BACTERIAL FACTORS INFLUENCING THE SETTLEMENT OF SHIP-FOULING ALGAE OF THE GENUS ENTEROMORPHA

by

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SUMMARY

The aim of this study was to assess the effects of selected marine bacteria upon the settlement of algae of the genus Enteromorpha.

The ship-fouling alga, Enteromorpha intestinalis, was chosen for the experimental work, and maintained in such a state to ensure the continuous supply of zoospores by means of a specially designed sea-water system and lighting regime. Experiments were carried out to select a suitable growth medium for zoospores produced by this species of Enteromorpha.

Bacteria were assessed for their ability to produce thin films on glass attachment substrates. These bacterial films were allowed to interact with suspensions of Enteromorpha intestinalis zoospores, and zoospore attachment and development were followed by optical microscopy. A counting method was developed for the enumeration of growing zoospores, two growth forms were observed, a filamentous form and a clumped, rhizoidal, form.

Some bacteria from the genera Pseudomonas and Alteromonas, and the Pseudomonas/Alteromonas group, were shown to encourage the settlement of zoospores. Other bacterial isolates from the genera Pseudomonas and Coryneform, and the Pseudomonas/Alteromonas group discouraged the settlement of zoospores.

It was found that a number of bacterial films continued to encourage the settlement of zoospores when the bacterial cells were killed. This also occurred with some of the bacterial isolates which discouraged the settlement of zoospores.

Cell-free bacterial extracts were shown to have little effect upon the settlement of zoospores, and there is evidence to indicate that the bacterial factor(s) that effect the settlement of zoospores may be located on the cell walls of the bacteria, or the extracellular polymers produced by these film-forming bacteria.

Copper, a common component of antifouling paints, inhibited the growth of the bacteria used in this study, but only at concentrations greater than normally achieved with copper-based antifouling paints.

KEY WORDS: Bacteria; Copper; Enteromorpha intestinalis; Marine Fouling; Zoospores.

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THIS THESIS IS DEDICATED TO MY FAMILY AND TO MY FRIENDS

CHAPTER 1

INTRODUCTION

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INTRODUCTION

1.1 Biofouling: an Historical Outline of the Problem and the Development of Antifouling Techniques.

Biofouling is the term applied to the unwanted growth of organisms upon structures of economic importance to man (Mitchell and Benson, 1981; Carson and Allsopp,1983). The term is normally reserved for structures submerged in an aqueous environment and hence the causative organisms of biofouling are usually aquatic animals, plants or protista.

In freshwater biofouling is minimal, the blockage of waterways by aquatic plants and the growth of slime being the main problems. The marine environment represents a totally different situation as it is much richer in fauna and flora and many species are specifically adapted to a sessile existence.

In early attempts to use the sea as a means of trade, communication and warfare, it is doubtful if mariners made any conscious efforts to prevent biofouling, as their main concern was to keep their vessels watertight.

As construction methods improved, a variety of materials were used to improve the structural strength of vessels and increase their waterproof properties. Metal cladding was added to certain areas of the hull, mainly the bow and the keel, and pitch was used for waterproofing (Acock, 1966).

Whether the intention went beyond the desire to improve the durability of their vessels, and was an attempt to prevent the growth of marine organisms can only be a matter of conjecture. Houghton (1970) states that both the Phoenicians and the Romans were aware of the dangers presented by the ship worm (Teredo navalis) and gribble (Limnoria lignorum). They used pitch, copper and lead to protect the submerged parts of their ships' hulls from attack by marine borers.

By the nineteenth century the sheathing of wooden hulls with copper plates had become common practice (Acock, 1966; Turner, 1971) and it had been realised that copper salts, produced by the corrosive action of sea-water upon these plates, deterred the settlement and growth of fouling organisms. As the primary intention of shipbuilders was to maintain the structural strength of vessels, which included providing protection against ship worm, a method was sought to prevent copper cladding from dissolving in sea-water. Acock (1966) pointed out that it was Sir Humphry Davy who solved the problem of the corrosion of copper cladding by the introduction of the sacrificial cathode of zinc or cast iron. This deterred corrosion but allowed the development of fouling communities, as toxic copper salts were no longer produced.

The advent of iron-hulled vessels in the middle of the nineteenth century introduced a new problem. Initially

copper plates were used to prevent fouling, but it was soon realised that serious corrosion of the iron hulls occurred (Morgenstern, 1978). This corrosion was due to the electrochemical couple which ensues when metals of different electrochemical potentials are immersed in sea-water. Eventually copper cladding had to be abandoned (Gitlitz, 1980), but as iron hulls corrode rapidly when exposed to sea-water (even without the enhancing effects of copper), and as there was an increasing need to control fouling, anticorrosive/ antifouling paints were developed.

Paint formulations that employed a variety of toxic compounds were developed. Arsenic, copper and lead salts were incorporated into paints based on linseed oil, which had good drying properties. Millscale, a hydrated iron oxide produced as a result of weathering during the fabrication of ships, was often only partially removed before the beginning of a painting programme and this frequently led to the failure of paint systems (Alcock, 1966).

Better methods of preparation and the replacement of linseed oil by synthetic polymers has greatly improved the adhesive properties of paints, but the toxological approach to the control of biofouling still remains (Saroyan, 1969; Morgenstern, 1978; Gitlitz, 1980).

Design restrictions inherent in the use of sail made it economically unnecessary to attempt to control biofouling

which only marginally reduced speed: the main factors governing the time of passage were the prevailing winds.

With the advent of steam-powered, iron-hulled vessels, time of passage became dependent upon the power-to-weight ratio of the vessel, and biofouling assumed a much greater importance, for any increase in the weight of the vessel, or in its frictional resistance as it moved through the water, resulted in an increase in fuel consumption and hence a financial loss to the owner.

The importance of biofouling in terms of increasing the operating costs of vessels was not initially realised by owners; and the idea still prevails that biofouling is a factor of secondary importance when compared with corrosion (Houghton, 1968).

Since the large increases in the price of fuel oil in the early seventies the situation has changed and even marginal improvements in performance can result in considerable financial savings (Banfield, 1974). It is difficult to quantify the extent of the financial burden which fouling organisms levy against the shipping world, but some estimates have been made.

Christie and Henson (1968) estimated that the annual operating losses for a reduction of speed of 0.15 knots would amount to £2,000 for a 20,000 to £6,000 for an

80,000 ton tanker: bearing in mind that these figures were quoted in 1968, and inflation and further rises in the price of oil would have increased these operating losses considerably. Many vessels now spend long periods in dock, where conditions are particularly favourable for the growth of fouling organisms (Christie and Evans, 1975). There can be little doubt that the cost of biofouling because of increased fuel consumption is rising. Gitlitz (1980) estimated that a large vessel, such as a tanker, travelling at 15 knots would consume fuel at the rate of 170 tons a day. Operation of this vessel for 300 days a year would result in an annual fuel bill in excess of 4 million U.S. dollars (assuming the 1980 price of fuel of approximately $80/ton). In such a case a reduction in efficiency of 30%, which can occur as a result of biofouling, would increase the operational cost by 1 million U.S. dollars (Gitlitz, 1980).

Reductions in speed of 1-10% are commonly encountered as a result of biofouling (Fischer et al., 1975), and improved methods of biofouling control could result in significant financial savings to fleet operators.

In the case of military vessels, costs due to biofouling are particularly high. Such vessels often spend up to 80% of their time in port, where fouling organisms abound, and thus acquire heavy biofouling. This was pointed out by Fischer et al., (1975), and it is suggested that the cost

of hull fouling alone adds 150 million dollars to the United States Navy's annual fuel bill.

The United States Navy is planning to reduce the steaming time of its vessels from 176 days per year in 1982 to 160 days per year in 1984, and to berth 22 of its older ships (Isaacson, 1983). This is almost certain to lead to even greater losses in efficiency when these ships are steaming, and hence increased fuel costs.

Military vessels have particular biofouling problems in terms of the effects that fouling organisms have upon sophisticated and expensive surveillance equipment. Even microfouling may result in an unacceptable decrease in the efficiency of this type of equipment (Montemarano and Cohen, 1976), and this produces a problem that is difficult to quantify in economic terms.

Within the past few years the biofouling of off-shore structures has emerged as a major concern of platform operators (Houghton, 1978). North Sea gas and oil operations require that operating platforms be structurally safe (Hardy, 1981), and biofouling presents a problem both in that it inhibits routine maintenance (Ralph and Troake, 1980) and increases corrosion (Ralph, Goodman and Picken, 1981). Pipe (1979A) outlines some of the problems of maintenance presented by fixed structures including corrosion and the increased mechanical load which biofouling causes the structure to bear.

Long term corrosion rates of steel structures are not accurately known (Southwell et al., 1974) and the effects of mechanical loading are not understood in detail. Fouling algae such as Enteromorpha, Ulva and Desmarestia have not, at present, been found to have any significant effect upon the loading of structures (Hardy, 1981), but organisms such as Desmarestia viridis which enhances corrosion by the production of free sulphuric acid can substantially affect the corrosion rates of off-shore structures (Hardy, 1981).

Protective iron oxide coverings, which are found on mild steel, may be substantially modified by algal growth and Enteromorpha has been shown to considerably modify the protective properties of these coatings (Edyvean and Terry, 1983).

The economics of the marine mercantile environment now make it increasingly important that biofouling be recognised as a major financial burden upon those who seek to find their living from the seas.

1.2 Major Organisms Involved in the Marine Biofouling Process

The world's seas and oceans provide a habitat for a rich variety of organisms, many of which are equipped to survive in harsh environments, even so only relatively few present any problem as regards fouling.

The problem caused by marine borers was mentioned in section 1.1. Strictly speaking, these are biodeteriogenic organisms (Jones et al., 1976), yet they do not present any significant problem as regards fouling. Most vessels in commercial use, and all off-shore structures, are now fabricated from ferrous metals or concrete, and are not prone to marine borer attack. Marine biodeteriogenic organisms are now recognised to be of fundamental importance in the corrosion process (Costello, 1969; Chandler, 1979; Pipe, 1979), and marine bacterial films produce microfouling that significantly reduces the efficiency of heat-exchange systems (Characklis et al., 1981; Pritchard, 1981).

Biofouling may be regarded as a specialised form of biodeterioration, in which little structural damage is caused but economic loss accrues, due to reduced efficiency of ships and other marine structures.

Algae, molluscs, polyzoa and turnicates are the main groups of organisms involved in marine biofouling (Christie and Evans, 1975; Yamaguchi, 1975; Hanson and Bell, 1976; Jones et al., 1976; Luther, 1976; Berk et al., 1981) and there is increasing evidence that marine bacteria play an important role in pre-conditioning surfaces for colonisation by plants and animals (Zobell and Allen, 1935; Crisp, 1974; Rayner, 1975; Cundell and Mitchell, 1977; Kirchman and Mitchell, 1983).

Fouling by crustacea, molluscs, polyzoa and turnicates can be adequately controlled by modern copper-based antifouling coatings, but the effective life of these coatings is relatively short, 14 to 18 months being a typical useful life for such formulations (Gitlitz, 1980).

The marine algae have now largely replaced marine animals as the major group of fouling organisms on ships protected by copper-based antifouling paints (Banfield, 1974). Algae of the genera Enteromorpha and Ectocarpus are the most common biofouling organisms found on the hulls of tankers (Christie, Evans and Callow, 1975; Fletcher and Chamberlain, 1975; Evans, 1981) and this has led to the use of organotin compounds in an attempt to control these organisms (Ghanem and el-Malek, 1978 (Part 1)).

That algal fouling presents the shipping industry with its most difficult problem regarding biofouling (Callow, Evans and Christie, 1976) is hardly surprising, as these organisms have evolved in the harsh environment of the inter-tidal zone. Here conditions are constantly changing and resilient organisms, capable of survival over a wide range of temperature, salinity and nutrient levels (Evans, 1981) have developed.

1.3 Contemporary Methods for the Prevention of Biofouling

Many methods have been tried in an attempt to prevent

or minimise the biofouling of structures exposed to the marine environment, but to date none has proved to be wholly satisfactory.

Copper and copper-based alloys have a high resistance to biofouling but have limited application as they are expensive and, under certain conditions, enhance the corrosion of ferrous metals. Heat exchangers and sea-water handling systems take advantage of the corrosion resistance, thermal conductivity and antifouling properties of copper and its alloys (Moreton, 1982).

Technetium-99 is a radioactive isotope which emits β rays with a mean energy of 100kV, and Spitsyn et al. (1982) suggest that this could be used, applied as a thin coating, to prevent biofouling. These authors point out, however, that little is known of the corrosion properties of this metal, and much more work is needed before any practical system could be developed.

At high concentrations, chlorine is effective in killing the larvae of fouling organisms, but it does not remove the adult stages (Karande et al., 1982). Chlorine dioxide has been used to prevent fouling microbial films in heat exchangers (Sussman and Ward, 1977), and electrolytic hypochlorination has proved effective in protecting ocean thermal current sensors from biofouling (Kretschmer et al., 1980).

Mechanical cleaning is useful in certain circumstances. Nickels et al., (1981) describe a method for cleaning titanium and aluminium pipes, in that water-driven brushes remove (or modify) bacterial films that develop in these pipes. Of course, the mechanical removal of fouling is a traditional method of fouling control, and sand-blasting is a common procedure for the removal of scale and fouling organisms prior to painting.

Painting of submerged (or partly submerged) structures is the most widely practised method of biofouling prevention (Saroyan, 1969; Milne, 1973), and the periodic replacement of anticorrosive and antifouling paint systems is an essential part of the maintenance programme of most vessels and offshore structures (Dekker and Happe, 1981; Reitsma, 1981). It is important that these two systems are compatible, and in particular that the antifouling coating does not cause a reduction in the effectiveness of the anticorrosive system.

Cuprous oxide is the principle toxicant employed in antifouling paints (Kronstein and Denninger 1976; Overmars et al., 1980; Smith, 1980; Applebee and Kingston, 1981). It has proven to be highly compatible with anticorrosive paints, and effective against a wide range of fouling organisms, with the exception of marine algae (Evans, 1981).

Organotin compounds, chiefly tributyltin oxide and triphenyltin fluoride (Engelhart, 1975; del la Court, 1980)

are increasingly being used to control algal fouling. Organometallic polymers, e.g. poly (tri-n-butyl methacrylate) can be used in situations where long life antifouling coatings are needed, and cost is not an overriding factor, military vessels being good examples (Castelli, 1977).

Three basic methods of toxicant release are used. The most common approach is the soluble matrix-type paint, in which insoluble particles of toxicant are dispersed in a soluble binder (Banfield, 1974), as the binder dissolves, new toxicant is exposed and the antifouling properties of the paint are maintained. Insoluble matrix paints contain a soluble toxicant which leaches out the paint and eventually leaves a 'honeycomb' of non-toxic binder (Evans, 1981).

Recently, paint systems have been developed in which the toxicant is chemically linked to the binder, dissolving out of the paint by means of an hydrolysis reaction (Yoshida, 1980). Whichever method is used, all paints have a limited life and replacement is normally required after 1 to 3 years (van Londen et al., 1975; Dempsey, 1981).

Thus it seems that the only long-term solution to the fouling problem lies in an understanding of the ecology of fouling communities, and the underlying mechanisms that operate in the attachment process.

1.4 Research Objectives

As outlined in the preceding sections, biofouling is a major and continuing problem for those who own and operate ships. Marine algae of the genus Enteromorpha constitute a particular problem, because of their resistance to copper-based antifouling formulations.

It is known that marine bacteria influence the settlement and metamorphosis in certain marine invertebrates (Kirchman et al., 1981), and Kirchman and Mitchell (1983) suggest that a lectin binding mechanism operates in the settlement of larvae of Janua (Dexiospira) brasiliensis, a marine invertebrate.

The main objective of the work presented in this thesis was to evaluate the effects of bacterial films upon the settlement and subsequent growth of zoospores produced by algae of the genus Enteromorpha. A controlled environmental system was designed and constructed enabling selected species of the genus Enteromorpha to be kept in a viable state under laboratory conditions.

Nutritional requirements for the settlement and growth of zoospores of Enteromorpha intestinalis were examined, and a suitable medium was selected, to be used in subsequent experiments involving bacterial films.

Selected bacterial strains, isolated by Carson (1980), were used in experiments designed to test the effects of thin bacterial films upon the settlement processes of Enteromorpha intestinalis. Techniques were also developed to count the numbers of Enteromorpha intestinalis germlings attached to glass substrates, both in the presence and absence of bacterial films, and the results subjected to statistical analysis.

Bacterial strains, selected for their ability to influence the settlement of Enteromorpha intestinalis were used to test the effects of certain physical and metabolic factors associated with bacterial films which may affect the settlement of Enteromorpha intestinalis. The effects of copper ions upon the growth of selected bacterial isolates was also assessed.

CHAPTER 2

COLLECTION, IDENTIFICATION AND MAINTENANCE OF GROWTH OF ALGAE OF THE GENUS ENTEROMORPHA

2.1 Introduction

Algae which inhabit the inter-tidal zone are well adapted to life in the marine environment, thus accounting for their success as fouling organisms (Russell, 1971). In particular, Enteromorpha spp. can tolerate large variations in salinity, temperature, oxygen tension, availability of nutrients and the presence of organic and inorganic pollutants (Russell, 1971; Fletcher and Chamberlain, 1975).

Species of the genus Enteromorpha have complex life cycles, and reproduce by means of asexual zoospores and/or sexual gametes (Bliding, 1964). Alternation of generations is common, and vegetative reproduction is also exhibited, in which excised portions of thalli develop into mature plants.

A prerequisite for the research presented in this thesis was that there should be a ready supply of fertile algal material for the production of zoospores and/or gametes of selected species of the genus Enteromorpha.

Although Enteromorpha spp. can produce zoospores at any time of year (Bliding, 1964), it has been reported (van den Hoek et al., 1979) that maximum sporulation occurs in the early spring and late autumn. These considerations, coupled with the fact that the research laboratory was situated in Central Birmingham, necessitated the development

of a controlled environmental system that reproduced conditions under which Enteromorpha spp. exhibited maximum spore production. As little information is available on the nutritional requirements of the reproductive stages of Enteromorpha spp., a medium which maintained the viability of zoospores and gametes, and which supported the settlement and subsequent growth of these reproductive stages had to be selected.

This chapter describes the design, construction and maintenance of a controlled environmental system, and the selection of a suitable medium for the growth and development of selected species of the genus Enteromorpha.

2.2 Development of a Controlled Environmental System for the Growth of Members of the Genus Enteromorpha at an Inland Site

In its natural habitat the genus Enteromorpha encounters a wide range of constantly changing environmental conditions (Daniel et al., 1980), and has thus evolved the ability to withstand large fluctuations in illumination, nutrient levels, oxygen tension, salinity and temperature (Russell, 1971). Even so, certain basic conditions must obtain for the successful cultivation of algae of this genus (Skinner, 1972; Chapman, 1973).

A prerequisite for the growth of members of the genus Enteromorpha is an adequate supply of sea-water, either

natural or artificial, maintained in such a manner as to facilitate the growth and reproduction of the chosen species (Bonotto, 1976). To avoid the excessive accumulation of bacteria, detritus, organic compounds and nitrogen, all of which can be detrimental to algal growth (Kinne, 1976), a filtration system must be provided.

Adequate aeration is essential, and this is best incorporated in the basic design of the water handling system (Kinne, 1976).

The optimum temperature range for the growth and development of Enteromorpha spp. lies in the range 18-20°C (Skinner, 1972), but precise temperature control is unnecessary, as members of the genus Enteromorpha are adapted to life in the inter-tidal zone, where there are wide daily fluctuations in temperature (Christie and Evans, 1975). However, temperature control can be accomplished either by means of a specialised cooling system (Scott, 1972), or by siting the water holding tanks in such a position that temperature fluctuations are minimal (Jones and Dent, 1970). Thermal insulating materials can also prove effective in preventing excessive temperature variation in the bulk of the water, and tanks and associated pipework should be lagged (Jones and Dent, 1970; Tenore and Huguenin, 1973).

Species of the genus Enteromorpha can grow over a wide range of salinities (Russell, 1971), and this makes the precise monitoring of salinity unnecessary. The volume of water in the system should, however, be periodically checked, and any losses due to evaporation should be made good - either by the addition of fresh water (Moore and Gray, 1970), or by the renewal of the total sea-water volume.

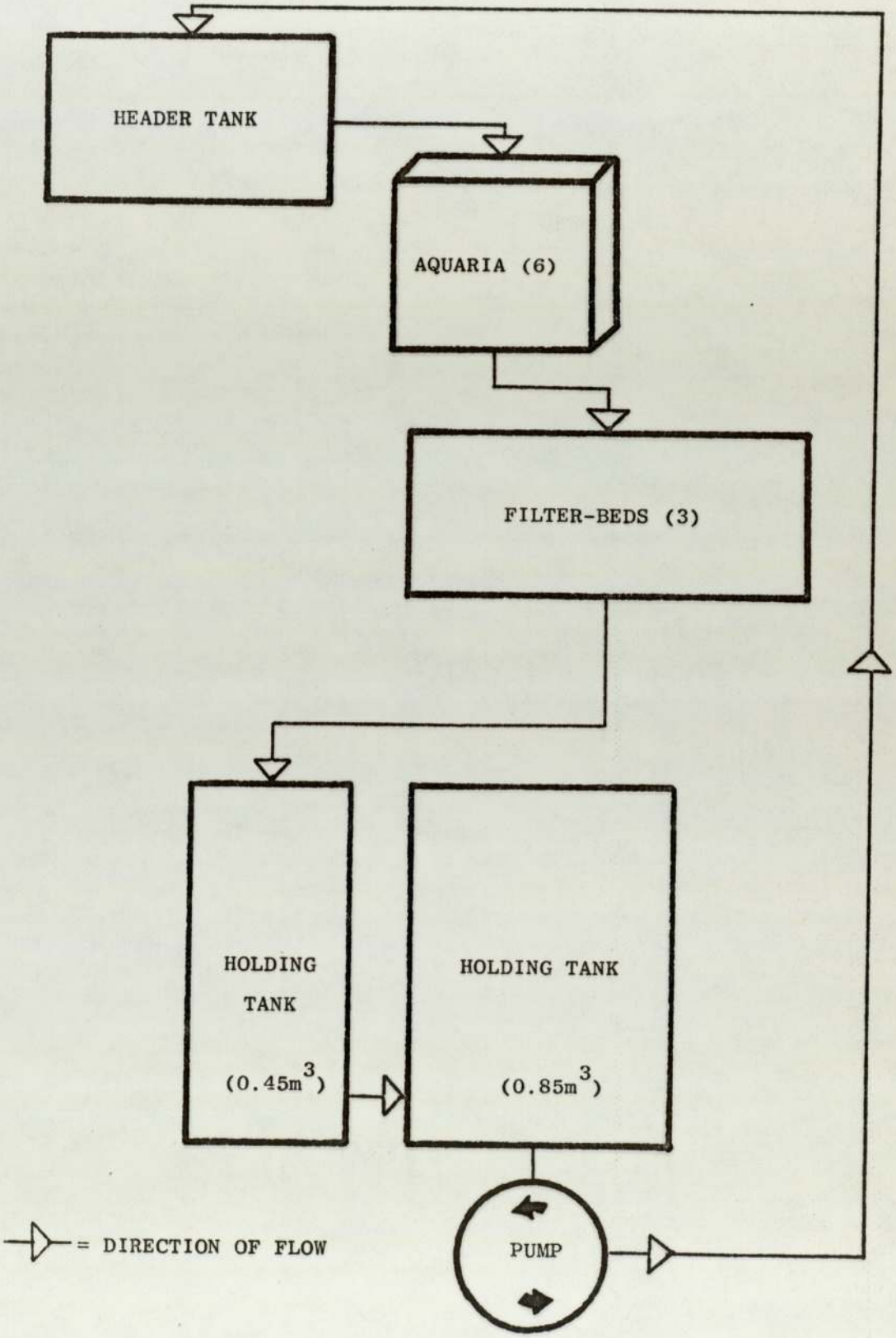
Finally, illumination must be controlled so that the intensity, periodicity and wavelengths of light correspond to the biochemical and physiological requirements of the plants (van Baalen and Edwards, 1973).

2.2.1 Development of a Recycling Sea-Water System

The sea-water handling system (Figure 1) was based upon two storage tanks, constructed from high density polyethylene, and had a total capacity of $1.2m^3$. These tanks were connected, by polyvinyl chloride (PVC) piping, to a 0.25 horsepower pump, having a lift capacity of $2.25m^3h^{-1}$ at 7m head. This gave, at maximum flow rates, a mean circulation time of 35 minutes.

Sea-water was pumped, through PVC piping, to a main header tank, which contained a coarse gravel filter-bed. This filter-bed removed large pieces of organic debris, and prevented sediment from reaching the algal growth area. A distribution network fed the sea-water into six

Figure 1: Sea-water handling system.



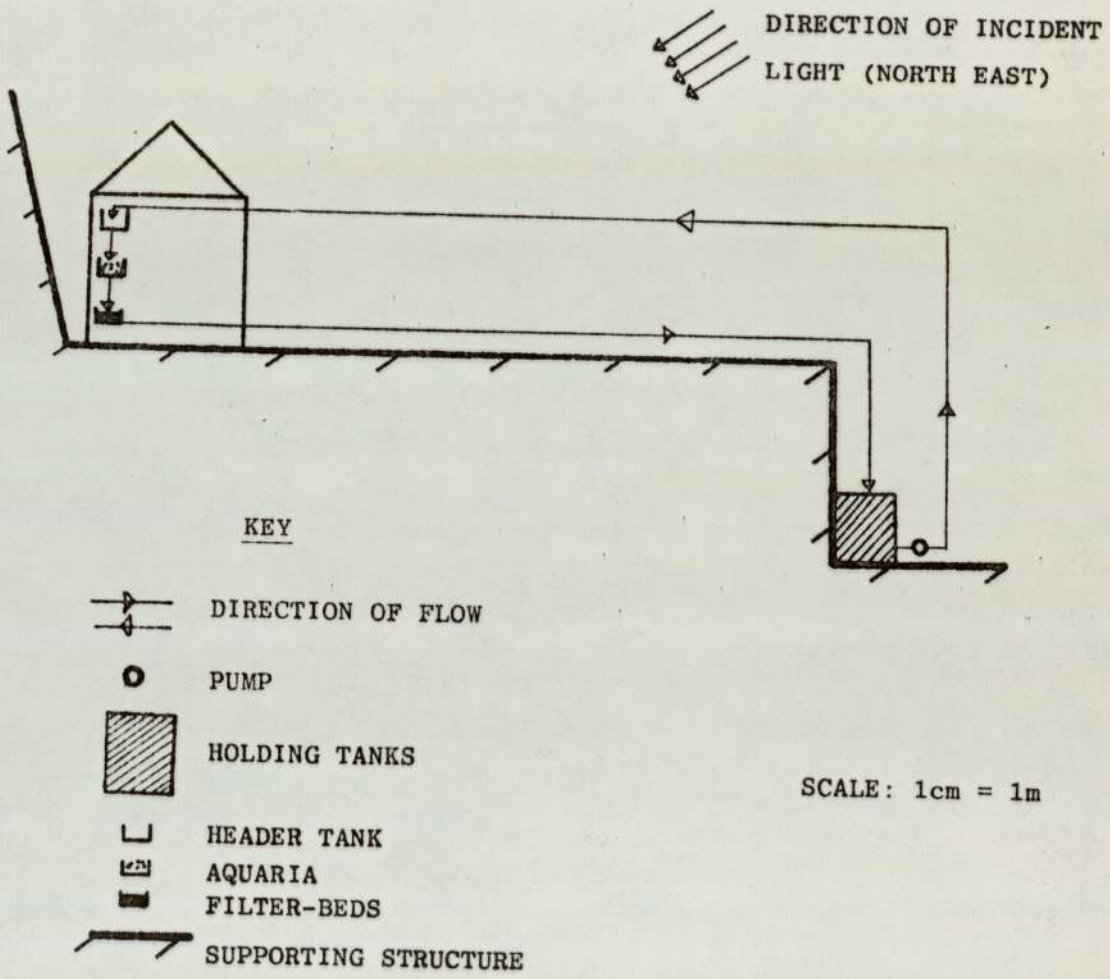
polycarbonate tanks, each having a surface area of $0.1m^2$. The flow rate into each tank was adjusted by means of ball valves situated at appropriate positions in the distribution network. These tanks served as holding areas for various species of Enteromorpha. Sea-water drained from these tanks through perforated PVC pipes into gravel filter-beds (Spotte, 1971), and returned to the main holding tanks by gravity, before re-circulation through the system.

The flow rate was adjusted to give a mean circulation time of about 1.5h in winter. In summer, with higher ambient temperatures, the flow rate was increased, to both reduce temperature and ensure adequate aeration.

Aeration was accomplished by allowing the sea-water to fall through the air at some points in the system. The entrances to the header tank, storage tanks and algal holding areas being the main aeration points. The system of perforated PVC pipes, located on the water feed into the filter-beds, ensured that the sea-water was oxygenated prior to filtration.

The algal holding area, header tank and filters were situated in a greenhouse, facing north-easterly. To ensure minimum temperature fluctuations in the bulk of the water, the main storage tanks were sited in a sheltered position (Figure 2), away from direct sunlight (Jones and Dent, 1970).

Figure 2: Location of water handling system.



The system was filled with freshwater, which was allowed to circulate for 24h, then emptied and refilled with fresh water. This cycle was repeated for 14 days, for the removal of excess plasticizer from new plastics (Carmignani and Bennett, 1976).

Sea-water was collected at 6-9 month intervals, starting in Spring 1980, from the Ministry of Defence Exposure Trials Station, Eastney, Portsmouth (Ordnance Survey, Sheet 196 (1974), Grid Reference SZ 684 001). Approximately $1m^3$ was collected, stored overnight in polypropylene containers, and transported to the laboratory in Birmingham within 24h.

2.2.2 Lighting Regime

The algal holding tanks were situated in a greenhouse, which had white screening applied to all glass areas, thus providing diffuse illumination. This was supplemented by six, 40W 'Grolux' fluorescent tubes (Thorn Lighting Limited). These fluorescent tubes are specifically designed to provide illumination suitable for the growth of plants, having a light flux of 810 lumens per tube, and a power output of 150mW between 650 and 680nm (Thorn, 1978), which is the range of the absorption maxima for chlorophyll (Bainbridge et al., 1965; Govindjee and Govindjee, 1974). The minimum light period was 12h, the day length in the winter being adjusted by means of a time switch incorporated into the lighting circuit.

