A STUDY OF VARIOUS ENVIRONMENTAL FACTORS ON THE GROWTH, ENCYSTMENT AND SURVIVAL OF FREE LIVING AMOEBAE

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ABSTRACT

Free-living amoebae (FLA) are soil organisms which have a worldwide distribution. Interest was raised when they were implicated in two fatal and several non-fatal infections in humans.

This investigation involved examination of the role and/or effect of several environmental factors on growth, encystment and cyst survival of FLA. The effect of K^{+}, Na^{+}, Mg^{+2}, Ca^{+2} and $Fe^{+2/+3}$ on the growth of four species of amoebae (*Naegleria gruberi, Naegleria fowleri, Acanthamoeba culbertsoni* and *Acanthamoeba castellanii*) was investigated. Inhibition of growth rate increased as the cation concentration was increased.

The roles of Mg^{+2} and Ca^{+2} in encystment were investigated and it was found that rather than being necessary, they were inhibitory.

The survival of cysts under low temperatures and high cation concentrations was studied. *Acanthamoeba* proved to be resistant to adverse conditions once encysted. *Naegleria* were not affected by high cation levels but were adversely affected by low temperatures.

A preliminary identification of two isolates from Ngawha Springs hot pools was undertaken showing both amoebae to be temperature tolerant *Naegleria* species.

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CHAPTER ONE: INTRODUCTION

1.1 General

Free living amoebae (FLA) are common soil organisms which are extremely widespread in distribution, having been isolated from many habitats and from every continent (Lawande et al., 1979; Bamforth, 1980; Brown et al., 1983; Sykora et al., 1983).

Interest in FLA was greatly increased when they were found as contaminants in tissue cultures of vero cells used for the production of polio vaccines (Jahnes et al, 1957; Culbertson et al., 1958). They were subsequently shown to be pathogenic for mice and other laboratory animals (Culbertson et al., 1959).

Since then FLA have been isolated from numerous sources and interest in their classification and pathogenicity increased (Chang, 1971b; Martinez, 1983).

The classification of FLA has been a controversial topic. Two major classification systems have been devised. Firstly Page (1967) used motility, ultrastructure, cyst morphology, nutrition, cytochemical characteristics and nuclear division as criteria to distinguish genera and species. The second system was based on study of nuclear structures and patterns of mitosis and division (Singh & Das, 1970). Page (1976) and other workers have since modified these two systems, and the criteria now also include flagellation tests, pathogenicity (determined by animal inoculation) and morphology (Chang, 1971b; Willaert, 1971).

The organisms of interest in this thesis belong to two genera, *Naegleria* and *Acanthamoeba*. *Naegleria* belong to the family *Vahlkamphidae* which have very active trophozoites, variable in size and shape with a conspicuous clear-haloed nucleus. The cytoplasm is granular and flows into the hyaline pseudopod, exhibiting standard amoeboid locomotion. The genus *Naegleria* is characterised by the possession of a non-feeding non-reproducing flagellate stage. The trophozoites differentiate under adverse conditions into the cyst form which has one smooth round wall (Willaert, 1971; Martinez et al., 1975; Page, 1976).

Acanthamoeba belong to the family *Acanthapodinae*, whose trophozoites are characterized by slender spine-like processes and slow, gliding movement. The cytoplasm is finely granular with one nucleus. *Acanthamoeba* lack the flagellate stage, but also possess a resistant cyst form. The cysts are spherical with a two-layered wall. The outer layer is wrinkled and the inner smooth (Bowers & Korn, 1968; Anderson & Jamieson, 1972b; Culbertson, 1975; Visvesvara & Balamuth, 1975; Visvesvara & Healy, 1975; Page, 1976; Cerva, 1977; Thong, 1980; Martinez, 1983).

Identification of amoebae to species level requires more detailed study. Criteria used include nutrition, indirect immunofluorescent antibody techniques, temperature tolerance and immunoperoxidase tests (Anderson & Jamieson, 1972b; De Jonckheere et al., 1974; Hadas et al., 1977; Nerad & Dagget, 1979; Stevens et al., 1980; Robinson & Lake, 1982; De Jonckheere et al., 1984).

1.2 Free Living Amoebae as Disease Agents

The first report of pathogenicity of FLA in humans came from Australia in 1965 when Fowler reported four cases of meningoencephalitis caused by amoebae (Fowler & Carter, 1965). Almost simultaneously, 5 American cases were described and the term Primary Amoebic Meningoencephalitis (PAM) was coined to describe the disease (Butt, 1966).

Originally these cases were all attributed to *Acanthamoeba* species, these being the amoebae initially isolated and identified as pathogens (Culbertson et al., 1959). Subsequent work, however, showed the organisms to be amoeboflagellates, therefore of the genus *Naegleria* rather than *Acanthamoeba* (Carter, 1982; Duma, 1982). The isolates were all found to be one species, named *Naegleria fowleri*, distinguishing it from the non-pathogenic *N. gruberi* (Carter, 1970).

Since this time there have been over 100 cases of PAM identified, though not all attributable to *N. fowleri* (Duma, 1972; Willaert, 1974).

