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ENVIRONMENTAL FACTORS AFFECTING ENCYSTMENT OF P.F.L.A. TOGETHER WITH DISINFECTION STUDIES

A thesis presented in partial
fulfilment of the requirements for the
degree of Master of Science in
Microbiology, at Massey University
Palmerston North, New Zealand

DAWN HARVEY

1986

ABSTRACT

Free living amoebae from the genera Naegleria and Acanthamoeba have been implicated in fatal and several non-fatal infections of the human central nervous system, and other organs. They can be isolated from a worldwide range of environments. The common occurance of these organisms in nature may be attributed to the ability to form resistant cysts to withstand adverse environmental conditions.

Research was performed to determine factors that will promote the encystment of amoebae, particularly Naegleria species. The parameters examined for the induction of encystment were: the type of substrate amoebae were growing on liquid or solid, the presence of bacteria, the cell concentration and nutrient availability and incubation at temperatures other than the optimal growth temperature.

Higher percentages of amoebae encysted on solid surface environments in comparison to the liquid media. In liquid media a greater percentage of trophozoites only formed a pre-encystment or roundform stage. The factor required for the complete encystment of roundforms was not present.

The encystment of Naegleria fowleri was not significantly influenced by the presence or absence of the bacterial species used (E. cloacae).

Encystment of Naegleria sp at different cell concentrations, using a nutrient media, a soil extract broth and a non-nutrient media was examined. Complete encystment of cells did not occur where nutrients were either high or absent, and the cell concentration was low.

The ability of trophozoites of N. fowerli, N. gruberi, A. culbertsoni, and A. castellanii to encyst at a range of temperatures from 4°C - 44°C was studied. The trophozoites of Acanthamoeba sp could encyst over a wider temperature range in comparison to the trophozoites of Naegleria sp.

The effect of disinfection using Baquacil was studied. Previously isolated strains of baquacil resistant N. fowleri, still had higher disinfection survival rates compared to sensitive strains. Resistance of trophozoites to Baquacil was not affected even after storage as a cyst. Baquacil resistant strains of N. fowleri were still sensitive to chlorination.

Disinfection of amoebic cysts using chlorine and Baquacil was investigated. Amoebic cysts require higher levels of disinfection for inactivation in comparison to trophozoites. Acanthamoeba cysts have a greater tolerance to chlorination than Naegleria sp.

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CHAPTER ONE: INTRODUCTION

1.1 The History of Free-Living Amoebae as Disease Agents:

In the last twenty years interest in the small free-living or limax amoebae has increased because of the discovery that Naegleria fowleri and various species of Acanthamoeba are pathogenic and capable of producing fatal meningoencephalitis in man (Culbertson et al, 1959,1965,1966; Fowler & Carter, 1965; Butt, 1968; Carter, 1970; Cursons & Brown, 1976). The diseases caused by pathogenic amoebae of the genera Naegleria and Acanthamoeba are referred to respectively as primary amoebic meningoencephalitis (PAM) (Culbertson, 1961; Butt, 1968) and granulomatous amoebic encephalitis (GAE). (Martinez, 1980, 1982).

In recent years there have been significant advances in the understanding of the epidemiology, pathogenesis and clinico-pathological characteristics of PAM and GAE.

GAE appears to be an opportunistic infection of the central nervous system (CNS) (Martinez, 1977, 1980, 1982, 1983; Garcia, 1983). GAE occurs in patients who are chronically ill, debilitated or those who are immunosupressed either by a systemic disease or a form of treatment e.g. radiation therapy. (Jager & Stamm, 1972; Bhagwandeen et al, 1975; Dagget et al, 1982; Garcia, 1983).

In contrast, Naegleria fowleri produces an acute fulminant, necrotizing haemorrhagic meningo-encephalitis, in healthy individuals with a recent history of water-related sports or swimming (Callicott et al, 1968; Duma et al, 1971; Carter, 1972; Chang, 1974; John, 1982; Dorsch et al, 1983).

Amoebic encephalitis was first detected in laboratory animals by Culbertson in 1958 during the production of polio vaccine (Culbertson et al, 1958). Amoebae were

discovered as a contaminant of kidney tissue culture. The contaminants were inoculated intracerebrally into mice and monkeys with the result some of the inoculated animals died from a fatal meningoencephalitis. The contaminants were originally identified as Acanthamoeba castellanii (Culbertson et al, 1959).

Amoebic Meningitis was first detected in man by Fowler and Carter (1965) in Australia. Butt (1966) described three fatal infections from Florida, U.S.A., and named the disease primary amoebic meningoencephalitis (PAM). A free-living amoebo-flagellate isolated from the brain and spinal fluid of a 16 year old victim of an unresponsive encephalitis was identified as belonging to the genus Naegleria. (Butt, 1968).

By 1970, the significance of PAM was well established but the identification of the aetiological agent had not been clarified. Carter (1970) described one of the amoeba isolates and showed it to be a previously unnamed species of Naegleria. The pathogenic species of Naegleria has been named N. fowleri after Malcolm Fowler.

The causative organisms of GAE are most probably Acanthamoeba sp (Martinez et al, 1977, 1980, 1982, 1983; Willaert etal, 1978 and Garcia, 1983). Acanthamoeba castellanii, A. culbertsoni, A. palestinensis, A. astronyxis have all been isolated from patients with GAE (Martinez 1980). It is of interest that Acanthamoeba sp have been isolated from cases of Keratitis (Nagington et al, 1974; Visvesvara et al, 1975) and respiratory diseases (Martinez et al, 1980).

Acanthamoeba sp and Naegleria sp have been associated with the bacterium Legionella pneumophila, the causative organism of Legionnaires' disease (Rowbotham, 1980, 1983a, 1983b). L. pneumophilia has been isolated from water of cooling towers (Morris et al, 1979; Dondero et al, 1980) and other aquatic environments. Free living amoebae have also been isolated from humidifiers (Shapiro et al, 1983). Legionnaires disease is contracted

following the inhalation of L. pneumophilia, which infect macrophage cells (Rowbotham, 1980, 1983[b]).

It is suggested that amoebae are a natural host for L. pneumophilia, and an amoebae full of Legionella could be the infective particle for man (Rowbotham, 1980).

1.2 Classification:

Several classifications of free living amoebae have been proposed (Page, 1967 [a], 1967 [b], 1976; Singh & Das, 1970; Chang1971). Singh believes that the main features to base the classification of amoebae are; nuclear structures and patterns of mitosis and division (Singh and Das, 1970). In his classification scheme Page uses the features of locomotive form and behaviour (motility); cytochemical features, ultrastr-ucture, cyst morphology, nutrition and nuclear division patterns during mitosis (Page, 1967 [a], 1967 [b], 1976). The classification proposed by Chang (1971) represents a combination of the ideas of Singh and Page and this is of most value for the classification of free-living amoebae.

The classification of an amoebae from Genus to species level requires very specific and precise mechanisms of identification. Techniques that are employed in the classification of amoebae to a species level include: indirect immunoflurorescent antibody (IFA) technique, incubation at 45°C, examination for disease produced experimentally in animals (Stevens et al 1980), exam-ination of cysts via electron microscopy (Lastovica, 1974; Schuster, 1975, 1979; Pussard and Pons; 1979).

Hadas et al (1977) used disc electrophoresis to determine its usefulness as a means of differentiating and classifying free living amoebae by comparison of protein bands. The method of determining iso-enzyme patterns for taxonomic criteria of free-living amoebae is widely used (De Jonckheere, 1981, 1982, 1983; Robin-son & Lake, 1982; Daggett and Nerad, 1983). De Jonckheere (1981) has described a new species of Naegleria,

N. australiensis sp. nov. based on morphological, physiological, serological and biochemical studies. Studies of immunoflurorescence revealed it was antigenically unique. Isoenzyme patterns of acid phosphatase and leucine amino peptidase showed a good way of differentiating a new pathogenic species from pathogenic N. fowleri.

1.3 Pathogenicity:

The olfactory neuroepithelium is the route of invasion in PAM due to N. fowleri (John, 1982; Garcia, 1983). Infection follows inhalation of water containing amoebae or flagellates (Chang 1971, 1974).

It has also been suggested that inhaling cysts during dust storms could lead to infection (Lawande and Duggan, 1979). Amoebae penetrate the nasal mucosa and the cribiform plate and travel along the olfactory nerves to the brain. Amoebae first invade the olfactory bulbs and then spread to the more posterior regions of the brain. Within the brain they provoke inflammation and cause damage to the tissue (Callicot et al1968, Carter, 1970, Chang, 1971 1974, Garcia, 1983).

Martinez et al,(1973) demonstrated experimentally the path through which the olfactory epithelium appears to be invaded. Invasion occurs primarily by direct penetration of sustentacular cells. Following intracellular invasion, if the host cell is destroyed the amoeba progresses inward. The final path of invasion is via schwann cells and along the perineural spaces of olfactory nerves of the submucosal plexus.

The mechanism of cytopathic action of N. fowleri remains a matter of controversy. Visvesvara and Callaway (1974) proposed that amoebae ingest the target cells by phagocytosis. Chang (1974) suggested that Naegleria released a cytolytic substance which Cursons and Brown (1976) stated may be a phospholipase. Hysmith and Franson

(1982) concluded that pathogenic Naegleria contain potent lipolytic enzymes that could contribute to the pathogenesis of Naegleria fowleri. Brown (1977, 1979 [a] and[b]), concluded that Naegleria injured target cells by direct contact with the host's cell, and there was no evidence of secreted cytotoxic factors by amoebae.

The site of a primary lesion in cases of Acanthamoeba sp infection appears to be from anatomical sites such as the lower respiratory tract (lungs), skin (skin ulcers) and eyes (corneal ulcers) (Nagington et al 1974, Gullet et al, 1979, Martinez et al 1980 [a] & [b]).

The involvement of the central nervous system (CNS) in GAE appears to be a secondary stage after haematogenous spread of Acanthamoeba sp from a primary lesion (Chang, 1974; Martinez et al 1977; Garcia, 1983; Martinez, 1983).

GAE due to Acanthamoeba sp usually occurs in debilitated individuals, with an impaired immune defence mechanism, e.g. diabetics, alcoholics, sufferers of Hodgkin's Disease and leukaemia, women during pregnancy, patients on immunosuppressive drugs or receiving radiation therapy, and without history of recent swimming (Duma et al, 1969; Chang, 1984; Martinez, 1980, 1983; Garcia, 1983).

It is thought that Acanthamoeba sp are part of the natural skin flora in humans. The opportunistic infections occur when people become immunosuppressed or immunologically deficient, and are susceptible to infection of a primary lesion with Acanthamoebae, (Martinez, 1980).

The cytotoxic action of Acanthamoeba sp to host cells probably results from the production of the cytolytic substance phospholipase, (McIntosh and Chang, 1971; Visvesvara and Balamuth, 1975).

1.4 Immunity:

Antibody to N. fowleri has been detected in surveys of normal human sera (Cursons et al 1980 (a & b); Reilly et al, 1982, 1983). The onset and duration of PAM is so rapid (John, 1982) that the IgG level in the bloodstream is unlikely to reach a titre level high enough to afford protection against N. fowleri (Cursons, et al, 1977). During infection with PFLA it is the CMI response that provides immunological protection in the host (Cursons et al 1977, Garcia, 1983, Martinez, 1983).

Cursons et al (1980) showed that both the in vitro macrophage inhibition test and the delayed hypersensitivity test showed responses to both heterologous and homologous antigens from pathogenic and non-pathogenic species of amoebae. These results led Cursons et al (1980) to hypothesize that environmental contact with non-pathogenic species of FLA can stimulate the immune system to be effective against pathogenic species.

Animal challenge studies have demonstrated that primates are relatively resistant to either intranasal or intravenous challenge with N. fowleri or A. culbertsoni (Culbertson, 1971; Wong et al 1975 [a] & [b]). When challenged by an intrathecal or intracerebral inoculation of amoebae, infection results.

Thong et al (1983) showed that mice are protected against N. fowleri infection by immunization with an amoeba-free supernatant from amoeba cultures. Protection is expressed mainly at the nasal mucosa, and results from the combined effects of CMI, and elimination of the organisms by shedding of necrotic epithelum. Immunization of mice with various antigenic preparations, live or formalinized N. fowleri or N. gruberi, by various routes (intravenous, intranasal or subcutaneously) significantly protects against a subsequent lethal challenge with N. fowleri (John et al, 1977; Haggerty & John, 1978, 1982; John,1982).

Intravenous immunization with N. gruberi afforded 65% protection against a challenge (John et al, 1977) and three intranasal immunizing doses with N. gruberi produced 88% protection against internasal challenge with N. fowleri (John, 1982). Serum IgA levels are greatly increased in mice given intranasal inoculations of live N. fowleri cells (Haggerty and John, 1978, 1982).

N. fowleri amoebae have been shown by immunofluorescence to cap and remove or internalize surface-bound antibody (Ferrante & Thong 1979). The ability of N. fowleri to remove antibody from the cell surface may enable the amoebae to counter the host's immune defenses.

Neutrophils from N. fowleri immunized mice are capable of killing amoebae (Ferrante & Thong, 1980). One method of killing is for a group of neutrophils to surround an amoeba and destroy it, presumably by contact and release of enzymes onto the amoeba cell membrane. A novel phagocytic process has been described in which neutrophils pinch off portions of an amoebae. Although unable to phagocytose an entire amoeba, several neutrophils are able to rupture an amoeba by pinching off and engulfing portions of it (Ferrante and Thong, 1980).

The significance of the immune system in controlling Acanthamoeba infections, is indicated that GAE usually occurs in debilitated and immunosuppressed individuals, (Martinez, 1980 [a] & [b], 1983).

1.5 Diagnosis:

The clinical symptoms in PAM due to N. fowleri are those associated with severe meningeal irritation: headache, nausea, vomiting and a stiff neck progressing rapidly to coma and death. Convulsions occur often. The incubation period of PAM may be as short as one or two days or upto two weeks. (Carter 1972, Chang, 1974, Garcia, 1983; Martinez, 1983). PAM typically occurs

in healthy young individuals who have a recent history of swimming in fresh water lakes or warm heated pools (Duma et al, 1969, 1971). Clinically PAM very closely resembles a fulminating bacterial meningitis, and laboratory findings are also similar, it is important to determine if the patient has a recent history of water-related activities (Carter 1972, Martinez et al, 1977). The clinical course in N. fowleri infection is rapid. The premortem diagnosis is established by finding trophozoites in the cerebrospinal fluid (CSF). (Carter, 1970; Martinez et al, 1977).

The examination of CSF by ordinary light microscopy is a very important laboratory procedure for the rapid diagnosis of PAM and GAE. Motile amoebae are readily seen in simple wet-mount preparations of CSF. Amoebae can be distinguished from other cells by their limax shape and progressive movement. Culture of amoebae from the CNS is very important for the definitive diagnosis. (dos Santos, 1970; Carter, 1970; Martinez et al, 1977; Martinez, 1983).

In PAM due to N.fowleri the CSF is purulent, tinged with pink due to erythrocytes from the blood. PAM is an acute, haemorrhagic necrotizing meningoencephalitis (John, 1982).

The leukocyte counts in the CSF in PAM varies from as low as 300/mm to as high as 26,000/mm. The protein concentration in the CSF is elevated, with high values of gamma globulin. The glucose value is very low as in bacterial meningitis. Amoebae are delicately refractile (dos Santos, 1970; Carter, 1970; Carter, 1972).

The CSF in cases of GAE due to Acanthamoeba sp may have a predominance of lymphocytes but until now no diagnosis of GAE has been made while the patient is still alive. (Martinez, 1983).

GAE due to Acanthamoeba sp occurs in chronically ill or debilitated individuals, or immunosuppressed patients without a history of recent swimming. The incubation

period is probably more than 10 days and the clinical course is subacute and chronic, making GAE hard to diagnose, and prolonged for several weeks. The clinical symptoms are mental abnormalities, meningism, localizing neurological signs and coma (Duma et al, 1978; Martinez, 1980).

In post-mortem diagnosis of the brain in cases of PAM due to N. fowleri the cerebral hemispheres are usually oedematous, markedly swollen with evidence of increased intracranial pressure (Duma et al, 1969). The cortex shows numerous focal superficial haemorrhages. Most of the lesions are in and around the frontal, temporal regions, base of the brain, and cerebellum. The olfactory bulbs are markedly haemorrhagic, surrounded by purulent exudate (Callicott, 1968).

Pockets of numerous trophozoites may be seen within oedematous neural tissue, with and without acute inflammatory cells. The trophozoites are usually located in the adventitial spaces. No cysts are present within CNS lesions (Duma et al, 1971 [a] & [b]).

The anatomic pathological characteristics of the brain from infection due to Acanthamoeba sp show the cerebral hemispheres might be characterized by severe oedema and softening associated with focal necrosis with recent haemorrhages as well as small abscesses. The posterior fossa structures, diencephalon thalmus and brainstem are usually the most affected areas (Duma et al, 1978).

The majority of the lesions of GAE are characterized by a chronic and granulomatous reaction, with several multinucleated giant cells. The invading amoebae invade from deep grey or white matter areas to the brain surface. Trophozoites and cysts are the amoebic forms observed,in scattered spaces and invading blood vessel walls (Martinez et al, 1980 [a] & [b]).

Acanthamoeba sp (A. castellanii and A. polyphaga) have been isolated from some eye lesions. Infection with Acanthamoeba sp is presumably aquired from the direct invasion of the ocular tissues. (Visvesvara & Healy,1975;

Nagington & Richards, 1976; Lund et al, 1978; Keys et al, 1980; Martinez, 1983). The amoebic trophozoites may be identified in direct smears of intraocular fluid or from the surface of the corneal ulcerations and may be cultured from these materials (Ma et al, 1981).

When the diagnosis of the causative amoebae requires identification to a species level, methods based on immunology of amoebae are employed, these include immunoelectrophoresis (De Jonckheere, 1982, 1983) immunoperoxidase (Culbertson, 1975) and indirect fluorescent antibody technique (De Jonckheere & Van de Voorde, 1974).

1.6 Treatment:

At present, no satisfactory treatment for PAM exists. Emetine, metronidazole and chloroquine and other antibiotics and chemotherapeutic drugs used in cases of E. histolytica infections and bacterial meningitis, are ineffective in free-living amoebic infections (Das, 1970; Stevens and Wilbert, 1980; Stevens et al, 1981).

Amphotericin B., is a drug of considerable toxicity, and is an antinaeglerial agent for which there is some evidence of clinical effectiveness. There have been three cases of PAM successfully treated with Amphotericin B, given intravenously and intrathecally (Apley et al, 1970; Anderson & Jamieson, 1972; Seidal et al 1982).

Amphotericin B and miconazole intravenously and intrathecally appear to be the drugs of choice for Naegleria sp (Ferrante, 1982). A synergistic or additive effect of those drugs has been demonstrated in vitro (Thong et al 1978; Ferrante, 1982).

The in vitro testing of a highly virulent human isolate of N. fowleri demonstrated that amoebae were extremely susceptible to amphotericin B(minimal inhibitory concentrated MIC, 0.15 ug/ml) somewhat susceptible to miconazole

(MIC 25ug/ml) and resistant to rifampin (MIC <100ug/ ml) (Stevens et al 1982). Mice were protected by treatment with lower doses of amphotericin B alone or in combination with miconazole (100 mg/kg) or rifampin (220 mg/kg). The most effective treatment for PAM is amphotericin B administered intravenously at a dose of 1mg per kg of body weight daily, with intrathecal inoculations of 0.1 mg on alternate days (Carter 1972).

Amphotericin B is a polyene compound that acts upon the plasma membrane, disrupting its selective permeability, and causing leakage of cellular components. When exposed to amphotericin B, amoebae round up, and do not form pseudopodia. Membrane related changes, evident by electron microscopy include enhanced nuclear plasticity, increased amount of smooth and rough endoplasmic reticulum, decreased food vacuole formation, and production of blebs of the plasma membrane (Schuster, 1979).

Tetracycline (Thong et al, 1978) and rifampin (1979) have been shown to act synergistically with amphotericin B to protect mice against N. fowleri infection. In the tetracycline study, chemotherapy was started 72 hours after the mice had been infected intranasally. Survival was 38% for amphotericin B - treated mice and 88% for mice treated with the amphotericin B - tetracycline combination (Thong et al, 1978).

The survival of a patient with a case of PAM relies on the early diagnosis, prompt intervention and initiation of antiamoebic therapy coupled with intensive supportive care (Seidal et al 1982).

No particular drug therapy has been demonstrably successful in infection involving Acanthamoeba sp, although several have shown promise (Nagington & Richards, 1976; Culbertson, 1981; Rowan-Kelly et al, 1982).

Acanthamoeba species, with few exceptions have exhibited remarkable resistance in vitro to numerous antiprotozoal, antimicrobial, antiviral and anticancer drugs (Casemore, 1970; Krishna Prasad, 1972; Stevens and O'Nell, 1974;

Chang, 1974; Duma & Finley, 1976). Hydroxystilbamidine, Ketoconazole, 5-fluorocytosine and gentamycin appear to be effective against Acanthamoeba (Casemore 1970; Duma and Finley, 1976).

In experimental Acanthamoeba meningoencephalitis in mice, Culbertson et al (1965) showed that sulphadiazine could effectively protect mice against this infection. Rowan-Kelly et al (1982) showed that sulphadiazine, while being therapeutic when given very early after infection, failed to protect mice once the amoebae had migrated and become established in the CNS. To date, the only human clinic case reported, where sulphadiazine has been used in treatment was the drug therapy used for an eye infection by Nagington et al (1974).

1.7 Occurence and Distribution :

Although it is a relatively rare disease PAM has been reported worldwide. To date there has been about 130 cases reported worldwide of infections due to N. fowleri and nearly 30 due to Acanthamoeba sp (Carter, 1970; Chang, 1974; Nagington et al, 1974; Nagington and Richards, 1976; Lund et al, 1978; Martinez, 1980, 1983; Garcia, 1983).

In the 17 cases reported from Czechoslovakia the infected persons came from 10 different localities, but all had used the same indoor swimming pool, which was filled with river water that had been adapted, treated, heated and chlorinated (Cerva and Novak, 1968; Cerva et al, 1968). In South Australia, where 18 cases have been reported, an epidemiological survey disclosed sources of F.L.A. pathogenic for mice, suggesting that nasal inoculation during swimming in polluted water was the most likely source of infection (Carter, 1969, 1970, 1972; Fowler & Carter, 1965).

The majority of cases of PAM (49 cases) and GAE (16 cases) have been reported from the U.S.A. (Duma et al)

1971 [a] & [b]; Jager & Stamm, 1972, Martinez et al [a] and [b], 1983; Stevens et al, 1981). Other areas that cases have been reported include; Great Britian (Apley et al 1970) New Zealand (Brown et al, 1983, Cursons & Brown, 1975; Cursons et al, 1976, 1978) Zambia (Bhagwandeen et al 1975) and Nigeria (Lawande et al, 1979)

Free-living amoebae are ubiquitous protozoa and widely dispersed in our environment (John 1982). F.L.A. have been isolated from samples coming from Antarctica (Brown et al, 1982) to Norway (Brown & Cursons, 1977) from sea level (Lastovica, 1980) to high-altitudes such as Titicaca Lake, 5000m above sea level, from Costa Rica (Chinchilla et al, 1979). Isolations have been recorded from a wide variety of environments such as samples of fresh water, air (Kingston & Warhurst (1968); Lawande et al, 1979) bottled mineral water (Rivera et al, 1981) dialysis machines or air conditioning systems, (Rowbotham, 1980) cooling and rinsing water in dental treatment units (Michel and Just, 1984) marine and brackish waters, ocean sediments (De Jonckheere et al, 1975; Brown and Cursons, 1977, 1979; Duma 1981) factory effluent (De Jonckheere, 1981; Dive et al, 1981; Duma, 1981) chlorinated swimming and domestic waters (Cerva, 1971; Anderson & Jamieson, 1977; De Jonckheere and Van de Voorde, 1977) sewage and sludge samples (Singh & Das, 1972; Lawande et al 1979) soil samples (Anderson & Jamieson, 1972; Cursons et al, 1978) thermal pools (Wellings et al, 1977; Brown et al 1983) and rivers and lakes (Wellings et al, 1977; Muscaro et al, 1981; Brown et al, 1983).

Several surveys have been made to try and associate the required environmental factors with the presence of F.L.A. (Duma 1981, Brown et al, 1983). Many of the isolations have been made from habitats manipulated or altered by man, this appears to be an important factor, from habitats subjected to warming, whether man-made or natural (John, 1982).

De Jonckheere et al (1975) investigated the distribution of pathogenic amoebae in Belgium and reported the isolation of pathogenic Naegleria only from thermally

polluted water. They suggested that thermally polluted but biologically healthy water is the primary site for N. fowleri proliferation (De Jonckheere and Van de Voorde, 1977).

There has been some disagreement on the role of thermal pollution in the distribution of thermophilic pathogens in the southern United States. Willaert and Stevens (1976) isolated pathogenic Naegleria in Florida only from thermally polluted waters. Wellings et al (1977) isolated pathogenic Naegleria from non-thermally associated waters in Florida and concluded that thermal pollution played little role in the maintenance of pathogenic Naegleria.

Temperature is a major environmental parameter selecting for PFLA in general and pathogenic Naegleria in particular. Independent studies conclude that N. fowleri is more prevalent in waters naturally or artifically heated above 25°C. (Wellings et al, 1977; Duma, 1981; Brown et al 1983). It appears that pathogenic Naegleria is selected for growth at the higher temperature range 25°C - 45°C, over nonpathogenic Naegleria sp (Tyndall, 1984).

Environmental factors other than temperatures selecting for the growth and persistence of N. fowleri are vague. Various physical and chemical analysis of waters from sites positive for N. fowleri have yet to reveal any obvious correlation. Duma (1981) found the greatest number of pathogenic Naegleria isolates was taken from waters which contained high concentrations of iron. In contrast to this Brown et al, 1983 found in his survey of the four uncontaminated pools, one had a very high iron content indicating high iron was inhibitory to PFLA. Two of the other uncontaminated pools had very high Na, C1, Ca levels and the fourth uncontaminated pool had very low cation levels.

A possible inhibitory effect of P. fluorescens and S. marcesens on the growth of N. fowleri was suggested

by Duma (1981). Where P. fluorescens was isolated from a lake, there was a correlation of no Naegleria species present.

The soil is the natural habitat of F.L.A. Amoebae can exist in the soil as a vegetative, trophozoite stage, or a resting, cyst state (John, 1982). The formation of cysts (encystment) enables F.L.A. to survive adverse environment conditions such as starvation, unfavourable temperature, pH, oxygen levels, dessication (Neff et al 1964; Bowers and Korn, 1969; Neff and Neff, 1972; Chiovetti 1976; Byers et al, 1980; Chiovetti and Bovee, 1982; Srivastava and Shukla, 1983). When environmental conditions are favourable, amoebae will excyst and resume the trophozoite stage (Neff et al, 1964, Chiovetti and Bovee, 1982).

1.8 Disinfection and Control Measures:

Disinfection of Municipal and recreational water is used to control the microbial quality of water, and hence the presence of F.L.A. in domestic and recreational water supplies (Engel et al, 1983).

Chlorine is the most commonly used chemical for the disinfection of water (White, 1972). Isolation of F.L.A. from chlorinated and domestic water supplies, is indicative that chlorination of water may not entirely eliminate the presence of PFLA (Cerva, 1971, De Jonckheere and Van de Voorde, 1976; Cursons et al, 1980). Amoebic cysts are more resistant to disinfection than trophozoites, and Acanthamoeba sp require higher levels of disinfection to become inactivated, than do Naeglerial sp (Derreumaux et al, 1974; Cursons et al, 1980).

The free available chlorine residual of drinking water in New Zealand varies from 0.1 - 0.2 mg l^{-1} and 0.5 mg l^{-1} - 0.8 mg l^{-1} for recreational water. Trophozoites of N. fowleri have been demonstrated to be susceptible to chlorination within the 0.5 mg l^{-1} - 0.8 mg l^{-1} range

Cursons et al, 1980). Anderson and Jamieson (1972) reported a case of PAM in South Australia, contracted from domestic bath water. They also reported that superchlorination to 10 mg l^{-1} had failed to eradicate Naegleria from a contaminated pool, there was no indication of the organic load in the water tested reported. Derreumaux et al,(1974) demonstrated that 0.5 mg l^{-1} of HOCI, the active disinfecting component of chlorine disinfection was able to eradicate both Naegleria sp and Acanthamoeba sp.

De Jonckheere & Van de Voorde (1976) found that an initial concentration of chlorine between 0.5 and 1.0 mg l^{-1} was cysticidal for Naegleria sp but that Acanthamoeba culbertsoni cysts were not inactivated by 40.0 mg l^{-1}. PFLA cysts inactivation in water is accomplished by the use of chlorine with dosages and residuals well above the required levels for inactivation of bacteria and viruses (Sproul et al, 1983).

The avoidance of contaminated water for recreational use, would appear to be the best control measure against contraction of PAM (Martinez, 1983)

2 MATERIALS

2.1 Ameoba Cultures Used

TABLE I Amoebae Cultures Used

SPECIES (spp)	Strain	Pathogenic	Source
Naegleria fowleri	NHI	+	NHI
Naegleria fowleri	MSM	+	MU
Naegleria fowleri	$MSMbr_2$	+	MU
Naegleria fowleri	$MSMbr_4$	+	MU
Naegleria gruberi	PL200f	-	NHI
Acanthamoeba culbertsoni	A-1	+	CCAP
Acanthamoeba castellanii	1501	-	IMTPL

+ = Positive
- = Negative
NHI = National Health Institute, Wellington, New Zealand.
MU = Massey University, Palmerston North, New Zealand.
CCAP = Culture Centre for Algae and Protozoa, England.
IMTPL = Institute de Medicine Tropicale Prins Leopold, Belgium.

2.2 Bacteria Cultures Used

SPECIES	Gram Reaction	Source
Enterobacter cloacae	-	MU
Escherichia coli	-	MU

- = Negative
MU = Massey University, Palmerston North, New Zealand.

2.3 Broths for Axenic Cultivation of Amoebae

2.3.1 Pages Amoeba Saline. (PAS) (Page, 1967) for diluting out either Naegleria spp or Acanthamoeba spp, and as a base for CYM medium.

ng/l

NaCl	=	1.2 g
kH_2PO_4	=	1.36 g
$MgSO_4.7H_2O$	=	0.04 g
$Na_2H\ PO_4$	=	1.42 g
Thaimine HCL	=	0.01 g
d - Biotin	=	0.02 g
Vitamin B_{12}	=	add till mixture is pink
L - methionine	=	0.895 g
Distilled water	=	1.0 litre

pH = 6.8

Combine the above compounds, this solution must be diluted by a factor of 1/10 before use with amoebae cultures. To sterilize, autoclave at 103.4 kPa (121°C) 15 minutes.

2.3.2 **CYM medium** (Cursons et al, 1978), for the cultivation of Naegleria spp.

Glucose	=	10.0 g
Difco Yeast Extract	=	5.0 g
Difco Casitone	=	10.0 g
Pages Amoeba Saline	=	1.0 litre

pH 6.8

Dispense in 4.5 ml aliquots into universal bottles and autoclave 103.4 KPa, 121°C, for 15 minutes.

Add aseptically 0.5ml of cocktail (see 2.3.3) to 4.5ml of sterile CYM.

2.3.3 **CYM cocktail**

Bovine serum	=		8.0 ml
Haemin	=		1.6 ml
Penicillin/Streptomycin	=	($2x10^5$ units cm^{-3}) of each =	1.4 ml
$CaCl_2.6H_2O$ ($2.7x10^{-3}$M)(0.6g/1)	=		4.0 ml

Mix the above and filter sterilize using 0.2 um filters. Store at 4°C.

2.3.4 **Acanthamoeba Ion Solution** (Stevens & O'Dell, 1973) the base medium for Neff broth.

$MgSO_4.7H_20$	2.47 g
$CaCl_2.2H_s$	0.11 g
KH_2PO_4	2.7 g
Ferric citrate	0.34 g
Biotin	0.02 g
Thiamine HCL	0.01 g
Vitamin B_{12}	$0.1x10^{-4}$g

Make up to 1 litre, and dilute the solution by a factor of 1/10 before use. Autoclave at 103.4 KPa (121°C) for 15 minutes.

2.3.5 **4% Neff Medium** (Stevens & O'Dell, 1973) for the cultivation of Acanthamoeba spp.

Difco Proteose peptone	=	40 g
Glucose	=	15 g
Difco Yeast Extract	=	7.5 g
Acanthamoeba ion solution	=	1 Litre

Dispense in 4.5 ml aliquots into universal bottle and auto clave at 103.4 KPA, 121°C, for 15 minutes. Before use for culturing add 0.2ml of penicillin/streptomycin solution ($2x10^5$ units CM^{-3}).

2.4 **Bacterial Growth Medium**

2.4.1 **Brain Heart Infusion Agar** for the cultivation of E.cloacae and E. coli.

Difco Bacto Brain Heart Infusion	=	37.0 g
Agar	=	20.0 g
Distilled water	=	1 litre

pH 6.8

Autoclave at 103.4 KPa, 121°C, for 15 minutes.

2.4.2 **Brain Heart Infusion Broth** for the cultivation of E. cloacae and E. coli.

Difco Bacto Brain Heart Infusion	=	37.0 g
Distilled Water	=	1 Litre

pH 6.8

Dispense in 10ml aliquots into universal bottles. Autoclave at 103.4 KPa, 121°C, for 15 minutes.

2.5 Disinfection Solutions

2.5.1 Water

Water used was Milli-q-water which is distilled and deionized.

2.5.2 Chlorine

A solution of BDH sodium hypochlorite (NaOC1) was diluted to the required concentrations of chlorine, with sterile chlorine free milli-q-water.

2.5.3 Baquacil

A solution of Baquacil (Polyhexamethylene biguanide hydrochloride) was obtained from ICI N.Z. Limited. Baquacil was diluted to the desired concentrations using 200 ppm hard water.

2.5.4 200 ppm Hard Water

Solution (1):

$CaC1_2.2H_2O$	10.769	g
$MgC1_2.6H_2O$	6.782	g
Distilled deionized water	100	ml

Solution (2):

$NaHCO_3$	5.603	g
Distilled deoinozed water	100	ml

For the preparation of 200 ppm hard water take 2ml of solution (1) and 4 ml of (2) and make upto 1 litre with distilled deionized water.

2.5.5 Preparation of Baquacil Standard Solutions

Transfer 1 ml of Baquacil into a 1 litre flask and make upto a litre volume using 200 ppm hard water. This is solution A.

Then into each of a series of 100 ml flasks add A (see chart) and make upto 100 ml volume with 200 ppm hard water.

1	2	3	4	5	6	Flask Number
1	2	4	6	8	10	Number of ml of Solution A
10	20	40	60	80	100	Approximate ppm Bacquacil

2.6 Reagents for the Chemical Analysis of Disinfection Solutions:

2.6.1 Baquacil Indicator Solution (Alford pers. Comm. 1980) for the estimation of Baquacil in aqueous solution.

Gelatin = 2.5 g
Eosine Y solution = 0.6%
Distilled Water = 260 ml

Dissolve 2.5g of gelatin in a little warm distilled water and make upto 250 ml. Add to this 10 ml of a 0.6% Eosine Y solution prepared by dissolving 0.6g Eosine Y in 100 ml distilled water.

2.6.2 Phosphate Buffer Solution (Palin, 1974) for DPD chlorine level determination.

Na_2HPO_4 = 24g
KH_2PO_4 = 46g
Disodium ethylene diamine tetra acetate dihydrate = 0.8 g
Mercuric chloride (mgC1) = 0.02g
Distilled Water = 1 Litre

Dissolve Na_2HPO_4 and KH_2PO_4 in distilled water. Combine this solution with 100 ml distilled water in which Disodium ethylene diamine tetra acetate dihydrate has been dissolved. Make up the volume to 1 litre, add MgC1.

2.6.3 N,N- Diethyl-p-phenylene diamine (DPD) indicator solution (Palin, 1974) for chlorine level determination.

{DPD oxalate 1 g
or {p-amino-N:N-diethylaniline sulfate 1.5 g
1 + 3 H_2SO_4 acid 8 ml
Disodium ethylene diamine tetra acetate dihydrate = 0.2 g
Distilled water = 1 litre

Dissolve DPD oxalate or p-amino-N:N- diethylaniline sulfate in distilled water containing 1+3 H_2SO_4 acid and disodium ethylenediamine tetra acetate dihydrate. Add distilled water, and store in brown stoppered bottle, discard when solution is coloured.

2.6.4 Standard Ferrous Ammonium Sulfate (FAS) Titrant (Palin, 1974) for chlorine level determination.

$Fe(NH_4)_2(SO_4)_2.6H_2O$	=	1.106g
1+3 H_xSO_4 acid	=	1 ml
Boiled and cooled distilled water	=	1 litre

Dissolve $Fe(NH_4)_2(SO_4)_2.6H_20$ in distilled water containing H_2SO_4 acid, make up volume to 1 litre with distilled water.

2.6.5 Potassium Iodide Solution (Palin, 1974) for chlorine level determination.

KI	0.5 g
Boiled & cooled distilled water	100 ml

Dissolve KI in distilled water, store in a brown stoppered bottle and discard when solution turns yellow.

2.7 Encystment Media

2.7.1 Pages Ameoba Saline (PAS) Agar (Page, 1967 modified)

PAS (see materials 2.3.1)	=	1 litre
Agar	=	20 g

Autoclave at 103.4 KPa, 121°C, 15 minutes.

2.7.2 Pages Amoeba Saline Bacto Agar (PASB) (Cursons, 1978 modified from Fulton, 1970) for plague counting of Naegleria spp, and Acanthamoeba spp.

PAS (see 2.3.1)	=	1 litre
Agar	=	20 g
Difco Bacto-peptone	=	2.0 g

Autoclave at 103.4 KPa, 121°C, 15 minutes.

2.7.3 Soil Extract Broth (SEB) 100%

Soil	=	1 kg
Distilled water	=	1 litre

Autoclave soil in water 103.4 KPa, 121°C, 15 minutes. Centrifuge 6000 rpm 15 minutes. Retain and reautoclave supernatant.

2.7.4 Soil Extract Broth (SEB) 25%

Garden Soil = 500 g
Distilled water = 1 litre

Autoclave soil in water 103.4 Kpa, 121°C, 15 minutes. Centrifuge 6000 rpm, 15 minutes. Retain and reautoclave supernatant.

2.7.5 Soil Extract Broth (SEB) plus Penicillin and Streptomycin.

S.E.B. 100% (see 2.7.3) = 25 ml
Penicillin/streptomycin ($2x10^5$ units cm^{-3}) of each = 0.5 ml
Add penicillin/streptomycin solution aseptically to SEB.

2.7.6 Soil Extract Agars (SEA)

Percentage soil Extract Agar	10	20	40	60	80	10
Required volume of SEB (2.7.3) ml	100	200	400	600	800	10
Required volume distilled water ml	900	800	600	400	200	-

To the required volumes of soil extract broth and distilled water add 20 g of agar. Autoclave 103.4 Kpa, 121°C, 15 minutes.

2.8 Miscellaneous Solutions:

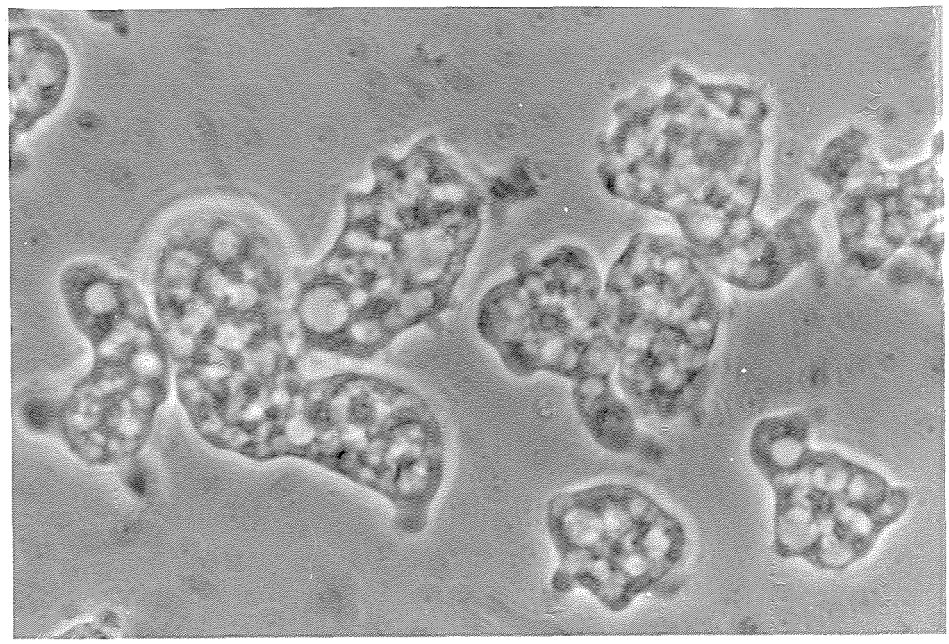
2.8.1 Sodium Phosphate Buffer 0.01M

Na_2HPO_4 = 14.2 g
$NaH_2PO_4.2H_2O$ = 18.0 g
Distilled water = 1 litre
pH 7.2

Autoclave at 103.4 Kpa, 121°C, 15 minutes.

Plate 1 Trophozoite stage of Naegleria fowleri (NHI)

Plate 2 Trophozoite stage of Naegleria gruberi (PL200f)



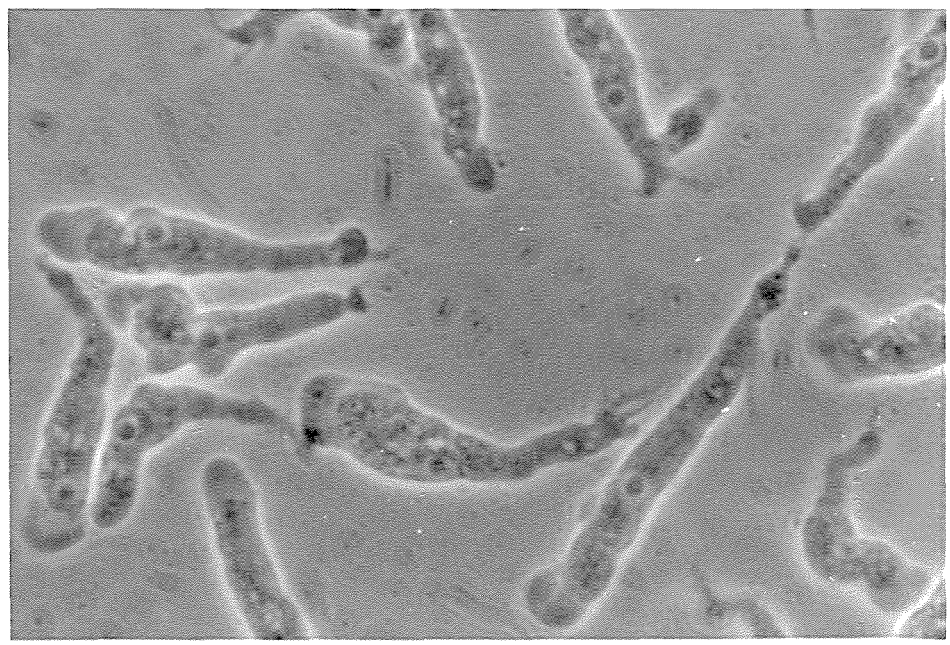
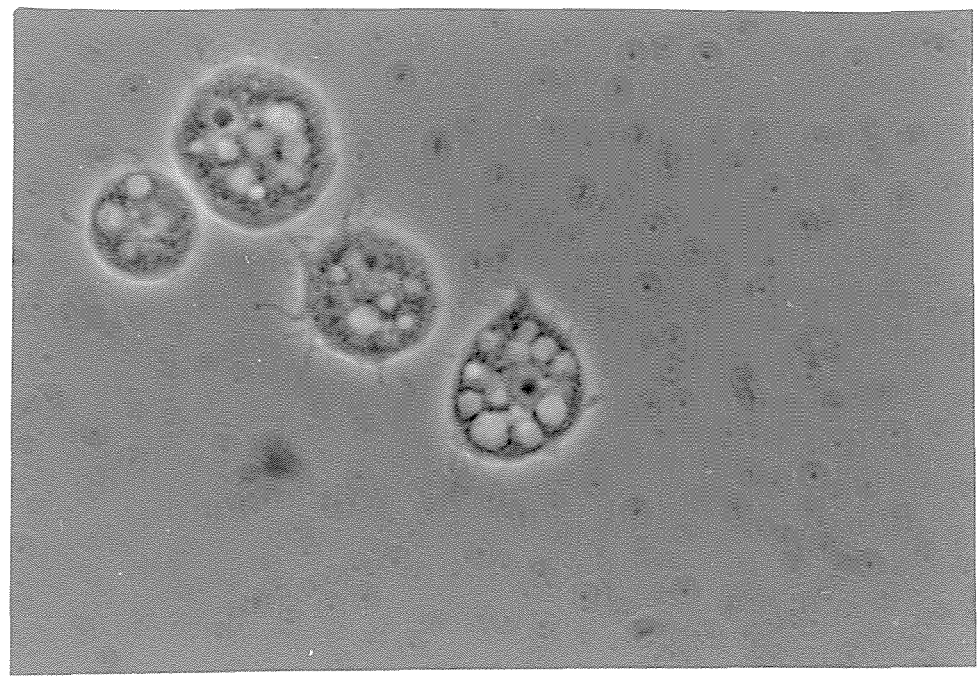


Plate 3 Trophozoite stage of Acanthamoeba culbertsoni (AI)

Plate 4 Trophozoite stage of Acanthamoeba castellanii (1501)



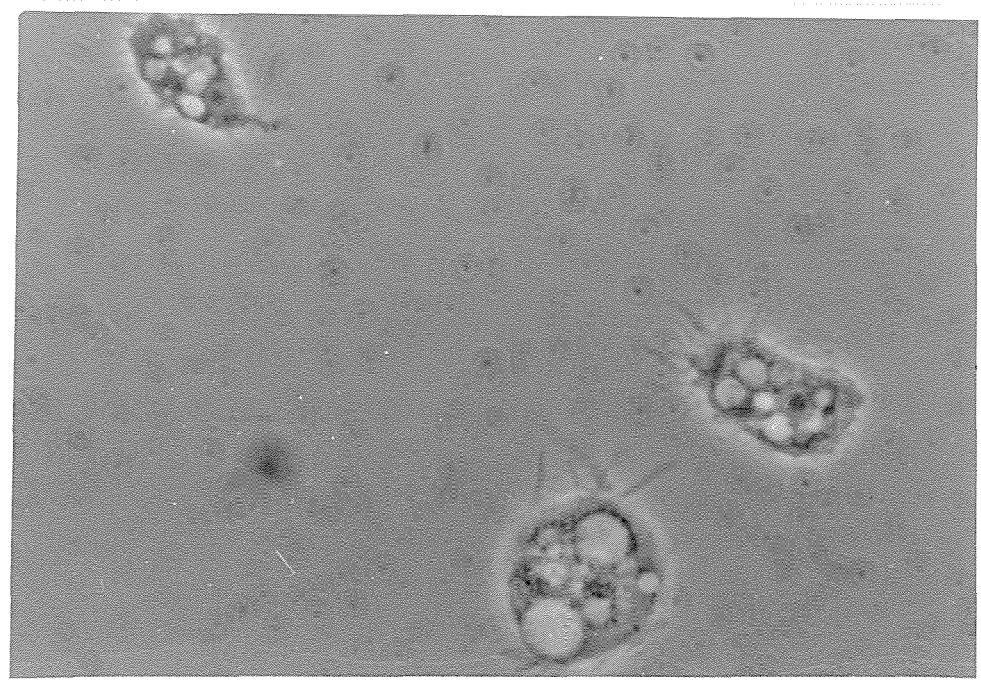
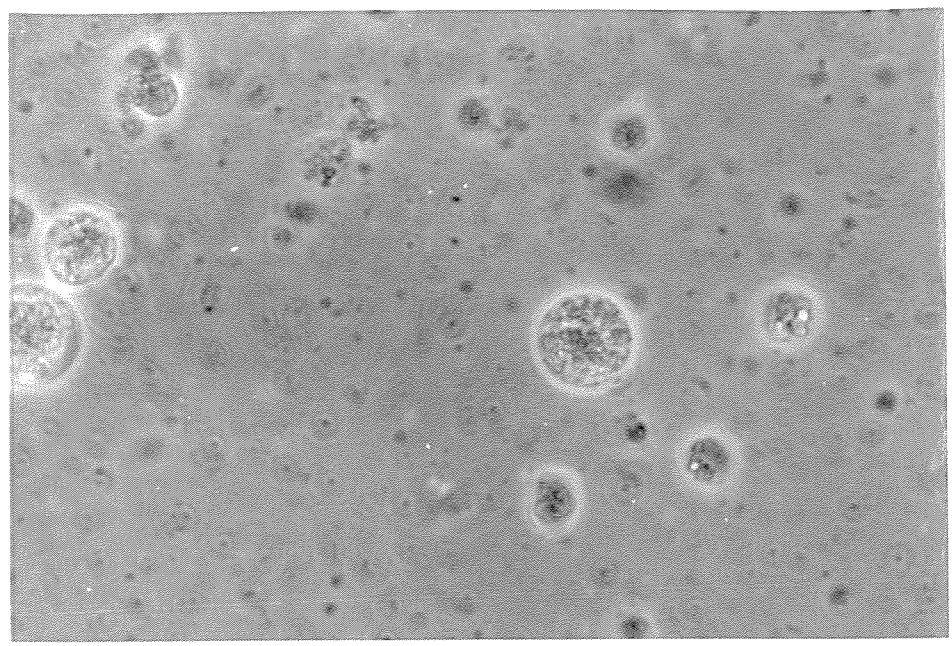


Plate 5 Roundform stage of Naegleria fowleri (NHI)

Plate 6 Roundform stage of Naegleria gruberi (PL200f)



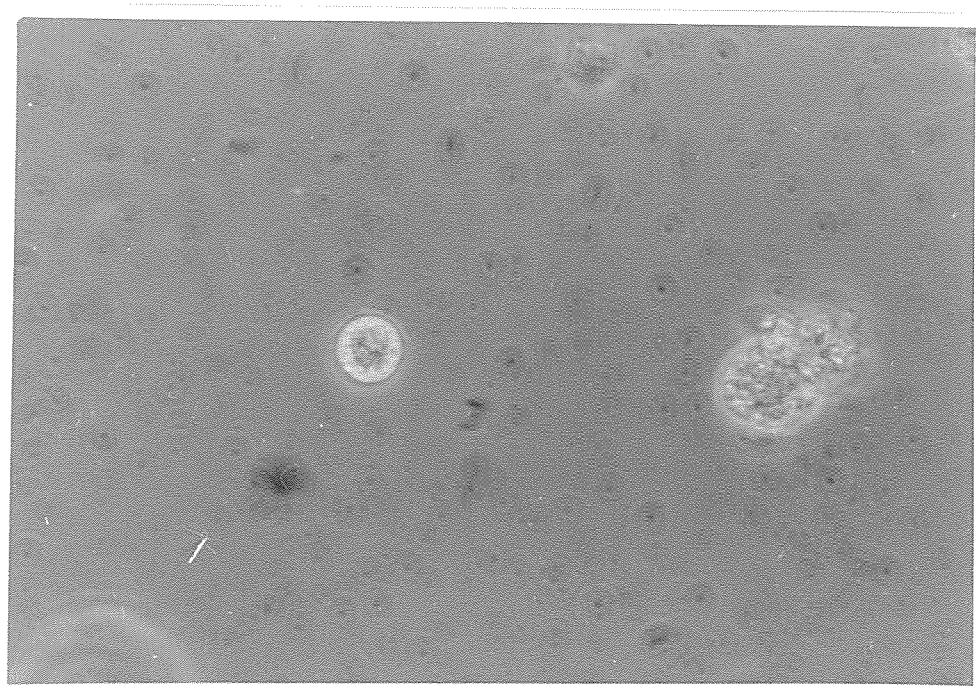
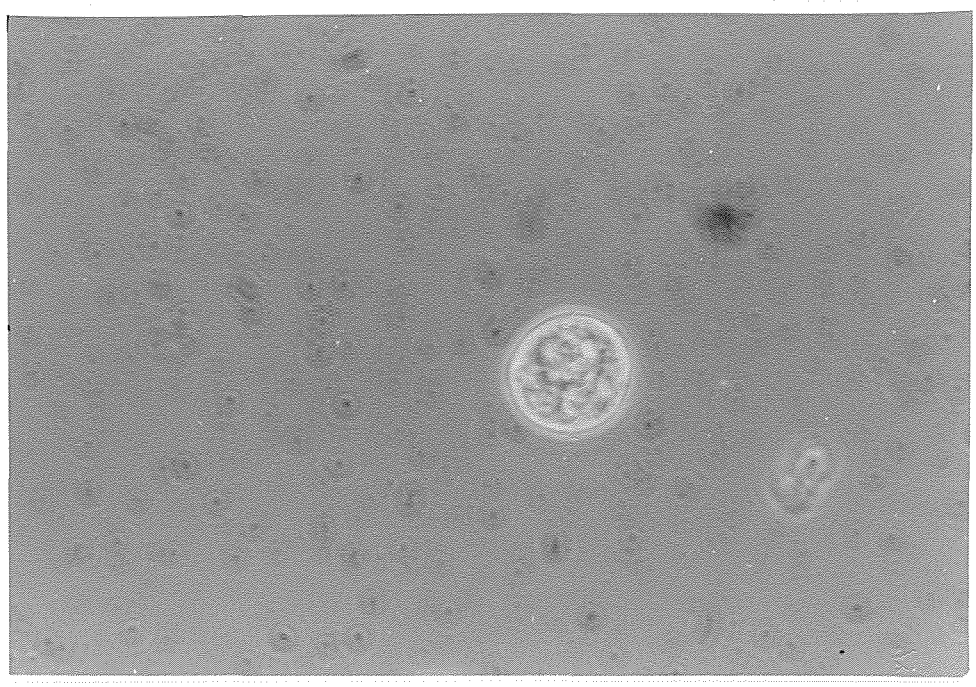


Plate 7 Roundform stage of Acanthamoeba culbertsoni (AI)

Plate 8 Roundform stage of Acanthamoeba castellanii (1501)



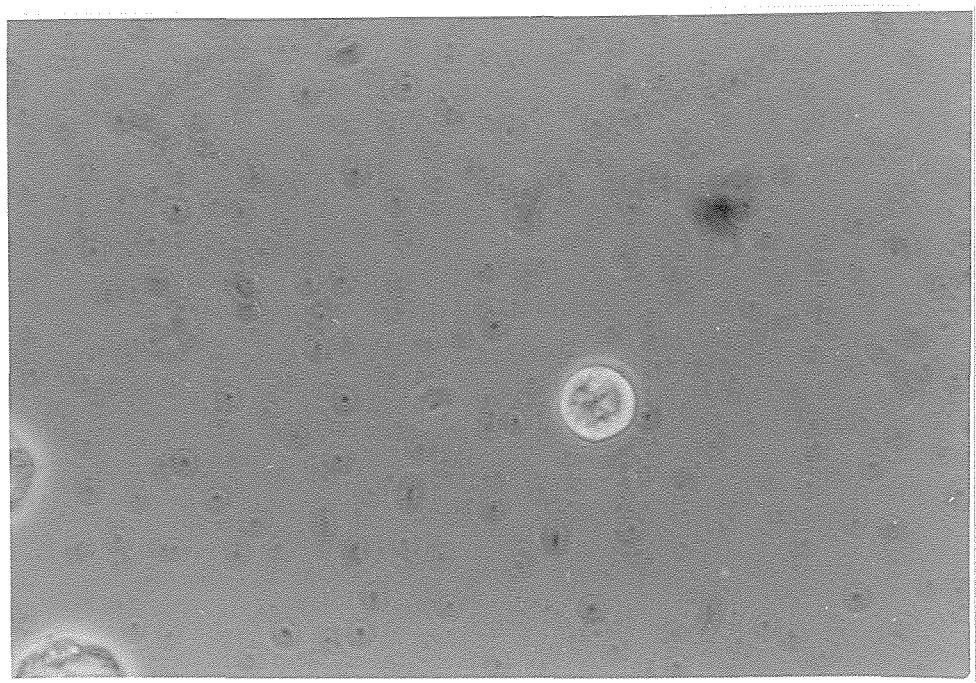
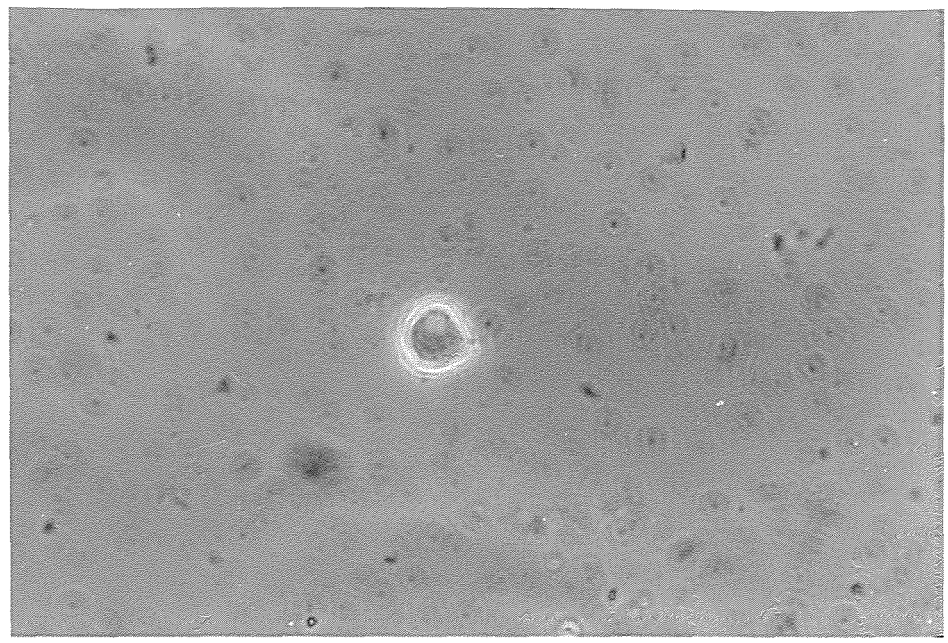


Plate 9 Cyst stage of Naegleria fowleri (NHI)

Plate 10 Cyst stage of Naegleria gruberi (PL200f)



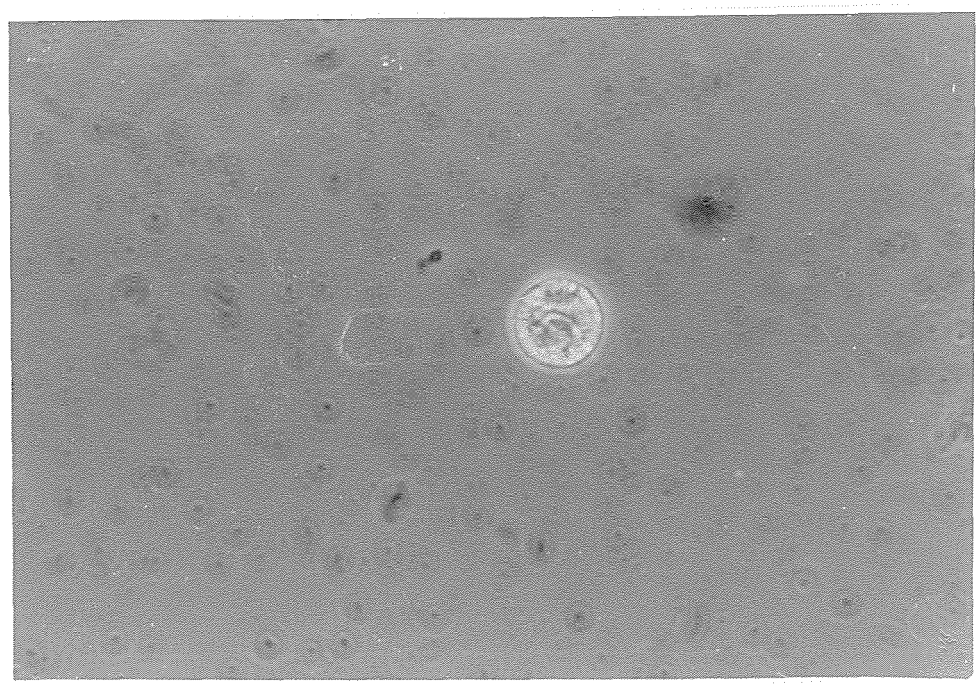
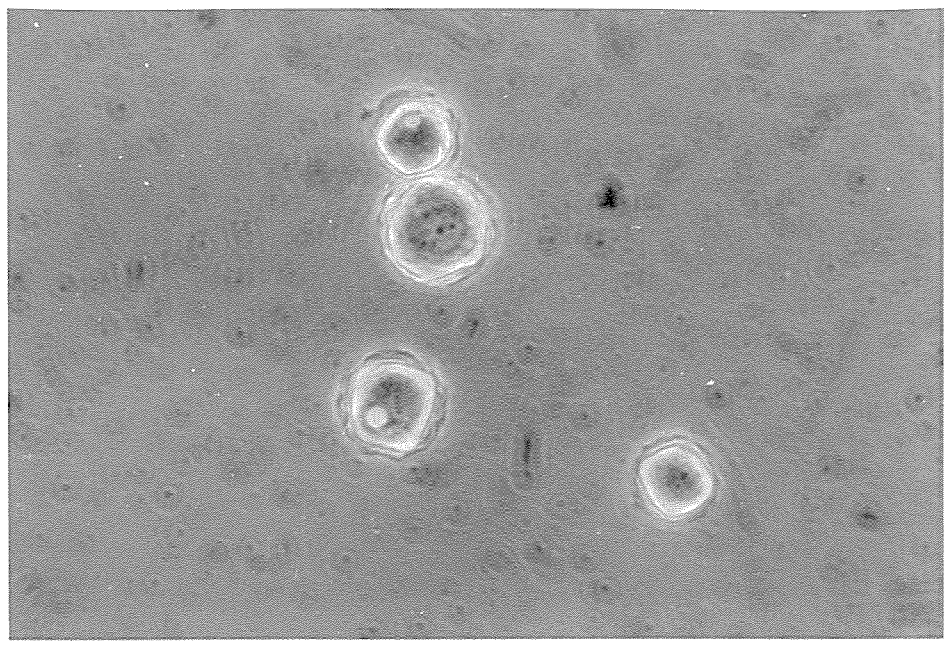
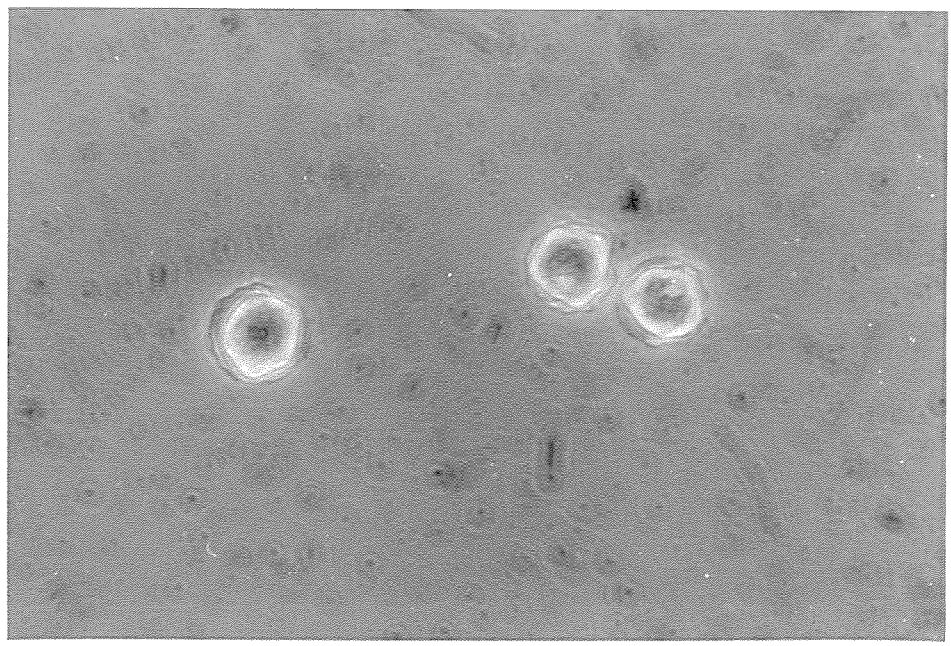


Plate 11 Cyst stage of Acanthamoeba culbertsoni (AI)

Plate 12 Cyst stage of Acanthamoeba castellanii (1501)





3 METHODS:

3.1 Sterilization

Non analytical glassware was sterilized at 103.4 Kpa, 121°C, 15 minutes. Analytical glassware was sterilized by dry heat at a minimum of 160°C, 120 minutes.

