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

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ABSTRACT

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

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

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

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

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

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CHAPTER ONE: INTRODUCTION

1.1 General

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

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

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

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

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

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

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

1.2 Free Living Amoebae as Disease Agents

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

1.3 Occurrence and Distribution of Free Living Amoebae

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

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

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

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

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

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

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

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

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

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

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

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

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

Table 1. Chemical Composition of Water; Comparison

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

1.4 The Aims of this Project

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

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

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

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

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

CHAPTER TWO: MATERIALS

2.1 Amoebae Cultures Used

2.1.1 Stock cultures

Table 2.

Species	Strain	Pathogenicity	Source
Naegleria fowleri	NHI	+	NHI
Naegleria gruberi	PL200f	-	NHI
Acanthamoeba castellanii	1501	-	CCAP
Acanthamoeba culbertsoni	A1	+	IMTPL

+ = positive
- = negative
NHI = National Health Institute, Wellington, New Zealand
CCAP = Culture Centre of Algae and Protozoa
IMTPL = Institut de Medicine Tropicale Prince Leopold, Belgium

2.1.2 Unknown isolates

Table 3

Strain	Source
PR2448	Ngawha springs
PR2449	"

2.2 Bacteria Culture Used

Enterobacteria cloacae: source, Massey University.

2.3 Media for Axenic Cultivation of Amoebae

2.3.1 Pages Amoeboid Saline (PAS) (Page, 1967).

Base for CYM and for diluting amoebae

NaCl	1.2	g
KH_2PO_4	1.36	g
$MgSO_4.7H_2O$	0.04	g
Na_2HPO_4	1.42	g
Thaimine HCl	0.01	g
d-biotin	0.02	g
Vitamin B_{12}	add till mix is pink	
L-methionine	0.895	g

Make up to 1 litre and dilute 1/10 before use.
Autoclave at 103.4 KPa (121°C) 15 minutes.

2.3.2 Acanthamoeba Ion Solution (Stevens & O'Dell, 1973)

Base for Neff

$MgSO_4.7H_2O$	2.47	g
$CaCl_2.2H_2O$	0.11	g
KH_2PO_4	72.0	g
Ferric citrate	0.34	g

Make up to 1 litre and dilute 1/10 before use.
Autoclave at 103.4 KPa (121°C) 15 minutes.

2.3.3 CYM (Cursons et al., 1978)

for culture of *Naegleria* spp.

Difco casitone	10.0	g	pH 6.8 - 7.0
Glucose	10.0	g	
Difco yeast extract	5.0	g	
PAS solution	1	litre	

Dispense 4.5 ml into universal bottles and autoclave 103.4 KPa 15 minutes.
Add 0.5 ml cocktail.

Cocktail

Bovine serum	8	ml
Haemin	1.6	ml
Pen/Strep ($2x10^5$ units cm^{-3})	1.4	ml
$CaCl_2.6H_2O$ ($2.7x10^{-3}$M)(0.6 g/l)	4.0	ml

Mix and filter sterilise, 0.2 um filters.

2.3.4 Neff (Stevens & O'Dell, 1973)
for culture of *Acanthamoeba* spp.

Difco proteose peptone	40	g
Glucose	15	g
Difco yeast extract	7.5	g
Ac ion solution	1	litre

Dispense in 4.5 ml lots into universal bottles and autoclave 103.4 KPa 15 minutes.
Add 0.2 ml Pen/Strep. (2×10^5 units cm^{-3}).

2.4 Media for Monoxenic Amoebic Culture

2.4.1 PAS Agar

for encystment of amoebae

1 litre PAS + 20 g agar
Autoclave 103.4 KPa 15 minutes.

2.4.2 PASB Agar

for growth of amoebae

1 litre PAS + 20 g agar + 2 g bactopeptone.
Autoclave 103.4 KPa 15 minutes.

2.5 Miscellaneous Media

2.5.1 Brain Heart Infusion

for culture of *E. cloacae*

Broth: 37 g BHI + 1 litre water

Agar: 37 g BHI + 20 g agar + 1 litre water

2.5.2 Soil Extract Broth 100% (SEB)

Soil	1 kg
Water	1 litre

Autoclave 103.4 KPa 15 minutes.
Centrifuge 6000 rpm 15 minutes. Decant supernatant and re-autoclave.

2.5.3 Soil Extract Broth 50% (SEB)

Soil	500 g
Water	1 litre

Autoclave 103.4 KPa 15 minutes.
Centrifuge 6000 rpm 15 minutes. Decant supernatant and re-autoclave.

2.5.4 Soil Extract Agar

Distilled water	900 ml
SEM	100 ml
Agar	20 g

Autoclave 103.4 KPa 15 minutes.

2.5.5 Neff Agar (CYM Agar)

(CYM) Neff media	1 litre
Agar	20 g

Autoclave 103.4 KPa 15 minutes.

2.5.6 Neff/50 Agar (CYM/50)

(CYM) Neff medium	500 ml
Distilled water	500 ml
Agar	10 g

Autoclave 103.4 KPa 15 minutes.

2.5.7 Phosphate Buffer

Na_2HPO_4 (0.01M)	14.2 ml
NaH_2PO_4 (0.01M)	12.43 ml

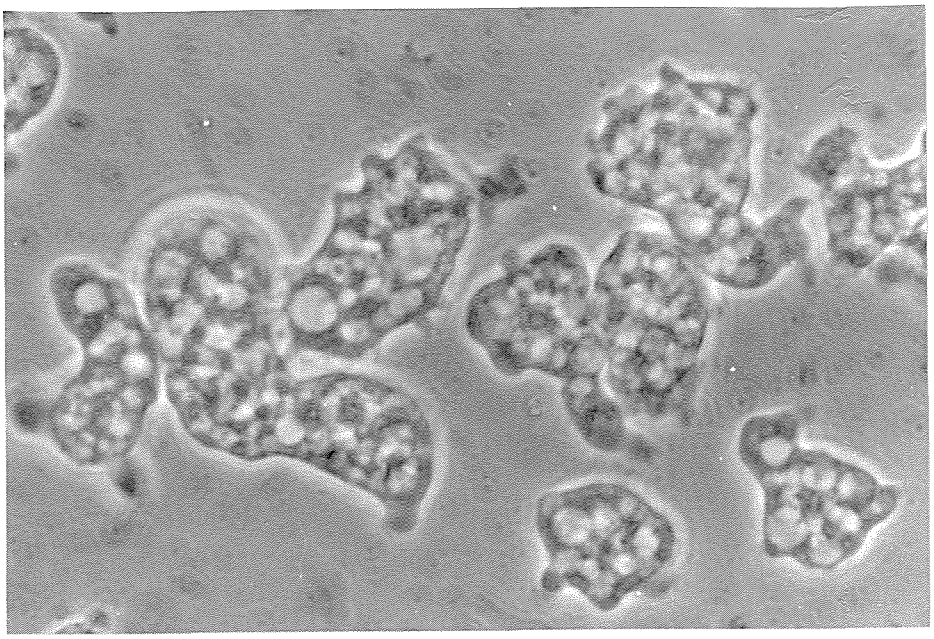
pH 7.5

2.5.8 Sodium Lauryl Sulphate

Sodium lauryl sulphate	25 g
Distilled water	100 ml.

Plate 1. Trophozoite stage of *Naegleria fowleri* (NHI).

Plate 2. Trophozoite stage of *Naegleria gruberi* (PL200f).



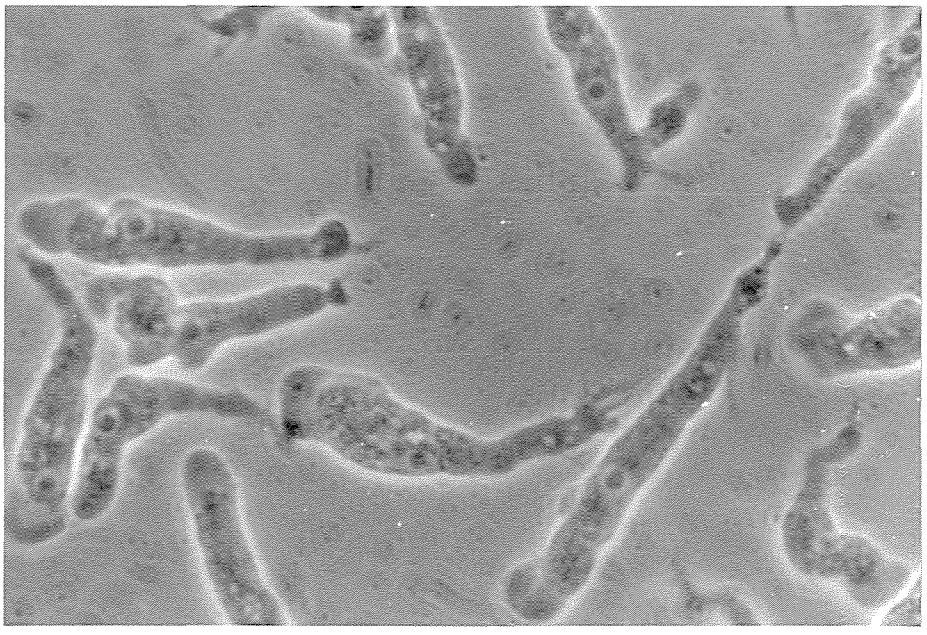
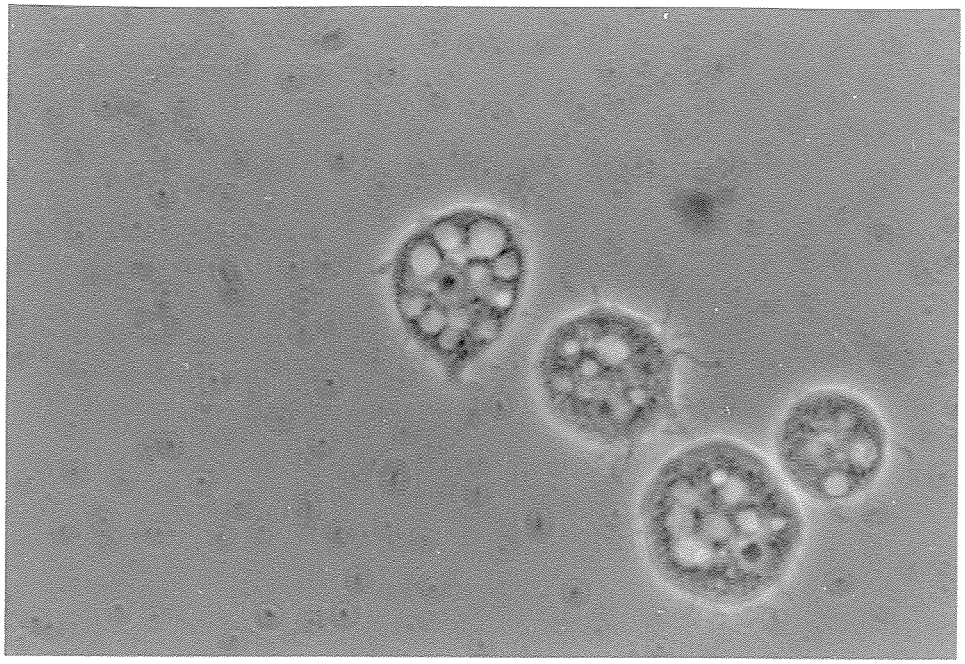


Plate 3. Trophozoite stage of *Acanthamoeba culbertsoni* (AI)

Plate 4. Trophozoite stage of *Acanthamoeba castellanii* (1501).



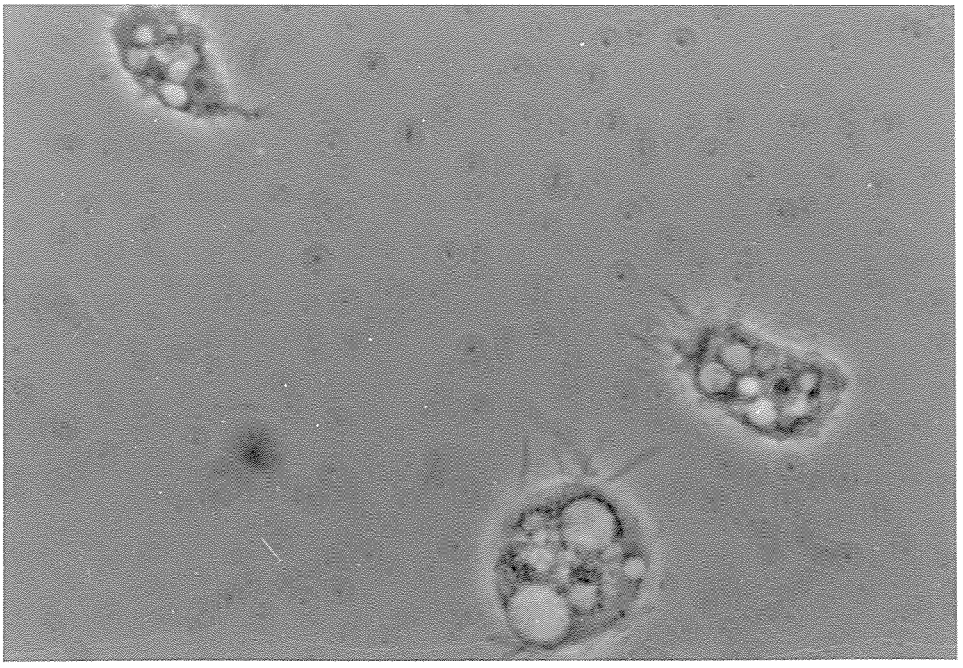
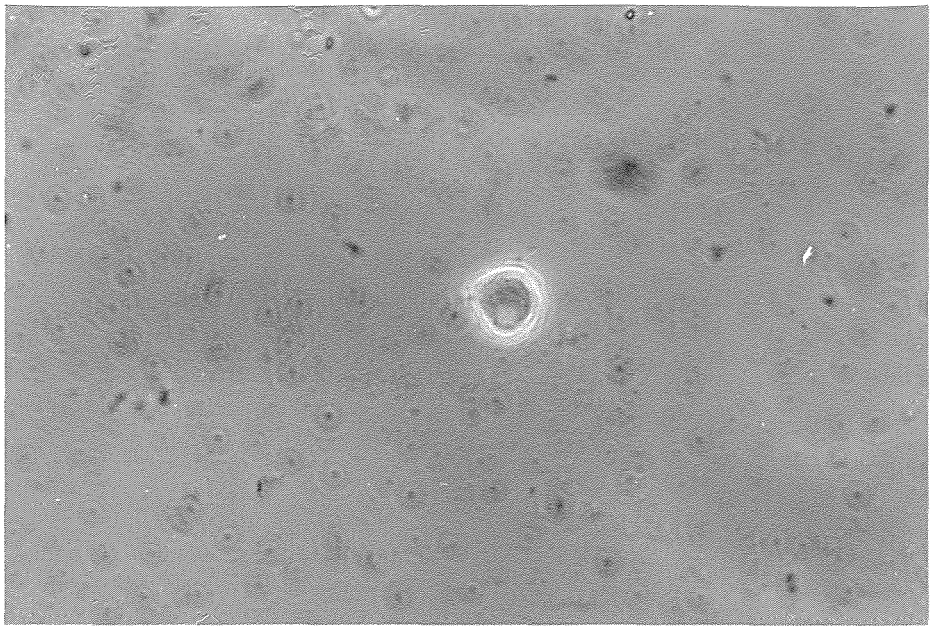


Plate 5. Cyst stage of *Naegleria fowleri* (NHI).

Plate 6. Cyst stage of *Naegleria gruberi* (PL200f).



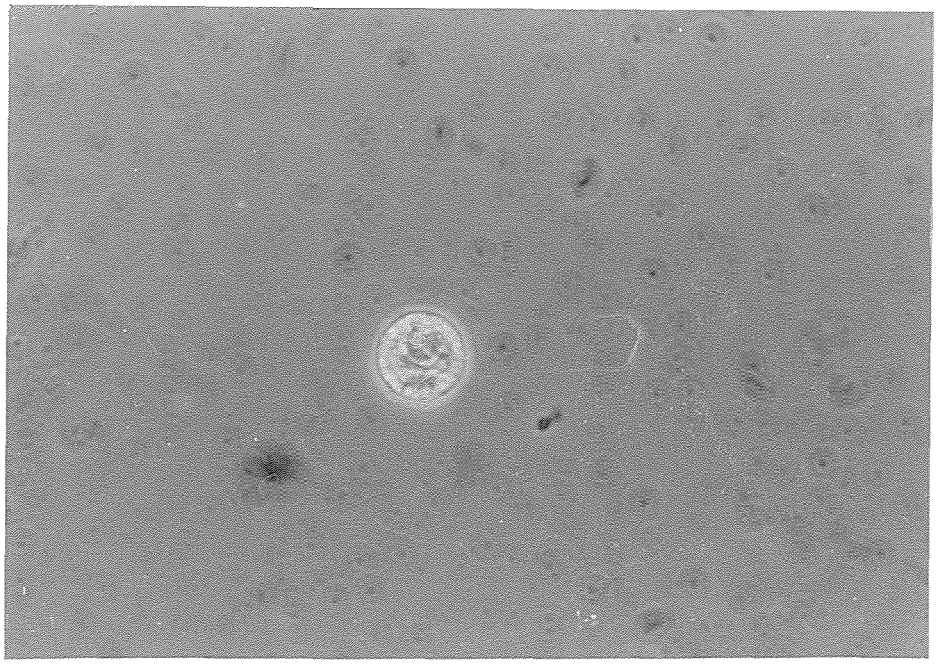
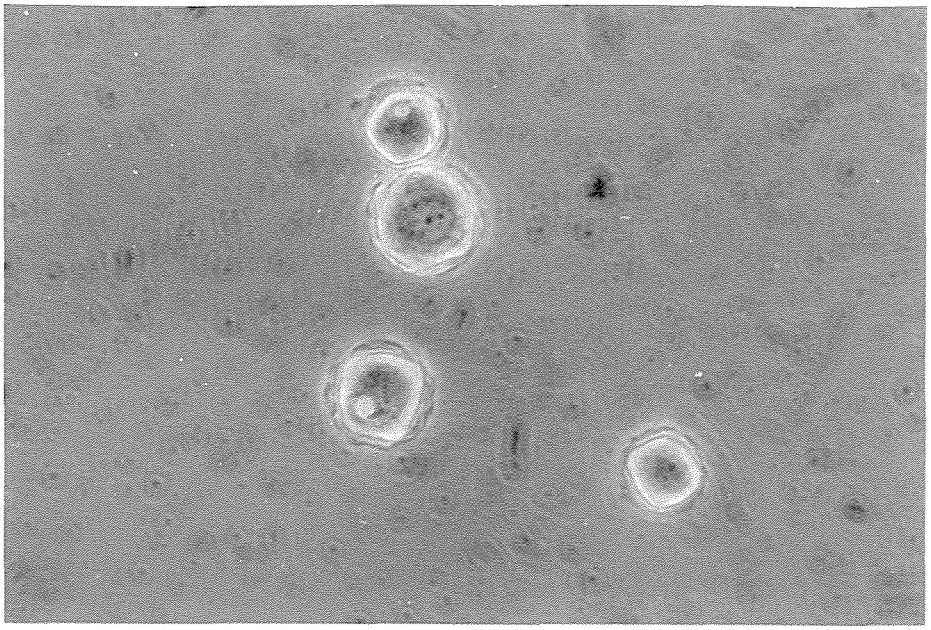


Plate 7. Cyst stage of *Acanthamoeba culbertsoni* (AI).

Plate 8. Cyst stage of *Acanthamoeba castellanii* (1501).



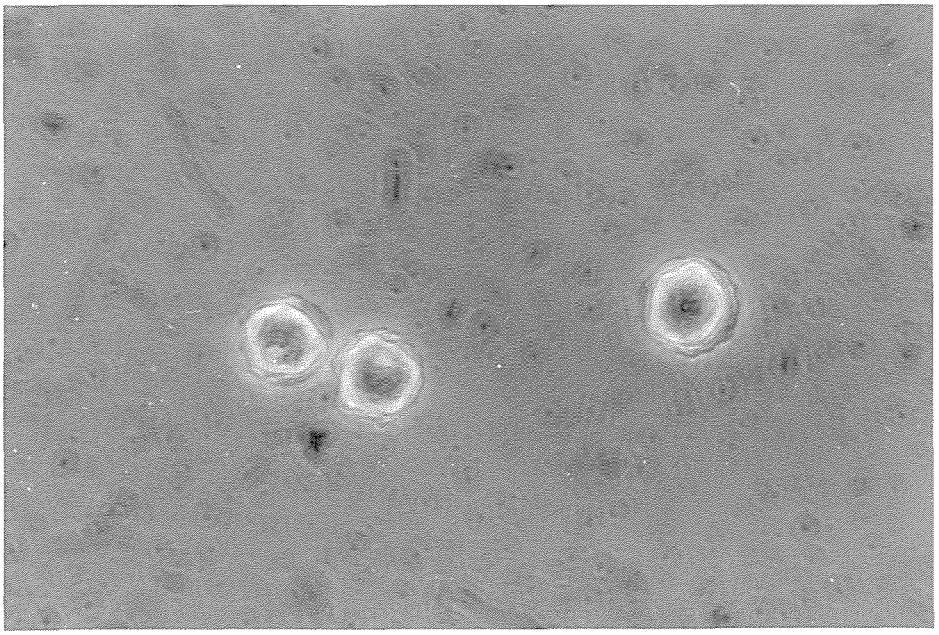
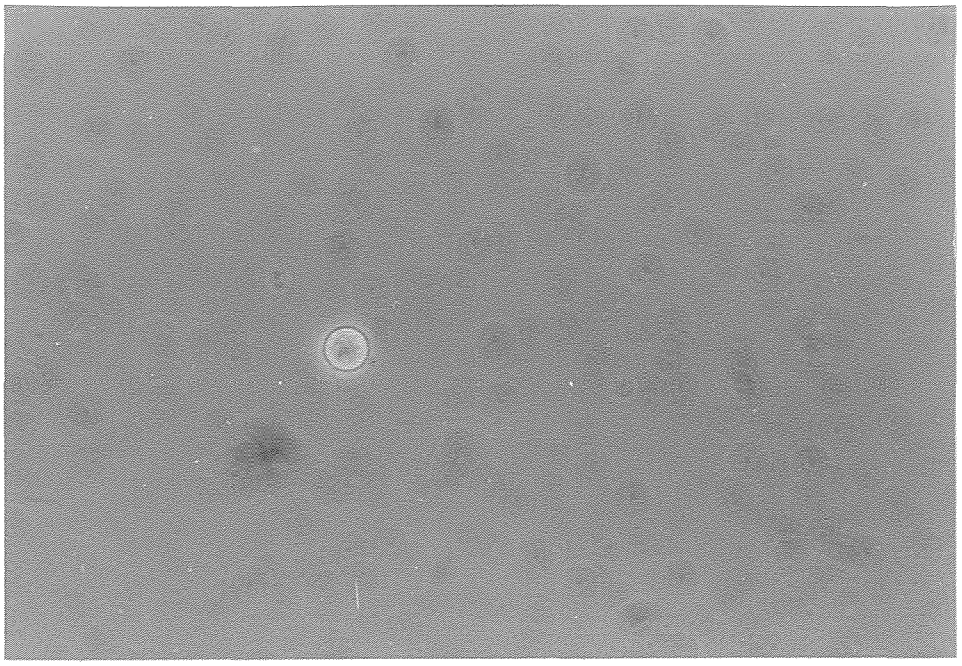


Plate 9. Round form of *Naegleria fowleri* (NHI).

Plate 10. Round form of *Naegleria gruberi* (PL200f).



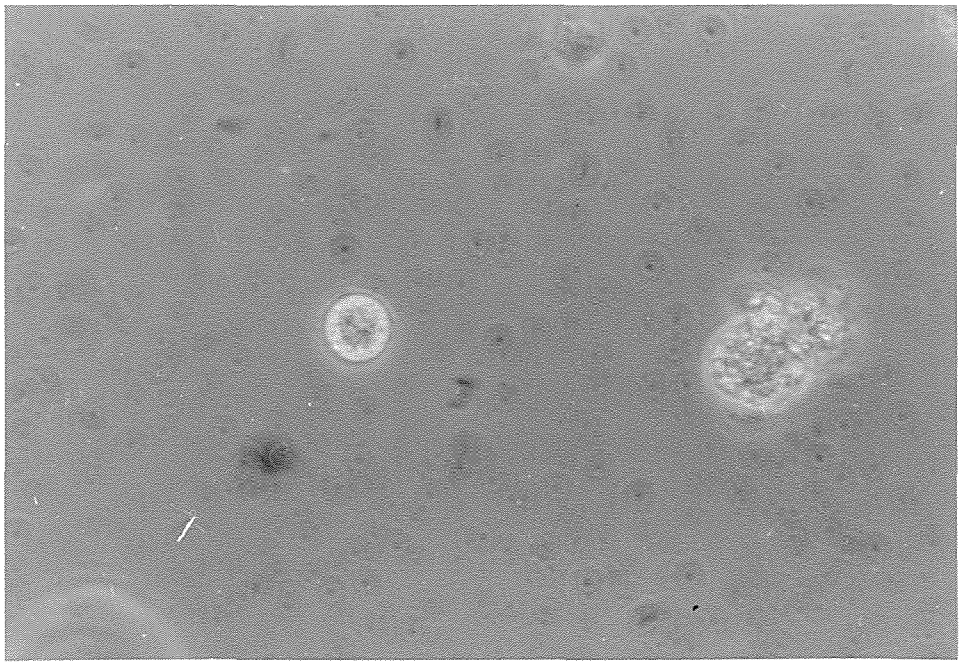
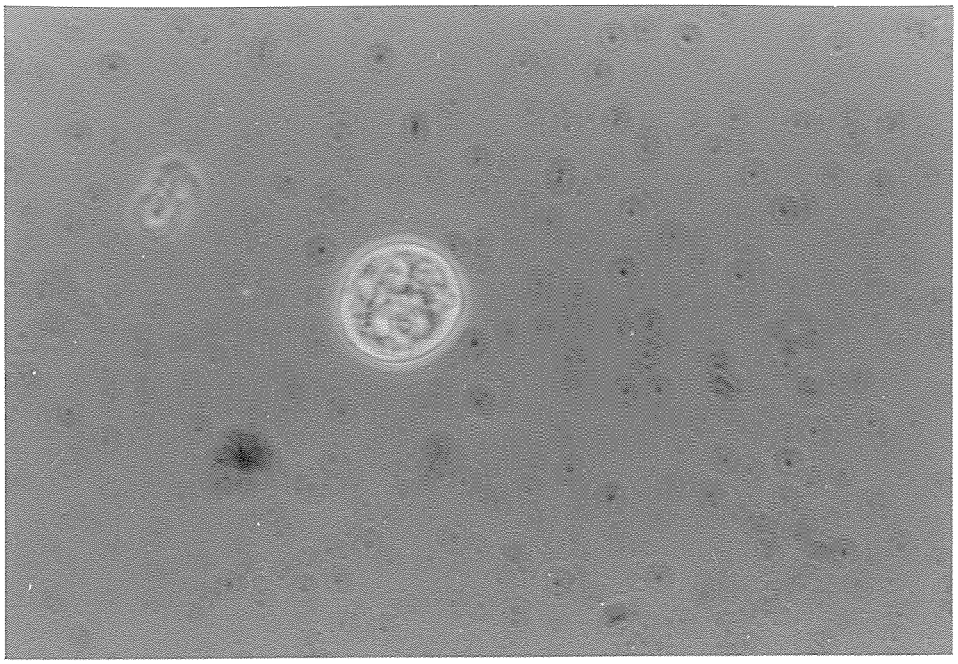
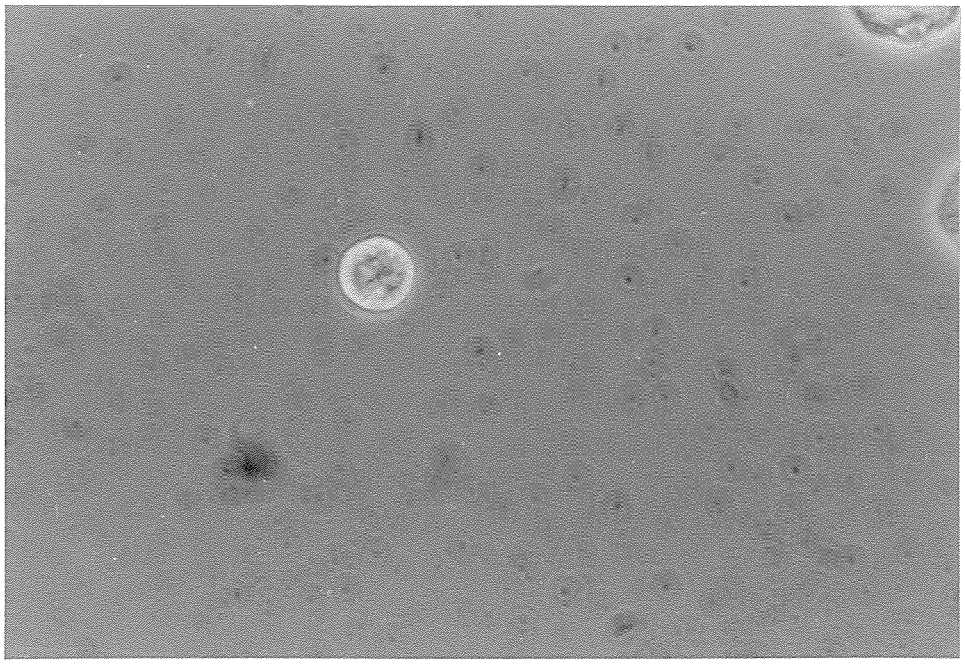


Plate 11. Round form of *Acanthamoeba culbertsoni* (AI).

Plate 12. Round form of *Acanthamoeba castellanii* (1501).





CHAPTER THREE: METHODS

3.1 Testing the Effect of Some Ions on Amoebic Growth

Chemical analysis (see Appendix) was undertaken on the media used to determine the initial levels of Ca^{+2}, Mg^{+2}, K^{+}, Na^{+} and $Fe^{+2/+3}$ ions. Using these initial concentrations, the following final concentrations were achieved by adding chloride salts of the above ions.

Final concentration Ca^{+2}, Mg^{+2}, K^{+}, Na^{+} (mM)

0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100

Final concentration Na^{+} (% w/v)

0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5

Final concentration Fe^{+2}, Fe^{+3} (mg l^{-1})

0, 1, 2, 3, 4, 5

All tests were carried out in 125 cm^3 kimax flasks containing 49 ml growth media (Neff for *Acanthamoeba* spp and CYM for *Naegleria* spp.). Duplicate flasks were set up containing each concentration of cation. The flasks were plugged with cotton wool, sealed with parchment paper and rubber bands and sterilized by autoclaving.

The inoculum was prepared by washing a 48-hour culture of trophozoites (see Appendix) twice in PAS and resuspending in deionised water. The concentration of amoebae was determined by direct count under phase contrast in a haemocytometer. The amoebae suspension was then diluted in water to a concentration of 5×10^4 cm ml^{-1}. Each flask was inoculated with 1 ml of the diluted amoebae giving a concentration of 1×10^3 amoebae ml^{-1}.

The flasks were incubated at 37°C for pathogens and 30°C for non-pathogens on rotating shakers. Duplicate samples were removed from each flask every 24 hours and three counts were made on each sample using the haemocytometer. This gave a total of twelve separate counts daily for each set of conditions, and was continued for 7-9 days.

Results were graphed as time vs log no. amoebae and the growth rate calculated after 48 hours. Growth rates were then graphed against cation concentrations to compare the effect of each cation.

To determine whether Ca^{+2} ions are amoebicidal for *N. fowleri* trophozoites independent of ionic strength, the above experiments were repeated using PAS and water instead of growth media. Ca^{+2} concentrations used were 0, 40, 50, 60, 70 mM.

3.2 Production of Cysts in Liquid Media

Chemical analysis was undertaken to determine initial levels of Mg, Ca, K, Na and Fe in PAS, SEB, Neff and CYM. To 49 ml PAS, $MgCl_2$ was added to give the final concentrations of 0, 0.1, 0.3, 0.5% Mg^{+2} ions. $MgCl_2$ was also added to water, $CaCl_2$ to water and PAS, to give the above concentrations.

All tests were undertaken in 125 ml kimax flasks in 50 ml volumes.

24-hour amoebae cultures were washed twice, suspended in deionised water and diluted to a concentration of 5×10^5 trophozoites ml^{-1}. One ml was added to each flask, giving the initial concentration of 1×10^5 amoebae ml^{-1}.

The flasks were then incubated at 37°C for pathogenic and 30°C for non-pathogenic organisms. The flasks were incubated standing and the experiment repeated with incubation on rotating shakers.

At 24-hour intervals duplicate samples were removed and observed under phase contrast in a haemocytometer. The percentages of cysts, round forms and trophozoites were determined three times for each sample.

Cysts can be distinguished from trophozoites and round forms by the possession of a distinct cyst wall which refracts the light to give a bright yellow colour. Round forms (pre-encystment stage) are distinguished from trophozoites by the lack of pseudopodia and vacuoles,

and the perfect spherical shape. They are distinct from cysts due to the lack of the cyst wall.

Results were graphed; % cation vs time, and % encystment vs cation concentration.

A soil extract medium (SEM) was prepared (2.5.2) to determine any cationic factors present in soil which may play a role in encystment. Concentrations of Mg^{+2}, Ca^{+2}, K^{+}, Na^{+} and $Fe^{+2/+3}$ were determined both before and after 7 days in the medium.

125 ml flasks containing 50 ml sterile SEM were inoculated in duplicate to give 1×10^{5} trophozoites ml^{-1}. Two samples were removed every 24 hours and the percentage of cysts, round forms and trophozoites determined and graphed vs time.

3.3 Production of Cysts on Solid Media

The agars used were PASB, 10% SEA, CYM, 50% CYM, Neff and 40% Neff.

Trophozoites from 24-hour cultures were washed twice and resuspended in PAS to a concentration of 5×10^{4} trophozoites ml^{-1}.

To each plate was added 4 ml PAS, and 1 ml amoebae suspension. The plates were then incubated, at $37^{o}C$ for pathogens and $30^{o}C$ for non-pathogens.

Samples were removed daily, after complete mixing of the liquid on the agar, for 7 days.

The percentage cysts, round forms and trophozoites were determined by direct count under phase contrast, and results graphed vs time.

At 3-day intervals 2 ml PAS were added to each plate to prevent drying out.

In order to determine any utilization of Ca, Mg, K, Na or $Fe^{+2/+3}$ during encystment, the liquid on top of the agar was analysed both before and after encystment. To counteract any dehydration, and

therefore increased cation concentrations, the solution was removed, made up to 5 ml, centrifuged for 15 min at 2000 rpm and analysed in a flame photometer (see Appendix).

3.4 Cyst Survival

3.4.1 Temperature

The standard method for storage of amoebae is in the cyst form on agar slopes, sealed and stored at room temperature.

Slopes of PAS agar were prepared by cooling 12 ml sterile agar in universal bottles at a 45^{o} angle.

A 24-hour culture of amoebae was washed and resuspended in PAS. A PAS agar plate was then inoculated with 1 ml of amoebae culture, 0.1 ml *E. cloacae* (overnight culture) and 4 ml PAS. This was then incubated overnight at the appropriate temperature. The cells were then harvested, washed once and resuspended in 4 ml PAS. 1 ml of this suspension + 0.1 ml *E. cloacae* were inoculated onto a PAS agar slope and incubated overnight in a sloped position. Thirty-six slopes were stored at each of the following temperatures: 15, 10, 4, -10^{o}C. Two slopes were removed from each temperature each month for eight months.

Each slope was washed thoroughly with PAS and the washings transferred onto 2 PASB plates containing 0.1 ml *E. cloacae* and 4 ml PAS. The slopes were rinsed again and the rinsings transferred to another PAS plate containing 0.1 ml *E. cloacae* and 4 ml PAS. The plates were then incubated for 1-10 days at appropriate temperatures. Daily checks were made under phase contrast for viable trophozoites.

In the case of negative results, 0.05 ml samples were removed and dropped into the centre of a PASB plate covered in a lawn of *E. cloacae*. The plates were inverted and incubated at appropriate temperatures for up to 2 weeks. Daily checks were made for plaque formations where motile trophozoites had ingested bacteria, leaving a clear zone of agar.

Where both methods were negative, a negative result was recorded.

3.4.2 Cyst survival in liquid media

Duplicate 125 ml flasks were set up containing 49 ml of water at the following cation concentrations:

0-100 mM Mg^{+2}, Ca^{+2}, K^{+}, Na^{+}, and 0-5 mg l^{-1} Fe^{+2} and Fe^{+3}.

Amoebae were grown in liquid media overnight, washed twice and resuspended in PAS. PAS plates containing 4 ml PAS and 0.1 ml *E. cloacae* were inoculated with 1-2 ml of the amoebae solution. The plates were incubated for 1-4 nights at the appropriate temperatures until 80-100% encystment was obtained (determined by direct count).

The plates were then washed thoroughly with PAS, the washings centrifuged and resuspended in PAS. The amoebae were then washed once in SLS which destroyed trophozoites, round forms and any remaining bacteria. Cysts remained viable and were washed twice to remove the SLS. Flasks were inoculated with 1 ml of cysts suspension at a dilution which gave a concentration in the flask of 1×10^{4}. The flasks were then incubated at appropriate temperatures.

At 24-hour intervals duplicate samples were removed. 0.1 ml E. cloacae plus 0.1 ml sample (undiluted and 1:10 dilution) were spread onto PASB agar plates, which were inverted and incubated at appropriate temperatures. Concentration of viable cysts in the flask was estimated by counting the number of plaques formed in the bacterial lawn on the PASB agar. It is assumed that each plaque originated from one cyst.

Results were graphed as cysts 0.1 ml^{-1} (plaques) vs time.

3.5 Preliminary Identification of *Naegleria* Isolates

3.5.1 Morphology

Morphology of amoeba trophozoites, flagellate and cystic stages was examined under phase contrast microscopy and compared with similar examination of known species *N. fowleri, N. gruberi, A. castellanii* and *A. culbertsoni*.

3.5.2 Temperature tolerance

Routine subculturing was undertaken for all isolates prior to testing. 0.5 ml aliquots of amoebae were added to 4.5 ml medium in universal bottles and were incubated without shaking in a closed water-bath set at 45°C. Daily samples were counted in duplicate in a Neubauer haemocytometer under phase contrast. After seven days 0.5 ml samples were removed and subcultured into 4.5 ml fresh medium which was then also incubated at 45°C stationary in a closed water bath. Samples were counted daily and the results graphed as log no. amoebae vs days.

CHAPTER FOUR: RESULTS

4.1 Effect of Cation Concentration on Growth of Amoebae

4.1.1 Effect of Na, Mg, Ca and K

Figures 1-4 show the effect of cation concentrations on the growth rate of FLA. The cations tested were Ca^{+2}, Mg^{+2}, K^{+} and Na^{+}. The R_2 values shown in Table 4 indicate that there is a strong correlation between concentration and growth rate.

Ca^{+2} had the greatest inhibitory effect on all species. Extrapolation indicates that Ca^{+2} may be amoebicidal at 40-50 mM for *N. fowleri* and 30-40 mM for *N. gruberi*.

With the exception of *N. fowleri*, Na^{+} was the least inhibitory cation. *N. fowleri* was affected least by K^{+}, whereas Mg^{+2} and Na^{+} had similar effects.

Na^{+2} and K^{+} had similar effects on *N. gruberi* and the *Acanthamoeba* species.

The effect of all ions on the two *Acanthamoeba* species closely resembled each other compared with *Naegleria* species which differed in all except the effect of Ca^{+2}.

The following legend applies to Figures 1-4:

▲ = Ca^{+2}
● = Mg^{+2}
■ = K^{+}
◆ = Na^{+}

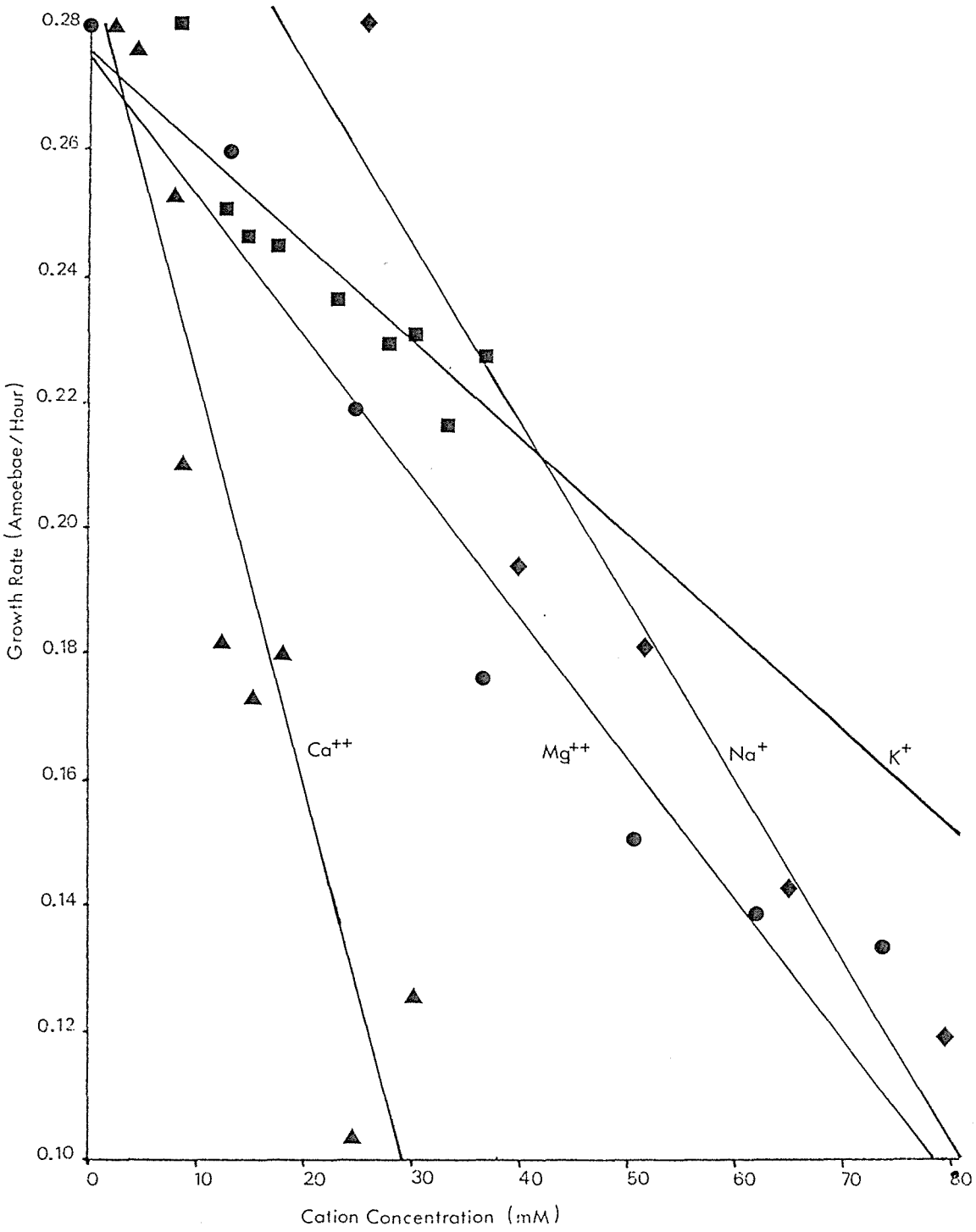


Figure 1. The effect of cation concentration on growth rate of *N. fowleri*.

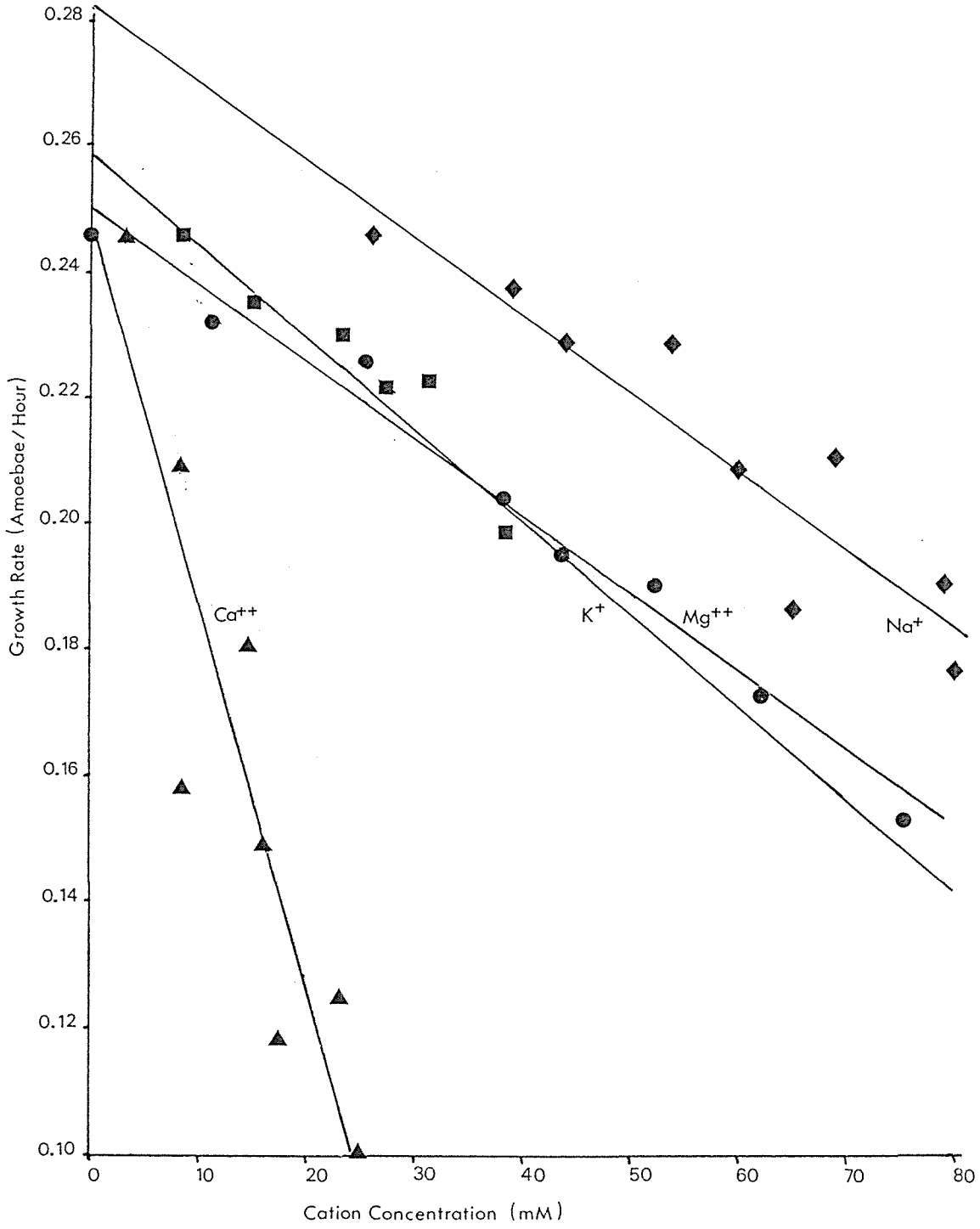


Figure 2. The effect of cation concentration on growth rate of *N. gruberi*.

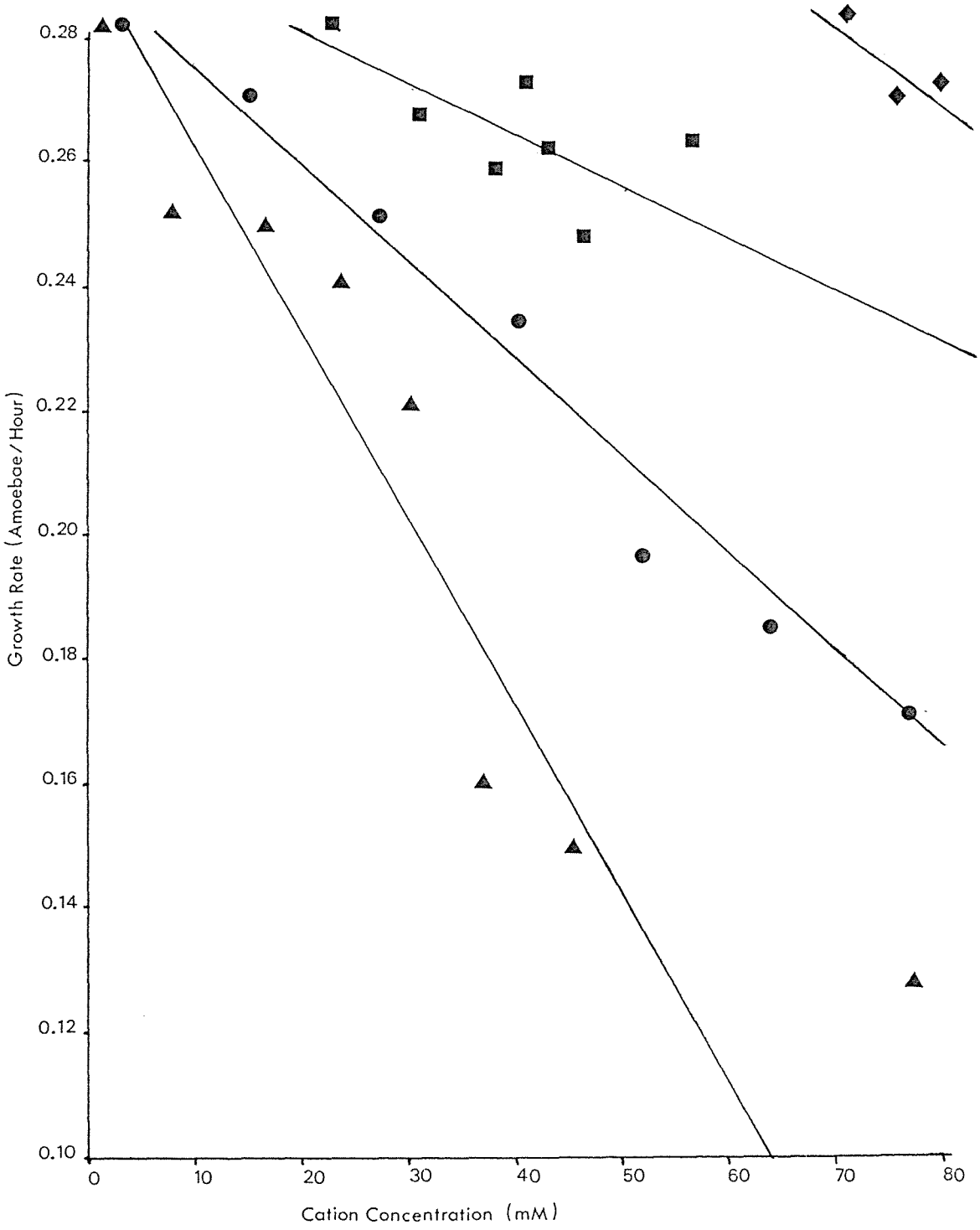


Figure 3. The effect of cation concentration on growth rate of *A. culbertsoni*.

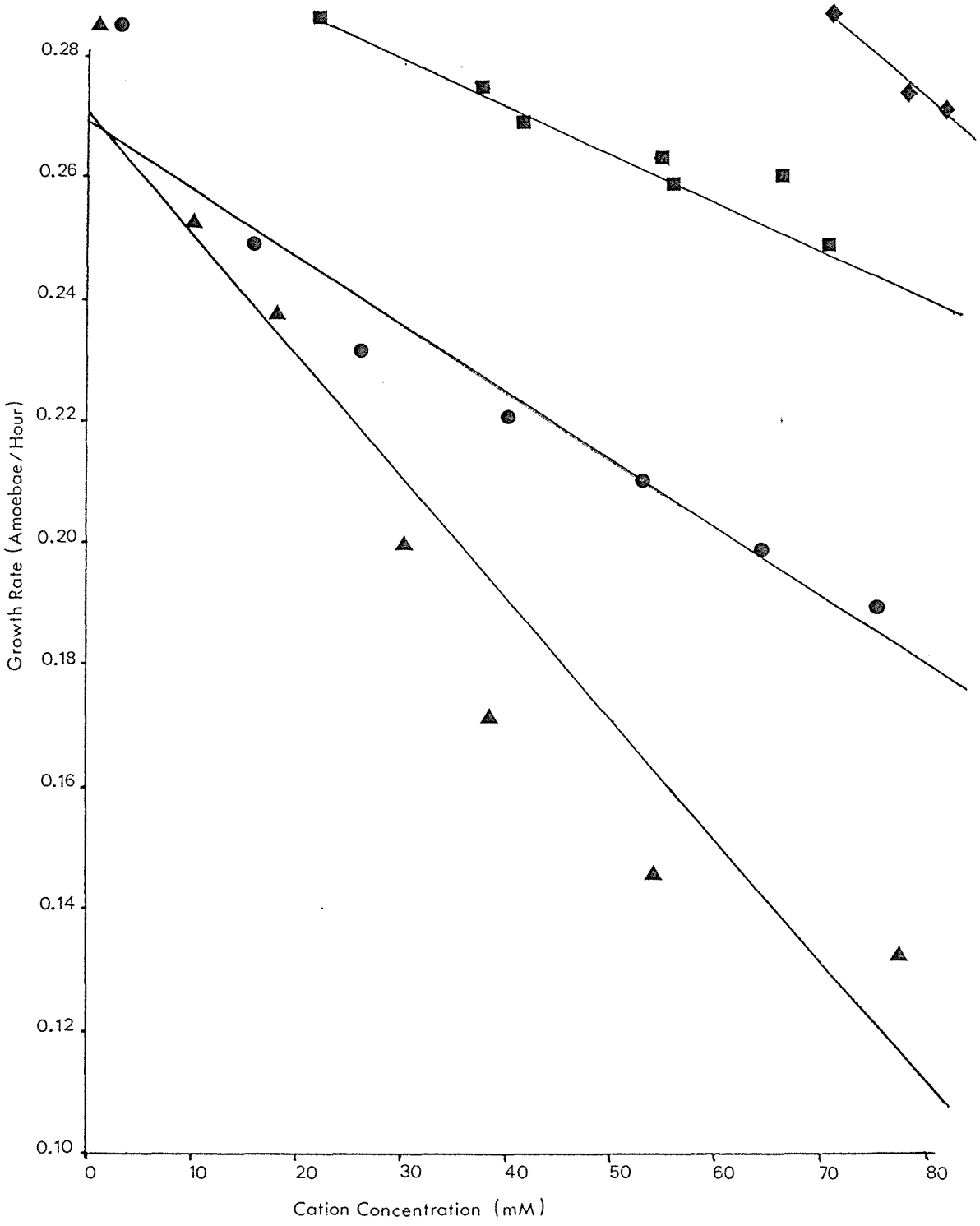


Figure 4. The effect of cation concentration on growth rate of *A. castellanii*.

Table 4. Linear Regression Analysis (y = A + Bx)

Data Set	Regression line equation	Correlation coefficient (e_2)
Figure 1		
Ca	y = 0.288 - 0.00648x	0.885
Mg	y = 0.274 - 0.00217x	0.949
K	y = 0.275 - 0.00154x	0.871
Na	y = 0.332 - 0.00288x	0.923
Figure 2		
Ca	y = 0.245 - 0.00585x	0.811
Mg	y = 0.250 - 0.00126x	0.989
K	y = 0.258 - 0.00144x	0.916
Na	y = 0.282 - 0.00122x	0.839
Figure 3		
Ca	y = 0.291 - 0.00300x	0.907
Mg	y = 0.292 - 0.00162x	0.975
K	y = 0.294 - 0.000766x	0.487
Na	y = 0.371 - 0.00129x	0.825
Figure 4		
Ca	y = 0.271 - 0.00204x	0.917
Mg	y = 0.270 - 0.00115x	0.892
K	y = 0.302 - 0.000776x	0.922
Na	y = 0.370 - 0.00122x	0.908

4.1.2 Effect of NaCl

Figures 5-8 show the effect of 0-1.5% NaCl on the $\log_{10}$ number of amoebae.

N. fowleri was inhibited markedly at concentrations of 0.75% or greater but relatively unaffected at 0.25 and 0.5%. *N. gruberi* was also more affected at 0.75% or greater.

Acanthamoeba species were both affected by the increase in NaCl concentration, but the $\log_{10}$ number of amoebae dropped only gradually as NaCl percentage increased.