2.2.3 Discussion

In the development of a controlled environmental system of the type described in the preceding sections, one of the major considerations is the choice of materials for the water system. Pipework can be obtained in a variety of materials, but many are not suitable for use in a circulating sea-water system. Brass, copper and bronze are readily corroded by sea-water, and produce toxic copper compounds (Dyer and Richardson, 1962), and many plastics contain toxic and inhibitory materials (Blankley, 1973). Unplasticised PVC has been found to be a suitable non-toxic material (Gilpin-Brown, 1970), and so was used for all pipework, valves and associated fittings. Blankley (1973) has provided data on the toxic and inhibitory effects of various materials and concluded that polycarbonate and white polyethylene were non-toxic. Algal holding tanks were constructed from clear polycarbonate, and the main storage tanks were manufactured from high density polyethylene.

The minimum temperature to which Enteremorpha spp. were exposed was 11°C, whilst the maximum temperature did not exceed 26°C. The growth of Enteromorpha spp. did not seem to be affected by these temperature fluctuations, and algal fronds remained viable for up to 4 months.

Daylight fluorescent tubes were used by Skinner (1972) in her experiments on an Enteromorpha species, whilst

Partington and Jennings (1971) favoured the use of 'Grolux' fluorescent tubes. In this study these were used to supplement natural daylight, and proved to be highly effective in maintaining the growth and reproductive capacity of members of the genus Enteromorpha. Van den Hoek et al (1979) have given the best indication of the most suitable day length, and report that Enteromorpha spp. have their highest growth rates in early summer. A minimum day length of 12h was adopted in winter, which kept the algal photoperiod near to that naturally occurring in early summer.

The equipment was in continuous operation for three years; it needed little maintenance, and proved to be an economical method for sustaining the growth and reproductive capacity of members of the genus Enteromorpha.

2.3 Collection and Identification of Selected Species of the Genus Enteromorpha

The identification of members of the genus Enteromorpha to the species level presents some difficulties. Newton in her 'Handbook of the British Seaweeds' (Newton, 1931) states the following:-

> "The separation of the species of Enteromorpha presents difficulties on account of the variation in habit due to environmental conditions and the age of the plant. The specific distinctions therefore are not always clearly marked, and perplexing intermediate forms may frequently be found".

This situation is little changed today and the classification of the genus Enteromorpha is still obscure and difficult (van den Hoek et al., 1979). The survey published by Bliding in 1964 is an invaluable aid to the identification of members of the genus Enteromorpha, and this work was used throughout this study.

The difficulty in the classification of the genus Enteromorpha is reflected in the literature on Enteromorpha spp. as fouling organisms. Thus Russell (1971); Banfield (1974); Fry (1975); Luther (1976); Taylor and Evans (1976); Evans (1981) and Hardy (1981) all cite Enteromorpha spp. as fouling organisms, but do not identify the species. Fletcher and Chamberlain (1975) cite Enteromorpha intestinalis as the most widely used test alga in fouling studies, and this is certainly one of the simplest to identify to species level.

The intestinalis group of the genus Enteromorpha, as defined by Bliding (Bliding, 1964), contains the following members:-

Enteromorpha compressa (Linnaeus), variety compressa (Greville). Enteromorpha compressa, variety usneoides (Bonnemaison). Enteromorpha intestinalis (Linnaeus), variety intestinalis (Link). Enteromorpha intestinalis (Linaeus), variety asexualis (Bliding). Enteromorpha intestinalis (Linnaeus), variety asexualis (Bliding), form cornucopiae (Lyngbye).

However, classification at the varietal level is extremely difficult and subject to errors, due to the complex intermediate forms that often occur (Newton, 1931).

In this study, plants of Enteromorpha intestinalis (Linnaeus) Link variety intestinalis (classified according to the method of Bliding, 1964) were collected from three sites:-

1) Hobbs Point at Pembroke Dock (Ordnance Survey, Sheet 159 (1974), Grid Reference SM 968 041).
2) Devil's Point at Plymouth (Ordnance Survey, Sheet 201 (1974), Grid Reference SX 459 534).
3) Black Pill, Swansea (Ordnance Survey, Sheet 159 (1974), Grid Reference SN 620 906).

All algal material was collected from upper to middle shore locations. These sites were chosen as they contained abundant colonies of Enteromorpha intestinalis, and were easily accessible.

The algae were collected by removing them from the substrate to which they were attached, placing them in containers of sea-water and transporting them to the laboratory in Birmingham within 12 hours of collection.

2.4 Initiation of Sporulation

Healthy fronds of Enteromorpha intestinalis were removed from the algal holding tanks, washed in sea-water, drained,

and dried between filter-papers in plastics Petri plates at 4°C for 18 hours (Fletcher and Chamberlain, 1975). At the end of this period the strands were placed into sterile sea-water (autoclaved at 103.4kPa for 15 minutes) where they released large numbers of gametes or zoospores.

2.5 Selection of a Growth Medium for Enteromorpha intestinalis

A variety of media have been proposed for growing marine algae (McLachan, 1973), but only relatively few have been used for the growth of species of the genus Enteromorpha. Erd-Schreiber medium (Schreiber, 1927) was used by Partington (1972) and Jennings (1971), and by Skinner (1972), in experiments on unspecified species of Enteromorpha. In a histochemical investigation of the settlement processes in Enteromorpha intestinalis, Fletcher and Chamberlain (1975) used Erd-Schreiber medium for the growth of zoospores produced by this species. Fries (1975) noted the effects of the artificial sea-water ASP6 (Provasoli et al., 1957) upon the early growth of Enteromorpha compressa and Enteromorpha linza, whilst Bliding (1964) gives Kylin's modification of Schreiber's solution as a general growth medium for members of the genus Enteromorpha. This section describes experiments designed to select the most appropriate growth media for Enteromorpha intestinalis.

2.5.1 Materials and Methods

Healthy fronds of Enteromorpha intestinalis were removed from the algal holding tanks, dried between filter-papers, weighed and placed into sterile Erlenmer flasks containing 300cm^3 aliquots of sterile medium. The media used were:-

1) ASP6 (Provasoli et al., 1975)
2) Erd-Schreiber medium (Schreiber, 1927)
3) Kylin's modification of Schreiber's solution (Bliding, 1964).

In addition sea-water (aged for several months (McLachlan, 1973)) was supplemented with inorganic nitrogen and pyrophosphate as follows:-

A) Sea-water plus ammonium nitrate (0.05 to 2.0g dm^{-3}).
B) Sea-water plus di-sodium di-hydrogen pyrophosphate (0.05 to 2.0g dm^{-3}).
C) Sea-water plus ammonium nitrate and di-sodium di-hydrogen pyrophosphate (both at 0.05 to 2.0g dm^{-3}).

The supplemented sea-waters A, B and C were sterilised by autoclaving at 103.4kPa for 15 minutes, and 300cm^3 aliquots were dispensed into conical flasks containing known masses of Enteromorpha intestinalis.

The fronds of Enteromorpha intestinalis were incubated at 15-25°C (ambient temperature), under natural lighting, supplemented by 'Grolux' fluorescent tubes, thus providing

an 18 hour day-length regime, for 28 days. At the end of the incubation period the algal fronds were removed from the flasks, dried and weighed.

2.5.2 Results and Discussion

The results are presented in Tables 1,2,3 and 4; where the mean mass of five replicate samples of Enteromorpha intestinalis at the beginning of the experiment, and after 28 days of incubation are shown (95% confidence limits are also given, see Appendix 1 for details of statistical method).

Enteromorpha intestinalis grown in unsupplemented sea-water was, for the most part, green and viable after 28 days, but a loss of mass of almost 14% had occurred (Tables 1,2,3 and 4), indicating lack of active growth in this medium. When sea-water was supplemented with inorganic nitrogen, pyrophosphate, or both, at levels in excess of 0.05g dm^{-3} there was a marked decrease in the mass of Enteromorpha intestinalis fronds after 28 days incubation (Table 3).

When Enteromorpha intestinalis fronds were grown in sea-water supplemented with 0.05g dm^{-3} of ammonium nitrate all fronds were green and healthy in appearance, and produced viable spores after 28 days of incubation (Table 1). There was also an increase in mass of 45%, indicating

continued growth of Enteromorpha intestinalis in this medium. The addition of pyrophosphate alone produced less marked results (Table 2), with a mass gain of only 23% for the same incubation period.

There appears to be no advantage in the addition of pyrophosphate and inorganic nitrogen (compared with the effects of inorganic nitrogen alone), as the mass gain when both additional nutrients are present, is 44.44% (Table 3). Media ASP6 and Erd-Schreiber produce no better results than unsupplemented sea-water (Table 4), with many healthy green fronds, and only a small loss in mass.

Kylin's modification of Schreiber's solution (KSM) is prepared by adding $10cm^3$ of a solution of 1% sodium nitrate and 0.2% di-sodium hydrogen phosphate, plus two drops of a 1% solution of ferric citrate, to $1dm^3$ of sterile sea-water (the solutions are added aseptically, after autoclaving, as precipitation occurs if they are autoclaved together. They may be autoclaved separately at 103.4kPa for 15 minutes). KSM has inorganic nitrogen added at $0.2g\ dm^{-3}$ and phosphate at $0.02\ gm^{-3}$.

When $0.05g\ dm^{-3}$ of ammonium nitrate is added to sea-water, the greatest increase in mass of Enteromorpha intestinalis occurs (Table 1). As KSM contains approximately half the additional inorganic nitrogen (sodium nitrate containing about half the level of inorganic

TABLE 1 Growth of Enteromorpha intestinalis in sea-water: The effects of additional inorganic nitrogen

Nitrogen: added as NH_4NO_3 ($g\ dm^{-3}$)	(mM)	Initial mass of Enteromorpha intestinalis (g)*	Mass of Enteromorpha intestinalis after 28 days (g)*	Change in mass of Enteromorpha intestinalis (as % of initial mass)
2.00	25.000	2.33 ± 0.17	0.52 ± 0.06	-76.68
1.00	12.500	2.11 ± 0.15	1.50 ± 0.17	-28.91
0.50	6.250	2.00 ± 0.20	1.62 ± 0.19	-19.00
0.05	0.625	2.00 ± 0.23	2.90 ± 0.27	+45.00
Zero	Zero	2.20 ± 0.21	1.90 ± 0.31	-13.64

* Mean of five replicates, with 95% confidence limits

TABLE 2 Growth of Enteromorpha intestinalis in sea-water: The effects of additional pyrophosphate

Pyrophosphate: added as $Na_2H_2P_2O_7$ ($g\ dm^{-3}$)	(mM)	Initial mass of Enteromorpha intestinalis (g)*	Mass of Enteromorpha intestinalis after 28 days (g)*	Change in mass of Enteromorpha intestinalis (as % of initial mass)
2.00	9.000	1.37 ± 0.19	0.43 ± 0.1	-68.61
1.00	4.500	1.22 ± 0.15	0.92 ± 0.87	-24.59
0.50	2.250	1.18 ± 0.11	0.98 ± 0.62	-16.95
0.05	0.225	1.00 ± 0.17	1.23 ± 0.19	+23.00
Zero	Zero	2.2 ± 0.21	1.9 ± 0.31	-13.64

* Mean of five replicates, with 95% confidence limits

TABLE 3 Growth of Enteromorpha intestinalis in sea-water: the effects of additional inorganic nitrogen and pyrophosphate

Nitrogen: added as NH_4NO_3 (g dm^{-3})	Pyrophosphate: added as $Na_2H_2P_2O_7$ (g dm^{-3})	Initial mass of Enteromorpha intestinalis (g)*	Mass of Enteromorpha intestinalis after 28 days (g)*	Change in mass of Enteromorpha intestinalis (as % of initial mass)
2.00	2.00	1.09 ± 0.15	0.19 ± 0.05	-82.57
1.00	1.00	1.15 ± 0.12	0.25 ± 0.37	-78.26
0.50	0.50	1.14 ± 0.17	0.37 ± 0.43	-67.54
0.05	0.05	1.17 ± 0.11	1.69 ± 0.29	+44.44
Zero	Zero	2.2 ± 0.21	1.9 ± 0.31	-13.64

* Mean of five replicates, with 95% confidence limits

TABLE 4 Growth of Enteromorpha intestinalis in various media

Media	Initial mass of Enteromorpha intestinalis (g)*	Mass of Enteromorpha intestinalis after 28 days (g)*	Change in mass of Enteromorpha intestinalis (as % of initial mass)
ASP6	2.10 ± 0.24	1.85 ± 0.26	-11.9
Erd-Schreiber	1.90 ± 0.2	1.65 ± 0.22	-13.6
Kylin 's modification of Schreiber's solution	1.85 ± 0.25	2.64 ± 0.24	+42.7
Sea-water + 0.05g dm^{-3} NH_4NO_3	2.00 ± 0.24	2.90 ± 0.27	+45
Sea-water + 0.05g dm^{-3} $Na_2H_2P_2O_7$	1.00 ± 0.17	1.23 ± 0.19	+23
Sea-water + 0.05g dm^{-3} Na_4NO_5 + 0.05g dm^{-3} $Na_2H_2PO_7$	1.17 ± 0.11	1.69 ± 0.29	+44.44
Sea-water	2.20 ± 0.32	1.90 ± 0.31	-13.64

* Mean of five replicates, with 95% confidence limits

nitrogen compared with ammonium nitrate) as that which was found to encourage maximum growth of Enteromorpha intestinalis, the results with KSM compare well with those found for the addition of ammonium nitrate to sea-water.

Phosphate does not seem to be a limiting factor for the growth of Enteromorpha intestinalis in sea-water, as the addition of pyrophosphate alone produces a maximum increase in mass of 23%, whereas the addition of 0.05g dm^{-3} of ammonium nitrate results in a 45% increase. It is therefore probable that nitrogen is the limiting factor for the growth of Enteromorpha intestinalis in sea-water used in these experiments.

2.6 Chapter Conclusions

Enteromorpha intestinalis may successfully be grown at an inland site, providing that a suitable environmental system is provided.

Kylin ' s modification of Schreiber's solution (Bliding, 1964) is a suitable medium for the cultivation of Enteromorpha intestinalis. As this medium is minimally fortified it is less probable that the results presented in subsequent chapters are due to abnormal growth patterns of Enteromorpha intestinalis, which could occur with an enriched medium.

Kylin's modification of Schreiber's solution was used as the sole medium for the growth of Enteromorpha intestinalis and is referred to by the abbreviation KSM in subsequent chapters.

CHAPTER 3

GROWTH OF BACTERIAL FILMS

3.1 Introduction

When a surface is submerged in sea-water bacterial attachment occurs (Corpe, 1970), with the eventual development of a mucilaginous film, which is the result of extracellular polymer secretion by the attached bacteria (Marshall, 1981). This film, normally referred to as the primary film, is rapidly colonised by other marine organisms, e.g. diatoms, invertebrate larvae and algal spores, resulting in an even thicker secondary film.

Research has shown that the formation of the primary film occurs in two phases (Marshall, 1976). Carson (1980) demonstrated that there was an initial twelve-hour period, during which the surface properties of the attachment substrate are relatively important in the selection of the type of bacteria which attach, and a secondary period (exceeding 12 hours) during which the surface properties of the attachment substrate are modified. During this secondary period a more diverse bacterial film develops, and it has been demonstrated that after 12 hours immersion the surface properties of the attachment substrate play a less important role in influencing the type of bacteria present in the primary film (Carson, 1980).

Marine bacteria are known to influence the settlement of certain fouling organisms, notably the marine invertebrate Janua (Dexiospira) brasiliensis (Kirchman et al., 1981; Kirchman and Michell, 1983), but bacterial films have not

previously been demonstrated to affect the settlement of fouling algae of the genus Enteromorpha.

This chapter describes a method for the production of bacterial films on glass attachment substrates. Bacterial films produced by this method were later used in experiments to determine the effects of such films on the settlement of Enteromorpha intestinalis.

3.2 Selection of Bacteria

Marine bacteria had been isolated and identified to genus level during a previous research programme (Carson, 1980). These isolates (from glass, paint, resin and steel attachment-substrates) were available as freeze-dried cultures, coded according to the following scheme (Carson, 1980, page 128).

> "The isolates were coded so that the time of immersion and substrate could be readily identified, and a typical code such as 212SB2 indicates that the sample was taken on the 2nd day of December (212), at the second 12 hour sampling period of the day (B), and it was a steel substrate; the remaining digit identifies the number of the isolate (1-6) for that particular sampling time, 212SB."

The decision to use these isolates was based on four criteria:-

1) The isolates represented bacteria generally known to colonise surfaces immersed in sea-water.

2) They were available in pure culture.

3) Methods of isolation were known.

4) Most isolates had been previously classified to genus level.

A number of isolates (Table 5) were selected from genera isolated during the first 12 hours of immersion of an attachment substrate, when bacterial numbers on glass attachment substrates are in the region of 2.48×10^3 cells cm^{-2} to 5.46×10^3 cells cm^{-2} (Carson, 1980), and the bacterial film is relatively thin. Section 3.3. provides the rationale for the choice of glass as the attachment substrate used.

TABLE 5 List of Genera, Isolation Times and Isolation Codes

Isolation Code*	Isolation Time (hours)*	Genus*
3011G1	12	Unknown
3011G2	12	*Pseudomonas* sp.
3011G4	12	*Pseudomonas* sp.
1412GA2	4	*Alteromonas vaga*
1412GA3	4	*Alteromonas* sp.
712P2	12	*Pseudomonas/Alteromonas* Group
712P3	12	*Pseudomonas/Alteromonas* Group
712P4	12	*Coryneform* sp.
1512PA2	4	*Coryneform* sp.
1512PA6	4	*Coryneform* sp.
712R1	12	*Micrococcus* sp.
712R2	12	*Pseudomonas/Alteromonas* Group
712R3	12	*Benekea* sp.
1512RA4	4	*Pseudomonas/Alteromonas* Group
1512RB5	8	*Coryneform* sp.
1412SA2	4	*Alteromonas* sp.
1412SA3	4	*Pseudomonas/Alteromonas* Group

* Carson (1980).

3.3 Choice of Attachment Substrate

An important consideration in the choice of attachment substrates was to produce bacterial films to investigate the effects of microbial films upon the settlement and growth of zoospores of Enteromorpha intestinalis.

A major disadvantage of opaque attachment substrates is that epifluorescence microscopy is the best method for the enumeration of viable organisms attached to such substrates (Duddridge, 1981). This technique can be used successfully for the enumeration of bacteria on surfaces, but presents difficulties when counting relatively large organisms, such as the developing zoospores (germlings) of Enteromorpha intestinalis. When counting Enteromorpha intestinalis germlings, relatively large areas have to be scanned to obtain statistically valid results, which is a time-consuming and tedious task when using epifluorescence microscopy. Also, as the germlings develop so does the microbial film, and this introduces counting errors, as it is difficult to distinguish Enteromorpha intestinalis germlings from the bacterial film with which they become associated.

The use of glass as an attachment substrate alleviated these problems, and normal bright-field microscopy was used to enumerate the numbers of attached Enteromorpha intestinalis zoospores. Zoospore development was followed by this method, and the results recorded photographically.

Glass coverslips (No.3) were chosen as the attachment substrates for the production of bacterial films, and in later experiments on the settlement and development of Enteromorpha intestinalis zoospores.

3.4 Growth of Bacteria in Kylin's Modification of Schreiber's Solution (KSM)

The selection of KSM as the growth medium for Enteromorpha intestinalis has been described in Chapter 2. In this section the effects of KSM medium upon the growth of the bacteria shown in Table 5 are assessed.

3.4.1 Materials and Methods

The bacteria shown in Table 5 were available as freeze-dried cultures (Carson, 1980). These were reconstituted with Johnson's marine broth (Johnson's marine agar (Johnson, 1968), without the agar (Carson, 1980)), and a $1cm^3$ inoculum added to $100cm^3$ of the same medium contained in an Erlenmeyer flask. Incubation was for 12 hours, at $19^{\circ}C$.

After incubation, the cells were centrifuged out of suspension, washed in sterile sea-water, re-suspended and counted, using a Helber chamber. The total number of cells was adjusted to approximately $10^7 cm^{-3}$, either by centrifugation and re-suspension in a suitable volume of sterile sea-water, or by dilution with sterile sea-water.

A 1 cm^3 inoculum (10^7 cells) was added to 4cm^3 of KSM in a 'Repli dish' compartment (the Repli dish has 25 compartments, 1.8cm^2, with a capacity of 6cm^3, they are manufactured from clear polystyrene, and supplied sterile by Sterilin Limited, Teddington, U.K.). Incubation was at 19^oC for 12 hours. 0.1cm^3 samples were taken after approximately 5 minutes of incubation, and then at hourly intervals until the end of the incubation period. These samples were diluted with KSM in a range of dilution steps from 10^{-2} to 10^{-4}, and plated by the spread-plate technique (as described by Postgate, 1969; except that 0.1cm^3 was plated) onto Johnson's marine agar (Johnson, 1968) in 1cm^3 and 0.1cm^3 aliquots. Five replicate plates were made for each dilution step, and for each dilution aliquot. The plates were incubated until colonies were visable, and the number of colonies per plate counted.

3.4.2 Results and Discussion

Under ideal conditions a Poisson distribution should be obtained when bacteria are plated onto a suitable medium (Fisher et al., 1922), but this should be checked by an appropriate statistical test (Jones, 1979). The ratio of the variance to the mean of a set of sample values will approximate to unity if there is agreement with a Poission series, and any departure from unity can be assessed by reference to a table of chi squared (Elliott, 1971).

The chi squared variance to mean ratio test for a small sample (Appendix 1 was used to check the distribution of colonies of bacteria within each series of replicate plates, and the distributions were found to conform to the Poisson series, at the 95% probability level (P 0.05).

The coefficient of variation (q.v., Appendix 1) may be used to compare the relative variability of a series of replicate samples (Daniel, 1978; Elliott, 1971), if the underlying distribution from which the samples are drawn is random (Jennison and Wadsworth, 1939). As conformity with the Poisson series indicates that a distribution is random (Petrie, 1978), and the colony counts conformed to a Poisson distribution, the coefficient of variation was calculated for the colonies on each series of replicate plates, and was found to be less than 5%, which indicates an acceptable level of precision for the colony counts (Jennison and Wadsworth, 1939).

The colony counts were converted to viable cells cm^{-3}, by multiplying by the appropriate dilution factor, and plotting the results as $\log_{10}$ viable cells cm^{-3} (Y-axis), against time (X-axis) to produce the growth curves shown in Figures 3-19.

A general feature of all the bacterial growth curves is that the increase in viable cell numbers is not in excess of two log cycles. This suggests that there was

some limiting factor for microbial growth in this medium. The small volume of medium used, and the lack of additional carbon source in KSM were the most probable limiting factors.

After 12 hours most of the culture had entered the 'death phase', and there was a rapid decline in viable cell numbers for all cultures except isolate 712R1, a Micrococcus sp. (Figure 13); isolate 1512RB5, a member of the Coryneform group (Figure 17), and isolate 1412SA3, a member of the Pseudomonas/Alteromonas group (Figure 19), all of which continued in the stationary phase.

These results indicate that the maximum period that could usefully be used for the preparation of bacterial films was in the region of 12 hours, after this, cell death was occurring among the microbial population and the possibility of coherent film formation was greatly reduced.

Figure 3: Growth curve for bacterial isolate 3011G1 (genus unknown) in KSM.

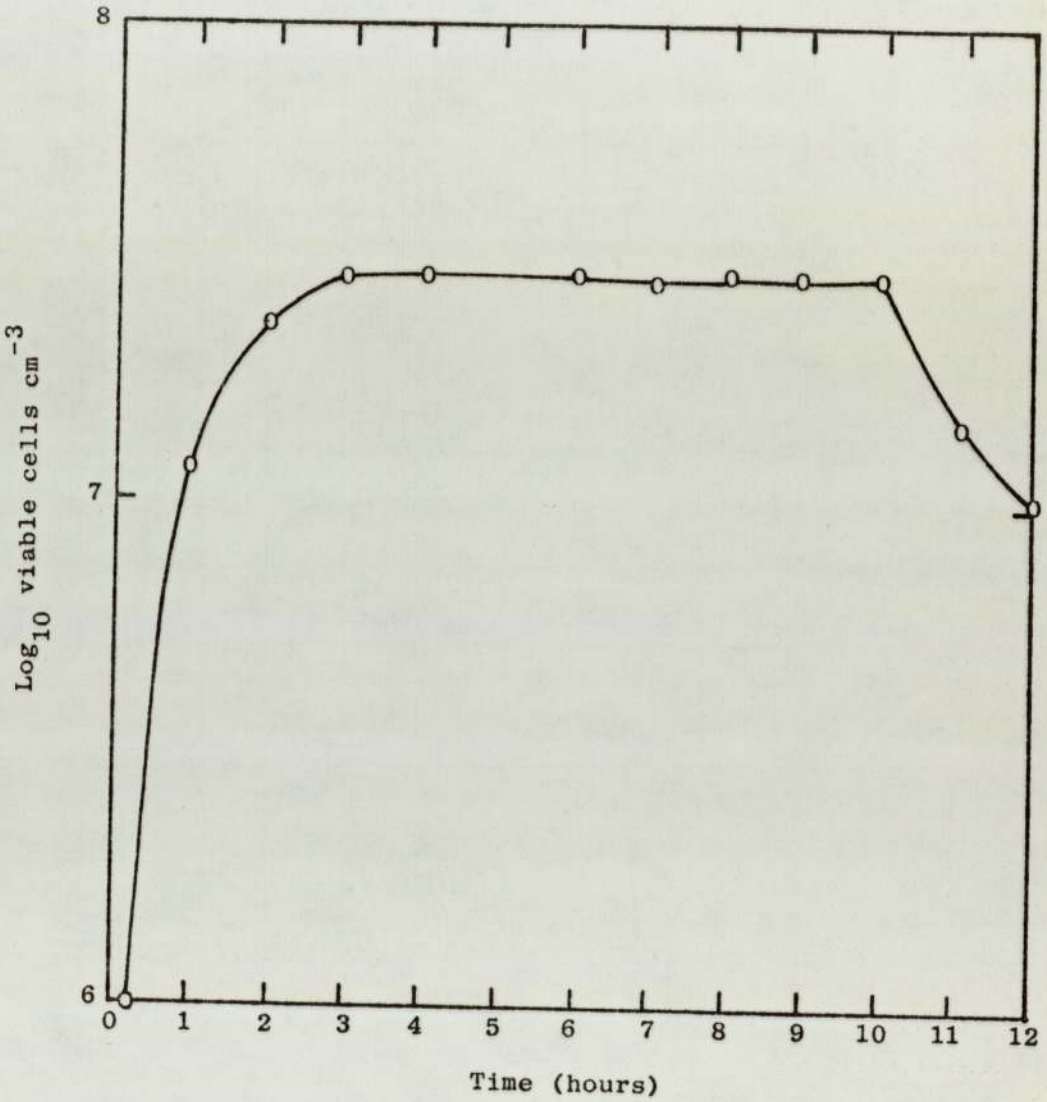


Figure 4: Growth curve for bacterial isolate 3011G2 (*Pseudomnas* sp.) in KSM.

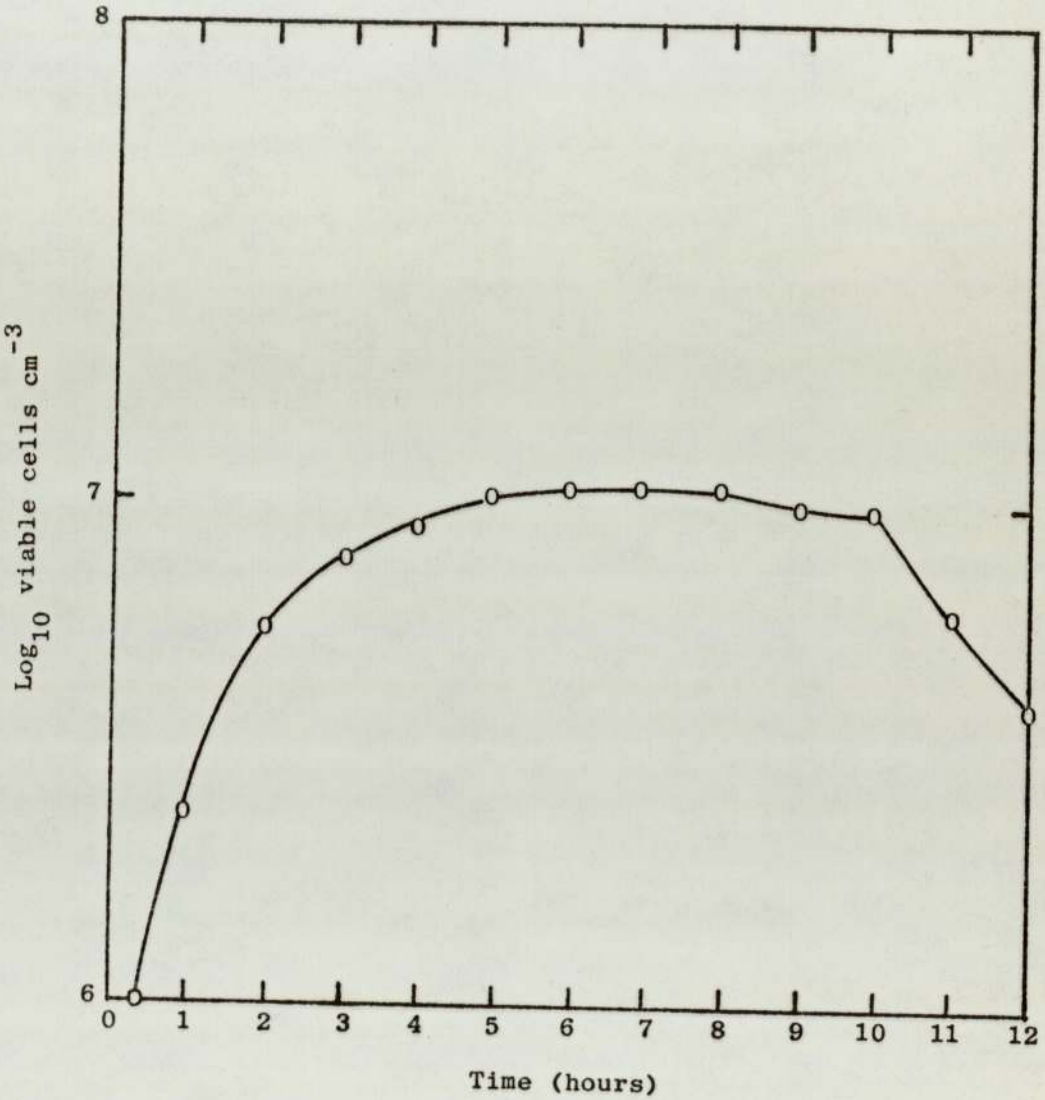


Figure 5: Growth curve for bacterial isolate 3011G4 (*Pseudomonas* sp.) in KSM.