3.2 Axenic Culture Technique

3.2.1 Maintenance of amoeboe stock cultures

All axenic cultures of Naegleria species were cultured in CYM medium, A. culbertsoni (A-1) and A. castellanii (ISO1) were cultured in Neff medium. To subculture amoebae cultures 0.5 ml of a culture was inoculated into 4.5 ml of the appropriate medium. Pathogenic species were incubated at 37°C and subcultured every 24 hours, and non-pathogenic species were incubated at 30°C and subcultured every 48 hours. Cultures were incubated in universal bottles on rotary gyroshakers (150 rpm).

3.2.2 Bacteria Culture

Bacterial stock cultures of E. cloacae and E. coli are held by Microbiology Department, Massey University. Bacteria were plated out from stock slopes for single colonies onto BHI agar plates, and incubated overnight at 37°C. A colony from the BHI plate was inoculated into 10 ml of sterile BHI broth, and incubated overnight at 37°C.

3.3 Storage of Amoebae Cultures

Amoebae are stored in the cyst stage, on PAS agar slopes in universal bottles. The cultures are resloped every six months.

To prepare slopes 12 ml of PAS agar is dispensed in universal bottles. After autoclaving the bottles are cooled on a slope.

To store amoebae as cysts; a 24 hour axenic culture of each species held was centrifuged at 1500 rpm for 10 minutes, washed with 5ml of sterile PAS and re-centrifuged. The cultures were washed three times. Onto a PAS agar plate 1.0 ml of washed culture was inoculated with 0.1ml of E. cloacae culture and 4ml PAS. The plates were incubated at 30°C for non-pathogens and 37°C for pathogenic species

for 1 - 2 days. The amoebae were harvested from plates, washed and resuspended in 4ml PAS. PAS agar slopes were inoculated with 1ml harvested amoebae and 0.1 ml of E. cloacae and incubated overnight in sloped position. Stock slopes are stored at room temperature.

3.4 Production of Disinfectants

3.4.1 Chlorine

A stock solution of sodium hypochlorite (BDH) was diluted to the required concentration using sterile, chlorine free, deionized distilled water.

3.4.2 Baquacil

A stock solution of baquacil was obtained from I.C.I. (NZ) Limited and diluted to the appropriate concentration, using sterile 200 ppm hard water.

3.5 Chemical Analysis of Disinfectants

Chlorine solutions were analysed by the DPD method (Palin 1974). Baquacil was analysed by the method written by I.C.I. (N.Z.) Limited. A standard solution is made by adding 1 ml of indicator solution (materials **2.6.1**) to 10 ml of 200 ppm hard water. To 10 ml of test solution 1 ml of indicator solution is added. The solutions are read using a spectrophotomer at absorbance of 540nm. Baquacil concentrations are calculated from a standard curve of absorbance at 540 nm vs Baquacil concentrations.

3.6 Disinfection Tests

Disinfection of Amoebic Trophozoites using Baquacil as the Disinfection (Modific. Dawson et al 1983).

A quality of 10 ml of the axenic test culture was centrifuged at 1200 rpm for 10 minutes. The pellet was resuspended in sterile 0.01 M phosphate buffer and then re-centrifuged this constitutes one wash. The culture was washed three times and then counted using a phase contrast microscope and Zeiss cell counting chamber. The cell concentration was adjusted to 5×10^5 amoebae cells ml^{-1}.

Disinfection levels were monitored before and after incubation of the test period using a 10 ml volume.

One ml of washed amoebae of known cell number was inoculated into 24 ml of 0.1 M phosphate buffer in, 125 ml kimax flask, containing the desired concentration of disinfectant. The amoebae were incubated at the appropriate temperature and time period on a gyrating shaker 120 r.p.m.

Fifteen ml of the disinfection test was filtered through a sterile 5um cellulose acetate millipore filter. There was no neutralizing agent for Baquacil available. The filter was aseptically removed from the millipore apparatus and washed in 5 ml of sterile PAS saline solution. From the washing solution 0.1 ml was plated onto a bacterial lawn of 0.2ml E. cloacae. The plates were incubated at 30°C for non-pathogenic species and 37°C for pathogenic species and after 1-2 days incubation studied for the formation of amoebic plaques. A plaque is a clear area in the bacterial lawn created by amoebae feeding on the bacteria. Plaques are counted as representing the number of amoebae surviving disinfection.

3.6.2 Disinfection by Chlorine of Amoebic Trophozoites

Amoebae from axenic cultures were centrifuged, washed and counted as in method (3.6.1).

1 ml of washed amoebae was inoculated into 114 ml of deionised distilled water containing the required chlorine concentrations, in a 250 ml Erlenmeyer flask. The flasks were then incubated at the required temperature at a time period on a rotating shaker 120 rpm.

A sample of 1 ml of test solution was removed after the incubation period, a crystal of sodium thiosulphate $Na_2S_2O_3.SH_2O$ was added to neutralize the chlorine from the neutralized sample 0.1 ml was plated onto a bacterial lawn of E. cloacae on PASB agar. The plates were incubated and the number of amoebic plaques formed were counted.

The required volume from the disinfection test solution, was used for chemical analysis by titration of the chlorine level as in method 3.5.

3.6.3 Disinfection of Amoebic cysts

A 24 hour axenic culture of amoebic trophozoites was washed and resuspended in sterile PAS saline solution.

To induce amoebae to encyst, 1 ml of amoebae suspension plus

4 ml PAS were plated onto PAS agar. Plates were incubated at 37°C for pathogenic species and 30°C for non-pathogens.

Amoebic cysts were harvested from agar plates, washed and counted. For the disinfection tests cysts were inoculated into a test flask containing the required concentration of 1×10^4 cysts ml^{-1}, the volume in each flask was 1 litre. Flasks were incubated at the required temperature on a rotary shaker 120 rpm. At the required time intervals samples were removed for disinfection level analysis (method **3.4**). A volume of 0.1 ml of sample was plated onto a bacterial lawn of E. cloacae on PASB agar. Plates were incubated (according to method **3.6.1** and **.2**) and the number of bacterial plaques forming counted.

Where the disinfection assays were run for a period of upto 5 days, disinfection levels were boosted, when required to the desired level.

3.7 Encystment Experimentation

3.7.1 Encystment of Amoebae in a Liquid Broth Environment

Axenic cultures of amoebae were centrifuged at 1200 rpm for 10 minutes and washed using sterile PAS saline three times.
The cell concentration established using phase contrast microscope and a haemocytometer. Amoebae were inoculated into duplicate 125 ml kimax flasks containing:

Flask (i) 25 ml 100% soil extract broth (SEB)
Flask (ii) 25 ml 100% SEB + 1 ml penicillin/streptomycin solution.
Flask (iii) 25 ml 25% SEB.
Flask (iv) 25 ml 25% SEB + 1 ml penicillin/streptomycin colution.

The cell concentration in all flasks was 5×10^5 amoebae ml^{-1}. Flasks were incubated at 37°C on rotary shakers 120 rpm.

Duplicate samples were removed from each flask daily. Cell counts differentiating for trophozoites, roundforms and cysts in the total cell population were made triplicate percentages of each cell morphological type were calculated.

Visually the three forms can be distinguished by various morphological features: (for photographs see materials section)

Trophozoites: are motile, have pseudopodia and food vacuoles.

Roundforms: are a pre-encystment phase, these are round in shape, are not motile, have a thin wall surrounding the cell.

Cysts: round in shape and usually occur in clumps, have a thick cell wall which refracts the microscopic illuminating light to a bright yellow. Cysts do not have pseudopodia or food vacuoles.

3.7.2 Encystment of Amoebae on solid nutrient surfaces with and without the presence of Bacteria

Axenic cultures were centrifuged, washed and counted as in method (3.7.1). The amoebae were resuspended in sterile PAS solution at a concentration of 2×10^6 trophozoites ml^{-1}.

The following nutrient agars were inoculated with 4 ml of sterile PAS solution plus 1 ml of amoebae suspension:-

PASB and 20,40, 60, 80, and 100% soil extract agar, all with and without 0.2ml of E. cloacae bacteria.

Plates were incubated at 37°C and duplicate samples were removed every 24 hours from each plate.

Cells counts were performed on samples and the percentage trophozoites, round forms and cysts for each cell population determined, using phase contrast microscope and cell counting chambers.

3.7.3 Effect of Cell concentrations and Nutrient availability on Encystment of Amoebae:

Trophozoites from a 24 hour axenic culture of amoebae were centrifuged, washed and counted as in method (3.7.1).

For each liquid media used, a set of four 125 ml kimax flasks all containing 25 ml of the same broth were inoculated with 1 ml of amoebae, to give four different cell concentrations. Amoebae were at cell concentrations:

Flask (i) 1×10^6 amoebae ml^{-1}.
Flask (ii) 1×10^5 amoebae ml^{-1}.
Flask (iii) 1×10^4 amoebae ml^{-1}.
Flask (iv) 1×10^3 amoebae ml^{-1}.

Each set of four flasks was experimentally set up in duplicate.

Flasks were incubated at 37°C for pathogenic species and 30°C for non-pathogens.

Duplicate samples were taken daily from each flask. Triplicate counts to determine percentages of trophozoites, roundforms, and cysts in the cell population were made. Counts were made using a haemocytometer with phase contrast microscope.

3.7.4 Effect of Temperature on Encystment of Amoebae

Trophozoites were grown in axenic culture for 24 hours then harvested via centrifugation for 10 minutes at 1200 rpm. The cultures were washed three times and resuspended in sterile PAS solution at a cell concentration of 4×10^6 amoebae ml^{-1}.

A 10% SEA plate was inoculated with 4 ml PAS and 1 ml amoebae suspension. Duplicate plates were incubated at the following temperature, 4°C, 15°C, 25°C 30°C, 37°C and 44°C.

Daily, duplicate samples were taken from each plate and cell counts using phase contrast microscope and a cell counting chamber made. The percentage of trophozoites, roundforms and cysts in each cell population was calculated.

To prevent plate dehydration at incubation at higher temperatures, sterile PAS was added at the required intervals.

CHAPTER FOUR: Results

4.1 Encystment of Amoebae in Liquid Nutrient Broth.

Fig. 1 shows in 100% soil extract broth the percentage of trophozoites dropped from 98% to nil over a period of 2 days. The percentage of roundforms (a pre-encystment phase) peaked at 95% on day 2 and cells had started to encyst by day 3. The maximum number of cysts counted was 25% by day six.

In Fig. 2 the overall cell number drops over the initial 2 days of incubation but stabilizes between days 3 and 6 at 5.3 log 10 cells ml^{-1} (1.9×10^5 ml^{-1}). The number of trophozoites decreases over the first 2 days. The number of cysts increases over 3-5 days of incubation and then levels at 4.6 $\log_{10}$ cysts ml^{-1} (3.9×10^4 cysts ml^{-1}).

In Fig. 3 the percentage trophozoites declined to zero by day 2. The percentage of roundforms peaked on the second day of incubation and fluctuated until day 5 where it stablized to 63%. Cysts appeared as 45% of the cell population on the third day of incubation, peaked on day four at 55% and then stablized to 37% of the population.

Fig. 4 shows that total cell number drops after the second day and stabilizes at 5.3 $\log_{10}$ cells ml^{-1} (1.99×10^5 cells ml^{-1}).The number of trophozoites drops after day 2. Roundforms are stable at 5.2 $\log_{10}$ cells ml^{-1} (1.98×10^5 cells ml^{-1}) until day 4. Cysts peak at 4.9 $\log_{10}$ ml^{-1}(7.94×10^4 cells m^{-1}) on day 5.

Fig. 5 & 6 illustrate the total cell number decreases after 3 days incubation to 4.6 $\log_{10}$ cells ml^{-1} (3.98×10^4 cells ml^{-1}) and stablizes around 5.0 $\log_{10}$cells ml^{-1} (1×10^5 cells ml^{-1}) after day 4. Trophozoites are still present in the cell population until day 3 of incubation. Flagellates appear as 10% of the population on day one. The Naegleria flagellate is a transient, non-feeding, non-dividing form (John D.T. 1982). Roundforms appear as 45%, 4.8 $\log_{10}$ cells ml^{-1} (6.3×10^4 ml^{-1}) on day one and decrease as cysts which are present on day 2 as 55%, 4.9 $\log_{10}$ cells ml^{-1} (7.9×10^4 cells ml^{-1}) increase.

In **Fig. 7 & 8** the number of cells decreases to 5.3 $\log_{10}$ cells ml^{-1} (1.99 x 10^5 cells ml^{-1}). The percentage of trophozoites has decreased to zero after day 2 and the number of roundforms peaks at 85%, 5.2 $\log_{10}$ cells ml^{-1} (1.58 x 10^5 cells ml^{-1}). Cysts appear in the cell population on day 5 of incubation at 53%, 4.6 $\log_{10}$ cells ml^{-1} (3.98 x 10^4 cells ml^{-1}).

4.2 Encystment of Amoebae on solid Nutrient surfaces with and without the presence of Bacteria

In **Fig. 9 & 10** the total number of cells decreases till day 3 to 5.25 $\log_{10}$ cells ml^{-1} (1.77 x 10^5 cells ml^{-1}), and then increases and peaks on day 5 at 5.75 $\log_{10}$ cells ml^{-1} (5.6 x 10^5 cells ml^{-1}). Trophozoites are a high percentage of the cell population for the 7 days incubation. The decrease in number of trophozites during days 1-3 is coupled with the formation of roundforms. Cysts are present by day 4 of incubation at a low percentage 5-10%, 4.25 $\log_{10}$ cells ml^{-1} (1.77 x 10^5 cells ml^{-1}).

Fig. 11 & 12 show the total cell number decreases to 5.3 $\log_{10}$ cells ml^{-1} (1.99 x 10^5 cells ml^{-1}) on day 2 of incubation and peaks at 6.75 $\log_{10}$ cells ml^{-1} (5.6 x 10^6 cells ml^{-1}) by day 6. The trophozoites in the population percentage drop to 80% on days 1-3, roundforms appear as 15-20% of the cell population, cysts are present on day 4 at 5% of the cell population.

In **Fig. 13 & 14** the total cell number decreased from 6.75 $\log_{10}$ cells ml^{-1} (5.6 x 10^6 cells ml^{-1}) to 5.9 $\log_{10}$ cells ml^{-1} (7.9 x 10^5 cells ml^{-1}). The percentage trophozoites remained high over 7 days 100 - 95%. Cysts appeared on day 2 and were at a constant low percentage of the cell population.

Fig. 15 & 16 show the total cell number decreased from 7.0 $\log_{10}$ cell ml^{-1} (1 x 10^7 cells ml^{-1}) to 5.8 $\log_{10}$ cells ml^{-1} (6.3 x 10^5 cells ml^{-1}) by day 7. The trophozoites remained high 100-90% till day 4 of incubation, after which the percentage declined to 60% by day 7. Cysts were 5% of the population on day 2 and peaked at 40% on day 7.

In Fig 17 & 18 the total cell number declined over the period of incubation from 6.75 $\log_{10}$ cells ml^{-1} (5.6×10^6 cells ml^{-1}) to 5.6 $\log_{10}$ cells ml^{-1} (3.98×10^5 cells ml^{-1}). Roundforms were 20% of the cell population on day one and were not in the cell population after day 2. Cysts formed by day one and peaked on day 3 as 30% of the cell population.

Fig. 19 & 20 show the total cell number decreases from 6.5 $\log_{10}$ cells ml^{-1} (3.16×10^6 cells ml^{-1}) to 5.6 $\log_{10}$ cells ml^{-1} (3.98×10^5 cells ml^{-1}). Trophozoites are a high proportion 100-85% of the population for 6 days. Roundforms are present on day one only and cysts appear on day 2 and peak on day 4 as 15%, 5.0 $\log_{10}$ cysts ml^{-1} ($1x\ 10^5$ cysts ml^{-1}) and decline to 5% by day 6.

In Fig. 21 & 22 the total cell number declined from 6.5 $\log_{10}$ cells ml^{-1} (3.1×10^6 cells ml^{-1}) to 5.1 $\log_{10}$ cells ml^{-1} (1.25×10^5 cells ml^{-1}) on day 4. The greater percentage of cells were trophozoites - 75% on day 4. Cysts were present by day one and peaked at 25% on day 4.

In Fig. 23 & 24 the total cell number decreased from 6.75 $\log_{10}$ cells ml^{-1} (5.6×10^6 cells ml^{-1}) to 5.4 $\log_{10}$ cells ml^{-1} (2.5×10^5 cells ml^{-1}) on day 6. Trophozoites declined to 77% by day 3 and increased to 89% by day 6. Cysts were 10% of the population on day 2 peaked at 21% on day 3 and stabilized at 10% for the last 3 days of incubation.

In Fig. 25 & 26 the total cell number declines from 7 $\log_{10}$ cells ml^{-1} (1×10^7 cells ml^{-1}) to 5.4 $\log_{10}$ cells ml^{-1} (2.5×10^5 cells ml^{-1}) and increased after day 3. The percentage of trophozoites in the population dropped to 45% on day 4. Cysts appeared in the cell population on day 2 at 24%, 5.25 $\log_{10}$ cells ml^{-1} (1.77×10^5 cysts ml^{-1}) and peaked on day 4 at 55%.

Fig. 27 & 28 show the total cell number decreased from 6.75 $\log_{10}$ cells ml^{-1} (5.6×1^{-6} cells ml^{-1}) to 6.1 $\log_{10}$ cells ml^{-1} (1.25×10^6 cells ml^{-1}). The percentage trophozoites decreased to 45% on day 3 of incubation and peaked to 95% on day 4. Cysts appeared in the cell population on day 2 at 2%, peaked at 55% day 3 and declined day 4.

In Fig. 29 & 30 the total cell number decreased from 6.75 $\log_{10}$ cells ml^{-1} (5.6 x 10^6 cells ml^{-1}) to 6.2 $\log_{10}$ cells ml^{-1} (1.58 x 1-^{6} cells ml^{-1}). The percentage trophozoites decreased to 45 on day 3 of incubation and increased by day 4. Cysts appeared in the cell population at 30% on day 2 and peaked at 55% day 3.

Fig. 31 & 32 show the total cell number decreased from 6.9 $\log_{10} ml^{-1}$ (7.9 x 10^6 cells ml^{-1}) to 6.1 $\log_{10}$ cells ml^{-1} (1.25 x 10^6 cells ml^{-1} (1.25 x 10^6 cells ml^{-1}). The percentage trophozoites decreased to 40% on day 3 and increased on day 4. Cysts appeared in the cell population on day 2 at 28% and peaked on day 3 at 60%.

Fi.g 33 & 34 show the total cell number ml^{-1} decreased from 6.85 $\log_{10}$ (7.07 x 10^6 cells ml^{-1}) to 5.5 $\log_{10}$ cells ml^{-1} (5.16 x 10^5 cells ml^{-1}) on day 7. The percentage trophozoites decreased from 100 to 10 on day 7. Cysts had formed in the cell population by day 2 and peaked at 90% on day 5 and decreased to 80% on day 7.

In Fig. 35 & 36 the total cell number ml^{-1} decreased from 6.85 $\log_{10}$ to 5.6 $\log_{10}$ (7.07 x 10^6 to 3.98 x 10^5 cells ml^{-1}) on day 7. Trophozoite percentage dropped from 100 to 25 after 7 days incubation. Cysts were present in the cell population by day 2 at 18% and peaked at 75% on day 7.

4.3 The Effect of Cell Concentrations and Nutrient Availability on Encystment of N. gruberi

In Fig. 37 & 38 the total cell number dropped from 6 $\log_{10}$ to 5.6 $\log_{10}$ cells ml^{-1} (1 x 10^6 - 3.9 x 10^5 cells ml^{-1}) on day 6 and total cell lysis occurred by day 7. The percentage trophozoites declined from 100% to 0 over seven days incubation. Roundforms counted for 15% of the cell population on day 1 and peaked at 100% on day 6, day 7 total cell lysis occurred. No cysts were formed.

Fig. 39 & 40 show the total cell number dropped from 5 $\log_{10}$ cells ml^{-1} (1 x 10^5cells ml^{-1}) to 4 $\log_{10}$ cells ml^{-1} (1 x10^4 cells ml^{-1}) over 3 days of incubation. Day 4 total cell

lysis had occurred. Trophozoites had decreased to nil, by day 2 roundforms peaked on days 2 and 3 at 100% and lysed by day 4. There was no encystment.

4.4 The Effect of Cell Concentrations and Nutrient Availability on Encystment of N. fowleri

In **Fig. 41 & 42** the total cell number increased from 6 $\log_{10}$ cells ml^{-1} (1×10^6 cells ml^{-1}) to 7.25 $\log_{10}$ cells ml^{-1} (1.78×10^7 cells ml^{-1}) on day 5, the total cell number decreased after day 5. Trophozoites decreased in percentage from 100% to nil over 7 days incubation. Roundforms were present in the population as 10% on day one peaked to 100% on day 8 and lysed by day 9. No cysts were formed.

Fig. 45 & 46 show that the total cell number ml^{-1} increased from 4 $\log_{10}$ cells ml^{-1} (1×10^4 cells ml^{-1}) to 6.75 $\log_{10}$ cells ml^{-1} (5.6×10^6 cells ml^{-1}) on day 6. Trophozoites peaked at 100% for the first 3 days of incubation, and decreased to nil on day 9. Roundforms were present in the population on day 4 and peaked at 100% on day 9 of incubation. No encystment of cells occurred.

In **Fig.47 & 48** the total number of cells ml^{-1} increased from 3.3 $\log_{10}$ cells ml^{-1} (1.9×10^3 cells ml^{-1}) to peak at 6.9 $\log_{10}$ cells ml^{-1} (7.9×10^6 cells ml^{-1}) on day 7 and then decreased. Trophozoites peaked at 100% of the population from day 1 till day 4 of incubation and decreased to nil on day 9. Roundforms were present as 5% of the cell population on day 4 and peaked at 100% day 9. No cysts were formed.

Fig. 49 & 50 show the total number of cells ml^{-1} decreased from 6 $\log_{10}$ cells ml^{-1} (1×10^6 cells ml^{-1}) to 5.5 $\log_{10}$ cells ml^{-1} (3.1×10^5 cells ml^{-1}) over 7 days incubation. Trophozoites peaked at 95% and decreased to nil on day 4. Roundforms peaked at day 2 of incubation at 72%. Cysts were counted on day 2 of incubation and peaked at 60% on day 7.

Fig. 51 & 52 show that the total cell number decreased over the 4 day incubation period until cell lysis occurred. The percentage trophozoites from 100-0 over the first two days of incubation. Roundforms peaked on days 2 and 3. No cysts were formed.

In Fig. 53 & 54 the total cell number decreased over 3 days until cell lysis occurred. The percentage of trophozoites decreased from 100 to nil over the first 2 days of incubation. Roundforms peaked at 100% on the second day of incubation. No cysts were formed.

Fig. 55 & 56 show the total cell number decreased until day 3 of incubation where total cell lysis had occurred. The percentage trophozoites decreased from 100% to nil on day 2 of incubation. Roundforms peaked at 100% on day 2. No cysts were formed.

In Fig. 57 & 58 the same trends were shown as in Fig. 55 & 56.

4.5 Effect of Temperature on Encystment of Amoebae

In Fig. 65 the trophozoites remained a high percentage over 5 days incubation. Cysts appeared in the cell population on day 3, and peaked at 25% on day 4. Roundforms appeared and peaked at 5% on day 3.

When incubated at 44°C on 10% soil extract agar, trophozoites of N. fowleri had lysed and were not detectable after 24 hours incubation.

Fig. 66 shows the percentage trophozoites remained 100% over the first 8 days of incubation, and decreased to 0 percent by day 16 of incubation. Roundforms appeared at 80% of the population on day 12 and decreased to 30% by day 20. Cysts were present as 35% of the population on day 16 and peaked at 70% day 20 of incubation.

Fig. 67 illustrates trophozoites decreased from 100% to 55% of the cell population over 8 days incubation. Roundforms

peaked at 10% of the population percentage on day 2 of incubation. Cysts were 28% of the population on day 3 and increased to 45% after 8 days of incubation at 15°C.

Fig. 68 shows the percentage trophozoites decreased from 100% to 25% after 8 days incubation at 25°C. Cysts increased from 10% on day one to 75% of the cell population. There were no roundforms present.

In Fig. 69 the percentage trophozoites decreased to 64% after 4 days of incubation at 30°C and then peaked again at 90% on day 7. Cysts were 12% after 24 hour incubation and peaked at 36% on day 4 of incubation at 30°C.

In Fig. 70 the percentage trophozoites remained at 100% until day 3. Trophozoites were 0% of the cell population on day 4 of incubation. Cysts were present at 65% of the cell population and peaked at 100% on day 4.

When incubation at 44°C on 10% SEA after 24 hours incubated cells of A. castellanii were not countable and lysis had occurred.

In Fig. 71 Trophozoites remained a very high percentage of the cell population 100% after 8 days incubation, decreasing to 60% after 20 days incubation. Cysts appeared in the cell population after 12 days incubation at 10% and peaked at 40% after 20 days. Roundforms on day 15 of incubation were present as 12% and peaked at 15% on day 16.

Fig. 72 shows the percentage trophozoites declined from 100% on day one of incubation to 0% on day 4. Cysts appeared as 90% on day 2 of incubation and peaked at 100% on day 4.

In Fig. 73 trophozoites decreased from 100% to 10% of the cell population after 24 hours of incubation at 25°C and were not present by day 3 of incubation. Cysts were 90% of the cell population on day 1 of incubation and peaked at 100% on day 2.

In Fig. 74 the percentage trophozoites decreased from 100 to 0 over 2 days incubation at 30°C. Cysts were counted as

90% of the population after 24 hours incubation and peaked at 100 percent on day 2.

Fig. 75 shows trophozoites decreased from 100-0% of the cell population over 4 days incubation at 37°C. Cysts were 60% of the cell population after 24 hours incubation and peaked at 100% after 3 days incubation

When cells of A. culbertsoni were incubated at 44°C, after 24 hours of incubation 100% of the cell population had rounded up and by day 2 total cell lysis had occurred.

When cells of N. gruberi were incubated at 4°C on 10% soil extract agar after 24 hours of incubation total cell lysis had occurred.

Fig. 76 illustrates during 16 days incubation trophozoites were the highest percentage of the cell population. Roundforms were present at a low 5% from days 8 to 14. Cysts peaked at 30% on day 15 of incubation only.

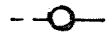
In Fig. 77 the percentage trophozoites decreased from 100% to 0 over 9 days incubation at 25°C. Roundforms counted as 25% after 24 hours incubation and peaked at 80% on day 5 of incubation. Cysts peaked at 66% on day 9 of incubation

Fig. 78 shows trophozoites remained 100% of the cell population until day 3 of incubation, after 6 days trophozoites were no longer present in the cell population. Roundforms appeared at 48% on day 3 of incubation and peaked at 100% on day 6. No cysts were formed.

When cells of N. gruberi were incubated at temperatures 37°C and 44°C on soil extract agar, after 48 hours incubation cells had rounded up and after 3 days total cell lysis had occurred.

In Figures 1-78 the following legend applies;

Trophozoites = 

Roundforms = 

Cysts = 

Flagellates = 

Log_{10} of the number of cells ml^{-1} = 

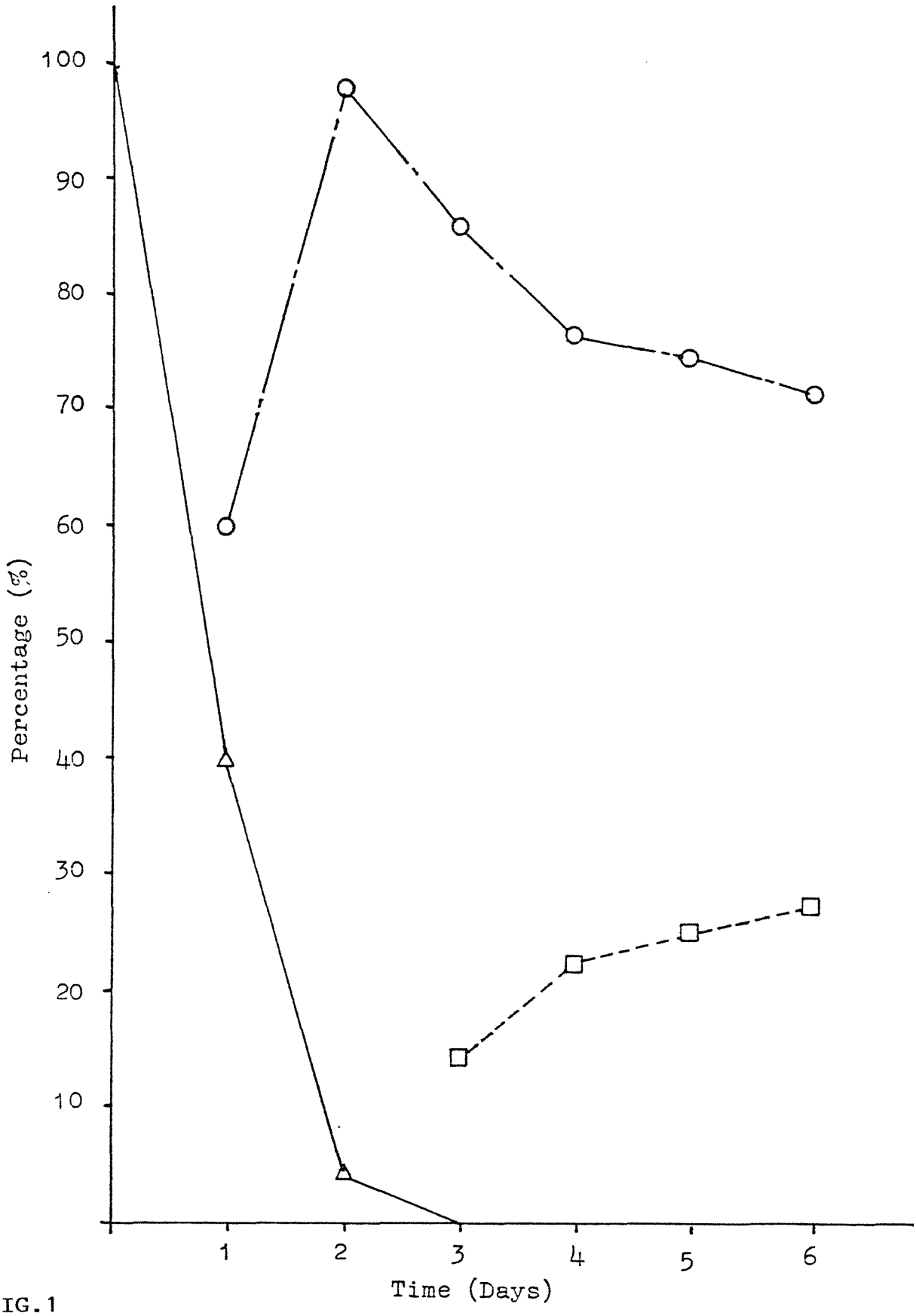


FIG.1

Percentage Trophozoites, round forms and cysts of N. Fowleri during 6 days incubation in 100% soil extract broth.

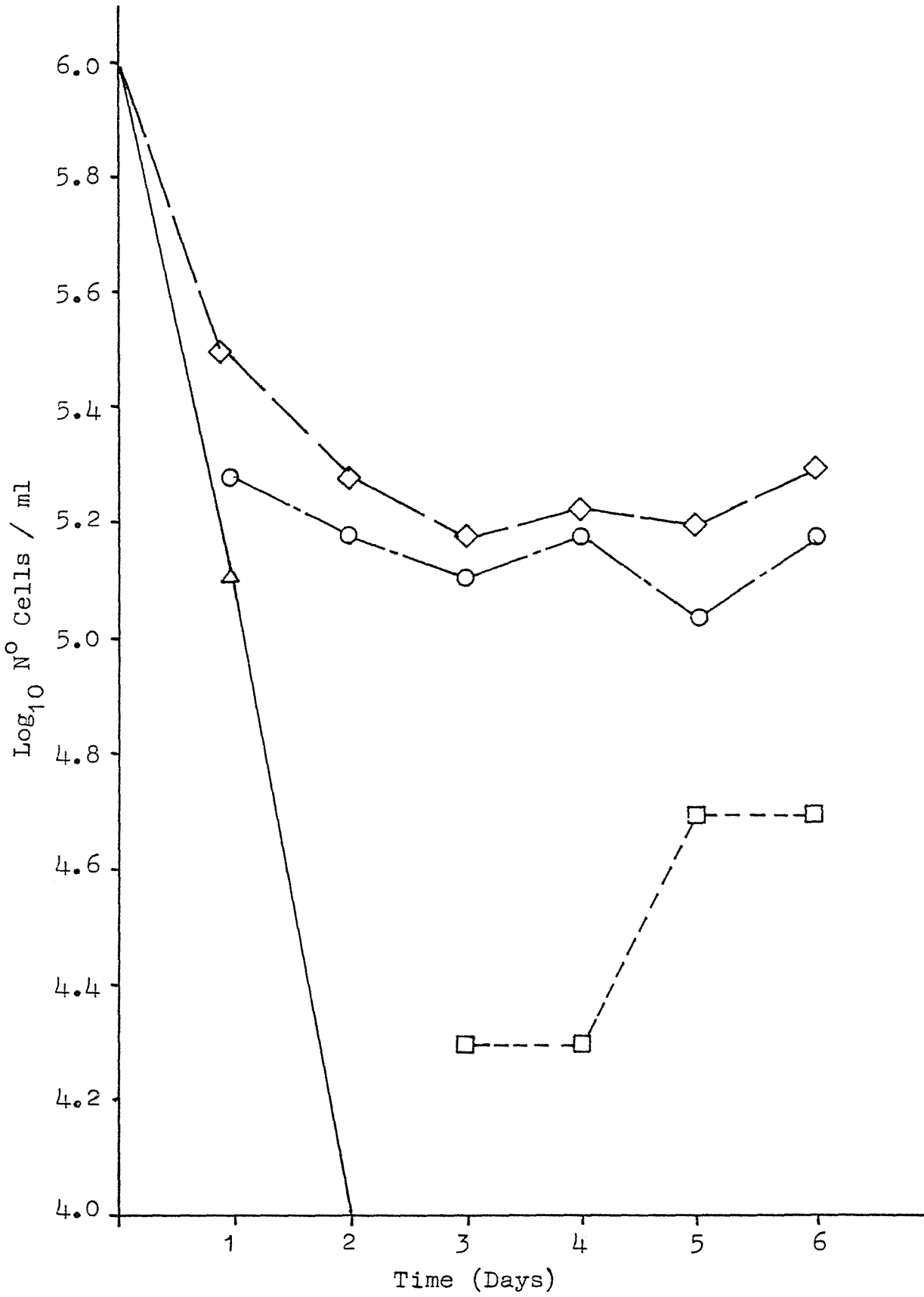


FIG.2

Log 10 of number of trophozoites, round forms and cysts of N. fowleri during 6 days incubation in 100% soil extract broth.

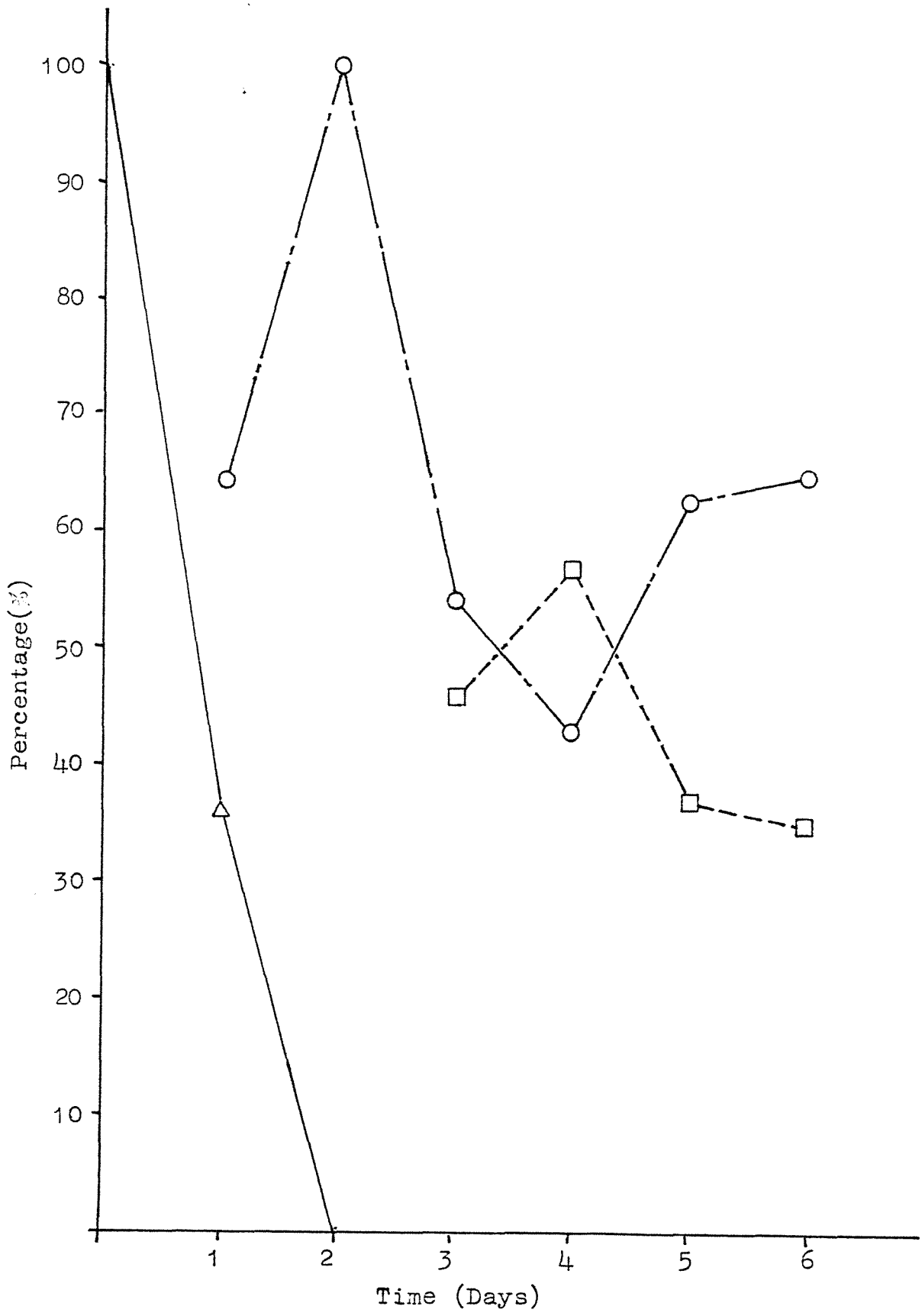


FIG.3

Percentage trophozoites, round forms, and cysts of N. fowleri during 6 days incubation in 100% soil extract broth plus penicillin and steptomycin.

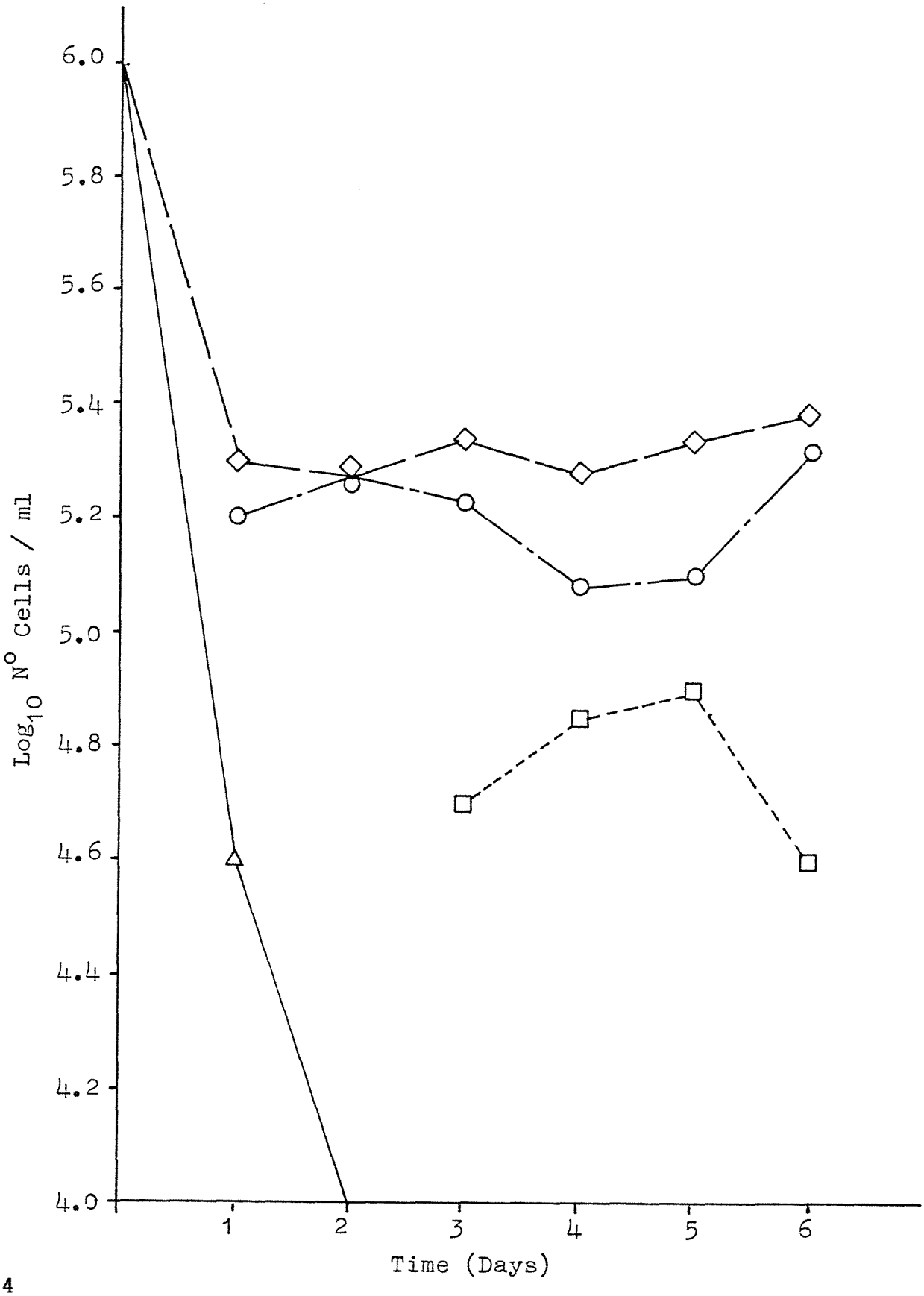


FIG. 4

Log 10 of number of trophozoites, round forms and cysts of N. fowleri during 6 days incubation in 100% soil extract broth plus penicillin and streptomycin.

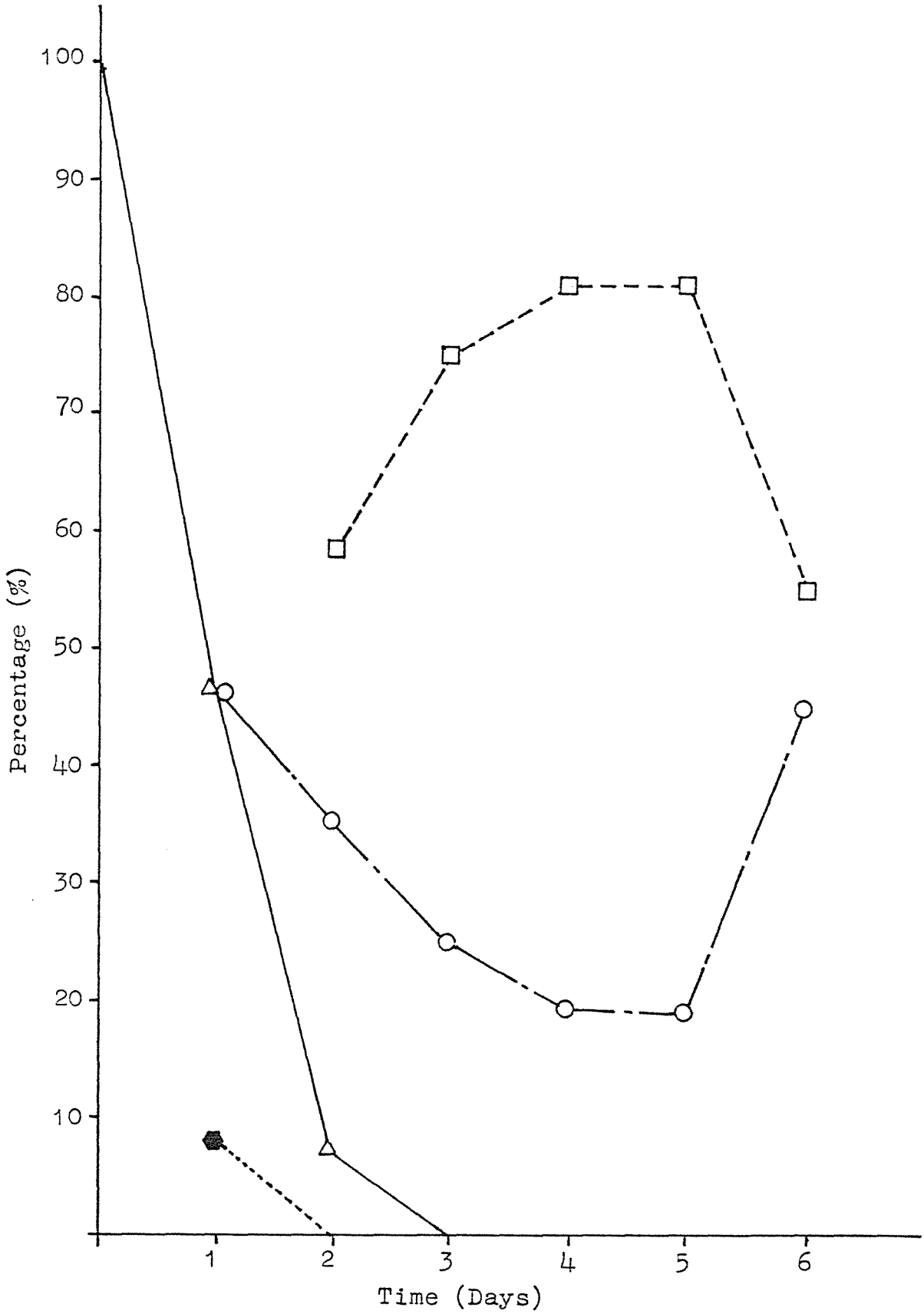


FIG. 5

Percentage Trophozoites, round forms and cysts of N. fowleri during 6 days incubation in 25% soil extract broth.

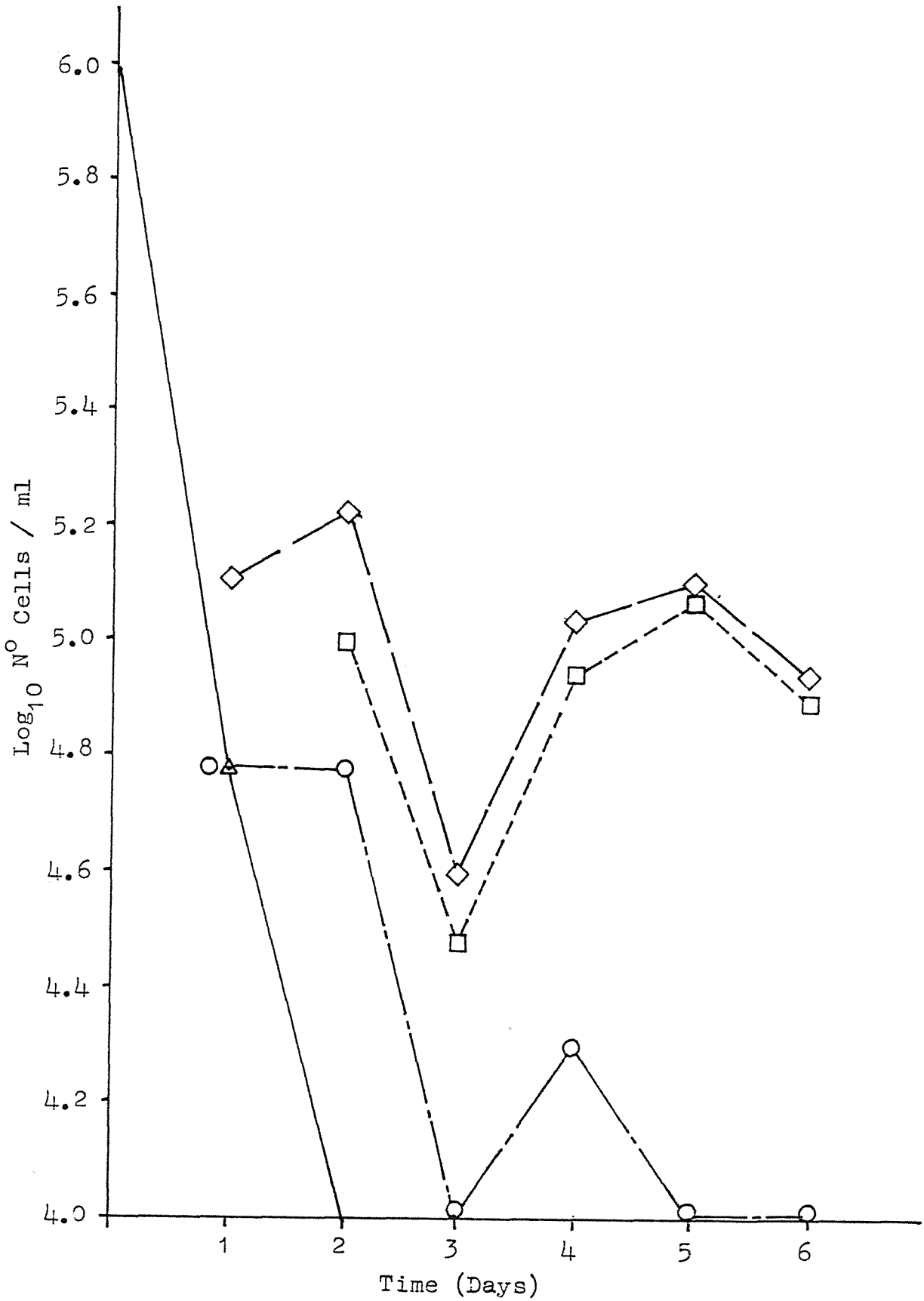


FIG.6

Log 10 of number of trophozoites, round forms and cysts of N. fowleri during 6 days incubation in 25% soil extract broth.

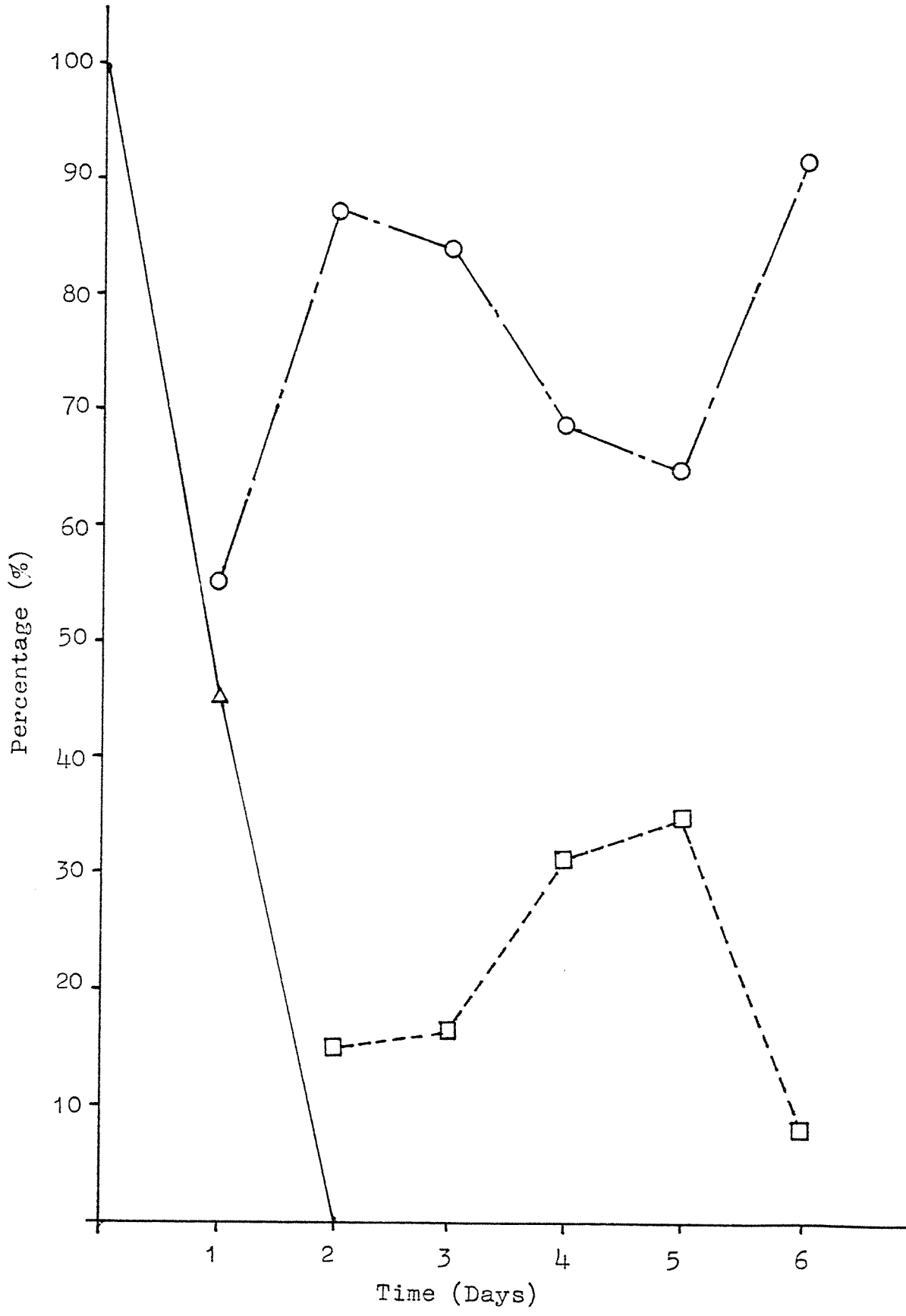


FIG.7

Percentage trophozoites, round forms and cysts of N. fowleri during 6 days incubation in 25% soil extract broth plus penicillin and streptomycin.

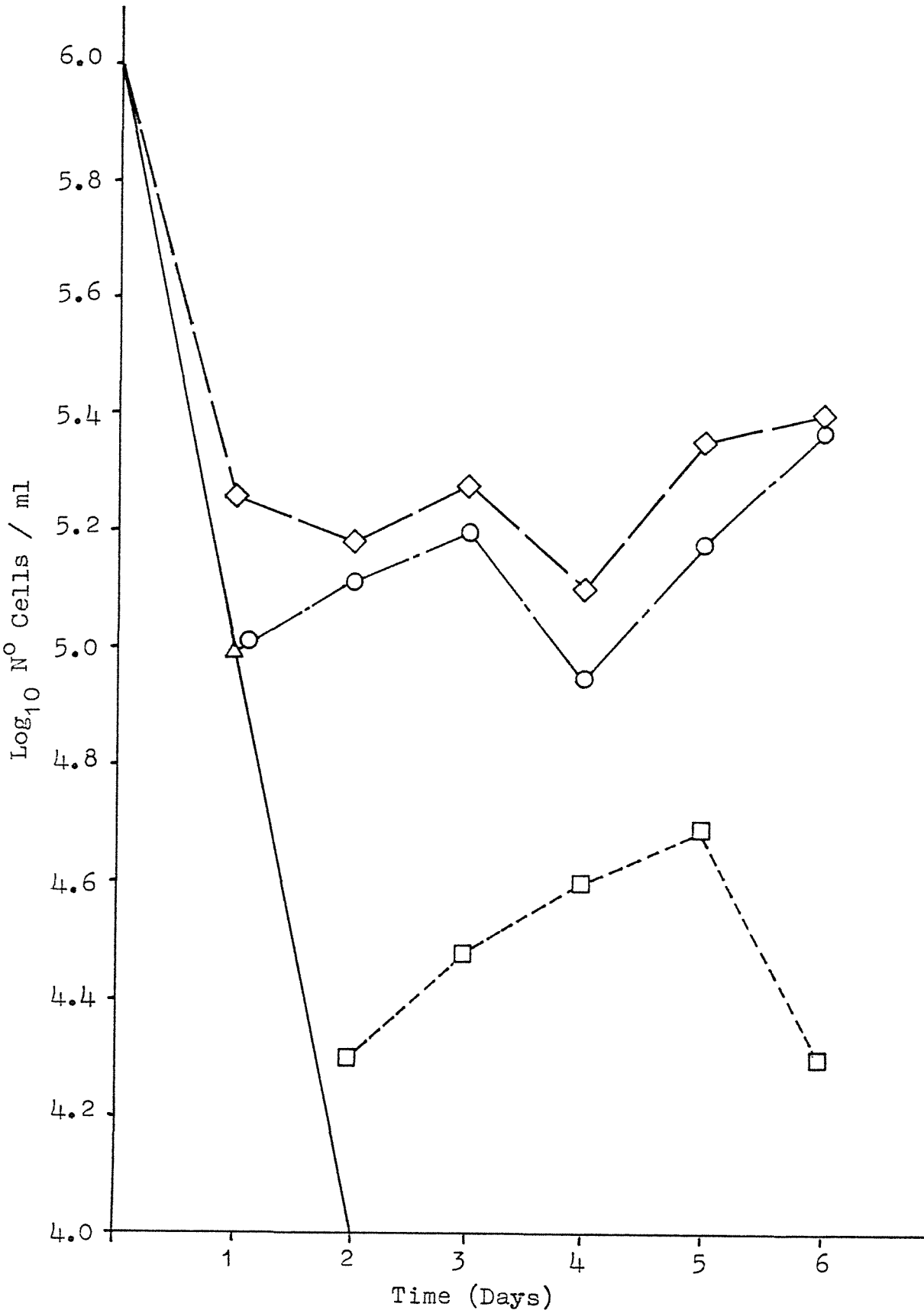


FIG.8

Log 10 of number of trophozoites, round forms, cysts of N. fowleri during 6 days incubation in 25% soil extract broth plus penicillin and streptomycin.

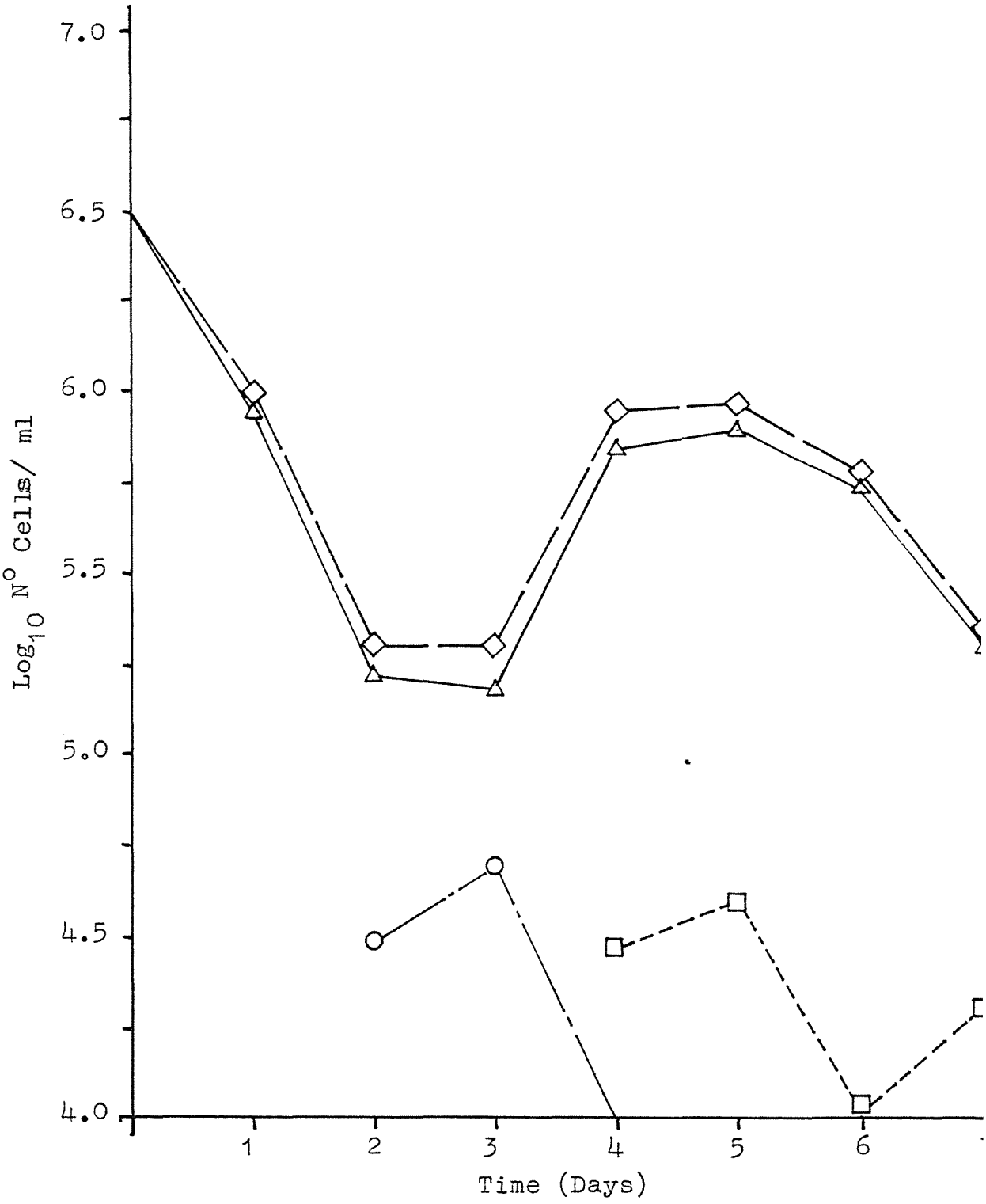


FIG.9

Log 10 of number of trophozoites, round forms and cysts during 7 days incubation on PASB agar.