The following legend applies to Figures 5-8:

△ 0.0% NaCl	◇ 0.75	■ 1.25
□ 0.25	▲ 1.0	● 1.5
○ 0.50		

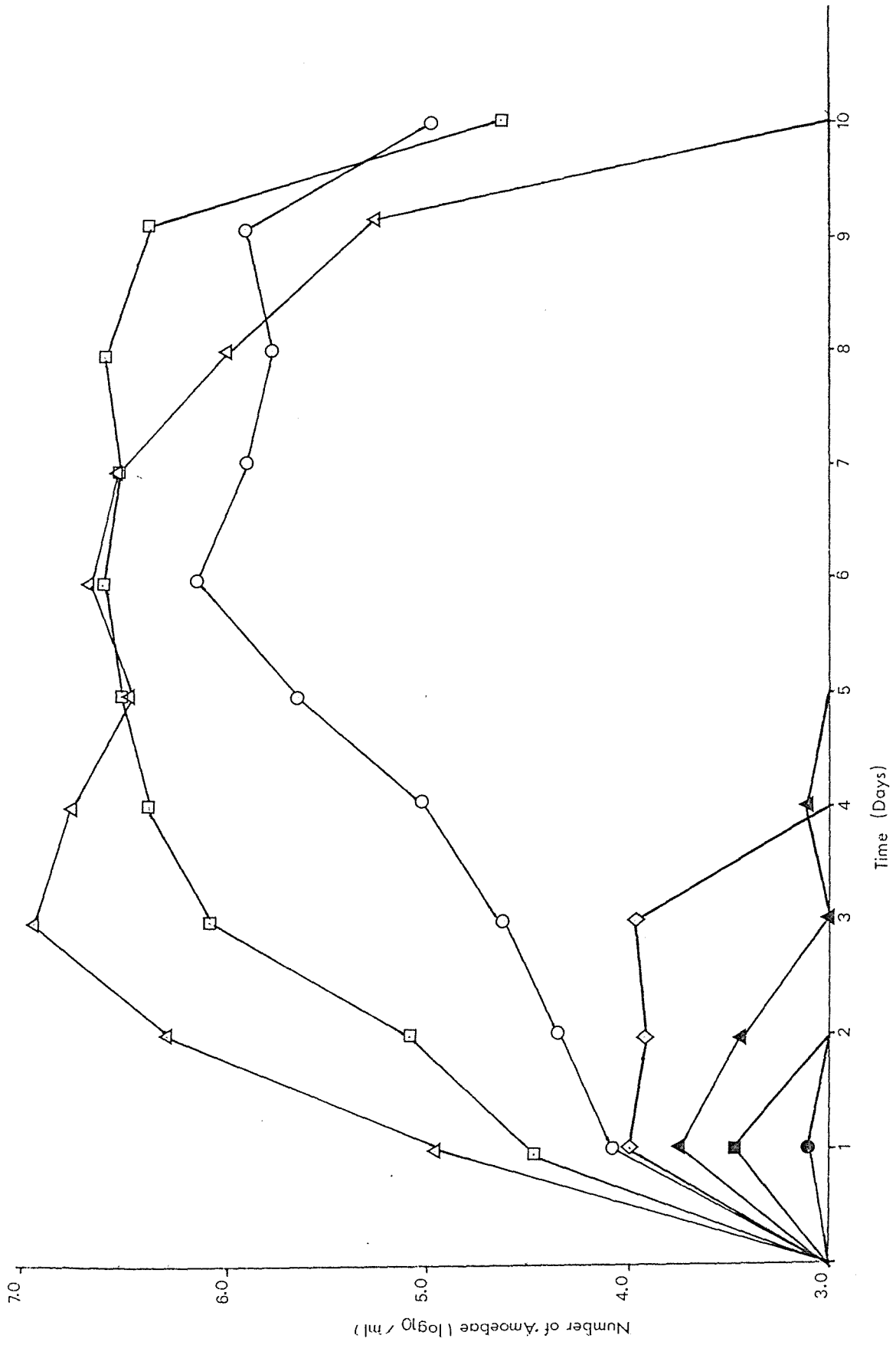


Figure 5. The effect of NaCl concentration on growth rate of *N. fowleri*.

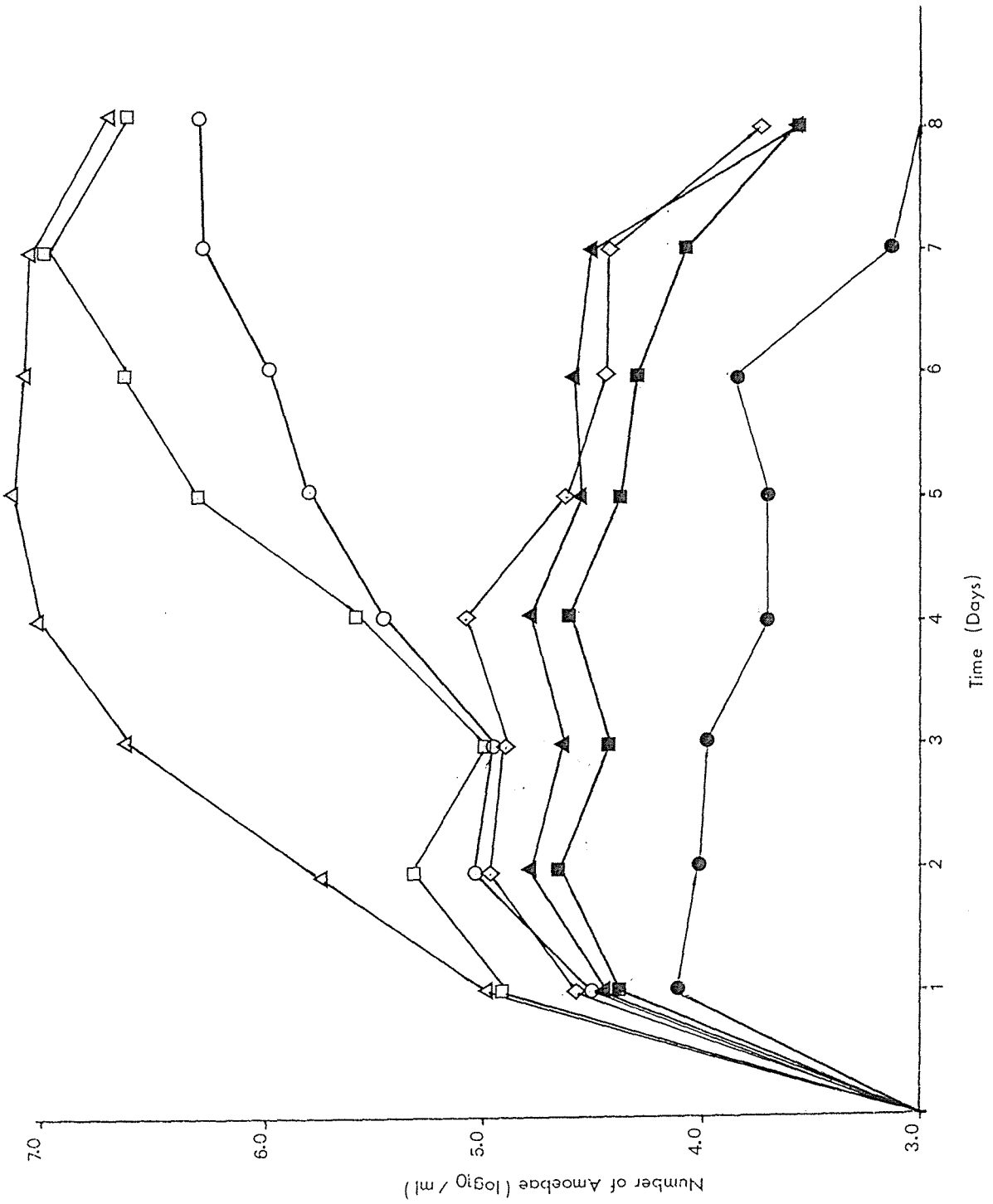


Figure 6. The effect of NaCl concentration on growth rate of *N. gruberi*.

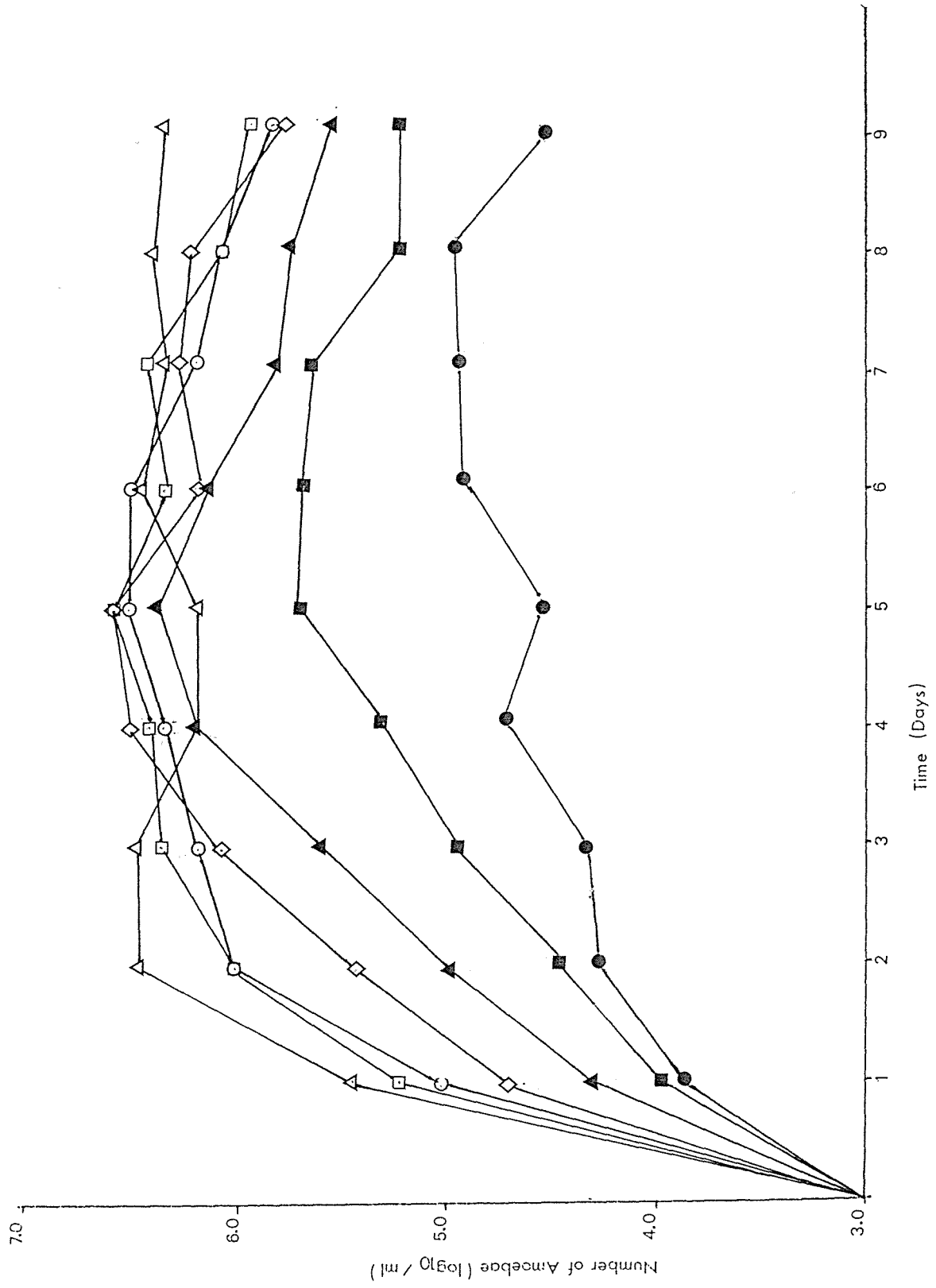


Figure 7. The effect of NaCl concentration on growth rate of *A. culbertsoni*.

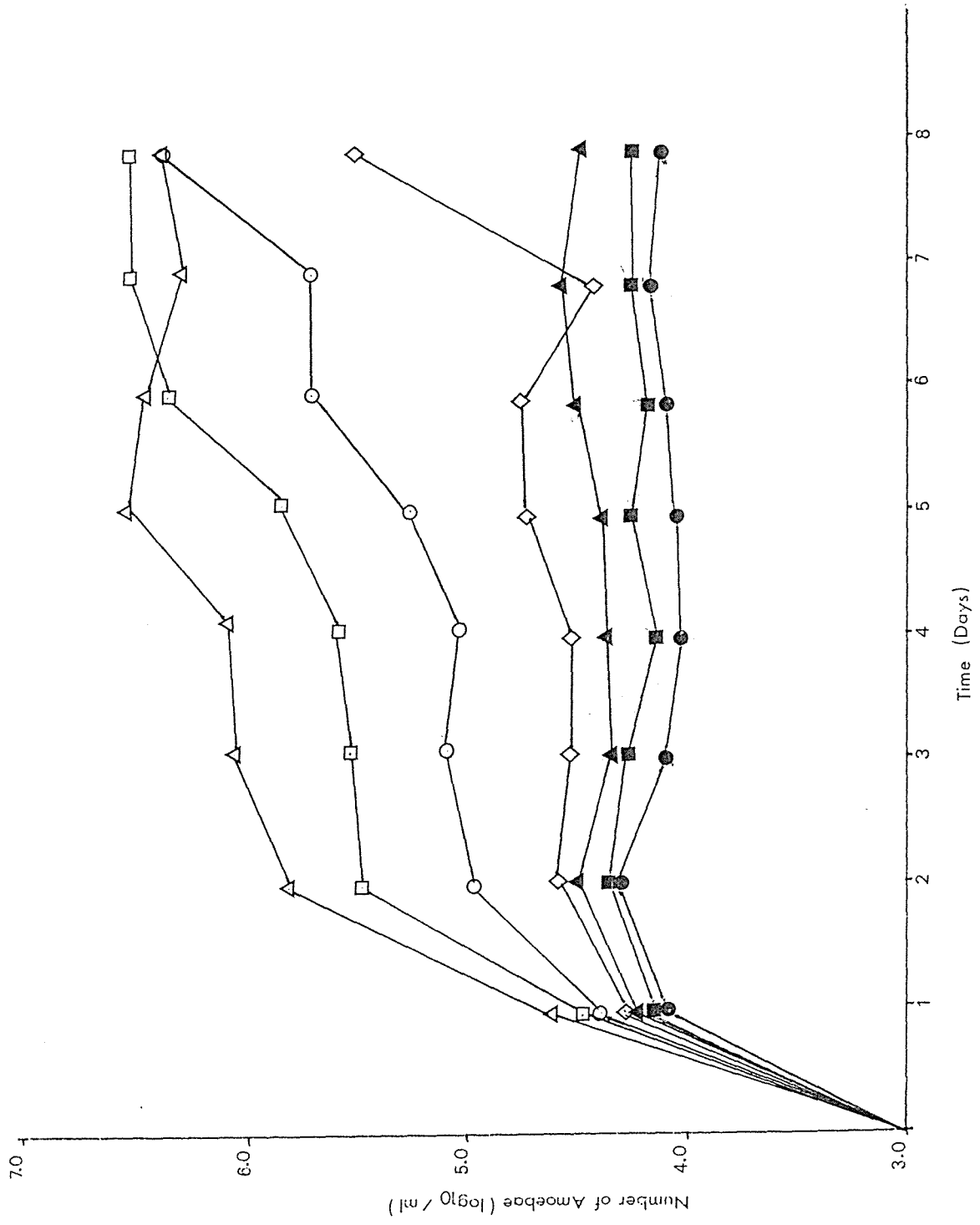


Figure 8. The effect of NaCl concentration on growth rate of *A. castellanii*.

4.1.3 Effect of Fe^{+2}, Fe^{+3} on growth of amoebae

Figures 9-12 show the effect of 1-5 mg l^{-1} Fe^{+2} and Fe^{+3} on growth of amoebae trophozoites.

Figures 9, 10 and 11 show decreasing growth rates corresponding to increasing iron levels.

In all four figures Fe^{+2} and Fe^{+3} are shown to have similar effects but of differing magnitude.

Figure 9 shows that Fe^{+3} had more inhibitory effect on *N. fowleri* trophozoites than Fe^{+2} did. The reverse is true for both *N. gruberi* and *A. culbertsoni* (Figures 10 and 11 respectively), where Fe^{+2} had the greater inhibitory effect.

Both Fe^{+2} and Fe^{+3} had a stimulatory effect on the growth of *A. castellanii* (Figure 12). The stimulation increased with increased concentration of Fe^{+3}, but reached a peak at 3 mg l^{-1} Fe^{+2}.

There is no significant correlation between species of the same genus, nor is there any correlation with pathogenicity.

The following legend applies to Figures 9-12:

● = Fe^{+3}
○ = Fe^{+2}

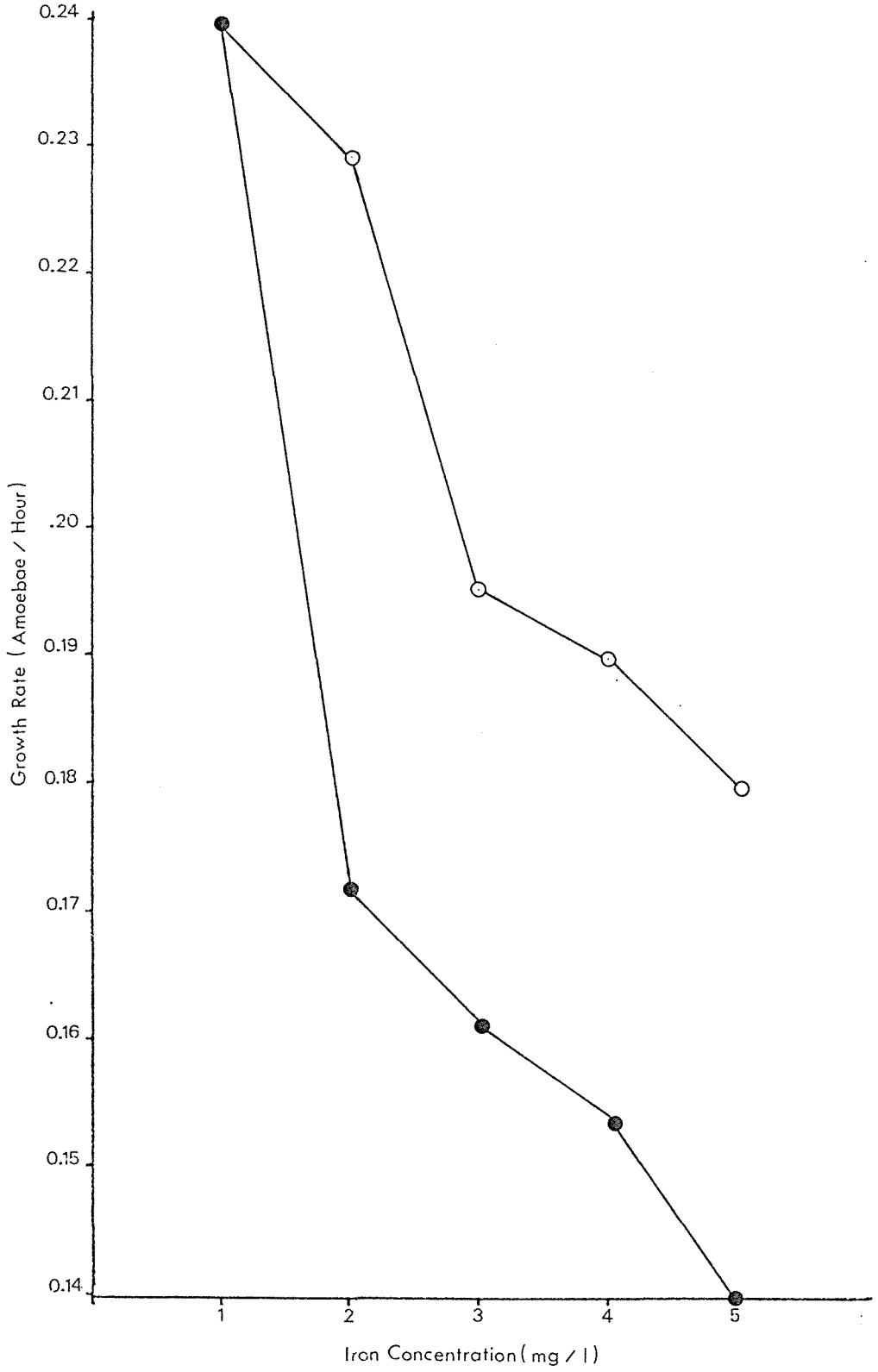


Figure 9. The effect of iron concentration on growth rate of *N. fowleri*.

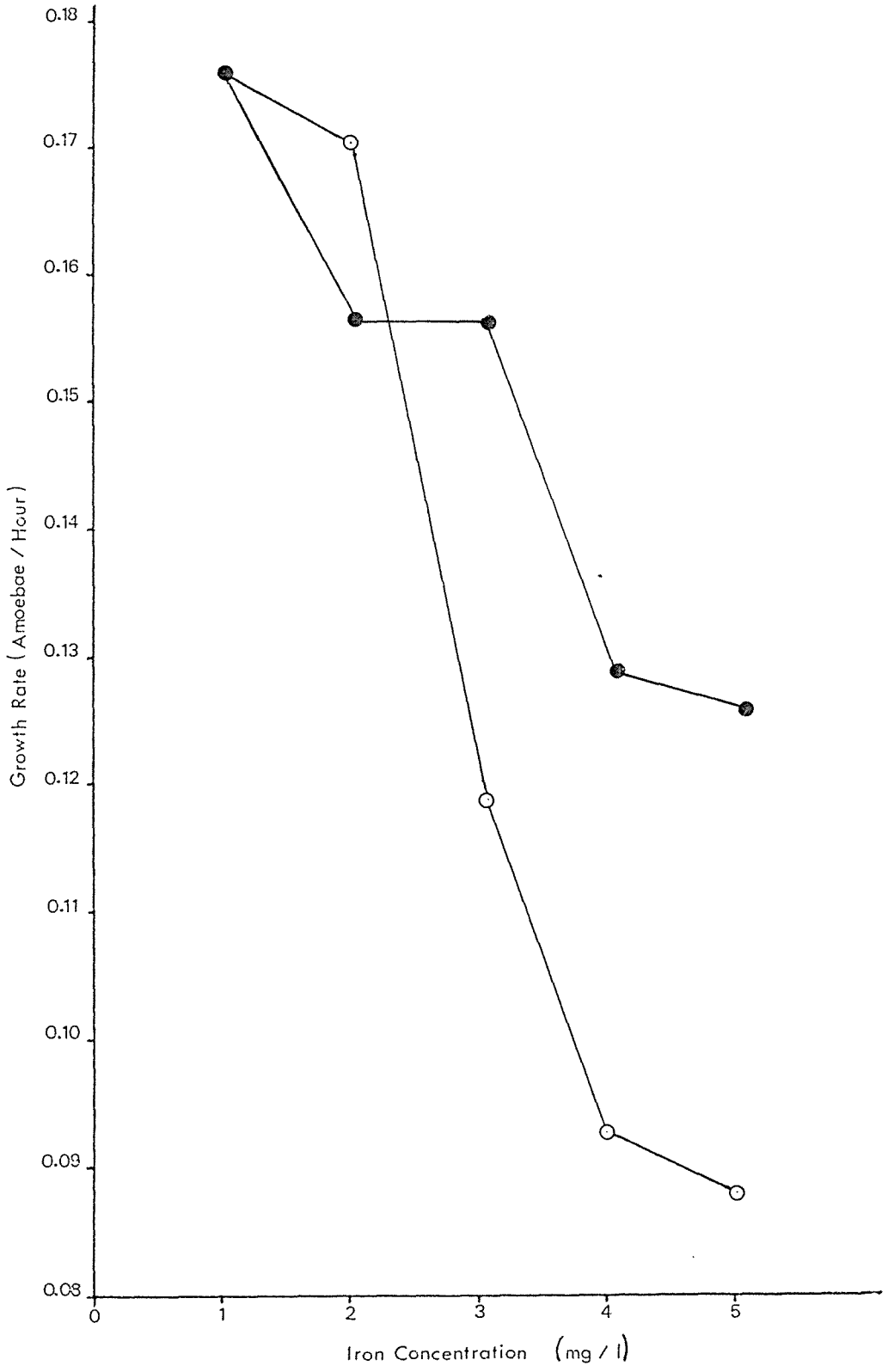


Figure 10. The effect of iron concentration on growth rate of *N. gruberi*.

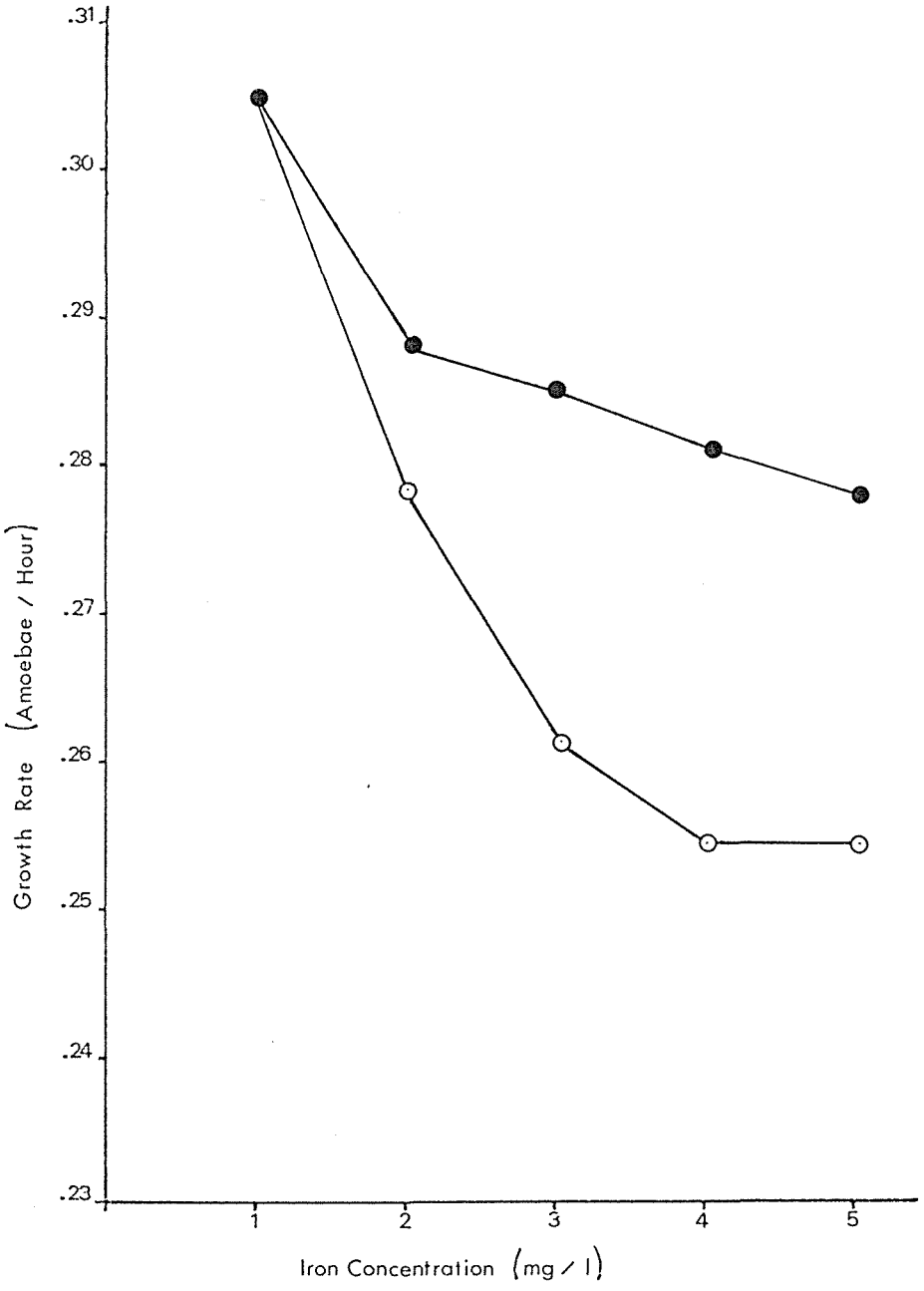


Figure 11. The effect of iron concentration on growth rate of *A. culbertsoni*.

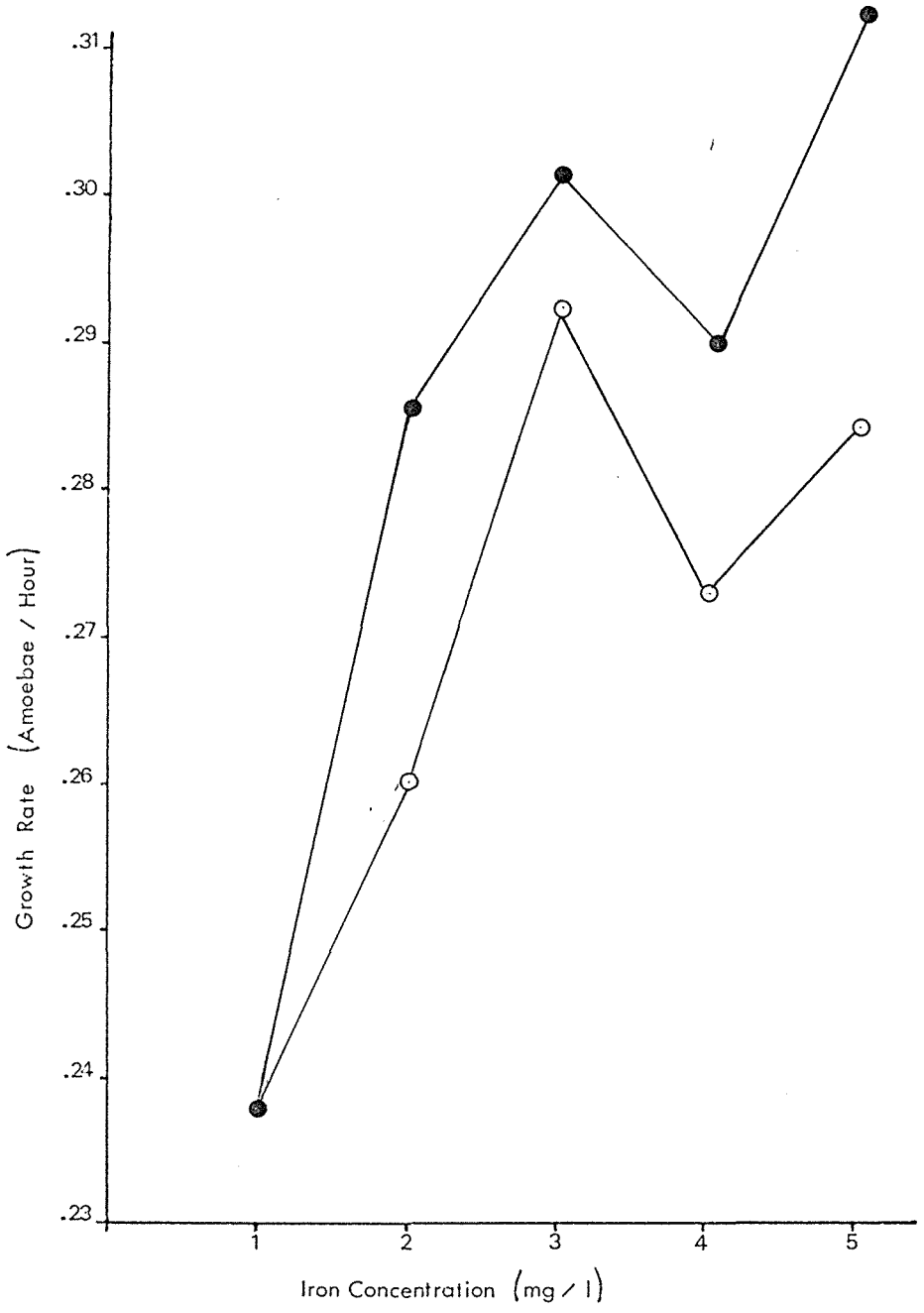


Figure 12. The effect of iron concentration on growth rate of *A. castellani*.

4.1.4 Effect of Ca^{+2} on growth of *N. fowleri* trophozoites

Figures 13 and 14 show the effect of Ca^{+2} at a range of concentrations on the trophozoites of *N. fowleri*. Figure 13 illustrates the experiment undertaken in pure water, and Figure 14 PAS as the basal medium.

In both cases, an increase in Ca^{+2} concentration correlates with a decrease in amoebae numbers. An amoebicidal concentration is regarded for these purposes as the concentration required to give a 2 x $\log_{10}$ decrease in amoebae numbers, i.e. from 5.0 to 3.0 log no. amoebae ml^{-1}. 70 mM Ca^{+2} was amoebicidal after three days in water and after two in PAS. As the concentration of Ca^{+2} decreased, the time required to reach the same level of amoebae was increased. At 60 mM Ca^{+2} three days were required to give a 2 $\log_{10}$ drop in water, and five days were required in PAS. Five days were required in water at 50 mM, and six days were required at the same level in PAS.

The difference between water and PAS was greatest at 40 mM.

The inhibitory/amoebicidal effect of Ca^{+2} was delayed in PAS at 50 and 60 mM, but was enhanced at 40 mM.

The following legend applies to Figures 13 and 14:

× = 0 mg^{1-1} Ca^{+2}
■ = 40
▲ = 50
● = 60
○ = 70

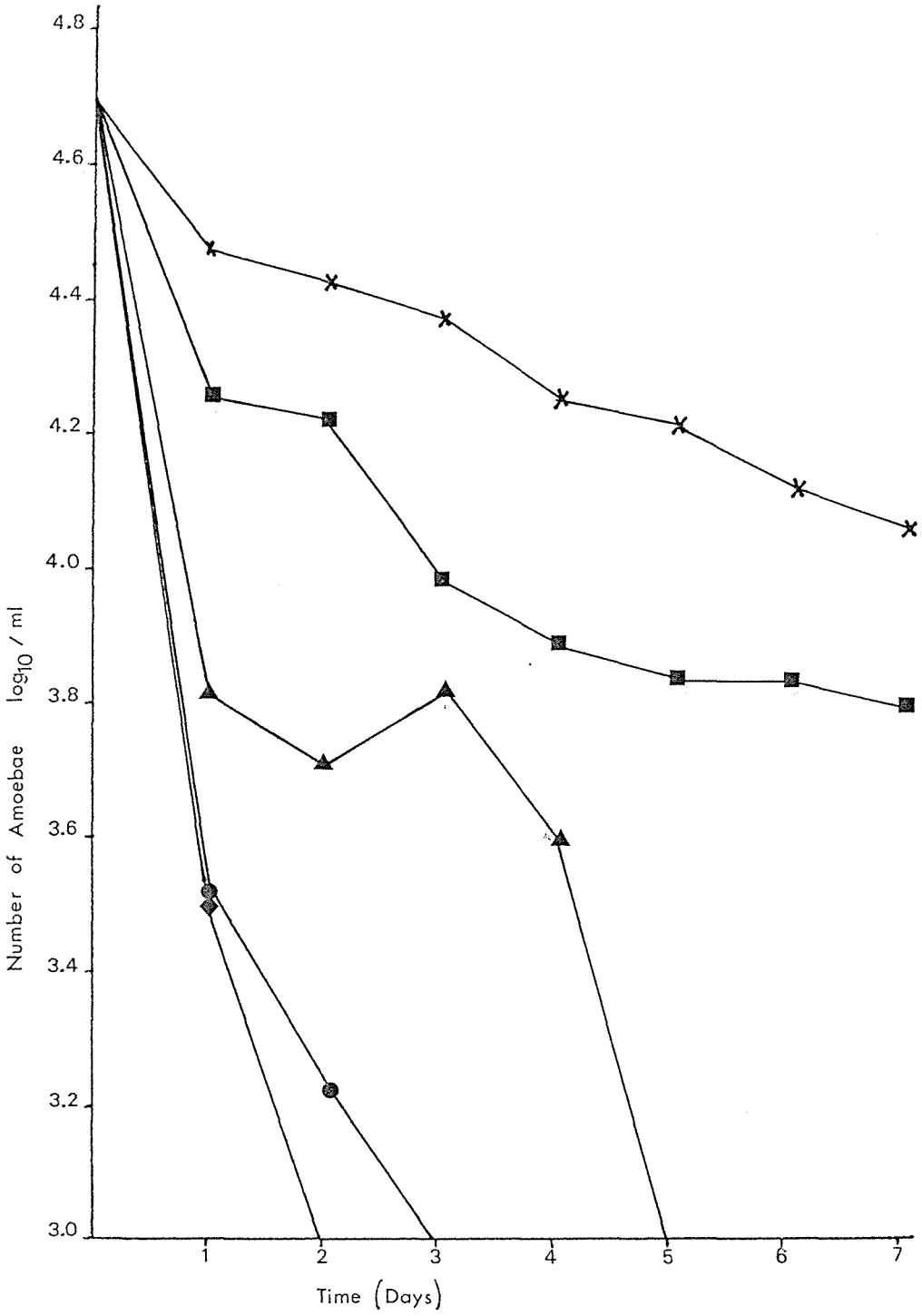


Figure 13. The effect of Ca^{+2} concentration on growth rate of *N. fowleri* in water.

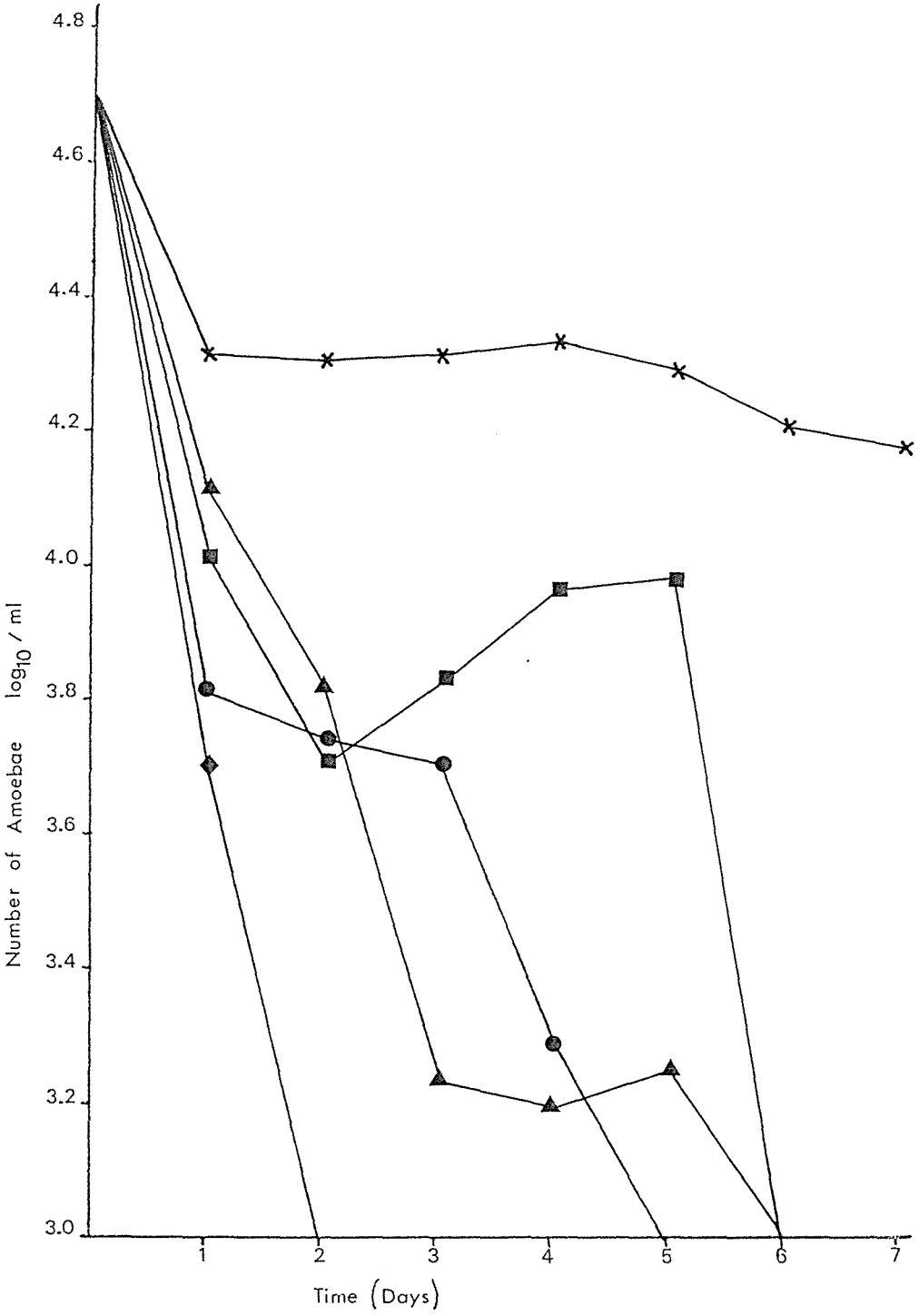


Figure 14. The effect of Ca^{+2} concentration on growth rate of *N. fowleri* in PAS.

4.2 Encystment in Liquid Media

4.2.1 Effect of Ca^{+2} and Mg^{+2} on encystment

The following graphs (Figures 15 - 30) show the encystment produced by each species under the following four sets of conditions:

1) Deionised water + 0.1, 0.2, 0.4 mM Mg^{+2}
2) PAS + 0.1, 0.2, 0.4 mM Mg^{+2}
3) Deionised water + 0.1, 0.2, 0.4 mM Ca^{+2}
4) PAS + 0.1, 0.2, 0.4 mM Ca^{+2}

N. fowleri (NHI) encysted best in pure water with no additives. 88% encystment was achieved, and no round forms were observed. 0.2 mM Mg^{+2} in water produced 50% encystment, but no encystment was produced in any other case, other than minimal levels which did not remain. Round forms were produced in all water experiments, regardless of encystment. PAS, however, produced no encystment or round forms. The time after which trophozoites declined in PAS is related to concentration of cation present.

N. gruberi (PL200f), (Figures 19-22), as for *N. fowleri* (Figures 15-18) produced no cysts in PAS. However, unlike *N. fowleri*, no lasting level of cysts was produced in water either. 26% encystment was produced in 0.1 mM Ca^{+2} in water but this level dropped as round forms increased in proportion. Round forms were produced in most cases, reaching higher levels in the presence of Ca^{+2} or Mg^{+2}.

A. culbertsoni (A1) (Figures 23-26) produced the best encystment in pure water where 100% cysts were produced. A maximum of 15% round forms was observed. PAS alone produced 86% encystment with a maximum of only 22% round forms. Encystment reached levels of 44, 45 and 40% in 0.1, 0.2 and 0.4 mM Mg^{+2} in water, respectively, and 68, 45 and 51% in 0.1, 0.2 and 0.4 mM Ca^{+2} in water. In PAS plus Mg^{+2} 94, 100 and 100% round forms were produced in concentrations of 0.1, 0.2 and 0.4 mM. A maximum of 12% encystment was recorded in 0.1 mM but decreased as round forms increased. In PAS plus 0.1, 0.2 and 0.4 mM Ca^{+2}, 80, 100 and 100% round forms were produced, with cyst levels reaching maximums of 29, 3 and 0, respectively.

A. castellanii (1501) (Figures 27-30) produced 100% encystment in both water and PAS after 5 and 6 days respectively. No round forms were produced. Water + Mg^{+2} produced only minimal levels of cysts and round forms, the highest level of encystment of 7% in 0.1 mM Mg^{+2} and the highest level of round forms of 72% in 0.4 mM Mg^{+2} in PAS produced higher levels of encystment than Mg^{+2} in water. 0.1, 0.2 and 0.4 mM produced cyst levels of 74%, 42% and 9% respectively. In 0.2 and 0.4 mM the levels of round forms increased with levels of cysts and in the latter case round forms reaching 100% and in the former, the cyst numbers dropping off as the round forms increased.

Deionised water is the best encystment medium for all species, followed by PAS for *Acanthamoeba*. *Naegleria* could not encyst in PAS. PAS plus Mg^{+2} and Ca^{+2} caused high levels of encystment of 01, encystment of A1 higher than 68% on addition of Mg or Ca to PAS and water. Ca^{+2} had more effect on A1 than Mg^{+2}. Mg^{+2} in water at 0.2 mM was the best encystment medium after pure water for *N. fowleri*, and Ca^{+2} 0.1 mM in water was best for PL200f.

The following legend applies to Figures 15-30:

△ = 0 mgl^{-1} Ca^{+2} or Mg^{+2}
□ = 0.1
○ = 0.2
◇ = 0.4

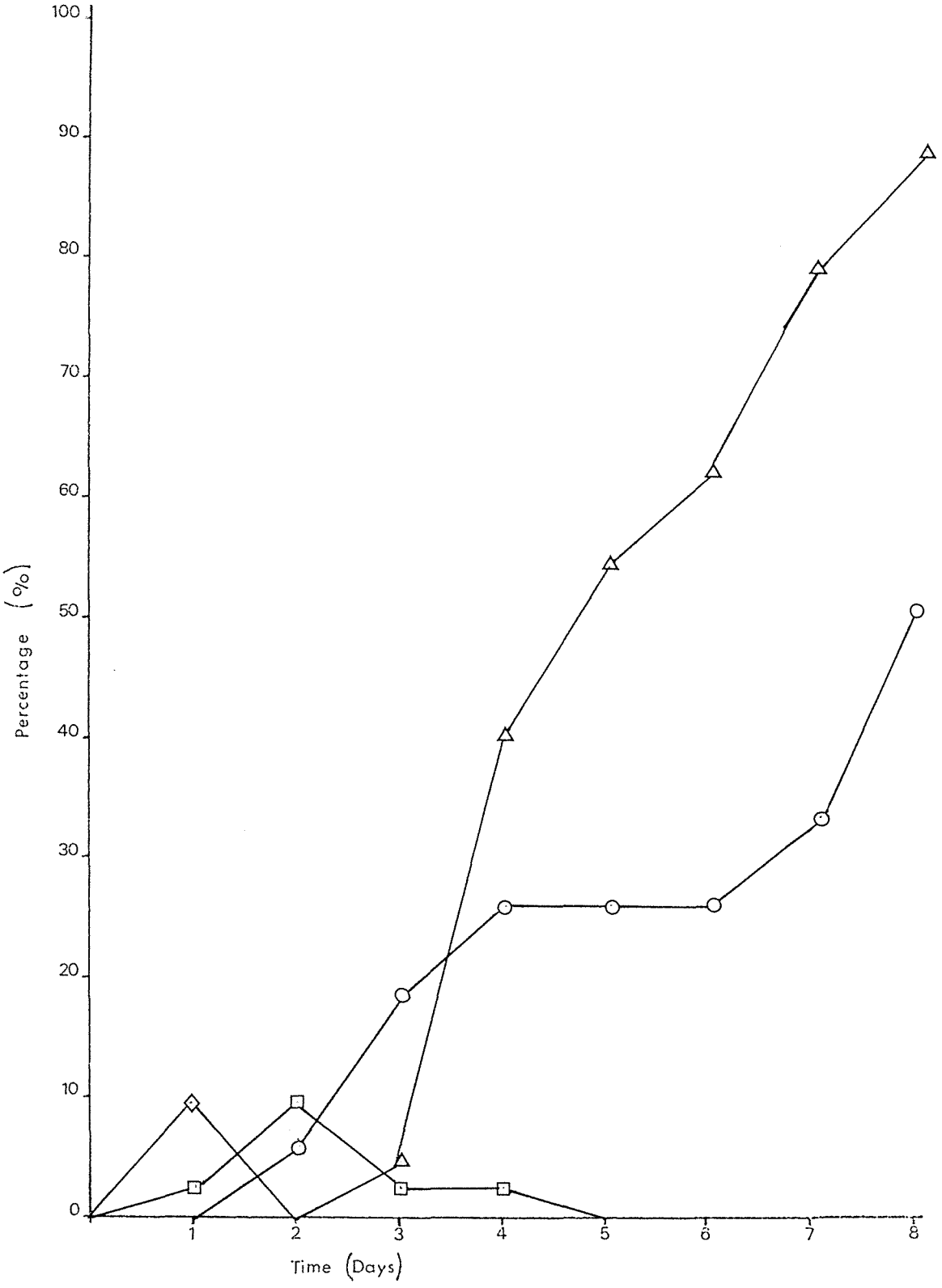


Figure 15a. The effect of Mg_{+2} concentration in water on encystment of *N. fowleri*. Encystment levels.

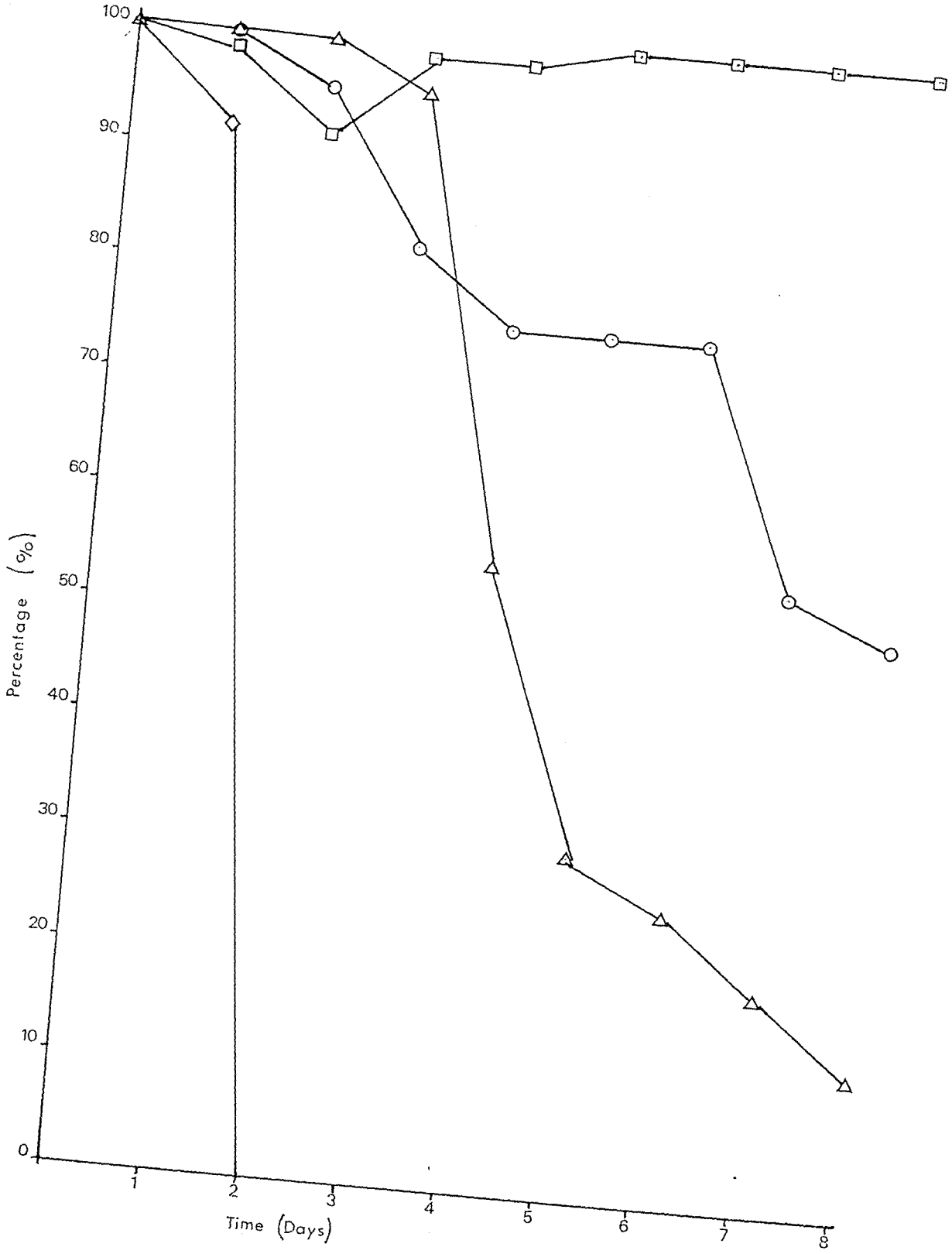


Figure 15b. The effect of Mg_{+2} concentration in water on encystment of *N. fowleri*. Trophozoite levels.

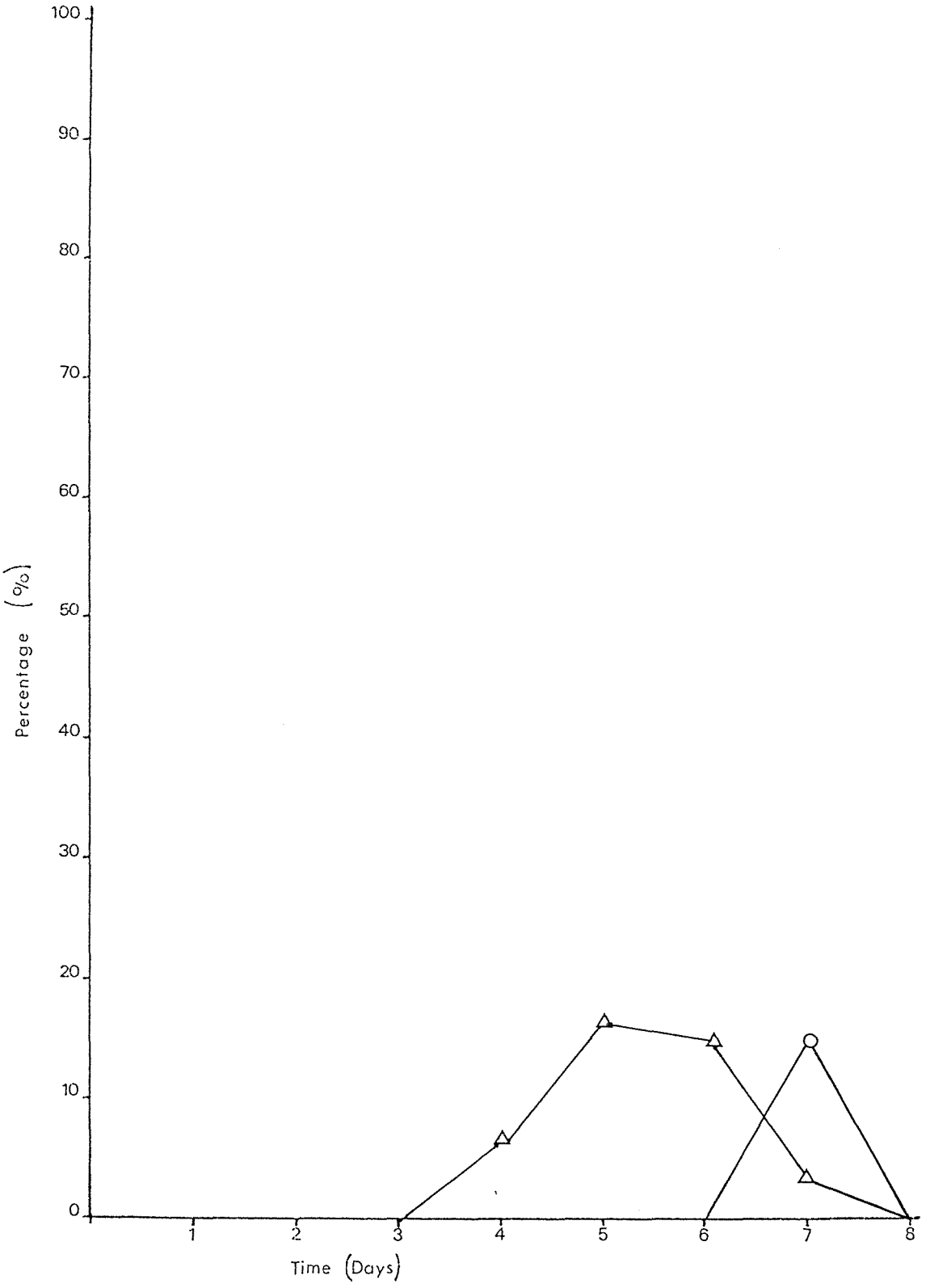


Figure 15c. The effect of Mg^{+2} concentration in water on encystment of *N. fowleri*. Round form levels.

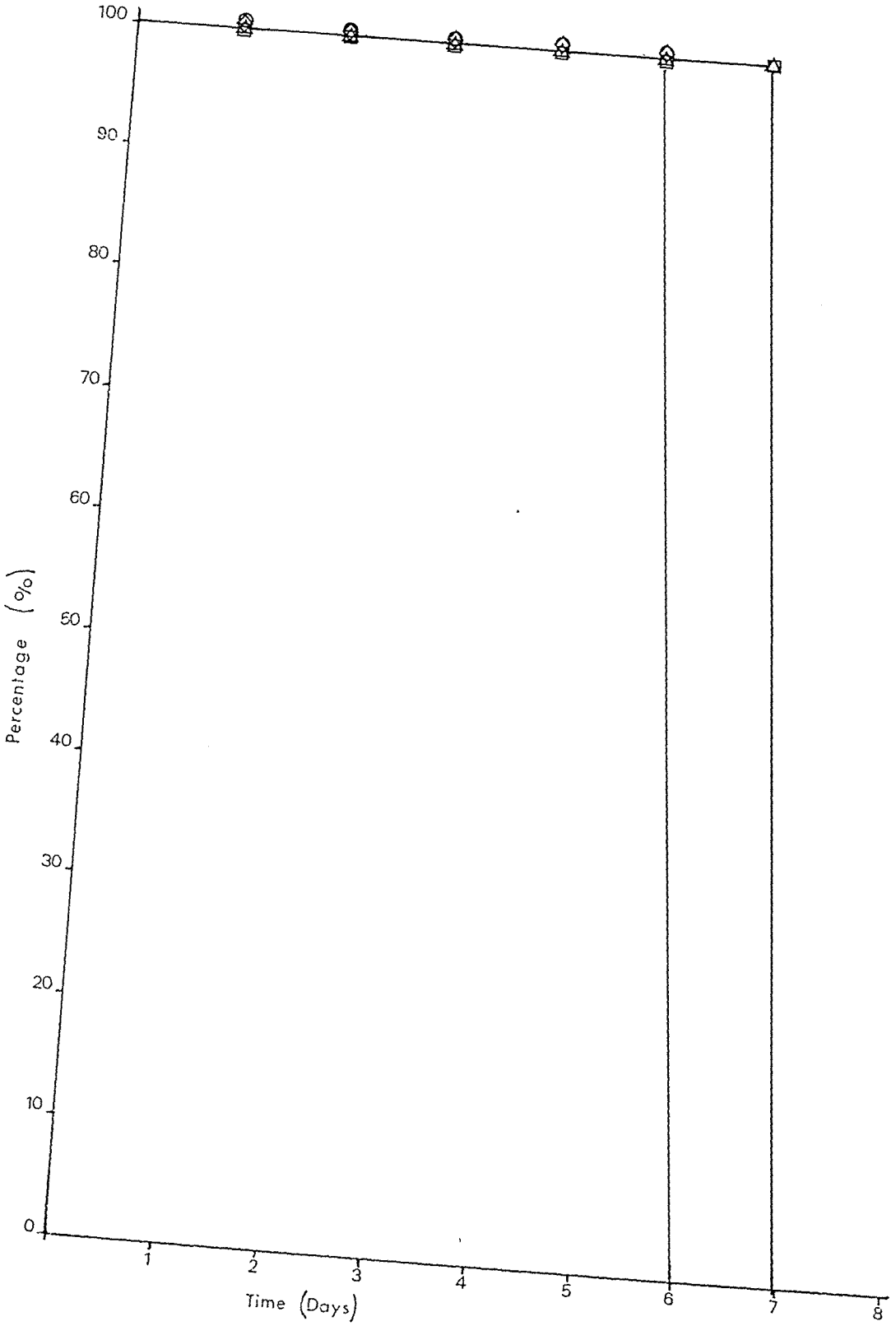


Figure 16. The effect of Mg^{+2} concentration in PAS on encystment of *N. fowleri*.