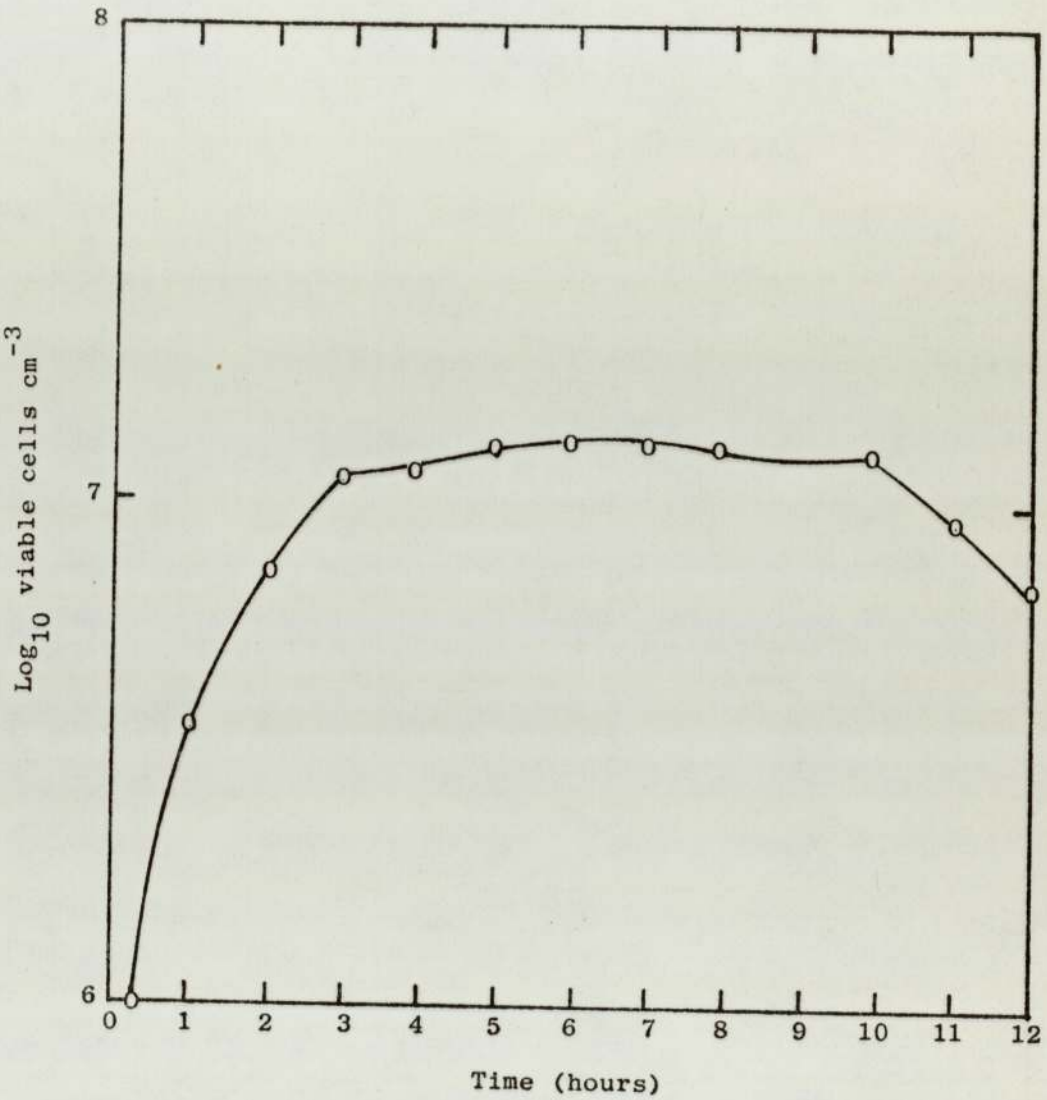


Figure 6: Growth curve for bacterial isolate 1412GA2 (*Alteromonas vaga*) in KSM.

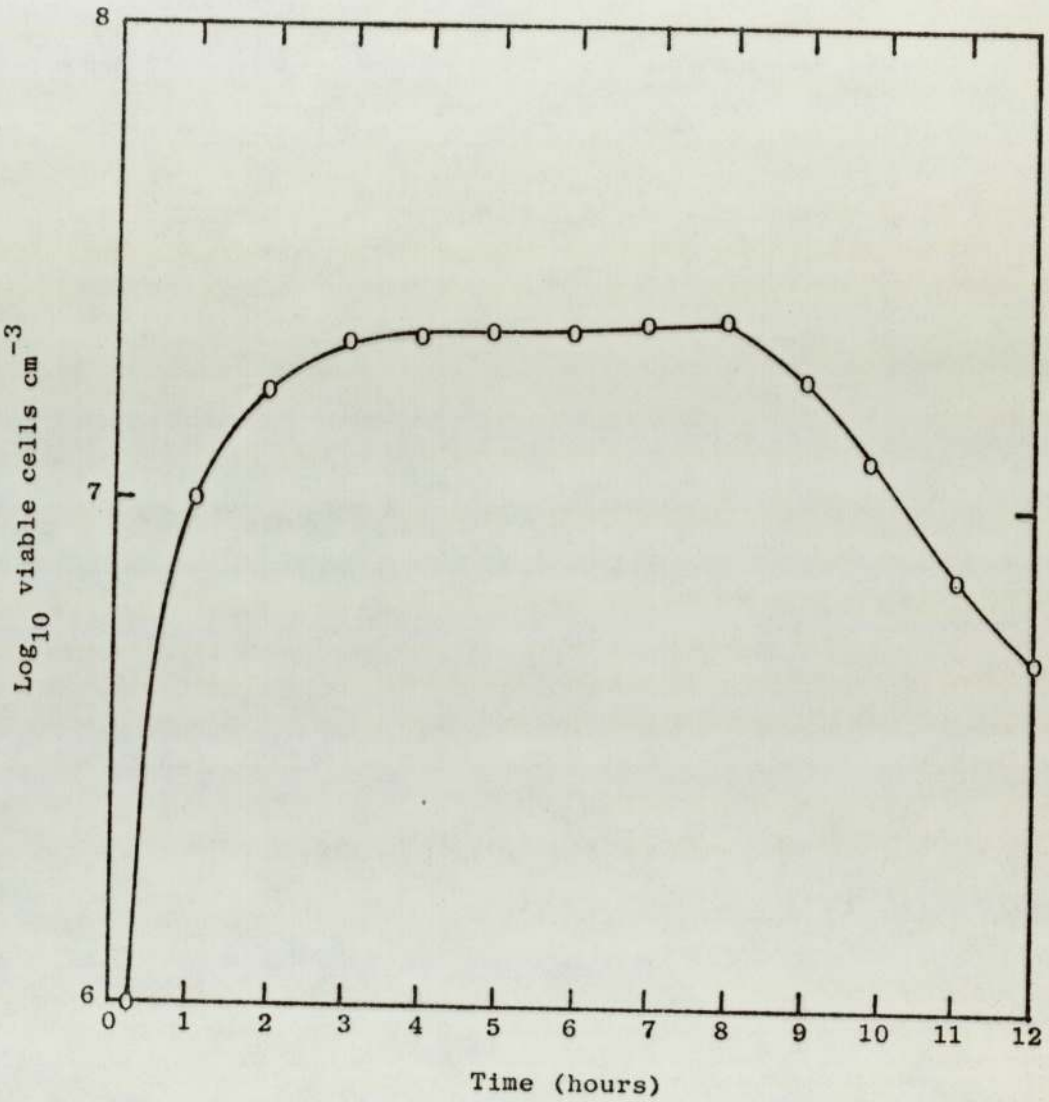


Figure 7: Growth curve for bacterial isolate 1412GA3 (*Alteromonas* sp.) in KSM.

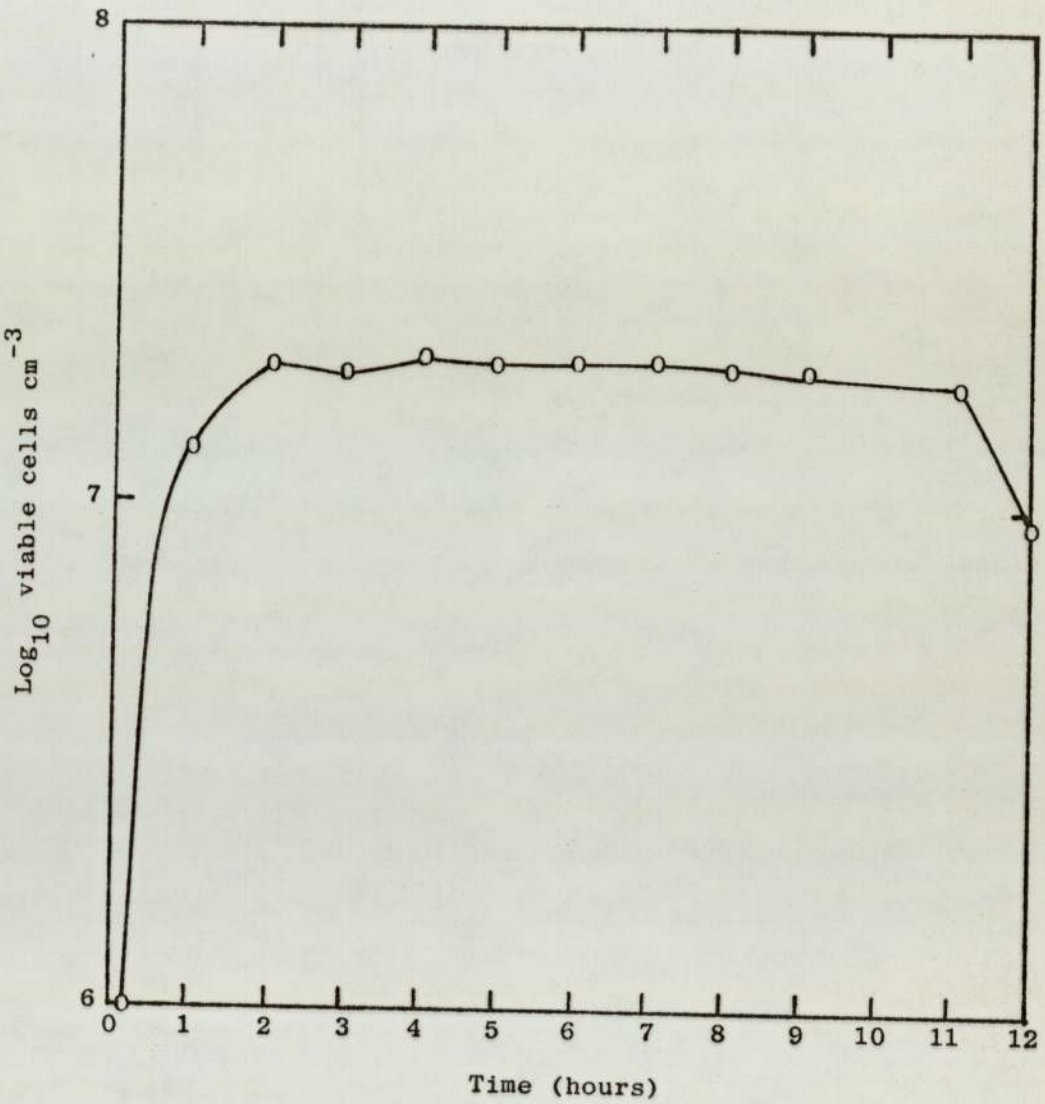


Figure 8: Growth curve for bacterial isolate 712P2 (*Pseudomonas/Alteromonas* group) in KSM.

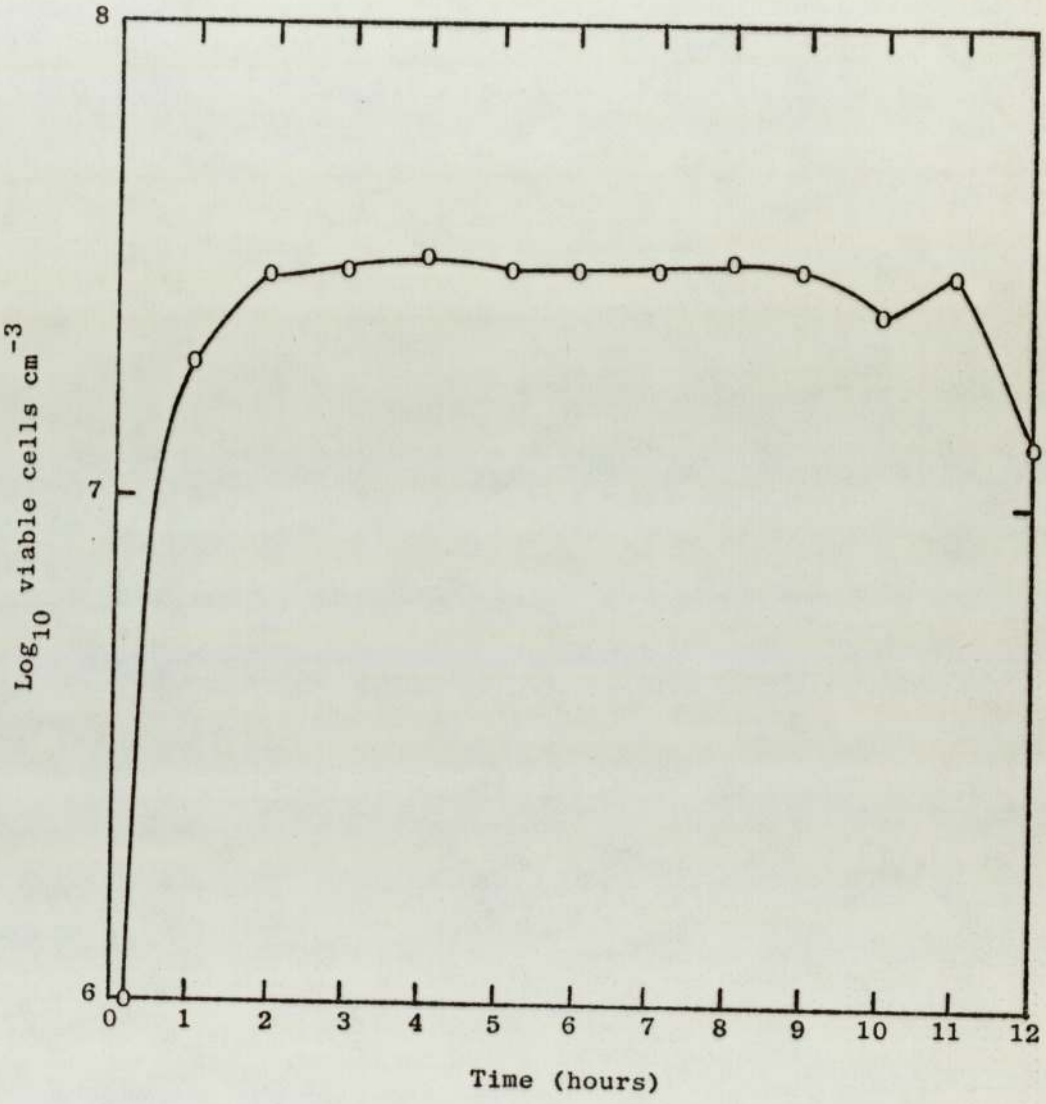


Figure 9: Growth curve for bacterial isolate 712P3 (*Pseudomonas/Alteromonas* group) in KSM.

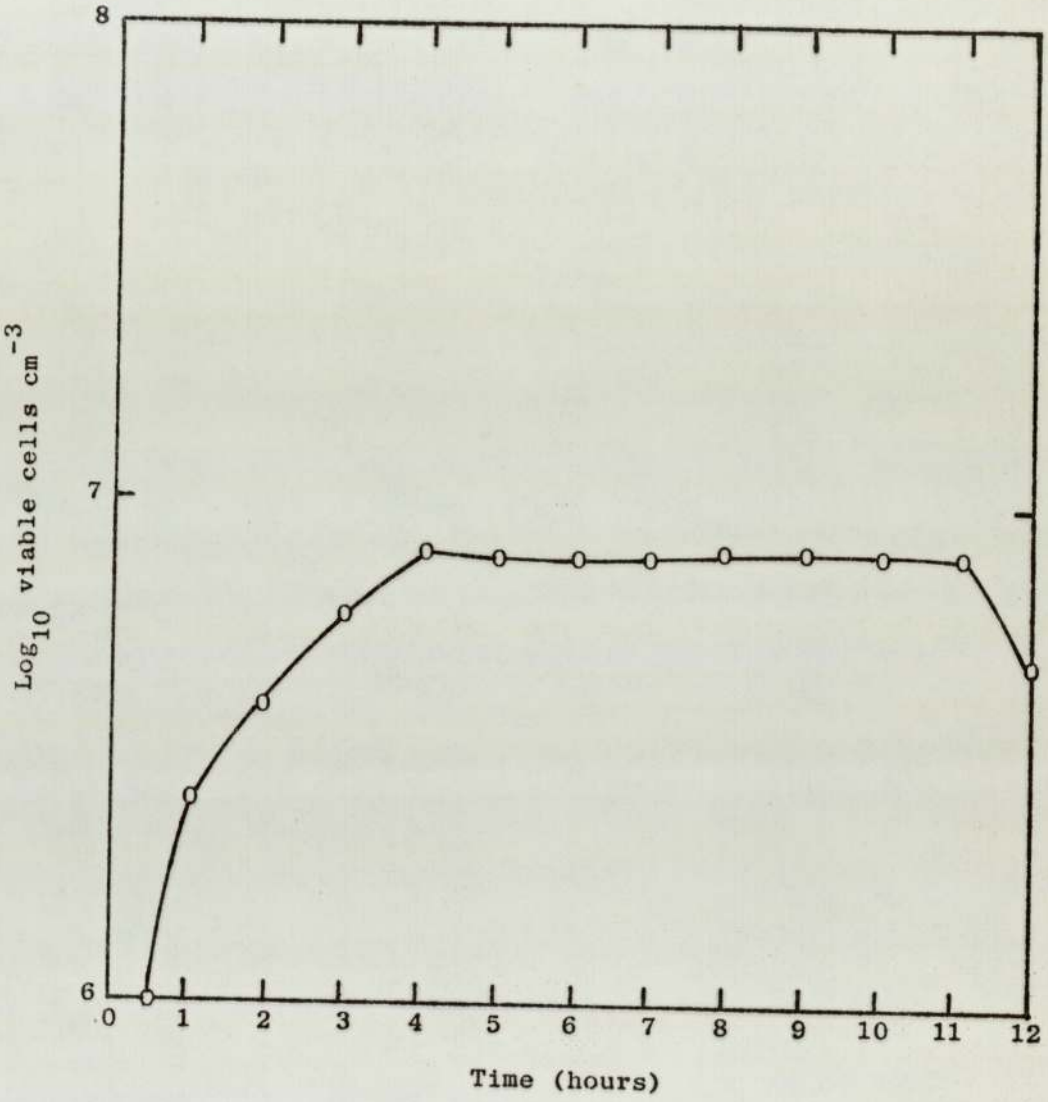


Figure 10: Growth curve for bacterial isolate 712P4 (*Coryneform* group) in KSM.

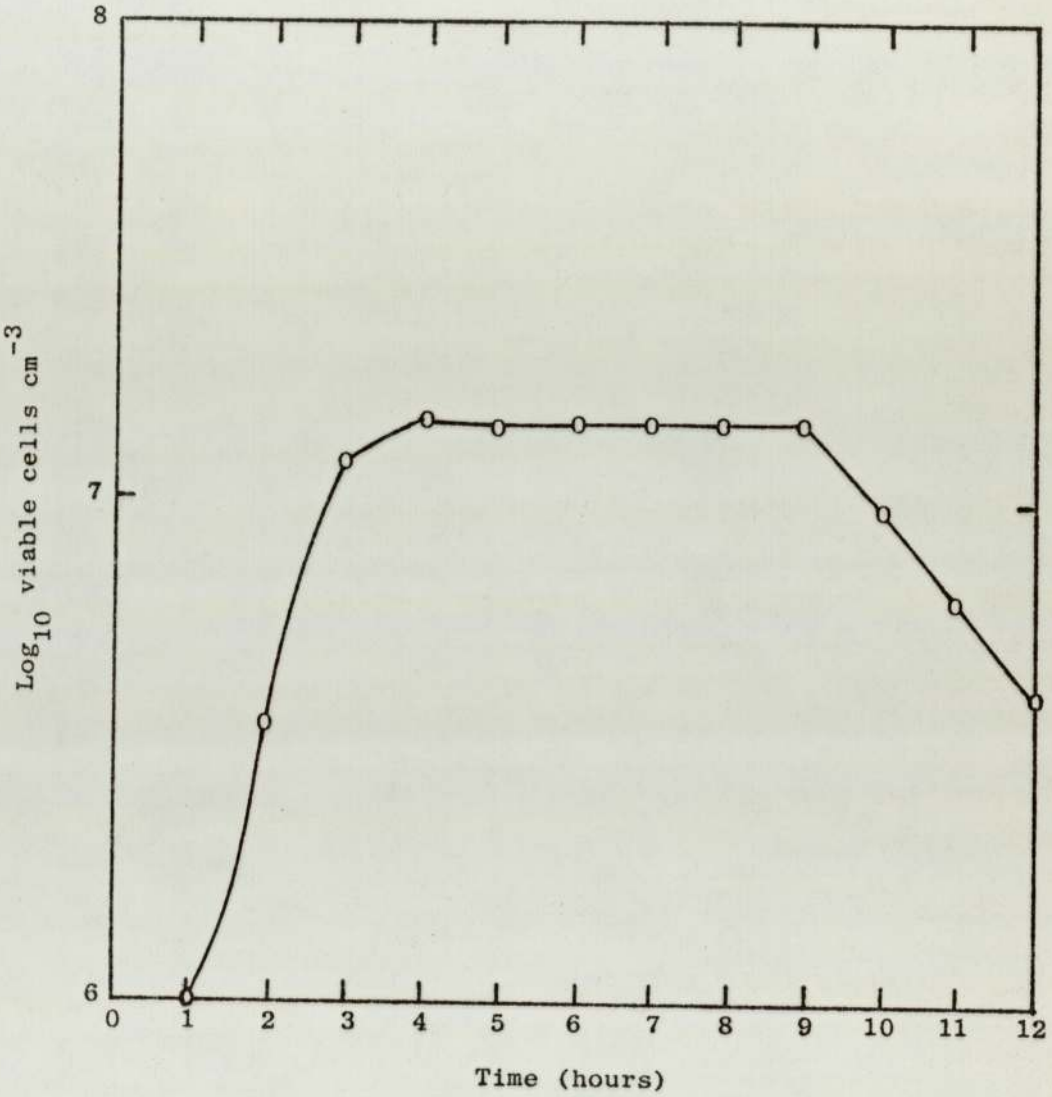


Figure 11: Growth curve for bacterial isolate 1512PA2 (*Coryneform* group) in KSM.

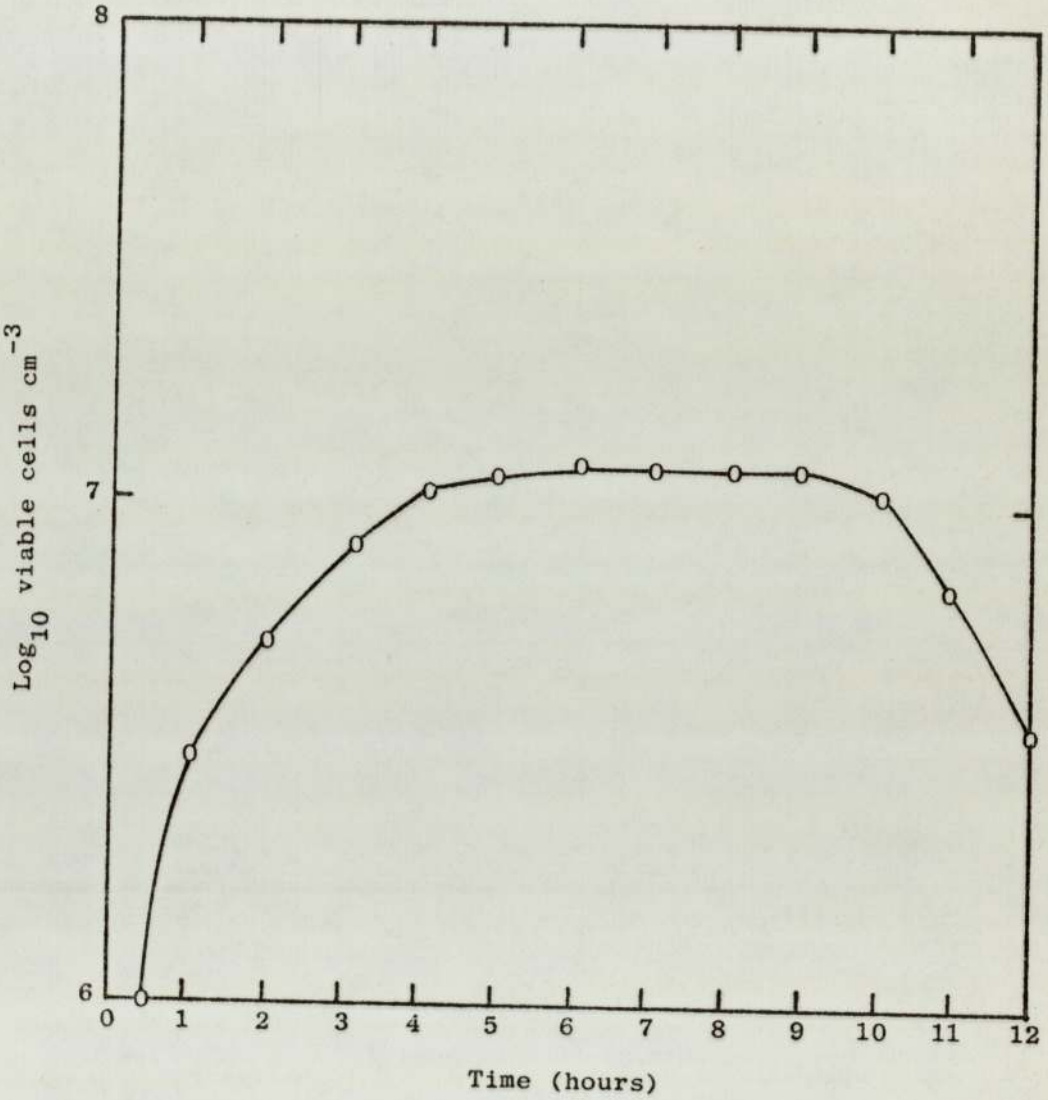


Figure 12: Growth curve for bacterial isolate 1512PA6 (*Coryneform* group) in KSM.

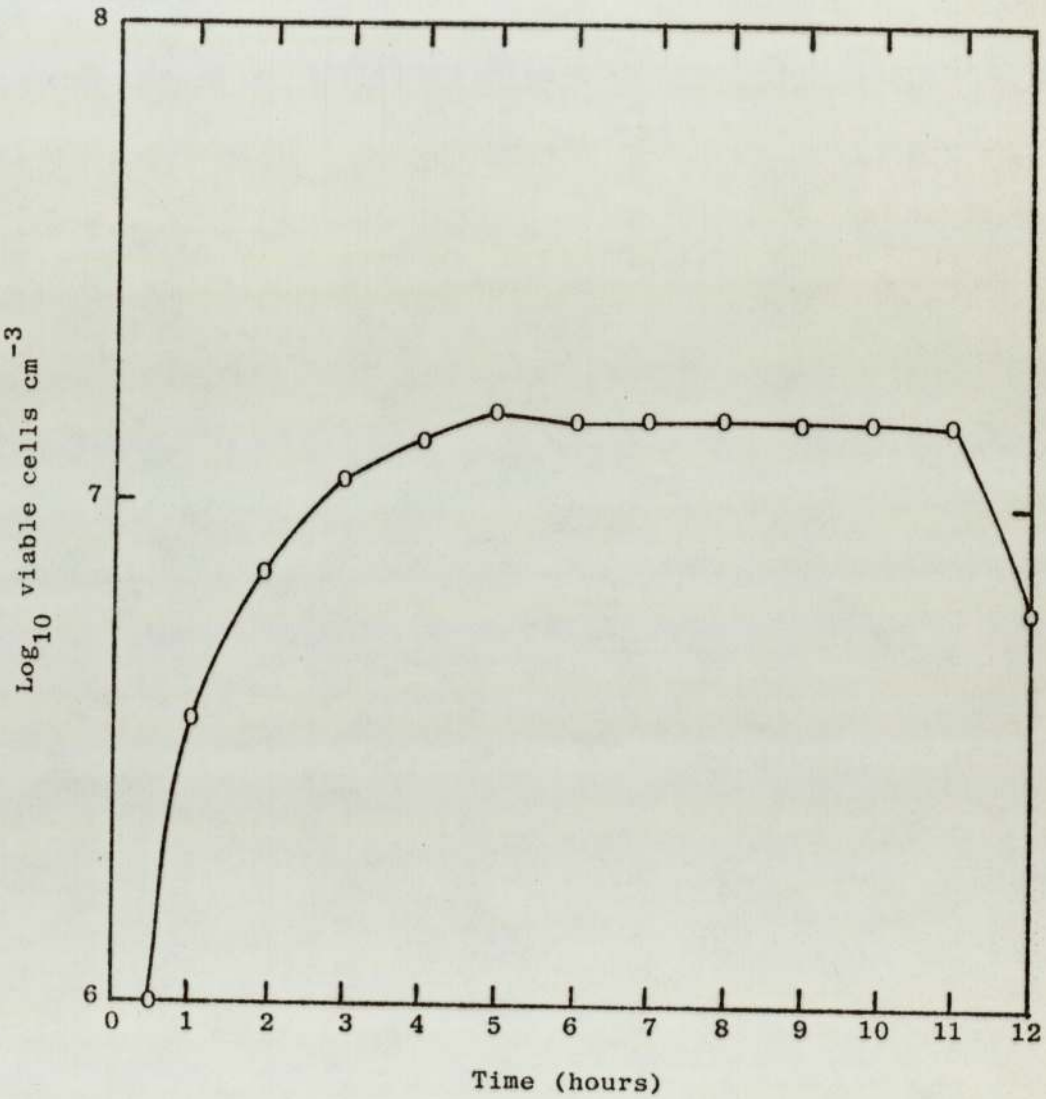


Figure 13: Growth curve for bacterial isolate 712R1 (*Micrococcus* sp.) in KSM.