N. fowleri

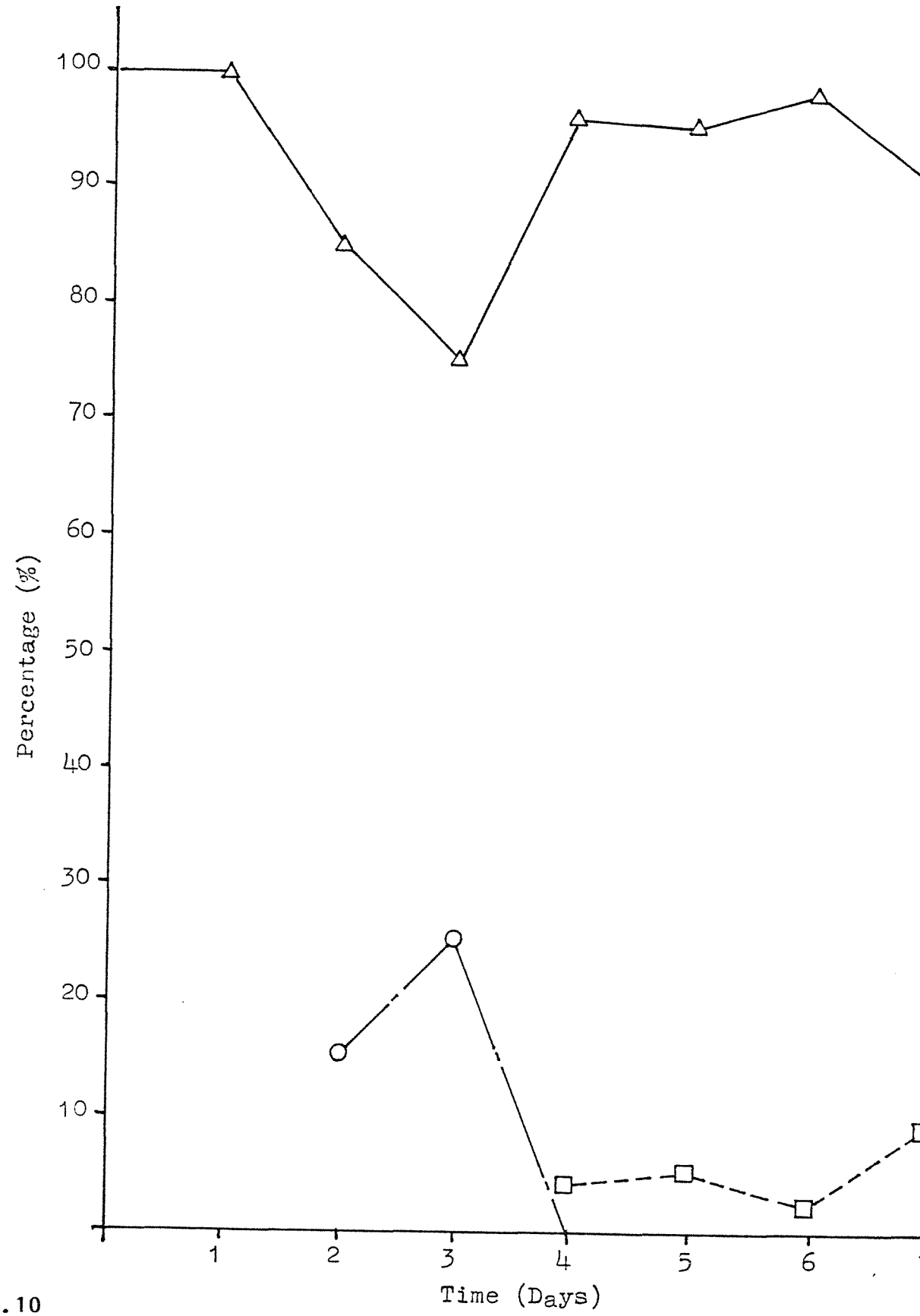


FIG.10

Percentage trophozoites, round forms and cysts during 7 days incubation on PASB agar.

N. fowleri

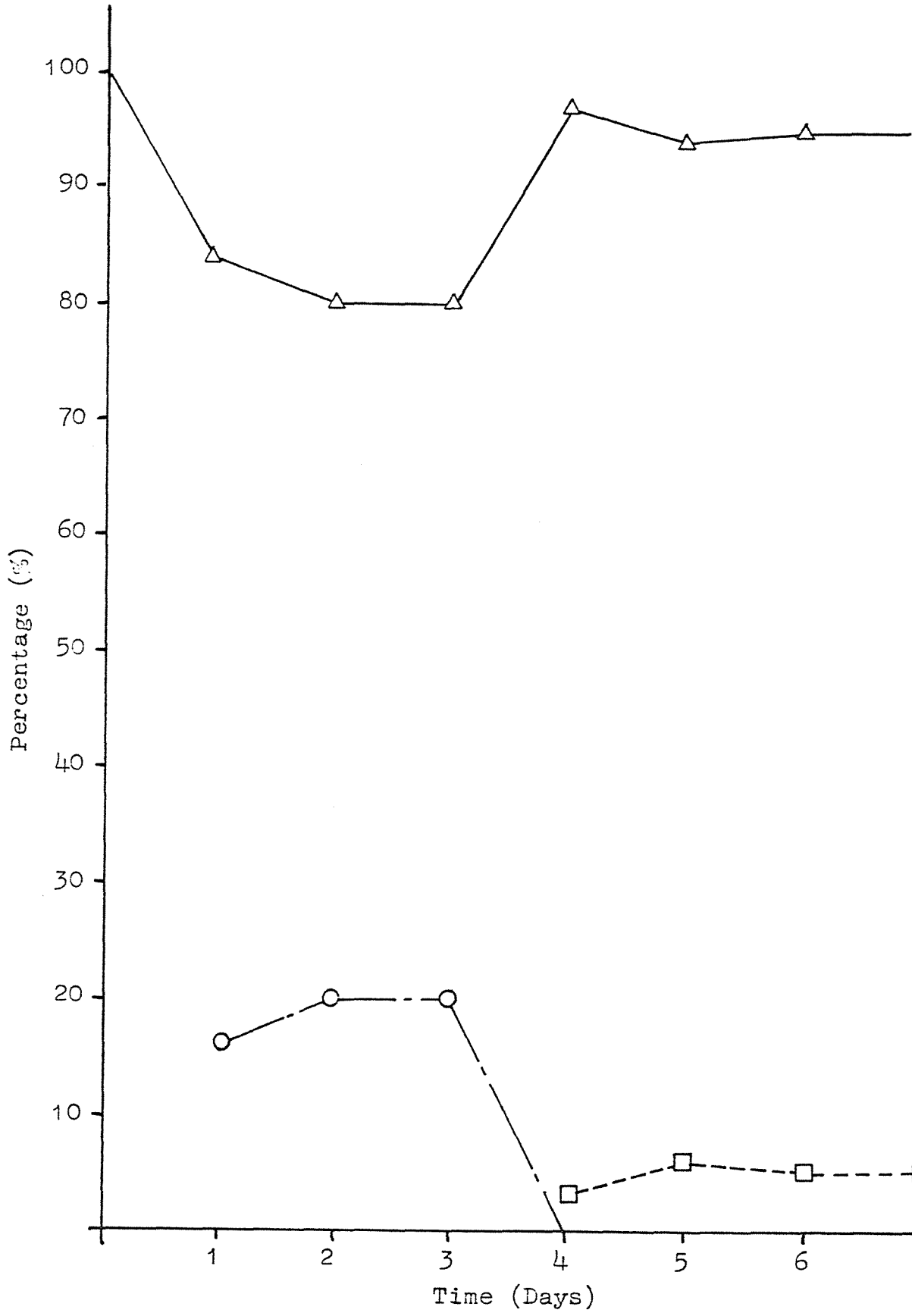


FIG. 11

Percentage trophozoites, round forms and cysts of N. fowleri during 7 days incubation on PASB agar plus E. cloacae

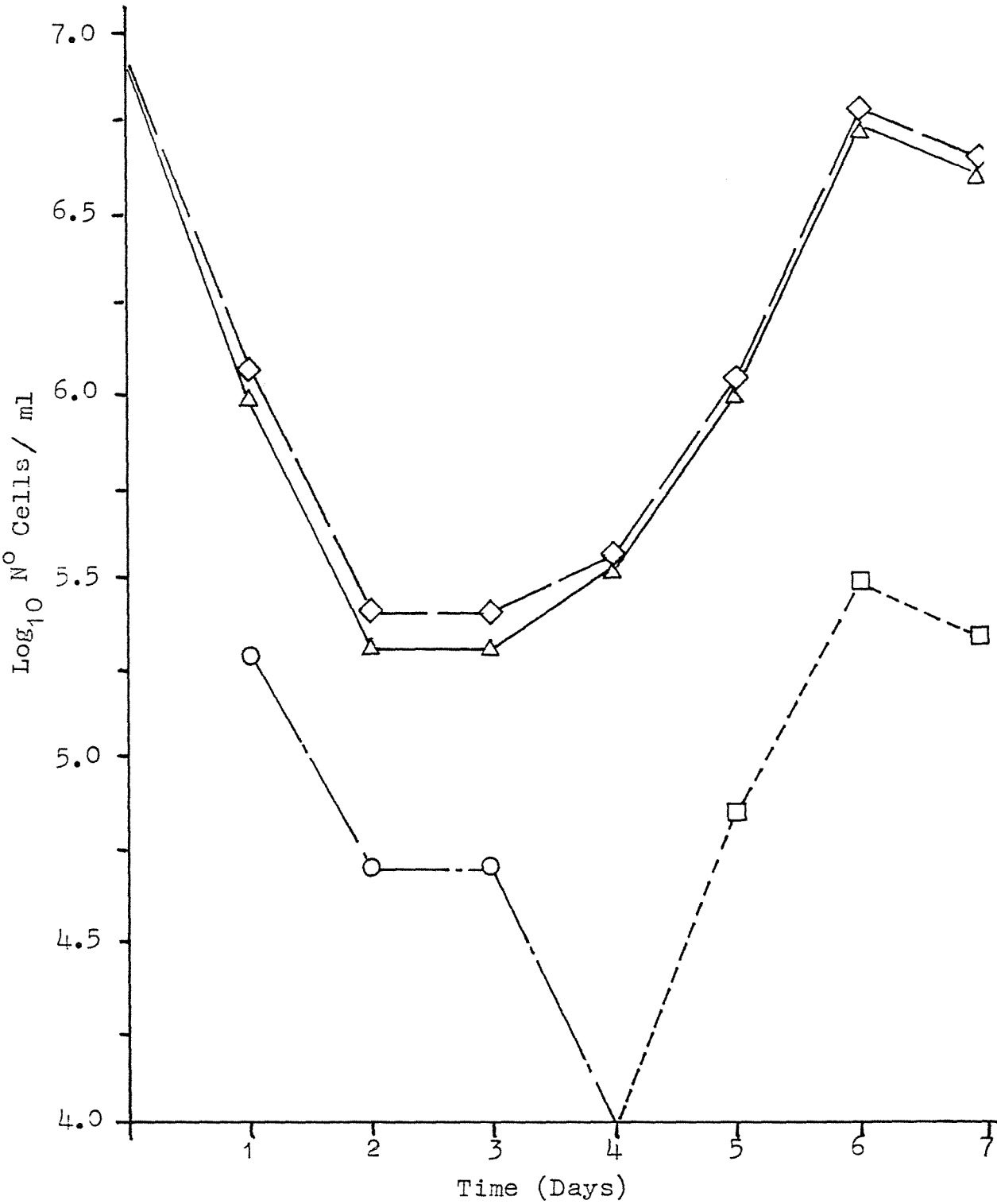


FIG.12

Log 10 of number of trophozoites, roundforms and cysts of N. fowleri during 7 days incubation on PASB agar plus E. cloacae

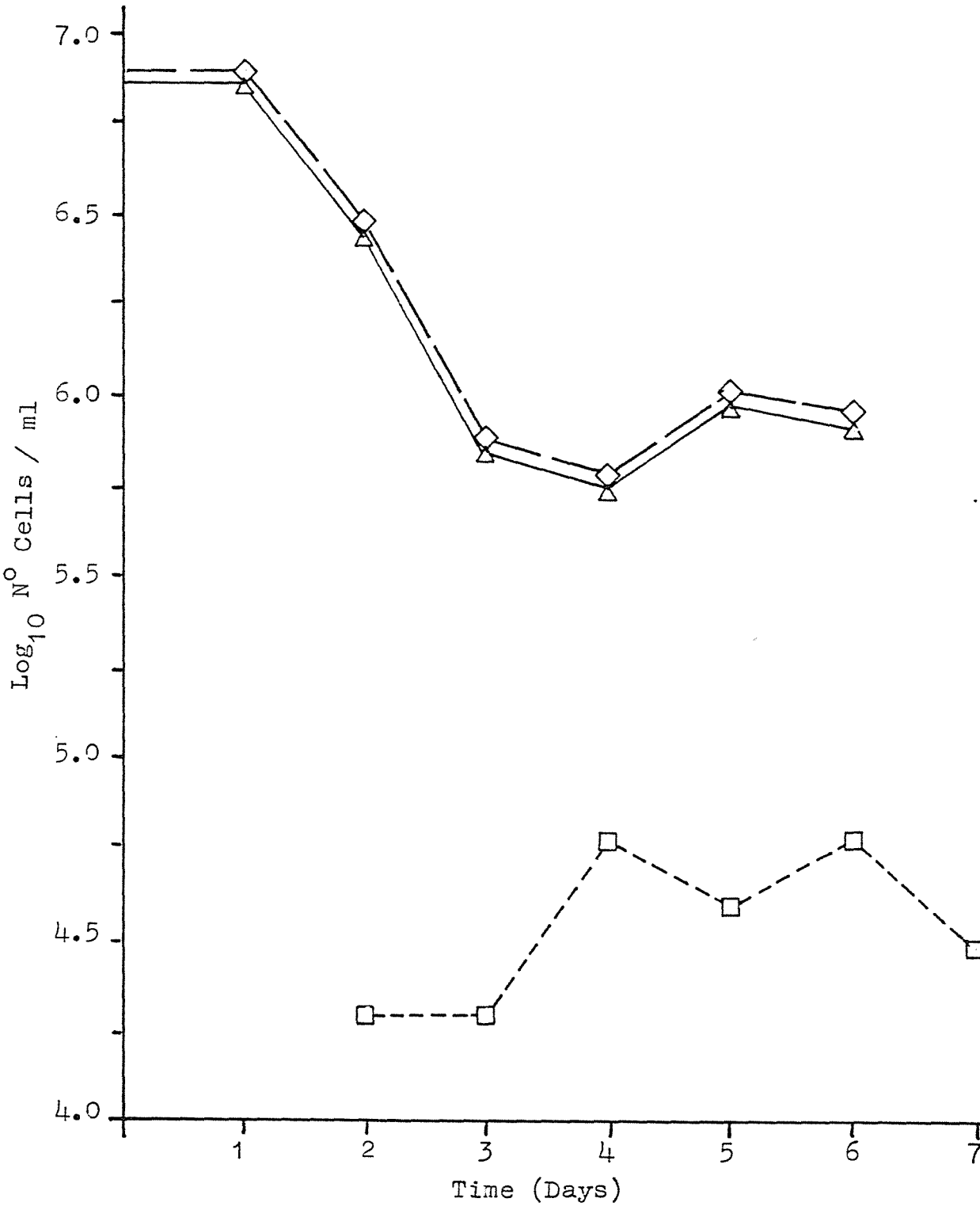


FIG.13
Percentage trophozoites, roundforms and cysts of N. fowleri during 7 days incubation on 10% soil extract agar.

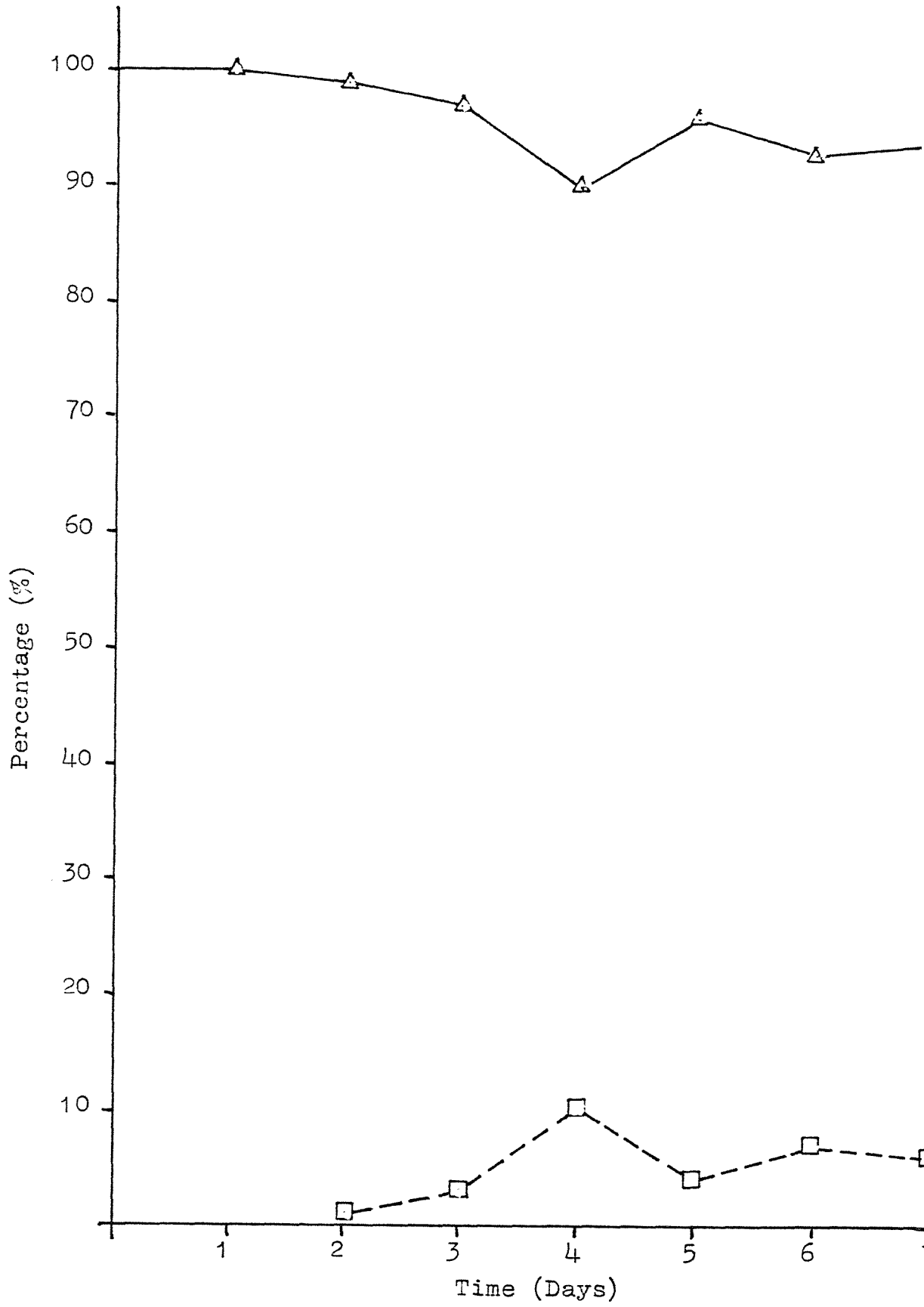


FIG.14

Log 10 of number of trophozoites, roundforms and cysts of N. fowleri during 7 days incubation on 10% soil extract agar.

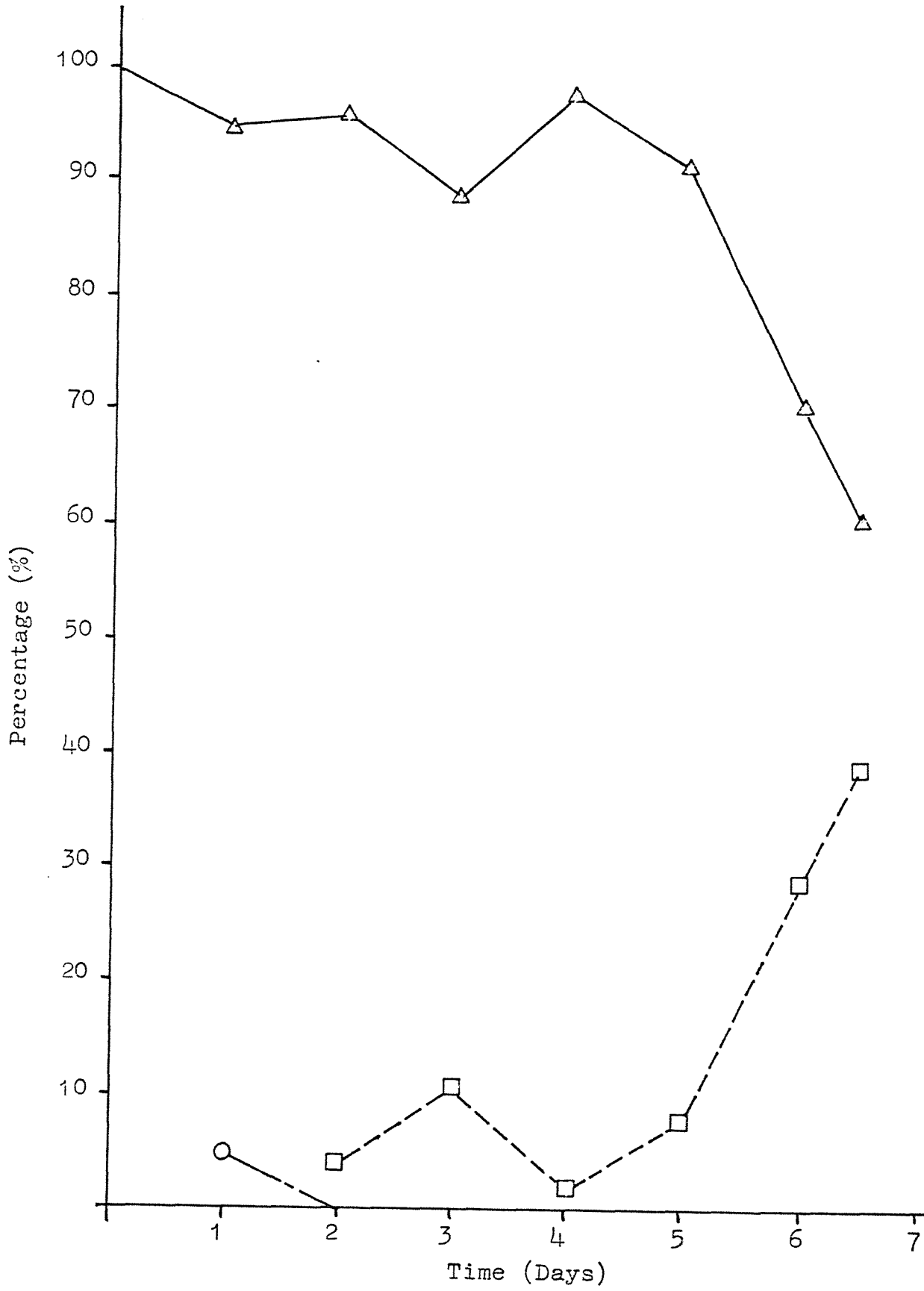


FIG.15

Percentage trophozoites, roundforms and cysts of N. fowleri during 7 days incubation on 10% soil extract agar plus E. cloacae.

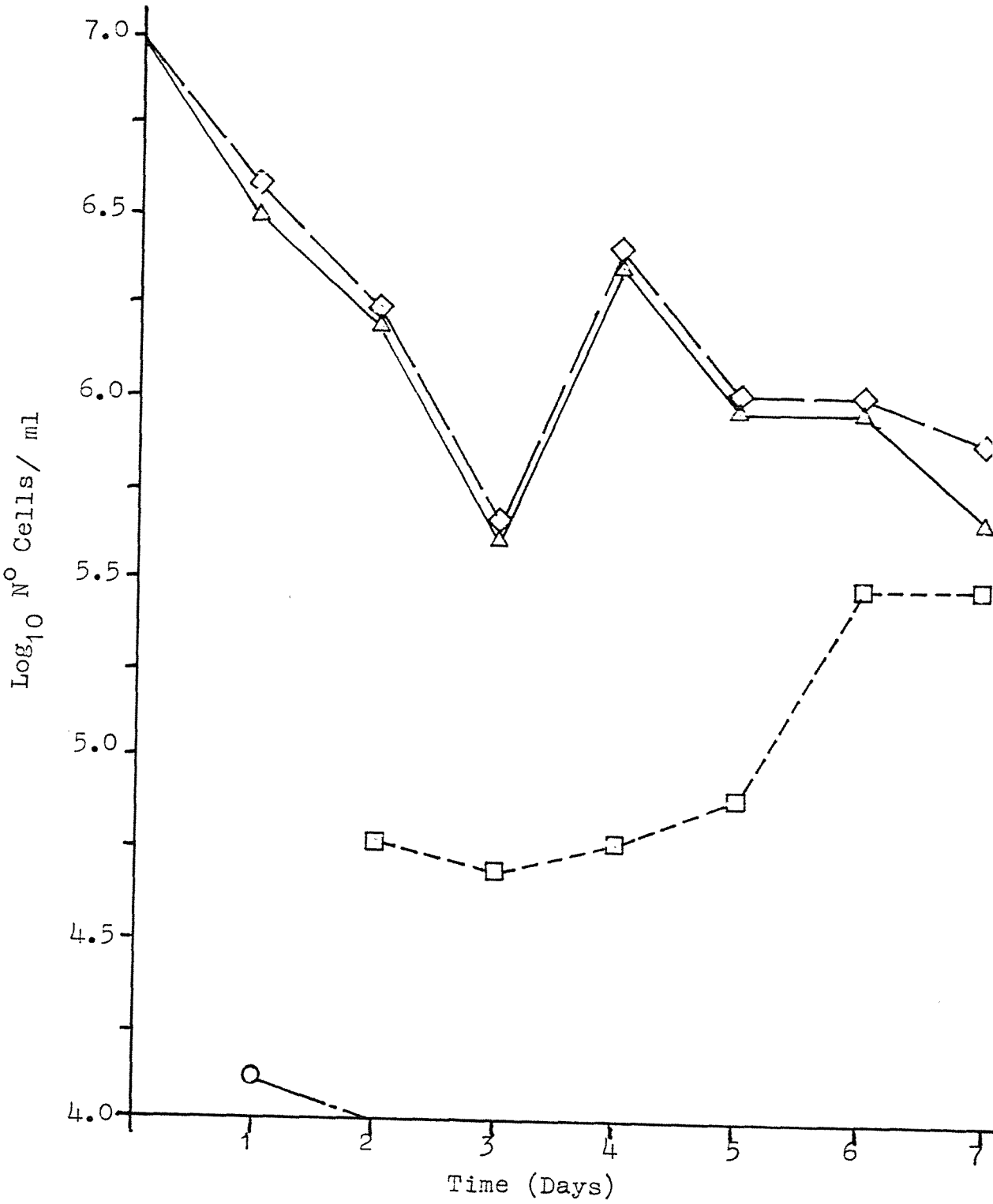


FIG. 16

Log 10 of number of trophozoites, roundforms and cysts of N. fowleri during 7 days incubation on 10% soil extract agar plus E. cloacae.

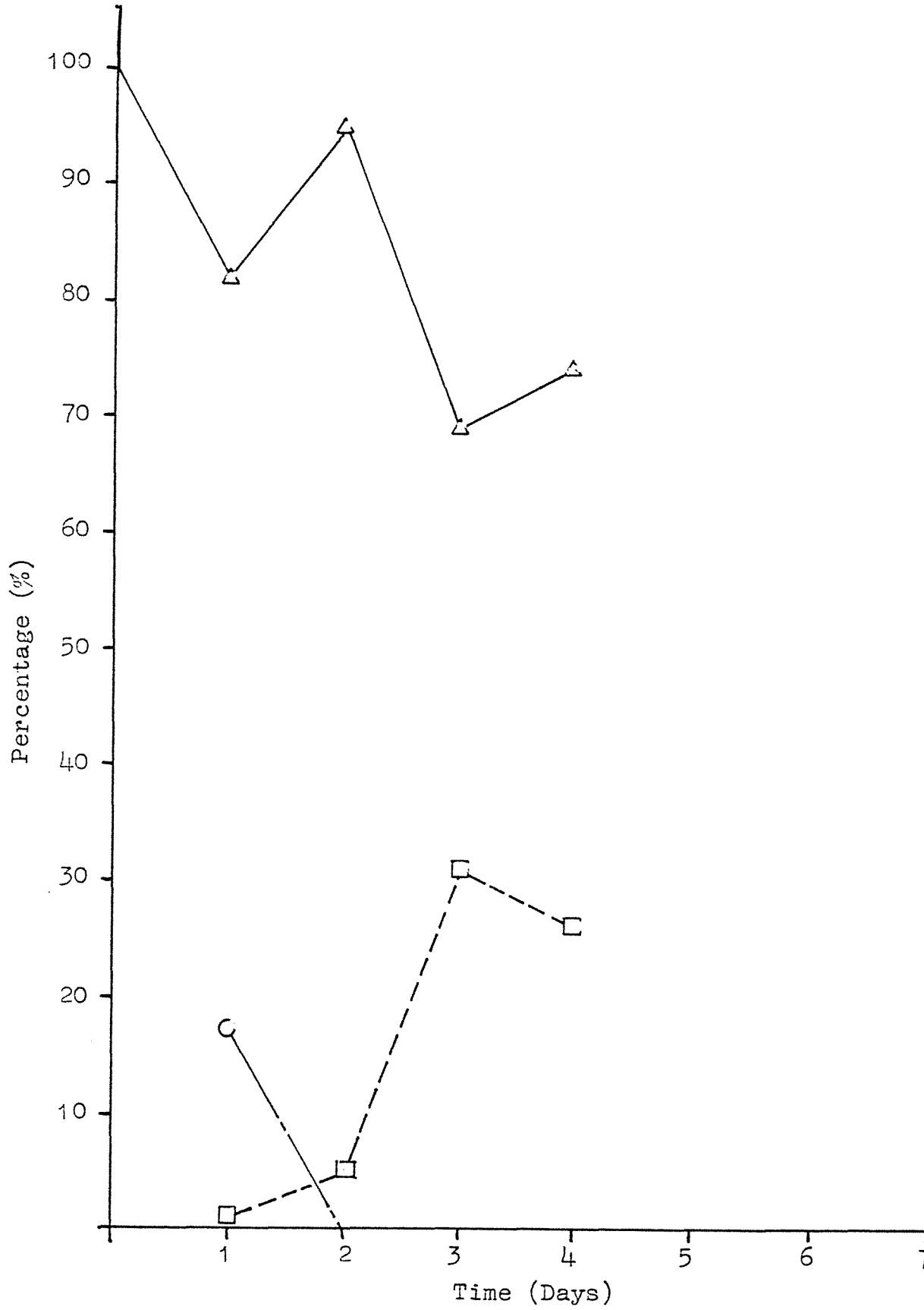


FIG. 17

Percentage trophozoites, roundforms and cysts of N. fowleri during 4 days incubation on 20% soil extract agar.

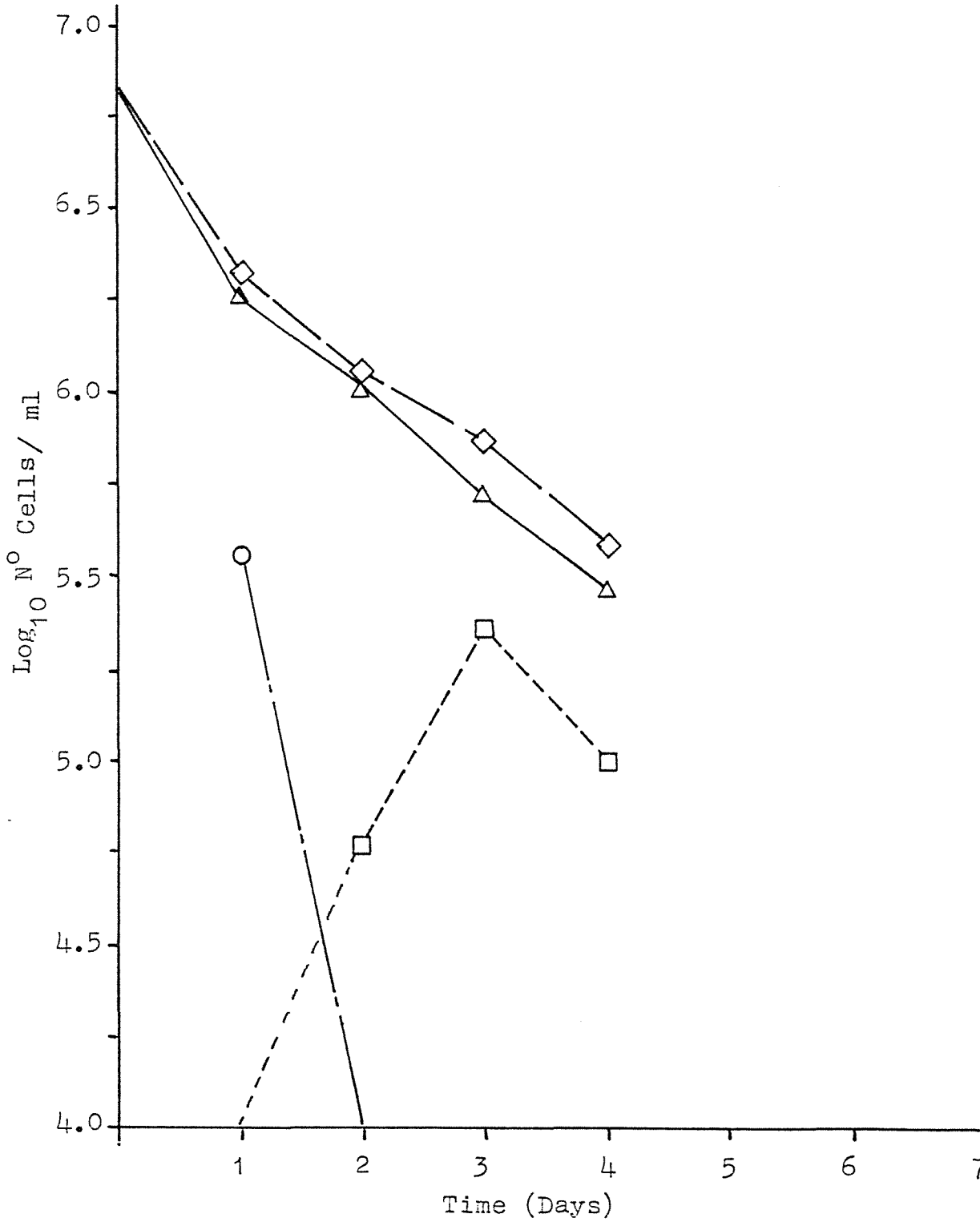


FIG. 18

Log 10 of number of trophozoites, roundforms and cysts of N. fowleri during 4 days incubation on 20% soil extract agar.

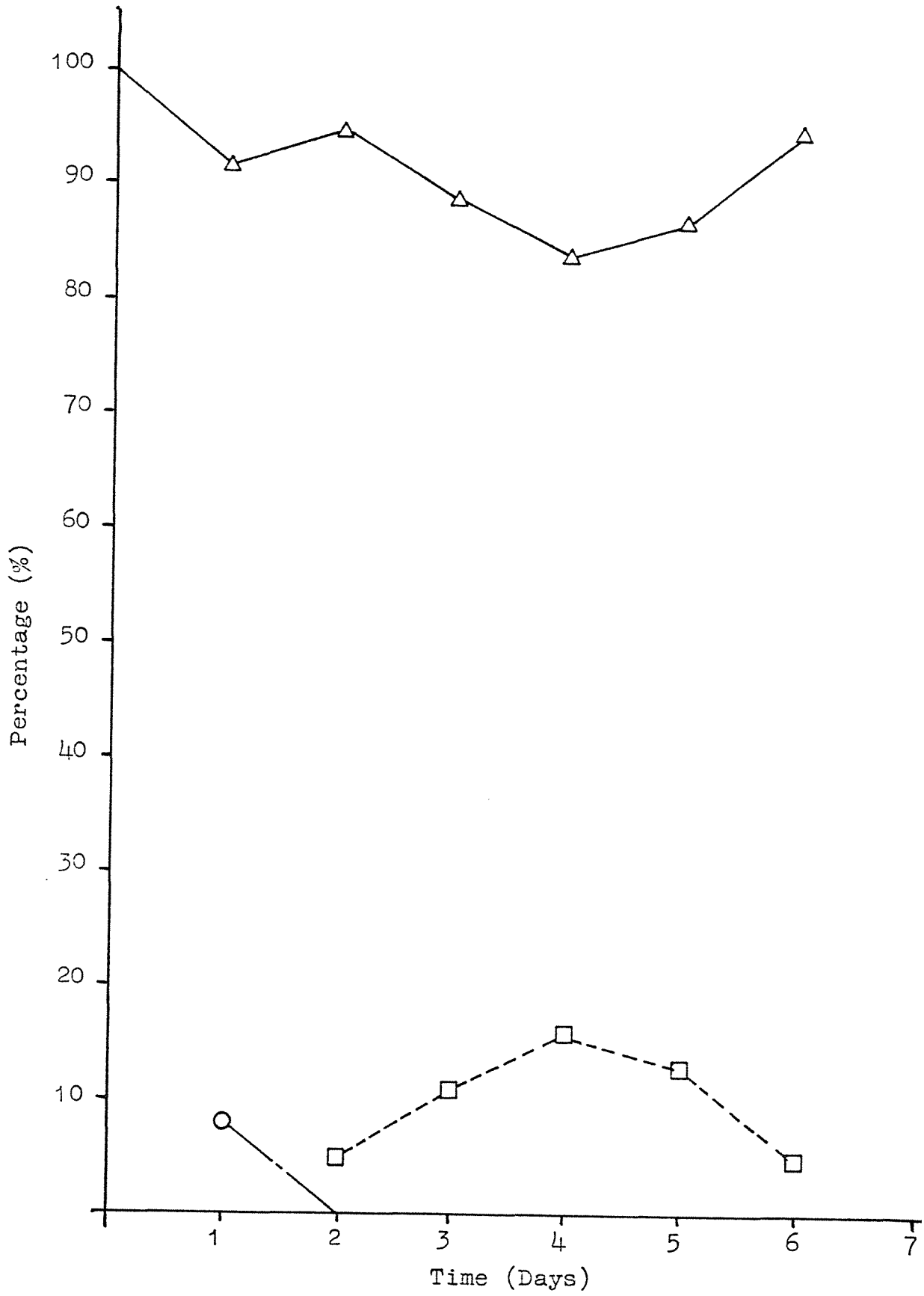


FIG.19
Percentage trophozoites, roundforms and cysts of N. fowleri during six days incubation on 20% soil extract agar plus E. cloacae.

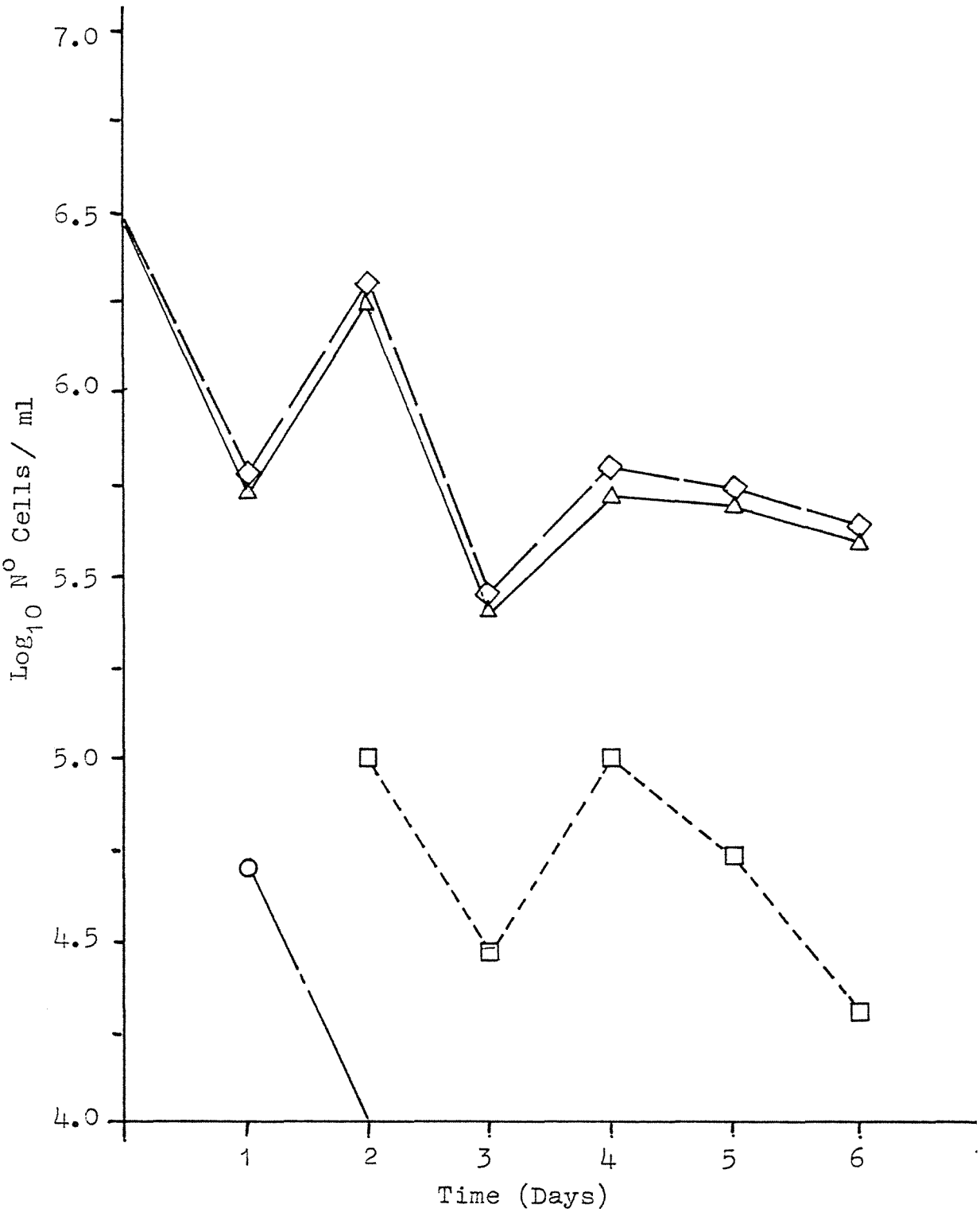


FIG. 20
Log 10 of number of trophozoites, roundforms, and cysts of N. fowleri during six days incubation on 20% soil extract agar plus E. cloacae.

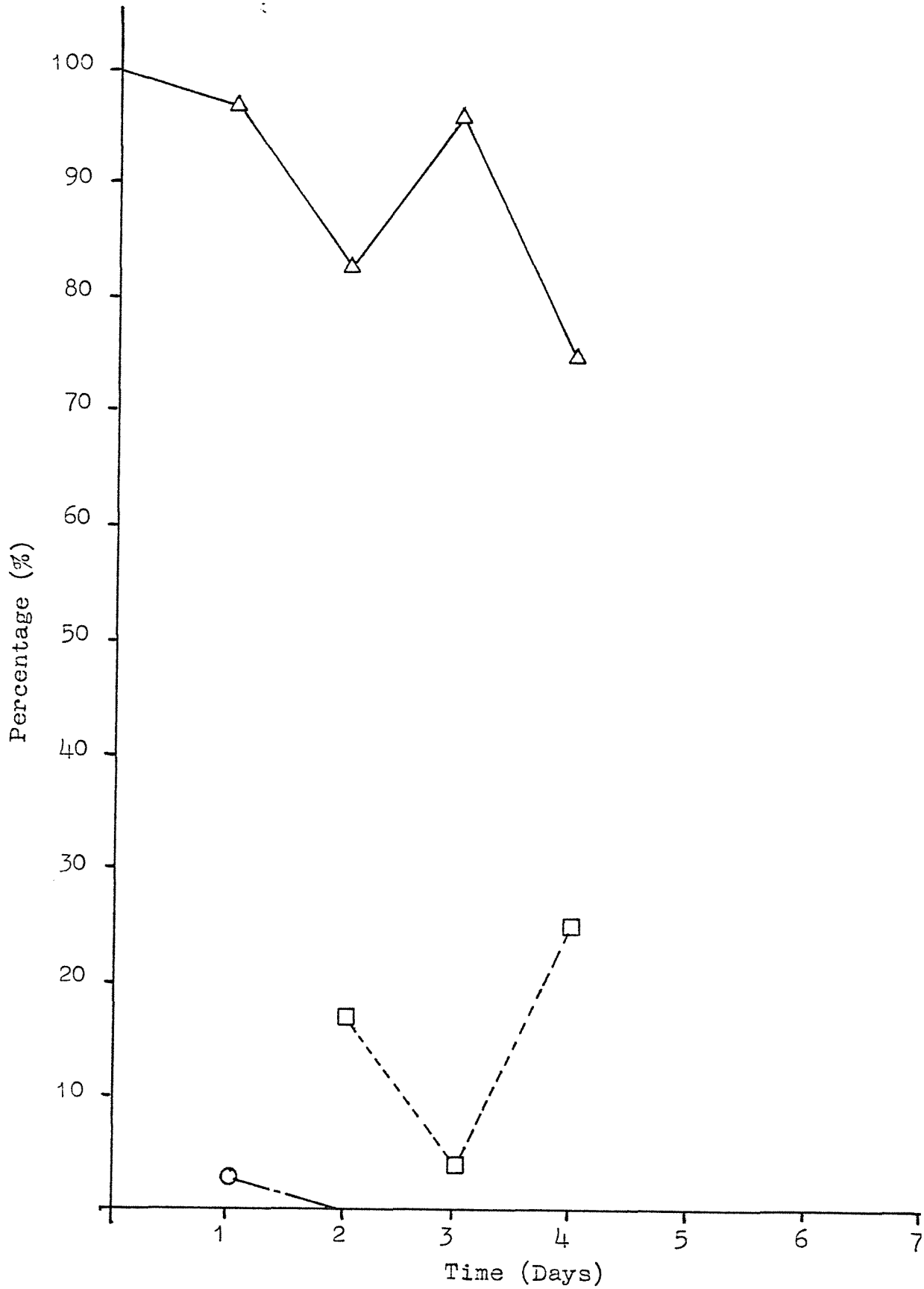


FIG.21

Percentage trophozoites, roundforms and cysts of N. fowleri during 4 days incubation on 40% soil extract agar.

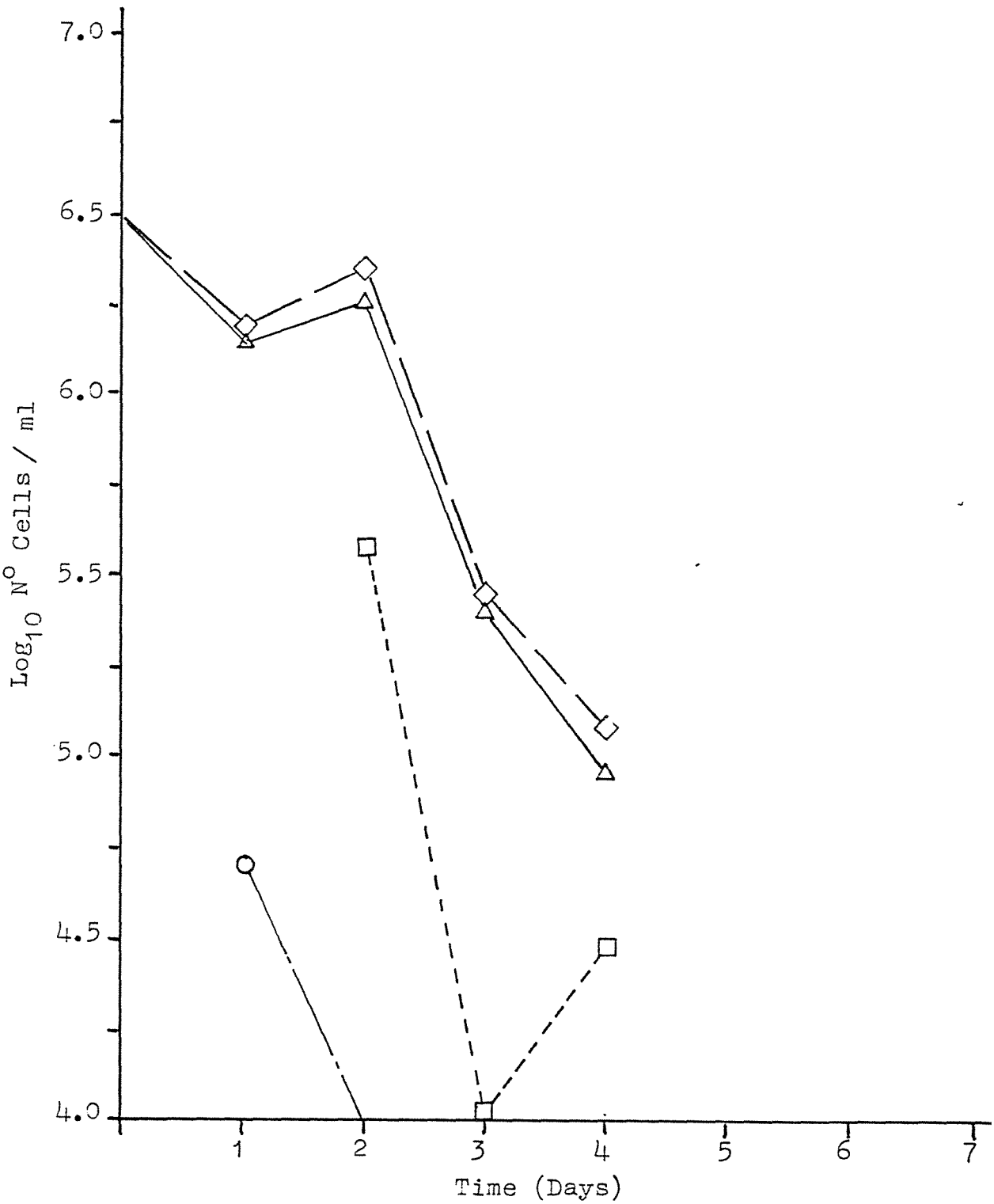


FIG.22
Log 10 of number of trophozoites, roundforms and cysts of N. fowleri during 4 days incubation on 40% soil extract agar.

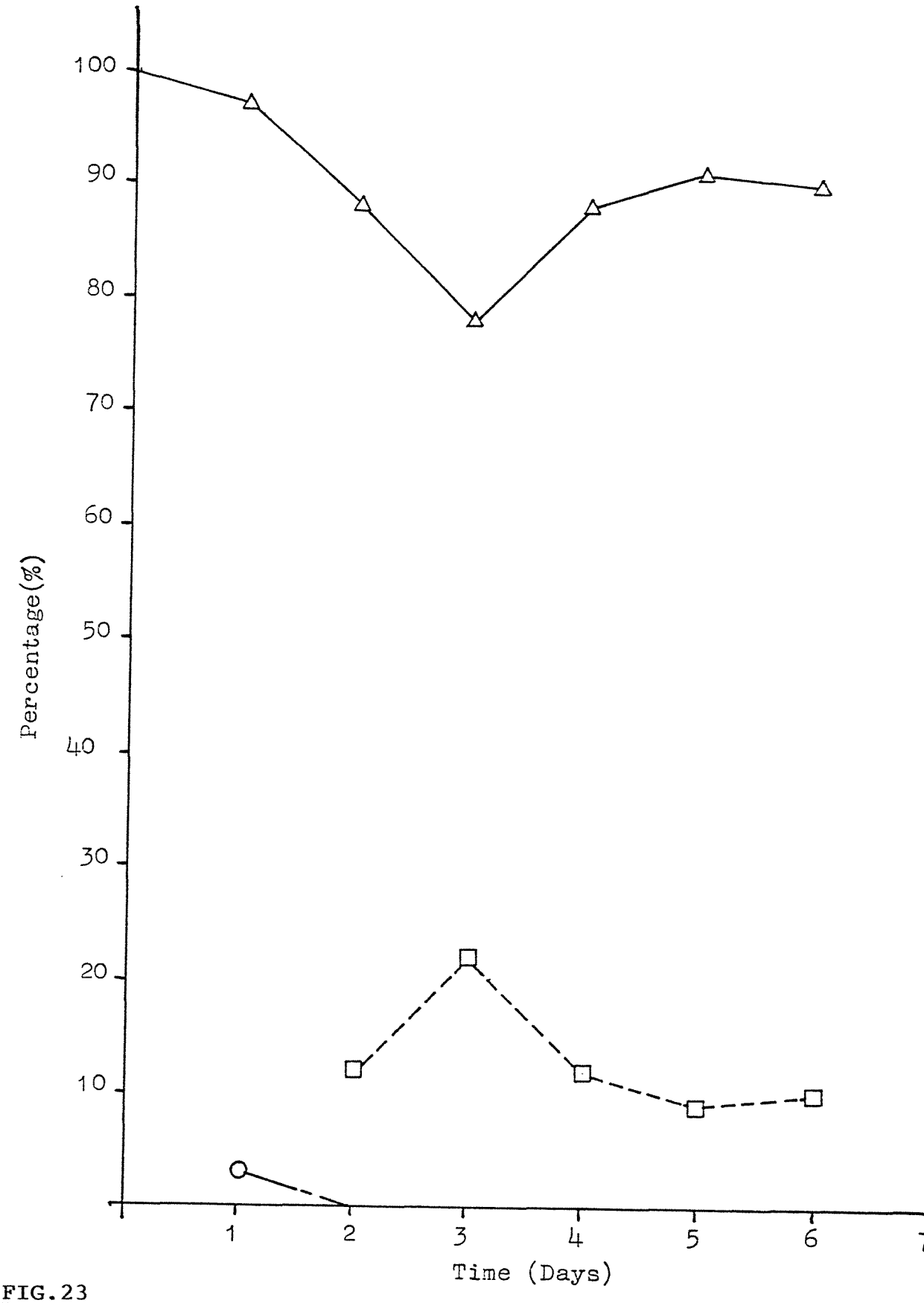


FIG.23

Percentage trophozoites, roundforms and cysts of N. fowleri during 6 days incubation on 40% soil extract agar plus E. cloacae.

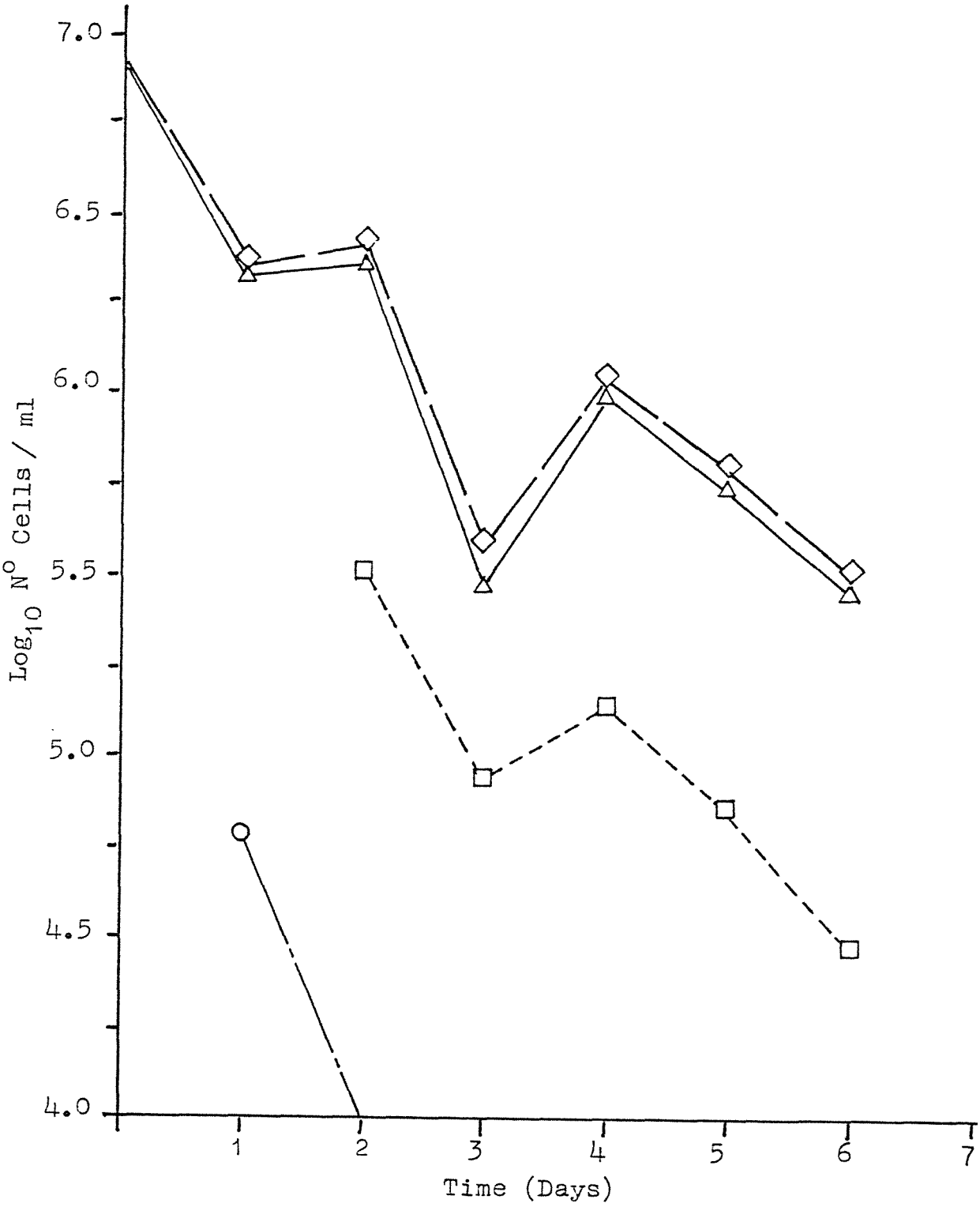


FIG.24

Log 10 of number of trophozoites, roundforms and cysts of N. fowleri during 6 days incubation on 40% soil extract agar plus E. cloacae.

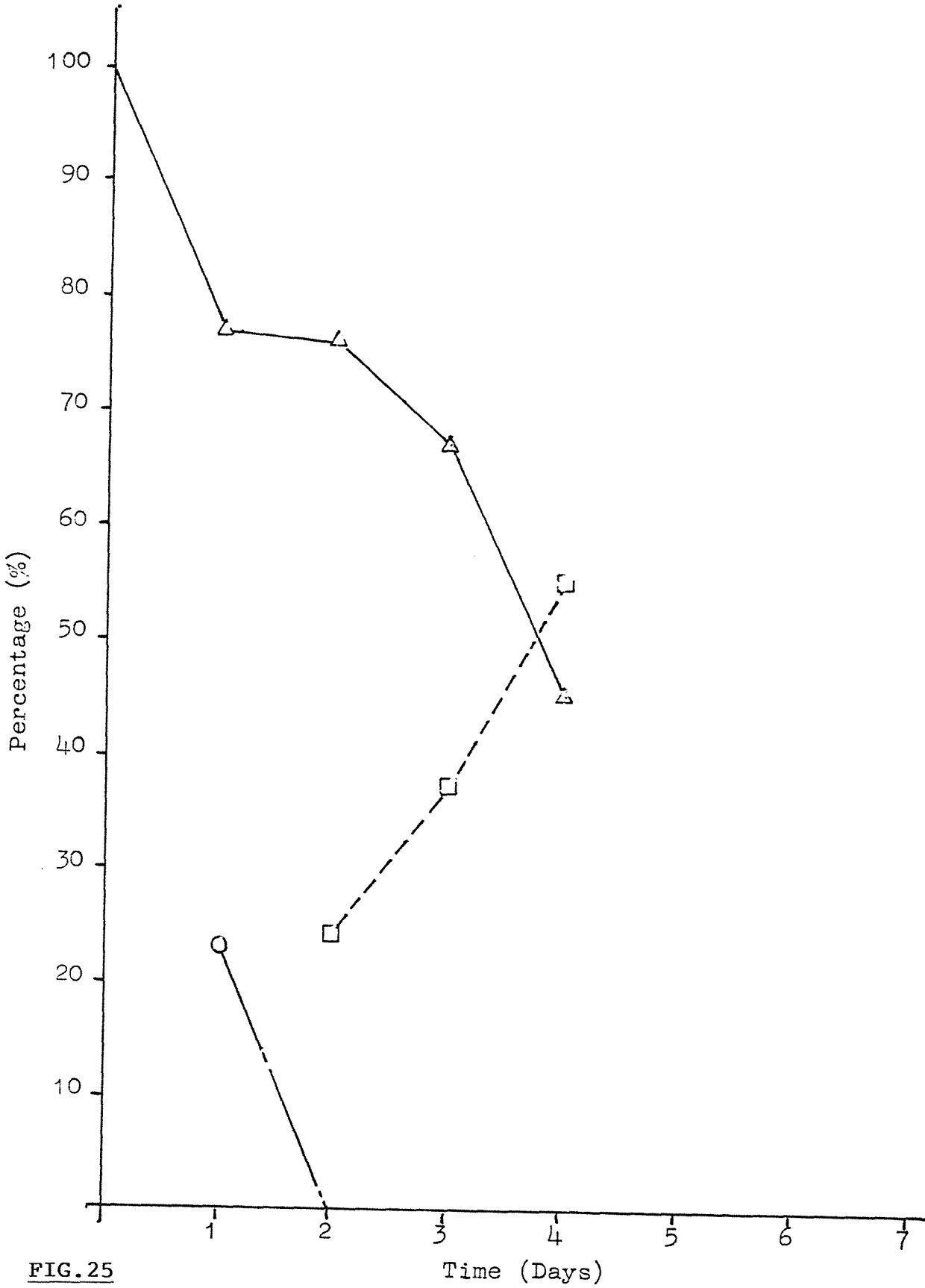


FIG.25

Percentage trophozoites, roundforms and cysts of N. fowleri during 4 days incubation on 60% soil extract agar.