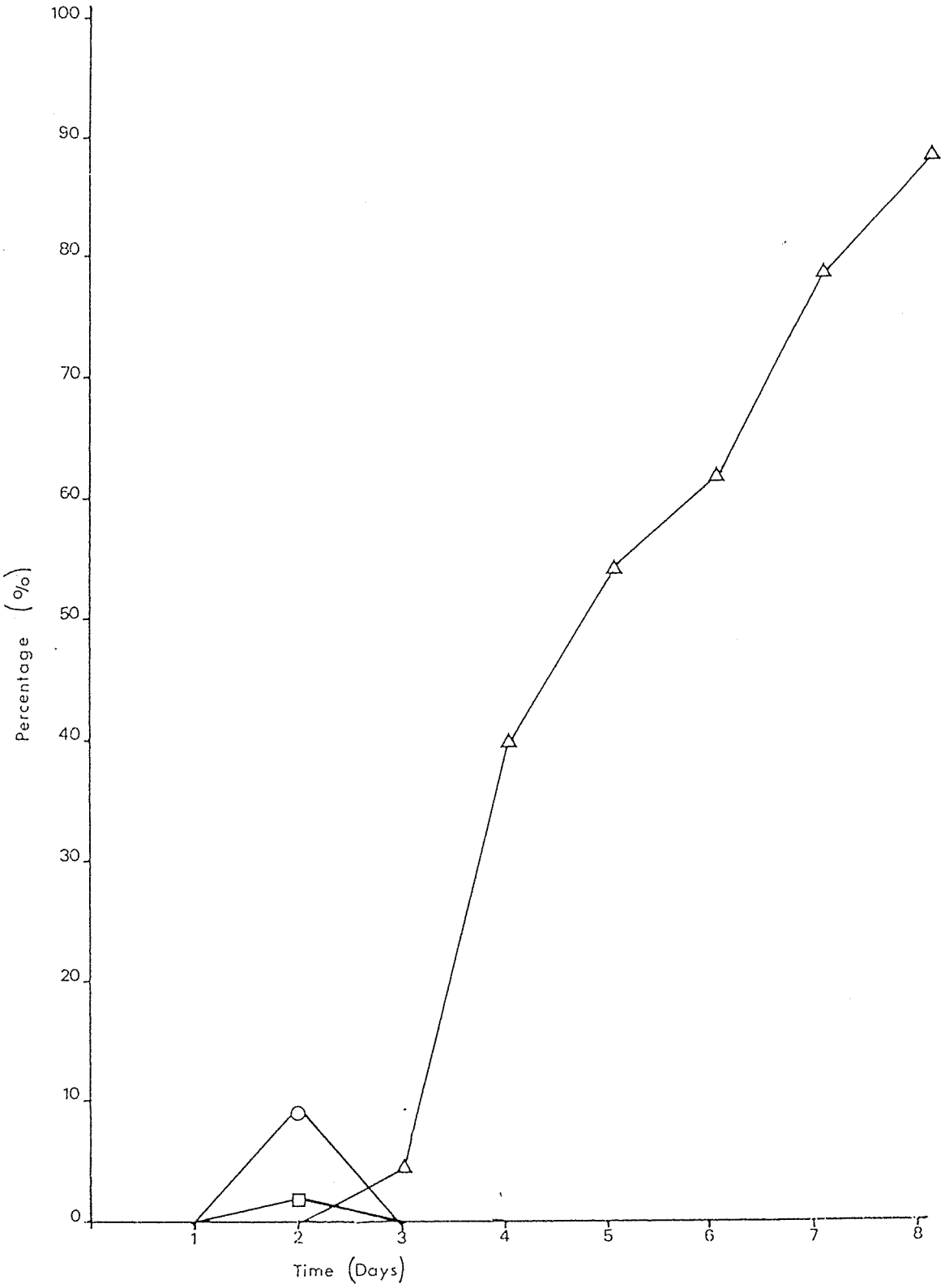


Figure 17a. The effect of Ca^{+2} concentration in water on encystment of *N. fowleri*. Encystment levels.

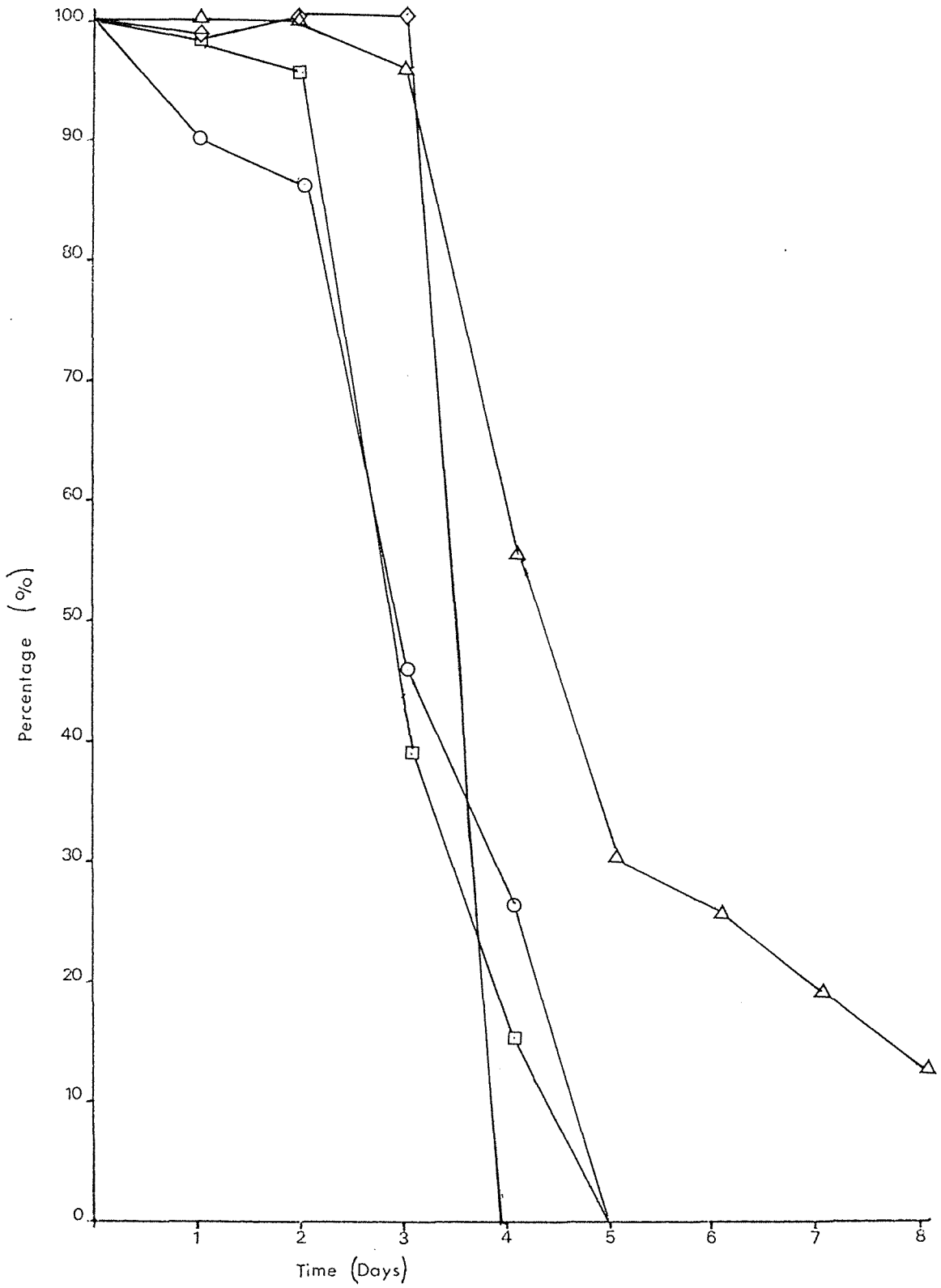


Figure 17b. The effect of Ca^{+2} concentration in water on encystment of *N. fowleri*. Trophozoite levels.

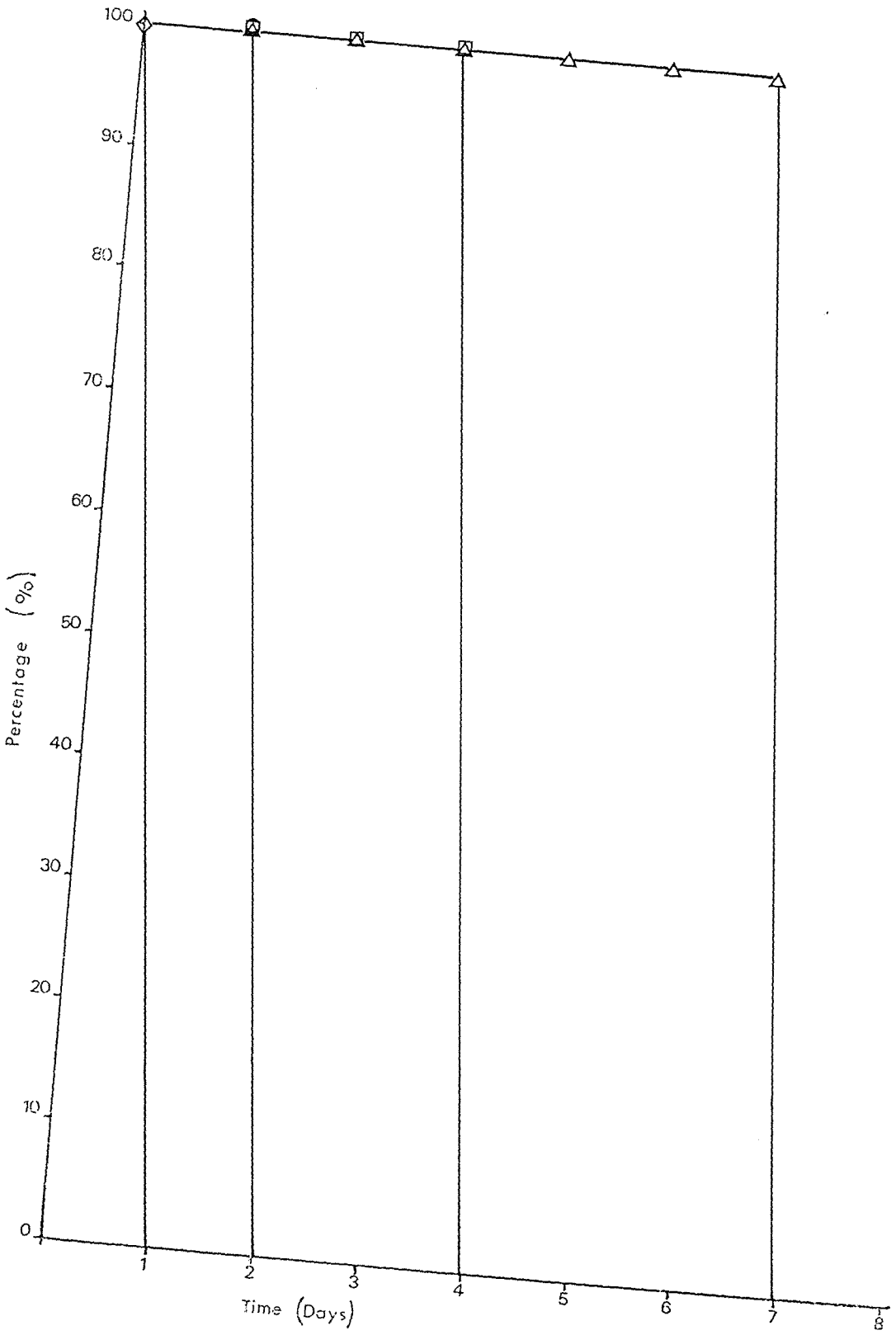


Figure 18. The effect of Ca^{+2} concentration in PAS on encystment of *N. fowleri*.

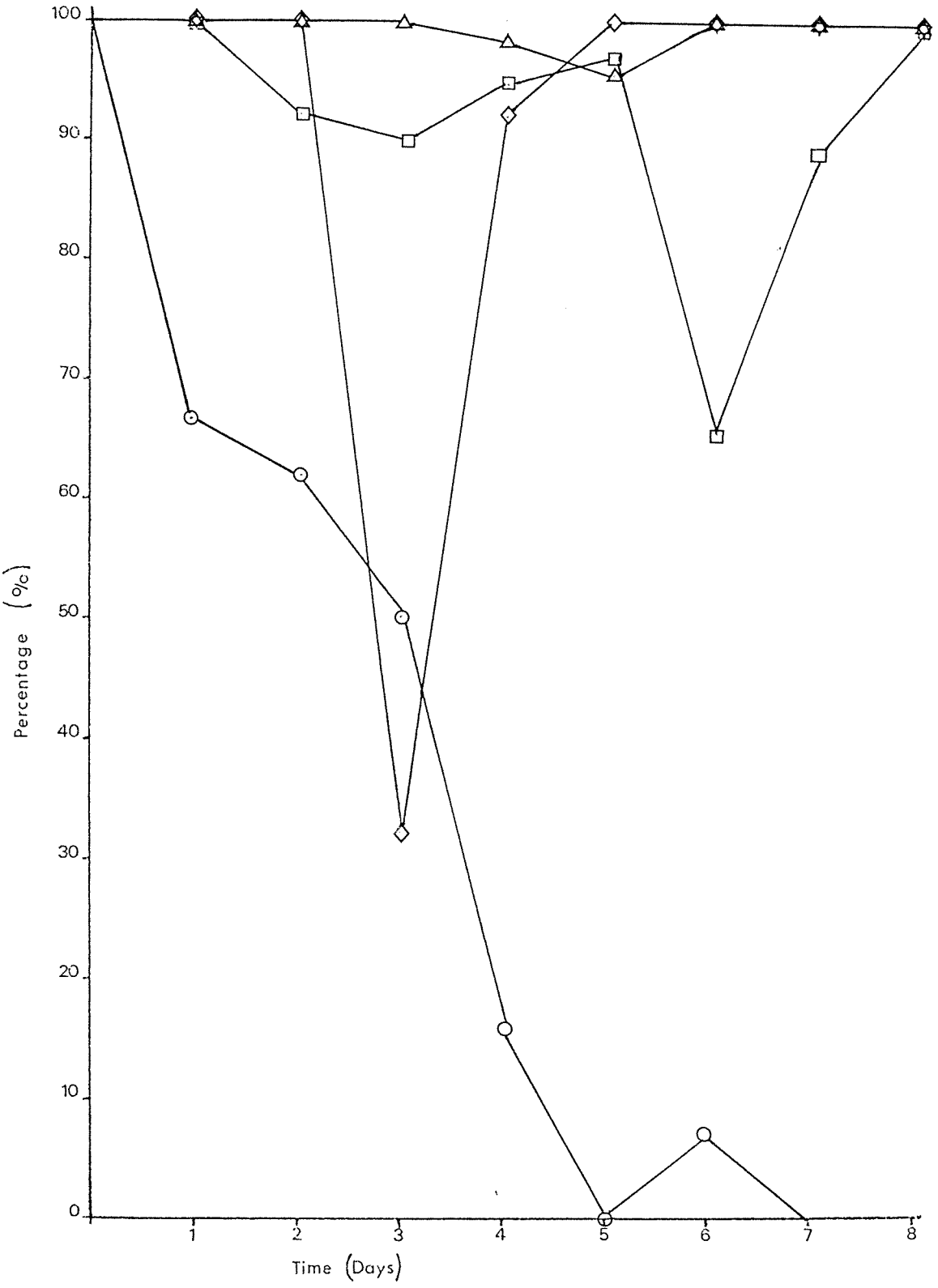


Figure 19b. The effect of Mg^{+2} concentration in water on encystment of *N. gruberi*. Trophozoite levels.

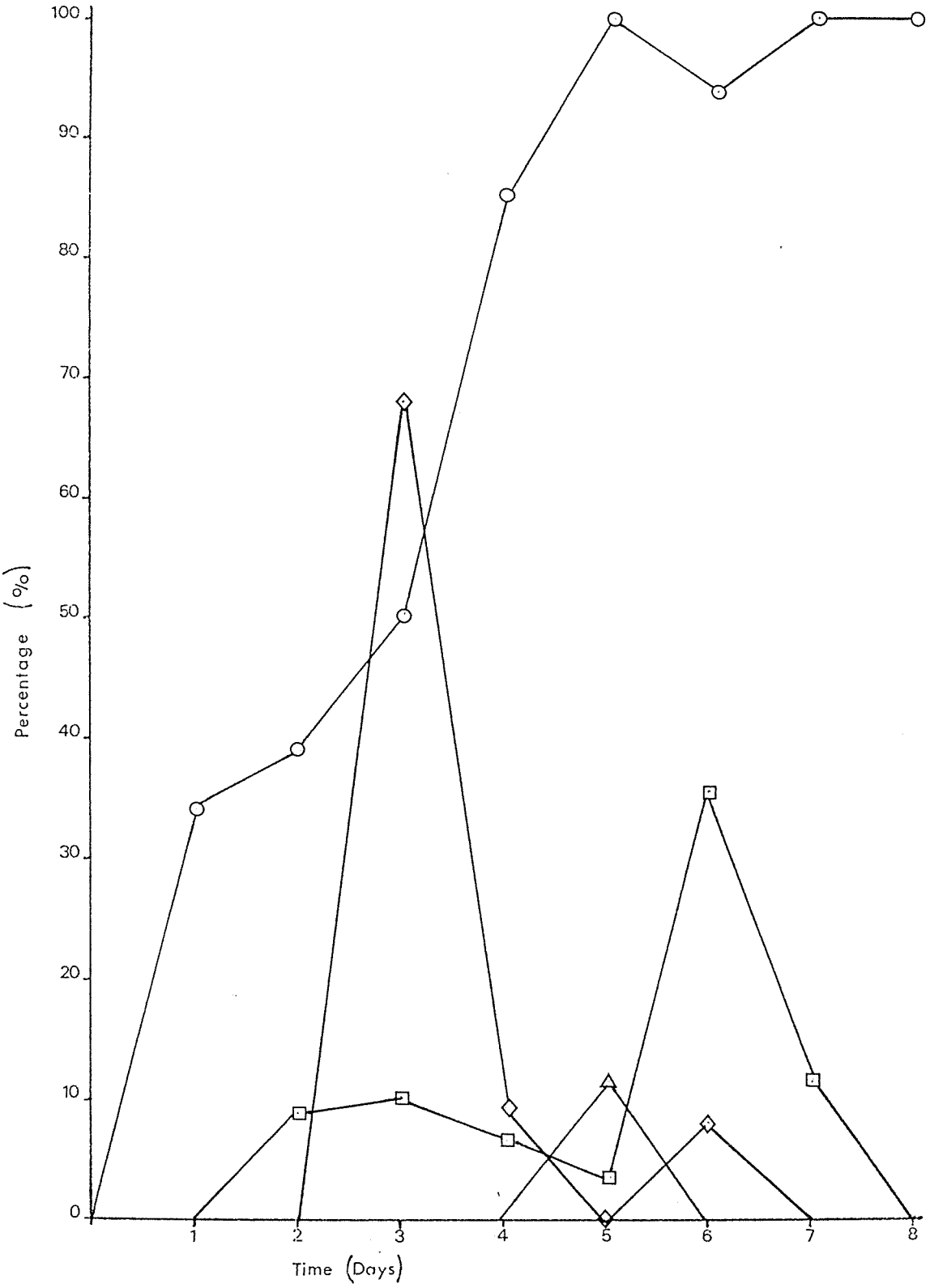


Figure 19c. The effect of Mg^{+2} concentration in water on encystment of *N. gruberi*. Round form levels.

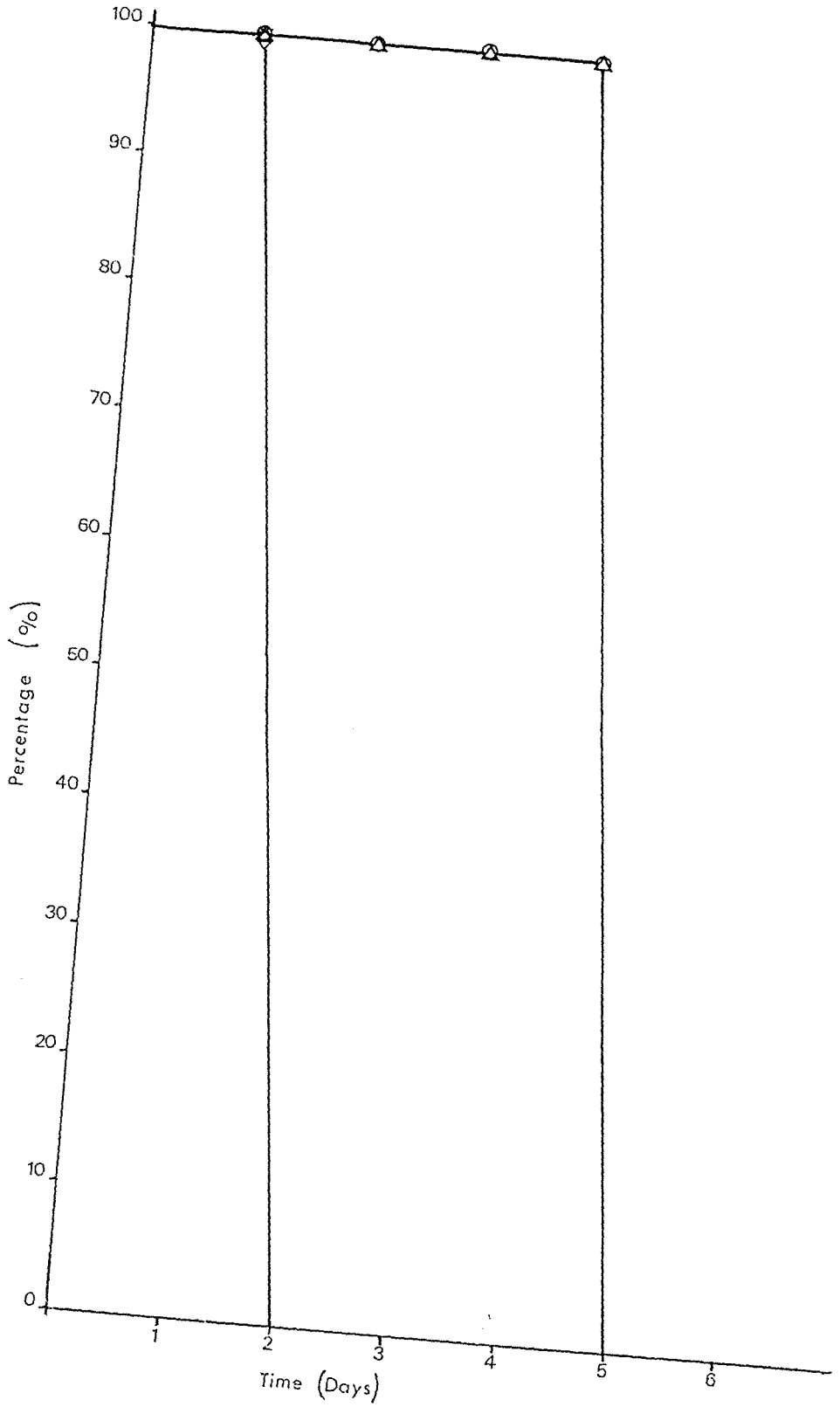


Figure 20b. The effect of Mg^{+2} concentration in PAS on encystment of *N. gruberi*. Trophozoite levels.

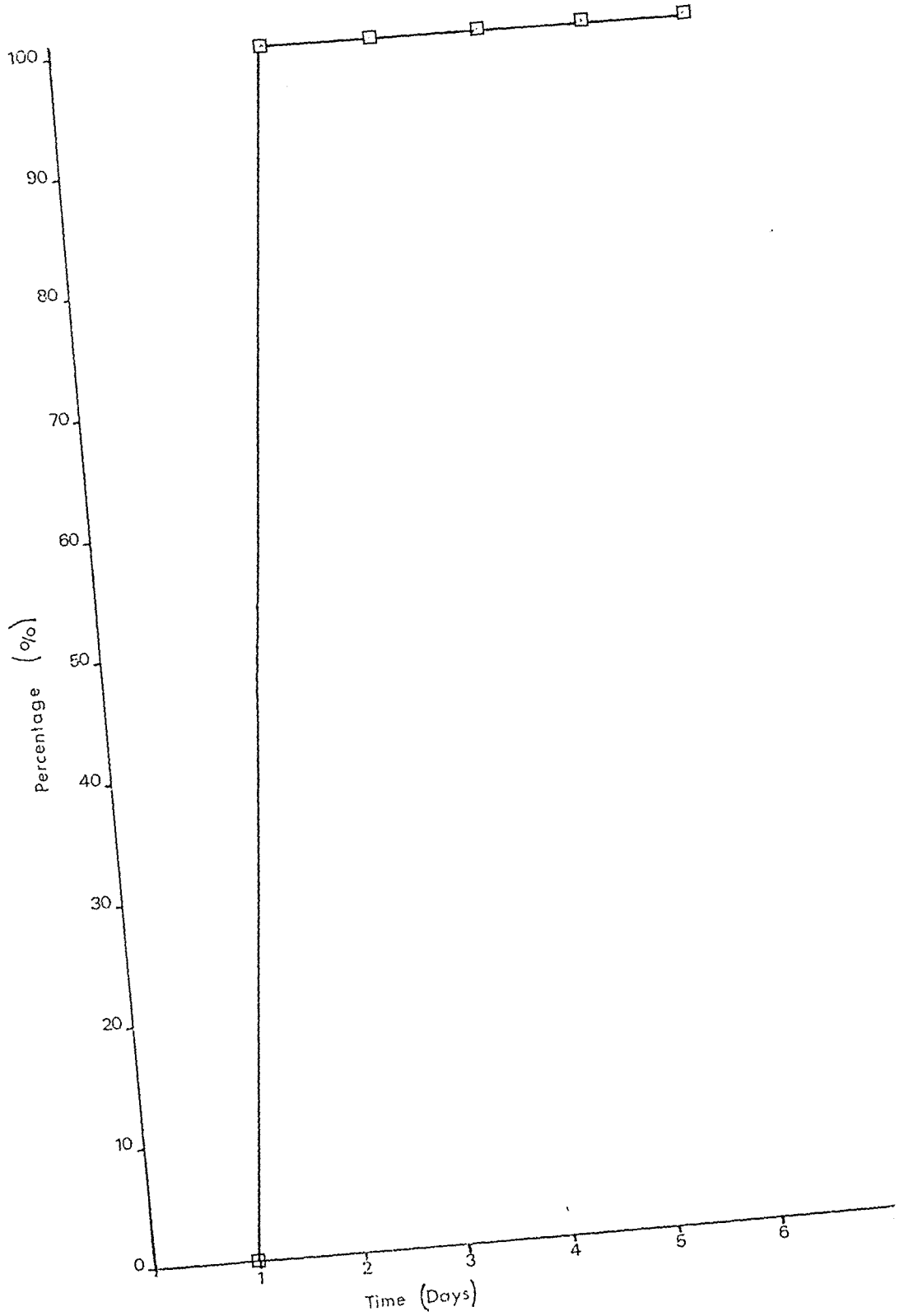


Figure 20c. The effect of Mg^{+2} concentration in PAS on encystment of *N. gruberi*. Round form levels.

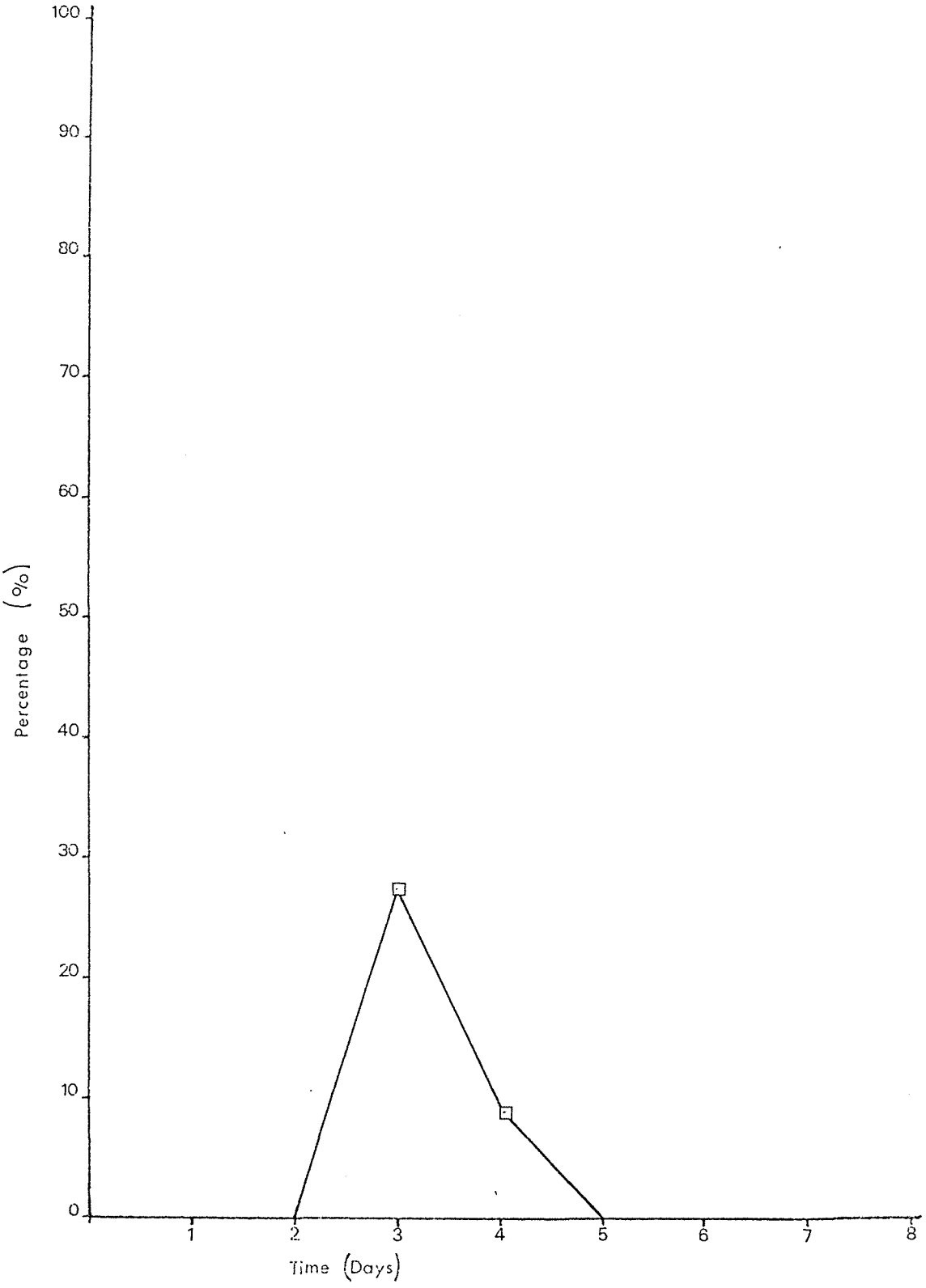


Figure 21a. The effect of Ca^{+2} concentration in water on encystment of *N. gruberi*. Encystment levels.

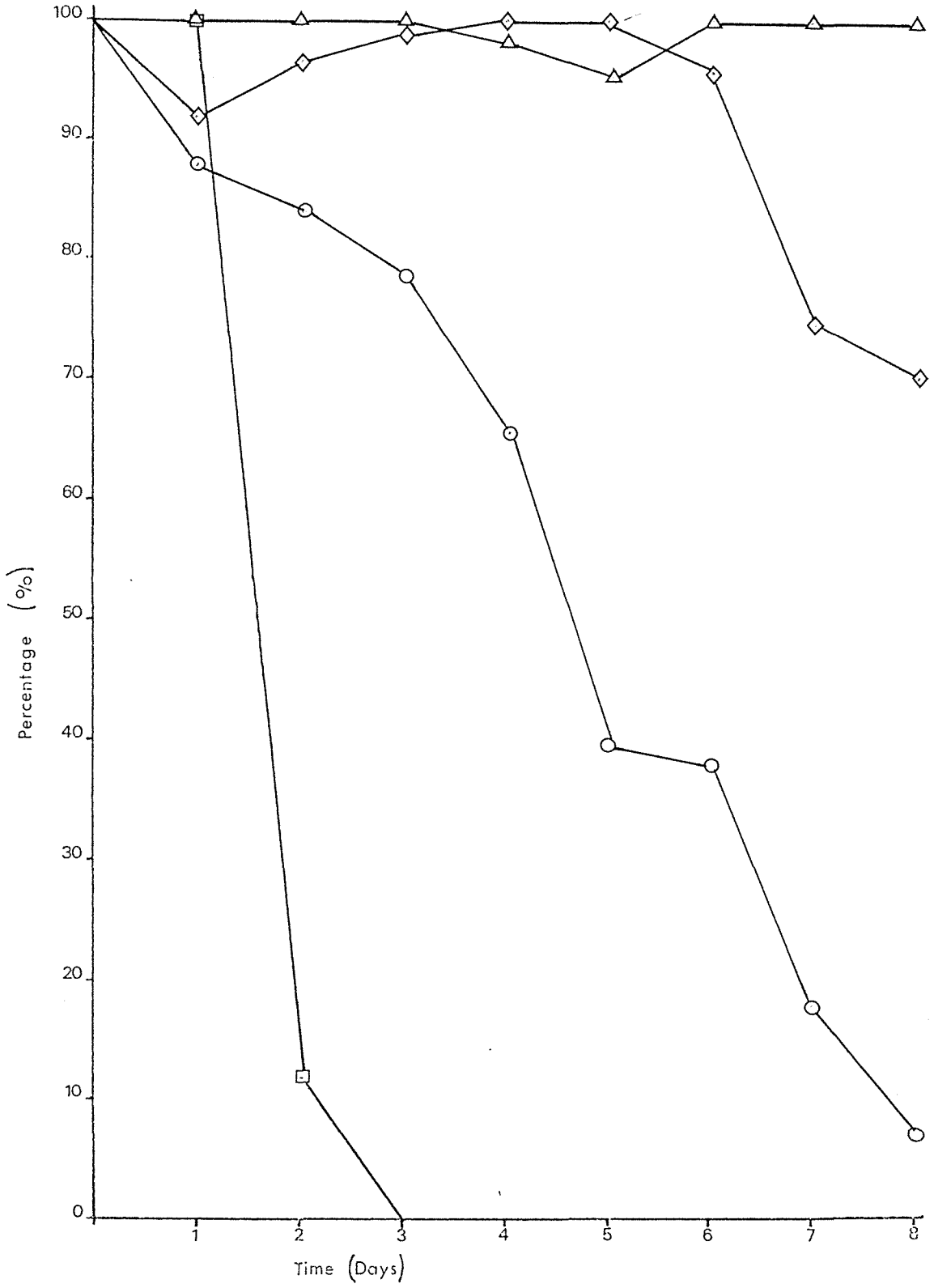


Figure 21b. The effect of Ca^{+2} concentration in water on encystment of *N. gruberi*. Trophozoite levels.

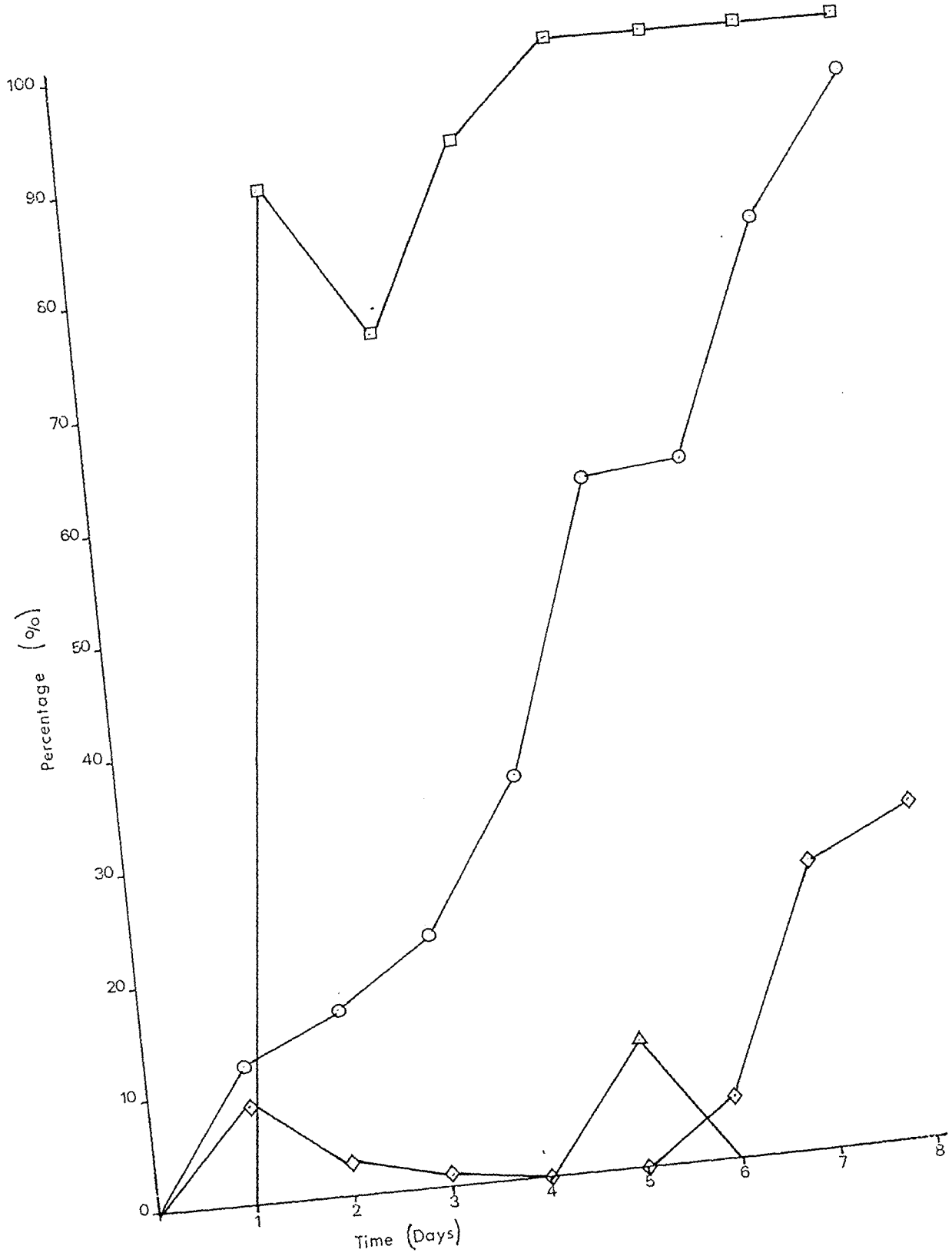


Figure 21c. The effect of Ca^{+2} concentration in water on encystment of *N. gruberi*. Round form levels.

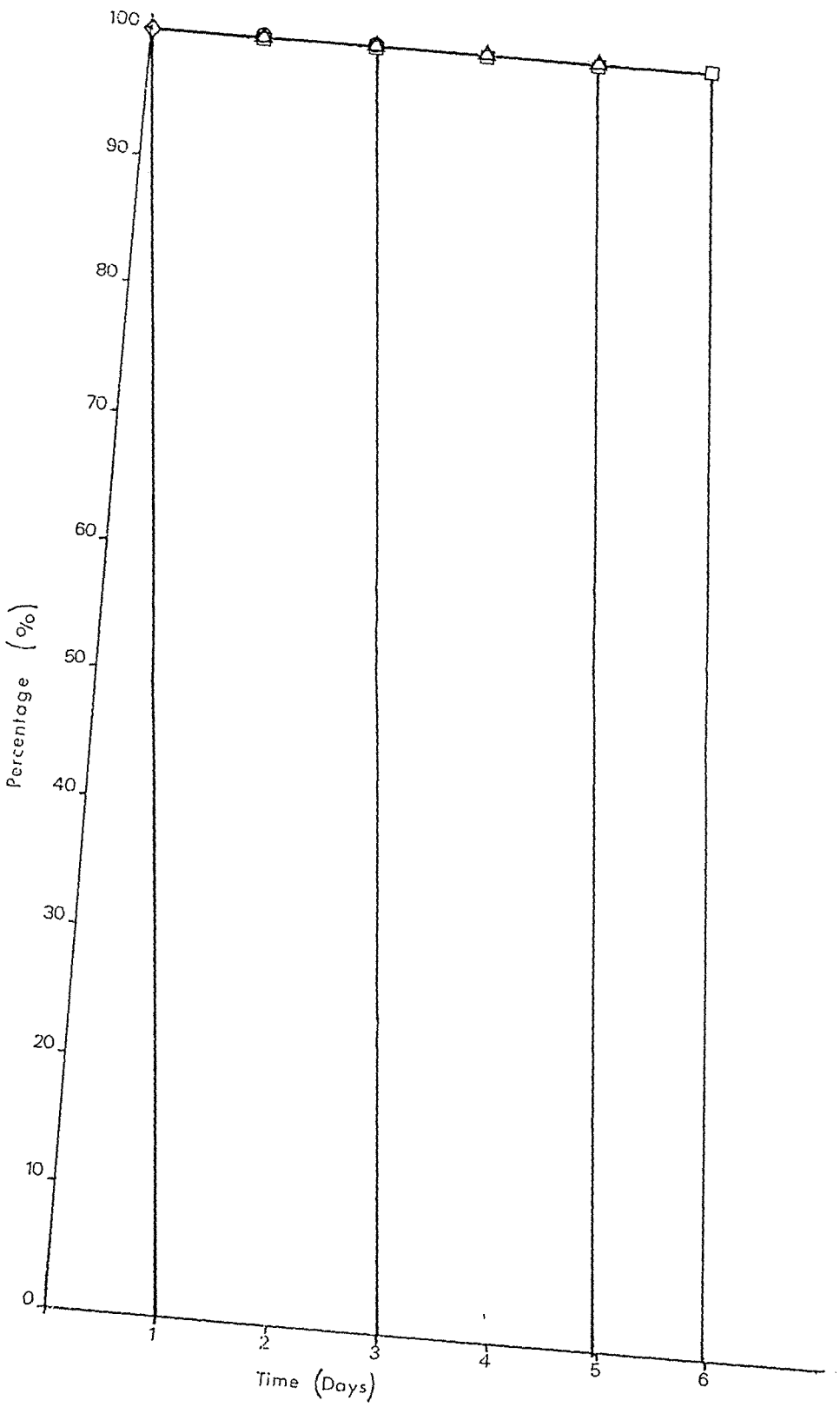


Figure 22. The effect of Ca^{+2} concentration in PAS on encystment of *N. gruberi*.

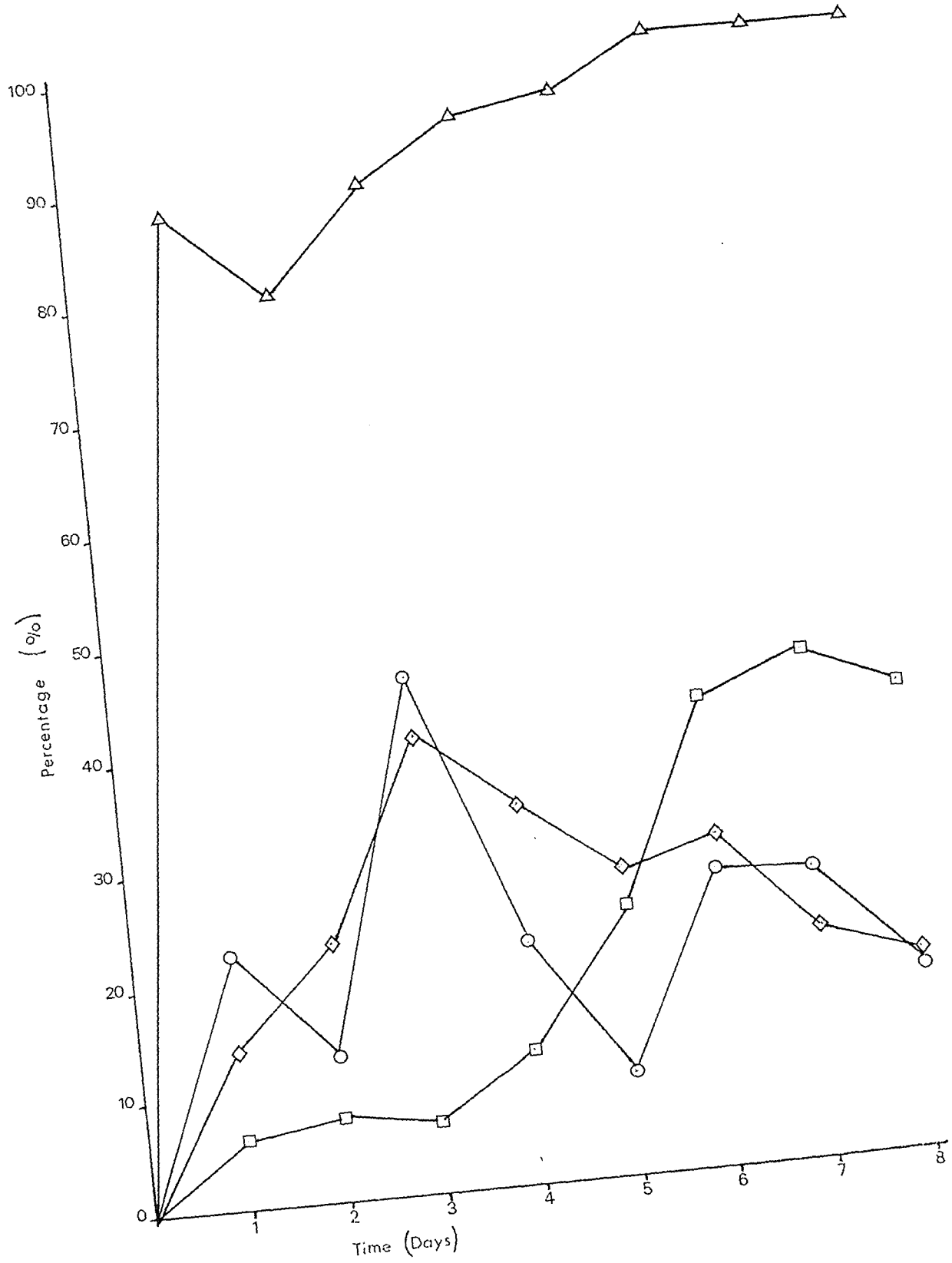


Figure 23a. The effect of Mg^{+2} concentration in water on encystment of *A. culbertsoni*. Encystment levels.

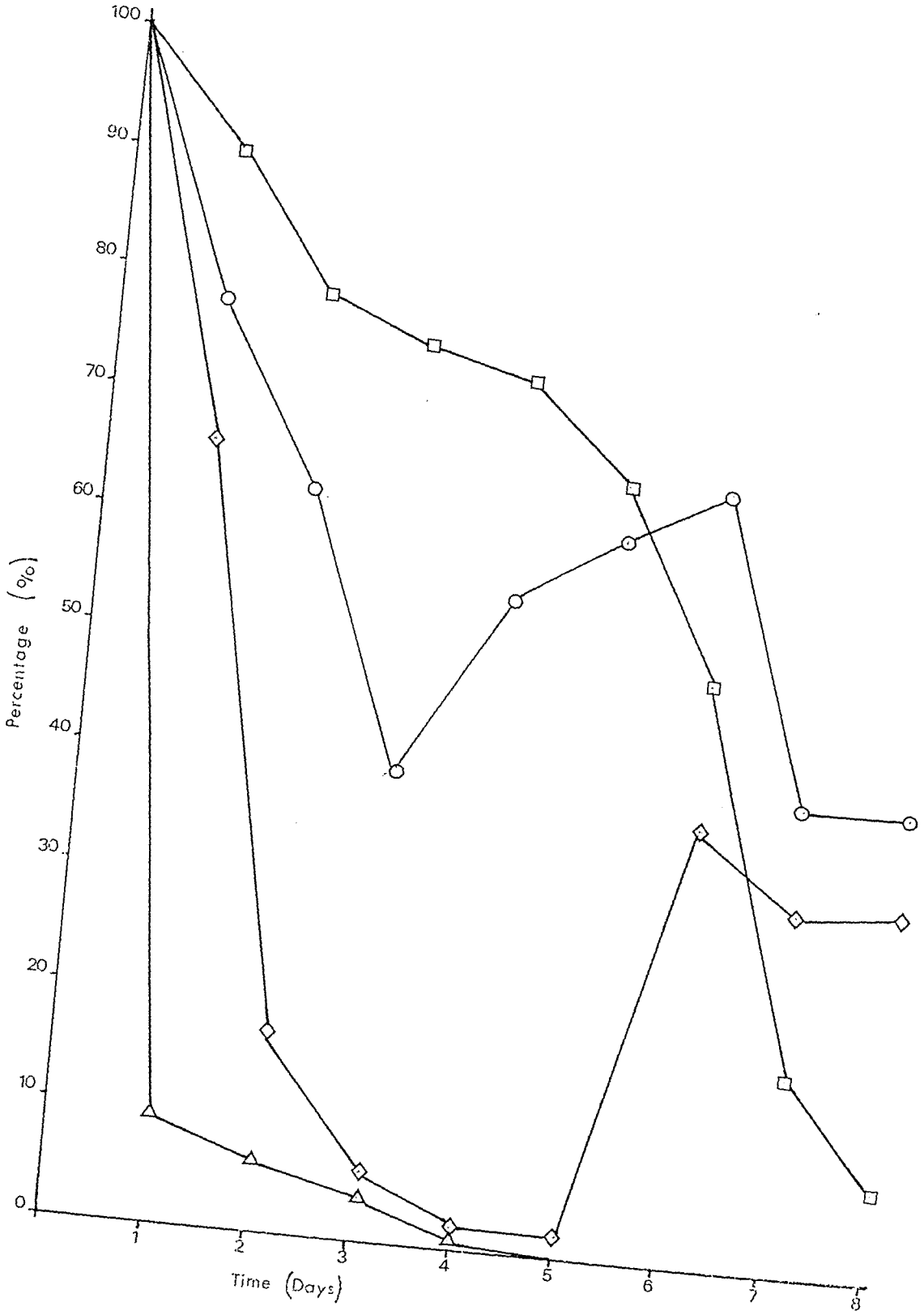


Figure 23b. The effect of Mg^{+2} concentration in water on encystment of *A. culbertsoni*. Trophozoite levels.

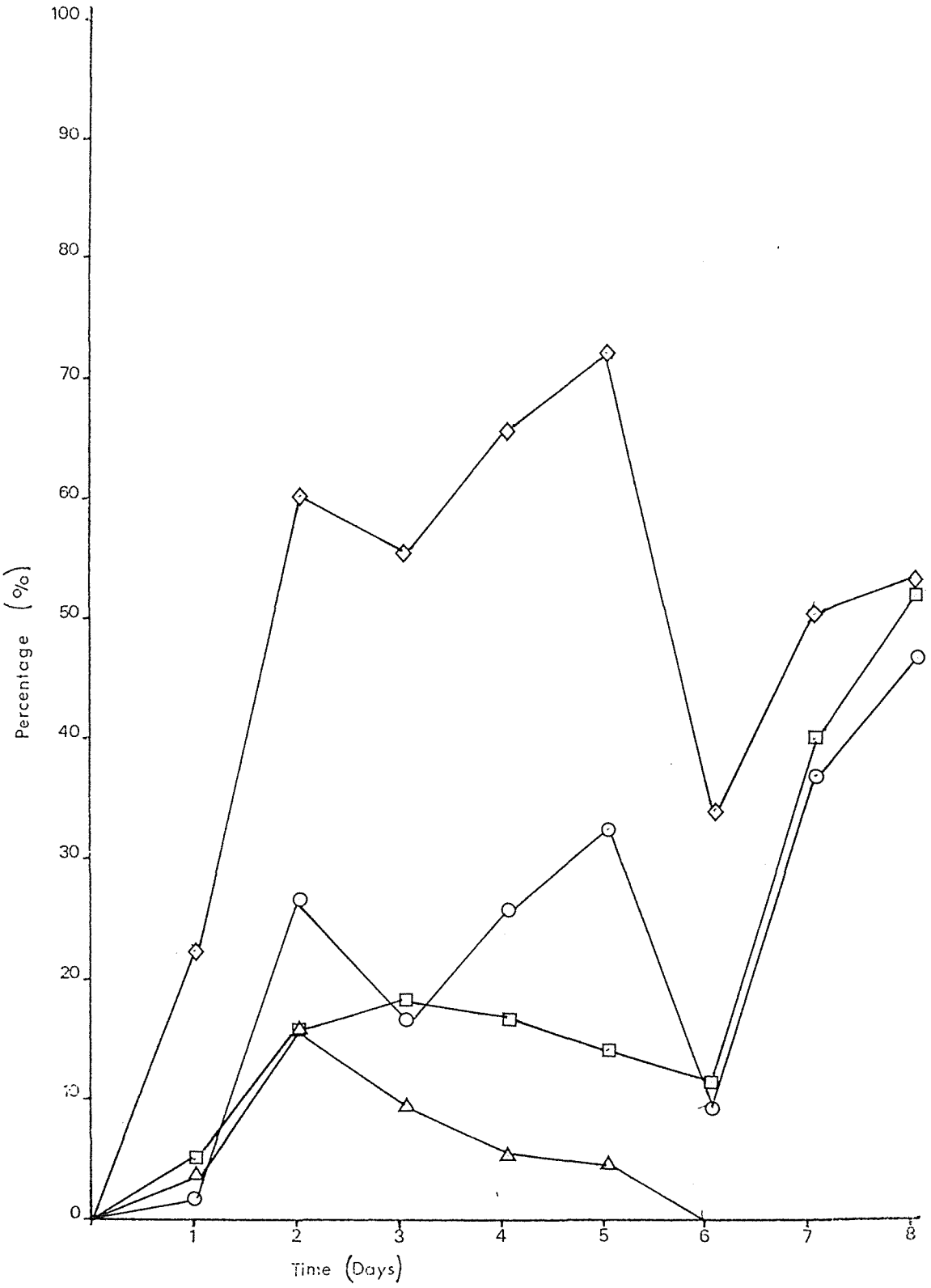


Figure 23c. The effect of Mg^{+2} concentration in water on encystment of *A. culbertsoni*. Round form levels.

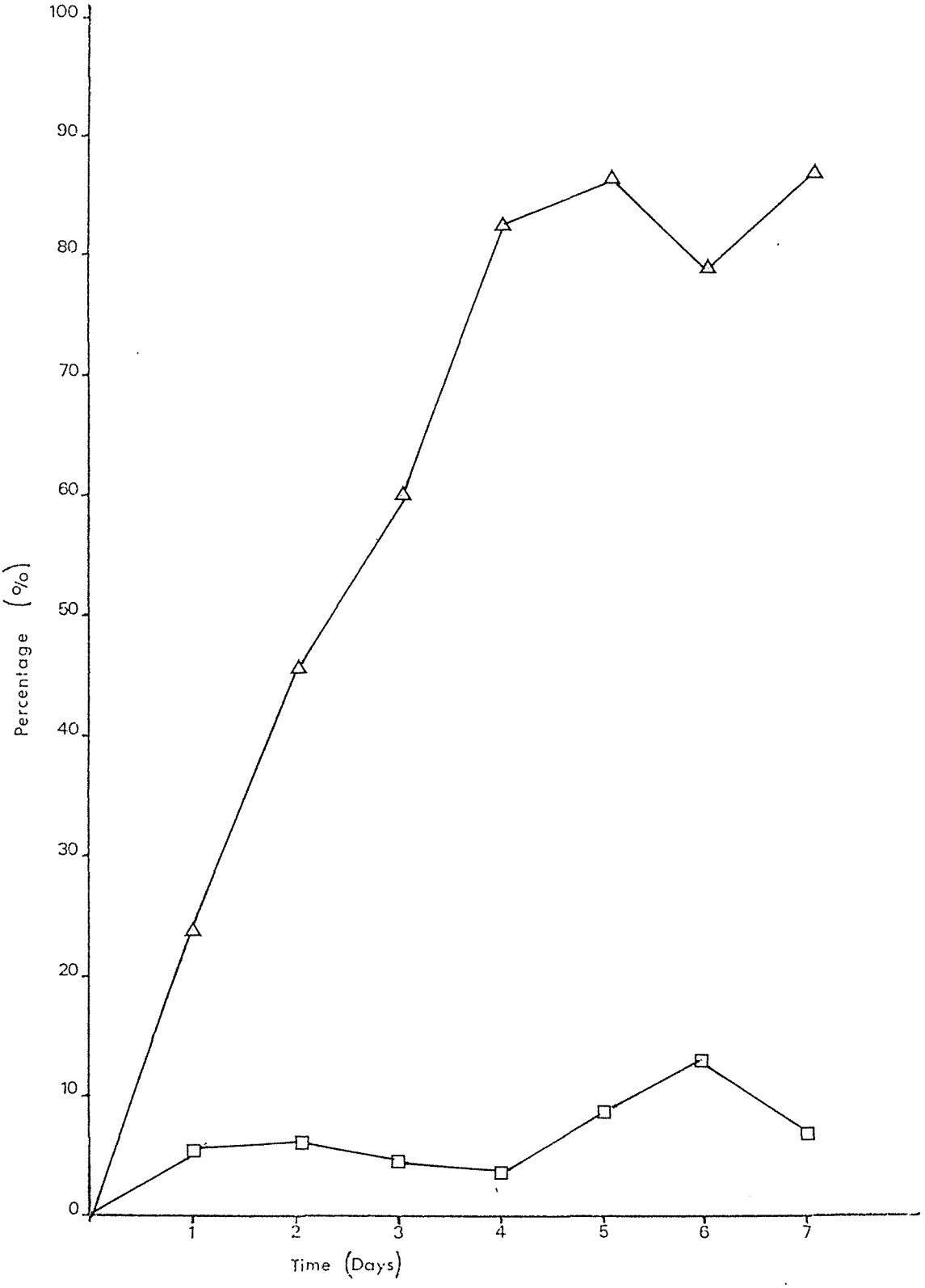


Figure 24a. The effect of Mg^{+2} concentration in PAS on encystment of *A. culbertsoni*. Encystment levels.

