

ROLE OF MARINE NITRIFYING BACTERIA IN A CLOSED SYSTEM WITH

Penaeus monodon

by

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INTRODUCTION

In recent years there has been widespread interest in rearing aquatic organisms of nutritional and commercial value (Calaprice, 1976). The most hopeful prospect for marine prawn culture in the United Kingdom (Wickins, 1976), the Americas (Hanson & Goodwin, 1977) and South Africa probably lies in intensive culture under controlled conditions. A closed system approach, in which a captive body of water is circulated, provides the scope for water quality management which results in maximum water utilization and minimal discharge. On the other hand, direct utilization of sea-water in open systems presents problems for aquaculture since this water is subjected to diurnal and seasonal fluctuations in temperature, salinity and turbidity, as well as contamination from industrial, agricultural and maritime sources. Furthermore, large mariculture farms release enormous amounts of organic wastes which result in eutrophication and could lead to environmental deterioration of coastal waters (Gerhardt, 1978).

It is well established that circulated sea-water develops an unusual ionic composition as a result of the metabolic activity of the prawns and of the nitrifying bacteria in the biological filter. The changes include elevated levels of ammonia, nitrite and nitrate and reduced pH. The presence of even sublethal levels of these nitrogenous compounds in closed systems have been found to affect growth of penaeid species (Wickins, 1976). Ammonia and nitrite, which rapidly accumulate in the water, are usually maintained at nontoxic levels by nitrification in the biological filters (Spotte, 1974; Johnson & Sieburth, 1974). The chemolithotrophic bacteria responsible for nitrification are presently classified by their cellular morphology and by the oxidation of either ammonia and nitrite (Watson, 1974). The predominant ammonia- and

nitrite-oxidizing bacteria isolated from natural environments are *Nitrosomonas europaea* and *Nitrobacter winogradskyi*, respectively (Watson *et al.*, 1981). Direct observation of nitrifying bacteria in natural environments, however, has been limited to studies involving light microscopy with immunofluorescent techniques (Fliermans *et al.*, 1974; Fliermans & Schmidt, 1975).

The electron microscopic observation of nitrifying bacteria is difficult in natural microcosms with low levels of nitrification and with the presence of sunlight and anaerobic conditions conducive to the enrichment of other bacteria with a similar ultrastructure. However, in closed systems with extremely active nitrification but poor light conditions, the occurrence of morphologically similar forms in numbers that could be easily detected by electron microscopy is unlikely (Johnson & Sieburth, 1976). Furthermore, the cyst-like colonies of the nitrifiers are unique and are not found with the methane-oxidizing bacteria with a similar ultrastructure (Davies & Whittenbury, 1970; Smith & Ribbons, 1970), whereas the thick cell wall of the cyanobacteria (Carr & Whitton, 1973) and the distinctive cell morphologies of the purple sulphur and purple nonsulphur bacteria (Pfennig, 1967) separate them from the nitrifiers. Therefore, closed systems with active nitrification provide the ideal environment to study the activities of nitrifiers in conjunction with their relative abundance, nature and diversity. In spite of the opportunity offered by closed systems, previous studies (Kawai *et al.*, 1965; Wickins, 1976; Gerhardt, 1978; Mevel & Chamroux, 1981) on nitrification have been primarily indirect observations on rates of ammonia and nitrite oxidation to nitrate (Johnson & Sieburth, 1976). Studies on the enumeration and identification of nitrifiers in closed systems have been seriously neglected. Kawai *et al.* (1964) included the enumeration of nitrifiers in their study on nitrification

while, in a qualitative study, an attempt to identify the *in situ* nitrifiers in closed systems (Johnson & Sieburth, 1976) was not very successful.

This study was undertaken to investigate the three basic aspects of nitrification necessary for the understanding of such a process in closed systems, viz., the oxidation of ammonia and nitrite to nitrate, and the enumeration and identification of the nitrifying bacteria. Prior to determining the concentrations of the nitrogenous compounds in the culture water, various methods were evaluated for their accuracy and reproducibility with both sea-water and culture water samples. This approach is necessary in order to gauge the accuracy of results obtained by such methods. Enumeration of nitrifying bacteria was preceded by an investigation on the effect of incubation time on the maximum most probable number estimate. Such an investigation was necessary because of the inconsistent approach to the enumeration of nitrifiers in previous studies (Wilson, 1927; Walker *et al.*, 1937; Lewis & Pramer, 1958; Molina & Rovira, 1964; Meiklejohn, 1965; Smith *et al.*, 1968). Incubation periods appear to have been chosen arbitrarily in previous investigations. Identification of nitrifying bacteria necessitates the isolation and purification of these organisms. Isolation of nitrifiers is a difficult and time-consuming task (Watson *et al.*, 1981) and could be the main reason for not being included in previous studies on nitrification. Since the success of this study depended upon the isolation and purification of these chemolithotrophs, this aspect is dealt with in detail.

The changes most likely to be associated with nitrification in a closed system were also monitored in the culture water. These included pH, dissolved oxygen and biochemical oxygen demand. Apart from a biological

sand filter, no other form of culture water treatment was effected during the investigation. The effect of growing the "sugpo" or jumbo tiger prawn, *Penaeus monodon* (Kinne, 1977) for 22 weeks in a captive body of sea-water was evaluated by comparing the survival and wet mass with those reported by other workers.

This study differs greatly from previous reports on nitrification in closed systems because both the "causes" and "symptoms" of this important detoxifying process are investigated. It is intended that the findings of such a study would aid culturists in exploiting the nitrifying potential of closed systems to its utmost.

I. LITERATURE REVIEW

A) SEA-WATER CULTURE SYSTEMS

Since the main objective of this study revolved around nitrifying bacteria, a detailed review of culture systems *per se* is outside the scope of this thesis. In view of this, an outline of the basic sea-water culture systems only will be dealt with because of their relevance to the topic.

There are two basic categories of aquatic culture systems, viz., still-water- and running-water systems. Numerous small organisms, such as bacteria, unicellular plants and a variety of small invertebrate animals, can be cultured successfully in still-water systems. However, many multicellular plants and animals require running-water. Among the running-water systems, three types can be distinguished: open-, semi-open- and closed systems. However, it should be pointed out that no experimental water system is entirely open, nor can it be completely closed because, over extended periods, some replacement of culture water is unavoidable (Kinne, 1976). A summary of Kinne's (1976) review of the principal characteristics of running sea-water culture systems, relevant to the present study, is presented.

1. Open

In open sea-water systems (flow-through systems) the culture medium is used once only. A continuous flow of sea-water from a natural source enters the culture container and is subsequently discarded. Such systems require a permanent supply of suitable natural sea-water and, hence, a location close to an unpolluted shore. These systems are used

for observation and maintenance of living organisms, e.g., teaching, demonstrations, food supply and stock cultures. In some cases, however, open systems may provide the only means for successful rearing and breeding, e.g., in situations where insufficient information is available on environmental or nutritional requirements of the animals cultivated, or in cases where commercial cultivation prevails. Pre-treatment of sea-water may be necessary for use in closed systems. Adjustments with regard to water temperature, gaseous content and turbidity can be achieved through heat exchange, aeration, sedimentation and filtration, respectively. In addition, disinfection, and the changing of nutrients, other substances or organisms may be desirable.

2. Semi-open

These systems combine the technological principles of open and closed systems to varying degrees. Depending upon the extent of water renewal or circulation, the characteristics of either open- or closed systems prevail. Semi-open systems are used either in coastal areas where tidal or weather conditions make a continuous supply of natural sea-water difficult, or in cases where culture water treatment becomes uneconomical unless part of the water is renewed. These systems are useful in public aquaria and other displays of aquatic animals, as well as in aquaculture farms where the total water capacity of the system is insufficient for long-term support of a high "animal load". When new sea-water becomes available, or when the water quality drops below minimum requirements, part of the circulated water is discarded and replaced by water freshly pumped from the sea.

3. Closed

In closed sea-water systems the culture water is continuously recycled. Culture-water circulation offers better possibilities for monitoring and controlling essential environmental factors than open and semi-open systems. Closed systems are to a great extent independent of the seasonal, physico-chemical and biological variations characteristic of sea-water, e.g., temperature, salinity, turbidity, organic substances, plankton components and pollutants, especially in coastal areas. They can be operated away from the sea and with artificial sea-water.

In a typical closed sea-water system (Fig. 1) water circulates through a culture tank, filter, reservoir, header or gravity tank and back to the culture tank. Circulation pumps are placed after the reservoir to avoid clogging. The reservoir provides an emergency water supply for 12 to 24 h in the case of a pump failure, while also acting as a settling tank.

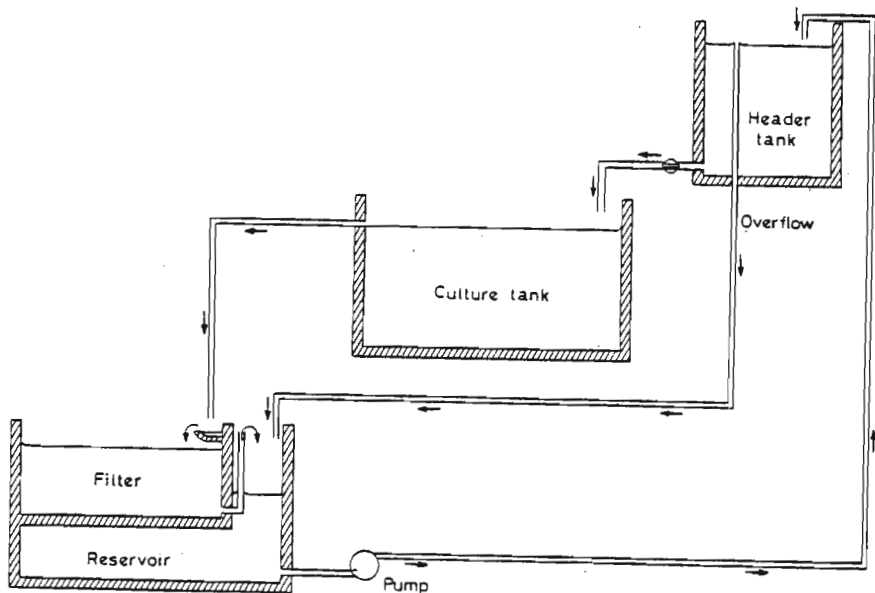


Fig. 1. Closed sea-water system (Kinne, 1976).

To prevent sudden overall pollution, to restrict the spread of disease and to facilitate water treatment, each culture tank should have its own circulatory system (Kelley & Moreno, 1961 - cited by Kinne, 1976). Such individual sea-water circulation has been employed successfully for many years in several public aquaria, e.g., at Wuppertal in the Federal Republic of Germany (Weidemann, 1943 - cited by Kinne, 1976). When the systems comprise less than 400 l, adequate water circulation can be provided by airlifts.

Some closed systems, e.g., the Marine Biological Laboratory at Plymouth, England, have no filter and operate a filterless system with special sedimentation tanks (Wilson, 1952 - cited by Kinne, 1976). Filterless closed systems have a lower carrying capacity than conventional types (Herald *et al.*, 1962 - cited by Kinne, 1976) but support filter-feeding animals more efficiently than the latter types. In closed systems, the cultivated organisms tend to modify the chemistry of the culture water. If extensive, the withdrawal of, e.g., oxygen or release of, e.g., metabolic end products may approach critical levels. The most obvious changes that occur in a captive body of sea water are:

- decrease in dissolved oxygen concentration and alkaline reserve;
- increase in carbon dioxide and nitrogenous compounds;
- increase in microbial numbers; and
- changes in pH.

In the sea, a balanced integration of production, transformation and re-mineralization of organic substances usually prevents any adverse changes in the flow of energy and matter. In closed systems, a comparable balance must be achieved and then maintained artificially. Most closed

systems contain representatives of only a few species isolated from a multitude of environmental and organismic interrelations. The cultivator must reconstitute acceptable environmental and nutritional conditions to counteract any detrimental influences on the culture medium. Closed seawater systems require continuous management of the basic influences which the environment and organisms exert upon each other, with the aim of maintaining the conditions within the tolerance limits of the organisms cultivated. In essence, closed-system operation means, above all, continuous culture water treatment. However, as the physico-chemical changes were monitored in the present study, no culture water treatment, apart from biological filtration, was carried out.

B) NITRIFYING BACTERIA

The biological oxidations of ammonia to nitrite, and nitrite to nitrate are collectively referred to as nitrification and are carried out in nature by two physiological groups of Gram-negative, chemolithotrophic bacteria. The organisms in both groups fix carbon dioxide via the Calvin cycle (Campbell *et al.*, 1966) for their major source of cell carbon. They derive their energy and reducing power from either the oxidation of ammonia (ammonia-oxidizing bacteria) or nitrite (nitrite-oxidizing bacteria). With the exception of a few strains of *Nitrobacter winogradskyi*, which can be grown chemoheterotrophically, the nitrifying bacteria are obligate chemolithotrophs. Nitrifying bacteria are found in soil, compost, sewage disposal systems, fresh water and marine habitats, and in most other aerobic environments where organic compounds are being mineralized (Watson *et al.*, 1981).

Investigators of nitrifying bacteria are faced with many problems not usually encountered with heterotrophic bacteria. The slow growth rate,

lack of a distinct colonial morphology and inability to metabolize organic compounds have made research on this group of chemolithotrophs extremely difficult. Since these difficulties are closely associated with the present study, the literature survey in this section includes the isolation, purification, enumeration and identification of nitrifying bacteria.

1. Isolation and purification

Enrichment cultures are necessary for the isolation of nitrifying bacteria because they cannot be isolated directly from source material. Although no organic compounds are added to the enrichment media, the nitrifying bacteria release organic compounds that can support the growth of heterotrophic bacteria. In enrichment cultures heterotrophic bacteria usually outnumber the nitrifying bacteria because the generation time of the latter is between 8-24 h, even under optimal conditions. This makes the isolation of nitrifying bacteria a very difficult and time-consuming task (Watson *et al.*, 1981).

Various attempts have been made to isolate and purify nitrifying bacteria (Meiklejohn, 1950; Lewis & Pramer, 1958; Watson, 1965; Soriano & Walker, 1968). The most frequently cited explanations for the difficulties encountered in obtaining nitrifying bacteria in pure culture are:

- autotrophic nitrifiers grow very slowly;
- heterotrophic contaminants in enrichment cultures develop at a rate equal to or greater than that of nitrifying bacteria;
- the liquid medium that is usually employed for the

growth of nitrifying bacteria contains insoluble carbonate to which the cell adsorbs, preventing dispersed growth; colonies formed on solid media are so small - equal to or less than 100 μ m in diameter - that they cannot be seen with the naked eye; and colonies of nitrifying bacteria cannot be distinguished morphologically from colonies of contaminating bacteria.

Numerous techniques reported for the purification of nitrifiers from enrichment cultures will be discussed.

a) Solid media

The isolation of nitrifying bacteria was first accomplished by Winogradsky (1890 - cited by Lewis & Pramer, 1958). Initially, a "negative plate" method was employed by inoculating gelatin plates and picking off from areas where no growth could be seen. Winogradsky (1891 - cited by Meiklejohn, 1950) abandoned the "negative plate" method and described a procedure whereby enrichment cultures were prepared in a liquid medium containing an ammonium salt and a carbonate. Both substances were replenished as they were utilized by the growing cultures. After prolonged incubation, motile bacteria appeared in the cultures and poured plates were made on an inorganic silica gel medium. Small colonies, which developed on this medium, were transferred into sterile liquid mineral media. The inoculated medium was incubated and the cultures were checked for purity and their ability to oxidize ammonia to nitrite. This purification procedure was used successfully by many workers (Boulanger & Massol, 1903; Bonazzi, 1919; Gibbs, 1919; Nelson, 1931; Kingma Boltjes, 1934, 1935 - all cited by Meiklejohn,

1950). The last three workers used, in addition, a micromanipulator to isolate nitrifying bacteria in pure culture.

Meiklejohn (1950) used a modification of the method by Winogradsky (1891 - cited by Meiklejohn, 1950) to isolate *Nitrosomonas* sp. in pure culture. This procedure had three essential steps:

the population of nitrifiers in a sub-culture of the twenty-fifth transfer was increased by successive additions of ammonium sulphate and calcium carbonate over a period of four months;
removal of the bacteria adhering to the calcium carbonate particles with a stream of carbon dioxide;
and
removal of samples to inoculate plates containing an inorganic silica gel medium.

After incubation at 25C for a fortnight, the plates were found to be covered with numerous tiny colonies of a peculiar, glassy, compact appearance and about 100 μ m in diameter. With the aid of a low power microscope objective, the position of a few, well-defined, single colonies was marked. These were picked off with the fine point of a Pasteur pipette and placed in an ammonium sulphate medium. After incubation at 25C for 14-21 d, five of the cultures were observed to oxidize ammonia to nitrite. Two of these cultures were free of heterotrophic contaminants and were assumed to be pure cultures of an ammonia-oxidizing bacterium.

Soriano & Walker (1968) reported the isolation and purification of ammonia-oxidizing autotrophs, *Nitrosomonas* sp. and *Nitrosocystis* sp.,

by using a purified agar medium and a method of picking colonies with glass capillary pipettes operated by a micromanipulator. A comparison between the dilution procedure and the agar plate procedure for the isolation of pure cultures of ammonia-oxidizing bacteria was also made by these workers. For the dilution procedure, serial dilutions were made in sterile media from an enrichment culture so that the final dilution contained an average of 1-2 organisms.ml⁻¹. Tubes containing 8 ml of medium were inoculated with 1 ml of the final dilution and incubated at 25C or 30C. At intervals during incubation tests were made for the presence of acid and nitrite. For the agar plate procedure, an inoculum consisting of a drop of either undiluted or diluted enrichment culture was spread over the surface of a dried agar plate. The plates were incubated at 25C or 30C until nitrite was detected on the agar and presumptive colonies of nitrifiers could be seen under the microscope at magnifications of 80x or 100x. Isolated microcolonies were sucked into capillary pipettes and transferred to tubes of sterile liquid media. Approximately 84 d were required to isolate a pure culture of *Nitrosomonas* sp. using the dilution technique whereas 47 d were required by the plating technique.

Harms *et al.* (1976) isolated *Nitrosovibrio tenuis* from a soil sample collected on the islands of Hawaii. An enrichment culture, prepared in a mineral salts medium, was incubated at 30C for 30 d and periodically analysed for changes in nitrite concentration. When all the ammonium was oxidized to nitrite, culture aliquots were transferred into sterile mineral salts medium. After two transfers *N. tenuis* was isolated in pure culture by plating on an inorganic agar medium. After approximately 135 d cells from single colonies were transferred and maintained in test tubes containing sterile mineral salts media.

b) Liquid media

According to Meiklejohn (1950) a different technique of isolation was used by Heubult (1929), Engel & Skallau (1937) and Bömecke (1939). These workers prepared enrichment cultures in a liquid mineral medium from which a very high dilution of the culture was made in the same medium, shaken for 1 h, and a large number of subcultures prepared in liquid media.

In 1955, Golberg & Gainey (cited by Lewis and Pramer, 1958) resolved conflicting views regarding the role of surface phenomena in nitrification by demonstrating that appreciable quantities of particulate matter were not essential for rapid nitrification in liquid media. This report was confirmed by similar results obtained by Engel & Alexander (1958). Following these reports, Lewis & Pramer (1958) attempted the isolation of *Nitrosomonas* spp. by dilution techniques using media as free from particulate material as possible. These workers found that by using a "particulate-free" medium the development of *Nitrosomonas* spp. in enrichment cultures was greater than that of associated heterotrophic bacteria. The ratio of the number of cells of *Nitrosomonas* spp. to that of heterotrophic bacteria was adequate to permit isolation of the former in pure culture by dilution techniques.

In 1971, Watson & Waterbury isolated two new nitrite-oxidizing bacteria, viz., *Nitrococcus mobilis* and *Nitrospina gracilis*, from a marine environment. Initial enrichment cultures were incubated for up to 3 months at room temperature and periodically analyzed for changes in nitrite and nitrate concentrations. When over half the nitrite was oxidized to nitrate the enrichment culture was serially diluted in a mineral salts medium containing sodium nitrite. *N. gracilis* was

isolated in pure culture after nine serial dilutions and *N. mobilis* after three serial dilutions.

Watson *et al.* (1971) isolated *Nitrosolobus multiformis* in pure culture from soil samples obtained from various parts of the world. Enrichment cultures were incubated at 25C for 30-60 d and periodically analyzed for changes in nitrite and nitrate concentration. When the concentration of either nitrite or nitrate increased, the enrichment culture was serially diluted in a mineral salts medium. The changes in nitrite and nitrate were similarly followed in the cultures and when nitrification was detected they were again serially diluted. *N. multiformis* was obtained in pure culture by repeated serial dilutions.

c) pH-stat cultures

Watson (1965) isolated *Nitrosocystis oceanus* in pure culture by using an initial enrichment culture. When nitrite production increased at an exponential rate, the organisms were inoculated into fresh medium in a shake flask. After repeated serial transfers in a liquid medium the crude culture was grown in a pH-stat fermentor. Culture growth continued until a population of 3×10^7 cells.ml⁻¹ was reached and the nitrite concentration was 0,2-0,3 g-at.NO₂-N.ℓ⁻¹. Usually, at this stage, the ratio of heterotrophic to autotrophic cells was 1:100. Once this ratio was obtained, the culture was serially diluted and 1-10 cells were inoculated into fresh medium and incubated in a shake flask at 25C for 2-3 weeks before nitrite could be detected. After 10 µg-at.NH₃-N.ℓ⁻¹ were oxidized, the entire contents of the flask were inoculated into nine volumes of medium in a pH-controlled vessel. Incubation at room temperature was allowed to continue until a concentration of 1×10^7 cells.ml⁻¹ was reached before being checked for purity.

In 1971 Watson reisolated *Nitrosospira briensis* from the soils of Crete, the Greek mainland and Switzerland. Enrichment cultures were incubated at 25C for 60-120 d and periodically analysed for changes in pH and nitrite and nitrate concentrations. When nitrification was detected, cultures were serially diluted in an inorganic salts medium. After several serial transfers, the number of heterotrophic bacteria exceeded the number of nitrifying bacteria and thus additional serial dilutions were useless. In order to obtain a culture with a greater proportion of nitrifying bacteria, the organisms were grown in a pH-stat fermentor operated on a semi-continuous flow basis. After 3 months the bacterial population in the fermentor was 5×10^7 cells.ml⁻¹. Ten litres of medium were used daily and the generation time of the nitrifiers was 24 h. After this prolonged incubation, the nitrifying bacteria outnumbered the heterotrophs 100:1 and pure cultures were obtained by serial dilution.

Koops *et al.* (1976) isolated a moderate halophilic ammonia-oxidizing bacterium, *Nitrosococcus mobilis*, from brackish water. An enrichment culture was incubated at 30C until ammonia had been oxidized quantitatively to nitrite. This enrichment culture served as an inoculum for a culture grown in a pH-stat fermentor. *N. mobilis* was isolated by plating serially diluted samples of the second enrichment culture. After 3 months incubation at 30C, single colonies were picked off from the plates and inoculated in liquid media before being checked for purity.

d) Antibiotics

Gould & Lees (1960) described an antibiotic-plating technique for the isolation of *Nitrobacter* sp. in pure culture. These workers reported

that the technique is simple and rapid and that with the appropriate choice of antibiotics it may eliminate every type of heterotrophic contaminant. The organism was isolated by initially preparing an enrichment culture from soil. A loop of the enrichment culture was then spread onto a matromycin-nitrite-agar plate and growth of the small, clear, colonies of *Nitrobacter* sp. was followed under the microscope at a magnification of 100x. When growth of the colonies was reasonably advanced, usually within 10 d of plating, single colonies were picked off and transferred to terramycin-nitrite-agar plates. The colonies of *Nitrobacter* sp. appearing on this second set of plates were then inoculated into a liquid medium. Nitrite oxidation in the inoculated medium proceeded rapidly and the culture was apparently pure.

Since nitrifying bacteria are usually outnumbered by heterotrophic bacteria in their habitats, the common objective in all the isolation methods discussed, is to obtain an enrichment culture in which the former group of bacteria outnumber the latter group. This is a tedious, time-consuming and, sometimes, impossible task. A failure to obtain a high ratio of nitrifiers: heterotrophic contaminants in enrichment cultures is possibly the main reason why some workers (Gibbs, 1919; Meiklejohn, 1950) have failed to obtain pure cultures of nitrifying bacteria. Some workers (Watson, 1965; Watson, 1971; Koops *et al.*, 1976) have overcome this problem by growing nitrifiers in pH-stat fermentors.

The subsequent step of purification has two basic variations, viz., the serial dilution of the enrichment culture either in a liquid medium or transfer of the various dilutions onto solid media. In the former technique, the heterotrophic contaminants are diluted out and pure cultures of the nitrifiers are obtained in the higher dilutions. However, a prerequisite for the success of this technique is that the nitrifying

bacteria must outnumber the heterotrophic contaminants. In the latter technique, isolated colonies are transferred, either manually or mechanically, to a liquid medium specific for the type of nitrifying bacteria being isolated. A disadvantage is that it is difficult to distinguish microcolonies of nitrifying bacteria from those of contaminant bacteria (Lewis & Pramer, 1958; Watson *et al.*, 1981).

In the survey presented, a completely different approach was employed by Gould & Lees (1960) in their attempt to obtain pure cultures of *Nitrobacter* sp. It is the only report where antibiotics were used to suppress heterotrophic contaminants in the purification of nitrifying bacteria. The use of antibiotics to obtain pure cultures of nitrifying bacteria required further investigation and was included in the present study. In addition, many of the methods discussed for the isolation and purification of nitrifying bacteria were used in an attempt to obtain pure cultures of ammonia- and nitrite-oxidizing bacteria from the closed system used in this study.

e) Purity

Cultural and observational criteria have been used to determine the purity of nitrifying bacteria (Table 1). Various organic media have been used to detect the presence of heterotrophic contaminants associated with isolates of nitrifying bacteria. Some investigators (Meiklejohn, 1950; Soriano & Walker, 1968; Harms *et al.*, 1976; Koops *et al.*, 1976) have used only one type of medium whereas others (Gould & Lees, 1960; Watson, 1965; Watson, 1971; Watson & Waterbury, 1971; Watson *et al.*, 1971) have used a wider range of media. The choice of a wider range of organic media to detect the presence of heterotrophic contaminants has obvious advantages. A disadvantage in using these media for

TABLE 1. Cultural and observational criteria reported for pure cultures of nitrifying bacteria

Cultural and observational criteria	I n v e s t i g a t o r s										
	Meiklejohn (1950)	Gould & Lees (1960)	Soriano & Walker (1968)	Watson (1965)	Watson (1971)		Watson & Waterbury (1971)	Watson <i>et al.</i> (1971)	Harms <i>et al.</i> (1976)		Koops <i>et al.</i> (1976)
Organic media used for the detection of heterotrophic contaminants	Nutrient agar	Nutrient agar	Peptone agar	NB	FTM	1 s	0,5 s	1 s	Yeast extract	5 g	0,5 g
		NB		1 s	TSB	0,25 s	0,5 s	0,25 s	Beef extract	5 g	0,1 g
		Peptone 0,5% + glucose 0,1%		1 s + 0,1% tryptone	ACB	0,5 s	0,5 s	0,5 s	Peptone	5 g	0,1 g
		Dilute inorganic medium + ammonium sulphate 0,05% + peptone or glucose 0,1%		1 s + 0,1% yeast extract	NB	-	0,25 s	-	MgSO ₄ .7H ₂ O	100 mg	-
				0,25 s					KH ₂ PO ₄	20 mg	-
				0,1% tryptone					FeSO ₄ .7H ₂ O	10 mg	-
				+ 0,1% yeast extract					Sea-water	250 ml	400 ml
		The last two media were used with and without agar		+ 0,1 % acetate					Tap-water	750 ml	-
				+ 0,1% glucose					Distilled water	-	600 ml
				+ 0,1% pyruvate							
			+ 0,1% lactate								
			0,1% NO ₃								
Observational criteria used to judge the purity of nitrifiers	Colonial morphology on inorganic silica gel medium	Not reported	Colonial morphology on inorganic agar medium	Phase-contrast- and electron microscopy							

s - strength; FTM - Fluid thioglycollate medium; TSB - Trypticase soy broth; ACB - AC broth; NB - Nutrient broth.

the isolation of heterotrophs is that it may not be possible to isolate all types of heterotrophic contaminants with the range of media reported in Table 1. Also, autotrophic contaminants cannot be detected by such means.

In order to overcome the disadvantages of the cultural criteria, observational tests have also been used to determine the purity of nitrifiers (Table 1). Meiklejohn (1950) and Soriano & Walker (1968) reported using the colonial morphology of nitrifying bacteria to determine the purity of their isolates. Although Gould & Lees (1960) claimed to have isolated pure cultures of nitrifiers, they did not report any observational criteria to determine purity of the cultures. Watson (1965), Watson (1971), Watson & Waterbury (1971), Watson *et al.* (1971), Harms *et al.* (1976) and Koops *et al.* (1976) used phase-contrast- and electron microscopy to observe the purity of nitrifying bacteria. According to Lewis & Pramer (1958) and Watson *et al.* (1981) microcolonies of nitrifying bacteria have no distinguishing features to separate them from contaminants. Therefore, the use of colonial morphology as a criterion for assessing the purity of nitrifiers by Meiklejohn (1950) and Soriano & Walker (1968) is questionable. The use of phase-contrast- and electron microscopy as observational criteria are more acceptable and widely used.

In this study both cultural and observational criteria were used to judge the purity of nitrifying bacteria.

2. Enumeration

Viable autotrophic nitrifying bacteria in environmental samples are usually enumerated by indirect most-probable-number (MPN) techniques

and different incubation periods have been used by various workers (Wilson, 1927; Walker *et al.*, 1937; Molina & Rovira, 1964; Meiklejohn, 1965). Most commonly, estimates of nitrifying populations in soils have been based upon examinations of the media 21 d after inoculation (Matulewich *et al.*, 1975). In marine environments, 60 d incubation periods have been used for both ammonia- and nitrite-oxidizers by Yoshida (1967 - cited by Matulewich *et al.*, 1975). However, an earlier qualitative study by Carey (1938) used incubation periods of up to 90 d. Ideally, the incubation period should last long enough to account for all nitrifying bacteria in the inoculum capable of growth under the conditions provided. Apart from the more commonly used MPN technique, direct methods of enumeration for nitrifying bacteria have also been reported (Fliermans *et al.*, 1974; Fliermans & Schmidt, 1975; Ward & Perry, 1980).

a) Indirect

The MPN technique is the most commonly used indirect means of enumerating nitrifying bacteria. Serial dilutions of the inoculum are made in the appropriate medium which is usually dispensed in test tubes to conserve space during incubation. The test tubes are incubated at temperatures between 20-28°C for varying lengths of time. Incubation periods have been chosen arbitrarily and vary in the different investigations (Wilson, 1927; Walker *et al.*, 1937; Molina & Rovira, 1964; Meiklejohn, 1965). At the end of the incubation period the test tubes positive for nitrification are recorded and the MPN determined from statistical tables, e.g., Fisher & Yates (1957).

The periods of incubation used by various investigators to enumerate nitrifying bacteria by the MPN technique are shown in Table 2. The

incubation period ranges from 14 to 113 d, with 21 d being most frequently used. In spite of reporting higher counts of *Nitrosomonas* sp. after an incubation of 35 d than at 21 d, Molina & Rovira (1964) unscientifically chose the latter period to enumerate nitrifiers in soil samples. The 113 d incubation period used by Matulewich *et al.* (1975) represents a significant increase over those reported in earlier investigations.

TABLE 2. Incubation periods used for the enumeration of nitrifying bacteria by the MPN technique

Investigator	Sample	Incubation period (d)	
		Ammonia-oxidizers	Nitrite-oxidizers
Wilson (1927)	Soil	21	-
Walker <i>et al.</i> (1937)	Soil	21	-
Lewis & Pramer (1958)	Soil	30	30
Molina & Rovira (1964)	Soil	21	21
Meiklejohn (1965)	Soil	14	14
Smith <i>et al.</i> (1968)	Soil	21	21
Matulewich <i>et al.</i> (1975)	Fresh water, plant roots, sediment, slime	113	113

Most probable numbers of nitrifying bacteria obtained by Molina & Rovira (1964), Meiklejohn (1965) and Smith *et al.* (1968) are shown in Table 3. These results indicate that the population of nitrite-oxidizing bacteria is much smaller than that of the ammonia-oxidizers. Although estimates of nitrifying bacteria were based on the MPN technique with 5

(Molina & Rovira, 1964), 2 (Meiklejohn, 1965) and 3 (Smith *et al.*, 1968) replicate tubes at each dilution, large variations were found between replicate samples as indicated by the standard deviation and fiducial limits.

TABLE 3. Numbers of nitrifying bacteria obtained by various investigators using the MPN technique

Investigator	Sample	Incubation (d)	Nos. of bacteria.g ⁻¹ sample	
			NH ₃ -oxidizers	NO ₂ -oxidizers
Molina & Rovira (1964)	Alfalfa rhizosphere	21	17 743 ± 5 514	4 414 ± 1 165
	Alfalfa roots	21	1 109 ± 281	314 ± 123
	Corn rhizosphere	21	11 389 ± 1 338	2 166 ± 687
	Corn roots	21	400 ± 37	67,9 ± 16
Meiklejohn (1965)	Heavy soils	14	7 730 ± 1 360	1 955 ± 875
	Light soils	14	2 402 ± 691	109 ± 46
	All soils	14	4 808 ± 858	943 ± 423
Smith <i>et al.</i> (1968)	Soil	21	565 000	1 870 000 }* 171 000 } 1 100 3 620 }* 332 }

*Upper and lower fiducial limits at P = 0,05.

