Studies on

Freshwater Thermophilic Amoebae in Natural and Man-Made Water Bodies in the Rockhampton Area

by

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ABSTRACT

Free-living amoebae (FLA) are an assemblage of heterotrophic, eukaryotic organisms, which survive in a range of specialised and extreme environments worldwide. A few, namely *Naegleria fowleri,* pathogenic *Acanthamoeba* spp. and *Balamuthia mandrillaris* may occasionally become parasites of humans and produce life and sight-threatening diseases that are difficult to treat effectively. This thesis presents the findings of a 12-month survey of 3 natural and 3 man-made water-bodies in the Rockhampton area. It was undertaken to determine if these thermophilic FLA were present in readily recoverable quantities using the sample volumes detailed in this study thereby reflecting high population densities which could pose a potential health hazard to humans using these water bodies recreationally. Thermophilic FLA were recovered from all sites with the majority being isolated in the warmer months of spring and summer. In addition, culture dynamics and desiccation experiments were conducted to study the response of FLA to laboratory manipulation of parameters that included food supply and the effects of prolonged desiccation with the objective being to predict survivability of FLA populations in response to changing environmental conditions. Food supply experiments showed that *N. fowleri* would survive as dormant cysts after consuming a finite food supply. Desiccation experiments conducted at 22°C showed that all of the five species of thermophilic FLA would survive to 14 weeks. This thesis demonstrated that thermophilic FLA are a common component of the microbiota of freshwater bodies in Rockhampton's tropical environment.

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DECLARATION

I certify that the text of this thesis is my original work and it does not incorporate any material previously submitted for a degree or diploma. To the best of my knowledge, it does not contain, without due reference, any material that was previously published or written by another person.

Signature Redacted

Signed: Dated: 11th November 2002

(P.J. Applegarth)

1.0 INTRODUCTION

1.1 HISTORICAL PERSPECTIVE

Three decades ago in Australia, Fowler and Carter described a new and rapidly fatal human brain disease caused by free-living amoebae (FLA) (Carter, 1972). Butt (1966) described several fatal cases in Florida with the same symptoms as those reported in the Australian case. It was noted then that the infection was acquired by intra-nasal instillation of water during swimming. Butt (1966) who was also the first researcher to recover pathogenic amoebae from an aquatic site associated with the Florida cases named this new disease Primary Amoebic Meningoencephalitis (PAM).

1.2 RECENT LITERATURE

Amoebae belong in the Kingdom PROTISTA, Phylum RHIZOPODA, Class Heteroblastea. They constitute an assemblage of heterotrophic eukaryotic organisms that are universally spread throughout the world. Amoebae are chemoheterotrophic and exist without a cell wall. Phagocytosis is one of the feeding methods used by amoebae. Ingested food particles (usually bacterial cells) are digested by the application of lysosomal enzymes in a phagolysosome. Nutrients are also absorbed from their immediate environment through the cell membrane. The life cycle of amoebae includes a trophozoite stage in which they feed, move and asexually reproduce. Reproduction is by binary fission.

When the environment becomes hostile the trophozoite form changes to a dormant, desiccation-resistant, non-feeding cyst stage. Less extreme environmental conditions combined with an adequate food and moisture supply results in reversion to the trophozoite form (Cano & Colomé, 1988).

Pathogenic free-living amoebae have been implicated in cases of serious human central nervous system (CNS) and ophthalmic disease (Ma *et al.*, 1990). These protozoa continue to stimulate significant interest in clinical medicine and protozoology resulting in the publication of numerous studies (Katz *et al.*, 2000; Cabanes *et al.*, 2001; Hiti *et al.*, 2002; Radford *et al.*, 2002; Shenoy *et al.*, 2002). The ubiquitous nature of these organisms and their ability to survive in many different aquatic environments and adverse conditions such as drought has promoted interest amongst epidemiologists (Brown *et al.*, 1983).

The initial section of this review will consider the historical perspective, morphology, characterisation and features of human infection by FLAs including clinical features and pathology. The main body of the review will then focus on the ecology of free-living amoebae (FLA), including environmental sources of FLA such as public swimming pools, reticulated water supplies and thermally elevated aquatic environments (artificial and natural). The review will conclude with a summation of the central issues of the topic including consideration of the value of the information revealed by the literature review in relation to this research project.

1.2.1 Morphology

The morphological stages of *Naegleria* spp. and *Acanthamoeba* spp. range from a highly motile multi-flagellated non-pathogenic form found in *Naegleria* spp. to a dormant, potentially infectious, desiccation resistant survival cyst found in both *Naegleria* spp. and *Acanthamoeba* spp. In between these stages is a feeding, motile trophozoite form.

***Naegleria* spp.** have three stages in their life cycles, namely, a vegetative trophozoite, a motile flagellate and finally a desiccation resistant cyst. The only human pathogen in this genus is *N. fowleri* which is thermophilic as it grows at temperatures up to 40°C - 45°C. In the trophozoite form it is a small (10 x 20 μm) amoeba. *Naegleria* spp. move by producing hyaline rounded swellings (lobopodia) that break rapidly from their surface. After the lobopodia form, granular cytoplasm flows into them. Lobopodia may form at any time and at multiple sites on the protozoan surface. The net effect is that of a cell whose shape is changing continuously. An example of a non-pathogenic species is *N. gruberi* with a preferred temperature range of 22°C - 35°C. (Lennette, 1985).

Another morphological feature of these amoebae is the presence of a contractile vacuole that regularly opens to the outside of the cell and then reforms rapidly. This feature, which is easily visible in the cytoplasm of the cell using light microscopy, is a rapid aid in the direct examination of pathology specimens. The finding of a contractile vacuole within a cell facilitates rapid, presumptive identification of amoebae within a population of leucocytes in a wet preparation of cerebrospinal fluid (CSF). In contrast, leucocytes

are of similar size and appearance to pathogenic amoebae but they have no contractile vacuole. The trophozoite form divides by mitosis and during this process, a complete nuclear membrane may be seen by electron microscopy (Ma *et al.*, 1990). A rounded structure called the uroid is found at the posterior end of *Naegleria* spp. and this structure sometimes includes trailing filaments (Lennette, 1985).

Biflagellate (non-feeding) and smooth wall cyst (dormant) stages form part of the normal life-cycle of *Naegleria* spp. These stages usually occur in response to altered habitat conditions such as low food supply or presence of a chemotherapeutic agent. The cysts are spherical and 7 - 15 μm in size (Lennette, 1985) with a double wall containing two or three pores that may be sealed with mucoid plugs. Cysts are not usually found in pathology specimens from humans with PAM as the patient usually dies before encystment occurs. This lack of cyst forms is due to the rapidity of development of the infection and the extremely high mortality (Ma *et al.*, 1990).

***Acanthamoeba* spp.** produce fine finger-like projections called acanthopodia on the surface of the trophozoite and these provide a means of motility. *Acanthamoeba* spp. do not produce a flagellate stage but will form a double walled cyst if environmental changes occur resulting in stress to the vegetative, feeding form. The cyst has a wrinkled outer wall called the ectocyst. The inner wall, known as the endocyst, may be round, polygonal or stellate (Lennette, 1985).

Page (1988) reported that cyst size varies amongst *Acanthamoeba* spp., and thus has taxonomic value, with the following mean size ranges being identified as follows:

A. hatchetti	13 μm
A. polyphaga; A. griffini	14 μm
A. castellanii	14 – 16 μm
A. culbertsoni	15 – 18 μm
A. rhysodes	16 – 18 μm
A. comandoni	18 - 25.3 μm
A. tubashi	23 μm

As can be seen from the above, size ranges overlap in many of the *Acanthamoeba* spp. Ostioles or pores are found at intervals in the cyst walls at the point where the ectocyst touches the endocyst. Different species have varying numbers of ostioles, for example: *A. castellanii* has 7; *A. astronyxis* has 5 and *A. hatchetti* has 3 or 4 (Ma *et al.,* 1990). The trophozoite in both *Naegleria* spp. and *Acanthamoeba* spp. is the only morphological form that is invasive, however the cysts may be considered potentially infectious as they can change to an invasive trophozoite form (Ferrante, 1991).

1.2.2 Characterisation

Laboratory identification and *in vitro* susceptibility testing of FLA have important implications for public health authorities concerned with minimising public exposure to

these amoebae in swimming pools and reticulated water as well as for health professionals managing individual cases of invasive amoebic disease of humans. Several features are used to fully characterise an amoeba strain with size, presence of intracellular organelles, temperature tolerance and life-cycle studies being significant identifying features. More involved study methods involve demonstration of zymodemes by iso-enzyme electrophoresis. This method is used to produce zymograms where enzyme patterns are used to identify species of free-living amoebae by comparison with reference strains. For examination of different groups of *Naegleria* spp. a range of enzymes need to be examined. This is due to the fact that the varying species are found in different but overlapping temperature habitats. An isolate can be identified by choosing appropriate enzymes after determining the isolate's temperature preference (Australian Water Quality Centre, 1992).

Nerad & Daggett (1979) also reported that non-pathogenic and pathogenic isolates of *Naegleria* spp. could be identified by starch gel electrophoresis. Of the 24 *N. fowleri* isolates studied by Nerad & Daggett (1979) each isolate produced an individual pattern of enzymes. These isolates included a mixture of clinical isolates and environmental isolates that had been collected from five countries (Belgium, Australia, Czechoslovakia, The United States of America and New Zealand). Using the technique of enzyme electrophoresis, Nerad & Daggett (1979) were able to separate known pathogenic isolates from the remaining species. These researchers postulated that high temperature tolerant *Naegleria* spp., which were also non-pathogenic, may prove to be previously undescribed species.

Progressing on from the above techniques, Szenasi *et al.* (1998) reported on alternative means of differentiating between pathogenic and non-pathogenic free-living amoebae. These methods involve molecular techniques including restriction endonuclease digestion of chromosomal DNA in digests of whole-cells or of mitochondrial DNA for nucleic acid sequence analysis; and iso-electric focusing and staining for acid phosphatase and propionyl esterase activity to facilitate iso-enzyme profile analysis. These researchers also discussed the use of monoclonal antibodies as a potentially promising means of differentiation between pathogenic and non-pathogenic strains. It was interesting to note that despite the advancement in techniques, Szenasi *et al.* (1998) suggested that identification methods still need further refinement in relation to complexity, time frames, specificity and reliability.

1.2.3 Ecology

Free-living amoebae exist in a broad range of very different and varied habitats and microenvironments. When considered as a group they are extremely cosmopolitan; living in soils, water (both fresh and marine), on plants and animals (submerged and aerial) and inside vertebrates. They may also be found at water-air, water-soil, water-animal and water-plant interfaces. The small size and mass of FLA cysts and trophozoites allows them to be easily suspended in air or water and thus they can be moved passively between different ecosystems by prevailing atmospheric conditions. The concentration of different species at any particular place is the result of factors such as: site conditions including the food supply; life-cycle stage at time of arrival; mode of arrival; and the

presence of other microbiota. The interactions of all these species produce an ecosystem that approaches an equilibrium world-wide (Rodriguez-Zaragoza, 1994).

The main food supply of FLA are indigenous bacteria, algae, fungi and other protozoa. The survival of FLA during extreme conditions such as drought or low food supply is dependent on two strategies, namely, the transition to a tough drought-resistant cyst form or the production of more numerous smaller amoebae. The latter technique is practiced by *Mayorella* spp. and *Amoeba* spp. Their survival advantage lies in their ability to find temporary and less concentrated food resources. Amoebae of the genera *Mayorella* and *Amoeba* do not encyst and, therefore, will not survive if the food supply completely disappears. Cyst-forming species of amoebae need food in sufficient quantity to start the encystation stage and to accumulate nutrient reserves (Rodriguez-Zaragoza, 1994).

One of the most important roles of amoebae in ecosystems is as predators of bacteria. This produces two significant implications for plants, firstly, for nutrient recycling and secondly, for the stimulatory effect that the FLA have on bacterial cycling and phosphorus and nitrogen fixation. Bacteria are one of the principal components in recycling nutrients in soil and aquatic ecosystems. Any biotic or abiotic event that radically alters bacterial density will influence the amount of plant nutrients available. Such events would include predation by bacteriotrophic amoebae. Some species of amoebae, for example: *A. castellanii* and *A. polyphaga* do not require preformed purines and pyrimidines and they can therefore participate directly in the nitrogen cycle (Rodriguez-Zaragoza, 1994). Steinert *et al.* (1998) presented an alternate view with

protozoans acting as environmental hosts for a range of waterborne bacterial pathogens such as *Mycobacterium avium* and *Legionella pneumophila.* These researchers found *L. pneumophila* living within the outer walls of the double-walled cysts of *A. polyphaga* and proposed that these sites may act to protect the bacteria at times of less favourable environmental conditions.

The rhizosphere is a significant protozoal habitat particularly for the gymnamoebae. These amoebae engulf entire bacterial cells in their feeding process and are dependent on a constant diet of essential amino acids and other substrates from their prey. Greater numbers of FLA are found in soil than in aquatic systems. This is probably due to the efficient method by which FLA are motile on particulate surfaces and thus prey on colonies of bacteria attached to soil particles (Rodriguez-Zaragoza, 1994). Consideration will now be given to research into varying ecological sources of FLA including environmental, swimming pools and thermally elevated aquatic environments.

1.2.4 Environmental Sources of Amoebae

In 1985, Kyle & Noblet studied the population of free-living amoebae in Willards Pond, a small farm water body located in the Piedmont region of Southern Carolina. The pond had a surface area of 1500 m^2, a mean depth of 1.9 m and a high nutrient level due mainly to runoff from surrounding cattle pastures. Stratification occurred in the vertical water columns that were composed of layers of phytoplankton and blue-green algae

(cyanoprokaryotes). Kyle & Noblet (1985) found no free-living amoebae in the phytoplankton layer but dense populations in the filamentous cyanobacterial layer. The amoebae used the cyanobacteria as a substrate for growth as did the bacteria that were also found living on the cyanobacterial mucilage.

In a later study at Willards Pond, Kyle & Noblet (1986) partitioned the aquatic ecosystem into three zones: benthic, planktonic and neustonic. They found that population densities of FLA reached the maximum in each aquatic zone in late summer. The littoral sediment (less than 0.5 m depth) was a major habitat for FLA. *Acanthamoeba* spp. and *Naegleria* spp. reached a maximum in August (mid-summer), *Hartmannella* spp. in July (early summer) and *Vahlkampfia* spp. in May (spring). Kyle & Noblet (1986) also found that FLA could be found in higher numbers in the surface film layer (neustonic) than in subsurface samples highlighting the importance of reliable sampling procedures. This surface layer is an area in water bodies where nutrients and many different micro-organisms such as protozoa, algae and bacteria are concentrated. Another significant finding concerned the ability of *Naegleria* spp. to transform to a flagellate stage. This ability conferred an advantage over other FLA in that *Naegleria* spp can move more rapidly to other less populated areas of the aquatic ecosystem (Kyle & Noblet, 1986).

The 1986 study by Kyle & Noblet determined that amoeba communities were denser in the detrital layer. This layer extended to a depth of 3.0 to 3.4 m during the summer period. *Naegleria* spp. were regularly recovered from these detrital layer communities. The authors suggested that *Naegleria* amoebae descend to below the detrital layer,

convert to a motile flagellate form and then return to a position in the neustonic layer. They proposed possible explanations for this occurrence as being associated with a decrease in food under the detrital layer; an anoxic situation with a redox potential less than -100 mV or the existence of a reducing situation in the hypolimnion. A hypolimnion forms at the lake bottom after thermal layering in the water column. In this layer, oxygen levels are lowered with a corresponding increase in methane, hydrogen sulphide and carbon dioxide. The result is that the pH is increased and protozoa recovered from profundal levels (at a depth of approximately 4.5 m) must be tolerant of such reducing conditions. Variations of the numbers of FLA in these deep sediments show that some FLA are not capable of surviving in this area. This study suggested that an anoxic habitat could be toxic to *Hartmannella* spp. and *Vahlkampfia* spp. but found that *Naegleria* spp. could tolerate anoxia only if hydrogen sulphide was absent. *Acanthamoeba* spp. were found to increase in number while anoxic conditions existed in the profundal layer. Cysts of *Acanthamoeba* spp. have been reported to be more tolerant of chemicals than cysts of other FLA. The increase in *Acanthamoeba* spp. numbers could be explained by *Acanthamoeba* spp. trophozoites actually growing under these extreme conditions (Kyle & Noblet, 1986).

Lloyd *et al.* (1981), made the observation that *A. castellanii* has evolved two mechanisms to protect against increased hydrogen sulphide levels. These are the oxidisation of hydrogen sulphide and the use of different electron transfer routes that are not affected by hydrogen sulphide. Climatic changes also helped the spread of FLA into varied

ecological niches (Lloyd *et al.,* 1981). Wind action and freshwater entry into the ponds were major contributors to the redistribution of FLA (Kyle & Noblet, 1986).

Whilst sampling aquatic environments in the Tulsa area of Oklahoma (USA) John & Howard (1995) recovered pathogenic amoebae during every month of the year. The majority of pathogenic amoebae were recovered in spring and autumn. The sampling revealed that the isolation rate was one pathogen for every 60 samples with one pathogen per 3.4 L of water including the following: " 38% *Naegleria australiensis*; 35% *Acanthamoeba* spp.; 18% *N. fowleri* and 9% Leptomyxid amoebae" (John & Howard, 1995). The pathogenic Leptomyxid species recovered was an undescribed species and not *B. mandrillaris* (Visvesvara *et al.,* 1993). In a study of free-living amoebae in Egypt, Hamadto *et al.* (1993), determined that FLA were present in swimming pools (32 % of samples), surface water (20 % of samples) and tap water (4 % of samples). In contrast, Sadaka *et al.* (1994) reported that sampling of varying water sources in Alexandria (Egypt) did not result in isolation of any amoebae from the drinking water, swimming pools, sea and lake water.

A study by *et al.* (1992), involving sampling of the water supply and dust samples from the home environment of 50 contact lens wearers, revealed that *Acanthamoeba* spp. were isolated from six bathroom cold water taps. These researchers suggested that the presence of limescale in the water provided a "... favourable microenvironment for amoebae". A Finnish study reported that colonisation by amoebae probably occurs in those parts of the water distribution system where the residual concentration of disinfectant is low, the

water delay is long and the water systems are old and poorly maintained (Vesaluoma *et al.*, 1995). Recently, Cabanes *et al.* (2001) calculated that, at a concentration of 10 *N. fowleri* per litre in river water, the PAM risk to humans swimming in that water was 1 chance in 8.5×10^8.

1.2.5 Amoebae in Swimming Pools

Due to the high probability of intranasal instillation of water during physical activity in swimming pools and the associated relative risk of contracting some form of invasive amoebic disease, public swimming pools have been popular sites for research into free-living amoebae. One of the earliest studies of this type was undertaken in an indoor pool in Czechoslovakia between October 1967 and January 1969 where sixteen cases of PAM had been reported between 1962 and 1965. Concentrations of 100 to 1000 amoebae per litre of pool water were found by the researchers (De Jonckheere, 1979). These concentrations are 10 to 100 times greater than reported by Cabanes *et al.* (2001) in an unnamed river in France. The results of their study are mentioned above.

Naegleria cysts are destroyed when the free chlorine level reaches 0.5 μg/mL whilst levels below this figure will only kill the trophozoite stage. Elemental iodine has similar activity to chlorine against *Naegleria* cysts and trophozoites. Other water disinfectants such as chlorine dioxide, Deciquam 222, ozone and Baquacil (polyhexamethylene biquanide hydrochloride) have been shown to have some amoebicidal properties

depending on the physical and chemical parameters of the water being treated (Cursons *et al.*, 1980; Dawson *et al.*, 1983).

Vesaluoma *et al.* (1995) determined that amoebae were present in 41 % of the Finnish pool water samples studied thus indicating a theoretical risk to swimmers and in particular to contact lens wearers. In samples where the free chlorine concentration was below the recommended level (46 % of the samples), amoebae were detected in 67 % of those samples. Interestingly, amoebae were found in a sample in which the free chlorine residue was as high as 3.3 μg/mL and even a free chlorine concentration of 4 μg/mL did not destroy the *Acanthamoeba* cysts after three hours of exposure. 71 % of the amoebae were isolated from pools in which the bacteriological quality was considered acceptable. Vesaluoma *et al.* (1995) found that there was no direct correlation between quality of swimming water and amoebae isolated. In contrast, Bottone *et al.* (1992) and Badenoch *et al.* (1990) suggested that amoebic pathogenicity might be enhanced by bacterial contamination although the mechanism of enhancement was not suggested.

A similar study undertaken in Mexico by Rivera *et al.* (1993) investigated the presence of pathogenic and free-living amoebae in swimming pools and physiotherapy tubs. Eight genera of amoebae were isolated including: *Glaeseria* spp., *Filamoeba* spp., *Vannella* spp., *Amoeba* spp., *Acanthamoeba* spp. (x 4 pathogenic strains), *Vahlkampfia* spp., *Hartmannella* spp. and *Naegleria* spp. Amoebae were recovered predominantly in spring and summer. Accordingly, the majority of PAM cases were diagnosed in summer. In waters where chloride levels were 5.31 μg/mL or greater, no amoebae were recovered.

The method for characterisation of amoebae isolated in that study involved morphology studies using taxonomic keys and isoelectric focusing of total proteins and iso-enzymes. This study showed the importance of removing organic matter from pools and adding active chloride greater than 1.5 μg/mL to remove FLA trophozoites. It was also demonstrated that the chloride usually used in pools and tubs did not destroy the majority of viable cysts of FLA (Rivera *et al.*, 1993).