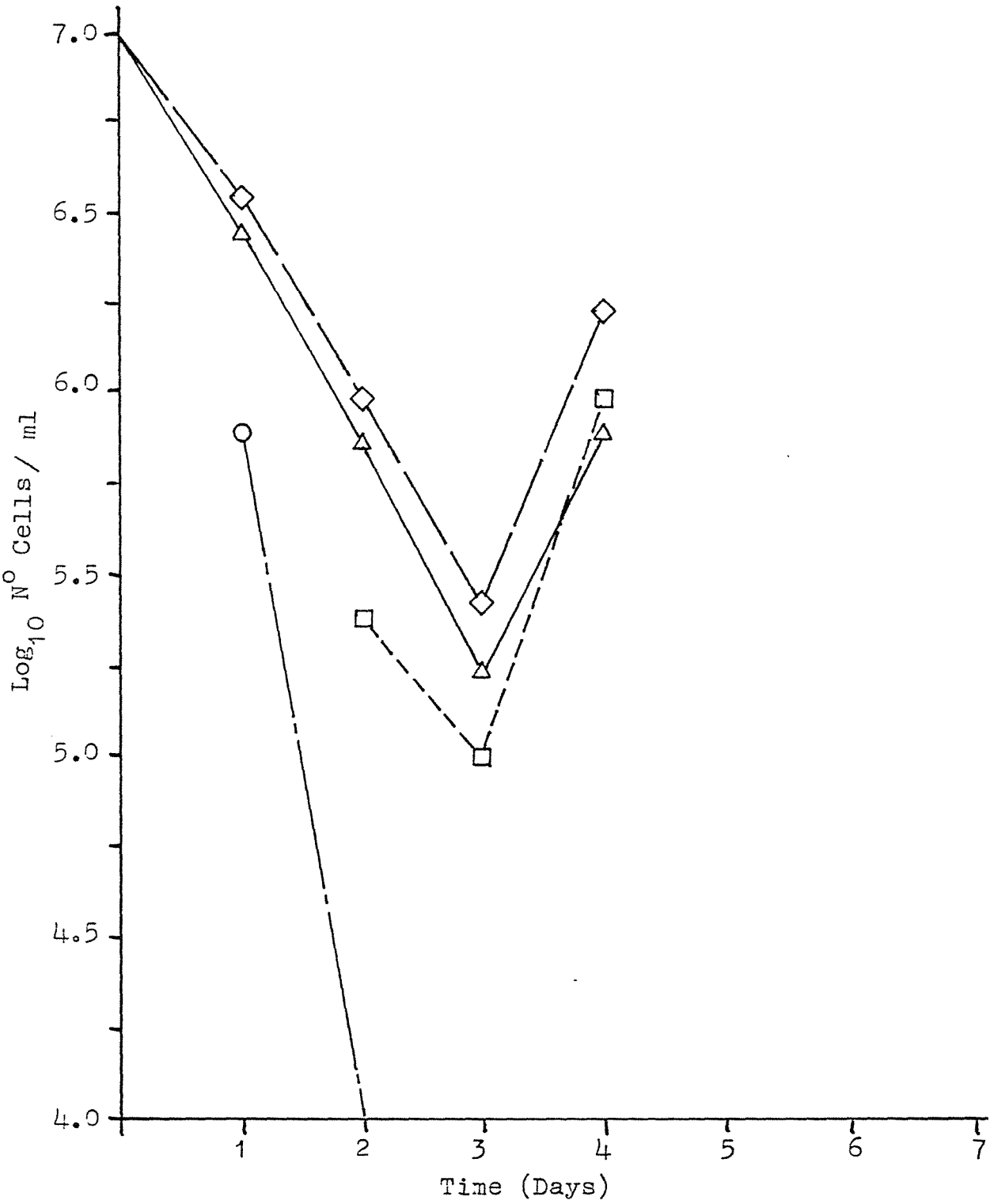


FIG. 26

Log 10 of number of trophozoites, roundforms and cysts of N. fowleri during 4 days incubation on 60% soil extract agar.

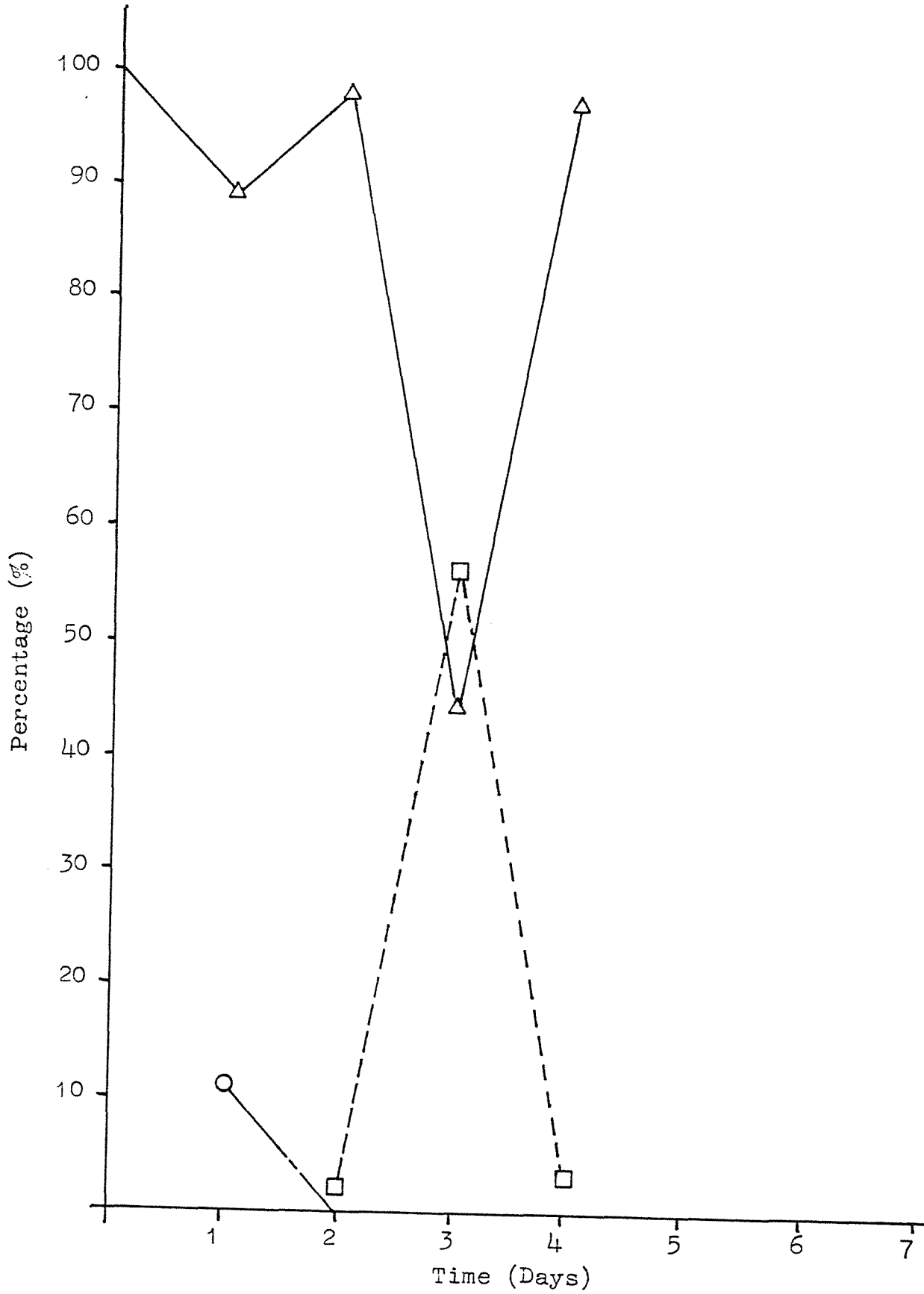


FIG. 27

Percentage trophozoites, roundforms and cysts of N. fowleri during 4 days incubation on 60% soil extract agar plus E. cloacae.

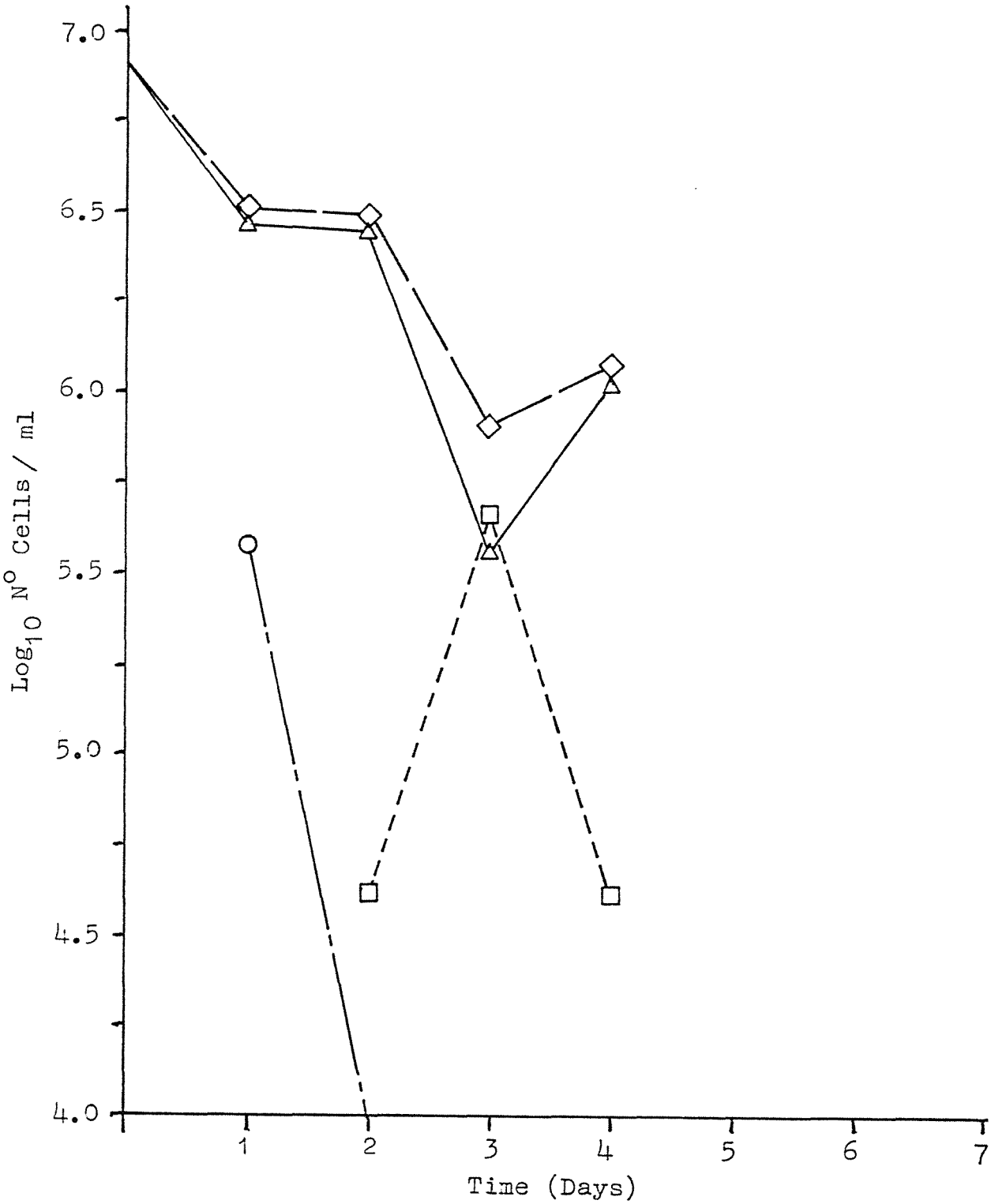


FIG.28

Log 10 of number of trophozoites, roundforms and cysts of N. fowleri during 4 days incubation on 60% soil extract agar plus E. cloacae.

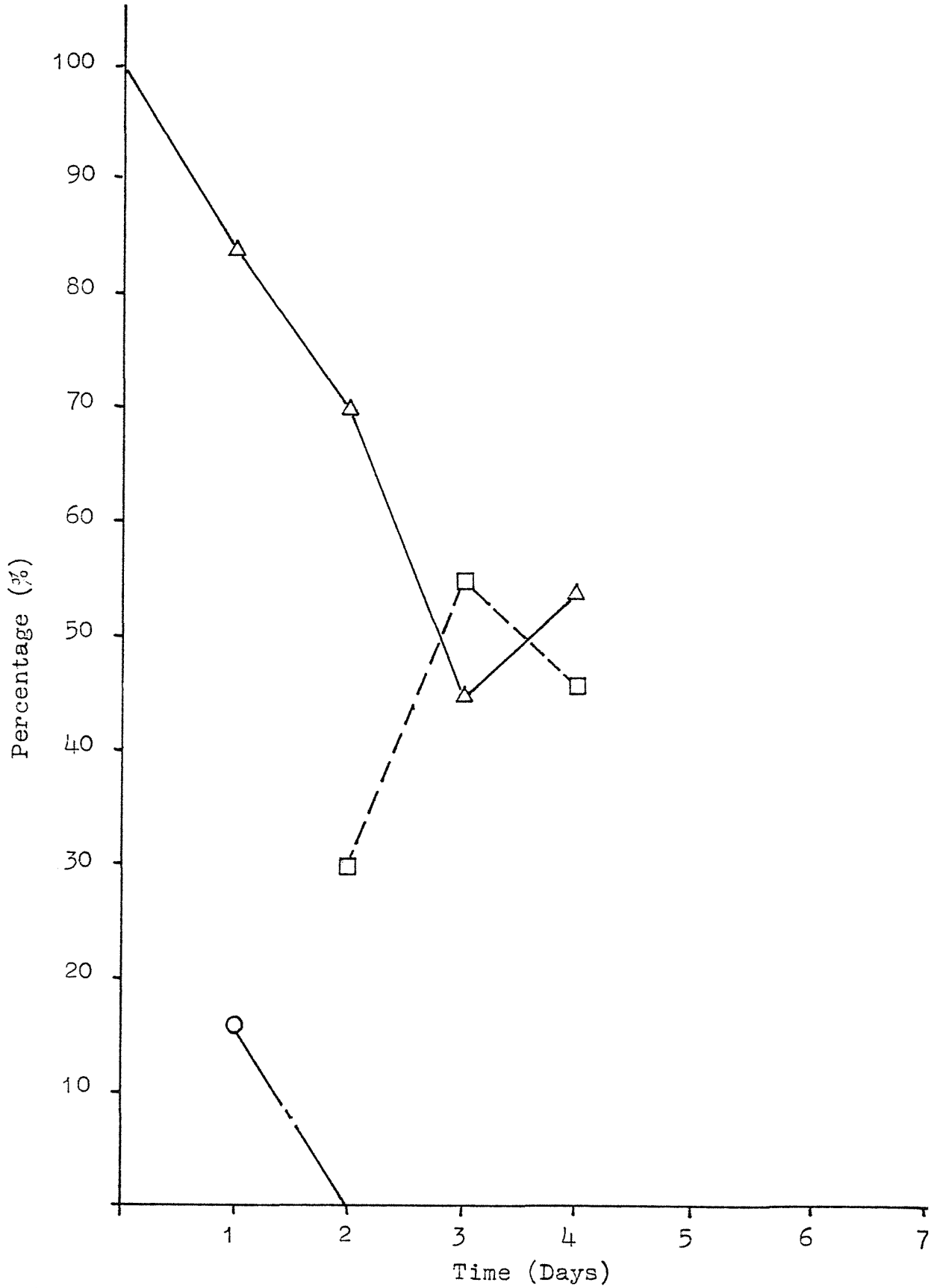


FIG.29

Percentage trophozoites, roundforms and cysts of N. fowleri during 4 days incubation on 80% soil extract agar, plus E. cloacae.

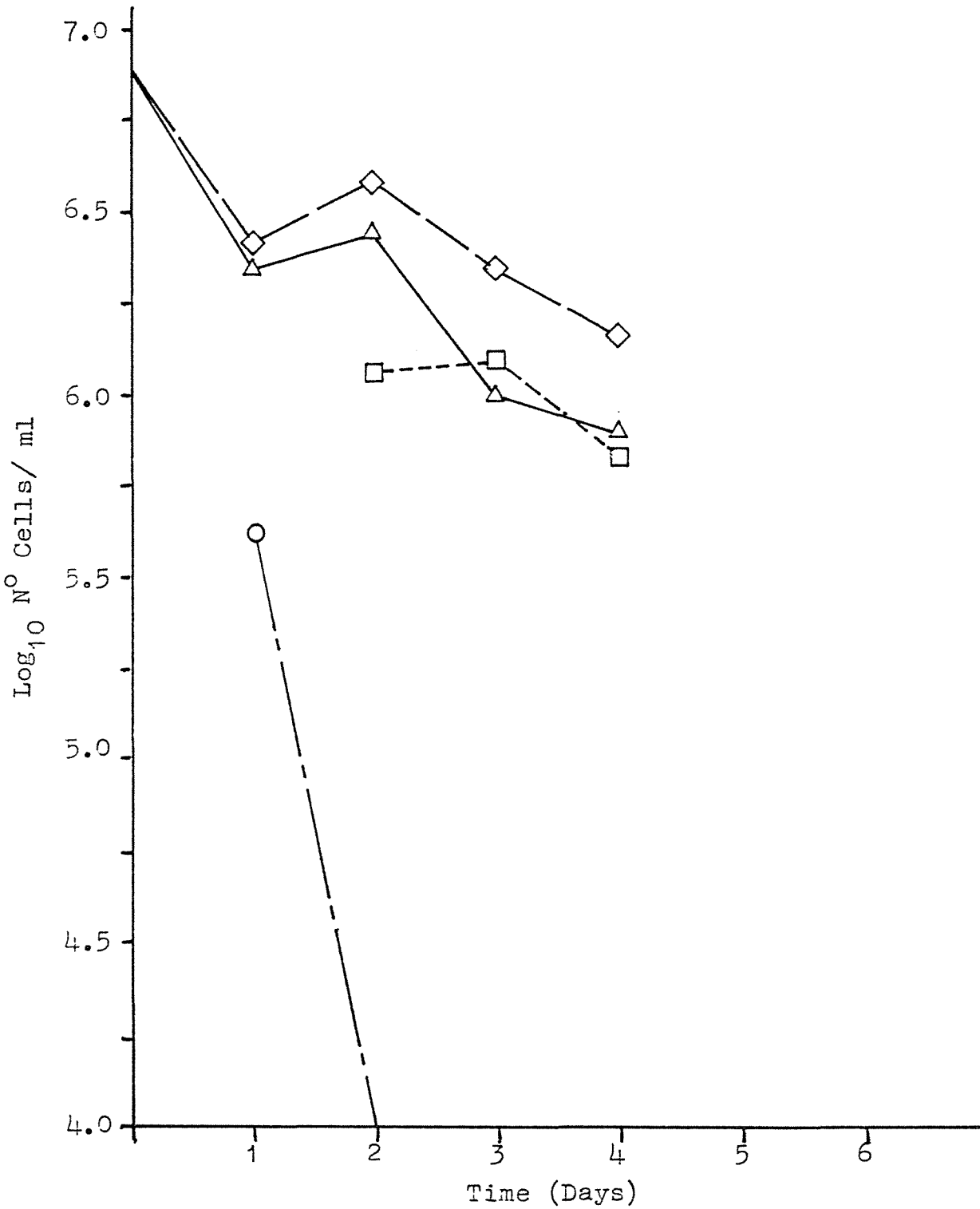


FIG. 30

Log 10 of number of trophozoites, roundforms and cysts of N. fowleri during 4 days incubation on 80% soil extract agar, plus E. cloacae.

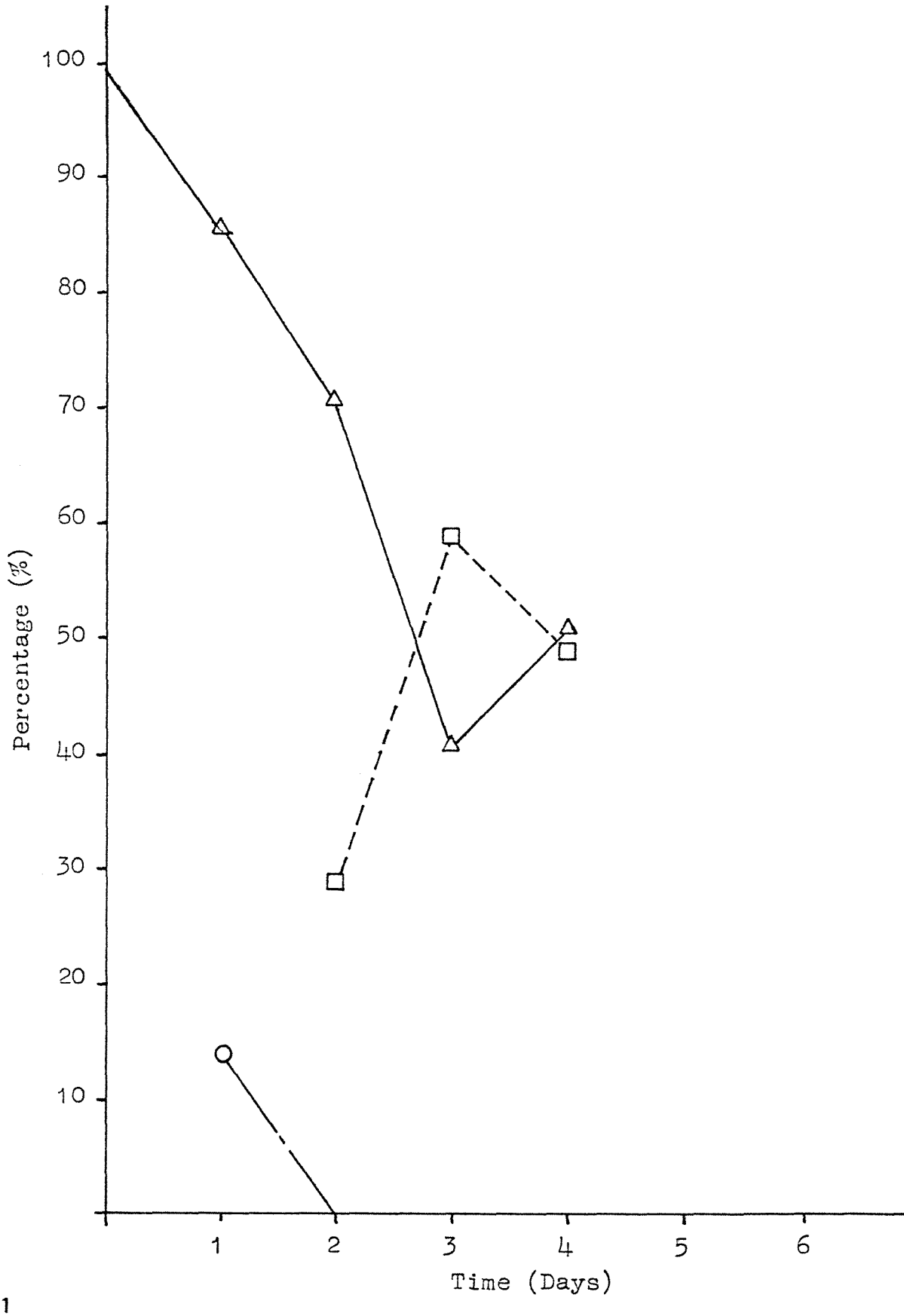


FIG.31

Percentage trophozoites, roundforms and cysts of N.fowleri during 4 days incubation on 80% soil extract agar.

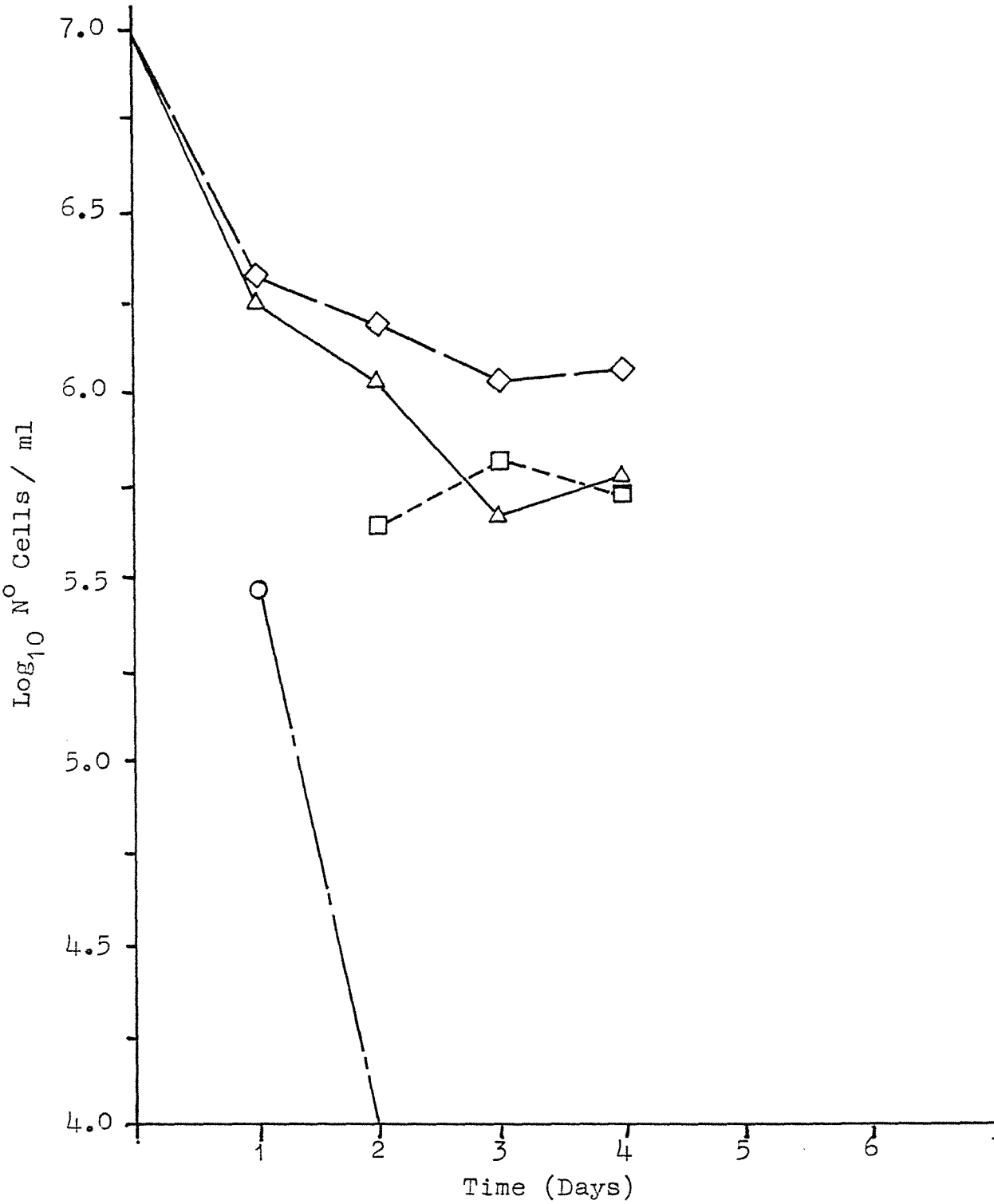


FIG.32

Log 10 of number of trophozoites, roundforms and cysts of N. fowleri during 4 days incubation on 80% soil extract agar.

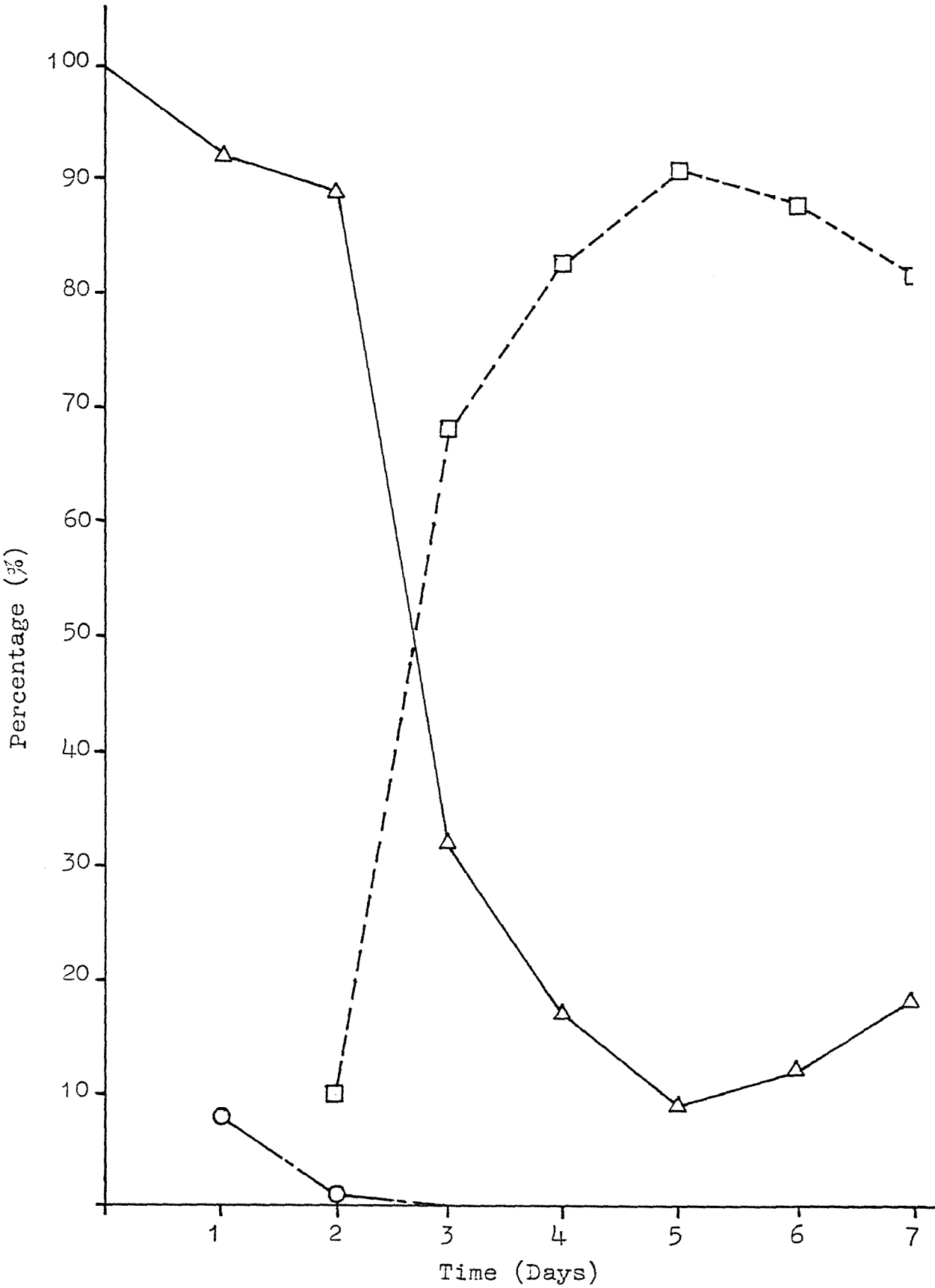


FIG.33

Percentage trophozoites, roundforms and cysts of *N. fowleri* during 7 days incubation on 100% soil extract agar.

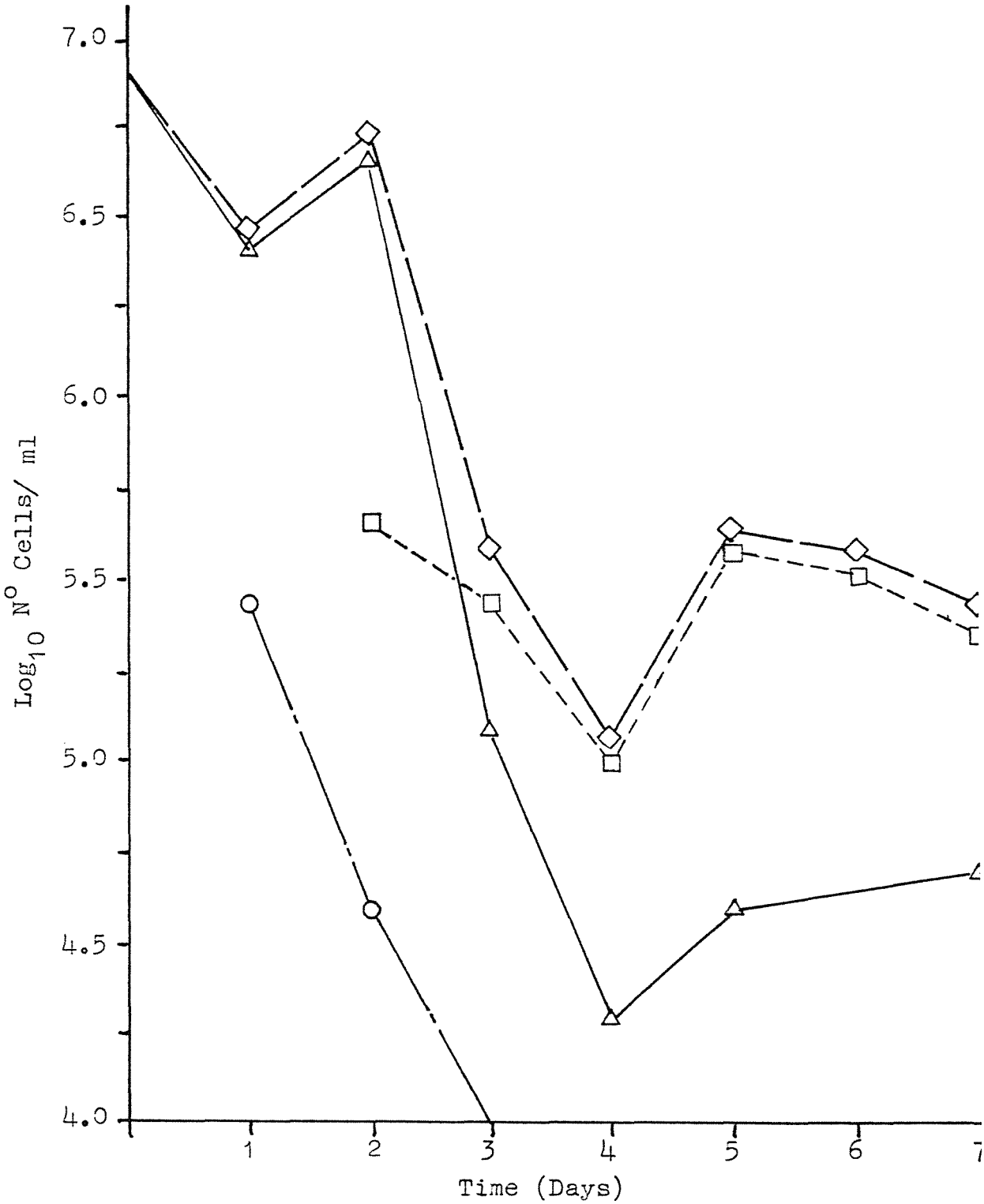


FIG.34

Log 10 of number of trophozoites, roundforms and cysts of N. fowleri during 7 days incubation on 100% soil extract agar.

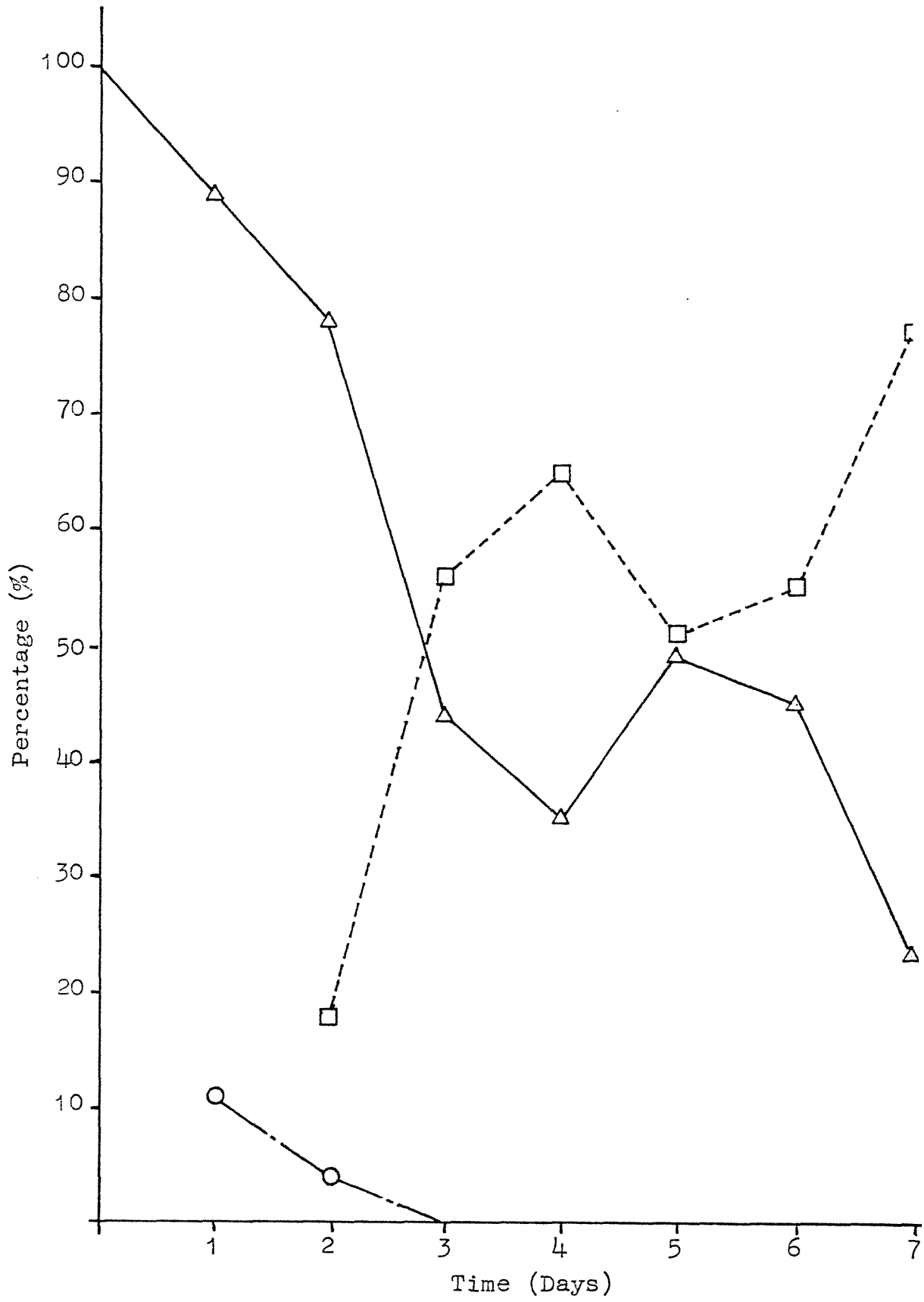


FIG. 35

Percentage trophozoites, roundforms and cysts of N. fowleri during 7 days incubation on 100% soil extract agar plus E. cloacae.

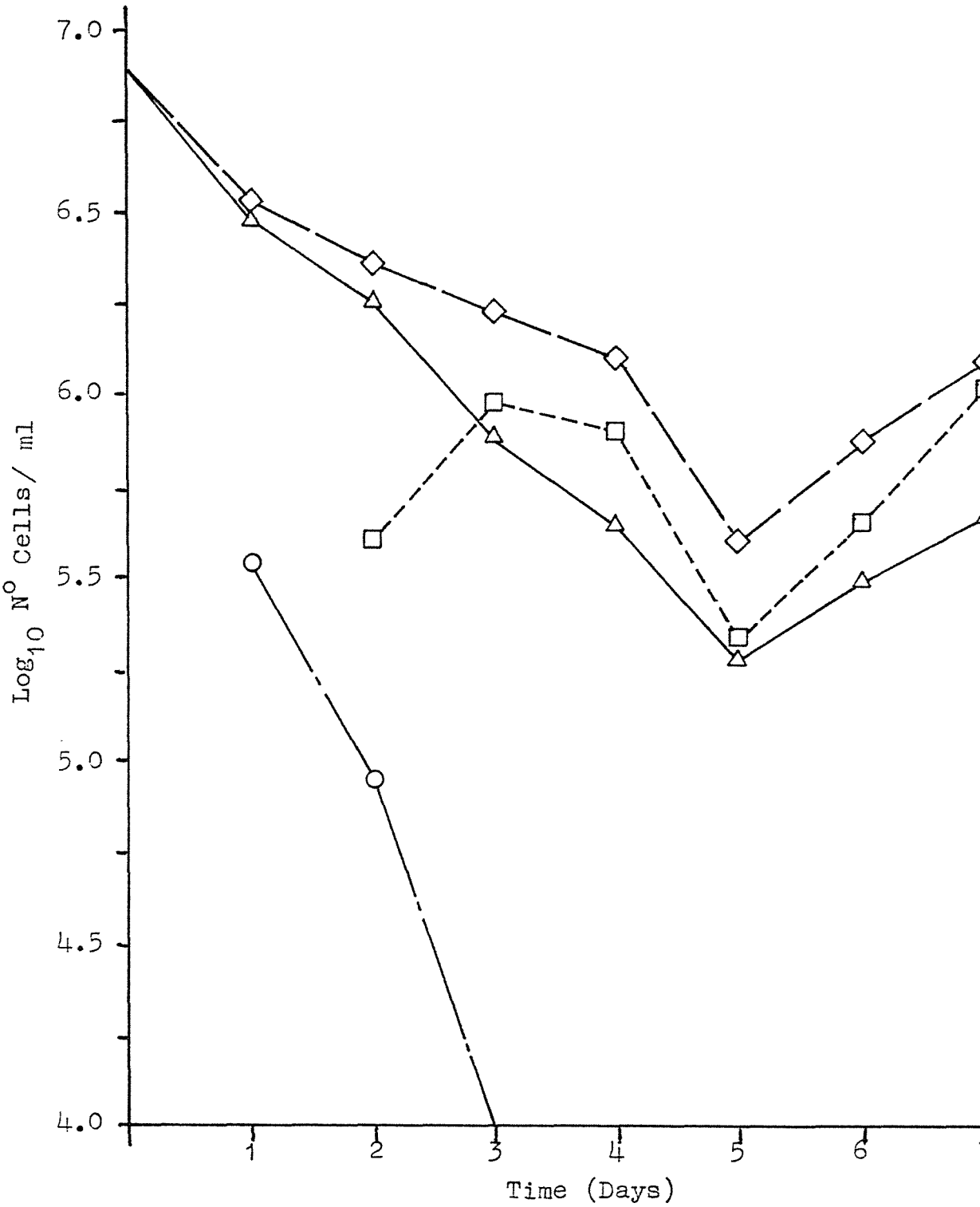


FIG. 36

Log 10 of number of trophozoites, roundforms and cysts of N. fowleri during 7 days incubation on 100% soil extract agar plus E. cloacae.

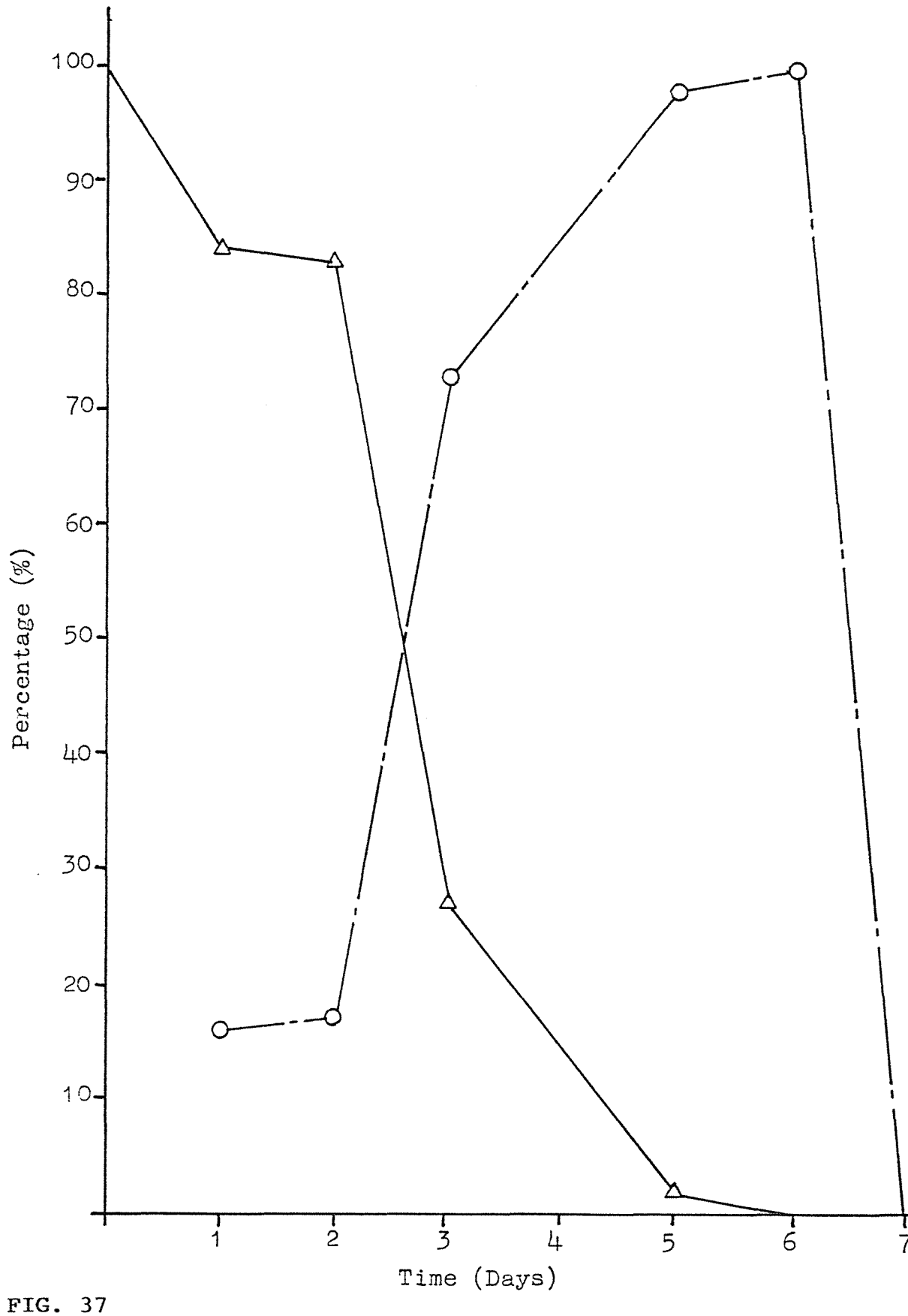


FIG. 37

The effect of population density on the percentage of trophozoites, roundforms and cysts of N. gruberi during 7 days incubation in 100% soil extract broth.

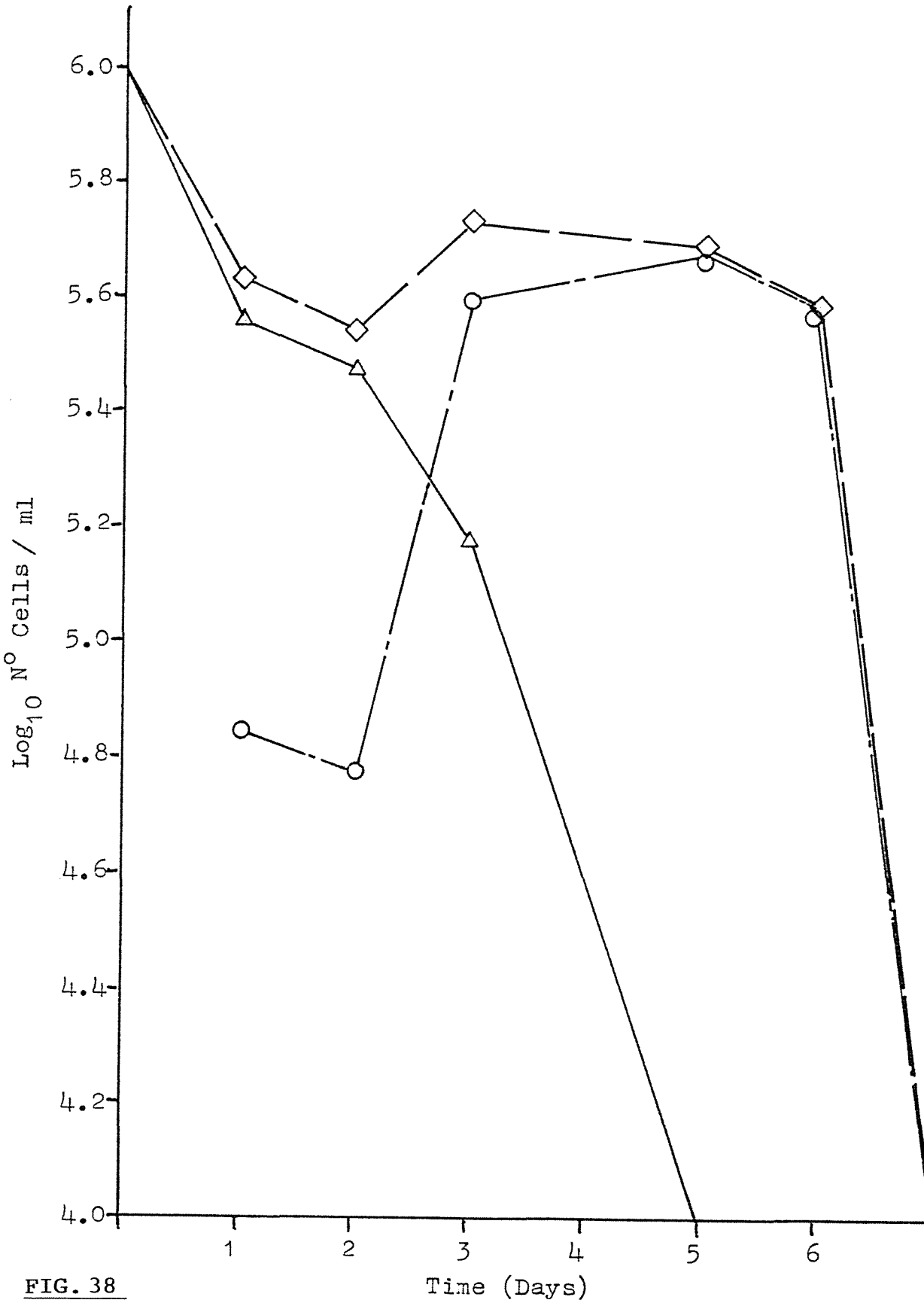


FIG. 38

The effect of population density on the number of trophozoites, roundforms and cysts of N.gruberi during 7 days incubation in 100% soil extract broth.

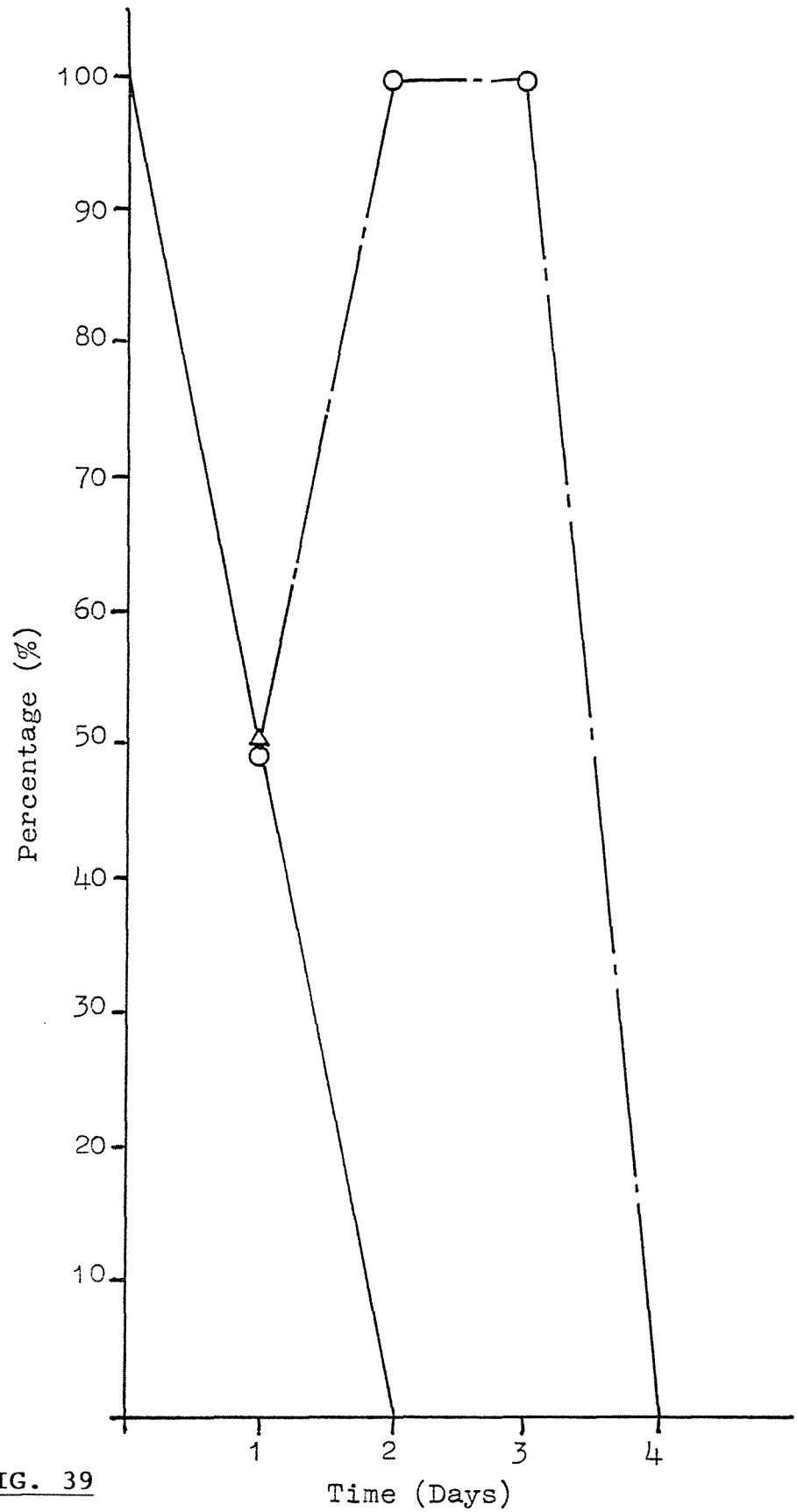


FIG. 39

The effect of population density on the percentage of trophozoites, roundforms and cysts of N.gruberi during 4 days incubation.

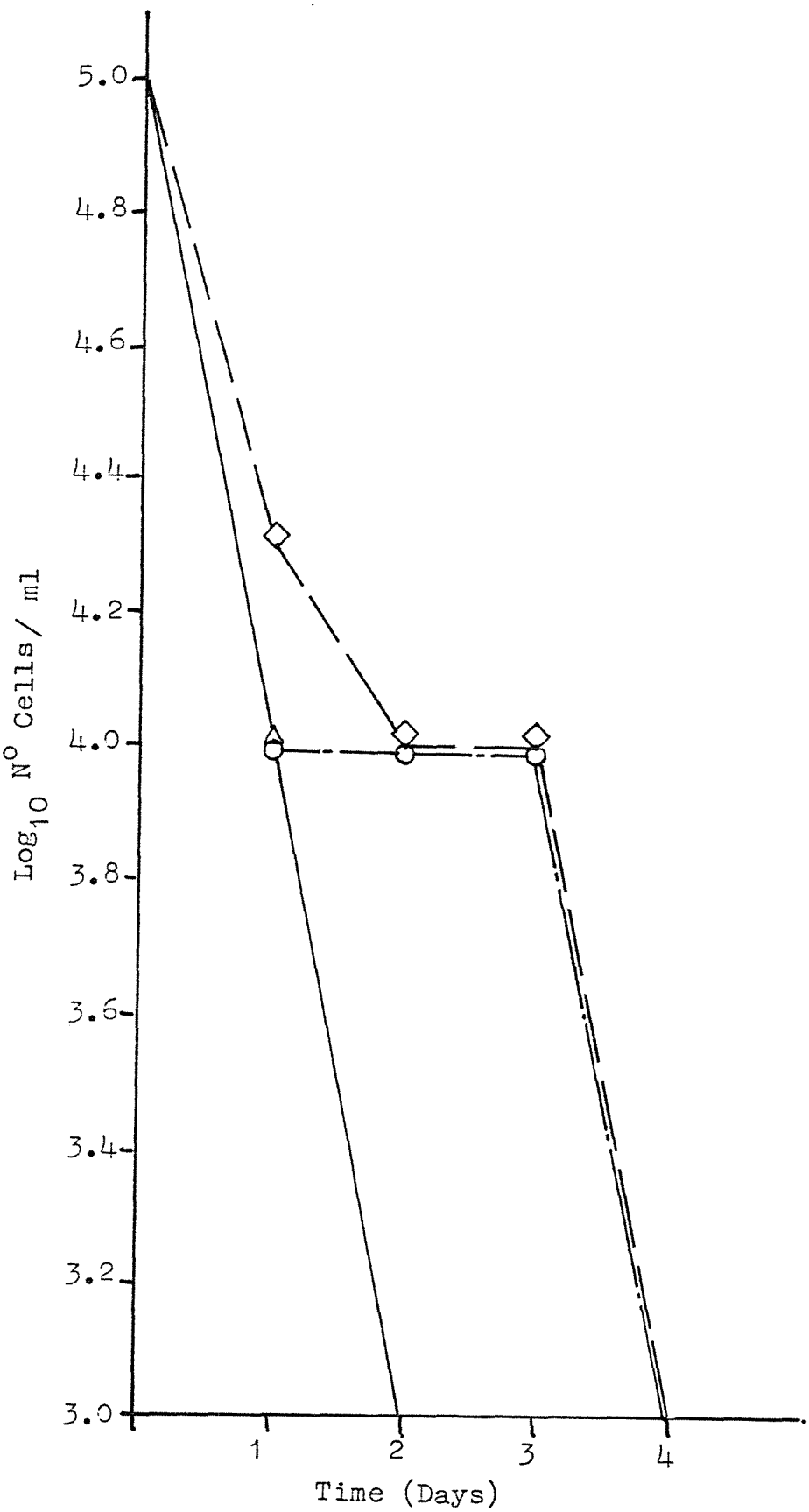


FIG.40.

The effect of population density on the number of trophozoites, roundforms and cysts of N. gruberi during 4 days incubation in 100% soil extract broth.

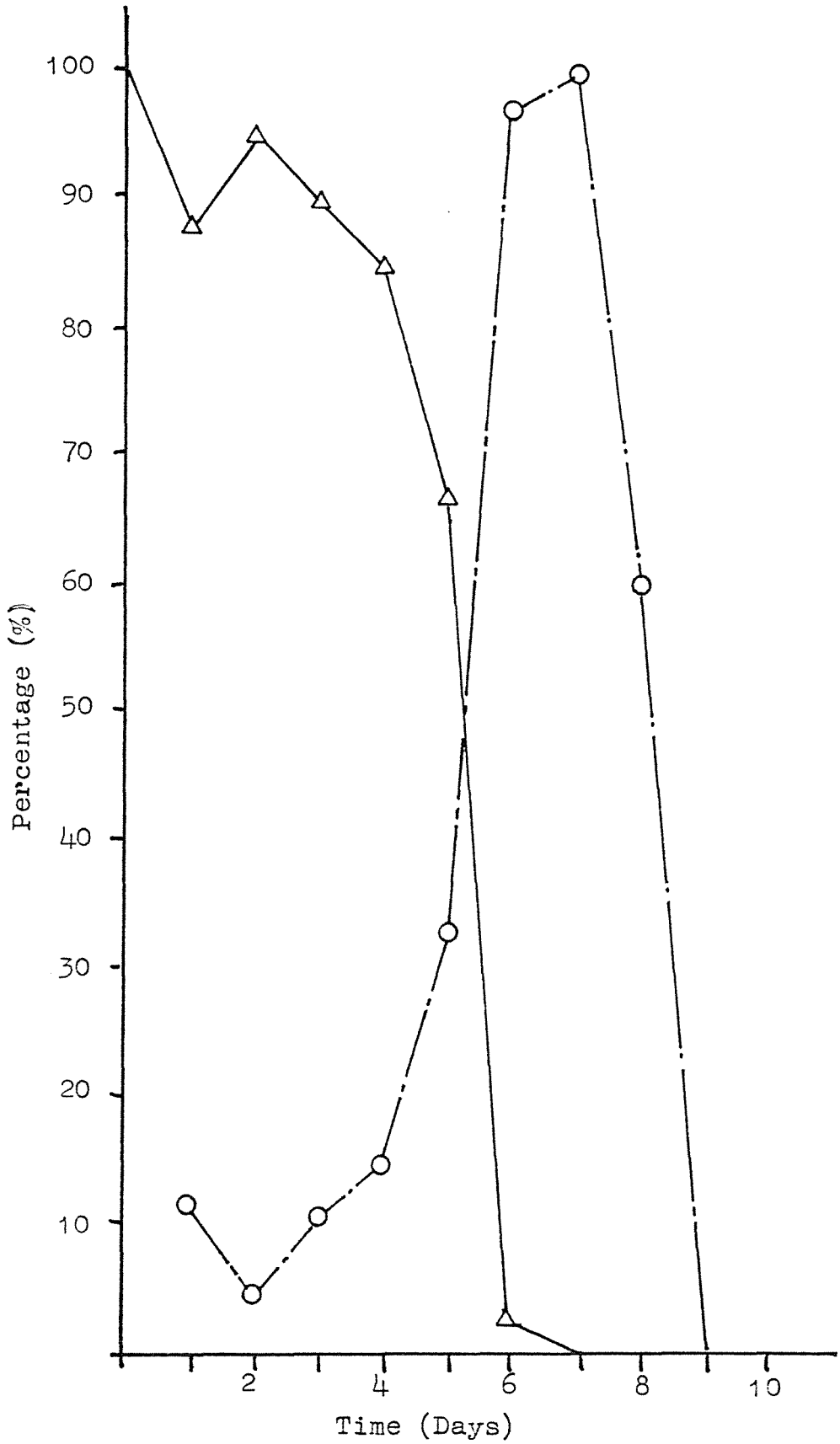


FIG. 41

Percentage Encystment of an inoculum of 1 x 10^6 cells ml^{-1} of N. fowleri during 9 days incubation in CYM media.

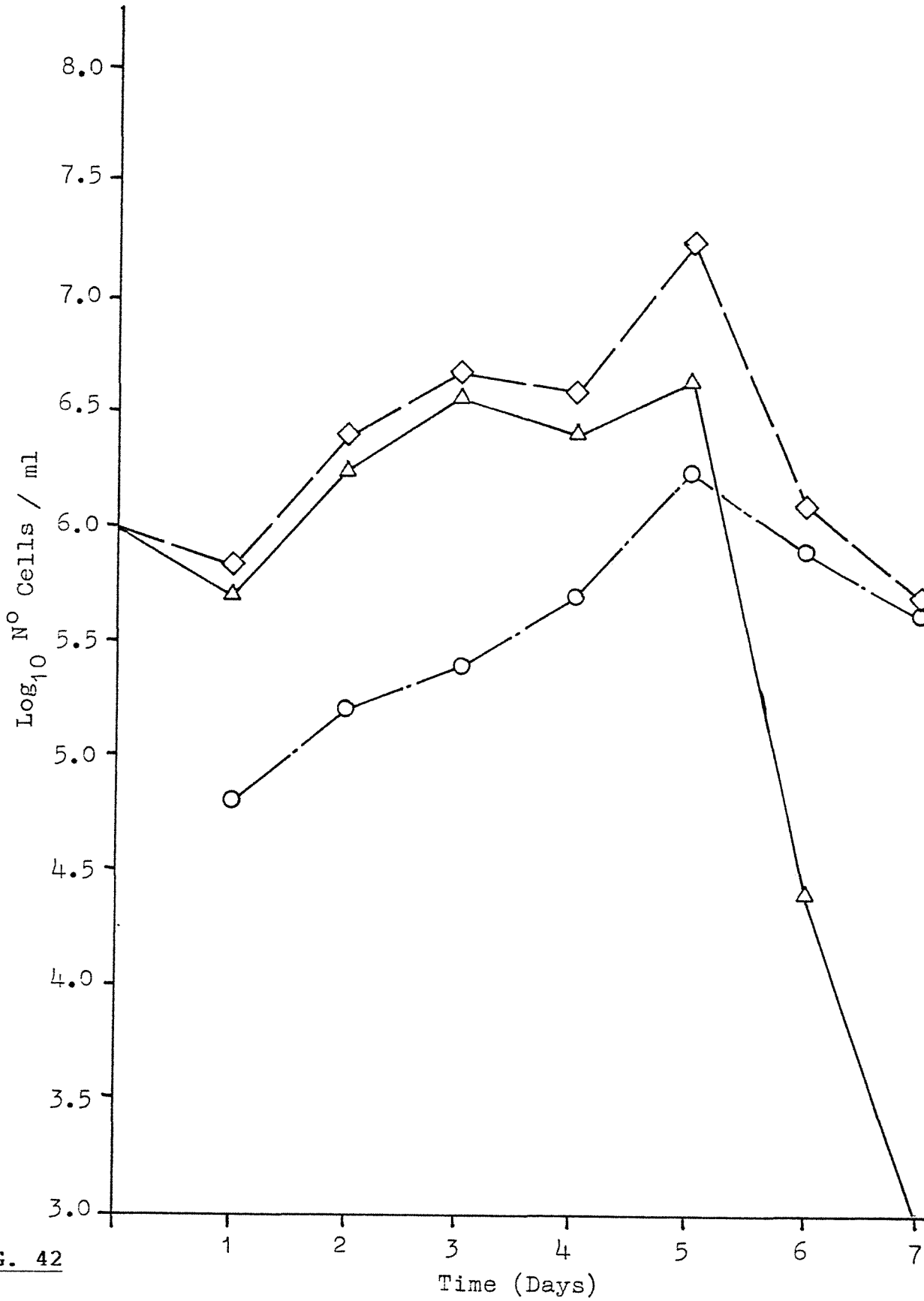


FIG. 42

Log 10 of the number of trophozoites, roundforms and cysts of an inoculum of 1 x 10^6 cells ml^{-1} of N. fowleri during 7 days incubation in CYM media.