It has been demonstrated that *Acanthamoeba* do in fact cause an encephalitis, the disease given the name Granulomatous Amoebic Encephalitis (GAE) to distinguish it from PAM (Martinez, 1982).

GAE has not been attributed to a single *Acanthamoeba* species, but several have been implicated. Still more have been shown to be pathogenic in animals (Byers, 1979; Duma et al., 1979; Chang, 1974; Dagget et al., 1982; Martinez, 1983).

N. fowleri is the only *Naegleria* species known to be pathogenic (Butt et al., 1968; Duma et al., 1969; Daggett et al., 1982; Garcia, 1983).

Although the incidence of these diseases is low, their importance lies in the fact that they are nearly always fatal (Butt et al., 1966; Duma et al., 1969; Chang, 1971d; Martinez, 1977; Garcia, 1983; Newsome & Wilhelm, 1983a).

PAM is more common than GAE (Garcia, 1983). This may be a reflection on the different epidemiological patterns, or on the relative pathogenicity of the organisms (Chang SL, 1971d).

PAM is an acute, fulminant disease which is rapidly fatal (Duma et al., 1969; Garcia, 1983; Martinez, 1983). After an incubation period of approximately 3-7 days symptoms begin to appear, and death usually occurs within the following week (Carter, 1972; Chang, 1974; Garcia, 1983).

PAM appears to be an opportunistic infection (Cerva et al., 1973). *N. fowleri* is a free living organism with no need for a host, i.e. is non-parasitic. Most patients have been young healthy people who have had some recent contact with water, often in a recreational sense (Duma et al., 1971; Singh & Das, 1972; Robinson & Lake, 1981; Stevens et al., 1981; John, 1982; Dorsch et al., 1983; Garcia, 1983; Newsome & Wilhelm, 1983a; Chang, 1974).

It is assumed that while in contact with water contaminated with *N. fowleri*, inhalation or aspiration enables the organism to gain access to the upper nasal passage (Duma et al., 1971; Anderson & Jamieson, 1982a; Singh & Das, 1972; Cerva et al., 1973; Chang, 1974; Lawande et al., 1979a; John, 1982; Dorsch et al., 1983; Newsome & Wilhelm, 1983a; Chang, 1974).

In the few cases where no contact with water has been established, inhalation of air-borne cysts could explain the entry of the organism into the nasal passage (Duma et al., 1969, 1971; Chang, 1974; Lawande et al., 1979 a & b; Dorsch et al., 1983).

The incidence of PAM increases during the summer months (Duma et al., 1969; Dorsch, 1982; John, 1982; Dorsch et al., 1983; Newsome & Wilhelm, 1983a). This fact could have two explanations. Initially, warm temperatures are more favourable to the proliferation of pathogenic amoebae, this stimulating an increase in the concentration of amoebae in a particular water source (Duma et al., 1969; Dorsch et al., 1983). Secondly, in the summer, recreational activity is increased. This has a two-fold effect. Primarily, sediment may be stirred up from the lake or pool bottom, dispersing more amoebae throughout the water. Increased numbers of people swimming in contaminated water also increases the probability of infection (Duma, 1981; John & Nussbaum, 1983).

Although *N. fowleri* has been isolated in several cases from nasal cavities of healthy people, there is no evidence that the disease is contagious (Cerva et al., 1973; John, 1982). The low incidence indicates the presence of host factors which may influence the progress of infection. For example, the presence of excess mucus, as present in non-specific respiratory infections, facilitates the movement of amoebae (Chang, 1974).

An alternative epidemiology has been proposed in adddition to the inhalation theory of entry to the body. It has been suggested that the effect of water is to wash amoebae, already present, further into the nasal cavity, thereby confirming the idea of opportunistic infection (Chang, 1974).

Once successfully introduced, the amoebae are phagocytosed by the cells of the nasal mucosa, and move from these cells through the cribiform plate, up the olfactory nerve and into the subarachnoid space (Garcia, 1983; Chang 1981a).

The nasopharynx becomes ulcerated and the olfactory bulbs become heavily infected. All along the path of invasion the nerve tissue is

inflamed, all tissues are haemorrhagic and massive necrosis occurs (Carter, 1972; Chang, 1974; Martinez, 1977; Thong, 1980; Garcia, 1983).

The movement of amoebae is largely due to their ability to phagocytose nerve and blood tissues. *N. fowleri* produces a cytotoxin which kills cells and thereby reduces fragment sizes, enabling easier phagocytosis and digestion (Carter, 1968; Chang, 1974; Thong, 1980; John, 1982; Chang 1971a). However the work of Brown (1977, 1979) disregards the presence of cytotoxin and emphasises phagocytosis.

In the early stages of infection, the patient suffers vague respiratory symptoms similar to those of any mixed respiratory disorder; i.e. headache, sore throat, discharging nose and respiratory distress. As the invasion of amoebae proceeds, olfactory problems develop and vomiting and fevers occur (Carter, 1972; Chang, 1974; Garcia, 1983).