1.2.6 Thermally Elevated Aquatic Environments

N. fowleri has been isolated from thermally elevated aquatic environments worldwide. However, the association between temperature and occurrence of amoebae remains inconclusive. Huizinga & McLaughlin (1990) reported that *N. fowleri* was isolated from the thermally polluted arm of a nuclear power plant reservoir and not from the ambient temperature arm of the reservoir. In contrast an investigation of thermal spas in northwest Spain observed no relationship between temperature and the presence or number of isolates of pathogenic amoebae per sample (Penas-Ares *et al.*, 1994). Penas-Ares *et al.* (1994) assessed the presence and pathogenicity of free-living amoebae in 12 thermal spas in northwest Spain. In 8 of the12 spas tested, 13 isolates of amoebae from 6 genera were isolated, namely: *Vahlkampfia, Flabellula, Naegleria, Acanthamoeba, Paramoeba* and *Lingulamoeba* spp. This group utilised the most probable number method to estimate the amoebae counts. Pathogenicity studies of the isolates demonstrated by death of mice after intracerebral and intranasal inoculation was only associated with the *Acanthamoeba* spp., chiefly *A. polyphaga.*

A study from the North Island of New Zealand investigated naturally heated thermal pools for the presence or absence of pathogenic free-living amoebae. In this study, Brown *et al.* (1983) determined that some non-pathogenic strains of amoebae such as *Vahlkampfia* were able to tolerate 45°C. *In vitro* studies using the bacterium *Enterobacter cloacae* as a nutrient source, Brown *et al.* (1983) established an inverse correlation between numbers of amoebae and bacteria.

A 1994 study of FLA in water sources in Alexandria, Egypt, found that continuous discharge of organic waste and sewage into canals provided the bacterial nutrients needed for amoebae to grow and thrive (Sadaka *et al.,* 1994). In addition, because factories release thermal pollution into these canals the authors found that the increased temperature provided ideal conditions for bacterial growth and consequent excystment and growth of FLA. Identification of the FLA isolated showed 9 species were present, namely, *N. fowleri, N. gruberi, A. rhysodes, A. glebae, A. astronyxis, A. culbertsoni, A. palestinensis, V. inornata* and *V. avara*. This study also attempted to culture amoebae from the nasal mucosa of 500 healthy children. Only six children were found to harbour FLA, viz. *N. gruberi* and *A. rhysodes* (Sadaka *et al.,* 1994).

1.2.7 Conclusion

This literature review has considered a range of international studies. Opinions and findings regarding the distribution and pathogenicity of free-living amoebae vary. Given the limited number of Australian studies in this field, further research involving local aquatic environments would be warranted. Considering the reported world-wide distribution and potential pathogenicity of free-living amoebae it could be suggested that the proposed study to isolate and characterise free-living amoebae from natural and man-made aquatic habitats in the Rockhampton area may prove beneficial to Public Health Authorities as a basis for comparison with the international situation. Free-living amoebae implicated in human infection will now be reviewed.

1.3 REVIEW OF FREE-LIVING AMOEBAE

Amoebic infections of the CNS may be caused by the parasitic amoeba *Entamoeba histolytica* or by the free-living amoebae *N. fowleri, Acanthamoeba* spp. and *B. mandrillaris. E. histolytica* produces a brain abscess following extra-intestinal invasion and spread via the blood and the vascular system (John, 1982). A new free-living leptomyxid amoeba classified as *B. mandrillaris* has been found to cause CNS disease. This organism, which was previously considered a harmless, soil-dwelling protozoan, should now be considered in the differential diagnosis of progressive or atypical childhood stroke (Griesemer *et al.,* 1994).

1.3.1 *Naegleria*

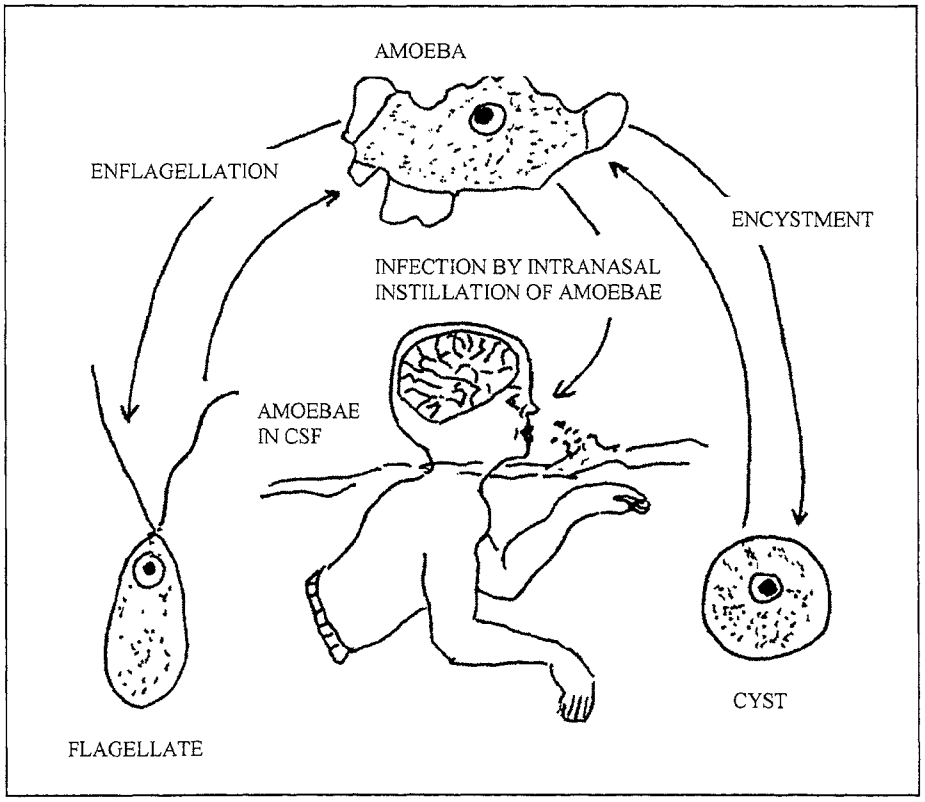
The genus ***Naegleria*** comprises free-living amoebae that are found in soil and freshwater environments worldwide. *N. gruberi*, which is non-pathogenic, was the first member of this genus to be described. A second species, *N. fowleri* causes PAM, a usually fatal disease of the CNS. *N. australiensis*, a thermophilic species that was originally recovered from floodwaters in Australia, is pathogenic for mice but non-pathogenic for humans (Marciano-Cabral, 1988). Other thermophilic species of *Naegleria* include *N. lovaniensis, N. italica* and *N. jamiesoni.*

Australian scientists (Dobson *et al.,* 1997) recently described four new *Naegleria* species with the proposed names*: N. carteri*, named in honour of Professor Rodney Carter who described the deadly brain disease PAM; *N. sturti*, named for Charles Sturt who was the original European explorer of the Murray River between 1829 – 1830; *N. morganensis* named after the town of Morgan on the Murray River, where it was first recovered from environmental samples; and *N. niuginiensis* that was named after its geographic origin. These new species do not kill laboratory mice nor do they colonise man-made environments, as do *N. fowleri and N. lovaniensis*. *N. carteri* is found in tropical areas of Australia, northern tributaries of the Murray-Darling River Basin and has been reported from areas of Sri Lanka. *N. morganensis* has only been found in the Murray-Darling Basin. *N. sturti* has been recorded from sites in Bangladesh, Sri Lanka, Bali and the Murray-Darling Basin. Relationships between the present and new species are confirmed by SSU rDNA sequences and allozyme analysis. *N. niuginiensis* and *N. morganensis* are

grouped together and *N. sturti* is linked with them but at a greater genetic distance. *N. minor* was the nearest species to *N. carteri* based on SSU rDNA sequences (Dobson *et al.*, 1997).

Naegleria spp. are amoeboflagellates classified in the family Vahlkampfiidae. Members of this family change from trophozoite forms to non-dividing, non-feeding flagellates. A desiccation resistant cyst is also part of the life cycle of *Naegleria* spp. (John, 1992). Illustration 1. (below) depicts the life cycle of *N. fowleri.*

Illustration 1. Life Cycle of *Naegleria fowleri* from John, 1992, p. 148



Cyst structure determined using both light and electron microscopy has been utilised for morphological characterisation of *Naegleria* spp. isolates. *N. gruberi* has an outer layer to its cyst wall that is not found in the cyst walls of *N. fowleri* and N. *jandini*. Pores filled

with mucoid plugs are found in the cyst wall. These pores are thicker in *N. gruberi* when compared with *N. fowleri.* It has been found that trophozoites of *N. australiensis, N. fowleri, N. gruberi* and *N. jadini* harbour *Legionella* spp. as endosymbionts. Approximately 1000 bacterial cells may be held inside each amoebic trophozoite (Rodriguez-Zaragoza, 1994).

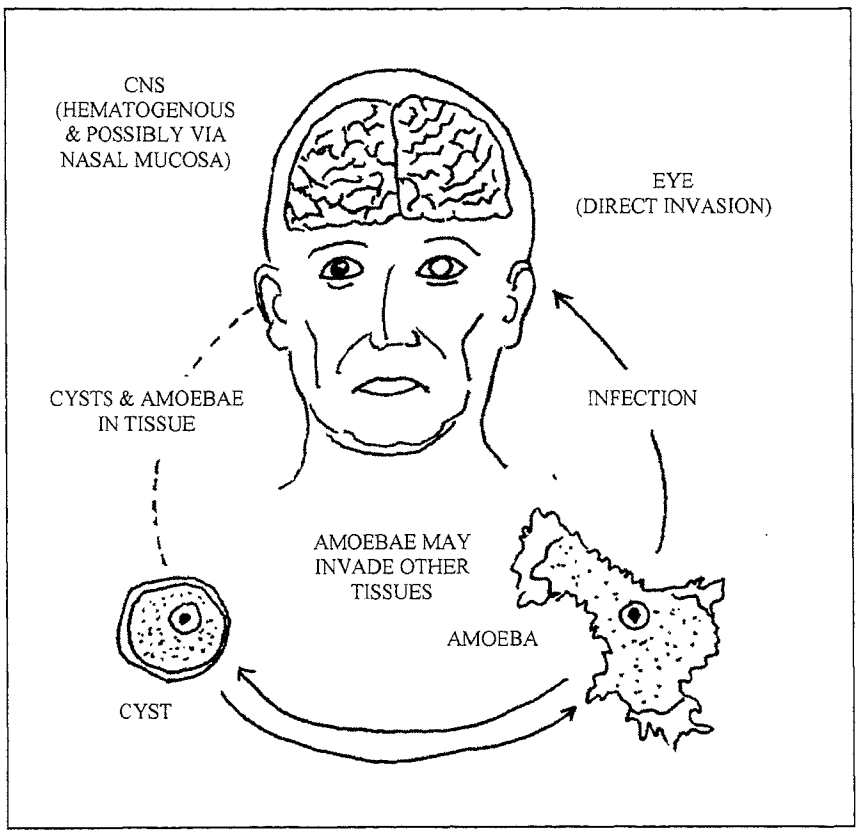
1.3.2 *Acanthamoeba*

Acanthamoeba spp. include both pathogenic and non-pathogenic strains. All known species of *Acanthamoeba* were originally considered to be harmless, soil and water dwelling FLA. This belief changed dramatically after Culbertson and colleagues discovered a species of *Acanthamoeba* that could kill laboratory inoculated animals (Culbertson *et al.*, 1959). This new pathogen was named *A. culbertsoni* by Singh & Das (1970). *Acanthamoeba* spp. can cause corneal ulcers in humans as well as a fatal brain disease known as Granulomatous Amoebic Encephalitis (GAE) thus creating considerable interest from the medical profession (Singh & Das, 1970).

Trophozoites of *Acanthamoeba* spp. form protective cysts if exposed to unfavourable environmental conditions such as a low food supply. Under food deprivation conditions, the organism produces cyst specific macromolecules and utilises trophozoite components as building blocks for the creation of the protective cyst structures. The cell wall of the cyst is synthesised and contains two products not found in the trophozoite, namely, cellulose and an acid-insoluble protein containing material (Weisman, 1976). *A.*

polyphaga, A. castellanii, A. rhysodes and *A. astronyxis* have obvious wrinkled ectocysts whilst *A. lenticulata, A. royreba* and *A. culbertsoni* have round ectocysts. Pores are found in the cell wall with the number of pores varying with differing species (Ma *et al.*, 1990). Illustration 2. (below) depicts the life cycle of *Acanthamoeba* spp.

Illustration 2. Life cycle of *Acanthamoeba* spp. from John, 1992, p. 149



1.3.3 *Balamuthia mandrillaris*

B. mandrillaris has been identified as a cause of GAE in humans and animals (Schuster & Visvesvara, 1996). *B. mandrillaris* infects both immunocompromised hosts as well as those with no immune system disorder. The disease process proves fatal after a minimum

of seven days (but may extend to months) after onset of symptoms. This organism has never been isolated from the environment and it is considered to be a fastidious organism that can only be grown in laboratory conditions if supplied with tissue culture cells (Griesemer *et al.*, 1994). *B. mandrillaris* has two stages in its life cycle, a trophic or feeding stage as well as a dormant cyst stage. After this amoeba infects its human host, both the motile trophic and resting cyst stages may be found in pathology specimens. The pathological effects associated with human infection by *B. mandrillaris, N. fowleri* and *Acanthamoeba* spp. will now be discussed.

1.4 AMOEBAE IMPLICATED IN HUMAN INFECTION

As stated previously, rapidly fatal CNS disease can be caused by amoebae which normally have free-living life-cycles. These bacterivorous, asexual organisms contribute to water ecology by removing bacteria (Cano & Colomé, 1988). Infection of the CNS can be caused by FLA, in particular *N. fowleri* and *Acanthamoeba* spp. that can be considered as opportunistic pathogens (Martinez *et al.*, 1980). Millions of humans bathe each swimming season in ponds, pools and lakes and only an unlucky few develop CNS amoebic disease. The other presentation of amoebic disease is Amoebic Keratitis. Amoebic CNS disease and *Acanthamoeba* keratitis will now be detailed.

1.4.1 Primary Amoebic Meningoencephalitis (PAM)

N. fowleri causes a type of CNS infection which was termed PAM by Butt (1966). The risk of contracting *N. fowleri* CNS disease is 1 case per 2.6 million water contacts (John, 1982). This condition generally leads to death in 3 - 7 days following onset of an acute fulminating meningitis (Carter, 1972). PAM usually occurs in children or young adults with a history of recent exposure to freshwater in a lake or pool. Infection occurs as a result of intranasal instillation of water containing amoebae or their flagellated form (John, 1982). Both Lawande *et al.*, (1979) and dos Santos (1970) suggested that amoebic cysts might be inhaled during dust storms and this could also lead to infection. Ma *et al.* (1990) supported these observation regarding modes of entry of FLA whilst also proposing that cysts within aerosols may be inhaled or aspirated and thus produce CNS disease.

Cabanes *et al.* (2001) aimed to quantify the risk of acquiring PAM after swimming in relation to the density of *N. fowleri* in the water. Their calculation for the probability of inhaling amoeba whilst swimming was based on the assumptions that the average volume of water inhaled during swimming is 10 ml and that the distribution of amoebae within the water follows a Poisson distribution. This group proposed that the risk of humans acquiring PAM can be calculated using a formula adjusted by a factor to reflect available epidemiological findings. Their example was that the risk was 8.5×10^{-8} at a concentration of ten *N. fowleri* per litre of water.

If a specimen of CSF is obtained by lumbar puncture, pathology results are commonly as follows: cloudy and blood-stained CSF under increased pressure; hundreds to thousands of white blood cells (usually neutrophils) per mL; CSF protein analysis shows an increased level whilst glucose levels are generally reduced. These results are also characteristic of bacterial meningitis thus requiring a careful search for an infecting organism using techniques such as: the Gram stain; direct bacterial and fungal antigen detection and wet mounts for amoebae and yeast cells. If the Gram stain of a purulent CSF specimen fails to demonstrate a causative bacterium, PAM should be considered in the provisional diagnosis. Motile amoebae may be observed in an unstained, non-refrigerated wet preparation of CSF from a patient with PAM. A drop of CSF from the same patient, allowed to dry and stained with hematoxylin may show an organism with a nucleus containing a large karysome (Ma *et al.*, 1990; Markell *et al.*, 1986).

A comprehensive patient history should be taken with particular consideration given to determination of recent freshwater exposure. Clinical symptoms include: positive Kernigs and Brudzinski's sign (CNS protective mechanisms), photophobia, agensia (altered taste sensation) and palosmia (altered sense of smell). Most patients become comatose and succumb to the disease in approximately 3 - 7 days as a result of cardio-respiratory arrest and pulmonary oedema. At post-mortem examination, the diagnosis is one of acute meningoencephalitis with numerous amoebae being seen in the grey matter (Ma *et al.*, 1990; Markell *et al.*, 1986).

The primary lesion in PAM occurs in the olfactory neuroepithelium. *Naegleria* organisms invade the CNS via active phagocytosis by the subtentacular cells within the olfactory neuroepithelium. Free-living amoebae track along the olfactory nerves that terminate at the olfactory bulb. This olfactory bulb is found in the subarachnoid space and is in very close contact with CSF. The subarachnoid space has an abundant blood supply and acts as the main dispersal point for the amoebae to migrate to distant parts of the CNS. Histological examination of the affected CNS demonstrates amoebae inside the olfactory mucosa and olfactory bulbs. Haemorrhagic necrosis of cerebral white and grey matters and a purulent exudate are also evident. Only trophozoites of *Naegleria* spp. are found in the affected tissue (Ma *et al.*, 1990).

The treatment of choice for PAM is the amoebicidal polyene antibiotic Amphotericin B. This therapeutic agent affects amoebae in the following ways:

- creation of autophagic vacuoles in the cytoplasm is enhanced;
- food vacuole numbers are reduced;
- nuclear shape is altered and mitochondrial abnormalities are produced;
- endoplasmic reticular and autophagic vacuole numbers are increased;
- pseudopod creation is prevented; and
- blebbing of the cell plasma membrane (John, 1982).

John (1982) found that a major drawback to the *in vitro* testing of prospective amoebicidal agents was that the susceptibility of *N. fowleri* and *N. gruberi* was affected by the composition of the medium supporting the growth of the amoebae under test. *In*

vitro testing of a virulent isolate of *N. fowleri* showed that Amphotericin B had a minimum inhibitory concentration (MIC) of 0.15 µg/mL when used as a challenge indicating that the isolate was susceptible to this chemotherapeutic agent. The MIC of Miconazole was found to be 25 µg/mL indicating a lower susceptibility for *N. fowleri*. Rifampicin's MIC at ≤ 100 µg/mL indicated resistance by *N. fowleri* (John, 1982).

Carter (1972) reported the efficacy of the anti-fungal preparation Amphotericin B in one case of PAM in a 14-year-old Central Queensland boy who lived in Mt. Morgan (a small town close to Rockhampton). After 2 days of treatment the patient's temperature returned to the normal range, however, after 5 days of treatment, a high number of amoebae were still present in the CSF. Amphotericin B was then administered intrathecally and intra-ventricularly using doses of 0.1 mg per day on alternate days. The patient subsequently recovered (Carter, 1972).

1.4.2 Granulomatous Amoeboencephalitis (GAE)

A second CNS disease known as GAE is caused by *Acanthamoeba* spp. that usually enter the CNS via the haematogenous route with the primary site of infection being a lesion of the skin or lungs. GAE usually presents in people with chronic or debilitating illness including immunosuppression. (Martinez *et al.,* 1980). Some patients who have died of GAE suffered from long-standing skin ulcerations that were later found to contain amoebic trophozoites and cysts. *Acanthamoeba* spp. may also enter the lungs in inhaled air or dust carrying cysts or trophozoites (Ma *et al.,* 1990).

The incubation period for GAE is not well understood. It may take weeks or months to initiate the disease (Ma *et al.*, 1990). Symptoms that develop during the illness may include headache, drowsiness, and alteration of the personality, altered mental state and eventually coma. Low-grade pyrexia is generally present and most cases report the presence of neck stiffness. The commonest cause of death is bronchopneumonia (Ma *et al.*, 1990). Histological examination of affected tissue shows granulomatous changes, haemorrhage and necrosis following GAE caused by *Acanthamoeba* spp. Cysts and trophozoites are found throughout the affected tissues but are concentrated in the peri-vascular spaces and walls of the blood vessels (Ma *et al.*, 1990).

1.4.3 Balamuthia mandrillaris infection

Until recently, only free-living amoebae from the genus *Naegleria* and *Acanthamoeba* have been implicated in CNS disease. *B. mandrillaris*, a newly described leptomyxid amoeba, has recently been found to also cause fatal CNS disease. This organism was first recovered from the brain of a pregnant mandrill baboon (*Papio sphinx)* that had died of meningoencephalitis (Greisemer *et al.*, 1994). Retrospective study of brain sections of many patients diagnosed with GAE showed that *B. mandrillaris* was the cause of their meningoencephalitis. In formalin preserved tissue sections, trophozoites and cysts of *B. mandrillaris* look like *Acanthamoeba* spp. particularly when using light microscopy. Greisemer *et al.* (1994) reported two fatal cases of amoebic meningoencephalitis caused by *B. mandrillaris* in children with recent exposure to freshwater. Their immune systems were functioning normally and initially amoebic CNS disease was not included in the

differential diagnosis. These authors noted however that *B. mandrillaris* may often be detected in the oral cavity and nasopharynx of healthy children. Similarly, Reed *et al.* (1997) reported a fatal case of GAE caused by *B. mandrillaris* in a 5 year old with a chronic skin lesion.

The finding that *B. mandrillaris* had caused infection in otherwise healthy children prompted Huang *et al.* (1999) to examine the possibility that humans may not have immunity to *B. mandrillaris.* This group found anti-*B. mandrillaris* antibody (IgG and IgM) titres at the following levels:

- Cord blood: Low antibody levels
- Adult sera: Antibody titres of 1:64 to 1:256.
- Children (1 - 5 yrs): Similar to adult titres.

