had been dead for approximately one month. The two carcasses mentioned appeared to have been dead one week at the time of discovery. . . .The distance between the most northerly carcass and the one found furthest south in the Grand Detour area was approximately 80 miles. Forty-seven carcasses were found dead within the Park boundary."

A couple of weeks later on September 4, Dr. Gallivan reported that despite continued aerial surveys over the affected regions no further carcasses had been detected. His final total of 217 carcasses buried west of the Slave River is well below the published figure of 269 (Choquette et al., 1972), however, in his September 4 report Dr. Gallivan went on to say that plans were in place to resurvey the regions in late autumn after the leaves had fallen and any carcasses found would be burned. While there is no direct information on any additional carcasses being found in the fall survey, Dr. L.G. Gould of the HAB was in the region the following summer and reported the final carcass total west of the river in the 1963 outbreak was 289.

Spread of anthrax continues - 1964. During the summer of 1964, regular anthrax

surveillance flights were conducted over the previously affected regions and by the middle of July a large outbreak was detected in the Grand Detour area with a few additional carcasses observed in the Hook Lake area. There is less data available on this and subsequent outbreaks in the HAB files as carcasses disposal operations were now being overseen by WBNP personnel. By chance, Dr. Gallivan was in the area at the time to perform anthrax vaccinations on bison in WBNP, and included information about this outbreak in his reports.

On August 17, Dr. Gallivan reported that disposal operations were almost complete and that the outbreak had spread west across the entire Central Park region and south across the Peace River into the Lake One region. A total of 306 carcasses were located in the four regions; 44 at Hook Lake, 202 at Grand Detour, 49 at Park Central and 11 at Lake One. The first deaths occurred in the Hook Lake and Grand Detour regions with the peak period of deaths between July 10 and 20 after which mortalities ended abruptly with no new carcasses discovered after July 22. Around this time carcasses were discovered in the Park Central regions where deaths continued until around August 4. No carcasses were reported in the Lake One region until August 1 and the last of the deaths appeared to have occurred in this region where the final carcasses were located around August 10. As in the previous year the affected areas were resurveyed in the fall and according to a letter from Mr. B.E. Olson, Superintendent of WBNP,

> "In the Grand Detour area we found 57 animals after the leaves shed off the trees, and these have all been limed, buried and/or burned. The same success has been achieved in the [Park Central region] . . . as well as a few animals found around or adjacent to Lake One south of the Peace River."

Unfortunately, the number of carcasses found in the Park Central and Lake One regions during the fall clean-up was never recorded.

It is worth noting the anthrax vaccination roundups which Dr. Gallivan had originally come north to take part in during the summer of 1964 never took place. Initially, the large and expanding anthrax outbreak commanded everyone's attention, and later in the summer large-scale flooding of the bison ranges left the herds inaccessible.

According to Dr. Gallivan the end of the outbreak coincided with the arrival of heavy and continuous rains in all of the affected regions flooding all low lying areas and making the terrain impassible for ground vehicles. The rain continued from mid-August through September with two or three heavy showers occurring daily and leaving hundreds of km^2 of prairie under water.

Water action has been demonstrated to passively transport anthrax spores in endemic regions of Kruger National Park, South Africa (De Vos, 1990) and has been implicated in spore transport in Canadian outbreaks (Heath and Brewitt, 1982; Dragon and Rennie, 1995). It is possible that the intensive flooding had a cleansing effect in the affected regions, flushing anthrax spores off low lying prairies and dispersing heavy concentrations of spores. This may explain why after three successive years of increasing numbers and geographical range of mortalities, the disease suddenly disappeared; no single cases or mass mortalities were observed during the summers of 1965 and 1966 despite favourable outbreak conditions and intensive surveillance. In subsequent outbreaks in the regions the disease has generally remained limited in scope.

Sporadic outbreaks - 1967 to 1971. Choquette et al. (1972) stated that between August 3 and September 7, 1967, 120 bison died of anthrax in the Lake One region. A letter to Dr. Lewis from Dr. Choquette of the CWS confirmed the first carcasses were found on August 3 but gives no indication of their condition nor when the outbreak could have started. A copy of a specimen record sent south with blood samples indicated the outbreak lasted until at least August 20 on which date positive samples were collected from a bison dead less than 24 hours.

A single case of anthrax was confirmed in a dead bison in the Lake One region in 1968 and a small localized outbreak involving 31 carcasses was discovered in the Hook Lake region in 1971 (Choquette et al., 1972). Dr. L. Kindt of the HAB was in the area vaccinating bison at the time of the 1971 outbreak. Kindt reported the first carcasses were found on June 22 and the outbreak was estimated to have begun around June 10. Fresh carcasses were found in the area until July 6 when the outbreak appeared to have terminated. In addition to the official tally, two carcasses were found on July 6 but their

condition suggested they were winter kills and, though disposed of with the rest, they were not sampled or considered anthrax deaths. However, on the same day searchers found the carcass of a bull bison which was in very poor shape showing signs of profuse watery diarrhea and still covered with a thick winter coat. It was believed this animal had also died of causes other than anthrax, but *B. anthracis* was subsequently isolated from anal and jugular swabs taken from the carcass.

Between 1962 and 1971, 1102 bison carcasses were found and disposed of during anthrax outbreaks in the Hook Lake, Grand Detour, Park Central and Lake One regions (Table 1). This figure is a minimal estimate of the actual losses due to anthrax. Even with resurveying the affected regions in autumn after the leaves had fallen, carcasses were still undoubtedly missed in areas of heavy brush or had long since been dismembered by scavengers.

PATHOLOGY AND SYMPTOMS IN BISON

In bison, anthrax is usually acute and rapidly fatal. The classic, often repeated, appearance of morbid bison during anthrax outbreaks was first reported by Novakowski et al. (1963),

> "The animals, head lowered, gaunt and drawn, feeding voraciously at times, were depressed and inordinately indifferent, whereas they should have been active and alert, as the outbreak occurred during the rut period. Most of the animals walked with difficulty, staggering at times, and exhibited a stiff-legged gait when running. Also, a swelling of the preputial and umbilical region was noted in many animals."

There has been a temptation, especially during outbreaks, to assume that all inactive, indifferent or limping bison are terminally ill with anthrax. However, these general traits are not prognostic indicators of a fatal anthrax infection. During outbreaks in northern Canada, a number of moribund bison were reported to have experienced a less acute form of the disease and recovered (Novakowski et al., 1963; Choquette et al., 1972; Gates et al., 1995). However, the number of animals recovering and their immunological

status afterwards has never been quantified. Stress due to environmental conditions associated with anthrax outbreaks, mechanical injury or other diseases may also cause the bison to behave in an abnormal "weakened" manner. Dr. Gallivan reported that two sick bison, one lame and the other weak, were shot during the 1963 outbreak. Upon post-mortem both were found to be afflicted with tuberculosis and neither exhibited any lesions suggestive of anthrax.

Acute anthrax appears to cause sudden death in bison, with no signs of struggle or flailing in the soil immediately surrounding the carcasses (Novakowski et al., 1963; Choquette et al., 1972; Gates et al., 1995). After death the carcass bloats rapidly causing the legs to splay in a "saw-horse" position. During the early outbreaks carcasses were often found with a bloody discharge from their mouth or anus (Novakowski et al., 1963; Choquette et al., 1972). Occasionally there was also a frothy white exudate from the mouth or nose. However, during the 1993 MBS outbreak no bloody exudates from the natural body openings were observed that could not be traced to post-mortem scavenging although one cadaver did have a frothy white discharge issuing from its nose (Gates et al., 1995). The discrepancy in reports of bloody discharge is one of the major differences between the early outbreaks around WBNP and the MBS outbreak and its cause remains unknown.

There are multiple references to bloody discharges from carcasses in the HAB files indicating the phenomenon was fairly common. Of particular interest is a 1963 field report from Dr. Gallivan which demonstrated the exudates were indeed a symptom of anthrax. While out surveying for carcasses, Dr. Gallivan came upon a yearling bull lying in the open prairie. The animal was very reluctant to move even with the helicopter hovering only a few feet above its head. The animal finally rose with great difficulty and slowly walked a short distance away. The aircraft landed and Dr. Gallivan got out to observe the animal while the pilot flew back to camp to get a rifle. A few minutes after the helicopter left the bull assumed a braced leg stance, commenced to sway and suddenly toppled to the ground. As Dr. Gallivan watched blood began to flow from the bull's nostrils. When the pilot returned with the rifle, the animal was shot and blood samples were taken from which

B. anthracis was later isolated.

Post-mortem examination of bison during anthrax outbreaks has been limited. During the 1962 outbreak eight morbid bison were shot for specimen collection and necropsy (Novakowski et al., 1963). All eight exhibited subcutaneous edematous swellings mostly in the underbelly area. In five of the animals there were petechiae on the spleen, although the organ was not enlarged and was normal in consistency. In seven of the animals the lymph nodes in the regions of swelling were hypertrophied and slightly hemorrhagic. The intestinal tracts of all the animals were congested but the consistency of the feces was normal. In two of the animals the rumen contained a large quantity of fluid. Other organs, such as the liver, pancreas, kidneys and bladder, were congested. In all cases the blood clotted normally.