A different approach of enumerating nitrifying bacteria was reported by Watson (1965). The number of a pure culture of *Nitrosocystis oceanus*

was estimated by the time taken to produce a given amount of nitrite. However, with this approach the following assumptions were made, viz., there was no lag phase; a constant amount of nitrite was produced per cell division; and that there was a constant generation time. The production of nitrite in this experiment was found to be $2 \times 10^{-6} \mu\text{g-at.NO}_2\text{-N.cell}^{-1}$. The cells had a fairly uniform generation time of 24 h when grown under optimal conditions. The theoretical relationship between the size of the inoculum and the period of incubation needed for the production of $2 \mu\text{g-at.NO}_2\text{-N}$ on the basis of the above assumptions is shown in Table 4. Experimentally, when 10^2 , 10^3 or 10^4 cells were inoculated in 1 l of medium, it took 14, 11 and 8 d, respectively, to produce $2 \mu\text{g-at.NO}_2\text{-N.l}^{-1}$, partially confirming the theoretical calculations.

TABLE 4. Time required to produce $2 \mu\text{g-at.NO}_2\text{-N.l}^{-1}$ with varying numbers of *Nitrosocystis oceanus* cells as an inoculum (Watson, 1965).

Inoculum (cells.l ⁻¹)	Estimated time to produce $2 \mu\text{g-at.NO}_2\text{-N.l}^{-1}$ (d)
1	21
10	18
10^2	14
10^3	11
10^4	8
10^5	4,25
10^6	1

Engel & Alexander (1958) determined the numbers of a pure culture of *Nitrosomonas europaea* by the MPN technique. Counts were also determined by plating on an agar medium. After 4 weeks incubation in a moist chamber the colonies were counted with the aid of a stereo-microscope.

b) Direct

In 1974, Fliermans *et al.* used immunofluorescence for the direct detection of *Nitrobacter agilis* and *N. winogradskyi* in natural environments. These workers demonstrated that fluorescent antibodies can be prepared for *Nitrobacter*, that these antibodies are specific and may be used to detect and study *Nitrobacter* in various environments.

Fliermans & Schmidt (1975) combined the specific detection of *N. agilis* and *N. winogradskyi* by immunofluorescence with the estimation of their metabolic activity by autoradiography. Bacterial numbers were determined by direct microscopic counts of fluorescent antibody (FA)- stained cells on membrane filters. Their results (Fig. 2) indicate that the use of combined FA-autoradiography to detect these bacteria in the natural environment is very sensitive. Bacterial activity was assessed with respect to a given isotope in both cultural and natural systems. This FA-autoradiography technique shows distinct promise in providing the microbial ecologist with a sensitive means of evaluating single-cell activity. The possibility of using this approach in complex natural environments to follow a particular bacterium of special physiological interest is worthy of further attention.

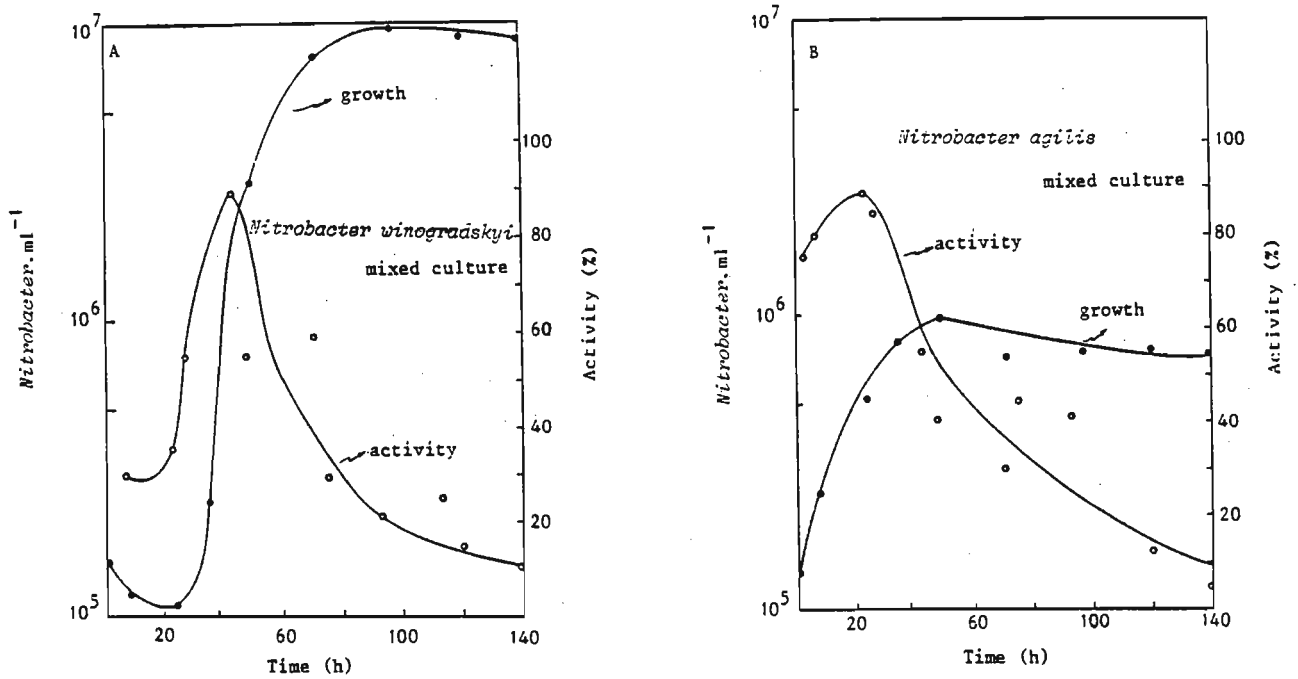


Fig. 2. Semilogarithmic plot of growth and relative activity measurements of (A) *N. winogradskyi* in a mixed culture with *N. agilis* and (B) *N. agilis* in a mixed culture with *N. winogradskyi*. Initial populations were approximately equal. *Nitrobacter* medium was amended with 1 μCi of $\text{NaH}^{14}\text{CO}_3 \cdot \text{ml}^{-1}$, and samples for activity and growth determinations were taken during the growth cycle (Fliermans & Schmidt, 1975).

Ward & Perry (1980) developed a FA-assay for a marine ammonia-oxidizing bacterium, *Nitrosocystis oceanus*. Antibodies to this organism were prepared and tested against pure cultures of marine, freshwater and soil ammonia-oxidizers, and against bacteria from natural sea-water samples. Cell counts of culture samples determined by the FA-assay agreed with haemocytometer and acridine orange counts (Fig. 3). Counting efficiency and reliability of the FA-assay were tested by comparing it with two independent methods of enumeration. Concentrated pure culture samples were counted using a haemocytometer and dilutions of these samples were counted by the FA-assay and by acridine orange direct counts.

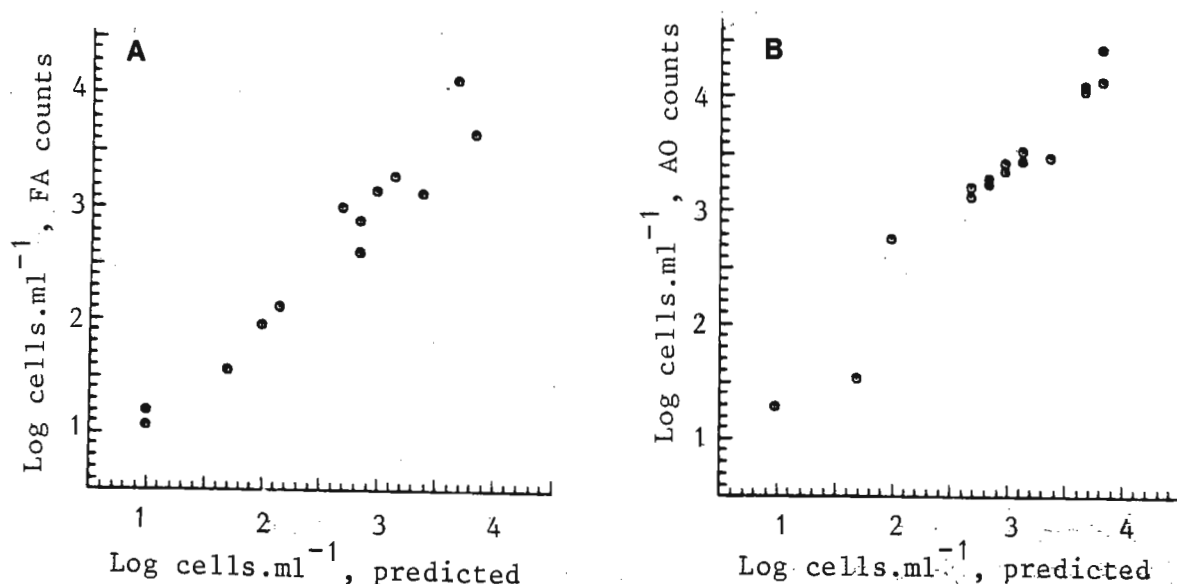


Fig. 3. Log-log transformed count data between calculated regression and predicted line with slope 1 and intercept 0 ($P \geq 0,95$). (A) FA vs predicted counts: $\text{cells.ml}^{-1} = 0,9761 \text{ FA} + 0,0839$, $\text{df} = 11$, $r = 0,9733$. (B) Acridine orange vs predicted counts: $\text{cells.ml}^{-1} = 1,003 \text{ acridine orange} + 0,3874$, $\text{df} = 15$, $r = 0,9677$ (Ward & Perry, 1980).

The slow growth rate, together with the minute and non-distinctive colonial morphology of nitrifying bacteria, makes it difficult to enumerate these organisms in natural environments. The technique of enumeration described by Watson (1965) is based upon certain assumptions which would be restricted to pure cultures under laboratory conditions. Engel & Alexander (1958) enumerated a pure culture of *Nitrosomonas europaea* by counting the colonies on an agar medium. This method cannot be applied to nitrifiers in natural environments because of their non-distinctive morphology and the fact that they would be overgrown by heterotrophic bacteria.

In comparison with culture techniques the FA-assay provides a highly selective, rapid, sensitive and direct method for the autecological study of specific microorganisms in natural environments. Together with the unique advantages of the assay in permitting the simultaneous

detection, identification and enumeration of a particular bacterium in a wide variety of ecosystems, is the disadvantage that dead or inactive cells are stained with FA as are viable cells (Fliermans & Schmidt, 1975). Another drawback of utilizing the FA-technique for enumerating natural heterogenous populations of nitrifiers is that it requires prior knowledge of the different types of nitrifying bacteria present in the sampled environment and also the availability of their specific antisera. These prerequisites were virtually impossible to meet in the present study and for this reason the FA-technique was not used for enumerating populations of nitrifying bacteria in the closed system. Therefore, the MPN technique, widely used for enumerating nitrifiers in natural environments, was the method of choice for this study. The accuracy and sensitivity of this technique for the quantification of nitrifiers is unknown because no standard exists. Qualitatively, the MPN technique provides no information on the nature of the chemolithotrophs present, nor on the species diversity of the nitrifying habitat (Fliermans *et al.*, 1974). In spite of these limitations the MPN technique appears to be the most satisfactory method available at present for the enumeration of nitrifying bacteria in their natural environments.

c) Effect of the length of incubation

Various lengths of incubation have been used to enumerate nitrifying bacteria in environmental samples by the MPN technique (Table 2). The ideal incubation period should last just long enough to account for all of the inoculum cells capable of growth under the conditions provided (Matulewich *et al.*, 1975). In the case of the MPN technique growth of nitrifying bacteria is detected indirectly by testing for either the oxidation of ammonia or nitrite in the culture medium.

The effect of incubation time on the MPN estimates of nitrifying bacteria was investigated by Matulewich *et al.* (1975). To encounter potentially disparate responses, different types of freshwater samples and sewage treatment plant effluents, which were discharged into a river, were used as inoculum sources. Serial dilutions of the inoculum sources were prepared in a phosphate buffer (pH 7.2) and portions were transferred to 10 replicate tubes per dilution for each type of medium, i.e., for ammonia- and nitrite-oxidizers. Incubation was at $28 \pm 1^\circ\text{C}$. The medium in each tube was examined at regular intervals according to the protocol in Table 5 for the oxidation of either ammonia or nitrite. The MPN estimates of nitrifying bacteria from the different sources of inoculum are shown in Table 5. In all the samples ammonia-oxidizers appeared to have completed their response within the experimental period of 113 d. The median period of incubation necessary to attain a stable maximum MPN estimate of this group was 25 d (range 20 to 55 d). Completion of the response was not observed for the nitrite-oxidizers.

In practice, the selection of appropriate periods of incubation must balance convenience against the purpose of the study. The disadvantages of an unnecessarily prolonged incubation are many, including the delayed acquisition of data that could aid in framing experimental questions. Too brief an incubation of either nitrifying group results in an underestimation of unknown and variable magnitude. The results (Table 5) of Matulewich *et al.* (1975), derived from various nonmarine aquatic habitats and effluents, suggest that a 35 d incubation period would generally be sufficient to attain the maximum estimate of ammonia-oxidizers possible under the conditions described. Obviously, it is difficult to make a specific recommendation for nitrite-oxidizers. These results also indicate that the incubation period reported in many investigations (Wilson, 1927; Walker *et al.*, 1937; Molina & Rovira,

TABLE 5. Effect of incubation time on estimates of nitrifying bacteria (adapted from Matulewich *et al.*, 1975)

Incubation time (d)	Estimate (MPN.ml ⁻¹ water or sediment)						Estimate (MPN.g ⁻¹ slime or plant material)					
	Water		Sediment 1		Sediment 2		Slime		Yellow pond lily		Pondweed	
	NH ₄ - oxid.	NO ₂ - oxid.	NH ₄ - oxid.	NO ₂ - oxid.	NH ₄ - oxid.	NO ₂ - oxid.	NH ₄ - oxid.	NO ₂ - oxid.	NH ₄ - oxid.	NO ₂ - oxid.	NH ₄ - oxid.	NO ₂ - oxid.
5	2,7	ND	329	ND	4,5	ND	16 300	ND	3 710	26,6	1 900	6,6
10	298	ND	49 300	79,2	3 990	150	64 500	54,6	271 000	147	24 800	1 690
15	2 750	ND	130 000	1 160	15 000	365	245 000	NC	2 220 000	3 360	363 000	3 840
20	3 290	ND	275 000	2 750	62 200	875	309 000	4 640	3 100 000	NC	581 000	NC
25	NC	0,9	NC	NC	101 000	2 280	464 000	5 760	NC	NC	NC	5 820
30	NC	3,3	NC	7 920	130 000	3 990	NC	13 700	NC	NC	NC	690
35	NC	4,0	NC	24 000	NC	6 220	NC	16 300	3 710 000	3 900	NC	48 000
40	NC	7,9	NC	NC	NC	15 000	NC	34 800	NC	NC	NC	NC
55	NC	27,5	NC	32 900	NC	27 500	NC	113 000	4 500 000	11 400	668 000	189 000
70	NC	62,2	NC	49 300	NC	NC	NC	NC	NC	NC	NC	248 000
103-113	NC	298	NC	62 200	NC	39 900	NC	198 000	NC	14 700	NC	350 000

ND, none detected.

NC, no change.

1964; Meiklejohn, 1965; Smith *et al.*, 1968) of nitrifying bacteria may have been too short to yield maximum estimates of these organisms. It would appear that nitrite-oxidizers have been more seriously underestimated than ammonia-oxidizers.

Lack of information in the literature reviewed makes a comparative evaluation with the findings of Matulewich *et al.* (1975) impossible. For this reason, and also to ascertain the effect of incubation time on the estimates of marine nitrifying bacteria by the MPN technique, a similar investigation was included in this work.

3. Taxonomy

The family *Nitrobacteraceae* Buchanan is listed in Part 12 (Gram-negative chemolithotrophic bacteria) of the eight edition of Bergey's Manual of

Determinative Bacteriology (Watson, 1974). Nitrifying bacteria are rod-shaped, ellipsoidal, spherical, spirillar or lobular cells without endospores. Flagella are subpolar or peritrichous and often absent. Cells derive their energy from the oxidation of ammonia or nitrite and satisfy their carbon needs by the fixation of CO_2 . Only a few strains of one species, *Nitrobacter winogradskyi*, have been shown to be facultatively chemoorganotrophic. The organisms are not parasitic. Nitrifiers are commonly found in soil, freshwater and sea-water. All the organisms placed in this family are obligate aerobes and none require organic growth factors. Cells are rich in cytochromes but no other pigments have been demonstrated.

a) Classification

The seventh edition of Bergey's Manual of Determinative Bacteriology (Starkey, 1957) lists the genera of ammonia- and nitrite-oxidizing bacteria shown in Fig. 4. All these were originally described by the Winogradsky's (Winogradsky, 1890; Winogradsky, 1892; Winogradsky, 1931; Winogradsky, 1935a; Winogradsky, 1935b; - cited by Watson, 1971). Many of the original descriptions were based on mixed cultures and were so brief that it is impossible to identify an organism from them. Some of the characteristics used for the separation of these bacteria into genera are variable and have resulted in the grouping of several morphologically unrelated strains into one genus. Moreover, none of the original strains was preserved in culture (Watson, 1971).

Subsequent to the work of the Winogradsky's, most investigators were able to isolate only members of the genus *Nitrosomonas* or *Nitrobacter*, and doubts were expressed as to the validity of the other genera (Imsenecki, 1946 - cited by Meiklejohn, 1950). Until the early 1960's, less than a dozen strains of nitrifying bacteria were preserved in pure culture, and

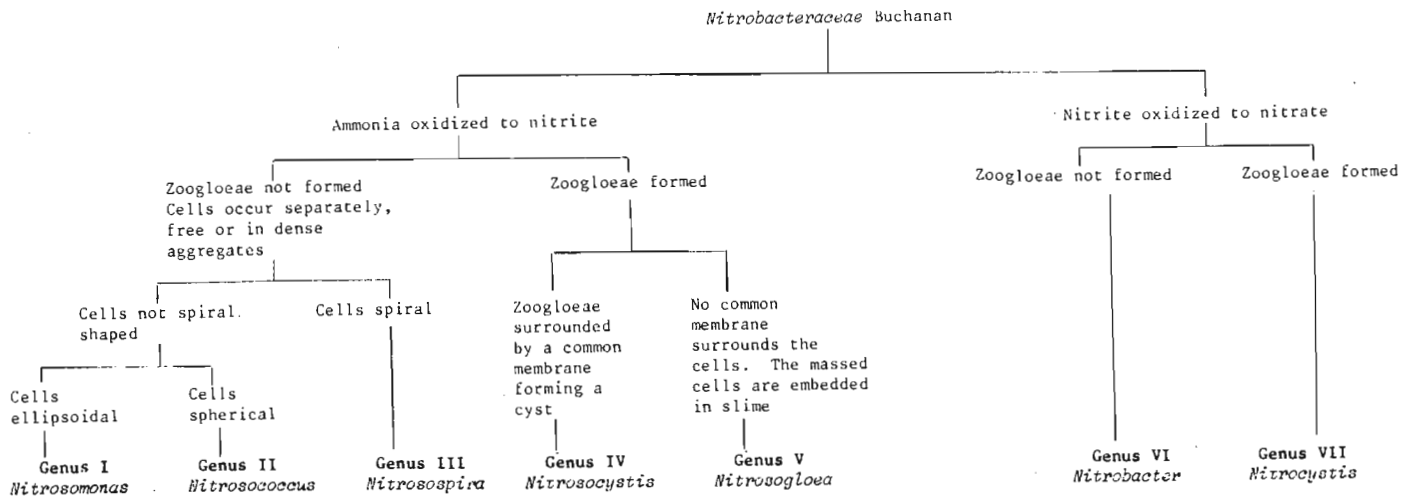


Fig. 4. Description of the genera of the family *Nitrobacteraceae* Buchanan in the seventh edition of Bergey's Manual of Determinative Bacteriology (adapted from Starkey, 1957).

all of them belonged either to the genus *Nitrosomonas* or *Nitrobacter*.

These organisms have a generation time of 8 h or more, making it difficult to isolate them in pure culture; thus few pure cultures existed.

Although the taxonomy of the bacteria in the family *Nitrobacteraceae* has long been in need of revision, the lack of cultures in the early 1960's had made such a task impossible (Watson, 1971).

Studies on nitrifying bacteria were initiated at the Woods Hole Oceanographic Institution, Massachusetts in 1957 and are continuing at present. During this period, thousands of enrichment cultures were made, and over 200 nitrifying bacteria were isolated in pure culture. These studies showed that several morphologically different types of nitrifying bacteria existed and indicated that not all the genera of nitrifying bacteria listed in Fig. 4 were valid. It was also found from these studies that the ability of cells to grow in clumps forming

zoogloea or cysts is a characteristic shared by several morphological types of ammonia- and nitrite-oxidizing bacteria (Watson, 1971). Thus, zoogloea and cyst formations were used by Starkey (1957) as taxonomic criteria, are not justifiable.

In 1971, Watson had obtained a sufficient number of pure cultures of nitrifying bacteria to warrant a revision of the classification of the family *Nitrobacteraceae* Buchanan. The proposed revision of the family *Nitrobacteraceae* Buchanan by Watson (1971) was accepted by the Judicial Commission of the International Committee on Systematic Bacteriology and is listed in the eighth edition of Bergey's Manual of Determinative Bacteriology (Watson, 1974). It is based entirely on the cellular morphology of the strains grown in pure culture, with the exception of *Nitrosococcus nitrosus* (Fig. 5).

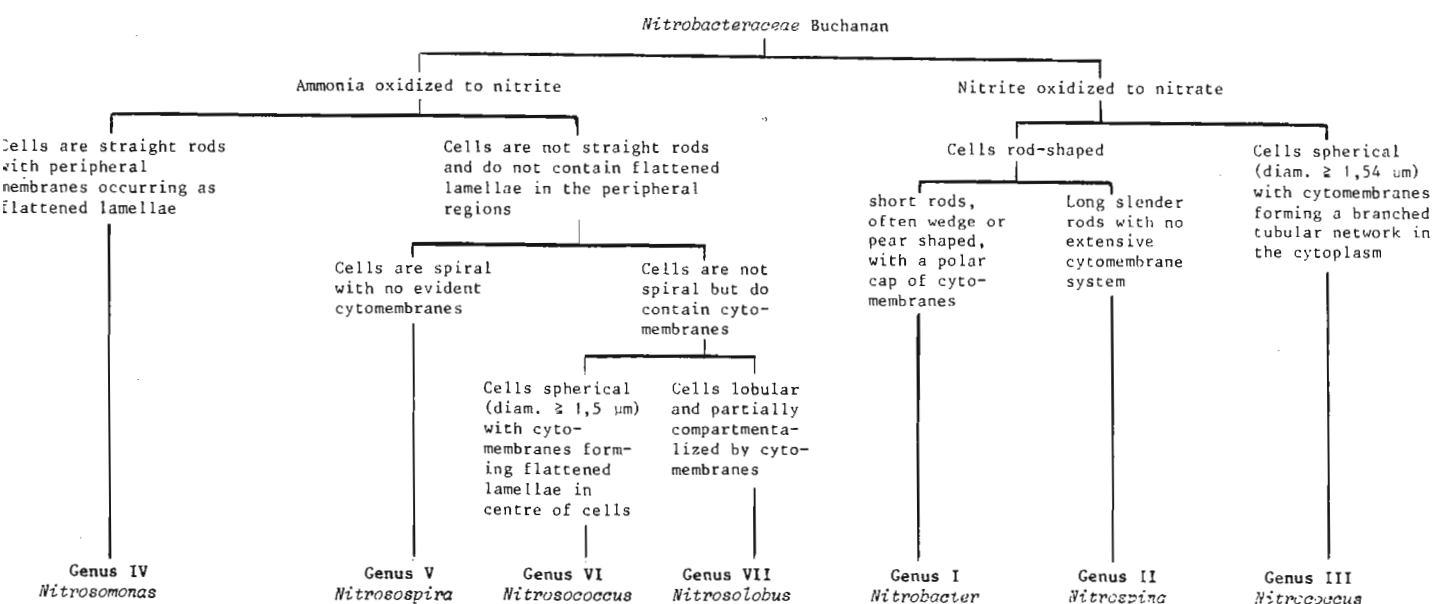


Fig. 5. Description of the genera of the family *Nitrobacteraceae* Buchanan in the eighth edition of Bergey's Manual of Determinative Bacteriology (adapted from Watson, 1974).

The classification in Fig. 4 by Starkey (1957) placed the nitrifying bacteria in seven genera: *Nitrobacter*, *Nitrosomonas*, *Nitrosococcus*, *Nitrospira*, *Nitrosocystis*, *Nitrosogloea* and *Nitrocystis*. Watson (1971) accepted the first four genera, combined *Nitrocystis* with *Nitrobacter*, placed *Nitrosocystis* and *Nitrosogloea* as genera *incertae sedis* and added three new genera: *Nitrospina*, *Nitrococcus* and *Nitrosolobus* (Fig. 5).

b) Identification

Ammonia- and nitrite-oxidizing bacteria are categorized taxonomically by their shape, size and arrangement of membranes within their cytoplasm (Tables 6 and 7). The cells may be rod-, spherical-, spiral- or lobular-shaped and many, but not all, possess extensive cytomembranes which are arranged characteristically in each species (Watson *et al.*, 1981).

TABLE 6. Characteristics of ammonia-oxidizing bacteria (adapted from Watson *et al.*, 1981)

Species	Cell morphology	Size (µm)	Cytomembranes	Habitat
<i>Nitrosomonas europaea</i>	Rod	0,8-1,0 x 1,0-2,0	Peripheral, lamellar	Soil, sewage, freshwater, marine
<i>Nitrosovibrio tenuis</i>	Curved rod	0,3-0,4 x 1,1-3,0	Lacking; occasional invagination of plasma membrane	Soil
<i>Nitrosococcus nitrosus</i>	Coccoid	1,5-1,7 (diameter)	Ultrastructure not examined	Soil
<i>Nitrosococcus oceanus</i>	Coccoid	1,8-2,2 (diameter)	Centrally located parallel bundle, lamellar	Obligately marine
<i>Nitrosococcus mobilis</i>	Coccoid	1,5-1,7 (diameter)	Peripheral, lamellar, occasionally intruding into centre of cell	Brackish water
<i>Nitrospira briensis</i>	Spiral	0,3-0,4 (diameter) 0,8-1,0 (amplitude)	Lacking	Soil
<i>Nitrosolobus multiformis</i>	Lobular	1,0-1,5 (diameter)	Internal, compartmentalizing the cell	Soil

TABLE 7. Characteristics of nitrite-oxidizing bacteria (adapted from Watson *et al.*, 1981)

Species	Cell morphology	Size (μm)	Cytomembranes	Habitat
<i>Nitrobacter winogradskyi</i>	Rod	0,6-0,8 x 1,0-2,0	Polar cap of flattened vesicles in peripheral region of the cell	Soil, freshwater, marine
<i>Nitrococcus mobilis</i>	Coccoid	1,5-1,8 (diameter)	Tubular cytomembranes randomly arranged throughout the cytoplasm	Marine
<i>Nitrospina gracilis</i>	Rod	0,3-0,4 x 2,6-6,5	Only occasional bleblike invaginations of the plasma membrane into the cytoplasm	Marine

In most cases multiplication is by binary fission, but *Nitrobacter winogradskyi* has a polar type of cell growth and multiplies by budding. In the larger spherical- and lobular-shaped cells the cytomembranes intrude into the inner region of the cells but in the short rod-shaped cells the cytomembranes are restricted to the peripheral regions. No extensive cytomembranes are evident in the elongated nitrifying bacteria (Watson, 1974).

In view of the fact that chemolithotrophs cannot be identified by the conventional methods used for heterotrophs, the presence of cytomembranes in nitrifying bacteria offers a useful means of identification (Tables 6 and 7). However, the identification of nitrifying bacteria is long and tedious and involves, sequentially, the isolation, purification and the growth and preparation of cells for electron microscopy. As already discussed, the isolation and purification is also time-consuming and difficult (Gibbs, 1919; Meiklejohn, 1950). Ultra-thin sections of the bacteria are required for electron microscopy. Cultures of nitrifying bacteria are grown for up to 3 months (Watson, 1971) before sufficient cells are obtained for processing for electron microscopy.

C) NITRIFICATION IN CLOSED SYSTEMS

The importance of chemolithotrophic bacteria lies in their ability to detoxify and transform some of the end products of metabolism of other organisms and to produce bacterial biomass for phagotrophic micro-organisms in their habitats. These chemolithotrophs include nitrifying, sulphur-oxidizing, iron-oxidizing and hydrogen bacteria. Nitrification is the process whereby ammonia, the end product of animal metabolism and ammonification, is oxidized through nitrite to nitrate by nitrifying bacteria (Sieburth, 1979).

Attempts to demonstrate non-autotrophic nitrification by seven open-sea bacteria proved unsuccessful; hence, Carlucci *et al.* (1970) consider that heterotrophic nitrification in the sea is probably of little ecological importance. According to Kinne (1976) non-biological processes play an insignificant role in the production of nitrite. Similar findings were obtained by Hamilton (1964) who reported that photo-oxidation of ammonia is insignificant in the sea. According to Hamilton (1964) photoreduction of nitrate to nitrite is more likely, but also of doubtful ecological importance. Today, it is generally accepted that nitrification is carried out predominantly by a group of chemolithotrophs, i.e. the nitrifying bacteria.

Sketchy and incomplete information has been published on nitrification in closed-systems. Some workers (Gerhardt, 1978; Mevel & Chamroux, 1981) failed to include the role of nitrifying bacteria in such investigations. For these reasons, the role of nitrifiers under such conditions formed an important aspect of this study.

1. Effect of different biological filtration treatments

In 1978, Gerhardt carried out a comparative study of different biological filtration treatments. Twenty juvenile *Penaeus indicus* Milne Edwards were cultured for approximately 2 months in 4 different types of sea-water circulatory systems: bacterial filtration, algal filtration, algal plus bacterial filtration and the exchange of 25% of the sea-water every 3 d. The bacterial- and algal plus bacterial filtration systems comprised of tanks equipped with a perforated false bottom which supported a filter medium made up of quartzite gravel chips (mean diameter 5 mm) and crushed oyster shell (mean chip size 2,5 mm). The latter system had, in addition, the circulating water passing over a tray containing *Rhizoclonium* sp. before flowing back into the tank. In the algal filtration system the algal tray, containing *Rhizoclonium* sp., represented the sole means of biological filtration as no gravel filter medium was present. The water exchange system served as controls. It had neither algae nor a gravel medium. Two separate trials were carried out with the different filtration systems.

In the first experimental trial all unionized ammonia values were within the "safe" level of $0,1 \text{ mg NH}_3\text{-N.l}^{-1}$ reported by Wickins (1976) for penaeid species (Fig. 6). However, in the second experimental trial the unionized ammonia concentrations in the algal filtration system exceeded the $0,1 \text{ mg NH}_3\text{-N.l}^{-1}$ "safe" level of Wickins (1976). However, Gerhardt (1978) observed that this increase in ammonia concentration, monitored over approximately 20 d, had no appreciable adverse effect on the prawns.

Gerhardt (1978) also reported that the filtration systems with gravel achieved nitrification in a shorter time-period and maintained lower ammonia stabilization levels than those systems without gravel

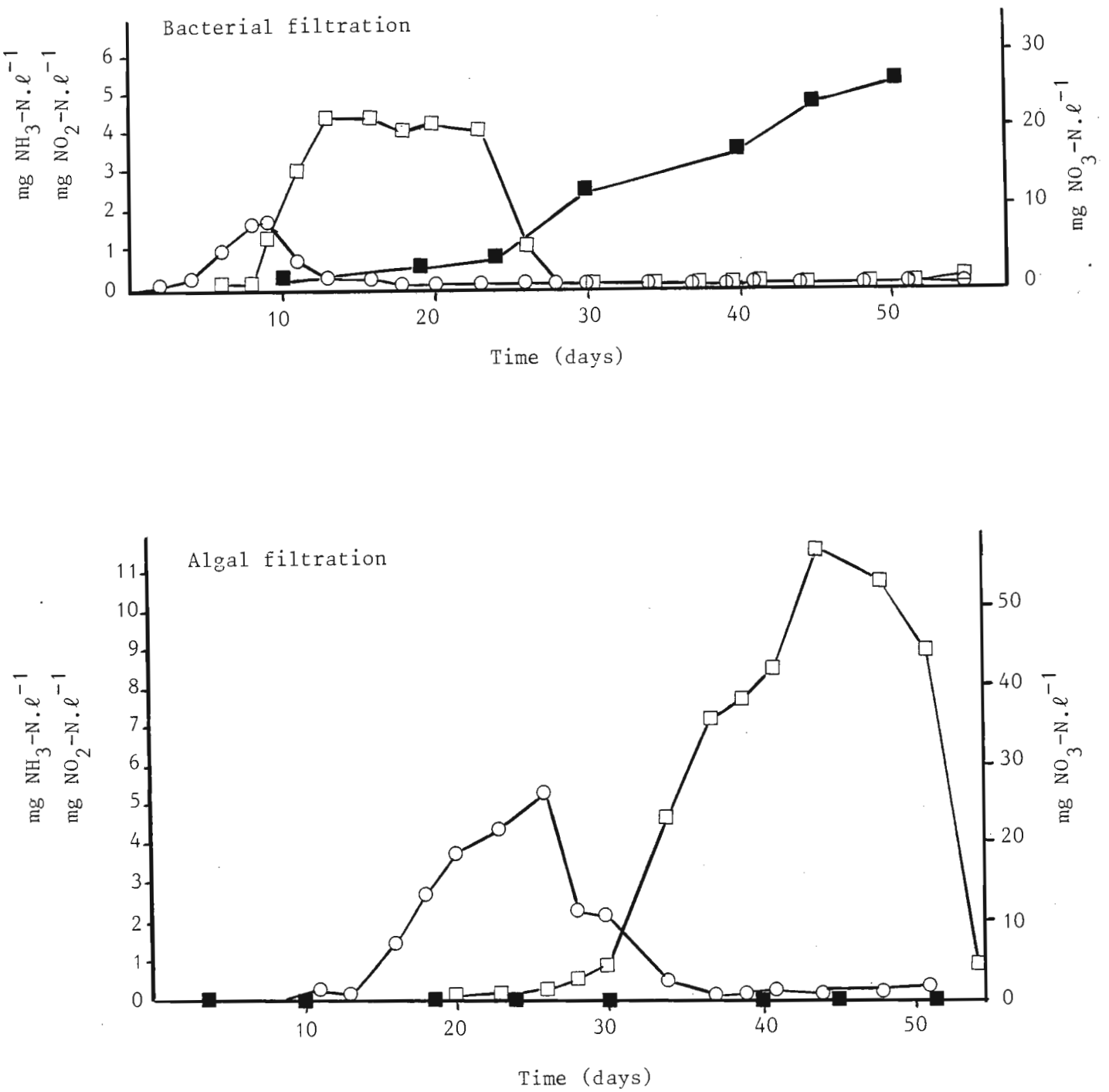


Fig. 6. Ammonia (—○—), nitrite (—□—) and nitrate (—■—) concentrations in bacterial and algal filtration systems (Gerhardt, 1978).