These results prompt speculation that environmental exposure with subsequent antibody formation is protective in the majority of cases. The isolated cases may be an example of an individual that does not produce antibodies at a protective level. In the two patients described in the study by Greisemer *et al.* (1994), examination of brain sections showed trophozoites concentrated around blood vessels and amoebae deep in the brain parenchyma. Amoebic cysts were also noted in one patient. *B. mandrillaris* forms a thick walled cyst which probably indicates a capacity for survival in the harshest of natural habitats. This protozoan is highly fastidious and needs to be supplied with living tissue culture cells before it can be grown *in vitro*. Chemotherapy for this infectious agent is unknown. Early diagnosis is associated with a higher probability of a more satisfactory

prognosis than seen in the two cases described above. In the absence of a proven infectious agent, brain biopsy should be considered to facilitate definitive diagnosis in conjunction with culture and *in vitro* susceptibility testing (Greisemer *et al.,* 1994).

1.4.4 Amoebic Keratitis

The *Acanthamoeba* spp. disease spectrum also includes a potentially sight-destroying infection of the human cornea that is known as Acanthamoebic Keratitis. Rapid diagnosis and treatment is essential to avoid ulceration of the cornea, loss of vision and potential blindness requiring enucleation (Lennette, 1985). Contact lens wear is associated with a higher risk of acquiring these sight-destroying organisms which have often been found in the storage cases of the contact lenses. Biofilms of microbes can cause disinfectant failure and also provide a food source for the amoebae (Ma *et al.,* 1990; Gray *et al.,* 1995).

Non-fermentative Gram-negative bacteria and coliforms are the favoured food source of *Acanthamoeba* spp. *Pseudomonas aeruginosa* which is a non-fermentative bacterium, usually kills *A. castellanii* and *A. polyphagia* trophozoites through production of an amoebicidal toxin (Gray *et al.,* 1995). Walochnik *et al.* (2000) reviewed eighteen cases of Keratitis associated with *Acanthamoeba* spp. between 1996 and 1999. These researchers reported that their findings supported the assumption that pathogenicity in *Acanthamoeba* is a fixed characteristic of certain strains.

The cornea has no direct blood supply and it is highly possible for cysts or trophozoites of *Acanthamoeba* spp. to infect and invade corneal tissue. Five species of *Acanthamoeba* are known to cause corneal ulceration namely *A. castellanii, A. culbertsoni, A. hatchetti, A. polyphaga* and *A. rhysodes* (Despommier *et al.,* 1994). If the cornea is traumatised, an accelerated disease process may proceed. Formation of a hypopyon with severe pain and loss of sight are diagnostic features. Corneal ulceration, corneal infiltration followed by clouding, iritis, and sometimes scleritis occurs (Dreibe *et al.,* 1988). If contact lens wearers develop this disease then the early symptoms are less pronounced and the disease continues to develop along the same path as the more acute form but at a slower rate. If no obvious damage to the cornea has occurred then early symptoms may suggest *Herpes simplex* infection. As the abscess continues to develop, a diagnostic feature becomes evident, namely, the formation of a circular, paracentral infiltrate with a clear interior. This circular formation precedes a more indurated erosion of the cornea. Raised corneal epithelial lines are another diagnostic indicator in *Acanthamoeba* keratitis. If scraped and then examined by histopathologic and culture techniques, these lines will be shown to contain *Acanthamoeba* trophozoites and cysts (Dreibe *et al.,* 1988).

Acanthamoeba keratitis was first reported in the United Kingdom in 1974 by Nagington *et al.* (1974). These infections produced chronic ulceration of the cornea that in one patient necessitated enucleation of the affected eye. *A. polyphaga* and *A. castellanii* respectively, were cultured from corneal tissue of these two patients (Nagington, 1975).

The first cases of *Acanthamoeba* keratitis in the USA were reported from Texas by Jones *et al.* (1975). Earlier cases of *Acanthamoeba* keratitis were often associated with eye trauma or exposure to contaminated water.

Since 1985, an increase in the number of amoebic eye infections has been recorded. This increase has been associated with the use of contact lenses particularly with soft contact lenses. Several risk factors have been noted to be associated with the use of contact lenses, namely, use of homemade saline solution, less frequent than recommended disinfection of lenses and the use of lenses during swimming (John, 1992). Saline solutions containing protein residue from contact lenses promote the growth of bacteria that are an excellent food source for amoebae. Amoebae are directly inoculated onto the cornea after they attach to the contact lens in the storage case (Despommier *et al.,* 1994).

The prognosis following a diagnosis of amoebic keratitis depends on many factors including speed of diagnosis, number of amoebae and susceptibility of the organism to the amoebicidal chemotherapeutic agent. Corticosteroids are always used in the therapeutic regime to manage the inflammation and to facilitate keratoplasty. In a study by Dreibe *et al.* (1988), excellent results were obtained after treating four patients with 1% Clotrimazole combined with Brolene (propamidine isethionate) and Neosporin (polymyxin B sulfate; bacitracin zinc & neomycin sulfate).

1.4.5 Conclusion

Free-living amoebae are a diverse group of protozoa. Their activity in aquatic and terrestrial ecosystems is ecologically important as modifiers of the size and make-up of the microbial community. A small number of FLA cause serious human infection when they become parasites of human tissue. Given the potentially serious implications in relation to management of risks to public health, the following research project was undertaken in the Rockhampton area in Central Queensland.

Aims for the project were to:

- determine the distribution of thermophilic FLA in natural and man-made water-bodies within the Rockhampton area
- determine if water temperature and rainfall have any influence on the distribution of thermophilic FLA in the water-bodies sampled
- identify isolated amoebae using culture and morphological characteristics;
- determine if scanning electron microscopy techniques could be used to study the ultra-structures of FLA cysts with the objective being to develop a technique for rapid identification of these FLA;
- investigate the variance in population dynamics of the human pathogen *N. fowleri* through manipulation of bacterial food supply; and
- determine the survivability of five species of free-living amoebae subjected to desiccation over 14 weeks at 22°C.

2.0 MATERIALS & METHODS

2.1 FIELD SAMPLING AND ISOLATION

A series of six sites in the Rockhampton (Central Queensland, Australia) area were utilised for water sample collections for this project (Table 1 below). Samples were collected at monthly intervals for 12 months using the protocol listed in 2.1.1. Both the sampling volume of 1000 mL and sampling method used were determined to be the most useful based on information from the Australian Water Quality Centre (1992) which suggested that a sample of 500 mL to 1000 mL was adequate if "... the density of amoebae... is expected to be many organisms per litre..." as would be anticipated in the six sites sampled in this survey. Site maps and photographs of all sites are shown in Illustrations 3 - 14. The depths of the water bodies were either measured using a wooden dipping pole or else recorded from data provided by Rockhampton City Council.

Table 1: Water Sampling Sites in the Rockhampton Area

Site	Location
1	Central Queensland University (CQU) Pond
2	Fitzroy River
3	Japanese Gardens Pond
4	Yeppen Yeppen Lagoon
5	Murray Lagoon
6	Kershaw Gardens

2.1.1 Sampling Protocol

Sample Collection:

- 1000 mL of water was collected from each site monthly for 12 months;
- Individual sterile, screw-top glass bottles were utilised;
- A sample was collected by dipping the bottle into the surface water and moving it forward slowly, ensuring that the water always flowed directly into the bottle thus avoiding contamination of the water by the collector's hand; and
- Bottles were filled to the shoulder with 5.0 cm of airspace above the water sample.

Field Records:

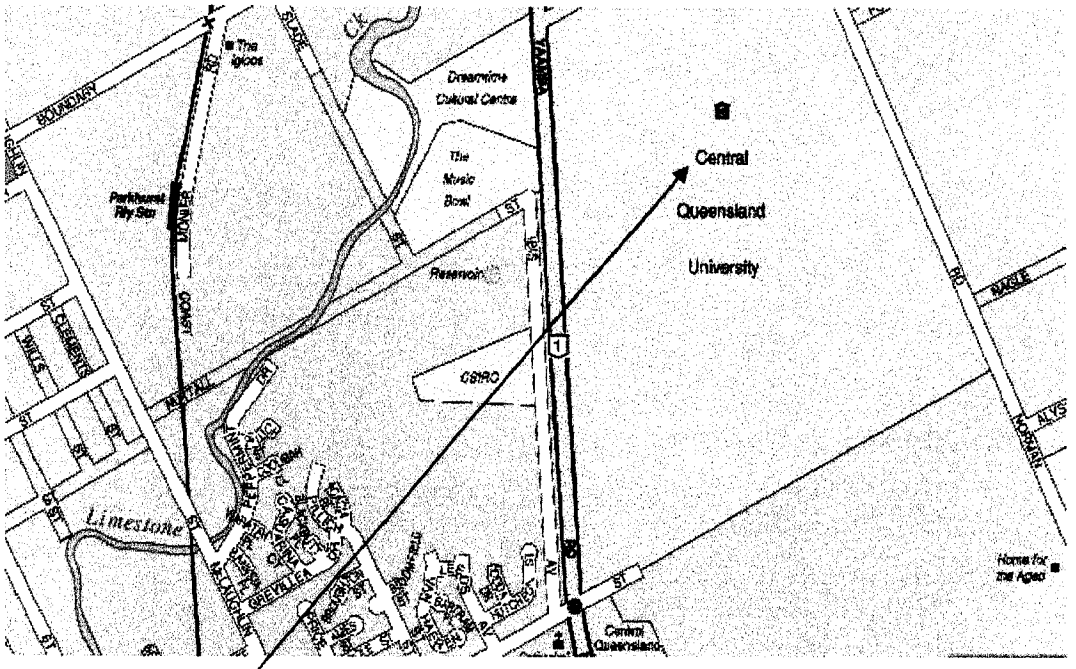
- Samples were labelled at the time of collection (location, date and time); and
- Site water temperature was recorded immediately after sample collection using a calibrated mercury-in-glass thermometer.

Sample Transport:

- Samples were transported to the CQU Microbiology Laboratory at ambient temperature in a thermally insulated container;
- Sample processing was commenced immediately on arrival at the laboratory; and
- Sample pH was recorded using a calibrated glass-electrode pH meter.

Site 1: CQU pond is an artificial water body that was created in 1978. It has a surface area of approximately 40 m^2 and an average depth of 1.5 m. Illustrations 3 and 4 (below) show the site map and photograph for sampling site 1.

Illustration 3: Site Map of Sampling Site 1 - Central Queensland University

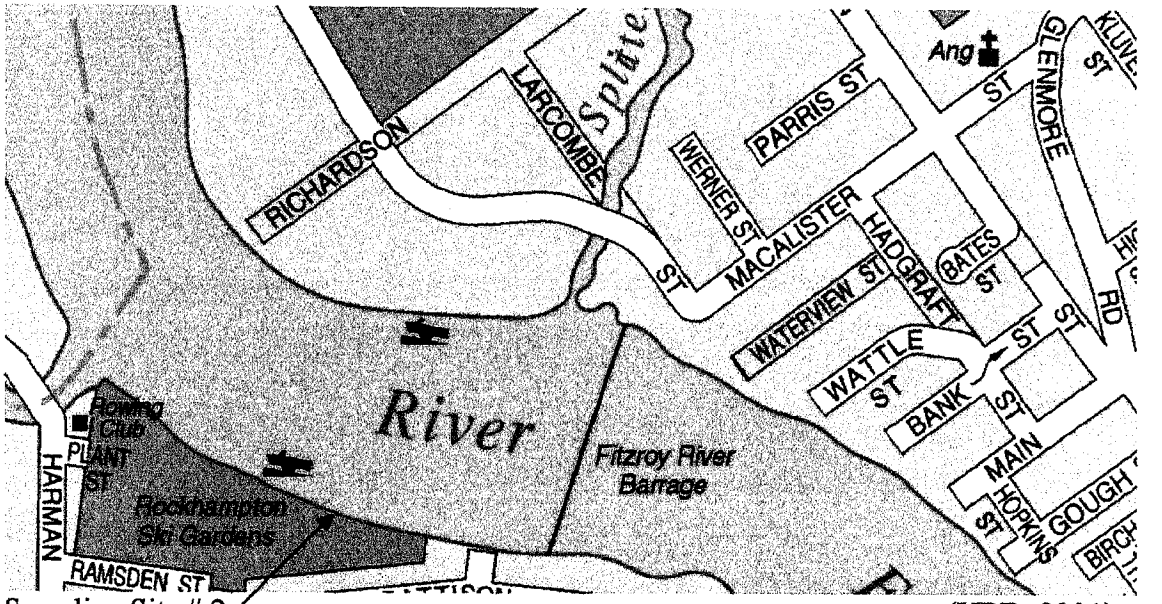


(UBD, 2001)

Illustration 4: Photograph of Sampling Site 1 - Central Queensland University

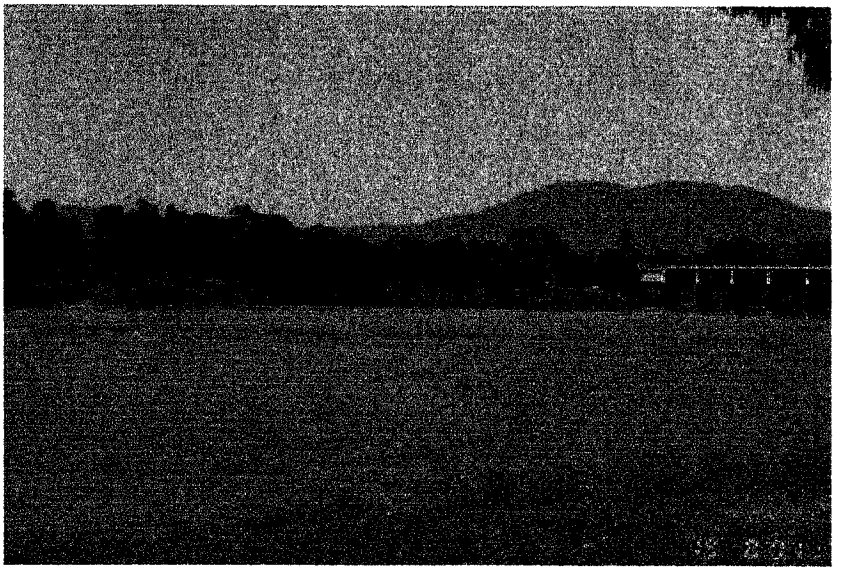
Site 2: The Fitzroy River is a natural water body with a catchment area of over 142,000 Km^2 (Rockhampton City Council, 2001). It flows through the city of Rockhampton via a barrage sited upstream from the city. All water samples were collected from the freshwater side of the barrage. The river is used extensively for recreation purposes. Illustrations 5 and 6 (below) show the site map and photograph of site 2.

Illustration 5: Site Map of Sampling Site 2 - Fitzroy River



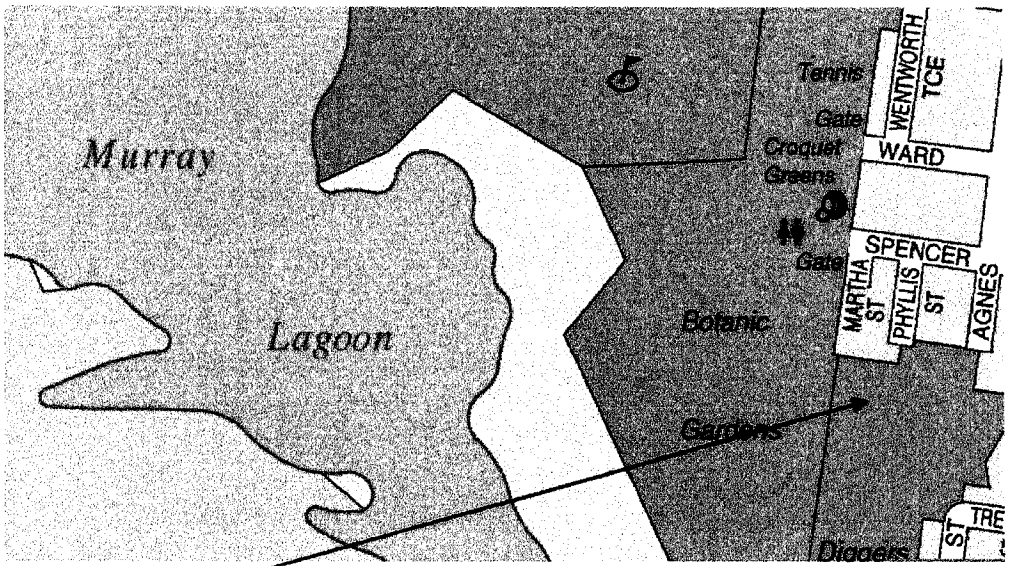
(UBD, 2001)

Illustration 6: Photograph of Sampling Site 2 - Fitzroy River



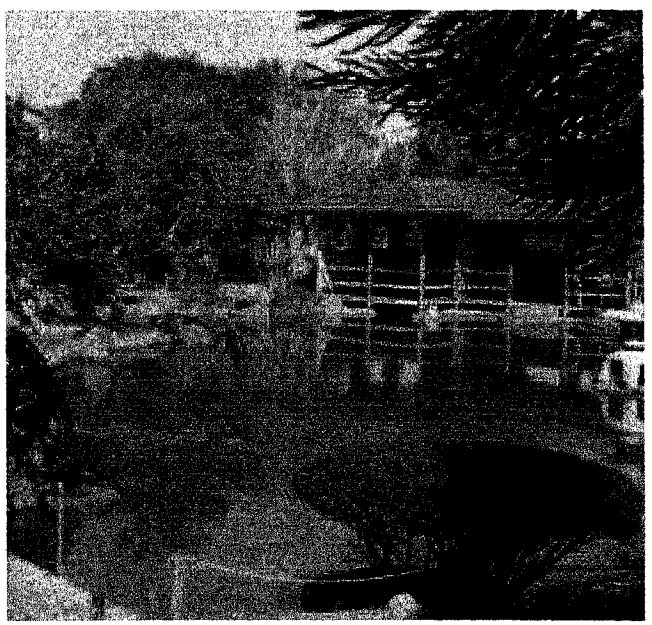
Site 3: The Japanese Gardens pond is a man-made water body which was created in 1981 in conjunction with Rockhampton's 'sister' city Ibusuki City (Japan). The pond has a surface area of approximately 200 m^2 and an average depth of 1 m. Illustrations 7 and 8 (below) show the site map and photograph of sampling site 3.

Illustration 7: Site Map of Sampling Site 3 - Japanese Gardens



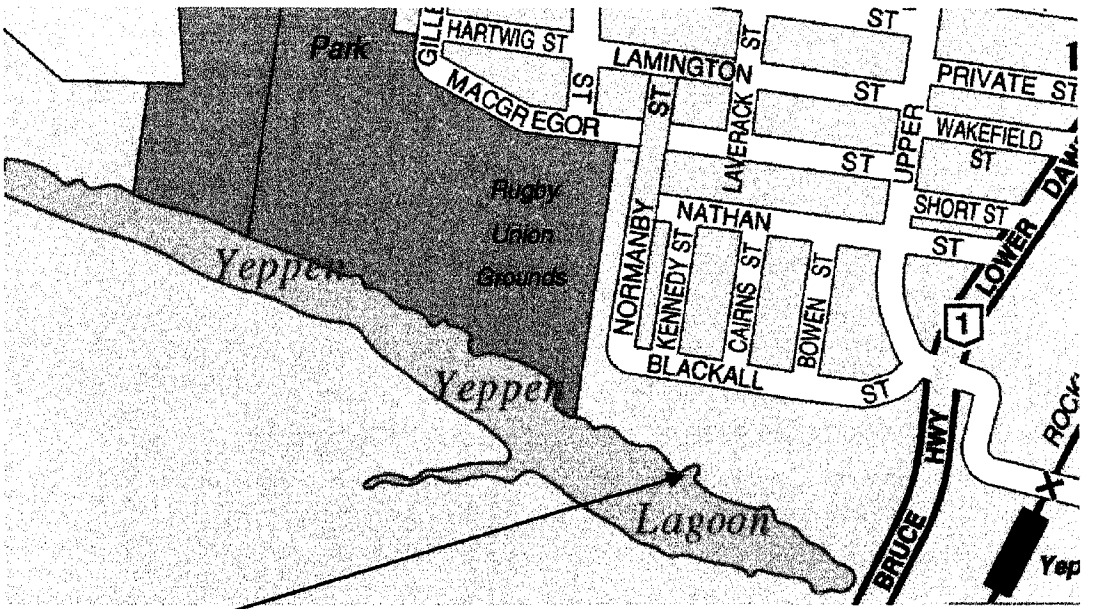
Sampling Site # 3 (UBD, 2001)

Illustration 8: Photograph of Sampling Site 3 - Japanese Gardens



Site 4: The Yeppen Yeppen Lagoon is a natural water body. It has a variable surface area (approx. 1500 m^2) and an average depth of 5 m. Illustrations 9 and 10 (below) show the site map and photograph for sampling site 4.