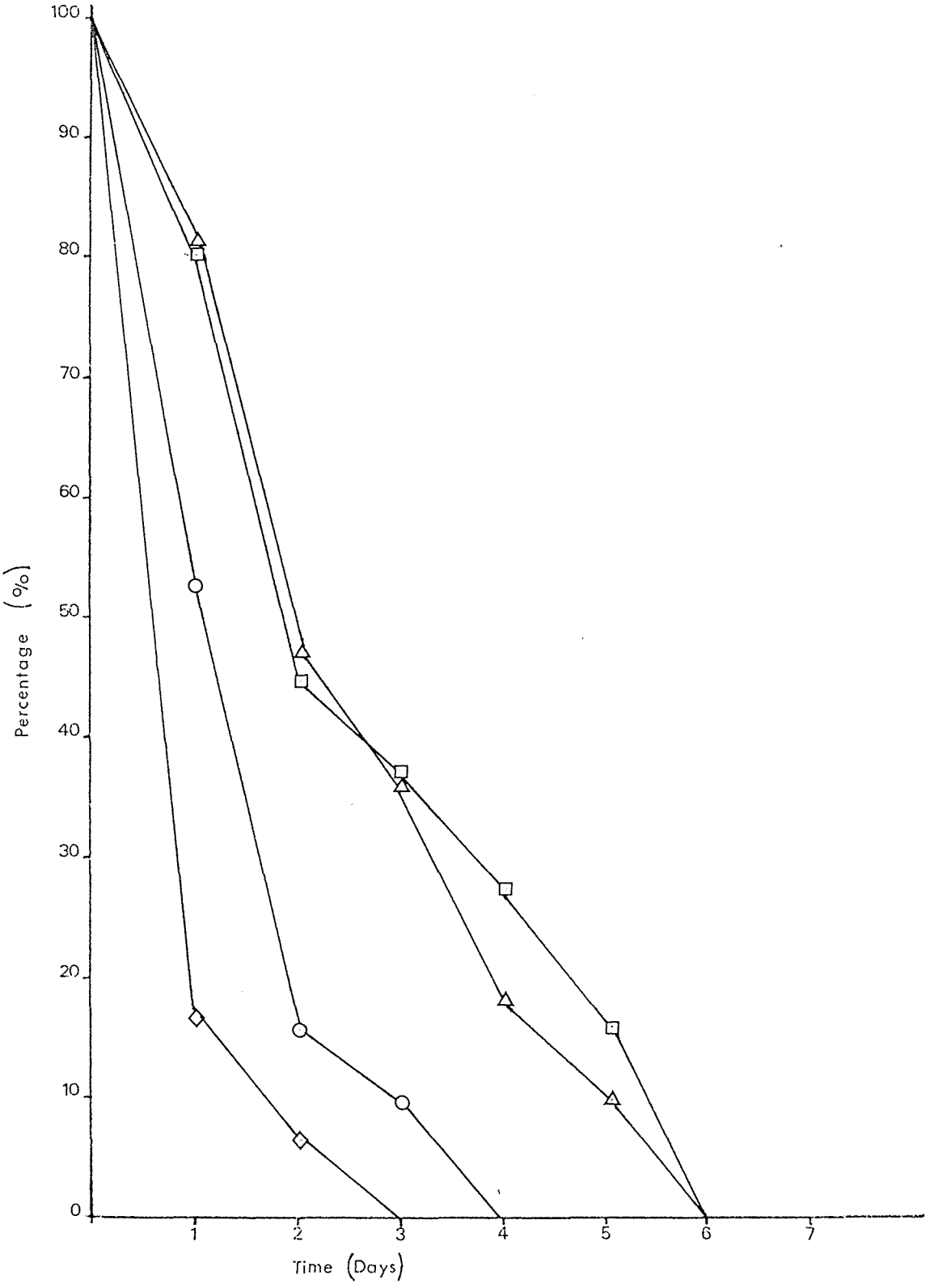


Figure 24b. The effect of Mg^{+2} concentration in PAS on encystment of *A. culbertsoni*. Trophozoite levels.

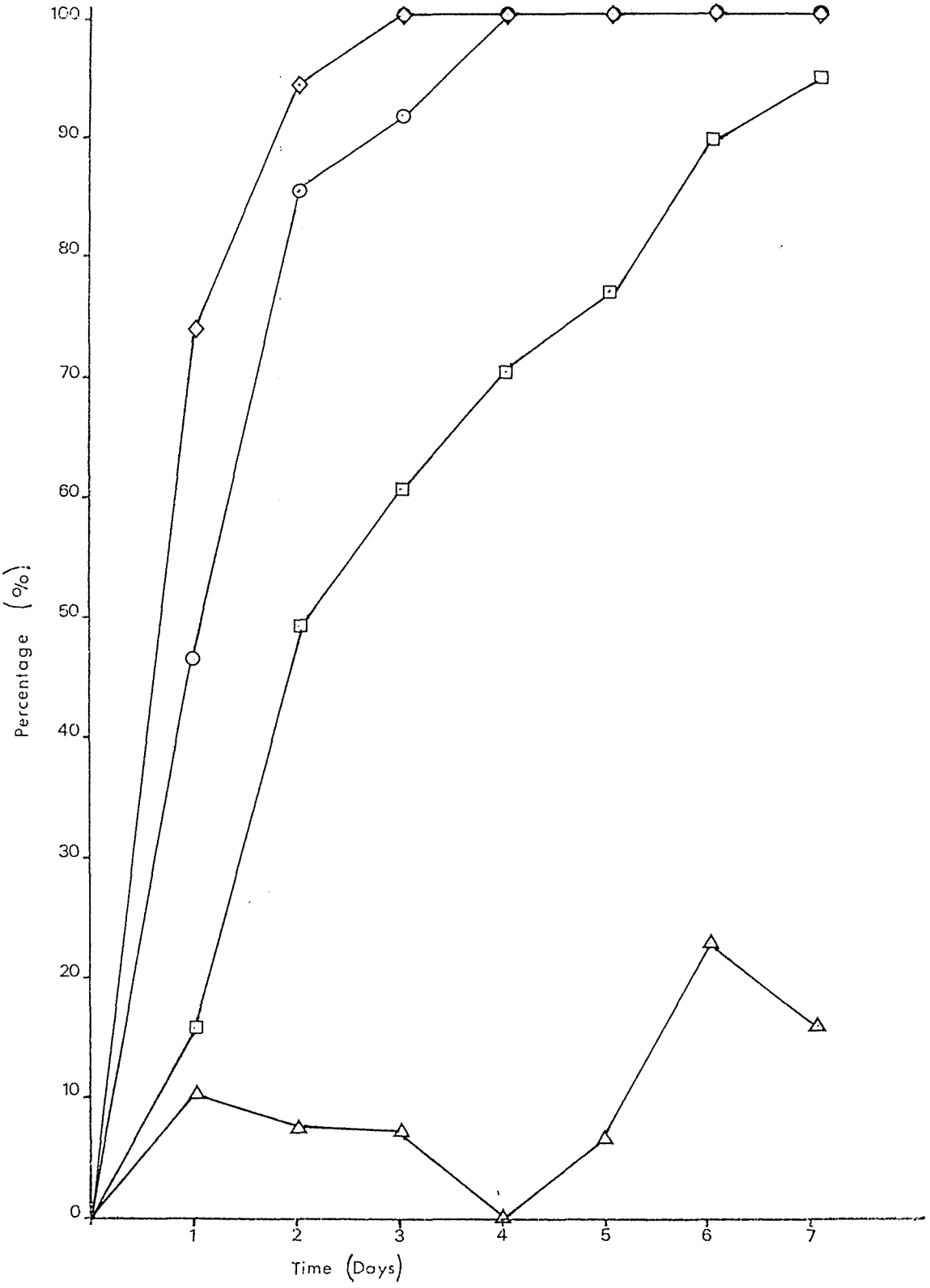


Figure 24c. The effect of Mg^{+2} concentration in PAS on encystment of *A. culbertsoni*. Round form levels.

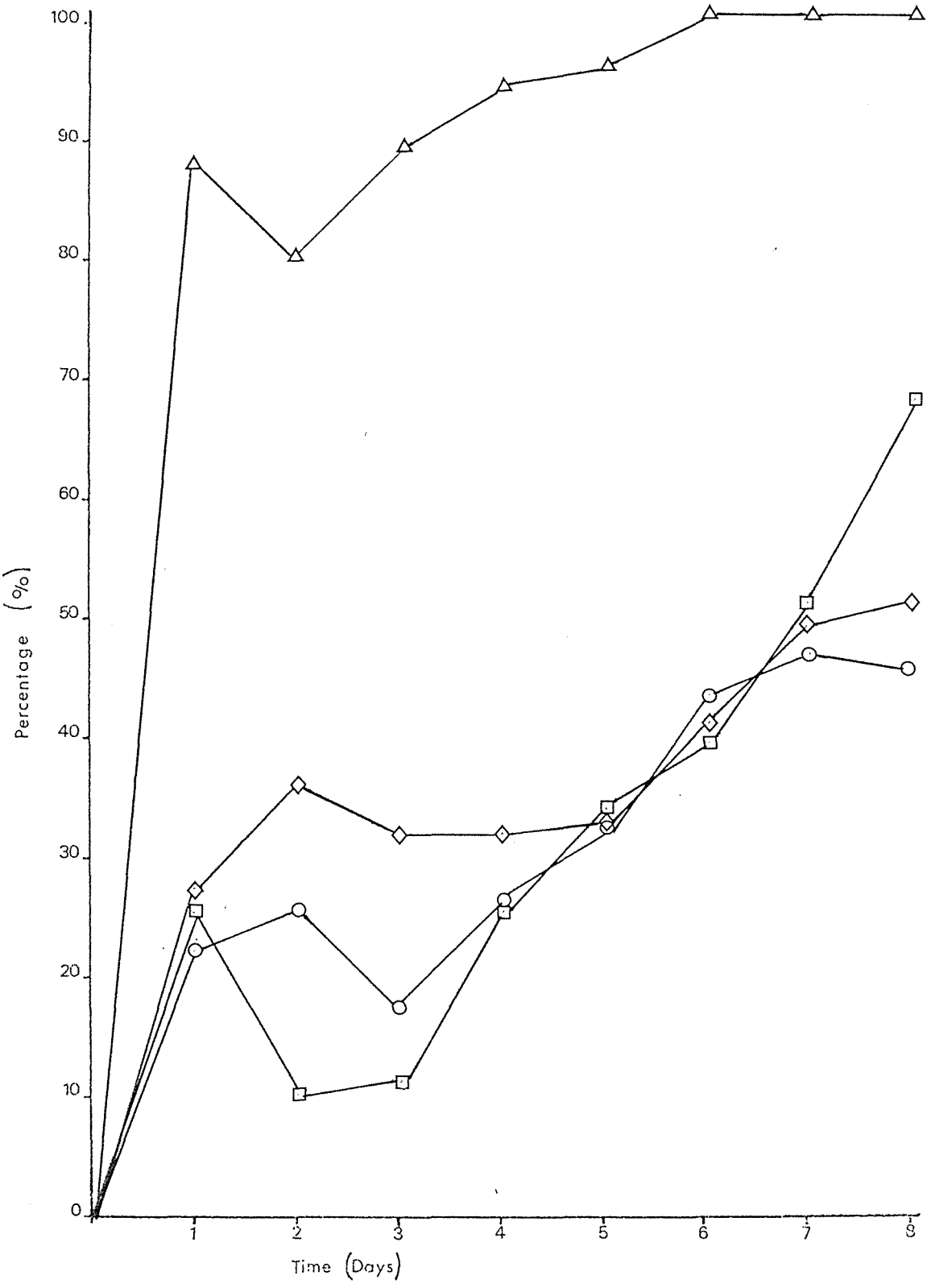


Figure 25a. The effect of Ca^{+2} concentration in water on encystment of *A. culbertsoni*. Encystment levels.

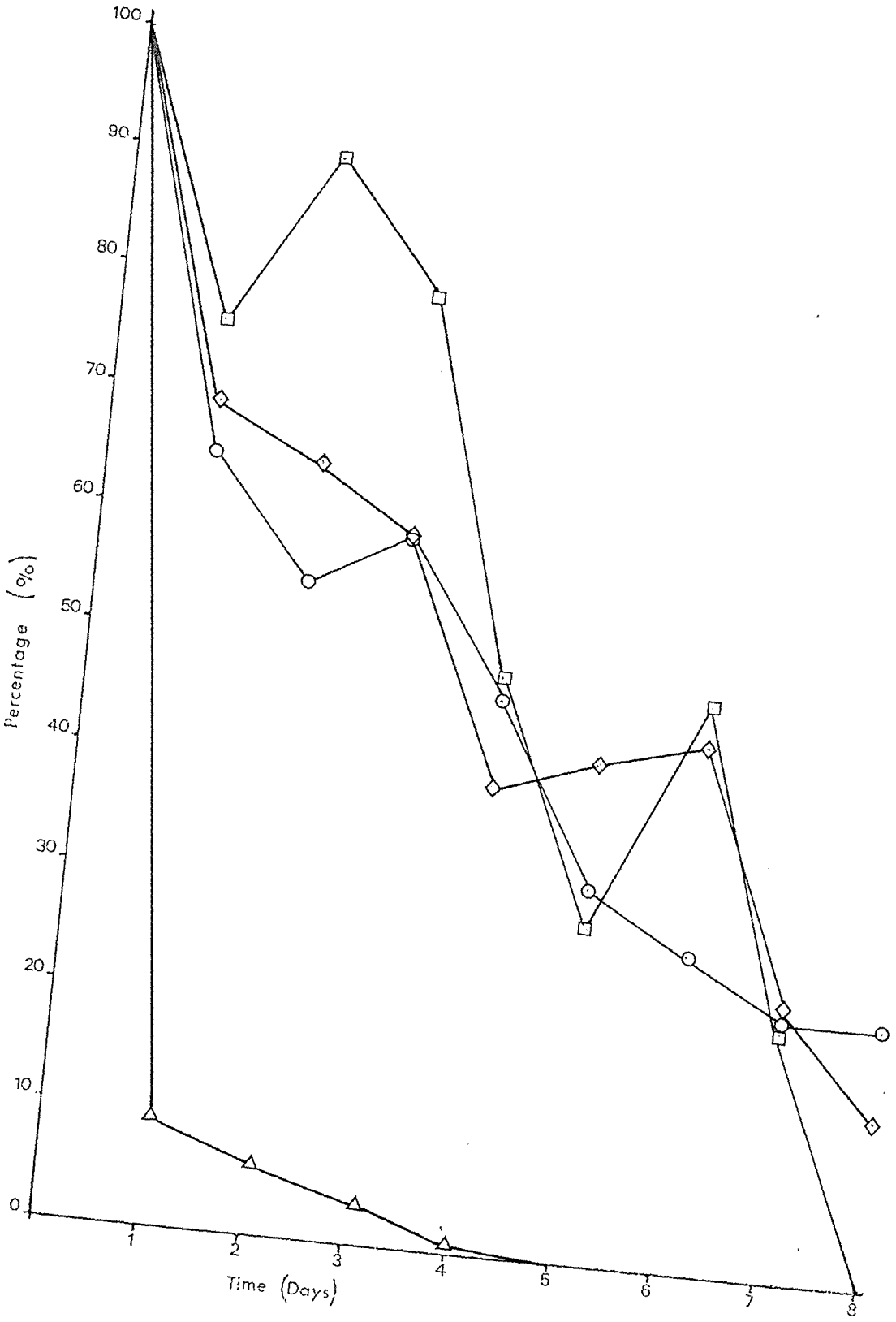


Figure 25b. The effect of Ca^{+2} concentration in water on encystment of *A. culbertsoni*. Trophozoite levels.

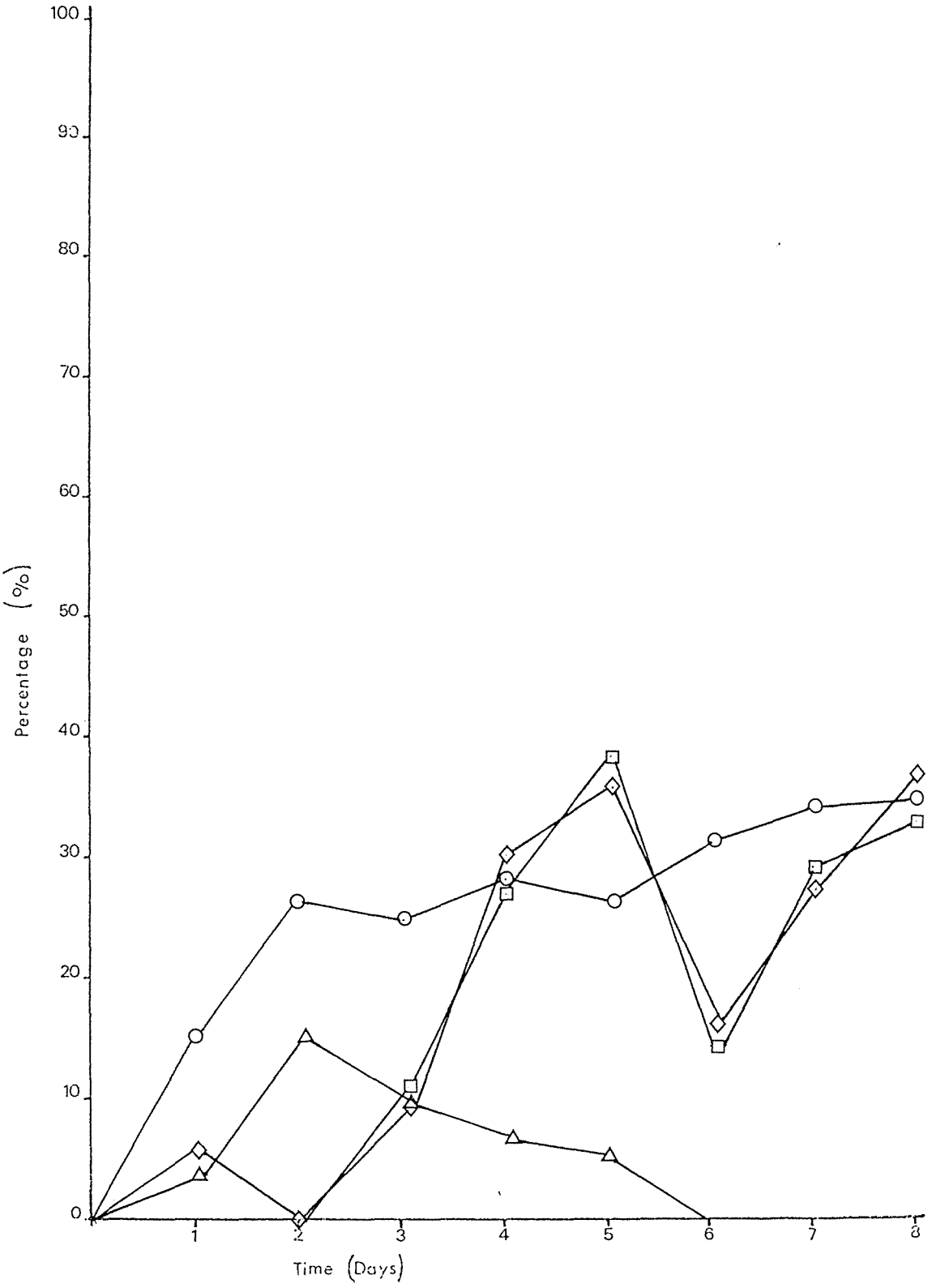


Figure 25c. The effect of Ca^{+2} concentration in water on encystment of *A. culbertsoni*. Round form levels.

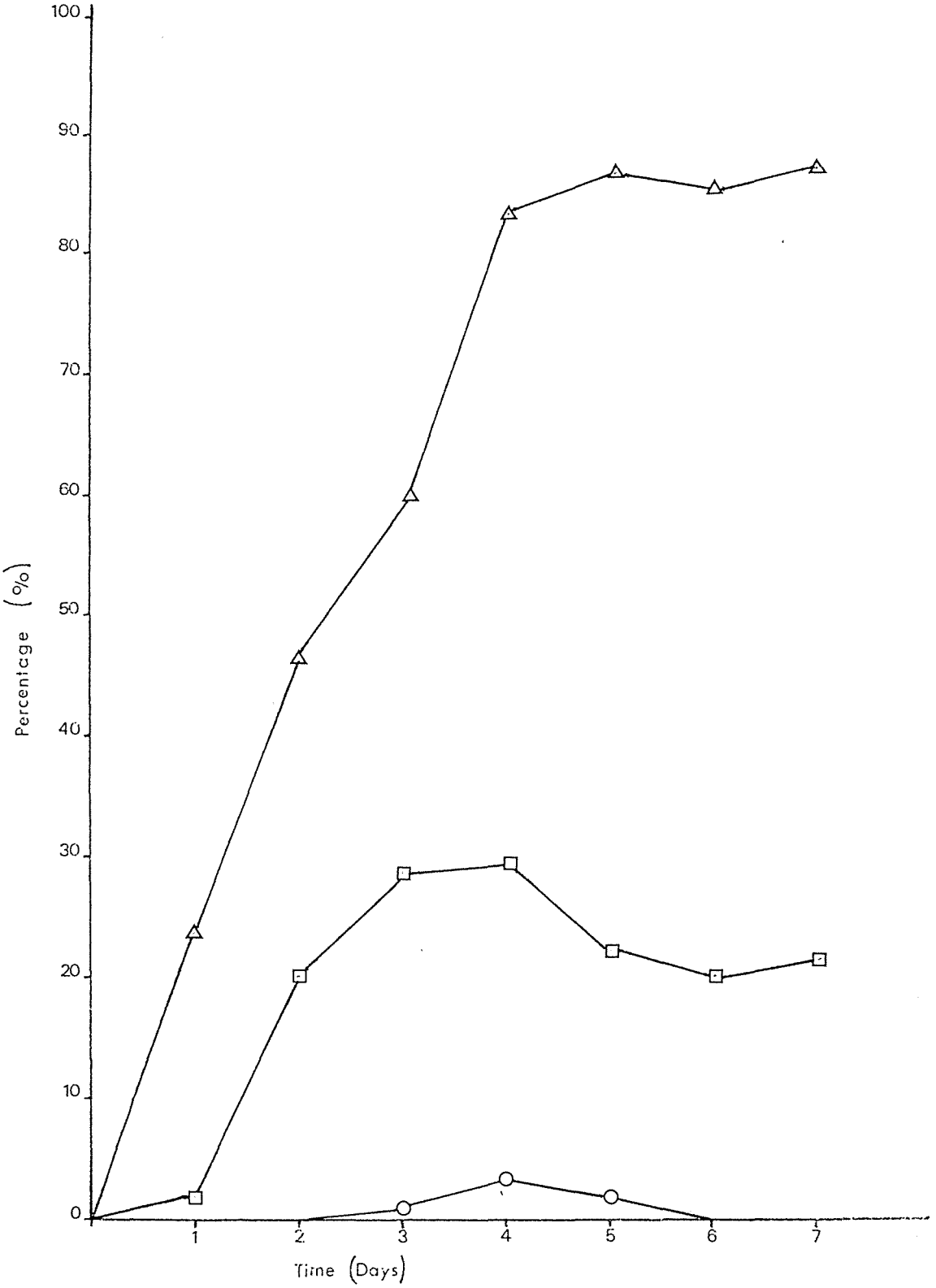


Figure 26a. The effect of Ca^{+2} concentration in PAS on encystment of *A. culbertsoni*. Encystment levels.

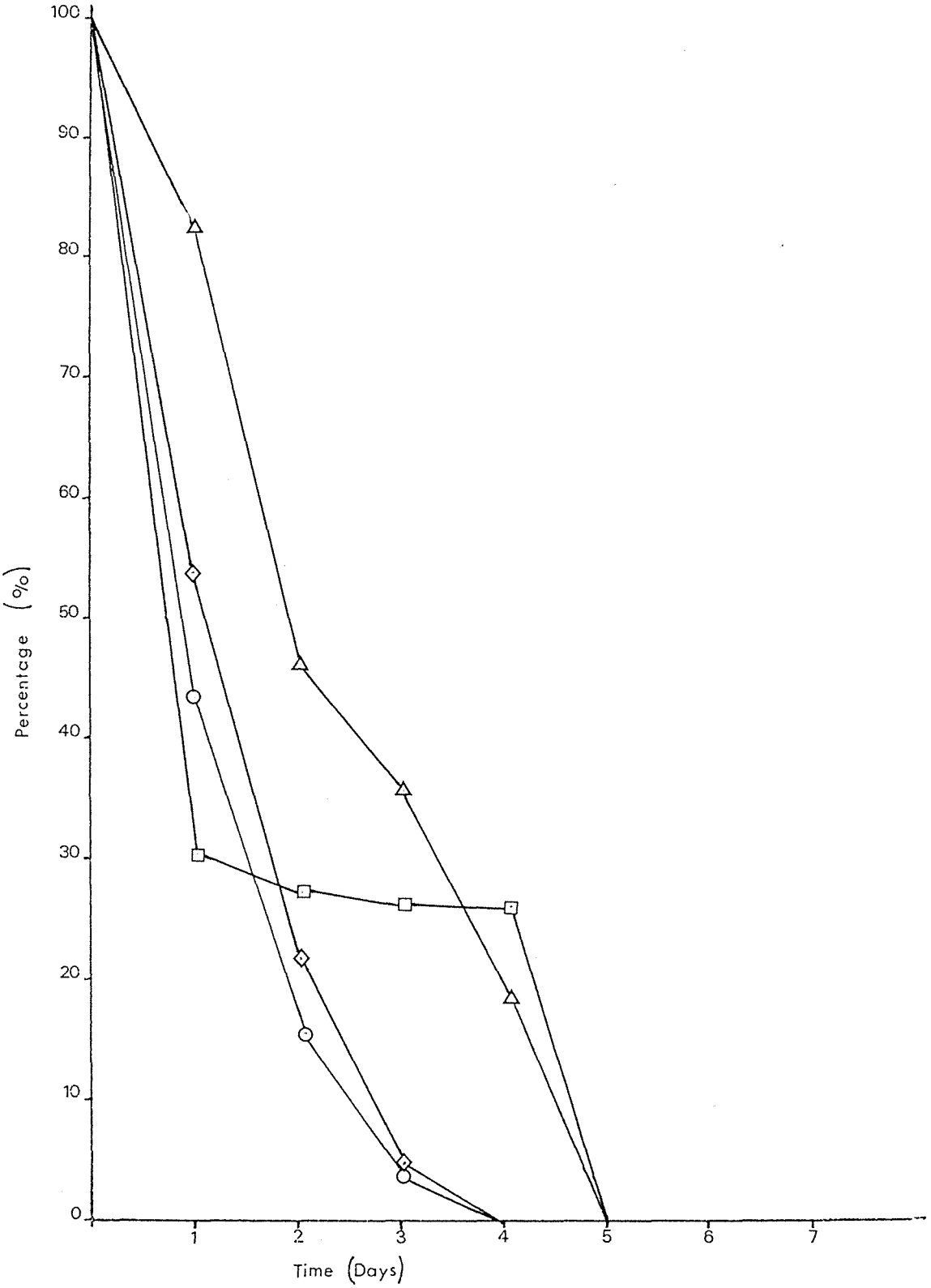


Figure 26b. The effect of Ca^{+2} concentration in PAS on encystment of *A. culbertsoni*. Trophozoite levels.

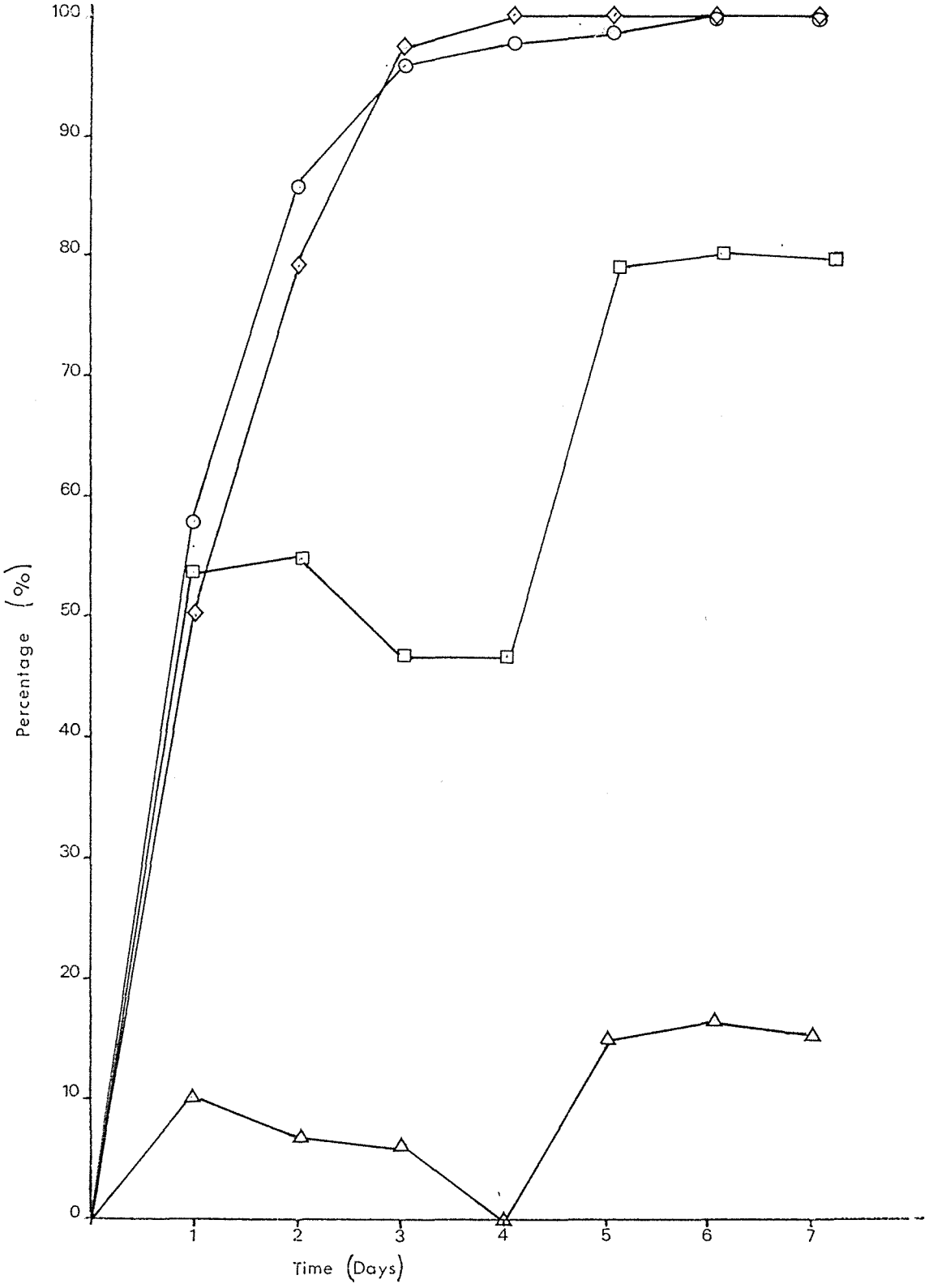


Figure 26c. The effect of Ca^{+2} concentration in PAS on encystment of *A. culbertsoni*. Round form levels.

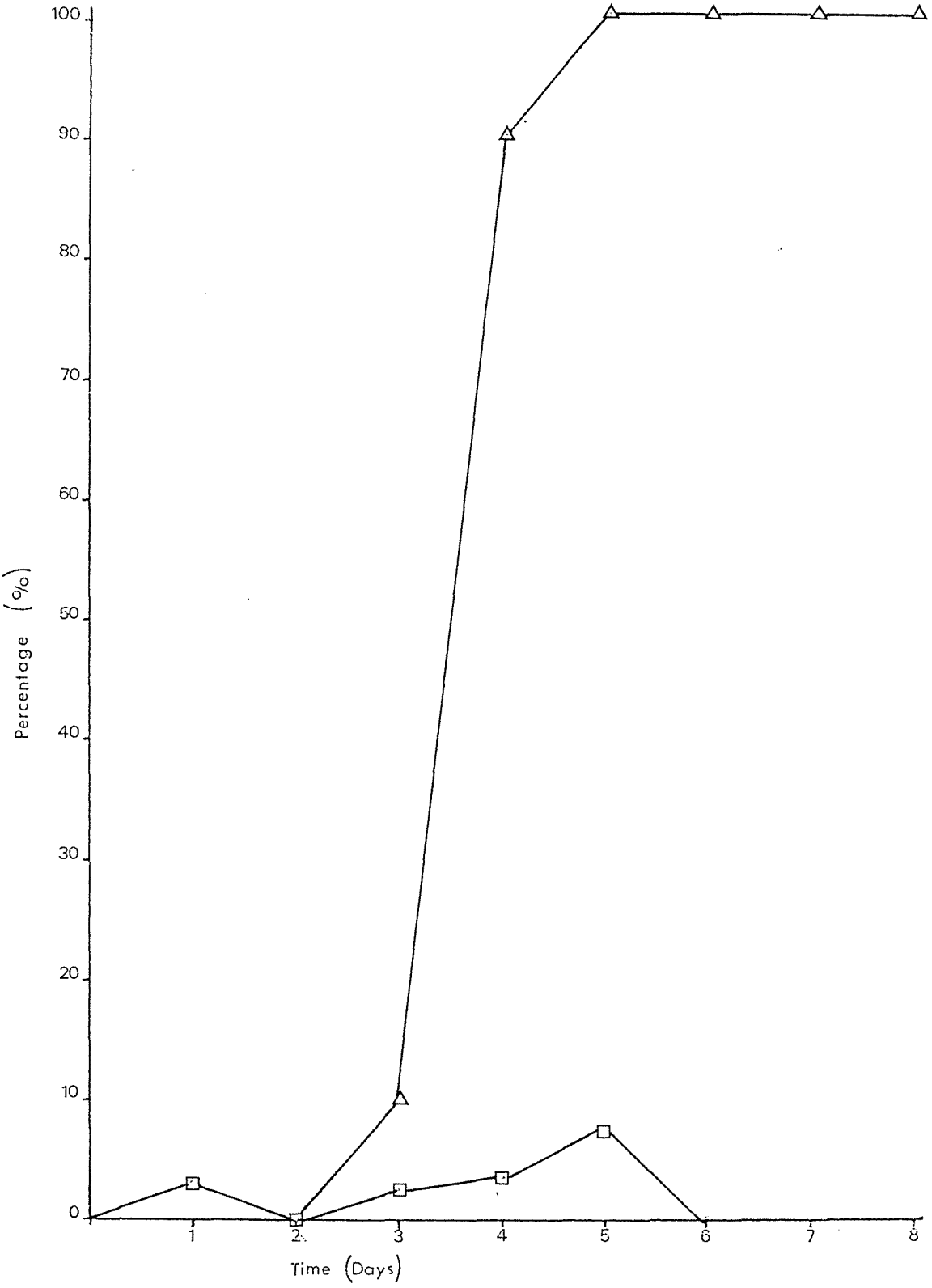


Figure 27a. The effect of Mg^{+2} concentration in water on encystment of *A. castellanii*. Encystment levels.

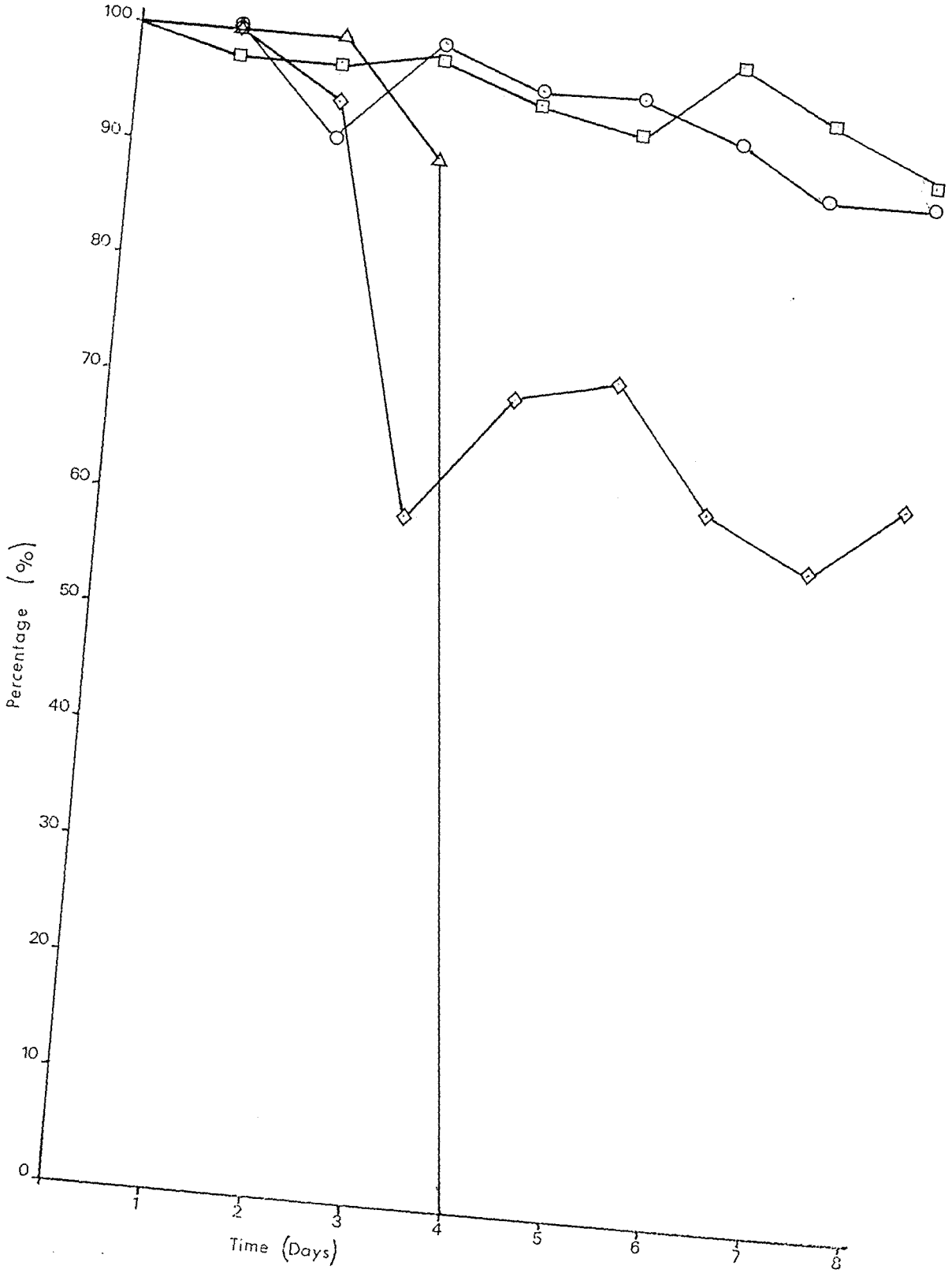


Figure 27b. The effect of Mg^{+2} concentration in water on encystment of *A. castellanii*. Trophozoite levels.

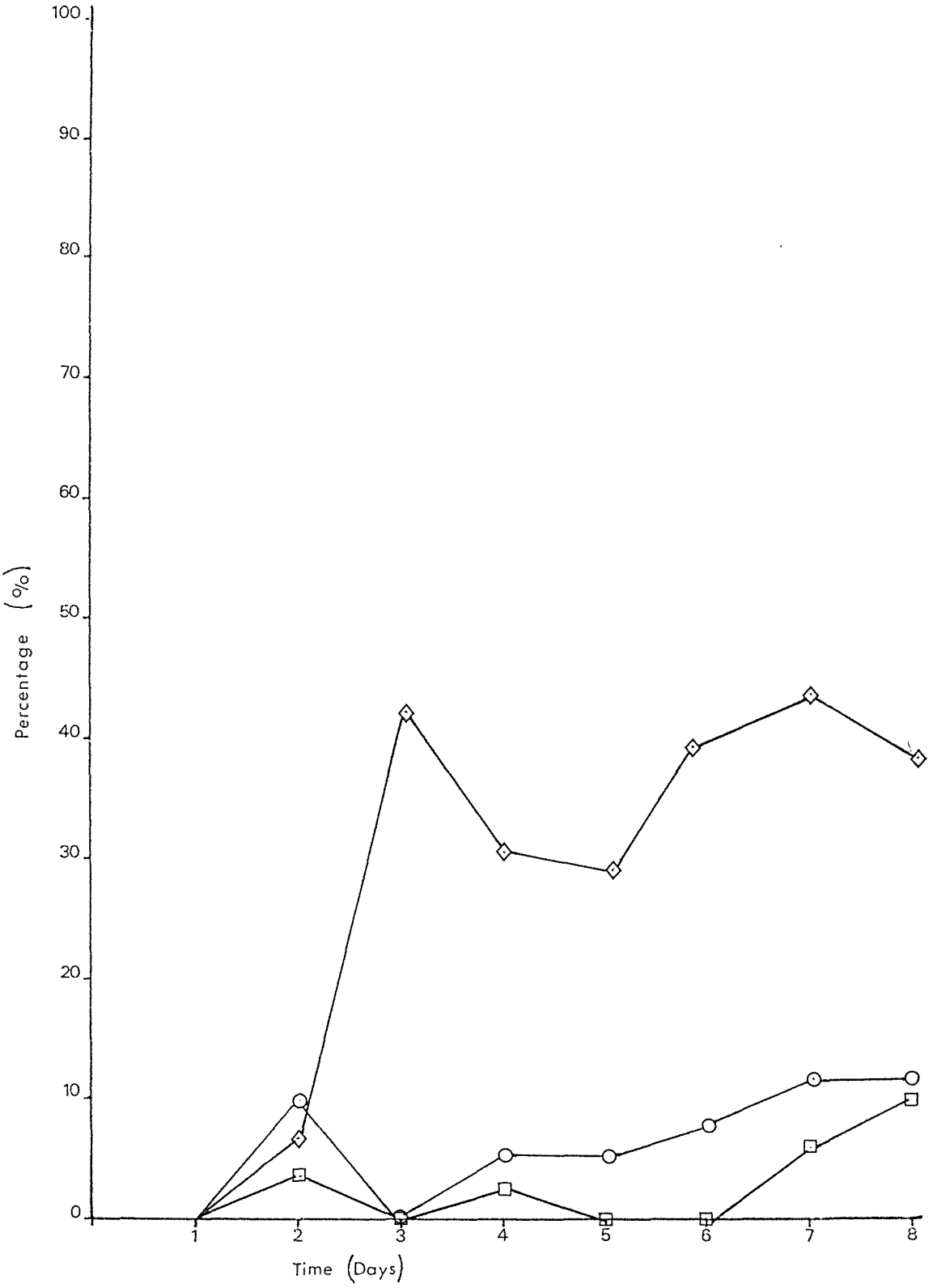


Figure 27c. The effect of Mg^{+2} concentration in water on encystment of *A. castellanii*. Round form levels.

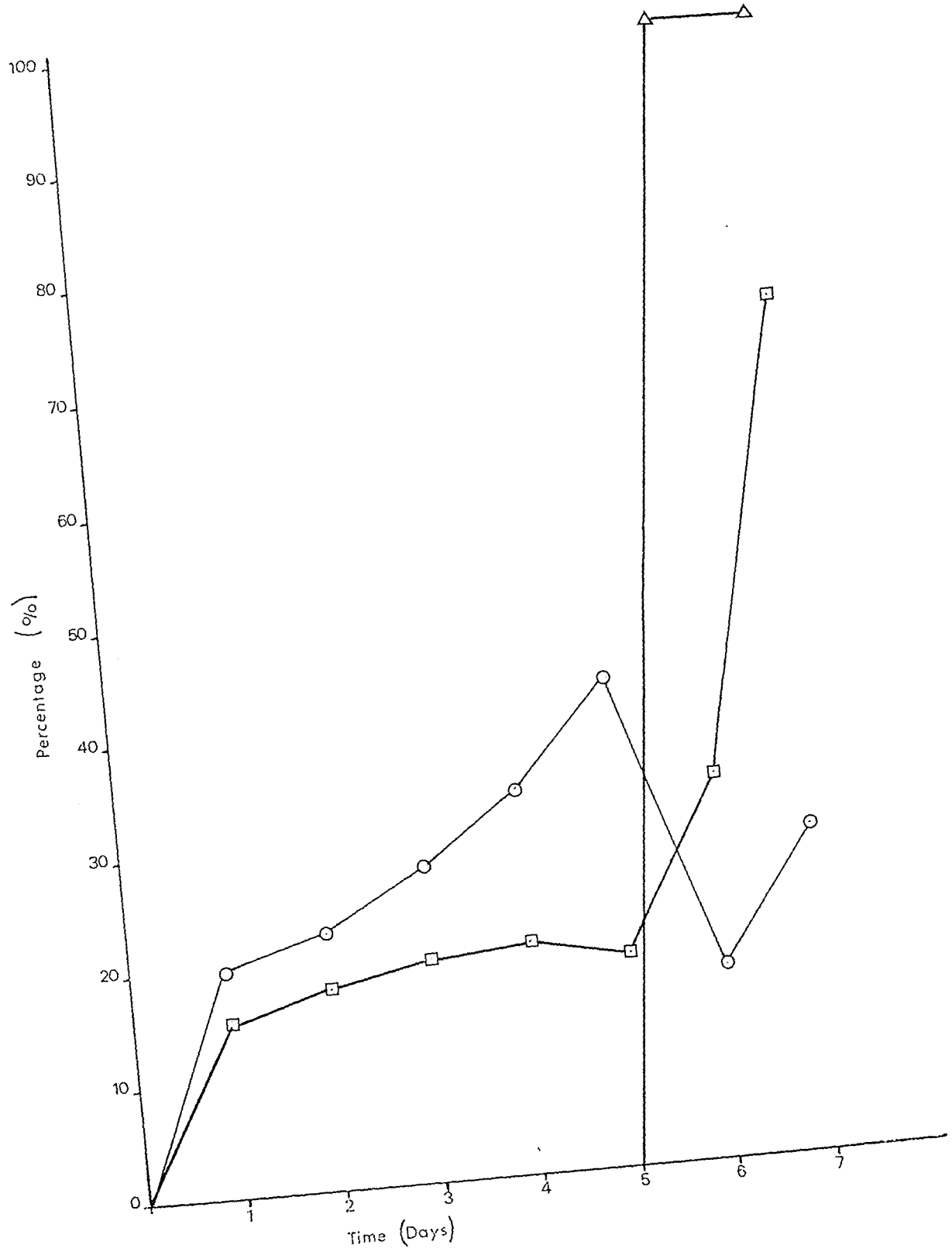


Figure 28a. The effect of Mg^{+2} concentration in PAS on encystment of *A. castellanii*. Encystment levels.

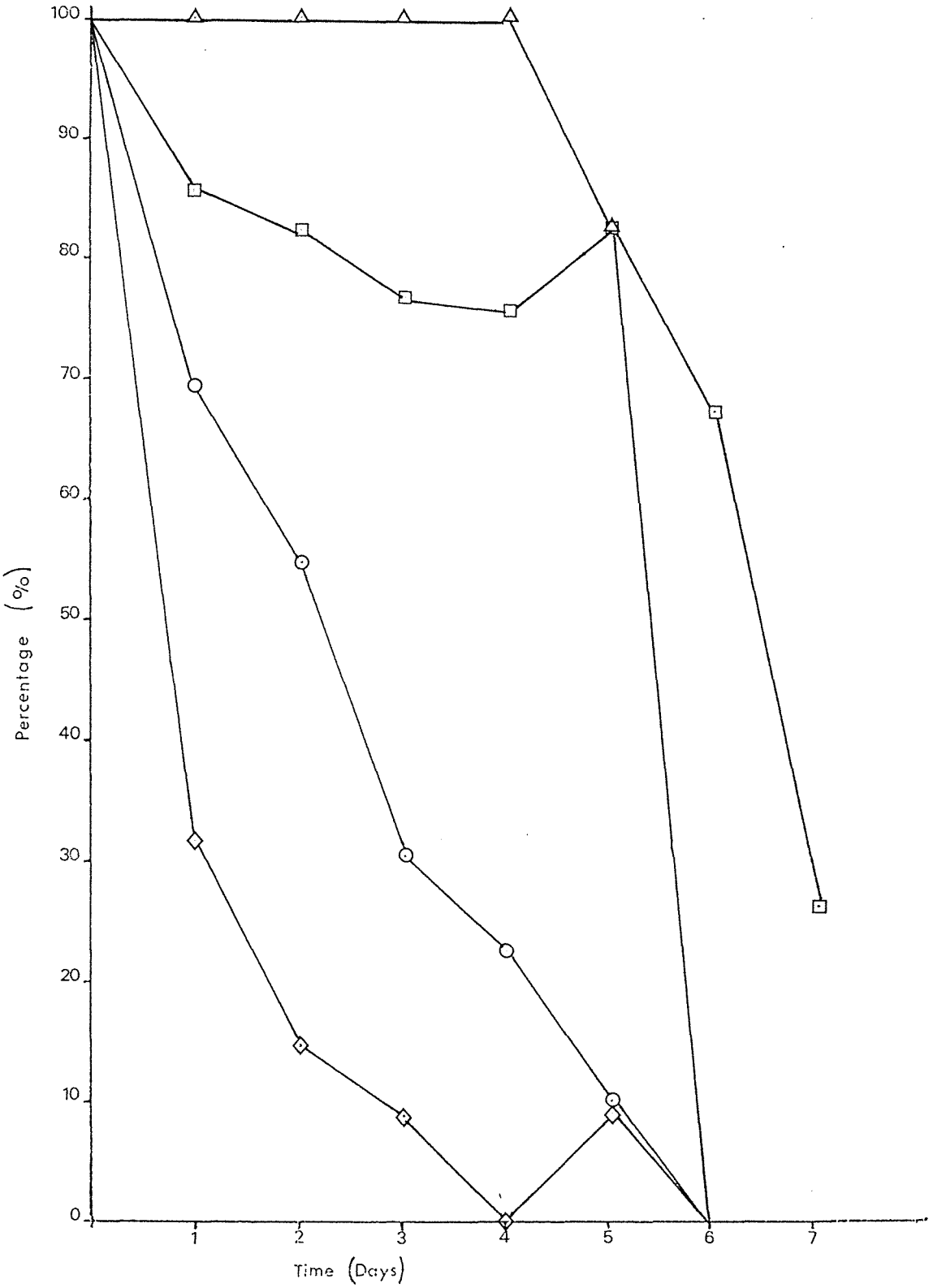


Figure 28b. The effect of Mg^{+2} concentration in PAS on encystment of *A. castellanii*. Trophozoite levels.

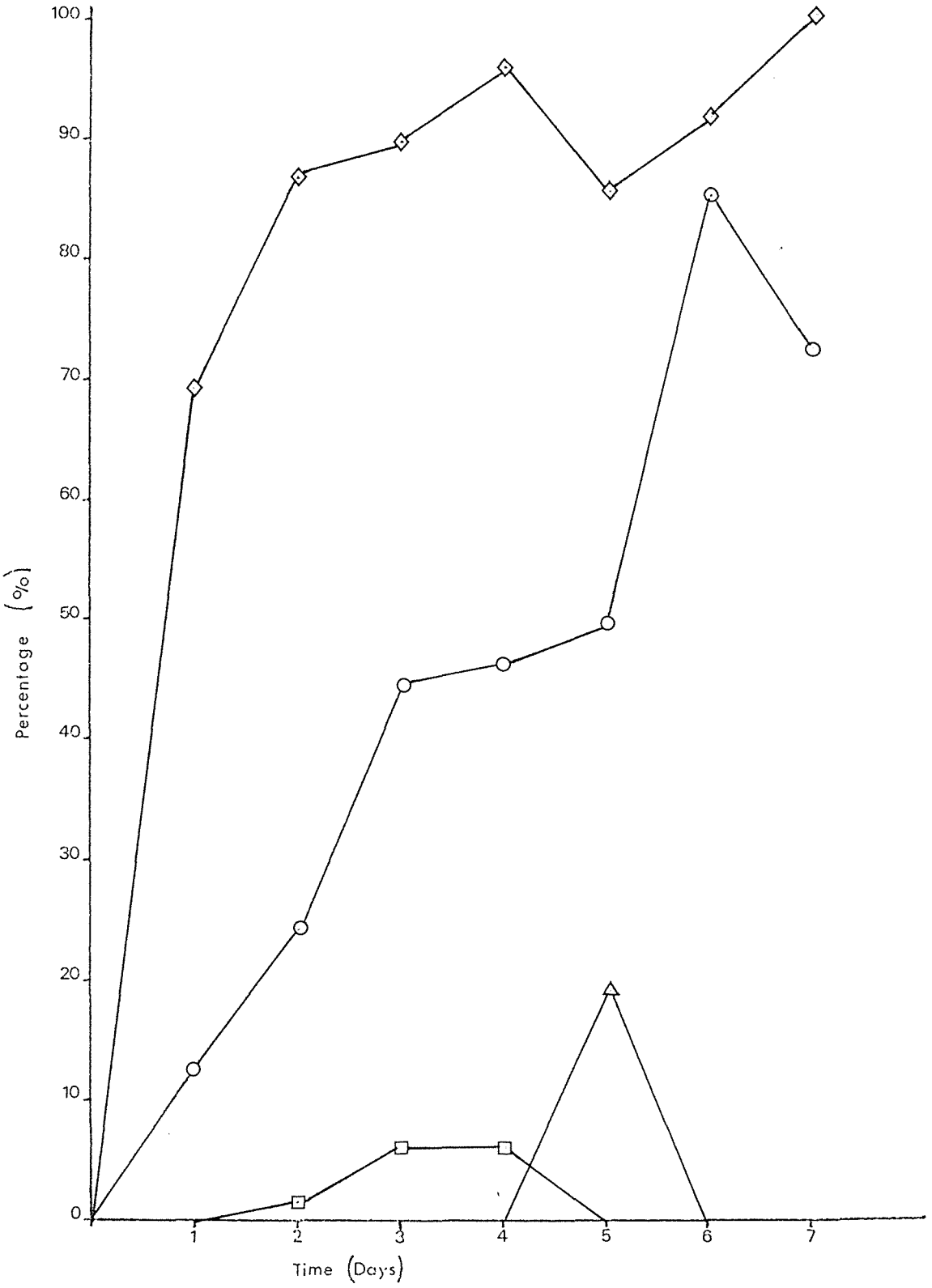


Figure 28c. The effect of Mg^{+2} concentration in PAS on encystment of *A. castellanii*. Round form levels.