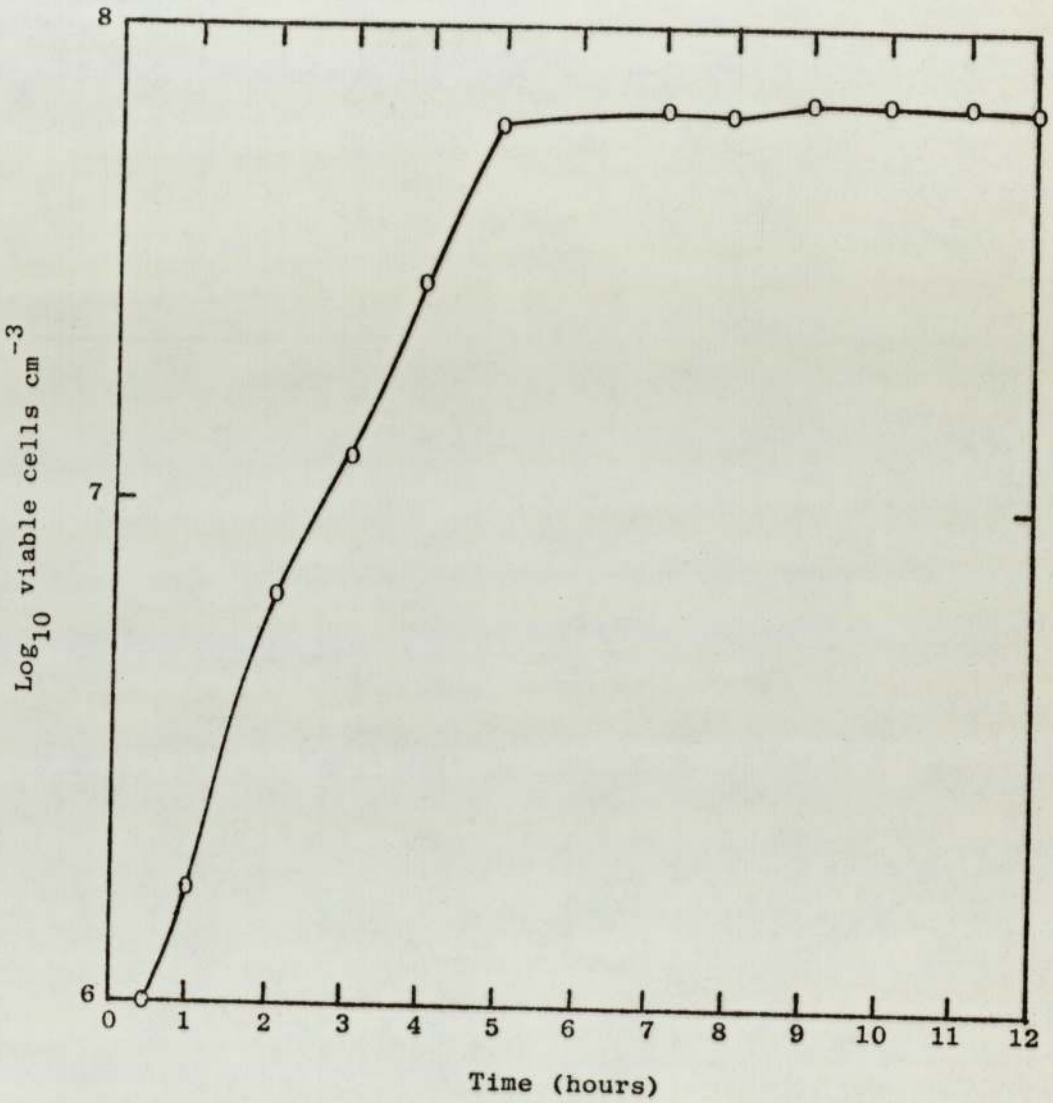


Figure 14: Growth curve for bacterial isolate 712R2 (*Pseudomonas/Alteromonas* group) in KSM.

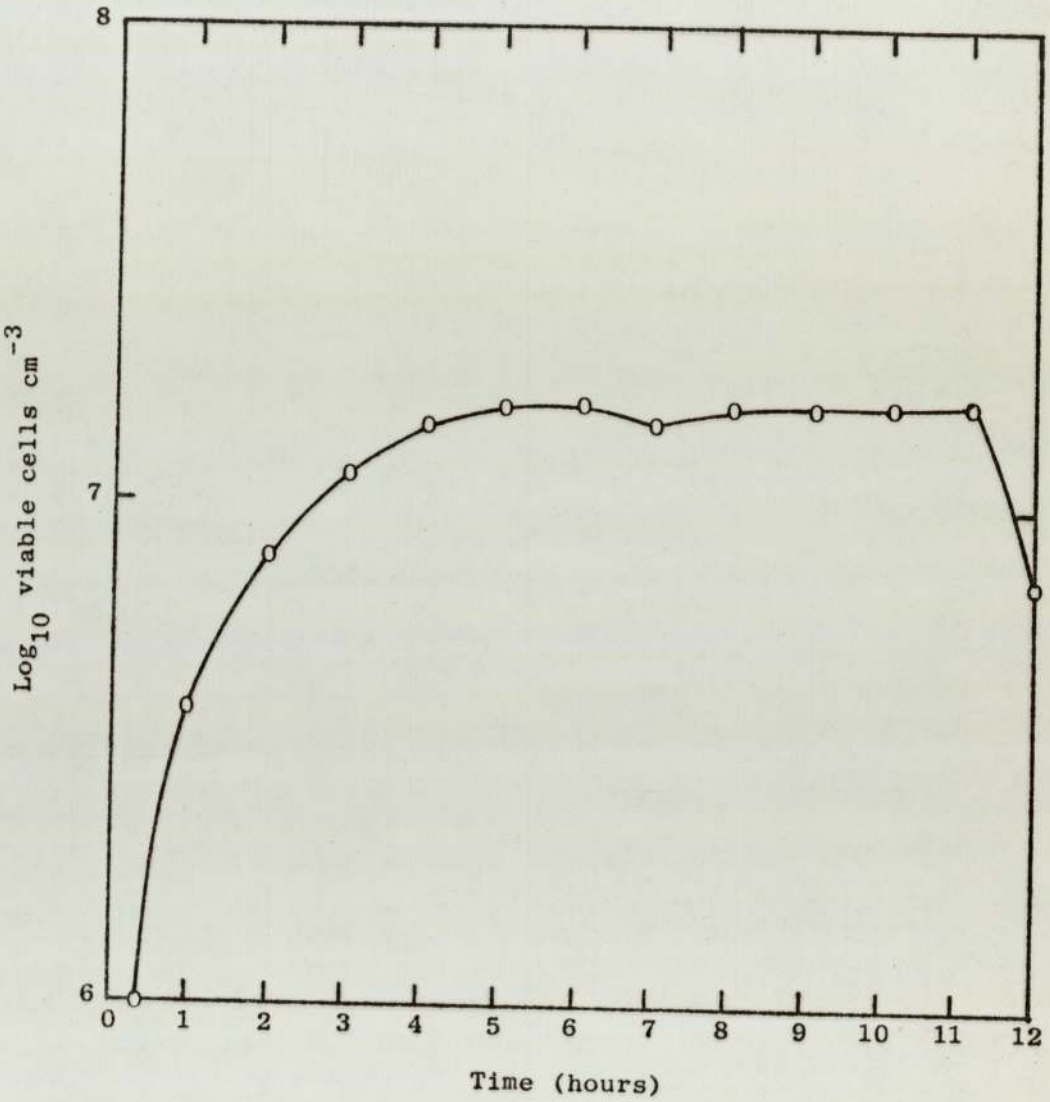


Figure 15: Growth curve for bacterial isolate 712R3 (*Benekea* sp.) in KSM.

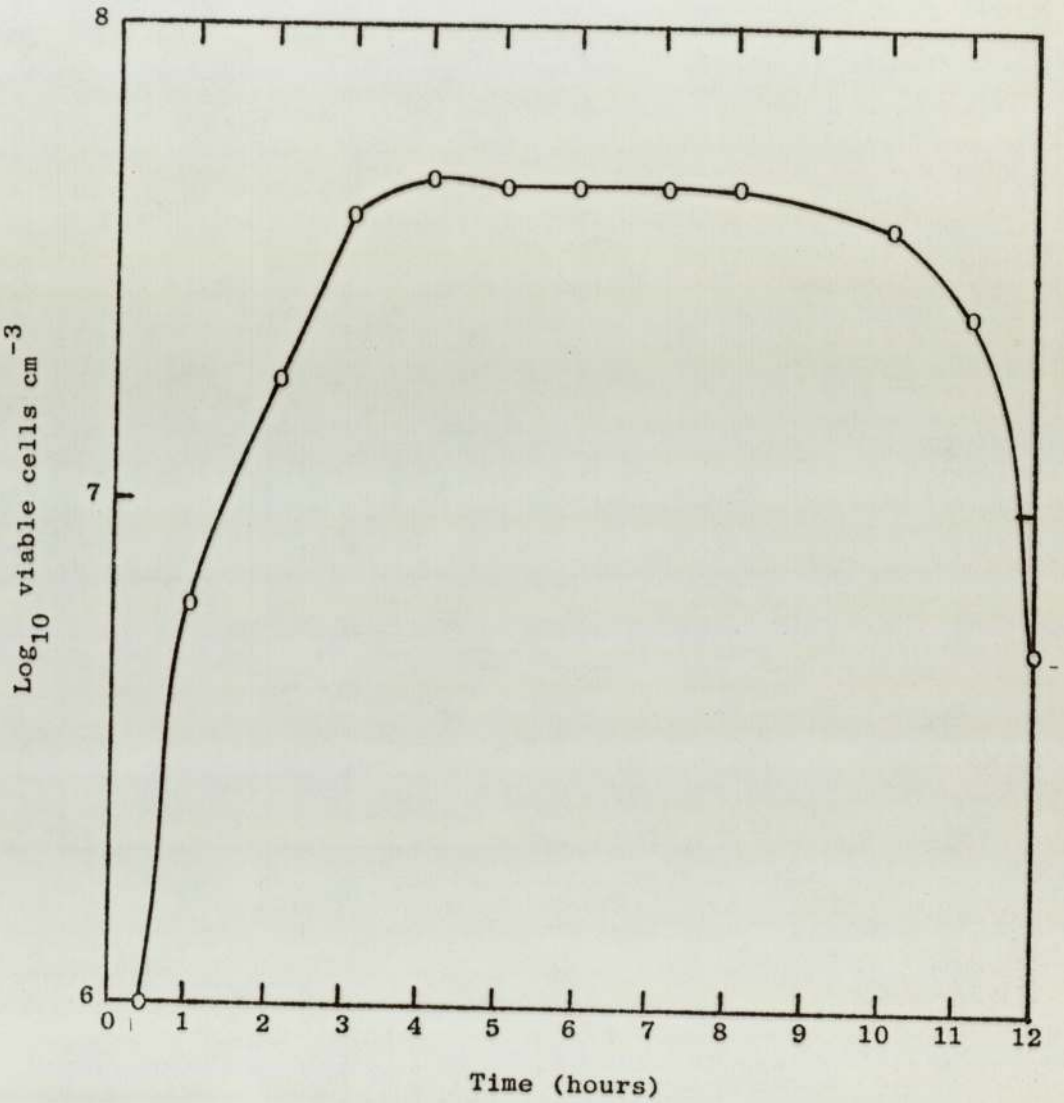


Figure 16: Growth curve for bacterial isolate 1512RA4 (*Pseudomonas/Alteromonas* group) in KSM.

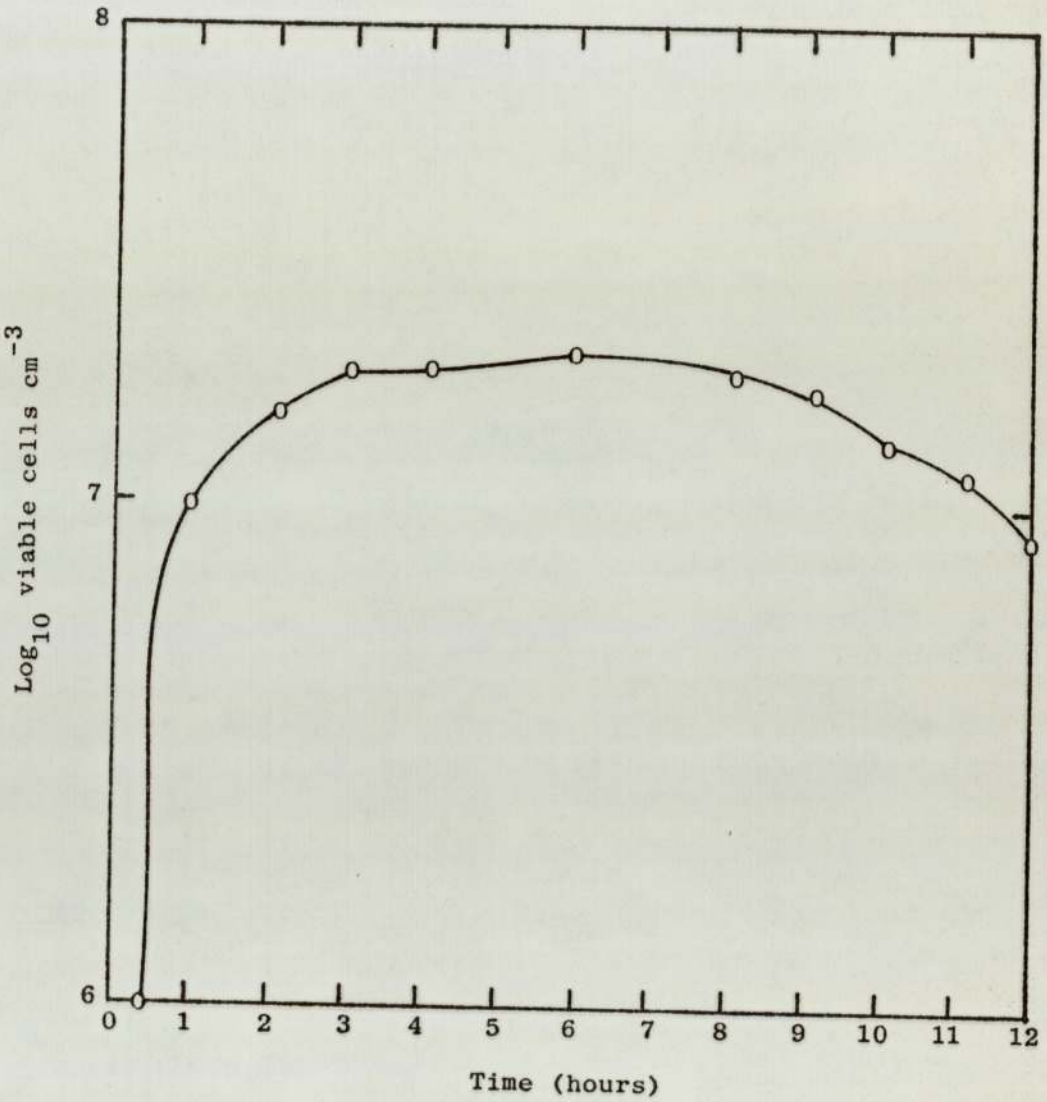


Figure 17: Growth curve for bacterial isolate 1512RB5 (*Coryneform* group) in KSM.

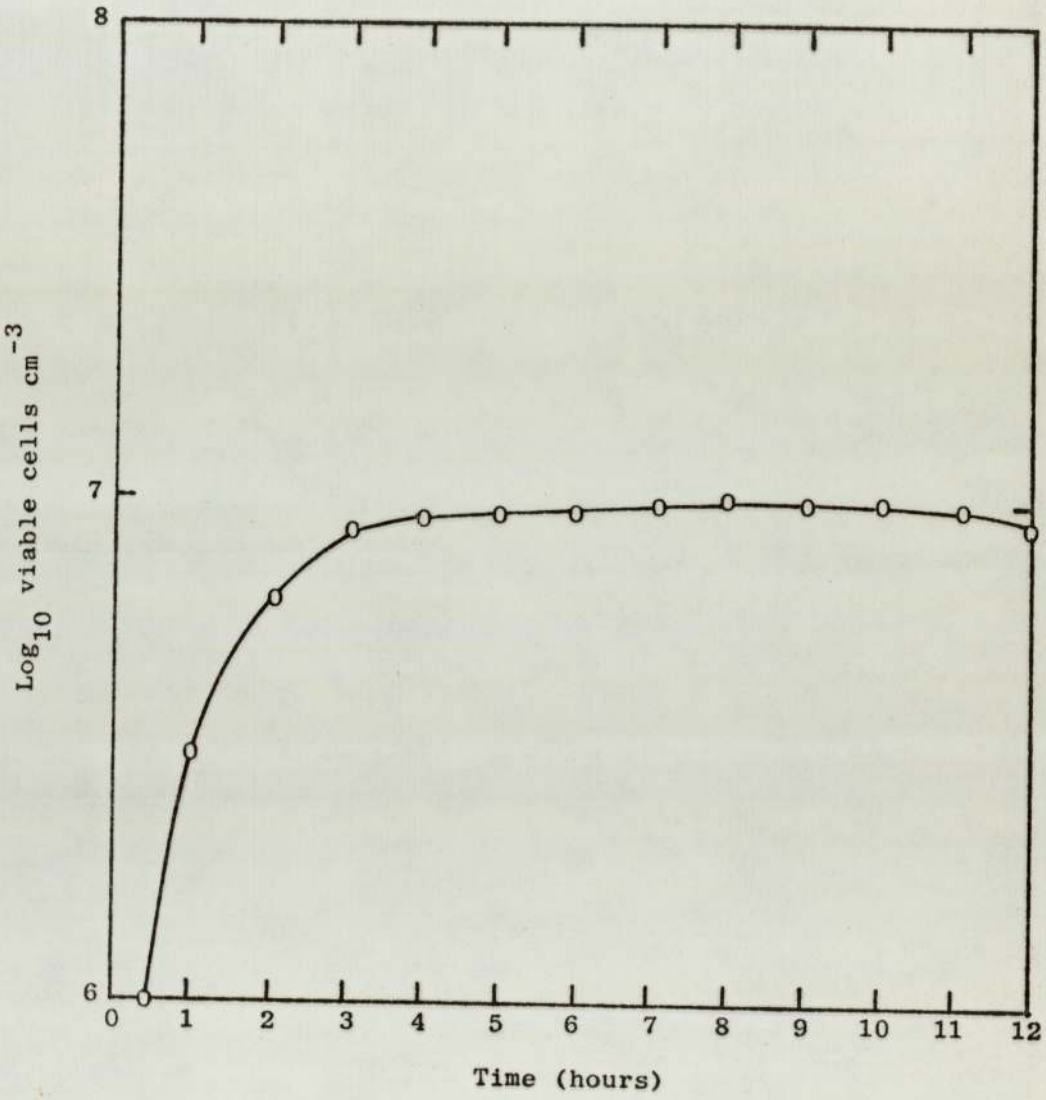


Figure 18: Growth curve for bacterial isolate 1412SA2 (*Alteromonas* sp.) in KSM.

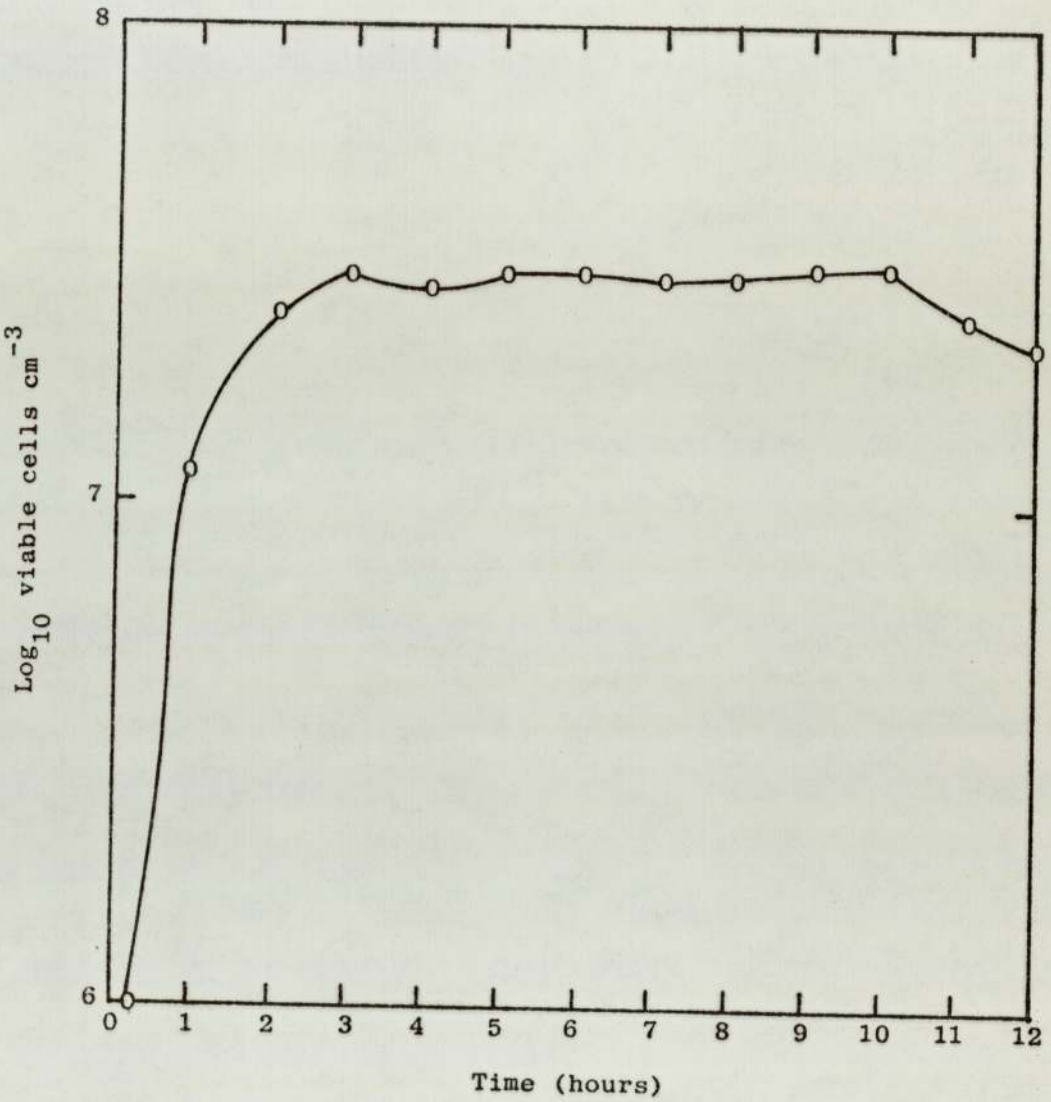
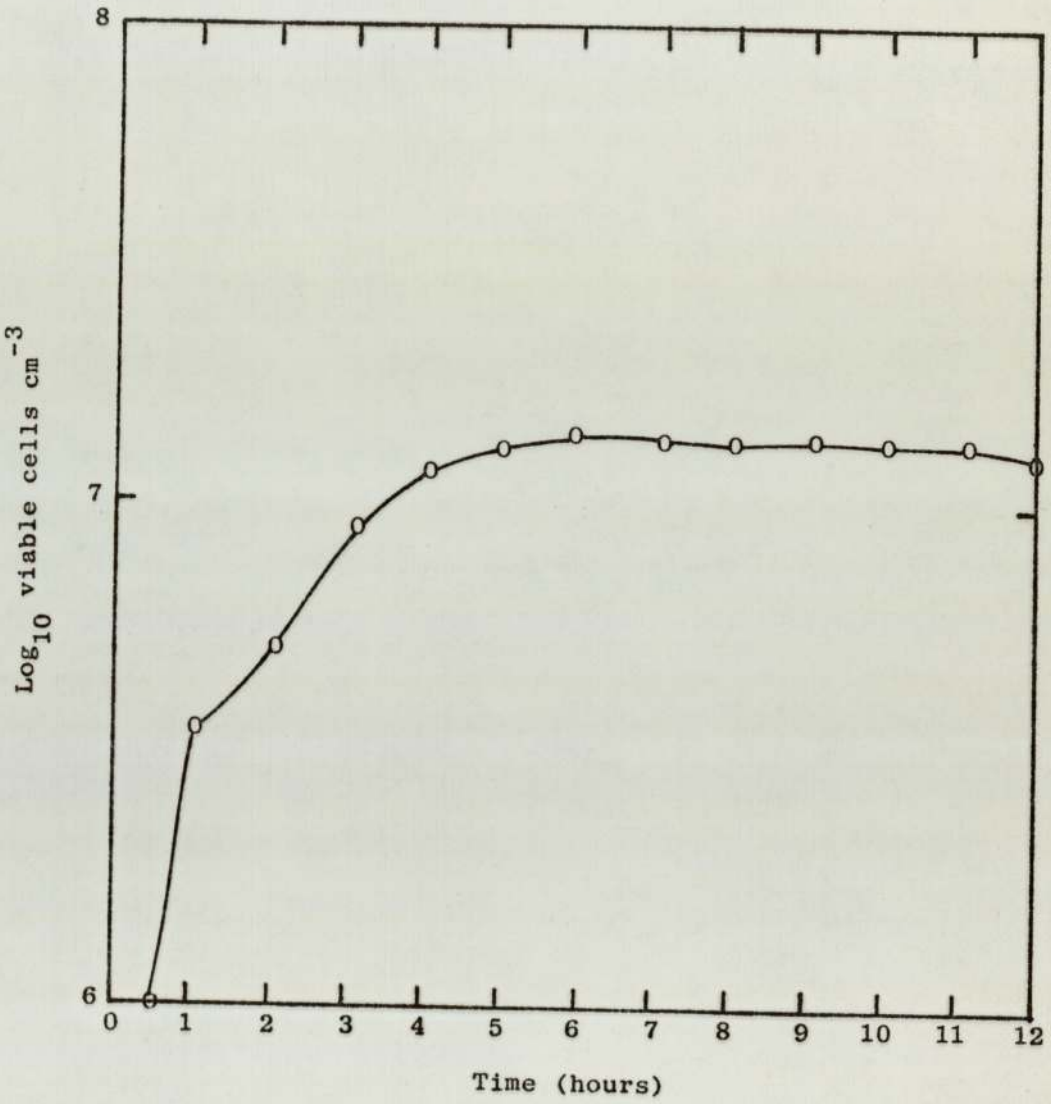


Figure 19: Growth curve for bacterial isolate 1412SA3 (*Pseudomonas/Alteromonas* group) in KSM.



3.5 Production of Thin Bacterial Films

After adhesion to a surface, marine bacteria rapidly synthesize a network of extracellular polymeric fibrils (Corpe, 1970; Marshall et al., 1971B; Colwell et al., 1980) and/or extracellular polysaccharide slime (Kirchman and Mitchell, 1983). Eventually a thick film of slime develops, that often detaches from the attachment substrate to leave the underlying surface exposed (Characklis, 1981).

Thus the surface of the attachment substrate may have areas of thick slime and closely adjacent areas of exposed surface. This is undesirable when investigating the effects of bacterial films on the settlement and growth of Enteromorpha intestinalis zoospores, as it is difficult to assess the relative effects of slime covered areas and exposed surface.

It was therefore necessary to develop a method for the production of thin bacterial films, that exhibited a minimum of exfoliation. This section describes the development of such thin bacterial films.

3.5.1 Materials and Methods

The number of viable bacterial cells occurring in open waters is considered to be low (Marshall, 1976), but the situation is somewhat different in coastal waters. Persoone and de Pauw, in a study of pollution in Ostend Harbour

(Persoone and de Pauw, 1968), estimated the numbers of bacteria in harbour water at the mouth of the Westerschelde to be between $5x10^3$ and $2x10^4$ cm^{-3}, whilst Carson found less than $10^3 cm^{-3}$ in waters at the Menai Bridge, North Wales (Carson, 1980), the area of isolation of the bacteria used in this study.

In the preparation of an inoculum for the production of bacterial films, it was decided that an inoculum of 10^3-10^4 cells cm^{-3} (expressed as total cells cm^{-3}) should be used. This decision was made for the following reasons:-

1) Natural sea-water from an unpolluted coastal region contains approximately this number of bacteria cm^{-3}.
2) Bacterial numbers in this range were found at the Menai Bridge, the site of isolation of the bacteria used in film production.

The bacteria shown in Table 5 were cultured and prepared as described in Section 3.4.1, except that the final inoculum (for inoculation into KSM) was adjusted to 10^3- 10^4 cells cm^{-3} (total cell count).

Coverslips (1.8cm^2, No3), which fitted the compartments of the 'Repli plate', were cleaned by washing in detergent ('Pyroneg' supplied by Diversey Limited, Northampton, U.K.), boiled in 40% nitric acid (chromic acid was not used, as this is known to adsorb onto glass and can be inhibitory to algal

growth (Blankley, 1973)), and washing with several changes of distilled water.

One pre-cleaned coverslip was placed in a 'Repli dish' compartment, 5 cm^3 of KSM was added along with an 0.1 cm^3 inoculum of bacteria and the procedure replicated. Incubation was at 19°C, for 12 hours, after which one of the coverslips was removed and examined by phase contrast microscopy.