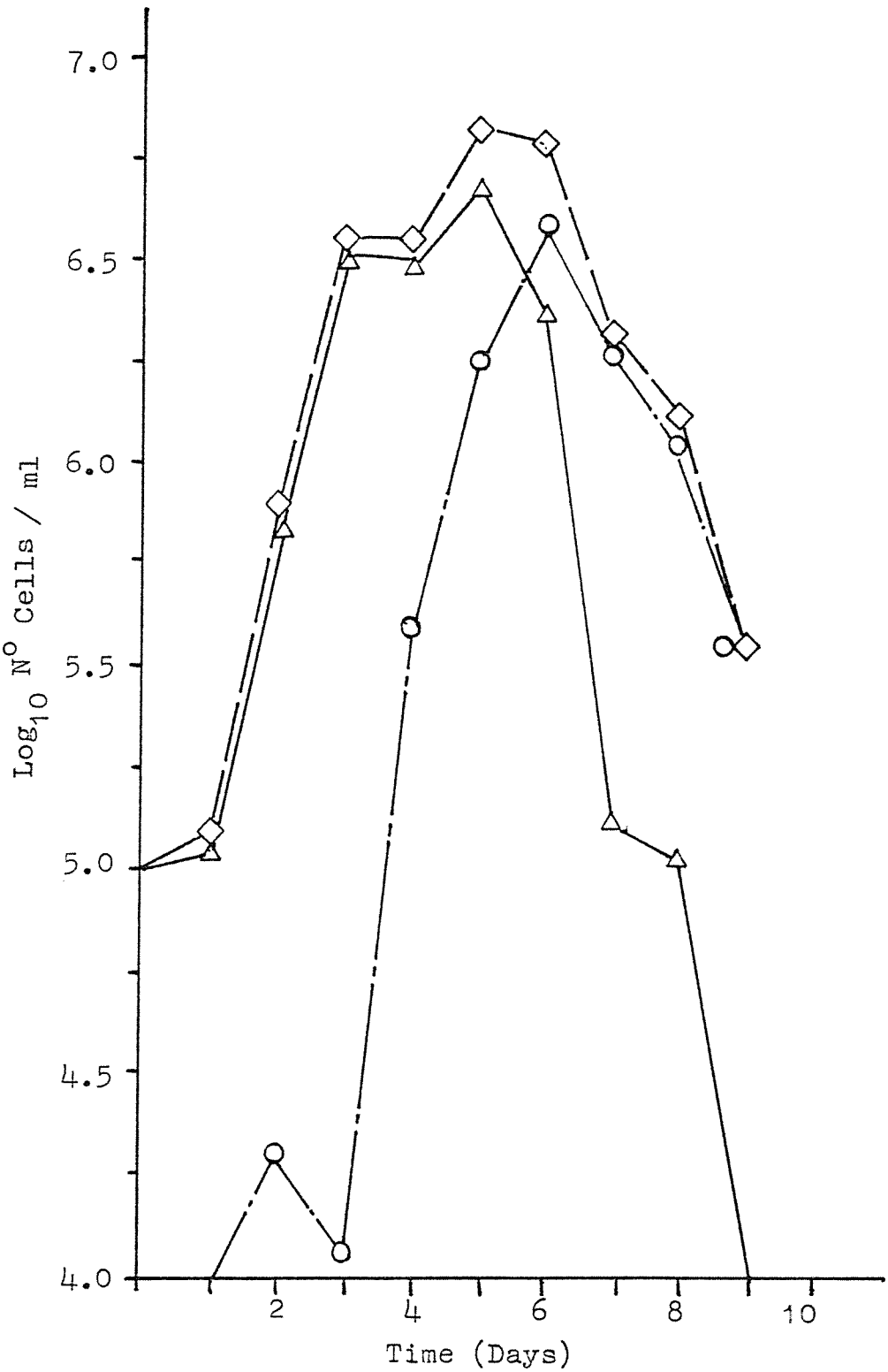


FIG.43

Log 10 of the number of trophozoites, roundforms and cysts of an inoculum of 1 x 10^6 cells ml^{-1} of N. fowleri during 9 days incubation in CYM media.

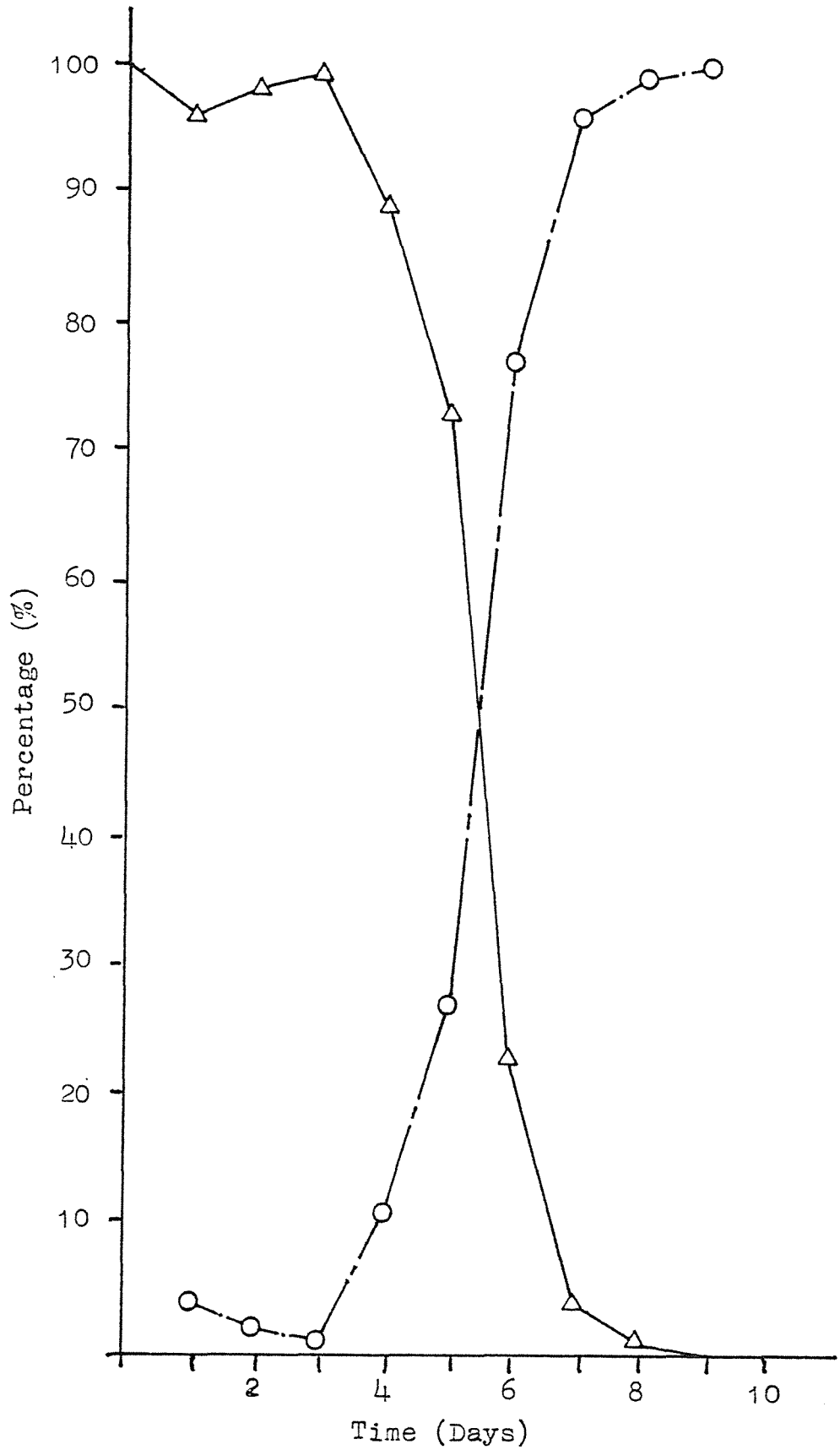


FIG. 44

Percentage encystment of an inoculum of 1 x 10^5 cells ml^{-1} of N. fowleri during 9 days incubation in CYM media.

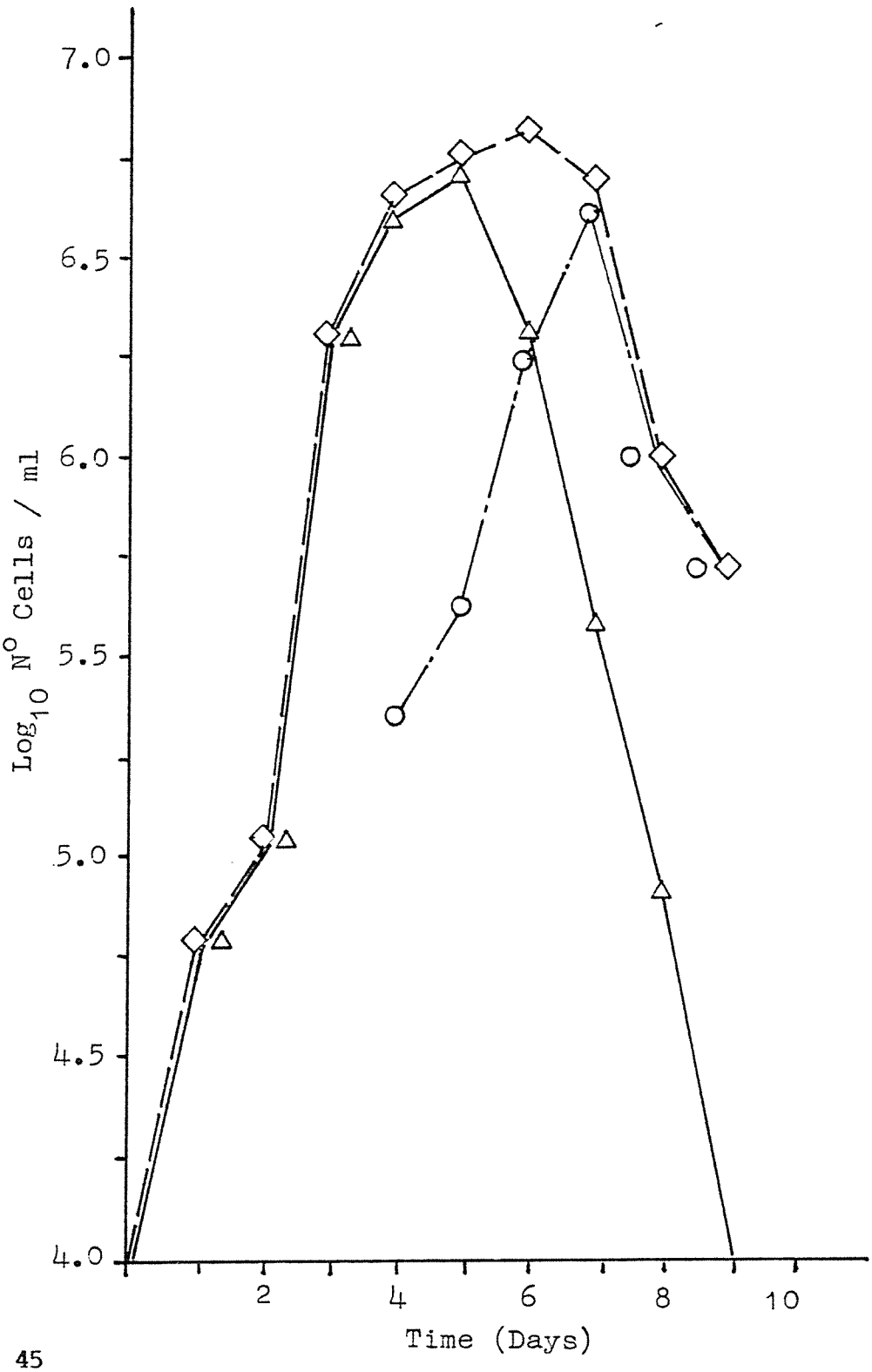


FIG. 45

Log 10 of the number of trophozoites, roundforms and cysts of an inoculum of 1 x 10^4 cells ml^{-1} of N. fowleri during 9 days incubation in CYM media.

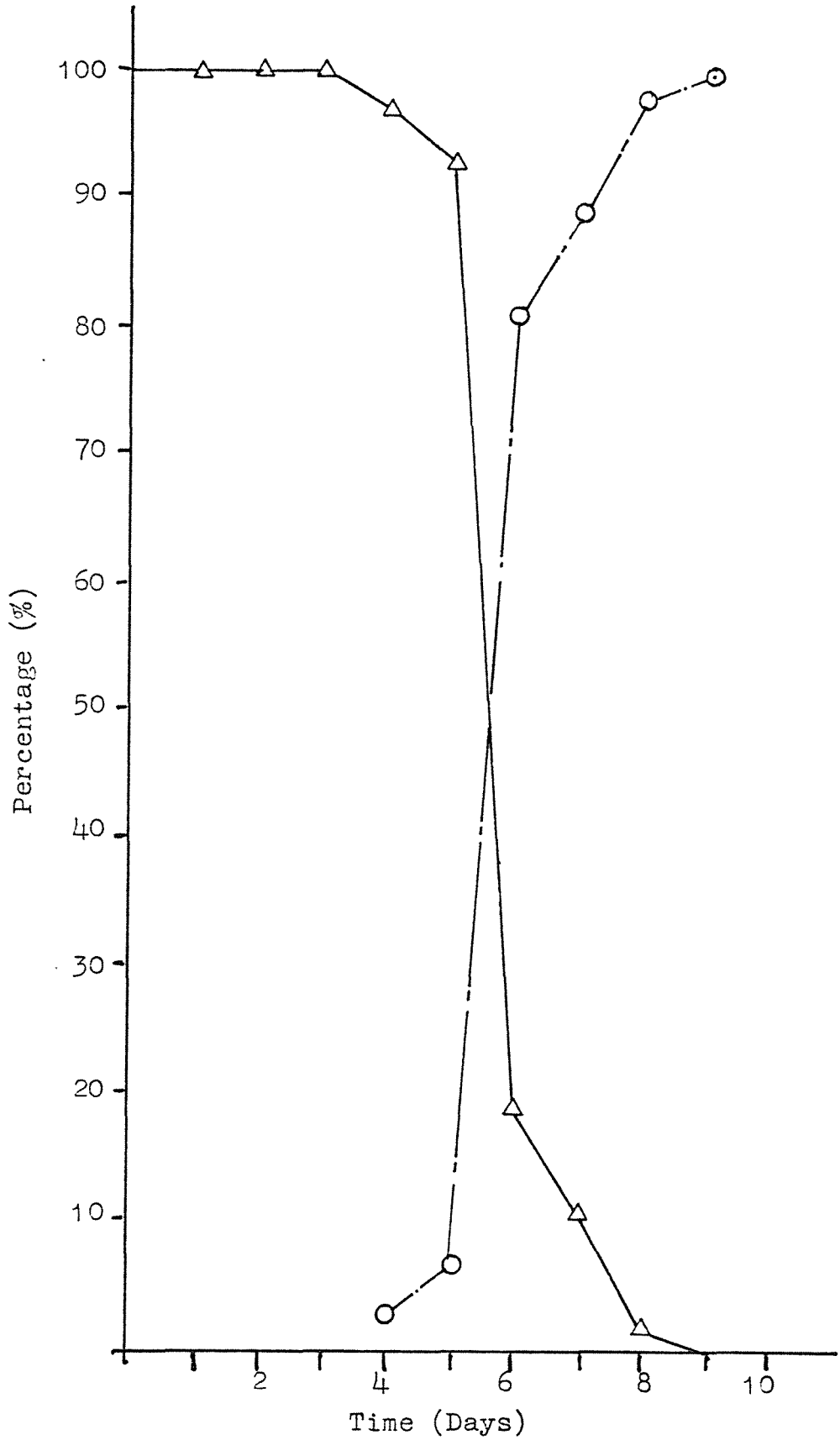


FIG. 46

Percentage encystment of an inoculum of 1 x10 ^{4} cells ml^{-1} of N. fowleri during 9 days incubation in CYM media.

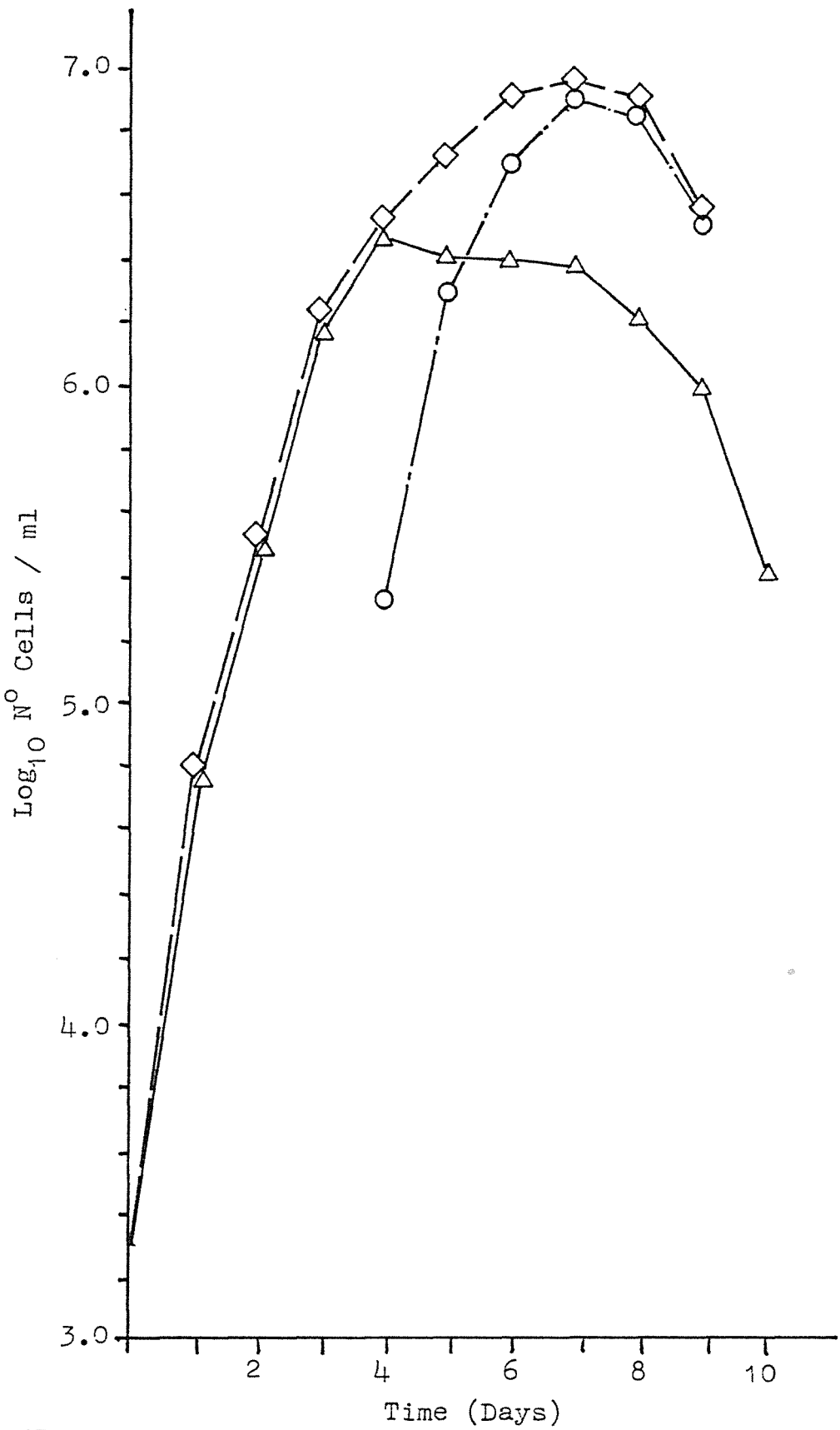


FIG. 47

Log 10 of the number of trophozoites, roundforms and cysts of an inoculum of 1 x 10^3 cells ml^{-1} of N. fowleri during 9 days incubation in CYM media.

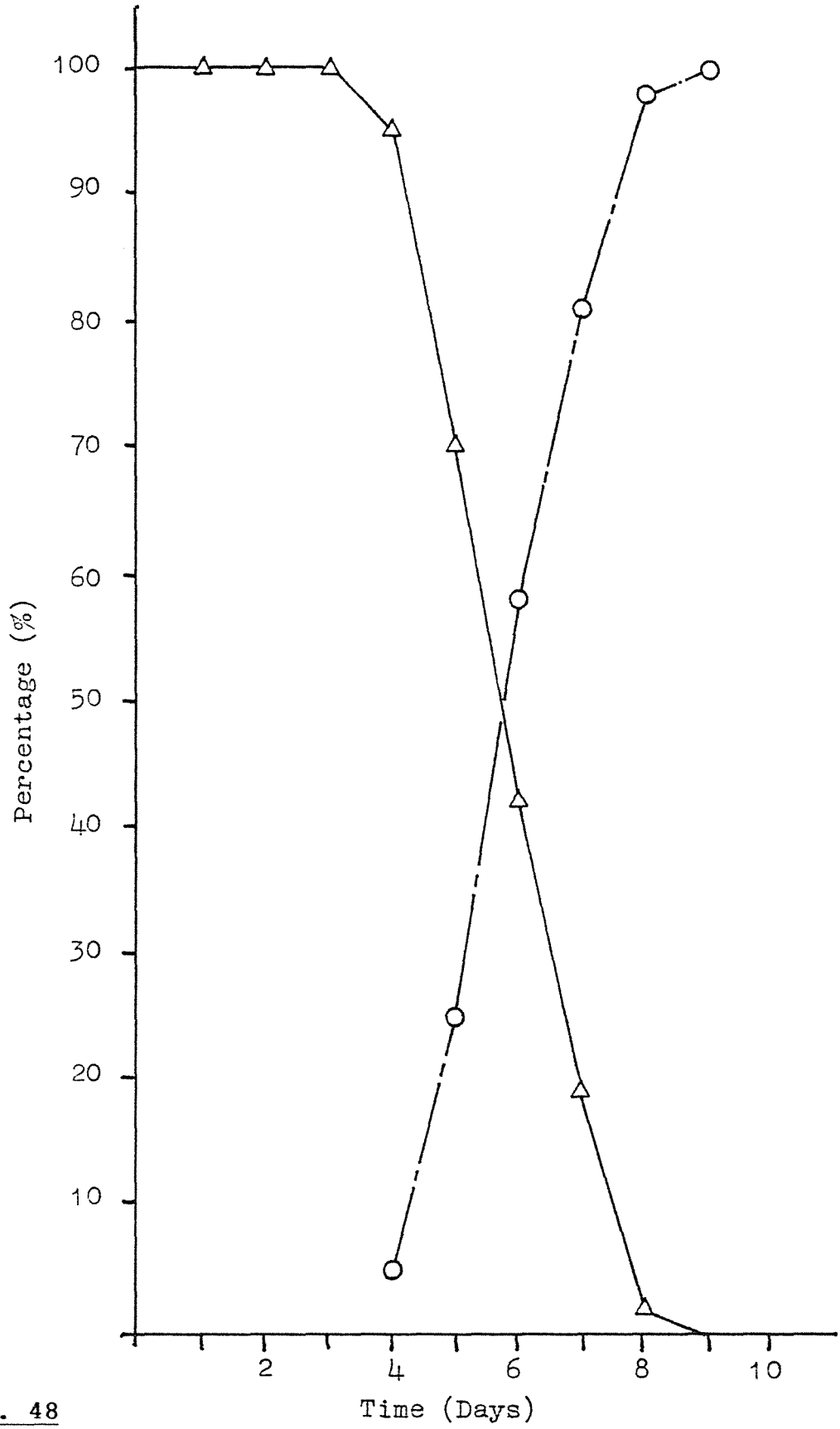


FIG. 48

Percentage encystment of an inoculum of 1 x 10^3 cells ml^{-1} of N. fowleri during 9 days incubation.

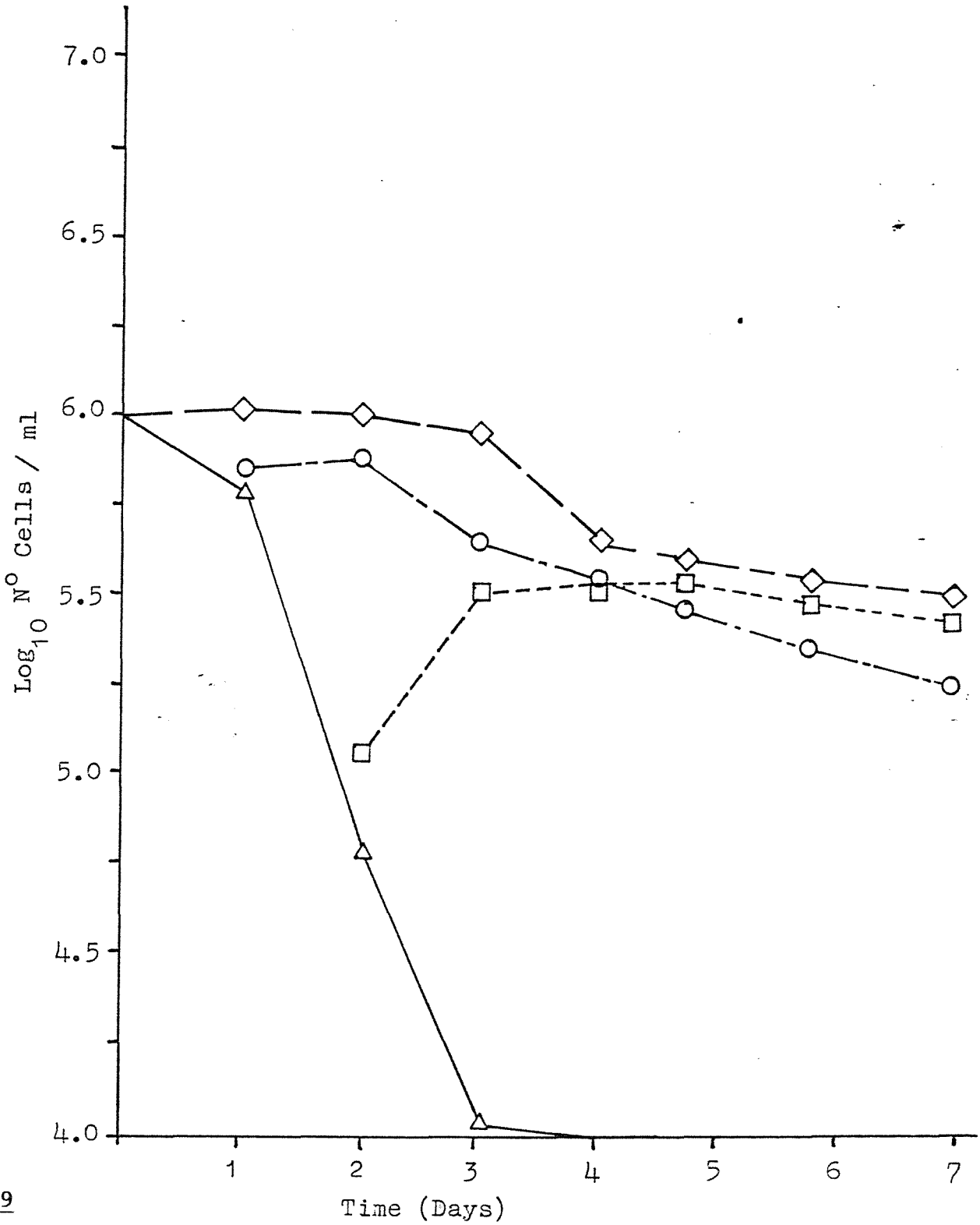


FIG.49

Log 10 of the number of trophozoites, roundforms and cysts of an inoculum of 1 x 10^6 cells ml^{-1} of N. fowleri during 7 days incubation in 100% soil extract broth.

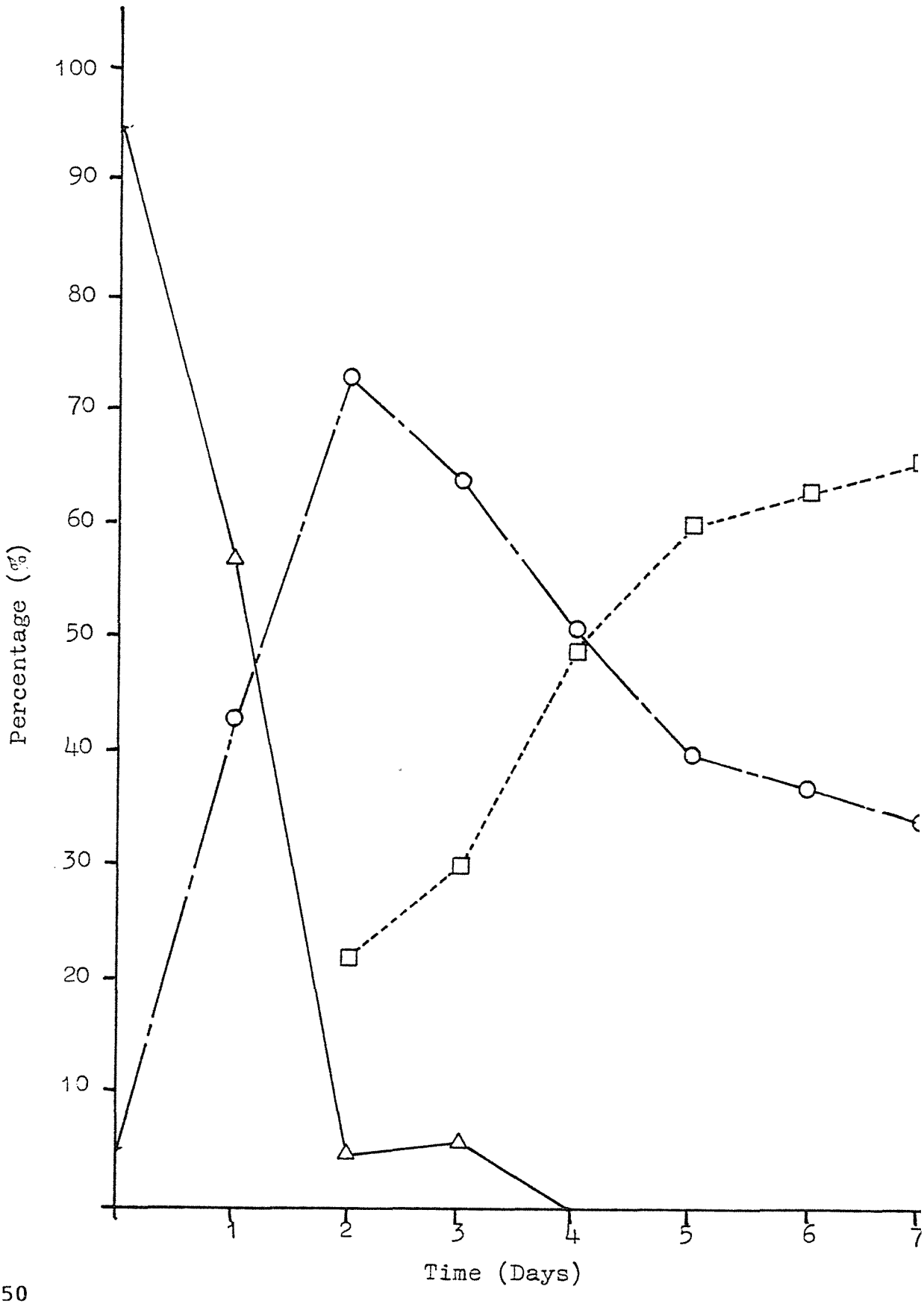


FIG.50

Percentage encystment of an inoculum of 1 x 10^6 cells ml^{-1} of N. fowleri during 7 days incubation in 100% soil extract broth.

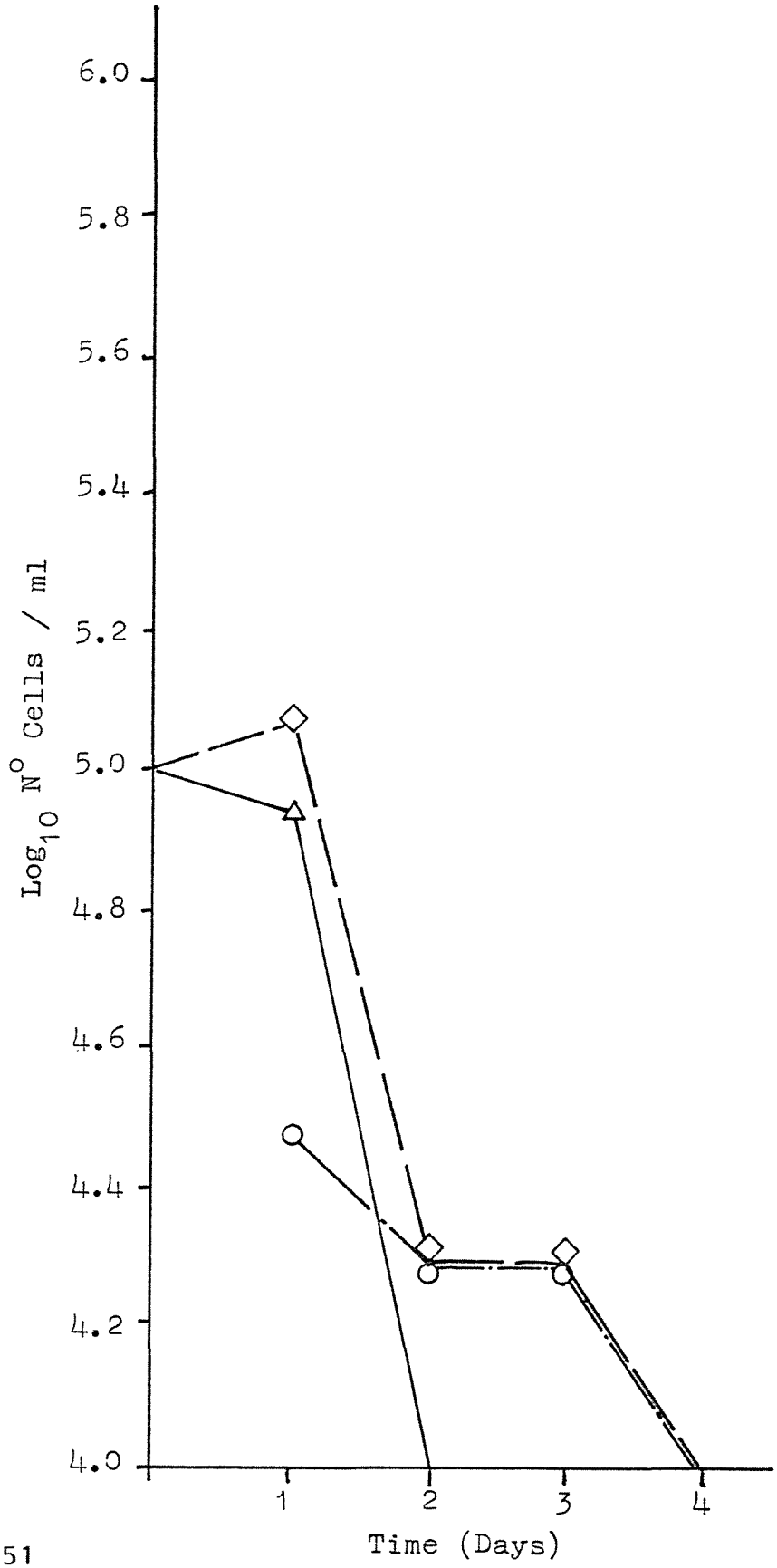


FIG. 51

Log 10 of the number of trophozoites, roundforms and cysts of an inoculum of 1 x 10^5 cells ml^{-1} of N. fowleri during 4 days incubation in 100% soil extract broth.

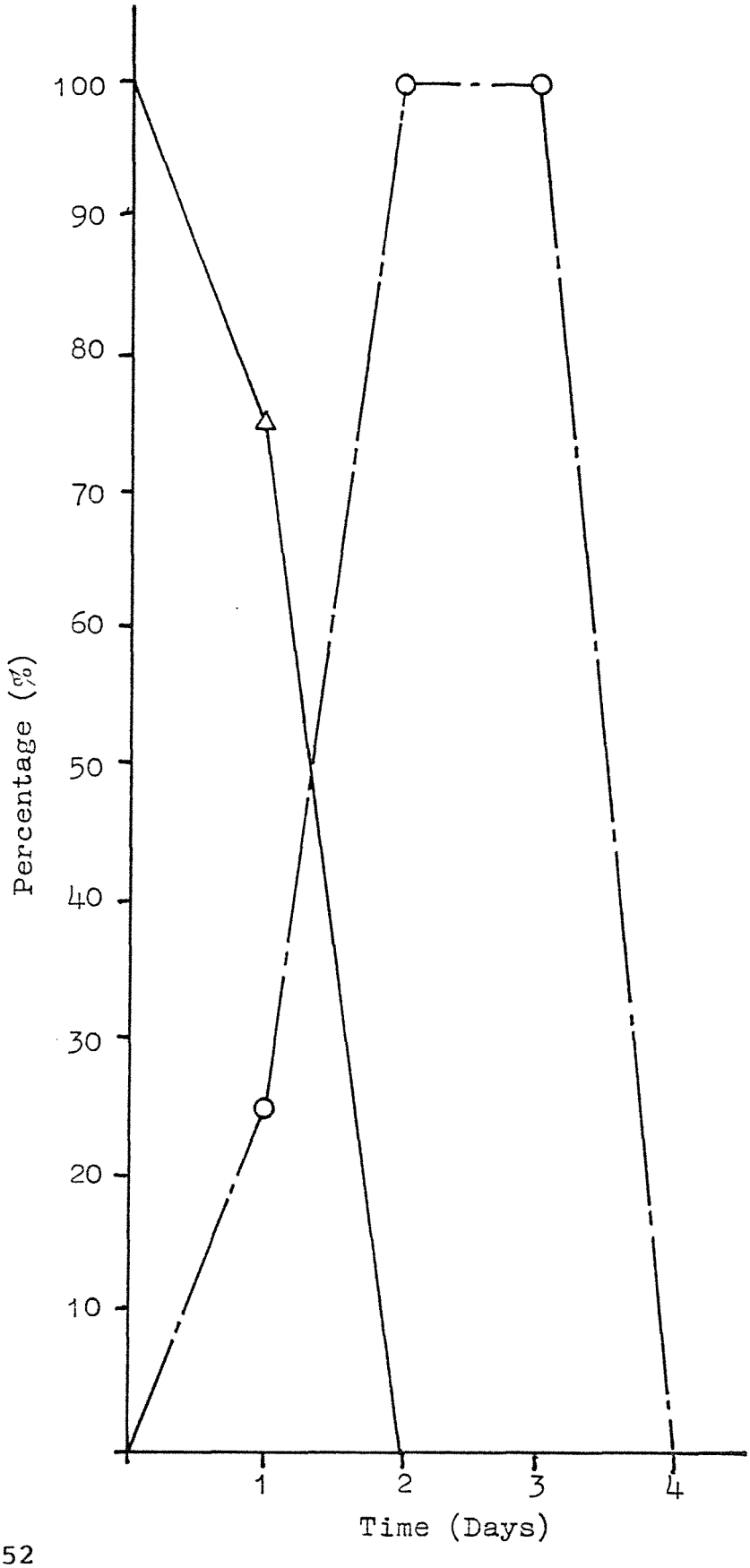


FIG.52

Percentage encystment of an inoculum of 1 x 10^5 cells ml^{-1} of N. fowleri during 4 days incubation in 100% soil extract broth.

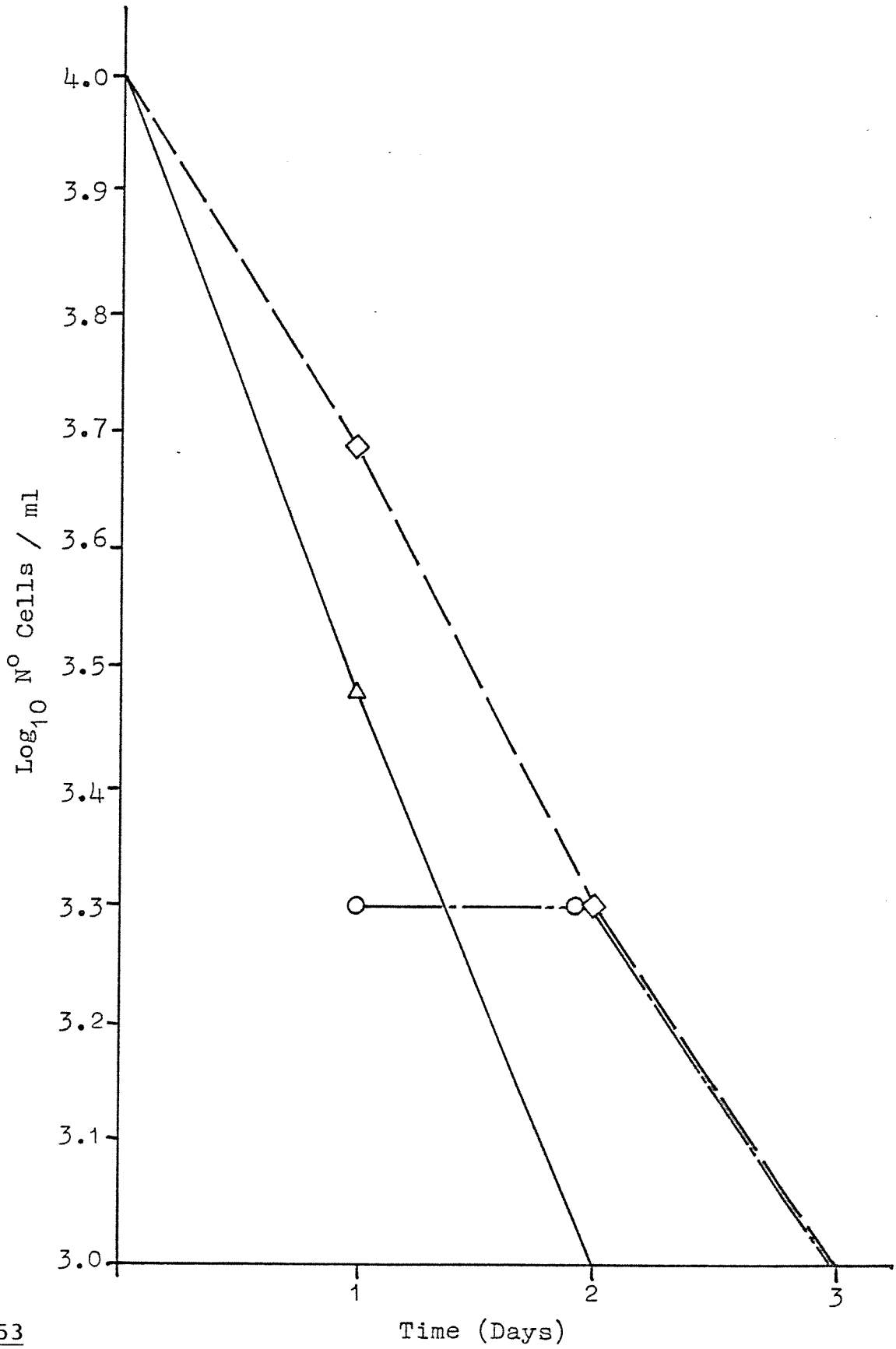


FIG. 53

Log 10 of the number of trophozoites, roundforms and cysts of an inoculum of $1x10^4$ cells ml^{-1} of N. fowleri during 3 days incubation in 100% soil extract broth.

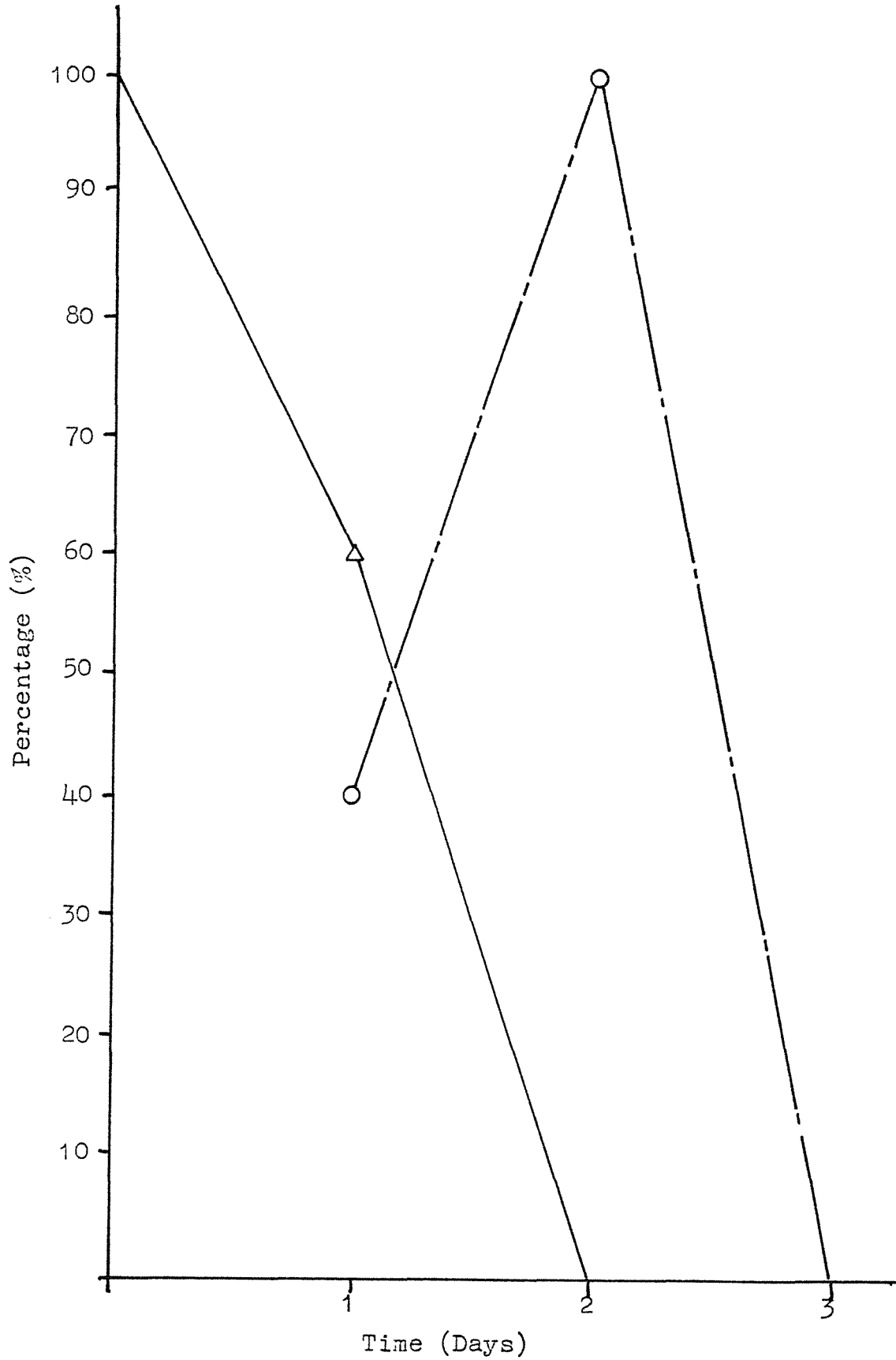


FIG.54

Percentage encystment of an inoculum of 1 x 10^4 cells ml^{-1} of N. fowleri during 3 days incubation in 100% soil extract broth.

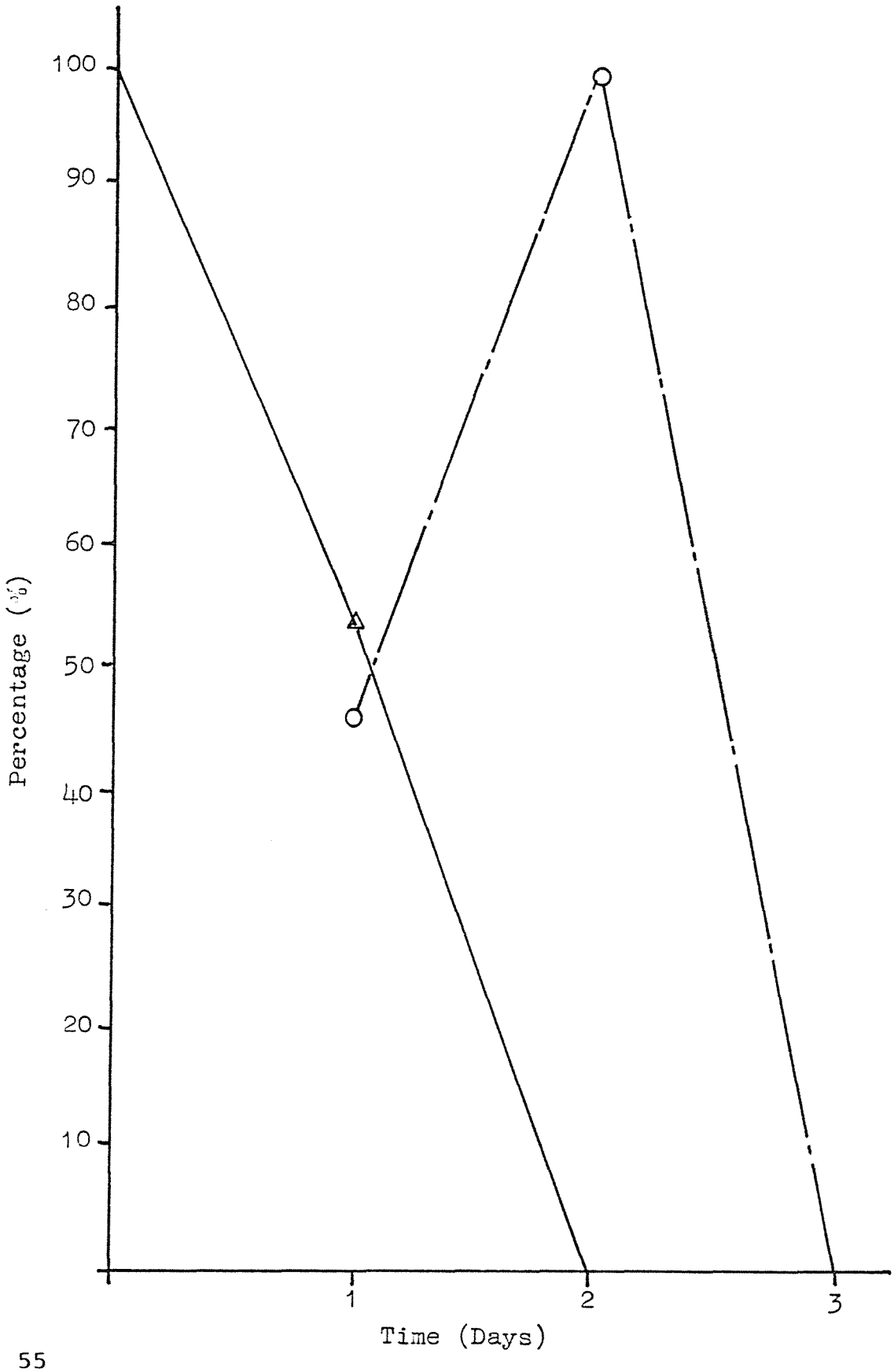


FIG. 55

Percentage encystment of an inoculum of 1 x 10^6 cells ml^{-1} in phosphate buffer pH 7, for 3 days.

N. fowleri

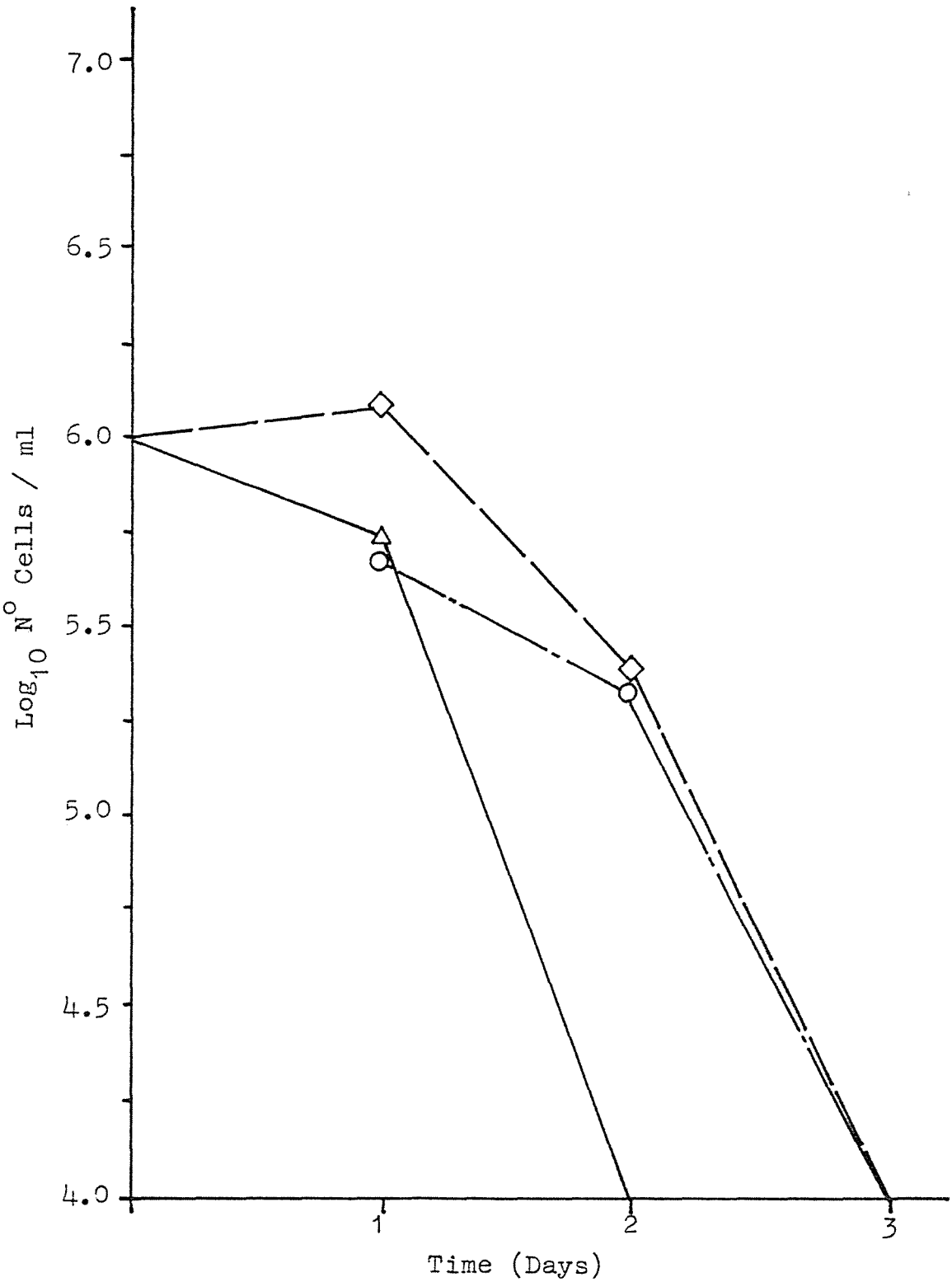


FIG. 56

Log 10 of the number of trophozoites, roundforms and cysts of an inoculum of 1 x 10^6 cells ml^{-1} of N. fowleri during 3 days incubation in phosphate buffer pH 7.

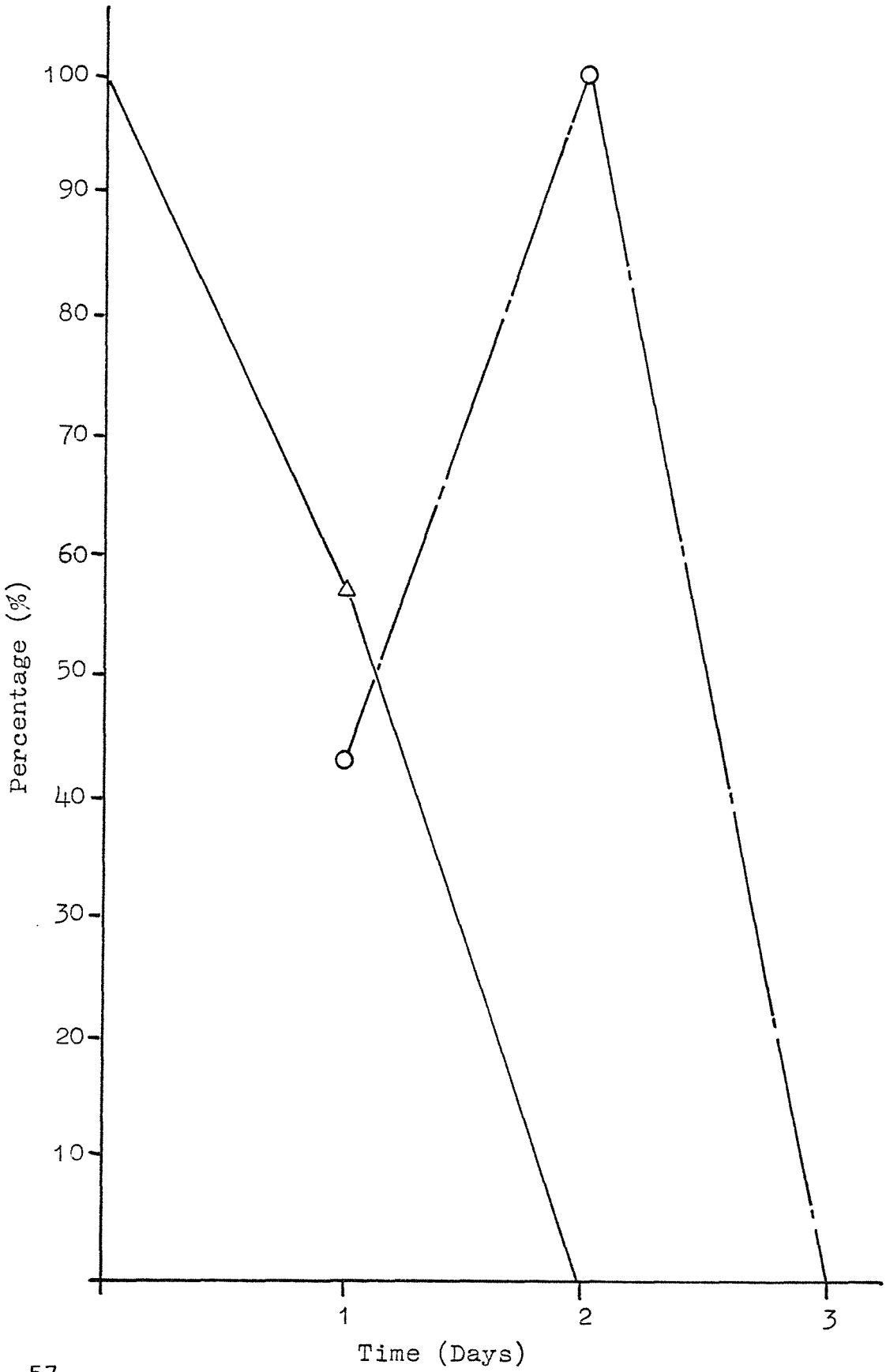


FIG. 57
Percentage encystment of an inoculum of 1 x 10^5 cells ml^{-1} in phosphate buffer pH 7, for 3 days.
N. fowleri

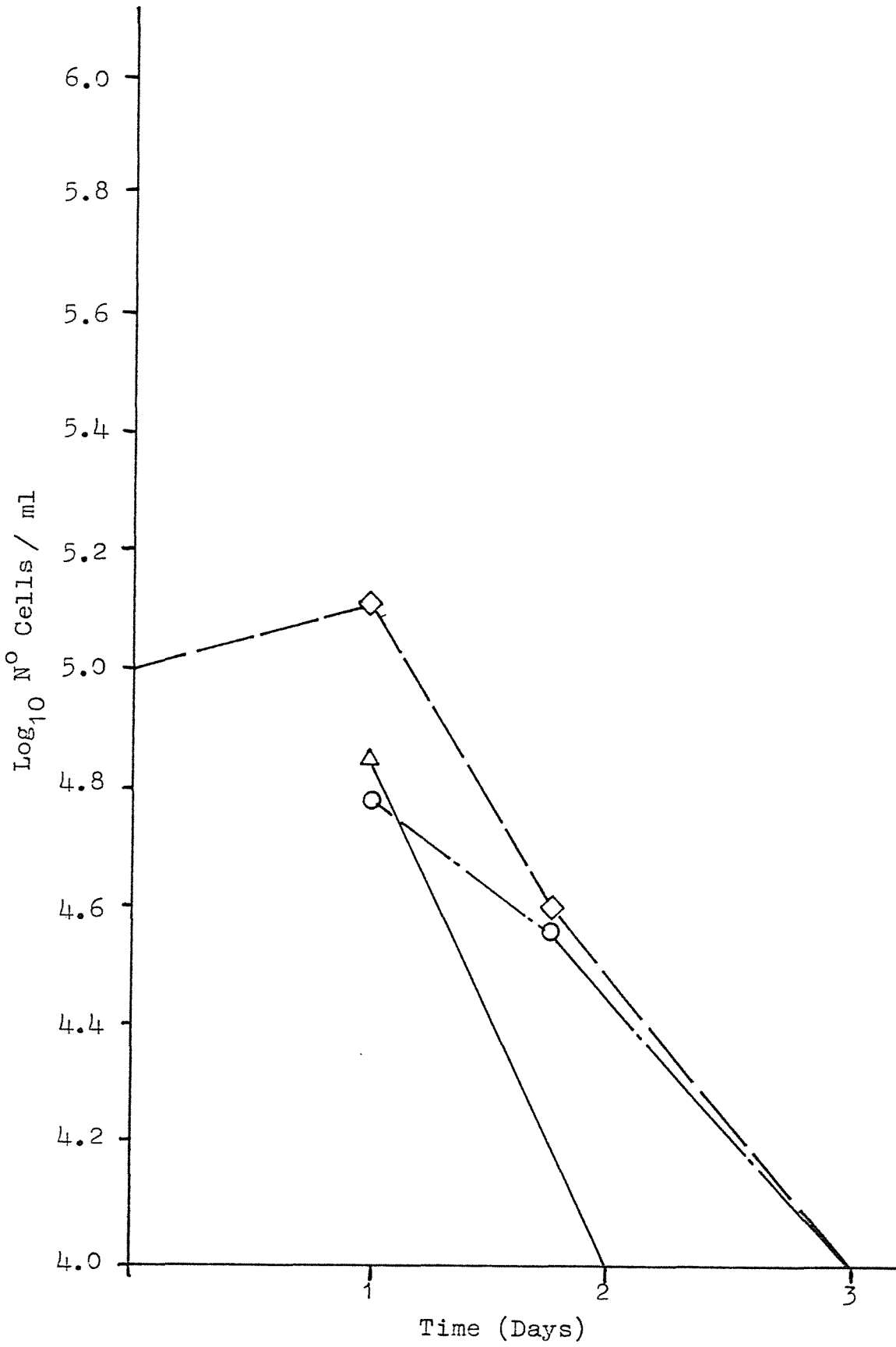


FIG. 58

Log 10 of the number of trophozoites, roundforms and cysts of an inoculum of 1 x 10^5 cells ml^{-1} of N. fowleri during 3 days incubation in phosphate buffer, pH 7.