(Fig. 6). This was probably due to the greater surface area offered for bacterial attachment by the gravel chips. Nitrite levels stabilized at low concentrations in the bacterial- and algal plus bacterial filtration systems but failed to decrease in the algal filtration- and water exchange systems (Fig. 6). Nitrate did not accumulate in the algal filtration system whereas, in the bacterial filtration system, nitrate began to accumulate and increased substantially. Gerhardt (1978) concluded that the filamentous algae effectively removed all the nitrate produced in the algal filtration system.

In his final assessment of the 4 recirculatory systems Gerhardt (1978) pointed out that only the increased nitrate levels (Fig. 6) in the bacterial filtration system and the inorganic carbon levels in the algal filtration system constituted any real difference among the different biological filtration systems. The bacterial filtration system was considered the most suitable and convenient since a system of water exchange is labour intensive, while the system involving algae has certain disadvantages, viz., loss of condition of algae which resulted in decreased removal of nitrate, and an antibiotic effect on nitrifying bacteria.

2. Ammonia, nitrite and nitrate profiles

In closed systems, the cultivated organisms tend to modify the chemistry of the culture water. If extensive, the withdrawal of, e.g., oxygen, or release of metabolic end products may approach critical levels. One of the most obvious changes that occur in a captive body of sea-water is the increase in nitrogen compounds (Kinne, 1976). Ammonia, the major metabolic waste of most aquatic animals, is among the first forms of nitrogen to appear in the culture water. Ammonia is extremely toxic to all life-forms, even in small concentrations. Fortunately, nitrifying

bacteria oxidize ammonia to nitrite, a less toxic state, and finally to relatively harmless nitrate (King & Spotte, 1974). To date, very few reports on nitrification in the presence of prawns in closed sea water systems have been reported.

In 1981, Mevel & Chamroux studied nitrification in the presence of *P. japonicus*. For 9 months, the bacteriological and chemical changes in the sea-water and sand filter were followed, as were the growth and mortality rates of the prawns. In the three tanks used in their study, tanks 2 and 3 contained equal numbers of prawns while tank 1 served as a negative control. Prior to introducing the prawns, each system was "conditioned" for 5 weeks by the daily addition of casein extract at a rate of 1,5 g.500 ℓ^{-1} .

After introducing the prawns into the tanks, ammonia was detected, followed sequentially by nitrite and nitrate (Fig. 7). This establishment of nitrification was not observed in the control tank without prawns. The results of the chemical analyses of the sand and water, shown in Fig. 7, indicate clearly that nitrification was established in tanks 2 and 3 as soon as the prawns were introduced into the aquarium. This is shown by the succession of the mineral forms of nitrogen which become sequentially oxidized from ammonia through nitrite to nitrate, the latter tending to accumulate in the environment. This phenomenon corresponds to the classic sequence of nitrification described in closed systems (Mevel & Chamroux, 1981).

It is clear from Fig. 7 that after reaching its maximum concentration, ammonia does not reappear in the water. Soon after excretion by the prawns it is oxidized by the nitrifying bacteria which are established in the system. The appearance of ammonia and nitrite corresponds to the

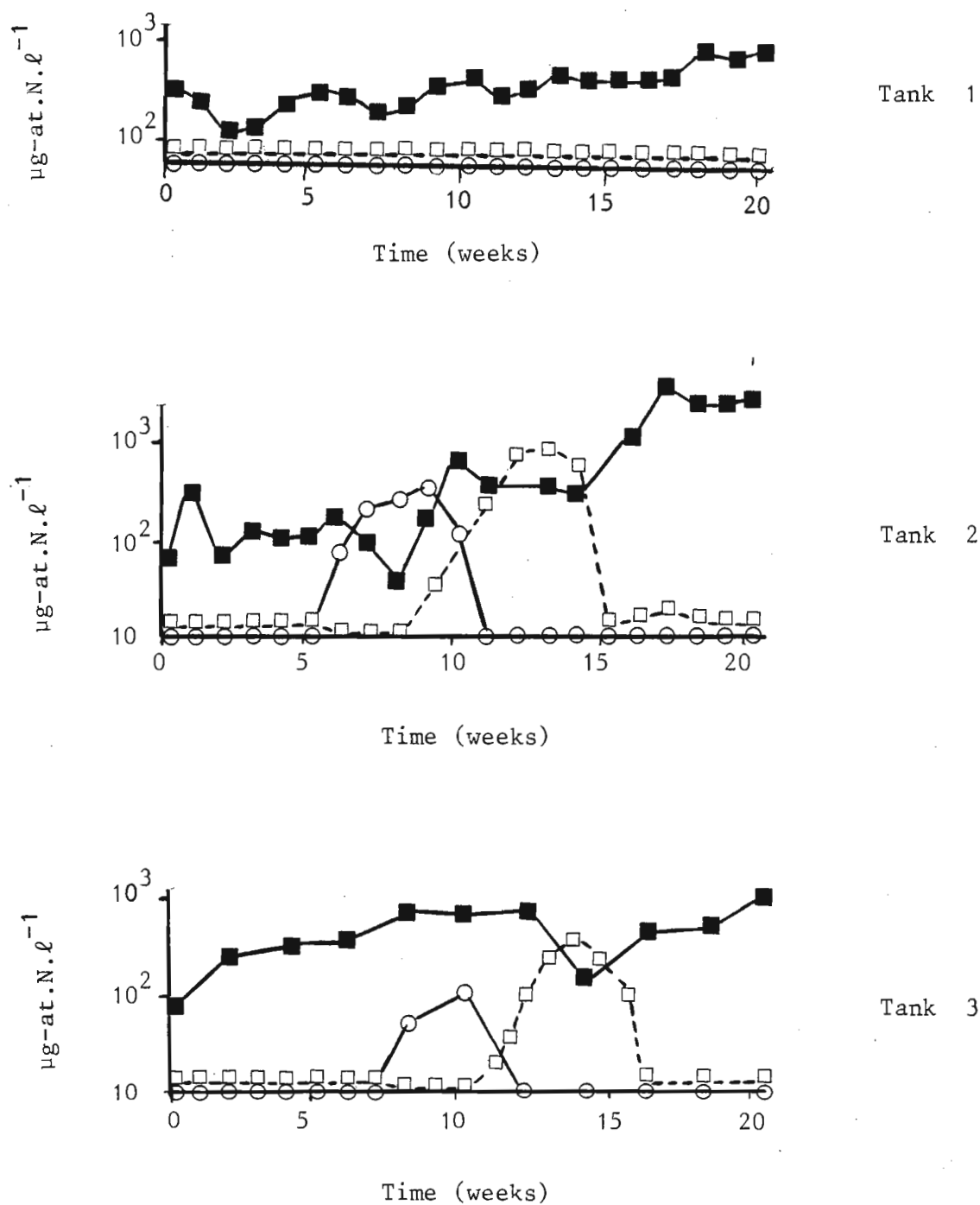


Fig. 7. Establishment of nitrification in the water and sand of the experimental ecosystems: \circ — \circ , $\text{NH}_3\text{-N}$; \square --- \square , $\text{NO}_2\text{-N}$; \blacksquare — \blacksquare , $\text{NO}_3\text{-N}$; (Mével & Chamroux, 1981). Tank 1 = control tank without prawns. Tank 2 and 3 = experimental tanks with prawns.

conditioning phase of the system, during which time the nitrifying populations establish themselves. When the system has an adequate nitrifying population, nitrate is the only nitrogenous salt which can be detected in the water.

Gerhardt (1978) reported a similar pattern of nitrification in the experimental system using a biological sand filter. These results are discussed earlier in this review (Fig. 6). Kawai *et al.* (1964) also obtained a similar pattern of nitrification in a closed system with seawater that was used to cultivate fish. The system required about two months before an equilibrium of nitrifying activity was reached, i.e., when the rates of oxidation of ammonia and nitrite were high enough to maintain these nitrogen compounds at a minimum stable level.

The above-mentioned reports on nitrification in the presence of prawns are based on the chemical analysis of ammonia, nitrite and nitrate in the culture water. No attempt was made to investigate either the numbers or types of nitrifying bacteria present during the investigation.

Although Mevel & Chamroux (1981) included a bacteriological analysis in their investigation on nitrification, this was confined to the total heterotrophic microflora and did not take into account the chemolithotrophic nitrifying bacteria. Gerhardt (1978) did not include any bacteriological analyses in his investigation. For any investigation on nitrification to be complete it should include, in addition to the chemical analysis of ammonia, nitrite and nitrate, bacteriological analyses to determine both the numbers and types of nitrifying bacteria in the environment under investigation. As pointed out already, the enumeration and identification of nitrifying bacteria are both difficult and time-consuming. It is possible, that for these reasons, the aspects of enumeration and identification have been conveniently omitted from previous studies on nitrification in closed systems.

The present study was undertaken to investigate the chemical and bacteriological aspects of nitrification that are necessary to obtain a better understanding of such events in a closed system.

D) DETERMINATION OF AMMONIA, NITRITE AND NITRATE IN SEA-WATER

Numerous methods have been reported for the determination of ammonia, nitrite and nitrate. However, relatively fewer methods have been reported for these determinations in sea-water, and even fewer methods are applicable to saline closed systems. An attempt was made to review and select methods that would be most appropriate for the detection of such nitrogenous compounds in the closed system under investigation.

1. Ammonia

In all the methods used for the determination of ammonia-nitrogen ($\text{NH}_3\text{-N}$) the sum of NH_4^+ and NH_3 is recorded. Most of the earlier procedures also included varying amounts of labile organic nitrogen compounds, such as trimethylamine and the amino acids in the determination (Koroleff, 1976).

Prior to 1965, determinations of ammonia in sea-water had rarely been performed as a routine procedure on oceanographic expeditions due to the lack of a rapid and sensitive method. In much of the early work, ammonia was determined in sea-water by distillation and Nesslerization. Later studies showed that this procedure yields erroneously high results because of the breakdown of organic nitrogen compounds at the high alkalinity and elevated temperature used for the distillation (Riley, 1965).

Some workers (Wattenberg, 1929; Cooper, 1933; Robinson & Wirth, 1934 - all cited by Riley, 1965) have determined ammonia in sea-water by the Nessler method which was modified to prevent the interference of magnesium salts either by complexing them with tartrate or removing them through a preliminary precipitation. Since the coloured complex formed with the Nessler's reagent is colloidal and tends to coalesce, these methods lack precision (Riley, 1965).

The highly sensitive pyridine-pyrazolone colorimetric method described by Kruse & Mellon (1951 - cited by Riley, 1965) for the determination of ammonia in industrial wastes has been applied by Atkins (1957 - cited by Riley, 1965) to the analysis of sea-water. The salts in sea-water were found to depress the absorbance obtained from a given quantity of ammonia by approximately 40% (Riley, 1965). In the interest of speed and simplicity, and to minimize the chance of contamination, the determination of ammonia in sea-water should be by a direct procedure rather than by distillation (Strickland & Parsons, 1960). The classical "direct" method of Wattenberg (1929 - cited by Riley, 1965) is tedious, time-consuming and has a poor sensitivity. Most of these criticisms are no longer valid in the extraction method of Kruse & Mellon (1953 - cited by Riley, 1965). However, the method is tedious and there is some uncertainty in the determination of ammonia concentrations below $0,5 \mu\text{g-at.NH}_3\text{-N.l}^{-1}$ (Strickland & Parsons, 1960).

Three procedures were chosen for further investigation, viz., the alkaline hypochlorite-arsenite method (Strickland & Parsons, 1960), the phenolhypochlorite method (Solórzano, 1969) and the ammonia electrode method (Orion Research Inc., 1978). According to Riley & Chester (1971), the first two methods are widely used for the analysis of ammonia in sea-water. The ammonia electrode method is recommended by Gilbert & Clay (1973) and Thomas & Booth (1973).

a) Alkaline hypochlorite-arsenite method

Strickland & Parsons (1960) evaluated the method of Richards & Kletsch (1964), prior to its publication, and found it sensitive, convenient and reliable. The procedure outlined by Strickland & Parsons (1960) contains only minor modifications of the original method. A major drawback of this procedure is that, in addition to ammonia, a considerable fraction of the nitrogen, present in dissolved amino acids, is also determined. The ammonia in sea-water is oxidized to nitrite by alkaline hypochlorite at room temperature and the excess oxidant is destroyed by the addition of arsenite. The nitrite is then determined by the method of Shinn (1941) as applied to sea-water by Bendschneider & Robinson (1952). The procedure of Strickland & Parsons (1960) is reported to have a range of detection between $0,1-10 \mu\text{g-at.N.l}^{-1}$ with a 10 cm cuvette.

At a concentration of $3 \mu\text{g-at.N.l}^{-1}$ the correct value lies in the range:

$$\text{mean of } n \text{ determinations} \pm 0,25.n^{-\frac{1}{2}} \mu\text{g-at.N.l}^{-1}$$

At a concentration of $1 \mu\text{g-at.N.l}^{-1}$ the correct value lies in the range:

$$\text{mean of } n \text{ determinations} \pm 0,11.n^{-\frac{1}{2}} \mu\text{g-at.N.l}^{-1}.$$

The time required for maximum oxidation of ammonia depends on salinity and temperature. In distilled water, 1,5 h is required, whereas oxidation is slower in sea-water and 3,5 h is allowed for complete oxidation between 20-30°C. Only about 5% of any urea nitrogen in the water is oxidized but a considerable fraction (approximately 80%) of the nitrogen in some amino acids is recorded as ammonia. Any nitrite initially present in the samples is unchanged by the analytical procedure.

Thus a correction for its presence can be made accordingly (Strickland & Parsons, 1960).

b) Phenolphychlorite method

The blue colour of indophenol obtained at high pH by the reaction of ammonia, phenol and hypochlorite was first reported by Berthelot (1859 - cited by Koroleff, 1976). Since then many modifications have been attempted (Solórzano, 1969). Applications of this method to the direct determination of ammonia in sea-water have failed because of the interference of magnesium and calcium ions. Riley (1953) overcame this problem by using the reaction for the analysis of ammonia in distillates of sea-water. Although the procedure is reproducible and sensitive, it has the disadvantage of requiring distillation.

Newell (1967) developed a method using chloramine-T, instead of hypochlorite, to produce the blue indophenol colour with ammonia. The method is lengthy and involves extraction of the colour by n-hexanol (Solórzano, 1969). Emmet (1968 - cited by Solórzano, 1969) produced the blue colour of indophenol after buffering the solution at pH 9,5. This method, although reproducible, has poor sensitivity and requires extreme attention to detail for its success (Solórzano, 1969).

The phenolphychlorite method of Solórzano (1969) uses high pH but eliminates interference due to precipitation by complexing magnesium and calcium with citrate. No distillation or extraction is necessary and a batch of determinations can be completed within an hour. Working conditions are not critical and there is no interference from other trivalent forms of nitrogen. The procedure has a detectability range of 0,1 to 10 $\mu\text{g-at.N.l}^{-1}$ with 10-cm cuvettes. The standard deviation

of a set of samples containing a concentration of $3 \mu\text{g-at.NH}_3\text{-N.l}^{-1}$ is $0,07 \mu\text{g-at.N.l}^{-1}$. Solutions of amino acids and urea in sea-water were added to sea-water samples at a concentration of $3 \mu\text{g-at.N.l}^{-1}$.

Negligible responses were obtained, the absorbances being equivalent to less than $0,1 \mu\text{g-at.N.l}^{-1}$ of ammonia, from glycine, alanine, lysine, histidine, arginine, tyrosine, glutamic acid and urea. No colour resulted from the addition from nucleic acids (Solórzano, 1969).

c) Ammonia electrode method

The ammonia selective electrode uses a hydrophobic gas permeable membrane to separate the sample solution from that of the internal electrode. The membrane prevents ionic species from entering the internal solution, thus eliminating any false response due to dissolved ions. However, high levels of ions can affect the solubility of ammonia to some degree and must be diluted. Dissolved ammonia in the sample solution diffuses through the membrane until the partial pressure of ammonia is the same on both sides of the membrane. In any given sample the partial pressure of ammonia will be proportional to its concentration (Orion Research Inc., 1978).

Thomas & Booth (1973) investigated the use of an Orion Ammonia Electrode Model 95-10 in the determination of ammonia in surface waters, sewage samples and sea-water. These workers claimed that, in addition to being inexpensive, the ammonia electrode offers the following advantages: minimal sample and reagent preparation prior to analysis; a wide concentration range; precision and accuracy comparable to accepted methods; and speed (maximum of $5 \text{ min.sample}^{-1}$). A comparison was made between the electrode method and the indophenol blue method on a Technicon Auto Analyzer, using river and sewage

samples. The results differed by an average of 1,2% and 4,6% for river and sewage samples respectively. Sea-water samples were spiked with various concentrations of ammonium chloride in order to investigate the ability to recover ammonia in a saline environment. As shown in Table 8, the mean recovery was 97,1% which was in agreement with the 98% recovery obtained with river samples.

TABLE 8. Ammonia measurement in saline waters (Thomas & Booth, 1973)

Concentration of spike added (mg.NH ₃ -N.l ⁻¹)	Concentration of spiked samples mg.NH ₃ -N.l ⁻¹	Recovery (%)
0	0,027	
0,030	0,052	83,3
0,050	0,079	104,0
0,080	0,109	103,0
0,100	0,131	104,0
0,200	0,215	94,0
0,500	0,500	94,6
		97,1 Mean

In order to test the precision of the electrode, seven replicates at four different concentrations were run in the following order: high, low, high intermediate and low intermediate. The precision expressed as standard deviation was $\pm 0,038$ at $1,002 \text{ mg.NH}_3\text{-N.l}^{-1}$; $\pm 0,003$ at $0,132 \text{ mg.NH}_3\text{-N.l}^{-1}$; $\pm 0,017$ at $0,768 \text{ mg.NH}_3\text{-N.l}^{-1}$; and $\pm 0,007$ at $0,188 \text{ mg.NH}_3\text{-N.l}^{-1}$. Comparatively, the precision of the indophenol blue method was $\pm 0,040$; $\pm 0,008$; $\pm 0,030$; and $\pm 0,009 \text{ mg.NH}_3\text{-N.l}^{-1}$, respectively. Thomas & Booth (1973) concluded that the precision of

the electrode method was comparable to that of the indophenol blue method.

The accuracy of the electrode method was also tested by Thomas & Booth (1973) by running two series of seven spiked replicates at two different concentration levels and, from this, calculating the percent recovery. The low and the low intermediate river water samples from the precision determination, mentioned above, were spiked with 0,20- and 0,50 mg.NH₃-N.ℓ⁻¹. As seen in Table 9 the accuracy of the electrode method is equivalent to that of the indophenol blue method at comparable concentration levels.

TABLE 9. Accuracy of the ammonia electrode method (Thomas & Booth, 1973)

Method	Concentration of sample (mg.NH ₃ -N.ℓ ⁻¹)	Concentration of spike added (mg.NH ₃ -N.ℓ ⁻¹)	Concentration of sample + spike (mg.NH ₃ -N.ℓ ⁻¹)	Recovery (%)
Electrode	0,188	0,50	0,659	96
	0,132	0,20	0,301	91
Indophenol blue	0,224	0,50	0,674	93
	0,163	0,20	0,340	94

In 1973, Gilbert & Clay used an Orion Ammonia Electrode Model 95-10 for the determination of ammonia in aquarium- and sea-water. Electrode reproducibility was evaluated using samples with varying ammonia levels, from different marine life display tanks at the new England Aquarium. The results of these workers indicate that the relative precision of

these analyses was fairly constant over a wide concentration range. The standard deviation ranged from $\pm 0,11$ at 4,68 ppm NH_3 to $\pm 0,003$ at 0,045 ppm NH_3 . Expressed as a percentage of the mean, the standard deviation was 2% and 7% respectively. It is clear from the results of Thomas & Booth (1973) and Gilbert & Clay (1973) that the electrode method provides an accurate means of analyzing ammonia in sea-water. However, Gilbert & Clay (1973) reported that analyses at ppb and sub-ppb levels are time-consuming and that direct analysis by this method is impractical.

2. Nitrite

a) Griess-Ilosvay method

Several titrimetric-, colorimetric- and gasometric methods have been proposed for the determination of nitrites (Burns, 1970 - cited by Tabatabai, 1974). Among these methods, the well-known colorimetric reaction of Griess-Ilosvay has proved the most specific, sensitive and accurate method available for the determination of nitrites in soil extracts and water samples. This method is based on the reaction of nitrites with a primary aromatic amine in an acidic solution with the formation of an intermediate diazonium salt. The diazonium salt is then treated with an aromatic compound containing amino or hydroxyl substituents to form a conjugated azo dye that can be measured photometrically (Tabatabai, 1974).

Details of the Griess-Ilosvay technique used by different workers vary somewhat and there are a number of discrepant statements concerning the relative efficiency of the several modifications (Warrington, 1881; Ilosvay, 1881; Weston, 1905 - all cited by Barnes & Folkard,

1951). These differences relate to the composition of the reagents, particularly with respect to their acid content, rate of colour development, and intensity and stability of the coloured dye (Barnes & Folkard, 1951). With the objective of recommending a rapid, sensitive and reliable method, Barnes & Folkard (1951) compared variations of the Griess Ilosvay technique described by various workers (Rakestraw, 1936; Wattenberg, 1937; American Public Health Association, 1942 - all cited by Barnes & Folkard, 1951; Rider & Mellon, 1946). The colour development in the method of Wattenberg (1937) is slow, with maximum intensity being reached only after 90 min. The other 3 methods require about 15 min for full colour development. The Rider & Mellon method (1946) is the most sensitive. The maximum absorption is 18% greater than that given by the Wattenberg method (1937) and 15% greater than the other two methods. In assessing the efficiency of the various techniques examined, Barnes & Folkard (1951), considered the time taken to reach a stable maximum, the value of this maximum, i.e., the sensitivity, and its reproducibility. The Rider & Mellon method (1946) was found to be superior in all respects to the other methods examined.

All the methods examined by Barnes & Folkard (1951) determine nitrites by diazotizing sulphanilic acid and coupling with α -naphthylamine to produce a red azo dye. Shinn's method (1941) for the determination of nitrites uses sulphanilamide as the diazotizing reagent and N-(1-naphthyl)-ethylenediamine hydrochloride as the coupling reagent. The method proposed by Shinn (1941) depends upon the addition of a sufficient excess of sulphanilamide for the complete utilization of all nitrites present. It is claimed that this method gives a colour that is more rapidly developed and more stable. The range of nitrite determination by this procedure is between 0,0025-0,005 mg nitrite in a final volume of 50 ml.

Barnes & Folkard (1951) modified the method of Shinn (1941) by changing the concentrations of the diazotizing and coupling reagents. The resultant rate of colour development is extremely rapid with full colour being obtained in 10 min at 25C. This is relatively much faster than the procedure of Rider & Mellon (1946) while the absorption is greater by about 5%. The absorption is also greater than that in Wattenberg's technique (1937 - cited by Barnes & Folkard, 1951) by 23%. Satisfactory calibration curves were obtained by Barnes & Folkard (1951) for the range 0 to $121 \mu\text{g}.\text{NO}_2\text{-N}.\ell^{-1}$.

Bendschneider & Robinson (1952) applied Kershaw & Chamberlain's (1942) modification of the Shinn (1941) procedure to the determination of nitrite in sea-water. Calibration curves were prepared over the range 0,05 to 1,00 $\mu\text{g-at}.\text{NO}_2\text{-N}.\ell^{-1}$. The results indicated that the colour produced is directly proportional to the nitrite concentration in conformity with Beer's law. Bendschneider & Robinson (1952) also showed that their proposed method is much more sensitive than the then accepted method which used sulphanilic acid and α -naphthylamine as reagents. In addition, salinity had no effect on colour intensity. Identical optical densities were obtained for nitrite solutions of equal concentration in either distilled water or synthetic sea-water as confirmed by Grasshoff (1976a). The method of Bendschneider & Robinson (1952) is now generally accepted for nitrite analysis in sea-water (Strickland & Parsons, 1960; Grasshoff, 1976a). According to Strickland & Parsons (1960), this procedure can detect nitrite within the range of 0,01 to 2,5 $\mu\text{g-at}.\text{N}.\ell^{-1}$ with 10 cm cuvettes.

The correct value at the 1 $\mu\text{g-at}.\text{N}.\ell^{-1}$ levels lies in the range:

$$\text{mean of } n \text{ determinations} \pm 0,032.n^{-\frac{1}{2}} \mu\text{g-at}.\text{N}.\ell^{-1}.$$

The correct value at the $0,3 \mu\text{g-at.N.l}^{-1}$ levels lies in the range:

$$\text{mean of } n \text{ determinations} \pm 0,023.n^{-\frac{1}{2}} \mu\text{g-at.N.l}^{-1}.$$

b) Nitrogen oxide electrode method

Orion Research Inc. has made available another method for the determination of nitrite by developing the Nitrogen Oxide Electrode Model 95-46. Tabatabai (1974) used the nitrogen oxide electrode to determine nitrite in soil extracts and non-saline water. The electrode procedure was evaluated by comparing its results for nitrite, in the above-mentioned samples, with those obtained by the colorimetric method of Griess-Ilosvay as modified by Barnes & Folkard (1951). Figures 8 and 9 show the results obtained by the electrode procedure as plotted against the $\text{NO}_2\text{-N}$ values obtained by the colorimetric procedure for soil extracts and water samples, respectively.

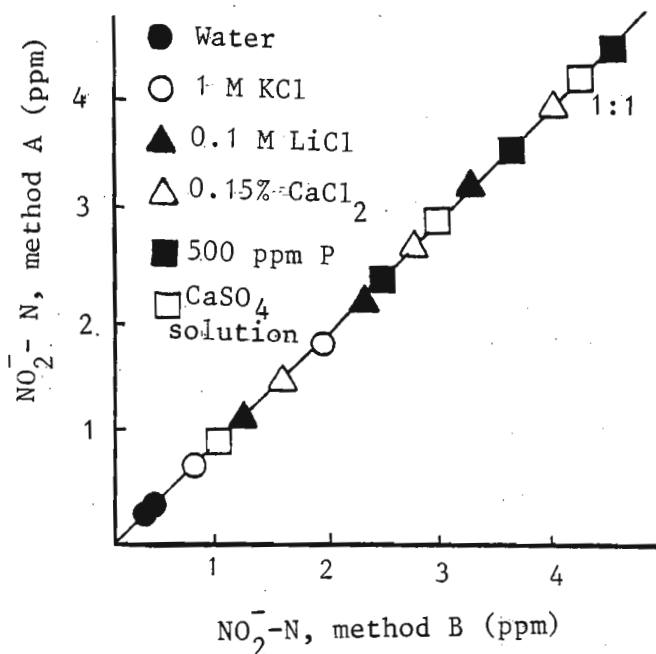


Fig. 8. Comparison of $\text{NO}_2\text{-N}$ values obtained for soil extracts by the electrode method² (A) and Griess-Ilosvay method (B) (Tabatabai, 1974).

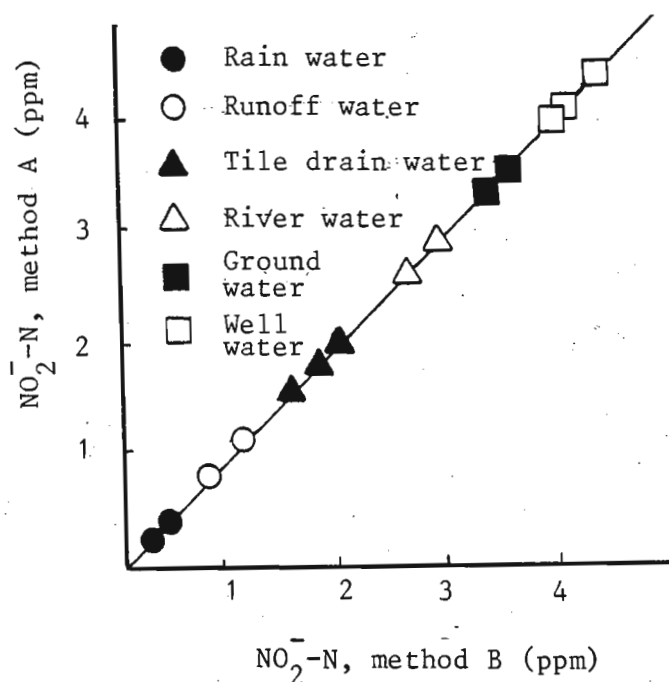


Fig. 9. Comparison of $\text{NO}_2\text{-N}$ values obtained for water samples by the electrode method (A) and Griess-Ilosvay method (B) (Tabatabai, 1974).

The electrode procedure gave a quantitative (99,2-100,8%) recovery of nitrite added to the various soil extracts and water samples studied. No interference was detected in analyses of NaNO_2 solutions containing 0,5, 1,0 or 5,0 ppm of $\text{NO}_2\text{-N}$ when these solutions were made 0,1 M with respect to the following cations and anions: NH_4^+ , Na^+ , K^+ , Ag^+ , Ca^{2+} , Mg^{2+} , Cu^{2+} , Hg^{2+} , NO_3^- , Cl^- , SO_4^{2-} and PO_4^{2-} . The standard deviation ranged from 0,34 to 1,2 as a percentage of the mean of six replicates (Tabatabai, 1974).

3. Nitrate

Determinations of nitrate in sea-water is much more difficult than in fresh water, owing to interference by chloride ions which prevent the use of most of the commoner colorimetric reagents such as phenol-disulphonic acid or 2,4-xyleneol (Mullin & Riley, 1955).

Much of the early work on the determination of nitrate in sea-water was based on its reduction to ammonia which was separated by distillation and determined by Nessler's method (Riley, 1965). Harvey (1926 - cited by Riley, 1965) has criticized these methods on the grounds that the drastic conditions usually used for the reduction and distillation can cause the decomposition of amino acids and other organic nitrogen compounds present in the water.

Owing to interference from dissolved inorganic and organic compounds, it does not appear to be feasible to determine nitrate photometrically in sea-water by using its strong absorption band in the far ultra-violet region (Riley, 1965). Such a procedure, devised by Armstrong (1963), measures the absorbance of nitrosyl chloride spectrophotometrically. While this procedure is good for small samples containing high concentrations of nitrate, the use of concentrated sulphuric acid and lack of sensitivity limit its use in routine analysis (Wood *et al.*, 1967).

In addition to interference by chloride ions, most of the colorimetric reagents commonly used for the determination of nitrate in fresh water lack the sensitivity necessary for the determination of small concentrations present in sea-water. The absorptiometric methods which have been described for the direct determination of nitrate in sea-water are based on its reaction in a medium of strong sulphuric acid with certain readily oxidized organic compounds such as diphenylamine, diphenylbenzidine, resorcinol and strychnidine, which lead to the formation of intensely coloured oxidation products (Riley, 1965). However, no reliable and trouble-free method has been developed with these colorimetric reagents (Grasshof, 1976b).

The most sensitive and generally applied methods for nitrate determination in sea-water are based on the reduction of nitrate to nitrite which is then determined by means of the formation of an azo dye. The reduction can be performed by either a homogenous or heterogenous reaction (Grasshoff, 1976b). A method based on the homogenous reduction of nitrate by hydrazine in the presence of copper ions as catalyst has been proposed by Mullin & Riley (1955). The reduction of nitrate in a heterogenous system using either metallic zinc or cadmium has been used by Morris & Riley (1963) and Wood *et al.*, (1967). Reduction of nitrate with zinc powder is sensitive to temperature and it is also necessary to centrifuge or filter each sample (Wood *et al.*, 1967). Morris & Riley (1963) found that the reduction with zinc is not very reproducible and that only 85-90% of the nitrate is reduced to nitrite. Mullin & Riley (1955) used hydrazine, in the presence of cupric ion as a catalyst, to reduce nitrate to nitrite. This method requires 24 h at constant temperature for the reduction and is erratic and unreliable in the presence of high nitrite concentrations (Morris & Riley, 1963).