The replicate coverslip was placed face down on a plate of Johnson's marine agar (Johnson, 1968) and the coverslip and plate incubated at 19°C, for up to 36 hours, after which the plates were assessed for bacterial growth.

3.5.2 Results and Discussion

On examination by phase contrast microscopy a thin, coherent film of slime and bacteria was observed adhering to the glass surface. This covered most of the area of the coverslip, except for the edges, which had no slime coating. This was seen for all bacterial isolates tested.

Viability after 12 hours was checked by examining the agar plates. Colonies of bacteria had grown on the surface of the agar in contact with the coverslip, and around the edge of the coverslip; this was found for all bacterial isolates tested.

These results indicate that this was a suitable method for the production of bacterial films, and that the viability of the bacteria was maintained during film formation.

3.6 Chapter Conclusions

Of the 17 bacterial isolates investigated all produce growth curves which show an increase in cell numbers not exceeding two log cycles, when incubated in KSM under the conditions stated. Furthermore, the death phase occurred within 12 hours for most isolates, except 712R1; 1512RB5 and 1412SA3. This provided a guide to the length of incubation for the production of bacterial films.

It was possible to produce films of all 17 isolates chosen for this study, and these isolates maintained their viability during the incubation period used for film production.

CHAPTER 4

ASSESSMENT OF CERTAIN MARINE BACTERIA FOR THEIR EFFECTS UPON THE ATTACHMENT AND GROWTH OF ENTEROMORPHA INTESTINALIS ZOOSPORES

4.1 Introduction

A method for the production of thin bacterial films was described in Chapter 3. This chapter details the use of these films in experiments designed to investigate the effects of bacterial films upon the settlement and growth of zoospores of Enteromorpha intestinalis.

The quantification of algal growth in general, and on surfaces in particular, presents some difficulties, and various methods have been adopted by many workers to overcome these problems. Young (1978) used critical point drying and transmission electron microscopy to examine bacteria collected at the air/water interface. Favali et al. (1978) used transmission electron microscopy and ethanol dehydration to investigate algal growth on plastics films. These methods are suitable for the enumeration of attached bacteria and algae when small areas are examined, but are tedious and time consuming when large numbers of cells need to be counted, which is often so when statistical comparisons between two groups are to be made.

Dry weight, packed cell volume, and the measurement of optical density (or cell suspensions) can be used to study growth rates (Sorkin, 1973), but do not provide information on the spatial distribution of algae attached to surfaces. Errors may also arise from the presence of extraneous matter, and if the difference between two populations is small, the results can be difficult to

interpret. These disadvantages also apply to the use of pigment analysis (as described by Hansmann, 1973) as an indicator of growth.

The advantages of direct counting by optical microscopy were outlined by Lund et al., (1958), who argued that by using this method there was no doubt about the number of organisms present, that errors do not arise from the presence of detrital material, and the results of counts could be subjected to statistical analysis.

An additional advantage of direct counting is the ease with which living and dead cells may be distinguished. Actively growing Enteromorpha intestinalis germlings are bright green due to the presence of chlorophyl; dead germlings are brown, as the chlorophyll has been partially, or completely, degraded.

This chapter describes a method for the direct counting of actively growing Enteromorpha intestinalis germlings, settled upon bacterial films or clean glass attachment substrates. The results of these counts were subjected to statistical analysis, and are presented in the results and discussion section.

4.2 Materials and Methods

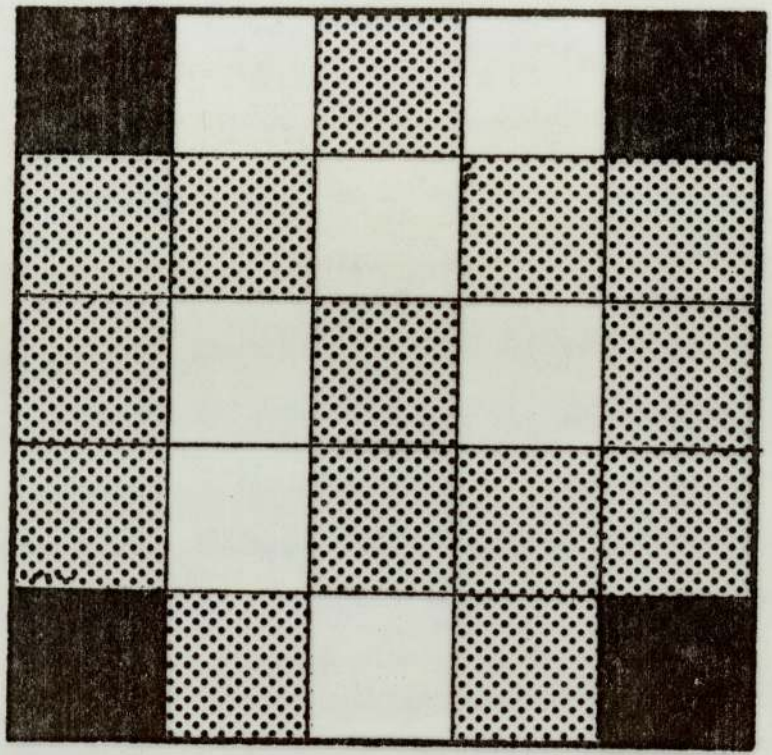
Bacterial films were produced as described in Chapter

3, Section 3.5.1, for each of the bacterial isolates shown in Section 3.2, Table 5. Fourteen replicate films were made for each of the bacterial isolates.

Each of the coverslips, that served as attachment substrates for the bacterial films, was placed in a Repli dish compartment with $4cm^3$ of Kylin's modification of Schreiber's solution (KSM). A coverslip, cleaned by washing in detergent, ('Pyroneg'), boiling in 40% nitric acid and washing in several changes of distilled water, was placed in each of 7 other compartments. These served as controls, allowing Enteromorpha intestinalis zoospore attachment to a film-free surface to be assessed. This arrangement is shown in Figure 20.

A zoospore suspension was produced from fertile fronds of Enteromorpha intestinalis by the method given in Chapter 2, Section 2.4. In this case $1dm^3$ of sterile KSM was added to 500g of dry Enteromorpha intestinalis, and allowed to stand for 20 minutes. The liquid was decanted, and a sample taken for examination in a Helber chamber by phase-contrast microscopy, at a magnification of x 400. Enteromorpha intestinalis zoospores were observed, mixed with detrital material, and this suspension was used as the zoospore inoculum. $1cm^3$ of zoospore suspension was added to each compartment of the Repli dish, and settlement of Enteromorpha intestinalis zoospores was encouraged by incubation in the dark for 18 hours, at $19^{o}C$.

Figure 20: Distribution of experimental and control surfaces in Repli Dish compartments (plan).



Glass coverslips coated with bacteria (experimental).

Glass coverslips not coated with bacteria (control).

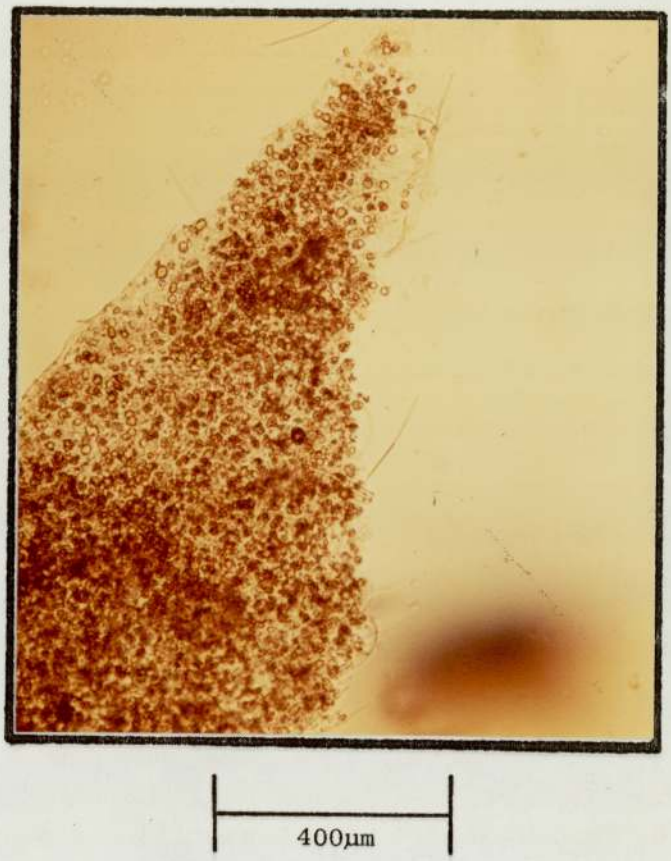
Cells not used in experimental trials.

At the end of the incubation period, the coverslips were examined for attached Enteromorpha intestinalis zoospores, using phase-contrast microscopy (x 400). Attached Enteromorpha intestinalis zoospores were taken as an indication that settlement had occurred, and the coverslips (with attached zoospores) were maintained at 15-25^{o}C and illuminated by daylight, supplemented with 'Grolux' fluorescent tubes, providing an 18 hour day length for 8 weeks; the KSM being changed for fresh KSM every two to three days.

During this time a thick film developed on all the coverslips (Figure 21). This thick film was composed of unicellular algae, diatoms, bacteria and Enteromorpha intestinalis germlings. Removal of this thick, but loosely attached, film with a mounting needle, allowed inspection of the firmly attached Enteromorpha intestinalis germlings.

At this stage the Enteromorpha intestinalis germlings were small, and counting could only be accomplished by use of a binocular microscope at x 40 magnification. In order that the whole area of a coverslip could be systematically covered, a holder was constructed from glass microscope slides and wedged into a Petri plate containing KSM (Figure 22a). This held a coverslip firmly, and the coverslip and holder could be moved using a conventional mechanical stage. This prevented the Enteromorpha intestinalis germlings from drying out, and allowed the coverslips to be scanned according to the pattern shown in figure 22b (Lund et al., 1958).

Figure 21: Thick film of unicellular algae, diatoms and bacteria.



(Thomas and Allsopp, 1983)

Figure 22a: Coverslip holder.

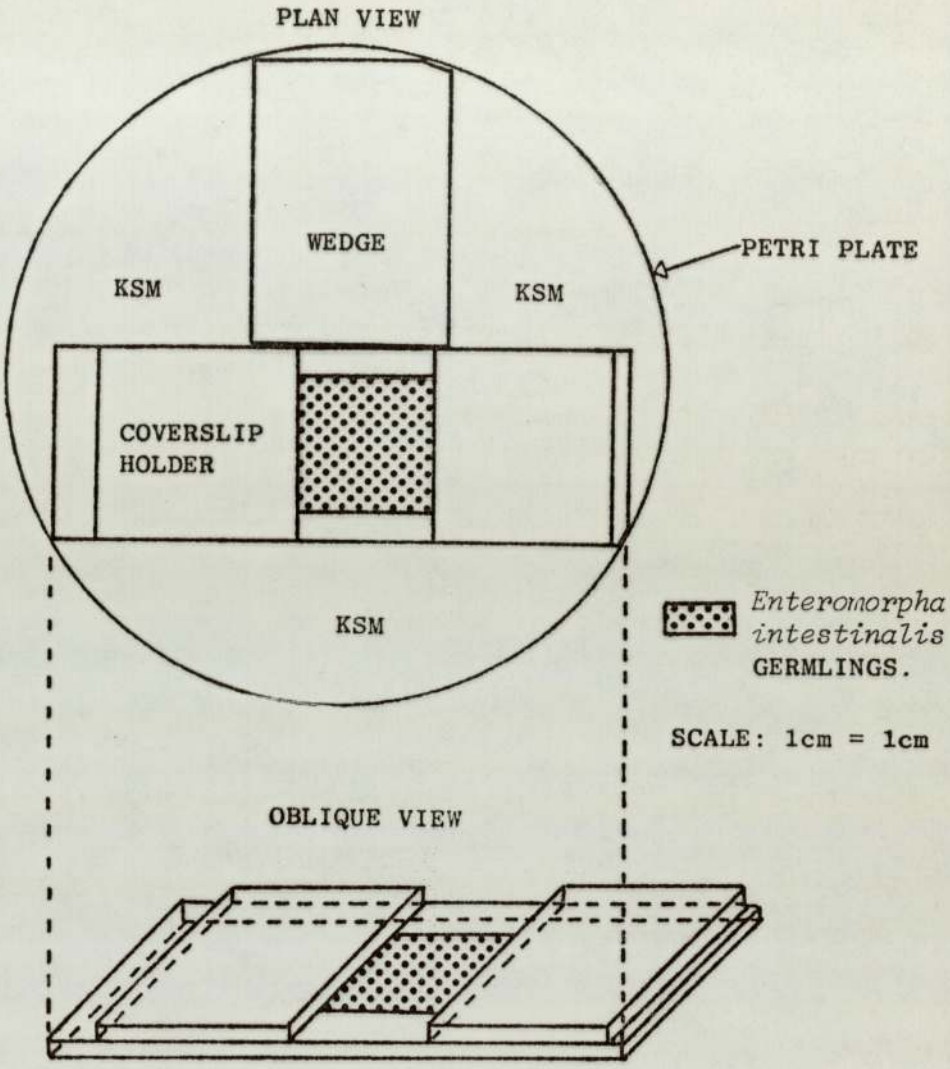
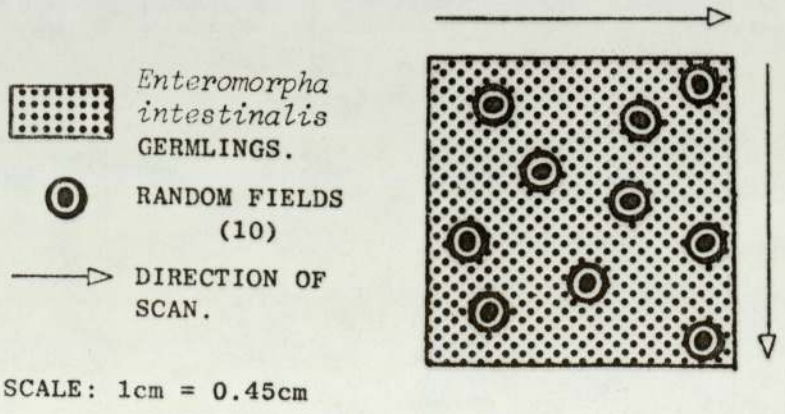


Figure 22b: Coverslip scanning pattern.



The number of Enteromorpha intestinalis germlings in each of 10 fields, having a total area of 4.7cm^2, was counted for each coverslip. The choice of 10 fields for counting represented a compromise between the practicality of counting large numbers of replicate coverslips, and the necessity to record sufficient Enteromorpha intestinalis germlings to permit statistical analysis.

Two distinct patterns of growth were observed:-

1) Single filaments (Figure 23).

2) Multi-filament clumps (Figure 24).

These were recorded separately so that a full statistical analysis could be carried out. Figure 25 shows the tip of an actively-growing Enteromorpha intestinalis filament, which was typical of Enteromorpha intestinalis germlings (filaments and clumps) seen during the course of these experiments.

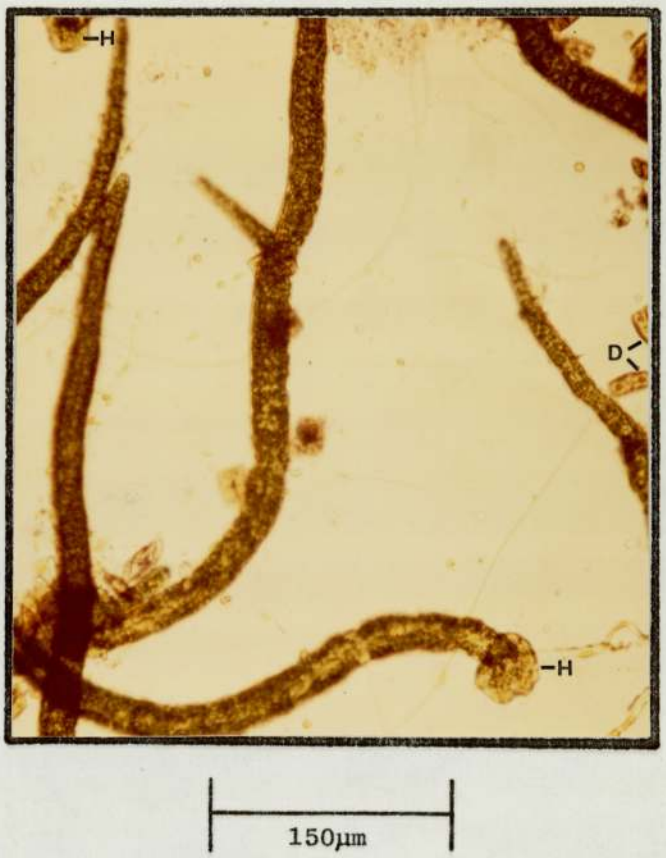
Due to the two separate growth forms of Enteromorpha intestinalis germlings, filaments, clumps and filaments plus clumps were scored separately for subsequent statistical analysis.

4.3 Results and Discussion

4.3.1 Introduction

In tables 6 to 8 the columns headed 'experimental group' provide the values for Enteromorpha intestinalis germlings

Figure 23: *Enteromorpha intestinalis* filaments, showing holdfasts and attached diatoms of the genus *Acnanthes*.



D = DIATOMS H = HOLDFASTS

(Thomas and Allsopp, 1983)

Figure 24: *Enteromorpha intestinalis* multi-filament clump.

(Thomas and Allsopp, 1983)

settled on glass attachment substrates. These attachment substrates were pre-treated by the growth of the bacterial isolate indicated in the column headed 'isolate code'. The columns headed 'control group' refer to glass attachment substrates not pre-treated by the growth of a bacterial film.

The data were obtained by recording the number of Enteromorpha intestinalis germlings on each of the replicate coverslips, for both the experimental and control group. In the case of the experimental groups the number of replicate attachment substrates was never less than 13, and in most cases, 14. For the control groups there were 7 replicates in each case.

Replicates were scored by counting the number of Enteromorpha intestinalis germlings in each of the 10 fields (see section 4.2), and these numbers allowed frequency distributions to be plotted for experimental and control groups that proved to be significant.

TABLE 6 Mean and Variance Values for *Enteromorpha intestinalis* germlings (Filaments plus Clumps), Experimental and Control Groups

	EXPERIMENTAL GROUP		CONTROL GROUP	
ISOLATE	MEAN	VARIANCE	MEAN	VARIANCE
3011G1	130	4356	11	64
3011G2	92	1369	73	1024
3011G4	7	36	110	36481
1412GA2	73	1936	82	1521
1412GA3	31	2116	136	73984
712P2	31	625	46	256
712P3	66	1369	37	576
712P4	92	3481	128	576
1512PA2	25	1225	8	121
1512PA6	83	2500	101	81
712R1	50	3364	29	324
712R2	76	5041	67	5041
712R3	69	2304	71	4624
1512RA4	42	2916	6	196
1512RB5	47	2401	18	196
1412SA2	7	100	2	9
1412SA3	36	1849	12	441

TABLE 7 Mean and Variance Values for *Enteromorpha intestinalis* germlings (Filaments), Experimental and Control Groups.

	EXPERIMENTAL GROUP		CONTROL GROUP	
ISOLATE CODE	MEAN	VARIANCE	MEAN	VARIANCE
3011G1	114	3136	9	49
3011G2	80	900	68	841
3011G4	6	36	99	30276
1412GA2	60	1296	68	1024
1412GA3	29	1764	117	53824
712P2	25	441	38	225
712P3	56	961	32	441
712P4	80	2401	113	576
1512PA2	20	961	7	81
1512PA6	67	1600	87	49
712R1	43	2401	26	289
712R2	64	3721	57	3600
712R3	59	1681	62	3600
1512RA4	36	2209	5	121
1512RB5	38	1849	12	144
1412SA2	4	36	2	9
1412SA3	33	1600	9	289

TABLE 8 Mean and Variance Values for *Enteromorpha intestinalis* germlings (Clumps), Experimental and Control Groups.

	EXPERIMENTAL GROUP		CONTROL GROUP	
ISOLATE CODE	MEAN	VARIANCE	MEAN	VARIANCE
3011G1	16	144	2	9
3011G2	12	81	5	16
3011G4	1	1	11	324
1412GA2	12	81	14	81
1412GA3	3	16	19	1600
712P2	4	25	7	9
712P3	10	49	6	16
712P4	12	144	16	16
1512PA2	3	16	1	4
1512PA6	15	100	14	9
712R1	7	81	3	2
712R2	12	121	11	121
712R3	10	64	9	81
1512RA4	6	64	2	9
1512RB5	9	49	6	9
1412SA2	3	16	0	0
1412SA3	3	9	2	16

4.3.2 Results, Statistical Analysis and Discussion

Tables 6 to 8 show the mean and variance (Appendix 1) values for Enteromorpha intestinalis germlings settled on experimental and control attachment substrates. Elliott (1971) considered there to be three relationships between the mean and variance, indicating one of three possible distributions:-

1) Random distribution - variance equal to mean.
2) Regular distribution - variance less than mean.
3) Contagious distribution - variance greater than mean.

Table 6 gives the mean and variance values for Enteromorpha intestinalis germlings (filaments plus clumps) present on experimental and control attachment substrates after 8 weeks. In general, the variance is greater than the mean, and the underlying distribution from which these samples were taken appears to be of the contagious type for the experimental and control groups. The matched control for bacterial isolate 1512PA6 (a Coryneform sp.) has a variance less than the mean, suggesting a regular distribution. This type of distribution is often seen when the individuals in a population are relatively crowded (Elliott, 1971), and could represent a variation in the zoospore inoculum, as all other control groups seem to be distributed in a contagious manner. Some variation in the spore inoculum is inevitable, as there is no simple way in which the numbers of Enteromorpha intestinalis zoospores may be standardised - the number of viable zoospores, capable of settlement, can

only be determined by the matched control method adopted here, and assessment can only be made at the end of the experiment.

When Enteromorpha intestinalis filaments are considered (Table 7) the situation is much the same, with the variance to mean ratios indicating a contagious distribution for experimental and control groups; with the sole exception of the matched control to bacterial isolate 1512PA6, where the ratio of the variance to the mean indicates a regular distribution.

The picture is less clear in the case of Enteromorpha intestinalis clumps (Table 8). The majority of values give a variance greater than the mean, indicating a contagious distribution, whilst the matched controls for bacterial isolates 1512PA6 and 712R1 (a Micrococcus sp.) have variance less than the mean, indicating a possible regular distribution, that may again be explained in terms of a variation in the original zoospore inoculum. The experimental group for bacterial isolate 3011G4 (a Pseudomonas sp.) has a mean of 1 and a variance of 1, which indicates a random distribution, but these values are low and do not allow firm conclusions to be drawn. The matched control for bacterial isolate 712P4 (a Coryneform sp.) also shows a random distribution (mean 16, variance 16). The significance of random, regular and contagious distributions

will be returned to later when considering the chi-squared test for agreement with a Poisson distribution.

The tendency towards a contagious distribution indicated in the analysis shown in Tables 6,7 and 8 is frequently seen in natural populations (Elliott, 1971), which is a reflection of the fact that environmental factors are not evenly distributed. In this case there may be a tendency for Enteromorpha intestinalis zoospores to congregate, due to unevenness of the attachment substrate surface, differential absorption of ions or nutrients, and other unknown factors.

An alternative explanation for contagion in this species is that there is a general tendency for Enteromorpha intestinalis zoospores to associate with each other. This is known for other species (Elliott, 1971), and could also explain the formation of the multi-filament clumps shown in Figure 24.

In distinguishing a difference between an experimental group and a control group a decision has to be made as to the choice of the most suitable statistical procedure. Parametric tests assume a particular underlying parent distribution, e.g. a normal distribution, and that the mean and variance for each sample group (experimental and control) are the same. Non-parametric methods require no such assumptions (Elliott, 1971), but are lower in power-efficiency; power-efficiency being the ability of a test to reject the

hypothesis that there is no difference between two groups when in fact, there is, a difference. Thus parametric tests are to be preferred when the underlying distribution of a parent population is known.

The chi-squared variance to mean ratio test (Appendix 1) is a suitable method for testing the distribution of data points in a sample against goodness of fit to a Poisson series. If the data fit a Poisson distribution parametric tests may be used, if not then the simplest solution (which avoids lengthy transformations of data) is to use non-parametric methods.

In general, but with some exceptions, the data for experimental and control groups did not conform to a Poisson distribution ($P = 0.05$). One exception was the Enteromorpha intestinalis found on experimental attachment substrate 3011G4, where the clumps of germlings were distributed according to a Poisson distribution. This was the only experimental group to have randomly distributed Enteromorpha intestinalis germlings, but its matched control did not demonstrate randomness. The control groups for bacterial isolates 712P2, 712P4, 712R1 and 1512RB5 all showed random distributions for Enteromorpha intestinalis clumps, but not for filaments or filaments plus clumps. Only the matched control for bacterial isolate 1512PA6 showed random distribution for Enteromorpha intestinalis filaments, clumps, and filaments plus clumps. These

findings agree, in general, with those seen by inspection of the tables of means and variances, and whilst they do not prove contagion as a general feature of Enteromorpha intestinalis settlement, they do indicate that in the majority of instances Enteromorpha intestinalis germlings were not distributed in a random manner.

Non-parametric statistical methods seem to be the most suitable procedures for the analysis of the present data. There are instances, however, when parametric tests, with their greater power-efficiency, may be used. Parker (1979) suggests the use of the t-test, and provides a formula (Appendix 1) to test the differences between the means of two small samples when the population variances are not assumed to be equal.

The Mann-Whitney U-test (Appendix 1) is a non-parametric alternative to the t-test, with a power-efficiency never less than 86% (Elliott, 1971). This test was used to compare the median values (parametric tests compare mean values, non-parametric tests compare median values) of the experimental groups with those of their corresponding matched controls. Table 9 summarises the results of the t-test for significant values of t; whilst Table 10 shows significant results for the Mann-Whitney U-test. Comparison of the two tables indicates a general agreement between the results of the two tests, and it appears that either test is suitable for the analysis of this type of data.

TABLE 9

Summary of Significant Values of t-test Results for Enteromorpha intestinalis Germlings Settled on Live Bacterial Films

ISOLATE CODE	FILAMENTS PLUS CLUMPS	FILAMENTS	CLUMPS
3011G1	***	***	***
3011G2			**
1512RA4	*	*	
1412SA2			*

* = Significant at P=0.05

** = Significant at P=0.01

*** = Significant at P=0.001

TABLE 10

Summary of Significant Mann-Whitney U-test Results for Enteromorpha intestinalis Germlings Settled on Live Bacterial Films

ISOLATION CODE	FILAMENTS PLUS CLUMPS	FILAMENTS	CLUMPS
3011G1	**	**	**
3011G2			*
3011G4	*		
712P2			*
712P4		*	*
1512RA4		*	
1412SA2			*

* = Significant at P=0.05

** = Significant at P=0.01

There is a highly significant difference between the experimental group and its matched control for bacterial isolate 3011G1 (genus unknown), the results are significant at the P=0.01 level for both the t-test and the Mann-Whitney U-test. This is the case for Enteromorpha intestinalis filaments plus clumps, filaments, and clumps. Reference to detailed tables of t (Pearson and Hartley, 1966; 1972) shows that the calculated value of t for isolate 3011G1 exceeds the tabulated value of t at P=0,001; such a result is considered very highly significant (Elliott, 1971). In such a case the odds are 1 in 1000 that the differences between the means are due to chance alone.

With bacterial isolate 3011G2 (a Pseudomonas sp.) only Enteromorpha intestinalis clumps show a significant difference between the mean (P=0.01) and median (P=0.05) values for experimental and control groups. Bacterial isolate 1512RA4 (a member of the Pseudomonas/Alteromonas group) shows a significant (P=0.01) difference between the means of experimental and control groups for Enteromorpha intestinalis (filaments plus clumps, and filaments), as measured by the t-test; but the Mann-Whitney U-test detects a significant difference (P=0.05) only for Enteromorpha intestinalis filaments. Bacterial isolate 1412SA2 (an Alteromonas sp.) shows a significant difference (P=0.05) between the means and the medians, of experimental and control groups for Enteromorpha intestinalis clumps only.

In all the above cases the mean of the experimental group exceeds the mean of the control group, this implies that films of these bacteria, when present during the initial stages of settlement of Enteromorpha intestinalis germlings, encourage the settlement and/or subsequent growth of Enteromorpha intestinalis.

Bacterial isolates 3011G4 (a Pseudomonas sp.), 712P2 (a member of the Pseudomonas/Alteromonas group) and 712P4 (a Coryneform sp.) all show significant differences between the median values of Enteromorpha intestinalis germlings when experimental and control attachment substrates are compared using the Mann-Whitney U-test (P=0.05) (3011G4, filaments plus clumps; 712P2, clumps; 712P4, filaments, clumps). As the median values of the control groups exceeds the median values for the experimental groups, it may be inferred that these bacterial isolates discourage the settlement and/or subsequent growth of Enteromorpha intestinalis.

Table 11 summarises the results for bacterial isolates that encourage or discourage the settlement of Enteromorpha intestinalis. Table 12 presents those bacterial isolates which did not have any significant effects upon the settlement of Enteromorpha intestinalis.

There appears to be no obvious relationship between isolates that show positive, negative or no effect upon the settlement of Enteromorpha intestinalis; except that the

majority of Coryneform isolates tested showed no effect upon the settlement of Enteromorpha intestinalis. Neither time of isolation, nor the type of substrate from which the bacteria were originally isolated (Carson, 1980) seemed to correlate with an isolate's ability (or inability) to effect the settlement of Enteromorpha intestinalis.