Illustration 9: Site Map of Sampling Site 4 - Yeppen Yeppen Lagoon

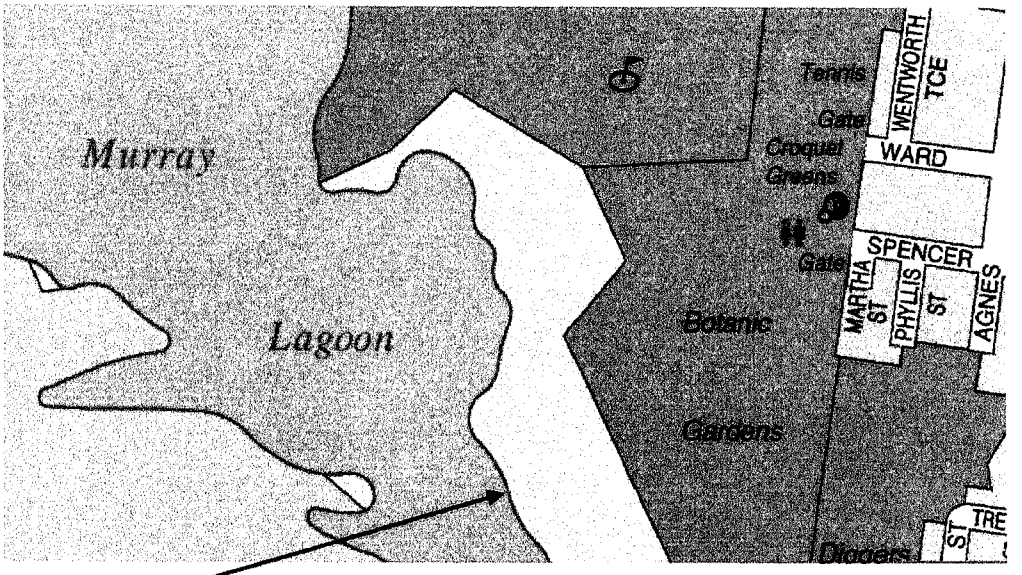


Sampling Site # 4 (UBD, 2001)

Illustration 10: Photograph of Sampling Site 4 - Yeppen Yeppen Lagoon

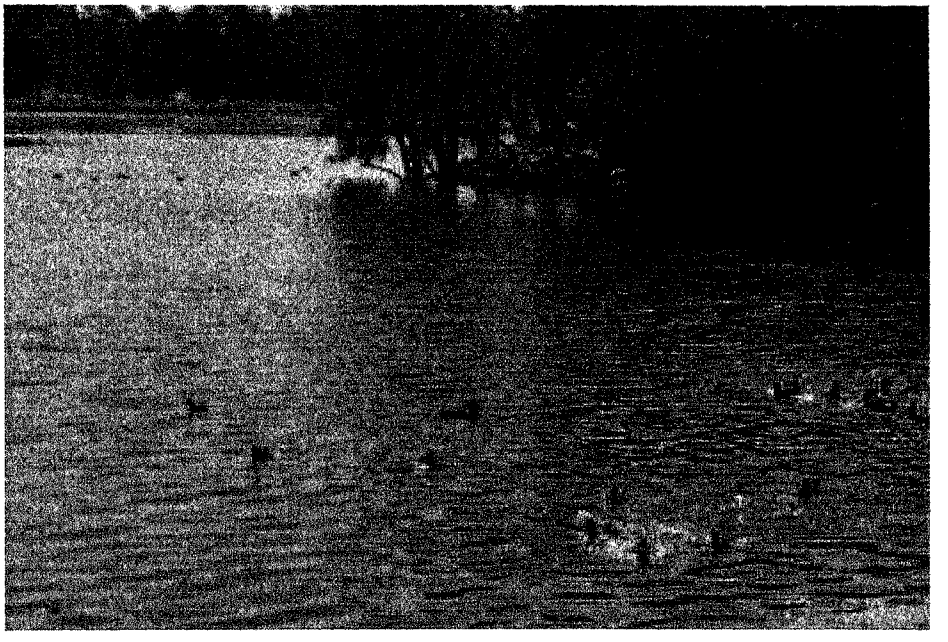
Site 5: The Murray Lagoon is a natural water body. It has a variable surface area (approx. 1400 m^2) and an average depth of 2 m. Illustrations 11 and 12 (below) show the site map and photograph for sampling site 5.

Illustration 11: **Site Map of Sampling Site 5 - Murray Lagoon**



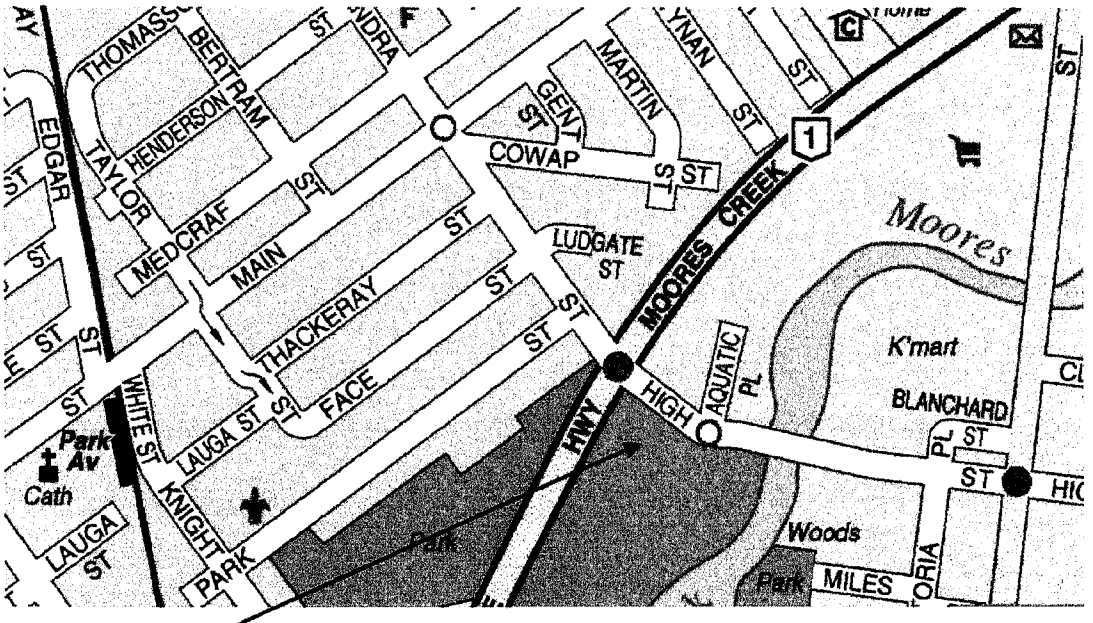
Sampling Site # 5

Illustration 12: **Photograph of Sampling Site 5 - Murray Lagoon**



Site 6: The Kershaw Gardens pond is an artificial water body that was created in 1990. It has a surface area of approximately 200 m^2 and an average depth of 1.5 m. Illustrations 13 and 14 (below) show the site map and photograph for sampling site 6.

Illustration 13: **Site Map of Sampling Site 6 - Kershaw Gardens**



Sampling Site #6 (UBD, 2001)

Illustration 14: **Photograph of Sampling Site 6 - Kershaw Gardens**

On return to the laboratory, samples were mixed vigorously and then decanted into five 200 mL conical bottom sterile centrifuge tubes and centrifuged at 2200 x g for 10 minutes. The supernatant was removed by vacuum aspiration and the remaining sediment was collected from each sample. 200 µL of concentrates were left in the bases of the centrifuge tubes following the vacuum aspiration process. The concentrate from each of the five tubes was transferred into a sterile 10 mL plastic screw-top tube using a sterile transfer pipette. This provided a total of 1000 µL of concentrate from each sample. A diluent was prepared using a suspension of 1×10^8 colony forming units (cfu) of *Escherichia coli* (ATCC 35218 strain) in sterile distilled water. The concentration of the bacterial suspension was established by optically comparing the suspension with a 0.5 McFarland Standard (NCCLS, 2000). Table 2 (below) summarizes water sample volumes and dilution factors used.

Table 2: Water Sample Volumes and Dilution Factors

Volume of sample concentrate (mL)	Volume of E. coli suspension (mL)	Dilution factor
0.5	0.5	1/2
0.25	1.0	1/5
0.125	1.125	1/10

Each diluent and specimen concentrate was mixed gently by inversion and each half of each diluent/specimen concentrate mix was spread (utilising a bent sterile glass spreader) over a separate water agar plate. Plates were labelled with the sample code (comprising site code; date of collection; dilution used) and the temperature of incubation (42 or

44°C). These temperatures were chosen to facilitate isolation of thermophilic amoebae that were considered to be of Public Health interest. All culture plates were sealed into transparent plastic sleeves and placed into either the 42°C or 44°C incubator as determined by the plates labelling code. Plates were checked for amoebic plaques after 2 and 4 days and any plaques detected were numbered, recorded and the location on the agar surface was marked on the reverse side of the petri dish using a black, permanent marking pen.

Plaques were examined microscopically to ascertain their location on the agar surface and thus facilitate marking of that site. Each marked site was excised from the agar plate using a sterile scalpel blade. This 3 mm x 5 mm piece of agar was then placed upside down on a fresh water agar plate that was labelled with the isolates identification code. This code included temperature, date of isolation, sample source, plaque number and diluent used. Three drops of an *E. coli* suspension (ATCC 35218 strain) was then applied to the agar block to provide a nutrient source for the growing amoebae. This culture technique was repeated on two separate occasions until a single amoeba could be cloned for propagation as a pure culture. This pure amoeba culture was transferred to a 7 mL McCartney bottle containing a non-nutritive agar slope and one drop of *E. coli* suspension and labelled with the isolate identification code. After 2 days in the 37°C incubator, this slope was placed in a cold room at 4°C to facilitate long-term storage of the amoebae isolates.

2.1.2 Identification

Data collected on all organisms were compared with information presented by Page (1988) as summarised below in Table 3:

Table 3: *Naegleria* Species Identification Data (Page, 1988)

Characters	*Naegleria fowleri*	*Naegleria lovaniensis*	*Naegleria australiensis*
Growth at:			
42°C	+	+	+
45°C	+	+	-
Cyst Diameter (μm)	7 - 15	9.6 - 13	~ 11.6
Known pathogenicity:			
In mice	+	-	+
In humans	+	-	-

The identification techniques utilized in this project involved determination of:

- temperature tolerance;
- cyst size; and
- transformation to flagellate stage.

Temperature tolerance is considered an important taxonomic characteristic for identification of *Naegleria* spp. and *Acanthamoeba* spp. (Australian Water Quality Centre, 1992). The methods used to determine temperature tolerance included use of two replicate cultures of actively growing amoebae isolates spread onto 9 cm water agar plates

with an *E. coli* lawn as a food source. Plates were incubated at temperatures of 42°C and 45°C for this experiment. Note: The higher temperature of 45°C was intentionally varied from the 44°C in the incubation of environmental samples to facilitate identification according to the temperature ranges published by Page (1988). The technique which follows was used for each amoebae species isolated:

- Two 9 cm water agar plates were covered with a lawn of *E. coli* by applying 0.5 mL of an *E. coli* suspension onto the centre of each plate. The suspension was then spread evenly over the surface of the plate using a bent sterile glass spreader.
- These plates were then allowed to dry at room temperature for 30 minutes.
- 100 μL of a water suspension of the 48 hour cloned amoebae culture was placed near the center of the bacterial lawn.
- The plates were labeled with the isolate number and incubation temperature.
- Each plate was sealed inside a clear plastic sleeve to minimise drying.
- Plates were then incubated at the designated temperature (42°C or 45°C).
- Cultures were examined after 48 to 72 hours and scored as either positive (+) or negative (-).
- Positive scores were recorded for those cultures where the growth front of the amoebae had moved at least 10 mm.
- Negative scores were applied to those cultures that had died.
- Cultures with growth less than 10 mm were re-incubated for a further 24 hours and then scored as either positive or negative as above.

Cyst sizes were estimated using a compound light microscope fitted with a calibrated sizing graticule in the eyepiece. A glass cover slip was placed onto a water agar plate covered with cysts of the amoebae under study and the plate was then placed under the microscope objective to enable sizing to be determined. Ten cysts were examined and the average size recorded for each isolate.

Flagellate conversion was assessed to test amoebae isolates for their ability to convert from a trophozoite to a flagellate form. The technique involved use of a 48 hour, 9 cm water agar culture with a dense surface population of trophozoites:

- A thin layer of distilled water was applied to the surface of each plate.
- The plates were then incubated at 37°C for 2 hours.
- Plates were then examined under the microscope at hourly intervals for 4 hours to determine the presence or absence of motile flagellates.

2.2 CULTURE DYNAMICS

A culture of *N. fowleri* amoebae was studied to determine the effects on its growth using two types of food sources namely: viable (live) and non-viable (dead) bacterial cells. As the same cultures of *N. fowleri* were supplied with identical suspension densities of bacterial cells on identical water agar plates, then the only difference lay in the viability status of the *E. coli* suspension. Amoebae growing on the finite food source (dead bacteria) were hypothesised to form cysts sooner due to nutrient depletion. Viable bacteria, in contrast, would act as a longer acting food resource due to their ability to

undergo binary fission. The experiment was undertaken over seven days with the numbers of cysts and trophozoites being counted at one, three, five and seven days after incubation at 37°C.

In this experiment, a 1 x 10^8 cfu/mL suspension of *E. coli* (ATCC 35218 strain) was utilised as the nutrient source for the amoebae. Half of the suspension was heat treated to make it non-viable. This treatment process involved heating 20 mL of the suspension to 80°C in a microwave oven for a total of 10 minutes. The bacterial suspension was mixed and the temperature was checked at 10-second intervals using a calibrated mercury-in-glass thermometer. The suspension was allowed to return to room temperature before being subjected to 2 repeats of the above treatment. After final cooling to room temperature, the suspension was checked for sterility by streaking 0.5 mL of the suspension onto a 5% horse-blood agar plate. This inoculum subsequently produced no growth after incubation at 37°C for 24 hours.

A total of four water agar plates with lawns of trophozoites and cysts were washed with sterile distilled water after the plate surfaces were gently rubbed using sterile glass spreaders to maximise the number of active trophozoites and cysts that could be collected. The 5 mL volume collected utilising this technique was centrifuged at 200 x g for 10 minutes. The supernatant was carefully removed with a sterile, plastic transfer pipette leaving 0.5 mL as sediment in the base of the centrifuge tube. This sediment was made up to 5 mL with 4.5 mL of sterile distilled water. The centrifugation process was repeated once more to obtain 1.0 mL of washed and concentrated trophozoites and cysts

and this concentrate was utilised to supply the inoculum for all of the six plates utilised for this experiment.

The final concentration of cysts and trophozoites in this 1.0 mL volume was determined to be on average 80000 cysts and 13000 trophozoites per mL using an Improved Neubauer Counting Chamber. This 1.0 mL of concentrated washed trophozoites and cysts was divided into two 0.5 mL aliquots. 1.0 mL of 1 x 10^8 cfu/ml suspension of viable *E. coli* cells was added to 0.5 mL of the washed trophozoites and cysts and 1.0 mL of 1 x 10^8 cfu/ml suspension of non-viable *E. coli* was added to the other 0.5 mL of the washed trophozoites and cysts giving a total of 1.5 mL of each combined cyst/trophozoite/ *E. coli* suspension.

Aliquots of 0.5 mL of each suspension of trophozoites and cysts and either viable or non-viable *E. coli* were spread over 9 cm water agar plates using sterile glass spreaders. A total of six, 9 cm water agar plates were used for this experiment (three using suspension of viable *E. coli* and three using non-viable *E. coli*). These plates were allowed to dry before being sealed in individual plastic bags to minimise desiccation of the agar plate and placed at random locations within a 37°C incubator. The numbers of trophozoites and cysts were estimated on alternate days for a total of seven days at a magnification of x100 with the aid of a reference grid randomly marked on the base of each agar plate. Cyst and trophozoite numbers on the agar plate surface were estimated as follows:

- A 5 cm x 5 cm square was marked at random on the base of each agar plate.
- This square was further bisected into two rectangles 5 cm x 2.5 cm.
- Each 5 cm x 2.5 cm was then divided into 5 equal rectangles
- Each of the resulting 10 rectangles were examined under x100 magnification
- The total numbers of trophozoites and cysts counted on each of the 10 rectangles, on every plate, were averaged to provide an estimate of the relative abundance of cysts and trophozoites present on a 1 cm x 2.5 cm section of the agar surface.

2.3 SEM

Two techniques were utilized to prepare amoebic cysts of *N. fowleri* for study of structural features including pores and pore rims using a Jeol 5300LV Scanning Electron Microscope. The amoebic cysts were coated with a 5 - 7 nanometre (nm) layer of gold to facilitate imaging of the surface topography. The first preparation technique involved desiccation in a silica gel desiccator and the second technique used drying in a critical point drying apparatus.

2.3.1 Desiccator

A culture of the particular amoeba isolate under study by SEM was allowed to encyst by incubating a clonal culture of the isolate on a water agar plate enclosed in a plastic sleeve at room temperature (22°C) for 7 days. A concentrated sample of the cysts from this isolate was achieved by gently scraping the surface of the culture plate on which three

drops of sterile water had been added. This enabled a collection of cysts to be aspirated into a 1 mL sterile plastic transfer pipette. One drop (50 μL) of the aspirated suspension was allowed to drop onto the centre of a 25 x 25 mm piece of filter paper. This paper was allowed to dry for 30 minutes before a 4.0 x 4.0 mm section was cut from the centre of the paper. This 4.0 x 4.0 mm piece of filter paper was then placed into an open plastic container (to enable isolate labelling) and then dried for 48 hours in a silica gel desiccator.

2.3.2 Critical Point Drying

The second technique used as a preparation process for amoebic cysts involved use of Critical point drying (CPD) in a Polaron E3000 apparatus The critical point drying method avoids damage to soft organic structures by preventing the formation of a liquid/gas interface, thus the delicate specimen is not exposed to surface tension forces (Bio-Rad Laboratories, c. 1987). The cysts were concentrated onto 4 mm x 4 mm squares of filter paper (using the same technique as discussed in 2.3.1) and then placed inside the specimen cage with a gauze lid attached. The system was then flushed with liquid CO_2 and the drying cycle initiated.

2.3.3 Sputter coating

The cysts layered on to 4 mm x 4 mm squares of filter paper (prepared as noted in 2.3.1 and 2.3.2) were mounted onto aluminium stubs using double-sided tape and then placed

into a Polaron SC513 gold-coating instrument. A vacuum of 10^{-1} Torr was generated in the instrument to allow a 5 - 7 nm gold layer to coat the specimens using an evaporative gold sputter technique.

2.4 DESICCATION STUDIES

Five different species of amoebae (*N. fowleri, N. australiensis, N. carteri, Willaertia magna* and *Acanthamoeba* sp.) were used in this aspect of the study. A single clone of each isolate was identified microscopically at x400 magnification and cut from the water agar plate and inverted onto another sterile 9 cm diameter water agar plate to that the clone came into direct contact with the surface. Three drops of a 1 x 10^8 cfu/mL *E. coli* suspension were then added to the block of medium. The plate was then sealed inside a plastic bag and incubated for 48 hours at 37°C after which the plate was observed for a growth front containing amoebic trophozoites. The growth front was then gently washed off the surface of the medium with 0.5 mL of sterile distilled water and the washings were collected in a sterile 10 mL container. The volume of these washing was increased to 2 mL by addition of sterile distilled water. A further 2 mL of the 1 x 10^8 cfu/mL *E. coli* suspension was added to the 2 mL of amoebae suspension and gently mixed. This amoebae/*E. coli* suspension was poured over a fresh water agar plate to create a lawn of amoebae. This lawn plate was then allowed to dry at room temperature.

After the drying process was completed, 4.0 mm diameter ceramic 'fishspine' electrical insulation beads were spread evenly over the entire surface of the agar plate. This plate was then sealed in a plastic bag and returned to the 37°C incubator for three weeks. This procedure was repeated for each of the five amoeba species used in this study. After three weeks, the 'fishspine' beads were removed from the media and divided into two lots each of 14 beads. The beads were stored either at 22°C on a laboratory bench or at 22°C in a desiccator.

At weekly intervals, for 14 weeks, one bead containing one of each of the test species was removed from the desiccator (10 beads per week in total). Each of these beads was then placed onto the centre of a fresh water agar plate and three drops of 1×10^8 cfu/mL *E. coli* suspension were applied to the bead before sealing in a clear plastic sleeve for incubation at random places in a 37°C incubator. After two days the plates were examined microscopically to determine the presence or absence of amoebic plaques at the site of placement of the bead. These data were scored as a plus (+) or minus (-) according to the presence or absence of amoebic plaques. No attempt was made to quantify the number of trophozoites as it was considered to be technically infeasible. If one cyst or multiple cysts excysted in the presence of a food stimulus, then the short generation time would ensure a plaque would form rapidly. In both examples, 2 days post inoculation of the bead and incubation, an observer would not be able to determine if one or multiple cysts had excysted. Due to this inability to accurately assess each beads population of amoebae, the plus (+) or minus (-) methodology of scoring the plates was adopted.

3.0 RESULTS

3.1 FIELD SAMPLING AND ISOLATION

Figures 1 - 6 (below) display the thermophilic amoebae isolates cultured from the three natural and three man-made water bodies sampled over the 12-month period from July 1997 to June 1998.