During the 1962 outbreak, an autopsy was performed on a fresh carcass by Dr. Lewis (Novakowski et al., 1963). The published account reported edematous swellings and a bloody discharge from the body openings. There was some degree of rigor mortis in the bloated animal. The spleen was twice its normal size and hemorrhagic, and the other organs were congested. The blood was dark and failed to clot. Among the HAB files are Dr. Lewis' notes on the post-mortem and the original specimen record which expand on the truncated published account. Dr. Lewis estimated the animal had died approximately 7 hours before examination and had not even had time to completely bloat. He observed a generalized septicemia with hemorrhaging and yellow gelatinous exudates under the skin. The lymph glands were enlarged and hemorrhagic and the spleen was twice its normal size and almost black in colour. The liver was congested and there was a bloody discharge from the animal's anus. *Bacillus anthracis* was successfully isolated from spleen, liver, lymph and gelatinous exudate specimens collected from the carcass.

A post-mortem was performed by CWS personnel on the first carcass found during the 1963 epizootic. The biologists noted the cadaver was extremely bloated and had a bloody discharge issuing from its nose but had no significant edematous swelling. The blood of the carcass was dark and failed to coagulate. Upon necropsy some subcutaneous hemorrhaging was noted and there were hemorrhagic petechiae on the liver.

The spleen had ballooned to six times its normal size and was filled with non-coagulated blood. *Bacillus anthracis* was successfully isolated from ear and spleen specimens taken from the carcass.

Together, the limited necropsies indicate bison infected with anthrax experience a terminal hemorrhagic septicemia. There is generalized hemorrhage throughout the body accompanied by subcutaneous gelatinous edema. The lymph nodes, especially near areas of edematous swelling, are hypertrophied and hemorrhagic. The blood is dark and often fails to clot. The spleen is enlarged and darkened and the other viscera may be congested or display hemorrhagic petechiae. Carcasses bloat rapidly after death and rigor mortis is often incomplete. There may be variable discharges of blood or white froth from the natural body openings and serosanguinous fluid may accumulate in the digestive tract or abdominal cavity. Although these symptoms are suggestive of anthrax in bison, especially when accompanied by mass-mortality, they are not diagnostic. Only bacteriological isolation of *B. anthracis* from the carcass is truly conclusive for the disease.

OTHER SPECIES

Although the northern anthrax outbreaks appeared to selectively affect bison, individual cases of anthrax have also been diagnosed in moose (*Alces alces*). Anthrax in moose has only been recorded near the end of the largest epizootics in 1963, 1964 and 1993 (Choquette, 1970; Gates et al., 1995). This suggests the disease "spilled over" into the moose due to high levels of contact between moose and bison carcasses directly or to large-scale contamination of the local environment with spores from the numerous carcasses. Dr. Norton reported a single moose carcass was found by clean-up crews during the 1962 epizootic. The animal's date of death was estimated around August 12, near the end of the epizootic. Unfortunately, the carcass was quite decomposed precluding the collection of specimens for bacteriological investigation and its actual cause of death remained unknown.

Despite large-scale scavenging during the outbreaks, no cases of anthrax have been observed in northern carnivore and omnivore species. The northern outbreaks

provide a very large, readily accessible source of contaminated food that attracts many scavenging species, but there has been no evidence of disease in these species despite the fact that they literally gorge themselves on the carcasses. The carcass of a black bear (*Ursus americanus*) was found at Hook Lake near the end of the 1962 outbreak, but advanced decomposition precluded the collection of specimens for culture to determine whether anthrax played a role in its death.

Anthrax spores have been isolated from the digestive tracts of ravens (*Corvus corax*) and herring gulls (*Larus argentatus*) shot while feeding on bison carcasses but the birds did not show any signs of disease (Broughton, 1987; Gates et al., 1995). Because of these findings, intestinal carriage by avian scavengers has been implicated in the spread of *B. anthracis* spores between the northern epizootic regions (Choquette et al., 1972). There has been little sampling of non-avian scavengers for carriage of anthrax in northern Canada. Dr. Gallivan reported anal and oral swabs were collected from the carcass of a black bear approximately 48 hours after it had been shot feeding on a carcass during the 1971 epizootic but the culture results were negative. During the same epizootic, oral and anal swabs were collected from a wolf that had been shot but *B. anthracis* was not isolated. During the 1962 outbreak Dr. Norton reported that a fox (*Vulpes vulpes*) showing no signs of fear and acting strangely had approached one of the clean-up crews. The workers feared the animal was rabid and shot it. The whole carcass was subsequently tested for both anthrax and rabies, but the laboratory was unable to isolate either microbe. The fox had undoubtedly been feeding on bison carcasses and may have been carrying spores on its fur or in its intestinal tract, but diagnostic testing was done only on the blood and spleen. The role of scavengers as mechanical vectors of anthrax spores during epizootics remains unclear.

Biting insects have also been implicated in the transmission of anthrax in northern Canada (Pyper and Willoughby, 1964; Cousineau and McClenaghan, 1965; Broughton, 1992). In their correspondence, Drs. Lewis, Norton and McClenaghan all felt that while insects were not responsible for the initiation of the outbreaks, they may have been involved in its spread and maintenance. Horseflies (Tabanidae) were particularly singled

out as possible vectors of disease. Towards the end of the 1962 clean-up operations field workers collected numerous insect species from carcasses and sent them south for bacteriological testing. The collection included mosquitoes and deerflies but no horseflies which had disappeared the week before collection with the arrival of cooler weather. *Bacillus anthracis* was not isolated from any of the insect species submitted.

DEPOPULATION PROGRAMS

After the 1964 epizootic, an attempt was made to create a buffer zone in the Grand Detour region. WBNP and CWS officials believed that mixing between bison in the northeast portion of the Park and Hook Lake region occurred only occasionally and theorized that the spread of anthrax could be contained by a buffer zone between the two groups; even though anthrax deaths had already occurred in bison within WBNP itself.

Van Camp and Calef (1987) reported that in November 1964 and March 1965, 189 and 333 bison, respectively, were slaughtered in the Grand Detour region. However, within a few months a similar number of bison had moved back into the depopulated area. Health of Animals Branch veterinarians were present at both slaughters to inspect the slaughter from a meat safety and animal welfare perspective . The field reports of the veterinarians confirm the number of animals killed in the March 1965 hunt but are at odds on the number of bison culled during the November hunt. One report listed that 173 bison were slaughtered in November while a second stated that closer to 250 animals were killed. In a letter written after both slaughters Mr. Phillips of the Department of Northern Affairs and National Resources (NANR) gave an official total of 554 bison for both culls. This final number appears to be the most accurate, however, it is unknown how the total breaks down between the two culls.

Dr. Gallivan reported that bison were also shot by NANR personnel during the 1971 outbreak in an effort to minimize the risk of the outbreak spreading beyond the Hook Lake area. Between July 1 and July 4 at least 44 bison were shot in the region. The carcasses were not processed by clean-up crews but were left on site to be consumed by scavengers.

VACCINATION ROUNDUPS

An anthrax vaccination program was initiated in the bison herds of Hook Lake and WBNP in 1965 (Carbyn et al., 1993). A Sterne-type live spore vaccine was used on the animals at the same dosage and immunization schedule (once a year) as for cattle. Herds of bison were directed with a helicopter into permanent corrals constructed at Hook Lake and at Hay Camp, Lake One and Sweetgrass Station in the Park. The animals were run through a chute system where they were branded, vaccinated and then released. Between 1965 and 1977, a total of 26,977 bison were vaccinated against anthrax in northern Canada (Table 2).