TABLE 11

Bacterial Isolates which Encourage or Discourage the Settlement of Enteromorpha intestinalis

		Enteromorpha intestinalis settlement	
ISOLATION CODE	GENUS	ENCOURAGED	DISCOURAGED
3011G1	Unknown	+	
3011G2	Pseudomonas sp.	+	
3011G4	Pseudomonas sp.		+
712P2	Pseudomonas/Alteromonas group		+
712P4	Coryneform sp.		+
1512RA4	Pseudomonas/Alteromonas group	+	
1412SA2	Alteromonas sp.	+	

TABLE 12

Bacterial Isolates which show no Effects upon the Settlement of *Enteromorpha intestinalis*

ISOLATION CODE	GENUS
1412GA2	*Alteromonas vaga*
1412GA3	*Alteromonas* sp.
712P3	*Pseudomonas/Alteromonas* group
1512PA2	*Coryneform* sp.
1512PA6	*Coryneform* sp.
712R1	*Micrococcus* sp.
712R2	*Pseudomonas/Alteromonas* group
712R3	*Benekea* sp.
1512RB5	*Coryneform* sp.
1412SA3	*Pseudomonas/Alteromonas* group

The variation in morphology of Enteromorpha intestinalis, that is seen in the formation of filaments and clumps, is difficult to explain. A possible explanation could be based upon the work of Fries (1975), who found abnormal growth-forms in Enteromorpha linza grown in axenic culture. When bacteria were added to these cultures normal growth ensued. These abnormal growth forms consisted of multi-filament masses, but it is difficult to equate these multi-filament masses with the multi-filament clumps found during the course of the experiments presented here, as in this case the cultures were not axenic.

An alternative explanation is that Enteromorpha intestinalis germlings do not change their morphology, but groups of spores tend to aggregate, each one developing into a single filament. If the spores were closely packed they would give the appearance of a multi-filament clump. The mean number of clumps (Table 8) tends to be less than the mean number of filaments (Table 7), for experimental and control groups in general and it is likely that there is a clumping of individual Enteromorpha intestinalis germlings against a background of singular settled germlings. This is a common form of contagious distribution (Elliott, 1971).

As previously described (section 4,3,1), the number of Enteromorpha intestinalis germlings per microscope field were counted; this allowed frequency distributions to be

plotted, these are shown in Figures 26 to 47. The majority of these frequency distributions are positively skewed, suggesting a negative binomial distribution. Agreement with a negative binomial distribution would be indicative of a contagious distribution for the settlement of Enteromorpha intestinalis. The statistic U is a measure of the difference between the sample estimate of variance and the expected variance in a negative binomial distribution (Elliott, 1971 - see Appendix 1 for formula). The data presented in Figures 26 to 47 were tested using this method, but did not conform to the negative binomial distribution. This does not disprove contagion, as there are several other mathematical models which describe a contagious distribution, and the pronounced tendency for Enteromorpha intestinalis germlings to form clumps is highly suggestive of a contagious distribution.

4.4 Chapter Conclusions

The majority of isolates tested did not effect the settlement of Enteromorpha intestinalis under the experimental conditions used.

In the case of bacterial isolate 3011G1 (genus unknown) there was a highly significant (P=0.001) difference between the means of the experimental and control groups, when subjected to the t-test. This was confirmed by the Mann-Whitney U-test, where the differences between the median

values of the experimental and control groups was very significant (P=0.01). As the means and medians of the experimental group exceeded the means and medians of the control group, bacterial isolate 3011G1 appears to encourage the settlement of Enteromorpha intestinalis.

Bacterial isolate 3011G2 (a Pseudomonas sp.) also appeared to enhance the settlement of Enteromorpha intestinalis, although these results were significant only at the P = 0.01 (t-test), and P=0.05 (Mann-Whitney U-test) levels.

Bacterial isolate 1512RA4 (a member of the Pseudomonas/ Alteromonas group) and bacterial isolate 1412SA2 (an Alteromonas sp.) significantly encouraged the settlement of Enteromorpha intestinalis at the P=0.05 level as indicated by the Mann-Whitney U-test.

In the case of bacterial isolates 3011G4 (a Pseudomonas sp.), 712P2 (a member of the Pseudomonas/Alteromonas group) and 712P4 (a Coryneform sp.), the median values of the control groups exceeded those of the experimental groups. These results were significant at the P=0.05 level when subjected to the Mann-Whitney U-test. This indicated that these isolates discouraged the settlement of Enteromorpha intestinalis.

Enteromorpha intestinalis germlings appeared as filaments or clumps, this difference in growth form could be due to aggregation of the germlings.

For experimental and control groups which showed statistically significant differences between their means or medians, the frequency distributions were positively skewed, but did not follow a negative binomial distribution.

Figure 26: Frequency distribution of *Enteromorpha intestinalis* filaments plus clumps on glass coated with bacterial isolate 3011G1 (genus unknown).

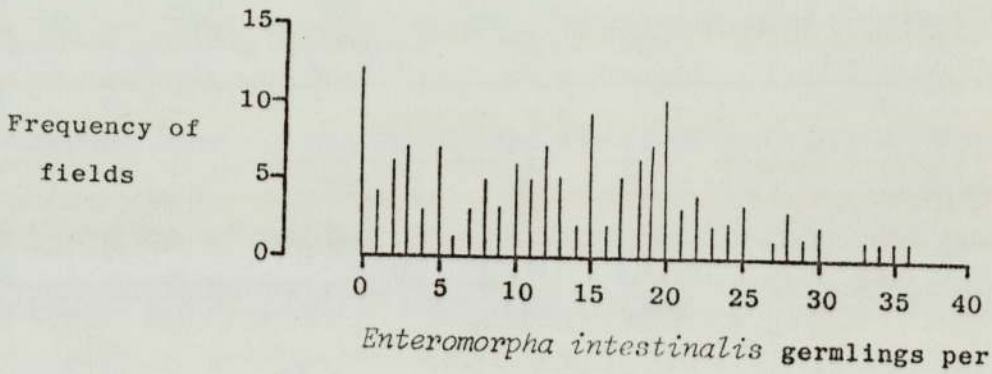


Figure 27: Frequency distribution of *Enteromorpha intestinalis* filaments plus clumps on glass. Matched control for bacterial isolate 3011G1 (genus unknown).

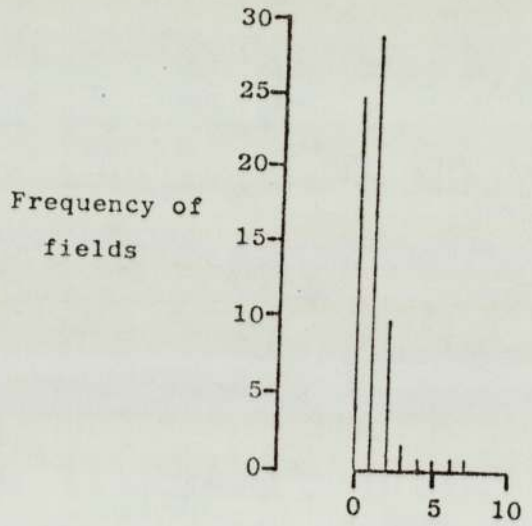


Figure 28: Frequency distribution of *Enteromorpha intestinalis* filaments on glass coated with bacterial isolate 3011G1 (genus unknown).

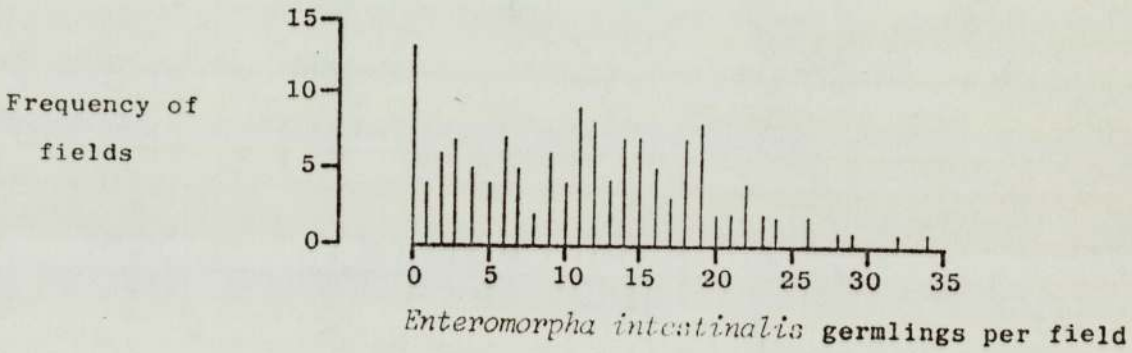


Figure 29: Frequency distribution of *Enteromorpha intestinalis* filaments on glass. Matched control for bacterial isolate 3011G1 (genus unknown).

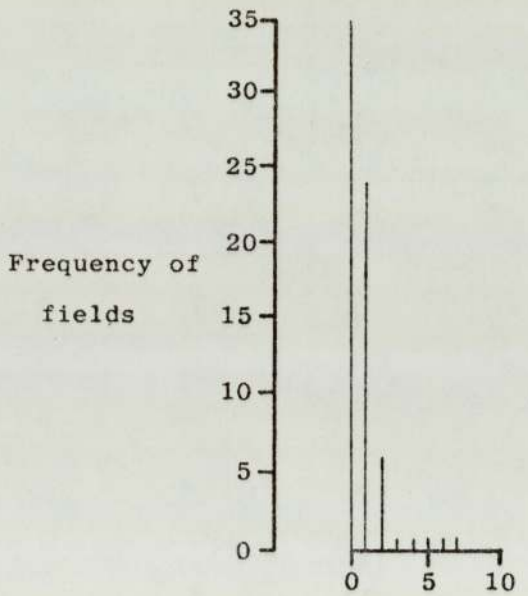


Figure 30: Frequency distribution of *Enteromorpha intestinalis* clumps on glass coated with bacterial isolate 3011G1 (genus unknown).

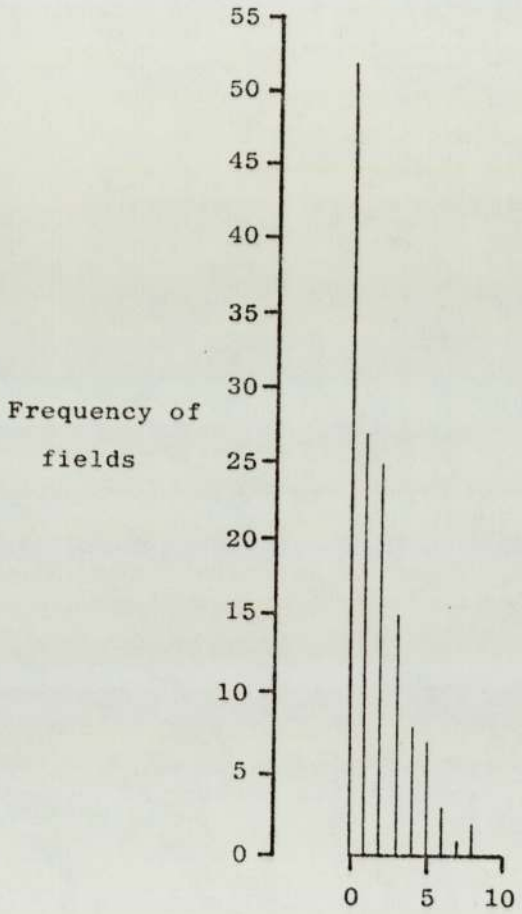


Figure 31: Frequency distribution of *Enteromorpha intestinalis* clumps on glass. Matched control for bacterial isolate 3011G1 (genus unknown).

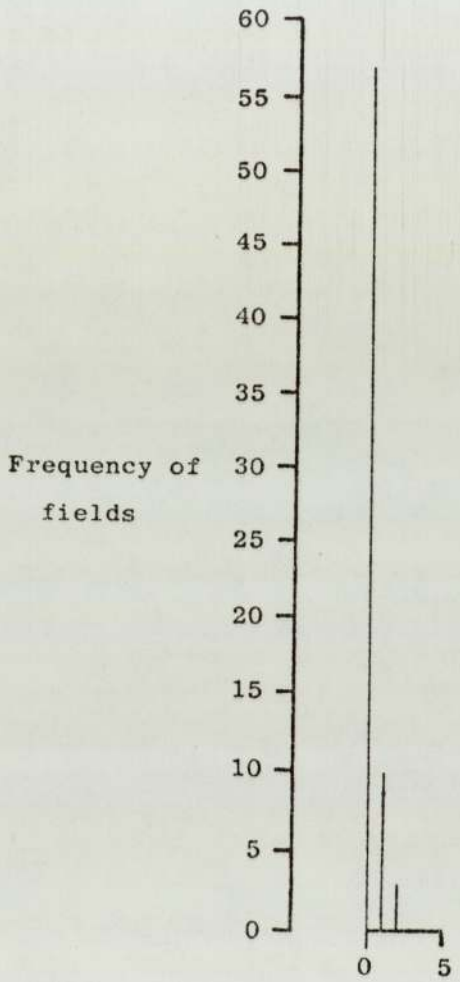


Figure 32: Frequency distribution of *Enteromorpha intestinalis* clumps on glass coated with bacterial isolate 3011G2 (*Pseudomonas* sp.).

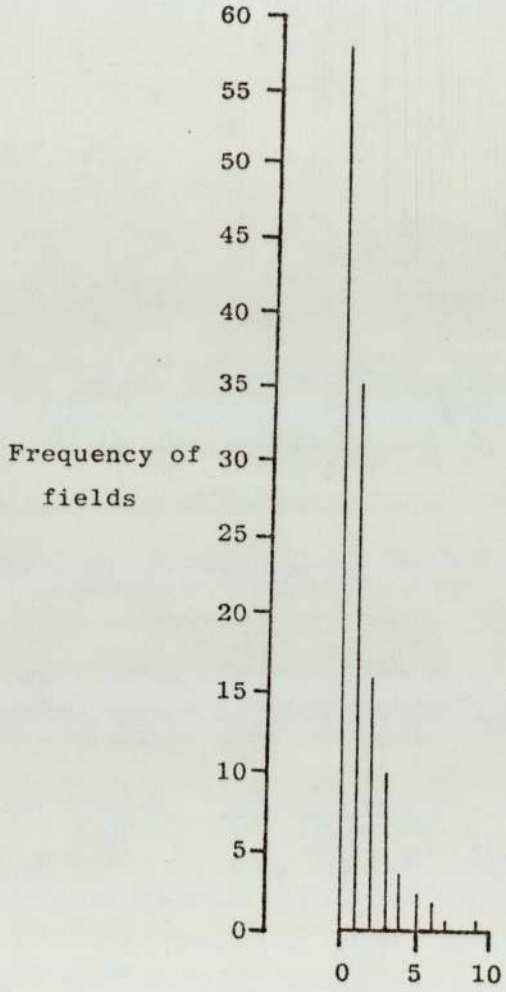


Figure 33: Frequency distribution of *Enteromorpha intestinalis* clumps on glass. Matched control for bacterial isolate 3011G2 (*Pseudomonas* sp.).

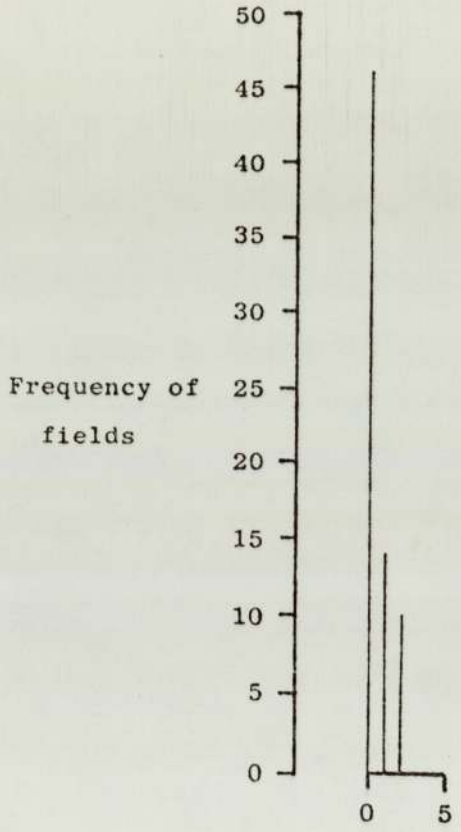


Figure 34: Frequency distribution of *Enteromorpha intestinalis* filaments plus clumps on glass coated with bacterial isolate 3011G4 (*Pseudomonas* sp.).

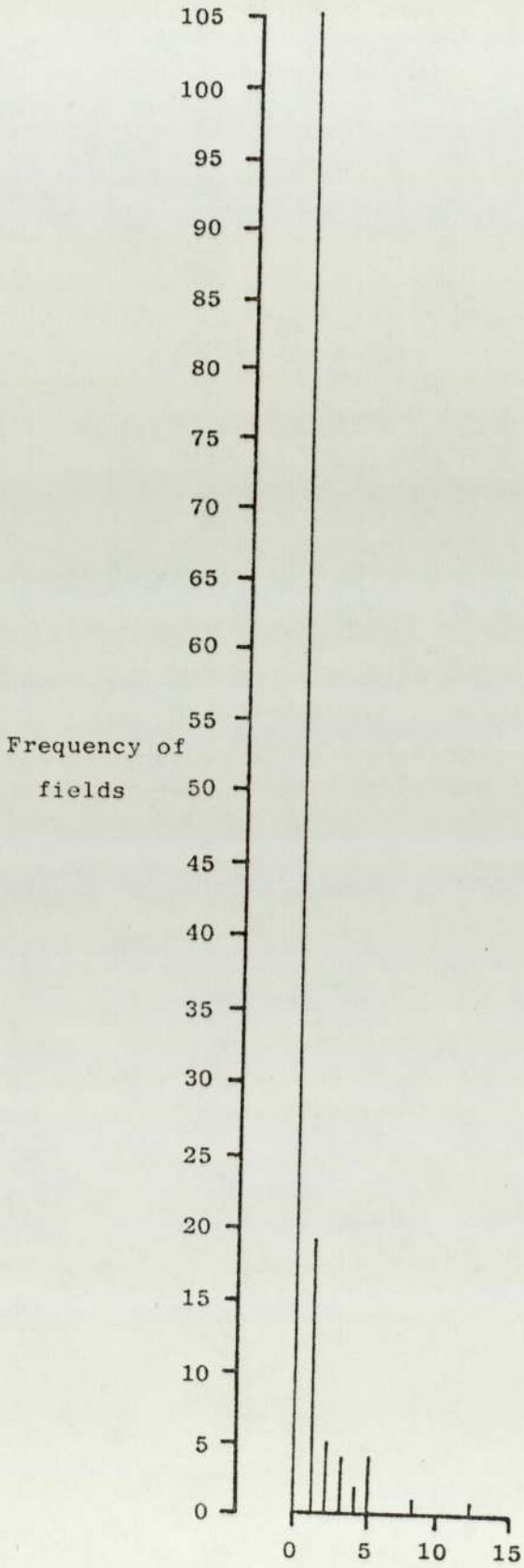


Figure 35: Frequency distribution of *Enteromorpha intestinalis* filaments plus clumps on glass. Matched control for bacterial isolate 3011G4 (*Pseudomonas* sp.)

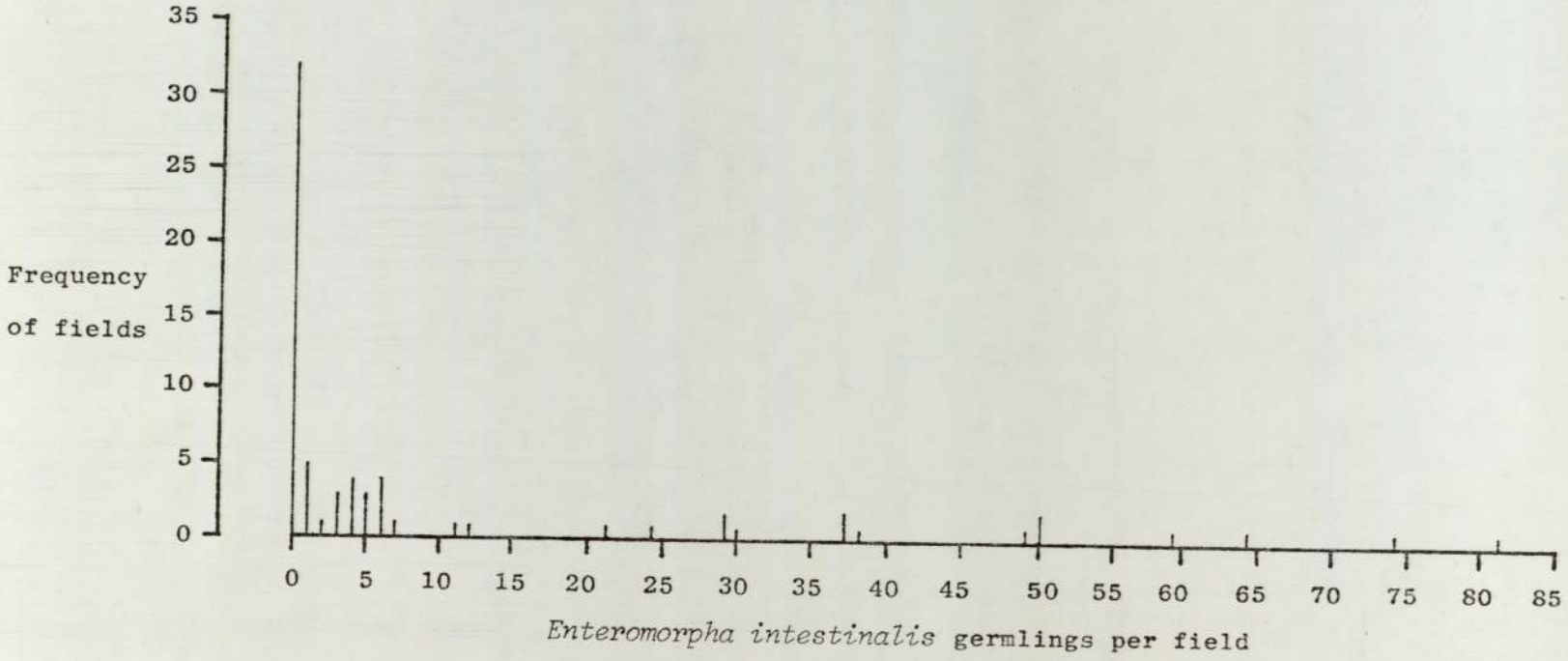


Figure 36: Frequency distribution of *Enteromorpha intestinalis* clumps on glass coated with bacterial isolate 712P2 (*Pseudomonas/ Alteromonas* group).

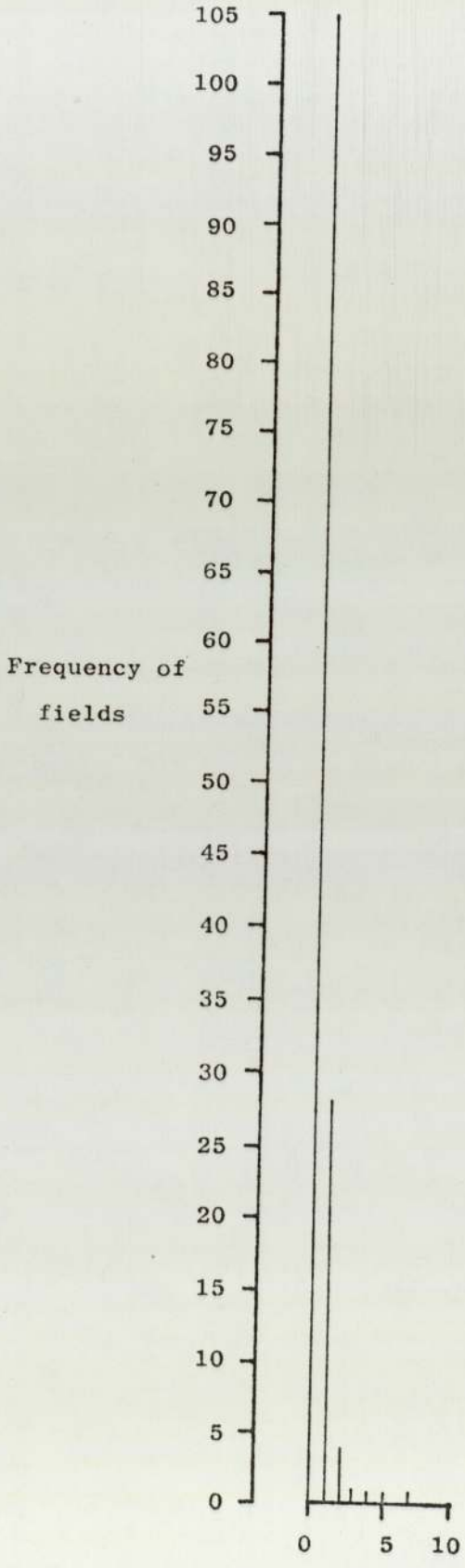


Figure 37: Frequency distribution of *Enteromorpha intestinalis* clumps on glass. Matched control for bacterial isolate 712P2 (*Pseudomonas/Alteromonas* group).

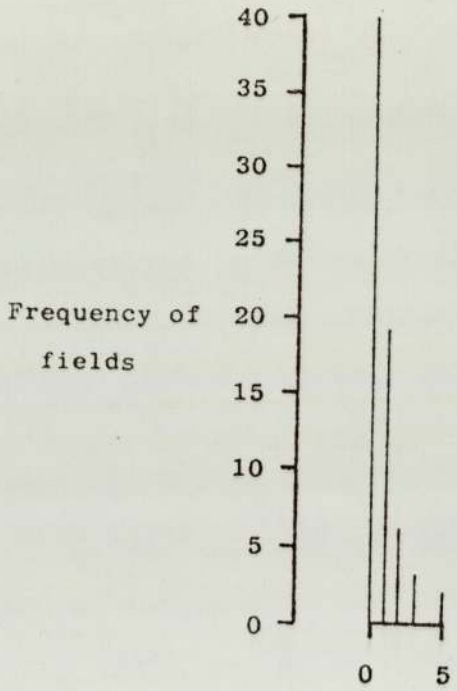


Figure 38: Frequency distribution of *Enteromorpha intestinalis* filaments on glass coated with bacterial isolate 712P4 (*Coryneform* sp.).

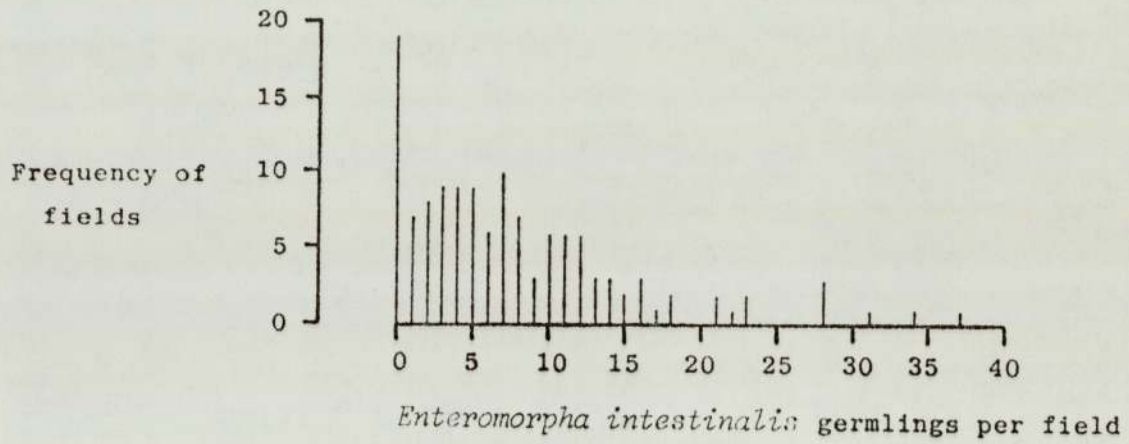


Figure 39: Frequency distribution of *Enteromorpha intestinalis* filaments on glass. Matched control for bacterial isolate 712P4 (*Coryneform* sp.).

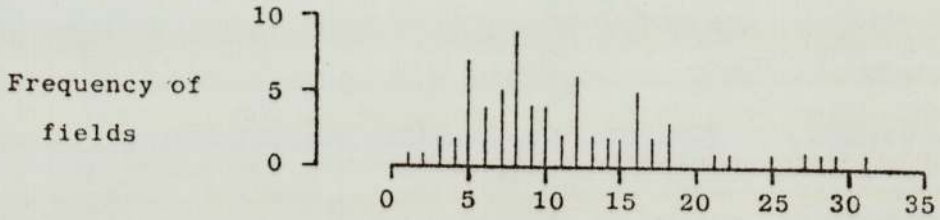


Figure 40: Frequency distribution of *Enteromorpha intestinalis* clumps on glass coated with bacterial isolate 712P4 (*Coryneform* sp.).

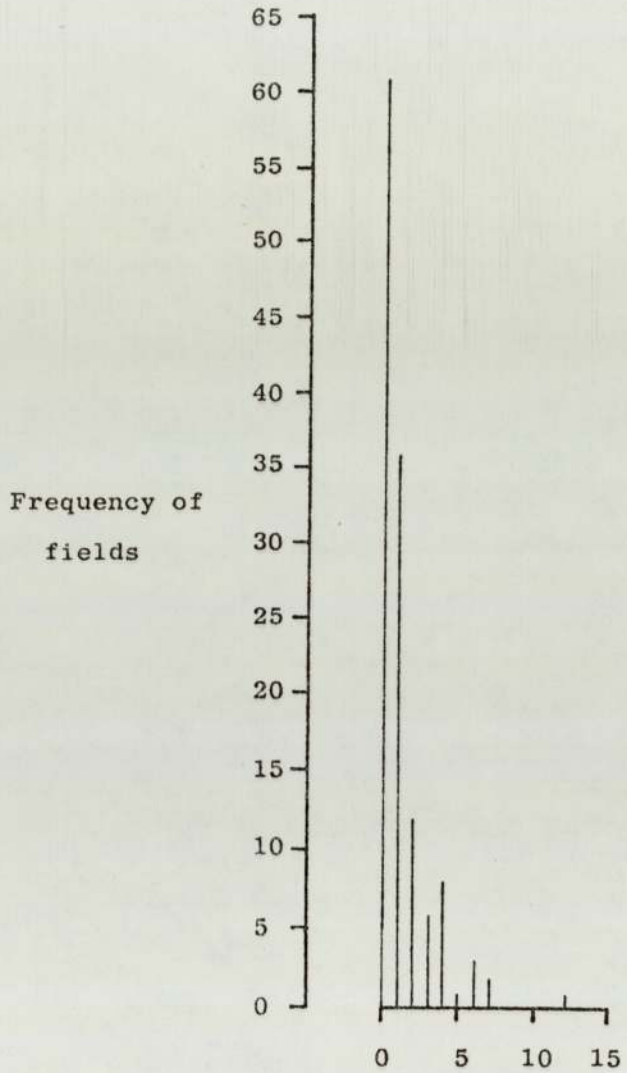


Figure 41: Frequency distribution of *Enteromorpha intestinalis* clumps on glass. Matched control for bacterial isolate 712P4 (*Coryneform* sp.).