The use of cadmium has proved to be advantageous in the routine analysis of nitrate in sea-water (Wood *et al.*, 1967). The method of Morris & Riley (1963) reproducibly reduces $91 \pm 1\%$ of the nitrate to nitrite when samples are percolated through a column of amalgamated cadmium filings. This method has negligible salt error, minimal temperature effect and there are no interfering ions in open sea-water. The column decreases in efficiency with use because of the formation of $\text{Cd}(\text{OH})_2$, and possibly CdCO_3 (Wood *et al.*, 1967).

Grasshoff (1964 - cited by Wood *et al.*, 1967) modified the method of Morris & Riley (1963) by lengthening the reducing column and treating the metal with an alkaline ammonium chloride solution to complex the oxidized Cd^{++} ion, thus prolonging the life of the column. This

procedure was further refined by Strickland & Parsons (1965) by replacing the ammonium chloride with the tetrasodium salt of ethylenediamine tetra-acetic acid (EDTA) for the column conditioner. Washing of the cadmium filings with nitric acid before amalgamation was also suggested by Strickland & Parsons (1965). This appears to pit the Cd, thus providing a greater surface area.

The method described by Wood *et al.* (1967) uses copper as the cathode, rather than mercury, in the reduction of nitrate to nitrite. EDTA is used to complex the Cd^{++} ion and nitrite determination is carried out according to the method of Shinn (1941) as applied to sea-water by Bendschneider & Robinson (1952). Wood *et al.* (1967) have claimed the method to be accurate for nitrate concentrations from 0,05 to $60 \mu\text{g-at.N.l}^{-1}$. This is the concentration range of nitrate normally found in sea-water. The concentration levels and standard deviations for ten replicates at each level are:

$$40,00 \pm 0,26 \mu\text{g-at.NO}_3^--\text{N.l}^{-1}, \text{ using 1 cm cuvettes}$$

$$20,00 \pm 0,12 \mu\text{g-at.NO}_3^--\text{N.l}^{-1}$$

$$1,00 \pm 0,04 \mu\text{g-at.NO}_3^--\text{N.l}^{-1}, \text{ using 10 cm cuvettes.}$$

From the literature, the method of Wood *et al.* (1967) seemed most appropriate for the analysis of the closed system water in this study. Nevertheless, this method was evaluated for its application to the closed system water before being selected as the method of choice for nitrate analysis.

II. MATERIALS AND METHODS

A) DESIGN OF THE CLOSED SYSTEM

The extremely corrosive nature of sea-water determined the type of material to be used in the closed system. With reference to cost and anti-corrosive properties, plastic was the obvious choice with metals being excluded. However, sea-water has the ability to dissolve a wide variety of toxic substances (Atz, 1964) such as dioctyl phthalate plasticizer which leaches from new plastic in contact with water (Carmignani & Bennett, 1976). Since most aquatic animals are extremely sensitive to such substances (Gerhardt, 1978) an attempt was made to leach these toxic substances from materials used in the closed system. Leaching was carried out by circulating 500 l sea-water in the closed system (Fig. 10) for 4 weeks. Thereafter, this water was discarded. The closed system comprised 8 replicate tanks, a reservoir, a coarse sand filter with a foam prefilter, and a circulation system (Fig. 10).

1. Tanks

Low density polyethylene tanks with dimensions of 30(W) x 36(H) x 79(L) cm were used. Each tank has a capacity of 85 l. The tanks were lined on the outside with black plastic sheets to reduce light penetration and thereby limiting algal growth in the culture water. A 7 mesh.cm⁻¹ plastic sheet was placed on top of each tank to prevent the prawns from jumping out and minimize macro-contamination from the surroundings.

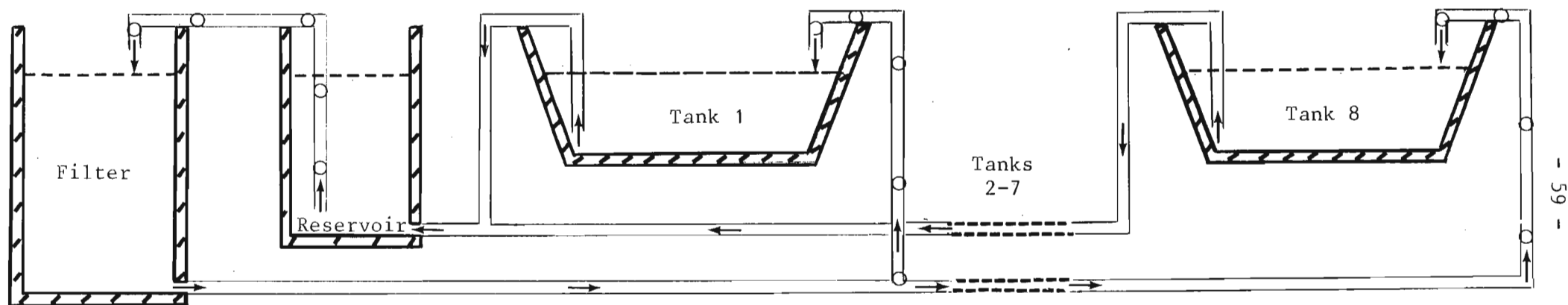


Fig. 10. Closed system: → - indicates direction of water circulation;
 O - indicates airlift.

2. Circulation

Circulation was achieved through a system of airlifts and siphons (Fig. 10). Airlifts were used to return filtered sea-water to the individual tanks. Siphons were linked to the reservoir and thus maintained the water levels in the tanks and reservoir in equilibrium. The circulation pipes were made of polyvinylchloride, either 2 cm or 5 cm in diameter. A siphon was created and maintained in each tank by using a piece of silicon tubing, 1 cm in diameter, which then entered a polyvinylchloride pipe. Air for lifting water was obtained from a central compressed air supply. As a precaution, this air was filtered through a cartridge filter assembly [Ultra-Gard (Pty) Ltd] designed to remove oil and particulate matter. This system circulated the culture water at a rate of $106,6 \text{ l.h}^{-1}$.

3. Culture water

Sea-water was obtained from the Durban Dolphinarium and Aquarium which pumps sea-water from the Indian Ocean. Six hundred litres of sea-water, diluted with distilled water to obtain a salinity of 30‰ and filtered through Whatman No. 1 filter paper, were added to the closed system. Salinity was monitored daily with a YSI (Yellow Springs Instrument Co., Inc.) Model 33 S-C-T meter fitted with a conductivity cell and a precision YSI thermistor temperature sensor combined into a single probe. Salinity was maintained at 30‰ by the addition of distilled water to compensate for daily evaporation losses of approximately 2 l. Control-O-Mat aquarium heaters [Aquarium Accessories (Pty) Ltd] were used to maintain the culture water temperature at $28 \pm 2^\circ\text{C}$.

4. Filter

Circulating sea-water from the tanks entered a reservoir before being air-lifted into the filter (Fig. 11). The filter comprised of coarse sand particles, 2-5 mm in diameter, placed 20 cm deep in a plastic container, 30(H) x 29(D) cm. This container was placed within a larger plastic container, 40(H) x 31(D) cm, which collected the filtered water before it circulated back to the tanks. The base of the smaller container was perforated and a 7 mesh.cm⁻¹ plastic sheet prevented the loss of sand particles from the filter. A 12 mm thick sheet of open cell foam plastic made of polyester [Sondor Holdings (Pty) Ltd] was placed on top of the sand filter and acted as a prefilter. This foam prefilter was highly efficient in preventing the sand filter from blocking up with debris. In addition, this prefilter was re-usable after cleaning.

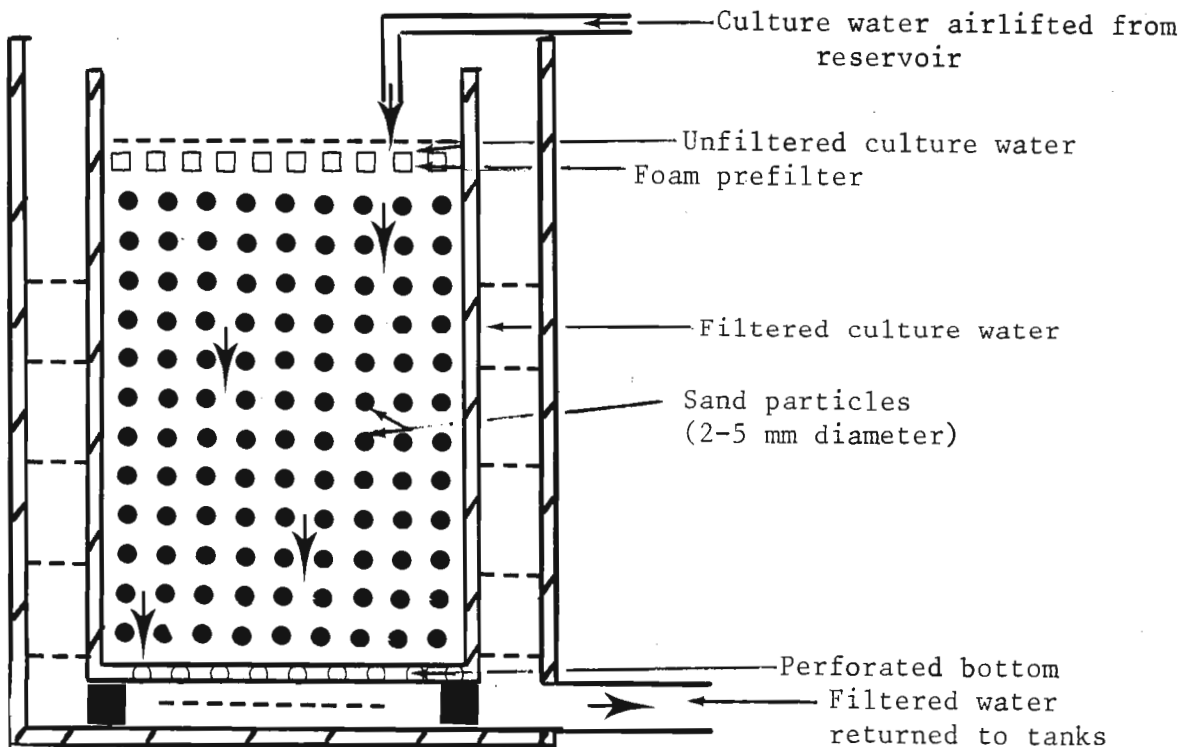


Fig. 11. Biological filter.

5. *Penaeus monodon*

Juvenile *P. monodon* Fabricius were supplied by D. Cook from the Fisheries Development Corporation Prawn Research Unit at Amatikulu, Natal. Juveniles were used for this study because they readily accept formulated pellets and move into an exponential growth phase (D. Cook, 1980 - pers. comm.). The prawns were transported by road in 2 x 30 l plastic buckets, each containing 20 l of sea water saturated with oxygen. Each bucket contained 27 prawns and the journey took approximately 2 h. Two prawns died in transit. The average wet mass of the juveniles was 0,68 g within a range of 0,5-1,0 g. Six juveniles were transferred to each of the eight experimental tanks. This initial stocking density of 17 g.m^{-2} is considered as semi-intensive aquaculture (D. Cook, 1980 - pers. comm.). This stocking density was chosen since the wet mass could be expected to rise to $\pm 350 \text{ g.m}^{-2}$ at the end of the experimental period when the wet mass of the prawn would be $\pm 13 \text{ g}$ (D. Cook, 1980 - pers. comm.). Four prawns were kept in a separate tank for use as replacements in case of accidental loss of the experimental prawns.

The prawns were fed twice daily with "Higashi-Maru" prawn pellets at a rate of 3% of their wet mass. These pellets, imported from Japan and supplied by the Fisheries Development Corporation Prawn Research Unit at Amatikulu, were used as the principal source of food for the prawns. Once weekly, this diet was substituted with I & J pet food (Irvin & Johnson Ltd) which is deboned fish. This was fed at a rate of 10% of the wet mass of the prawns. Every morning unconsumed food was removed from the tanks to minimize fouling of the culture water. Growth was monitored by determining the wet mass of the prawns.

B) PHYSICO-CHEMICAL ANALYSIS OF THE CULTURE WATER

Physico-chemical analyses were carried out fortnightly, for 22 weeks, on the culture water to monitor changes in water quality. These included determinations of the pH, dissolved oxygen (DO), biochemical oxygen demand (BOD) and ammonia-, nitrite- and nitrate concentration. Prior to determining the concentrations of nitrogenous compounds in the culture water, various methods were evaluated for their accuracy and reproducibility. Freshly collected sea-water and 16 week old culture water served as samples for these evaluations. Thereafter, the most appropriate method was chosen for the respective determinations in the culture water. Samples for chemical analyses were taken randomly from each tank after gentle agitation of the culture water. In order to determine whether or not one sample per culture tank was representative of that tank, five samples were taken from each of three tanks during the experimental trial. The standard deviations of these samples, expressed as a percentage of the mean, were: 4,1% for ammonia by the method of Solórzano (1969); 3,75% for nitrite by the method of Strickland & Parsons (1960); and 4,92% for nitrate by the method of Wood *et al.* (1967). These results justified collecting only one sample per tank for the analysis of these compounds in the culture water. For these analyses, the following precautions were taken to minimize contamination from extraneous sources:

analytical reagents were used wherever possible;

double, glass distilled water, which was passed through a Milli-Q system, was used for the chemical analyses and in the washing of glassware;

all glassware were soaked in an alkaline cleanser (5% Extran MA 01) for 24 h, rinsed in tap water and then soaked in two changes of distilled water.

1. pH

Ten millilitre samples of culture water were collected randomly from 3 different tanks. In order to obtain a representative sample the water in the sample tank was carefully agitated before sampling. The pH was determined immediately after collection by using an Orion Model 601/ Digital Ionalyzer fitted with a combination glass electrode.

2. Dissolved oxygen

DO was determined 5 cm below the water surface at the following positions in the closed system, viz., the point at which filtered sea-water entered the tank, at random points in the tank, and the reservoir. These measurements were carried out monthly throughout the experimental period.

Measurements of DO were obtained with a YSI Model 54A Dissolved Oxygen Meter fitted with a YSI 5739 Dissolved Oxygen Probe. The probe has a thin permeable membrane which is stretched over the polarographic sensor to isolate the sensor elements from the environment while allowing gaseous exchange. When a polarizing voltage is applied across the sensor, oxygen that has passed through the membrane reacts at the cathode causing a flow of current. The membrane passes oxygen at a rate proportional to the pressure difference across it. These measurements were corrected for salinity according to the following formula (Yellow Springs Instrument Co., Inc., 1978):

$$A = M[1,0 - \frac{[Cs/Co(Sf - So)]}{Sf}]$$

Where:

A = Actual DO of sample in ppm

M = Measured DO with instrument

Co = Salinity of sea-water (36,11‰)

Cs = Salinity of sample

Sf = DO of saturated fresh water at 760 mm pressure and
at the sample temperature

So = DO of saturated ocean water at 760 mm pressure and
at the sample temperature

3. Biochemical oxygen demand

The method of Colwell *et al.* (1975) was used to determine the BOD of the culture water. Water samples were collected fortnightly from each tank for 22 weeks for BOD analysis. Two dilutions (5% and 15%) of the samples were prepared with dilution water and incubated for 5 d in the dark at room temperature. The dilution water was filter sterilized sea-water saturated with oxygen. DO was measured according to the procedure described in B) 2 above.

4. Ammonia

The term "ammonia" is used in different contexts in biological literature and hence requires definition. Ammonia is defined as the gas NH_3 , and ammonia nitrogen, as nitrogen in NH_3 or ammonium, NH_4^+ . The latter combines with a number of elements but in biological excretions occurs as ammonium hydroxide, which dissociates in a reversible reaction into ammonia and water. In most biological studies, and in the present study, the term "ammonia" refers to both NH_3 and NH_4^+ .

Three methods, viz., the phenolhypochlorite - (Solórzano, 1969), alkaline hypochlorite-arsenite- (Strickland & Parsons, 1960) and the electrode method (Orion Research Inc., 1978), were evaluated for the determination of ammonia.

a) Phenolhypochlorite method

Principle

The reaction of ammonia, phenol and hypochlorite at high pH, produces the blue colour of indophenol which absorbs strongly at 640 nm.

Reagents

Phenol-alcohol solution: 10 g phenol dissolved in 100 ml

95% v/v ethyl alcohol.

Sodium nitroprusside solution: 1 g sodium nitroprusside

dissolved in 200 ml distilled water and stored in an amber bottle for not longer than one month.

Alkaline solution: 100 g tri-sodium citrate and 5 g sodium hydroxide dissolved in 500 ml distilled water.

Sodium hypochlorite solution: a solution of laboratory grade sodium hypochlorite was made up to 1,5 N. This solution decomposes slowly and its strength was tested monthly as follows:
2 g potassium iodide were added to a 150 ml Erlenmeyer flask containing 50 ml distilled water, 1 ml hypochlorite solution and 0,5 ml concentrated hydrochloric acid. This mixture was then titrated with a thiosulphate solution (12,5 g sodium thiosulphate in 500 ml distilled water) until no yellow colour remained. The hypochlorite solution should be discarded when less than 12 ml of thiosulphate solution is used (Strickland & Parsons, 1960).

Oxidizing solution: 100 ml alkaline solution was mixed with 25 ml hypochlorite solution and used within 2 h.

Potassium bromide solution: 1,5 g potassium bromide dissolved in 250 ml distilled water.

Standard ammonia solution: 0,1 g anhydrous ammonium sulphate, previously dried at 110°C for 1 h, dissolved in 1000 ml distilled water. One millilitre chloroform was added as a preservative. Five millilitres of this solution (approximately 7,5 $\mu\text{g-at.N}$) were transferred to a 500 ml volumetric flask containing 10 ml potassium bromide solution, and diluted to volume with distilled water. The resulting ammonia concentration is equivalent to 15 $\mu\text{g-at.N.l}^{-1}$.

Procedure

Two millilitres phenol solution, 2 ml sodium nitroprusside solution and 5 ml oxidizing reagent were added successively to 50 ml sample. The sample was mixed thoroughly after each addition. The colour was allowed to develop at room temperature (22-30°C) for 1 h and the absorbance recorded at 640 nm in a Beckman Model 25 spectrophotometer with a 1-cm path-length cuvette. A standard graph was prepared in the concentration range from 0,1 to 10 $\mu\text{g-at.N.l}^{-1}$. Ammonia concentrations of unknown samples were determined directly from the standard graph.

b) Alkaline hypochlorite-arsenite method

Principle

The ammonia in sea-water is oxidized to nitrite by alkaline hypochlorite at room temperature and the excess oxidant destroyed by the addition of arsenite. The nitrite then reacts with sulphanilamide in an acid solution. The resulting diazo compound reacts with N-(1-naphthyl)-ethylene-diamine and forms a highly coloured azo dye with peak absorbance at 543 nm.

Reagents

Sodium hydroxide solution: 330 g sodium hydroxide pellets dissolved in 2000 ml distilled water.

Sodium hypochlorite solution: refer to the phenolhypochlorite method in 4 a) above.

Sodium arsenite solution: 20 g arsenic trioxide and 30 g sodium hydroxide pellets dissolved in 100 ml distilled water.

The solution was cooled and diluted to 500 ml with distilled water.

Potassium bromide solution: 1,5 g potassium bromide dissolved in 250 ml distilled water.

Oxidizing reagent: for sea-water samples, 0,75 ml sodium hypochlorite solution was added to 100 ml sodium hydroxide solution.

for distilled water standards and blanks, 1,5 ml sodium hypochlorite solution was added to 100 ml sodium hydroxide solution.

These solutions were used within 2 h.

Acidifying solution: Concentrated hydrochloric acid was diluted with an equal volume of distilled water. Fifty millilitres distilled water and 10 ml oxidizing reagent were added to a 125 ml Erlenmeyer flask containing 2 ml sodium arsenite solution and three drops of a bromothymol blue indicator solution. This solution was then titrated with the diluted hydrochloric acid until the colour changed from blue to yellow. Titrations were carried out in triplicate and varied by $< 0,1$ ml of acid. The mean volume of the acid was recorded to the nearest 0,05 ml. If x ml (about 7-8 ml) acid was used, 2,2 g sulphanilamide was dissolved in 200 ($x + 0,15$) ml acid and diluted to 2000 ml with distilled water. This solution was freshly prepared whenever a new sodium hydroxide solution was used.

N-(1-naphthyl)-ethylenediamine dihydrochloride solution: 0,50 g of the dihydrochloride was dissolved in 500 ml distilled water.

Standard ammonia solution: refer to the phenolhypochlorite method in 4 a) above.

Procedure

Ten millilitres oxidizing reagent were added to a 150 ml Erlenmeyer flask containing 50 ml sample, standard or blank. This solution was mixed and allowed to stand at room temperature (20-30C) for 3,5 h. The flask was covered with parafilm to reduce contamination by atmospheric ammonia. Two millilitres sodium arsenite solution and 10 ml acidifying solution were then added with successive mixing. After a period of between 3-8 min, 1 ml naphthyl-ethylenediamine solution was added and the contents of the flask mixed immediately. Absorbance was measured between 10-120 min later with a Beckman Model 25 spectrophotometer. Absorbance readings were carried out in a 1 cm path-length cuvette against a distilled water reference at 543 nm. Determinations were carried out within the range 0,1 to 10 $\mu\text{g-at.N.l}^{-1}$. The measured absorbance was corrected by that of a reagent blank and the ammonia-nitrogen concentration calculated from the expression:

$$\mu\text{g-at.N.l}^{-1} = F \left[E - \frac{0,65 \cdot C}{F'} \right]$$

where:

E is the corrected absorbance;

C is the concentration of nitrite in the sample ($\mu\text{g-at.N.l}^{-1}$);

F' is the factor described in the nitrite method in 5 a) below; and

F is the factor $\left[F = \frac{3,0}{E_s - E_b} \right]$

where

E_s is the mean extinction of 4 standard ammonia solutions, each containing 3,0 $\mu\text{g-at.N.}\ell^{-1}$.

E_b is the mean extinction of the 4 blank solutions which contained distilled water and 1 ml potassium bromide solution.

c) Ammonia electrode method

Principle

The ammonia electrode uses a gas-permeable membrane to separate the sample solution from the internal electrode solution. Dissolved ammonia in the sample solution diffuses through the membrane until the partial pressure of ammonia is the same on both sides of the membrane. In any given sample the partial pressure of ammonia will be proportional to its concentration.

An Orion Model 95-10 Ammonia Electrode was used. The electrode potential was measured with an Orion Model 701 A Digital pH/mv meter. Sample agitation, throughout the procedure, was achieved on a magnetic stirrer using a Teflon-coated stirring bar. A piece of styrofoam insulated the samples from any heat generated by the magnetic stirrer. Ammonia determinations were carried out by the direct measurement technique described in the Instruction Manual for the ammonia electrode (Orion Research, Inc., 1978).

Reagents

10 M Sodium hydroxide solution: 40 g sodium hydroxide pellets dissolved in 80 ml distilled water and then diluted to 100 ml in a volumetric flask.

0,1 M Ammonium chloride standard solution: 0,535 g ammonium chloride, previously dried at 110C for 1 h, dissolved in 50 ml distilled water and then diluted to 100 ml in a volumetric flask.

Procedure

Serial dilutions of the 0,1 M ammonium chloride solution were made in distilled water to prepare 10^{-2} M, 10^{-3} M and 10^{-4} M standard solutions. The electrode was placed in 100 ml of 10^{-3} M standard to which 1 ml 10 M NaOH was added. With the function switch set to REL MV the reading on the meter was adjusted to zero with the calibration control. Thereafter, the electrode was rinsed with distilled water and placed in 100 ml of 10^{-4} M standard containing 1 ml 10 M NaOH. The electrode potential was recorded after a stable reading was displayed. This procedure was repeated for the 10^{-2} M standard solution. A calibration curve was prepared by plotting the millivolt reading (linear axis) against concentration (log axis) on standard 4-cycle semi-logarithmic paper. After rinsing, the electrode was placed in 100 ml sample containing 1 ml 10 M NaOH. The millivolt reading was recorded and the sample concentration was determined from the calibration curve. A slope of 58 ± 2 mv is acceptable for a calibration curve obtained as described above.

5. Nitrite

Two methods, viz., the Griess-Ilosvay method (Strickland & Parsons, 1960) and nitrogen oxide electrode method (Orion Research Inc., 1979), were

evaluated for the determination of nitrite.

a) Griess-Ilosvay method

Principle

Nitrite reacts with sulphanilamide in an acid solution, producing a diazo compound which reacts with N-(1-naphthyl)-ethylenediamine to form a highly coloured azo dye. This is measured spectrophotometrically at 543 nm.

Reagents

Sulphanilamide solution: 5 g sulphanilamide, dissolved in 50 ml concentrated hydrochloric acid and 300 ml distilled water. This was then diluted to 500 ml with distilled water.

N-(1-naphthyl)-ethylenediamine dihydrochloride solution: refer to the alkaline hypochlorite-arsenite method in 4 b) above.

Standard nitrite solution: 0,345 g anhydrous sodium nitrite, previously dried at 110°C for 1 h, was dissolved and then diluted with distilled water to 1 ℓ in a volumetric flask. This solution was stored in a dark bottle with 1 ml chloroform as preservative. Ten millilitres of this solution ($1\text{ ml} \equiv 5\text{ }\mu\text{g-at.N}$) were diluted to 1 ℓ with distilled and used the same day. One millilitre of the working solution was equivalent to $1,0\text{ }\mu\text{g-at.N.}\ell^{-1}$ in 50 ml distilled water.

Procedure

One millilitre sulphanilamide reagent was added to 50 ml sample in a 125 ml Erlenmeyer flask. This was mixed and allowed to react for

2-10 min. One millilitre naphthyl-ethylenediamine solution was then added and the solution mixed immediately. Between 10-120 min later, the absorbance of the solution was measured at 543 nm in a 1-cm path-length cuvette against distilled water. The measured absorbance was corrected by subtracting both turbidity and reagent blanks. To obtain the turbidity blank 1 ml sulphanilamide solution was added to 30 ml sample. Reagent blanks were prepared with distilled water as described for the samples. Nitrite-nitrogen concentration ($\mu\text{g-at.N.l}^{-1}$) was calculated from the expression:

$$\mu\text{g-at.N.l}^{-1} = \text{corrected absorbance} \times F'$$

where:

$$F' = \frac{2,00}{E_s - E_b}$$

and

E_s is the mean extinction of the 4 standard solutions; and

E_b is the mean extinction of the two blanks described below.

Four standard solutions, containing 2,0 ml of the dilute nitrite solution, were each made up to 50 ml in a volumetric flask. Two 50 ml aliquots of distilled water served as blanks. Nitrite determinations on these six solutions were carried out as outlined for the samples.

b). Nitrogen oxide electrode method

Principle

The nitrogen oxide electrode uses a hydrophobic gas-permeable membrane to separate the sample solution from the internal electrode solution. The gaseous anhydrides of nitrous acid, from an acidified nitrite-containing sample, diffuse through the membrane until the partial

pressures of the nitrogen oxides (NO , NO_2 , N_2O_3 and N_2O_4) are the same on both sides of the membrane. In any given sample the partial pressure of the gaseous anhydrides will be proportional to their concentration.

An Orion Model 95-46 Nitrogen Oxide Electrode was used. Electrode potential was measured with an Orion Model 701 A Digital pH/mv meter, Sample agitation, throughout the procedure, was achieved with a magnetic stirrer and a Teflon coated stirring bar.

Reagents

0,1 M Sodium nitrite solution: 0,69 g anhydrous sodium nitrite, previously dried at 110C for 1 h, was dissolved and diluted to 100 ml with distilled water in a volumetric flask.

Acid buffer solution: 190 g sodium sulphate dissolved in 800 ml distilled water in a 1 l volumetric flask. Thereafter, 53 ml concentrated (96-97%) sulphuric acid were added slowly and the solution diluted to volume with distilled water.

Procedure

The 0,1 M sodium nitrite solution was serially diluted with distilled water to prepare 10^{-3} M, 10^{-4} M and 10^{-5} M standard solutions. Ten millilitres acid buffer were added per 100 ml standard. The electrode was placed in the 10^{-5} M standard with the function switch of the meter set to mv and the electrode potential recorded as soon as a stable reading was displayed. The electrode was then rinsed with distilled water and the procedure repeated to obtain the electrode potential for the 10^{-4} M and 10^{-3} M standards. The millivolt readings (linear axis) of the 3 standards were plotted against concentration

(log axis) on standard 4-cycle semilogarithmic paper. Samples were transferred to a 150 ml beaker and 10 ml acid buffer were added to each 100 ml sample. The millivolt reading was recorded and the concentration of nitrite determined directly from the standard calibration curve.

6. Nitrate

Only the method of Wood *et al.* (1967) warranted evaluation for the determination of nitrate.

Principle

The nitrate sample, treated with a solution of tetrasodium ethylenediamine tetra-acetate, is reduced to nitrite in a column of copperized cadmium filings. Nitrite is then determined by diazotization according to the Griess-Ilosvay method in 5 a) above.

Reagents

Nitrate standard solution: 0,1264 g potassium nitrate previously dried at 110C for 1 h, was dissolved in distilled water and diluted with the same to 250 ml. Two drops of chloroform served as a preservative. One millilitre of this solution is equivalent to 5 µg-at.N.

Nitrite standard solution: 0,0863 g sodium nitrite, previously dried at 110C for 1 h, was dissolved in distilled water and diluted to 250 ml. Two drops of chloroform served as a preservative. One millilitre of this solution is equivalent to 5 µg-at.N.

Copper sulphate solution, 0,08 M: 20 g hydrated copper sulphate, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, were dissolved in 1 l distilled water.

EDTA solution, 0,1 M: 38 g of the tetrasodium salt of ethylenediamine tetra-acetic acid were dissolved in distilled water and diluted to 1 ℓ.

HCl, 2 N: 85 ml of 12 N HCl were diluted to 500 ml with distilled water.

HNO₃, 0,3 N: 10 ml of 15,4 N HNO₃ were diluted to 500 ml with distilled water.

HCl, 0,0015 N: 0,125 ml of 12 N HCl was diluted to 1 ℓ with distilled water.

Column wash solution: 1 ml of the EDTA solution added to 50 ml of 0,0015 N HCl.

Preparation of columns

Cadmium granules were cut and sifted to obtain a fraction that was retained on a 0,5 mm screen but passed through a 2 mm screen. Approximately 40 g of this fraction were washed with 2 N HCl in a separatory funnel and then rinsed with distilled water. This was followed by washing with 0,3 N HNO₃, rinsing with distilled water and then washing with 2 N HCl to remove the NO₃ ion. A thorough rinsing with distilled water followed. The cadmium was then treated with 100 ml of the copper sulphate solution in a special washing bottle (Fig. 12). It was well shaken and then flushed with distilled water, taking care not to expose the copperized cadmium to air.

A small plug of glass-wool was placed in the bottom of the column (Fig. 13) which was then filled with distilled water. The copperized cadmium was introduced slowly by inverting the copperizing vessel (Fig. 12) into the column reservoir and tapping the column during the process to settle

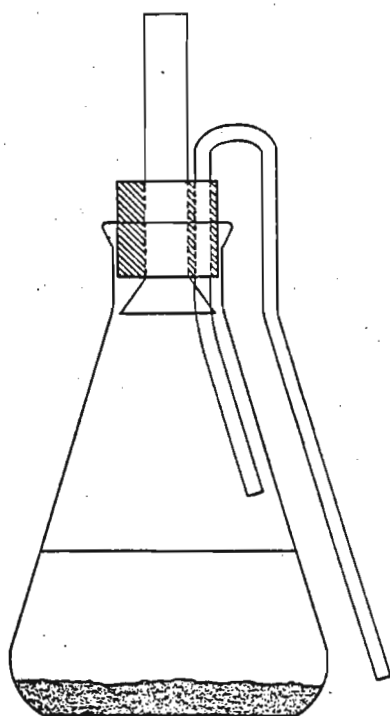


Fig. 12. Vessel used to introduce copperized cadmium to the column after treating with copper sulphate (Wood *et al.*, 1967).

the cadmium. To prevent the column from running dry the cadmium was not filled above the discharge tip (Fig. 13). Suction was used to remove the copper and fine cadmium that settled on top of the column. A small glass-wool plug placed on top of the column acted as a filter. The column was then washed with approximately 50 ml of the wash solution and allowed to stand at least 24 h before use. During this period the wash solution was renewed 4 times. Four litres of distilled water containing $60 \mu\text{g-at. NO}_3\text{-N.l}^{-1}$ and 20 ml EDTA solution. l^{-1} were passed through the column to condition it for the optimal reduction of nitrate.