Once established within the subarachnoid space, the organisms invade both the meninges and the grey matter. The meninges are severely involved showing massive inflammation and necrosis, associated with symptoms characteristic of bacterial meningitis such as stiff neck and mental abnormalities (Carter, 1972; Chang, 1974; Garcia, 1983). Encephalitis is secondary to meningitis and extensive but superficial lesions are formed in both the grey and white matter (Duma et al., 1969; Martinez, 1975).

On post-mortem analysis, organisms are found only as trophozoites, concentrated mainly in the perivascular spaces, meninges, olfactory bulbs and in all lytic sites (Garcia, 1983). The inflammatory exudate contains elevated numbers of polymorphonuclear leucocytes, eosinophils, macrophages and neutrophils (Martinez, 1977, 1983).

Ante-mortem diagnosis can be done by sampling the cerebrospinal fluid and examining directly under a phase contrast microscope. Amoebae appear distinct from leucocytes and macrophages as motile trophozoites with distinct hyaline and uroid regions (Apley et al., 1970; Carter, 1972; John, 1982; Garcia, 1983; Martinez, 1983). The protein levels and leucocyte numbers in the CSF are elevated, and glucose levels depressed (Garcia, 1983). An alternative method of diagnosis of infection is to culture a drop of the CSF on a lawn of bacteria. Presence of amoebae is

indicated by a clearing of the lawn as bacteria are digested (Duma, 1972).

If suspicion is high, diagnosis is straightforward. Amoebic involvement should be suspected in any cases of meningitis where bacteria are not demonstrated in the CSF. However, the low incidence of this disease decreases the index of suspicion and therefore the likelihood of diagnosis (Apley et al., 1970; Thong, 1980; John, 1982; Garcia, 1983; Martinez, 1983).

Successful treatment relies on immediate action, and with regard to the rapid course of the disease, quick diagnosis is therefore imperative (Cotter, 1973; Cursons & Brown, 1976; Carter, 1978; Lawande et al., 1979b; Thong, 1980; Cain, 1981; Stevens et al., 1981; Seidel et al., 1982a; Garcia, 1983; Martinez, 1983).

There have been only four cases of PAM which have been successfully treated (Apley et al., 1970; Anderson, 1973; Seidel, 1982a). Amphotericin B, a fungicide, is the drug of choice in such treatments (Stevens et al., 1981). Apley et al. (1970) reported the survival of two PAM patients after intravenous administration of Amphotericin B, daily for ten days. The third patient successfully treated was given Amphotericin B both intravenously and intraventricularly (Anderson & Jamieson, 1972a). In the fourth case, following the lack of success of these treatments in other patients, Amphotericin B was administered in conjunction with Miconazole and Rifampin. Amphotericin B and Miconazole were given both intravenously and interthecally, and Rifampin administered orally. This treatment continued ten days (Seidel et al., 1982b).

Although both *in vivo* and *in vitro* experiments have found successful antinaeglerial drugs and drug combinations, no drug regime has been found which is consistently successful (Cotter, 1973; Cursons & Brown, 1976; Thong et al., 1978; Lee et al., 1979; Seidel et al., 1982b; Dorsch et al., 1983; Garcia, 1983). The four effective regimes have been repeated without success in other patients.

Unsuccessful treatments could be due to several factors: inter-strain differences (Stevens et al., 1981; Dorsch et al., 1983); route of administration (Stevens et al., 1981); poor penetration of CSF (Dorsch

et al., 1983) and host factors. Amphotericin B binds to the amoebic membrane causing leakage of cell components. The amoebae round up and rupture or disintegrate. In lag phase the drug is amoebicidal but in log phase is only inhibitory. The drug is highly toxic and must be administered carefully and the patient monitored constantly (Duma et al., 1976; Thong et al., 1978; John, 1982).

GAE, in contrast to PAM, takes a chronic form rather than acute. This is due to the stimulation of a granulomatous reaction which is absent in PAM (Jager & Stamm, 1972; Chang, 1974; Martinez, 1977; Garcia, 1983; Martinez 1983).

GAE appears to be an opportunistic infection, but there is little known about the epidemiology of the disease (Duma et al., 1969; Martinez, 1979;: Duma, 1981; Martinez, 1983). *Acanthamoeba* species form part of the normal fauna of healthy people. Entry to the body as a pathogen is usually facilitated by injury, such as a break in the skin, eye irritation or disturbance of the normal fauna (Chang, 1974; Sawyer et al., 1982). A primary granulomatous lesion then develops at the site of infection and the organisms are restricted to the site by a fibrous wall (Carter, 1972; Griffin, 1972; Nagington et al., 1974; Babington, 1977; Lund et al., 1978; Byers, 1979; Daggett et al., 1982; Martinez, 1983). All cases of GAE have occurred after haematogenous spread from a primary lesion (Duma et al., 1969;: Chang, 1974; Duma & Finley, 1976; Martinez, 1977; Garcia, 1983; Martinez, 1983).