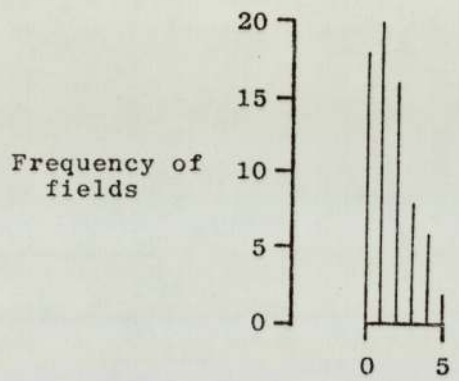


Figure 42: Frequency distribution of *Enteromorpha intestinalis* filaments plus clumps on glass coated with bacterial isolate 1512RA4 (*Pseudomonas/Alteromonas* group).

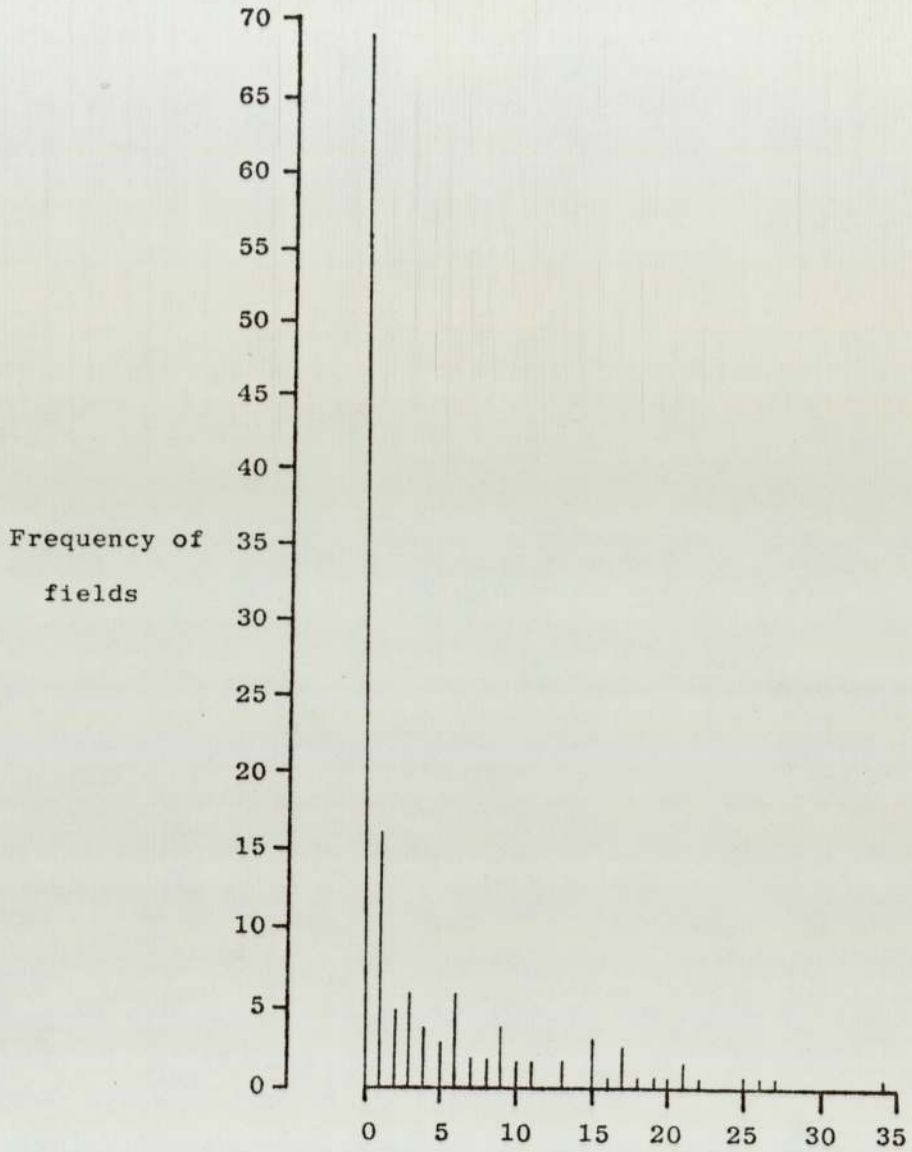


Figure 43: Frequency distribution of *Enteromorpha intestinalis* filaments plus clumps on glass. Matched control for bacterial isolate 1512RA4 (*Pseudomonas/Alteromonas* group).

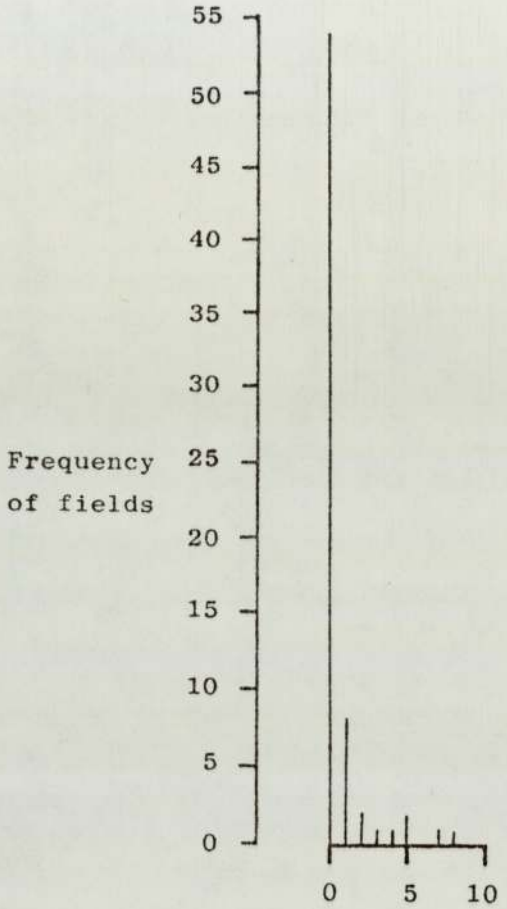


Figure 44: Frequency distribution of *Enteromorpha intestinalis* filaments on glass coated with bacterial isolate 1512RA4 (*Pseudomonas/Alteromonas* group).

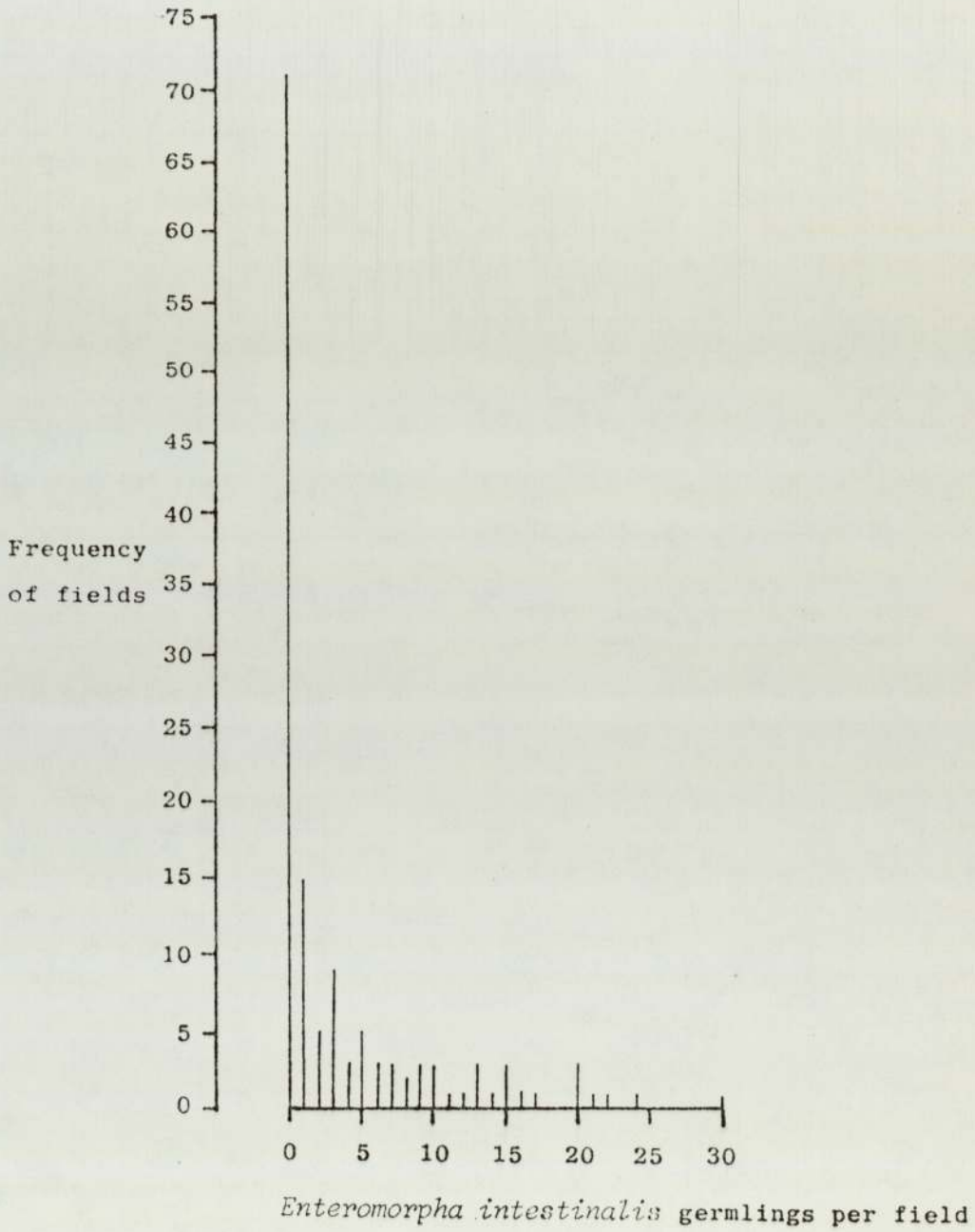


Figure 45: Frequency distribution of *Enteromorpha intestinalis* filaments on glass. Matched control for bacterial isolate 1512RA4 (*Pseudomonas/Alteromonas* group).

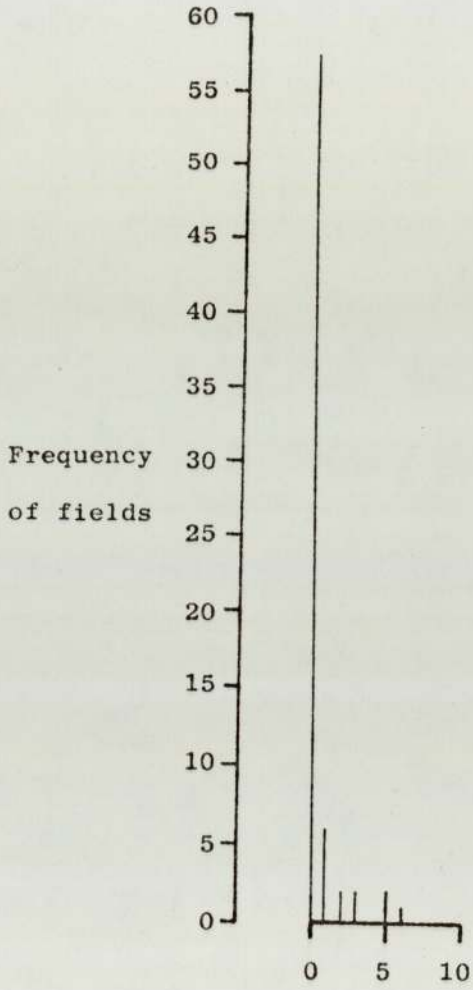


Figure 46: Frequency distribution of *Enteromorpha intestinalis* clumps on glass coated with bacterial isolate 1412SA2 (*Alteromonas* sp.).

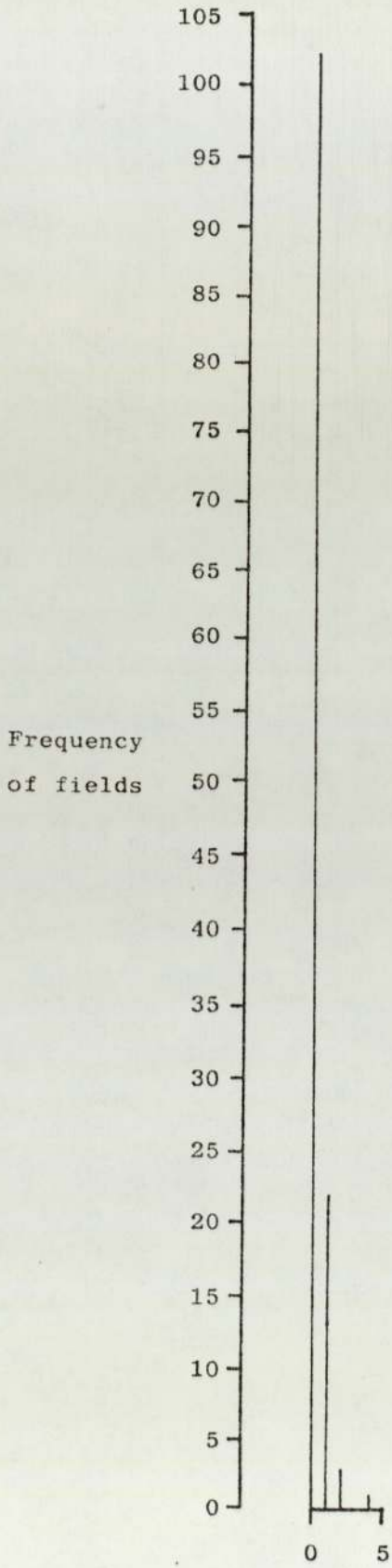
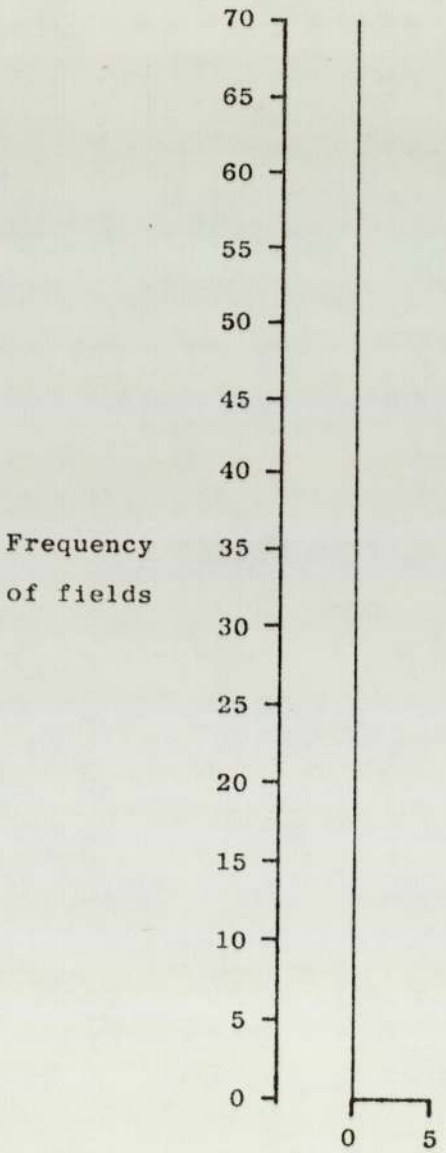


Figure 47: Frequency distribution of *Enteromorpha intestinalis* clumps on glass. Matched control for bacterial isolate 1412SA2 (*Alteromonas* sp.).



CHAPTER 5

THE EFFECTS OF FORMALIN-KILLED BACTERIAL FILMS, AND CELL-FREE BACTERIAL EXTRACTS, UPON THE SETTLEMENT AND GROWTH OF ENTEROMORPHA INTESTINALIS ZOOSPORES

5.1 Introduction

The experiments described in Chapter 4 produced data which indicated that some of the bacterial isolates collected by Carson (1980) effected the settlement and/or subsequent growth of Enteromorpha intestinalis zoospores.

This chapter describes the methodology, and presents the results of experiments designed to test the effects of formalin-killed cells and cell-free extracts upon the settlement and/or subsequent development of Enteromorpha intestinalis zoospores.

5.2 Effects of Formalin-Killed Bacterial Films Upon the Settlement and Growth of Enteromorpha intestinalis Zoospores.

Marine bacteria capable of adhering to surfaces do so by the production of extracellular polymeric materials (Fletcher and Floodgate, 1973; Marshall, 1981). Haack and McFeters (1982) investigated the effects of metabolites excreted by epilithic algae upon the growth of heterotrophic bacteria, and concluded that the bacteria utilised dissolved organic substances, produced by the epilithic algae, as their main carbon source. At present there appears to be little further work published on the interaction between algae and bacteria, apart from studies on bacterial populations colonising the surface of algae, but more information is available on the interaction between marine invertebrates and bacteria. Kirchman and Mitchell (1983), presented data that suggested

that the inducer for metamorphosis of Janua (Dexiospira) brasiliensis larvae is located on the extracellular polymer produced by bacterial films. They further noted that the induction of metamorphosis in Janua (Dexiospira) brasiliensis was not effected by pre-treatment of the bacterial films with formalin, suggesting that film-forming bacteria do not have to be metabolically active to affect metamorphosis in this intervertebrate species (Kirchman and Mitchell, 1983).

Corpe (1970), used formalin to kill bacterial cells used for the preparation of an acid polysaccharide, and found that glass slides coated with this polysaccharide fouled more rapidly than uncoated slides. This suggests that the polymer structure was not affected by treatment with formalin. To test the hypothesis that dead bacterial films could still affect the settlement of Enteromorpha intestinalis, formalin-killed bacterial films were prepared for use in settlement experiments with Enteromorpha intestinalis zoospores.

5.2.1 Materials and Method

Bacterial films were produced as described in Chapter 3, section 3.5.1, for each of the isolates that produced a significant effect upon the settlement of Enteromorpha intestinalis zoospores (Chapter 4, section 4.3.2, Table 11). Twenty replicate films were made for each bacterial isolate; 14 for use in settlement experiments, and 6 to assess the effectiveness of the bacterialcidal action of formalin.

Formalin is a 40% aqueous solution of formaldehyde (HCHO), and is considered to act as an alkylating agent, inactivating microorganisms by reacting with the carboxyl, amino, hydroxal and sulphahydril groups of proteins (Phillips, 1952).

The bacterial films (attached to coverslips) were placed in formalin (40% HCHO) for 30 minutes, removed, drained and washed several times in sterile sea water. Seven clean coverslips (not coated with a bacterial film) were treated in a similar manner.

Fourteen of the film-coated coverslips were stored in sterile sea water at $4^{\circ}C$, whilst the remaining film-coated coverslips were placed, film side down, onto plates of Johnson's Marine Agar (Johnson, 1968) and incubated for 36 hours at $19^{\circ}C$. None of the formalin-treated films produced viable colonies after this incubation period; and accordingly formalin sterilisation was adjudged to be a suitable method for the production of dead bacterial films.

All the coverslips were treated as described in Chapter 4, section 4.2; ie. they were placed in a Repli-dish, innoculated with algal spore suspension, and inclubated at $15-25^{\circ}C$ for 8 weeks. The number of Enteromorpha intestinalis germlings was assessed by the counting procedure described in Chapter 4, section 4.2, and the results recorded for statistical analysis.

5.2.2 Results and Discussion

In Tables 13 to 15 the columns headed 'Experimental Group' provide the mean and variance values for Enteromorpha intestinalis germlings settled on glass attachment substrates coated with a formalin-killed bacterial film. The columns headed 'Control Group' refer to glass attachment substrates not coated with a formalin-killed bacterial film.

Enteromorpha intestinalis germlings again showed a tendency to form filaments and clumps; in this case the variance was always greater than the mean, indicating an overall tendency towards contagion (Elliott, 1971), whilst the chi-squared variance to mean ratio test indicated that the data did not conform to a Poisson distribution.

Table 16 summarises the results of the t-test (Parker, 1979) and the Mann-Whitney U-test (Elliott, 1971), for those isolates which showed significant differences between the means of medians of experimental and control groups.

When subjected to the t-test, formalin-killed films of bacterial isolate 3011G1 (genus unknown) show a significantly higher mean number of Enteromorpha intestinalis germlings (filaments plus clumps), when compared with a control group. A significant difference between the numbers of Enteromorpha intestinalis filaments settled on formalin-killed films of bacterial isolate is also indicated; but there was no difference in the mean numbers of Enteromorpha intestinalis

TABLE 13

Mean and Variance Values for Enteromorpha intestinalis Germlings (Filaments plus Clumps), Formalin-Killed Bacterial Films

	EXPERIMENTAL GROUP		CONTROL GROUP	
ISOLATION CODE	MEAN	VARIANCE	MEAN	VARIANCE
3011G1	46	1024	14	121
3011G2	65	2209	47	729
3011G4	91	3481	92	1444
712P2	146	5776	93	1369
712P4	143	6400	100	2401
1512RA4	54	1521	25	625
1412SA2	68	2116	39	1681

TABLE 14

Mean and Variance Values for *Enteromorpha intestinalis* Germlings (Filaments), Formalin-Killed Bacterial Films

	EXPERIMENTAL GROUP		CONTROL GROUP	
ISOLATION CODE	MEAN	VARIANCE	MEAN	VARIANCE
3011G1	37	1225	9	169
3011G2	56	2025	41	841
3011G4	64	2601	91	1296
712P2	137	6084	88	1369
712P4	132	6084	81	2116
1512RA4	45	1369	17	484
1412SA2	60	1936	31	1444

TABLE 15

Mean and Variance Values for Enteromorpha intestinalis Germlings (Clumps), Formalin-Killed Bacterial Films

	EXPERIMENTAL GROUP		CONTROL GROUP	
ISOLATION CODE	MEAN	VARIANCE	MEAN	VARIANCE
3011G1	10	81	4	25
3011G2	9	100	6	36
3011G4	26	256	15	121
712P2	9	81	5	25
712P4	14	121	37	2304
1512RA4	9	81	8	64
1412SA2	8	81	8	64

TABLE 16

Summary of Statistically Significant Results for *Enteromorpha intestinalis* Germlings Settled on Formalin-Killed Bacterial Films

		t-test		Mann-Whitney U-test	
ISOLATE CODE	GENUS	Filaments plus Clumps	Filaments	Filaments plus Clumps	Filaments
3011G1	UNKNOWN	**	*	*	*
712P2	*Pseudomonas*/ *Alteromonas* Group	*			
1512RA4	*Pseudomonas*/ *Alteromonas* Group	*	*		

* = Significant at P = 0.05

** = Significant at P = 0.01

clumps between experimental and control groups. These results were confirmed by the Mann-Whitney U-test, and are similar to the results produced by live films of bacterial isolate 3011G1. The results indicate that metabolic activity of bacterial isolate 3011G1 is not required for the encouragement of settlement of Enteromorpha intestinalis.

A similar conclusion can be reached for bacterial isolate 1512RA4 (a member of the Pseudomonas/Alteromonas group), although these results should be viewed with caution as a significant difference is detected only by the t-test, and not confirmed by the Mann-Whitney U-test.

In the experiments with live bacterial films (Chapter 4), bacterial isolate 712P2 (a member of the Pseudomonas/ Alteromonas group) appeared to discourage the settlement of Enteromorpha intestinalis clumps.

In the case of formalin-killed films of this bacterial isolate, the t-test indicated a significant difference between the means of experimental and control groups for Enteromorpha intestinalis filaments plus clumps but not for filaments or clumps when scored separately. The mean number of Enteromorpha intestinalis filaments plus clumps settled on formalin-killed films of bacterial isolate 712P2 was greater than the mean number of Enteromorpha intestinalis filaments plus clumps settled on control attachment substrates and this indicated that zoospore attachment was encouraged by formalin-killed films of bacterial isolate 712P2.

This difference between live and dead films of bacterial isolate 712P2 could indicate that continued bacterial metabolism is required to discourage the settlement of Enteromorpha intestinalis, whilst the presence of dead cells, or extracellular polysaccharide, is sufficient to encourage algal settlement. When cells of isolate 712P2 are metabolically active they could excrete products that prevented or inhibited zoospore attachment.

The Mann-Whitney U-test did not indicate a difference in the median values between experimental and control groups for isolate 712P2, but this test does have a lower power-efficiency than the t-test (see Chapter 4, section 4.3.2).

Formalin-killed films of the remaining bacterial isolates did not show any significant differences between mean or median values of experimental and control groups. This could be an indication that continued metabolism is required for these isolates to effect the settlement of Enteromorpha intestinalis. This was investigated by the experiments described in section 5.3

5.3 Effects of Cell-Free Bacterial Extracts Upon the Settlement and Growth of Enteromorpha intestinalis Zoospores

Christie et al. (1970) present data that suggest that zoospores of Enteromorpha intestinalis are susceptible to attack by proteolytic enzymes.

Young and Mitchell (1973) investigated the chemotactic response of mature oyster (Crassostera virginica) larvae, and found that a motile marine Pseudomonad elicited a positive chemotactic response in oyster larvae.

The above authors suggest that settlement processes in marine algae and marine invertebrates may be affected by extracellular products of microbial metabolism.

The experiments described in this section were designed to investigate the effects of bacterial cell-free extracts upon the growth and settlement of Enteromorpha intestinalis zoospores.

5.3.1 Materials and Method

There are many methods for the preparation of cell-free extracts, and these are discussed in detail by Hughes et al. (1971), who state that:-

> "...there is no approach other than the empirical one in choosing a method for disintegrating microbes for a specific problem."

The method adopted in the experimental work described in this section was that of solid shear by hand grinding by means of a chilled pestle and mortar.

For each of the bacterial isolates that produced a significant effect upon the settlement and/or subsequent growth of Enteromorpha intestinalis zoospores (Chapter 4,

section 4.3.2, Table 11), a bacterial culture was prepared as described in Chapter 3, section 3.4.1, paragraph 1.

After incubation, the bacterial cells were centrifuged out of suspension, washed in sterile sea-water and filtered. The filtered cells were weighed, and 1g (wet-weight) was mixed with 4g of fine, washed, white sand. This mixture was ground in a mortar and pestle (chilled to $0^{o}C$), eluted with $20cm^3$ of sterile sea-water and filtered through a 0.2 m membrane filter (Whatman catalogue No. 7182 002) to remove cell wall fragments.

Twenty-one clean glass coverslips were placed in the cells of a Repli-dish, and $3cm^2$ of Kylin's modification of Schreiber's solution (KSM) was added to each cell. A 1 cm^3 aliquot of bacterial extract was added to each of 14 Repli plate cells, 7 further compartments containing a coverslip, but did not receive an aliquot of bacterial extract.

An Enteromorpha intestinalis zoospore suspension was prepared, as described in Chapter 4, section 4.2, paragraph 3; and a $1cm^3$ inoculum added to each of the Repli dish cells, which were then incubated at $15-25^{o}C$ for 8 weeks. The number of Enteromorpha intestinalis germlings as assessed by the counting procedure described in Chapter 4, section 4.2, and the results recorded for statistical analysis.

5.3.2 Results and Discussion

In Tables 17 to 19 the columns headed 'Experimental Group' provide the mean and variance values for Enteromorpha intestinalis germlings settled on glass attachment substrates in the presence of $1cm^3$ of a bacterial cell-free extract. The columns headed 'Control Group' refer to glass attachment substrates incubated without the addition of bacterial cell-free extracts.

In all the experimental and control groups the variance is greater than the mean. This is the case for Enteromorpha intestinalis filaments, clumps and filaments plus clumps; again showing a tendency towards a contagious distribution of Enteromorpha intestinalis germlings on glass attachment substrates, under the experimental conditions used.

The chi-squared variance to mean ratio test indicated that the data were not in agreement with a Poisson distribution. This indicates that non-parametric tests are more reliable indicators of differences between control and experimental groups than parametric tests.

The non-parametric Mann-Whitney U-test (Elliott, 1971) and the t-test (Parker, 1979) were performed on the experimental and control groups, and the results are summarised in Table 20. In general there were no significant differences between the means, or medians, of experimental and control groups.

TABLE 17

Mean and Variance Values for Enteromorpha intestinalis Germlings (Filaments plus Clumps) in the Presence of Cell-Free Bacterial Extracts

	EXPERIMENTAL GROUP		CONTROL GROUP	
ISOLATION CODE	MEAN	VARIANCE	MEAN	VARIANCE
3011G1	47	1156	59	4624
3011G2	35	1225	59	4489
3011G4	11	121	19	361
712P2	64	3025	44	484
712P4	57	2304	48	1156
1512RA4	91	1225	73	1024
1412SA2	41	625	26	729

TABLE 18

Mean and Variance Values for *Enteromorpha intestinalis* Germlings (Filaments) in the Presence of Cell-Free Bacterial Extracts

	EXPERIMENTAL GROUP		CONTROL GROUP	
ISOLATION CODE	MEAN	VARIANCE	MEAN	VARIANCE
3011G1	40	784	51	3481
3011G2	31	1296	50	3249
3011G4	25	6241	12	324
712P2	59	2704	25	841
712P4	51	1849	41	1444
1512RA4	79	841	68	841
1412SA2	33	576	24	784

TABLE 19

Mean and Variance Values for *Enteromorpha intestinalis* Germlings (Clumps) in the Presence of Cell-Free Bacterial Extracts

	EXPERIMENTAL GROUP		CONTROL GROUP	
ISOLATE CODE	MEAN	VARIANCE	MEAN	VARIANCE
3011G1	7	49	9	100
3011G2	4	25	8	121
3011G4	5	25	8	49
712P2	4	25	18	441
712P4	6	36	7	81
1512RA4	12	81	5	16
1412SA2	8	36	2	9

TABLE 20

Summary of Statistically Significant Results for *Enteromorpha intestinalis* Germlings Settled on Glass Attachment Substrates in the Presence of Cell-Free Bacterial Extracts

Bacteria from which Cell-Free Extract Obtained		t-test	Mann-Whitney U-test
ISOLATION CODE	GENUS	CLUMPS	CLUMPS
712P2	*Pseudomonas/ Alteromonas* Group		*
1512RA4	*Pseudomonas/ Alteromonas* Group	*	
1412SA2	*Alteromonas*		*

* = Significant at P = 0.05

The t-test indicated a difference between the means of the experimental and control groups for cell-free extracts of bacterial isolate 1512RA4 (a member of the Pseudomonas/ Alteromonas group), but this was only seen with Enteromorpha intestinalis clumps (Table 20). In this case the mean of the experimental group exceeded the mean of the control group, which indicates that cell-free extracts of this bacterial isolate encourage settlement and/or subsequent growth of Enteromorpha intestinalis clumps, but this was not confirmed by the Mann-Whitney U-test.