Procedure

One millilitre of the EDTA solution was added per 50 ml sample in a 50 ml stoppered measuring cylinder and shaken. Any excess sample or

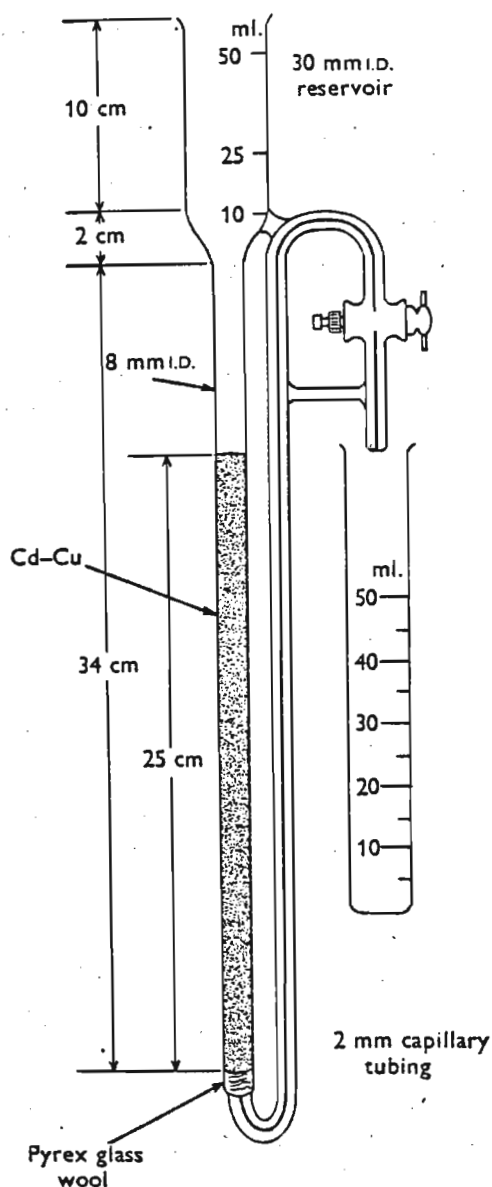


Fig. 13. Column used to reduce nitrate to nitrite in sea-water, with collecting cylinder (Wood *et al.*, 1967).

wash solution was removed from the column reservoir with suction to about 5 mm above the cadmium. A 3-4 ml portion of the sample was added to the column reservoir to rinse out the previous sample. Approximately 2 ml were allowed to percolate into the column before removing the remaining solution by suction. The remainder of the sample was then added to the reservoir and a 50 ml graduated measuring cylinder was placed under the column discharge tip. At the 50 ml level the flow rate was approximately $6 \text{ ml} \cdot \text{min}^{-1}$ or $1 \text{ drop} \cdot \text{sec}^{-1}$. Several 5 ml portions of the sample, passing through the column, were used to rinse the collecting

cylinder until a total volume of 20 ml had been discarded. The next 15 ml of the reduced sample were collected and diluted to 30 ml with water. If the nitrate concentration was less than $10 \mu\text{g-at.N-}\ell^{-1}$, 30 ml sample were collected and not diluted. The reduced sample was then treated as a nitrite sample and its concentration determined according to the method described in 5 a) above. The reduction efficiency (E) of the column was determined by comparing the corrected absorbance (Ac) of a nitrite standard with the Ac of a reduced nitrate standard of the same concentration:

$$E = \frac{\text{Ac, NO}_3}{\text{Ac, NO}_2}$$

Blanks and standards were treated in the same way as the samples. The blank solution was prepared by adding 1 ml EDTA solution to 50 ml 0,0015 N-HCl. The absorbance of the blank solution in a 1-cm path-length cuvette should not exceed 0,010. A correction was made for any nitrite initially present in the sample by subtracting this value from the total nitrite concentration obtained through the reduction procedure.

C) ISOLATION AND PURIFICATION OF NITRIFYING BACTERIA

Nitrifying bacteria cannot be isolated directly from source material (Watson *et al.*, 1981). Therefore, enrichment cultures were necessary to obtain pure cultures from the culture water. Dilution- and plating techniques incorporating the use of a micromanipulator were used for this purpose. In addition antibiotics were also used in an attempt to obtain pure cultures of nitrifiers. Both ammonia- and nitrite oxidizing bacteria were isolated and purified by using the same techniques. Obviously, different media were used for these two groups of

nitrifiers (Table 10).

Materials

TABLE 10. Media used for the isolation of marine nitrifying bacteria

	Ammonia-oxidizers				Nitrite-oxidizers			
	*Medium A (Watson, 1965)		*Medium B (Kimata <i>et al.</i> , 1961)		*Medium C (Watson & Waterbury, 1971)		*Medium D (Kimata <i>et al.</i> , 1961)	
Distilled water	250	ml	250	ml	300	ml	250	ml
Sea-water	750	ml	750	ml	700	ml	750	ml
(NH ₄) ₂ SO ₄	1 320	mg	30	mg				
NaNO ₂					69	mg		
KNO ₂							30	mg
MgSO ₄ ·7H ₂ O	200	mg			100	mg		
CaCl ₂ ·2H ₂ O	20	mg			6	mg		
KH ₂ PO ₄			2	mg			2	mg
K ₂ HPO ₄	114	mg			1 740	μg		
Chelated iron (13%)	130	mg			1	mg		
Fe-EDTA chelated			200	μg			200	μg
Na ₂ MoO ₄ ·2H ₂ O	1	μg			30	μg		
MnCl ₂ ·4H ₂ O	2	μg			66	μg		
CoCl ₂ ·6H ₂ O	2	μg			0,6	μg		
CuSO ₄ ·5H ₂ O	20	μg			6	μg		
ZnSO ₄ ·7H ₂ O	100	μg			30	μg		
Phenol red 0,04%	3,25	ml	3,25	ml				
pH	7,8		7,8		8,0		8,0	

*Original medium modified.

Modified Griess reagent (Horwitz, 1975)

Solution 1: 0,5 g sulphanilic acid dissolved in 150 ml 15% (v/v) acetic acid.

Solution 2: 0,1 g α -naphthylamine was boiled in 20 ml distilled water until dissolved. This was poured, while still hot, into 150 ml 15% acetic acid.

Solutions 1 and 2 were mixed, filtered and stored in a brown glass bottle.

Medium E

Medium A (Table 10 - Materials), without any $(\text{NH}_4)_2\text{SO}_4$ added, was solidified with 1,5% Noble agar (Difco) and poured into petri-dishes. Five millilitres of 1% (w/v) $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$, prepared according to the procedure of Vogel (1961), were added to the solidified medium to obtain an even distribution of $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$ over the agar surface. Excess moisture was removed by drying the medium at 40C. The petri-dishes were inoculated with evenly distributed drops of enrichment cultures.

1. Enrichment cultures

Enrichment cultures were made fortnightly, throughout the experiment, in either medium A (Watson, 1965) and B (Kimata *et al.*, 1961) for ammonia-oxidizers or medium C (Watson & Waterbury, 1971) and D (Kimata *et al.*, 1961) for nitrite-oxidizers. All of these media (Materials - Table 10) were filter-sterilized through a MF-Millipore GS membrane filter with a pore size of 0,22 μm . Filter sterilization was preferred to steam sterilization to overcome the problem of precipitation which occurred in the latter procedure. Every batch of medium was checked

for sterility by inoculating 5 ml of medium into a test tube containing 10 ml 0,25 strength nutrient broth. This was incubated for 3 weeks at 30C and checked visually and microscopically for contaminants.

Inocula for enrichment cultures were culture water samples obtained from the different tanks throughout the experimental period. The contents of the sample tank were carefully mixed to obtain a representative sample. A 10% inoculum was used in both test tubes and Erlenmeyer flasks. These enrichment cultures, the former as stationary- and the latter as shake cultures, were incubated in the dark at 28-30C for upto 12 months. In addition, the inocula were also serially diluted in test tubes to isolate both the dominant and fastest-growing organisms. In a dilution series, the fastest-growing organisms will predominate in the lower dilutions, while the dominant organisms will predominate in the higher dilutions (Watson *et al.*, 1981). Enrichment cultures were also grown in a fed-batch type reactor. (Refer to E) 1 below for details.

Growth of the ammonia-oxidizing bacteria was monitored by one or more of the following procedures:

oxidation of ammonia in the culture media which was determined by the phenolhypochlorite method (Solórzano, 1969); increase in nitrite concentration which was determined by adding a drop of Griess reagent (Materials) to two drops of culture medium. The intensity of the red colour is proportional to the nitrite concentration. In the presence of nitrite oxidizing bacteria the nitrite may be oxidized to nitrate and may not be detected; lowering of the pH. This was indicated by a change in the phenol red indicator from red to yellow. Oxidation

of ammonia results in a lowering of the pH of the culture medium;

the breakdown of magnesium ammonium phosphate. Enrichment cultures were inoculated onto medium E (Materials) containing magnesium ammonium phosphate. The growth of ammonia-oxidizing bacteria produced zones of clearing which are caused by the utilization of ammonia from magnesium ammonium phosphate;

turbidity of the culture medium after 3-4 months incubation. During this prolonged period of incubation the pH was adjusted with 10% potassium carbonate (K_2CO_3) and medium B had to be replenished with ammonium sulphate at a final concentration not exceeding $30 \mu g. \ell^{-1}$;

microscopic examination of the enrichment cultures. The cultures were examined by phase-contrast microscopy and also Gram-stained.

Growth of nitrite-oxidizing bacteria was monitored by one or more of the following procedures:

oxidation of nitrite. One drop of Griess reagent (Materials) was added to 0,1 ml of the culture medium. A colourless reaction indicated the complete oxidation of nitrite while a pink colour indicated the presence of nitrite. Quantitative determinations of nitrite were carried out according to the procedure outlined in B) 5 above;

increase in nitrate concentration as a result of nitrite oxidation. Nitrate concentrations in the culture media were measured according to the procedure outlined in

B) 6 above;

turbidity of the medium (although heterotrophs could also contribute to the turbidity). After 3-4 months incubation the enrichment cultures became slightly turbid. During this prolonged incubation, medium C and D were replenished with sodium nitrite to a final concentration not exceeding 30 mg.l^{-1} . The pH did not require any adjustment because nitrite oxidizing bacteria do not alter the pH of the culture medium (Watson *et al.*, 1981); microscopic examination of the enrichment cultures. The cultures were examined for their Gram reaction and by phase-contrast microscopy.

2. Purification

a) Dilution techniques

Serial dilutions were carried out from turbid enrichment cultures that oxidized ammonia. Ten-fold dilutions were prepared in test-tubes containing either medium A and B or C and D for the ammonia- and nitrite oxidizers, respectively. The tubes were incubated at 28-30°C for up to 4 months. The highest dilution showing oxidation of ammonia was again serially diluted and the procedure repeated until pure cultures were obtained. To determine whether the culture was free of heterotrophic contaminants, 1 ml of the culture medium was added to 10 ml aliquots of three types of organic media which included 0,25 strength nutrient broth, 0,50 strength fluid thioglycollate and 0,25 strength trypticase soy broth.

The organic media were made up in 75% sea-water and 25% distilled water. If no growth was obtained in any of the organic media after 3 weeks

incubation at 28-30C, it was assumed that the culture was free of heterotrophic contaminants. The cultures were then examined microscopically to determine whether all the bacteria present were morphologically similar. Turbid cultures were examined with a phase-contrast- as well as a scanning electron microscope. If the culture comprised only one morphological type of cell, it was assumed to be pure.

b) Plating techniques

The isolation of pure cultures of nitrifying bacteria was also attempted on agar media. Medium A and C were solidified with one of 3 solidifying agents: 1,5% agar (Difco), 1,5% Special Agar-Noble (Difco) and 0,75% agarose (Merck). Turbid enrichment cultures were either streaked directly onto the respective agar medium or serially diluted in filter sterilized sea-water and thereafter 0,1 ml spread onto the agar plate with a sterile bent glass rod. The plates were incubated at 28-30C in a petri-dish cannister for approximately 4 months before discarding. The petri-dish cannister prevented the agar medium from drying during the prolonged incubation period. Agar plates were examined weekly both visually and microscopically. Numerous slow-growing colonies were picked off at various stages of growth and transferred to either medium A or C depending upon the type of nitrifier. Microscopic colonies were picked off with the aid of a Leitz-Micromanipulator and a Laborlux II microscope mounted on a common base plate. The isolated colonies were incubated in test-tubes containing either medium A or C for upto 4 months. Colonies that oxidized either ammonia or nitrite were checked for purity as described in 2 a) above.

c) Use of antibiotics

In addition to the above-mentioned dilution- and plating techniques antibiotics were incorporated into solid media to reduce or eliminate heterotrophic contaminants. Two antibiotic solutions, oxytetracycline ($400 \mu\text{g.ml}^{-1}$) and albamycin T ($800 \mu\text{g.ml}^{-1}$) were filter sterilized through a MF-Millipore GS membrane filter with a pore size of $0,22 \mu\text{m}$. Five milliliters of an antibiotic solution were pipetted into a sterile petri-dish and mixed with 15 ml molten (50C) medium A or C containing either 0,75% agarose or 1,5% Special Agar-Noble. Turbid enrichment cultures were serially diluted in filter sterilized sea-water and 0,1 ml inoculated onto the antibiotic medium and spread with a sterile bent glass rod. The petri-dishes were incubated at 28-30C for up to 4 months. Microcolonies were picked off with the aid of a micromanipulator and checked for purity as before.

Prior to incorporating the antibiotics into the solid media their effect was evaluated on both the nitrifying bacteria and the heterotrophic contaminants. Various dilutions (final concentration of $0,1-4000 \mu\text{g.ml}^{-1}$) of the filter sterilized antibiotics were made in medium A or C. One millilitre of an actively growing enrichment culture, having a total cell concentration of approximately $10^7 \text{ cells.ml}^{-1}$ was inoculated into 9 ml of each of the different dilutions and incubated at 28-30C. These cultures of nitrifying bacteria were then checked for their ability to oxidize ammonia or nitrite and compared with a control culture not containing any antibiotic. The minimum inhibitory concentration (MIC) required to inhibit the growth of the heterotrophic contaminants was determined in the following manner. Various dilutions (final concentration of $0,1-4000 \mu\text{g.ml}^{-1}$) of the filter sterilized antibiotics were prepared in 0,25 strength nutrient broth made up in sea-water. One

millilitre of an actively growing enrichment culture (having a total cell concentration of approximately 10^7 cells.ml⁻¹) was inoculated into 9 ml of each of the different dilutions. The cultures were incubated at 28-30C for 2 weeks and examined for turbidity.

Bacterial counts were obtained with a Thoma (Weber Scientific International Ltd.) bacterial counting chamber having a depth of 0,02 mm.

D) ENUMERATION OF NITRIFYING BACTERIA

Nitrifying bacteria were enumerated in each tank and in the biological filter of the closed system throughout the experimental period. The influence of incubation time on the numbers of nitrifying bacteria was also determined. This was done in order to ascertain the period of incubation required to enumerate marine nitrifying bacteria. Only one such study has been reported by Matulewich *et al.* (1975) who worked on non-marine nitrifying bacteria.

1. Closed system

Counts of the viable ammonia- and nitrite oxidizing bacteria were determined by the MPN technique. Samples were collected fortnightly from each tank and the biological filter. Prior to sampling, the water in the sample tank was carefully mixed with a glass rod to obtain a homogenous sample with minimum disturbance to the aquarium animals. Eight replicate samples, i.e., one from each tank, were collected with sterile pipettes. Three replicate samples were collected from the biological filter, approximately 5 cm below the sand surface, with sterile pipettes. One millilitre of each sample was serially diluted in test tubes containing 9 ml aliquots of medium A (for ammonia oxidizers) and

medium C (for nitrite oxidizers). The concentration of the energy source was reduced to 30 mg.l^{-1} for both media. This would reduce the incubation time for enumeration, since only complete oxidation of NH_3 or NO_2 were scored as positive, and would minimize any toxic effects that a higher concentration of NH_3 or NO_2 would have on the nitrifiers (Sieburth, 1979; Watson *et al.*, 1981). The tubes were incubated at 28-30C and the medium checked for complete oxidation of NH_3 and NO_2 at intervals of 10, 20, 30, 60 and 120 d after inoculation. Ammonia was determined by the phenolhypochlorite method (Solórzano, 1969) and nitrite with the Griess reagent. Control test tubes containing either medium A or medium C were inoculated with 1 ml sterile sea-water and incubated with each batch of samples. The numbers of nitrifying bacteria were estimated from the statistical tables of Fisher & Yates (1957).

2. Influence of incubation time

Ammonia-oxidizers

Five different samples were used for this evaluation. Samples 1-3 were obtained at different periods during the fortnightly sampling of water from the closed system. Samples 4 and 5 were also obtained from the closed system but were maintained in shake flasks for 18 months prior to evaluation. Serial ten-fold dilutions, up to 10^{-10} , of each sample were prepared in filter-sterilized sea-water. Each of 5 replicate test tubes containing 9 ml of medium A (energy source reduced to 30 mg.l^{-1}) was then inoculated with 1 ml of each dilution of the sample. The test tubes were incubated at 28-30C and the culture medium was tested every 5 d for 130 d for the oxidation of ammonia. To reduce sample loss, 0,1 ml was removed and tested for ammonia every 5 d. Control test tubes containing 9 ml aliquots of medium A were inoculated with

1 ml sterile sea-water and incubated with the different samples. Test tubes showing complete oxidation of ammonia were taken as positive for the presence of ammonia-oxidizers and the numbers of ammonia-oxidizers obtained from the statistical tables of Fisher & Yates (1957).

Nitrite-oxidizers

The influence of incubation time on the numbers of nitrite-oxidizing bacteria was determined according to the procedure outlined above for ammonia-oxidizing bacteria. Seven different samples were used for this evaluation. Samples 1-3 were obtained at different periods during the fortnightly sampling of water from the closed system. Samples 4-7 were also obtained from the closed system but were maintained in shake flasks for 18 months prior to evaluation. Medium C (energy source reduced to 30 mg.l^{-1}) was used instead of medium A and nitrite was determined as before.

E) IDENTIFICATION OF NITRIFYING BACTERIA

Nitrifying bacteria are categorized taxonomically by their shape, size and arrangement of membranes within their cytoplasm (Tables 6 and 7).

Materials

Modified veronal-acetate buffer (Palade, 1952 - cited by Glauert, 1965)

Stock solution : 2,89 g barbitone sodium and 1,15 g anhydrous sodium acetate were made up to 100 ml with distilled water.
This buffer is stable and keeps for months at 4C.

Working solution: one part of stock solution added to 3 parts of filter-sterilized sea-water and used at pH 7,5.
Discard after 1 d.

Lead citrate (Reynolds, 1963 - cited by Glauert, 1965)

Lead citrate was prepared by mixing 1,33 g $\text{Pb}(\text{NO}_3)_2$, 1,76 g $\text{Na}_3(\text{C}_6\text{H}_5\text{O}_7) \cdot 2\text{H}_2\text{O}$ and 30 ml distilled water in a 50 ml volumetric flask. The solution was shaken vigorously for 1 min and then allowed to stand for 30 min with intermittent shaking to ensure complete conversion of lead nitrate to lead citrate. Eight millilitres of 1 N NaOH were then added and the solution diluted to 50 ml with distilled water. This solution has a pH of 12 and is stable for 6 months.

1. Repeated fed batch culture

To obtain sufficient cells for taxonomic purposes, the ammonia- and nitrite-oxidizing organisms were grown as a repeated fed batch culture, i.e., a batch culture in which growth is prolonged by a continuous supply of nutrients and the periodic removal of portions of the culture. A Gallenkamp Modular Fermentor with a 1 l culture vessel was used. Since inadequate aeration limits the growth of nitrifiers (Gould & Lees, 1960), the culture medium was kept saturated with oxygen throughout the growth of the organisms.

Ammonia-oxidizers were grown in filter sterilized medium A. Both the ammonia- and nitrite concentrations were monitored throughout the growth of these organisms. Peristaltic pumps supplied fresh medium to the culture vessel with the simultaneous removal of an equivalent volume of the culture medium from the vessel. This prevented both the

build up of toxic nitrite levels and depletion of ammonia. The supply of fresh medium was controlled to avoid diluting out the organisms from the culture vessel. The pH was maintained at 7,8 with sterile 0,03 M K_2CO_3 pumped by the modular pH-controller. The phenol red indicator in medium A served as an additional check for pH control. Ammonia-oxidizing bacteria were maintained for 2-3 months until the cell concentration was approximately $10^7 \cdot ml^{-1}$. Nitrite-oxidizing bacteria were grown in medium C. The nitrite- and nitrate concentrations were monitored throughout the growth of these organisms. Nitrate has been reported to inhibit the growth of nitrite-oxidizing bacteria at concentrations of $2000 \mu g NO_3-N \cdot ml^{-1}$ (Gould & Lees, 1960). Fresh medium was supplied by peristaltic pumps which simultaneously removed an equivalent volume of the culture medium. This prevented the build-up of toxic nitrate levels. The flow rate of fresh medium was controlled to prevent the bacteria from being diluted out of the culture vessel. pH control was not necessary as the nitrite-oxidizers do not alter the pH of the culture medium (Watson *et al.*, 1981). The cultures were maintained for 3-4 months until the cell concentration was approximately $10^7 \cdot ml^{-1}$.

The fermentor vessel was inoculated with 5% of an actively growing culture for both types of nitrifiers. If the inoculum was smaller or in the stationary phase of growth, there was a very long lag phase and in some instances, failed to grow. Pure cultures were checked for purity throughout the period of growth. Bacterial counts were obtained with a Thoma bacterial counting chamber.

The repeated fed batch cultures, while still in the logarithmic phase of growth, were used for all morphological and ultrastructural studies.

2. Morphology

Cells grown in the Gallenkamp fermentor were centrifuged at 15 000 x g in a Beckman J2-21 centrifuge for 15 min at 4C. The pellet, obtained from 20 ml of culture medium, was washed twice in filter-sterilized sea-water and finally resuspended in 1 ml of the sea-water. This was then examined by phase-contrast- and scanning electron microscopy. Gram- and negative stains of the pellet were also prepared and examined by light- and electron microscopy, respectively. The colonial morphology of the nitrifying bacteria grown on agar media was studied by phase-contrast microscopy and a stereo-microscope (Wild Photomakroskop M400) fitted with a Photoautomat MPS 55 for photography. Old enrichment cultures that had been maintained in shake flasks for 18 months were also examined by the above-mentioned procedures. The same procedures were used for both ammonia- and nitrite-oxidizing bacteria.

a) Phase-contrast microscopy

One millilitre of the above-mentioned pellet suspension was fixed with a drop of 1% formaldehyde solution. A small drop of culture suspension was placed on a microscope slide and covered with a coverslip. The bacteria were viewed with a Zeiss Invertoscope D microscope fitted with Neofluar Ph 2 phase contrast objectives. Photographs were taken with a Zeiss Contarex camera attached to the microscope.

b) Negative staining

A cell suspension of approximately 10^9 cells.ml⁻¹ is required for negative staining. The cells grown in the fermentor vessel had a concentration of approximately 10^7 cells.ml⁻¹ and had to be concentrated

for this technique. Centrifugation was not considered because of possible damage to flagella. The cells were concentrated on a Nuclepore polycarbonate membrane filter as described in 2 c) below. Three to 4 drops of filtered sea-water were then used to resuspend the bacteria from the filter surface. One drop of this turbid suspension was placed on a formvar coated grid, with a Pasteur pipette. After 3 min excess moisture was removed by touching the edge of the grid with filter paper. The cells were stained by floating the grid (specimen side down) on a drop of 1,0% phosphotungstic acid (Polaron Equipment Ltd.), pH 7,0, for 2,5 min. The optimum staining time and phosphotungstic acid concentration were determined by varying both parameters. Excess moisture was removed by touching the edge of the grid with filter paper. The specimen was viewed with a Philips EM 301 transmission electron microscope using an acceleration voltage of 60 kv and magnifications of between 5000-20 000.

c) Scanning electron microscopy

The cells were centrifuged at 15 000 x g in a J2-21 centrifuge at 5°C for 15 min. The pellet was resuspended in 1 ml of filtered sea water and transferred onto a 13 mm, 0,22 μ m Nuclepore polycarbonate membrane filter by injecting the cell suspension through a stainless steel filter holder supporting the membrane filter. At the same time, a vacuum of approximately 800 mbar was drawn through the outlet of the filter holder to ensure good adherence of the cells to the filter and the removal of excess moisture.

Fixation

The filter was placed on a Whatman No. 17 5,0 cm diameter filter pad saturated with 1% glutaraldehyde in veronal acetate buffer (Materials),

pH 7,5. After fixing for 2 h the membrane filter was transferred to a filter pad saturated with veronal acetate buffer for 15 min to wash off excess fixative. This step was repeated before dehydration.

Dehydration

This was carried out in 25%, 50%, 75% and 100% ethyl alcohol for 1 x 20 min in each of the first three concentrations and 2 x 20 min in the last. The membrane filter was transferred onto filter pads saturated with the appropriate alcohol concentration. Petri-dishes were used to prevent the filter pads from drying out. Thereafter, the membrane filter, together with the filter pad, was placed in a vacuum desiccator containing silica gel. A vacuum of 800 mbar was applied for 1 h.

Gold coating was carried out in a Polaron E5000 Specimen Coating Unit and the specimen was viewed with a Philips SEM 500 scanning electron microscope.

3. Ultrastructure

a) Fixation

Cells were prefixed in 0,2% glutaraldehyde in the growth medium for 1 h with gentle agitation in an orbital incubator at 30C. The glutaraldehyde concentration was increased to 1,0% and the cells were incubated for another hour. After prefixation the cells were pelleted by centrifugation at 15 000 x g in a Beckman J2-21 centrifuge for 15 min at 5C, washed 4 times in veronal-acetate buffer (pH 7,5), resuspended in 1,0% osmium tetroxide (OsO_4) in the same buffer, and kept overnight on ice. The next morning the cells were pelleted by centrifugation, washed twice in veronal-

acetate buffer and resuspended in 0,25% uranyl acetate (aqueous) for 1,5 h. Subsequently, the cells were washed twice in veronal-acetate buffer and finally enrobed in 3% Noble Agar (Difco). The agar containing the cells was placed in a petri-dish over ice and cut into 1 mm² blocks.

Towards the latter part of this study, cells were also prefixed with glutaraldehyde containing malachite green (0,1% w/v). This prefixation was carried out according to the above-mentioned procedure. Malachite green combined with glutaraldehyde has been recommended as a fixative for bacterial cell membranes by Kushnaryov *et al.* (1980).

b) Dehydration

The agar blocks were transferred to vials containing 2 ml distilled water and dehydrated by adding 38 ml of absolute ethyl alcohol, dropwise, to achieve a final concentration of 95%. This was followed by 4 x 15 min washes in absolute ethyl alcohol.

c) Embedding

A "One-Shot" Spurr Low Viscosity Kit (Polaron Equipment Ltd.) of medium hardness was used as the embedding medium. This embedding medium avoids the use of a transitional solvent such as propylene oxide after dehydration in either alcohol or acetone. The advantage of using a low viscosity embedding medium is that it facilitates the rapid infiltration of dehydrated specimens. Infiltration was carried out in 25%, 50% and 75% of the embedding medium in absolute alcohol for 1 h in each solution. This was followed by transferring the blocks to 100% of the embedding medium in which they were left overnight. The next morning the blocks were transferred to Taab (TAAB Laboratories Ltd.) embedding capsules

(8 mm diameter, truncated pyramid, polythene, size 00) containing fresh embedding medium. The capsules were then placed in a vacuum desiccator for 2 h, under a vacuum of 800 mbar, to remove any air bubbles from the embedding medium. Polymerization was carried out overnight at 70°C.

d) Preparation of thin sections

Thin sections were cut with glass knives made with a LKB Knifemaker Model 7800. Commercially available troughs (LKB TRUFS) were attached to the knives with molten paraffin wax. The specimen was sectioned with a Reichert OmU3 Ultramicrotome and thin sections were collected directly in distilled water. Crinkled sections were flattened by passing a chloroform saturated applicator stick over them without direct contact. Gold coloured sections, with a thickness of 90-150 nm on the Peachey colour scale (Peachey, 1958 - cited by Glauert & Phillips, 1965), were collected onto a 200 mesh copper support grid.

e) Section staining

Thin sections were stained with lead citrate (Materials). All solutions used in the procedure were filtered through a 0,45 µm Sartorius membrane filter immediately before use.

Lead citrate

An alkaline lead stain was obtained by the use of lead citrate which is soluble in high concentrations in basic solutions. The lead citrate solution (Materials) was prepared according to Reynolds (1963 - cited by Glauert, 1965). Sections were stained in a petri-dish containing potassium hydroxide pellets. This precaution was taken to prevent the contamination of sections by the formation of a precipitate which occurs

when the staining solution reacts with carbon dioxide from the atmosphere (V. Bandu, University of Natal, 1982 - pers. comm.). The staining solution, must be used without disturbing the precipitate at the bottom of the storage bottle. The grids were floated (section side down) on a drop of the lead citrate solution for 15 min and then washed immediately with 0,02 N sodium hydroxide followed by washing in distilled water. Excess moisture was removed by touching the edge of the grid with a piece of filter paper.

III. RESULTS

A) EVALUATION OF METHODS FOR THE DETERMINATION OF AMMONIA, NITRITE AND NITRATE

The completion of this aspect of the study was necessary before selecting the most appropriate method for the analysis of these nitrogenous compounds in the culture water samples collected fortnightly throughout the experiment.

1. Ammonia

Evaluation of the phenolhypochlorite- (Solórzano, 1969), alkaline hypochlorite-arsenite- (Strickland & Parsons, 1960) and electrode method (Orion Research Inc., 1978) was based on the recovery of a range of spikes (various concentrations of ammonia) added to sea-water and culture water samples. The difference between a sample with and without a spike is expressed as a percentage of the spike added (Tables 11, 12, 13). As a result of its initially high ammonia concentration, the culture water was diluted within the range of detection by the two spectrophotometric methods. This was not necessary for the electrode method which has a wider range of detection.

The accuracy of the phenolhypochlorite method (Solórzano, 1969), shown in Table 11, was superior to the other two methods (Tables 12, 13) for both sea-water and culture water samples. The alkaline hypochlorite-arsenite method (Strickland & Parsons, 1960) produced a consistently higher recovery in sea-water, while the opposite was true for the culture water. Although the ammonia electrode method (Orion Research Inc., 1978) has a greater accuracy than the alkaline hypochlorite-arsenite method (Strickland & Parsons, 1960), the former method lacks sensitivity for very low concen-

TABLE 11. Accuracy of the phenolhypochlorite method (Solórzano, 1969)

Sample	*Conc. of sample ($\mu\text{g-at.NH}_3\text{-N.l}^{-1}$)	Conc. of spike added ($\mu\text{g-at.NH}_3\text{-N.l}^{-1}$)	*Conc. of sample + spike ($\mu\text{g-at.NH}_3\text{-N.l}^{-1}$)	Recovery (%)
Culture water (diluted 30x with distilled water)	5,575	0,100 1,000 1,500 2,000 3,750 7,500	5,670 6,565 6,995 7,365 8,875 10,050	95,0 99,0 94,0 89,5 88,0 65,7
Sea-water (undiluted)	1,875	0,100 1,000 1,500 2,000 3,750 7,500	1,960 2,765 3,235 3,685 5,165 9,125	85,0 89,0 90,7 90,5 87,7 96,7

*mean of five replicates.

TABLE 12. Accuracy of the alkaline hypochlorite-arsenite method (Strickland & Parsons, 1960)

Sample	*Conc. of sample ($\mu\text{g-at.NH}_3\text{-N.l}^{-1}$)	Conc. of spike added ($\mu\text{g-at.NH}_3\text{-N.l}^{-1}$)	*Conc. of sample + spike ($\mu\text{g-at.NH}_3\text{-N.l}^{-1}$)	Recovery (%)
Culture water (diluted 30x with distilled water)	5,851	0,100 1,000 1,500 2,000 3,750 7,500	5,923 6,308 6,440 6,607 7,042 9,013	72,0 45,7 39,3 37,5 31,8 42,2
Sea-water (undiluted)	0,537	0,100 1,000 1,500 2,000 3,750 7,500	0,657 1,715 2,208 2,965 5,118 10,047	120,0 117,8 111,4 121,4 122,2 126,8

*mean of five replicates.

TABLE 13. Accuracy of the ammonia electrode method (Orion Research Inc., 1978)

Sample	*Conc. of sample ($\mu\text{g-at.NH}_3\text{-N.l}^{-1}$)	Conc. of spike added ($\mu\text{g-at.NH}_3\text{-N.l}^{-1}$)	*Conc. of sample + spike ($\mu\text{g-at.NH}_3\text{-N.l}^{-1}$)	Recovery (%)
Culture water (undiluted)	44	10 000 1 000 100	9 000 1 150 120	89,6 110,6 76,0
Sea-water (undiluted)	0,85	10 000 1 000 100	9 000 875 80	90,0 87,4 79,2

*mean of five replicates.

trations of ammonia. The phenolhypochlorite method (Solórzano, 1969), selected for the determination of ammonia in the culture water, was used within the range $0,1\text{-}10 \mu\text{g-at.NH}_3\text{-N.l}^{-1}$.

2. Nitrite

The nitrogen oxide electrode- (Orion Research Inc., 1979) and the Griess-Ilosvay method (Strickland & Parsons, 1960) were evaluated for both sea-water and culture water samples as described for the evaluation of ammonia. Unfortunately, the electrode method could not be assessed because of its poor performance. Erratic results were obtained with this method and a replacement electrode from the suppliers also produced poor results. The unsatisfactory electrode performance can be seen when the calibration curve obtained in the evaluation of this method is compared with that of the expected calibration curve (Fig. 14). The slope of the calibration curve obtained in this study is $\sim 26 \text{ mv}$ whereas that of a typical calibration curve is $\sim 58 \text{ mv}$. This method was eventually abandoned since further attempts at success proved futile.