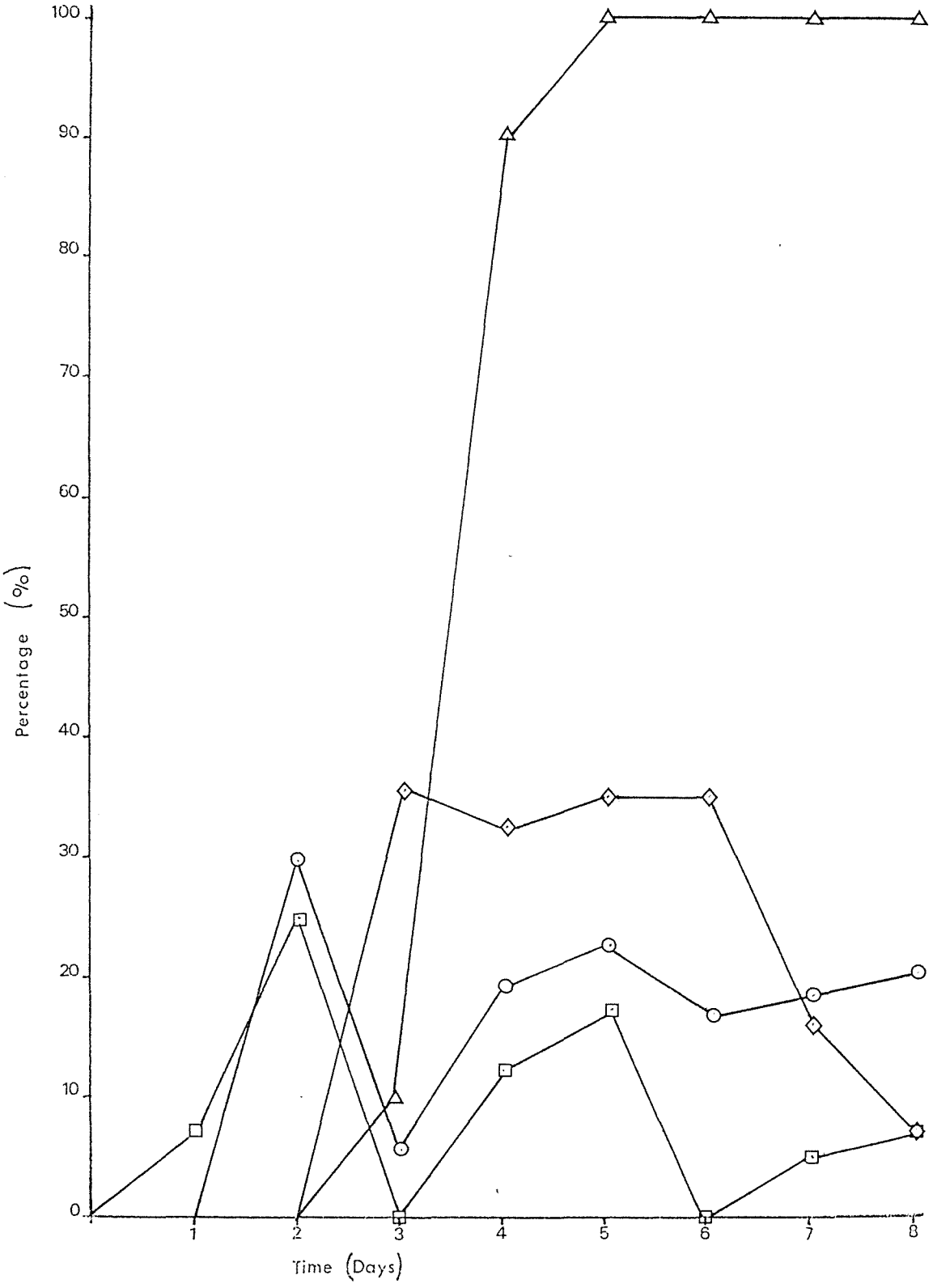


Figure 29a. The effect of Ca^{+2} concentration in water on encystment of *A. castellanii*. Encystment levels.

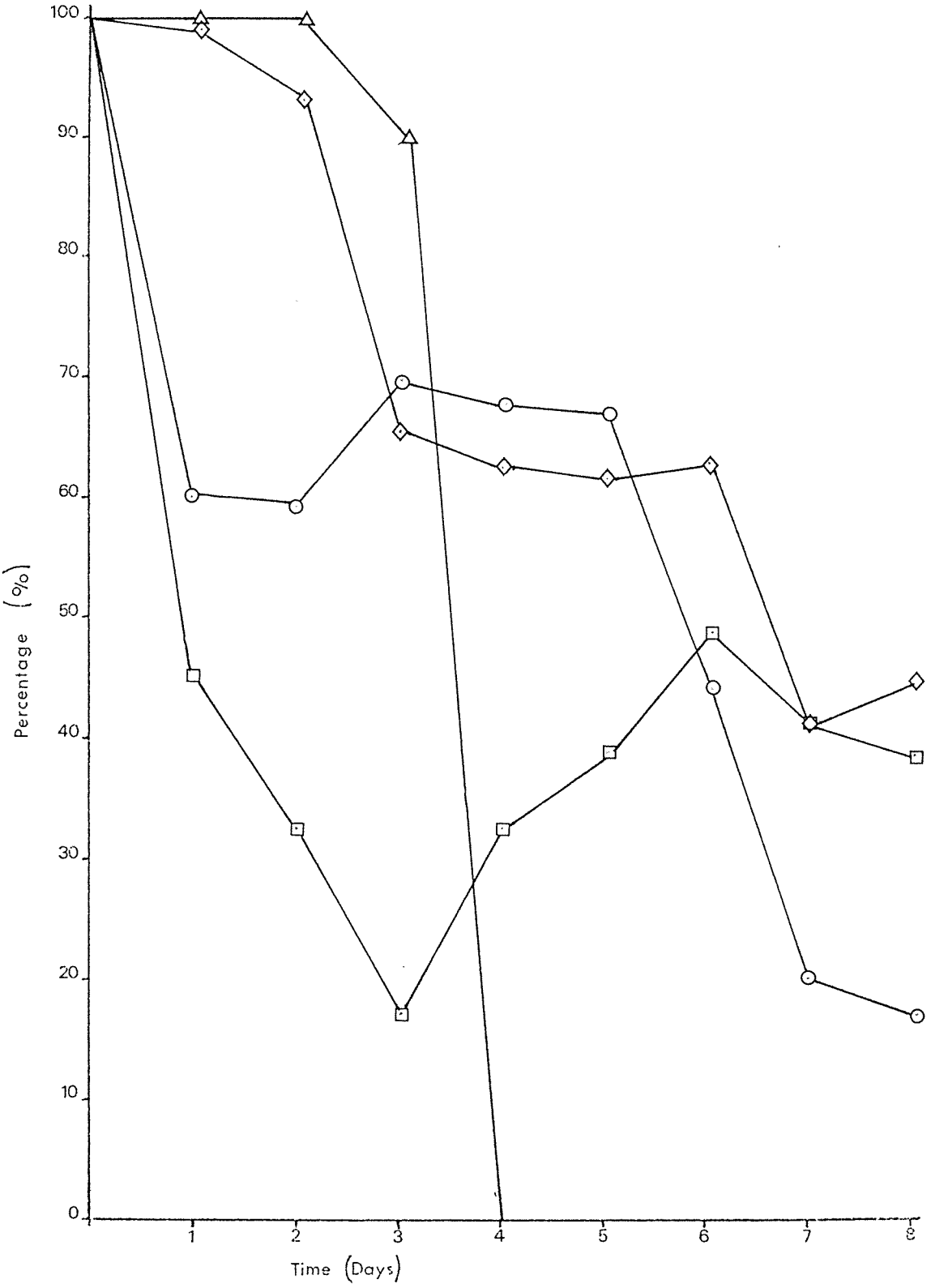


Figure 29b. The effect of Ca^{+2} concentration in water on encystment of *A. castellanii*. Trophozoite levels.

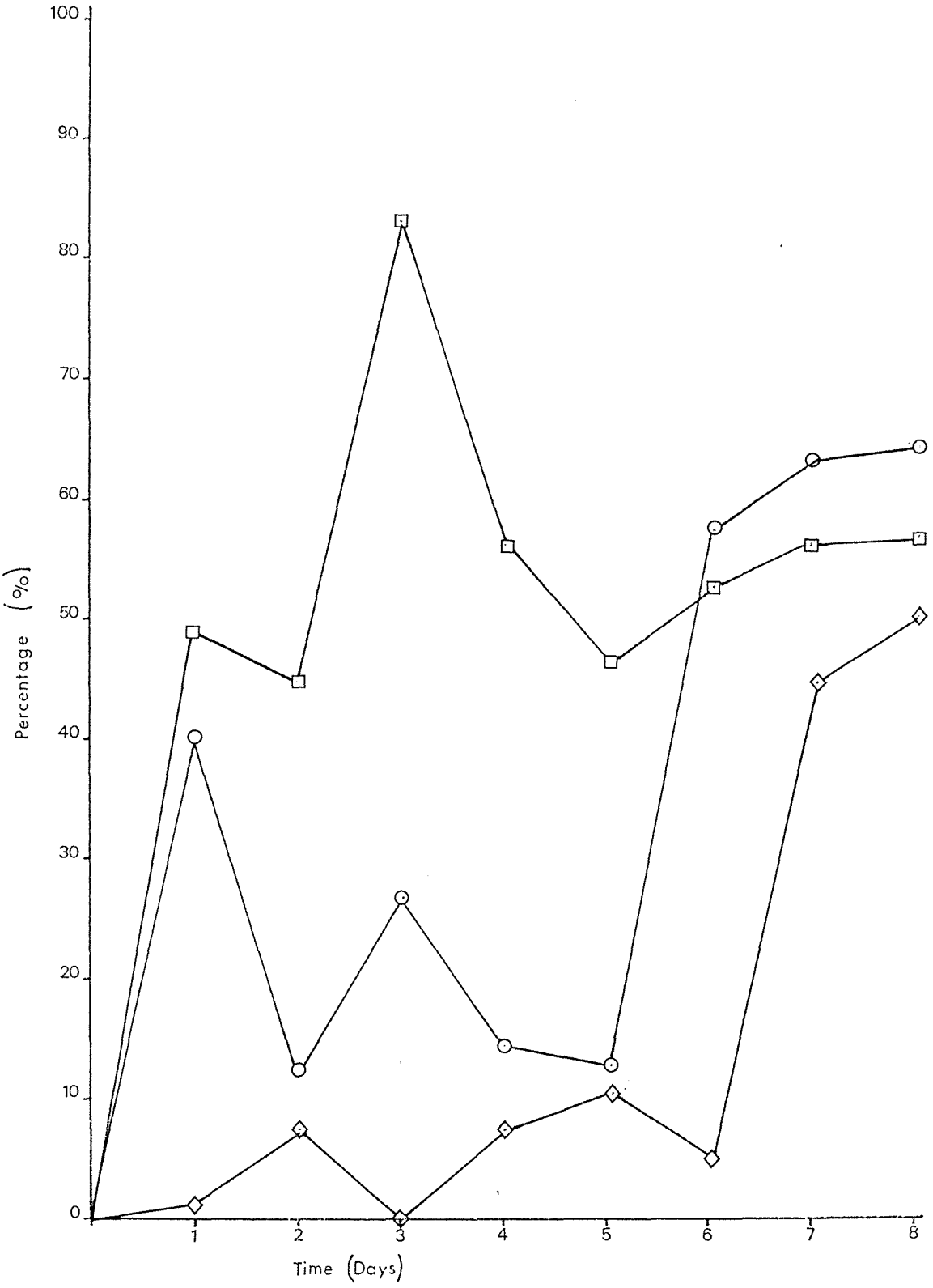


Figure 29c. The effect of Ca^{+2} concentration in water on encystment of *A. castellanii*. Round form levels.

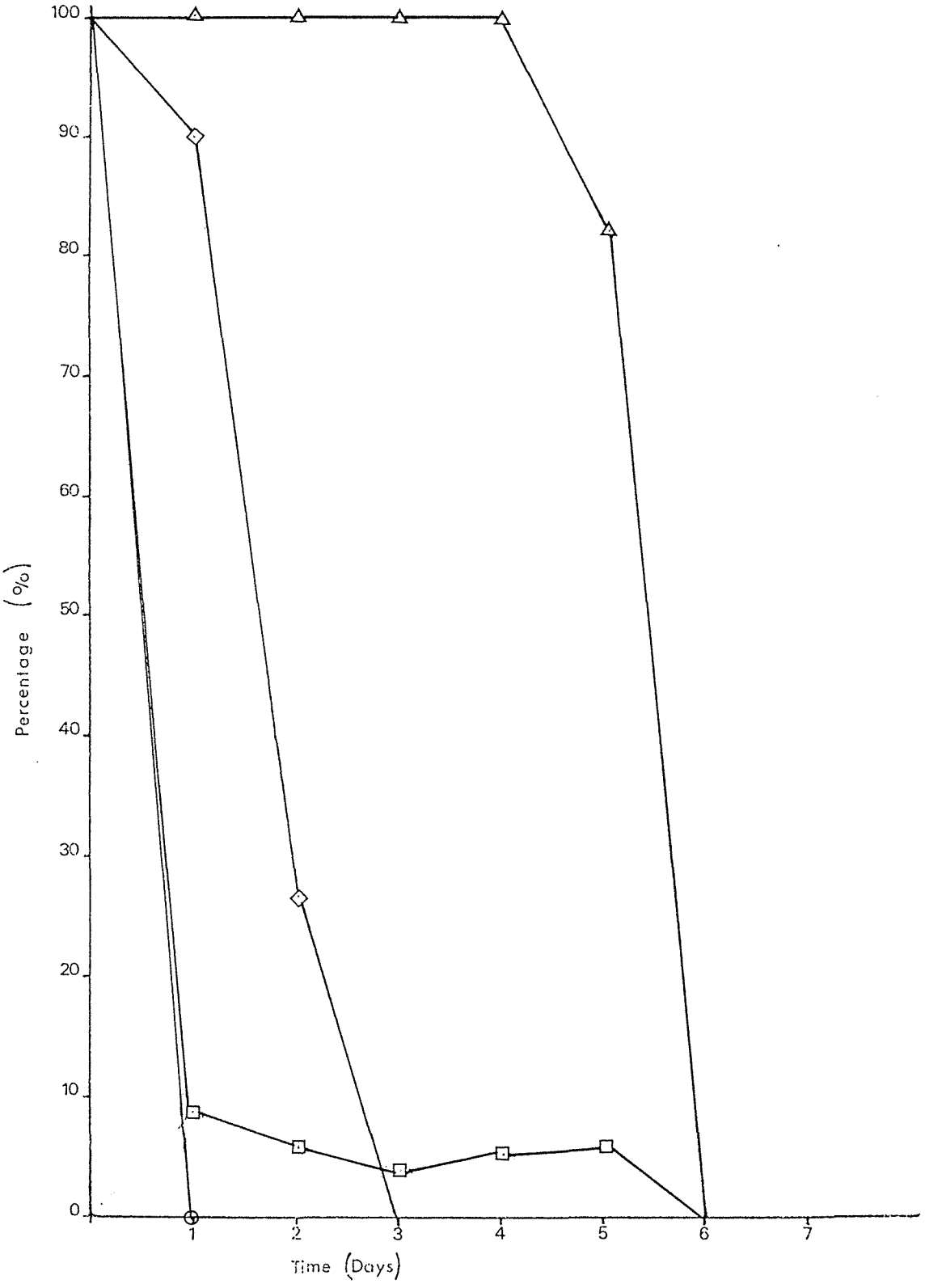


Figure 30b. The effect of Ca^{+2} concentration in PAS on encystment of *A. castellanii*. Trophozoite levels.

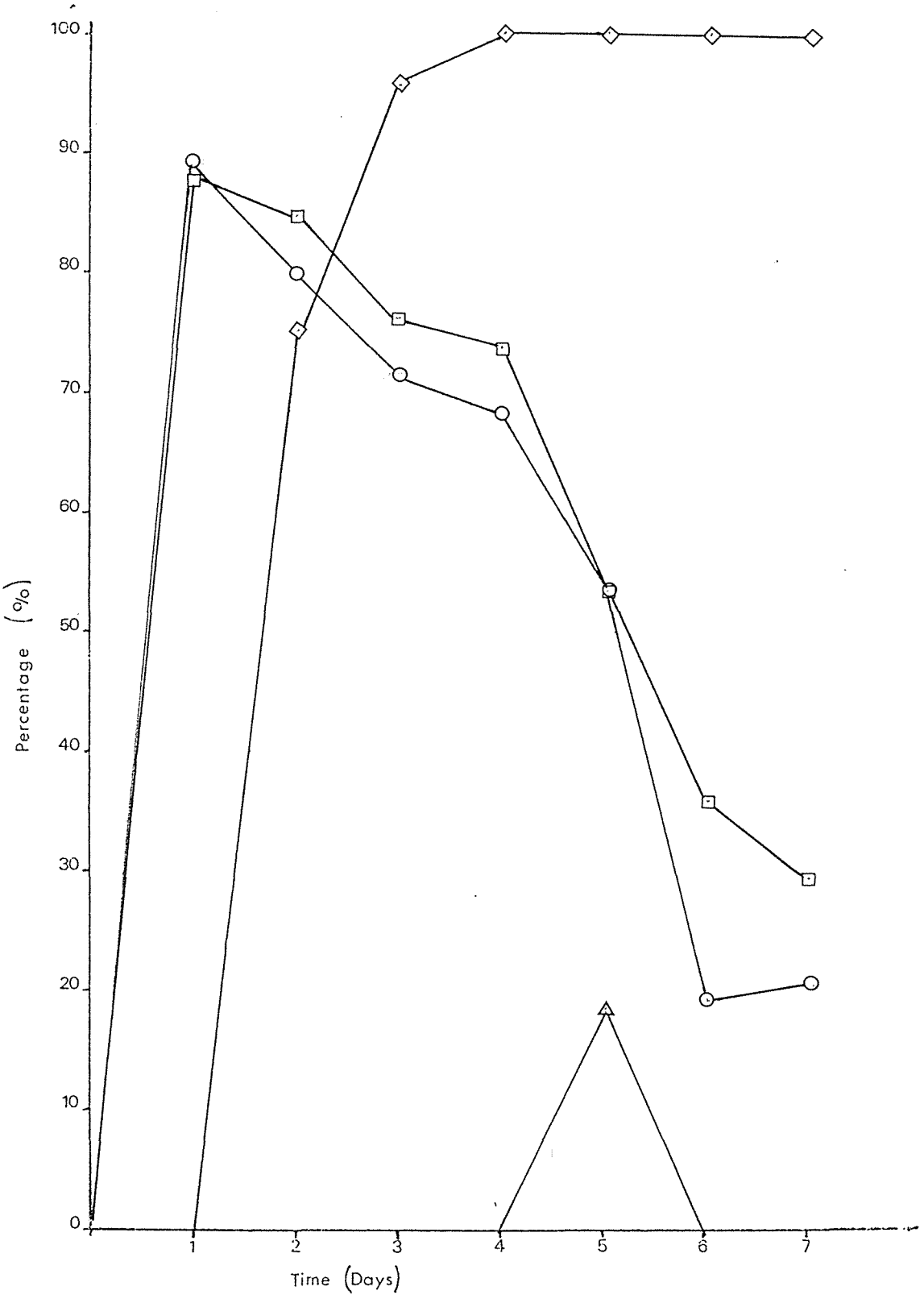


Figure 30c. The effect of Ca^{+2} concentration in PAS on encystment of *A. castellanii*. Round form levels.

Table 5. Chemical Analysis of Basal Media

	PAS	Neff	CYM	SEB
Ca	10	22	10	96
Mg	7	57	7	18
K	30	840	306	160
Na	98	1575	590	18
$Fe^{+2/+3}$	1	1	1	1

4.2.2 Encystment in soil extract broth

N. fowleri encysted to 100% after two days in both 100 and 50% SEB (Figures 31-32). Round forms only produced at 13% on the first day in 50%. *N. gruberi* produced a maximum of 2 and 3% encystment in 50 and 100% SEB (Figures 33-34). Round forms were produced reaching peaks of 76 and 43% after 6 and 5 days in 50 and 100% respectively. After 7 and 6 days respectively all amoebae had disappeared.

A. culbertsoni (A1) produced 100% encystment in 50% SEB after 6 days, but only reached a maximum of 84% in 100% SEB (Figures 35-36). Trophozoite levels in both dropped steadily and both round forms and cysts were produced. Round forms peaked at 39 and 50% in 50 and 100% respectively but dropped away as cyst numbers increased.

A. castellanii (01) showed fluctuating proportions of cysts and trophozoites (Figures 37-38). No round forms were produced. Encystment reached 100% after 7 days in 50% SEB, and a peak of 76% after 6 days in 100%.

Naegleria species responded similarly to 50 and 100% SEB, but 50% SEB produced higher levels of *Acanthamoeba* encystment than 100%.

SEB produced higher levels of encystment than water or PAS for both *Naegleria* species.

Encystment of A1 in 50% SEB reached 100% after 6 days, which is equal to A1 in pure water and better than PAS. 100% SEB did not produce as high encystment levels as water, but was approximately the same as PAS.

1501 reached 100% encystment after 5 days in pure water, 6 days in PAS and 7 days in 50% SEB. 100% SEB was not as effective as pure water, PAS or SEB (50%) at producing encystment but produced a similar level to 0.1 mM Mg^{+2} in PAS, 0.1 and 0.2 mM Ca^{+2} in PAS (approximately 75%). However, this level was attained earliest in SEB.

The following legend applies to Figures 31-54:

- ● Cysts
- ○ Round forms
- ■ Trophozoites

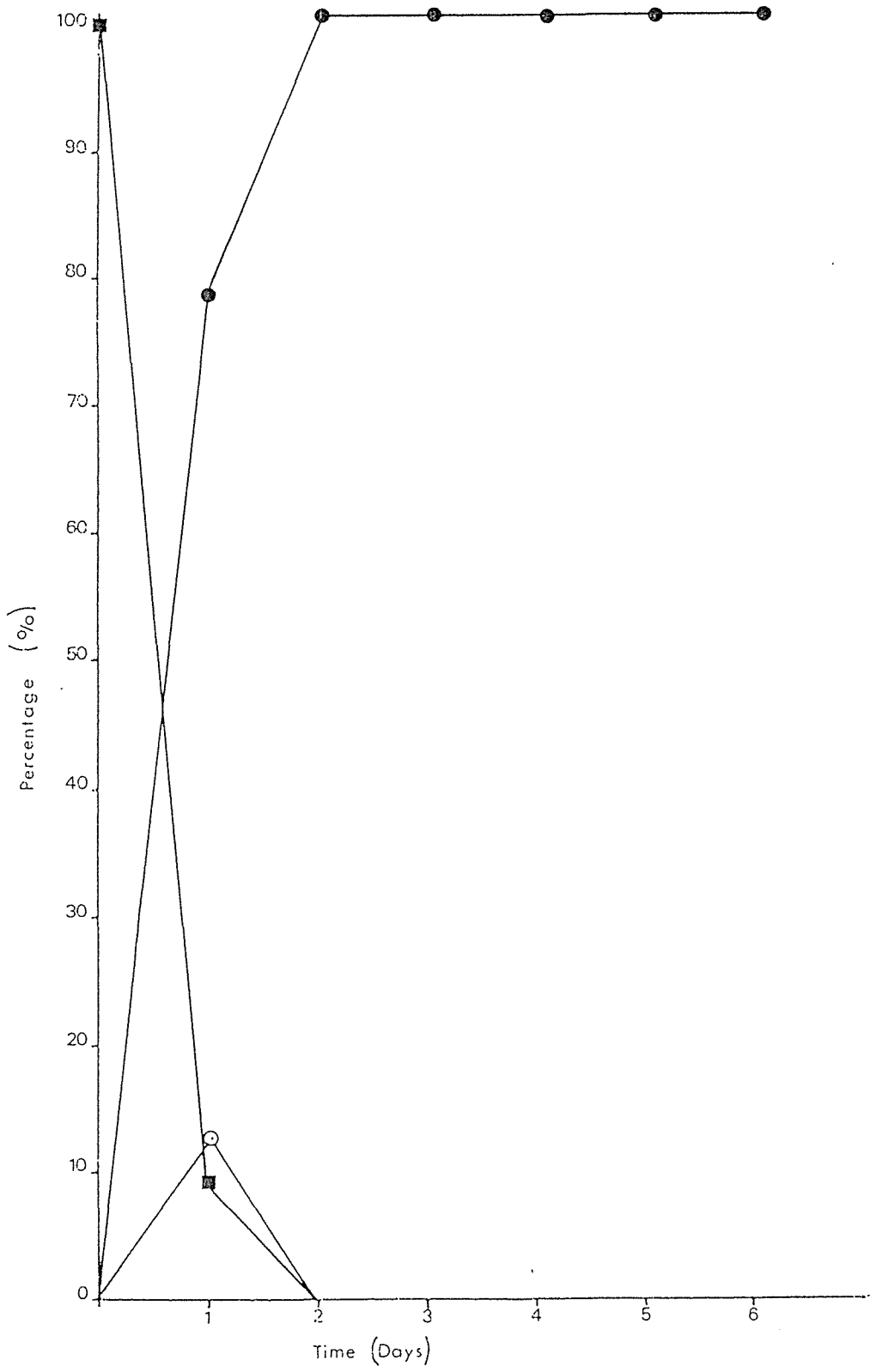


Figure 31. Encystment of *N. fowleri* in 50% SEB.

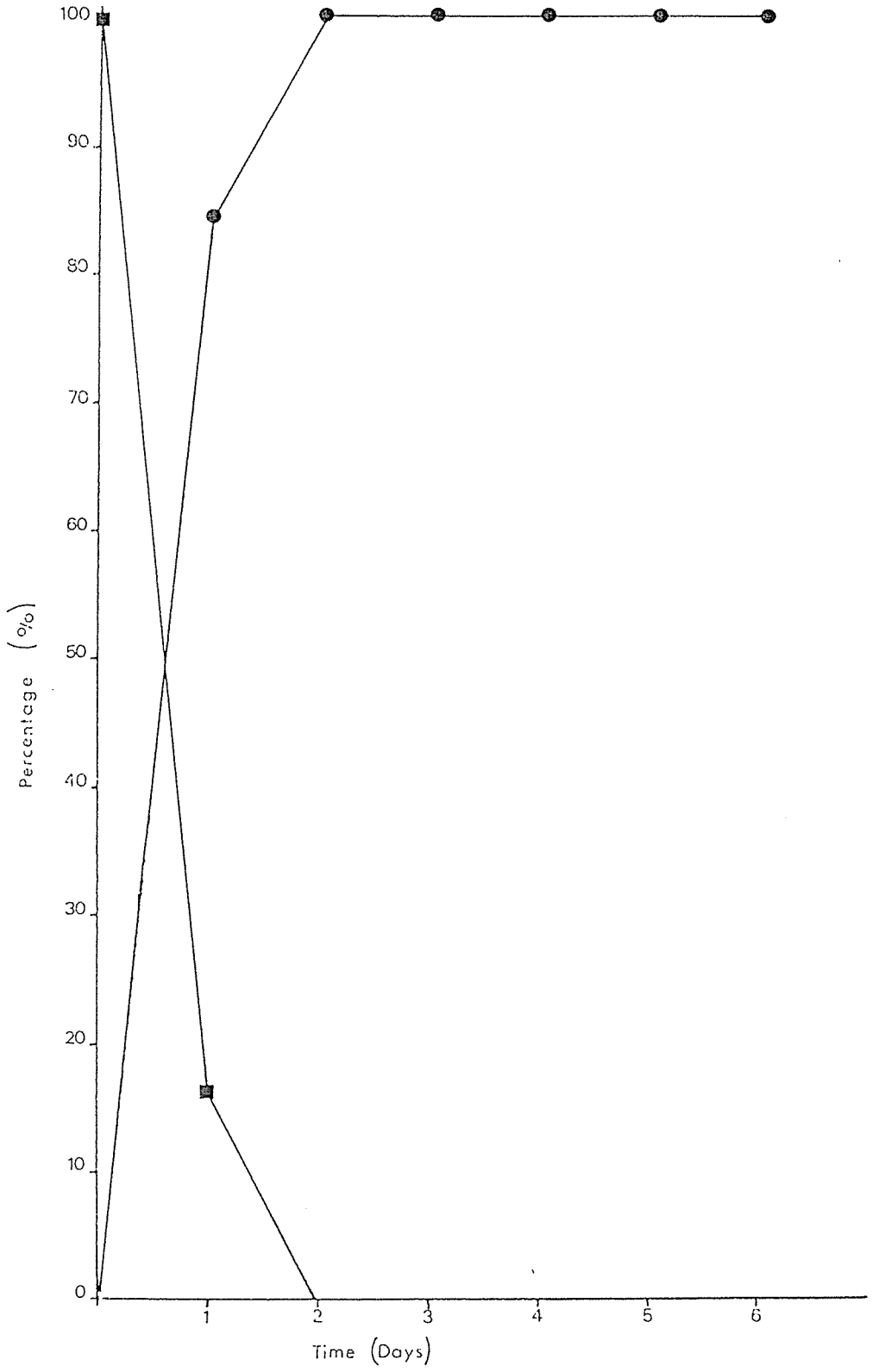


Figure 32. Encystment of *N. fowleri* in 100% SEB.

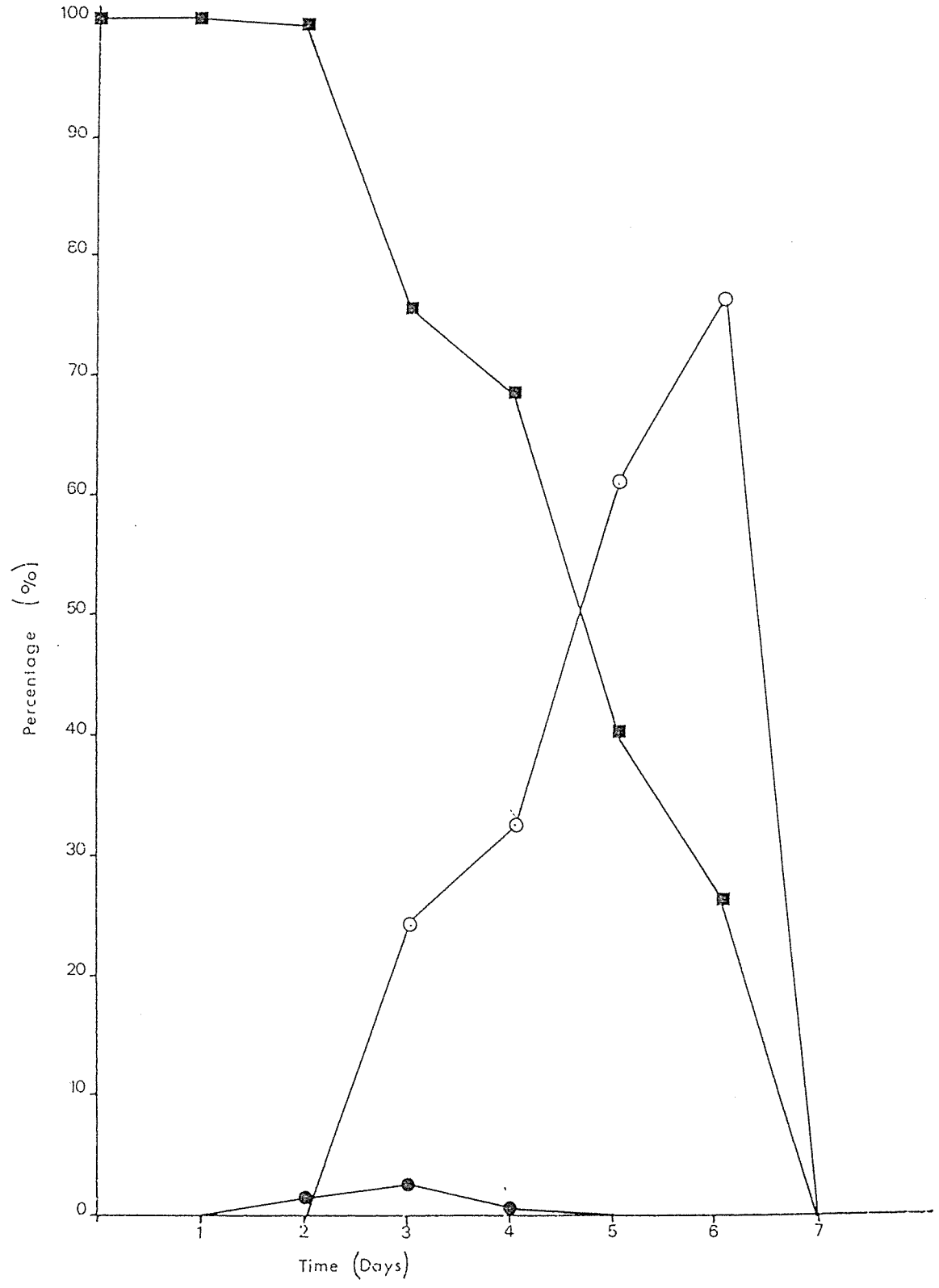


Figure 33. Encystment of *N. gruberi* in 50% SEB.

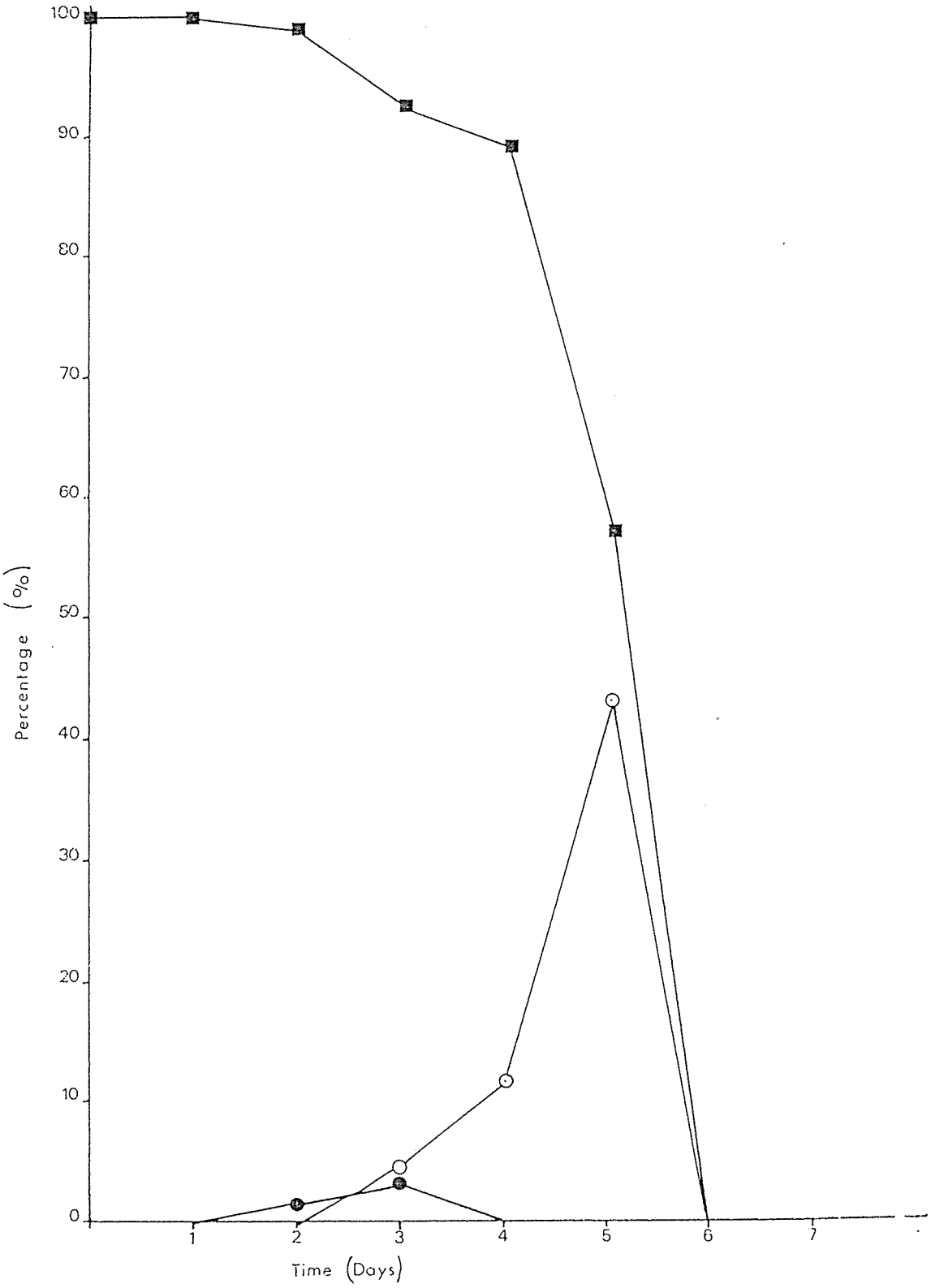


Figure 34. Encystment of *N. gruberi* in 100% SEB.

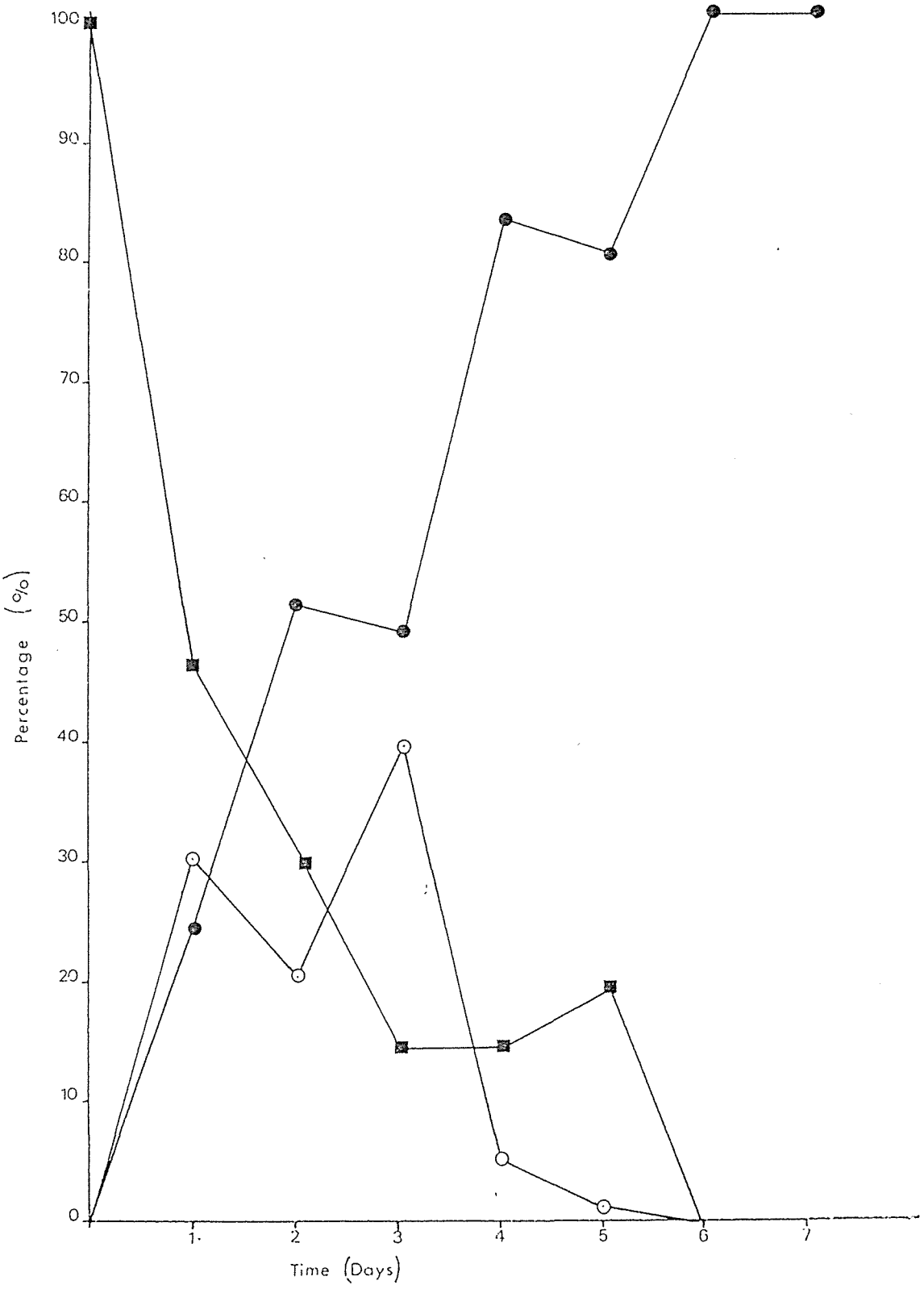


Figure 35. Encystment of *A. culbertsoni* in 50% SEB.

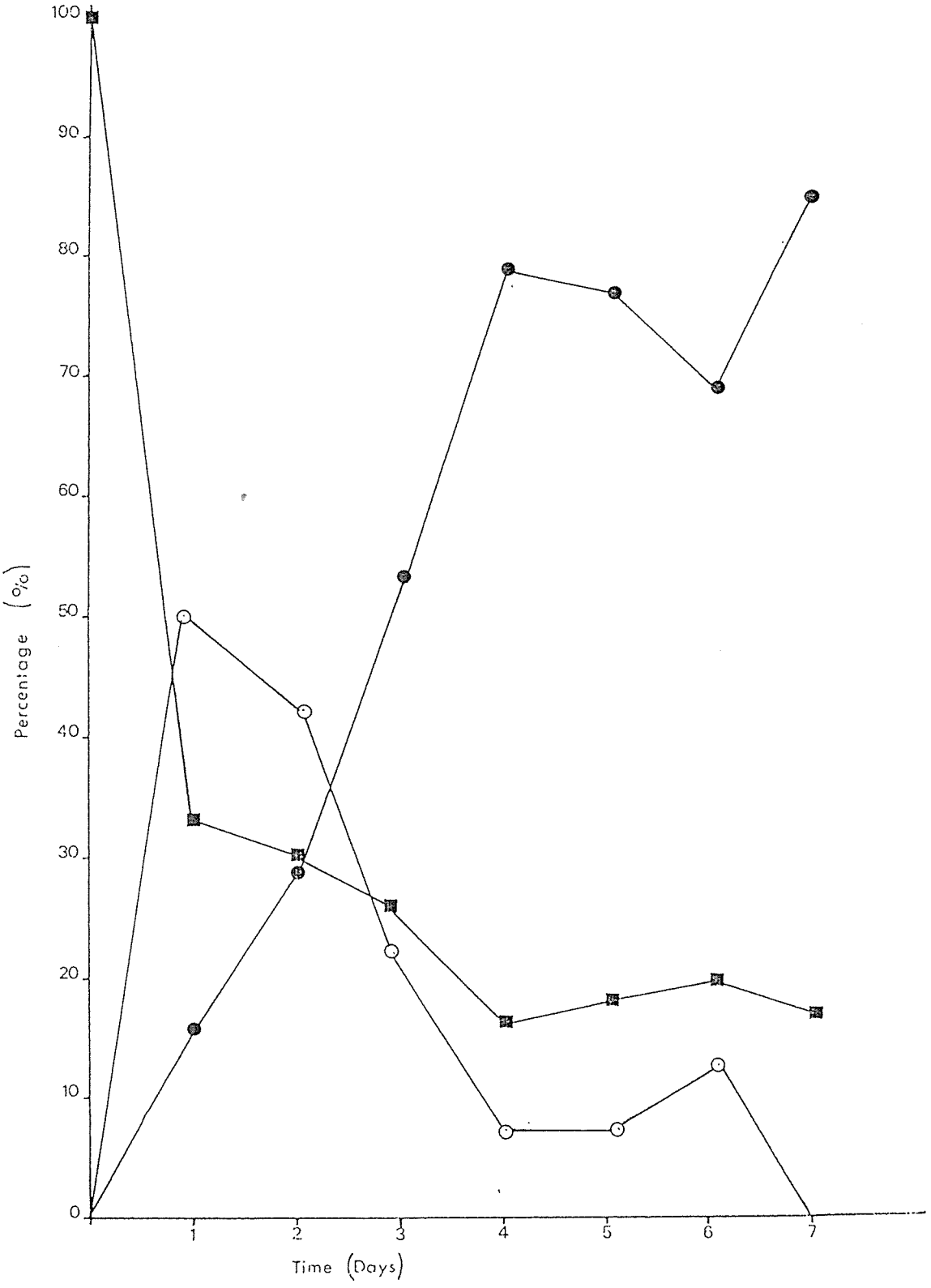


Figure 36. Encystment of *A. culbertsoni* in 100% SEB.

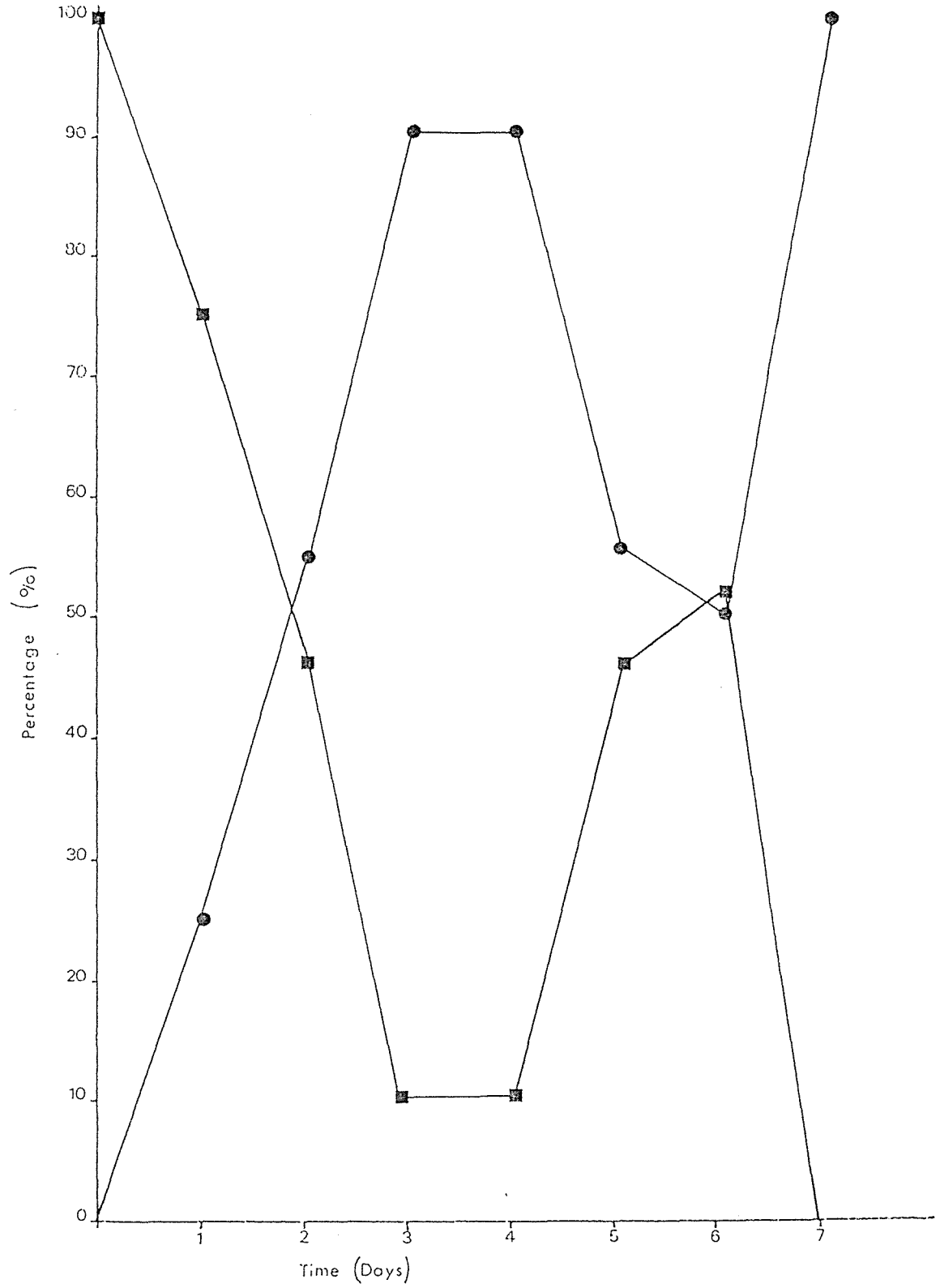


Figure 37. Encystment of *A. castellanii* in 50% SEB.

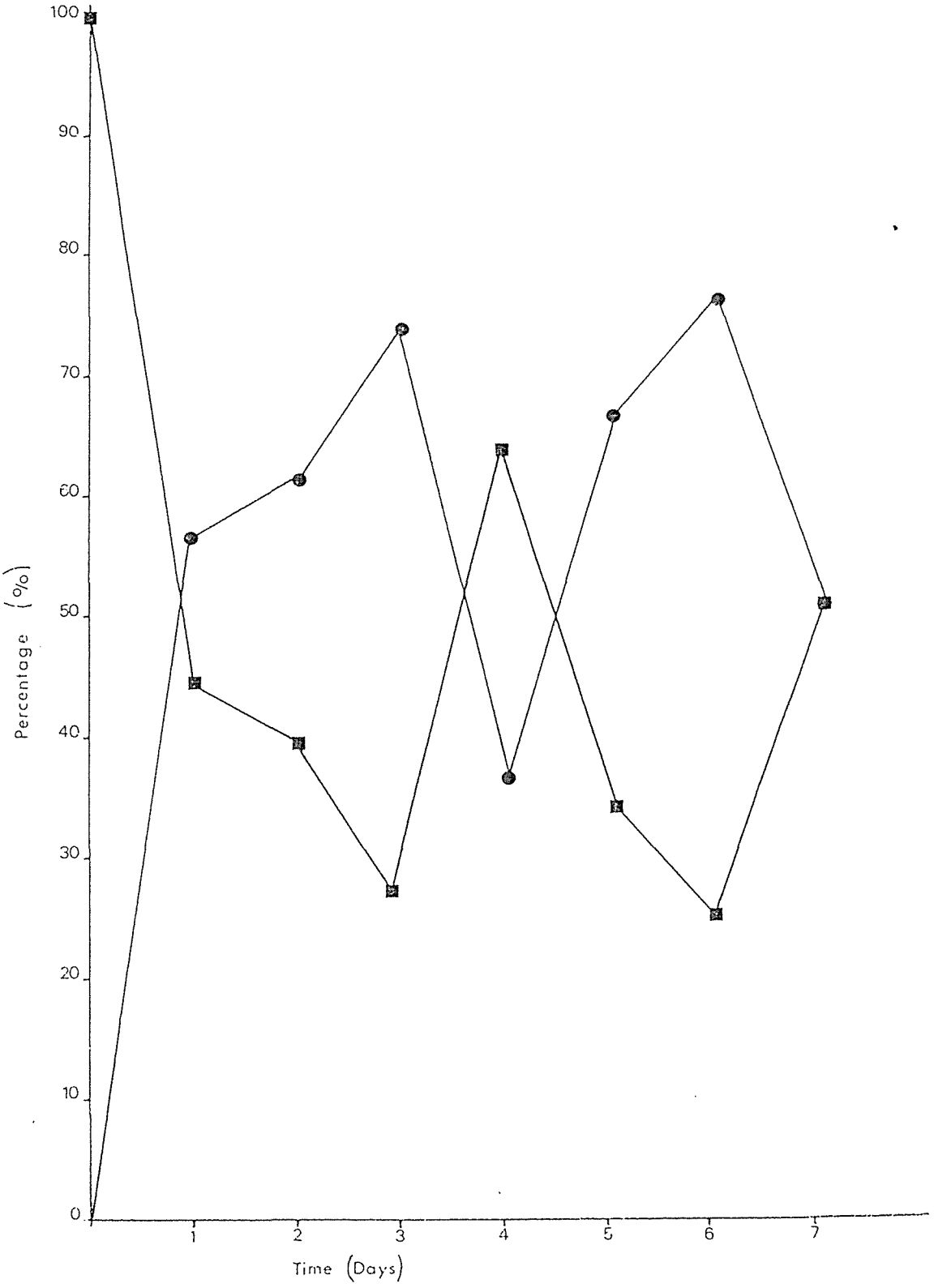


Figure 38. Encystment of *A. castellanii* in 100% SEB.

4.3 Encystment on Solid Media

4.3.1 *N. fowleri* (NHI) (Figures 39-42)

Figure 39 shows the encystment of *N. fowleri* on PASB agar. Encystment reached 100% after six days.

SEA produced 85% encystment after five days, after which an equilibrium was established with the trophozoite level of 15% (Figure 40).

Round forms were not formed on either PASB agar or SEA. Encystment was minimal on both CYM and CYM/50 agar (Figures 41-42). Maximum levels of 6 and 12% were attained. Round forms were produced and reached levels of 29 and 20 on CYM/50 and CYM respectively.

More cysts were produced on low nutrient agar, and more round forms, which did not encyst, were produced on high nutrient agar.

4.3.2 *N. gruberi* (PL) (Figures 43-46)

N. gruberi produced only minimal levels of cysts on all four media. No round forms were produced on any media. Maximum encystment occurred on CYM/50 agar where 33% was reached. Levels of 3% and 8% were reached on the low nutrient agars and CYM respectively.

4.3.3 *A. culbertsoni* (A1) (Figures 47-50)

100% encystment was achieved in both PASB and SEA, at 7 and 5 days respectively (Figures 47-48). No round forms were produced.

Round forms were present after 3 days on both nutrient agars Neff and Neff/50. Figure 49 shows that encystment reached 70% after 7 days on Neff/50 due to a decrease in round forms on the 6th and 7th days. On Neff agar encystment reached a maximum of 10% and round forms reached 24%.

4.3.4 *A. castellanii* (1501) (Figures 51-54)

100% encystment was only achieved on SEA (Figure 52). PASB agar only stimulated 95% encystment (Figure 51). On PASB agar, round forms were produced but disappeared after 48 hours to be replaced by cysts. Round forms were not produced on SEA. The nutrient medium did not stimulate good encystment. On Neff and Neff/50 an equilibrium situation appeared after 6 and 7 days respectively with steady levels of trophozoites, cysts and round forms.

Encystment reached 39% on Neff/50 and 12% on Neff agar.

Both *Acanthamoeba* species and *N. fowleri* showed best encystment on low nutrient agar.

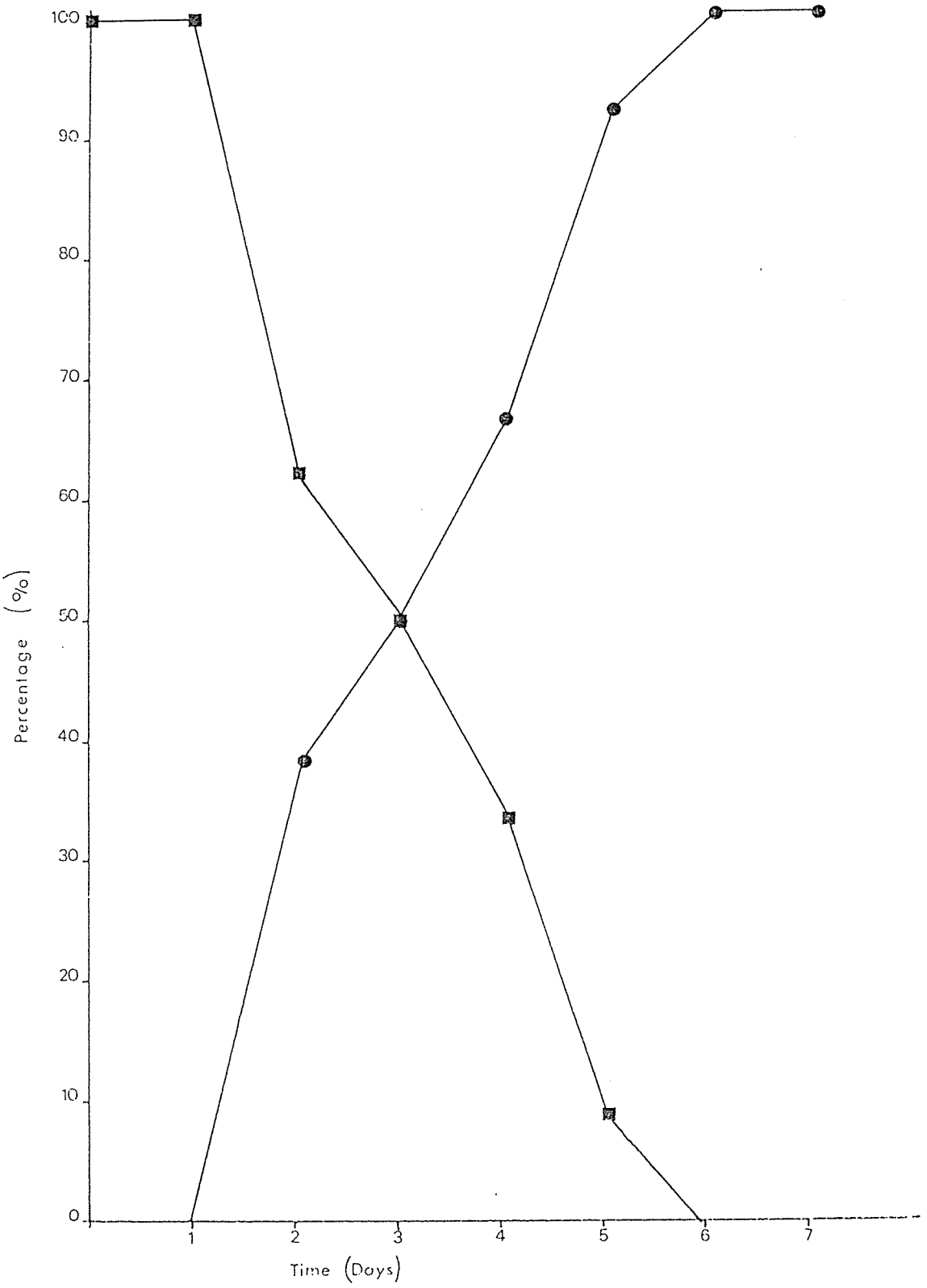


Figure 39. Encystment of *N. fowleri* on PASB agar.