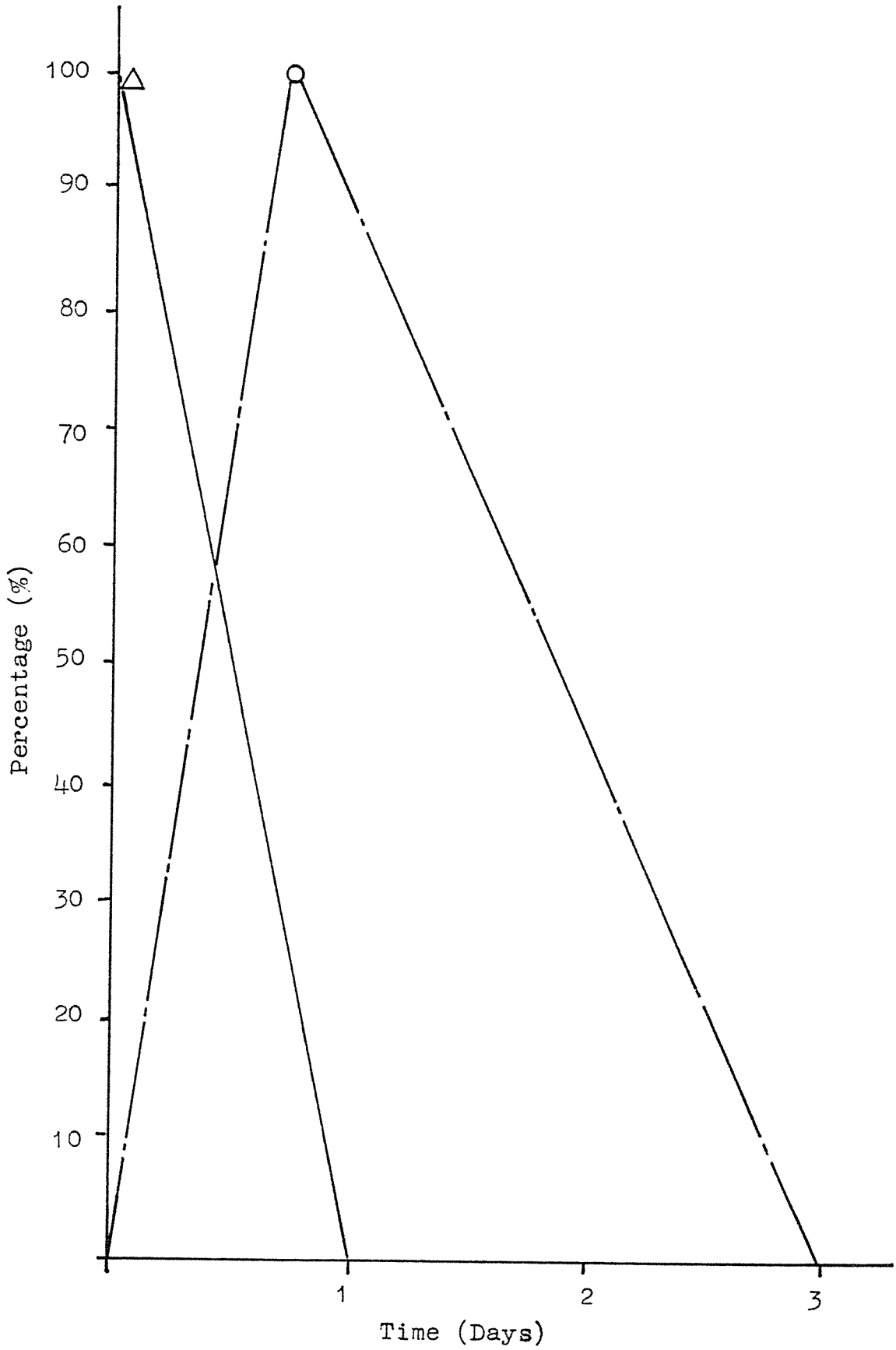


FIG. 59

Percentage trophozoites, roundforms and cysts during 3 days incubation on 10% soil extract agar at 4°C.

N. fowleri

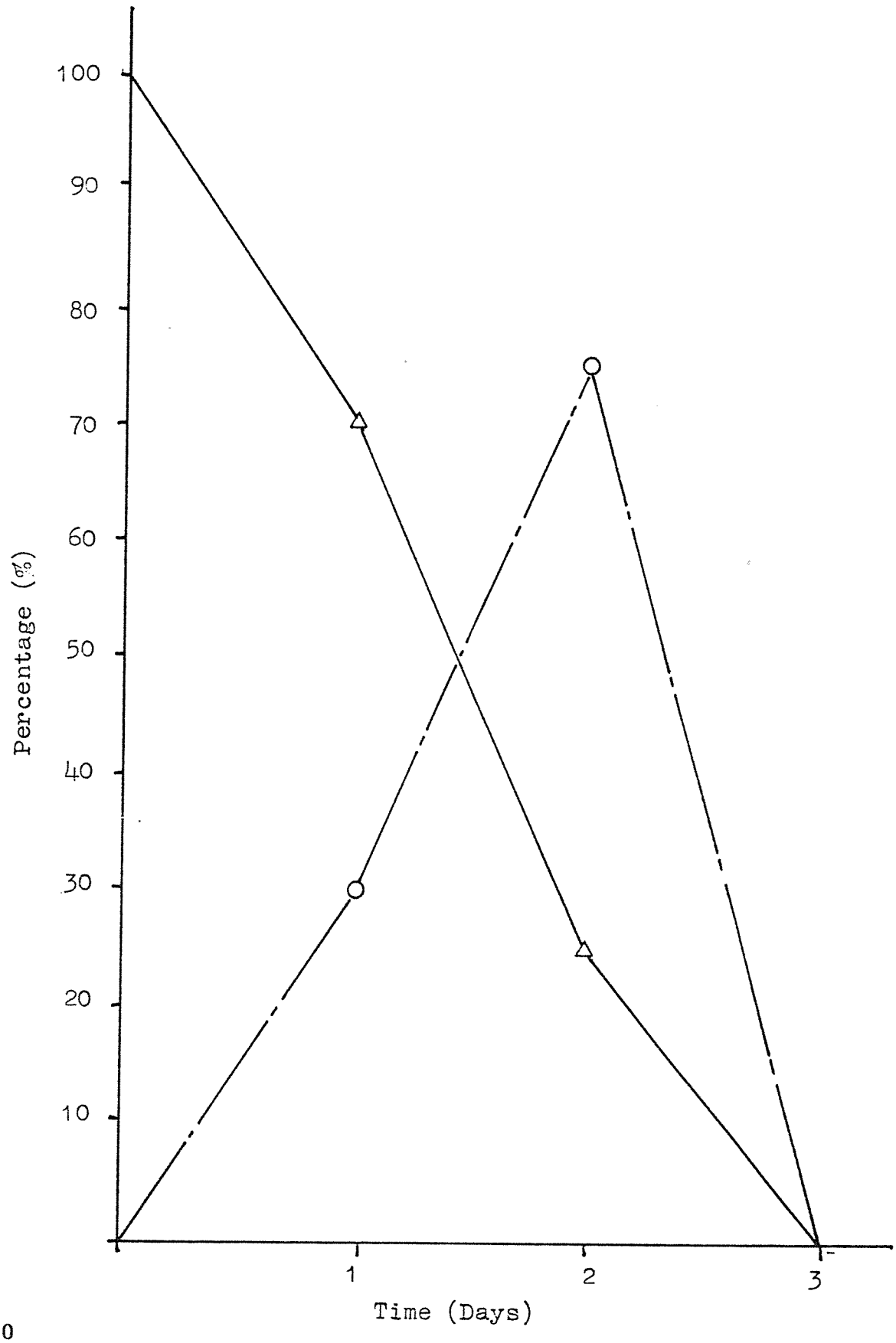


FIG.60

Percentage trophozoites, roundforms and cysts during 3 days incubation on 10% soil extract agar at 15°C.

N. fowleri

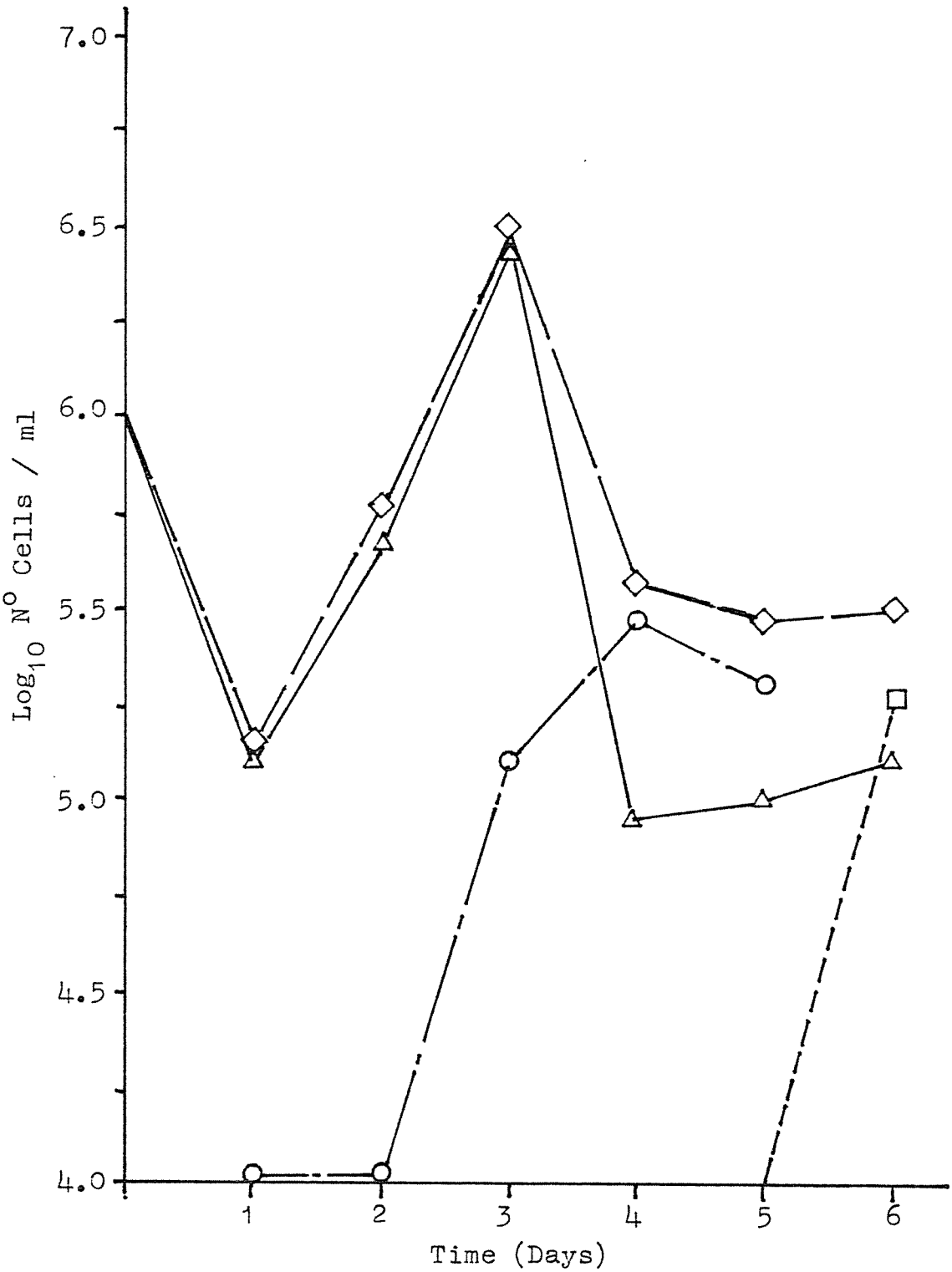


FIG.61

Log 10 of the number of trophozoites, roundforms and cysts during 6 days incubation on 10% soil extract agar at 25°C.

N. fowleri

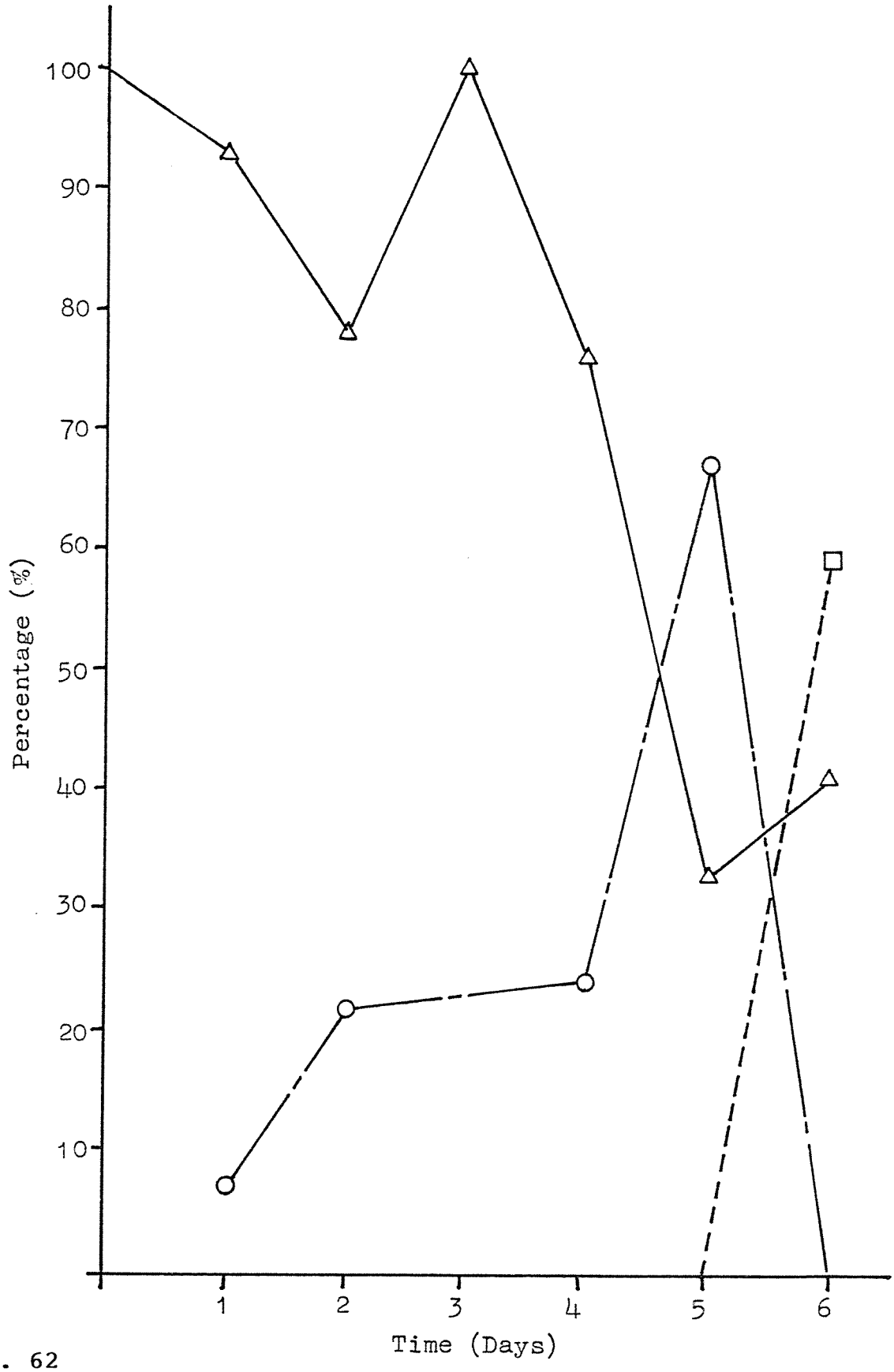


FIG. 62

Percentage trophozoites, roundforms and cysts during 6 days incubation on 10% soil extract agar at 25°C.

N. fowleri

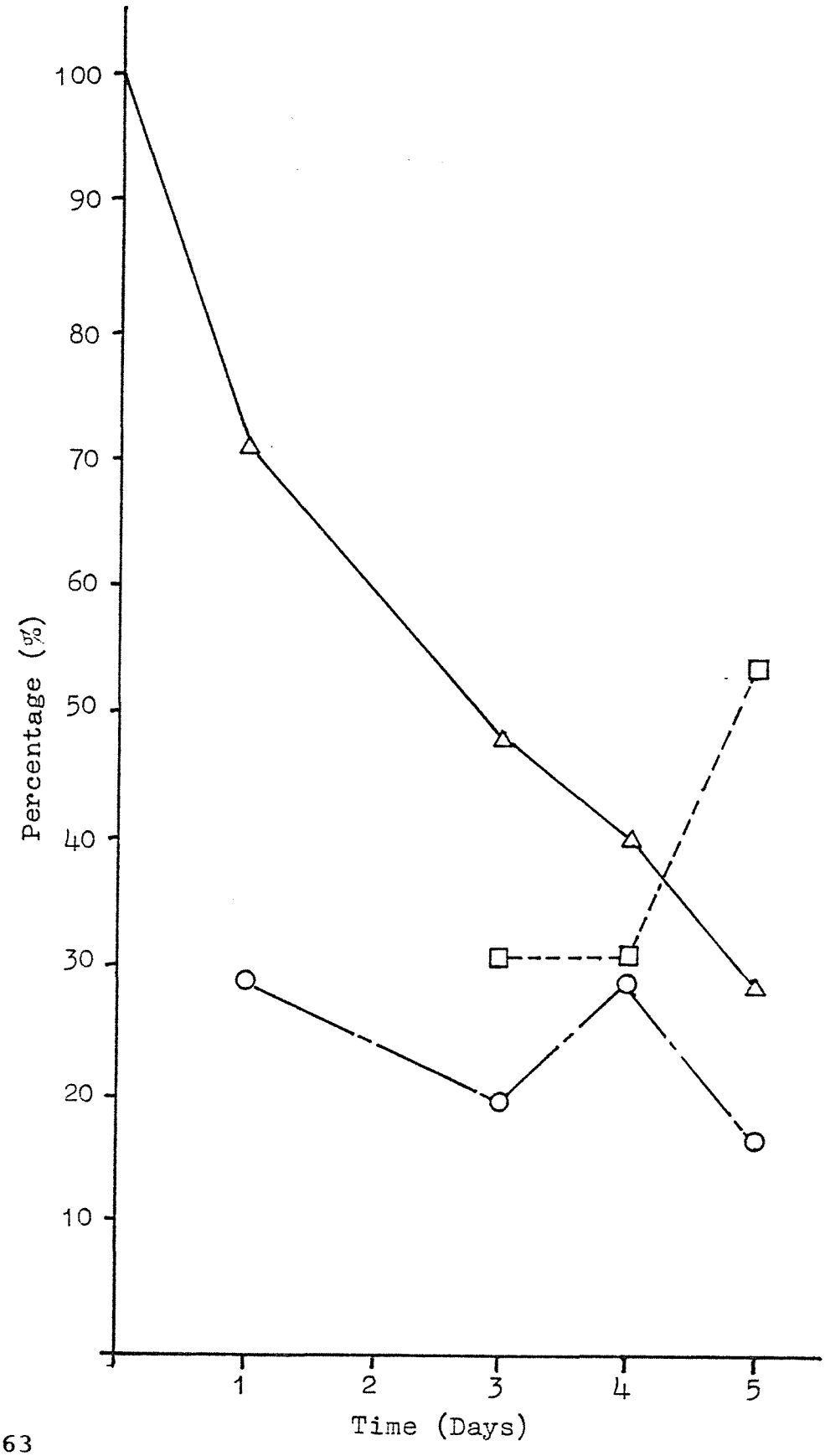


FIG. 63

Percentage trophozoites, roundforms and cysts of N. fowleri during 5 days incubation on 10% soil extract agar at 30°C.

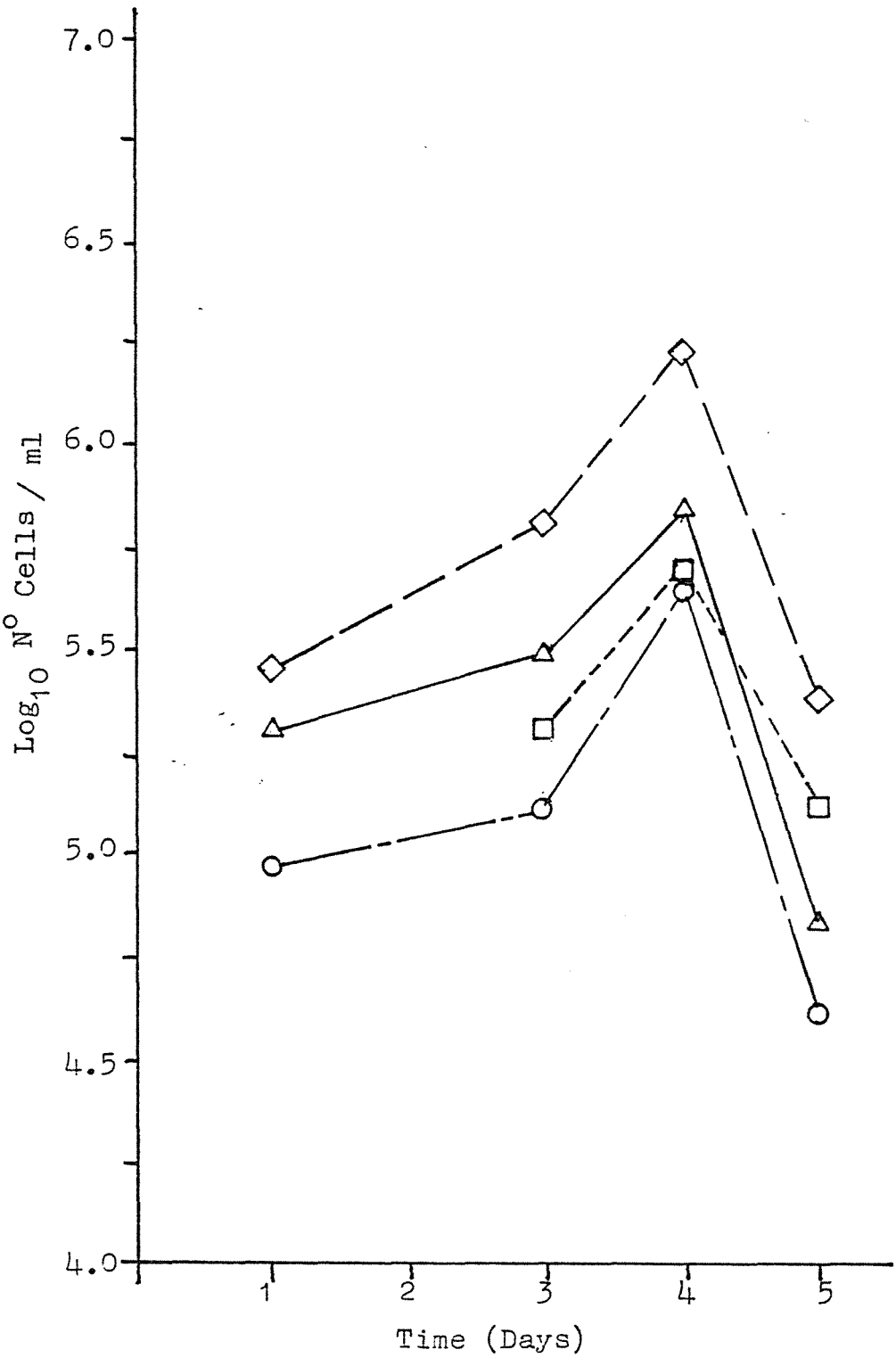


FIG.64

Log 10 of the number of trophozoites, roundforms and cysts of N. fowleri during 5 days incubation on 10% soil extract agar at 30°C.

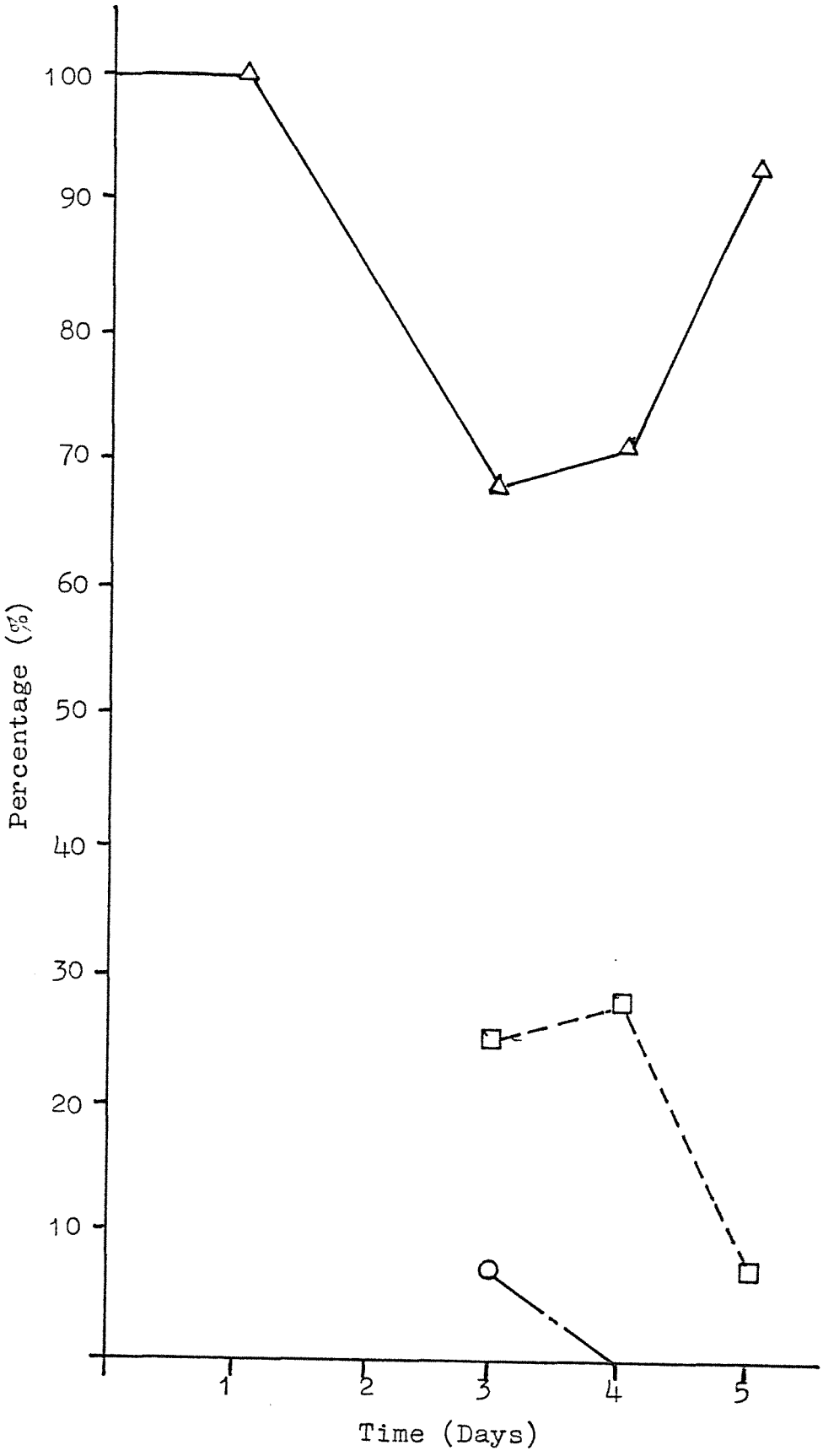


FIG.65

Percentage trophozoites, roundforms and cysts of N. fowleri during 5 days incubation on 10% soil extract agar at 37°C.

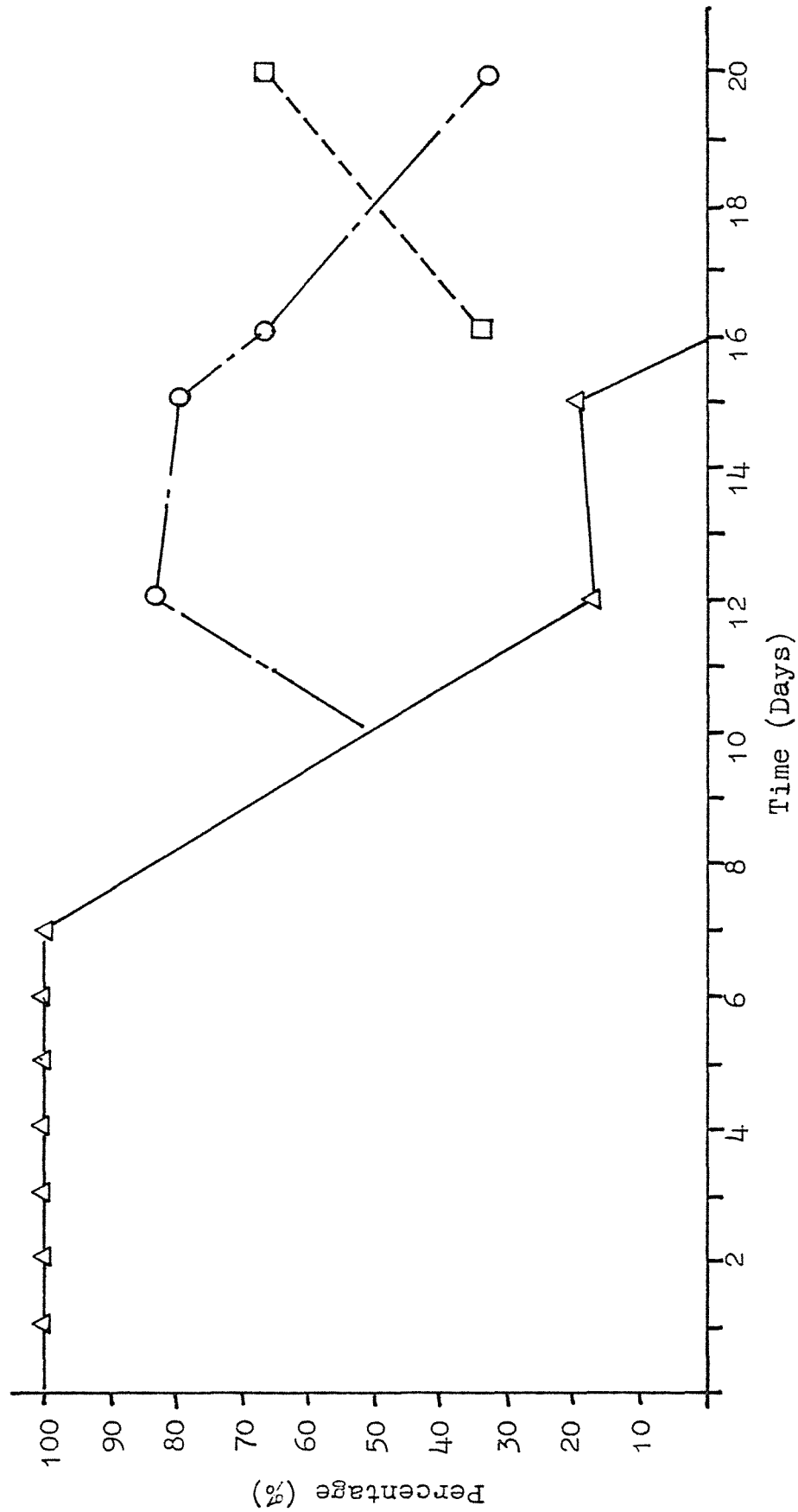


FIG. 66

Percentage trophozoites, roundforms and cysts of Acanthomoeba castellanii during 20 days incubation at 4°C on 10% soil extract agar

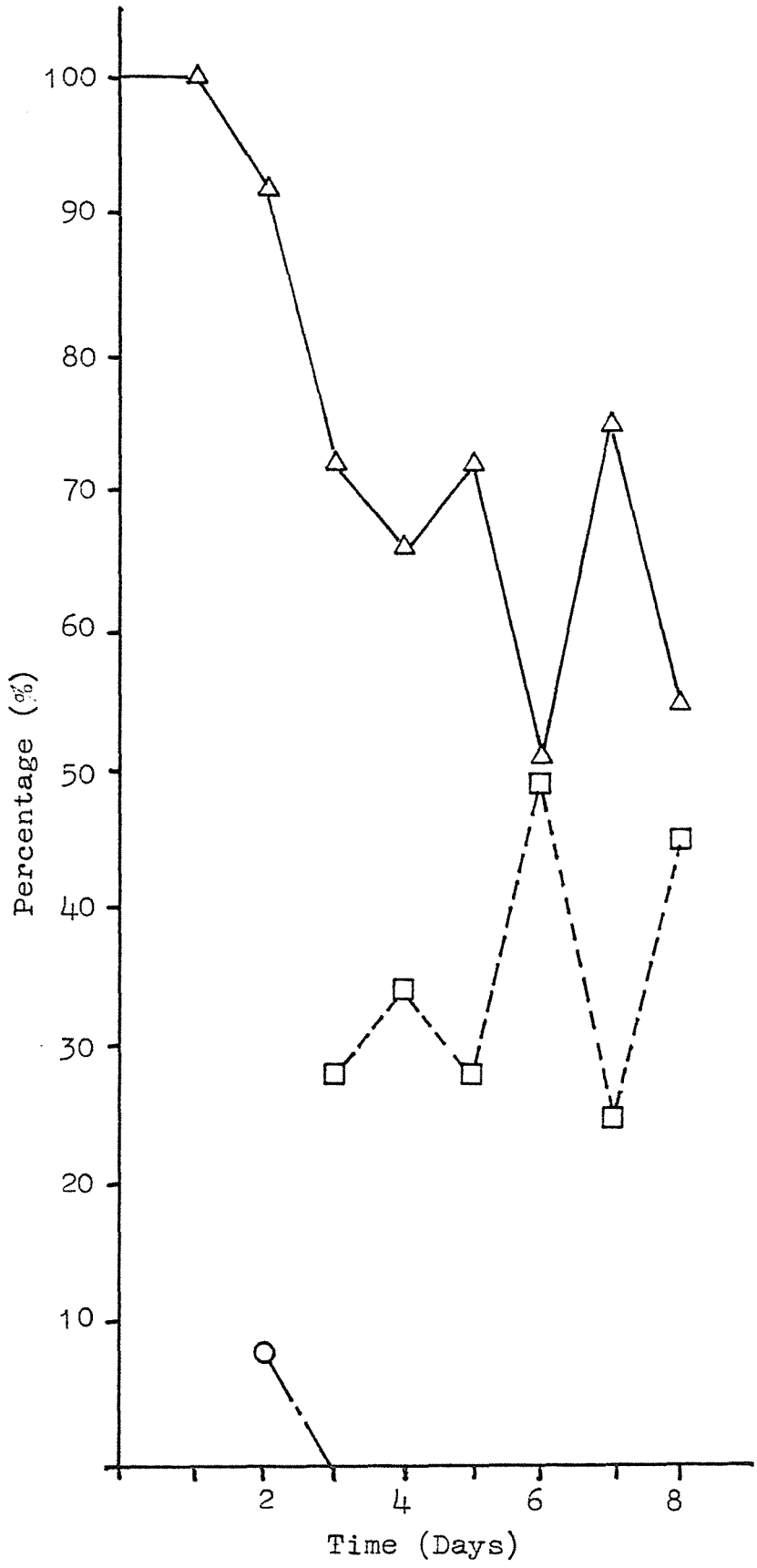


FIG.67

Percentage trophozoites, roundforms and cysts of A. castellanii during 8 days incubation on 10% soil extract agar at 15°C.

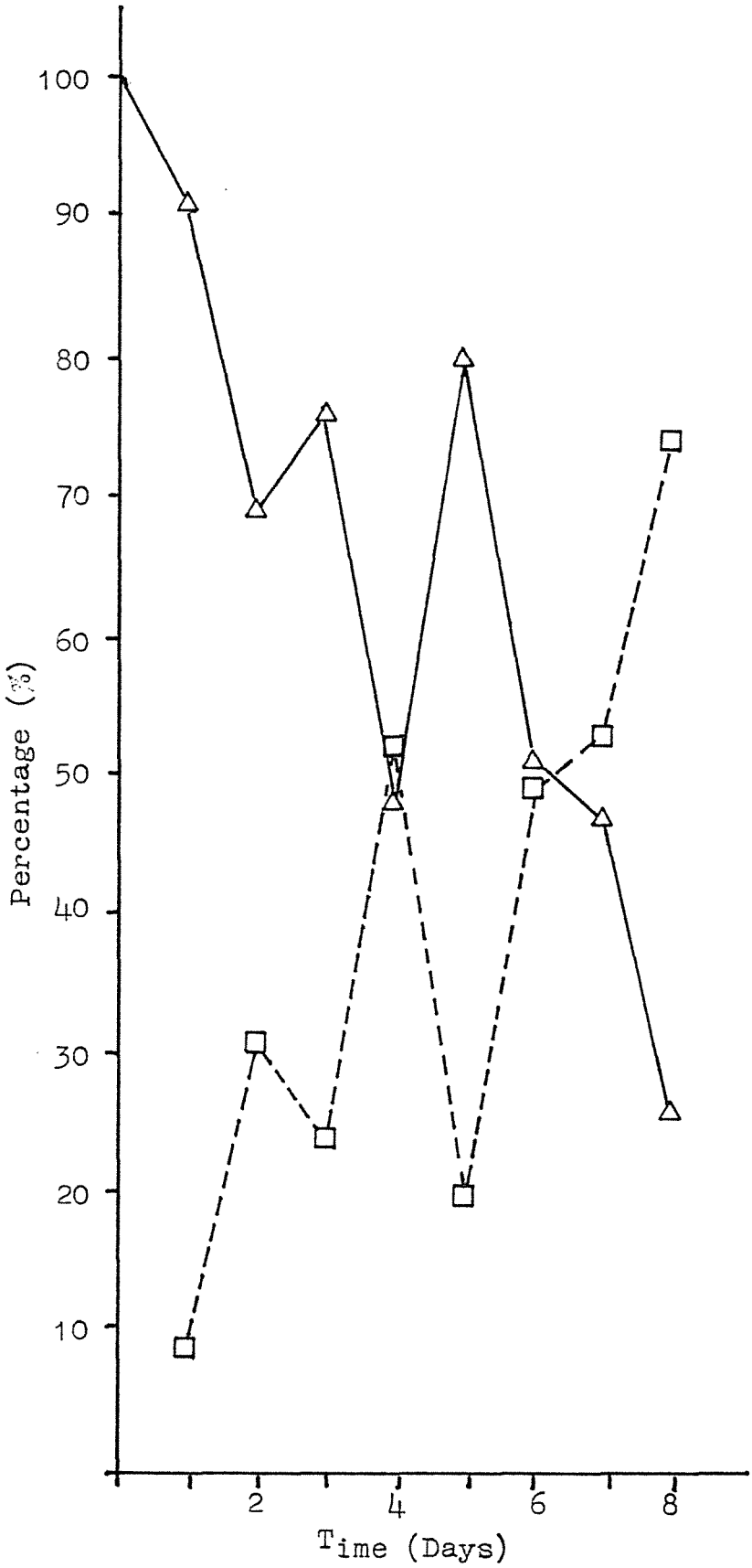


FIG. 68

Percentage trophozoites, roundforms and cysts of A. castellanii during 8 days incubation on 10% soil extract agar at 25°C.

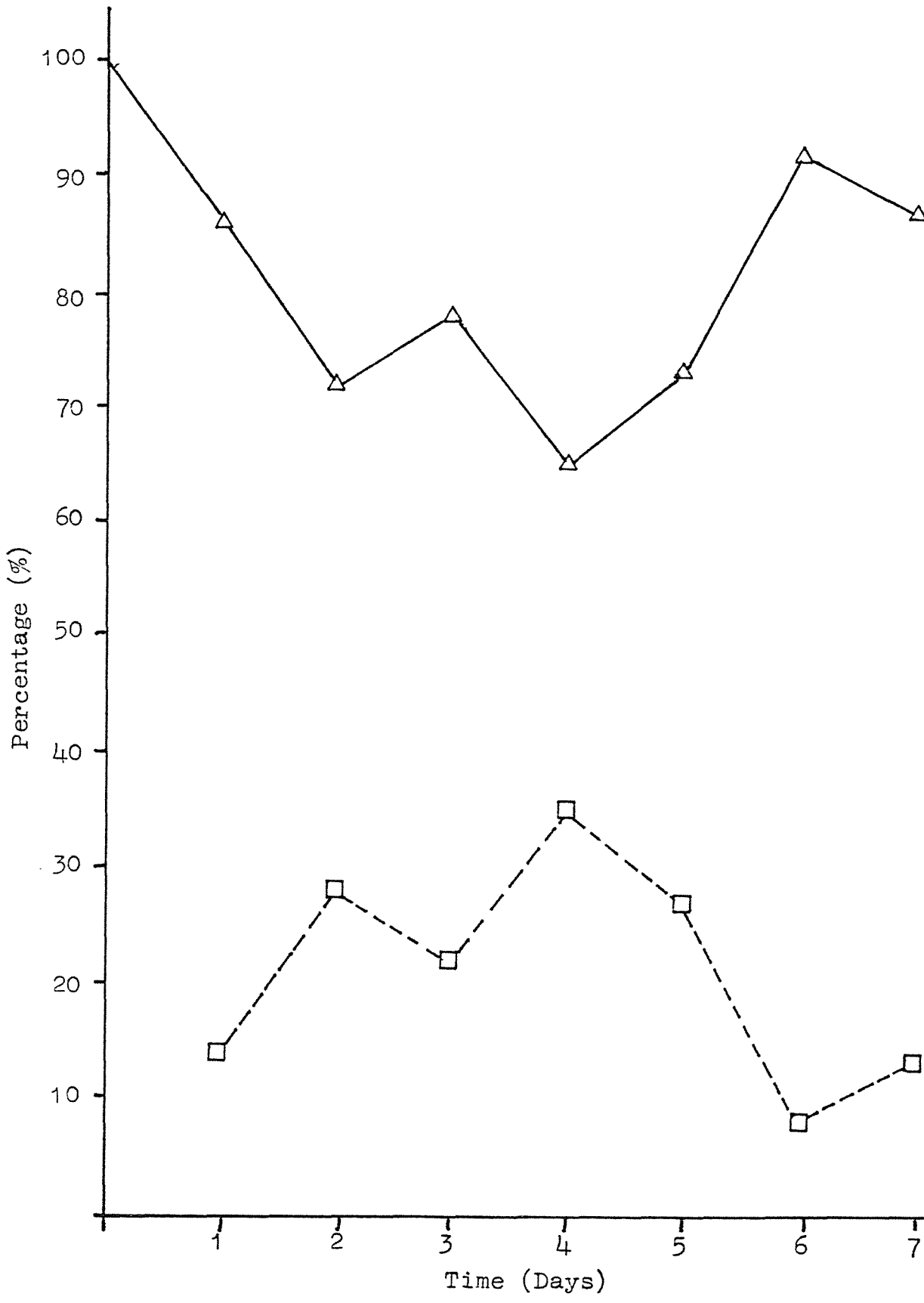


FIG.69

Percentage trophozoites, roundforms and cysts of A. castellanii during 7 days incubation on 10% soil extract agar at 30°C.

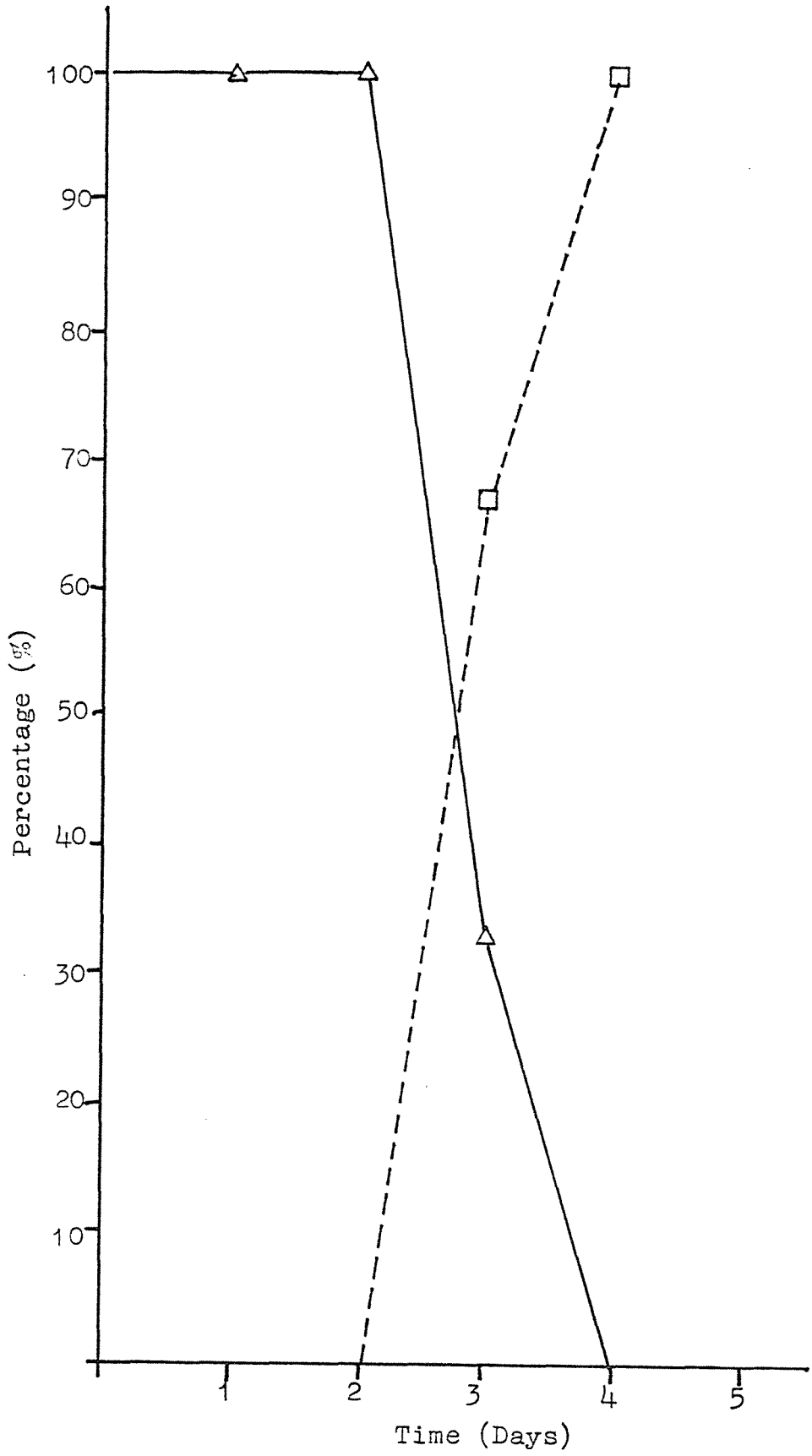


FIG. 70

Percentage trophozoites, roundforms and cysts of A. castellanii during 5 days incubation on 10% soil extract agar at 37°C.

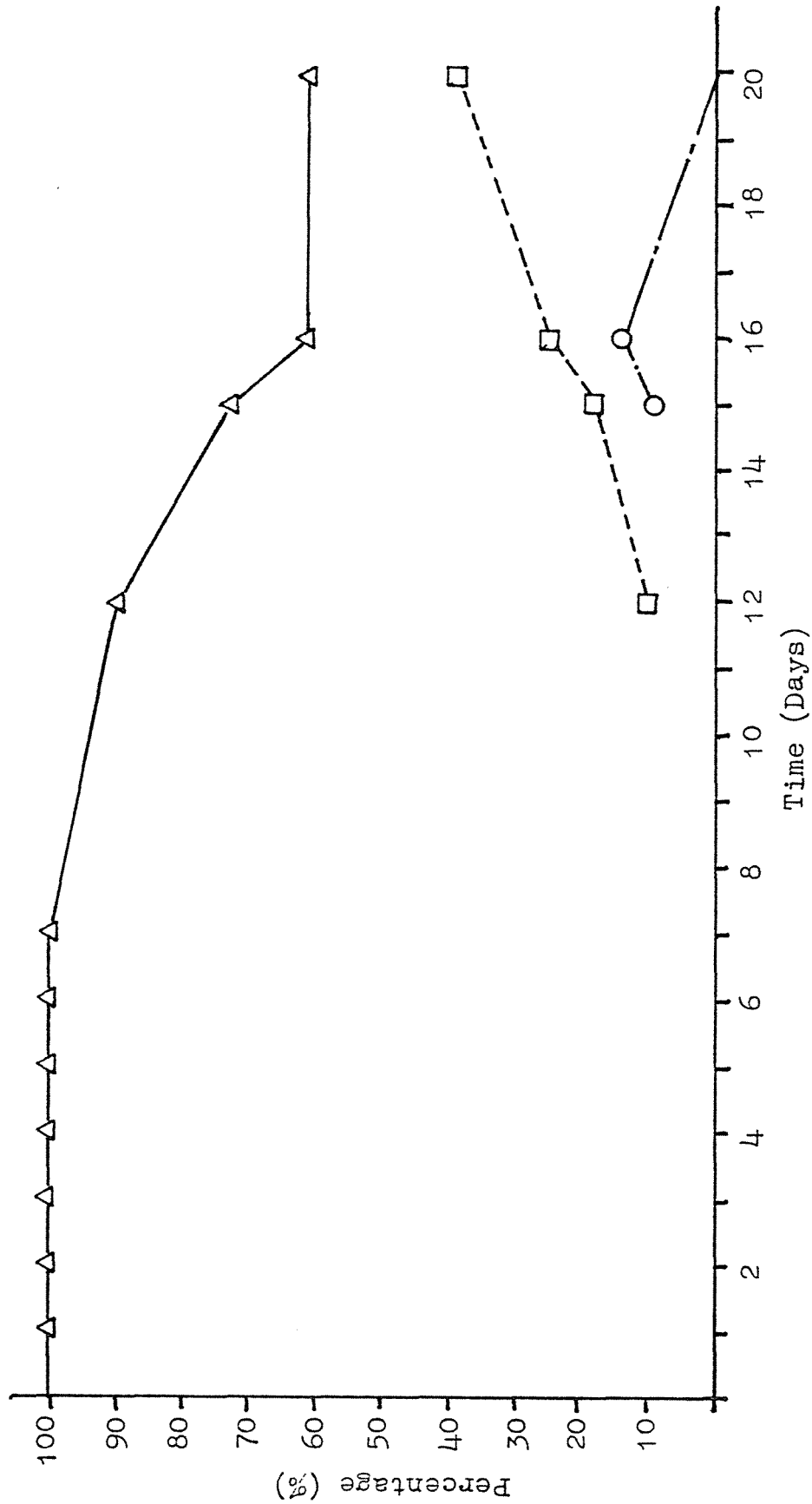


FIG. 71

Percentage trophozoites, roundforms and cysts of A. culbertsoni during 20 days incubation on 10% soil extract agar at 4°C.

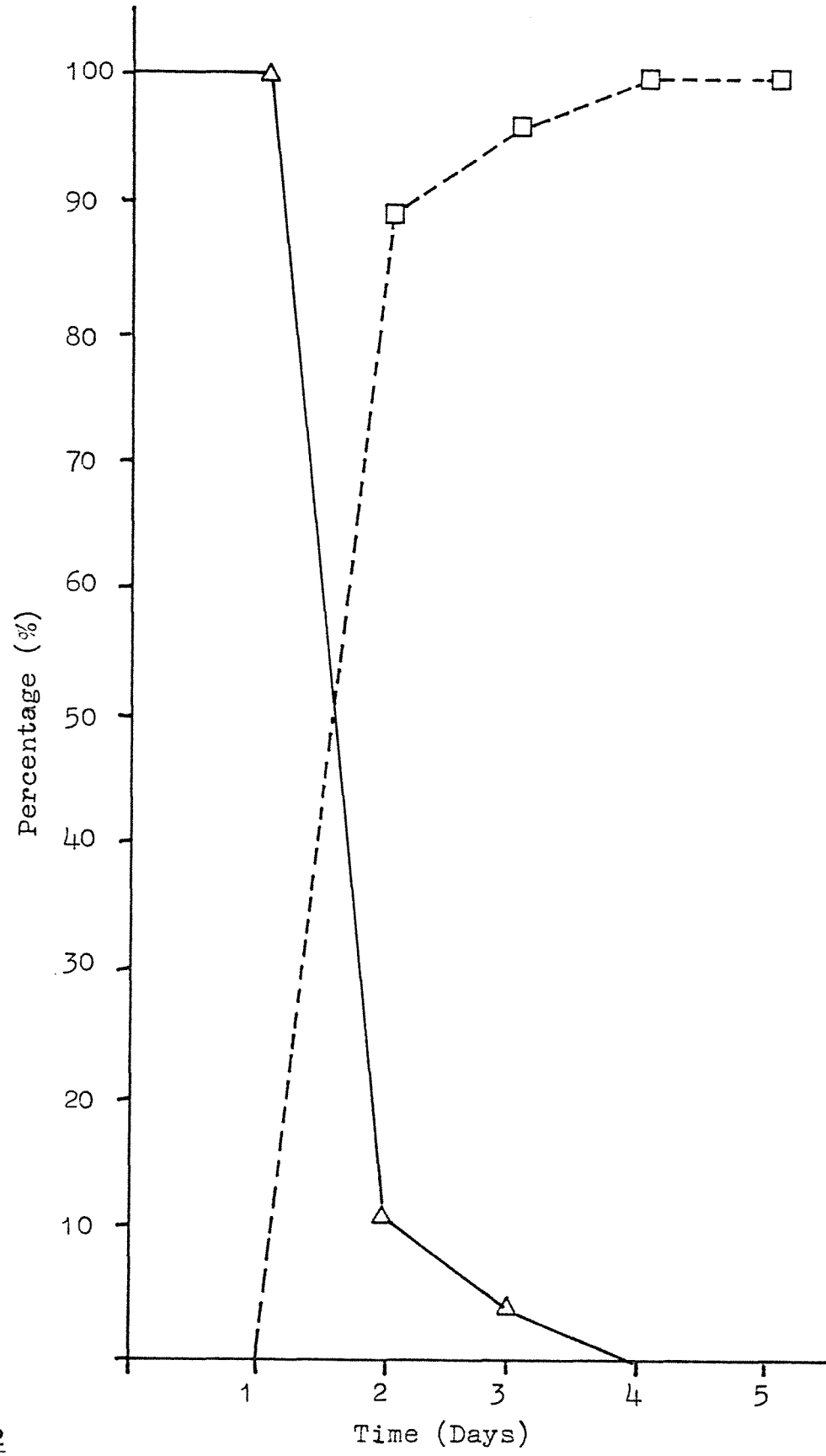


FIG. 72

Percentage trophozoites, roundforms and cysts of A. culbertsoni during 5 days incubation on 10% soil extract agar at 15°C.

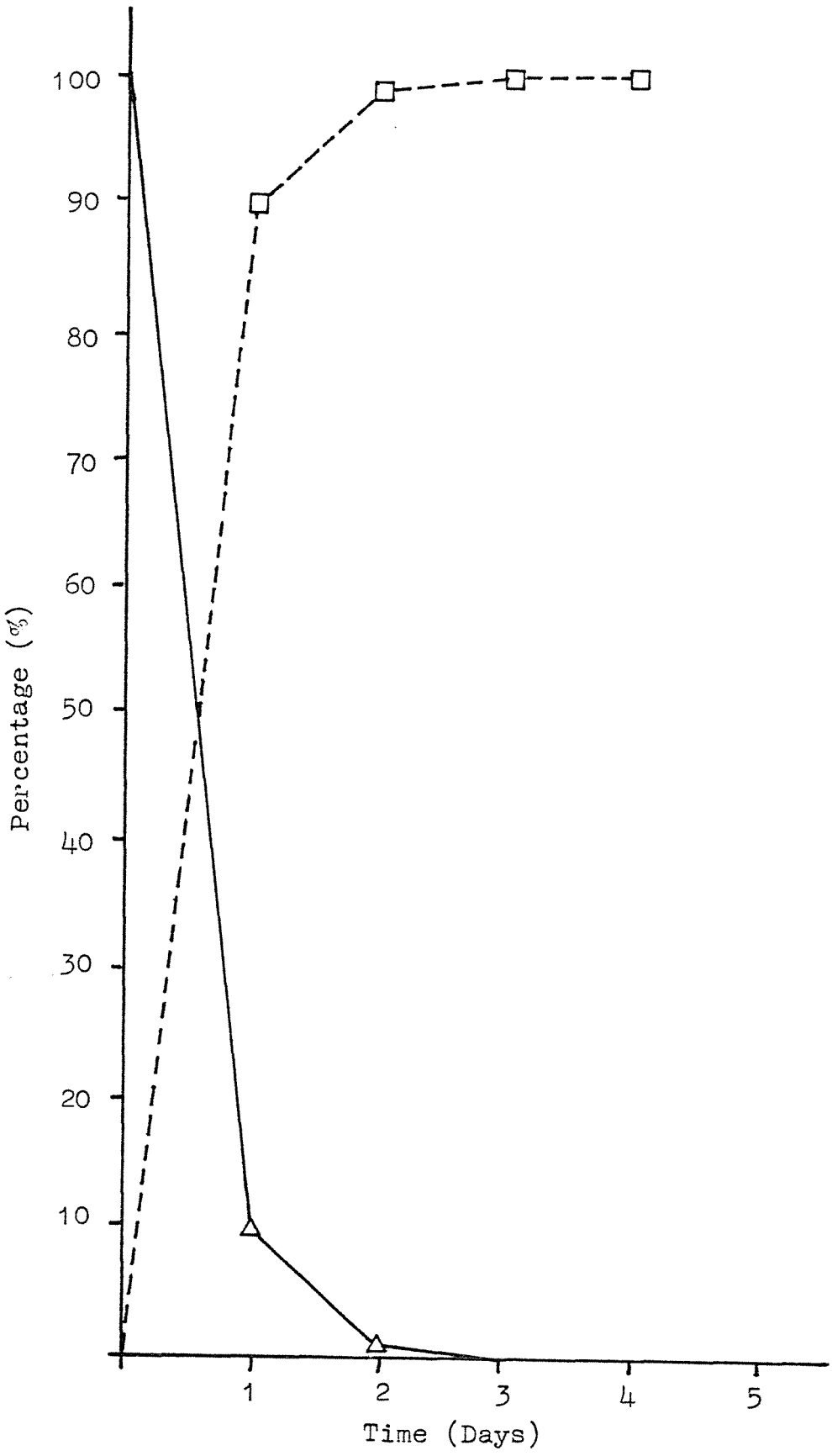


FIG. 73

Percentage trophozoites, roundforms and cysts of A. culbertsoni during 5 days incubation on 10% soil extract agar at 25°C.

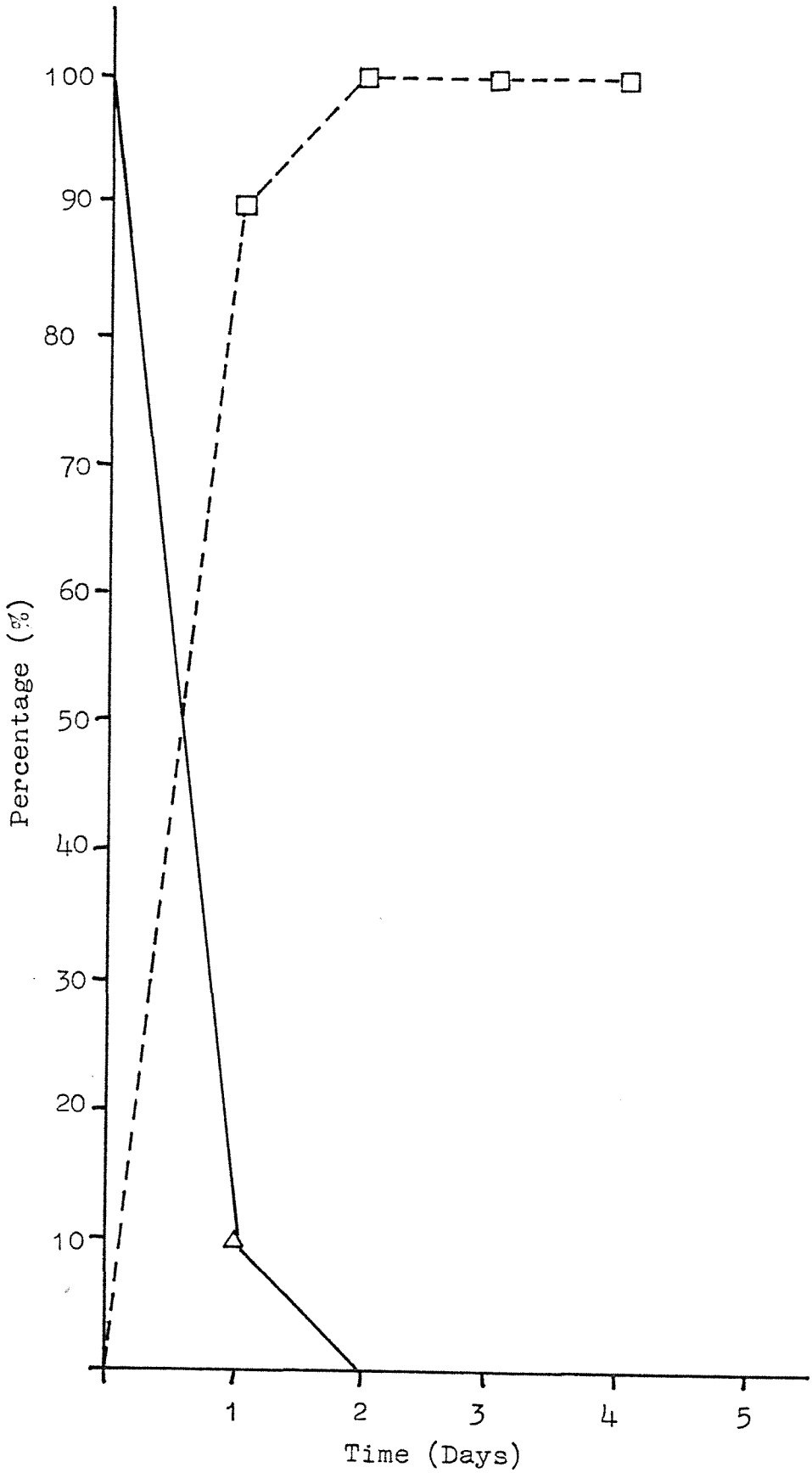


FIG. 74

Percentage trophozoites, roundforms and cysts of A. culbertsoni during 5 days incubation on 10% soil extract agar at 30°C.