The common factor in all cases of GAE is that every patient was in some way immunocompromised. Patients include diabetics, chronic alcoholics and pregnant women, as well as sufferers of Hodgkin's Disease and Leukaemia. All of these present a stress on, and/or prevent the efficient action of, the immune system. Alcoholism and diabetes reduce the migration of white blood cells, so reducing the speed and efficiency of the immune response. Patients undergoing immunosuppressive or radiation therapy are also highly susceptible to GAE (Griffiths et al., 1966; Duma et al., 1969; Jager & Stamm, 1972; Daggett et al., 1982; Garcia, 1983; Martinez, 1977, 1979, 1983). Due to these deficiencies in the immune system, particularly of the cellular response, the primary lesions are not sealed off adequately. This allows the organisms to move unrestric-

ted throughout the body via the blood (Duma et al., 1969; Chang, 1974; Duma & Finley, 1976; Martinez, 1977; Garcia, 1983).

The term GAE specifically refers to secondary lesions produced in the Central Nervous System. Once the organisms have gained access to the CNS, they centre in the brain where they cause localized and well-confined lesions in both grey and white matter (Duma et al., 1969; Chang, 1974; Martinez, 1983).

GAE may remain in a subacute or asymptomatic form for some time, progressively worsening as more tissue becomes involved. Because of the insidious onset of the disease, the incubation time is uncertain, but is greater than ten days (Martinez, 1979, 1983; Garcia, 1983).

The symptoms are initially vague and signs of meningitis and mental abnormalities develop sometimes only a week before death. As with PAM, the patient eventually becomes comatose and dies due to oedema and hernia of the brain (Duma et al., 1969; Martinez, 1979, 1983).

The inflammatory response to GAE is considerable, with copious amounts of exudate produced in the subarachnoid spaces. This is contrary to PAM, where the immune and inflammatory responses are minimal. The inflammatory reaction produces elevated levels of multinucleated giant cells, lymphocytes, monocytes and eosinophils (Duma et al., 1969; Martinez, 1977, 1979).

GAE can be diagnosed by direct phase contrast microscopic examination of the CSF. However, no diagnosis has ever been made ante-mortem. As with PAM, a high degree of awareness of the disease is required before it is suspected and thereby diagnosed. Diagnosis is extremely difficult in the early stages of the disease (Chang, 1974; Martinez, 1979).

Acanthamoebic infections are generally resistant to any drug regime despite limited success *in vitro* with gentamycin, 5-fluorocytosine and metronidazole (Casemore, 1970; Duma & Finley, 1976). However, one patient has reportedly recovered from GAE after treatment with orally-administered metronidazole, trimethoprin and sulphanethoxazole, and onetine chloraquine administered parenterally. After each subsequent

relapse chloroquine phosphate was administered three times daily and the patient recovered completely (Duma, 1972).

Acanthamoebae are responsible for a large number of infections which are more common but less serious than GAE. Acanthamoebic lesions have been found in all major organs but are particularly common in the eyes, lungs and on the skin (Carter, 1982; Griffin, 1983; Nagington et al., 1974; Babington, 1977; Lund et al., 1978; Byers 1979; Dagget et al., 1982; Martinez, 1983). Less common are infections of the nose, ears and throat. In one case a bone marrow graft became infected with Acanthamoebae, but no other reports of bone infection have been seen (Byers, 1979; Borochovitz et al., 1981; Daggett et al., 1982).

All Acanthamoebic infections appear to be opportunistic. The host has usually been injured or in some way debilitated, and *Acanthamoeba*, already part of the normal fauna, can cause further injury by infection. In this way, subclinical and asymptomatic infections could be quite common (Martinez, 1977; Lund et al., 1978).

PFLA are not restricted in their pathogenicity to humans alone. Acanthamoebae have been isolated from, and implicated in the formation of visceral lesions of a dog (Ayers et al., 1972), pneumonic lesions of bulls (McConnell et al., 1968) and a buffalo (Voelker et al., 1977), and from a renal granuloma in freshwater goldfish (Taylor, 1977). Both *Acanthamoeba* and *Naegleria* are frequently found in association with fish. Several freshwater fish kills have been attributed to PLFA. Found as part of the normal fauna, *Naegleria* are facultative dwellers in the gill mucosa of freshwater fish. *Acanthamoeba* appear to be ubiquitous on the host, both freshwater and marine fish (Stevens et al., 1977; Taylor, 1977; Voelker et al., 1977; De Jonckheere, 1979a). In all cases no single *Acanthamoeba* species has been implicated.

Naegleria species are not known to cause or be involved in any infection besides PAM. However, myocarditis is often associated with PAM, though no amoebic involvement has been proven. Subclinical infections are possible, considering the frequency of isolation of *Naegleria* species from apparently healthy individuals.

Interest has been increased recently concerning the role of PFLA in humidifier diseases such as Legionnaires' Disease. Legionnaires' Disease is contacted by inhalation of the bacterium *Legionella pneumophila*. The dispersal of the bacterium is facilitated by air-conditioning and humidifier systems. The organisms have been commonly isolated from such systems, the source usually being the water storage facility. PFLA have been isolated from such places, and have been independently linked with mild respiratory diseases such as extrinsic allergic alveolitis. Similar symptoms have been reproduced in factory workers by challenging with extracts of both *Naegleria* and *Acanthamoeba* (Edwards et al., 1976; Friend et al., 1977; Shapiro et al., 1983).