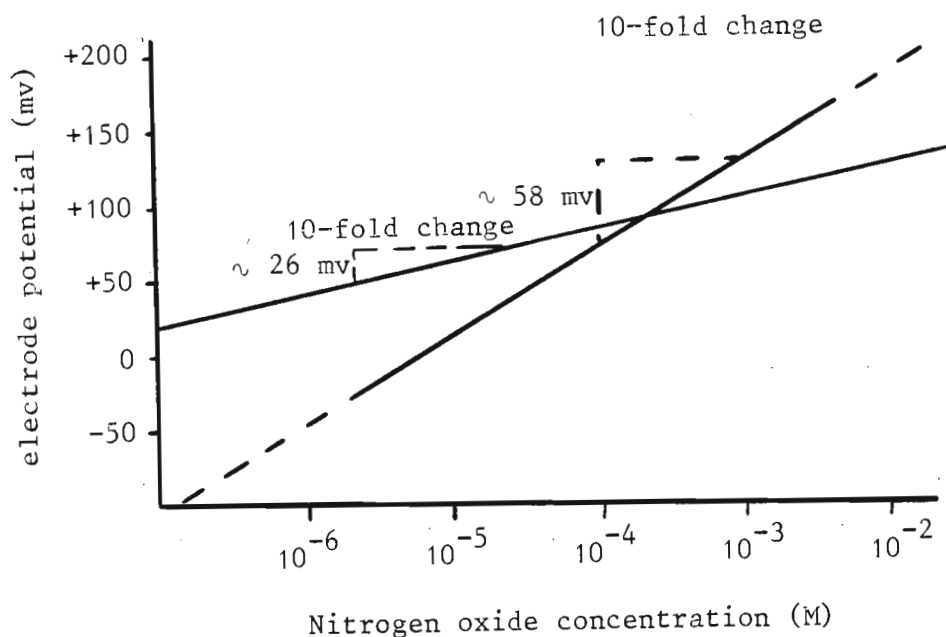


Fig. 14. Calibration curves for the determination of nitrite with the nitrogen oxide electrode (Orion Research Inc., 1979).
 —: expected response of the electrode.
 -----: response obtained with the electrode used in the present study.

The accuracy of the Griess-Ilosvay method (Strickland & Parsons, 1960) decreases at nitrite concentrations $< 1,0 \mu\text{g-at.N.l}^{-1}$ (Table 14). The average recovery of the spike, within the range $1-10 \mu\text{g-at.NO}_2\text{-N.l}^{-1}$, was 98,9%. This method was therefore adopted for the determination of nitrite in the culture water and used within the range $1-10 \mu\text{g-at.NO}_2\text{-N.l}^{-1}$.

3. Nitrate

Only sea-water samples were used for the evaluation of the method of Wood *et al.* (1967). Culture water samples were not used because a very high dilution was required to obtain a nitrate concentration within the range of detection of this method. Such a highly diluted sample would lower or mask the effect of any interfering substances in the culture water on the procedure. Two equivalent sets of standard nitrite and

TABLE 14. Accuracy of the Griess-Ilosvay method (Strickland & Parsons, 1960)

Sample	*Conc. of sample ($\mu\text{g-at.NO}_2\text{-N.l}^{-1}$)	Conc. of spike added ($\mu\text{g-at.NO}_2\text{-N.l}^{-1}$)	*Conc. of sample + spike ($\mu\text{g-at.NO}_2\text{-N.l}^{-1}$)	Recovery (%)
Sea-water	0,0329			
		0,10	0,1520	119,1
		0,50	0,4550	84,4
		1,00	1,0198	98,7
		1,50	1,5079	98,3
		2,00	2,0000	98,4
		3,00	2,9998	98,9
		4,00	4,013	99,5
		15,00	14,2641	94,9
Culture water	8,744			
(diluted 2x		0,10	8,839	95,0
with distilled		0,50	9,276	106,4
water)		1,00	9,701	95,7
		1,50	10,197	96,9
		2,00	10,705	98,1
		3,00	11,701	98,6
		4,00	12,685	98,5

*mean of five replicates.

nitrate solutions were prepared in sea-water. The concentrations of the nitrite and reduced nitrate standards were then determined by the Griess-Ilosvay method (Strickland & Parsons, 1960). The reduction efficiency of the copperized-cadmium column is based on the concentration of nitrite determined in the reduced nitrate standard relative to the concentration of nitrite determined in an equivalent nitrite standard (Table 15). The average reduction efficiency was 95,14% within the range 1-60 $\mu\text{g-at.NO}_3\text{-N.l}^{-1}$. The reduction efficiency was adequate for this study and nitrate in the

culture water samples was determined by this method in the afore-mentioned concentration range.

TABLE 15. Reduction efficiency of the copperized-cadmium column

*Conc. of std. solns. ($\mu\text{g-at.N.l}^{-1}$)	+Conc. of NO_3 ($\mu\text{g-at.N.l}^{-1}$)	*Conc. of NO_2 ($\mu\text{g-at.N.l}^{-1}$)	Reduction efficiency %
0,1	0,119	0,139	85,6
0,5	0,446	0,490	91,0
1,0	0,875	0,950	92,1
2,0	1,870	1,930	96,9
10,0	9,070	9,430	96,2
20,0	19,090	19,570	97,5
30,0	27,930	29,350	95,2
40,0	36,920	38,820	95,1
50,0	45,350	48,330	93,8
60,0	54,900	58,240	94,3

Results based on the mean of 5 replicates

*Std. solns. of NO_2 and NO_3 made in sea-water.

+ NO_2 determined after reduction

* NO_2 determined in std. NO_2 soln.

B) PHYSICO-CHEMICAL ANALYSIS OF THE CULTURE WATER

Sea-water is an unusually complex medium with a natural buffering action. When removed from the ocean and used in sea-water systems holding heterotrophs, its life supporting capacity tends to decrease. The major alterations include an increase in nitrogen- and organic compounds, and a decrease in DO and pH (Kinne, 1976). Since no attempt was made to counteract these changes, such alterations were expected in the captive body of sea-water used in this study.

1. pH

Fig. 15 represents the pH profile of the circulating culture water during the experimental period of 22 weeks. The initial pH of 8,16 dropped by 1,39 pH units to 6,77 at the end of this study. A relatively faster decrease of 1,01 pH units occurred during the first 10 weeks while a decrease of only 0,39 pH unit was experienced over the last 12 weeks. In closed systems a gradual decline in pH is the rule. This can be attributed to respiration by animals and bacteria, the oxidation of metabolic wastes, and the effect of accumulated nitrate ions (Atz, 1964).

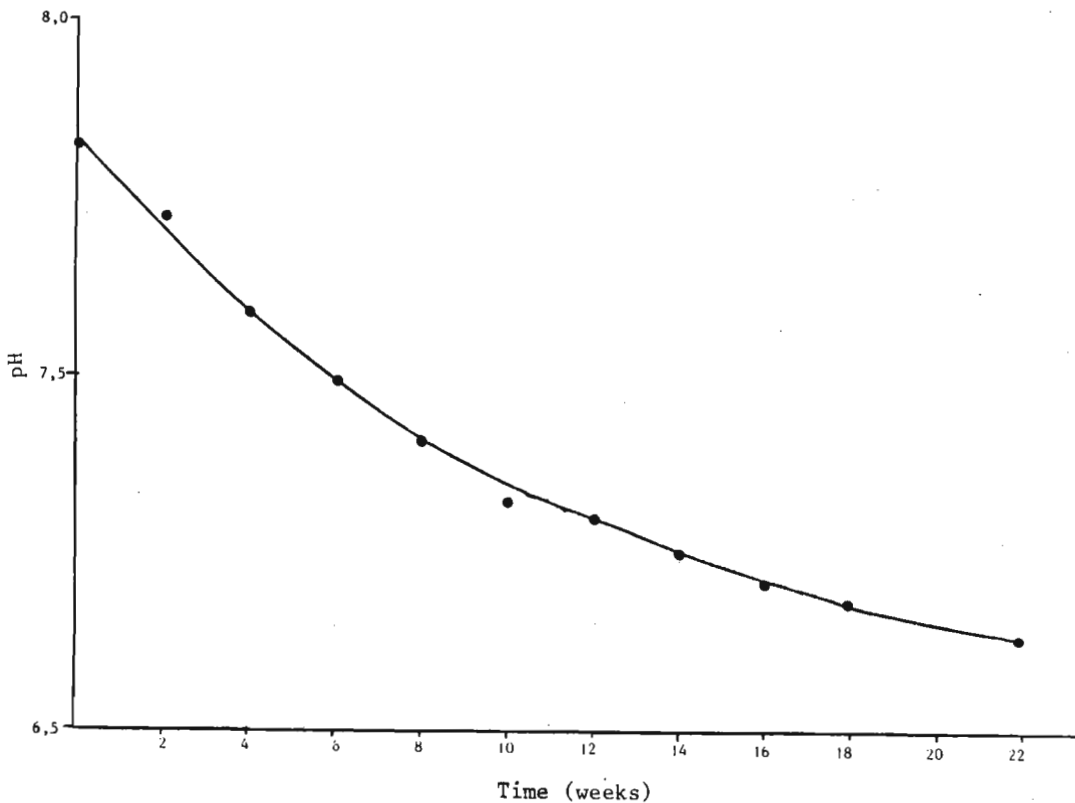


Fig. 15. pH of the culture water during the experimental period.

2. Dissolved oxygen

The DO values at different positions in the closed systems are given in Table 16. These values represent the mean of five, monthly determinations

during the experimental trial. The DO pattern was the same throughout the study. Calculation of oxygen saturation values is based on an oxygen solubility of $5,5 \text{ mg.}\ell^{-1}$ in the culture water (Rand *et al.*, 1976). The high DO content at the inlet and above the sand filter is due to the culture water being airlifted while the lower DO content of the water in the reservoir and exit of the sand filter is probably due to oxygen consumption by the aquarium animals and microorganisms, respectively. It is extremely difficult to compare the results in Table 16 with DO values obtained in other closed systems (Wickins, 1976; Gerhardt, 1978; Mevel & Chamroux, 1981) because of differences in experimental conditions. However, Wickins (1976) reported an 83% saturation for culture water in a closed system while Mevel & Chamroux (1981) found that the microflora of the sand filter decreased DO levels in the circulating water. The results in Table 16 confirm the reports by these workers.

TABLE 16. Dissolved oxygen values at various points in the closed systems

Sampling point	Dissolved oxygen	
	($\text{mg.}\ell^{-1}$)	Saturation (%)
Point at which filtered water returns to the tank, i.e., inlet	4,95	90,0
Random points in the tank away from the inlet	4,30	78,2
Above the sand filter	4,83	87,8
Exit of sand filter	3,36	61,1

3. Biochemical oxygen demand

Fig. 16 represents the BOD of the culture water over a period of 22 weeks. The BOD increases steadily for 12 weeks, decreases sharply and continues to increase until the end of the experiment. Since nitrification was not suppressed these values express the amount of oxygen utilized by micro-organisms to transform degradable carbon and nitrogen compounds. Unfortunately, these results cannot be compared with other closed systems because of a lack of such reports. However, since BOD is a measure of degradable carbon these results are in agreement with reports of elevated levels of organic and inorganic carbon in closed systems (King & Spotte, 1974; Kinne, 1976; Wickins, 1976; Gerhardt, 1978; Mevel & Chamroux, 1981).

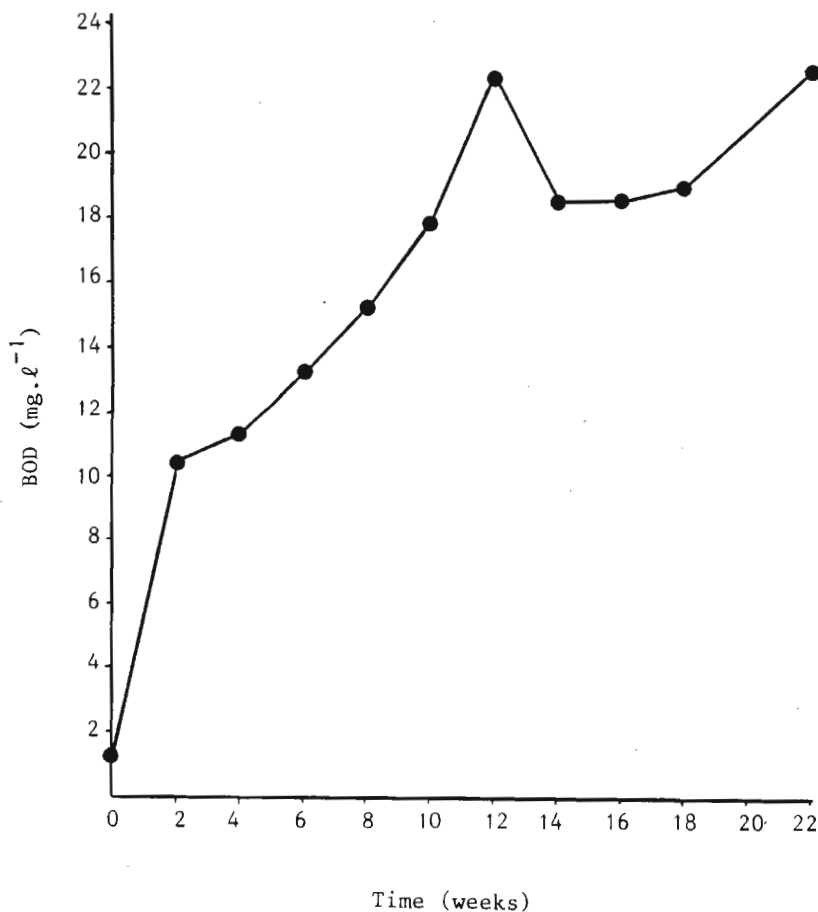


Fig. 16. Biochemical oxygen demand of the culture water.

4. Ammonia, nitrite and nitrate

The profile of the ammonia, nitrite and nitrate concentrations in the culture water (Fig. 17) clearly indicates the establishment of nitrification. This is shown by the early appearance of ammonia which is followed

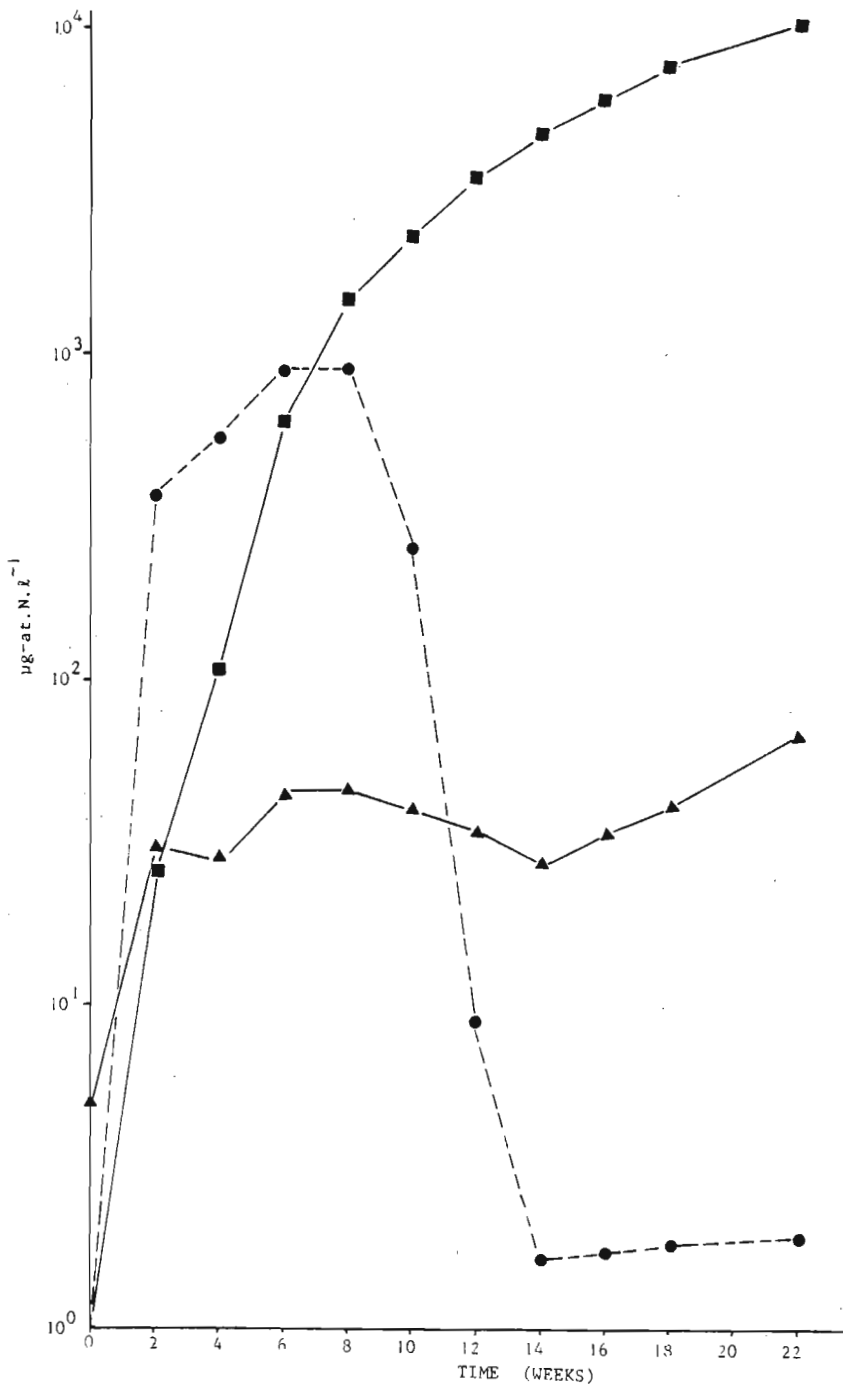


Fig. 17. Ammonia (—▲—), nitrite (---●---) and nitrate (—■—) concentrations in the culture water.

by nitrite and then nitrate which accumulates in the closed system.

This phenomenon corresponds to the classic sequence of changes of nitrification in closed systems (Gerhardt, 1978; Mevel & Chamroux, 1981).

During the experiment, ammonia fluctuates between 4,8 and 68,7 $\mu\text{g-at.N.l}^{-1}$ while nitrite increases until it reaches a maximum of 891 $\mu\text{g-at.N.l}^{-1}$ after 8 weeks. A rapid decrease occurs in the following 6 weeks before nitrite is maintained at $< 2 \mu\text{g-at.N.l}^{-1}$ up to the end of the experiment.

The initial slow rate of nitrate accumulation is increased with the concomitant increase in nitrite oxidation. Nitrate reaches a maximum concentration of 9898,1 $\mu\text{g-at.N.l}^{-1}$ at the end of 22 weeks.

C) ENUMERATION OF NITRIFYING BACTERIA

1. Closed system

Most probable number estimates of nitrifiers in both the sand filter and tank culture water are shown in Fig. 18. The numbers of ammonia-oxidizers in the filter reach their maximum after 8 weeks while 10 weeks are required for nitrite-oxidizers. After reaching their maximum numbers, the nitrifying population fluctuates until the end of the experiment. The maximum numbers of nitrifiers detected in the tank culture water is more than five hundred times lower than that of the filter. In spite of this difference, a similar pattern was observed for the numbers of nitrifying bacteria in both the filter and culture water. Kawai *et al.* (1964) also found a larger population of nitrifiers in the sand filter than the culture water. Although, Mevel & Chamroux (1981) did not include nitrifying bacteria in their study, higher numbers of aerobic bacteria were found in the sand filter than the culture water. The reason for this phenomena is that bacteria attach to the surfaces of the sand grains (Saeki, 1948 - cited by King & Spotte, 1974) and thereby accumulate in the sand filter.

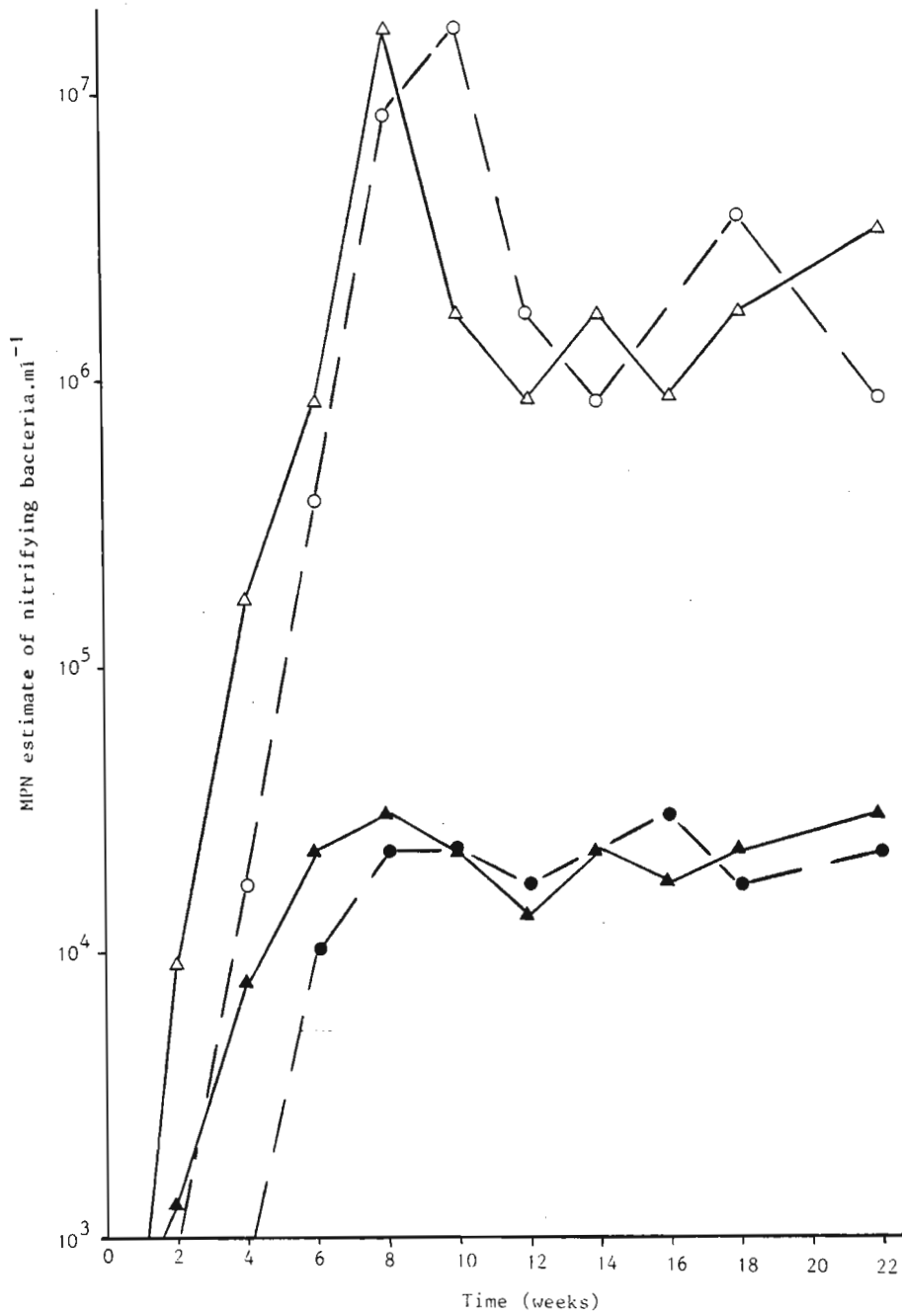


Fig. 18. Estimates of nitrifying bacteria in the sand filter and tank culture water (—△—, filter sand ammonia-oxidizers; —▲—, culture water ammonia-oxidizers; ---○---, filter sand nitrite-oxidizers; ---●---, culture water nitrite-oxidizers).

2. Influence of incubation time

The effect of incubation time on the estimates of ammonia-oxidizers and nitrite-oxidizers is shown in Tables 17 and 18, respectively. The large differences obtained among the various samples were expected since

they were not replicates. Maximum estimates of ammonia-oxidizers were obtained after a mean incubation period of 15 d within a range of 10 to 20 d. The mean incubation period required for maximum estimates of nitrite-oxidizers was 36,4 d within a range of 20 to 65 d. Only the report by Matulewich *et al.* (1975) is available for a comparative evaluation of the results obtained in this study. These workers found that an incubation period of 25 d was necessary to obtain maximum estimates of ammonia-oxidizers while nitrite-oxidizers required longer than 113 d (Table 5). It is evident from both studies that ammonia-oxidizers require a shorter incubation period than nitrite-oxidizers. Furthermore, these results (Tables 5, 17, 18) indicate that the period of incubation required for the maximum MPN estimate of nitrifiers varies with different samples and environments.

TABLE 17. Effect of incubation time on estimates of ammonia-oxidizers

Incubation time (d)	Estimate (MPN.ml ⁻¹) in various samples				
	Culture water	Culture water	Enrichment culture	Enrichment culture	Enrichment culture
5	3	17	4	7	114
10	4 498	44 978	1 137	44 978	1 137 627
15	17 258	172 583	NC	1 725 838	4 497 799
20	NC	NC	NC	NC	17 258 379
25	NC	NC	NC	NC	NC
30	NC	NC	NC	NC	NC
130	NC	NC	NC	NC	NC

NC - no change.

TABLE 18. Effect of incubation time on estimates of nitrite-oxidizers

Incubation time (d)	Estimate (MPN.ml ⁻¹) in various samples						
	Culture water	Culture water	Culture water	Enrichment culture	Enrichment culture	Enrichment culture	Enrichment culture
5	ND	ND	ND	ND	27	5	7
10	ND	ND	ND	ND	1 726	74	450
15	ND	ND	ND	27	11 376	1 138	4 498
20	74	ND	11	736	172 584	17 258	11 376
25	173	5	27	11 763	1 137 627	NC	NC
30	NC	27	173	NC	NC	1 725 838	NC
35	NC	114	270	NC	NC	NC	NC
40	1 138	450	7 362	NC	NC	NC	NC
45	1 726	1 726	113 763	NC	NC	NC	NC
50	NC	NC	NC	NC	NC	NC	NC
55	NC	NC	NC	NC	NC	NC	NC
60	NC	2 704	NC	NC	NC	NC	NC
65	NC	17 258	NC	NC	NC	NC	NC
70	NC	NC	NC	NC	NC	NC	NC
130	NC	NC	NC	NC	NC	NC	NC

ND - none detected.

NC - no change.

D) GROWTH OF *Penaeus monodon*

Three prawns died during the experimental period. These were replaced by the prawns kept for this purpose. The mortality represents 6,25% of the total number used for the study. Considering the fact that two deaths resulted from "jump-outs" and the third from cannibalism, none of the deaths can be attributed to adverse effects of the culture water. The mean wet mass of *P. monodon* is compared to that obtained by other workers in semi-open systems (Table 19). Although grown in a closed system for 22 weeks, the wet mass of the prawns in this study compares favourably with those obtained in other experimental trials.

TABLE 19. Comparison of the mean wet mass of *P. monodon* obtained by various investigators

Investigator	Period of growth (d)	Initial stocking density (g.m ⁻²)	Culture system	Mean wet mass (g)
Forster & Beard (1974)	152 (approx.)*	33,2-83,0	Semi-open	12,95
Forster & Beard (1974)	152 (approx.)*	5,0-12,5	Semi-open	25,43
Doug Cook (pers. comm.)	172 ⁺	> 50,0	Semi-open	7,1
Doug Cook (pers. comm.)	178	10,0	Semi-open	15,82
Present study	173 ⁺	17,0	Closed	12,87

*Estimate of the period of growth because the age of the prawns not reported.

⁺Prawns used in these studies were from the same spawn.

E) CULTURAL CHARACTERISTICS AND IDENTIFICATION OF NITRIFYING BACTERIA

1. Cultural characteristics

a) Enrichment cultures

Enrichment cultures of both groups of nitrifiers were maintained for 3 years with regular transfers to fresh media. Shake flask cultures of ammonia-oxidizers were transferred monthly while those of nitrite-oxidizers were transferred every 3 months. These cultures were transferred to fresh media because of the accumulation of nitrite (> 700 mg.ℓ⁻¹) and nitrate (> 2 000 mg.ℓ⁻¹) which inhibited the growth of ammonia- and nitrite-oxidizers, respectively. Enrichment cultures in test tubes took twice as long to reach these inhibitory concentrations of nitrite and nitrate. These findings were only applicable to enrichment cultures having only one group of nitrifying bacteria, i.e., either ammonia-

oxidizers or nitrite-oxidizers. Growth of the nitrifying bacteria was negligible at pH 6 or lower and optimal at pH 7,6-8,0.

b) Cysts and zoogloea

Aggregates of nitrifiers were frequently seen to occur in enrichment cultures. These aggregates are referred to as either cysts or zoogloea. Although a clear distinction does not exist between these two types of aggregates, Watson *et al.* (1981) described a zoogloea as having loosely associated cells embedded in a soft slime layer and a cyst as closely packed and compressed cells firmly embedded in and surrounded by a tough slime layer. Scanning electron micrographs of aggregates of ammonia-oxidizers (Fig. 19) and nitrite-oxidizers (Fig. 20) cannot be categorized

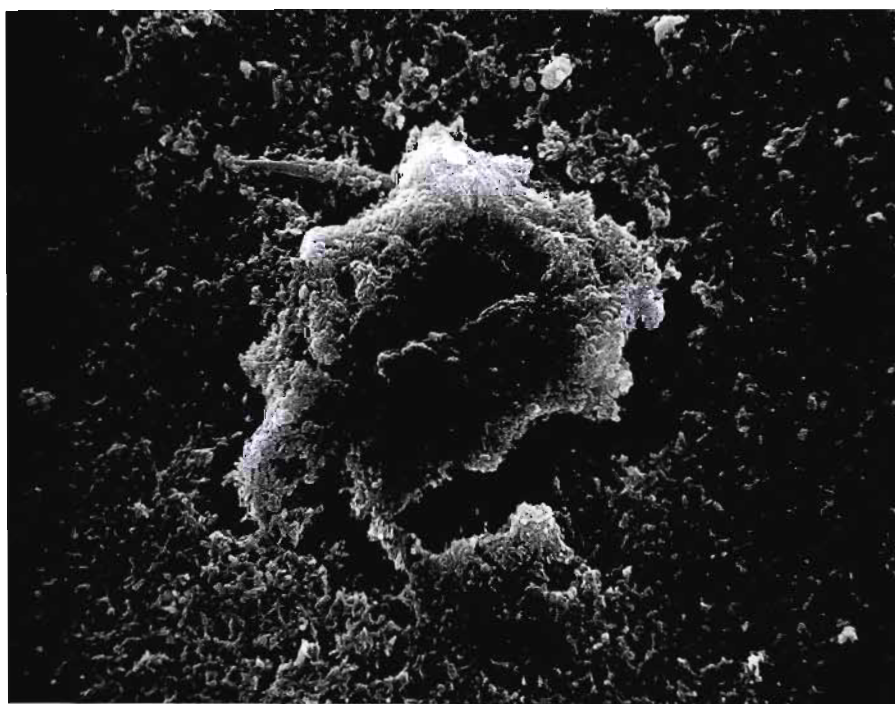


Fig. 19. Scanning electron micrograph of an aggregate of ammonia-oxidizing bacteria (4 687 x).

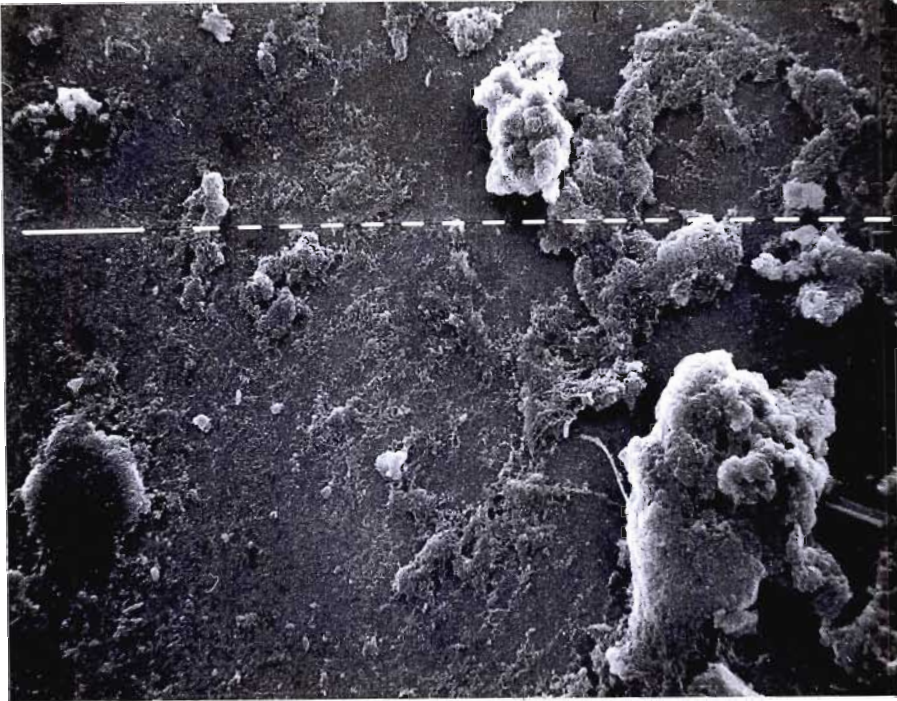


Fig. 20. Scanning electron micrograph of an aggregate of nitrite-oxidizing bacteria (1 200 x).

in either group according to the description by Watson *et al.* (1981). Even transmission electron micrographs (Figs. 21 and 22) may fail to provide a clear-cut separation of the two different types of aggregates. Fig. 21 shows small groups of cells within a large aggregate. The distorted shape of the cells in Fig. 22 indicates that the cells may be compressed and embedded in a tough slime layer.



Fig. 21. Section of an aggregate of ammonia-oxidizing bacteria (11 040 x).