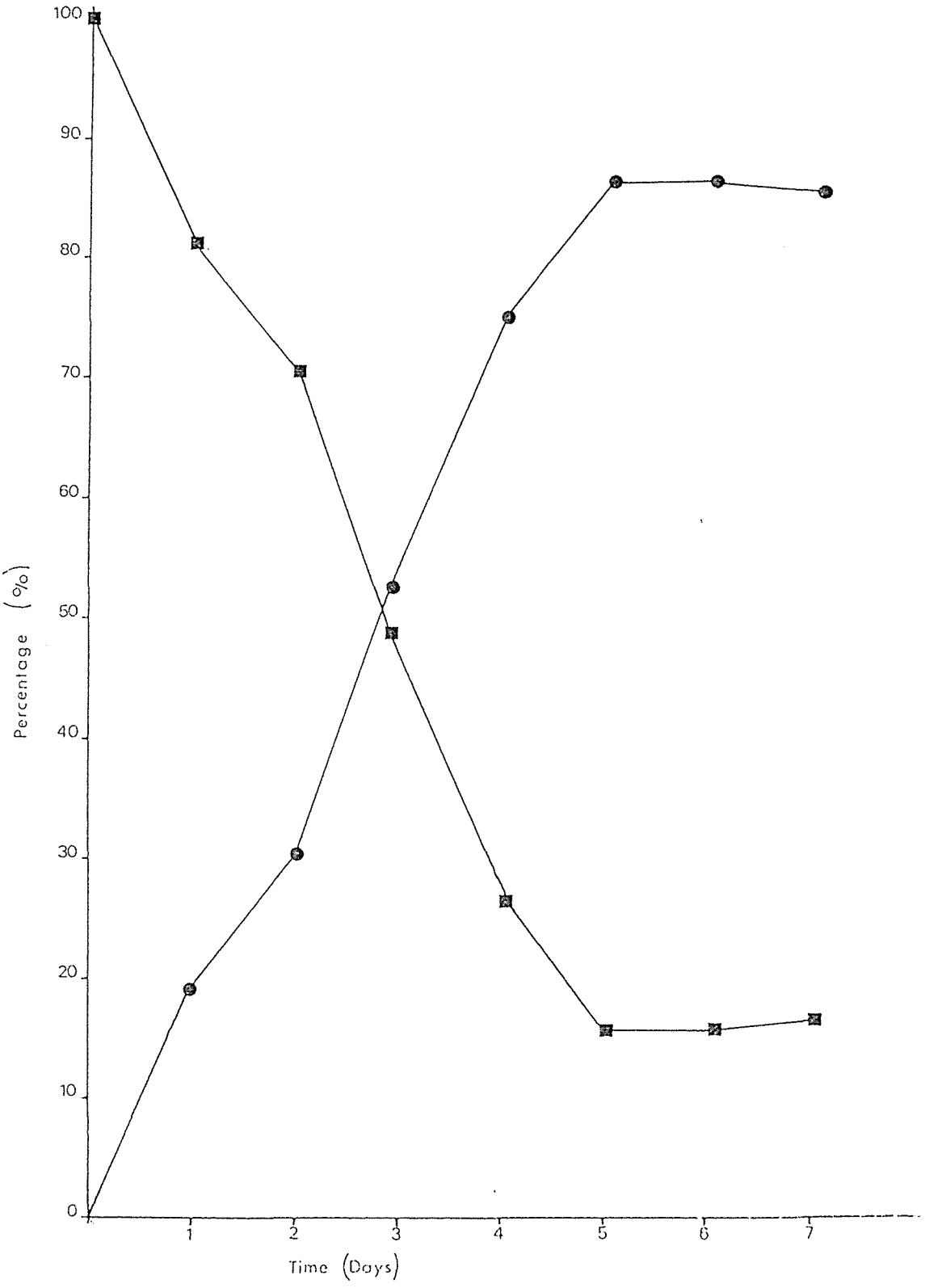


Figure 40. Encystment of *N. fowleri* on SEA.

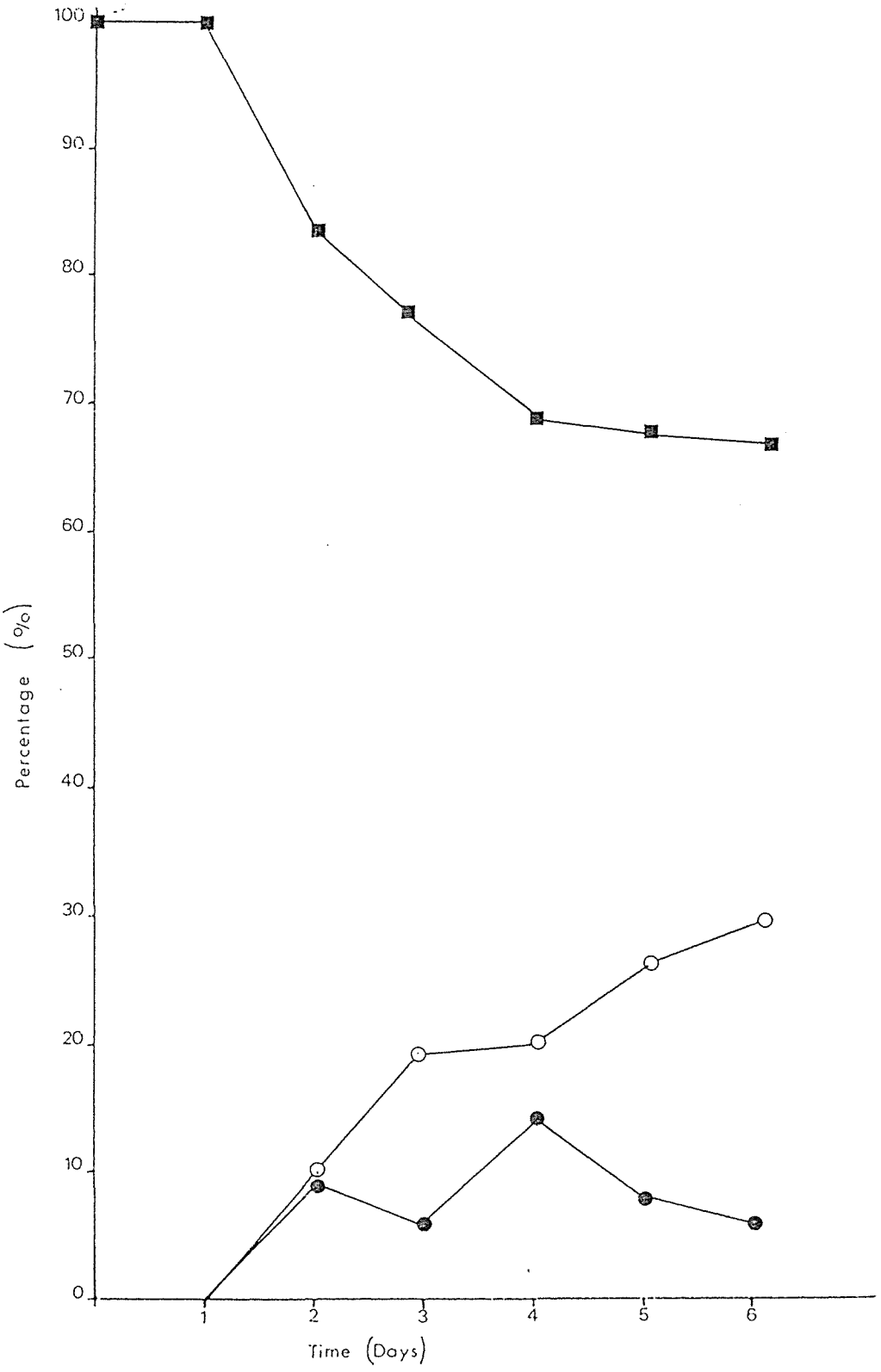


Figure 41. Encystment of *N. fowleri* on CYM/50 agar.

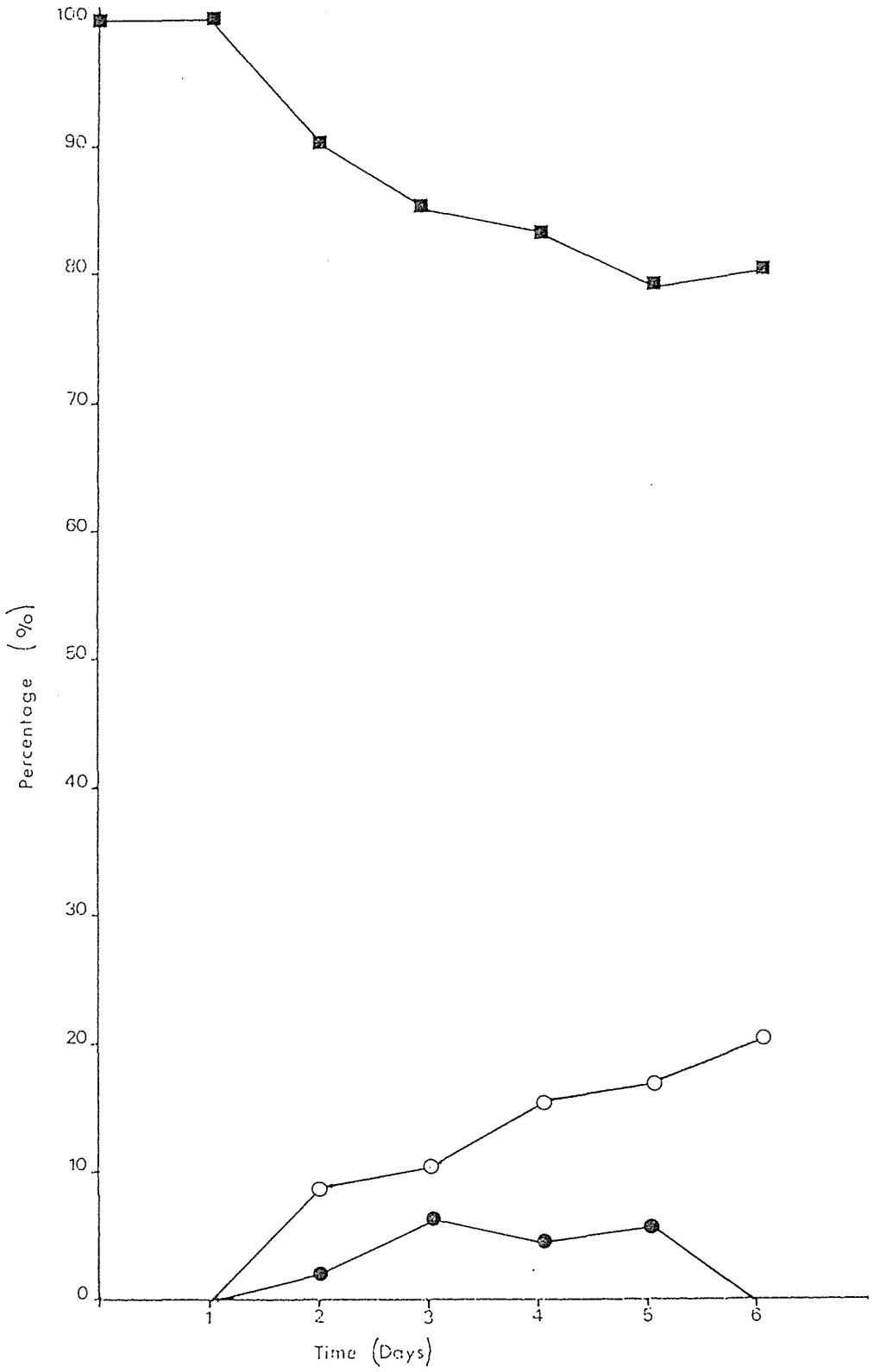


Figure 42. Encystment of *N. fowleri* on CYM agar.

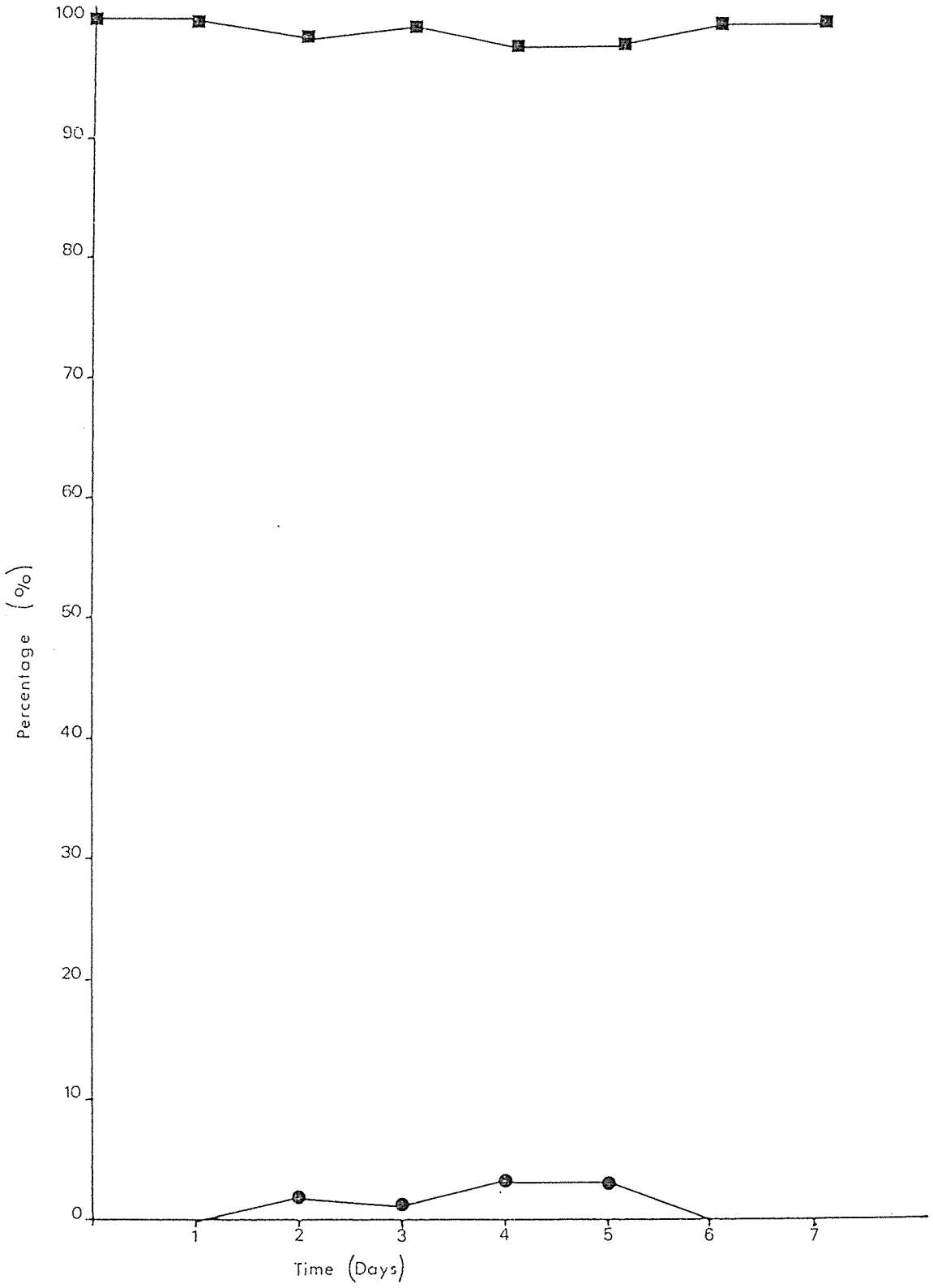


Figure 43. Encystment of *N. gruberi* on PASB agar.

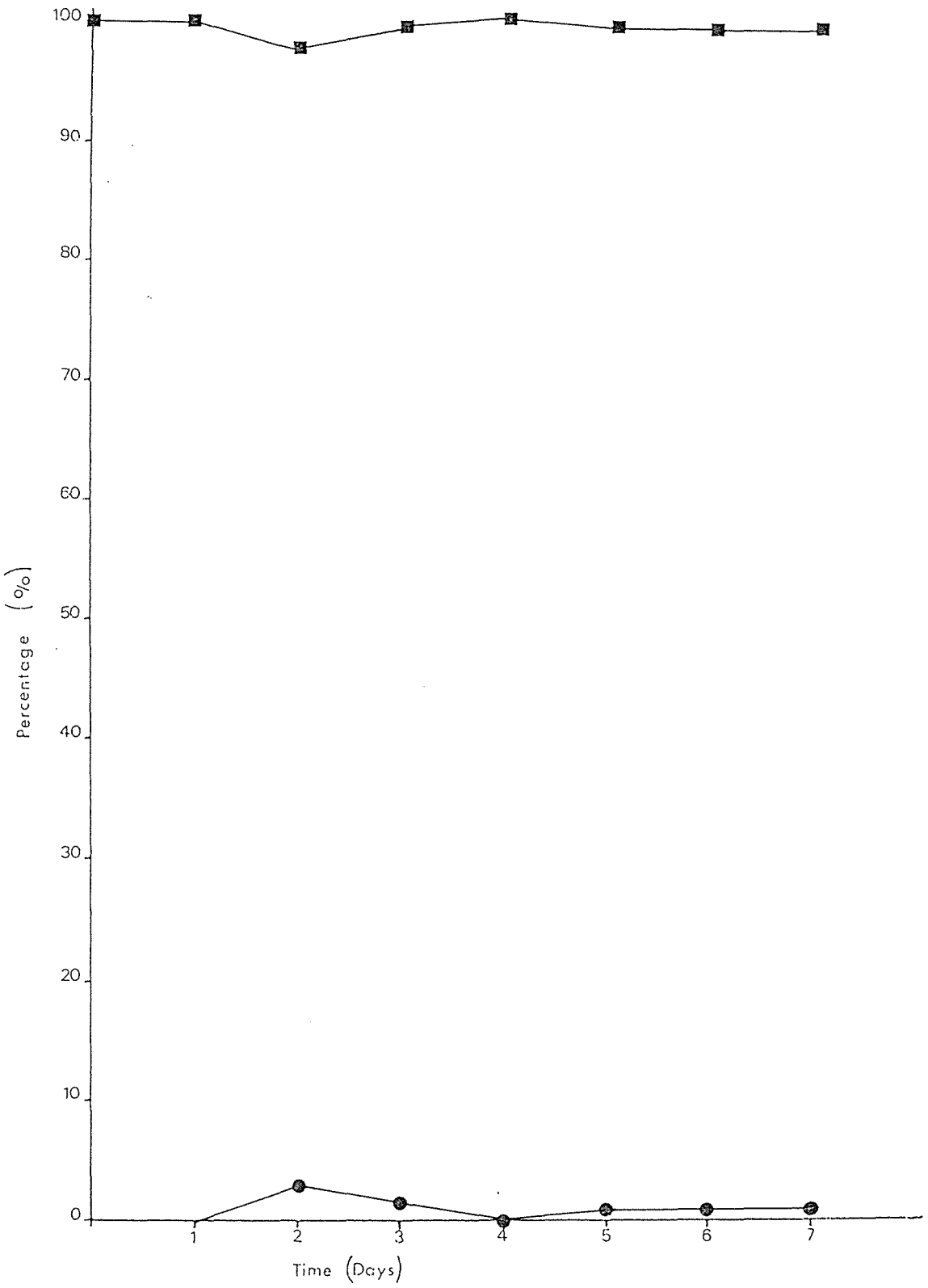


Figure 44. Encystment of *N. gruberi* on SEA.

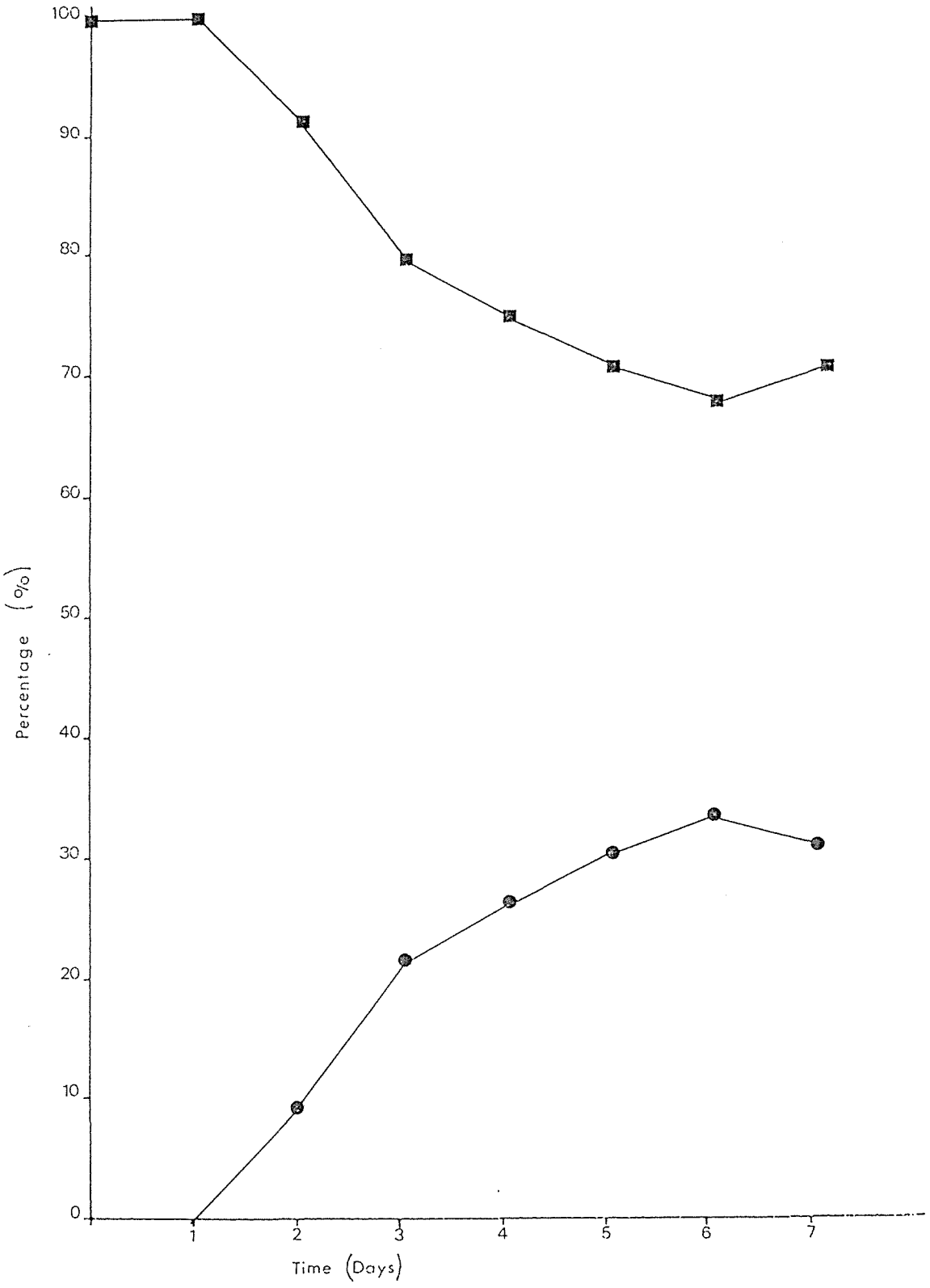


Figure 45. Encystment of *N. gruberi* on CYM/50 agar.

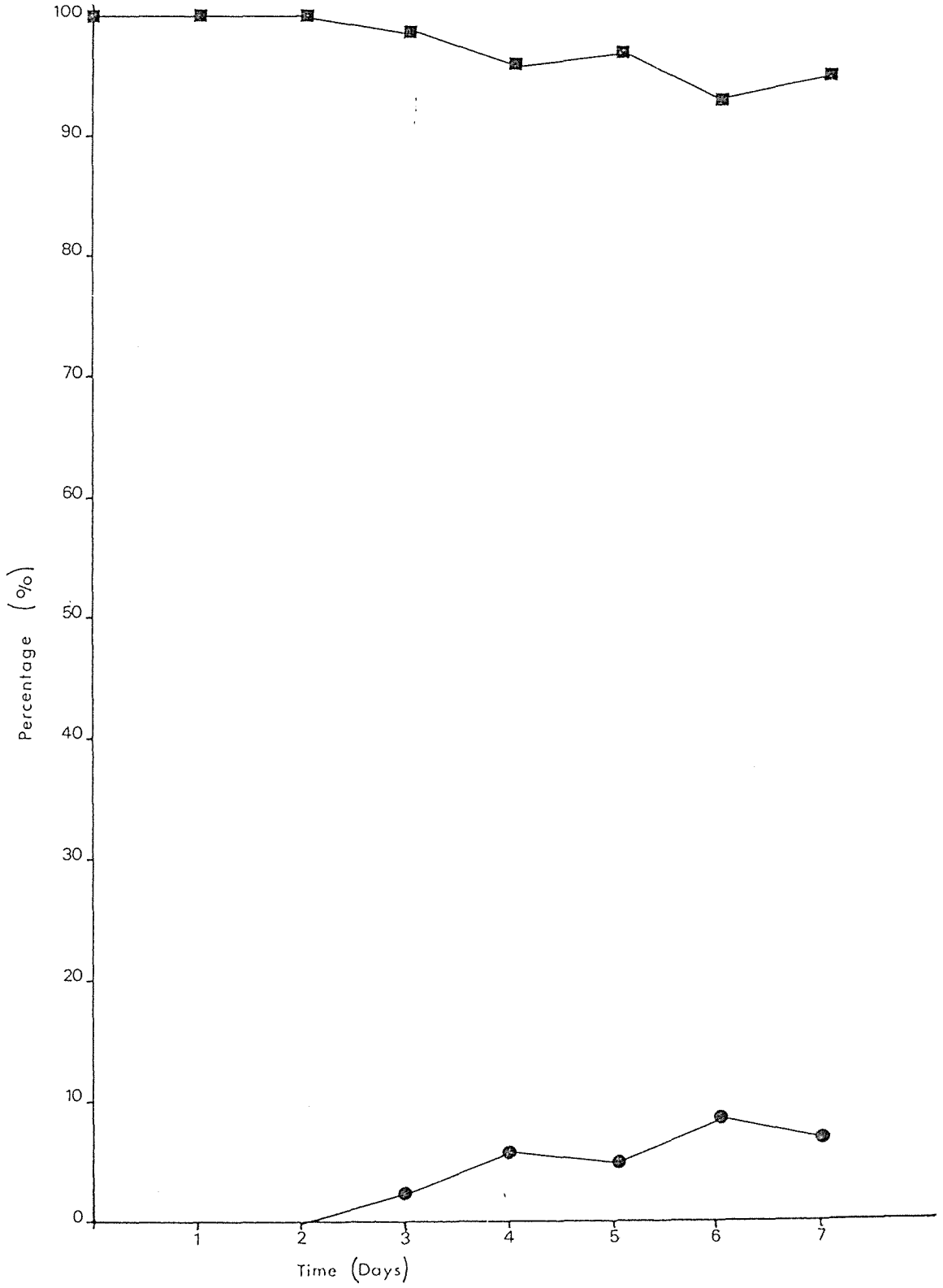


Figure 46. Encystment of *N. gruberi* on CYM agar.

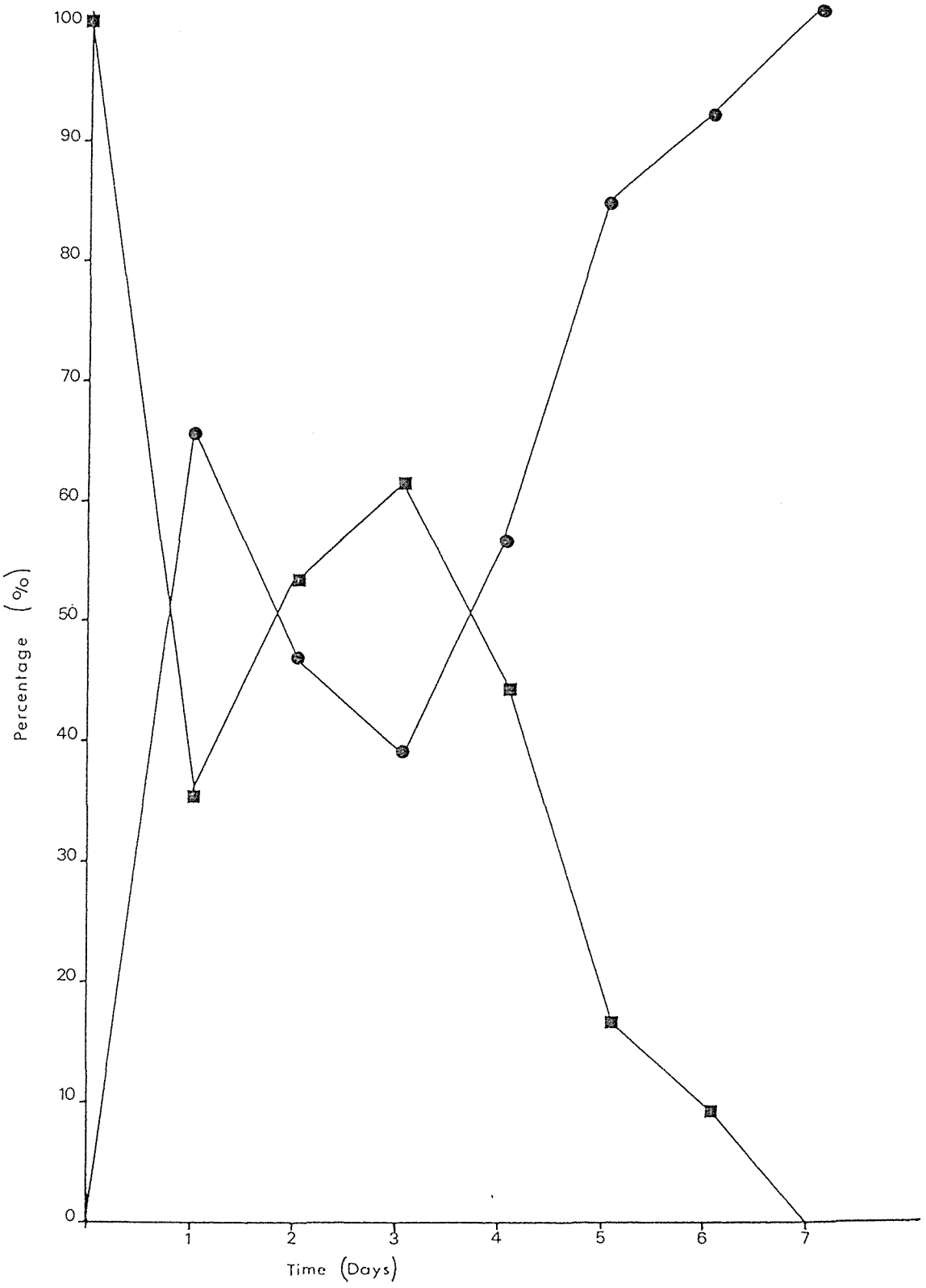


Figure 47. Encystment of *A. culbertsoni* on PASB agar.

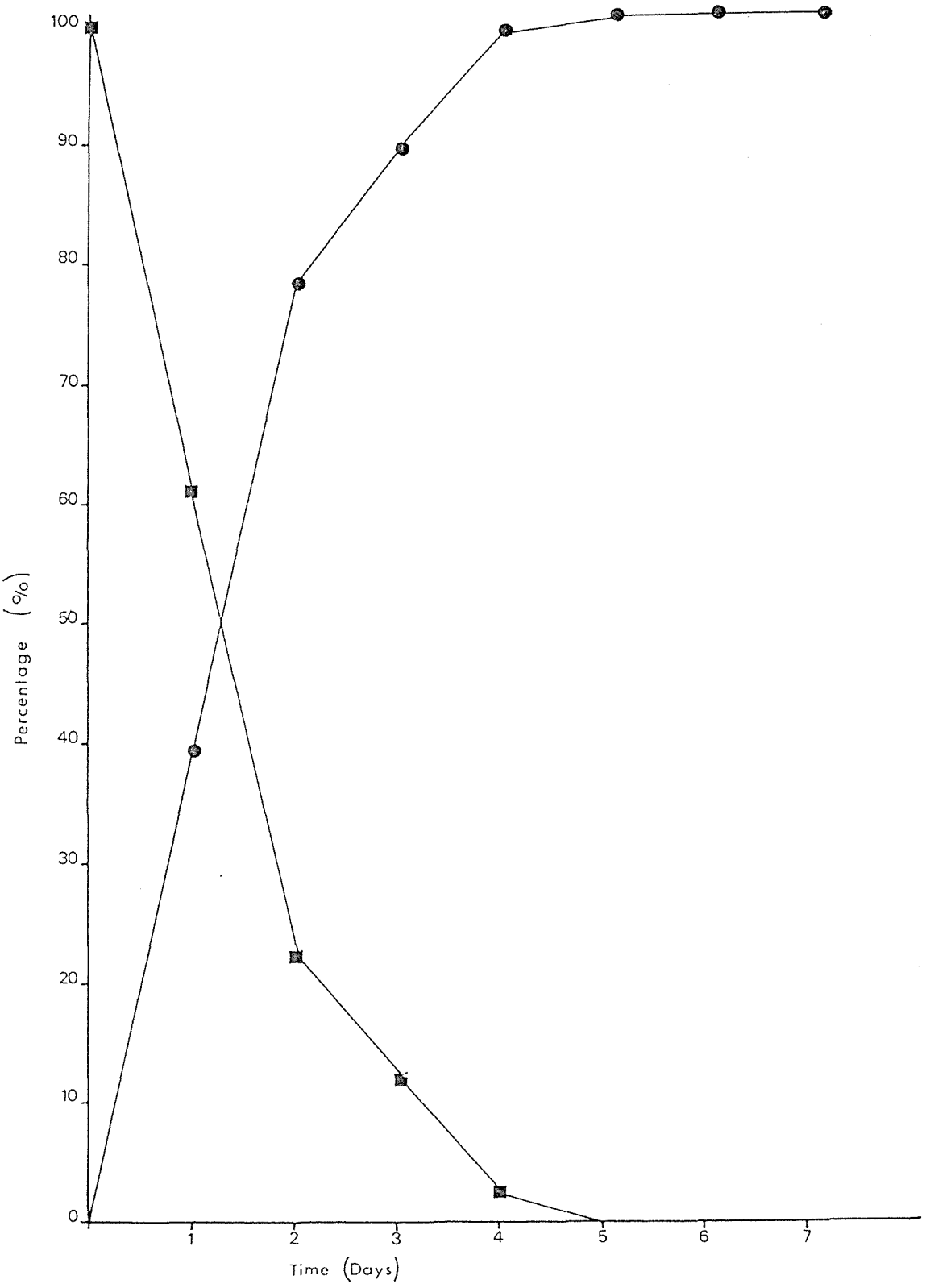


Figure 48. Encystment of *A. culbertsoni* on SEA.

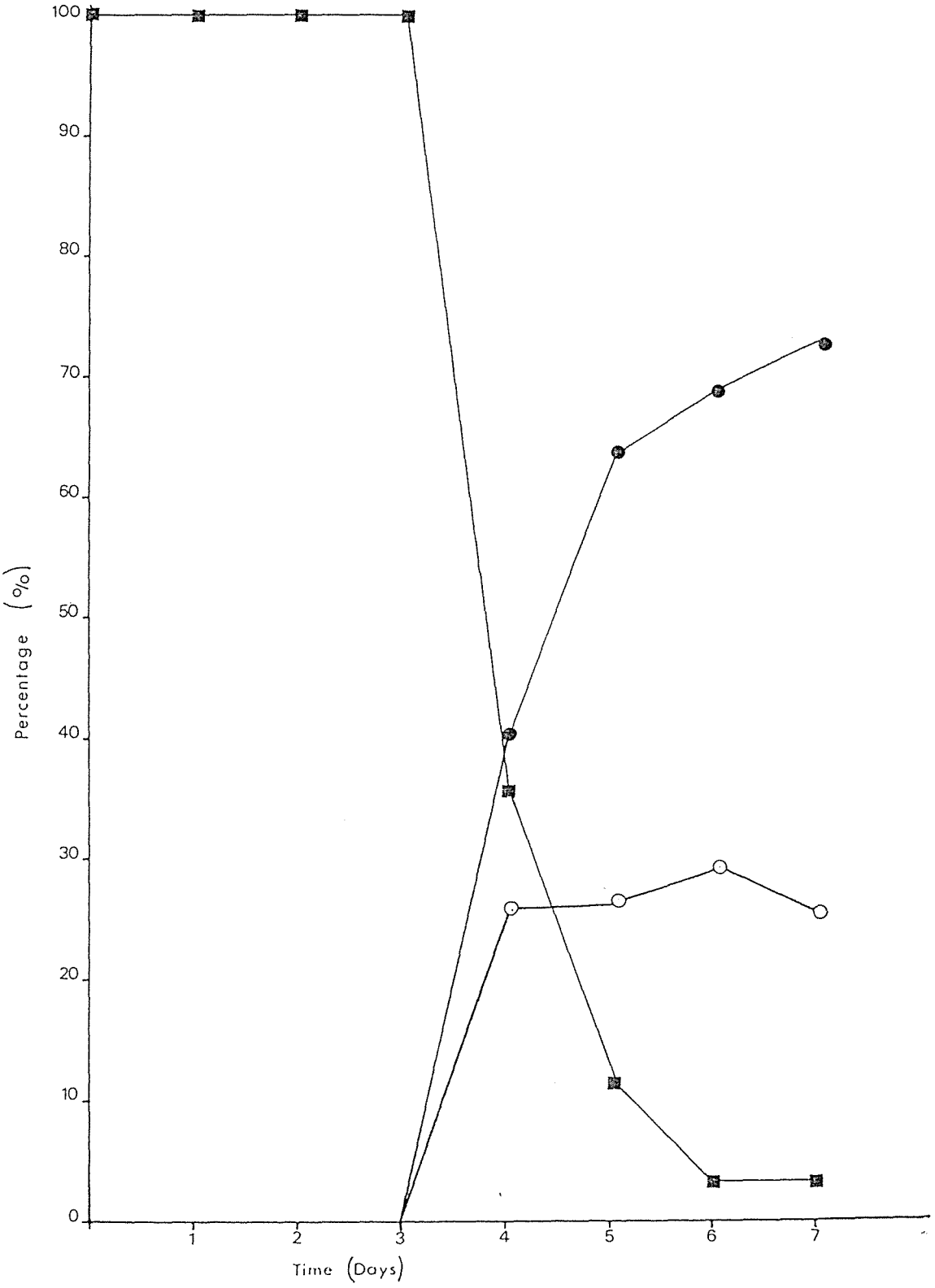


Figure 49. Encystment of *A. culbertsoni* on Neff/50 agar.

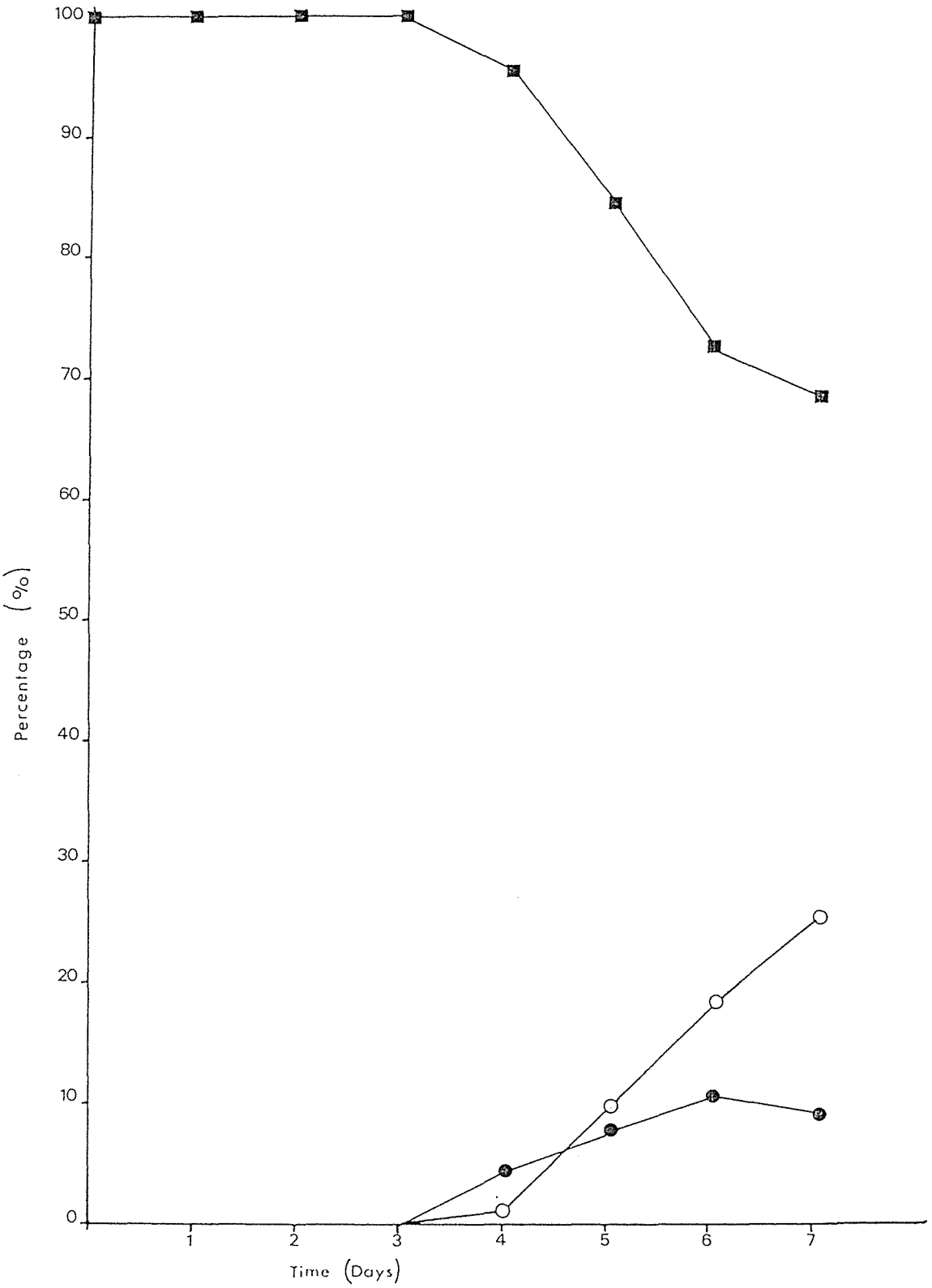


Figure 50. Encystment of *A. culbertsoni* on Neff agar.

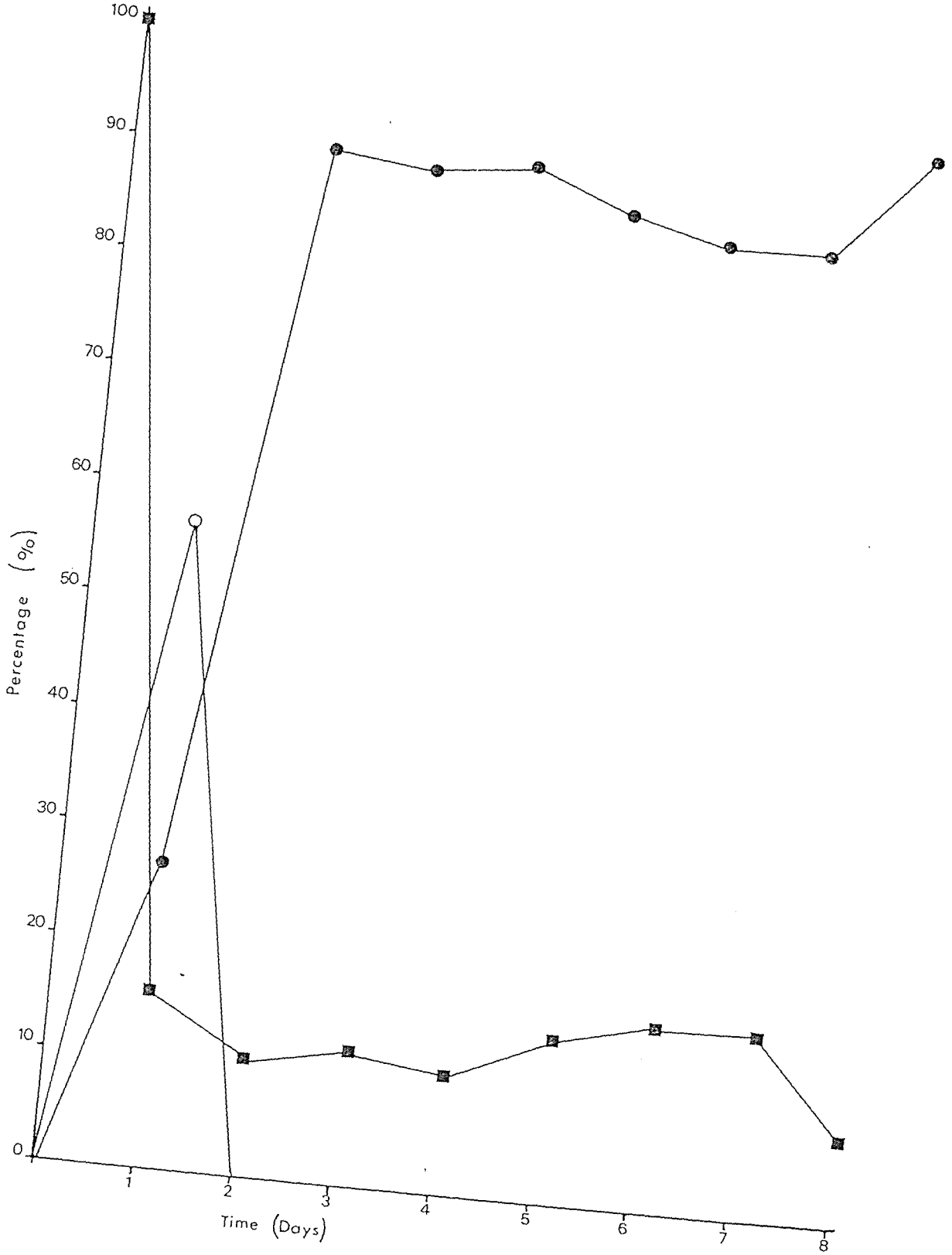


Figure 51. Encystment of *A. castellanii* on PASB agar.

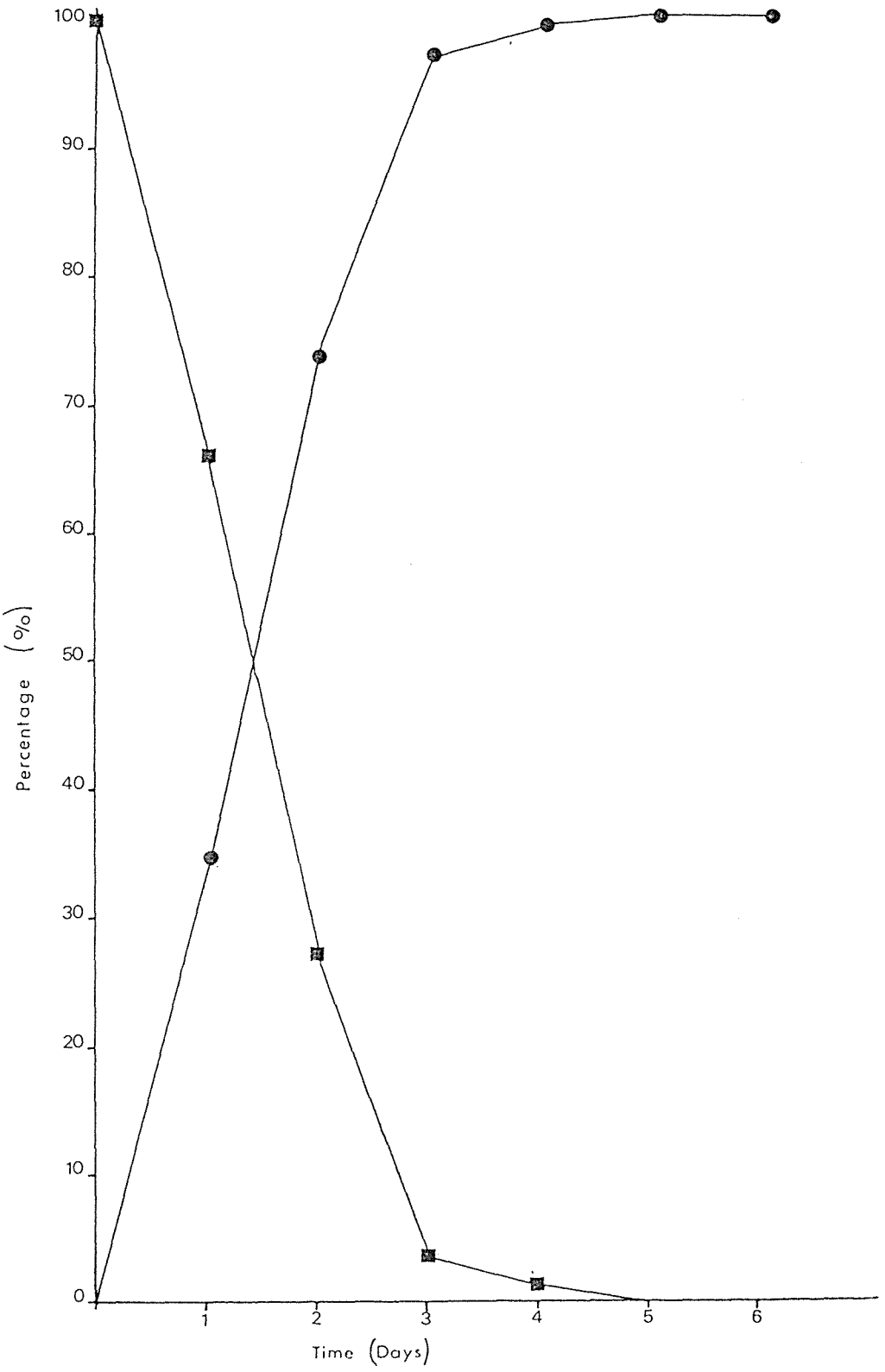


Figure 52. Encystment of *A. castellanii* on SEA.

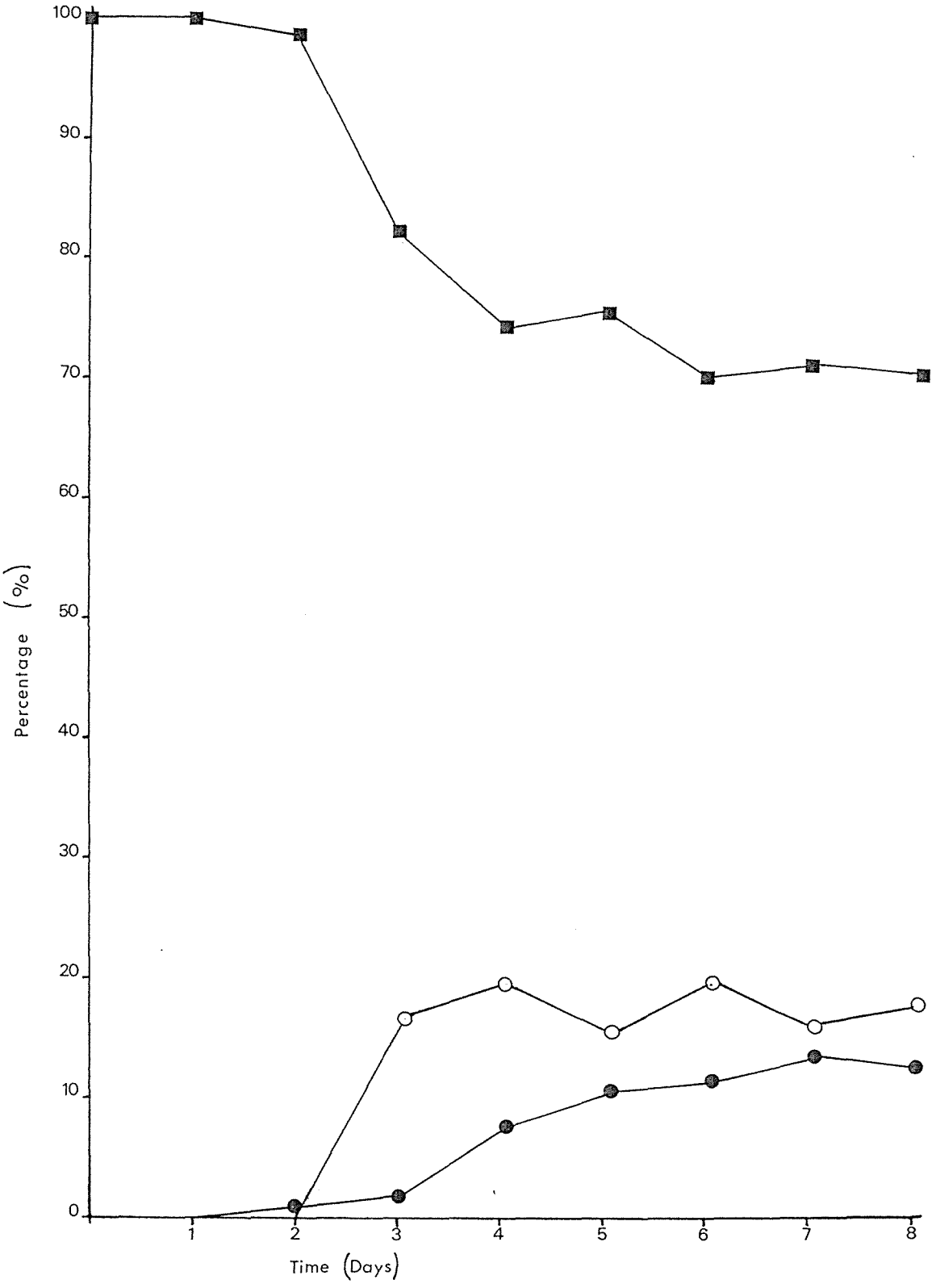


Figure 54. Encystment of *A. castellanii* on Neff agar.

4.3.5 Chemical analysis before and after growth on SEA and PASB

The levels of Ca and Mg in PASB were lower than in SEA. The levels of Na and iron were higher. Levels of K were approximately the same, and remained the same after growth had occurred, in both media and after all species.

The levels of cations in SEA remained the same after growth by both Acanthamoebae species and PL, i.e. were not incorporated in any living cells. After growth of NHI, the levels of Ca^{+2}, Mg^{+2} and $Fe^{+2/+3}$ were higher than the initial levels.

After growth of Acanthamoebae in PASB, the Ca and Mg levels were elevated, to the same level present both before and after growth in SEA. The levels of $Fe^{+2/+3}$ were depressed, but K and Na remained constant.

After growth of *Naegleria* spp. in PASB the Ca and Mg levels were also elevated, to approximately the level in SEA. No other changes occurred.

After growth of all species in PASB the levels of Ca and Mg had roughly doubled, reaching the approximate level present both before and after growth of all species in SEA.

Table 6. Chemical Analysis of Media Before and After Growth

	SEA (sterile)	After A1	After O1	After NHI	After PL
Ca	74	72	76	118	82
Mg	16	19	18	25	18
K	31	30	29	35	28
Na	72	68	82	74	88
$Fe^{+2/+3}$	0.3	0.4	0.4	0.8	0.4
	PASB (sterile)				
Ca	33	74	82	118	80
Mg	7	13	18	27	19
K	36	31	33	37	26
Na	104	128	100	116	90
$Fe^{+2/+3}$	1	0.5	0.3	1	1

4.4 Cyst Survival

4.4.1 Temperature

Tables 7 and 8 show the effect of temperature on the viability of storage slopes.

At room temperature (20°C), cysts of all species remained viable for at least 12 months (Table 7).

At 10°C and 15°C all cysts remained viable for at least 8 months (Table 8).

Acanthamoeba cysts survived 8 months at -10°C and 4°C. *N. fowleri* remained viable for 6 months at 4°C, and *N. gruberi* survived only 2 months at the same temperature. *N. fowleri* survived -10°C for 4 months and *N. gruberi* only survived one month.

Acanthamoeba showed more resistance to low temperatures than *Naegleria*. *N. gruberi* was more susceptible than *N. fowleri*, the pathogen.

Table 7. Viability of Cysts stored at Room Temperature

Species	Strain	Viability at 20°C (months)
N. fowleri	NHI	12
N. gruberi	PL200f	12
A. castellanii	1501	12
A. culbertsoni	A1	12
Unknown	PR2448	18
	PR2449	18

Table 8. Viability of Cysts stored at Various Temperatures

Species (strain)	Temp.(°C)	Months							
		1	2	3	4	5	6	7	8
N. fowleri (NHI)	-10	+	+	+	+	–	–	–	–
	4	+	+	+	+	+	+	–	–
	10	+	+	+	+	+	+	+	+
	15	+	+	+	+	+	+	+	+
N. gruberi (P1200f)	-10	+	–	–	–	–	–	–	–
	4	+	+	–	–	–	–	–	–
	10	+	+	+	+	+	+	+	+
	15	+	+	+	+	+	+	+	+
A. culbertsoni (A1)	-10	+	+	+	+	+	+	+	+
	4	+	+	+	+	+	+	+	+
	10	+	+	+	+	+	+	+	+
	15	+	+	+	+	+	+	+	+
A. castellanii (1501)	-10	+	+	+	+	+	+	+	+
	4	+	+	+	+	+	+	+	+
	10	+	+	+	+	+	+	+	+
	15	+	+	+	+	+	+	+	+

4.4.2 Effect of cation concentration on cyst survival in liquid media

Figures 55-62 show the effect of cation levels on the survival of amoebic cysts in water.

Na ions had the most detrimental effect on survival of *Naegleria* cysts, followed by Ca^{+2} and Mg^{+2}. K had the least effect, similar to the control. Increased iron levels had a slightly inhibitory effect on *Naegleria* spp., with very little difference between Fe^{+2} and Fe^{+3}, except at the 3 mg l^{-1} concentration. The inhibitory effect of iron was more pronounced on *N. fowleri* than *N. gruberi*.

Acanthamoebae were more resistant to the concentrations used of all cations, *A. culbertsoni* especially. Ca had a marginally greater detrimental effect than others, followed by Mg, Na and K in order of effect.

Increased iron levels had a harsher effect on *A. culbertsoni* than *A. castellani*. There was little difference between the effect of ferric and ferrous iron levels except at the 4 mg l^{-1} level.