L. pneumophila has been demonstrated as parasitic for FLA; the bacterium is phagocytosed but not killed by the amoebae (both *Acanthamoeba* and *Naegleria*), and can reproduce within the amoebae. The amoebae are not killed by this parasitism for some time, though become less motile and more vacuolated. There is a risk therefore that parasitized amoebae could be distributed via humidifier systems and inhaled, thereby introducing a larger inoculum of *L. pneumophila* than is normal, and increasing the risk of disease (Babington, 1977; Rowbotham, 1980; Dagget et al., 1982; John, 1982).

The immunology of PAM is largely unknown. The short duration of the disease allows no time for an immune response to become effective. The time required to produce effective antibody is more than the course of infection, from contraction to death. Therefore, the CMI response is relied on by the host during the infection (Carter, 1969; Cursons et al., 1977; Martinez, 1983). However, antibodies are believed to be important in host resistance to PAM.

It has been shown that there are several antigens which are shared by different species of *Naegleria* (i.e. group specific). However, the majority are species specific, and are, mainly, surface polysaccharide antigens (Culbertson, 1971; Fulton, 1971; Josephson et al., 1977). Antibody to group specific antigens protect mice and rabbits against challenge with *N. fowleri* (Anderson & Jamieson, 1972a; John et al., 1977; Thong, 1980; Dorsch, 1982; Ferrante & Smyth, 1984). Antibody to *Naegleria* antigens have been found in serum of healthy people, which indicates two things. Firstly, that these people have been in contact

with a *Naegleria* species in such a way as to raise antibody. Secondly, that as antibodoy to non-pathogenic *Naegleria* can protect animals from infection with *N. fowleri*, these people may be resistant to PAM (Anderson & Jamieson, 1972a; Thong, 1980; Dorsch, 1982; Martinez, 1983). This suggestion is supported by the fact that the prevalence of PAM in higher age groups is less. An older person is at less risk of contacting PAM, which could be due to more contact with non-pathogenic organisms raising a protective antibody titre (Martinez et al., 1975; Cursons et al., 1977; John et al., 1977; Thong, 1980; De Jonckheere, 1982; Dorsch, 1982).

Because of the rapid course of the disease, the role of host factors such as age, antibody titre, etc. in resistance to disease is important, but CMI response is most important during the course of infection (Carter, 1969; Cursons et al., 1977; Dorsch et al., 1983; Martinez, 1983). This is emphasised by the work of John (1982) which showed that *N. fowleri* is able to cap and internalise surface-bound antibody to avoid host defences.

Less is known about the immunology of GAE. GAE only occurs in debilitated and immunosuppressed people, particularly those in which the cell mediated immunity is depressed. The role of antibody is unknown. Antibodies to many *Acanthamoeba* species have been found throughout the normal population, probably produced in response to commensal organisms (Cursons et al., 1977).

Antibody to *Acanthamoeba* species has also been found in patients with respiratory diseases (Visvesvara et al., 1975). Antibody titres in a patient with GAE have never been recorded, but it is assumed that in a chronic infection such as this, antibody would play a role.

The epidemiology of GAE, i.e. haematogenous spread from unconfined primary lesion to CNS, suggests however that the CMI response is the more important (Visvesvara et al., 1975; Cursons et al., 1977).

The lack of successful treatment and diagnosis of these diseases puts the practical emphasis on prevention and control as opposed to therapy (Dorsch, 1982). Prophylaxis is basically aimed at removing the risk of infection by controlling or reducing amoebae numbers in the

environment to safe levels. The most important method available is disinfection.

It is known that *N. fowleri* is susceptible to chlorination as the trophozoite stage. A free residual level of 0.5 mg l^{-1} chlorine is enough to give a 99% kill within thirty minutes (Culbertson, 1971; Robinson, 1975; De Jonckheere & Van de Voorde, 1976; Lyons & Kapur, 1977; Walters et al., 1981; Dorsch, 1982; Johns, 1982; Garcia, 1983; WHO, 1983). At 0.3 mg l^{-1} free chlorine, the normal level in swimming pools, N. fowleri numbers are controlled (De Jonckheere & Van de Voorde, 1976; Chang, 1978; Cooper & Bowen, 1982; Dorsch et al., 1983).

Chlorine levels govern the distribution and density of *N. fowleri*. If there is no chlorination, the amoebae will establish themselves (Cooper & Bowen, 1982). This was demonstrated when an overland water pipeline was implicated in the South Australian cases of PAM. Once an adequate chlorination system was installed, which ensured adequate chlorine levels even at the end of the pipeline, where previously it had been nil, the amoebae numbers were controlled and the risk of PAM much decreased (Walters et al., 1981; Dorsch, 1982; Dorsch et al., 1983).

One study showed that even superchlorination failed to eradicate *N. fowleri* (Anderson & Jamieson, 1972a). However the free residual chlorine level was not considered and it is possible that the organic load was too high to leave a free residual high enough to inhibit *N. fowleri* (Dorsch, 1982; Dawson & Brown, 1985). *N. fowleri* is more susceptible to chlorination than the non-pathogenic *N. gruberi* (De Jonckheere & Van de Voorde, 1976).