While detailed records were kept of the number of bison successfully vaccinated during each roundup, the records for the total number of animals herded and the number of handling-related mortalities were less complete, especially earlier in the program. As a result, it is difficult to assess the impact of the roundups. The minimum number of animals herded during the roundups was 33,653 head, and included calves, yearlings and bison which had already been vaccinated in previous roundups that year. Although these latter groups were not vaccinated they could not be cut from the herd beforehand and were subjected to the same handling procedures and risk of injury as the rest of the herd. The mean mortality rate for roundups where deaths were observed was 2.0% of the animals herded. Extrapolating this value to roundups where the number of deaths was not recorded provides an estimated total mortality of 828 animals.

The extrapolation assumes a relatively constant rate of mortality between roundups. Dr. H.C. Hopf of the HAB indicated in his field reports in 1965 that mortality rates differed significantly between the Hook Lake and Lake One corrals, as the latter had a large boggy area at its entrance where many bison went down and were trampled. He also reported that during some of the roundups the animals were herded over great distances and arrived at the corrals exhausted which lead to increased mortalities. In 1972, Dr. G.A. Baux of the HAB reported that the mortality rate increased with the number of bison held in the corral. The large number of animals herded during the 1972 Sweetgrass Station roundup resulted in overcrowding in the corral and an elevated

mortality rate of 5.9%.

The estimates of round-up related mortalities only take into account cases that were readily observable by roundup personnel. Wardens believed that only a small portion of roundup-related deaths were actually observed (Carbyn et al., 1993). While physical injury such as trampling losses and goring wounds could have been rapidly fatal and easily noticed, other deaths, such as those due to exertional myopathies or separation of calves from their dams could have occurred days later and would not have been detected.

For most of the roundups the vaccinated bison were horn- or hip-branded for subsequent identification, however, there is no published data available on the percentage of bison revaccinated year to year. Dr. Choquette of the CWS mentioned in a letter to Dr. Lewis that only two (1.3%) of the 149 bison vaccinated at Hook Lake in September 1965 had been vaccinated the previous March. Dr. Gould estimated in a field report that approximately half of the 1414 bison vaccinated at Sweetgrass Station in February 1966 were revaccinates based on the presence of a horn-brand. The vaccination program at Hook Lake later that year could only identify 49 (8.5%) of the bison as revaccinates. Identification of the Hook Lake revaccinates was difficult because the animals vaccinated the previous year had been hip-branded and for most animals this area was obscured by a thick winter coat which had not yet shed. Overall, the HAB files suggest, at least for the first few years of the program, the revaccination rate was never greater than 50%.

Personnel involved in the vaccination program stated that it was unlikely that more than a third of the bison in the areas of the corrals were vaccinated in any given year (Choquette and Broughton, 1967). However, it was felt that the vaccination rate attained would likely prevent a large explosive outbreak in these areas through herd immunity (Choquette and Broughton, 1967; Choquette, 1970). It is difficult, however, to evaluate the success of the vaccination program. During the years the program was run, only one case of anthrax was recorded in the areas receiving coverage. By the 1971 outbreak the vaccination program had been scaled back to roundups at Sweetgrass Station only and animals in the affected Hook Lake region had not been vaccinated in five years (Carbyn et al., 1993). Sterne (1959) noted that whenever any large-scale immunization is undertaken

without strict nonimmuinized controls, it becomes impossible to statistically prove that a decrease in disease prevalence is due to the immunizations. The bison vaccination program was eliminated in 1977 to counter public criticism of mounting bison losses due to the roundups and to save money on what was perceived to be an ineffective program (Carbyn et al., 1993).

According to the HAB files at least 1102 bison died of anthrax between 1962 and 1971. Another 283 animals died of anthrax during outbreaks in 1978, 1991 and 1993 (Broughton, 1987, 1992; Carbyn et al., 1993; Gates et al., 1995). A further 598 animals were also killed during depopulation programs in the Grand Detour and Hook Lake regions and a minimum estimate of 828 animals died during anthrax vaccination roundups. These secondary deaths must also be taken into account in assessing anthrax's impact on the northern bison herds. The final total of 2811 bison mortalities is based only on observed carcasses, and is certainly an underestimate of the actual total losses incurred.

It has been over twenty years since anthrax was last observed in the Hook Lake, Grand Detour and Lake One regions and it may be tempting to assume these regions are no longer endemic for the disease. However, all five of the affected areas are remote and experience very little human activity from the end of June to September when outbreaks have typically occurred (the so-called anthrax season). The Hook Lake and Grand Detour regions have not received systematic surveillance for the disease since the mid-1980s. Today the two regions are subject to a single yearly herd composition study at the beginning of the anthrax season and are unmonitored for the rest of the summer. In contrast during the anthrax season fire patrol/anthrax surveillance flights are flown bi-weekly over the endemic areas in the Park and Sanctuary. The herds of WBNP have also been the subject of an extensive tuberculosis and brucellosis study for the last three years during which time they were closely monitored with radio telemetry and all observed mortalities were investigated. Similarly, in the three years following the outbreak in the Sanctuary, its herds were intensely monitored with the help of radio-collars and all observed carcasses were sampled for anthrax.

Even without apparent outbreaks it is very likely *B. anthracis* remains in the

regions. The microbe is capable of producing highly resistant spores which may remain viable and infective for decades in the soil before coming into contact with and infecting a new host. Because of this all five northern regions must still be considered endemic for the disease and the resident bison herds remain at risk. Coincidently, at the time of this writing a road survey crew working in the Park to the west of the Lake One region discovered 12 dead bison. Park wardens collected specimens from the animals to ship south for bacteriological examination and identified ten more carcasses in the general area. To no one's surprise *B. anthracis* was isolated from the carcass specimens. By the end of August, the carcasses of 106 bison and 3 moose had been found along the Peace River within the Park.

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Table 1. Number of bison carcasses found during anthrax outbreaks in northern Canada.

Year	Region affected					All Regions
	Hook Lake	Grand Detour	Park Central	Lake One	Mackenzie Bison Sanctuary	
1962	281	-	-	-	-	281[a]
1963	15	242	47	-	-	304
1964	44	259	49	11	-	363
1967	-	-	-	120	-	120[b]
1968	-	-	-	1	-	1[b]
1971	33	-	-	-	-	33
1978	12	27	40	-	-	79[c]
1991	-	-	32	-	-	32[d]
1993	-	-	-	-	172	172
Total	385	528	168	132	172	1385

[a] Novakowski et al., 1963.

[b] Choquette et al., 1972.

[c] Broughton, 1987.

[d] Broughton, 1992.

[e] Gates et al., 1995.

Table 2. Bison vaccinations against anthrax at Hook Lake and in Wood Buffalo National Park from 1965 to 1977.

Date	Location	Animals herded	Animals vaccinated	Known deaths[b]
March 1965	Sweetgrass	1623	1512	18
March 1965	Hay Camp	-	249	-5
May 1965	Lake One	2215	1441	111
May 1965	Hook Lake	-	700	7
September 1965	Hay Camp	-	149	-3
October 1965	Hay Camp	-	109	-2
1965	Sweetgrass	-	206	-4
February 1966	Sweetgrass	1455	1414	41
June 1966	Hook Lake	-	577	-12
June 1966	Lake One	3587	2173	-72
June 1968	Sweetgrass	-	210	-4
July 1968	Sweetgrass	-	744	-15
July 1969	Sweetgrass	-	3021	-60
February 1970	Sweetgrass	-	595	-12
June 1970	Sweetgrass	3921	2857	44
June 1971	Sweetgrass	947	779	18
June 1972	Sweetgrass	5500	3612	326
June 1972	Hook Lake	1154	870	23
February 1973	Sweetgrass	1084	1056	7
June 1973	Sweetgrass	3463	2887	16
June 1975	Sweetgrass	386	337	3
June 1976	Sweetgrass	1110	933	17
June 1977	Sweetgrass	648	546	8
Total		33653[a]	26977	639 (828)

[a] Where the number of animals herded was unknown, the number of animals vaccinated was substituted.

[b] Values in parentheses are extrapolations based on a mean mortality rate of 2.0%.

Evaluation of Extraction and Spore Purification Methods for Selective Recovery of Viable *B. anthracis* Spores

D.C. Dragon and R.P. Rennie

ABSTRACT

PLET medium was evaluated for recovery of spores of a number of environmental and clinical *Bacillus* isolates. The medium was inhibitory to most non-*anthracis Bacillus* but failed to distinguish *B. anthracis* ATCC 4229 from *B. mycoides* MU 711/84, *B. thuringiensis* QC 12093, *B. subtilis* 1A289 and environmental strains of *B. pumilus* and *B. circulans*. The addition of 5% horse blood to PLET medium improved its ability to differentiate *B. anthracis* from other strains but decreased its inhibitory capacity making the modified medium unsuitable for environmental screening. Ethanol purification of spores was less sensitive to incubation time than the heat purification regularly used in spore isolation. With seeded sterile soil samples, high specific gravity sucrose plus nonionic detergent solutions extracted significantly more spores than de-ionized water alone. However, in further comparisons both sucrose plus detergent and water extraction proved equally sensitive at detecting anthrax spores in spiked and naturally seeded soil specimens.