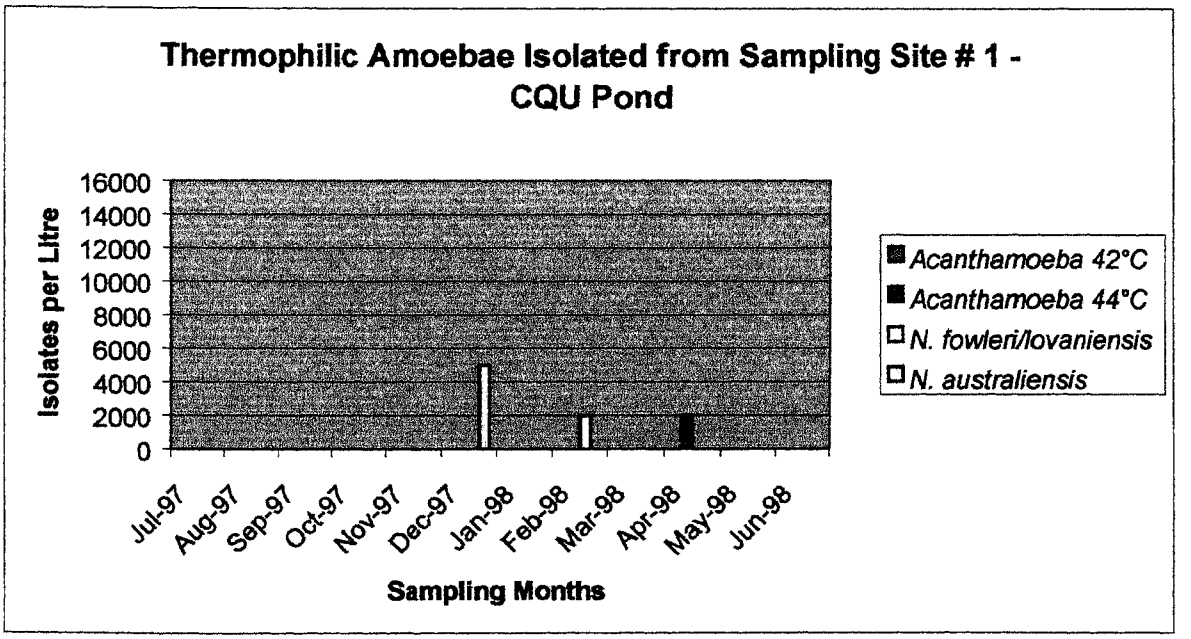


Figure 1: Thermophilic Amoebae Isolated from Sampling Site 1

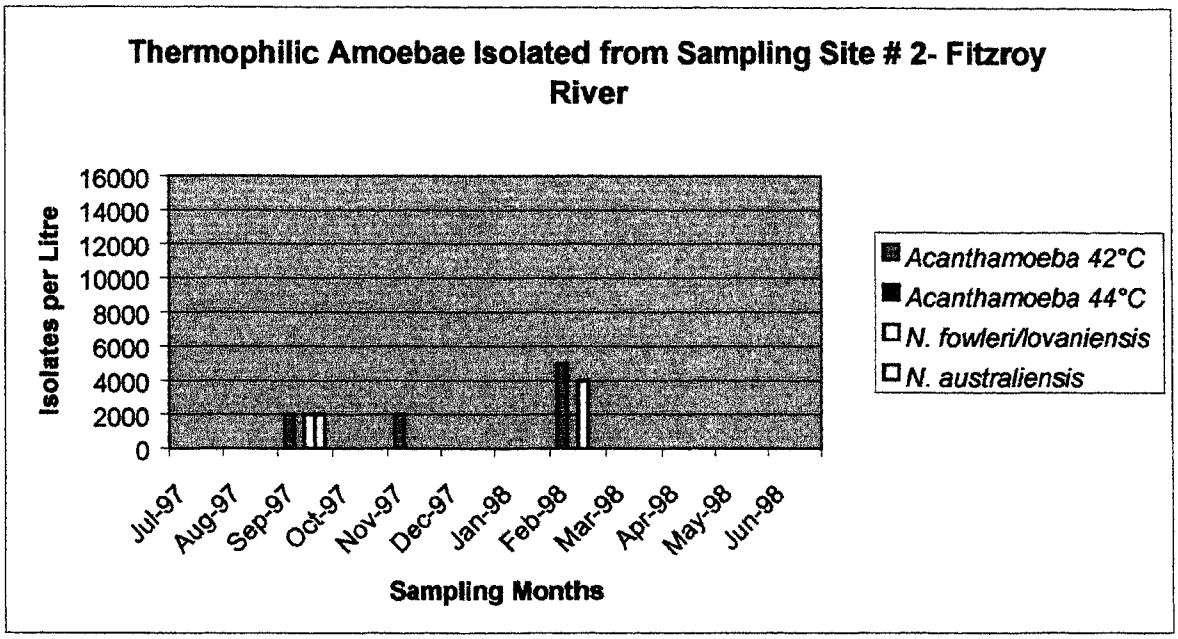


Figure 2: Thermophilic Amoebae Isolated from Sampling Site 2

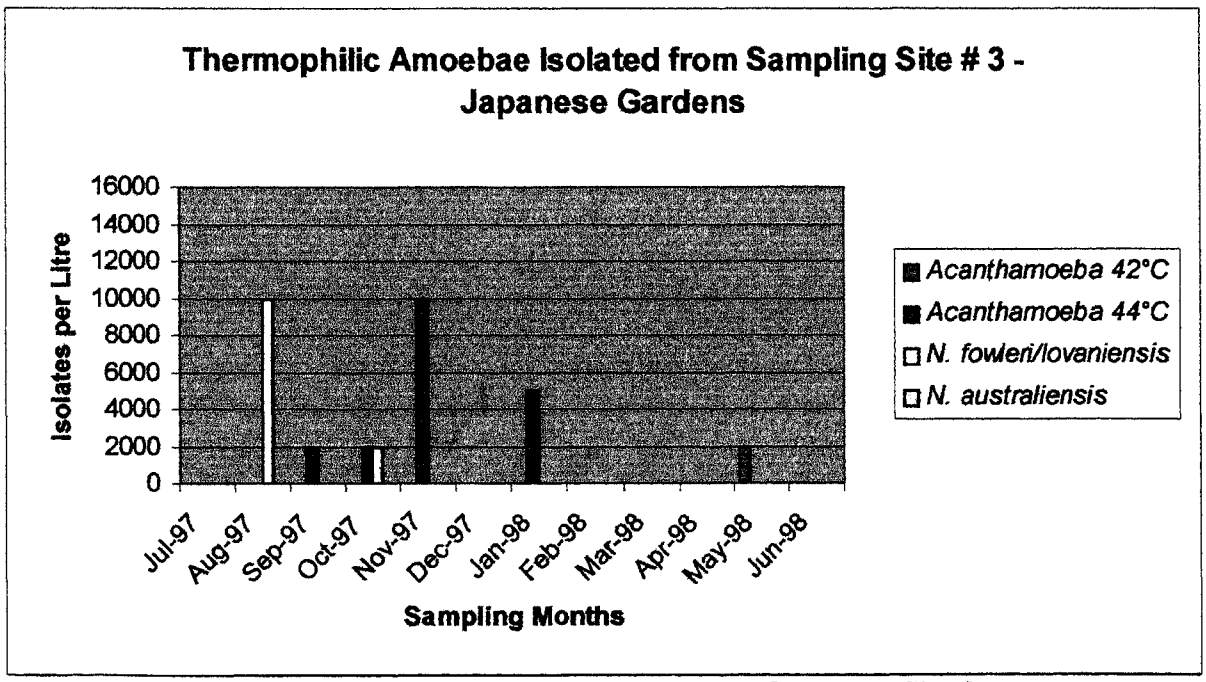


Figure 3: Thermophilic Amoebae Isolated from Sampling Site 3

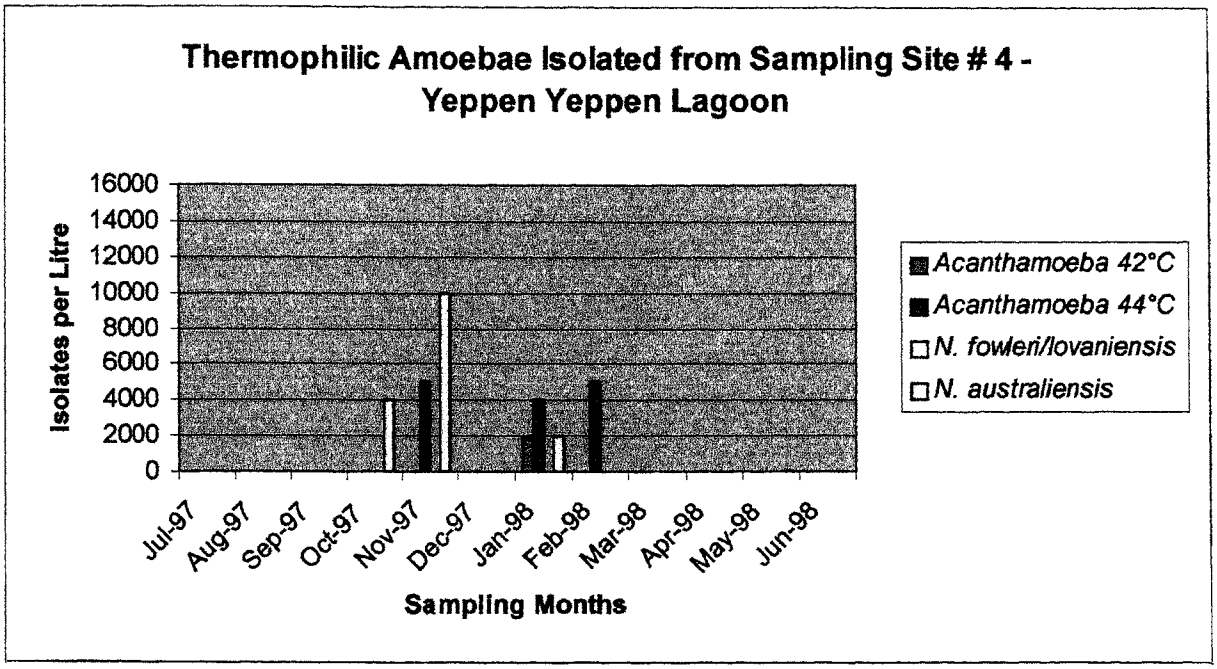


Figure 4: Thermophilic Amoebae Isolated from Sampling Site 4

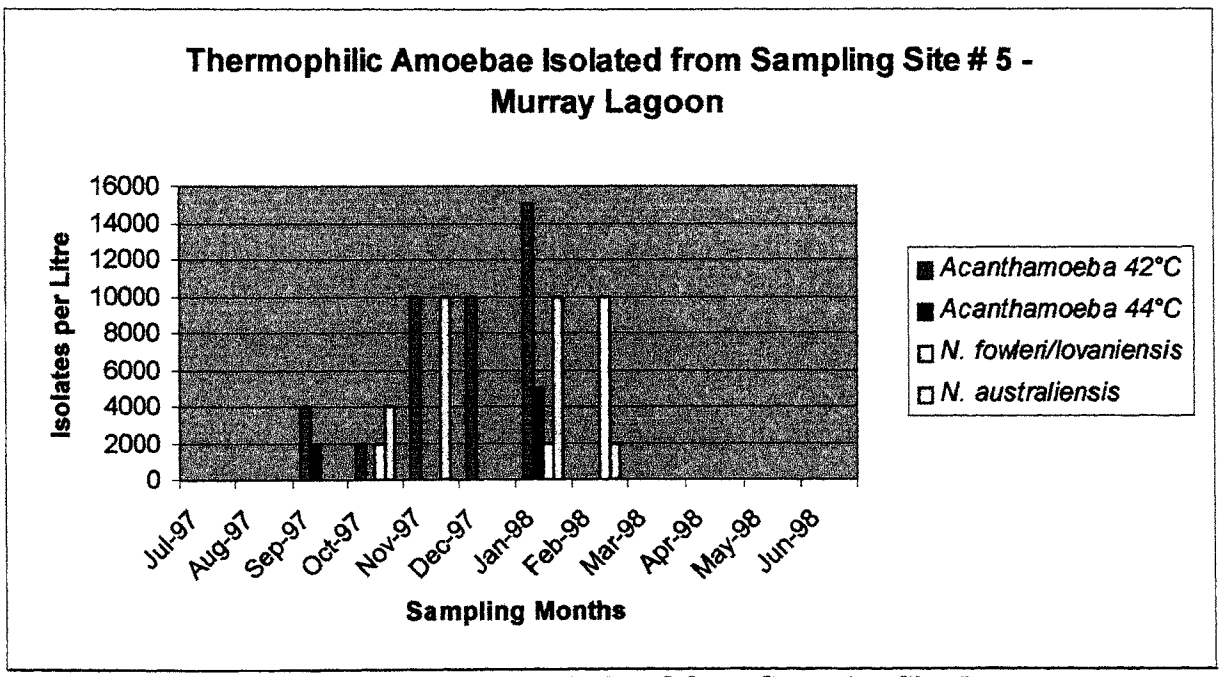
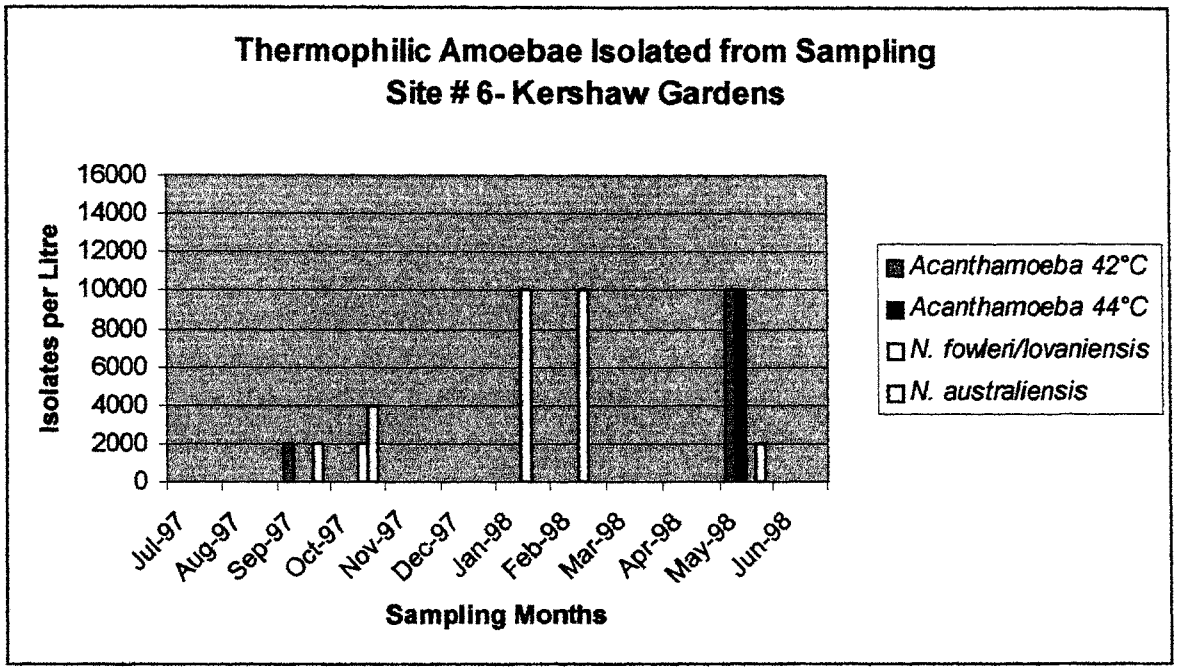


Figure 5: Thermophilic Amoebae Isolated from Sampling Site 5

Figure 6: Thermophilic Amoebae Isolated from Sampling Site 6



Illustrations 7 and 8 (below) display the rainfall and average temperatures recorded by the Rockhampton Bureau of Meteorology during the same time period as the survey samples were collected. The highest monthly temperature was recorded in February 1998 with a maximum of 33.4°C. No thermophilic amoebae isolates were recovered from the water bodies during the coolest months of July 1997 with a maximum of 22.9°C and minimum of 10.9°C and June 1998 with a maximum of 25.1°C and minimum of 10.8°C.

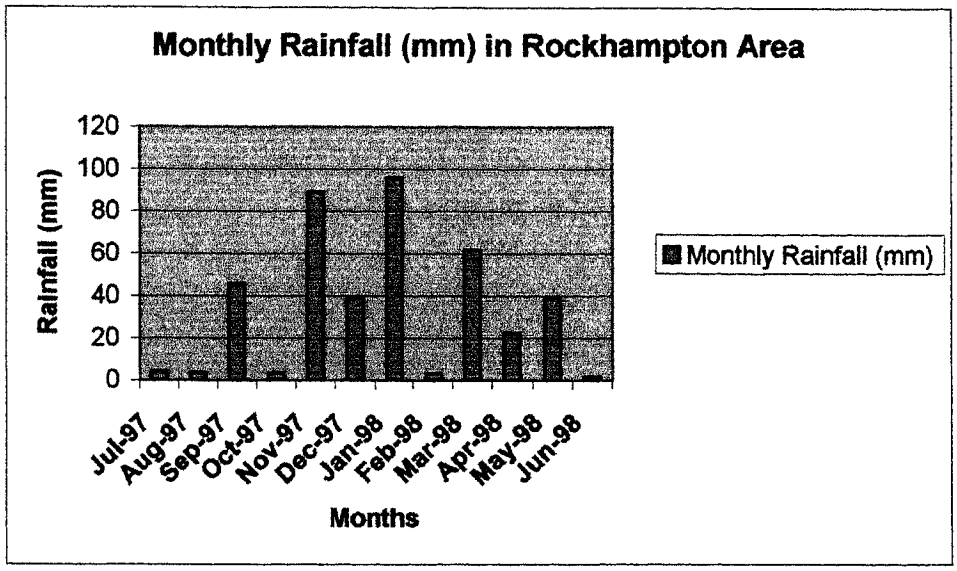


Figure 7: **Monthly Rainfall in Rockhampton Area During the Survey Period (Rockhampton Bureau of Meteorology, 2001)**

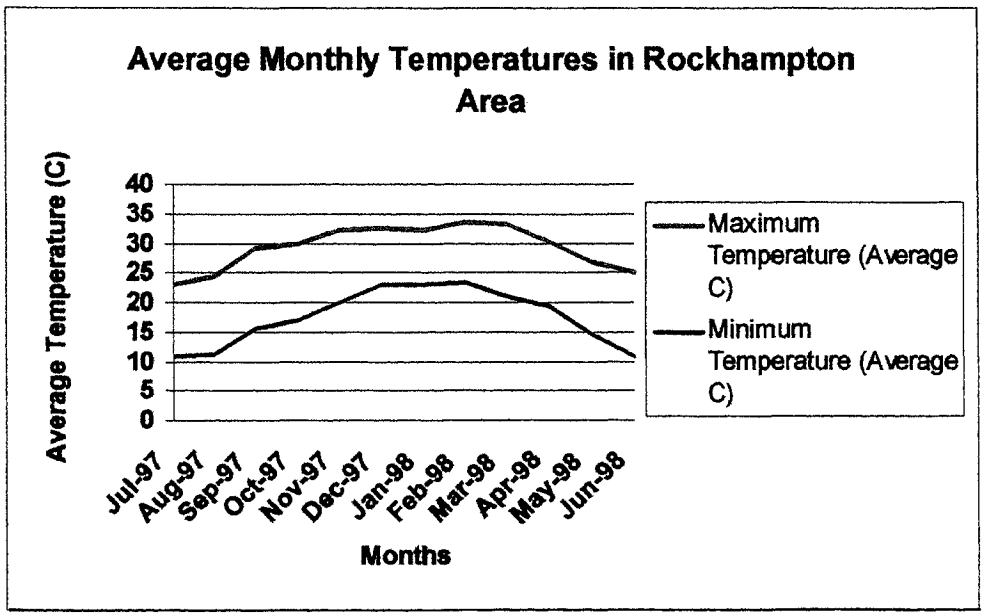


Figure 8 **Average Monthly Temperatures in Rockhampton Area During the Survey Period (Rockhampton Bureau of Meteorology, 2001)**

Figures 9 & 10 (below) present the pH values and temperatures recorded from each water sample collected during the 12-month survey.

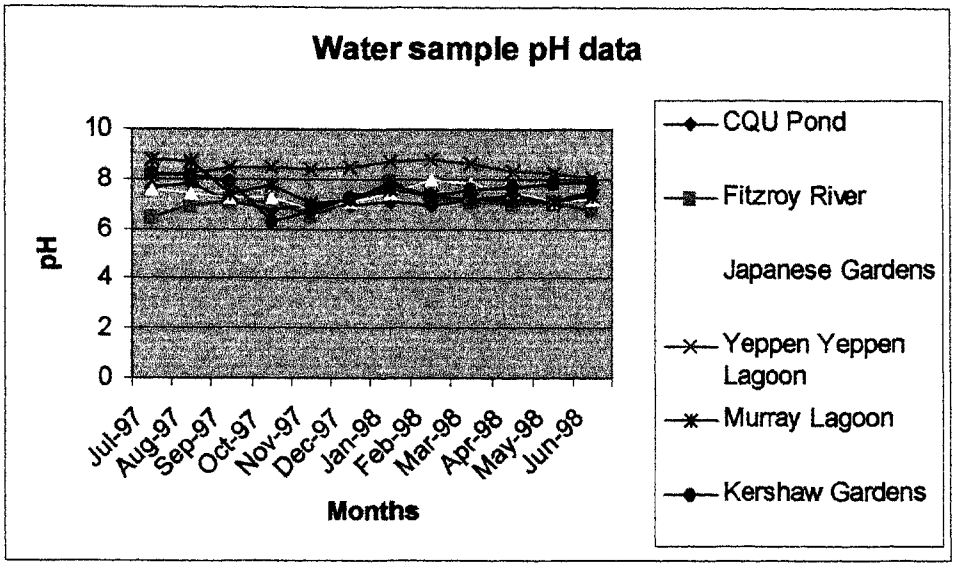


Figure 9: Water Sample pH Readings

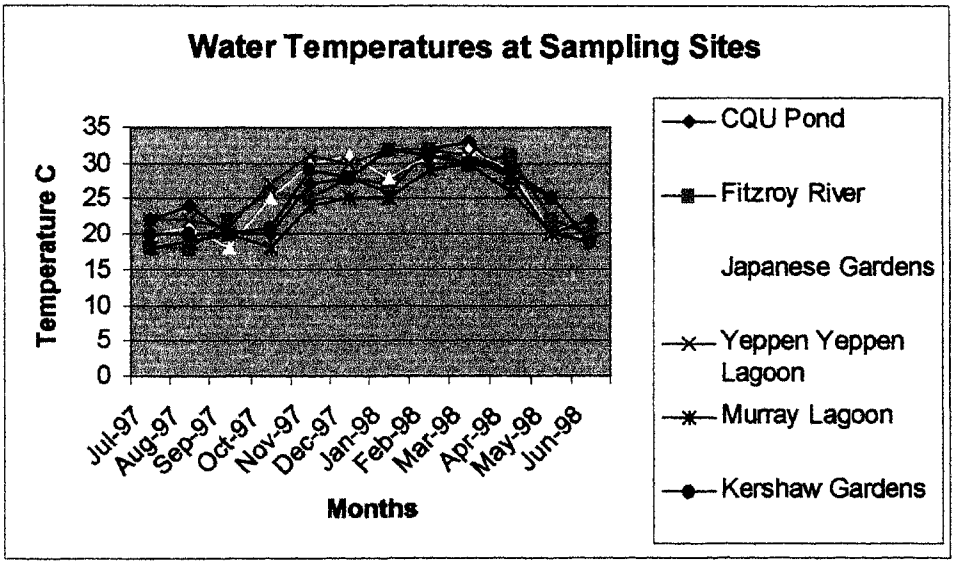


Figure 10: Water Temperatures Recorded at Sampling Sites

Thermophilic isolates from the CQU pond, when compared with those from the Japanese Gardens, Yeppen Yeppen Lagoon, Murray Lagoon and the Kershaw Gardens show a lower total number of successful samplings as well as less isolates per month. A fresh-water inflow-regulating valve automatically controls the water level of this pond.