However, *Acanthamoeba* species are far more resistant to chlorination than either *Naegleria* species. *Acanthamoeba* cysts survive levels higher than those which can safely be used in swimming pools (De Jonckheere & Van de Voorde, 1977; Lyons & Kapur, 1977; Garcia, 1983). However, as with *Naegleria*, cysts increase in susceptibility to chlorination as they age, so prolonged superchlorination could kill any trophozoites and old cysts, keeping the amoebae population in check (Robinson, 1975; De Jonckheere & Van de Voorde, 1976).

Although amoebae levels can be somewhat controlled in man-made situations, in natural situations eg. lakes, thermal pools, rivers, chlorination is not practical. This is mainly due to the amount of chlorine necessary for such volumes of water, and to counteract the organic load of such water before a high enough free residual level could be reached. Although prophylaxis relies partly upon surveillance of areas to determine the risk, and chlorination to control this risk, public awareness and education of the public about the disease are the main control measures which can be taken (Culbertson, 1971; Thong, 1980; Dorsch, 1982; John 1982; Garcia, 1983).

1.3 Occurrence and Distribution of Free Living Amoebae

FLA are ubiquitous organisms, readily isolated from most soils, particularly from the upper soil and litter layers (Griffin, 1968; Chang, 1971b; Duma, 1971; Cursons et al., 1977; De Jonckheere & Van de Voorde, 1977; Stevens et al., 1977; Lawande et al., 1979a; Bamforth, 1980; John, 1982; Brown et al., 1983; Garcia, 1983; Sykora et al., 1983; Umeche, 1983; WHO, 1983). They comprise 50% of the total protozoan fauna in the soil (Culbertson, 1971; Stevens et al., 1977; Bamforth, 1980). Isolation decreases in flooded soils, due to a dilution or washout effect. However, moisture is required for locomotion, and for the dissolution of nutrients and minerals from the soil. Amoebae live in the shallow surface film of water around soil particles and leaf material. The two major features of this habitat are a surface to crawl against and encyst onto, and available oxygen (Bamforth, 1980; Umeche, 1983).

PFLA have also been isolated from numerous sources of water including lakes and rivers (Griffin, 1968; Chang, 1971b; Cursons et al., 1977; Lyons et al., 1981; John, 1982; Shapiro et al., 1983), swimming pools (Chang, 1971b; Cursons et al., 1977; De Jonckheere, 1979b; Thong, 1980; WHO, 1983), thermal pools (Willaert & Stevens, 1976; Wellings et al., 1977; Thong, 1980; Duma, 1981; John, 1982; Shapiro et al., 1983; WHO, 1983), fish tanks (De Jonckheere, 1979a), sewage and factory effluents (Chang, 1971b; Cursons et al., 1977; Thong, 1980; Daggett et al., 1982; John, 1982; WHO, 1983), tap water (Chang, 1971b; Anderson & Jamieson, 1973; De Jonckheere & Van de Voorde, 1977; Bamforth, 1980; Walters et al., 1981; John, 1982; WHO, 1983), water baths (Cotter, 1973)

and humidifier systems (Edwards et al., 1976; Babington, 1977; Rowbotham, 1980; Thong, 1980). *Acanthamoeba* have also been isolated from brackish water, ocean sediments and the sea (Sawyer et al., 1977; Davis et al., 1978).

Considering soil as their primary habitat, the introduction of amoebae to these places is explained by several means. The most common method of distribution is via run-off or washout of soil during rain and floods (Sawyer et al., 1977). Amoebae can also be distributed as cysts, carried with dust particles through the air. In fact isolation from the air is not uncommon (Culbertson, 1961; Griffin, 1968; Kingston & Warhurst, 1968; Lyons & Kapur, 1977; Martinez, 1983). Alternatively, soil containing amoebae may adhere to bathers' feet and thereby be introduced to swimming pools and rivers (Chang, 1971b; De Jonckheere, 1979).

Most thermal pools have concreted surrounds, and risk of contamination is therefore minimal. However, in some cases the pools have mud bottoms and sides, and amoebae can be introduced by stirring up the mud or by leaching the surrounding soil (Duma, 1981).

PFLA have also been isolated from many biological habitats, including animal tissues and lesions (McConnell et al., 1968; Taylor, 1977; John, 1982). They appear to be especially associated with aquatic animals. *Naegleria* have been isolated from freshwater fish and molluscs (Kingston & Taylor, 1976; Taylor, 1977; John, 1982). *Acanthamoeba* have been demonstrated as part of the normal fauna of both marine and freshwater fish (Taylor, 1977; John, 1982). A pseudocyst containing *Naegleria* was found in the faeces of a water snail, but no infection was determined (Kingston & Taylor, 1976; John, 1982).

Although both *Naegleria* and *Acanthamoeba* have been isolated from human throats, ears, eyes and noses, in the majority no pathological signs could be associated with the amoebae (Wang et al., 1967; Lengy et al., 1971; Schumaker et al., 1971; Cerva et al., 1973; Josephson et al., 1977; Martinez, 1979, 1983; Chang, 1974).

The major environmental factor influencing the distribution of FLA is temperature (Wellings et al., 1977b; Duma et al., 1979; Thong, 1980).