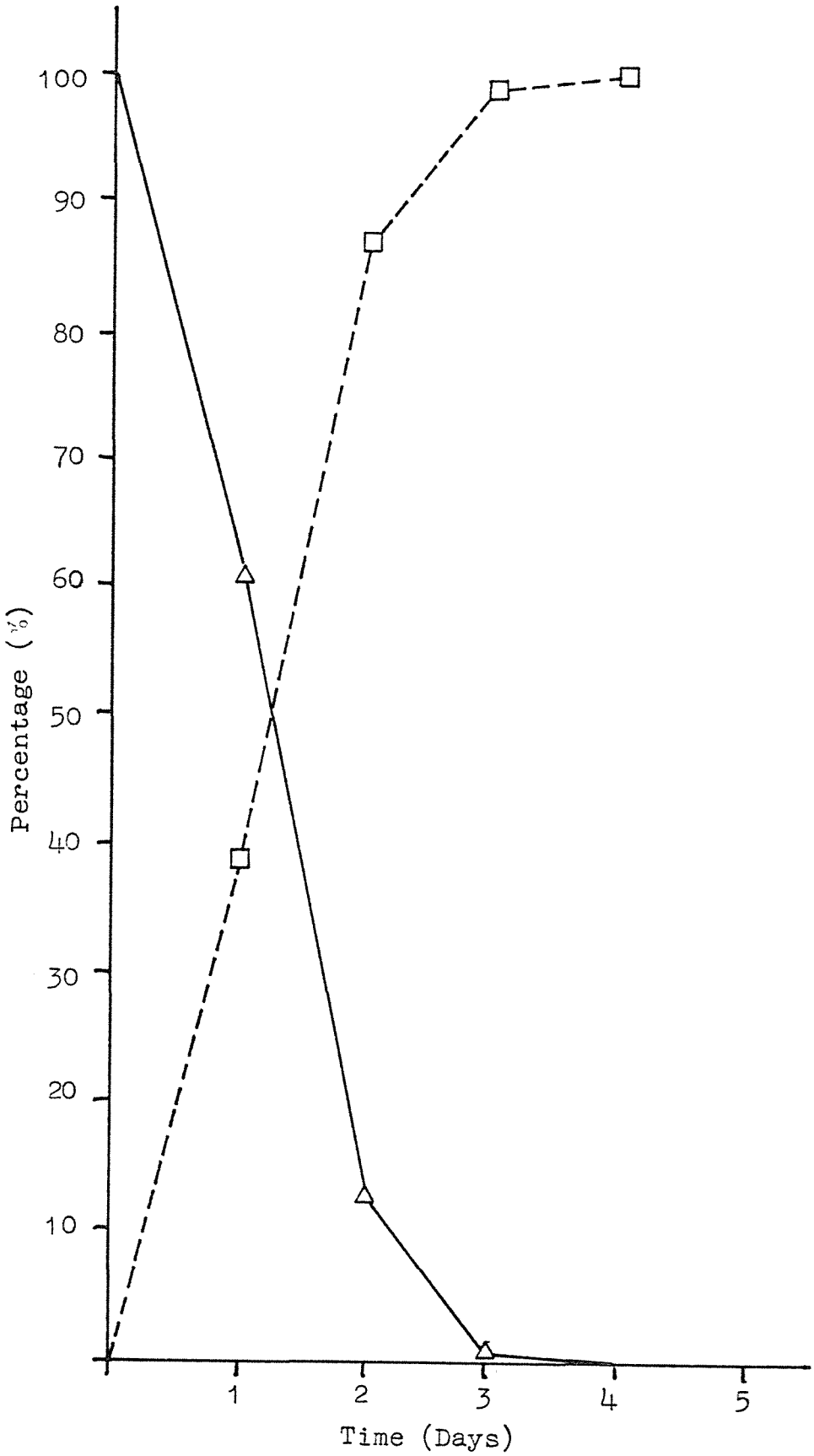


FIG. 75

Percentage trophozoites, roundforms and cysts of A. culbertsoni during 5 days incubation on 10% soil extract agar at 37°C.

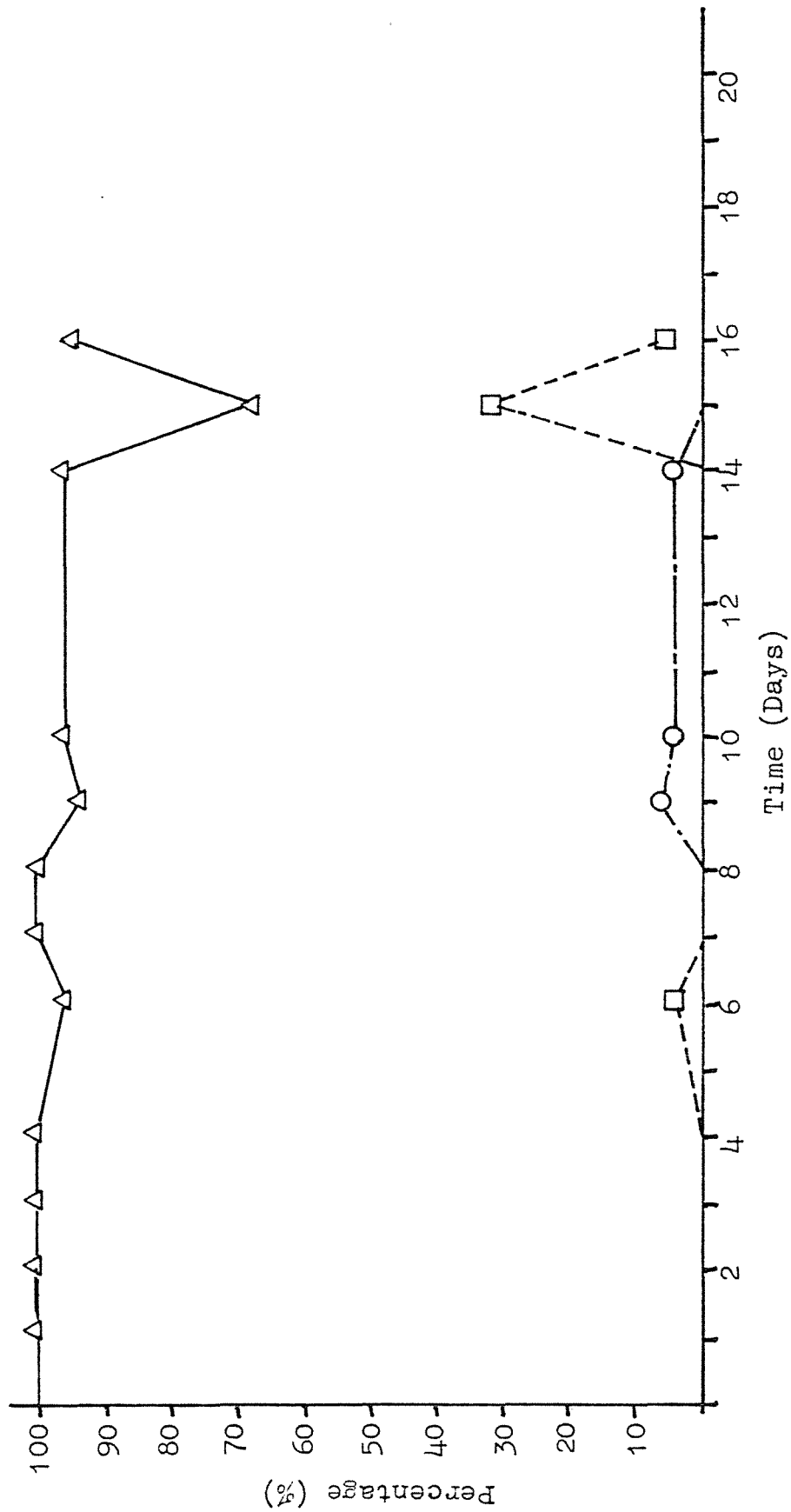


FIG. 76

Percentage trophozoites, roundforms and cysts of N. gruberi during 16 days incubation on 10% soil extract agar at 15°C.

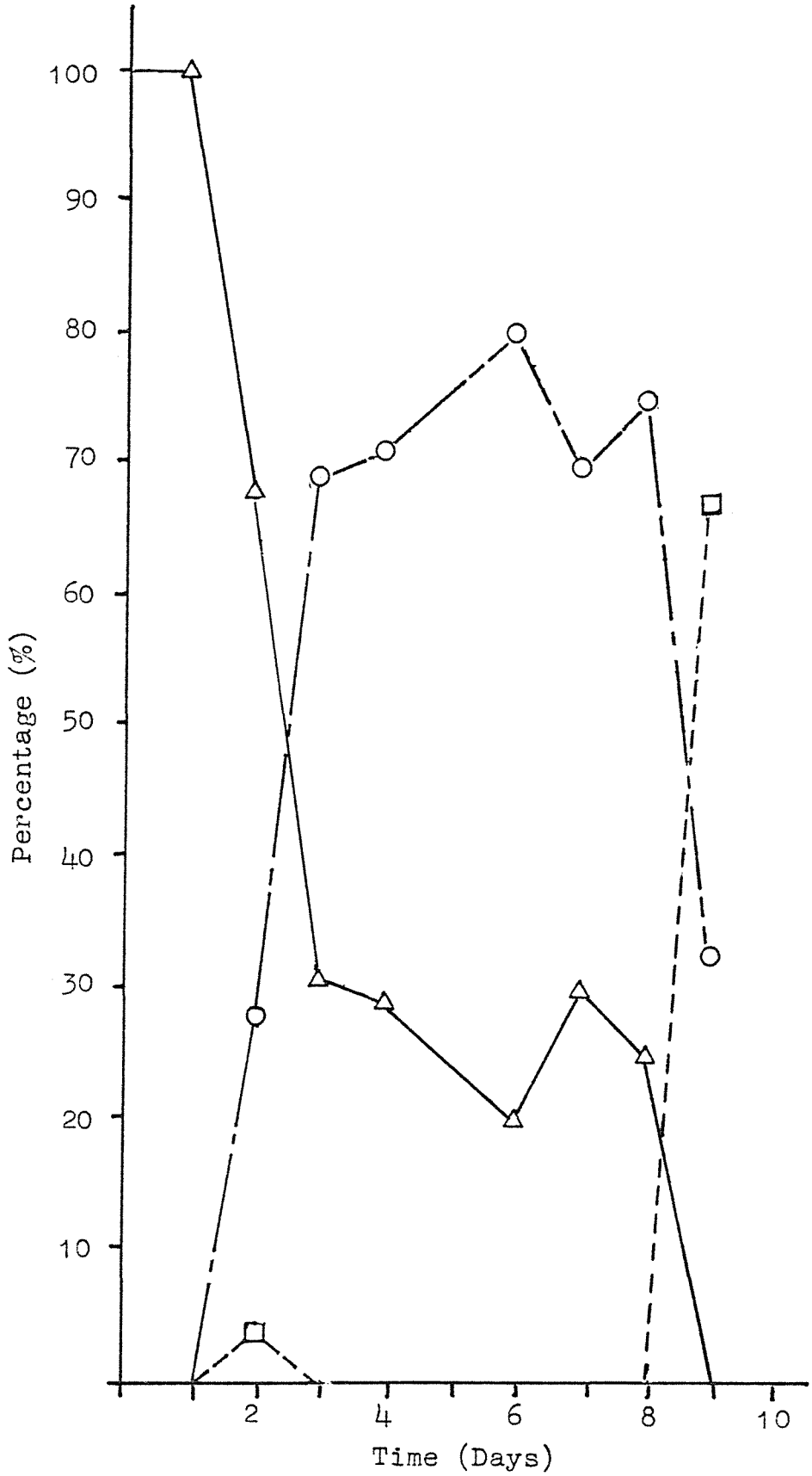


FIG.77

Percentage trophozoites, roundforms and cysts of N. gruberi during 9 days incubation on 10% soil extract agar at 25°C.

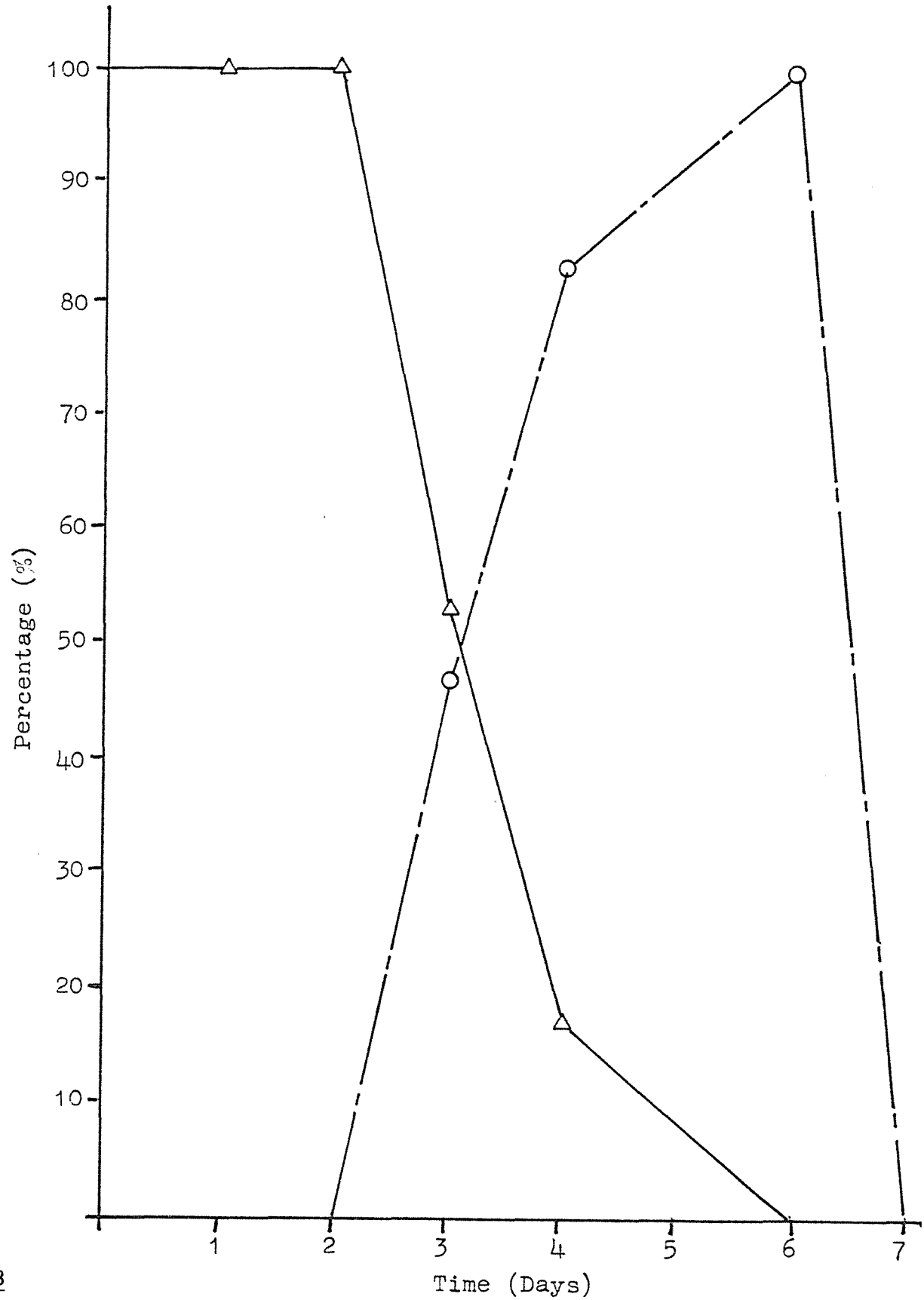


FIG. 78

Percentage trophozoites, roundforms and cysts of N. gruberi during 7 days incubation on 10% soil extract agar at 30°C.

4.6 Disinfection of Pathogenic Free-Living Amoebae

In **Fig. 79** the F.A.C. level initially dropped during chlorination of A. castellanii (01) from 4 mg to 1.9 mg l^{-1}, reaching 0.5 mg ℓ^{-1} F.A.C. after 1½ hours. After 3 hours incubation the F.A.C. level was boosted to 1 mgl^{-1} for A. culbertsoni the F.A.C. level dropped from 4 mgl^{-1} to 1.3 mg l^{-1} and declined to 0.8 mgl^{-1} after 5 hours. Graph is to indicate the fluctuating free chlorine levels during the process of disinfection.

The recommended level for disinfection with Baquacil is 50 mgl^{-1}. In **Fig. 80** the two Baquacil resistant strains $MSMbr_2$ and $MSMbr_4$ both have high percentage survival 78% and 65% respectively, the other Naegleria strain MSM has 10% survival at the 50 mgl^{-1} concentration. A. culbertsonii (A1) trophozoites have a 30% disinfection survival with 50 mgl^{-1} Baquacil. 75 mgl^{-1} Baquacil was amoebicidal for MSM and A1, $MSMbr_2$ had a % survival of 45 and $MSMbr_4$ a 32% survival. At a Baquacil concentration of 100 mgl^{-1} $MSMbr_2$ had a 40% survival rate and Br_4 an 8% survival rate.

In **Fig. 81** the percentage survival rate for $MSMbr_4$ at 50 mgl^{-1} after 6 months encystment and then excystment was 40% lower than before encystment. The percentage survival rates were 10% lower than the initial values for disinfection at 75 mgl^{-1} and 100 mg^{-1}.

Fig. 82 shows the Baquacil resistant strain MSMbr showed lower survival percentages at all chlorine concentrations with the exception of 0.4 mgl^{-1} of free available chlorine and 1.2 mgl^{-1} which was amoebicidal for MSM.

In **Fig. 83** A1 trophozoites have a higher range of tolerance to chlorine, in comparison to MSM. A1 has a 10% survival rate at 1.3-1.4 mgl^{-1} free available chlorine and a low 3% survival at 2mgl^{-1}.

Fig. 84 shows cysts of A1 had a 50% survival rate after 100 minutes of chlorination, this decreased to 40% after 160

minutes chlorination. After 240 minutes of chlorination there were not any detectable viable cysts of A1. Cysts of (O1) had a higher percentage survival, 85% after 100 minutes, 75% after 240 minutes and 67% after 6 hours chlorination.

In **Fig. 85** after 20 minutes disinfection cysts of NHI had a 20% survival, after 40 minutes disinfection this had declined to a 5% survival of amoebae. No viable cysts were detected after 60 minutes disinfection. PL had 60% cyst survival after 20 minutes disinfection and 10% survival after 40 minutes disinfection. No viable cysts detected after 60 minutes.

In **Fig. 86** cysts of (01) after 60 minutes disinfection in 50 mgl^{-1} Baquacil had 80% survival rate, this was the percentage survival after 240 minutes disinfection. After 30 minutes disinfection A1 cysts had a 25% survival rate, this decreased to 18% after 240 minutes.

Fig. 87 shows the percentage survival for A1 cysts remained around 10% during 5 days cysts were disinfected with 50 mgl^{-1} Baquacil. O1 cysts decreased in survival percentage from 62% on day 2 to 38% on day 5. Baquacil was not cysticidal at this level.

Fig.88 shows the percentage survival of PL cysts decreased to 90% after 4 hours disinfection in Baquacil. After 4 hours disinfection in Baquacil NHI cysts had a percentage survival of 82%.

In **Fig. 89** the percentage survival of PL cysts over 5 days disinfection in Baquacil declined from 90% after day 1 of disinfection to 80%. NHI cysts decreased in survival percentage from 78% to 69% on day 5 of disinfection.

In Figures 79-89 the following legend applies:

	TEST	CONTROL
N. fowleri ($MSMbr_2$) trophozoites =		
N. fowleri ($MSMbr_4$) trophozoites =		
N. Fowleri (MSM) Sensitive trophozoites =		
N. fowleri (MSMbr) trophozoites =		
A. culbertsoni (A1) trophozoites =		
A. culbertsoni (A1) cysts =		
A. castellanii (01) cysts =		
N. fowleri (NH1) cysts =		
N. gruberi (PL) cysts =		

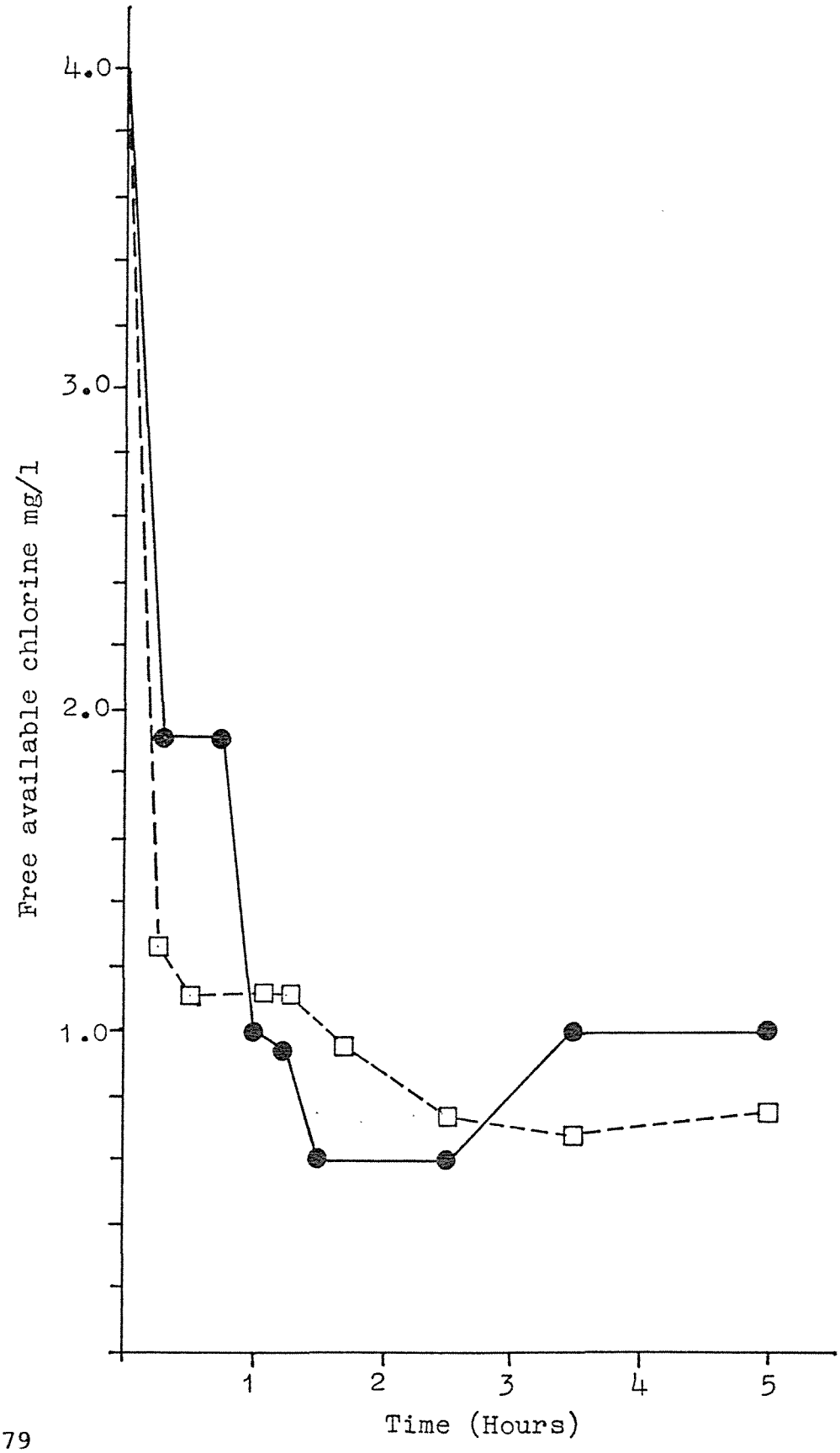


FIG.79

Levels of free available chlorine during chlorination of A. castellanii and A. culbertsoni cysts

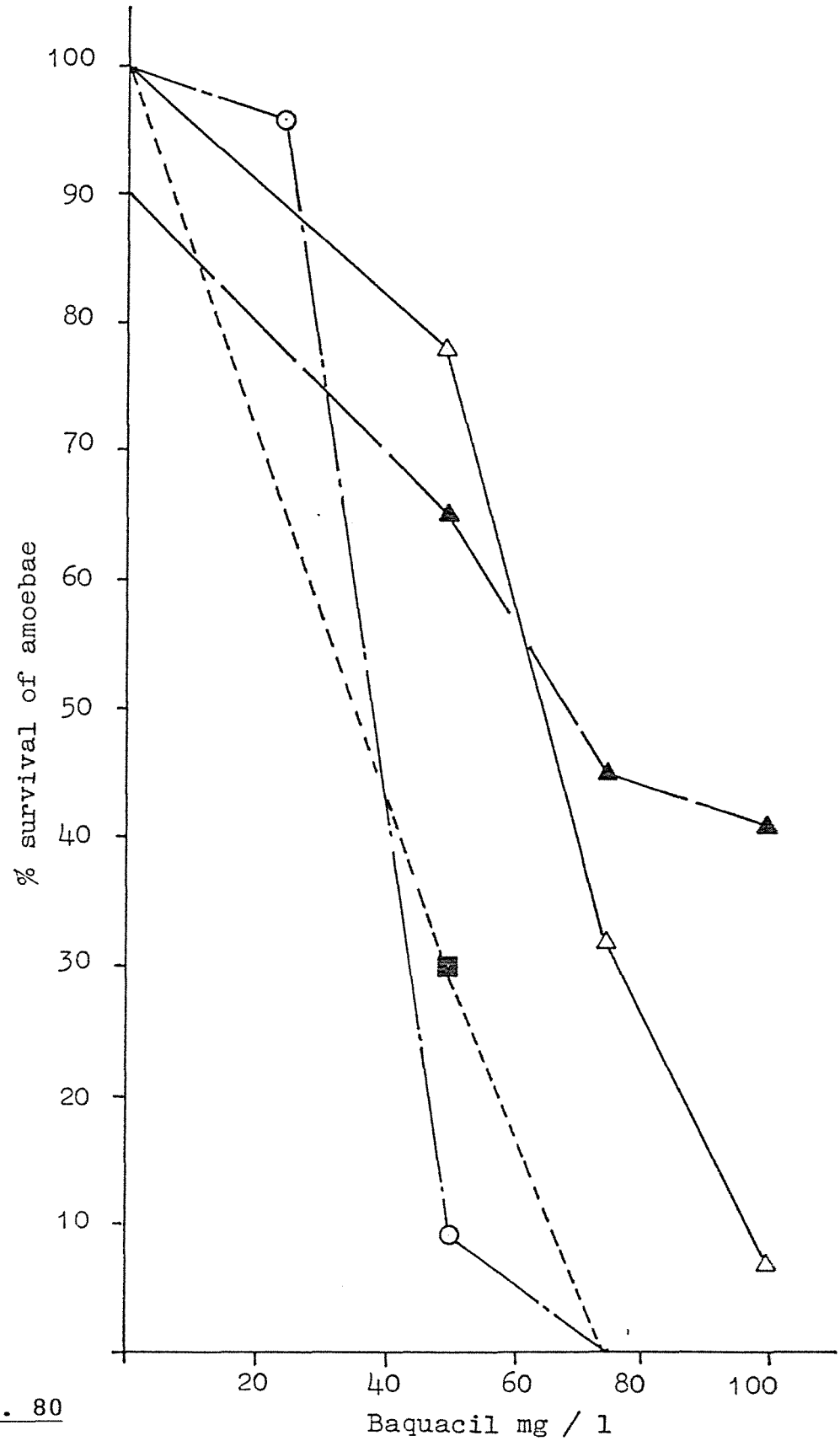


FIG. 80

The effect of Baquacil on Baquacil resistant and Baquacil sensitive amoebae for 30 minutes at 37°C.

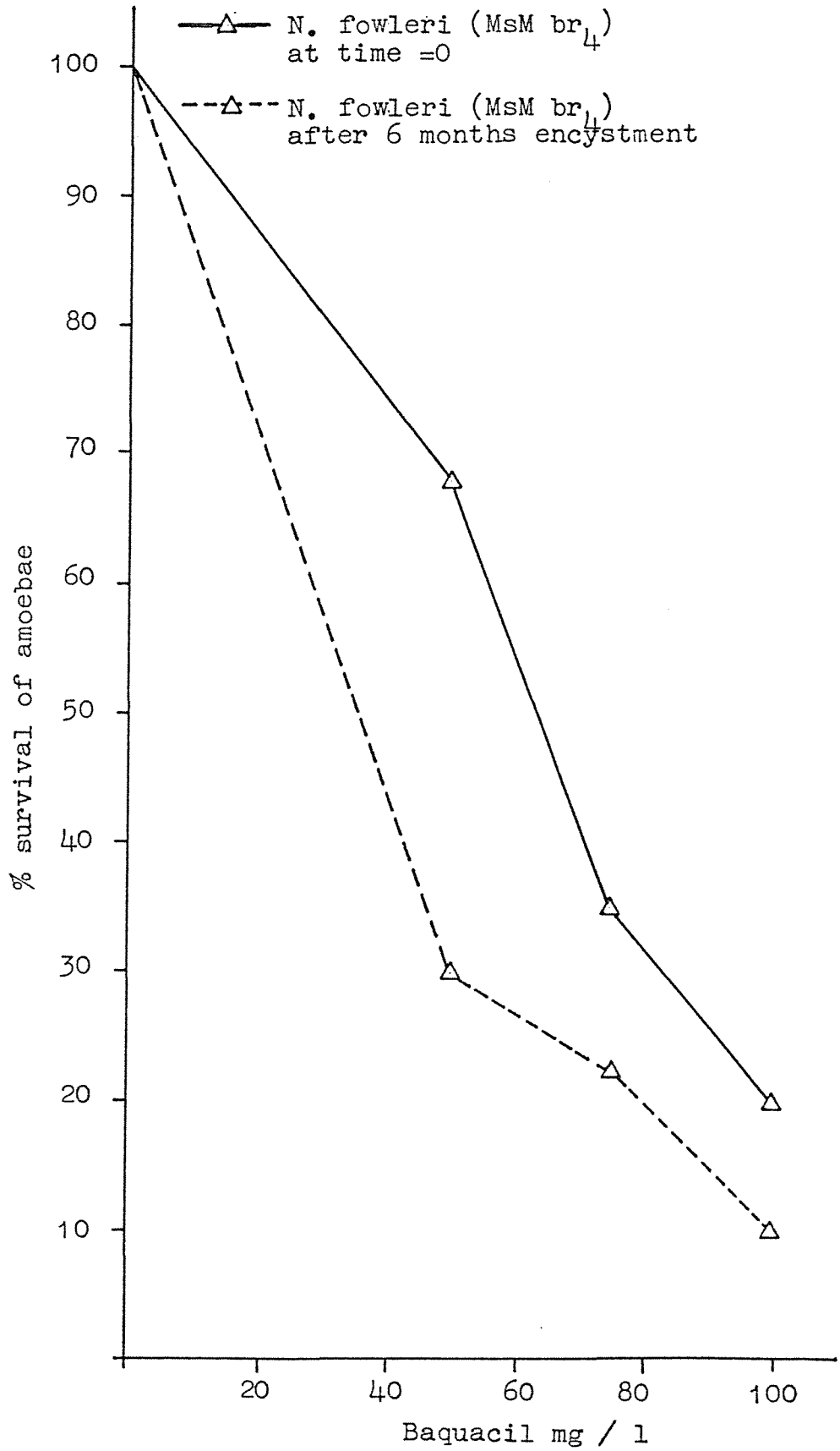


FIG.81

The effect of Baquacil on N. fowleri MsMbr$_4$ after 6 months encystment.

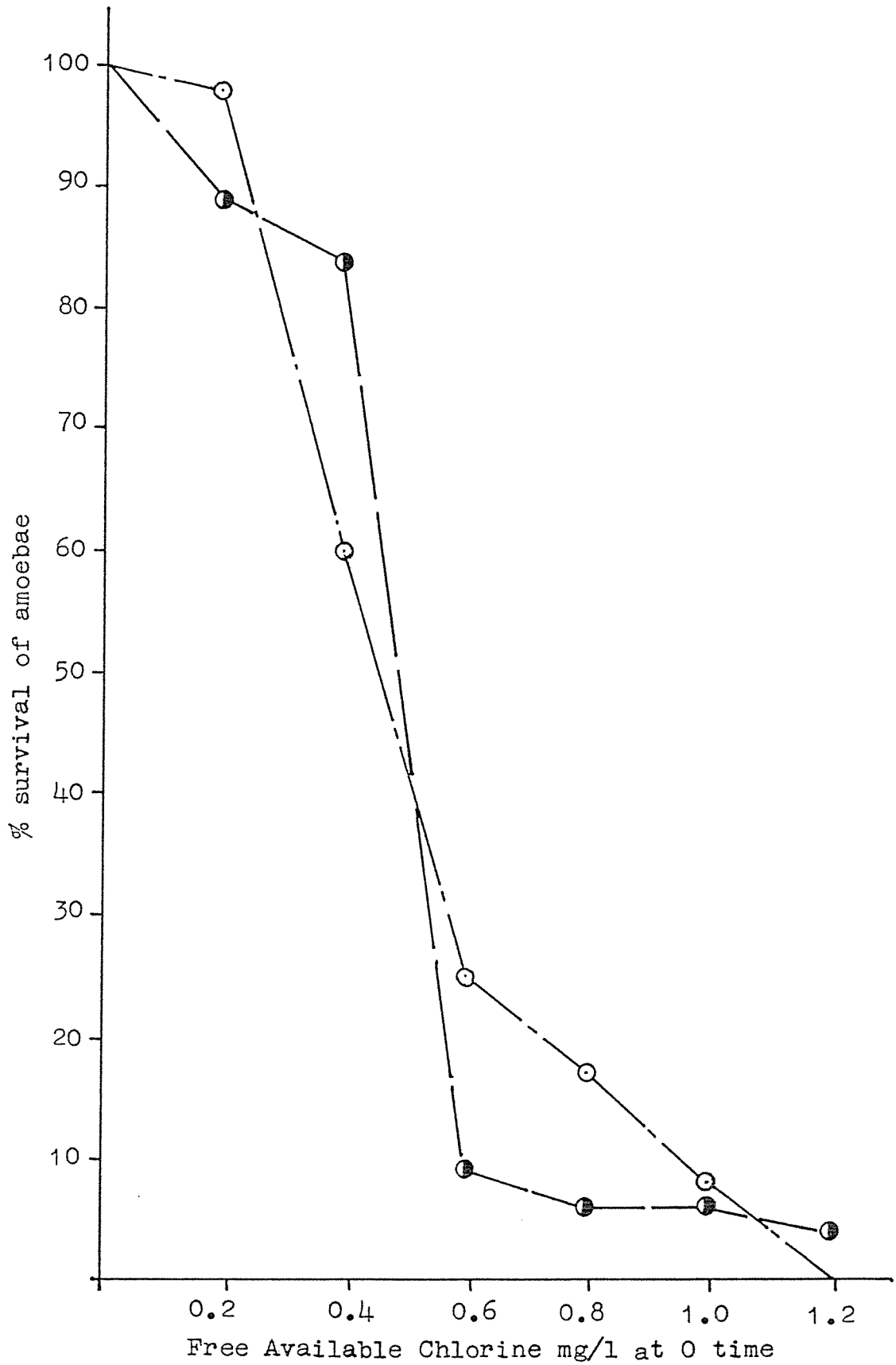


FIG. 82

The effect of chlorine on Baquacil resistant and Baquacil sensitive strains of N. fowleri MsM for 30 minutes at 37°C.

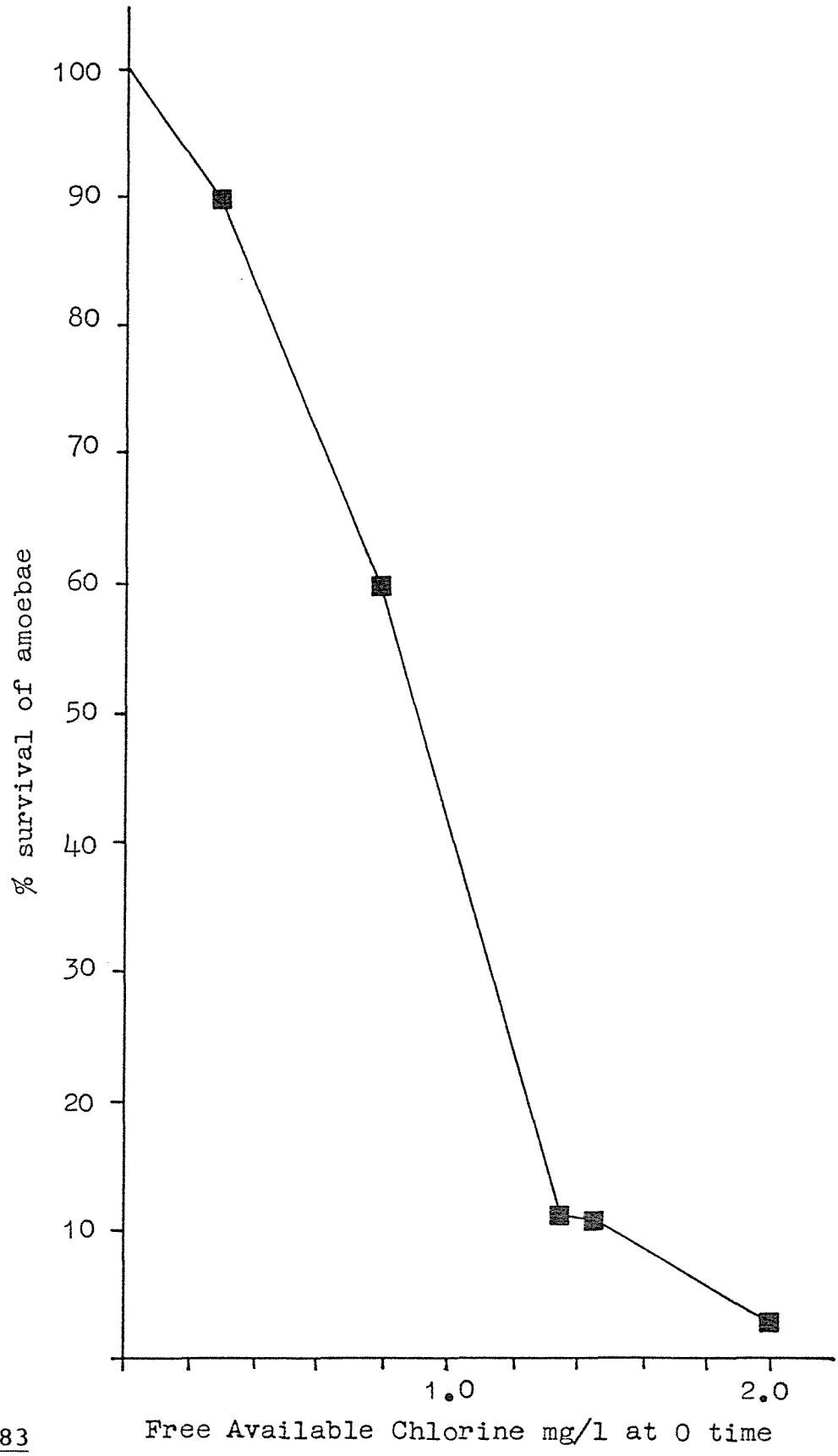


FIG.83

The effect of chlorine on A. culbertsoni (AI) Trophozoites for 30 minutes at 37°C.

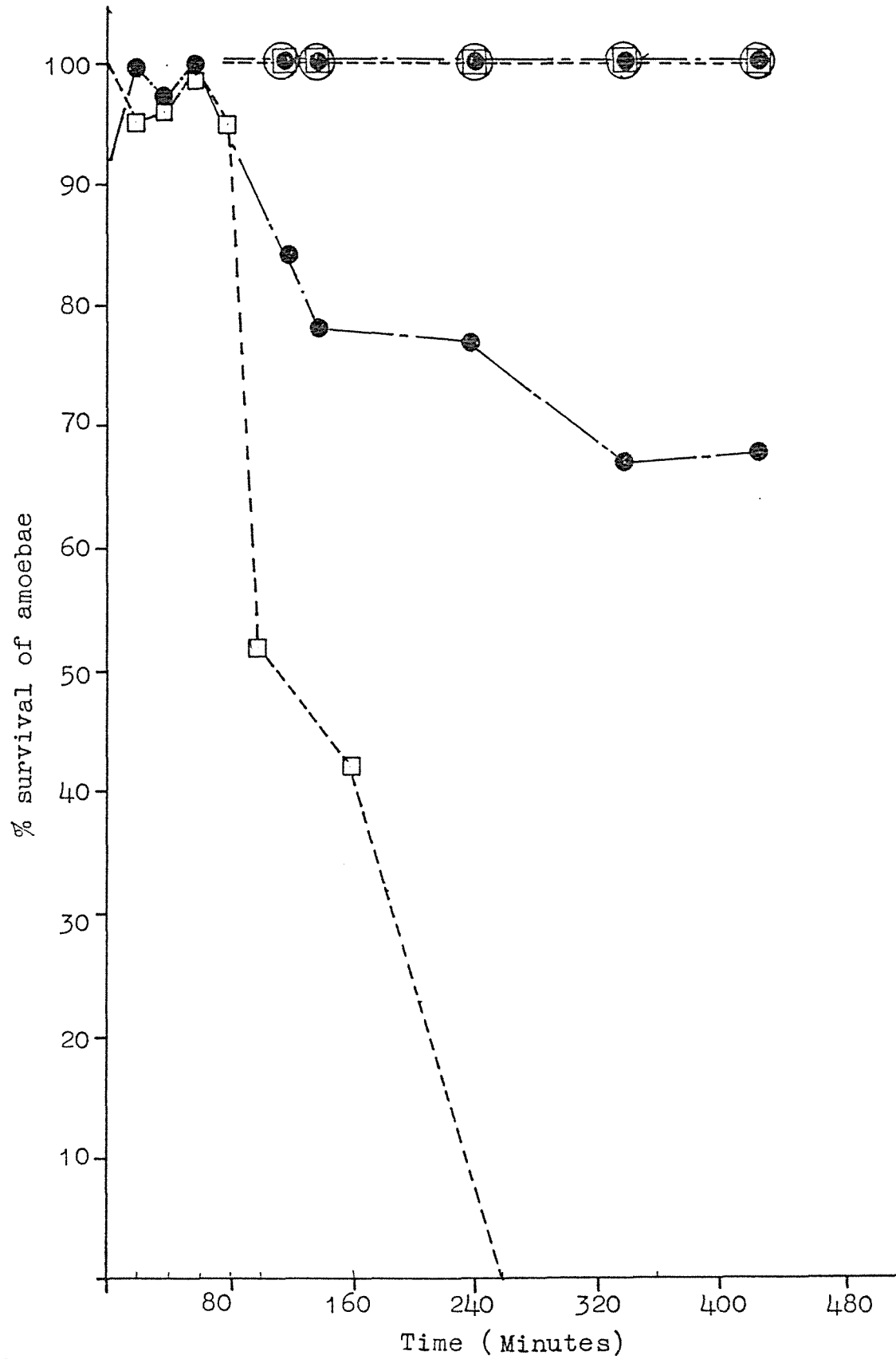


FIG. 84

Chlorination of A. culbertsoni (A1) and A. castellanii (O1) cysts with a free available chlorine level of 2mg l^{-1} for 6 hours.

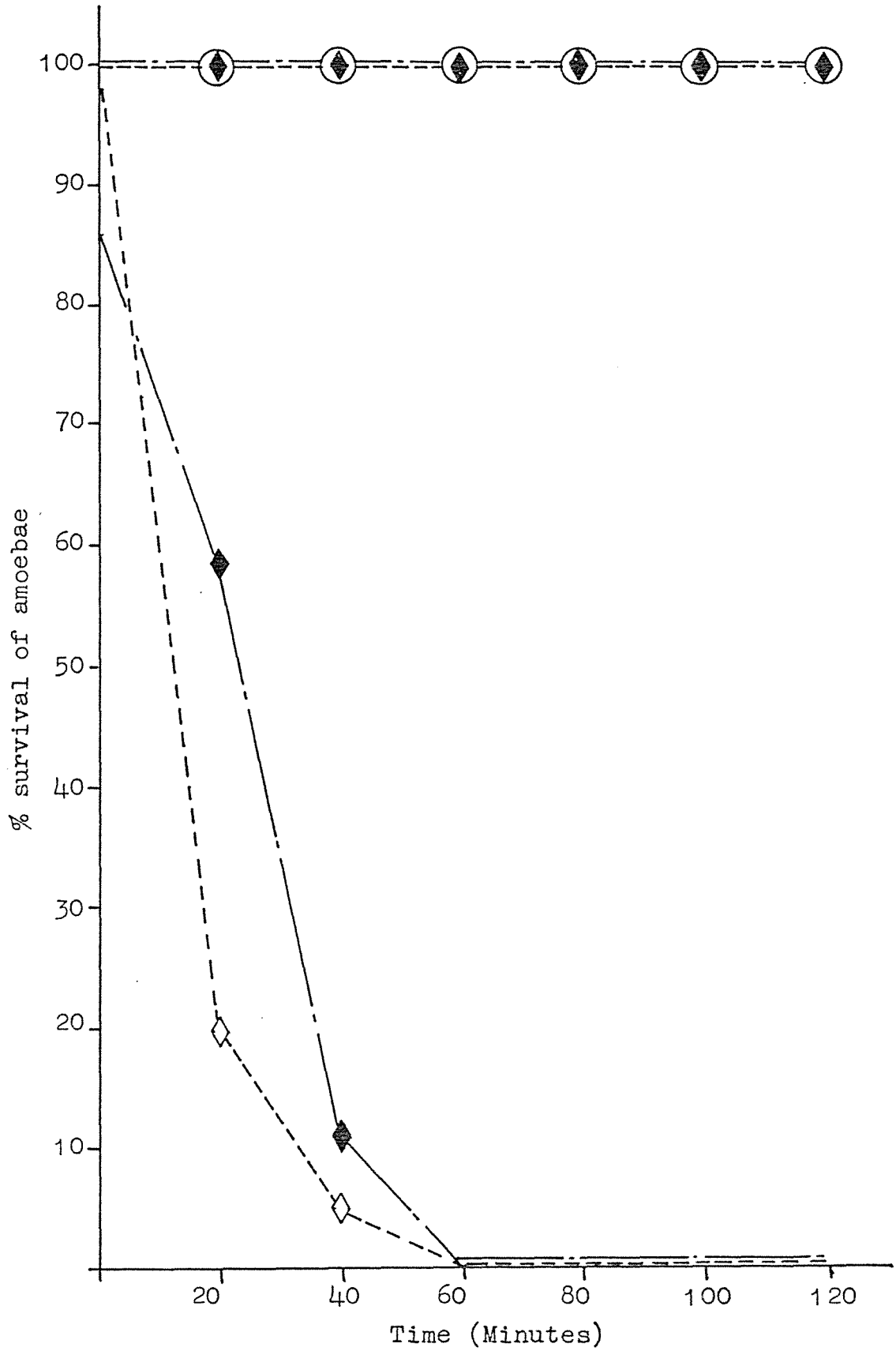


FIG.85

Chlorination of N. gruberi (PL) and N. fowleri (NHI) cysts with a free available chlorine level of 2 mg l^{-1} for 2 hours.

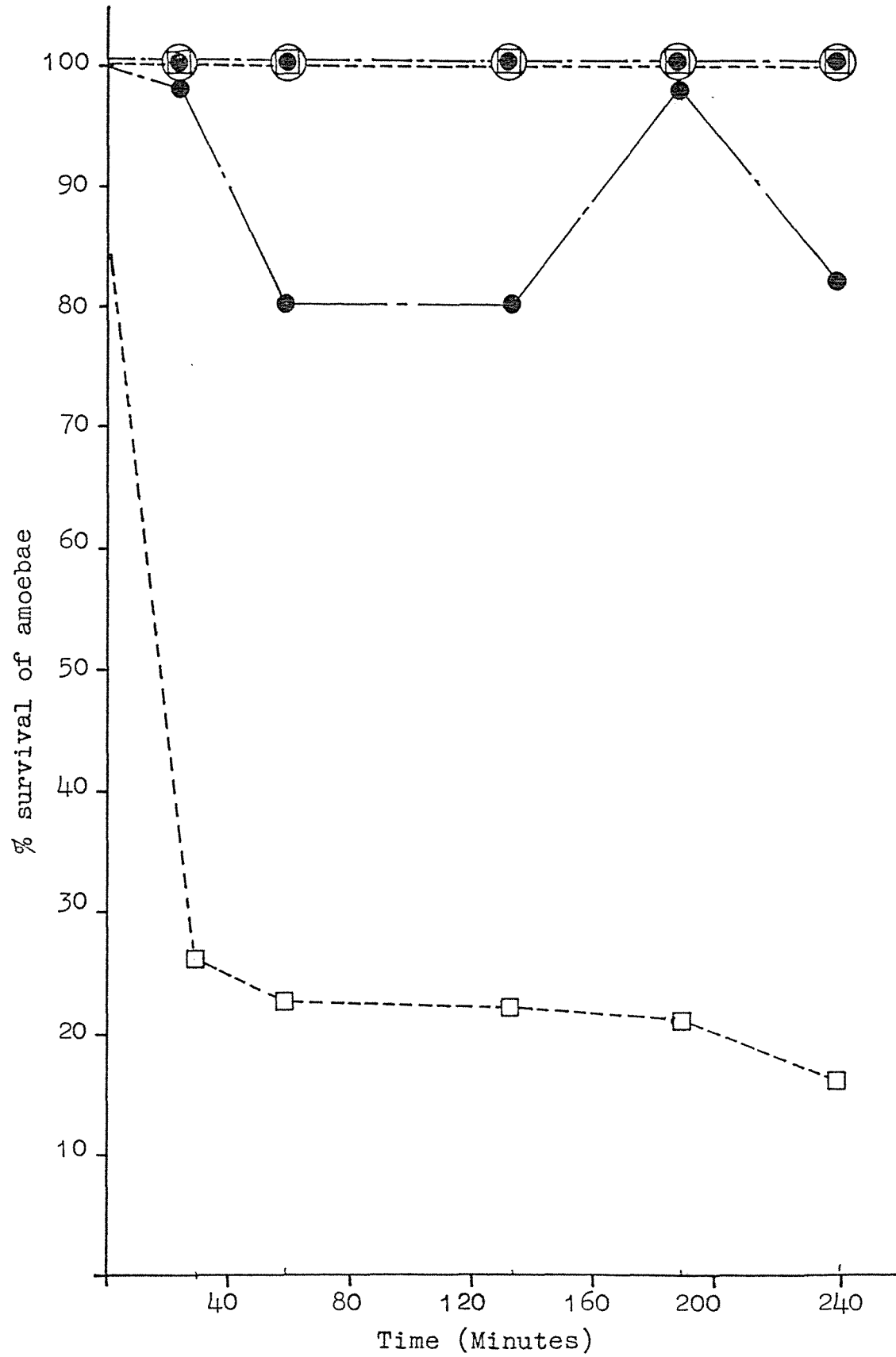


FIG. 86

Disinfection of A. culbertsoni (A1) and A. castellanii (O1) cysts in 50 mg l^{-1} baquacil for 4 hours

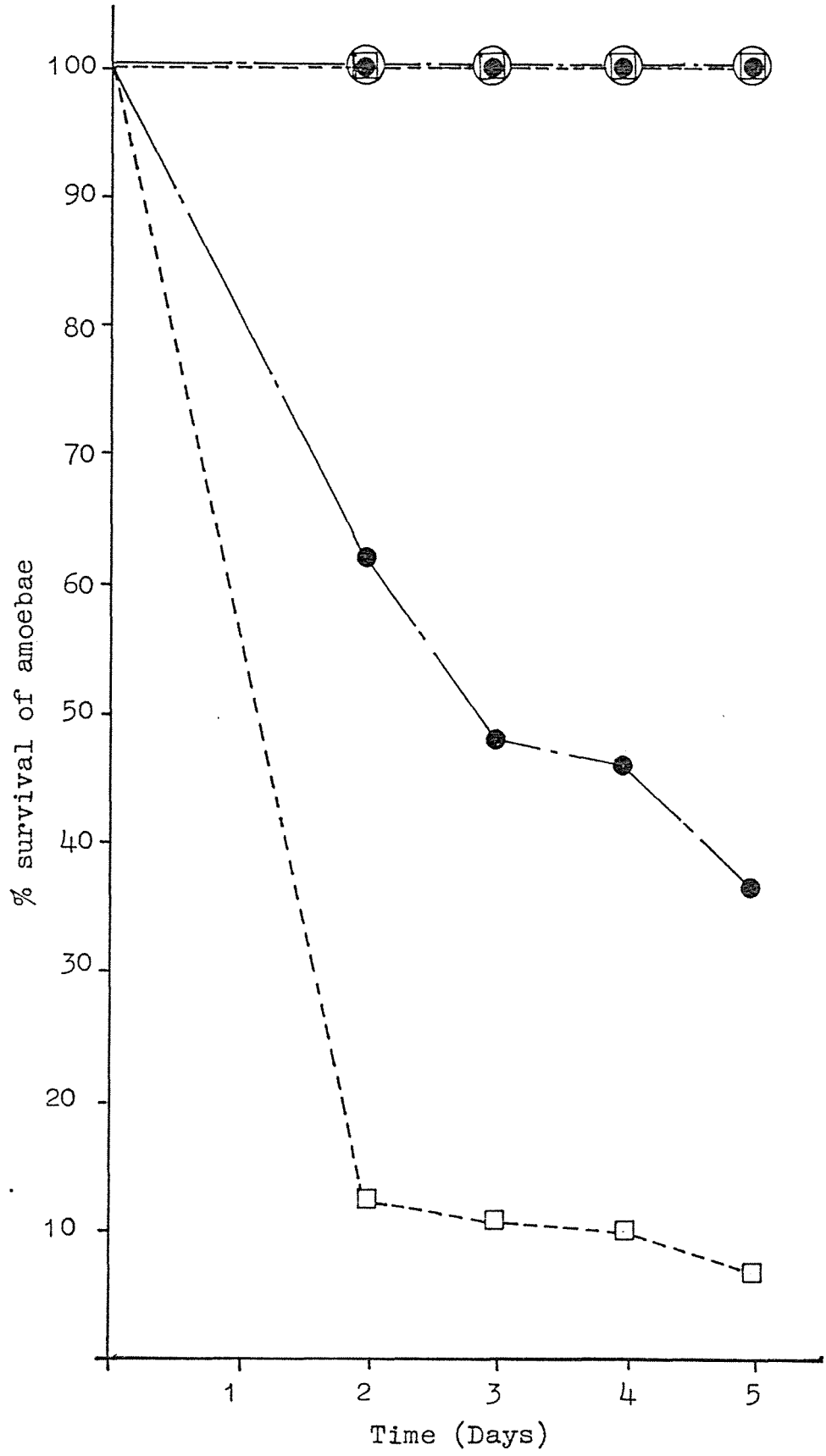


FIG.87

Disinfection of A. culbertsoni (A1) and A. castellanii cysts in 50 mg l^{-1} Baquacil for 5 days.

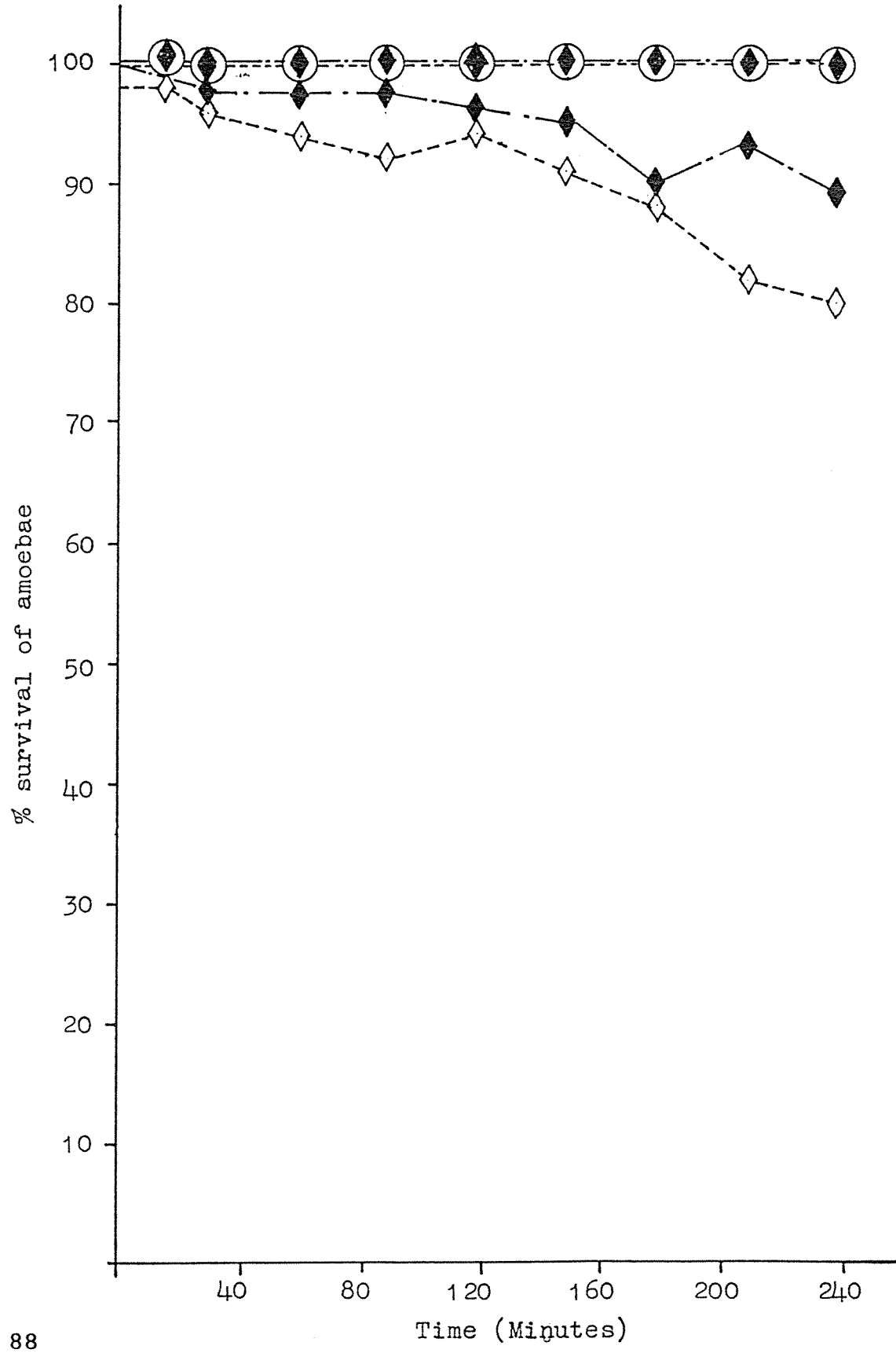


FIG. 88
Disinfection of N. fowleri (NHI) and N. gruberi (PL) cysts in 50 mg l^{-1} Baquacil for 4 hours.

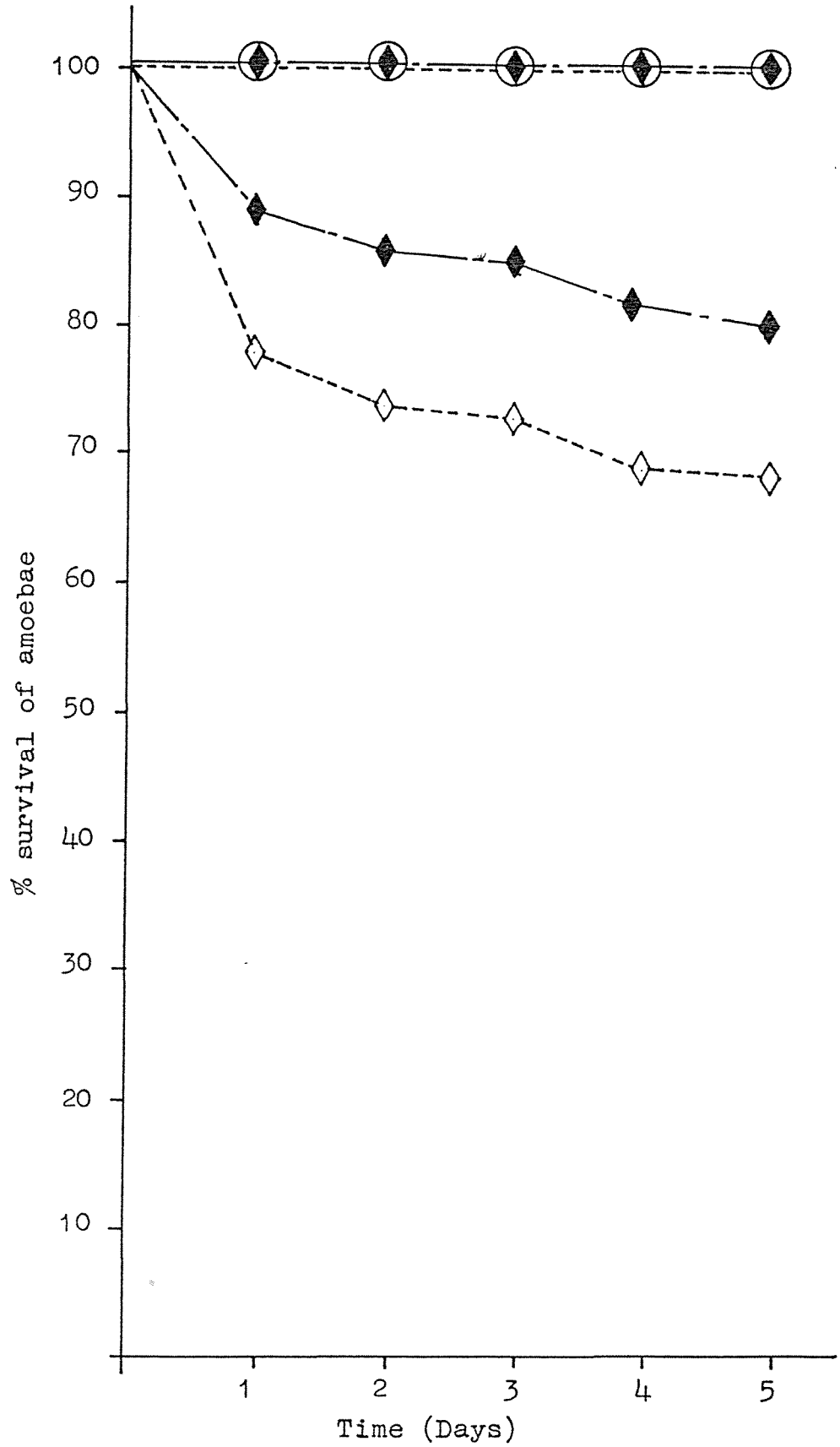


FIG. 89

Disinfection of N. fowleri (NHI) and N. gruberi (PL) cysts in 50 mg l^{-1} Baquacil for 5 days.