The following legend applies to Figures 55-58:

× = Control
▲ = Ca^{+2}
◆ = Na^{+}
● = Mg^{+2}
■ = K^{+}

The following legend applies to Figures 59-62:

× = Control
◆ = 1 mg l^{-1}
● = 2
■ = 3
▲ = 4
□ = 5

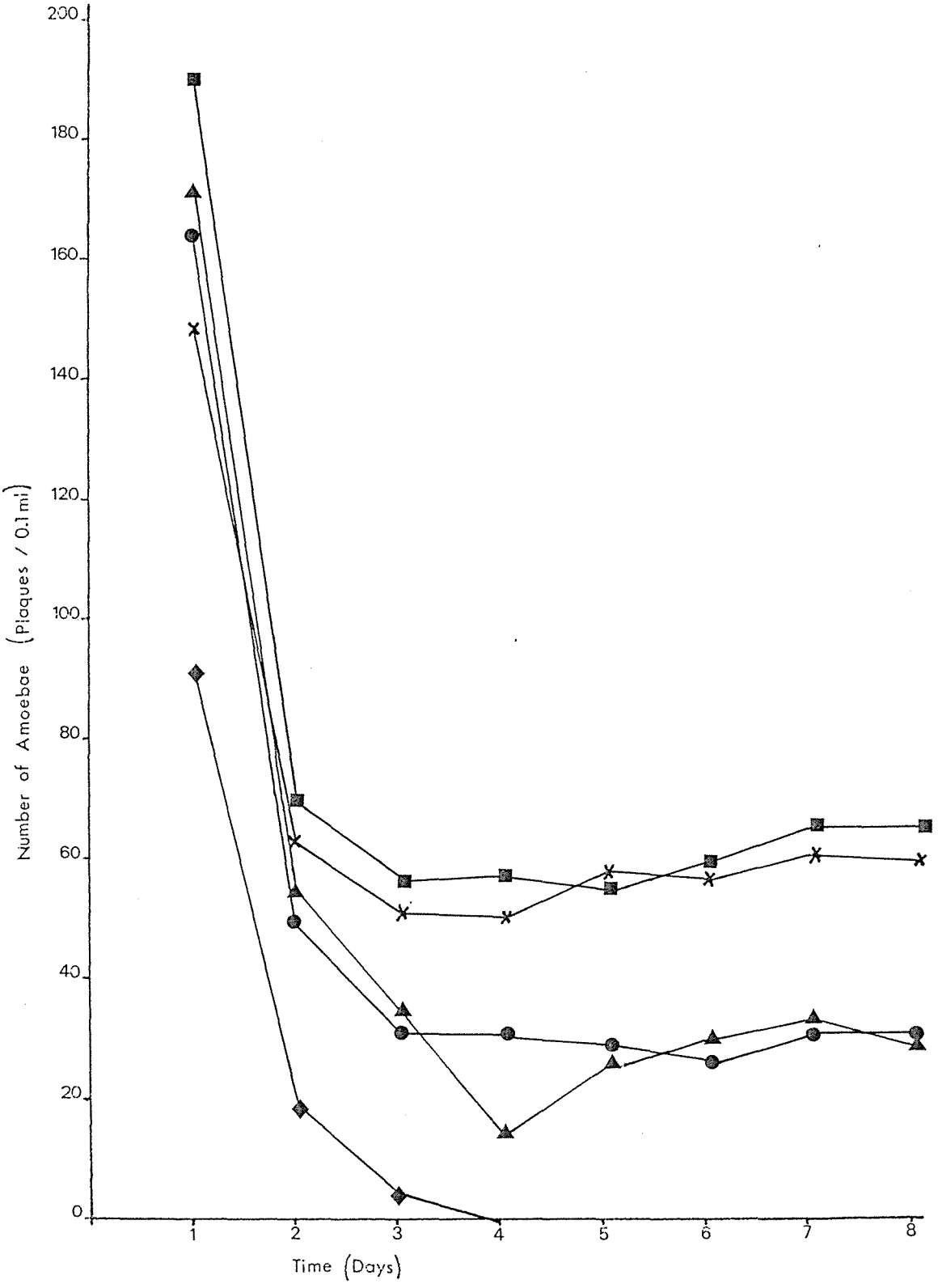


Figure 55a. Effect of cation concentration on survival of *N. fowleri* cysts: 600 mgl^{-1} of cation.

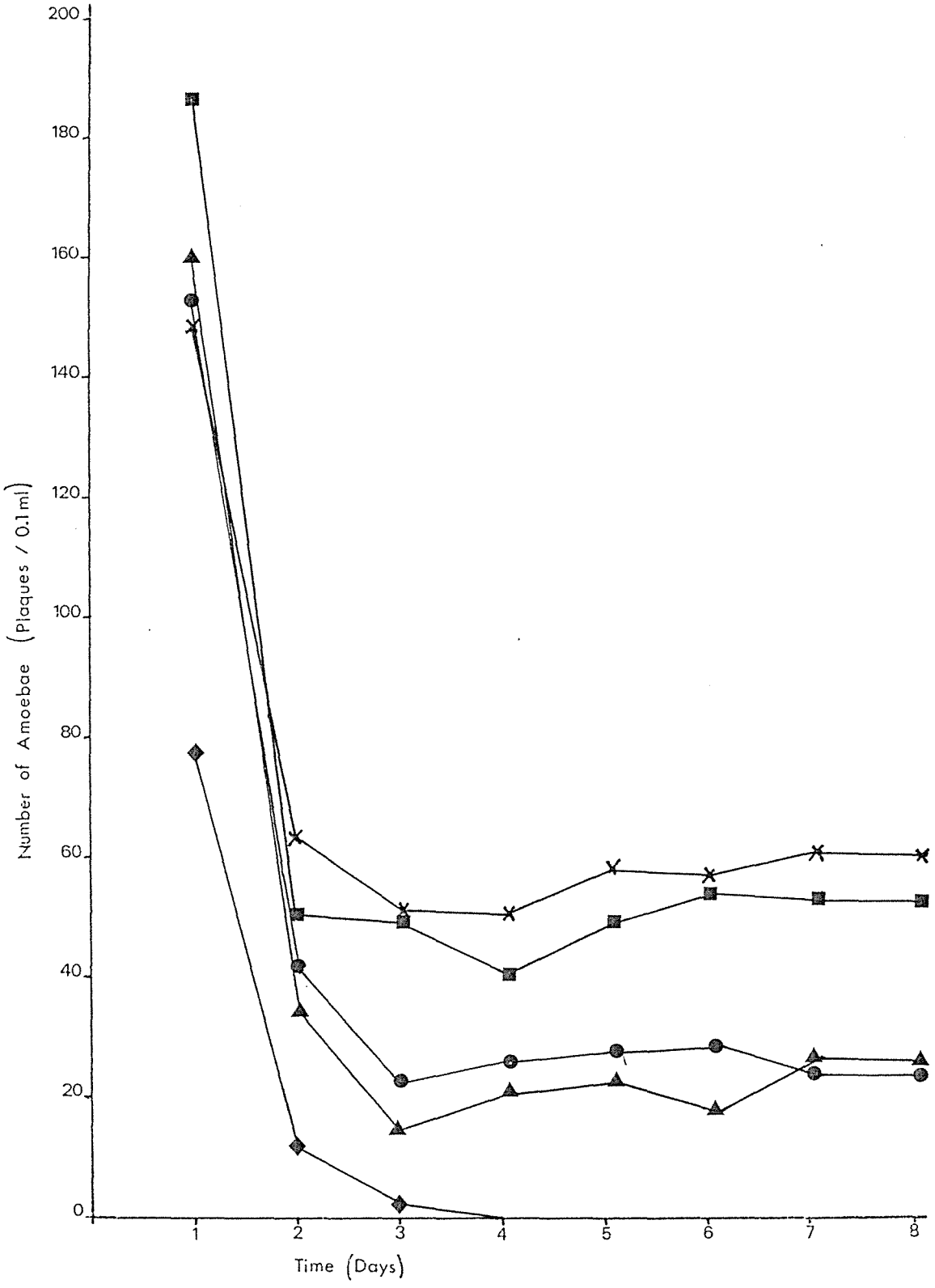


Figure 55b. Effect of cation concentration on survival of *N. fowleri* cysts: 1200 mgl^{-1} of cation.

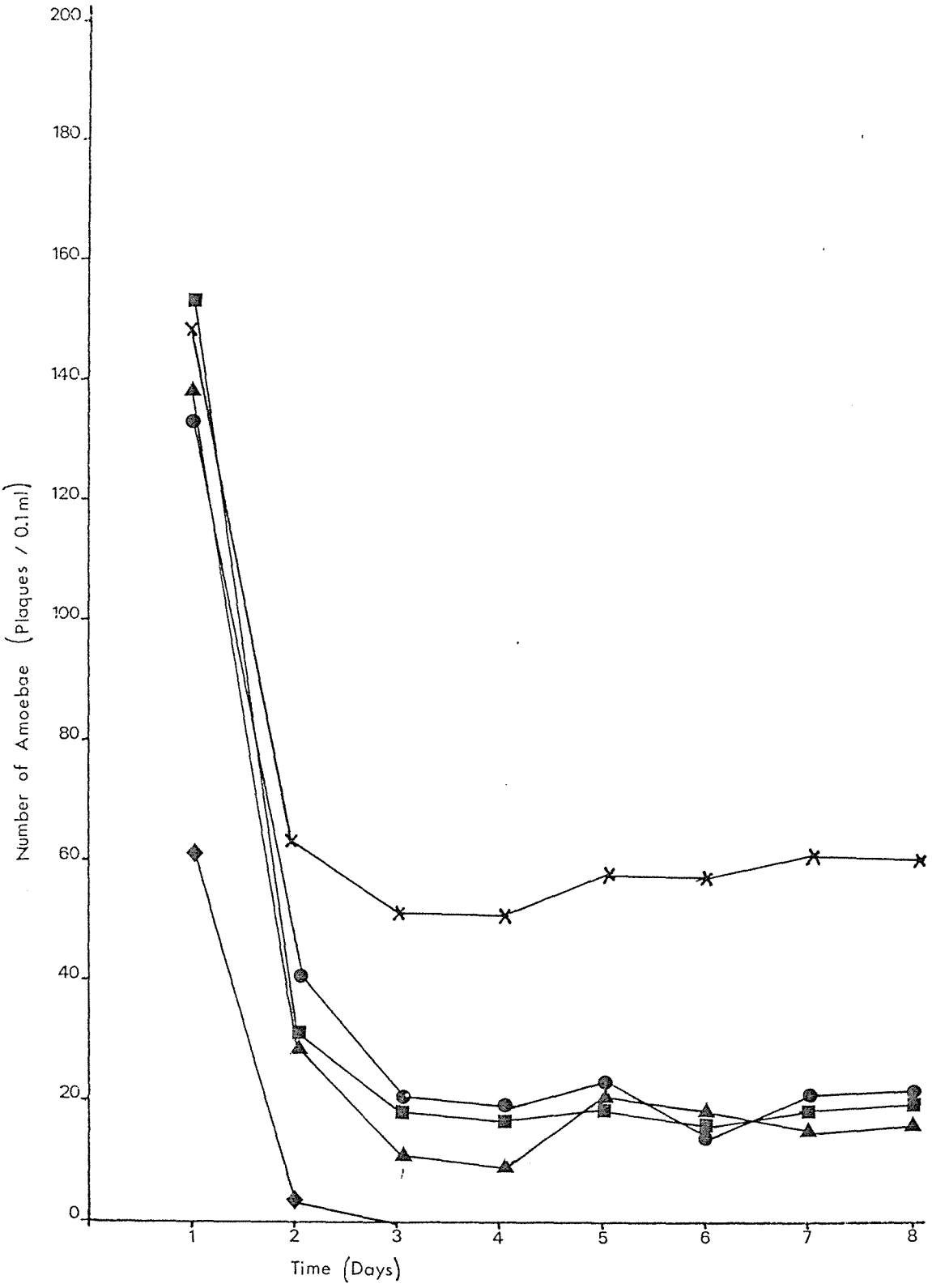


Figure 55c. Effect of cation concentration on survival of *N. fowleri* cysts: 1800 mgl^{-1} of cation.

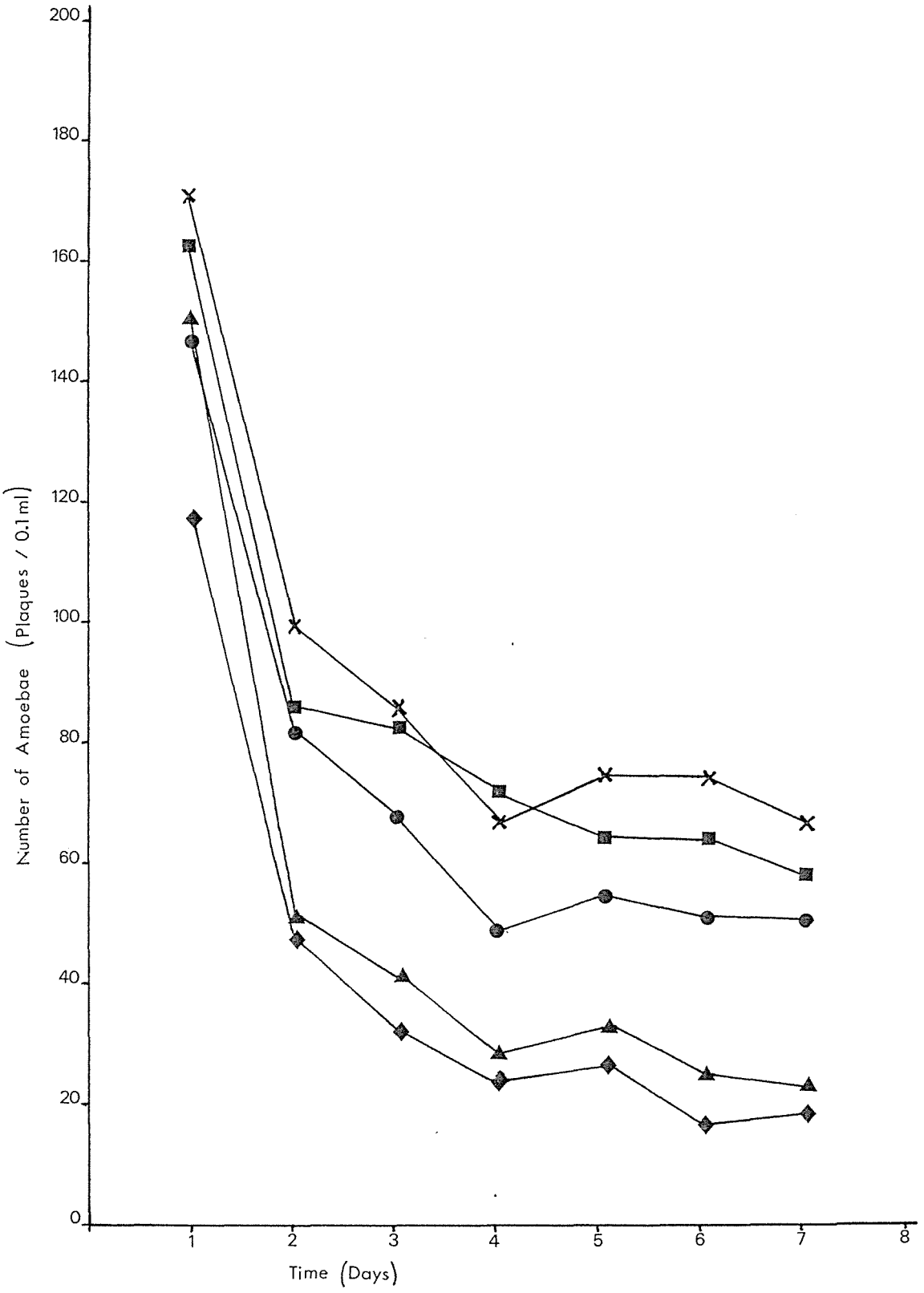


Figure 56a. Effect of cation concentration on survival of *N. gruberi* cysts: 600 mgl^{-1} of cation.

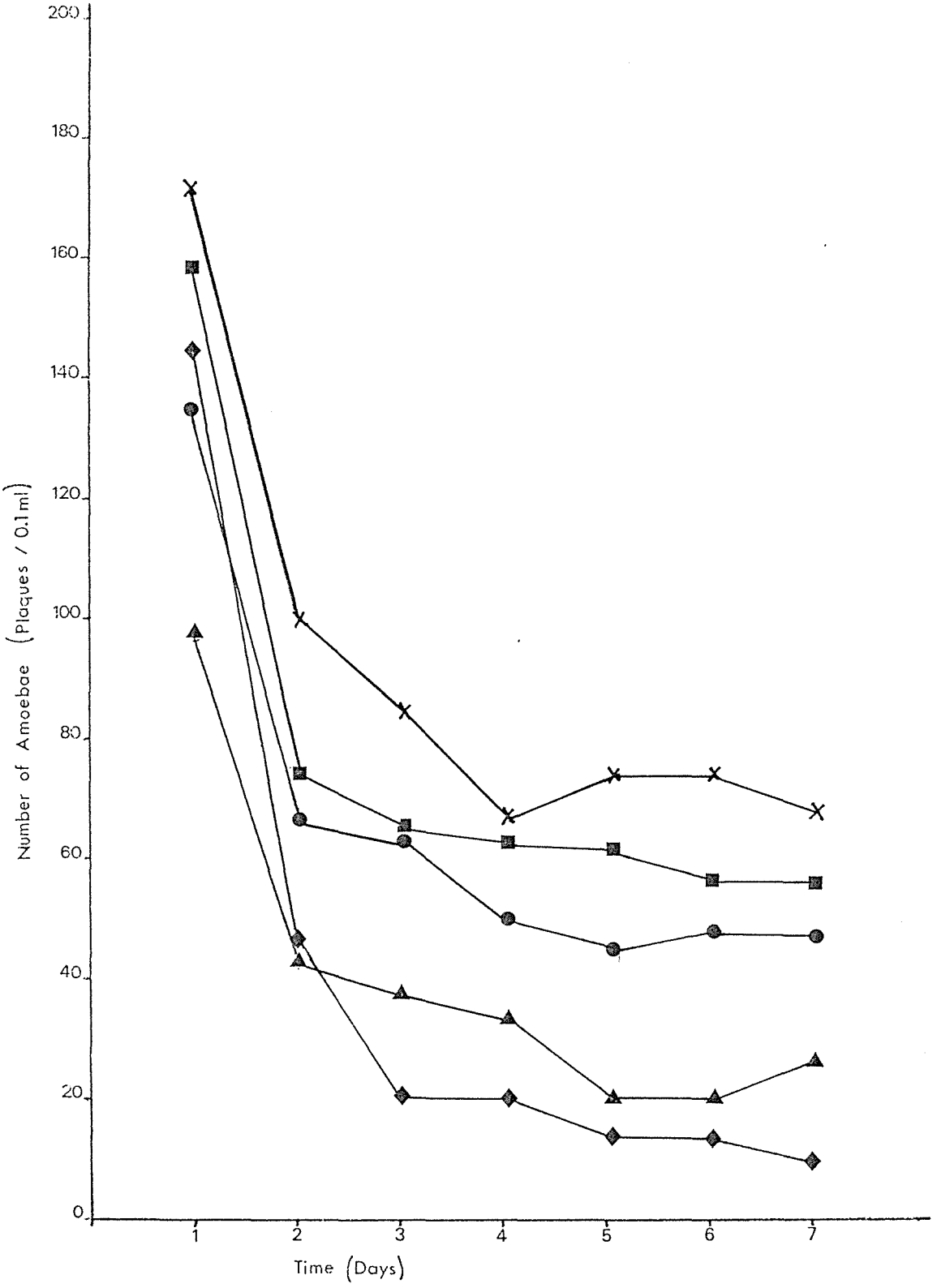


Figure 56b. Effect of cation concentration on survival of *N. gruberi* cysts: 1200 mg l^{-1} of cation.

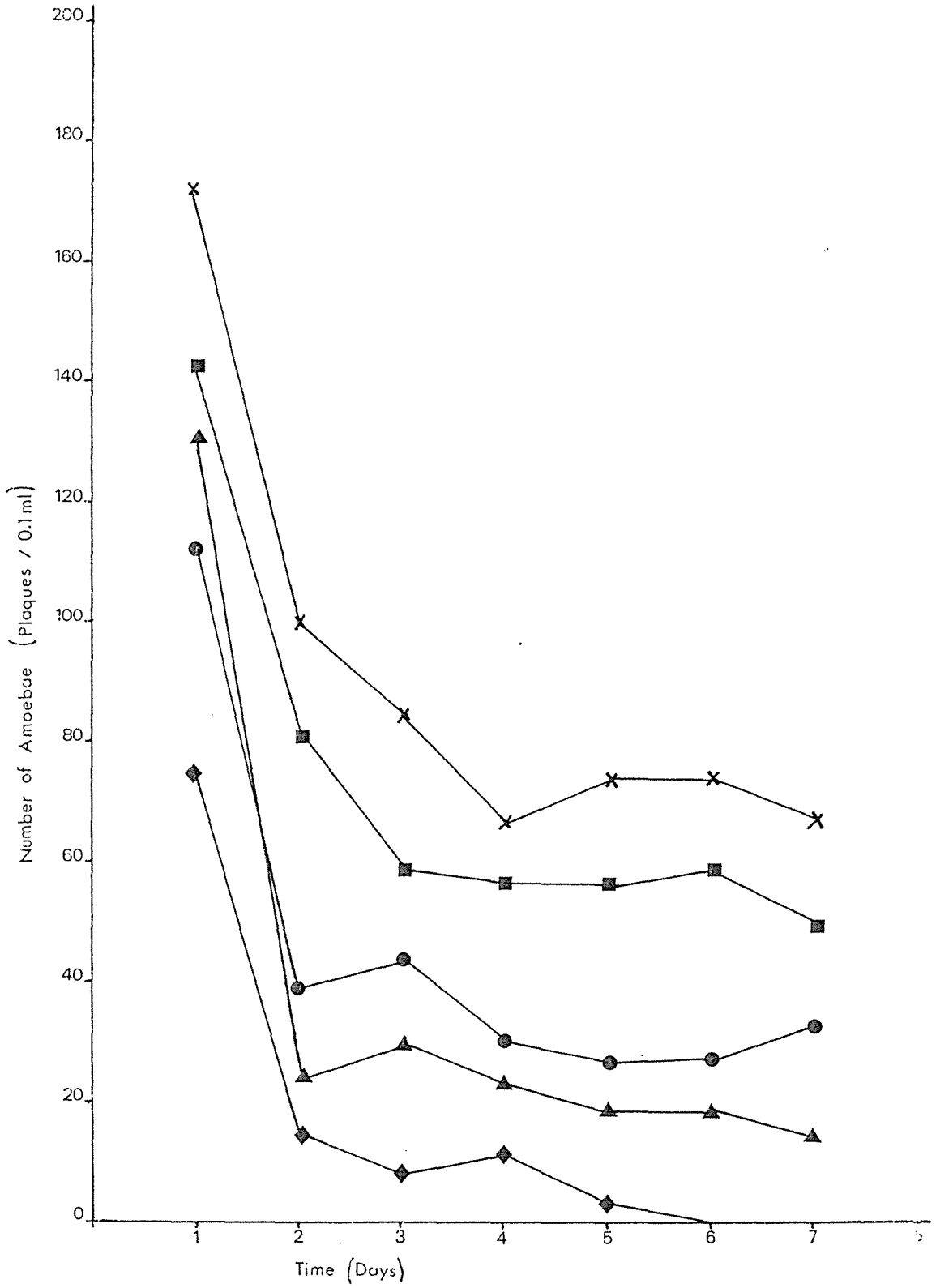


Figure 56c. Effect of cation concentration on survival of *N. gruberi* cysts: 1800 mg l^{-1} of cation.

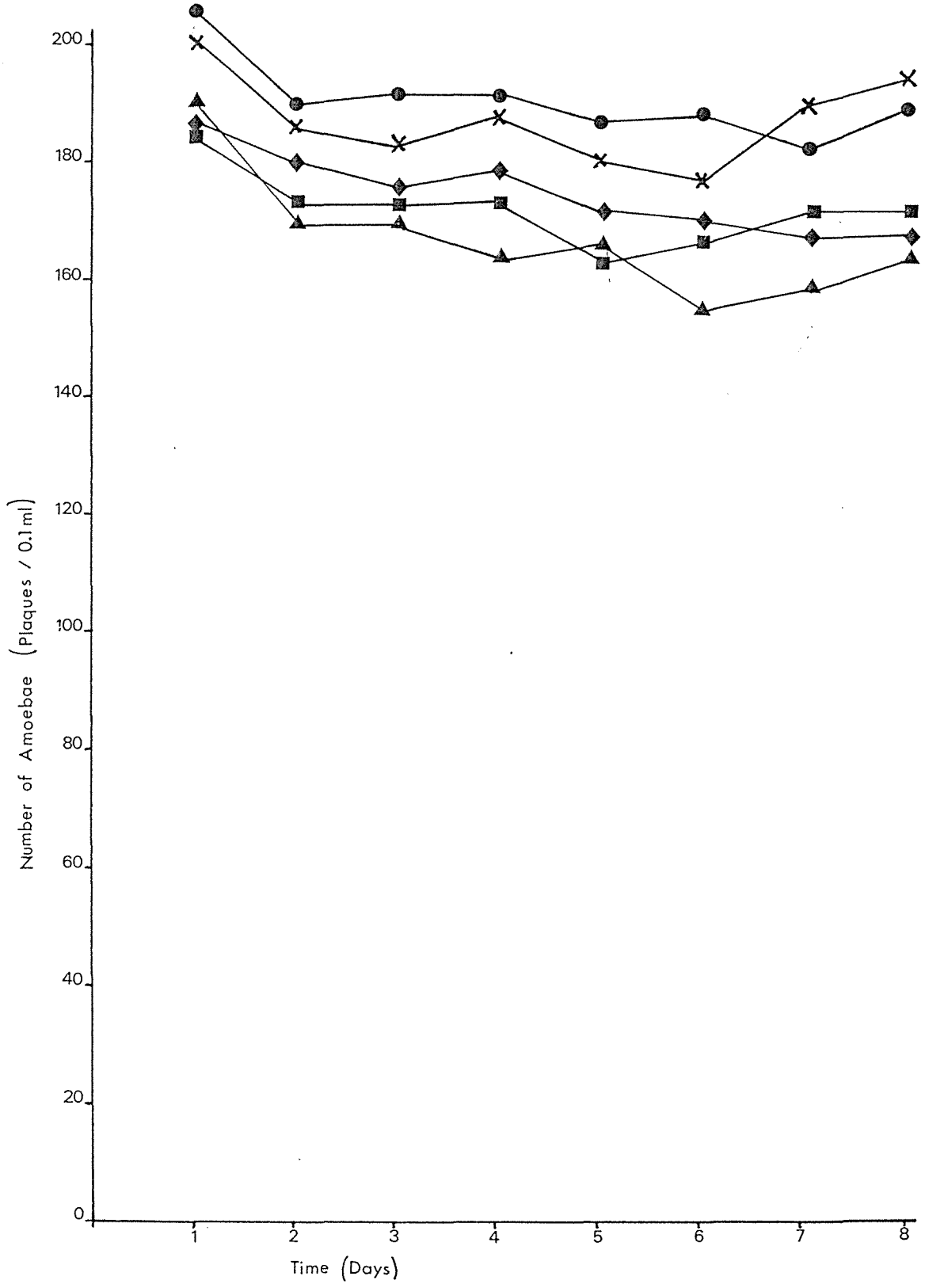


Figure 57a. Effect of cation concentration on survival of *A. culbertsoni* cysts: 600 mgl^{-1} of cation.

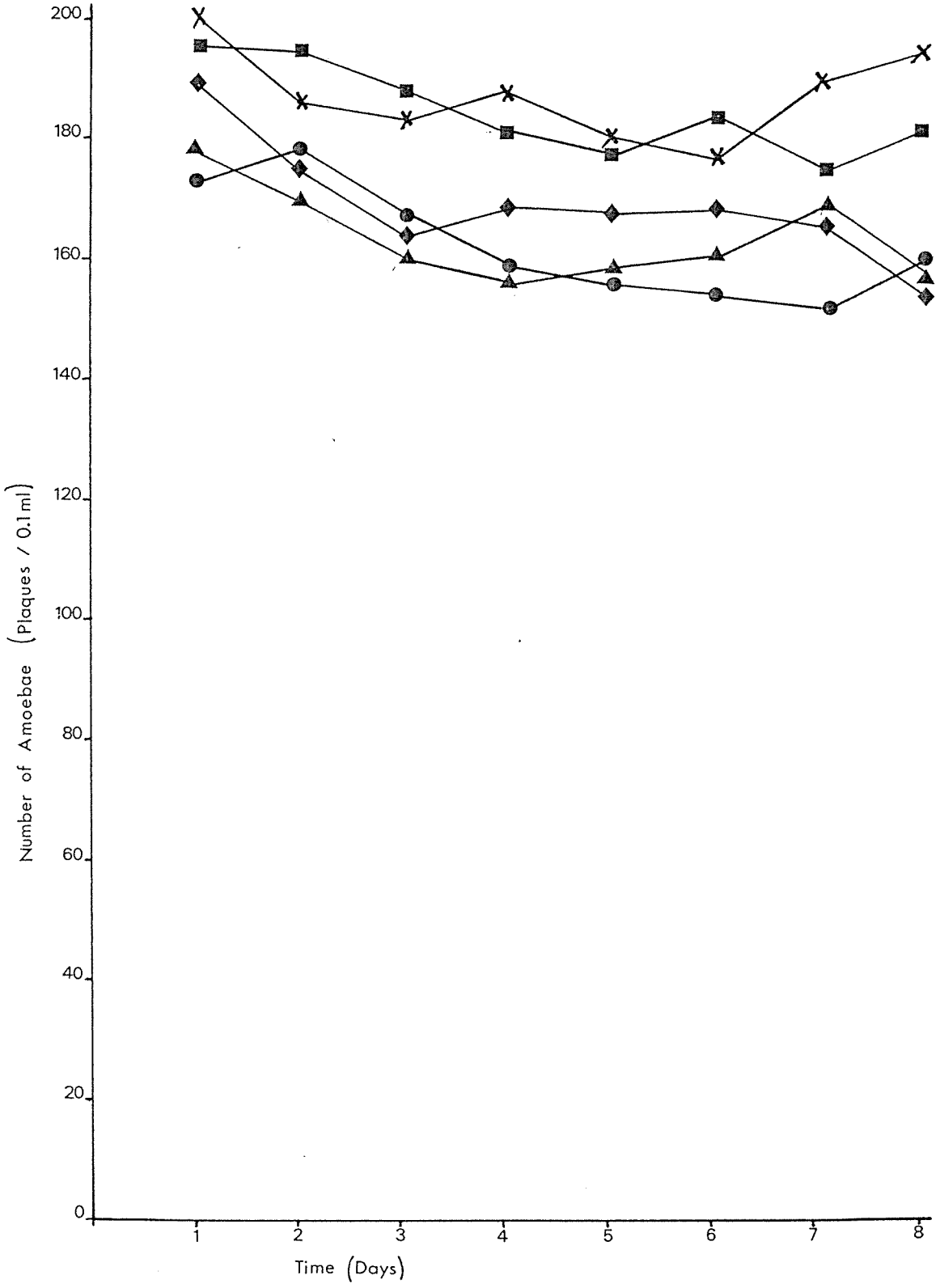


Figure 57b. Effect of cation concentration on survival of *A. culbertsoni* cysts: 1200 mgl^{-1} of cation.

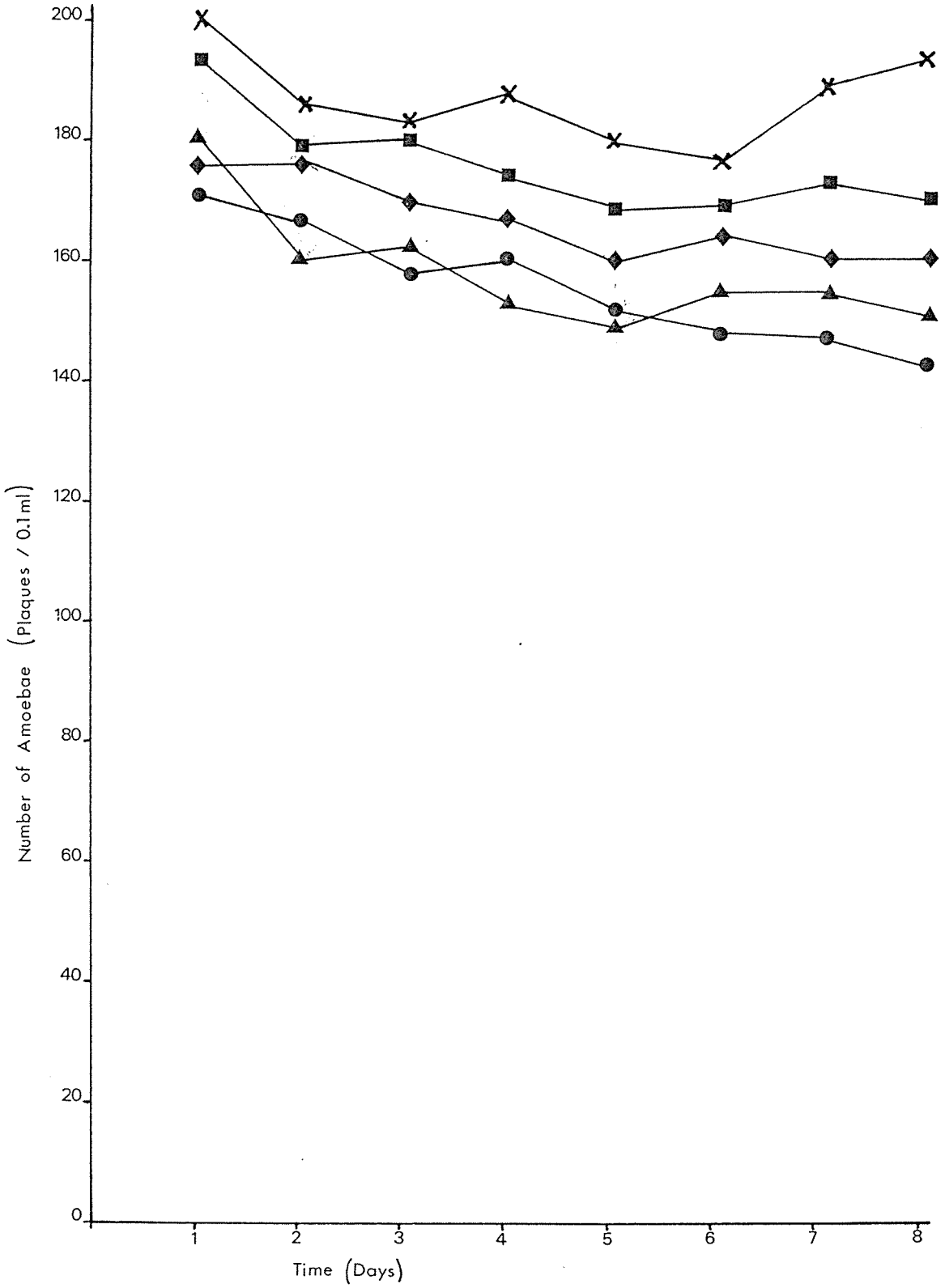


Figure 57c. Effect of cation concentration on survival of *A. culbertsoni* cysts: 1800 mgl^{-1} of cation.

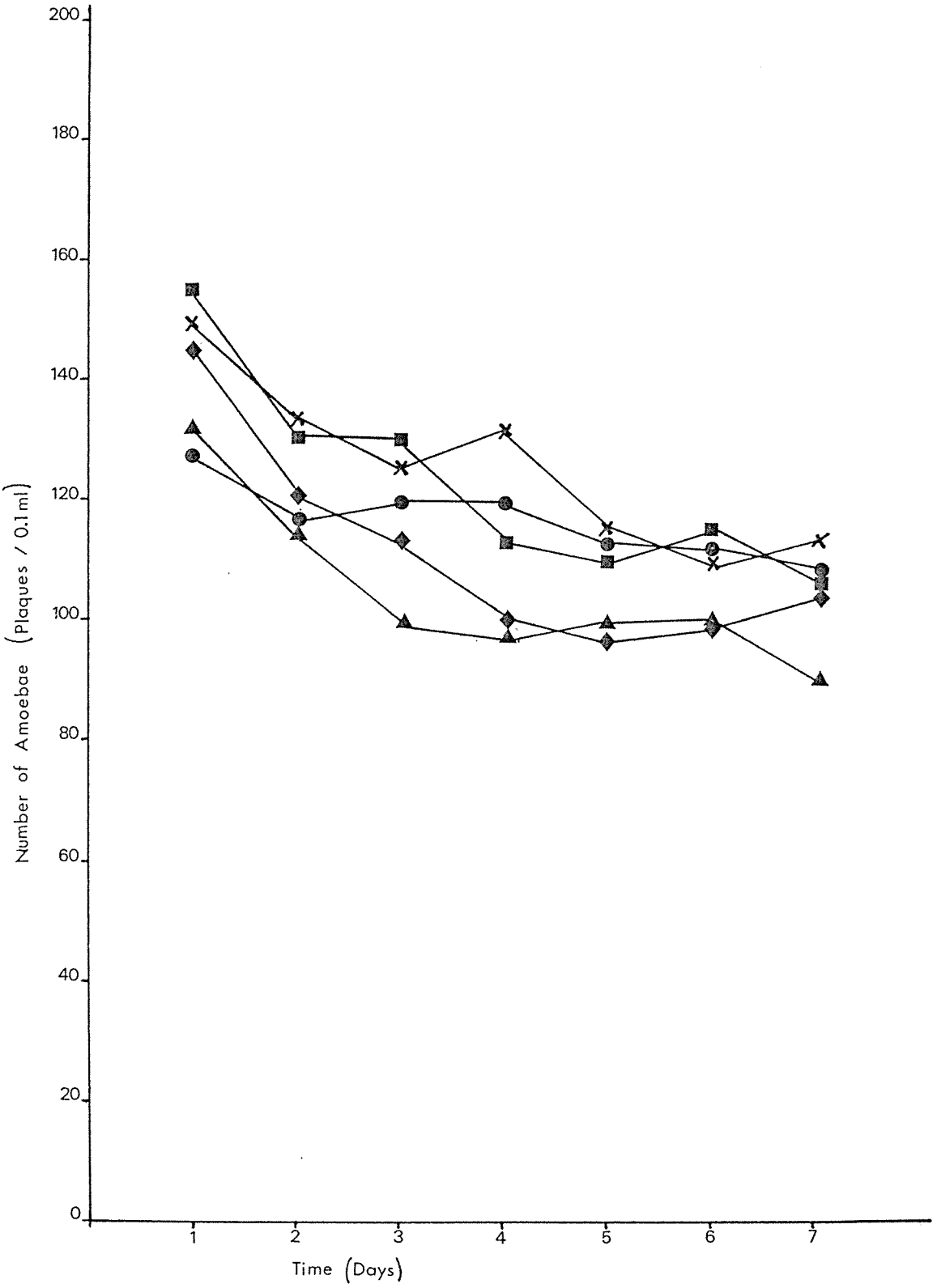


Figure 58a. Effect of cation concentration on survival of *A. castellanii* cysts: 600 mgl^{-1} of cation.

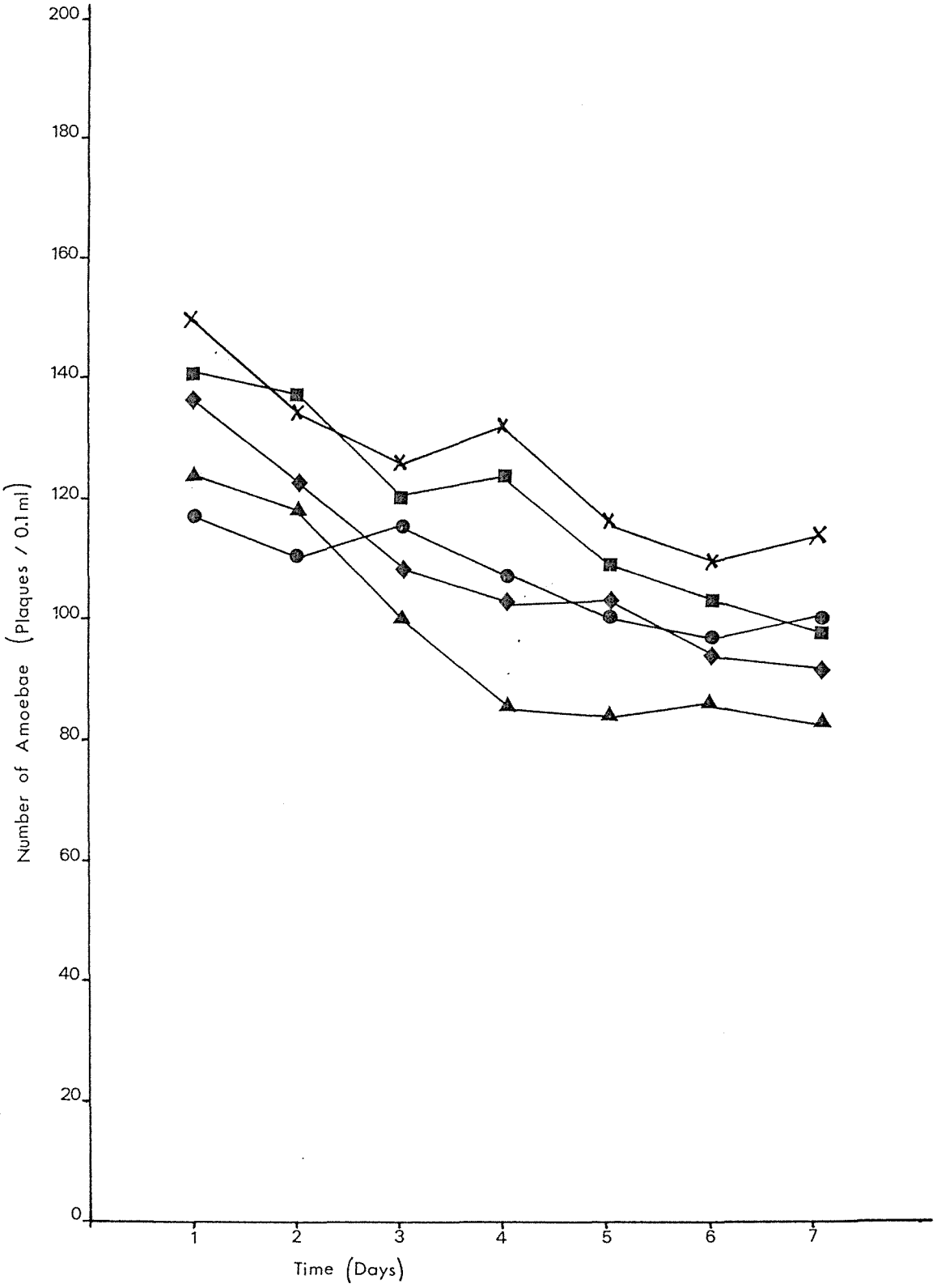


Figure 58b. Effect of cation concentration on survival of *A. castellanii* cysts: 1200 mgl^{-1} of cation.

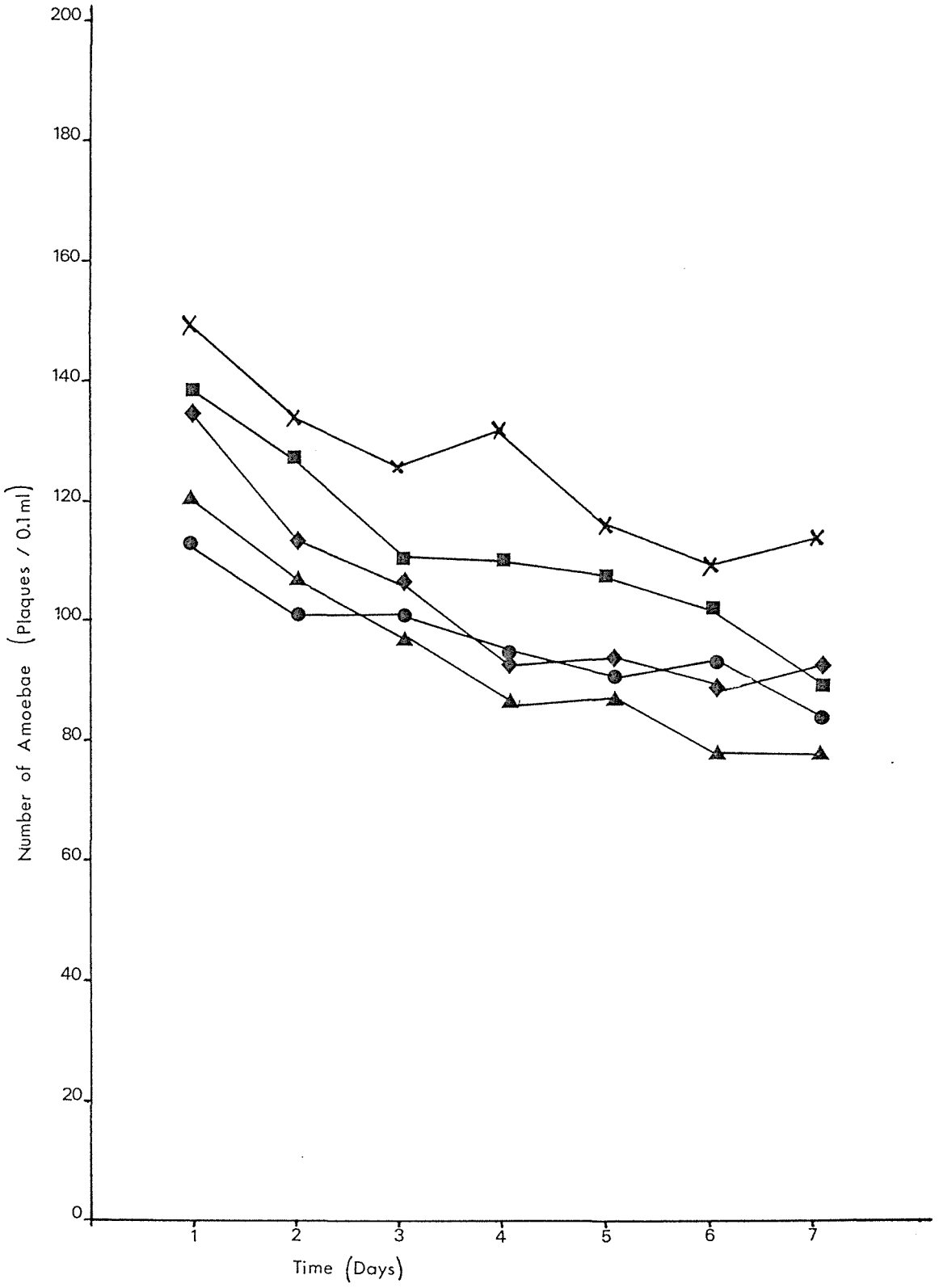


Figure 58c. Effect of cation concentration on survival of *A. castellanii* cysts: 1800 $mg\,l^{-1}$ of cation.

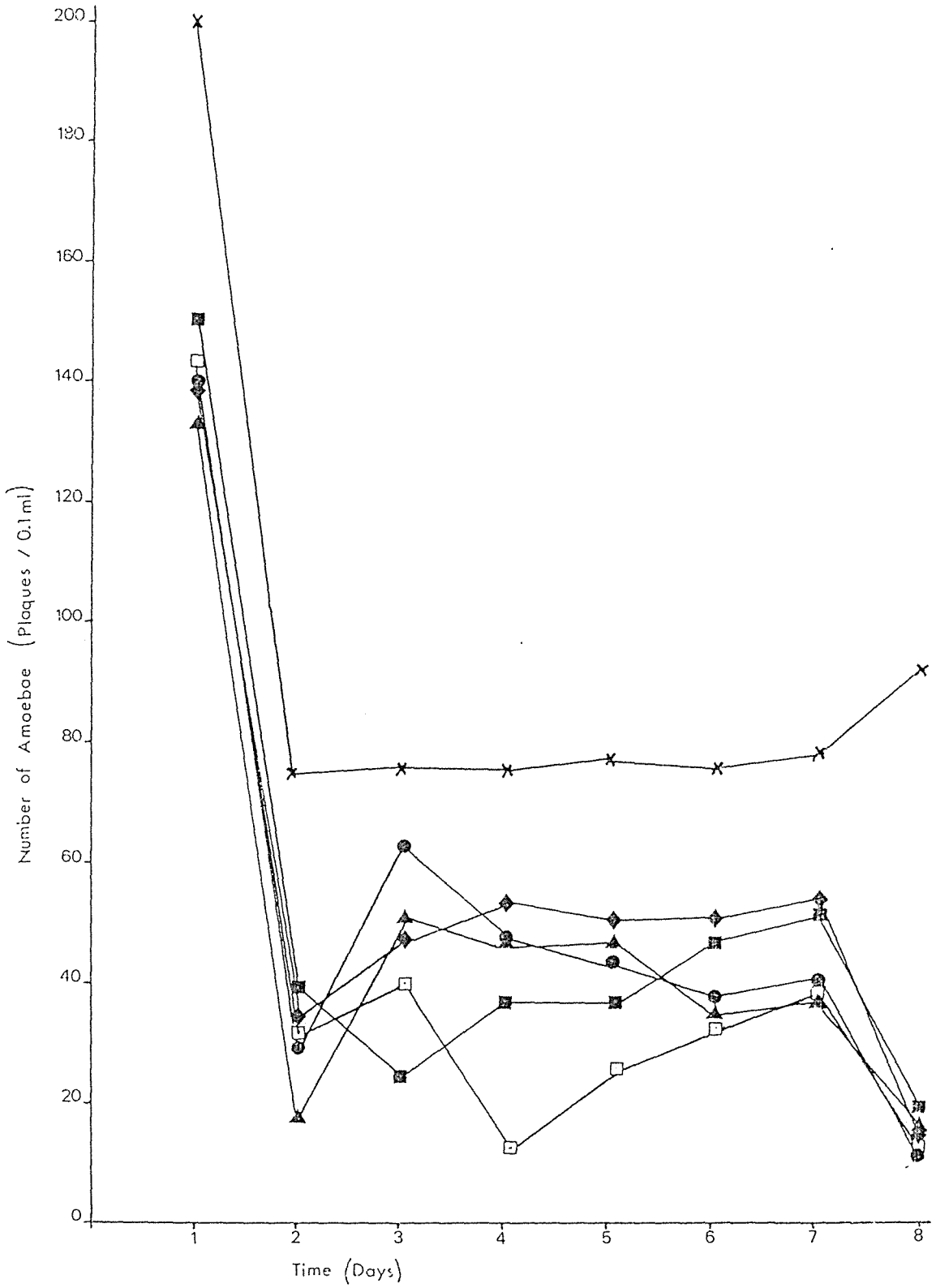


Figure 59a. Effect of iron concentration on survival of *N. fowleri* cysts: Fe^{+3}.

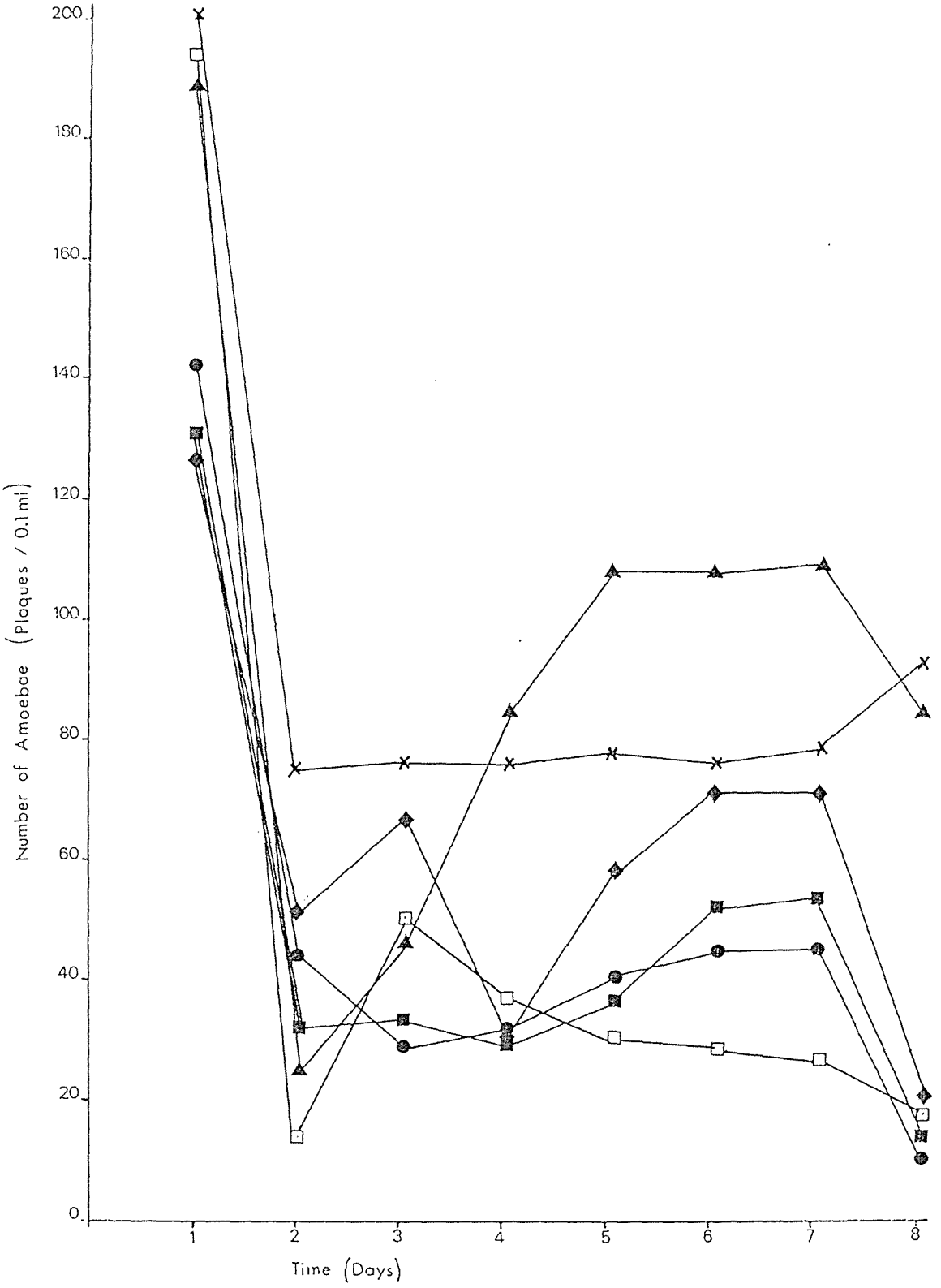


Figure 59b. Effect of iron concentration on survival of *N. fowleri* cysts: Fe^{+2}.

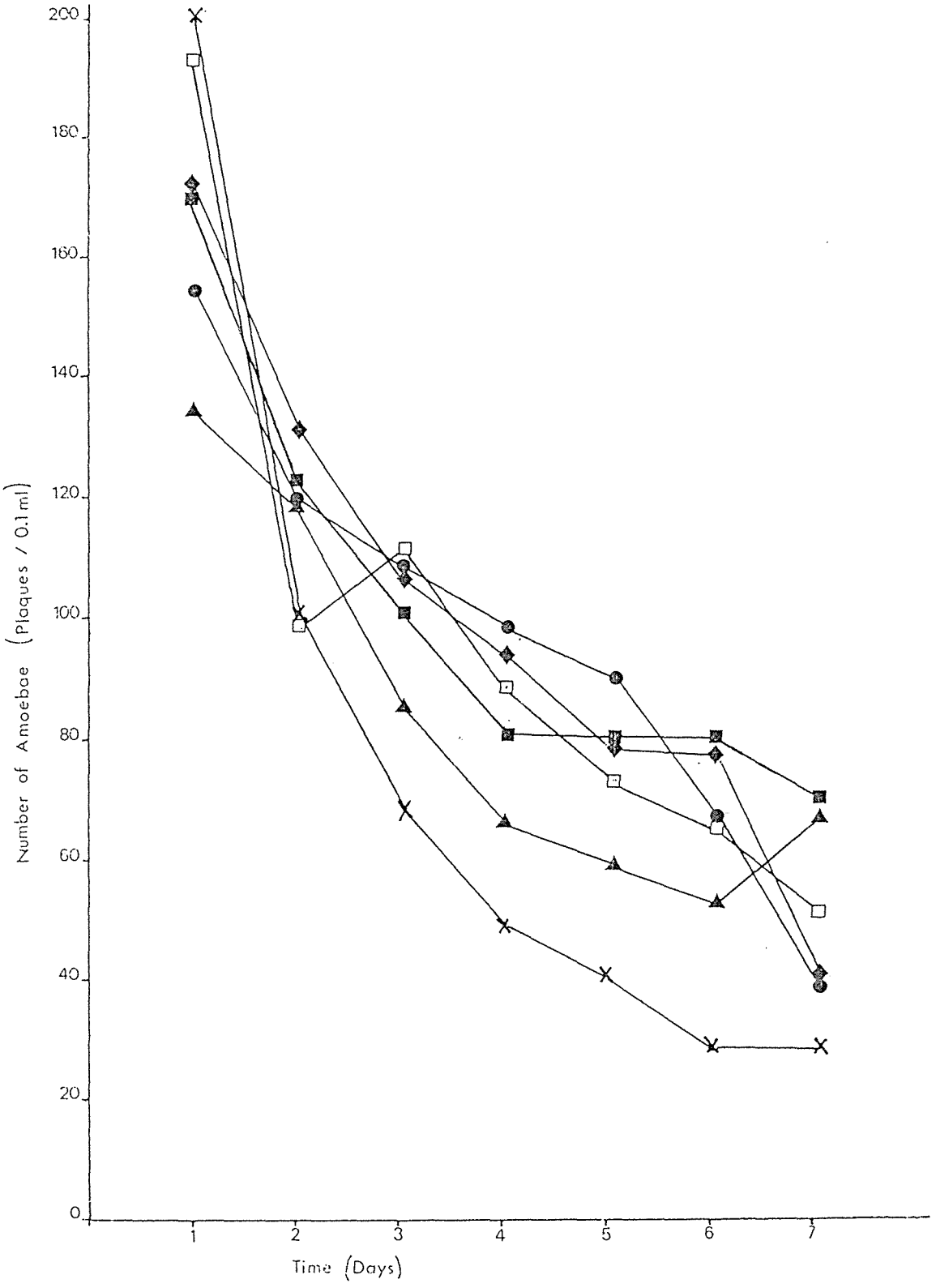


Figure 60a. Effect of iron concentration on survival of *N. gruberi* cysts: Fe^{+3}.