The Fitzroy River also displayed significantly less thermophilic amoebae activity over the 12-month period of the survey. These results could be the consequence of the significantly greater volume and depth of flowing water particularly when compared with the Yeppen Yeppen Lagoon and Murray Lagoon that are land-locked and only experience fresh water inflows from rainwater.

The Japanese Gardens pond displays a moderate population of thermophilic amoebae. This is also an ornamental pond like the CQU pond. However, the Japanese Gardens is different from the CQU pond in that it has a larger surface area, minimal plant life, mostly full sun exposure during the day and a large population of fish. The average depth is 1 metre and an artificial waterfall continually circulates and aerates the water. Waste products from the fish population would contribute organic material to the pond and which should promote a bacterial population. This in turn would support the numbers of amoebae, including thermophiles, detected during the 12-month survey.

The Kershaw Gardens pond has many similar features to the Japanese Gardens pond including: man-made construction, large surface area, artificial waterfall and full exposure to sunlight. They differ in that the Kershaw Gardens pond has increased plant-

life. The Kershaw Gardens pond had two episodes of isolation of *N. fowleri/N. lovaniensis* in January and February 1998 whereas this species was isolated from the Japanese Gardens pond in August and in October 1997. The presence of water plants and possibly more fish could be significant factors that promote colonisation of the water by thermophilic amoebae.

The remaining two lagoons, namely the Yeppen Yeppen Lagoon and the Murray Lagoon had recoverable quantities of thermophilic amoebae in the warmest months of the year. These lagoons have very similar physical characteristics. Both have populations of fish, eels and turtles that would contribute to the high levels of organic material present within the water. Mixed populations of amoebae were detected. However, the Murray Lagoon had higher thermophilic amoeba population numbers over a longer period when compared with the Yeppen Yeppen Lagoon.

The field sampling and isolation data were statistically analysed (using the computer software application SPSS - Version 10.1) against the following Null Hypotheses:

(1) There is no seasonal variation in the number of *N. fowleri/lovaniensis* recovered

(2) There is no seasonal variation in the number of *N. australiensis* recovered

(3) There is no seasonal variation in the number of *Acanthamoeba* 42°C recovered

(4) There is no seasonal variation in the number of *Acanthamoeba* 44°C recovered

(5) There is no seasonal variation in the TOTAL number of amoebae recovered

(6) There is no correlation between the number of *N. fowleri/lovaniensis* recovered each month and the maximum temperature

(7) There is no correlation between the number of *N. australiensis* recovered each month and the maximum temperature

(8) There is no correlation between the number of *Acanthamoeba* 42°C recovered each month and the maximum temperature

(9) There is no correlation between the number of *Acanthamoeba* 44°C recovered each month and the maximum temperature

(10) There is no correlation between the TOTAL number of amoebae recovered each month and the maximum temperature

(11) There is no correlation between the number of *N. fowleri/lovaniensis* recovered each month and the minimum temperature

(12) There is no correlation between the number of *N. australiensis* recovered each month and the minimum temperature

(13) There is no correlation between the number of *Acanthamoeba* 42°C recovered each month and the minimum temperature

(14) There is no correlation between the number of *Acanthamoeba* 44°C recovered each month and the minimum temperature

(15) There is no correlation between the TOTAL number of amoebae recovered each month and the minimum temperature.

(16) There is no correlation between the TOTAL number of amoebae recovered each month and rainfall.

1st attempt: Hypotheses 1 - 5: General Linear Model

Between-Subjects Factors

		Value Label	N
SEASON	1.00	Winter	3
	2.00	Spring	3
	3.00	Summer	3
	4.00	Autumn	3

Multivariate Tests[c]

Source	Dependent Variable	Value	F	Hypothesis of df	Error df	Sig.
Intercept	Pillai's Trace	.857	7.513[a]	4.000	5.000	.024
	Wilks' Lambda	.143	7.513[a]	4.000	5.000	.024
	Hotelling's Trace	6.011	7.513[a]	4.000	5.000	.024
	Roy's Largest Root	6.011	7.513[a]	4.000	5.000	.024
Season	Pillai's Trace	1.526	1.811	12.000	21.000	.113
	Wilks' Lambda	.079	1.814	12.000	13.520	.147
	Hotelling's Trace	4.943	1.510	12.000	11.000	.251
	Roy's Largest Root	3.264	5.713[b]	4.000	7.000	.023

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	*N. fowleri/lovaniensis*	243666666.7[a]	3	81222222.222	1.740	.236
	N. australiensis	244666666.7[b]	3	81555555.556	3.375	.075
	Acanthamoeba 42C	182666666.7[c]	3	60888888.889	1.928	.205
	Acanthamoeba 44C	116250000.0[d]	3	38750000.000	1.000	.441
Intercept	*N. fowleri/lovaniensis*	243000000.0	1	243000000.00	5.207	.052
	N. australiensis	300000000.0	1	300000000.00	12.414	.008
	Acanthamoeba 42C	385333333.3	1	385333333.33	12.136	.008
	Acanthamoeba 44C	270750000.0	1	270750000.00	6.987	.030
Season	*N. fowleri/lovaniensis*	243666666.7	3	81222222.222	1.740	.236
	N. australiensis	244666666.7	3	81555555.556	3.375	.075
	Acanthamoeba 42C	182666666.7	3	60888888.889	1.918	.205
	Acanthamoeba 44C	116250000.0	3	38750000.000	1.000	.441
Error	*N. fowleri/lovaniensis*	373333333.3	8	46666666.667		
	N. australiensis	193333333.3	8	24166666.667		
	Acanthamoeba 42C	254000000.0	8	31750000.000		
	Acanthamoeba 44C	310000000.0	8	38750000.000		
Total	*N. fowleri/lovaniensis*	860000000.0	12			
	N. australiensis	738000000.0	12			
	Acanthamoeba 42C	822000000.0	12			
	Acanthamoeba 44C	697000000.0	12			
Corrected Total	*N. fowleri/lovaniensis*	617000000.0	11			
	N. australiensis	438000000.0	11			
	Acanthamoeba 42C	436666666.7	11			
	Acanthamoeba 44C	426250000.0	11			

a. R Squared = .395 (Adjusted R Squared = .168)
b. R Squared = .559 (Adjusted R Squared = .393)
c. R Squared = .418 (Adjusted R Squared = .200)
d. R Squared = .273 (Adjusted R Squared = .000)

The first attempt at analysis by classifying the data into 4 seasons showed that there was not enough variability to detect differences among the seasons resulting in a failure to reject the null hypotheses 1-5. See below for further modified results.

2nd Attempt General Linear Model: Seasons Redefined into 2 Categories

Autumn/Winter (March - August) & Spring/Summer (September - February)

Between-Subjects Factors

		Value Label	N
HALFYEAR	1.00	Autumn/Winter	6
	2.00	Spring/Summer	6

Multivariate Tests[b]

Source	Dependent Variable	Value	F	Hypothesis of df	Error df	Sig.
Intercept	Pillai's Trace	.854	10.221[a]	4.000	5.000	.024
	Wilks' Lambda	.146	10.221[a]	4.000	5.000	.024
	Hotelling's Trace	5.841	10.221[a]	4.000	5.000	.024
	Roy's Largest Root	5.841	10.221[a]	4.000	5.000	.024
HALFYEAR	Pillai's Trace	.736	5.621[a]	12.000	21.000	.113
	Wilks' Lambda	.237	5.621[a]	12.000	13.520	.147
	Hotelling's Trace	3.212	5.621[a]	12.000	11.000	.251
	Roy's Largest Root	3.212	5.621[a]	4.000	7.000	.023

a. Exact Statistic
b. Design: Intercept + HALFYEAR

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	*N. fowleri/lovaniensis*	96333333.333[a]	1	96333333.333	1.850	.204
	N. australiensis	176333333.3[b]	1	176333333.3	6.739	.027
	Acanthamoeba 42C	133333333.3[c]	1	133333333.3	4.396	.062
	Acanthamoeba 44C	90750000.000[d]	1	90750000.000	2.705	.131
Intercept	*N. fowleri/lovaniensis*	243000000.0	1	243000000.0	4.667	.056
	N. australiensis	300000000.0	1	300000000.0	11.465	.007
	Acanthamoeba 42C	385333333.3	1	385333333.3	12.703	.005
	Acanthamoeba 44C	270750000.0	1	270750000.0	8.070	.018
HALFYEAR	*N. fowleri/lovaniensis*	96333333.333	1	96333333.333	1.850	.204
	N. australiensis	176333333.3	1	176333333.3	6.739	.027
	Acanthamoeba 42C	133333333.3	1	133333333.3	4.396	.062
	Acanthamoeba 44C	90750000.000	1	90750000.000	2.705	.131
Error	*N. fowleri/lovaniensis*	520666666.7	10			
	N. australiensis	261666666.7	10			
	Acanthamoeba 42C	303333333.3	10			
	Acanthamoeba 44C	335500000.0	10			
Total	*N. fowleri/lovaniensis*	860000000.0	12			
	N. australiensis	738000000.0	12			
	Acanthamoeba 42C	822000000.0	12			
	Acanthamoeba 44C	697000000.0	12			
Corrected Total	*N. fowleri/lovaniensis*	617000000.0	11			
	N. australiensis	438000000.0	11			
	Acanthamoeba 42C	436666666.7	11			
	Acanthamoeba 44C	426250000.0	11			

a. R Squared = .156 (Adjusted R Squared = .072) b. R Squared = .403 (Adjusted R Squared = .343)
c. R Squared = .305 (Adjusted R Squared = .236) d. R Squared = .213 (Adjusted R Squared = .134)

Hypothesis 1: There was no significant difference in the numbers of *N. fowleri/lovaniensis* measured in spring and summer compared with autumn and winter ($F(1,10)=1.850, p=.204$, ns).

Hypothesis 2: There was a **significantly** greater number of *N. australiensis* measured in spring and summer compared with autumn and winter ($F(1,10)=6.739, p=.027$).

Hypothesis 3: There was no significant difference in the number of *Acanthamoeba* 42°C measured in spring and summer compared with autumn and winter ($F(1,10)=4.396, p=.062$, ns).

Hypothesis 4: There was no significant difference in the number of *Acanthamoeba* 44°C measured in spring and summer compared with autumn and winter (F(1,10)=1.850,p=.204, ns).

Hypothesis 5: A multivariate ANOVA demonstrated **significantly** greater numbers of total amoeba measured in spring and summer compared with autumn and winter (Pillai's Trace, F(4,7)=5.621,p=.024).

Correlations

Correlations

		Max. Temp	Min. Temp	RAINFALL
N. fowleri/lovaniensis	Pearson Correlation	.285	.355	-.163
	Sig. (1-tailed)	.185	.129	.306
	N	12	12	12
N. australiensis	Pearson Correlation	.377	.326	.594
	Sig. (1-tailed)	.114	.150	.021
	N	12	12	12
Acanthamoeba 42C	Pearson Correlation	.392	.451	.723
	Sig. (1-tailed)	.104	.071	.004
	N	12	12	12
Acanthamoeba 44C	Pearson Correlation	.422	.449	.642
	Sig. (1-tailed)	.086	.071	.012
	N	12	12	12

Correlations

		Max. Temp	Min. Temp	RAINFALL
TOTAL AMOEBAE	Pearson Correlation	.494	.533	.570
	Sig. (1-tailed)	.051	.037	.027
	N	12	12	12

Hypothesis 6: There was no significant correlation between the numbers of *N. fowleri/lovaniensisi* recovered each month and the maximum temperature (r(10)=.285,p=.185,ns).

Hypothesis 7: There was no significant correlation between the numbers of *N. australiensis* recovered each month and the maximum temperature (r(10)=.377,p=.114,ns).

Hypothesis 8: There was no significant correlation between the number of *Acanthamoeba* 42°C recovered each month and the maximum temperature (r(10)=.392,p=.104,ns).

Hypothesis 9: There was no significant correlation between the number of *Acanthamoeba* 44°C recovered each month and the maximum temperature (r(10)=.422,p=.086,ns).

Hypothesis 10: There was **a marginally significant** correlation between the total number of amoeba recovered each month and the maximum temperature (r(10)=.494,p=.051).

Hypothesis 11: There was no significant correlation between the numbers of N. *fowleri/lovaniensis* recovered each month and the minimum temperature (r(10)=0.355,p=.129,ns).

Hypothesis 12: There was no significant correlation between the numbers of *N. australiensis* recovered each month and the minimum temperature (r(10)=.326,p=.150,ns).

Hypothesis 13: There was no significant correlation between the number of *Acanthamoeba* 42°C recovered each month and the minimum temperature (r(10)=.451,p=.071,ns).

Hypothesis 14: There was no significant correlation between the number of *Acanthamoeba* 44°C recovered each month and the minimum temperature (r(10)=.449,p=.071,ns).

Hypothesis 15: There was **a significant** correlation between the total number of amoeba recovered each month and the minimum temperature (r(10)=.533,p=.037).

Hypothesis 16: There was **a significant** correlation between the total number of amoeba recovered each month and rainfall (r(10)=.570,p=.027).

This analysis confirms that there was a statistically significant increase in the total numbers of thermophilic amoebae recovered during the warmer months of the sampling period. This result was not unexpected, given the preferred temperature range of these organisms.

3.1.1 Identification

Identification studies were performed by examination of the isolates using light microscopy to collect morphological data on the trophozoites and cysts. The 12-month sampling survey produced 55 isolates of different thermophilic amoebae were recovered (Tables 5 – 10). Based on the techniques utilized for this project, complete speciation was not always possible (Example: Some isolates were only able to be identified as *N. fowleri/ lovaniensis*). *Acanthamoeba* spp. were identified by cyst morphology and the absence of the flagellated stage. A summary of the isolates collected during the 12-month survey is presented below in Table 4:

Table 4: Amoebae Collected in 12-Month Survey of 6 Sampling Sites

Species	Number of Isolates	% of Isolates
N. fowleri/N. lovaniensis	11	19.3
N. australiensis	12	21.8
Acanthamoeba spp	23	41.8
Non-viable amoebae	9	15.8
Total of Isolates Collected	**55**	

The following tables (5 - 10) summarise identification data for all viable, thermophilic amoebae isolated from the six sampling sites over the 12-month study. Cyst shape was abbreviated in all tables as either: S - Spherical or P - Polygonal.

Table 5: Site 1: CQU Pond - Thermophilic Amoebae Isolates

No.	Month	Cyst Size (μm)	Cyst Shape	42°C	45°C	Flagellation	Identification
1	12/97	14	S	+	+	+	*N. australiensis*
2	02/98	12	S	+	+	+	*N. fowleri/lovaniensis*
3	04/98	16	P	+	-	-	*Acanthamoeba* spp.

Table 6: Site 2: Fitzroy River- Thermophilic Amoebae Isolates

No.	Month	Cyst Size (μm)	Cyst Shape	42°C	45°C	Flagellation	Identification
1	09/97	15	S	+	-	-	*Acanthamoeba* spp.
2	09/97	12	S	+	-	+	*N. australiensis*
3	09/97	12	S	+	+	+	*N. fowleri/lovaniensis*
4	11/97	13	P	+	-	-	*Acanthamoeba* spp.
5	02/98	20	S	+	-	-	*Acanthamoeba* spp.
6	02/98	15	S	+	+	+	*N. fowleri/lovaniensis*

Table 7: Site 3: Japanese Gardens- Thermophilic Amoebae Isolates

No.	Month	Cyst Size (μm)	Cyst Shape	42°C	45°C	Flagellation	Identification
1	08/97	10	S	+	+	+	*N. fowleri/lovaniensis*
2	09/97	15	P	+	-	-	*Acanthamoeba* spp.
3	10/97	13	S	+	+	+	*N. fowleri/lovaniensis*
4	10/97	15	P	+	-	-	*Acanthamoeba* spp.
5	11/97	17	P	+	-	-	*Acanthamoeba* spp.
6	01/98	16	S	+	-	-	*Acanthamoeba* spp.
7	05/98	13	P	+	-	-	*Acanthamoeba* spp.

Table 8: Site 4: Yeppen Yeppen Lagoon- Thermophilic Amoebae Isolates

No.	Month	Cyst Size (μm)	Cyst Shape	42°C	45°C	Flagellation	Identification
1	10/97	12	S	+	-	+	*N. australiensis*
2	11/97	20	P	+	-	-	*Acanthamoeba* spp.
3	11/97	13	S	+	-	+	*N. australiensis*
4	01/98	13	S	+	-	+	*N. australiensis*
5	01/98	15	P	+	-	-	*Acanthamoeba* spp.
6	01/98	15	P	+	-	-	*Acanthamoeba* spp.
7	02/98	13	P	+	-	-	*Acanthamoeba* spp.

Table 9: Site 5: Murray Lagoon- Thermophilic Amoebae Isolates

No.	Month	Cyst Size (μm)	Cyst Shape	42°C	45°C	Flagellation	Identification
1	09/97	15	P	+	-	-	*Acanthamoeba* spp.
2	09/97	15	P	+	-	-	*Acanthamoeba* spp.
3	10/97	17	P	+	-	-	*Acanthamoeba* spp.
4	10/97	10	S	+	+	+	*N. fowleri/lovaniensis*
5	10/97	12	S	+	-	+	*N. australiensis*
6	11/97	16	P	+	-	-	*Acanthamoeba* spp.
7	11/97	10	S	+	-	+	*N. australiensis*
8	12/97	12	P	+	-	-	*Acanthamoeba* spp.
9	01/98	16	P	+	-	-	*Acanthamoeba* spp.
10	01/98	20	P	+	-	-	*Acanthamoeba* spp.
11	01/98	13	S	+	+	+	*N. fowleri/lovaniensis*
12	01/98	12	S	+	-	+	*N. australiensis*
13	02/98	15	S	+	+	+	*N. fowleri/lovaniensis*
14	02/98	14	S	+	-	+	*N. australiensis*

Table 10: Site 6: Kershaw Gardens- Thermophilic Amoebae Isolates

No.	Month	Cyst Size (μm)	Cyst Shape	42°C	45°C	Flagellation	Identification
1	09/97	20	P	+	-	-	*Acanthamoeba* spp.
2	09/97	10	S	+	-	+	*N. australiensis*
3	10/97	12	S	+	+	+	*N. fowleri/lovaniensis*
4	10/97	11	S	+	-	+	*N. australiensis*
5	01/98	15	S	+	+	+	*N. fowleri/lovaniensis*
6	02/98	12	S	+	+	+	*N. fowleri/lovaniensis*
7	05/98	14	P	+	-	-	*Acanthamoeba* spp.
8	05/98	16	P	+	-	-	*Acanthamoeba* spp.
9	05/98	13	S	+	-	+	*N. australiensis*

3.2 Culture Dynamics

This experiment demonstrated the effects of feeding a *N.fowleri* population a diet of viable *E. coli* versus non-viable *E. coli*. Figures 11 and 12 (below) present these results.