Non-pathogenic free living amoebae can be isolated constantly, year round from most water sources. Pathogens, however, are isolated with higher frequency in spring and summer than in winter or autumn (Carter, 1978; Sykora et al., 1983).

Low temperatures select for non-pathogenic species. At 4°C pathogenic *N. fowleri* trophozoites become non-viable. Temperatures lower than 12°C are inhibitory to *N. fowleri*, and at this temperature the amoebae encyst to survive (Carter, 1969; Robinson & Lake, 1981; Sykora et al., 1983).

Conversely, high temperatures select for pathogenic species (Carter, 1969; di Menna et al., 1969; Stevens et al., 1977; Delattre, 1981; Newsome & Wilhelm, 1983a). At temperatures higher than 21°C pathogenic species proliferate. Temperatures greater than 40°C select for pathogens. At 45°C *N. Gruberi* trophozoites become non-viable, but *N. fowleri* trophozoites can survive in water at 49°C (Chang, 1958; Carter, 1969; Robinson & Lake, 1981; WHO, 1983). As the temperature changes so the predominant amoebae species change (Robinson & Lake, 1981).

There are several theories considering the action of temperature on amoebic growth. Increased temperature is known to alter the physical properties of solutions, and can affect the toxicity of chemicals such as chlorine. It is also suggested that temperature may affect membrane permeability and diffusion rates. Low temperatures slow the movement of pathogenic species (Cairns et al., 1975; King et al., 1983.)

Fluctuating temperatures such as are produced by periodic thermal discharges appear overall to be more detrimental to amoebic growth than either high or low temperatures (Wellings et al., 1977b). However, from most habitats with permanently elevated temperatures, PFLA can be readily isolated. Continuous thermal effluents from industrial sites are advantageous to pathogenic amoebic growth. The elevated temperatures enable amoebae to proliferate, and the wash-off action of the continuous outflow aids in dispersal of amoebae. Secondly, the addition of hot water to a river, lake etc. will raise the local water temperature,

Table 1. Chemical Composition of Water; Comparison

	Contaminated pools concentration range	Te Puia	Butchers	Waingoro	Morere
Na	0 - 200	4400	6100	-	-
K	>45	-	-	-	0.07
Mg	> 6	-	-	-	0.1
Ca	5 - 11	550	3900	-	0.75
Fe	< 1	-	-	2.6	-
Cl	0 - 249	8100	15600	-	-
pH	6 - 7.7	-	-	-	9.6
$T^{o}C$	18 - 42	-	-	-	46
Pathogenic isolations	+	-	-	-	-

In Morere pool, the combination of high pH, high temperature and low cation levels could explain the lack of amoebae. However, neither pH nor temperature are sufficient alone to explain their absence.

It was concluded that the extreme levels of cations could be inhibitory, if not amoebicidal, in Te Puia and Butchers pools (Brown et al., 1983).

Iron is necessary for the growth of *Naegleria* species. Duma (1981) in a survey found that higher numbers of amoebae were obtained from lakes with elevated iron and manganese levels. In another American survey it was found that 14 out of 16 cases of PAM in one area were contracted in the vicinity of an iron smelter, indicating higher levels of the organism there (John, 1982).

Experiments using microbial iron chelators have demonstrated that lack of iron is inhibitory to *Naegleria fowleri*. At increased temperatures or higher levels of iron, the amount of chelators produced tends to be lower, so more iron is available for amoebae. The role of iron, however, is unknown (Newsome & Wilhelm, 1983b). Iron levels also

have an effect on Acanthamoebae. If iron is removed, then respiration becomes cyanide-sensitive (Hrynewiecka et al., 1980).

Bacteria are the primary food source of amoebae in nature (Odell et al., 1974). Isolation of amoebae is generally increased in habitats which also have high bacterial numbers (Carter, 1978; Cooper & Bowen, 1982; Shapiro et al., 1983).

Of three pools tested in the Waikato district, amoebae were found in the two which had high organic loads due to contamination from soil seepage, septic tanks and flooding. No amoebae were isolated from the third which had concrete pools, treated water, and correspondingly low bacterial numbers (di Menna et al., 1969). Brown et al. (1983) showed coliform numbers to be related to isolation of amoebae.

In one survey no amoebae were isolated from unpolluted (i.e. clean with no organic load) water. It appears that thermal, but biologically healthy, not sterile water is best for isolation of amoebae (De Jonckheere & Van de Voorde, 1977; Wellings et al., 1977a; Carter, 1978; Newsome & Wilhelm, 1983a).

Oxygen, pH and moisture are also important. Oxygen is necessary for growth of amoebae (Neff et al., 1958; Byers, 1979; Haight & John, 1982). The optimum pH for *N. fowleri* is 6.5, and for *N. guberi* 6.0 - 6.5 (Cerva, 1978).

Acanthamoeba trophozoites are much more tolerant of extremes in environmental conditions than *Naegleria* trophozoites. The adaptability and survival of the organism relies not only on the tolerance of the trophozoite but on the ability to form resistant and tolerant cysts (Culbertson, 1971; Corliss & Esser, 1974; Weisman, 1976; Byers, 1979).