INTRODUCTION

In nature *Bacillus anthracis* forms endospores which are highly resistant to many physical forces and degradative agents. These attributes may be useful for isolation of the organism from the environment, either from animal carcasses or from soil or other environmental samples.

The method of choice for detection of *B. anthracis* spores in soil and other environmental specimens is selective culture on PLET (polymyxin, lysozyme, ethylenediaminetetraacetic acid, thallium acetate) medium developed by Knisely (1966). Thallium cations in the medium are thought to be selective for the growth of *B. anthracis* in the presence of EDTA. Despite its widespread use in epidemiological studies and in

diagnostic laboratories, there have been few in-depth studies of PLET medium and the extraction method employed. Manchee et al. (1981) suggested a sensitivity limit of 3 spores/g of *B. anthracis* from environmental samples. The value was a theoretic limit based on sample dilutions, and assumed that water was able to extract all spores present and that all anthrax spores would germinate equally on the medium. Although polymyxin and lysozyme in the medium inhibit non-*Bacillus* contaminants, vegetative contamination is not generally a problem as samples are heat treated prior to inoculation at temperatures high enough to kill most vegetative cells but sub-lethal for spores. Concerns have been raised over the suitability of heat treatment for the purification of spores. Heat treatment will not destroy thermophilic contaminants and it has been noted that heat resistance varies among the spores of *Bacillus* species (Norris et al., 1981).

This study was designed to investigate ways to improve the standard anthrax spore isolation procedure at either the extraction, purification or culturing steps by utilization of specific characteristics of *B. anthracis* spores and vegetative cells. These included lack of haemolysis on blood containing medium, high buoyant density of bacterial endospores and the effect of detergents on separation of spores from soil particles. *Bacillus* spores are also resistant to ethanol while most vegetative micro-organisms are not and treatment with 50% ethanol was tested as an alternative to heat purification.

MATERIALS AND METHODS

Bacterial strains. Environmental isolates of *B. brevis*, *B. cereus*, *B. circulans*, *B. licheniformis*, *B. pumilus* and *B. subtilis* were kindly donated by Karen Emde of the University of Alberta Civil Engineering Environmental Laboratory. Avirulent, toxin-negative *B. anthracis* ATCC 4229 was generously provided by the Animal Diseases Research Institute (ADRI), Canadian Food Inspection Agency, Lethbridge, Alberta. All remaining *Bacillus* strains used in the experiments (Table 1) as well as *Pseudomonas aeruginosa* S169-90 were obtained from the clinical isolate collection of the Microbiology and Public Health Laboratory, University of Alberta, Edmonton, Alberta.

***Bacillus* endospore production.** Endospore stocks of each *Bacillus* strain were prepared

using modified Tarr's sporulation agar (Tarr, 1933; Claus and Berkeley, 1986). The sporulation medium was formulated as follows: 1.5 g sucrose, 1.5 g dibasic ammonium phosphate, 1.5 g sodium chloride, 0.75 g magnesium sulfate heptahydrate, 7.5 g monobasic potassium phosphate, 75 mg manganese sulfate monohydrate, 150 mg calcium chloride dihydrate, 2 mg ferric chloride hexahydrate, 1500 ml de-ionized water and 11.25 g Bacto-agar. The medium was autoclaved for 20 min at 121°C then allowed to cool to 50°C and the pH was adjusted to 7.4. The stocks were incubated for 14 days at 37°C.

Spore stocks were isolated and washed twice with 10 ml of 0.85% physiological saline. At each wash the spores were centrifuged at 5100 x g for 10 min at 4°C and vortexed for 30 s to re-suspend the spores. Slide smears were prepared from each stock and treated with Gram and spore stain to confirm the presence of endospores with minimal vegetative contamination (Hendrickson, 1985). Spore stocks were stored in saline at 4°C until required.

PLET culture. PLET medium was formulated according to Knisely (1966) and Carman et al. (1985), except 300 U/ml of chicken egg white lysozyme (Sigma, St Louis) was used instead of 40 μg/ml. The medium was stored at 4°C and checked weekly against positive (avirulent *B. anthracis* ATCC 4229) and negative (*B. subtilis* ATCC 6633) controls to insure that it retained its selectivity. Aliquots of each *Bacillus* spore stock were inoculated to plates of sheep blood agar (SBA), PLET and PLET supplemented with 5% de-fibrinated horse blood. All cultures were incubated at 37°C and observed one and two days after inoculation.

Isolation of *B. anthracis* spores from seeded sterile soil. Avirulent *B. anthracis* ATCC 4229 spores were added to autoclaved samples of potting, wallow and field soil and used to test the efficacy of sucrose solutions of various specific gravity with and without 0.5% (v/v) Triton X-100 (Sigma, St Louis) or Nonidet P-40 (Sigma, St Louis) for recovery of spores. All wash solutions were autoclaved for 20 min at 121°C which did not effect the specific gravity of the sucrose solutions. The potting soil was a commercially available, acidic, general-use soil comprised of 50% peat and 50% loam. The wallow and field soil types were collected from an open glacial lake bed surrounding Falaise Lake in the

Mackenzie Bison Sanctuary, Northwest Territories, site of a large anthrax epizootic in 1993 (Gates et al., 1995). Both soil types were alkaline, clay based and calcareous due to a high content of crushed mollusk shells. The wallow soil came from within a bison wallow where the rolling and pawing of the animals had denuded the area of vegetation.

Aliquots (2.5 g) of each soil were autoclaved for 25 min at 121°C. After cooling, 0.5 ml of a diluted saline suspension of *B. anthrcis* ATCC 4229 containing 2-8 x 10^5 spores was seeded to each sample and the samples were shaken vigorously to mix. The seeded specimens were stored in the dark for three days at room temperature prior to extraction experiments.

For extraction, 30 ml of wash solution was added, the sample was shaken vigorously for one min and then agitated on a shaker for 15 min at 75 rpm. The samples were then centrifuged at 850 x g for 45 s to settle surface foam. With sucrose wash solutions, the top three ml of supernatant were transferred to a new tube containing six ml of filtered 1% bovine serum albumin in 0.01 M phosphate buffered saline, pH 7.2 (BSA/PBS) to reduce the specific gravity and allow collection of spores by centrifugation. For non-sucrose solutions the top three ml of supernatant were transferred to an empty tube. The new tubes were gently vortexed and then centrifuged at 5100 x g for 10 min. The supernatant was discarded and the pellet was re-suspended in one ml of 1% BSA/PBS.

In experiments with seeded sterilized soil samples no heat or ethanol purification was performed and the samples were plated on SBA rather than PLET in order to maximize germination of the spores recovered. The SBA plates were incubated for 24 hr at 37°C when the colonies were counted and the percentage of spores recovered was calculated. The sensitivity limits of PLET medium with the standard water or 1.22 g/ml sucrose plus 0.5% Triton (sucrose/Triton) extraction were compared using sterilized 2.5 g wallow and field soil samples seeded in a dilution series with 10^4 to 10^1 *B. anthracis* ATCC 4229 spores. Extraction was as described above except the samples were plated on PLET medium.

Bactericidal and sporicidal effects of ethanol- and heat-purification and

sucrose/Triton extraction. Stocks of *B. anthracis* ATCC 4229 spores and overnight vegetative cultures of *B. cereus* ATCC 14579 and *Ps. aeruginosa* S169-90 were prepared in 1% BSA/PBS. The concentration of each stock was determined by serial dilution and overnight incubation on SBA plates. Multiple 1.0 ml aliquots of each stock were heated for 20 min in a 63°C water bath. A second set of 1.0 ml samples were centrifuged at 5100 x g for 10 min and the supernatant was replaced with 1.0 ml of filtered 50% ethanol. The microbes were re-suspended in the ethanol and gently agitated for one hr at room temperature. The samples were then centrifuged as before and the ethanol was replaced with 1.0 ml 1% BSA/PBS. A third set of 1.0 ml samples were centrifuged and the pellets were re-suspended in 350 µl of 1.22 g/ml sucrose/Triton. The samples were gently agitated for 15 min at room temperature and then 700 µl of 1% BSA/PBS was added to each tube to reduce the specific gravity of the samples. The samples were spun down and the pellets were re-suspended in 1.0 ml 1% BSA/PBS. Samples from all three treatments were then serially diluted, plated on SBA and incubated overnight at 37°C.