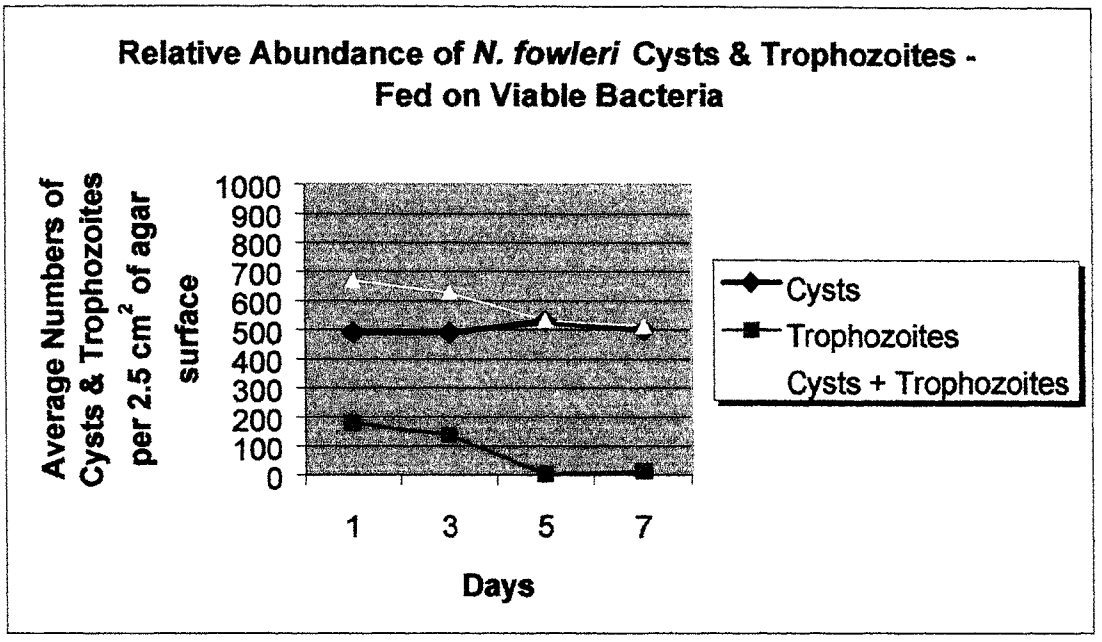


Figure 11: Relative Abundance of *N. fowleri* Fed on Viable Bacteria

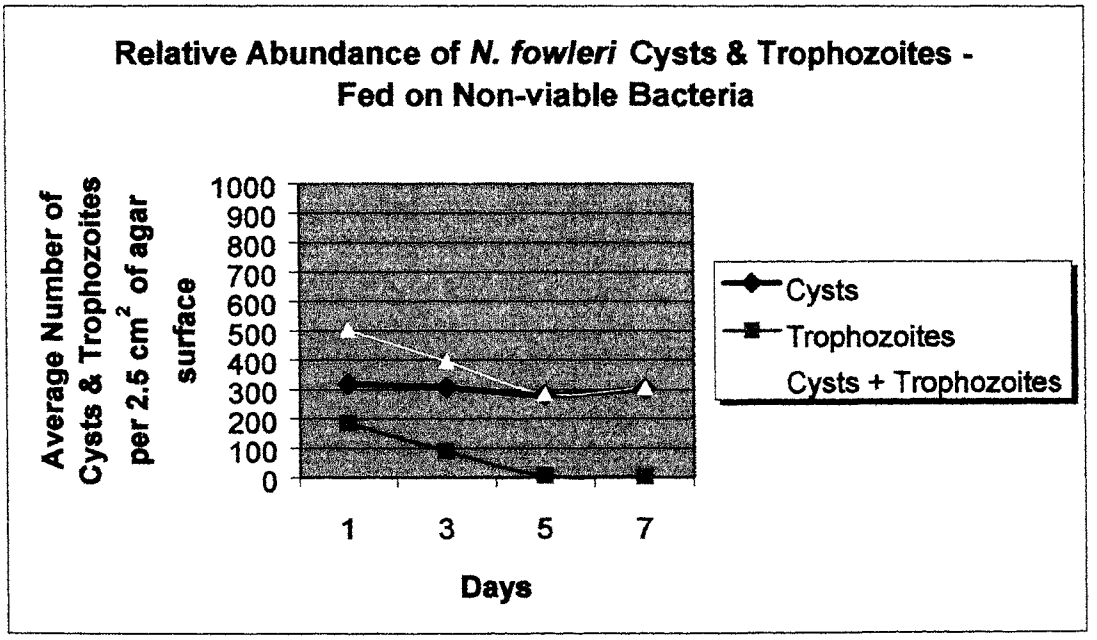


Figure 12: Relative Abundance of *N. fowleri* Fed on Non-viable Bacteria

The complete data produced as a result of the Culture Dynamics experiment is set out below in Table 11:

Table 11: Relative Abundance of *N. fowleri* fed on Live and Non-viable *E. coli*

Relative Abundance of *N. fowleri* fed on Live and Non-viable *E. coli*																				
Section	1		2		3		4		5		6		7		8		9		10	
	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T
Day 1																				
Plate 1 (V)	600	250	400	125	400	100	500	100	600	200	450	150	650	125	500	200	700	150	500	100
Plate 2 (V)	350	150	450	250	120	75	675	150	725	225	475	100	480	150	365	130	530	215	495	200
Plate 3 (V)	425	250	475	190	440	200	330	219	500	150	525	210	530	250	560	250	400	200	570	300
Plate 4 (NV)	175	125	200	175	200	200	250	100	260	150	270	100	245	100	300	160	300	125	210	150
Plate 5 (NV)	130	200	250	145	110	135	250	250	160	200	300	150	300	220	400	200	400	250	275	150
Plate 6 (NV)	300	300	400	250	450	250	350	225	500	150	325	150	1375	225	325	200	285	225	250	275
Average V	458	217	442	188	320	125	502	156	608	192	483	153	553	175	475	193	543	188	522	200
Average NV	202	208	283	190	253	195	283	192	307	167	298	133	640	182	342	187	328	200	245	192
Day 3																				
Plate 1 (V)	800	100	650	50	500	60	800	300	600	500	650	40	680	400	550	30	450	50	480	40
Plate 2 (V)	250	100	280	70	520	90	600	100	680	80	420	120	320	120	550	80	400	160	300	110
Plate 3 (V)	300	400	200	400	340	100	600	150	500	80	380	50	600	100	450	120	380	50	440	90
Plate 4 (NV)	760	200	250	100	300	100	250	150	450	150	250	100	200	150	150	250	200	100	200	150
Plate 5 (NV)	250	80	400	90	200	30	250	100	220	60	300	70	350	60	200	60	420	100	500	80
Plate 6 (NV)	300	80	300	50	360	50	250	100	340	50	200	30	380	30	300	70	250	20	420	30
Average V	450	200	377	173	453	83	667	183	593	220	483	70	533	207	517	77	410	87	407	80
Average NV	437	120	317	80	287	60	250	117	337	87	250	66.7	310	80	217	127	290	73	373	87
Day 5																				
Plate 1 (V)	500	0	650	0	500	0	250	0	600	0	650	0	300	0	420	0	500	0	450	0
Plate 2 (V)	200	10	500	0	300	0	400	0	480	10	360	0	600	0	500	0	450	0	650	0
Plate 3 (V)	800	10	850	0	500	10	600	0	800	30	850	0	480	0	650	0	500	0	600	0
Plate 4 (NV)	200	0	150	0	250	0	150	0	200	0	300	0	400	20	250	30	300	10	200	20
Plate 5 (NV)	160	0	380	0	180	0	300	10	28	0	180	0	160	10	250	10	300	0	320	10
Plate 6 (NV)	250	0	150	0	220	0	320	0	250	10	420	10	500	0	500	0	640	0	520	10
Average V	500	6.7	667	0	433	3.3	417	0	627	13	620	0	460	0	523	0	483	0	567	0
Average NV	203	0	227	0	217	0	257	3.3	159	3.3	300	3.33	353	10	333	13	413	3.3	347	13
Day 7																				
Plate 1 (V)	600	20	680	10	500	50	500	60	400	0	600	40	500	30	560	30	350	10	600	0
Plate 2 (V)	200	20	420	20	250	20	680	10	200	10	300	20	750	10	840	10	500	0	660	10
Plate 3 (V)	500	0	580	0	450	0	800	0	420	0	600	10	640	0	300	0	300	0	320	0
Plate 4 (NV)	410	0	271	1	221	4	169	1	231	4	134	2	523	15	256	1	165	15	143	0
Plate 5 (NV)	335	5	396	3	253	1	248	5	359	10	135	2	375	2	412	6	275	2	326	1
Plate 6 (NV)	155	1	205	1	235	2	205	6	330	1	315	1	492	10	583	2	539	4	336	3
Average V	433	13	560	10	400	23	660	23	340	3.3	500	23.3	630	13	567	13	383	3.3	527	3.3
Average NV	300	2	291	1.7	236	2.3	207	4	307	5	195	1.67	463	9	417	3	326	7	268	1.3

(C = Cysts; T = trophozoites; V = Viable; NV = non-viable)

The culture dynamics data was statistically analysed (using the computer software application SPSS - Version 10.1) against the following Null Hypotheses:

(1a) There is no difference in the total number of amoebae (Trophozoites (T) + Cysts (C)) when grown on viable or non-viable bacteria.

(2a) There is no difference between the total number of amoebae T's and C's when grown on either viable or non-viable bacteria at day 7.

(3a) There is no difference between the rates of production of T's when grown on viable or non-viable bacteria.

(4a) There is no difference between the rates of production of C's when grown on viable or non-viable bacteria.

(5a) There is no difference between the rate of production of amoebae (T + C) when grown on viable or non-viable bacteria.

Hypothesis 1a: General Linear Model

Within-Subjects Factors

Measure: Measure_1

TIME	Dependent Variable
1	TANDC1
2	TANDC3
3	TANDC5
4	TANDC7

Between-Subjects Factors

		Value Label	N
BACTERIA	1	Living (Viable)	3
	2	Dead (Non-viable)	3

Tests of Within-Subjects

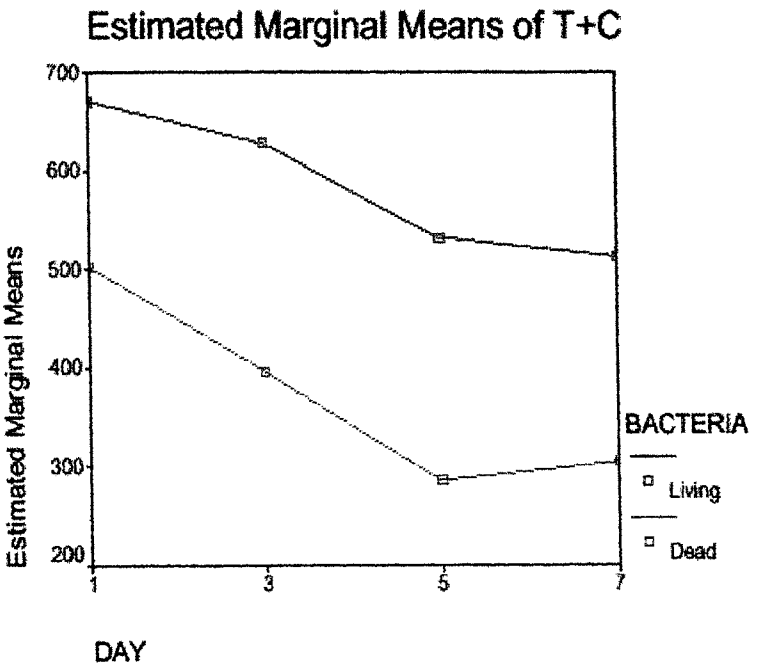
Measure: MEASURE_1

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
TIME Sphericity Assumed	134660.333	3	44893.444	6.182	.009
TIME * Sphericity Assumed	5308.333	3	1769.444	.244	.864
Error (TIME) Sphericity Assumed	87138.333	12	7261.528		

Tests of Between-Subjects Effects

Measure: MEASURE_1
Transformed Variable: Average

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	5506584.000	1	5506584.000	441.242	.000
BACTERIA	272214.000	1	272214.000	21.812	.010
Error	49919.000	4	12479.750		



Conclusion for Hypothesis 1a: A repeated measures ANOVA was calculated across time, with bacteria either viable (living) or non-viable (dead), as the between plates factor. As shown in the graph above, there were a greater number of total amoebae (T+C) when grown on viable (living) as opposed to non-viable (dead) bacteria ($F(1,4)=21.81, p=.005$, one-tailed).

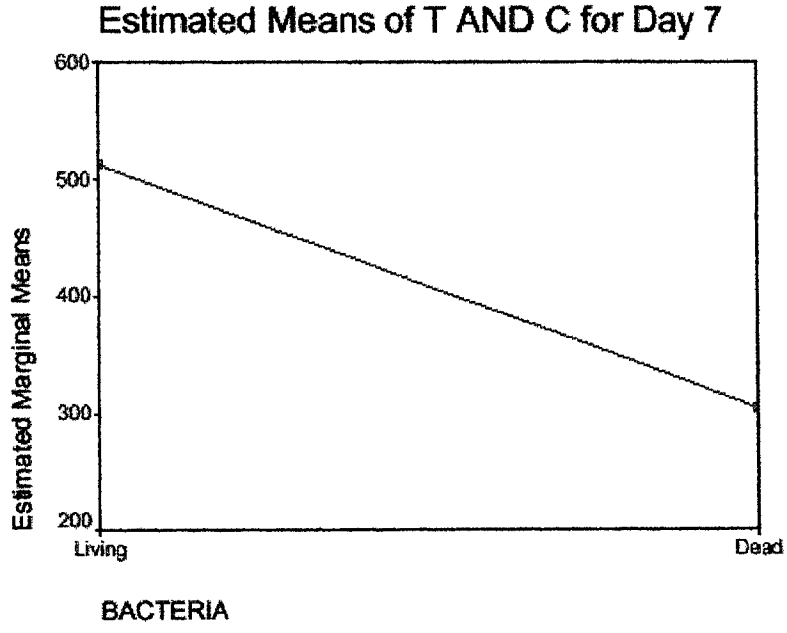
Hypothesis 2a: General Linear Model

Between-Subjects Factors

		Value Label	N
BACTERIA	1	Living (Viable)	3
	2	Dead (Non-viable)	3

Univariate Test Results

Source	Dependent Variable	Sum of Squares	df	Mean Square	F	Sig.
Contrast	TANDC1	41833.500	1	41833.500	3.208	.148
	TANDC3	79810.667	1	79810.667	8.719	.042
	TANDC5	90774.000	1	90774.000	8.685	.042
	TANDC7	65104.167	1	65104.167	40.271	.003
Error	TANDC1	52166.000	4	13041.500		
	TANDC3	36616.667	4	9154.167		
	TANDC5	41808.000	4	10452.000		
	TANDC7	6466.667	4	1616.667		



Conclusion for Hypothesis 2a: A multivariate ANOVA was calculated with bacteria (either living or dead) as the between factor. As shown in the graph above, a simple effect revealed that there were a greater number of total amoebae (T+C) when grown on viable (living) as opposed to non-viable (dead) bacteria on Day 7 ($F(1,4)=40.27, p=.002$, one-tailed).

Hypothesis 3a: General Linear Model

Within-Subjects Factors
Measure: Measure_1

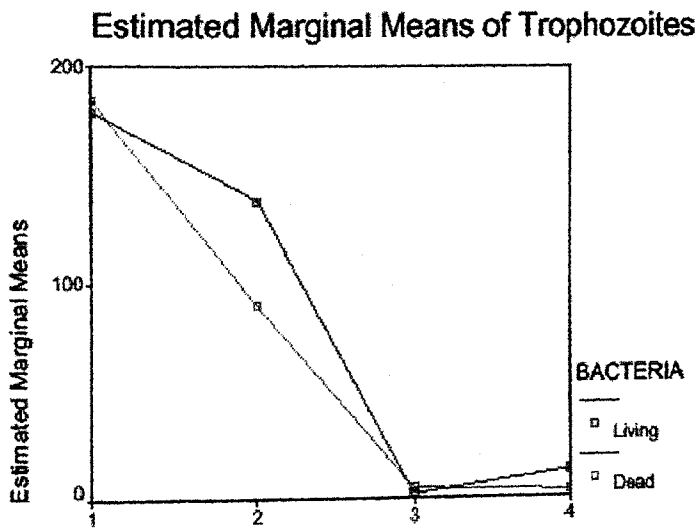
TIME	Dependent Variable
1	T1
2	T2
3	T3
4	T4

Between-Subjects Factors

		Value Label	N
BACTERIA	1	Living (Viable)	3
	2	Dead (Non-viable)	3

Tests of Within-Subjects
Measure: MEASURE_1

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
TIME Sphericity Assumed	134637.500	3	44879.167	43.731	.000
TIME * Sphericity Assumed	2781.000	3	927.000	.903	.468
Error (TIME) Sphericity Assumed	12315.000	12	1026.250		



Conclusion for Hypothesis 3a: As shown in the graph above, an interaction revealed no significant differences in the rate of production of trophozoites over time grown on either viable (living) or non-viable (dead) bacteria ($F(3,12)=0.90$, $p=.468$, ns).

Hypothesis 4a: General Linear Model

Within-Subjects Factors

Measure: Measure_1

TIME	Dependent Variable
1	C1
2	C3
3	C5
4	C7

Between-Subjects Factors

		Value Label	N
BACTERIA	1	Living	3
	2	Dead	3

Tests of Within-Subjects

Measure: MEASURE_1

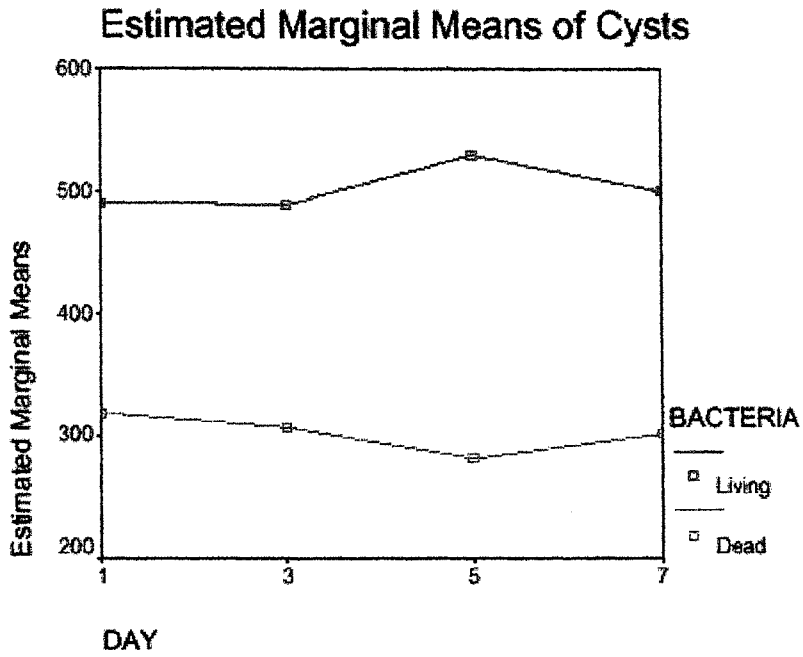
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
TIME Sphericity Assumed	226.833	3	75.611	.016	.997
TIME * Sphericity Assumed	5140.333	3	1713.444	.353	.788
Error (TIME) Sphericity Assumed	58172.833	12	4847.736		

Tests of Between-Subjects Effects

Measure: MEASURE_1

Transformed Variable: Average

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	3880104.167	1	3880104.167	355.133	.000
BACTERIA	241602.667	1	241602.667	22.113	.009
Error	43703.167	4	10925.792		



Conclusion for Hypothesis 4a: An interaction revealed no significant difference in the rates of production of cysts over time grown on either viable (living) or non-viable (dead) bacteria (F(3,12)=0.35, p=0.788, ns). However, there were significantly more cysts overall when grown on viable (living) as opposed to non-viable (dead) bacteria (F(1,4)=22.11, p=.004, one-tailed).

Hypothesis 5a: General Linear Model

Within-Subjects Factors

Measure: Measure_1

TIME	Dependent Variable
1	TANDC1
2	TANDC3
3	TANDC5
4	TANDC7

Between-Subjects Factors

		Value Label	N
BACTERIA	1	Living (Viable)	3
	2	Dead (Non-viable)	3

Tests of Within-Subjects

Measure: MEASURE_1

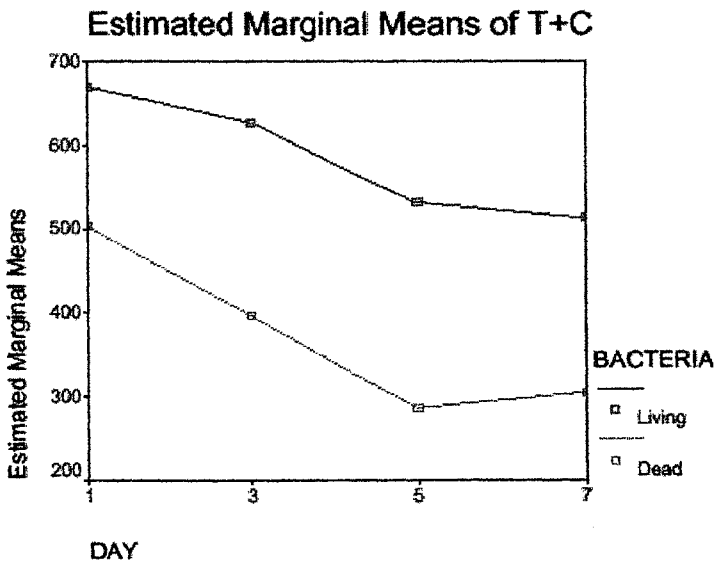
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
TIME Sphericity Assumed	134660.333	3	44893.444	6.182	.009
TIME * Sphericity Assumed	5308.333	3	1769.444	.244	.864
Error (TIME) Sphericity Assumed	87138.333	12	7261.528		

Tests of Between-Subjects Effects

Measure: MEASURE_1

Transformed Variable: Average

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	5506584.000	1	5506584.000	441.242	.000
BACTERIA	272214.000	1	272214.000	21.812	.009
Error	49919.000	4	12479.750		



Conclusion for Hypothesis 5a: As shown in the graph above, an interaction revealed no significant difference in the rate of production of total amoebae (T+C) when grown on either viable (living) or non-viable (dead) bacteria ($F(3,12)=0.24$, $p=.864$, ns). However, there was a significant overall decline in the quantity of amoebae over time ($f(3,12)=6.18$, $p=.009$, 2-tailed). In addition, per hypothesis 1, there were significantly more amoebae on the viable (living) bacteria plates ($F(1,4)=21.81$, $p=.005$, 1-tailed). The greater quantity of amoebae grown on viable bacteria was virtually entirely due to more cysts on those

plates (see results for Hypothesis 4a), rather than more trophozoites (see results for Hypothesis 3a). The decline in amoebae over time, in contrast, was virtually entirely due to the death of trophozoites and not cysts.

3.3 SEM

Results of this study were disappointing and no useful scientific data were obtained. This was due to the fact that amoeba cysts always appeared deformed with a crushed appearance predominating, It is possible the high vacuum of the gold coating process combined with the fragile characteristics of the cysts themselves and inadequate drying were the major reasons for these disappointing results. It would seem unlikely that SEM techniques could be routinely used for rapid identification of thermophilic amoebae.

3.3.1 Desiccator

The image shown below in Illustration 15 demonstrates an example of a SEM image attained after cysts were subjected to desiccation.