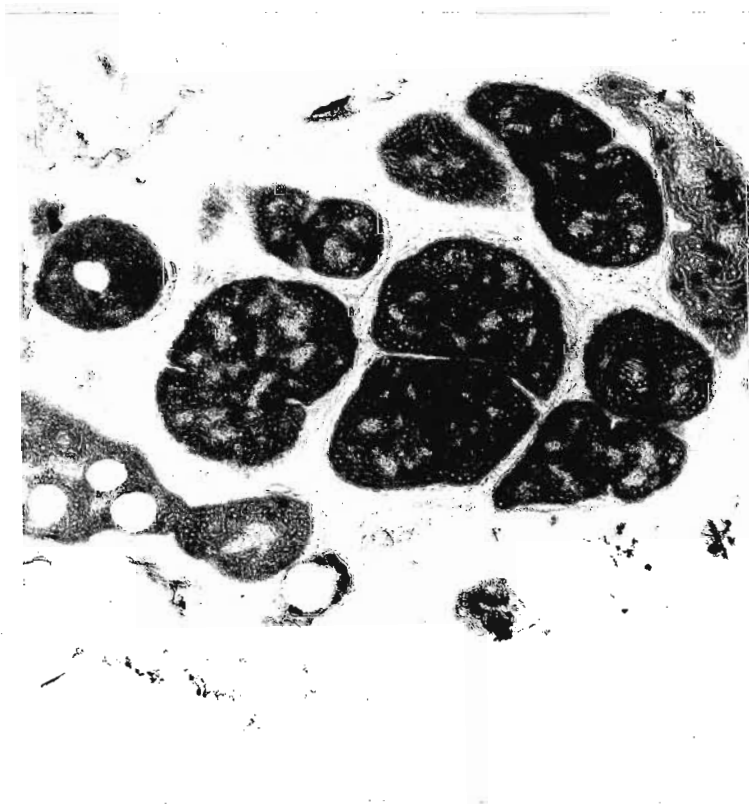


Fig. 22. Section of an aggregate of nitrite-oxidizing bacteria (18 400 x).

c) Colonial morphology

Microcolonies of nitrifying bacteria are slow-growing and were observed with a microscope within 1-2 weeks. Colonies are visible to the unaided eye after 1-3 months. The microcolonies appear colourless and are difficult to observe and can be easily missed by an inexperienced worker. In addition, these microcolonies lack any distinguishing features that could aid in their identification. Larger colonies, approx. 1,5 mm in diameter, were obtained if the agar medium was either buffered with 0,05 M HEPES for ammonia-oxidizers or replenished with NaNO_2 for nitrite-oxidizers. No distinguishing features were observed for these colonies (Figs. 23 and 24). In order to verify that the colonies were those of nitrifying bacteria and not contaminants, numerous colonies were transferred to the appropriate medium to test for the oxidation of either ammonia or nitrite.

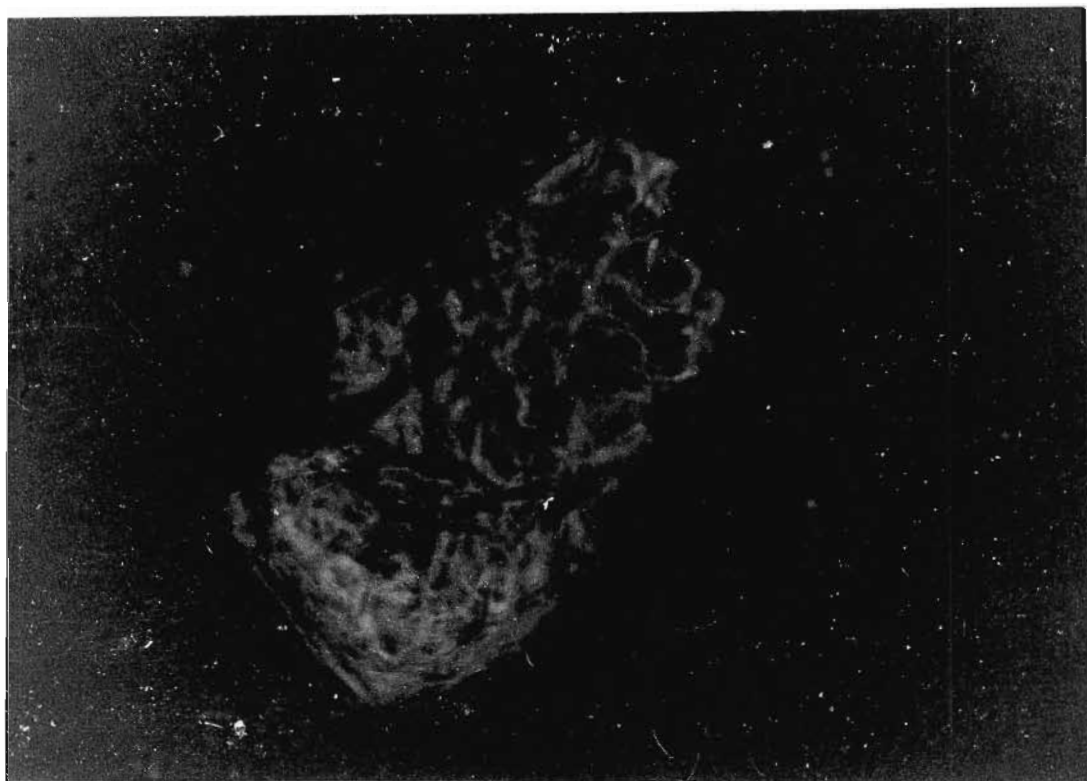


Fig. 23. Colonial morphology of ammonia-oxidizing bacteria (1 300 x).

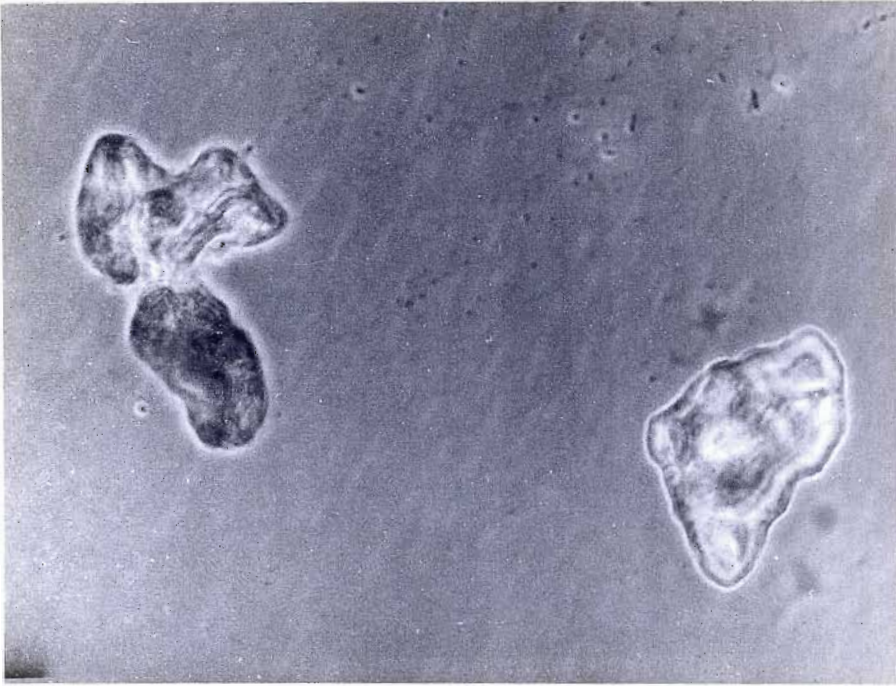


Fig. 24. Colonial morphology of nitrite-oxidizing bacteria (3 300 x).

d) Breakdown of magnesium ammonium phosphate

The breakdown of magnesium ammonium phosphate was used as an additional test to determine the ability of organisms to oxidize ammonia. The ammonium in this compound provides the energy source required by ammonia-oxidizers. Oxidation of the ammonium results in the breakdown of magnesium ammonium phosphate which in turn produces areas of clearing in the agar medium, 2-3 weeks after inoculation. These clear areas (Fig. 25b) are easily visible when compared with a control plate (Fig. 25a) inoculated with sterile sea water. When examined microscopically the action of the ammonia-oxidizers on individual particles of magnesium ammonium phosphate becomes more apparent. Figure 26a shows an intact particle at the very early stage of breakdown while that shown in Fig. 26b is at an advanced stage of breakdown.



Fig. 25. Appearance of the agar medium layered with magnesium ammonium phosphate after inoculation in (a) the absence and (b) presence of ammonia-oxidizers.

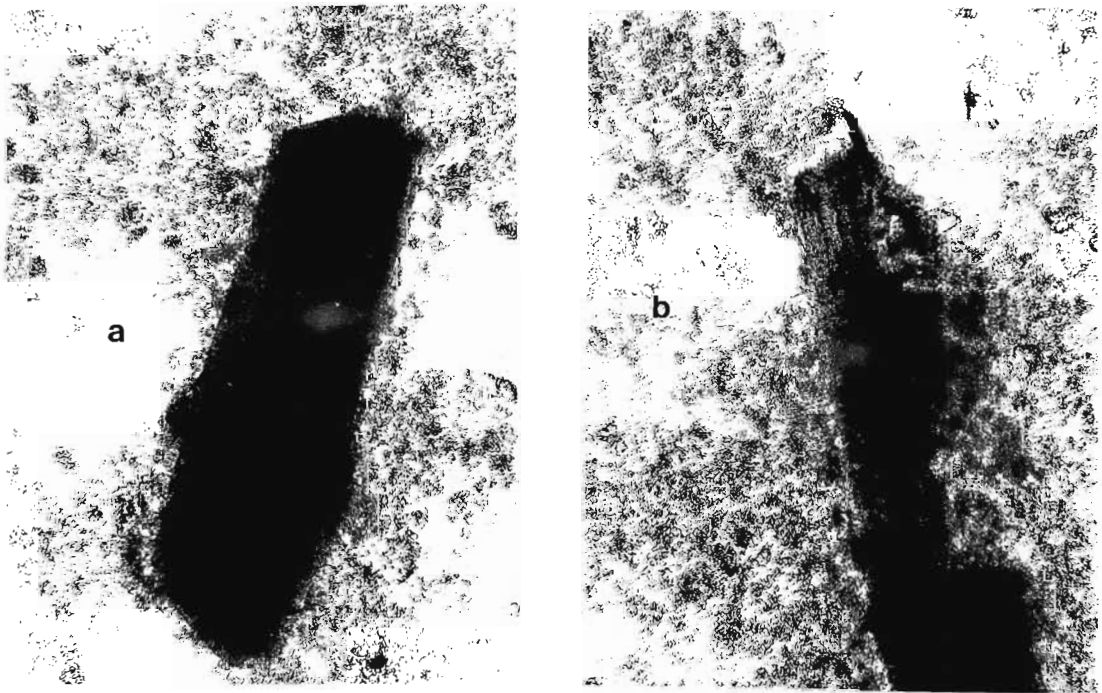


Fig. 26. Particle of magnesium ammonium phosphate at (a) an early and (b) advanced stage of breakdown by ammonia-oxidizing bacteria. (340 x)

e) Repeated fed batch culture

A much higher cell population of both types of nitrifiers were obtained with a filter-sterilized medium than with a steam-sterilized medium. The difference was approximately ten-fold. The reason for this phenomenon could be due to the "particle-free" medium in the former. A similar finding was reported by Lewis & Pramer (1958) who cultured *Nitrosomonas* spp. in a "particle-free" medium. During repeated fed batch culture the supply of air into the culture vessel results in a loss of water from the culture medium which is carried with the exhaust gases. Over prolonged periods of culture, a significant increase in salinity occurred. Figure 27 shows the effect of such an increase in salinity to 42‰ on ammonia-oxidizers. Gross deterioration and lysis of these cells are the results of such a change in salinity. The problems of water loss from the medium was overcome by using a 0,45 µm pore size hydrophobic filter at the exhaust port.

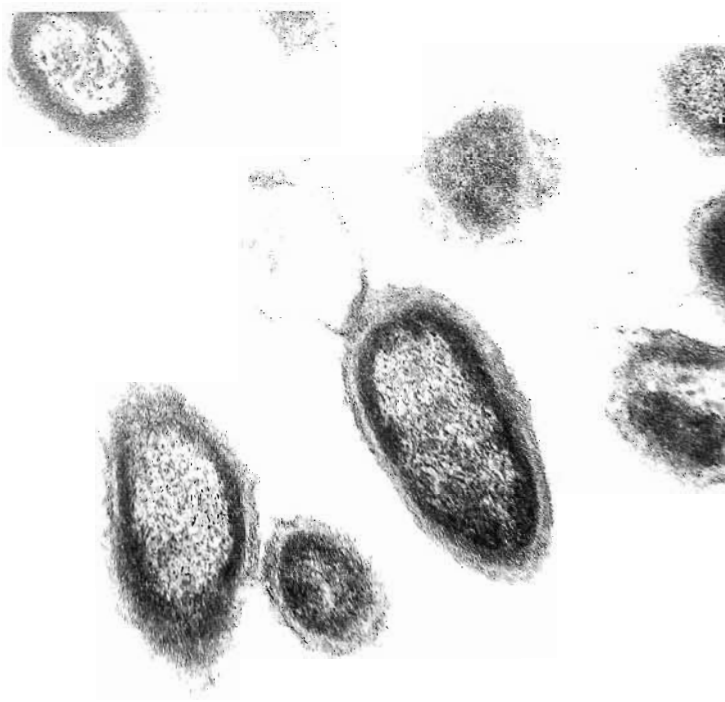


Fig. 27. Deterioration and lysis of cells as a result of an increased salinity in the growth medium (66 900 x).

2. Identification

Studies on the process of nitrification have primarily been indirect observations on rates of ammonia- and nitrite oxidation and nitrate production. Only one attempt to identify nitrifying bacteria, associated with nitrification in a closed system, has been reported (Johnson & Sieburth, 1976). These workers did not purify any of the nitrifiers and observed species of only *Nitrosomonas* and *Nitrosococcus*. In the present study, species of two genera of ammonia-oxidizers and three genera of nitrite-oxidizers were observed. These included species of *Nitrosomonas*, *Nitrosococcus*, *Nitrobacter*, *Nitrococcus* and *Nitrospina*. These five genera include all the genera of marine nitrifying organisms listed in the 8th Edn. of Bergey's Manual of Determinative Bacteriology (Watson, 1974) and by Watson *et al.* (1981). The organisms were identified on the basis of the characteristics described in Tables 6 and 7. *Nitrosomonas europaea* and *Nitrobacter winogradskyi* were obtained in pure culture. These two organisms were always dominant in enrichment cultures and numerous attempts to isolate species of the other three genera in pure culture proved futile.

a) Ammonia-oxidizers

Nitrosomonas europaea

Cytomembranes of this organism are arranged in the peripheral region of the cell (Figs. 28, 29, 30, 31). Figure 29 shows the concentric arrangement of cytomembranes in a cross-section of *N. europaea*. Three morphologically different strains were observed. The strain isolated in pure culture is rod shaped with rounded ends (Fig. 28) while the other two, also rod shaped, have pointed ends (Figs. 30, 31). Their respective dimensions are 1,85 x 0,65 μm and 2,24 x 0,60 μm . In one of these strains, the cells remain attached after division (Fig. 31). The existence of morphologically diff-

erent strains of *N. europaea* has also been reported by Watson *et al.* (1981). The pure culture of *N. europaea* is Gram-negative, 1,2 x 0,67 μ m in size, motile with one subpolar flagellum and occurs as single cells.



Fig. 28. Section of *N. europaea* showing the peripheral arrangement of cytomembranes (43 490 x).

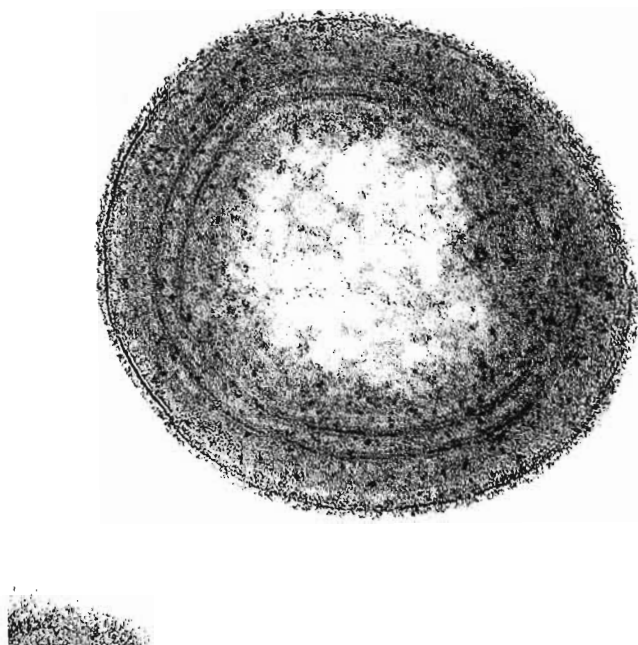


Fig. 29. Cross-section of *N. europaea* with a concentric arrangement of cytomembranes in the peripheral region (104 000 x).

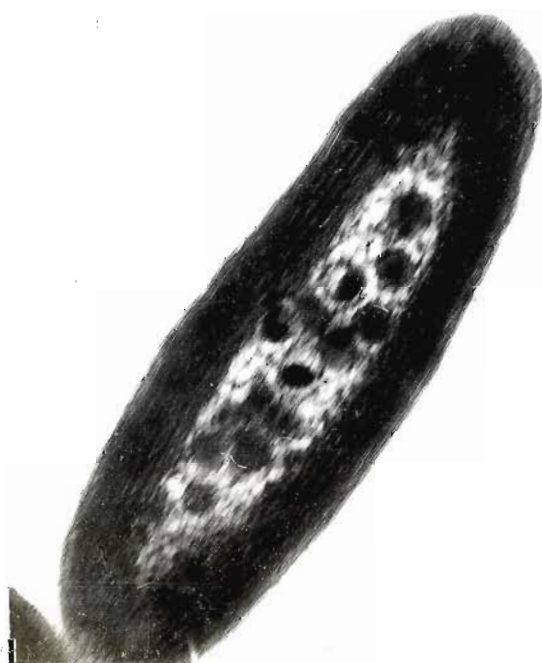


Fig. 30. Section of *N. europaea* with "pointed-ends" (53 527 x).



Fig. 31. Section of a strain of *N. europaea* which remains attached after cell division (33 454 x).

Nitrosococcus mobilis

The cells are spherical and 0,6 μm in diameter. Cytomembranes are peripherally arranged and intrude randomly into the cytoplasm (Figs. 32, 33). These cells were encountered in low numbers in crude enrichment cultures.

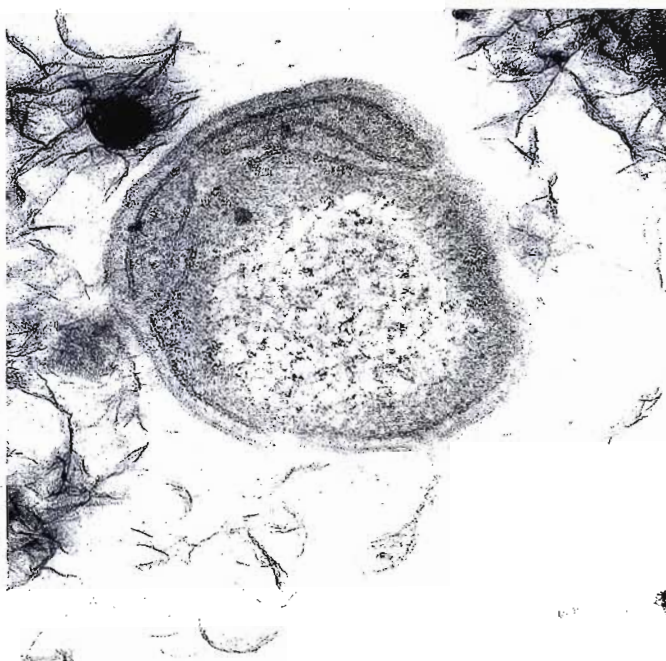


Fig. 32. Section of *N. mobilis* showing the arrangement of cytomembranes (86 981 x).

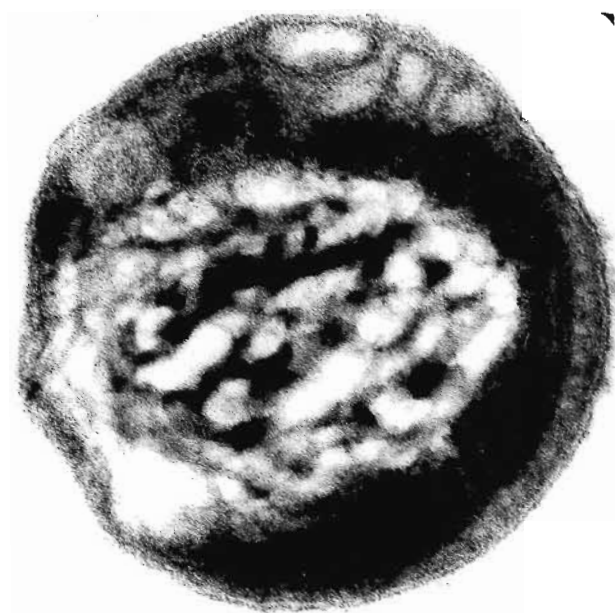


Fig. 33. Section of *N. mobilis* showing the arrangement of cytomembranes (140 509 x).

b) Nitrite-oxidizers

Nitrobacter winogradskyi

Members of this genus are Gram-negative, rod shaped, reproduce by budding, and have a polar cap of cytomembranes (Fig. 34). Cells are non-motile. These organisms are rich in cytochromes imparting a yellowish colour to cell suspensions. The strain of *Nitrobacter* isolated in this study, is not an obligate chemolithotroph and can be grown on a medium containing acetate as the carbon and energy source.

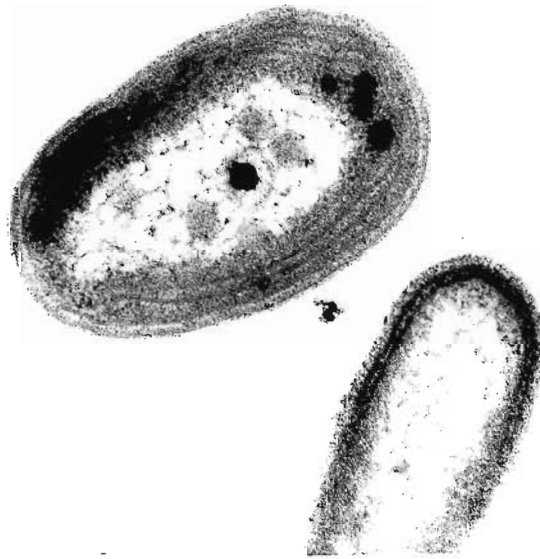


Fig. 34. Section of *N. winogradskyi* showing the arrangement of cytomembranes (53 527 x).

Nitrospina gracilis

These cells are long slender rods, $0.3 \times 1.6 \mu\text{m}$ in size, and lack an extensive cytomembrane system (Fig. 35). Occasional bleblike invagina-

tions reported for this organism (Watson & Waterbury, 1971) have not been found.

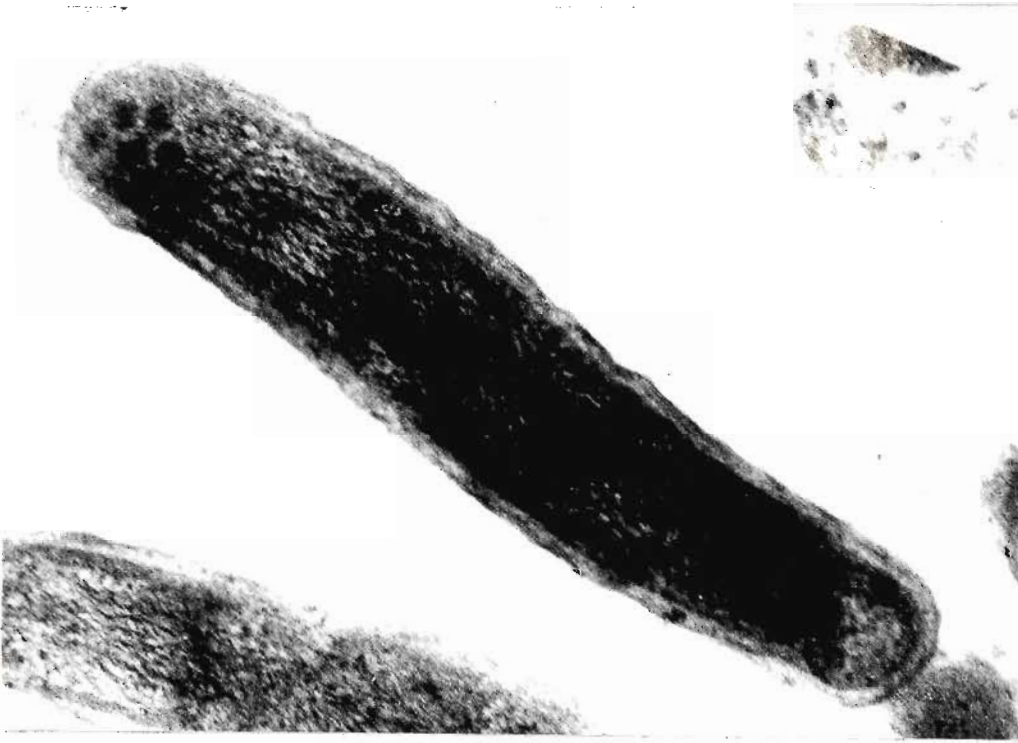


Fig. 35. Section of *N. gracilis* showing a lack of cytomembranes. (81 280 x).

Nitrococcus mobilis

Spherical cells, with a diameter of 1,25 μm were also observed in the culture water (Fig. 36). These cells possess distinct tubular cytomembranes randomly arranged within the cytoplasm. Cytomembranes of *N. mobilis* are very distinct in cells growing in aggregates (Figs. 24, 36) and less so in single cells (Fig. 38).

The effect of malachite green fixation can be seen by comparing Figs. 37 and 38. *N. mobilis* cells from the same culture were fixed with (Fig. 37) and without (Fig. 38) malachite green. Malachite green

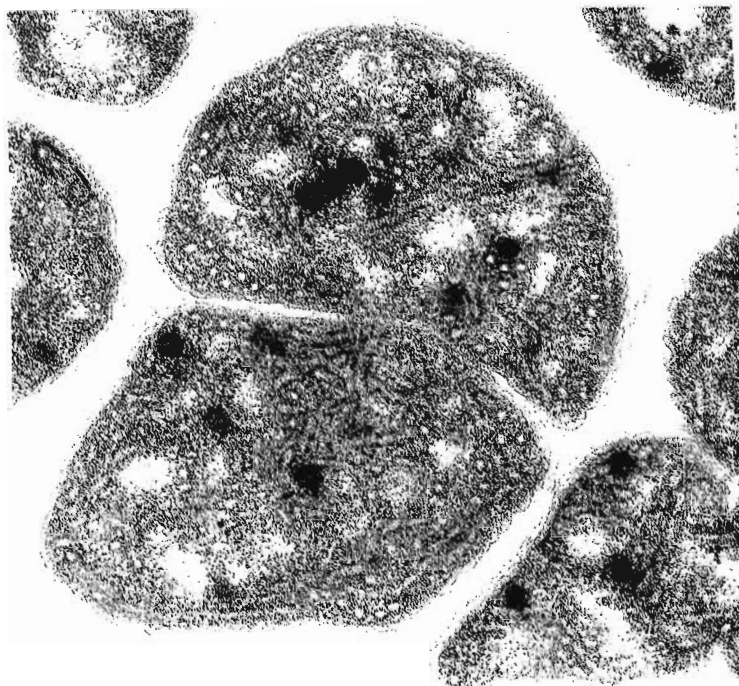


Fig. 36. Section of *N. mobilis* with randomly arranged tubular membranes (43 490 x).

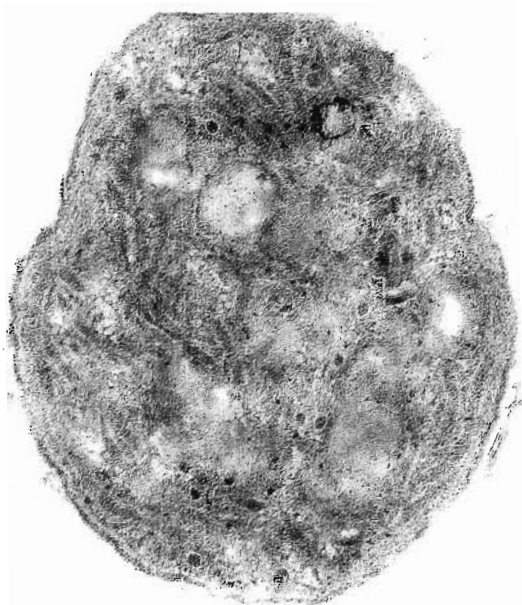


Fig. 37. Section of *N. mobilis* fixed with malachite green. Cytomembranes appear as electron dense areas (56 872 x).

enhances the appearance of cytomembranes. This effect is attributed to the lipid component of membranes which appear electron dense when fixed with malachite green (Kushnaryov *et al.*, 1980). The cytoplasmic membrane also appears as an electron dense layer. These results indicate the potential of using malachite green fixation to show the presence and arrangement of cytomembranes in nitrifiers. This technique would be extremely useful for cells lacking prominent cytomembranes.



Fig. 38. Section of *N. mobilis* fixed with glutaraldehyde and osmium tetroxide (82 340 x).

DISCUSSION

Previous studies on the process of nitrification in closed-systems and natural environments have been primarily indirect observations on the oxidation rates of ammonia and nitrite in the production of nitrate. *In situ* nitrifiers are usually enumerated by indirect cultural procedures using MPN techniques (Matulewich *et al.*, 1975). Direct examination of nitrifying bacteria in closed systems has been neglected in previous studies (Kawai *et al.*, 1965; Gerhardt, 1978; Mevel & Chamroux, 1981). In only one report on the direct examination of nitrifying bacteria, Johnson & Sieburth (1976) attempted to identify the *in situ* nitrifying population in closed systems. This investigation was not very successful as only two ammonia-oxidizing bacteria, *Nitrosomonas* and *Nitrosococcus*, were recognized in the culture water. Although these workers investigated closed systems in an active state of nitrification, they failed to identify any nitrite-oxidizers which must have been present. In addition, this investigation was only qualitative and restricted to the ultrastructure of the *in situ* microbiota. In view of such shortcomings in previous reports on nitrification in closed systems, the results obtained in the present study provides a more complete assessment of both quantitative and qualitative aspects of nitrification in such systems.

Active nitrification in the closed system is indicated by the numbers of nitrifying bacteria (Fig. 18) and the rate of nitrate accumulation from the oxidation of ammonia and nitrite (Fig. 39). Initially, the ammonia-oxidizers outnumbered the nitrite-oxidizers. After 8 weeks, both groups attained a common maximum MPN estimate before fluctuating until the end of the experiment. Kawai *et al.* (1964) also obtained an initially higher number of ammonia-oxidizers in a closed system. Thereafter, these workers reported that the nitrite-oxidizers were approximately 10x greater in

number than the ammonia-oxidizers for the remainder of the experiment. The fluctuating numbers of nitrifying bacteria (Fig. 18) is most probably due to the formation of aggregates in the culture water (Watson *et al.*, 1981). Since these bacteria commonly form aggregates, the enumeration of these organisms could produce such fluctuations in the MPN estimates.

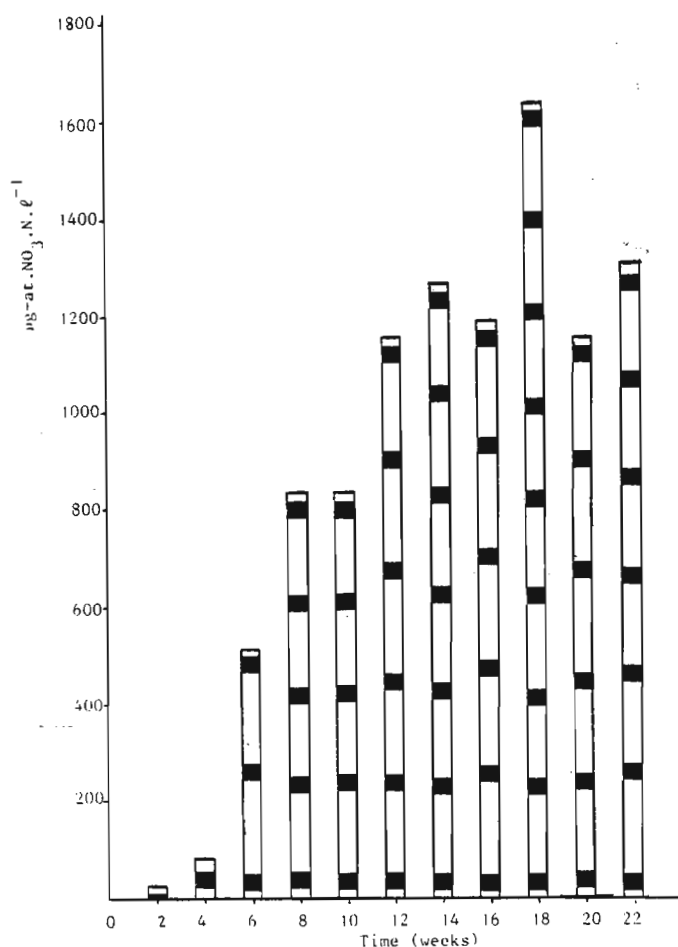


Fig. 39. Rate of nitrate accumulation in the closed system. These calculations are based on the data in Fig. 17.

The nitrification profile in Fig. 17 corresponds to the classical sequence of nitrification in closed systems (Roff, 1972; Gerhardt, 1978; Mevel & Chamroux, 1981). Figure 40 shows the pattern of nitrification reported by Roff (1972). A comparison of the results in Fig. 40 with those reported in the present study indicates the similarities in the successive appearance of the different nitrogenous compounds, i.e., ammonia followed by nitrite and finally nitrate, which accumulates in the environment.