Comparison of spore extraction and purification methods in non-sterilized soil samples. Water and 1.22 g/ml sucrose/Triton extraction solutions and heat and ethanol spore purification were compared for their ability to detect *B. anthracis* spores in positive and negative soil specimens collected from anthrax carcass disposal sites in northern Canada. Some of the specimens had been previously shown to contain *B. anthracis* spores by 1.22 g/ml sucrose/Triton extraction, ethanol purification and PLET culture. Negative soil specimens from disposal sites that yielded multiple positive samples were included in the tests in case they did contain anthrax spores that the first screening had missed. These experiments were performed under full Level III biosafety precautions in the anthrax diagnostic laboratory at ADRI, Lethbridge.

Eighteen soil specimens were divided into duplicate 3.0 g samples and extracted with 12 ml of 1.22 g/ml sucrose/Triton or de-ionized water. The extraction was as described above except the final isolated pellet was re-suspended in 1.0 ml of 50% ethanol and shaken one hr at 75 rpm at room temperature. The samples were then centrifuged at 5100 x g for 10 min and the ethanol was replaced with 1.0 ml of 1% BSA/PBS. One

hundred µl of each sample were spread on PLET plates which were then incubated for 48 hr at 37°C.

A second set of 18 soil specimens were divided into duplicate 3.0 g samples and extracted with 12 ml 1.22 g/ml sucrose/Triton. One set of samples was treated with 50% ethanol for one hr as described above. The other set was treated with heat to purify the spores present. The extracted pellet was re-suspended in 1.0 ml 1% BSA/PBS and heated for 20 min 63°C water bath. Afterwards, 100 µl aliquots of each sample were spread on PLET plates and incubated for 48 hr at 37°C.

The identity of colonies isolated from the non-sterilized soil specimens with a morphology similar to *B. anthracis* on the PLET plates was confirmed by lack of haemolysis on SBA, a zone of inhibition around a 10 µg penicillin G disc and development of mucoid, encapsulated colonies on bicarbonate agar as determined by Giemsa stain (Carman et al., 1985). Final identification was achieved through multiple-locus variable-number tandem repeat analysis which was performed by P. Corker, M. Hugh-Jones and P. Keim (Keim et al., 2000).

Statistical analyses. All statistical analyses were performed using SigmaStat 1.0 (Jandel Scientific, 1992) with the alpha value of each test set at 0.05. In experiments where a significant difference between treatment groups was detected via a one-way ANOVA, multiple pair-wise comparisons were performed using the Student-Newmann-Keuls method. In figures, the variance of each treatment is depicted by standard error.

RESULTS

Evaluation of PLET medium. PLET medium allowed few non-*anthracis Bacillus* strains to grow (Table III-5). *Bacillus anthracis* ATCC 4229 formed white, circular colonies two to three mm in diameter on the medium after overnight incubation. By 48 hr of incubation the colonies had domed up on the medium and were five to eight mm in diameter. None of seven *B. cereus* strains tested on the medium formed colonies after 48 hr of incubation, however, the strains tested from two other species of the *B. cereus*-group, *B. mycoides* MU711/84 and *B. thuringiensis* QC12093, both formed colonies after

one day that were indistinguishable from those of *B. anthracis* ATCC 4229. One of four *B. subtilis* strains tested grew within 24 hr into colonies morphologically similar to ATCC 4229. Environmental strains of *B. brevis* and *B. licheniformis* grew within 24 hr but remained as micro-colonies that were easily distinguished from ATCC 4229. Environmental isolates of *B. circulans* and *B. pumilis* formed colonies resembling ATCC 4229 on PLET medium but their formation was delayed; colonies were not observed until after 48 hr of incubation. Thus, if growth on the medium after 24 hours incubation at 37°C was included in the selection criteria, then PLET medium could differentiate *B. anthracis* ATCC 4229 from all test strains except *B. mycoides* MU 711/84, *B. thuringiensis* QC 12093 and *B. subtilis* 1A289. If incubation time was not included in the criteria, then the environmental isolates of *B. pumilus* and *B. circulans* would also be indistinguishable from *B. anthracis* ATCC 4229 on the medium. SBA recovered significantly more spores of *B. anthracis* ATCC 4229 than PLET medium ($p < 0.001$) even after 48 hr of incubation (Figure III-10).

On PLET medium supplemented with 5% horse blood, *B. anthracis* ATCC 4229 formed non-haemolytic, slightly irregular, cream colonies 2 to 3 mm in diameter after 24 hr incubation. Three strains of *B. cereus* formed colonies on the medium after 48 hr of incubation, however, the strains were readily distinguishable from *B. anthracis*; two formed β-haemolytic colonies and the third remained a micro-colony. *Bacillus mycoides* and *B. thuringiensis* also grew on the medium but their colonies were β-haemolytic. Blood supplemented PLET allowed more non-*anthracis* test strains to germinate and grow; 10 of 18 strains versus 7 of 20 strains tested on regular PLET medium. However, incubation on the supplemented medium allowed for the selective differentiation of *B. anthracis* ATCC 4229 from all other test strains. When the incubation was extended to 48 hr, differentiation between *B. anthracis*, *B. subtilis* 1A289 and the *B. pumilus* environmental strain was lost.

Extraction of *B. anthracis* spores from soil. Against field soil the combination of detergent and high specific gravity sucrose recovered significantly greater numbers of spores than de-ionized water, detergent or sucrose alone (Figure III-11; $p < 0.001$).

Increasing the specific gravity of the sucrose-detergent solutions resulted in increased spore recoveries. Sucrose-detergent solutions with a specific gravity of 1.18 g/ml recovered 1.5 to 2.0% of the seeded spores while 1.22 g/ml sucrose-detergent solutions recovered over 4.0% of the spores.

Against potting soil the extraction solutions recovered higher percentages of spores than against field soil (data not shown). Water recovered approximately 13% of the seeded spores while detergent only and sucrose (specific gravity up to 1.22 g/ml) only solutions recovered between 15 and 23% of spores. Sucrose-detergent solutions of 1.18 g/ml and 1.22 g/ml specific gravity recovered 22 to 28% of the seeded spores. Recoveries with the sucrose-detergent solutions were significantly greater than with water ($p < 0.001$). The various wash solutions recovered between 5 and 8% of the spores seeded to wallow soil (data not shown) and there was no significant difference in their spore recoveries ($p = 0.1831$).

Extraction with 1.22 g/ml sucrose/Triton was compared against standard water extraction in sterile field and wallow soil samples seeded with decreasing amounts of *B. anthracis* ATCC 4229 spores (Figure III-14). The extraction solutions were equally sensitive detecting down to 40 spores/g in both soil types.

Bactericidal and sporicidal effect of sucrose/detergent and heat and ethanol purification methods. The 1.22 g/ml sucrose/Triton extraction solution was tested against representative Gram-positive (*B. cereus* ATCC 14579) and Gram-negative (*Ps. aeruginaosa* S169-90) vegetative cells and *B. anthracis* ATCC 4229 spores to determine if high osmolarity sucrose plus detergent had a detrimental effect on the organisms (Figure III-15). At the same time the microbial stocks were treated with heat and ethanol to determine their bactericidal and sporicidal effects. Exposure to sucrose/detergent resulted in a 100-fold reduction in the concentration of the *B. cereus* and *Ps. aeruginosa* stocks. Treatment with heat and ethanol completely killed the *Ps. aeruginosa* stock and resulted in a 100-fold reduction in the *B. cereus* vegetative cells. The *B. cereus* vegetative cells were obtained from an overnight broth culture and it was thought the microbe would still be in a logarithmic growth phase with few spores present. However, subsequent spore

staining of the *B. cereus* stock revealed that approximately 1% of the vegetative cells contained fully formed endospores. It therefore appears that the three treatments killed the vegetative cells present in the *B. cereus* stock but not the spores. Neither exposure to sucrose/Triton, ethanol nor heat resulted in a change in the *B. anthracis* spore concentration.

Comparison of water and sucrose/Triton extraction, and heat and ethanol purification in naturally seeded soil samples. No significant difference was found between de-ionized water and 1.22 g/ml sucrose/Triton in extracting spores from soil samples collected from an anthrax endemic region; five of which had been demonstrated to be contaminated with anthrax spores. The organism was isolated from four of the five positive samples with sucrose-detergent and from two with water. There was no significant difference in the number of contaminant organisms observed with either wash solution (t-test; $p = 0.9846$); 141.2 ± 29.2 and 142.1 ± 31.4 contaminant colonies grew on PLET plates inoculated with water and sucrose/Triton extractions, respectively.