The cyst stage is a non-active non-feeding form which is produced in response to adverse conditions. In the natural environment encystment occurs in response to low temperature, dehydration, starvation and other inhibitory conditions. Once these conditions are removed, i.e. the environment becomes favourable again, excystment occurs and the amoebae resume trophozoite activities. Presence of Gram-negative

bacilli is one of the major stimuli for excystment (Butt, 1966; Culbertson, 1971; Carter, 1978).

Amoebae cysts can survive for at least six months at room temperature, and *N. fowleri* cysts can survive eight months at $4^{o}C$ (Warhurst et al., 1980; Larochelle & Gagnon, 1978; Byers, 1979; Biddick et al., 1983).

The encystment process has been studied only in *Acanthamoebae* species. The first stage of encystment is the production of round forms, then the amoebae round up, and vacuoles disappear as they discharge their contents. The changes which follow depend largely on the surrounding media, and also on the physiological state of the amoebae. Dry weight generally decreases, and protein is lost as the metabolism of the trophozoite switches over from carbohydrate breakdown to biosynthesis of cell wall components. This is related to an increase in cellular enzyme activity.

A double cyst wall is formed, composed largely of polysaccharides, cellulose in particular. Once the wall is formed, it takes approximately seventy-two hours before the cyst becomes fully resistant (Neff et al., 1964; Griffiths & Hughes, 1968; Bowers & Korn, 1969; Griffiths & Hughes, 1969 Raizada & Krishna, 1971; Corliss & Esser, 1974; Jeffery & Hawkins, 1976).

Encystment usually occurs after the stationary phase has been reached and the nutrient supply depleted (Neff et al., 1964; Neff & Neff, 1972; Corliss & Esser, 1974; Rotiroti & Stevens, 1975; Schuster, 1979).

Osmotic pressure is important in determining the speed and proportion of encystment. One theory suggests that specific ions have no role to play in encystment, simply osmotic pressure (Lasman, 1978).

However, other workers have stressed the importance of divalent cations, Mg^{+2} in particular. According to their work, encystment will not be initiated unless Mg^{+2} is provided and will only continue if Mg^{+2} is not limiting. In PAS with no added Mg^{+2}, only minimal encystment occurs, the Mg^{+2} being provided by lysis of trophozoites. However 90%

encystment can be achieved by the addition of 0.05M $MgCl_2$ (Neff et al., 1964; Griffiths & Hughes, 1967, 1968, 1969; Robinson, 1975; Lasman, 1978; Schuster, 1979). Mg^{+2} appears to decrease the loss of cell contents which occurs during starvation and encystment (Griffiths & Hughes, 1966, 1967, 1968). Mg^{+2} is probably also required to activate the enzymes necessary for encystment (Griffiths & Hughes, 1969) and stabilize the membrane (Griffith & Hughes, 1969). However, other studies have shown that increasing the Mg^{+2} concentration is inhibitory to encystment (Chagla & Griffiths, 1974; Lasman, 1978).

During encystment 90% of the internal K^{+} ions and 70% of the Na ions are lost from the cell. NaCl inhibits encystment at concentrations of 0.05M to 0.2M. The K:Na ratio decreases as a result of the loss of ions, and would be altered more by presence of either K or Na ions in the media (Klein, 1959; Griffith & Hughes, 1968).

In contrast with the knowledge about *Acanthamoeba* encystment, very little is known about the encystment of *Naegleria* species. To date it has been assumed that the processes are similar. *Naegleria* encyst in response to similar natural conditions, but have never been studied closely in the laboratory. They too have a "round form" or pre-encystment stage where presumably, they also switch their metabolism from growth and reproduction to encystment. In addition to starvation, temperature, crowding and moisture, *Naegleria* encyst in response to a decrease in CO_2 in the environment. This can be related to bacterial numbers, i.e. less bacteria, less fermentation and therefore less CO_2 production (Averner & Fulton, 1966).

Excystment of all species is via pores or ostioles in the cyst wall. Excystment is accompanied by an inflow of water, and can be stimulated by bacterial growth, and/or an increase in CO_2 concentration (Fulton & Dingle, 1967; Duma et al., 1969; Singh et al., 1970; Fulton, 1977; Schuster, 1979; Thong, 1980). *Naegleria* encyst after the culture has reached the stationary phase, and it is assumed that a necessity for Mg^{+2} and Ca^{+2} is the same as that for *Acanthamoeba* (Kadlec, 1974).

1.4 The Aims of this Project

Two major environmental surveys undertaken indicate temperature as the primary factor affecting distribution of PFLA (Wellings et al., 1971b; Duma, 1981). However, in the New Zealand survey mentioned previously, of 10 North Island thermal pools temperature could be disregarded as all pools were thermal. Therefore other factors must be involved.

The major aim of this project was to determine the role of the anomalous chemicals in the above survey, in growth and encystment of FLA (Brown et al., 1983(.

Most previous work that has been undertaken has not considered the role of these chemicals in an experimental way, merely after observation. Secondly this project aimed to elucidate the role of divalent cations in encystment because

(1) the literature is contradictory on this topic; and

(2) the encystment of *Naegleria* has not been adequately studied.