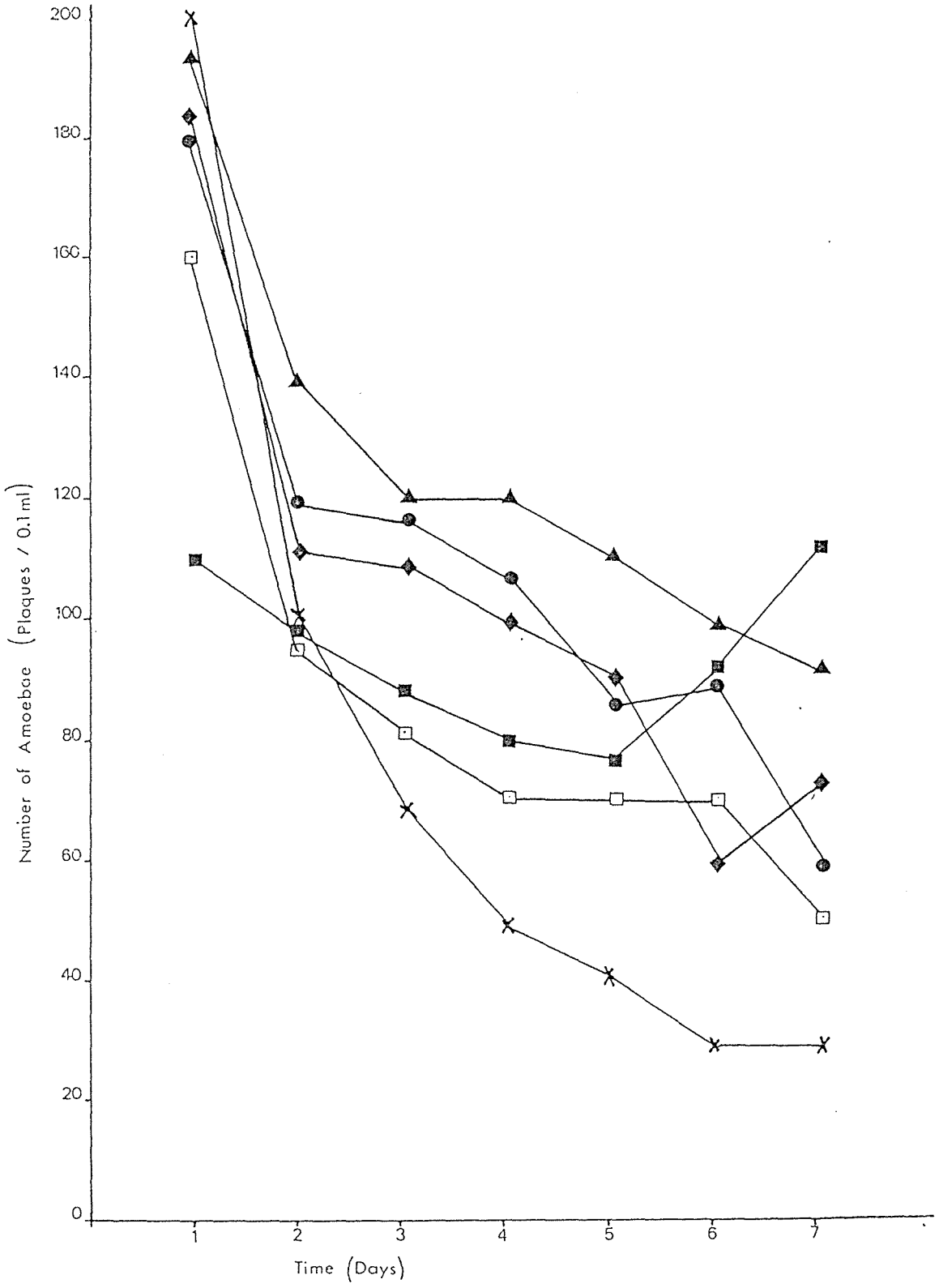


Figure 60b. Effect of iron concentration on survival of *N. gruberi* cysts: Fe^{+2}.

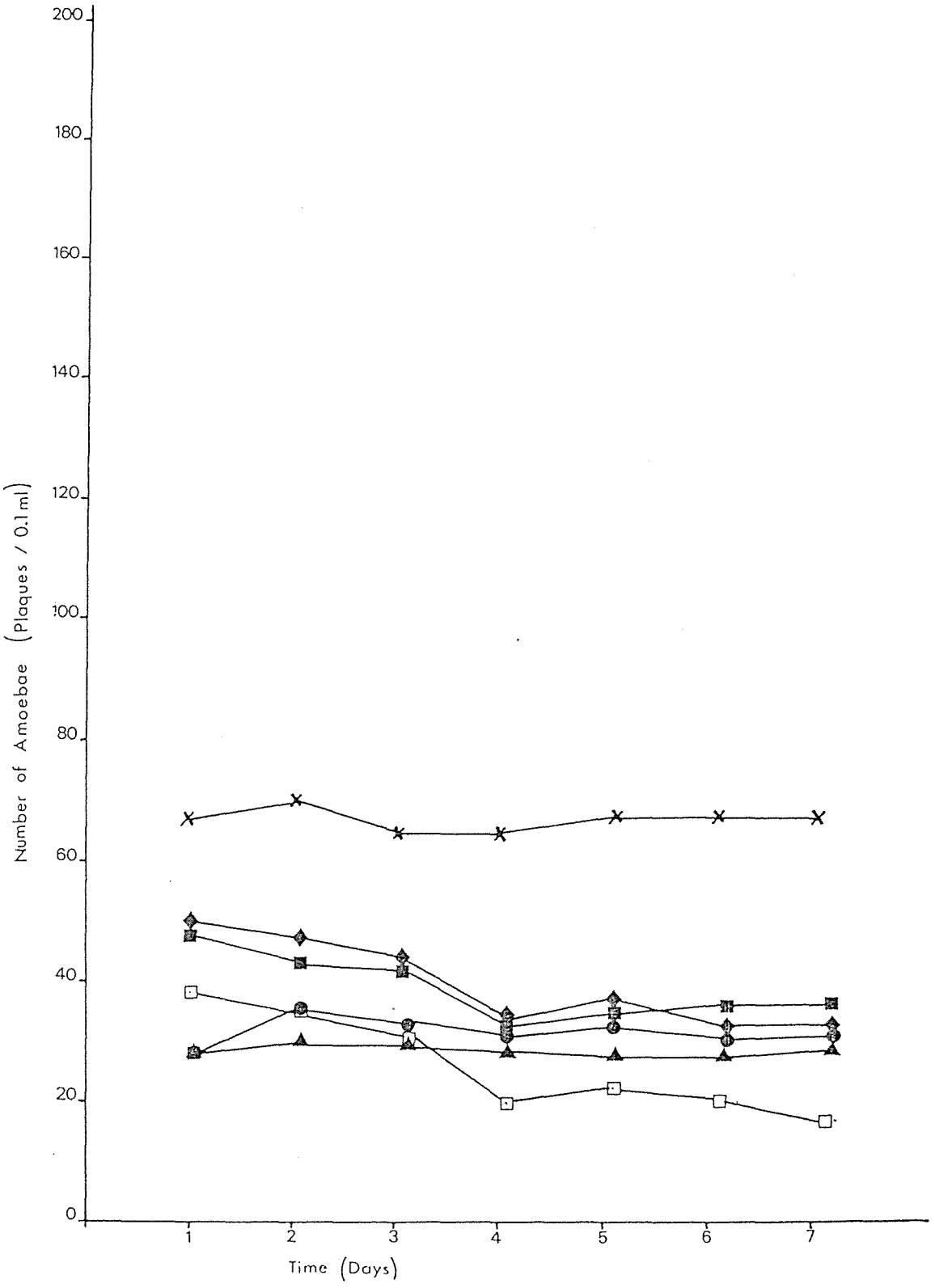


Figure 61a. Effect of iron concentration on survival of *A. culbertsoni* cysts: Fe^{+3}.

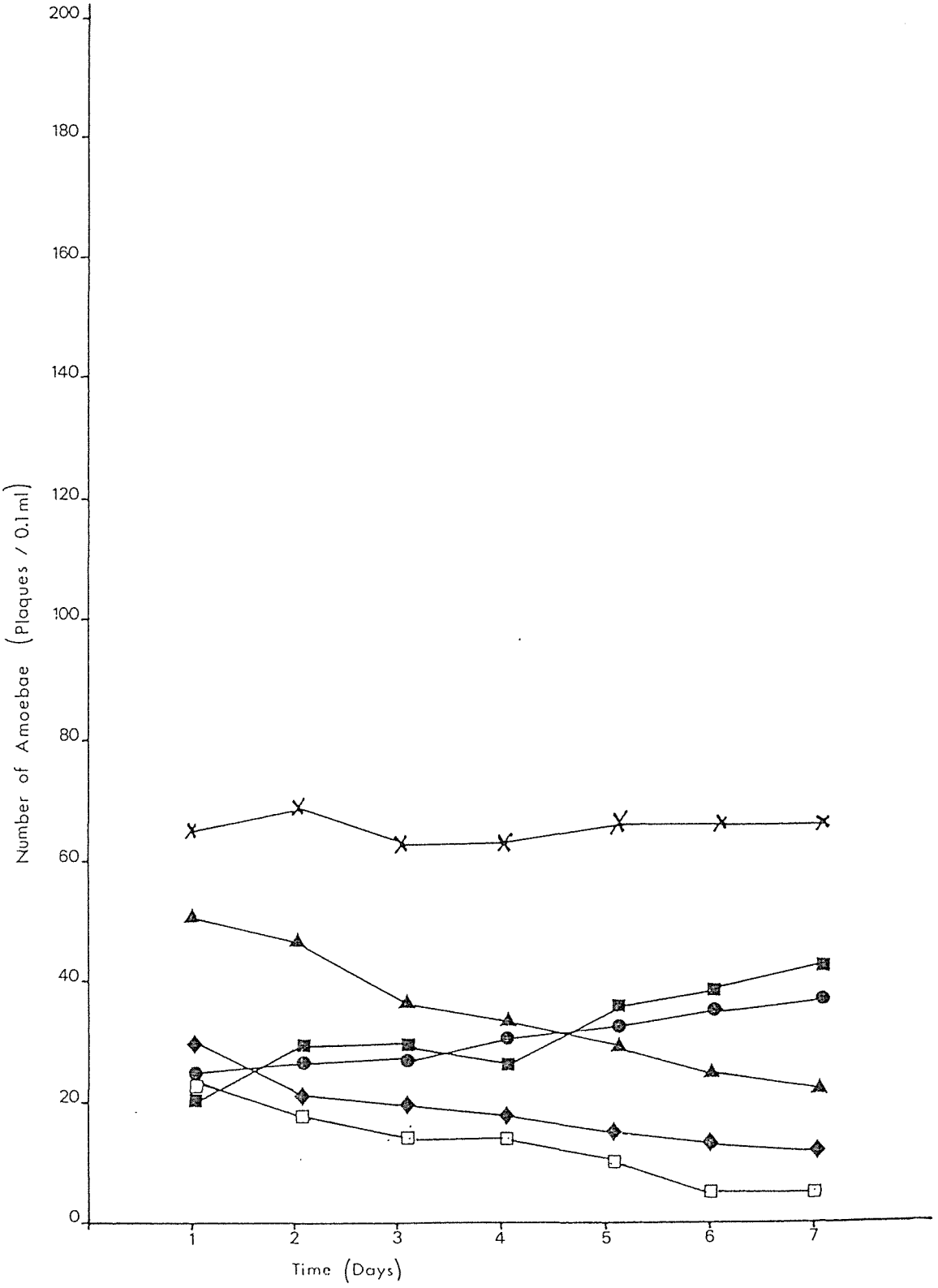


Figure 61b. Effect of iron concentration on survival of *A. culbertsoni* cysts: Fe^{+2}.

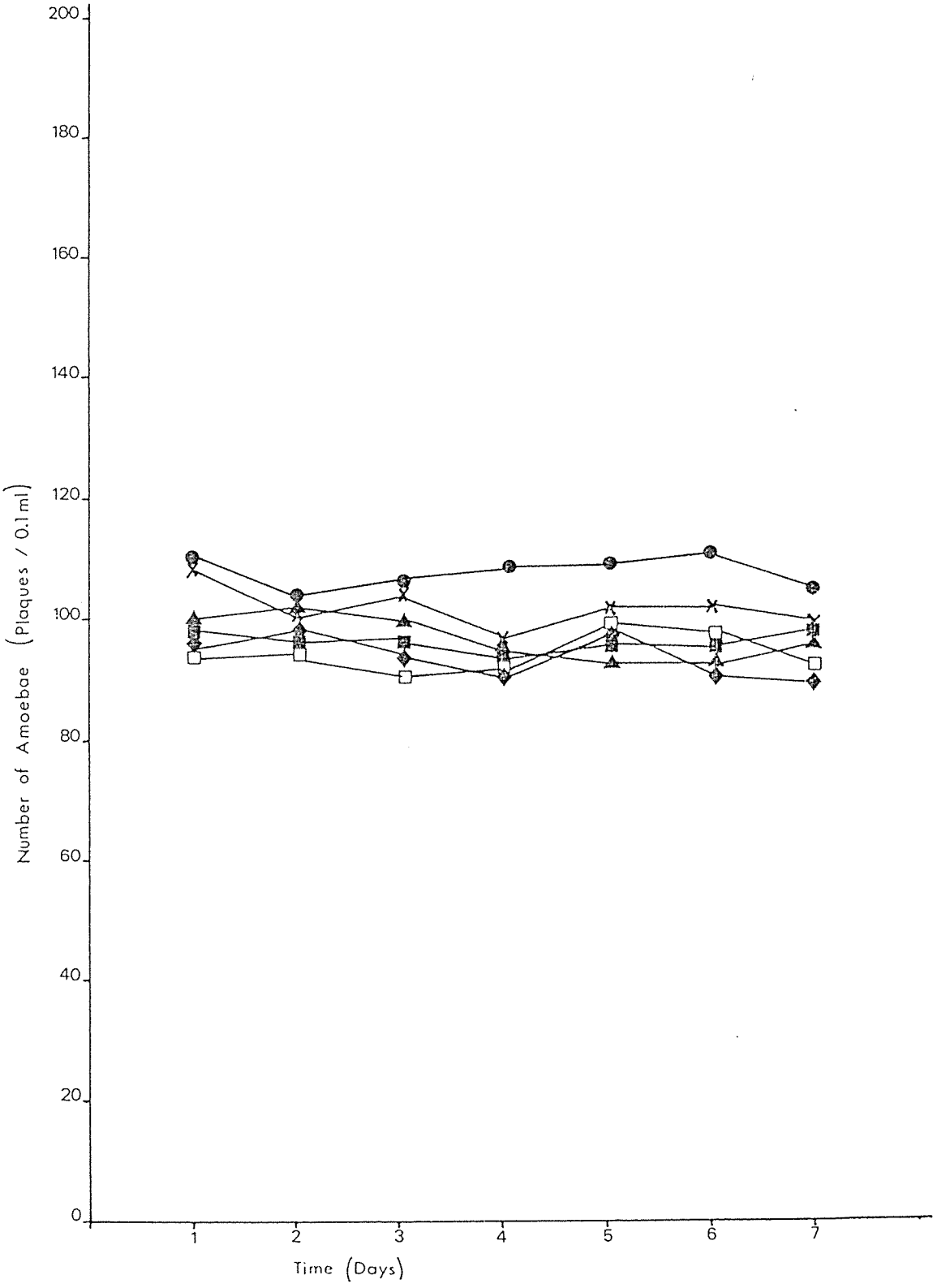


Figure 62a. Effect of iron concentration on survival of *A. castellanii* cysts: Fe^{+3}.

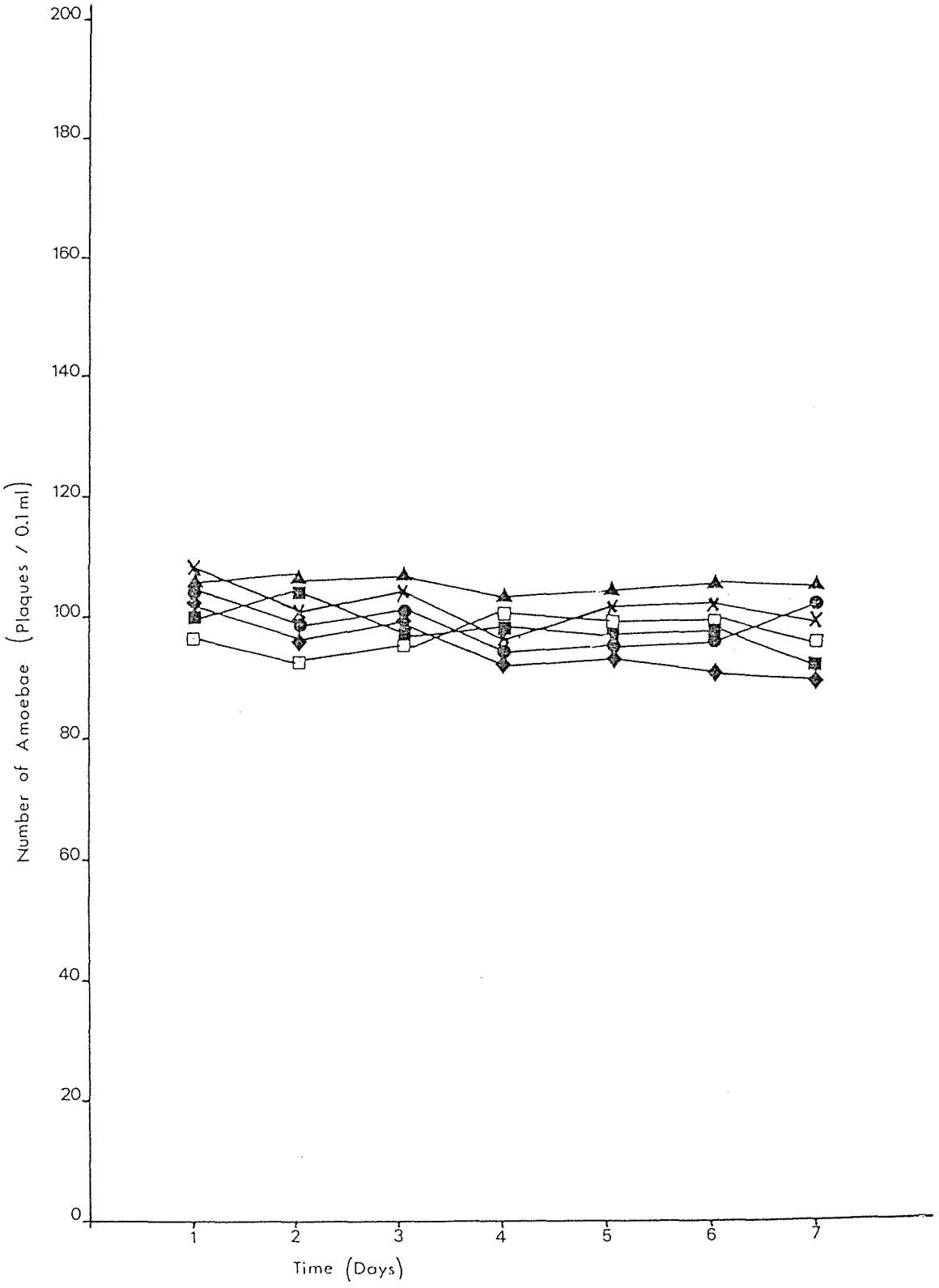


Figure 62b. Effect of iron concentration on survival of *A. castellanii* cysts: Fe^{+2}.

4.5 Preliminary Identification of Unknown Isolates

4.5.1 Morphology

By light microscopy it is not possible to distinguish *Naegleria* spp, except for the fact that *N. gruberi* are generally smaller (Plates 1, 2, 13 and 14).

PR 2448 and PR 2449 appeared identical. They had the characteristic clear-haloed nucleus, rapid pseudopodal movement and granular cytoplasm of naeglerial species. Flagellates of both species were observed but not photographed. The flagellates were similar to those produced by both *Naegleria* species.

In size PR 2448 and PR2449 resembled *N. fowleri* more than *N. gruberi*.

Therefore, they are members of *Naegleria* genus.

4.5.2 Temperature tolerance

N. gruberi PL200f was unable to multiply at 45°C and after 24 hours had formed cysts which remained at a steady level. These cysts were non-viable.

Both *N. fowleri* strains were able to survive and reproduce at 45°C, as were both the Ngawha Springs isolates.

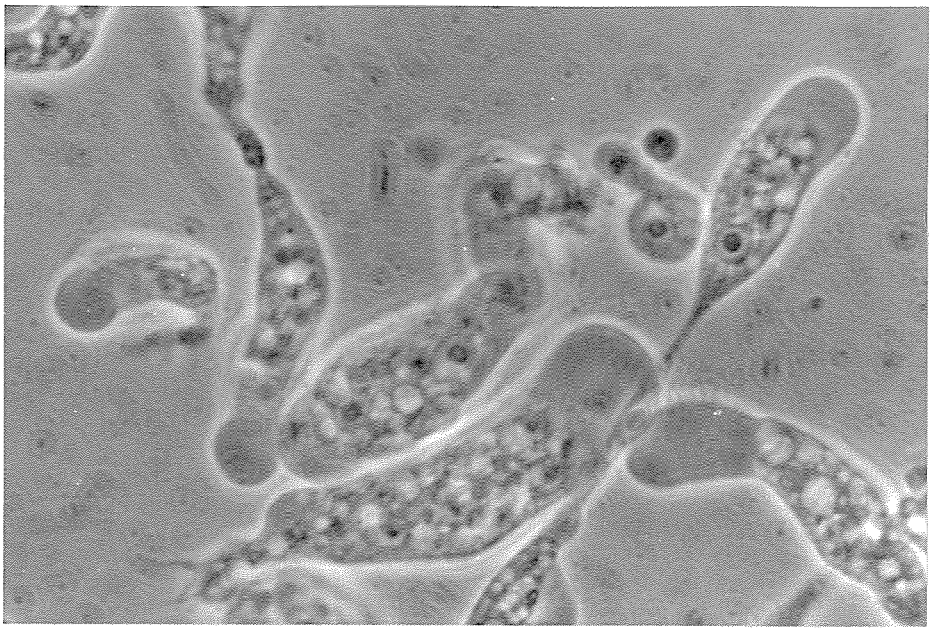
After addition to new media, *N. gruberi* (PL200f) was still unable to grow, but all other species increased their numbers.

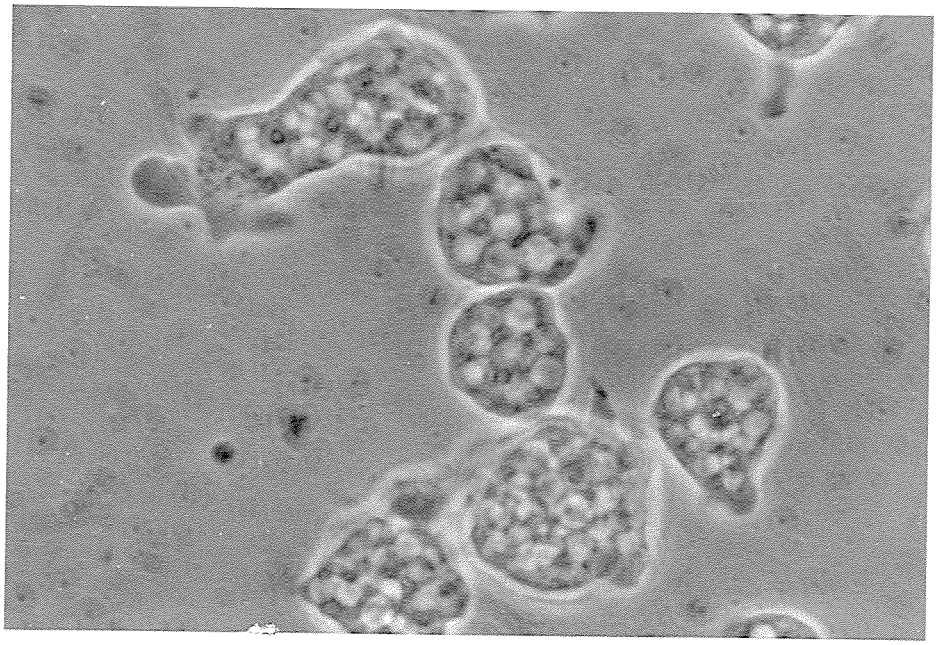
The following legend applies to Figure 63:

■ = *N. fowleri (NHI)*
● = *N. fowleri (MSM)*
○ = unknown PR2448
□ = unknown PR2449
▲ = *N. gruberi (PL)*
------ = indicates transfer of inoculum to new media.

Plate 13. Trophozoite stage of PR2448.

Plate 14. Trophozoite stage of PR2449.





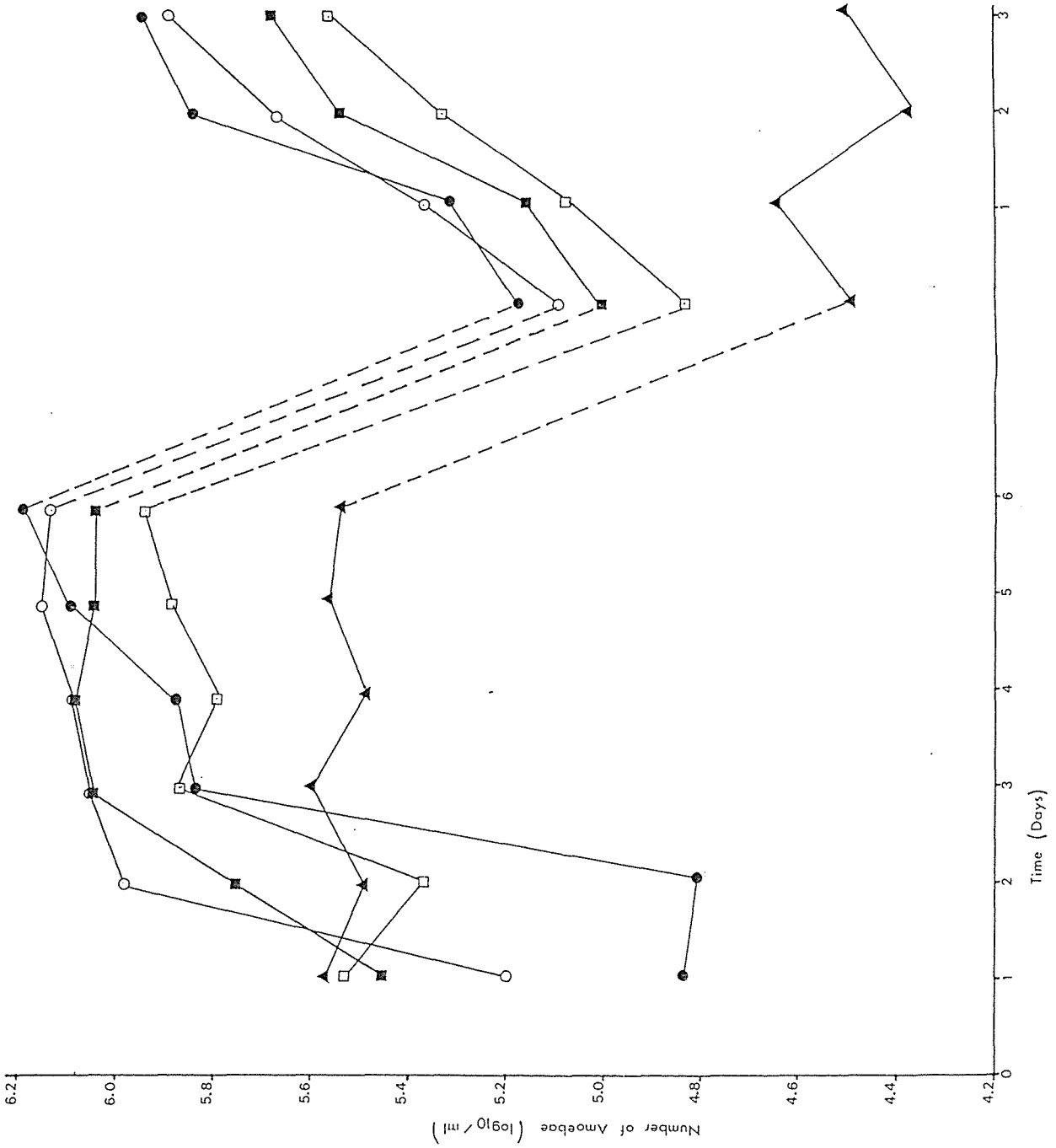


Figure 63. Growth of unknown Isolates, *N. fowleri* and *N. gruberi* at 45°C.

CHAPTER FIVE: DISCUSSION AND CONCLUSIONS

5.1 Cations and Growth

Cations are necessary to amoebae for several reasons, probably the most important being for osmoregulation (Griffiths et al., 1968; King et al., 1979; Larochelle et al., 1978; Neff et al, 1964; Williams, 1970; Willmer, 1963). The cell membrane is permeable to most inorganic ions, and these ions, especially Ca^{+2}, Mg^{+2}, K^{+} and Na^{+}, are necessary to maintain the osmotic balance of the cell (Larochelle et al., 1978a; Neff et al., 1964; Willmer, 1956). Levels of all four are maintained against electrical and concentration gradients in growth media. K^{+} is maintained at an intracellular (IC) : extracellular (EC) ratio of 30:1. The IC concentration of Mg^{+2} is also greater than the extracellular concentration. The IC levels of Ca^{+2} and Na^{+} are maintained at lower levels than the EC concentration (Klein, 1961; Schuster, 1979; Williams, 1970). There are several ways in which these levels are maintained.

(1) Cation binding: ions can be bound to internal cell structures or to the external cell membrane and are therefore held against gradient pressures (Klein, 1961; Schuster, 1979).

(2) Active transport: ions are actively, i.e. utilizing energy or enzyme systems, pumped into or out of the cell against the concentration gradient. This often, as in the case of the Na^{+}/K^{+} pump, involves the coupling of two mechanisms to keep the electrical gradient stable. The diffusion and electrical gradients are in opposition. In amoebae cells, the IC distribution of ions is governed more by electrical forces than by EC levels (Klein, 1959, 1961; Larochelle et al., 1978a; Schuster, 1979).

(3) Diffusion: ions move across the membrane according to the permeability of the membrane. Permeability can be altered by things such as changes in tonicity or osmotic pressure of the media, age or physiological condition of the amoebae (Griffiths et al, 1968; Klein, 1961; Larochelle et al., 1978a).

(4) Water movement: there are two processes by which water enters an amoeba cell:

(a) osmosis: is movement in response to osmotic pressure. This is a passive process (Byers, 1979; Larochelle et al., 1978a; Pal, 1972).

(b) pinocytosis: primarily a feeding mechanism, this is also a major source of water (Byers, 1979; Pal, 1972). Water entering the cell must equal water leaving (Pal, 1972). Water is pumped out of the cell via the contractile vacuole. When EC solute increases, i.e. osmotic pressure on the cell increases, water is pumped out at an increased rate (Pal, 1972).

(5) Leakage: in conditions of stress, eg. during encystment or starvation, there is an increase in membrane permeability and cell constituents leak out (Griffiths et al., 1968; Klein, 1961; Larochelle et al., 1978a). This process is accelerated in distilled water and decreased by solutions containing $MgCl_2$, or NaCl and KCl to a lesser degree (Griffiths et al., 1968; Willmer, 1956). Although Ca^{+2} does not appear to affect leakage, it does alter the membrane permeability when present EC (Larochelle et al., 1978).

Any increase in solute concentration may upset these mechanisms which are necessary for regulating the osmotic balance of the cell. Amoebae trophozoites are susceptible to lysis or plasmolysis if the osmotic balance is disturbed (Klein, 1961; Neff et al, 1958). Increased cation concentration showed an inhibitory effect on all amoebae, though less on *Acanthamoeba* species than *Naegleria*. Altering EC levels of cations could possibly upset the balance of these ions IC by any of the above mechanisms and so alter their utilization within the cell.

The concentrations of cations shown in Figures 1-4 are recorded in molarities in order to give direct compatability between the cations themselves. If the effect observed was due to charge, ionic strength or osmotic pressure, one would expect to see a relationship or similarity between the effects of Ca^{+2} and Mg^{+2}, and between Na^{+} and K^{+} at the same molarity. However no such similarities were observed. This suggests that the observed effects were due to the influence of a particular cation on the cell rather than solely the charge carried or the dissociation properties of the ion-bearing compound, eg. NaCl, $MgCl_2$. This requires the cells to be able to differentiate between different cations

and respond differently to each. Williams (1970) states that Na^+ and K^+ do have different roles within the cell and are not interchangeable. This conflicts, however, with Willmer (1963) who states there is no difference between them.

The following research has been carried out in *Acanthamoeba* cells only, not *Naegleria*. Na^+ and K^+ have similar roles within *Acanthamoeba* cells. They are both charge carriers which are weakly bound within the cell. They both activate IC enzymes and appear to be involved in locomotion. The independent role of K^+ is to neutralize anionic groups inside the cell, and the major role of Na^+ is to keep the osmotic balance (King et al., 1979; Williams, 1970). This suggests that Na^+ is pumped across the cell membrane in one direction or another according to the EC osmotic pressure. However, no Na^+ pump has been found in amoebae (Schuster, 1979). The Na^+/K^+ pump active in animal cells appears not to exist in amoeboid cells.

The results of this study suggest that there is no coupling pump system which involves any two of the cations tested. This does not exclude possible pumping mechanisms coupled with other ions or molecules.

Magnesium and Calcium ions are used to form and stabilize IC structures (Williams, 1970). Mg^{+2} affects the permeability of the membrane, presumably making it less permeable, preventing the leakage mentioned previously. However, it is also involved in control of IC enzymes (Griffiths & Hughes, 1968). EC Ca^{+2} can also affect membrane permeability by causing vesicles to be ejected (Larochelle & Gagnon, 1978; Williams, 1970). EC enzymes use Ca^{+2} as a co-factor (Williams, 1970). Increased EC Ca^{+2} levels have been shown to decrease locomotion (King et al., 1979). It appears that Ca^{+2} levels are finely balanced, as other studies have shown Ca^{+2} to be necessary for locomotion. A study about a flagellation factor Ψ and Ca^{+2} showed that Ca^{+2} is constantly swept into IC reservoirs from which it is released locally to allow movement in that region (Fulton, 1977).

Ca^{+2} is also involved in the IC regulation of Na^+ (Larochelle & Gagnon, 1978). Na^+, as mentioned previously, is important in maintaining the osmotic balance of the cell (Williams, 1970). Any

interference of this function by Ca^{+2} could be detrimental to the cell's survival, i.e. inappropriate Na^{+} levels, caused by Ca^{+2}, could lead to an upset of the osmotic balance. This is a possible mechanism for the inhibition of growth by Ca^{+2}. This inhibition is particularly marked with *Naegleria*. It is interesting to note that *Naegleria* are also very sensitive to high NaCl levels (Carter, 1979; Duma et al., 1969; Culbertson, 1971; Anderson & Jamieson, 1972a; Kadlec, 1974; Neff et al, 1958; Cerva, 1978; Robinson, 1975). Although the above roles of ions, and possible mechanisms of action apply only to *Acanthamoeba*, the current study shows similarities between the behaviour of *Naegleria* and *Acanthamoeba* to the same conditions. It is possible therefore that *Naegleria* have similar mechanisms in action and are affected in the same way.

The effect of Ca^{+2} is such that it could explain the lack of amoebae isolated from Te Puia and Butchers hot pools, especially as these pools also have high Na^{+} levels.

Acanthamoebae are much more resistant to high levels of salt (Figures 5-8; Culbertson, 1971; Kadlec, 1974; Lewis et al., 1979).

Naegleria appear to be more sensitive to most environmental conditions. Perhaps the mechanisms acted upon are more sensitive. It has been claimed that NaCl has disinfection value against *Naegleria*. At 0.7% it was claimed that *N. fowleri* was eradicated from a swimming pool (Anderson & Jamieson, 1972a). This study showed that at NaCl levels greater than 0.5%, amoeba numbers dropped markedly (Figures 5 and 6). However Figures 1-4 show that at lower concentrations Ca^{+2} is more effective at inhibiting amoeba growth and therefore possibly more effective as a disinfectant.

5.2 Encystment

Disinfection does not merely involve the eradication of amoebae trophozoites. Under adverse conditions amoebae encyst and become resistant to many things which affect the trophozoite such as starvation, dehydration, crowding (Corliss & Esser, 1974; Fulton, 1977; Fulton & Dingle, 1967; Neff et al., 1964; Rotiroti & Stevens, 1975; Schuster, 1979). Presumably the membrane is protected by the cyst wall. Osmotic

pressure has been proposed as the most important single factor influencing encystment (Corliss & Esser, 1974). On studies in liquid media on Acanthamoebae, Lorriman and Shafran (1978) showed that specific ions were not necessary for encystment to occur, but simply osmotic pressure. Under natural conditions (i.e. in the soil), dehydration causes an increase in osmotic pressure in the membrane causing encystment. The encystment could be due to either dehydration or increased osmotic pressure or a combination of the two (Bamforth, 1980).

Another school of thought suggests that specific ions, rather than total ionic strength, are required for encystment. Griffiths & Hughes (1967, 1968, 1969) showed that with *H. castellanii*, Magnesium caused maximum levels of encystment, but only did so without the presence of other ionic compounds. Chagla in 1974 working with the same species (renamed *A. castellanii*) found that although Mg is the only requirement for encystment, encystment occurred in any media as long as it was present.

Griffiths & Hughes (1967) found that $CaCl_2$ did not produce encystment, although Kadlec(1975), in a study involving several *Naegleria* and *Acanthamoeba* species found that $CaCl_2$ stimulated encystment. The experiments in Section 4.2.1 were designed to determine the effects of Ca^{+2} and Mg^{+2} on encystment in liquid media. In all cases pure (deionised) water produced the best levels of encystment. This medium is hypotonic to amoebae cells, thereby conflicting with both the ideas that osmotic pressure or Mg^{+2} and Ca^{+2} are necessary. *Naegleria* species could not encyst in PAS, and produced only low levels of cysts when Mg^{+2} and Ca^{+2} were added. Round forms (refer Plates 8-12 and page 159) were produced in larger number when Mg^{+2} or Ca^{+2} were added and appeared to be produced at the expense of encystment. Both *Acanthamoeba* species encysted well in PAS but like *Naegleria* encysted to a lesser degree when Mg^{+2} and Ca^{+2} were added.

It appears that as the ionic strength of the medium increases, encystment decreases although more round forms are produced. Contrary to many reports, (Griffiths & Hughes, 1967, 1968, 1969; Chagla, 1974; Kadlec, 1975) the present study has shown that Ca^{+2} and Mg^{+2} are not necessary for encystment to occur, but are inhibitory toward encystment. However the work of Carter (1979) and Lasman and Shafran (1978) provide

support for the current findings (Corliss & Esser, 1974). Lasman and Shafran (1978), working with *A. palistinensis* in liquid media, showed that specific ions are not necessary, but that encystment occurs in response to appropriate osmotic pressure. Mg^{+2}, K^{+} and Na^{+} all produced similar levels of encystment. Ca^{+2} has not been found to be good for producing cysts (Chagla et al., 1974; Lasman, 1978).

Amoebae are soil organisms, so attempts were made to test for encystment under more natural conditions using soil extract broth (SEB). All species except *N. gruberi* encysted to >80% in SEB. Contrary to the results shown in Figures 15-30, the results in SEB showed round forms produced prior to encystment, rather than in lieu of, which is more the expected pattern of encystment. The changes which occur during encystment depend to some extent on the media used, but the following three steps always occur (Chagla et al., 1974; Griffiths & Hughes, 1969; John, 1982):

(1) Rounding of cells to form a round form or pre-encystment stage (Neff et al., 1964; Bowers & Korn, 1969);

(2) Food vacuoles discharge their contents, and contractile vacuoles discharge water (Bowers & Korn, 1969);

(3) Metabolism switches from carbohydrate breakdown to cell-wall synthesis. Enzyme activity increases and there is a loss in protein and nucleic acid synthesis (Griffiths & Hughes, 1969; Raizada & Krishna, 1971; Neff & Neff, 1972; Jeffery & Hawkins, 1976).

The results suggest that the round forms produced in SEB are true pre-encystment forms which can contrive through the next two steps to become true cysts. However, in PAS or water with Mg^{+2} or Ca^{+2} added, the round form stage appears to prevent the formation of cysts. This could be due to the presence of something inhibiting the two final stages. Alternatively, the round forms produced may not be a pre-encystment form, or further, a factor necessary for encystment is absent. The third possibility is unlikely, as encystment proceeds in pure water which contains nothing more than the above media do.

Mg^{+2} is involved in the following cellular processes:

(1) membrane permeability (Griffiths & Hughes, 1968; 1969);
(2) leakage from the cell (Griffiths & Hughes, 1968; 1969);
(3) stability of the cell membrane (Jehan et al., 1975);
(4) control of lysis due to hypotonicity (Griffiths & Hughes, 1966)
(5) cyst-wall synthesis (Kadlec, 1974);
(6) enzyme activation (Griffiths & Hughes, 1968, 1969).

The first four roles involve action on the cell membrane. Step 2 in the encystment process involves the ejection of cell contents via membrane structures. As inhibition of encystment appears to occur at Step 2, the inhibitory action of Mg^{+2} could be due to an imbalance of the Mg^{+2} levels necessary for maintaining normal activity. The action of Mg^{+2} on decreasing leakage from the cell during encystment appears contrary to the fact that leakage always occurs during successful encystment. Stopping this leakage could effectively halt encystment.

Ca^{+2} is implicated in the formation of the cyst wall (Kadlec, 1974). However, Ca^{+2} also has an effect on the cell membrane when present EC (Williams, 1970). Ca^{+2} is linked with the ejection of vesicles through the cell membrane (Williams, 1970). Possibly the action of Ca^{+2} on encystment could be due to an increase in the expulsion of cell contents, i.e. the opposite effect to Mg^{+2}.

In Table 4, analysis of basal media shows that PAS (encystment of *Naegleria* was not observed with or without addition of Ca^{+2} or Mg^{+2}) has lower levels of Ca^{+2}, Mg^{+2} and K^{+} than SEB which produced good encystment. However water, which also produced good encystment, has no K^{+}, Mg^{+2} or Ca^{+2}. There is a possibility, however, as SEB is an undefined complex medium, that the ions may be bound in compounds which effectively remove them from solution, leaving the available ions at a negligible level. The level of Na^{+} in PAS is higher than that in water or SEB and so could be the only one, if any, of the cations tested to be significant for inhibition in basal media. During encystment the K^{+}:Na^{+} ratio drops, suggesting that there are critical levels of these ions in both active trophozoites and the encysting cell (Klein, 1959). any external excess of Na^{+} may change this critical ratio, as may an

external excess of Ca^{+2}, if, as suggested previously, it interferes with the regulation of Na^{+}. A change in the ratio of K^{+}:Na^{+} may therefore cause encystment to halt.

If, as suggested, Mg^{+2} and Ca^{+2} change the permeability of the membrane, the selective losses of 90% K^{+} and 70% Na^{+} may be altered, upsetting the critical ratio, and so inhibiting encystment.

On solid media, encystment generally proceeded to produce higher levels than in liquid media. Both *Acanthamoeba* and *Naegleria* species showed best encystment on low nutrient agars as opposed to high nutrient. This fact is supported by Srivastava & Shukla (1983), who found that *A. culbertsoni* will not encyst on nutrient-rich agar. *N. gruberi* however encysted best on nutrient agar.

High osmotic pressure has been proposed as an important encystment stimulus (Corliss & Esser, 1974; Lasman, 1978). However high nutrient agar exerts a higher osmotic pressure than low nutrient agar. The reason for low encystment on high nutrient agar could be based on the fact that as nutrients were freely available, there was no starvation stress and therefore no encystment. The results shown in Figures 39-54 and Section 4.3 all suggest that osmotic pressure is not the most important stimulus for encystment. In the case of section 4.3, starvation would appear to be more important (Neff et al., 1964; Rotiroti & Stevens, 1975).

Table 5 showed analysis of the supernatant off agar plates after growth had occurred. This was undertaken to determine whether any ions were utilized during growth and encystment. The results showed that with the possible exception of Fe and Acanthamoebae species in PASB agar, no ions were utilized, or incorporated into cells during growth and encystment. The levels of most ions actually rose, indicating that either some cells had died to release them,or that living cells ejected them (naturally or in response to the medium). This could occur in an effort to establish some sort of equilibrium with the surrounding medium.

5.3 Cyst Survival

The fact that Ca^{+2} is inhibitory to encystment of *N. fowleri* allows Ca^{+2} to still be considered as a possible disinfectant. However, once encysted, amoebae are very resistant to environmental conditions, and an effective disinfectant regime must eradicate cysts as well.

Acanthamoebae cysts, like their trophozoites, are more resistant to adverse conditions than *Naegleria*. A study by Chang (1978) showed the differing resistance of *N. fowleri* and *N. gruberi* to environmental conditions. Although he found *N. fowleri* to be more resistant to high tempratures when encysted, *N. gruberi* was more tolerant of low temperature extremes. However, Warhurst et al. (1980) found that *N. fowleri* can survive at least 8 months encysted at $4^{o}C$. A study by the author and colleagues (Biddick et al., 1984) showed *N. fowleri* cysts are capable of surviving 4 months at $-10^{o}C$ and 6 months at $4^{o}C$.

N. fowleri cysts, although the trophozoites are killed in 0.89% (physiological) saline, are able to survive in sea water (approximately 2%) (Carter, 1969). The cyst wall must therefore protect the amoebae from the effects of osmotic pressure and any other effects of salinity. The ability of the cyst wall to protect the amoebae against some factors, such as dessication, is under debate. Chang (1978) in an extensive study of cyst resistance to environmental conditions, found that both *N. gruberi* and *N. fowleri* are killed on drying. Similar observations have been repeated by Cain et al. (1981), John (1982), Warhurst et al. (1980) and Carter (1969). However Lawande et al. (1979a) found viable cysts in the dust of the desert in Nigeria and therefore claims they are resistant to dessication. He is supported by Lyons and Kapur (1977) and Duma et al. (1969) who also suggest that dessication does not decrease the viability of cysts. Very little work has been done on the ability of cysts to survive under different conditions and their ability to survive for any time under adverse conditions.

The standard method of storage of amoebae is in encysted form on slopes of agar, stored at room temperature (Byers, 1979). Table 6 shows that all four species can survive this treatment for at least 12 months. However, encystment in nature occurs generally in response to lowering temperatures as a protective mechanism, usually to survive over winter.

Excystment occurs in response to warmth of spring and summer. Encystment of *N. fowleri* occurs in response to 12-16^{o}C when the trophozoites are beginning to slow down and their survival decrease (Sykora et al., 1983). *N. gruberi* trophozoites, however, can survive in lower temperatures than this so encystment is not as imperative (Chang, 1978).

Once encysted, the organisms can presumably survive in this form until removal of the adverse condition occurs, eg. onset of spring/summer temperatures; overwintering must last all winter, at least 3 months under cold conditions. Considering the lower temperature tolerance of *N. gruberi* as opposed to *N. fowleri*, and the suggestion of greater resistance of *N. gruberi* cysts, the results of this study are surprising, showing *N. gruberi* to be far less tolerant of low temperatures than *N. fowleri*. Possibly *N. gruberi*, the trophozoite being more low-temperature-tolerant, has less need to encyst and therefore the encystment mechanism or the cyst itself are not as efficient or successful as those of *N. fowleri*.

The fact that both *N. gruberi* (PL) and *N. fowleri* (NHI) are New Zealand isolates, means that they would have rarely been subjected to such low temperatures. No environmental pressure was present requiring efficient encystment in response to low temperature.

Considering the ability of *N. fowleri* cysts to survive sea water concentrations of NaCl, compared with the intolerance of their trophozoites to similar conditions, the effect of cation concentration on cyst survival was not expected to be significant. However, the trends relating to the survival of trophozoites were expected to be much the same as those relating to survival of cysts, i.e. that Ca^{+2} would have the most inhibitory effect and K^{+} the least. The range was small, but for all cations tested, as concentration increased the inhibitory effect increased. Na^{+} eliminated all *Naegleria* amoebae and was the only ion to do so. This could be explained by suggesting that the cyst wall is selectively permeable, or that the small size of the Na^{+} ion is able to permeate the cyst wall. Under higher concentrations more Na^{+} may be able to enter the wall and affect the membrane. Acanthamoebae are more resistant to all conditions tested than *Naegleria*. This could be due to the possession of a very thick double-layered cyst wall, as opposed to Naegleria's single-layered wall.

5.4 Fe and Amoebae

In most growth media Fe is initially added as part of undefined substances such as serum, or bound in complex molecules like haemin. It has been established that there are interspecies differences in requirements for, and tolerance of, iron levels (Haight & John, 1980; O'Dell & Stevens, 1973). Both *Naegleria* species require iron in their environment. In a study by Newsome & Wilhelm (1983b) the need for iron in growth media of *Naegleria* was demonstrated. Iron chelators produced by bacteria were added to *N. fowleri* trophozoites and produced a decrease in growth rate of the amoebae. However when iron was added, the growth resumed at the normal rate.

In a survey of lakes in Virginia, Duma (1981) concluded that a high iron concentration may be important in determining the presence of *Naegleria*. John (1982) reports that 14 out of 16 cases of PAM in one area were from two lakes which received the effluent from an iron smelter. The water was often red from the iron content.

So although it appears that iron enhances amoeba growth and increases chances of isolating *N. fowleri*, its role is unknown. If Fe is removed from Acanthamoebae-growing media the respiration of the organism changes and becomes cyanide sensitive. A possible role or mechanism of action of iron on amoebae could therefore involve respiration (Hrynewiecka et al., 1980).

The media used for this study already contained 1 mg l^{-1} Fe. As Fe levels were increased growth rates of *A. culbertsoni* and *Naegleria* species were decreased. So although a certain level of Fe is necessary (Bard, 1974; Haight & John, 1982; Duma, 1981; John, 1982), anything above 1 mg l^{-1} is inhibitory.

A. castellanii, however, showed increased growth rate as iron levels increased, reaching a maximum of 3 mg l^{-1} In all cases there was no significant difference between the effect of Fe^{+2} and Fe^{+3}.

5.5 Conclusions

(1) Increase in cation levels decreased growth and encystment of all species of amoebae. The mechanisms for this action are not simply an upset of the osmotic or electrical gradient caused by ionic strength, osmotic pressure or charge on the outside of the membrane.

(2) Cells are able to distinguish between different ions. This suggests a different mechanism of action may work for each cation, possibly based on the role of the cation within the cell. Therefore inhibition of growth rate and/or encystment could be due to the following:

a) Na^{+} is responsible in *Acanthamoeba* cells for maintaining the osmotic balance of the cell. Ca^{+2} has an effect as the regulation of IC Na^{+} levels. The effect of excess Ca^{+2} could be due to disruption of this regulation which could in turn upset the osmotic balance.

b) Mg^{+2} prevents or decreases leakage from the cell which can occur in different growth phases and during encystment. Encystment in particular requires the discharge of certain cellular components. Any interference with this mechanism could inhibit encystment.

c) K^{+} and Na^{+} are lost during encystment in selective amounts. Any alteration of this ratio may inhibit encystment. Ca^{+2} and Mg^{+2} both have an effect upon the cell membrane. Any change in permeability could cause K^{+} and Na^{+} to be lost in different proportions and so inhibit encystment.

(3) Although the levels of cations in the media negatively influence growth and encystment of amoebae, once the amoebae are encysted they are relatively impervious to external ionic levels. The effect of Na^{+} on *Naegleria* is the only exception.

(4) Most research, and the above postulations, are based on *Acanthamoeba* species. Very little has been done on the osmoregulation of *Naegleria* species. However, it apears from the present study that

the mechanisms could be similar. This is a possible area for further research, i.e. the osmoregulation of *Naegleria* species and the role of cations within the cell.

(5) Temperature affects *Naegleria* species but not *Acanthamoeba* species, possibly due to a thinner cyst wall or the lack of environmental selective pressure towards the production of low temperature-tolerant cyst walls.

(6) Ca^{+2} is not suitable as a disinfectant as it fails to eradicate encysted amoebae.

(7) The results of this study can partially explain the absence of amoebae from Te Puia, Morere, Butchers and Waingaro hot pools (Table 1). The mechanisms of action are mentioned above.

a) Te Puia: extrapolating the Na^{+} graph (Figures 1-4) suggests that Na^{+} levels present in Te Puia pool would be inhibitory. This, in conjunction with the high Ca^{+2} level could explain the lack of amoebae.

b) Butchers: the extremely high Ca^{+2} levels could explain the absence of amoebae by affecting both encystment and growth.

c) Morere: the low cation levels could provide a hypotonic environment which could cause encystment but not support growth.

d) Waingaro: the elevated Fe level could explain absence of amoebae.

In encysted form amoebae generally clump together and/or adhere to surfaces. Therefore the likelihood of them being suspended in water is small, as the clumps tend to sink. Therefore the likelihood of inclusion in a sample of pool water for a test is also small. Most amoebae isolated will be trophozoites. If trophozoites are selected against by cation levels in the water they may encyst, sink and not be isolated. They would remain encysted until they die or the conditions causing encystment in the first place are removed.

(8) As iron levels increased, growth rates of *A. culbertsoni* and both *Naegleria* species were decreased. Presumably the level of iron in the growth media is the optimum level. *A. castelanii* increased growth as iron was added, so presumably iron has a different role in this species.

There is no significant difference in the effect of Fe^{+2} as compared to Fe^{+3}.

5.6 Preliminary Identification of Unknown Isolates

Under microscopic examination, the unknown Ngawha Springs isolates PR2448 and PR2449 appeared to be very similar to *Naegleria* species. They grew better in CYM (Naegleria Medium) than in Neff (Acanthamoeba medium).

Flagellation tests were not conducted, as both isolates produced flagellates spontaneously, giving a probable identification as *Naegleria*.

Naegleria vary markedly in size, shape, granulation and vacuolation depending on the condition of the amoebae (growth stage, nutrition etc.) and the media in which they are growing.

Observation under constant conditions, however, suggested the two isolates were more similar to *N. fowleri* than *N. gruberi*. Temperature tolerance tests have been used to determine the pathogenicity of *Naegleria* species (Griffin, 1972; Shapiro et al., 1983; Stevens et al., 1977; di Menna et al., 1969; Newsome & Wilhelm, 1983a; Delattre, 1981; Carter, 1969). However there are temperature tolerant non-pathogenic species. The only true test for pathogenicity is the ability to cause infection in animals.

The two isolates came from Ngawha Springs hot pool which has a temperature of 26°C. This temperature is within the range of both *N. gruberi* and *N. fowleri*. Both isolates grew equally well in both laboratory temperatures 30°C and 37°C, which are used generally for non-pathogenic and pathogenic isolates respectively. Griffin (1972), in a study of temperature tolerance and pathogenicity, found that temperature

tolerance is a better indicator of pathogenicity than animal tests, as the susceptibility of mice to infections varies markedly. However, the the likelihood of pathogenicity is increased as temperature tolerance increases. The WHO report in 1983 reported that viable *N. fowleri* trophozoites were found in a South Australian pipeline whose waters frequently reached 49°C. *N. gruberi* will not survive temperatures above 45°C.

Both isolates (Figure 63) follow the trend of *N. fowleri* species in response to temperatures of 45°C, whereas *N. gruberi* (PL) did not survive. So preliminary identification of PR2448 and PR2449 is temperature tolerant *Naegleria*, and not *N. gruberi*. However, gel electrophoresis and/or animal inoculation must be conducted for identification to species level.

APPENDICES

I. MAINTENANCE OF STOCK AMOEBAE CULTURES

Axenic cultures of *N. fowleri* (NHI) and *N. gruberi* (PL200f) in CYM medium and *A. castellanii* (1501) and *A. culbertsoni* (A1) in Neff medium were provided by C.J. Biddick, Massey University. 0.5 ml of each culture was added to 4.5 ml aliquots of fresh medium in 10 ml universal bottles. Pathogenic species were incubated at 37°C and non-pathogens at 30°C on rotary gyroshakers (150 rpm). Pathogens were subcultured every 24 hours and non-pathogens at 48 hour intervals.

II. MAINTENANCE OF STOCK E. CLOACAE CULTURES

Culture slopes of *E. cloacae* were provided by Mrs. F. Bacon, Massey University. *E. cloacae* was maintained on BHI agar. The plates were inoculated, incubated overnight at 37° and stored at 4°C. When required, a colony was picked off to inoculate 10 ml BHI broth, incubated overnight at 37°C and used the following day.

III. METHOD FOR CHEMICAL ANALYSIS OF MEDIA

Analyses for Na and K were carried out by flame photometry, and analyses for Ca, Mg and Fe were performed by atomic absorption spectroscopy. For the atomic absorption analysis, the sample and standard solutions contained lanthanium chloride (La concentration 1000 ug/ml) to eliminate any possibility of phosphate interference with the Ca and Mg determinations.

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