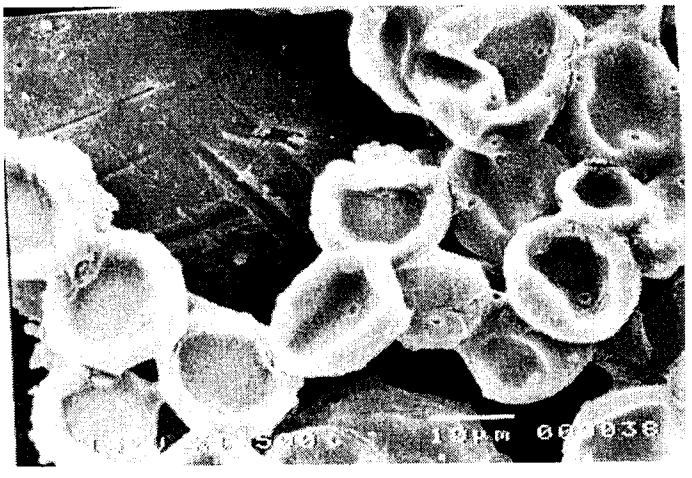


Illustration 15: SEM Image of Cysts of *Acanthamoeba* spp.

3.3.2 Critical Point Drying

The CPD process produced the amoebic cysts in a stabilised, dry form which was superior those produced by air-drying in a desiccator or under vacuum due to reduction in damage of the cysts. This method produced excellent images of cyst morphology however, the high voltages (15kV) required caused the target cyst to be vaporised before a corrected image could be captured for electronic storage.

3.3.3 Sputter coating

Images obtained showed a deformed cyst structure that was attributed to an affect of the vacuum process or inadequate drying. This study technique was not continued due to the significant morphological changes produced after a range of preparatory protocols were trialled.

3.4 DESICCATION STUDIES

The aim of the desiccation experiments was to examine the effect that prolonged drying (at room temperature 22°C and at room temperature 22°C in a silica gel desiccator) had on the long-term survival of cysts of selected amoebae (*N. fowleri; W. magna; Acanthamoeba* spp; *N. carteri*; *N. australiensis).* This experiment was designed to duplicate one of the conditions that an amoebic cyst may encounter in the environment,

for example, a pond that loses all of its water by evaporation. This would result in a transition from trophozoite to encysted forms that must then survive in the altered environment until the return of optimal growth and feeding conditions. Desiccation, of course, is not the only factor which would influence viability.

This experiment demonstrated the value of using an inert porous matrix such as the fishspine insulators beads as a 'carrier' of inoculum. This equates to a natural scenario when amoebae would congregate on inert inorganic or organic particles in their habitat. Tables 12 & 13 (below) present data from desiccation study experiments that were also statistically analysed using SSPS - Version 10.1(results follow on after Table 13).

Table 12: Desiccation Study at Room Temperature (22°C)

Trophozoites present: +
Trophozoites not seen: -

Week	1	2	3	4	5	6	7	8	9	10	11	12	13	14
W. magna	+	+	+	+	+	+	+	+	+	+	+	+	+	+
N. fowleri	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acanthamoeba spp.	+	+	+	+	-	+	+	+	+	+	+	+	+	+
N. australiensis	+	+	+	+	+	-	+	+	-	-	-	-	-	-
N. carteri	+	+	+	+	+	+	+	+	+	-	+	-	-	-

Table 13: Desiccation Study in a Desiccator at Room Temperature (22°C)

Trophozoites present:	+
Trophozoites not seen:	-

Week	1	2	3	4	5	6	7	8	9	10	11	12	13	14
W. magna	+	+	+	+	+	+	+	+	+	+	+	+	+	+
N. fowleri	+	+	+	+	+	+	+	+	+	+	-	+	+	+
Acanthamoeba spp.	+	+	+	+	+	-	+	+	+	+	-	+	+	+
N. australiensis	+	+	+	+	+	+	+	+	+	+	+	+	+	+
N. carteri	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Desiccation Study: Data for Survival at Room Humidity

		W. magna	*N. fowleri*	*Acanthamoeba* spp.
TIME	**Pearson Correlation**	-	-	.172
	Sig. (2-tailed)	-	-	.557
	N	14	14	14

Conclusion: Correlation of survival rates with time revealed that the *N. australiensis* was significantly prone to death due to desiccation over 14 weeks at room humidity (r= -.797, p=.001, 1-tailed). *N. carteri* was also significantly prone to eradication over 14 weeks at room humidity (r=-.745, p=.002, 1-tailed). *Acanthamoeba* spp. were not significantly prone to eradication over 14 weeks at room humidity (r=.172, p=.557, ns). Other amoeba were non-assessable due to high survival rates.

Desiccation Study: Data for Survival with Desiccation

		W. magna	*N. fowleri*	*Acanthamoeba* spp.	*N. australiensis*	*N. carteri*
TIME	**Pearson Correlation**	-	-.241	-.101	-	-
	Sig. (2-tailed)	-	.407	.730	-	-
	N	14	14	14	14	14

Conclusion: There was no significant tendency for any of the amoebae to be eradicated under desiccation conditions over the 14 weeks. *W. magna*, *N. australiensis* and *N.carteri* had perfect survival. There was no significant correlation between time and survival for *N.fowleri* (r=-.241, p=.407, ns) or *Acanthamoeba* spp. (r=-.101, p=.730, ns).

Desiccation Study: Difference between Room Humidity & Desiccation

		W. magna	*N. fowleri*	*Acanthamoeba* spp.	*N. australiensis*	*N. carteri*
TIME	**Pearson Correlation**	-	-.241	.022	-.797	-.745
	Sig. (2-tailed)	-	.407	.407	.001	.002
	N	14	14	14	14	14

Conclusion: *N. australiensis* were significantly more resistant to desiccation over time when compared with room humidity (r=-.797,p=.001, 2-tailed). In addition, *N. carteri* were significantly more survivable over time under desiccation when compared with room humidity (r=-.745, p=.002, 2-tailed). There were no significant differences for *Acanthamoeba* (r=.022, p=.942, ns) or *N. fowleri* (r=-.241, p=.407, ns). *W. magna* showed no adverse response to desiccation even after 14 weeks.

4.0 DISCUSSION

Two of the six aquatic sites sampled in this research, namely the Yeppen Yeppen and Murray Lagoons are particularly affected by high seasonal variations in temperature and rainfall, as they have no inflow of water other than rainwater run-off. Amoebae within these two lagoons may be subjected to significant water loss through dehydration when these lagoons enter a period of drying in drought periods. These amoebae would then have to rely on their cyst forms to survive until the next rainfall. Considering the robust nature of *N. fowleri* which was demonstrated to have a high survival rate in the desiccation experiments, it could be possible that this potential pathogen may survive a period of prolonged drought in a lagoon and when the water body is re-hydrated, dormant cysts will produce active trophozoites. This new generation of amoeba would bring with it a potential for human infection, as humans are attracted to ponds and lagoons during hot weather for recreational activities.

The laboratory techniques used to collect and concentrate the water samples prior to culturing the sediment proved to be a rapid and practical methodology to facilitate estimation of the thermophilic amoebae population present in the surface water. Use of SEM images of cyst structures for rapid identification and speciation of isolates proved unsuccessful possibly due to inadequate desiccation of the amoebic cysts. Specialised techniques such as iso-enzyme profile analysis and molecular methods are available to facilitate rapid identification of amoebae, however, these specialised techniques were not within the scope of this research project.

When related to the environment, results from the desiccation experiments suggest that all of the species tested are particularly robust and would survive at least 14 weeks of drought conditions. The return of water to a previously dehydrated pond should result in re-colonisation of the site by the pre-drought population of free-living amoebae. In retrospect, selecting 10 beads rather than a single bead for culture may have produced a more precise detection method to determine the presence or absence of viable amoebae. Future work in this area could incorporate this modification of the protocol.

The effects of variations in food quality on populations of amoebae cysts and trophozoites and the survival response of amoebae were demonstrated by the culture dynamics experiment. This experiment demonstrated no significant difference in the rate of production of total amoebae when grown on either living or dead bacteria. Despite the fact that initial inoculums were identical, a greater number of amoebae cysts were apparent after 1 day of incubation on the living bacteria. This increased encystment of amoebae in the early hours of this experiment could have been a response to overcrowding. As more and more amoebae compete for the abundant food supply, this overcrowding and the concurrent increase in metabolic wastes may have acted as a stimulus to the large trophozoite population to reduce to a more sustainable level through encystment. Amoebae populations could experience similar conditions if their habitats were adversely affected by drought. The subsequent reduction in available water would negatively impact upon the replication of their bacterial food supplies. Amoebae would encyst in response to this reduction in their food quality and quantity. The encysted forms

would provide the nucleus for a future population when optimal environmental conditions are restored.

The data produced during this 12-month survey of natural and man-made water bodies in the Rockhampton area demonstrated a statistically significant increase in populations of potentially pathogenic thermophilic amoebae over all sites sampled in the warmer spring/summer months when compared with the cooler autumn/winter months. Increased rainfall was associated with statistically significant increase in the numbers of amoebae recovered. Most rainfall was recorded during the spring/summer months. It could be suggested that the inflow of rainwater run-off into the water-bodies may result in increased concentrations of amoebae due to contamination of the water by soil particles colonised by thermophilic soil-dwelling amoebae.

Public Health authorities need to be aware of aquatic sites where thermophilic amoebae populations may reach high concentrations and subsequently place humans who are exposed to these organisms through water based activities at a greater risk of acquiring a potentially fatal disease. Recommendations for future study in this field include:

- Analysis of the relationship between bacterial and amoebae population numbers within the water bodies as a means of predicting risk without waiting for results of specialised culture and identification techniques; and
- Analysis of climatic conditions (rainfall and temperature) and their affect on the diversity of resident amoebae populations with emphasis on thermophilic pathogenic strains.

REFERENCES

Australian Water Quality Centre, 1992, *Analytical Methods Manual – Protozoology*

Badenoch R., Johnson A.M., Christy P.E. & Coster D.J., 1990, Pathogenicity of *Acanthamoeba* and a *Corynebacterium* in the rat cornea, *Archives of Ophthalmology*, **108**: 107 - 112

Bio-Rad Laboratories, c.1987, Polar on E3000/3100 Critical Point Drying Apparatus Operating Manual

Bottone E.J., Mayayag R.M. & Quershi M.N., 1992, *Acanthamoeba* keratitis: synergy between amebic and bacterial cocontaminants in contact lens care systems as a prelude to infection, *Journal of Clinical Microbiology*, **30**: 2447 - 2450

Brown T., Cursons R., Keys E., Marks M. & Miles M., 1983, The occurrence and distribution of pathogenic free-living amoebae in thermal areas of the North Island of New Zealand, *New Zealand Journal of Marine and Freshwater Research*, **17**: 59 - 69

Butt C., 1966, Primary amoebic meningoencephalitis, *New England Journal of Medicine*, **274**: 1473

Cabanes P.A., Wallet F., Pringuez E. & Pernin P., 2001, Assessing the risk of primary amoebic meningoencephalitis from swimming in the presence of environmental *Naegleria fowleri*, *Applied and Environmental Microbiology*, **67(7)**: 2927 - 2931

Cano R. & Colomé J., 1988, *Essentials of Microbiology*, West Publishing Company, Minnesota

Carter R., 1972, Primary amoebic meningo-encephalitis, *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **66(2)**: 193 - 213

Culbertson C.G., Smith J.W., Cohen H.K. & Minner J.R., 1959, Experimental infection in mice and monkeys by *Acanthamoeba*, *American Journal of Pathology*, **35**: 185

Cursons R.T., Brown, T.J. & Keys, E.A., 1980, Effect of disinfectants on pathogenic free-living amoebae: in axenic conditions, *Applied and Environmental Microbiology,* **40**: 62 - 66

Dawson M.W., Brown T.J. & Till D.G., 1983, The effect of baquacil on pathogenic free-living amoebae (PFLA) in axenic conditions, *New Zealand Journal of Marine Freshwater Research,* **17**: 305 311

De Jonckheere J., 1979, Studies on pathogenic free-living amoebae in swimming pools, *Bulletin De L'Institut Pasteur*, 77: 385 - 392

Despommier D., Gwadz R. & Hotez P., 1994, *Parasitic Disease, 3rd ed.,* Springer-Verlag, New York.

Dobson P.J., Robinson B.S. & Rowan-Kelly B., 1997, New thermophilic *Naegleria* species (*Herterolobosea, Vahlkampfiidae*) from Australia and Asia: Allozyme, morphometric and physiological characterisation, *ACTA Protozoologica* **36**: 261 - 271

dos Santos J.G., 1970, Fatal primary amebic meningoencephalitis: a retrospective study in Richmond, Virginia, *American Journal of Clinical Pathology*, **54**: 737 - 742

Dreibe W.T., Stern G.A., Epstein R.J., Visvesvara G.S., Adi M. & Komandina T., 1988, *Acanthamoeba* keratitis. Potential role for topical clotrimazole in combination chemotherapy, *Archives of Ophthalmology,* **106(9)**: 1196 - 1201

Ferrante A., 1991, Free-living amoebae: pathogenicity and immunity, *Parasite Immunology* **13**: 31 - 47

Gray T., Cursons R., Sherwan J. & Rose P., 1995, Acanthamoeba, bacterial, and fungal contamination of contact lens storage cases, *British Journal of Ophthalmology*, **79**: 601 - 605.

Greisemer D., Barton L., Reese C., Johnson P., Gabrielsen J., Talawar D. & Visvesvara G., 1994, Amoebic meningoencephalitis caused by *Balamuthia mandrillaris, Pediatric Neurology,* **10(3)**: 249 – 254

Hamadto H., Aufy S., El-Hayawan I., Saleh M. and Nagaty I., 1993, Study of free-living amoebae in Egypt, *Journal of the Egyptian Society of Parasitology* **23(3)**: 631 – 637

Hiti K., Walochnik J., Haller-Schober E.M., Faschinger C. & Aspock H., 2002 Viability of *Acanthamoeba* after exposure to a multipurpose disinfecting contact lens solution and two hydrogen peroxide systems., *British Journal of Ophthalmology*, **86**: 144 – 147.

Huang Z.H., Ferrante A. & Carter R.F., 1999, Serum antibodies to *Balamuthia mandrillaris*, a free-living amoeba recently demonstrated to cause granulomatous amoebic encephalitis, *Journal of Infectious Diseases,* **179(5)**:1305 - 1308

Huizinga H. & McLaughlin G., 1990, Thermal ecology of *Naegleria fowleri* from a power plant cooling reservoir, *Applied and Environmental Microbiology 56*(**7**): 2200 - 2205

John D., 1982, Primary amebic meningoencephalitis and the biology of *Naegleria fowleri*, *Annual Review of Microbiology* **36**: 101 - 123

John D.T, 1992, Opportunistically pathogenic free-living amoebae. In: Kreier J.P. & Baker J.R. (Eds), 1992, *Parasitic Protozoa, 2nd Edition, Volume 3,* Academic Press, San Deigo

John D., & Howard M., 1995, Seasonal distribution of pathogenic free-living amebae in Oklahoma waters, *Parasitology Research* **81(3)**: 193 – 201

Jones D.B., Visvesvara G.S. & Robinson N.M., 1975, *Acanthamoeba polyphaga* keratitis and *Acanthamoeba* uveitis associated with fatal meningoencephalitis, *Transcripts Ophthalmology Society, UK,* **95(2)**: 221 - 232

Katz J.D., Ropper A.H., Adelman L., Worthington M. & Wade P., 2000 A case of *Balamuthia mandrillaris* meningoencephalitis, *Archives of Neurology*, **57**: 1210 – 1213.

Kyle D. & Noblet G., 1985, Vertical distribution of potentially pathogenic free-living amoebae in freshwater lakes, *Journal of Protozoology,* **32(1)**: 99 - 105

Kyle D. & Noblet G., 1986, Seasonal distribution of thermotolerant free-living amoebae, *Journal of Protozoology,* **33(3)**: 422 - 434

Lawande R.V., Ogunkanmi A.E. & Egler L.J., 1979, Prevalence of pathogenic free-living amoebae in Zaria, Nigeria, *Annals of Tropical Medicine Parasitology,* **73(1)**: 51 - 56

Lennette E., 1985, *Handbook of Medical Microbiology, 4th Ed.*, American Society of Microbiology, Washington

Lloyd D., Kristensen B. & Degan H., 1981, Oxidative detoxification of hydrogen sulphide detected by mass spectrometry in the soil amoeba *Acanthamoeba castellanii, Journal of General Microbiology,* **126**: 167 - 170

Ma P., Visvesvara G., Martinez A., Theodore F., Daggett P. & Sawyer T., 1990, *Naegleria* and *Acanthamoeba* Infections: Review, *Reviews of Infectious Diseases*, **12(3)**: 490 - 513

Marciano-Cabral F., 1988, Biology of *Naegleria* spp., *Microbiology Reviews*, **52(3)**: 114 - 133

Markell E.K., Voge M. & John D.T., 1986, *Medical Parasitology, 7th Edition,* W.B. Saunders, Philadelphia

Martinez A.J., Garcia C.A., Halks-Miller M. & Arce-Vela R., 1980, Granulomatous ameboencephalitis presenting as a cerebral mass lesion, *ACTA Neuropathology (Berlin),* **5(2)**: 85 - 91

Nagington J., Watson P., Playfair T., McGill J., Jones B. & Steele A., 1974, Amoebic infection of the eye, *Lancet ii:* 1537 – 1540

Nagington J., 1975, Isolation of amoebae from eye infections in England, *Transcripts Ophthalmological Society U.K.*, **95**: 207 - 209

NCCLS, 2000, *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard - Fifth Edition*, Vol. 20 No. 2.

Nerad T.A. & Daggett P.M., 1979, Starch gel electrophoresis: an effective method for separation of pathogenic and non-pathogenic *Naegleria* strains, *Journal of Protozoology* **26**: 613 - 615

Page F.C., 1988, A New Key to Freshwater and Soil Gynamoebae, CCAP, The Ferry House, Cumbria, England.

Penas-Ares M., Paniagua-Crespo E., Madrinan-Choren R., Marti-Mallen M. & Arias-Fernandez M., 1994, Isolation of free-living pathogenic amoebae from thermal spas in N.W. Spain, *Water, Air and Soil Pollution,* **78**: 83 – 90

Radford C.F., Minassian D.C. & Dart J.K.G., 2002, *Acanthamoeba* keratitis in England and Wales: Incidence, outcome, and risk factors, *British Journal of Ophthalmology*, **86**, 536 – 543.

Reed R., Cooke-Yarborough C., Jaquiery A., Grimwood K., Kemp A., Su J. & Forsyth J., 1997, Fatal granulomatous amoebic encephalitis caused by *Balamuthia mandrillaris, Medical Journal of Australia* **21(7)**: 82 - 84

Rivera F., Ramirez E., Bonilla P., Calderon A., Gallegos E., Rodriguez S., Ortiz R., Zalvidar B., Ramirez P. & Duran A., 1993, Pathogenic and free-living amoebae isolated from swimming pools and physiotherapy tubs in Mexico, *Environmental Research,* **62**: 43 - 52

Rockhampton Bureau of Meteorology, 2001, Telephone Conversation, 1300hrs - 19th October

Rockhampton City Council, June 2001, *Community Services Directory* - Rockhampton City Council

Rodriguez-Zaragoza S., 1994, Ecology of free-living amoebae, *Critical Reviews in Microbiology,* **20(3)**: 225 - 241

Sadaka H., El Nassery S., Abou-Samra L. & Awadalla H., 1994, Isolation and identification of free-living amoebae from some water sources in Alexandria, *Journal of the Egyptian Society of Parasitology,* **24(2)**: 247 - 257

Schuster F.L. & Visvesvara G.S., 1996, Axenic growth and drug sensitivity studies of *Balamuthia mandrillaris*, an agent of amebic meningoencephalitis in humans and other animals, *Journal of Clinical Microbiology,* **34(2)**: 385 - 388

Seal D., Stapleton F. & Dart J., 1992, Possible environmental sources of *Acanthamoeba* spp. in contact lens wearers, *British Journal of Ophthalmology*, **76**: 424 - 427

Shenoy S., Wilson G., Prashanth H.V., Vidyalakshmi K., Dhanashree B. & Bharath R., 2002, Primary meningoencephalitis by *Naegleria fowleri* : First reported case from Mangalore, South India., *Journal of Clinical Microbiology*, **40**: 309 – 310.

Singh B.N & Das S.R., 1970, Studies on pathogenic and non-pathogenic small free-living amoebae and the bearing of nuclear division o the classification of the order of Amoebida, *Transcripts of Royal Society of Biology (London)* **259**: 435 - 476

Steinert M., Birkness K., White E., Fields B. & Quinn F., 1998, *Mycobacterium avium* bacilli grow saprozoically in coculture with *Acanthamoeba polyphaga* and survive within cyst walls, *Applied and Environmental Microbiology,* **64(6)**: 2256 - 2261

Szenasi Z., Endo T., Yagita K. & Nagy E., 1998, Isolation, identification and increasing importance of 'free-living' amoebae causing human disease, *Journal of Medical Microbiology,* **47(10)**: 939- 940

UBD, 2001, *Queensland Country Cities and Towns - Street Directory on CD-ROM,* Universal Press Pty Ltd, Brisbane

Vesaluoma M., Kalso S., Jokipii L., Warhurst D., Ponka A. & Tervo T., 1995, Microbiological quality in Finnish public swimming pools and whirlpools with special reference to free-living amoebae: a risk factor for contact lens wearers? *British Journal of Ophthalmology,* **79**: 178 - 181

Visvesvara G.S., Schuster F.L. & Martinez A.J., 1993 *Balamuthia mandrillaris*, NG, N Sp., agent of amebic meningoencephalitis in humans and other animals. *Journal of Eukaryotic Microbiology*, **40**: 504 – 514.

Walochnik J., Haller-Schober E-M, Kolli H., Picher O., Obwaller A. & Aspock H., 2000, Discrimination between clinically relevant and nonrelevant *Acanthamoeba* strains isolated from contact lens-wearing keratitis patients in Austria, *Journal of Clinical Microbiology*, **39(11)**: 3932 - 3936

Weisman R.A, 1976, Differentiation in *Acanthamoeba*, *Annual Review of Microbiology,* **30**: 189 - 211