5 DISCUSSION

5.1 Encystment of Amoebae in General:

In the laboratory life cycle of Naegleria, amoebae can remain as amoebae (trophozoites), they can encyst, or they can flagellate. Both encystment and flagellation are cellular differentiations (Fulton, 1972; Fulton, 1977; Fulton & Walsh, 1980). The process of encystment has barely been studied in Naegleria (Chiovetti, 1976), even though this process should be as easy to study experimentally as it has been in other small amoebae, such as Acanthamoeba (Neff et al, 1964; Bowers & Korn, 1969; Neff and Neff, 1972; Chagla & Griffiths, 1974; Weisman, 1976; Byers, et al 1980; Srivastava and Shukla, 1983).

The research presented and discussed is aimed to examine a wide range of parameters as potential stimuli for encystment of pathogenic free living amoebae (P,F,L,A.)

The Cell differentiation process most widely examined in N. gruberi is transformation of trophozoites to amoebae flagellates, opposed to encystment (Fulton, 1972; Fulton, 1977, Fulton & Walsh 1980) With the exception of John's work (1982) there have been few extensive studies of cell differentiation of N. fowleri.

The flagellates of Naegleria sp. usually have two anterior flagella, of equal length, an elongate, pear shaped body and a nucleus in the narrower anterior region (Page 1976; Fulton, 1972; Fulton, 1977).

Trophozoites of Naegleria are long and slender (8-15um) and have eruptive flowing movement via pseudopodium (Page, 1976; Fulton, 1977).

Naegleria cysts are spherical, often clumped closely together, and 7-10 um in diameter (Cater, 1970). N. fowleri cysts differ morphologically from N. gruberi, they have thinner walls and pores are not visible with the light microscope (Page, 1976; Schuster, 1975). At the ultrastructural level pores can be observed in both species of Naegleria cysts (Schuster, 1975). Pores in N. gruberi are surrounded by a collar and sealed by a mucoid plug, there is no collar seen in N. fowleri, and the plug is smaller (Schuster, 1975).

In Acanthamoeba species as in Naegleria species the first morphological change evident of encystment is a sudden rounding up of amoebae, "roundform" (see p.34) beginning in Acanthamoeba 5-6 hours after induction (Neff et al, 1964). The next stage in encystment is the formation of a young cyst in which the wall can not be seen with a light microscope (Neff et al, 1964). In the mature cyst a two layered wall is formed which reaches a final thickness ranging from (1.5 - 2.0 um) (Neff et al 1964).

Bowers and Korn in 1969 described the ultastructure of encysting cells of A. castellanii. The cyst wall is composed of two major layers, a laminar, fibrous exocyst and an endocyst of fine fibrils in a granular matrix.

Early in encystment the Golgi complex is enlarged and contained a densely staining material that appears to contribute to the generation of the cyst wall. Vacuoles containing cytoplasmic debris are present in encysting cells and some contents are deposited in the cyst wall. The nucleus releases small buds into the cytoplasm, and the nucleolus decreases to less than half its original volume. The cytoplasm volume is reduced by 80% and the water expulsion vesicle is the only cellular compartment without dense content in the mature cyst (Bowers and Korn, 1969).

5.2 Encystment of Amoebae in a Liquid Broth Environment:

The most widely used method for encysting Naegleria sp is incubation on a minimal agar plate, (Schuster, 1963, 1975; Kaushal and Shukla, 1975; Chiovetti, 1978;Chiovetti and Bovee, 1982). A higher percentage of N. fowleri cells encysted on 100% soil extract agar see **Fig. 33**, 90% compared to the 100% soil extract broth **Fig.1** 30%.

In contrast encystment of Acanthamoeba sp has mainly been achieved using an encystment liquid media (Neff & Neff, 1972; Chagla & Griffiths, 1974; Weisman, 1976; Byers et al 1980; Srivastava & Shukla, 1983). Srivastave and Shukla (1983) found that an inorganic non-nutrient,

liquid medium containing Na_2SO_4 (86 mM) and $MgSO_4$ (15mM) promoted good encystment of A. culbertsoni. Chagla and Griffiths, (1974) developed an encystment media for A. castellanii, these workers found the addition of 50 mM $MgCl_2$ to a normal growth media resulted in a complete encystation of cultures.

Raizada and Krishna Murti (1971) obtained encystment of A. culbertsoni in non-nturient agar medium containing NaCl $MgCl_2$ and taurine (86:15: 20 mM), and a liquid medium containing these components has achieved encystment.

In soil extract broth trophozoites of N. fowleri were transforming to the roundform stage, but some factor required to promote a rounded trophozoite to a mature cyst was absent. Fulton (1972, 1977) when studying transformation of N. gruberi trophozoites to amoebae flagellates found that one component of the medium yeast extract, controls whether differentiation occurs. The fractionation of yeast extract yielded two components that control differentiation, electrolytes and an unidentified factor.

The unidentified factor, can prevent differentiation at a concentration that does not increase the osmotic pressure of the solution. An increase in osmotic pressure, a decrease in nurients in the presence of oxygen and divalent salts induced encystment of Hartmannella rysodes (renamed Acantamoeba rysodes) (Coliss and Esser, 1974).

Metabolic inhibitors are widely used to interrupt various cellular processes and thereby help to elucidate the sequences of cellular events. (Neff & Neff, 1972). Puromycin is an inhibitor of transcription of messenger - RNA codes (Chiovetti and Bovee, 1982). When Bovee & Chiovetti (1982) exposed encysting amoebae N. gruberi to a concentration of puromycin 5×10^{-4}M no amoebae progressed in encystment beyond the rounded up stage. Puromycin had prevented the formation of enzyme systems required to metabolize and polymerize cellular materials into the structures of the cyst wall (Bovee and Chiovetti, 1982).

Inhibitor experiments suggest that differentiation in Naegleria involves not only a re-arrangement of pre-existing cell components but also a re-direction of cell metabolism to produce, in a programmed sequence, new RNA and protein molecules that are required for differentiation (Fulton, 1977). The stimuli required for the redirection of cell metabolism for complete encystment of N. fowleri cells was not present in 100% soil extract broth (see **Figs. 51-54**).

Neff and Neff (1972) found that the most effective inducers of differentiation in Acanthamoeba sp were inhibitors of DNA synthesis. These workers suggested certain deoxyribonucleosides may be corepressor molecules, which under adequate conditions of nutrition, inhibit differentiation. When these key molecules become depleted, cells accumulate in the stationary growth phase and differentiation may be induced (Neff and Neff 1972).

A low percentage nutrient soil extract agar 25% had a low percentage of N. fowleri cysts **(Fig. 23)** in comparison to the 25% soil extract broth **(fig. 5)**. These results support those of workers with Acanthamoeba sp who find a low nutrient liquid medium promotes good encystment (Neff and Neff, 1972; Chagla & Griffiths 1974; Weisman 1976; Byers et al 1980; Srivastava and Shukla,1983).

It is possible for Naegleria to encyst in a liquid or a solid surface environment, this factor alone does not govern differentiation of the cells (Fulton, 1972; Fulton, 1977; Fulton and Walsh, 1980). Parameters such as cell crowding and nutrient availability are suggested as a greater stimuli for encystment of amoebae (Griffiths and Hughes, 1968, 1969 Neff and Neff, 1972; Lasman and Shafran, 1978; Chiovetti and Bovee, 1982; Srivastava and Shukla, 1983).

5.3 Encystment of Amoebae on solid Nutrient surfaces with and without the presence of bacteria:

Protozoa are a major component of the soil community, and the major predators of certain soil bacteria (Barrett and Alexander, 1977).

Normal laboratory methods for encystment of Naegleria sp is, the trophozoites inoculated and incubated on a minimal nutrient agar with a bacterial species present (Singh, et al, 1964; Schuster, 1975; Jehan and Dutta, 1975; Chiovetti and Bovee, 1982).

Experiments using soil extract broth inoculated with the bacterial species E. cloacae proved that the bacterial growth was too rapid in comparison to the amoebae. Growth of amoebae in nutrient liquid media containing bacteria has not proved very satisfactory, since usually the by-products of bacterial growth inhibit amoebic growth (Fulton, 1977).

Hence soil extract agar gave preferable experimental conditions to the broth.

The results indicate that the encystment of N. fowleri does occur on PASB agar, with or without the presence of E. cloacae (**Figs. 9-12**). The number of cells encysting on PASB agar does not vary greatly with or without the presence of E. cloacae.

Results with the soil extract agars, (see **Figs. 17-36**) as with the PASB agars showed that cell ratios of trophozoites, roundforms and cysts did not vary greatly with or without the presence of E. cloacae.

The significance of bacteria in promoting differentiation of amoebae is most likely in the transformation of mature cysts to trophozoites i.e. excystment (Crump, 1950; Singh, et al, 1964, 1970; Jehan and Dutta, 1975)

Singh et al, 2958, found aqueous extract made from bacteria and fungi to cause good excystment of Schizopyrenus russelli, a soil amoebae. Excystment activity was found to be due to amino acis.

A species of Aerobacter [the generic name Aerobacter has been formally rejected, and new names including Enterobacter and Klebsiella appear in the literature (Fulton, 1977)]growing in nutrient agar produces an excystment factor which readily induces excystment of S. russelli cysts (Singh et al, 1964).

Results of work performed by Crump 1950, suggests that the requirement for the presence of bacteria in encystment and excystment may be an amoebae species dependent related factor. Crump (1950) found cysts of two soil amoebae were tested for the effects of the presence or absence of bacteria upon excystment. Excystment in one species was independent of the presence of bacteria, the other species was more sensitive and could not excyst without living bacteria.

5.4 Effect of Cell Concentrations and Nutrient Availability on Encystment of Amoebae:

When trophozoites of N. gruberi were inoculated into 100% soil extract broth (See **Figs. 37-40**) at concentrations of 1×10^6 cells ml^{-1} and 1×10^5 cells ml^{-1}, there was no observed encystment of these cells. These results agree with those of Chagla & Griffiths (1974), who found it was not possible to obtain complete encystment of A. castellanii in growth medium at the end of logarithmic growth when nutrients are limited and encystment should be promoted. A. culbertsoni has been reported as failing to undergo encystment in nutrient-rich media (Srivastava and Shukla, 1983).

In contrast to the results using N. gruberi, and those of Chagla & Griffiths (1974); and Srivastava & Shukla (1983), trophozoites of N. fowleri encysted in 100% soil extract broth (**fig. 49**) at a high cell concentration

of 1 x 10^6 trophozoites N. fowleri ml^{-1}.

In 100% soil extract broth inoculated with lower concentrations of N. fowleri (see **Figs. 51-54** inclusive) at 1 x 10^5 trophozoites ml^{-1} and 1 x 10^4 trophozoites ml^{-1} there was no encystment of N. fowleri.

It is possible that there is a requirement for some form of cell to cell communications for the transformation of roundforms to cysts (Chiovetti and Bovee, 1982). Hence the density of the cells is a critical factor for induction of encystation. Byers et al (1980), found in Acanthamoeba sp, A. castellanii, A. rhysodes, A.palestinensis, and A. astronyxis the rate of encystment varied with cell density at the time of starvation and was optimal at initial densities of 400-800 amoebae/mm^2.

The form of inter-cell communication could be via a chemical encystment inducing factor. Byers et al (1980) reported a factor enhancing initiation of encystment of Acanthamoeba sp was released into growth media following glucose-acetate starvation.

The chemical form of inter-cell communication theory is not supported by the results of work by Chiovetti & Bovee (1982). Working with N. gruberi, they found that a filtrate from a maximally encysting population of amoebae, had no effect on the rate of encystment of other populations. Neff et al (1964) concluded with Acanthamoeba sp encystment was not dependent upon the presence of other cells.

Klein (1959) found aerated mass cultures of Acanthamoeba in a liquid medium reached a population density of 3-4 x 10^6 cells ml^{-1}. The cells remained at this plateau for a period of 48-36 hours and then began to encyst.

As an alternative theory, to a communication between cells by some chemical factor, Chiovetti and Bovee (1982) have suggested the role of cell surface contact sites

as a form of communication for encystment induction. Initiator sites of N. gruberi were assumed to be terminators or side chains of protein molecules. Trypsin breaks peptide bonds, exposure of starving and crowded cells of N. gruberi to trypsin inhibited encystment of the amoebae (Chiovetti & Bovee, 1982).

The different results obtained with high cell concentrations of N. fowleri and N. gruberi (1×10^6 trophozoites ml^{-1}) inducing encystment in soil-extract broth may be due to different induction requirements of each species (Crump 1950, Griffiths & Hughes, 1968, Lasman & Shafran, 1978). Griffiths & Hughes (1968) found that encystment media developed for Acanthamoeba sp and H. rhysodes were unsatisfactory for the induction of encystment in H. castellanii. Lasman & Shafran (1978) concluded with Acanthamoeba sp one reason for the difference in nutrient requirements for encystment maybe due to species specificity.

Trophozoites of N. fowleri inoculated into an axenic liquid growth media CYM (**Figs. 42,43,45, and 47**) reached a maximal cell density after which, the cells rounded up and no encystment of N. fowleri trophozoites was observed.

In the growth media there has been a depletion of ions and energy source plus a build up of metabolic waste. Chagla and Griffiths (1974) suggest that encystation could be a consequence of the inhibition of cell division when nutrient concentrations are limiting growth. In the axenic liquid media environment the increase in cell density and depleting nutrients have stimulated N. fowleri to pre-encyst, (i.e. the formation of an immature cyst by the rounding up of a trophozoite). The second phase to transform into a mature cyst did not commence. The induction of encystment may require a two fold stimulus system. Trophozoites receive one stimulus and pre-encyst, then a second stimulus is required for pre-encystment forms to transform to mature cysts.

An example of an initial stimulus as illustrated from the results is cell crowding and nutrient depletion (Neff et al, 1964; Griffiths & Hughes, 1968; Chagla & Griffiths, 1974; Byers et al 1980; Chiovetti and Bovee, 1982).

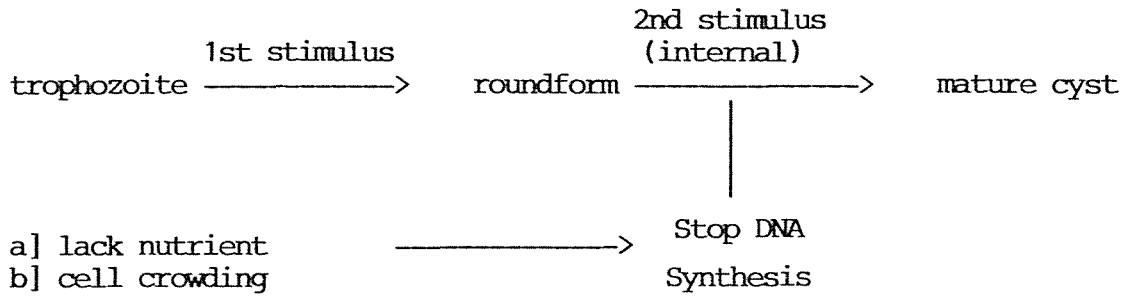
The second stimulus is required to trigger the formation of enzyme systems, which will transform the cell into a mature cyst (Neff & Neff, 1972; Weisman, 1976; Chiovetti and Bovee, 1982).

Raizada and Muriti (1971) found the induction of encystment in A. culbertsoni was accomplished by a 3-4 fold increase in the synthetic rate of cAMP, as judged by the incorporation of 8- (^{14}C) adenine into cellular cAMP.

The mechanism for cAMP action in encystment is not known (Weisman, 1976) cAMP has been found to induce good encystment of A. culbertsoni in solid as well as fluid media (Srivastava and Shukla, 1983) supporting the suggested intermediary role of cAMP in encystation. Generally cAMP acts via the protein kinase - mediated modification of enzymes and proteins and may influence transcriptional, translational and post-translational processes.

Neff and Neff (1972) conclude that with Acanthamoeba the failure to initiate or complete synthesis of its DNA may cause a vegetative cell to abandon its repeated cycle of growth and division and to initiate a new sequence of reactions leading to a differentiated state. It is likely that more than one transition point occurs in the cell cycle, when initiation of encystment occurs. When nuclear division and cell division take place are the most likely times (Chagla and Griffiths, 1974).

It is possible to diagramatically show the stages of encystment:



Chagla and Griffiths (1974) expressed encystment of A. castellanii as a two-part system in which there was a stage of initiation, followed by the stage of commitment. Neff et al (1964) concluded with Acanthamoeba sp there are two events in encystment (1) an inductive period followed by (2) a period of differentiation where specific new macromolocules are synthesized.

In a neutral pH, non-nutrient environment (phosphate buffer), trophozoites of N. fowleri did not encyst at any cell concentration (**Figs. 57-60**). An exogenous energy supply may be required by the cell for full encystment to eventuate. Chiovetti and Bovee (1982) found the induction of encystment of N. gruberi is not an energy requiring step. Subsequent energy requirements for cyst formation come from internal cell resources (Byers et al, 1977). During encystment there is a decrease in cellular contents of DNA, RNA, protein, lipid and glycogen, and an increase in cellulose (Potter and Weisman, 1970; Byers et al, 1977). Enzyme induction controls cellulose synthesis (Weisman, 1976). This is dependent on the occurence of both RNA and protein synthesis 8-14 hours from initiation of encystment, for the formation of cyst specific RNA and proteins required to transform the round form to the mature cyst, (Weisman, 1976).

5.5 The Effect of Temperature on the Encystment of Amoebae:

5.5.1 The Effects of Absolute Temperature on Encystment of Amoebae:

Experimental work performed on the encystment of amoebae

is usally at the optimum growth temperature for the organisms in question e.g.:

a] A. castellanii at 30°C (Griffiths and Hughes, 1968; Bowers and Korn, 1969; Neff and Neff, 1972; Chagla & Griffiths, 1974; Weisman, 1976; Martin and Byers, 1977; Byers et al, 1980).

b] A. culbertsoni at 37°C (Neff et al, 1964; Srivastava and Shukla, 1983).

c] N. gruberi at 30°C (Singh et al, 1964, 1970; Chiovetti, 1978; Chiovetti and Bovee, 1982).

d] N. fowleri at 37°C (Shuster, 1963, 1975).

A wide range of temperatures 4°C to 44°C were used for investigating the effects of an incubation temperature, other than the optimum growth temperature, on the encystment of amoebae.

The minimum temperature at which cells of N. fowleri encysted was 25°C, (**Fig. 63**) there was no encystment of N. fowleri cells incubated at temperatures below 25°C. Encystment of N. fowleri cells occured at 25°C, 30°C, (**Figs. 63-66**) and 37°C (**Fig.67**). No encystment was recorded at 44°C. This is an interesting observation as N. fowleri can tolerate temperatures above 37°C, to a maximum of 45°C (Griffin, 1972). The highest levels of encystment were recorded with cells incubated at the temperatures 30°C and 25°C which are just below the optimum growth temperature for N. fowleri, Trophozoites of the non pathogen N. gruberi encysted at 15°C and 25°C (**Figs. 79 & 80**).

There was no encystment recorded at 4°C, 30°C, 37°C and 44°C. Encystment did not occur at the normal growth temperature of N. gruberi, cells of N. gruberi were only seen to be in the pre-encystment roundform phase. These results do not agree with those of Chiovetti and Bovee (1982) who recorded encystment of N. gruberi when

incubated at 33°C. The difference in results may be accounted for by the use of two different types of plate media. Protease-petone-yeast-glucose (PYG) agar with Enterobacter aerogenes was used by Chiovetti and Bovee (1982). In this work N. gruberi was incubated on 10% soil extract agar plates with the absence of a bacterial species.

The trophozoites of A. culbertsoni encysted at 4°C, 15°C, 25°C, 30°C and 37°C (**Figs. 71-75**), and no encystment was recorded at 44°C. Srivastava and Shukla (1983) reported optimum encystment of A.culbertsoni was obtained using a standard encystment medium at pH 8.5-9.0 at 28°C ± 20°C with aeration by shaking.

Encystment of A. castellanii trophozoites was observed at 4°C, 15°C, 25°C, 30°C, and 37°C (**Figs. 68-73**). No encystment was recorded at 44°C. The greatest percentage of cells that had encysted was recorded at the cell population incubated at 25°C. This was the closest temperature cells need,to the optimum growth temperature for A. castellanii. Neff et al, (1964) working on the induction of differentiation in Acanthamoeba sp observed all phases of encystment are most rapid in the 30-32°C range. The upper temperature for encystment is 40°C, with the rounding up of cells and no completion of encystment at 42°C.

5.5.2 The Effects of Temperature fluctuation on Encystment of Amoebae:

A sudden change in temperature from the otpimal growth temperature can be used to induce differentiation of cells (Fulton, 1972, 1977; Srivastava and Shukla, 1983). The effects of fluctuation of environmental temperatures with regard to encystment of amoebae has not been widely studied. It has been demonstrated that fluctuating temperatures are detrimental to N. fowleri cysts, (Wellings, 1979) but no information submitted on the effect of fluctuating temperatures and encysting cells. A change in environmental temperatures is one

of many signals warning the cell of adverse conditions (Crump 1950), and thus stimulating encystment as a mechanism of protection.

Fulton (1972, 1977) studying differentiation of N. gruberi trophozoites to flagellates found that the process could be initiated simply by lowering the temperature of cultures by 30°C. Dingle (1979) used temperature shock to induce to flagellation of N. gruberi trophozoites. The maximum number of cells transform in the population when N. gruberi was temperature shocked at 38.2° $\pm$ 0.1°C and then returned to 19-22°C (Dingle, 1979)

A decline in temperatures within the viable temperature functioning range of N. fowleri of the cells will stimulate cells to encyst indicated by **Figs. 63-66**) The cells of Acanthamoeba show a wider range of temperature tolerance in comparison with those species of Naegleria.

A variance in temperatures higher and lower than the optimum growth temperature in Acanthamoeba (Neff et al 1964) also lowering the incubation temperature of A. culbertsoni from 28°C to 19°C was observed to have no favourable effect on encystment.

The slight variation of environmental temperature may indicate to the cell a change of seasonality, and induce encystment (Corliss and Esser, 1974). Seasonality of cyst production has been reported in some planktonic species of protozoa. The testate rhizopod Difflugia limnetica has been reported to have a cycle in which free swimming organisms sink to the bottom and encyst for winter at the end of summer. This is an area in which study on encystment and seasonality of amoebae can be furthered. Hibernation of Naegleria sp in lake sediments during winter has been shown to occur in Florida lakes (Wellings, 1979).

Enzymes controlling metabolic pathway required during encystment may have peak enzyme activity slightly above

or below the normal functioning cell temperature. The variance in the environmental temperature causes activation of enzymes. Encystment is consequently initiated as a result of metabolic activity within the cell. (Neff et al 1964, Weisman 1976, Fulton, 1972, 1977).

5.6 Disinfection of Amoebae:

In the last century chlorination has become the most widely used method of treating potable water supplies. Proper treatment by clarification and disinfection has has all but eliminated waterborne bacterial and viral pathogens. (White 1972). Pathogenic free-living amoebae (PFLA) have been, and continue to be isolated from chlorinated domestic and swimming waters, (Anderson and Jamieson, 1972; De Jonckheere & Van de Voorde, 1976; Lyons and Kapur, 1977; Dive et al 1978; Derr-Harf et al; Grillot Thomas-Ambroise, 1980; Walters et al, 1981; Esterman et al, 1984). Disinfection of amoeba via chlorination has been researched (De Jonckheere & Van de Voorde, 1976; Robinson 1978; Cursons et al, 1980; Walters et al 1981; Rubin et al 1983) also alternative methods for the disinfection of amoebae such as chlorine diozide (Cursons et al, 1980; Sproul et al, 1983); chlorinated cyanurates (Engel et al, 1983), Deciquam 222 and ozone (Cursons et al, 1980) and Baquacil (Dawson et al, 1983, 1984) have all been examined.

Baquacil is a 20% solution of polyhexamethylene biguanide hydrochloride (PHMB) which is recommended to be maintained at 50mg l^{-1} in swimming pools (Alford, pers. comm). Dawson et al, 1983 [a] & [b], 1984) found that species of amoeba had some disinfectant resistance towards the amoebicidal capacity of Baquacil.

When isolated baquacil resistant strains of N. fowleri were retested for disinfection by baquacil, strains $MsMbr_2$ and $MsMbr_4$ both showed higher percentages of survival, than other tested strains (see **Fig. 80**).

Increasing the concentration of Baquacil, decreased the survival rates of Baquacil resistant strains (**Fig.80**) but was not completely amoebicidal. These results are supported by Dawson et al, (1984).

Baquacil Resistant strains of N. fowleri cells were encysted, stored as cysts for a period of 6 months and excysted then tested for baquacil disinfection (See **Fig. 81**) There was still a level of Baquacil resistance expressed. The initial site of action of a disinfectant in amoebae is the cell membrane which is followed by a disruption to the function of the cell organelles. (White, 1972). During encystment and excystment there is a synthesis of new cell protein via expression of different codings on mRNA (Weisman 1976). It is possible that the resistance of N. fowleri strains $MsMbr_2$ and $MsMbr_4$ is caused by a mutation in the cell membrane, such that the entry of the active ingredient in Baquacil (PHMB) can not occur. The resistence of these N. fowleri strains is specific for Baquacil, and not disinfection in general, as these amoebae were sensitive to disinfection by chlorine when tested (**fig. 82**). Ions can bind to receptors on the cell membrane and then transfer into the internal cell (Schuster 1979). The active ingredient PHMB in Baquacil may bind falsely to a receptor replacing the passage of a required ion in the internal cell. A mutation of the receptor at the cell membrane, may prevent the entry of PHMB into the cell.

Disinfection of amoebic cysts showed Baquacil did not have cysticidal properties at the recommended level of 50 mg l^{-1} (**Figs. 86-89**). Amoebic cysts have a thick protective wall (Bowers and Korn, 1969; Schuster, 1975), which may serve as some form of mechanical protection against disinfection, as generally amoebic cysts require greater levels of disinfection for inactivation than trophozoites (De Jonckheere and Van de Voorde, 1976; Rubin et al, 1983).

In aqueous environments, uncombined chlorine, in the form of unionized hypochlorous acid (HOC1), is an extremely potent bactericidal and virucidal agent, even at concentrations of less than $0.1 mgl^{-1}$ (Ridgway and Olson, 1982). Chlorination involves breakpoint chlorination to establish and maintain an acceptable residual concentration,(free available chlorine see **Fig. 79**) for disinfection (White, 1972). In New Zealand the desired FAC level is 0.1 - 0.2 mg l^{-1} for potable water and from 0.5 - 0.8 mg l^{-1} for recreational waters (Cursons et al, 1980).

Trophozoites of N. fowleri received adequate disinfection when exposed to chlorine levels of 0.6 mg l^{-1} and greater. (See **Fig. 82**). These results are supported by Derreamaux et al (1974), Robinson, (1978); Cursons et al(1980) and Walters et al(1981). Trophozoites of Naegleria had a greater susceptibility to chlorine in comparison to trophozoites of Acanthamoeba species tested (**Fig. 83**). At a level of 1.2 mg l^{-1} and greater chlorine was amoebicidal to trophozoites of N. fowleri, trophozoites of Acanthamoeba had a 10% survival rate after disinfection. Cysts of Naegleria sp showed greater sensitivity to chlorination compared to Acanthamoeba (**Figs. 84 & 85**). De Jonckheere & Van de Voorde (1976) found that N. fowleri is more sensitive to chlorine than N. gruberi, and two Acanthamoeba strains are very resistant. They found both strains are insensitive to chlorine concentrations in domestic supplies and swimming pools, a free chlorine concentration of 4 mgl^{-1} does not destroy Acanthamoeba cysts of 3 hours contact time. Chang (1978) reported a chlorine residual of 2.5 mg l^{-1} is required for a 99.9% inactivation of cysts of N. fowleri in 10 minutes at pH 7.25. To achieve a 99.5% inactivation of N. gruberi cysts at a pH of 7 in 10 minutes required only 1.2 ml l^{-1} chlorine (Rubin et al 1983). These reported results are not totally supportive of each other (De Jonckheere & Van De Voorde, 1976; Chang , 1978; Rubin, et al 1983).

A comparison of the effectiveness of different disinfectants on the inactivation of amoebae was made by Cursons et al, 1980. The action of chlorine, chlorine dioxide, ozone and deciquam 222 (de-decyldimethyl-amonium bromide) were examined for amoebicidal properites. Of the four disinfectants examined deciquam 222 was the most effective amoebicide, next ranked chlorine, chlorine dioxide and ozone (Cursons et al,1980). Sproul et al (1983) found, in a comparison of the inactivation of N. gruberi cysts using chlorine and chlorine dioxide that chlorine dioxide was more effective in disinfection of cysts than chlorine. Chlorine dioxide required a 6.05 mg-min/L for a 99% inactivation and with free chlorine 12.1 mg-min/L was required (Sproul et al, 1983)

Engel et al (1983) conducted a study to examine the role of chlorinated cyanurates on the inactivation of N. gruberi cysts. Cyanuric acid (2,4,6 - trihydroxytri azine) is used as a chlorine stabilizer in swimming pools, but has been shown to inhibit the bacteriacidal effect of inhibiting free chlorine by the formation of chlorinated cyanurate species which have no measurable cysticidal effect.

BIBLIOGRAPHY:

ANDERSON, K. & JAMIESON, A. 1972
Primary Amoebic Meningoencephalitis.
Lancet 2: 902-03

APLEY J., CLARKE S.K.R., ROOME, A.P.C.H., SANDRY S.A., SAYGI G., SILK B., & WARHURST D.C. 1970.
PAM in Britian
Br. Med. J. 1:596-9.

BARRETT R.A., & ALEXANDER M. 1977.
Resistance of cysts of amoebae to microbial decomposition.
App. & Environ. Microbiol. 33: 670-674

BHAGWANDEEN, S.G., CARTER R.F., NAIK K.G., & LEVITT D. 1975.
A case of Hartmannellid Amoebic meningo-encephalitis in Zambia.
Am. J. Clin. Path,63: 483-492

BOWERS B. & KORN E.D. 1969.
The fine structure of A. castellani (Neff strain) II Encystment
J. Cell Bio 41: 786-805

BROWN T. 1977
Observations by light microscopy on the cytopathgenicity of N. fowleri in mouse embryo cell cultures.
J. Med. Micro 11: 249-59.

BROWN T. 1979 [a]
Observations by immunofluorescence microscopy and electron microscopy on the cytopathogenicity of N. fowleri in mouse embryo cell cultures.
J. Med. Micro 12:363-71.

BROWN T. 1979 [b]
Inhibition by amoeba specific antiserum and by Cytochalasin B of the cytopathogenicity of N. fowleri in mouse embryo cell cultures.
J. Med. Micro 12: 355-62.

BROWN T.J. & CURSONS R.T.M. 1977.
Pathogenic free-living amoebae (P.F.L.A.) from frozen swimming areas in Oslo, Norway.
Scand. J. Infect. Dis., 9:237-240.

BROWN T.J., CURSONS R.T.M. & KEYS E.A. 1982.
Amoebae from Antarctic soil and water.
App. & Environ. Microbiol 44:491-493.

BROWN T.J., CURSONS R.T.W., KEYS E.A., MARKS M., & MILES M. 1983
The occurence and distribution of Pathogenic free-living amoeba in thermal areas of the North Island of New Zealand.
N.Z. J. Marine & F.W. Res 17:59-69.

BUTT C.G. 1966
Primary Amoebic Encephalitis.
N. Eng J. Med. 274:1473-76.

BUTT C.G., BARO C., & KNORR R.W. 1968
Naegleria sp identified in amoebic encephatitis.
Am. J. clin. Path 42: 568-574

BYERS T.J., AKINS R.A., MAYNARD G.J., LEFKEN R.A., & MARTIN S.M. 1980
Rapid growth of Acanthamoeba in defined media, induction of encystment by glucose-acetate starvation.
J. Protozool 27: 216-19.

CARTER R.F. 1969.
Description of a Naegleria sp isolated from two cases of PAM and of the experimental pathological changes induced by it.
J. Path. 100: 217-44.

CALLICOTT H.H., JONES M.M., NELSON E.C., dos SANTOS J.G., UTZ J.P., DUMA R.J., MORRIS J.V. 1968
Meningoencephalitis due to pathogenic free-living amoeba.
J. Amer. Med. Ass. 206: 579-582

CARTER R.F. 1970
Description of a Naegleria sp isolated from 2 cases of PAM and of the experimental pathological changes induced by it.
J. Path 100: 217-44.

CARTER R.F. 1972
Primary amoebic meningoencephalitis: An appraisal of present knowledge.
Trans. R. Soc. Trop. Med. Hyg. 66: 193-213.

CASEMORE D.P. 1970
Sensitivity of Hartmanella (Acanthamoeba) to 5-fluorocytosine, hydroxystilbamidine, and other substances.
J. Clin. Path 23: 649-52.

CERVA L. 1971.
Studies of Limax amoebae in a swimming pool.
Hydrobiologia 38: 141-61.

CERVA L. & NOVAK K. 1968.
Amoebic Meningoencephalitis: 16 fatalities.
Sci 160: 92

CERVA L., NOVAK K., & CULBERTSON C.G. 1968
An outbreak of acute fatal amoebic meningoencephalitis
Am. J. Epi. 88: 436-44.

CHAGLA A.H., & GRIFFITHS A.J. 1974
Growth and encystation of A. Castellanii.
J. Gen. Micro 85: 139-45.

CHANG S.L. 1971
Small Free living amoebae: Cultivation quantitation, identification, classification, pathogenesis and resistence.
Curr. tops. comp pathobiol 1:201-54.

CHANG S.L. 1974
Etiological, pathological, epidemiological and diagnostical considerations of PAM.
Critical Reveiws in Micro 3: 135-159.

CHANG S.L. 1978
Resistance of pathogenic Naegleria to some common physical and chemical agents.
App. Environ. Microbiol 35: 368-375.

CHINCHILLA M., CASTROS E., ALFARO M. & PORTILLA E. 1979.
Amoebas de vida libre product oras de meningoencetalitis Primeros hallazgos en Costa Rica.
Rev. Latinoam de Microbiol 21: 135-142.

CHIOVETTI R. 1976
Re-encystment of the amoeboflagellate.
Trans. Amer. Micros. Soc. 95: 122-24.

CHIOVETTI R. 1978
Encystment of Naegleria gruberi (Schardinger).
1. Preparation of cysts for Electron Microscopy.
Trans. Amer. Micros. Soc. 97:245-249.

CHIOVETTI R. & BOVEE E.C. 1982
Initiation of encystment by multiple contacts among starving amoebae. N.gruberi (Schardinger 1899).
Acta. Protozool: 21: 149-55.

CORLISS J.O. & ESSER S.C. 1974
Comments on the role of the cyst in the life cycle and survival of free living protozoa.
Trans. Am. Micros Soc., 93: 578-93.

CRUMP L.M. 1950.
The Influence of the Bacterial Environment on the Excystment of Amoebae from Soil.
J. Gen. Microbiol 4: 16-21.

CULBERTSON C.G. 1961
Pathogenic Acanthamoeba (Hartmannella)
Am. J. Clin. Path. 35: 195-202.

CULBERTSON C.G. 1971
Pathogenicity of Soil Amoebae.
Ann. Rev. Microbiol 25: 231-54.

CULBERTSON C.G. 1975
Soil amoeba infection. Specific indirect immunoezymatic (peroxidase) staining of formalin-fixed paraffin sections.
Am. J. Clin. Pathol 63: 475-82.

CULBERTSON C.G. 1981
Amoebic meningoencephalitis p. 28-53
In H. Schonfeld (ed); Antibiotics and Chemotherapy Vol. 30 Skarger, Basel.

CULBERTSON C.G.; SMITH J.W. & MINNER J.R. 1958.
Acanthamoeba: Observations on animal pathogenicity.
Science 127: 1506.

CULBERTSON C.G., ENSMINGER P.W., OVERTON W.M. 1965
The isolation of additional strains of pathogenic Hartmannella sp (Acanthamoeba).
Am. J. Clin. Path 43: 383-87.

CULBERTSON C.G., ENSMINGER P.W., & OVERTON W.M. 1966
Hartmannella (Acanthamoeba): Experimental chronic granulomatous grain infections produced by new isolates of low virulence.
Am. J. of Clin. Pathol. 46: 305-314.

CULBERTSON C.G., SMITH J.W., COHEN E.V.M. & MINNER J.R. 1959.
Experimental infection of mice and monkeys by Acanthamoeba.
Am. J. of Pathol XXXV (35): 185-197.

CURSONS R.T.M., BROWN T.J. 1975
The 1968 N.Z. Cases of PAM-Myxomycete or Naegleria?
N.Z. Med. J. 82: 123-125.

CURSONS R.T.M. & BROWN T.J. 1975
Identification and Classification of the Aetiological Agents of primary amoebic meningo-encephalitis.
N.Z. J. Mar. Freshwater Res 10: 245-262.

CURSONS R.T.M., BROWN T.J. & KEYS E.A. 1976
PAM contracted in a thermal tributary of the Waikato River - Taupo: Case Report.
N.Z. Med. J. 84: 479- 481.

CURSONS R.T.M, BROWN T.J. & KEYS E.A. 1977
Immunity to PFLA
Lancet Oct. 1977 875 - 76.

CURSONS R.T., BROWN T.J. & KEYS E.A. 1978
Diagnosis & Identification of the Aetiological Agents of PAM.
N.Z. J. MED. Lab. Technol 32:11-14.

CURSONS R.T.M., BROWN T.J., & KEYS E.A. 1980
Effect of Disinfectants on P.F.L.A. in Axenic conditions.
Appl. Env. Microbiol 40: 62-66.

CURSONS R.T.M., BROWN T.J., & KEYS E.A., MORIARTY K.M. & TILL D. 1980
Immunity to P.F.L.A.: Role of CMI.
Infect Immun 29: 409-10.

CURSONS R.T.M., BROWN T.J., KEYS E.A. MORIARTY K.M. & TILL D. 1980
Immunity to P.F.L.A.: Role of Humoral Antibody.
Infect. Immun. 29: 401-07.

DAGGETT P.M., NERAD T.A. 1983.
The biochemical identification of Vahlkamfid ameobae.
J. protozool. 30: 126-28.

DAGGETT P.M., SAYER T.K., NERAD T.A. 1982
Distribution and possible interrelationships of pathogenic and non pathogenic Acanthamoeba from aquatic environments.
Microb. Ecol. 8: 371-86.

DAS S.R. 1970
Chemotherapy of exptal amoebic meningoencephalitis in mice infected with N.erobia.
Trans Roy. Soc. Trop Med. Hyg. 65: 106-7.

DAWSON M.W., BROWN T.J., BIDDICK C.J., TILL D.G., 1983 [b]
The effect of Baquacil on Pathogenic free living amoebae PFLA 2. In simulated natural conditions - in the presence of bacteria and/or organic matter.
N.Z. J. Marine & F.W. Res. 17: 313-320

DAWSON M.W., BROWN T.J., BIDDICK C.J. AND TILL D.G. 1982
The effect of Baquacil on pathogenic free-living amoebae (PFLA) 3. Increased Baquacil concentration and exposure time in the presence of bacteria.
N.Z.J. Marine & F.W. Res. 18: 53-56

DAWSON MW. BROWN T.J., TILL D.G. 1983 [a]
The effect of Baquacil on pathogenic free-living amoebae (PFLA). 1. In axenic conditions.
N.Z.J. Marine & F.W. Res. 17: 305-311.

De JONCKHEERE J.F. 1981
Naegleria Australiensis sp Nov.
another Pathogenic Naegleria from water.
Protistologica: (3) 423-429.

De JONCKHEERE J.F. 1981
Pathogenic and non-pathogenic Acanthamoebae sp in thermally polluted discharges and surface waters.
J. Protozool 28: 56-59.

De JONCKHEERE J.F. 1982
Isoenzyme patterns of pathogenic and non-pathogenic Naegleria sp. Using Agarose Isoelectric focusing.
Ann. Microbiol. (Inst. Pasteur) 133: 319-342.

De JONCKHEERE J.F. 1983
Isoenzyme and total protein analysis by agarose isoelectric focusing and taxonomy of the genus Acanthamoeba.
J. Protozool 30: 701-06.

De JONCKHEERE & VAN DE VOORDE 1976.
Differences in the destruction of cysts of pathogenic and non pathogenic Naegleria and Acanthamoeba by chlorine.
Appl. Environ. Microbiol 31: 294-297.

De JONCKHEERE J.F., VAN de VOORDE H. 1977.
The Distribution of N. fowleri in man-made thermal waters.
Am. J. Trop. Med. Hyg. 26: 10-15.

De JONCKHEERE J., Van DIJCK P., VAN de VOORDE H. 1974
Evaluation of the indirect fluorescent antibody technique for identificationof Naegleria species.
App. Micro 28: 159-64.

De JONCKHEERE J.F., Van DIJCK P., Van De VOORDE H. 1975
The effect of thermal pollution on the distribution of N. fowleri.
J. Hyg. 75: 7-13.

DERREUMAUX A.L. & JADIN J.B., WILLAERT E. & MORET R. 1974.
Action of Chlorine on Water Amoebae.
Ann. Soc. Belge. Med. Trop. 54: 415-428.

DERR-HARF C., MOLET B., SCHRIEBER J., KREMER M. 1978.
Epidemiology of Free living amoebae in the Waters of Strasbourg (France).
Ann. De. Parasitol 53: 467-477.

DINGLE A.D. 1979
Cellular and Environmental Variables determining the Numbers of flagella in Temperature shocked Naegleria gruberi.
J. Protozool. 26: 604-612.

DIVE D.G., LeCLERC H. De JONCKHEERE J., DELATTRE J.M. 1981
Isolation of N. fowleri from the cooling pond of an electric power plant in France.
Ann. Microbiol 132: 97-105

DONDERO T.J., RENDTORFF R.C., & MALLISON G.F. 1980.
An outbreak of Legionnaires' disease associated with a contaminated airconditioning cooling tower.
N. Eng. J. Med. 302: 365-370.

DORSCH M.M., CAMERON A.S., ROBINSON B.S. 1983.
The epidemiology and control of PAM with particular reference to South Australia.
Trans. Roy. Soc. Trop. Med. Hyg 77: 372-77.

dos SANTOS J.G. 1970
Fatal Primary Amoebic Meningoencephalitis: A retrospective study in Richmond, Virginia.
Am. J. clin. Pathol: 54: 737-742.

DUMA, R.J. (1981)
A study of Pathogenic free-living Amoebae in Fresh water Lakes in Virginia. Technical Rep. EPA - 600/S1 - 80 - 37, Health Effects Laboratory, Office of Research and Development, United States Environmental Protection Agency, Cincinati, Ohio. Feb. 1981. 131p.

DUMA, R.J. & FINLEY R. 1976.
In vitro susceptibility of pathogenic Naegleria and Acanthamoeba sp to a variety of therapeutic agents.
Antimicrob. Ag. Chemother., 10: 370-76.

DUMA R.J., FERRELL H.W., NELSON E.C. & JONES M.M. 1969.
Primary Amoebic Encephalitis.
J. Med. (New Eng(281: 1315-23.

DUMA R.J. ROSENBLUM W.I., McGEHEE, R.F. 1971.
PAM caused by Naegleria. Two new cases, Response to Amphotericin B, and a review.
Ann. Intern. Med. 74: 923-931.

DUMA R.J., SHUMACKER J.B. & CALLICOTT JH 1971.
Primary amoebic meningoencephalitis. A survey in Virginia.
Arch Environ. Health. 23: 43-48.

DUMA R.J., HELWIG W.B. & MARTINEZ A.J. 1978.
Meningoencephalitis and brain abscess due to free-living amoebae.
Annals. int. med. 88: 468-473.

ENGEL J.P., RUBINS A.J., AND SPROUL 1983.
Inactivation of N. gruberi cysts by chlorinated cyanurates.
Appl. Environ. Microbiol, 46: 1157-1162.

ESTERMAN A, RODER D.M., CAMERON S.A., ROBINSON B.S., WALTERS R.P., LAKE J.A., & CHRISTY P.E.
Determinants of the microbiological characteristics of Southern Australian Swimming Pools".
Appl. Environ. Microbiol 47: 325-328.

FERRANTE A. 1982
Comparative sensitivity of N. fowleri to amphotericin B. and amphotericin B. methyl ester.
Trans. Roy. Soc. Top. Med. Hyg. 76: 476-78.

FERRANTE A. & THONG Y.J. 1979
Antibody induced capping and endocytosis of surface antigen in N. fowleri.
Int. J. Parasitol 9: 599-601.

FERRANTE A. & THONG Y.J. 1980
Unique phagocytic process in neutrophil-mediated killing of N. fowleri.
Immunol. letters 2: 37-41.

FOWLER, M. & CARTER R.F. 1965
Acute Pyogenic Meningitis probably due to Acanthamoeba sp a preliminary report.
Brit. med J. 1965 2: 740-742.

FULTON C. 1970.
Amoebo-flagellates as Research Partners.
In Methods in Cell Biology IV: 341-476.
Prescott D.M. (ed) Academic Press N.Y. 514p.

FULTON C. 1972
Early events of cell differentiation in N. gruberi Synergistic control of electrolytes and a factor from yeast extract.
Dev. Biol 28: 603-19.

FULTON C. 1977.
Cell Differentiation in Naegleria gruberi.
Ann. Rev. Microbiol 31: 597-629.

FULTON C. & WALSH C. 1980
Cell differentiation and flagella elongation in N. gruberi.
J. Cell. Biol. 85: 346-60.

GRIFFIN J.L. 1972.
Temperature tolerance of pathogenic and non-pathogenic Free living amoebae.
Science 178: 869-70.

GRIFFITHS A.J. & HUGHES D.E. 1968.
Starvation and encystment of a soil amoeba H. castellanii.
J. Protozool 15: 673-77.

GRIFFITHS A.J. & HUGHES D.E. 1969
The physiology of excystment of H. castellanii.
J. Protozool 16: 93-99.

GRILLOT R. AND AMBROISE-THOMAS P. 1980.
Free-living amoebae in the Grenoble area swimming pools water. Influence of the "Winter/Summer" use of the sterilising procedure.
Rev. Epidem et Sante Pub 28: 185-207.

GULLET J., MILLS J. HADLEY K., PODEMSKI B. PITTS L., AND GELBER R. 1979.
Disseminated granulomatous Acanthamoeba infection presenting as an unusual skin lesion.
Am. J. Med. 67: 891-96.

HADAS E., KASPRZAK W., MAZUR T. 1977.
Electrophoretic characterization of small free-living amoebae.
Tropenmed. parasit 28: 35-43.

HAGGERTY R.M., JOHN D.T. 1978
Innate resistance of mice to experimental infection with N. fowleri.
Infect Immun, 20: 73-77.

HAGGERTY R.M. & JOHN D.T. 1983
Serum Agglutination and Immunoglobulin levels of mice infected with N. fowleri.
J. Protozool 29: 117-22.

HYSMITH R.M. & FRANSON R.C. 1982.
Elevated levels of Cellular and Extracellular Phospholipases from pathogenic N. fowleri
Biochemica et Biophysica Acta. 711 26-32.

KAUSHAL D.C. & SHUKLA 1975)
Effect of Bacterial and fungal extracts and Metabolic Inhibitors on Excystment of soil amoebae.
Ind. J. Exp Biol, 3: 375-377.

KLEIN R.L. 1959.
Transmembrane flux of K^{+2} in Acanthamoeba spp.
J. Cell Comp & Physiol, 53: 241-58.

JAGER B.V., STAMM W.P. 1972
Brain abcesses caused by Free living amoebae probably of the genus Hartmanella in a patient with Hodgkins disease.
Lancet 2: 1343-45.

JEHAN M, DUTTA G.P., KAUSHAL D.C., SHUKLA D.P. 1975
Effect of bacterial and fungal extracts & metabolic inhibitors on excystment of soil amoebae.
J. Expt Bid 3: 375-77.

JOHN D.T. 1982
PAM and the Biology of N. fowleri.
Ann. Rev. Microbiol. 36: 101-23.

JOHN D.T., WEIK R.R. & ADAMS A.C. 1977.
Immunization of mice against N. fowleri.
Infect & Immun: 16 817-820.

KEYS S.M., GREEN W.R., WILLAERT E. & STEVENS A.R. 1980
Keratitis due to A. Castellanii.
Arch. opthalmol 98: 475-79.

KINGSTON D. WARHURST D.C. 1968
Isolation of amoebae from the air.
I. Med. Micro 1: 27-36.

KRISHNA-PRASAD B.M. 1972
In Vitro Effects of drugs against pathogenic and non-pathogenic free living amoebae.
Ind. J. Expt. Biol. 10: 43-45.

LASMAN M. & SHAFRAN A.
Induction of encystment in A. palestinensis factors influencing cyst formation.
J. Protozool 25: (4) 489-491.

LASTOVICA A.J. 1974
Scanning Electron Microscopy of Pathogenic and non-pathogenic Naegleria cysts.
Int. J. Parasitol 4: 139-142.

LASTOVICA A.J. 1980
Isolation, distribution and disease potential of Naegleria and Acanthamoeba (Order; Amoebida) in South Africa.
Trans. R. Soc. Sth. Africa 44:269-278.

LAWANDE R.V. & DUGGAN M.B. 1979
PAM in Nigeria (report of 2 cases in children)
J. Trop Med. Hyg. 82: 134-36.

LUND O.F., STEFANI F.H. & DECHANT W. 1978
Amoebic keratitis: a Clinicopathological case report.
Brit. J. Opthalm. 62: 373-75.

LYONS T.B., KAPUR R. 1977.
Limax Amoebae in Public Swimming pools of Albany, Schenectady, and Rensselaer Counties, New York: their concentrations, correlations and significance.
Appl. Environ. Microbiol. 33: 551-555.

McINTOSH A.H. & CHANG R.S. 1971
A comparative study of four strains of Hartmannellid amoebae.
J. Protozool. 18: 632-6.

MA P.,WILLAERT E., JUECHTER B., STEVENS A.R. 1981
A case of Keratitis due to Acanthamoeba in New York, New York, and features of 10 cases.
J. Infect. Dis. 143: 662-667.

MARTINEZ A.J. 1977
Free living amoebic meningoencephalitides: comparative study.
Neurologia-Neurocirugia-Psiquiatia 18: 391-401

MARTINEZ A.J. 1980
Is Acanthamoeba encephalitis an opportunistic infection.
Neurology 30: 567-84.

MARTINEZ A.J. 1982
Acanthamoebiasis and immunosuppression. Case Report.
J. Neuropath and Exptal. Neu. 41: 548-57.

MARTINEZ A.J. 1983
Free-living amoebae: Pathogenic aspects. A review.
Protozool. Abstr. 7: 293-306.

MARTINEZ A.J., DUMA R.J., NELSON E.C. & MORETTA F.L. 1973
Experimental Naegleria Meningoencephalitis in mice.
Laboratory Investigation 29: 121-33.

MARTINEZ A.J.; GARCIA C.A., HALKS-MILLER M. & ARECEVEKI R.(1980a)
Granulomatous amoebic encephalitis presenting as a cerebral mass lesion.
Acta Neuropath 51: 85-91.

MARTINEZ A.J., SOTELO-AVILA, C. ALCAL H. & WILLAERT E.(1980b)
Granulomatous Encephalitis, Intercranial, Arteritis of Mycotic Ameurysm due to a free living amoeba.
Acta Neuropathol 49: 7-12.

MARTINEZ A.J., AVILA C.S. TAMOYO J.G., MORON T.J., WILLAERT E. & STAMM W.P. 1977.
Meningoencephalitis due to Acanthamoeba sp. Pathogenesis and clinicopathological study.
Acta neuropath (Berl) 37: 183-91.

MASCARO C., FLUVIA C., COSUNA A., GUEVARA D. 1981.
Virulent Naegleria sp isolated form a river in Caiz Spain.
J. Parasitol 67: 599

MATIN S.M. & BYERS T.J.
Evidence for Lysosomal Enzymes in Acanthamoeba and their activity changes during encystment.
Ohio J. Sci. 77 (1) 28, 1977.

MICHEL R. & JUST H.M. (1984)
Acanthamoeba, Naegleriae and other free-living amoebae in Cooling and Rinsing Water in Dental Treatment Units
Zentralbl. Bakteriol. Mikrobiol. Hyg. 1: 556 - 572

MORRIS G.K., PATLON C.M. & FEELEY J.C. 1979
Isolation of the Legionnaires' disease bacterium from environmental samples.
Ann. Intern. Med. 90: 664-666.

NAGINGTON J., WATSON P.G., PLAYFAIR J.J. 1974
Amoebic Infection of the Eye
Lancet 28:1537-1540.

NAGINGTON J. & RICHARDS J.E.
Chemotherapeutic compounds and Acanthamoeba from eye infections.
J. Clin Path. 29: 648-51.

NEFF R.J. & NEFF R.H. 1972
Induction of differentiation in Acanthamoeba by inhibitors.
Comptes. rendum es. trav. lab. Carlsberg 39: 111-168.

NEFF R.J., RAY S.A., BENTON W.F, WILBORN M. 1964
Induction of synchronous encystment (differentiation).
Meth. Cell Physiol. I: 55-86.

PAGE F.C. 1967 (a)
Taxonomic criteria for Limax amoebae, with descriptions of 3 new spp. of Hartmanella and 3 of Vahlkampfia.
J. Protozool 14: 499-21.

PAGE F.C. 1986 (b).
Redefinition of the genus Acanthamoeba with descriptions of three species.
J. Protozool 14: 709-724.

PAGE F.C. 1976
An illustrated key to Freshwater and Soil Amoebae.
FreshWater Biol. Assns. Scientific Publication No. 34.

PALIN A.T. 1974
Analytical control of water Disinfection with special reference to differential D.P.D. methods for chlorine, chlorine dioxide, bromine, iodine, ozone.
J. Instn. Wat. Engrs. 28: 139-154.

POTTER J.L. & WEISMAN R.A. 1971
Differentiation in Acanthamoebae: B Glucan synthesis during encystment.
Biochem. Biophys. Acat 237: 65-74.

PUSSARD M. & PONS R. 1979
Etude de pores kystiques de Naegleria (Vahlkampfidae-amoebida).
Protislologica 15: 163-175.

RAIZADA M.K., KRISHNA MURTI C.R. 1971
Changes in the activity of certain enzymes of Hartmanella (culbertson str. A1) during encystment.
J. Protozool 18: 115-19.

REILLY M.F., BRADLEY M.K., BRADLEY S.G. 1982.
Agglutination of N. fowleri by Human serum (41420)
Proc. Soc. Exptal. Biol. Med. 170: 209-12.

REILLY M.F., MARCIANO-CABRAL F., BRADLEY D.W., BRADLEY S.G. 1983
Agglutination of N. fowleri and N. gruberi by antibody in human serum.
J. Clin. Micro 17: 576-581.

RIDGWAY H.F. & OLSON B.H.
Chlorine Resistance Patterns of Bacteria from two drinking water distribution systems.
App. & Environ. Microbiol: 972-987.

RIVERA, F., GALVAN M. RABLES E., LEAL P., GONZALES L., AND LACY A.M. 1981
Bottled Mineral Waters polluted by Protozoa in Mexico.
J. Protozool 28: 54-56.

ROBINSON B. 1978
Effect of Chlorine on Naegleria fowleri.
Lib. ref. No. E.W.S. 78/16. Water & Sewage Treatment Branch.

ROBINSON B.S. & LAKE J.A. 1982
Identification of N. fowleri by enzyme electrophoresis.
Lib. ref. No. E.W.S. 82/24.

ROWAN-KELLY B., FERRANTE A., & THONG Y.J. 1982
The Chemotherapeutic value of Sulphadiazine in treatment of Acanthamoeba meningoencephalitis in mice.
Trans. R. Soc. Trop. Med. & Hyg. 76: 636-638.

ROWBOTHAM T.J. 1980
Preliminary report on the pathogenicity of Legionella pneumophila for freshwater and soil amoebae.
J. Clin. Pathol 33: 1179-83.

ROWBOTHAM T.J.1983
Isolations of Legionella pneumophilia from clinical specimens via amoebae, and the interaction of those and other isolates with amoebae.
J. Clin. Pathol 36: 978-86.

ROWBOTHAM T.J. 1983 (b)
Legionellae and Amoebae.
Proceedings of 2nd Int. Symposium 1983 published by A.S.M. pg 325-328.

RUBIN A.J., ENGEL J.P., SPROUL O.J. 1983
Disinfection of amoebic cysts in water with free chlorine.
J. WPCF 55: 1174-82.

SCHUSTER F.L. 1963
An Electron Microscopic study of the Amoebo-flagellate N. gruberi (Schardinger) II the cyst stage.
J. Protozool 10: 313-20.

SCHUSTER F.L. 1975
Ultrastructure of cysts of Naegleria spp. A comparative study.
J. Protozool22: 352-59.

SCHUSTER F.L. 1979
Small amoebae and amoeboflagellates.
Biochemistry and Physiology of Protozoa, Vol. 1 pg 215-285. New York, Academic 2nd Ed.

SCHUSTER F.L. & MANDEL N. 1984
Phenothiazine compounds inhibit in vitro growth of pathogenic Free Living Amoebae.
Antimicr. Agents & Chemother. 25: 109-112.

SEIDEL J.S., HARTMATZ P., VISVESVARA G.S., COHEN A., EDWARDS J. & TURNER J. 1982
Successful treatment of PAM
N. Eng. J. Med. 306: 346-48.

SHAPIRO M.A.,KAROL M.H.,KELETI G., SYKORA J.L., AND MARTINEZ A.J. 1983
The role of free-living amoebae occurring in heated effluents as causative agents of Human disease.
Wat. Sci. Tech: 15: 135-147.

SINGH B.N., & DAS S.R. 1970
Studies on pathogenic and non-pathogenic free living amoebae and the bearing of nuclear division on the classification of the order Amoebida.
Phil. Trans. R. Soc. (Lond): 259 : 435-476.

SINGH B.N., MATHEWS AND ANAND N. 1958
The Role of Aerobacter sp, Escherichia coli, and certain amino acids in the Excystment of Schizopyrenus russelli.
J. gen. Microbiol 19: 104-111.

SINGH B.N., SAXERALL, IYER S.S. 1964
Production of Viable sterile cysts of free living amoeba and role of bacteria on excystment.
Ind. J. Exp. Biol. 3: 110-112.

SINGH B.N., DUTTA T., DUTTA G.P. 1970
Factors inducing excystation in Free Living Amoebae.
Ind. J. Exp. Biol 9: 350-57.

SPROUL O.J., CHEN Y.S.R., ENGEL J.P., & RUBIN A.J. 1983.
Comparison of chlorine and chlorine dioxide for the inactivation of an amoebic cyst.
Env. Technol. Lett 4: 335-342.

SRIVASTAVA D.K. & SHUKLA O.P.
Encystment of A. culbertsoni by organic effectors.
Ind. J. Exp. Biol 21: 444-47.

STEVENS A.R. & O'DELL W.D. 1973
The influence of growth medium on Axenic cultivation of virulent and Avirulent Acanthamoeba
Proc. Soc. Exp. Biol. & Med. 143: 474-478.

STEVENS A.R., O'DELL W.D.
In vitro and Invivo activity of 5-fluorocytosine on Acanthamoeba.
Antimicrob. Agents & Chemother 6: 282-89.

STEVENS A.R., WILLAERT E. 1980
Drug sensitivity and resistance of 4 Acanthamoeba spp.
Trans. Roy Soc.Trop Med. Hyg. 74: 806-08.

STEVENS A.R., TYNDALL R.L., COUTANT C.C., WILLAERT E. 1977
Isolation of the etiological agent of PAM from artifically heated water.
APPL. Environ. Microbiol 34: 701-705

STEVENS A.R., De JONCKHEERE J.F. & WILLAERT E. 1980
N. Lovanesis new species: isolation and identification of 6 thermophilic strains of a new spp found associated with N. fowleri.
Intern. J. Parasitol 10: 51-64.

STEVENS A.R., STAULMAN ST., LANSEN M.A. CICHON M.J., WILLAERT E. 1981
PAM. A report of Two Cases and Antibiotic and Immunologic Studies.
J. of Infect. Dis. 143: 193-99.

THONG Y.H., ROWAN-KELLY B., FERRANTE A. 1979.
Treatment of experimental Naegleria meningoencephalitis with a combinatin of amphotericin B. and rifamycin.
Scand. J. Infect. Dis 11: 151-153.

THONG Y.H., ROWAN-KELLY B, FERRANTE A. & SHEPARD C. 1978.
Synergism between tetracycline and amphotericin B. in experimental amoebic meningoencephalitis.
Med. J. Aust. 1: 663-664.

THONG Y.H., CARTER R.F., FERRANTE A. & ROWAN-KELLY B. 1983.
Site of Expression of Immunity to N. fowleri in immunized mice.
Parasite Immunol 5: 67-76.

TYNDALL R.L. 1984.
Environmental isolation of Pathogenic Naegleria.
C.R.C. Critical Reviews in Environmental Control 13: 195-226.

VISVESVARA G.S. & CALLAWAY C.S. 1974
Light and Electron Microscopic observations on the ptahogenesis of N. fowleri in Mouse brain and tissue culture.
J. Protozool 21: 239-50.

VIVESVARA G.S. & BALAMUTH W. 1975
Comparative studies on related free living & pathogenic amoebae with special reference to Acanthamoeba.
J. Protozool 22: 245-56.

VISVESVARA G.S. & HEALY G.R. 1975
Comparative antigenic analysis of pathogenic free living Naegleria sp by the gel diffusion and immunoelectrophoresis techniques.
Infect. Immun. II: 95-108.

VIVESVARA G.S., JONES P.B. & ROBINSON N.M. 1975
Isolation Identification and Biological characterization of Acanthamoeba polyphaga from a Human eye.
Am. J. Trop. Med & Hyg. 24: 784-790.

WALTERS R.P., ROBINSON B.S. & LAKE J.A. 1981
Experiences in the control of Naegleria in public water supplies in S. Aust.
Proc. Aust. Water & Wastewater Ass. 9th Fed. Convert. 3.1 - 3.11.

WELLINGS F.M. 1979
Pathogenic Naegleria: distribution in Nature. Technical Rep. E.P.A. 600/1-79-081, Health Effects Laboratory Office of Research and Development, United States Environmental Protection Agency, Cincinati, Ohio. May 1979 1-79.

WELLINGS F.M., AMUSO P.T., CHANG S.L., & LEWIS A.L. 1977
Isolation and identification of pathogenic Naegleria from Florida lakes.
Appl. Environ. Microbiol 34: 661-667.

WELLINGS F.M., LEWIS A.J., AMUSO P.T. AND CHANG S.L. 1977
Naegleria and Water sports.
Lancet Jan 22: 199-200.

WEISMAN R.A. 1976
Differentiation in A. castellanii
Ann. Rev. Microbiol 30: 189-219.

WHITE G.C. 1972
Handbook of Chlorination
Van Nostrand Reinhold, New York 744p.

WILLAERT E. & STEVENS A.R. 1976
Isolation of pathogenic amoeba from thermal discharge water.
Lancet Oct, 1976: 741.

WILLAERT E., STEVENS A.R. & HEALY G.R. 1978
Retrospective identification of Acanthamoeba culbertsoni in a case of Amoebic meningoencephalitis.
J. Clin Pathol 31: 717-720.

WONG M.M., KARR S.L. Jr., BALAMUTH W.B. 1975 (a). Experimental infections with Pathogenic Free Living Amoebae in laboratory primate hosts: I (A) A study on susceptibility to N. fowleri. J. Parasitol 61: 199-208.

WONG M.M, KARR S.L. Jr., BALAMUTH W.B. 1975 (b). Experimental infections with pathogenic free living amoebae in laboratory primate hosts: I(B) A study on susceptibility to A. culbertsoni. J. Parasitol 61: 682-90.