There was no significant difference in the ability of heat and ethanol treatment to purify anthrax spores from soil samples collected from the endemic region; four of which were positive for anthrax spores. *Bacillus anthracis* was isolated from all four positive samples with ethanol and from two positive samples with heat treatment. There was no difference between the number of contaminants surviving either treatment (t-test; $p = 0.4184$); 161.1 ± 31.4 and 199.3 ± 34.5 contaminants developed on PLET plates after heat and ethanol treatment, respectively.

Even single colonies of *B. anthracis* stood out for easy identification amongst roughly 150 contaminating colonies after 48 hr of incubation on PLET. Prior to 48 hr, however, it was impossible to differentiate colonies, even when plates were viewed under a dissecting microscope.

DISCUSSION

Selectivity and sensitivity of PLET medium in the detection of *B. anthracis* spores. Published literature on the selectivity of PLET medium against non-*anthracis Bacillus*

species is limited. There has been little careful investigation in this area in the past 30 years. Knisely (1966) reported that the medium successfully recovered 21 of 22 *B. anthracis* strains after 24 hr of incubation at 37°C with the remaining strain recovered after 48 hr. The medium inhibited growth of 10 of 11 strains of *B. cereus* however the strain that did grow was morphologically indistinguishable from *B. anthracis*. The medium also failed to inhibit the growth of a "lysozyme-resistant" strain of *B. megaterium* and three strains of *B. subtilis* var. *niger* although Knisely did not state whether these strains could be morphologically distinguished from *B. anthracis*. All other *Bacillus* strains tested by Knisely; including five strains of *B. mycoides* and two strains of *B. thuringiensis*; failed to grow on the medium.

PLET medium used in this study allowed the growth of *B. anthracis* ATCC 4229 and inhibited all seven strains of *B. cereus* tested. However, the medium failed to inhibit strains from two other *B. cereus*-group species; *B. mycoides* and *B. thuringiensis*. The morphology of these two strains as well as *B. subtilis* 1A289 were indistinguishable from *B. anthracis* on the medium. Although only six environmental *Bacillus* isolates were tested, *B. pumilus* and *B. circulans* were also indistinguishable from *B. anthracis* ATCC 4229 on PLET. This finding raises concerns about differentiating *B. anthracis* from indigenous *Bacillus* species in environmental studies. Knisely (1966) only tested the medium against laboratory strains of non-*anthracis* species. Strains adapted to enriched culture medium in the laboratory may have lost the ability to scavenge cations or utilize thallium cations that was inherent to the wild parent strain. More laboratory studies of PLET medium should be performed against environmental isolates of non-anthrax *Bacillus* species that have received minimal culture in order to further address this issue.

Not all the anthrax spores germinated equally on PLET medium. Even after 48 hr of incubation, *B. anthracis* ATCC 4229 spore recovery on PLET was only 38% of the number recovered with nutrient-rich SBA from the same stock. McGetrick et al. (1982) also observed varying degrees of inhibition of *B. anthracis* spores on PLET medium; for five of 12 *B. anthracis* strains tested, PLET recovery of spore stocks was less than 40% of that recovered with enriched, non-selective medium. Thus, although PLET is selective for

B. anthracis, it is not an optimal recovery medium and may miss anthrax spores in a sample.

Although PLET medium was able to detect and differentiate *B. anthracis* ATCC 4229 spores from pure stocks in the laboratory after 24 hr of incubation, 48 hr of incubation was necessary to differentiate wild anthrax strains from contaminants in field samples. Thus, incubation time should not be used as a selective criterion for identification of *B. anthracis* on PLET in epidemiological studies. The addition of horse blood to PLET allowed for the differentiation of *B. anthracis* ATCC 4229 from all but two of the test strains; *B. subtilis* 1A289 and the *B. pumilus* environmental strain. However, the blood also decreased the selectivity of the medium possibly by providing cations that could chelate and inactivate EDTA in the medium or act as alternatives to thallium cations. The decreased inhibitory capacity of the supplemented medium increased the likelihood of *B. anthracis* colonies being overgrown and masked by contaminants and made the supplemented medium unsuitable for preliminary screening of specimens in epidemiological and environmental investigations.

***Bacillus anthracis* activation and purification.** One way to improve spore recovery is to pre-treat the sample to activate spores (Claus and Berkeley, 1986). The most common spore activator used is sub-lethal heat treatment. An alternative method for purifying spores from vegetative cells that is not commonly used is ethanol treatment (Holmes and Levinson, 1967). In our experiments, one hr incubation in 50% ethanol proved as effective a spore selective agent as 20 min at 63°C. Both treatments were equally bactericidal against Gram positive and Gram negative vegetative stocks yet neither reduced the viability of *B. anthracis* ATCC 4229 spores. Of interest, neither treatment improved spore recovery of *B. anthracis* (Fig. 4). Ethanol was as effective as heat in the purification of anthrax spores from non-sterile, naturally infected soil specimens and there was no significant difference in the number of spores isolated, the number of positive specimens identified or the number of surviving contaminants. Ethanol treatment, however, proved easier to perform and it was less time-sensitive; a 50% increase in incubation time did not significantly alter the outcome (data not shown).

***Bacillus anthracis* spore extraction from soil.** An earlier assumption (Manchee et al., 1981) that all anthrax spores could be recovered from an environmental sample with water were not borne out in this study. The percentage of seeded spores recovered with water from the three sterile soil types varied greatly suggesting that each had a different affinity for the spores.

It has been well established that *Bacillus* endospores, especially those of *B. cereus*-group species, are hydrophobic (Doyle et al., 1984; Koshikawa et al., 1989; Rönner et al., 1990; Wiencek et al., 1990). Husmark and Rönner (1990) identified that hydrophobic interactions were the predominant attractive force for spore adhesion to a variety of solid matrices. While no extraction solution allowed the recovery of all the seeded anthrax spores, solutions containing both non-ionic detergent (which disrupts hydrophobic interactions) and buoyant concentrations of sucrose to lift the freed spores improved spore recoveries, especially in the field soil. Although sucrose/detergent did not improve spore recoveries with wallow soil, this soil did not contain any loam (i.e., decaying plant matter) and the calcareous clay composition may contain few hydrophobic moieties that will bind spores. Similar results were obtained by Bowen et al. (1996) using a bi-phasic aqueous system with polyethylene glycol. Bowen suggested that the glycol disrupted hydrophobic interactions between anthrax spores and soil particles and then formed a buoyant hydrophobic layer into which the spores concentrated. Spore recoveries with the bi-phasic system were easier with simpler sandy soil than with loamy clay soil (similar to the field soil used here).

The number of positive anthrax samples from Northern Canada was limited and there were no significant differences observed in the extraction techniques tested with them. However, the experiments with seeded soil clearly demonstrated that sucrose-detergent extraction improved spore recovery in two of the soil types tested. Sucrose/detergent extraction and ethanol purification are viable additions to the PLET detection protocol and may improve the sensitivity of environmental screening for anthrax spores.

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Detection of Anthrax Spores in Endemic Regions of Northern Canada

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ABSTRACT

Bacillus anthracis spores were detected in 11 of 588 (1.9%) environmental specimens collected from anthrax endemic regions in northern Canada. All positive samples were associated with disposal sites of bison carcasses found during previous anthrax epizootics. Viable anthrax spores were found at three of six carcass burial sites surveyed in the Parson's Lake Road region of Wood Buffalo National Park and at four of eight carcass cremation sites examined in the Falaise Lake region of the Mackenzie Bison Sanctuary; no anthrax spores were recovered from decades-old anthrax mounds in the Hook Lake region. Anthrax spores were not recovered from 67 specimens collected randomly from bison wallows and meadows around Falaise Lake where carcass sites had not been reported. Anthrax spore concentrations of greater than 500 spores/g were associated with a red fox scat and soil specimens collected from a distinct bone bed found within cremation sites. At three of the four positive cremation sites, positive specimens were obtained only from within the bone bed.

INTRODUCTION

Bacillus anthracis is the causative agent of anthrax, an infectious, often fatal disease of wild and domestic animals, and man. *Bacillus anthracis* is global in its distribution with endemic regions on all continents except Antarctica. Central to the maintenance of the disease in an area is the bacterium's ability to form metabolically dormant endospores which are highly resistant to a number of environmental forces including temperature extremes, dessication, ultra-violet irradiation and many commonly used disinfectants. Anthrax spores may remain viable and infectious in the environment for years before coming into contact with a susceptible host and initiating a new cycle of disease.