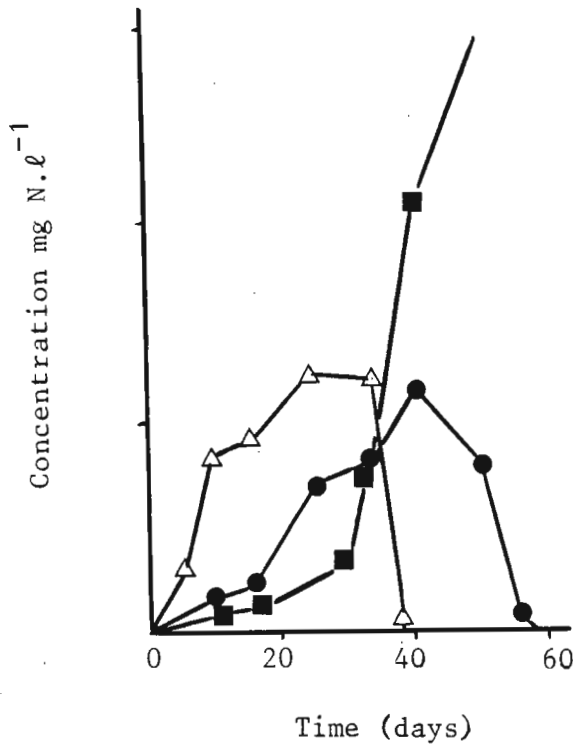


Fig. 40. Typical sequence of changes of inorganic nitrogen forms (NH₃, —△—; NO₂, —●—; NO₃, —■—) in a closed system using a biological filter (Roff, 1972).

An early appearance of adequate numbers of ammonia-oxidizing bacteria results in the rapid oxidation of ammonia which accumulates as nitrite (Fig. 41). The ammonia level in the culture water ranged between 4,8 and 68,7 $\mu\text{g-at.NH}_3\text{-N.l}^{-1}$ over the experimental period. The upper limit is equivalent to 2,5 $\mu\text{g-at.unionized NH}_3\text{-N.l}^{-1}$ and does not exceed the maximum acceptable level of 7 $\mu\text{g-at.unionized NH}_3\text{-N.l}^{-1}$ recommended as being non-toxic for *P. monodon* (Wickins, 1976).

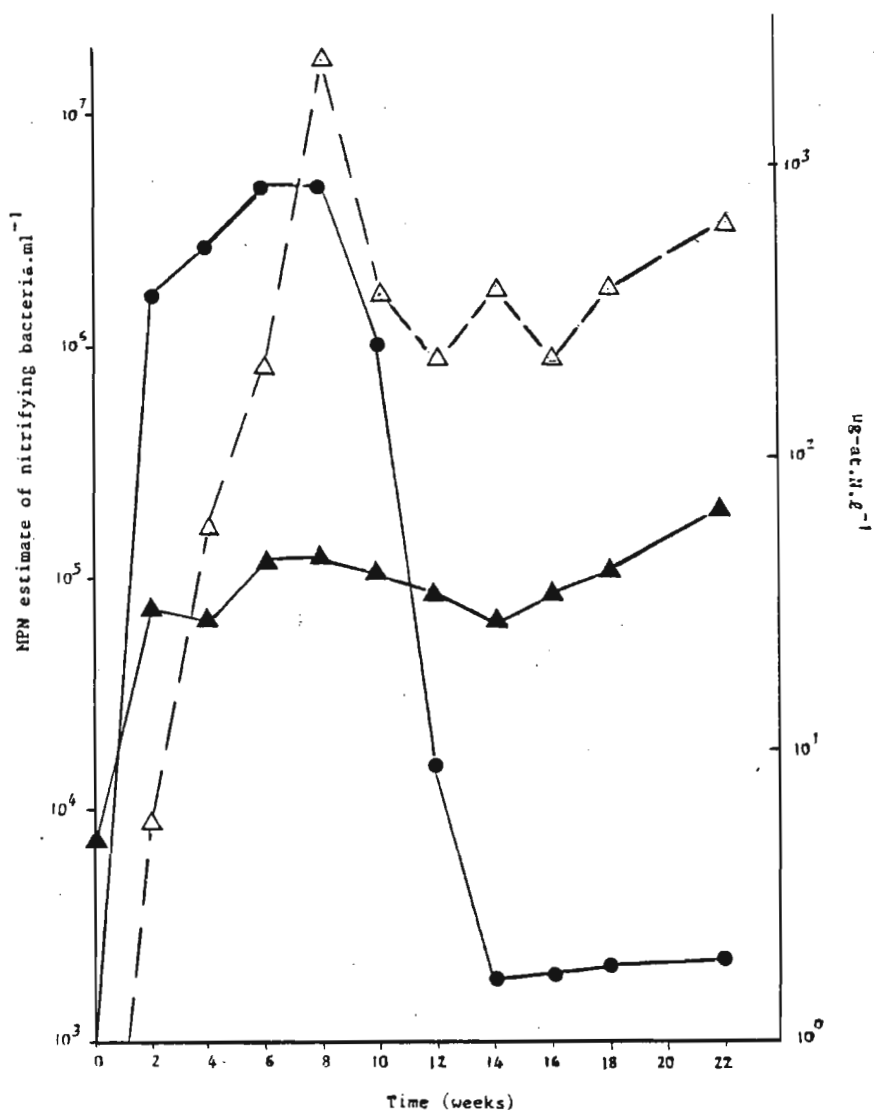


Fig. 41. Influence of the numbers of ammonia-oxidizers (--Δ--) on the oxidation of ammonia (-▲-) to nitrite (-●-). This data has been extracted from Figs. 17 and 18.

An accumulation of nitrite in the culture water is related to the initial slow growth and low numbers of nitrite-oxidizers (Fig. 42). An increase in the rate of nitrite-oxidation follows an increase in the numbers of nitrite-oxidizers. Thereafter, the nitrite concentration is maintained at a very low level, with a resultant steady accumulation of nitrate. Nitrite was detected within the range 0,2 to 891,0 $\mu\text{g-at. NO}_2\text{-N.}\ell^{-1}$ and nitrate within the range 0,4 to 989,1 $\mu\text{g-at. NO}_3\text{-N.}\ell^{-1}$ in the culture water. According to Wickins (1976), the growth of *P. monodon* was not affected by concentrations of 14 000 $\mu\text{g-at. NO}_3\text{-N.}\ell^{-1}$ after 3-5 weeks

exposure. This worker also reported that the growth of *P. indicus* was reduced by $\sim 50\%$ when exposed to $450 \mu\text{g-at.NO}_2\text{-N.l}^{-1}$ for 34 d. Mevel & Chamroux (1981) found that a nitrite concentration of $7 \mu\text{g-at.NO}_2\text{-N.l}^{-1}$ or more caused the death of *P. japonicus* in a closed system. This result should be interpreted with caution since many variables, e.g. fluctuating DO concentrations and high stocking density, were operative in this study. However, the elevated levels of nitrite observed in the present study, occurred for a short period and did not result in mortality of the prawns. Furthermore, no obvious adverse effects were observed on the growth of *P. monodon* (Table 19).

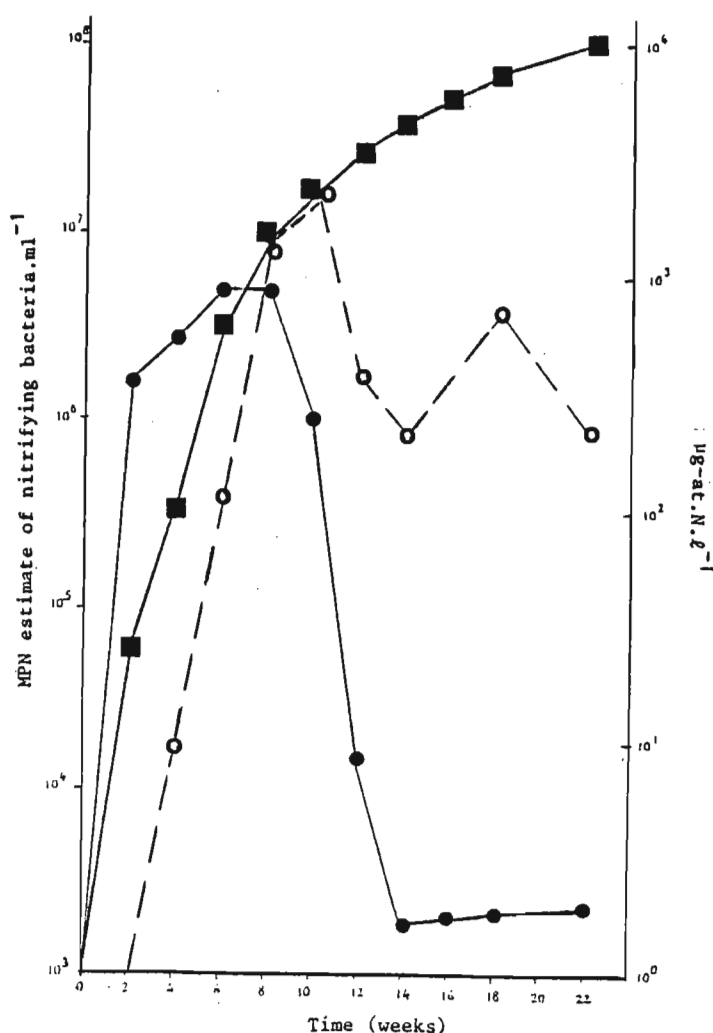


Fig. 42. Influence of the number of nitrite-oxidizers (--O--) on the oxidation of nitrite (-●-) to nitrate (-■-). This data has been extracted from Figs. 17 and 18.

Although the nitrifying population in the filter did not show any marked increase in growth after attaining their maximum numbers (Fig. 18) their nitrifying activity (Fig. 39) was sufficient to maintain ammonia and nitrite at fairly low levels. According to Saeki (1958 - cited by Kinne, 1976) the amount of nitrogen compounds assimilated by the filter sand bacteria ranges between 45 and 100% of the amount oxidized. The rate of increase is proportional to the increase of bacteria, while the oxidation rate is proportional to the mass of the bacteria. Hence, oxidation may proceed even when bacterial numbers have reached a "steady-state". Therefore, according to the results in Fig. 18, a significant assimilation of nitrogen compounds would occur during the first 8 weeks, while oxidation would proceed throughout the experiment.

The importance of adequate levels of DO on nitrification can be seen from the results by Mevel & Chamroux (1981) in Fig. 7. These workers attributed the decrease in nitrate concentration to denitrification stimulated by oxygen deficiencies in the closed system. It was also observed by these workers and Kawai *et al.* (1965) that nitrification increases and becomes intense at high oxygen concentrations. Gould & Lees (1960) also reported a similar effect on *Nitrobacter*. These findings emphasize the importance of adequate DO levels in the culture water of a closed system. In the present study, denitrification did not occur to any observable level (Fig. 42). The reason for this is that an oxygen deficiency was not experienced in the closed system (Table 16). Furthermore, the culture water percolating through the filter was almost saturated with oxygen and this did little to stimulate denitrification in the filter sand.

A decrease in DO of the culture water after percolating through the filter (Table 16) indicates oxygen consumption by the filter sand micro-

organisms (OCF). Since bacteria have the tendency to adhere to solid surfaces, particles of the sand filter provides the ideal substrate for bacterial colonization. A comparison of the ratio of nitrifiers in the sand filter to those in the tank culture water (Fig. 18) provides an indication of the larger population in the sand filter. Hirayama (1966) reported that OCF increases with the animal body mass, as do the concentrations of ammonia and nitrite. Since ammonia and nitrite are rapidly oxidized (Fig. 17), the rate of nitrate production (Fig. 39) would be indicative of the OCF in the present study. This is supported by Mevel & Chamroux (1981) who reported that OCF in their closed system increases with an increase in nitrification and *vice versa*.

Further evidence of a significant oxygen consumption by nitrifiers was presented by Gundersen & Mountain (1973). These workers observed that nitrifying bacteria use 21% of the total amount of oxygen consumed during organic decomposition, provided all the formed ammonia is completely oxidized to nitrate. Wickins (1976) reported that the oxidation of 1 mg $\text{NH}_3\text{-N}$ to nitrate requires 4.27 mg oxygen. The importance of adequate DO levels for nitrification in a closed system and the contribution by nitrifiers to the subsequent depletion as a result of OCF is supported by the above findings.

The objective of determining the BOD of the culture water (Fig. 16) was to measure the oxygen demand by microorganisms in the transformation of degradable carbon and nitrogen compounds. In assessing the quality of culture water in a closed system with active nitrification, the oxygen demand by carbonaceous and nitrogenous compounds should be considered together. Therefore, recommendations by Ruchhoft *et al.* (1946), Hurwitz *et al.* (1947), Buswell *et al.* (1950) and Young (1973) to suppress nitrification in the BOD test was not considered in this study. Other reasons

for not adopting nitrification suppression in the BOD test are:

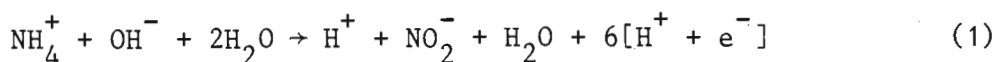
nitrification control is more appropriate when calculating the efficiency of organic waste removal in a biological treatment process (Young, 1973); carbonaceous control may also be inhibited by nitrification suppressants (Rand *et al.*, 1975); and since nitrifiers grow slowly in fresh domestic wastewater or industrial wastes, nitrification control has not been adopted as a routine step in the measurement of BOD (Young, 1973).

When compared to a BOD of 200-300 $\text{mg} \cdot \ell^{-1}$ for sewage (Colwell *et al.*, 1975) the BOD of the culture water (Fig. 16) indicates a relatively low level of pollution. Since the closed system was in an active state of nitrification (Fig. 39) the contribution to the BOD by nitrification must be significant. According to Young (1973), Montgomery & Borne (1966) and Wezernak & Gannon (1968) reported that wastewater containing 20 $\text{mg} \cdot \ell^{-1}$ $\text{NH}_3\text{-N}$ has a potential nitrogenous oxygen demand (NOD) of $\sim 87 \text{ mg} \cdot \ell^{-1}$. During intense nitrification in the closed system used in the present study $\text{NH}_3\text{-N}$ was oxidized at a mean rate of $117,1 \mu\text{g-at} \cdot \text{NH}_3\text{-N} \cdot \text{d}^{-1}$ to nitrate (Fig. 39). Thus the NOD for this quantity of $\text{NH}_3\text{-N}$ would be $\sim 7 \text{ mg} \cdot \ell^{-1}$. Therefore the significance of nitrification on BOD is clearly shown by the above example of NOD. This would also explain the parallel increase in BOD (Fig. 16) with the increase in nitrification (Fig. 39), especially during the early stages of the experiment.

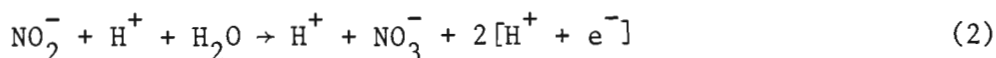
Changes in the pH of the closed system (Fig. 15) may be attributed to respiration by the prawns and bacteria, the oxidation of metabolic wastes, and the effect of accumulated nitrate ions (Atz, 1964). Gundersen &

Mountain (1973) reported that the oxidation of ammonia to nitrate by nitrifying bacteria in sea-water is accompanied by a drop in pH. This is explained by the fact that the formation of one mole of nitrate ion from one mole of ammonium ion is accompanied by the formation of exactly one mole of hydrogen ion. With $[H^+ + e^-]$ used to denote the reducing potential, these biochemical reactions can be written as follows:

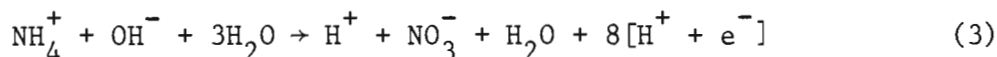
oxidation of ammonia:



oxidation of nitrite:



Overall:



Therefore, from equation (3), the end-product of nitrification is nitric acid. Since active nitrification occurred in the closed system significant amounts of nitric acid were formed (Fig. 39). This must obviously affect the buffering system of the culture water and would be reflected in its pH. This view is also shared by Gundersen & Mountain (1973). Wickins (1976) observed that the oxidation of excretory ammonia by nitrifying bacteria was largely responsible for the ionic changes that lower the pH in closed systems. Therefore, the decline in pH which occurred in the present study can be ascribed, in part, to active nitrification in the closed system.

Nitrification (Fig. 39) does not appear to be adversely affected by a decline in pH (Fig. 15). Although the nitrifiers were observed to grow optimally at pH 7,6-8,0 in culture, their nitrifying activities at pH 6,77 were sufficient to maintain the levels of ammonia and nitrite at

non-lethal levels (Wickins, 1976). The accumulation of $\sim 10\ 000\ \mu\text{g-at. NO}_3\text{-N}$ (Fig. 17) contributed significantly to the lowering of the initial pH of 8,16 to 6,77 over a period of 22 weeks. Although pH changes are normally counteracted in closed systems (Kinne, 1976), the results obtained in this study indicate that a decrease in pH to 6,77 can be tolerated by both prawns and nitrifying bacteria.

In view of the fact that this study was carried out in a closed system for 22 weeks, the mean wet mass obtained for *P. monodon* compares favourably with those reported in other experimental trials using semi-open systems (Table 19). As a result of water exchange, semi-open systems, although more labour intensive (Gerhardt, 1978), would provide culture conditions more conducive to nitrification than closed systems. It is possible that growth of the prawns would have improved with culture water treatment. This is supported by the report of Wickins (1976) that growth of *P. monodon* was reduced at low pH. At pH 6,45 growth was $\sim 42\%$ - while at pH 6,6 it was $\sim 78\%$ of controls grown at pH 8,0. However, in spite of a reduced growth, survival was 100% with the pH as low as 6,45. In the present study, survival of *P. monodon* was 93,75%. The mortality of 6,25% was not related to any detrimental effects of the culture water but to "jump-outs" and cannibalism. The survival rate was very good in comparison with that reported by Mevel & Chamroux (1981) for *P. japonicus*. These workers experienced death rates in excess of 30% in a closed system with a high animal load. The high mortality was caused by a build-up of nitrite in excess of $7\ \mu\text{g-at. NO}_2\text{-N.l}^{-1}$. Further research is necessary to investigate the growth of prawns under carefully monitored and controlled conditions of nitrification in closed systems. If such studies are undertaken, the stocking density must be taken into account since a lower stocking density is more conducive to growth (Table 19).

The accuracy and sensitivity of most methods will vary from one laboratory to another because of differences in experimental conditions. Since the final choice of a method is important for the accuracy of the results obtained, it is always good practice to evaluate any method before using it for the actual analysis of experimental samples. In the present study methods were chosen for the evaluation for the following reasons:

- ease of operation;
- low cost per sample;
- high degree of accuracy and sensitivity reported; and
- may be carried out in most small laboratories because no sophisticated equipment is required.

The phenolphosphorite method (Solórzano, 1969) offers the greatest accuracy and sensitivity for the determination of ammonia (Table 11). This finding is contrary to the report of Gilbert & Clay (1973) who observed the electrode method (Orion Research Inc., 1978) to be more accurate than that of Solórzano (1969). According to Riley & Chester (1971), the phenolphosphorite method (Solórzano, 1969) is widely used for the analysis of ammonia in sea-water. The results in this study (Table 11) indicate that this method is equally accurate for the analysis of ammonia in saline culture water from a closed system. Poor results (Table 12) were obtained with the alkaline hypochlorite-arsenite method (Strickland & Parsons, 1960) although a greater accuracy has been reported. Since a poor performance of this method was observed for both sea-water and culture water samples, it is unlikely that this was due to the presence of interfering substances in the latter samples.

The electrode method (Orion Research Inc., 1979) for nitrite could not be evaluated because of the erratic performance of the electrodes

(Fig. 14). This electrode performance was due to contamination of the internal filling solution (A. Pienaar, Labotec (Pty) Ltd., 1982 - pers. comm.). Several titrimetric, colorimetric and gasometric methods have been proposed for the determination of nitrite. Among these methods, the colorimetric method of Griess-Ilosvay (Strickland & Parsons, 1960) has proved the most specific, sensitive and accurate method for determination of nitrite in soil extracts and water samples (Tabatabai, 1974). The accuracy of this method (Table 14) for both sea-water and culture water samples has been verified in this study.

Direct determination of nitrate by colorimetric methods lacks reliability and sensitivity (Wood *et al.*, 1967). The alternative is an indirect method which reduces nitrate quantitatively to nitrite. This can then be determined by the sensitive method of Griess-Ilosvay (Strickland & Parsons, 1960). According to Wood *et al.* (1967), reduction of nitrate in a copperized cadmium column is greater than that obtained with zinc powder, hydrazine with cupric ions as catalyst, or cadmium alone. The reduction efficiency of the copperized cadmium column used in this study (Table 15) is far greater than that reported for any of the alternate methods of nitrate reduction (Wood *et al.*, 1967).

With the exception of the investigation by Matulewich *et al.* (1975), previous reports (Table 2) on the enumeration of nitrifying bacteria did not take into account the effect of incubation time on the maximum MPN estimates of these organisms. The results in Tables 5, 17 and 18 underline the importance of an adequate incubation period for the enumeration of nitrifiers. The results obtained in this study reaffirms the view of Matulewich *et al.* (1975) that the incubation periods used for nitrifiers in previous investigations may have been too short to yield maximum estimates of these organisms. Results from Tables 17 and

18 suggest that 20 d- and 65 d incubation periods are required to obtain the maximum MPN estimate of ammonia and nitrite-oxidizers, respectively. These incubation periods should be used as a guide for the enumeration of nitrifiers in closed systems. A generalization for the marine environment as a whole can only be made after further investigation of a wider range of marine samples. The results obtained in this study indicate that marine nitrifiers may require a shorter incubation period for enumeration than their non-marine counterparts. This may be especially true for non-marine nitrite-oxidizers that required an incubation period of longer than 113 d for the maximum MPN estimate (Matulewich *et al.*, 1975).

In practice, the selection of appropriate periods of incubation must balance convenience against the objectives of the study. Although the disadvantages of an unnecessarily prolonged incubation period are many, too brief an incubation of nitrifying bacteria results in an underestimation of unknown and variable magnitude. The period required for the maximum recovery of cells is a function of the physiological state of the inoculum as it influences the duration of the lag phase of growth, and of the growth rate once proliferation commences (Matulewich *et al.*, 1975). In the present study, nitrite-oxidizers that manifested themselves at high inoculum dilutions did so after a very long period of incubation. The phenomenon cannot be explained on the basis of unusually slow growth rates, which, contrary to observation, would have resulted in a gradual disappearance of substrate. It can be inferred, therefore, that these nitrite oxidizers passed through a prolonged lag phase.

The concentration of the energy source should also form an important consideration in the enumeration of nitrifiers from natural environments. Johnson & Sieburth (1976) reported that ammonia-oxidizers from closed

systems lysed in enrichment cultures containing 250 mg $\text{NH}_4 \cdot \ell^{-1}$. Therefore, a low concentration of the energy source is desirable. In this study 30 mg $\cdot \ell^{-1}$ of either $(\text{NH}_4)_2\text{SO}_4$ or NaNO_2 were used while Matulewich *et al.* (1975) used either 500 mg $(\text{NH}_4)_2\text{SO}_4$ or 100 mg KNO_2 in a litre of medium. However, no conclusion can be drawn on the effects of the different concentrations of the energy source without further investigation.

Problems facing investigators in the isolation and purification of nitrifying bacteria have been stressed in the literature review. However, it should be mentioned that these problems were also encountered in this study. Pure cultures of *Nitrosomonas europaea* and *Nitrobacter winogradskyi* were obtained with dilution and plating techniques, and also with the use of antibiotics. Greatest success was achieved with enrichment cultures grown in the fed-batch reactor. Since these enrichment cultures are provided with optimal growth conditions, viz., pH, temperature, oxygen saturation, energy source and agitation, the nitrifiers are able to outgrow the heterotrophic contaminants. This is the fundamental objective in obtaining pure cultures of nitrifying bacteria. The micromanipulator is a useful tool because single microcolonies of presumptive nitrifiers can be isolated at an early stage of growth. This technique would, to a certain extent, overcome the slow growth of these microcolonies.

Oxytetracycline and albamycin T successfully inhibited the growth of heterotrophic contaminants in enrichment cultures, and thereby assisted in the purification of nitrifiers. This finding together with that of Gould & Lees (1960) indicate the potential of using antibiotics to purify nitrifying bacteria. Although Gould & Lees (1960) used matro-mycin and terramycin to obtain pure cultures of *Nitrobacter*, these

workers did not attempt to explain the effect of the antibiotics on the nitrite-oxidizers. The simplest explanation would be that nitrifiers lack the uptake mechanisms for antibiotics. Since antibiotics have common uptake mechanisms for certain organic substrates, these mechanisms would not be present in chemolithotrophic bacteria.

Nitrosomonas europaea and *Nitrobacter winogradskyi* were the dominant nitrifiers in the enrichment cultures. Consequently, these organisms were obtained in pure culture while this could not be done with the slow growers. Attempts were made to isolate the slow growers from lower dilutions of enrichment cultures by inoculating solid media. This was in vain because the lack of a distinct colonial morphology and the confluent growth of heterotrophic contaminants made it difficult to obtain pure cultures of the slow growing nitrifiers. The results obtained in the present study indicate the advantage of growing enrichment cultures in fed-batch reactors. Antibiotics, if used judiciously, could play an important role in obtaining pure cultures of nitrifiers. This aspect requires further investigation. Also, further research is required on cultural conditions that would stimulate the growth of the less dominant nitrifiers, thereby aiding in their purification.

Species of all the known genera of marine nitrifying bacteria were observed in the culture water. The organisms obtained in pure culture, *Nitrosomonas europaea* and *Nitrobacter winogradskyi* are the most commonly encountered nitrifiers in natural environments. It appears that they were also the dominant nitrifiers in the closed system. Watson *et al.* (1981) reported the existence of three morphologically different strains of *Nitrosomonas europaea*. All three strains were observed in this study (Figs. 28, 30, 31). Two species of *Nitrosococcus*, viz., *N. oceanus*

and *N. mobilis* have been reported for the marine environment. Only the latter species has been found in the culture water (Figs. 32, 33). The description of this species is based on only one isolate cultured from the water of the North Sea (Koops *et al.*, 1976).

The species description of *Nitrococcus mobilis* (Fig. 37) is based on one isolate from South Pacific Ocean waters (Watson & Waterbury, 1971). The description of *Nitrospina gracilis* (Fig. 35) is based on one marine strain isolated from the South Atlantic Ocean waters (Watson & Waterbury, 1971). Difficulties in isolating the less dominant nitrifiers, viz., *Nitrosococcus mobilis*, *Nitrospina gracilis* and *Nitrococcus mobilis* are shown by the fact that the species description of these organisms is based on single isolations only. Also, this study indicates that species of marine nitrifying bacteria are common to the different oceans of the world. It should also be borne in mind that other nitrifiers, not previously isolated, may have been overlooked because of a lack of extensive cytomembranes or because of their extremely low numbers. Isolation of new species of nitrifiers will always remain a fertile area for future research.

This study highlights the significance of nitrification in a closed system by investigating both the physico-chemical and biological components of nitrification. With the exception of the biological filter, no further treatment of the culture water was necessary to maintain ammonia, nitrite and nitrate at non-lethal levels for 22 weeks. Evidence for the role of nitrifying bacteria in maintaining these nitrogenous compounds at non-lethal levels is provided in the form of the enumeration and identification of these organisms. The high rate of nitrification requires that pH is controlled. This would stimulate both the growth of *P. monodon* and the nitrifying bacteria. Although recommendation for closed system aquaculture is that the culture water

should be "seeded" with an adequate population of nitrifying bacteria. The inoculum for this purpose must be in the log phase of growth as this would avoid an unnecessarily long lag phase. Efficient seeding of the culture water would effectively reduce high levels of ammonia and nitrite that are characteristic of the classical sequence of nitrification (Figs. 17, 40).

It is hoped that the results of this investigation will assist culturists in exploiting the presence of nitrifying bacteria in closed systems. Judicious manipulation of these organisms would prevent the build-up of nitrogenous compounds, especially ammonia and nitrite, to toxic levels. Optimal conditions for growth, viz., pH and DO can be maintained with relatively little effort. These conditions appear to be the principal requirements for active nitrification in the presence of ammonia and nitrite. The advantages of closed systems over open systems are many and have been discussed earlier. Nitrate accumulation is very significant in closed systems and the disposal of untreated prawn effluent into natural aquatic environments would result in eutrophication of the water. Therefore, culturists should utilize the nitrate rich effluent for algal growth since algae forms part of the natural foods of prawns. This would improve the water quality for either recycling or disposal, while being of mutual interest to both aquaculture and the environment.

The objective of this study was to use a closed system as a model to investigate both quantitative and qualitative aspects of nitrification, viz., the oxidation rates of ammonia and nitrite to nitrate, and the enumeration and identification of the associated nitrifying population. Results from this investigation indicate that this objective has been successfully achieved. Detoxification of the culture water is clearly

demonstrated by the profile of the nitrogenous compounds and by the numbers and diversity of the nitrifying bacteria. Evidence of culture water changes as a result of nitrification is also presented. These changes include decreased pH and DO levels and an increased BOD.

In conclusion, it would be appropriate to pay tribute to the nitrifying potential of the chemolithotrophs that made possible the successful rearing of *P. monodon* for 22 weeks in a closed system without any water exchange.

SUMMARY

A closed system, stocked with *Penaeus monodon*, was used as a model to study the role of marine nitrifying bacteria. *P. monodon* was successfully grown for 22 weeks without any exchange of the culture water. A survival of 93,75% was recorded while the mean wet mass increased from 0,68 g to 12,87 g. The low mortality occurred from "jump-outs" and cannibalism and therefore was not related to any adverse effects of the culture water. Both the survival and wet mass of *P. monodon* compared favourably with those obtained in the more conducive semi-open systems.

The successful rearing of *P. monodon* in the closed system is attributed to the detoxifying ability of nitrifying bacteria present in the system. A classical pattern of nitrification was observed in the culture water, i.e., the appearance of ammonia followed successively by nitrite and nitrate, with the latter accumulating in the culture water. Ammonia-oxidizers reached their maximum numbers in the filter after 8 weeks while 10 weeks were required for nitrite-oxidizers. The maximum MPN estimate of either group of nitrifiers in the filter was $1,73 \times 10^7 \text{ ml}^{-1}$ while the maximum MPN estimate of their counterparts in the culture water was $\sim 500\times$ lower.

The MPN estimate of the nitrifying bacteria was related to the oxidizing patterns of ammonia and nitrite. Ammonia and nitrite were detected within the range 4,8-68,7 and 0,15-891 $\mu\text{g-at.N.l}^{-1}$, respectively. Nitrate reached a maximum concentration of 9898,1 $\mu\text{g-at.N.l}^{-1}$ at the end of the experiment. Ammonia, which is very toxic to aquarium animals, did not reach toxic levels during the experiment. Since toxic levels of nitrite and nitrate for *P. monodon* are not very well documented, such conclusions cannot be drawn for the nitrite and nitrate concentrations in the culture water.

Results obtained on the effect of incubation time on the maximum MPN estimate of nitrifying bacteria indicate that nitrite-oxidizers require a longer incubation period than ammonia-oxidizers. The former group of nitrifiers required a mean incubation period of 36,4 d within a range of 20 to 65 d, while the latter group required a mean incubation period of 15 d within a range of 10 to 20 d. These results also indicate that the enumeration of marine nitrifying bacteria in closed systems may require a shorter incubation period than their nonmarine counterparts.

Various methods for the determination of ammonia, nitrite and nitrate were evaluated for their accuracy and reproducibility. These evaluations were carried out with both sea-water and culture water samples. The results obtained indicate that it is necessary to check the accuracy and reproducibility claimed for any method because of differences in experimental conditions. The phenolhypochlorite- (Solórzano, 1969), Griess-Ilosvay- (Strickland & Parsons, 1960) and copperized cadmium reduction method (Wood *et al.*, 1967) were chosen for the determination of ammonia, nitrite and nitrate, respectively.

The slow growth of nitrifiers made it difficult to separate them from the faster growing heterotrophic contaminants. Pure cultures of the dominant ammonia-oxidizers, *Nitrosomonas europaea*, and nitrite-oxidizers, *Nitrobacter winogradskyi*, were obtained by using both dilution and plating techniques. These organisms were also obtained in pure culture by incorporating the antibiotics, oxytetracycline and albamycin T, in the culture medium. The micromanipulator served as a useful tool in isolating single microcolonies of the nitrifying bacteria. This assisted greatly in the purification of these organisms. Species of all known genera of marine nitrifiers were recognized from their ultrastructural morphology. These genera included *Nitrosomonas*,

Nitrosococcus, *Nitrobacter*, *Nitrococcus* and *Nitrospina*. However, one marine species, *Nitrosococcus oceanus* was not observed in the culture water.

The greatest change observed in the culture water was that of pH which decreased from 8,16 to 6,77 at the end of the experiment. This decrease occurred as a result of the nitric acid produced during nitrification. DO levels were maintained at 90% saturation in the tanks while the OCF decreased DO levels to 61,1% after the culture water percolated through the sand filter. The BOD did not exceed $23 \text{ mg} \cdot \ell^{-1}$ during the experiment. The influence of nitrification on the BOD values is shown from the NOD of $\sim 7 \text{ mg} \cdot \ell^{-1} \cdot \text{d}^{-1}$.

The objective of investigating the various aspects of nitrification in a closed system was achieved in this study. Results obtained in this study show that closed systems provide an ideal environment for such a study which is not feasible in natural ecosystems. It is hoped that the nitrifying potential of the chemolithotrophs, as highlighted in this study, will assist culturists in overcoming the toxic effects of nitrogenous compounds in closed systems.

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