In Canada, a large epizootic of the disease occurred in the free-roaming bison

(*Bison bison*) herds of the Hook Lake region of the Northwest Territories (NT) during the summer of 1962 (Figure I-2). Over the next two summers, anthrax outbreaks continued in the Hook Lake region and spread, first across the Slave River into the Grand Detour and Park Central regions and later across the Peace River to the Lake One region. Between 1962 and 1991, eight epizootics of anthrax were recorded in these four regions of the NT and northern Alberta resulting in the deaths of over 1200 bison and several moose (*Alces alces*) (Broughton, 1992; Dragon and Elkin, 2001). In 1993 a large epizootic occurred in the Mackenzie Bison Sanctuary (MBS) where 169 bison and three moose carcasses were found concentrated in five main areas: Slave Point, Boulogne Lake, Falaise Lake, Calais Lake and Mink Lake (Gates et al., 1995).

Research on anthrax in northern Canada has been limited to field observations during epizootics and cataloguing of carcasses. The source and movement of *B. anthracis* outside of the bison host is almost totally unknown. Bacteriological studies of environmental specimens from several of the northern endemic regions were initiated in order to determine the extent of anthrax spore contamination, to examine the ecology of *B. anthracis* spores and to assess carcass disposal methods.

MATERIALS AND METHODS

Environmental sampling and specimen storage. Between 1992 and 1997, field trips were undertaken to anthrax endemic regions in northern Canada to collect environmental specimens from carcass disposal sites and bison habitat. In August 1992 and July 1993, specimens were collected from anthrax mounds in the Hook Lake region and from burial sites along Parson's Lake Road (PLR) in central Wood Buffalo National Park (WBNP). Samples collected from the Park were obtained under research permit 92-13 (WBNP, Environment Canada; Environmental assessment registry number WB 92-32). In July of 1994 and 1997, trips were made into the Falaise Lake region of the MBS to collect specimens from carcass disposal sites under the authority of NT wildlife research permits WL001018 and WL001547. Soil specimens were also collected randomly from bison wallows and open meadows not associated with known anthrax carcass sites.

Each carcass site was visually surveyed and roughly mapped. A central wooden peg was hammered into the ground and environmental specimens were collected from around the site recording the distance and bearing of each relative to the wooden peg using a tape measure and compass. During surveying of disposal sites, the area was considered contaminated and appropriate safety precautions were taken. Personnel collecting specimens were vaccinated with the US anthrax vaccine preparation (Michigan Department of Public Health, Lansing, Michigan) and wore protective clothing comprised of rubber boots, disposal coveralls with hood and elastic cuffs, double-layered latex gloves, and a full-face HEPA-filtered M-95 respirator.

Environmental specimens collected were comprised of a heterogeneous mixture which included soil, charred bone, bison hair, animal feces, maggot casings, vegetation and ash. Samples were collected using disposable plastic spoons and were taken only to a maximal depth of five cm. The samples were carefully transferred to labelled Universal bottles; approximately 15 ml (3 to 12 g) of sample was collected in each bottle. The sample bottles were stored in a secure facility until such time as they could be screened for viable anthrax spores.

Spore extraction and *B. anthracis* identification. Bacterial endospore extraction and screening was carried out under full Level III biosafety precautions in the anthrax diagnostic laboratory at the Animal Diseases Research Institute (ADRI), Canadian Food Inspection Agency, Lethbridge, Alberta. Prior research by our laboratory with spores from an avirulent strain of *B. anthracis* indicated that flotation extraction with a high specific gravity sucrose plus Triton X-100 solution may be more effective at isolating spores than standard water extraction in soil types similar to those of the surveyed endemic regions (Dragon and Rennie, 2001). Research with avirulent spores also demonstrated that ethanol purification of spores from vegetative contaminants was as effective as standard heat treatment yet less stringent with regard to pre-incubation and exposure time. Flotation extraction and ethanol purification were used in conjunction with culture on PLET (polymyxin, lysozyme, ethylenediaminetetraacetic acid, thallium acetate) medium to isolate viable *B. anthracis* spores from the environmental specimens. The

spore extraction methods employed were as reported in a previous paper (Dragon and Rennie, 2001). PLET cultures were incubated at 37°C for 48 hr to optimize recovery of anthrax spores.

The identity of colonies isolated from the non-sterilized soil specimens with a morphology similar to *B. anthracis* on the PLET plates was confirmed by lack of haemolysis on blood agar plates, a zone of inhibition around a 10 μg penicillin G disc and development of mucoid, encapsulated colonies on bicarbonate agar as determined by Giemsa stain (Carman et al., 1985). Final identification was achieved through multiple-locus variable-number tandem repeat analysis (MLVA) which was performed by P. Corker, M. Hugh-Jones and P. Keim (Keim et al., 2000).

RESULTS

Screening of environmental specimens. One-hundred-sixty-nine environmental specimens were collected from five anthrax mounds in the Hook Lake area. Although the mounds were at least 14 yr old, they were still clearly visible on the surrounding flat meadows. Two mounds possessed sinkholes in their crowns and a third had a fresh burrow at its base although there was not sign of bone excavation. The mounds had multiple bison wallows about their bases which allowed for the collection of standing water even when the rest of the meadows appeared dry.

Bison carcasses were buried along PLR in predominantly old-growth pine and spruce forest during the 1991 anthrax epizootic. Six burial sites within 30 m of the road were sampled and 157 environmental specimens were collected. All six sites were in areas of loose-packed sand and, except for a few small sinkholes, appeared intact and undisturbed.

During the 1993 MBS epizootic bison carcasses were incinerated with either coal or wood and the remains were left unburied. One year after the epizootic, the cremation sites were covered in ash with the charred remains of logs about their perimeter. At each site, slightly offset from the centre, was a small bed of charred bones. At six of eight sites surveyed, there were thick mats of bison cape hair and large concentrations of maggot

casings underneath the bone bed. Although sometimes singed, the hair and casings were intact and undamaged. Four years after cremation, deciduous herbs and shrubs had overgrown the sites. One-hundred-ninety-five specimens were collected from eight cremation sites in the Falaise Lake region. In addition, 23 samples from bison wallows and 44 soil samples were collected from areas of meadow around the lake not associated with carcass sites.

After spore extraction and purification, samples were spread on PLET agar. Although colonies were observed on the plates after overnight incubation, it was impossible to differentiate colonies morphologically until after 48 hr of incubation. Of the 588 specimens screened with PLET medium, 174 (29.6%) exhibited domed, circular, white colonies four to eight mm in diameter that were morphologically similar to *B. anthracis* (Table III-8). The highest percentage of specimens with *anthracis*-like colonies were from Falaise Lake cremation sites and meadow samples while the lowest percentage was recovered from Hook Lake anthrax mounds.

Despite the selectivity of PLET medium, contamination of the plates was observed. The mean number of contaminants per plate, including *B. anthracis*-like colonies that were later discounted through confirmatory tests, was 191.2 ± 132.0, 12.7 ± 49.7, 160.0 ± 133.8 and 157.9 ± 118.8 for specimens from Hook Lake, Parson's Lake and Falaise Lake cremation sites and meadow samples, respectively. A one-way ANOVA test utilizing pairwise multiple comparisons via the Student-Newman-Keuls method demonstrated that significantly fewer contaminants were isolated with PLET medium from PLR specimens than from samples of the other regions ($P < 0.001$). PLET cultures of PLR specimens were also less likely to show any microbial growth after 48 hr of incubation. Eighty-six (54.8%) Parson's Lake samples failed to yield any colonies compared to 3 (1.8%) of Hook Lake samples, 11 (5.6%) of Falaise Lake carcass site specimens and none of the Falaise meadow samples.

Bacillus anthracis-like colonies from the PLET were picked and tested with blood agar plates and penicillin discs. Only 25 of the specimens yielded isolates that, like *B. anthracis*, were non-haemolytic and were inhibited to some degree by penicillin (range 7

to 40 mm zones of inhibition). None of the Hook Lake samples yielded non-haemolytic isolates that were inhibited by penicillin, while only three specimens each from PLR and the Falaise meadow and 19 specimens from the Falaise cremation sites exhibited colonies of this phenotype. None of the isolates from the three remaining Falaise meadow specimens formed mucoid colonies on bicarbonate plates after overnight incubation in 10% CO_2. Isolates from the three remaining PLR specimens and 11 of the 19 remaining Falaise cremation samples developed heavily mucoid colonies on the bicarbonate plates that were consistent with *B. anthracis*. Giemsa stained smears prepared from the bicarbonate cultures of 11 of the mucoid samples displayed rods with thick enveloping purple capsules compatible with *B. anthracis*. All of these eleven isolates were later confirmed to be *B. anthracis* via MLVA.

Location of positive *B. anthracis* isolates. The 11 *B. anthracis* positive specimens came from seven carcass disposal sites; three burial sites along PLR (50% of the sites in the region surveyed) and four (50%) cremation sites around Falaise Lake (Table III-9). No positive samples were isolated from Hook Lake anthrax mounds or Falaise meadow specimens. However, the sample size of meadow specimens was small and they were collected from an area roughly a km away from the closest positive disposal site.

In six (54.5%) of the eleven positive specimens, *B. anthracis* was detected at the sensitivity limit of the extraction/PLET culture procedure; 2 spores/g; represented by a single colony on the PLET medium. At five (71.4%) of the seven positive carcass disposal sites *B. anthracis* was isolated from a single environmental specimen. High levels of anthrax spores were associated with a red fox (*Vulpes vulpes*) scat and soil specimens collected from within the bone bed of cremation sites.

Site 16 was right along side PLR and was sampled in both 1992 and 1993 for a total of 47 specimens. The site has a small mound towards its centre approximately 60 cm high. The sole positive sample from this site, containing 800 anthrax spores/g, was a red fox scat found on top of the mound in 1992. The site was intact and no bison or other animal remains were observed in its immediate vicinity. Sites 22 and 23 were both located away from PLR under the forest canopy. From 22 and 25 specimens collected from sites

22 and 23, respectively, only a single *B. anthracis* colony was isolated. The positive specimen from site 22 was collected at the very edge of the disturbed sand while the positive specimen from site 23 was obtained from near the centre of the disturbed area.

At three of the four positive cremation sites at Falaise Lake, anthrax spores were closely associated with the charred bone bed. Sites 37 and 89 each yielded one positive sample with approximately 70 and 1200 viable anthrax spores/g, respectively, from within their bone beds. Site 41 yielded the most positive specimens with four, all of which were associated with the bone bed. As depicted in Figure III-29, the highest concentration of anthrax spores was found near the centre of the bone bed and the concentration of viable anthrax spores appeared to decrease toward the bed's periphery. Two positive samples representing 2 spores/g each were found at site 142. Both were located on the south side of the site well away from the bone bed.

DISCUSSION

The anthrax spore concentration of each positive sample was calculated from the number of *B. anthracis*-like colonies formed on PLET plates taking into account the percentage of isolates screened in confirmatory tests that proved to be *B. anthracis*. The theoretical sensitivity limit of the extraction and PLET culture procedure based on sample dilution was 2 spores/g, however, this assumes that the extraction procedure was able to isolate every anthrax spore in a given sample and that the spores were completely germinable on the PLET medium. Both assumptions are unlikely. Previous studies have demonstrated anthrax spores can form strong adhesions to soil particles and other solid matrices (Cole et al., 1984; Doyle et al., 1984; Bowen et al., 1996). While the flotation extraction method employed in this study was shown to be more effective at isolating anthrax spores than water from spiked soil samples, it was never successful at recovering 100% of the inoculated spores and recovery rates between soil types were variable (Dragon and Rennie, 2001). Compared to culture on enriched media, spores of several *B. anthracis* strains have been found to have reduced germinability on PLET medium (McGetrick et al., 1982; Dragon and Rennie, 2001). Therefore, the anthrax spore

concentrations listed in Table III-9 for positive environmental specimens can only be considered rough estimates of the true concentrations. However, relative to each other, the concentrations can still be used to identify high and low levels of anthrax spores in the environment.

Of the 588 environmental specimens collected, 11 (1.9%) were shown to contain viable *B. anthracis* spores. All 11 were directly associated with the disturbed area of carcass disposal sites in the PLR and Falaise Lake regions. The variance in anthrax recovery rates between the surveyed regions probably has more to do with differences in soil type and the amount of time elapsed between spore deposition and sample collection than with differences in carcass disposal methods. Hook Lake mounds were between 14 and 30 years old when sampled while PLR burial and Falaise cremation sites were less than two years old when samples were collected. The discrepancy in age allows a much greater opportunity for spores at the Hook Lake sites to have been either inactivated or dispersed by environmental forces. Although the PLR and Falaise sites were approximately the same age when sampled, the sandy soil of the PLR region may be poorly suited for the maintenance of spores (Dragon and Rennie, 1995).

Bacillus anthracis spores have been recovered from the feces of black-backed jackals (*Canis mesomelas*) and spotted hyenas (*Crocuta crocuta*) collected from around carcass sites during active anthrax epidemics in Etosha National Park, Namibia, and Luangwa Valley, Zambia (Turnbull et al., 1989, 1991; Lindeque and Turnbull, 1994). While carnivorous mammals have been suspected of intestinal carriage and dissemination of anthrax spores in northern Canada, the successful isolation of *B. anthracis* from the red fox scat is the first time it has been demonstrated bacteriologically.

The other two positive burial sites encountered in the PLR region had barely detectable levels of anthrax spores. It is difficult to interpret these findings. In several parts of the world, recurrent anthrax outbreaks have been associated with low-lying, alkaline soils rich in organic matter (Van Ness, 1971; Hugh-Jones and Hussaini, 1975). It was originally believed that these soil conditions influenced vegetative anthrax bacilli and allowed for cycles of germination, growth and resporulation resulting in an overall

increase in spore concentration (Van Ness, 1971). However, vegetative *B. anthracis* have very specific nutrient and physiological requirements and are unlikely to survive outside of a host. Instead, the specific soil factors linked to endemic areas may reflect environmental conditions that aid in maintaining anthrax spores at the site and prolonging their viability (Dragon and Rennie, 1995).

Burial site specimens from PLR contained significantly fewer endospores from *Bacillus* species able to grow on PLET medium than the other two regions surveyed. It is possible that the sandy soil was unsuitable for holding spores and any present are quickly removed via water action. The acidity of the soil may have reduced the long-term viability of spores formed at the site (Dragon and Rennie, 1995). It is also possible the PLR habitat supported a large number of *Bacillus* species whose spores were unable to germinate and grow on PLET medium and the perceived difference between the regions is an artifact introduced by the isolation protocol.

The majority of positive *B. anthracis* spore isolates from Falaise Lake were associated with the bone beds of cremation sites. The beds were comprised of charred shards of vertebrae and ribs that suggest the bulk of the carcass had rested above these beds prior to burning. The beds often contained thick mats of bison cape hair and masses of maggot casings under the bones. Cord wood and coal were piled on top and beside the body, dosed with aviation fuel and lit. Burning times were unknown and possibly quite variable. The bulk of the body would insulate the ground underneath from the intense heat and flame. The higher concentration of anthrax spores in the centre of the bone bed at site 41 compared to the edges is most likely the result of decreased protection of spores from the destructive effects of fire nearer the edges of the zone.

Overall, the level of anthrax spore contamination in the three endemic regions surveyed appeared very low. All positive samples were obtained in the immediate vicinity of carcass disposal sites within the area disturbed by clean-up operations. A study in the Falaise Lake region of wallow and meadow samples not associated with known carcass locations failed to detect any *B. anthracis* spores, however, these areas were not surveyed as intensively as the disposal sites and the results are not conclusive. The low recovery of

anthrax spores from some carcass disposal sites (one spore isolated from 25 samples) illustrates the need to thoroughly survey a site before declaring it anthrax free. High concentrations of anthrax spores in the environment of northern Canada, however, appear limited to scavenger fecal matter and anthrax carcass sites. Ecological studies of anthrax spores with PLET medium in African endemic regions have also demonstrated an association of high spore concentrations with scavenger feces and soil around carcass sites and the bacterium was only rarely found in environmental specimens not associated with carcasses (Turnbull et al., 1989, 1991; Lindeque and Turnbull, 1994).

While sporadic outbreaks have proven concentrations of anthrax spores high enough to cause disease in bison are obviously present somewhere in the endemic regions, it is unknown if the levels of spores found here represent a sufficient dosage to cause disease. Neither the infectious dosage of anthrax spores for bison nor how the animal contracts the disease is known. Bison may acquire the disease through inhalation of aerosolized spores during wallowing or may ingest lethal levels of spores while grazing. Further complicating the matter is the possibility of seasonal transient stresses, such as the rut, which may compromise bison immunologically thereby reducing the infectious dosage required (Gainer and Saunders, 1989).

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