The formation of Enteromorpha intestinalis clumps appeared to be encouraged by cell-free extracts of bacterial isolate 1412SA2 (an Alteromonas sp.), but discouraged by cell-free extracts of bacterial isolate 712P2 (a member of the Pseudomonas/Alteromonas group); in both cases the median values of experimental and control groups were significantly different ($P = 0.05$) when subjected to the non-parametric Mann-Whitney U-test.

Thus it is only Enteromorpha intestinalis clumps which appear to be affected by cell-free bacterial extracts. This is in contrast to the effects of dead bacterial films, which affect the formation of Enteromorpha intestinalis filaments and filaments plus clumps: but not Enteromorpha intestinalis clumps. With live bacterial films (Chapter 4) Enteromorpha intestinalis filaments, clumps and filaments plus clumps all appear to be affected by the presence of certain bacterial isolates (Chapter 4, Section 4.3.2, Tables 9 and 10).

5.4 Chapter Conclusions

It appears that the major bacterial factors influencing the settlement of Enteromorpha intestinalis are associated with the cell wall, or extracellular polysaccharide , of the bacterial isolates tested.

There is some indication that a cellular fraction may influence the incidence of Enteromorpha intestinalis clumps, but this was only observed with a minority of the bacterial isolates used.

CHAPTER 6

THE EFFECTS OF Cu^{2+} UPON THE GROWTH OF SELECTED BACTERIAL ISOLATES IN SEA WATER AND IN KYLIN'S MODIFICATION OF SCHREIBER'S SOLUTION (KSM)

6.1 Introduction

Copper compounds are the most common toxicants incorporated into antifouling paints (Kronstine, 1975). Studies have been conducted on copper tolerance in marine fouling organisms (Overnell, 1975; Salenko et al., 1976; Hall, 1980; Hall, 1981; Seeliger and Cordazzo, 1982), but little information is available on the level of copper released by antifouling formulations under normal operating conditions. Mesich and Huff (1973), estimated that 10μg cm^{-2} day^{-1} is a commonly achieved leaching rate, and that this leaching rate effectively prevents the growth of most fouling organisms.

Goodman et al., (1976) devised a rapid screening test for copper tolerance in the ship fouling alga Enteromorpha compressa, and found that the growth of this alga was inhibited by more than 75% in the presence of 0.25mg dm^{-3} of Cu^{2+}.

It has been suggested (Marszalek et al., 1979; Dempsey, 1981) that films of marine bacteria (particularly those producing extracellular polysaccharides) may prevent the toxicant reaching the target organism. The effects of copper compounds upon film-forming marine bacteria are not well documented. Duddridge et al., (1981) found that the numbers Pseudomonas fluorescens cells that attached to brass attachment substrates were lower than on stainless steel, mild steel and aluminium attachment substrates which suggests that copper inhibits the formation of bacterial films.

This chapter presents data from experiments designed to test the effects of divalent copper upon those marine bacteria that were found to have a significant effect upon the settlement and subsequent growth of Enteromorpha intestinalis.

6.2 Effects of Cu^{2+} Upon the Growth of Selected Bacterial Isolates in Sea Water.

Cuprous oxide, the major component of antifouling paints (Kronstine, 1975), dissolves in sea water to form cuprous chloride according to the following equation (de la Court and de Vries, 1973):-

$$\tfrac{1}{2}\ Cu_2O + H^+ + 2Cl^- \quad CuCl_2^- + \tfrac{1}{2}H_2O$$

Cuprous chloride is almost insoluble in water (Lowry and Cavell, 1939), and has a very low solubility, 1.3 ppm, in sea water (de la Court and de Vries, 1973). This low solubility of cuprous salts in sea water makes it difficult to use such compounds when investigating the effects of high levels of copper ions on marine organisms.

Goodman et al. (1976) used cupric chloride, which has a higher solubility in sea water, to investigate the effects of copper ions upon the growth of an unidentified species of Enteromorpha, and found that this provided a suitable bio-assay for the effects of copper on ship-fouling algae.

In the experiments described in this section, cupric chloride was used to investigate the effects of copper (Cu^{2+}) ions upon the growth of selected bacterial isolates.

6.2.1 Materials and Method

For each of the bacterial isolates found to have a significant effect upon the settlement and growth of Enteromorpha intestinalis (Chapter 4, section 4.3.2, Table 11), a culture was prepared by incubation in Johnson's Marine Broth for 12 hours at 19°C (see Chapter 3, Section 3.4.1, paragraph 1 for the full method).

After incubation the cells were centrifuged out of suspension, washed in sterile sea water, re-suspended in sterile sea water and counted using a Helber chamber. The number of cells was adjusted to approximately 10^6 - 10^7 cm^{-3}, either by centrifugation and re-suspension in a suitable volume of sterile sea water or by dilution with sterile sea water.

Cupric chloride was dissolved in water and filtered through an 0.2 m membrane filter (Whatman catalogue No. 7182 002); sterilising it sufficiently for the purpose of these experiments. Aliquots of this solution were aseptically added to sterile sea water, such that the final amount of cupric chloride added was 0, 5, 10, 50, 100 or 500mg dm^{-3} with respect to Cu^{2+}.

One hundred cm^3 of these solutions were placed, separately, into conical flasks and innoculated with a $1cm^3$ aliquot of bacterial suspension (containing approximately 10^6-10^7 cells). This was repeated for each of the cupric chloride/sea water solutions, and for each of the bacterial isolates. The flasks were incubated at 19°C for 12 hours,

after which samples were taken, passed through a suitable dilution-series of sterile sea water, and plated by the spread-plate technique onto plates of Johnson's Marine Agar (Johnson, 1968). Five replicate plates were made for each dilution step, and the plates incubated at 19°C until colonies were visible. The plates were counted and the number of colonies per plate recorded for subsequent analysis.

6.2.2 Results and Discussion

The results are presented in Figures 48 to 54, each datum point represents the mean of five replicate plates. The coefficient of variation for each series of replicate plates was 5% or less, which is considered to be an acceptable level of precision for colony counts (Jennison and Wadsworth, 1939).

Figures 48 to 54 show that for all bacterial isolates tested there was a decrease in viable cell count of about one log cycle in the presence of 5mg dm^{-3} Cu^{2+}, ie. a 90% reduction in viable cell numbers. Between 5 and 100mg dm^{-3} Cu^{2+} there was less of a reduction in viable cell numbers, but when cupric chloride was present at 500mg dm^{-3} (with respect to Cu^{2+}) virtually all bacterial cells had been killed.

Bacterial isolate 3011G4 (a Pseudomonas sp.) appeared to be particularly sensitive, in that there was a marked reduction in viable cell count between 50 and 100mg dm^{-3} Cu^{2+}. This could indicate that this bacterial isolate is particularly sensitive to copper salts, and it is interesting

Figure 48: Effects of Cu^{2+} on bacterial isolate 3011G1 (genus unknown) when grown in sea-water.

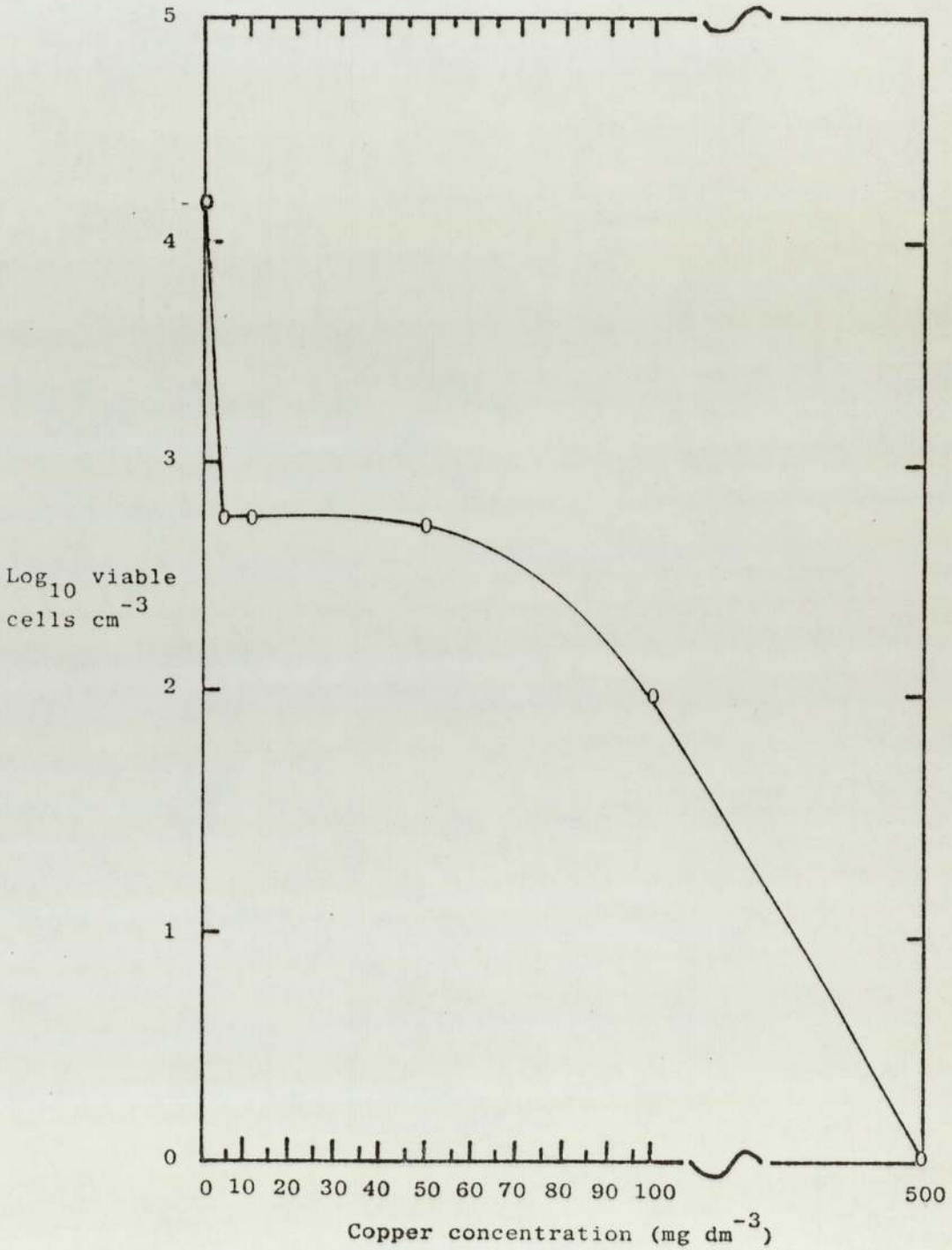


Figure 49: Effects of Cu^{2+} on bacterial isolate 3011G2 (*Pseudomonas* sp.) when grown in sea-water.

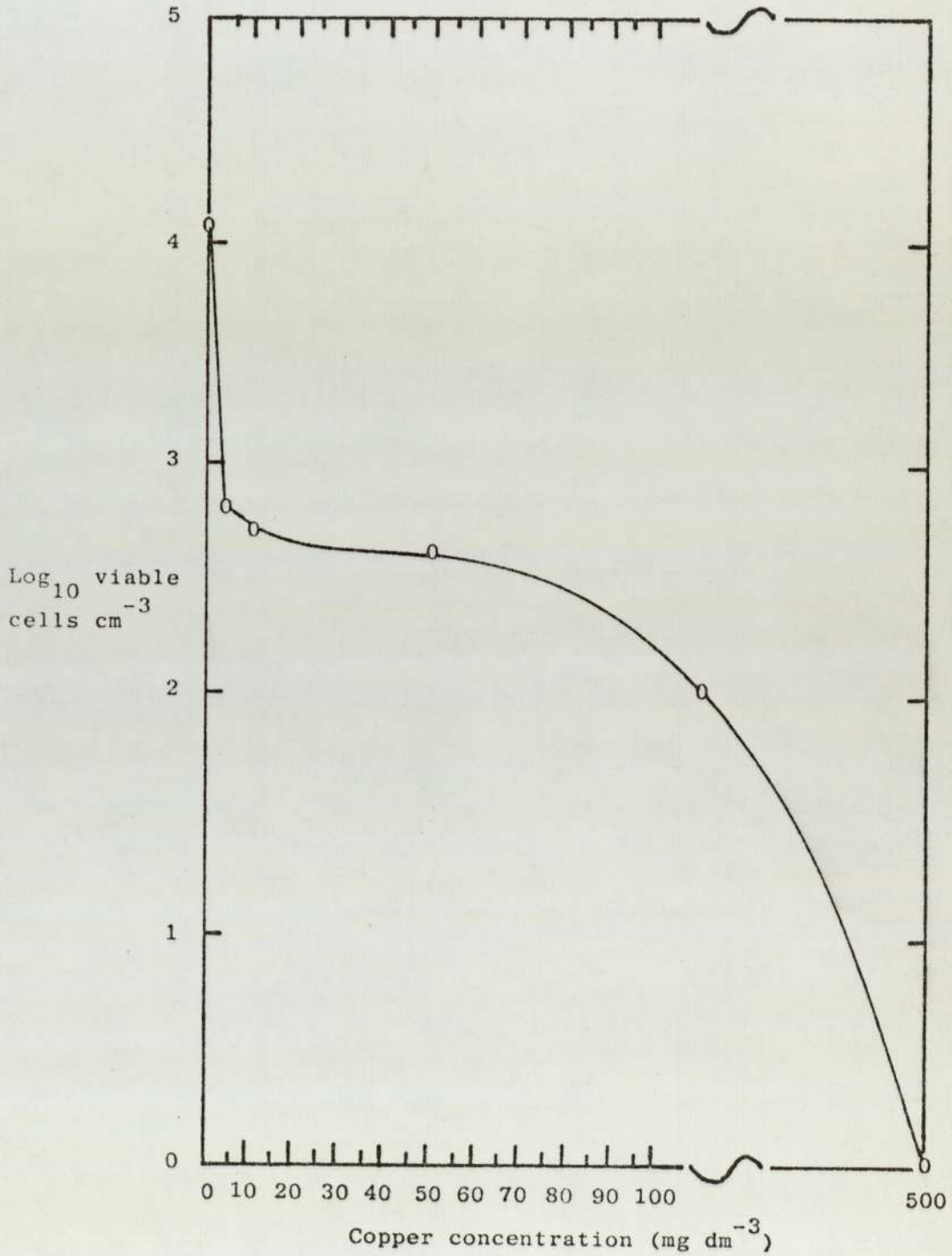


Figure 50: Effects of Cu^{2+} on bacterial isolate 3011G4 (*Pseudomonas* sp.) when grown in sea-water.

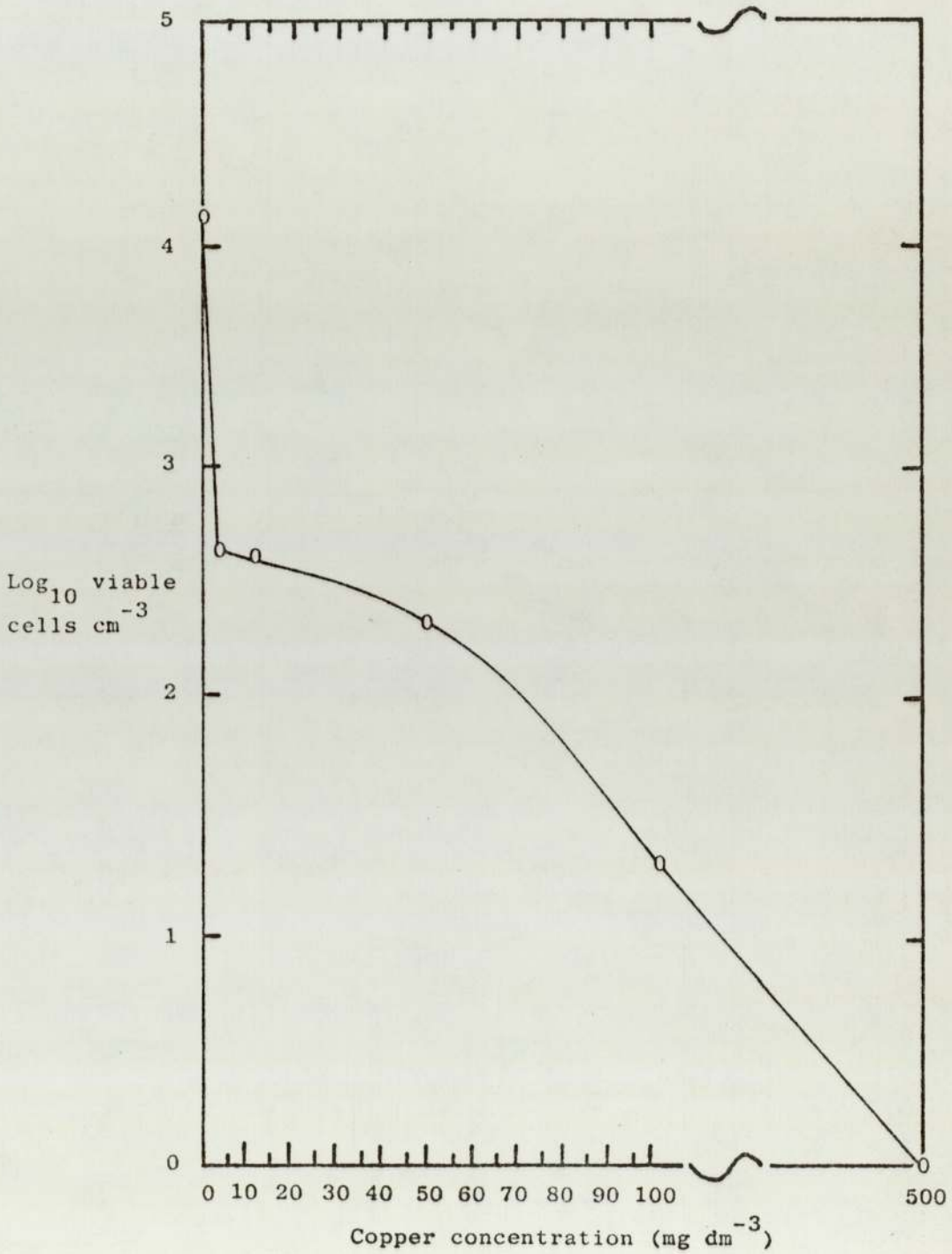


Figure 51: Effects of Cu^{2+} on bacterial isolate 712P2 (*Pseudomonas/Alteromonas* group) when grown in sea-water.

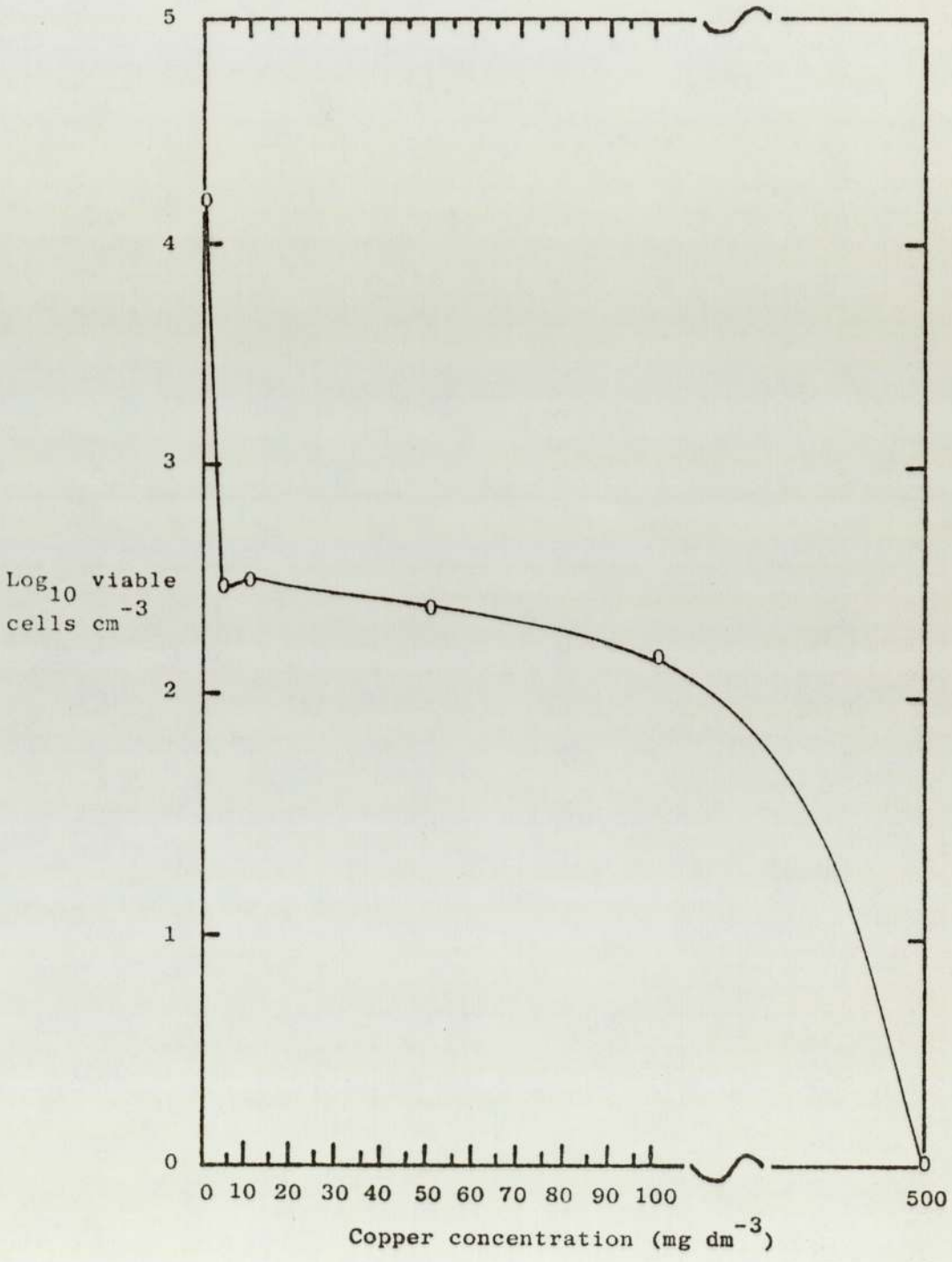


Figure 52: Effects of Cu^{2+} on bacterial isolate 712P4 (*Coryneform* sp.) when grown in sea-water.

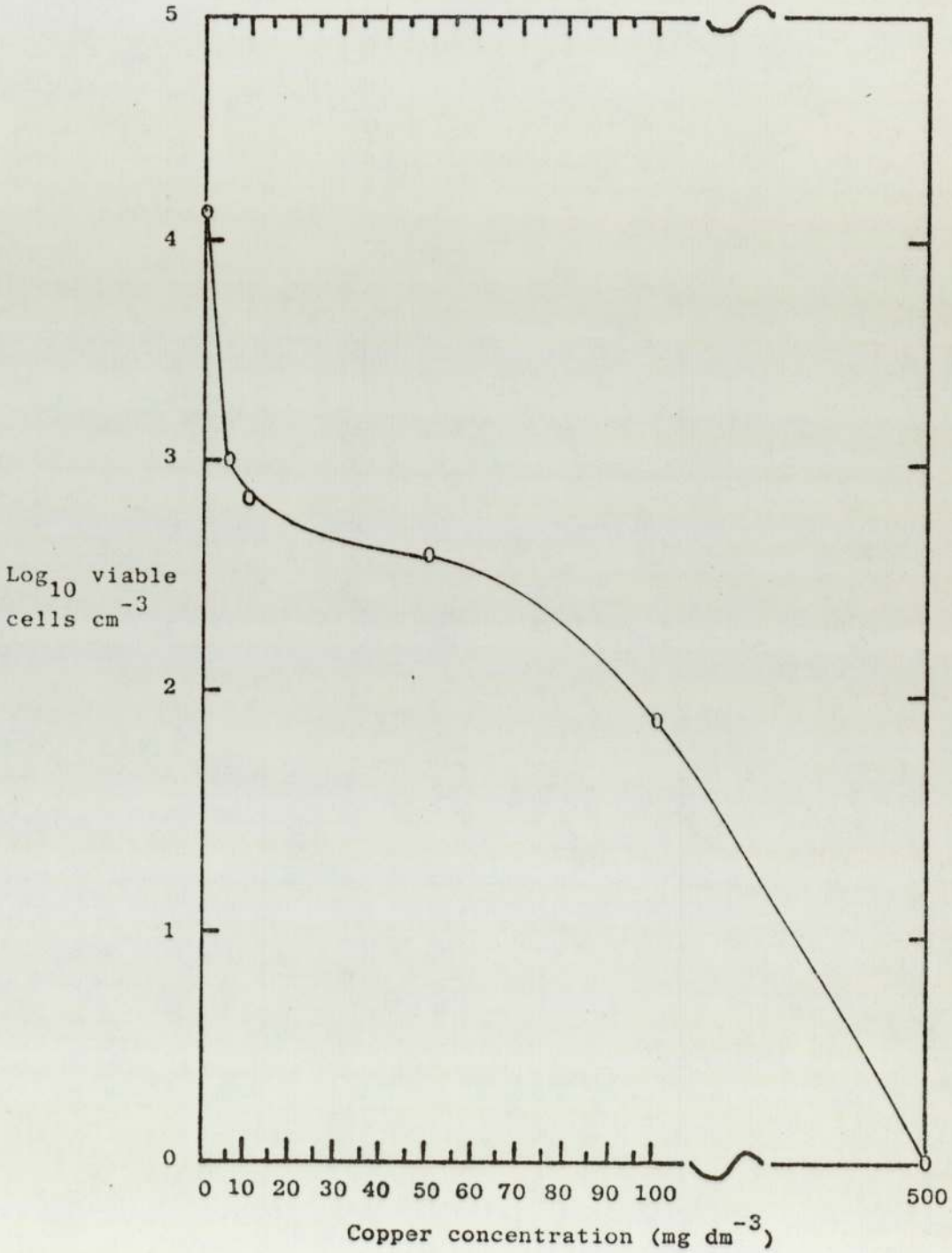


Figure 53: Effects of Cu^{2+} on bacterial isolate 1512RA4 (*Pseudomonas/Alteromonas* group) when grown in sea-water.

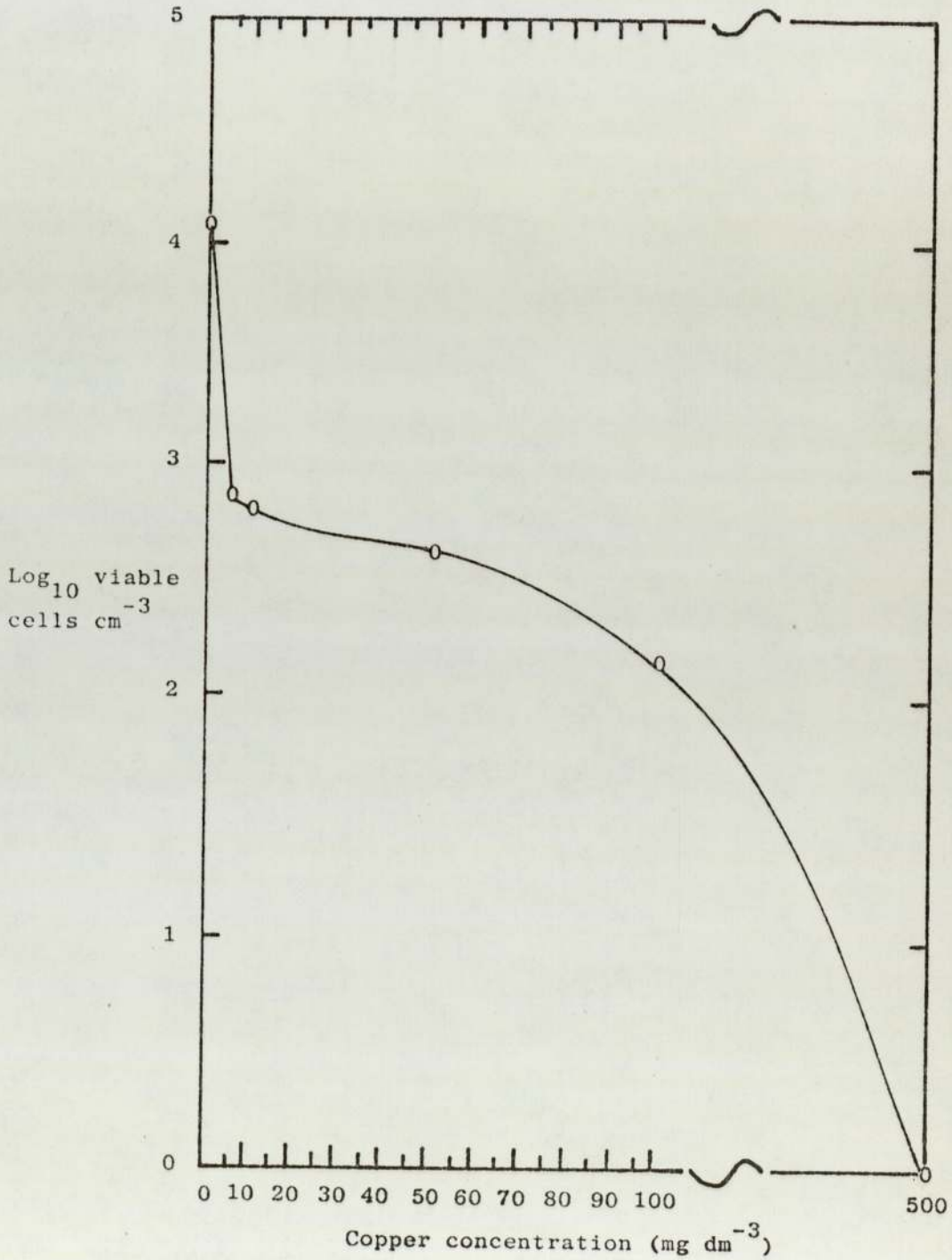
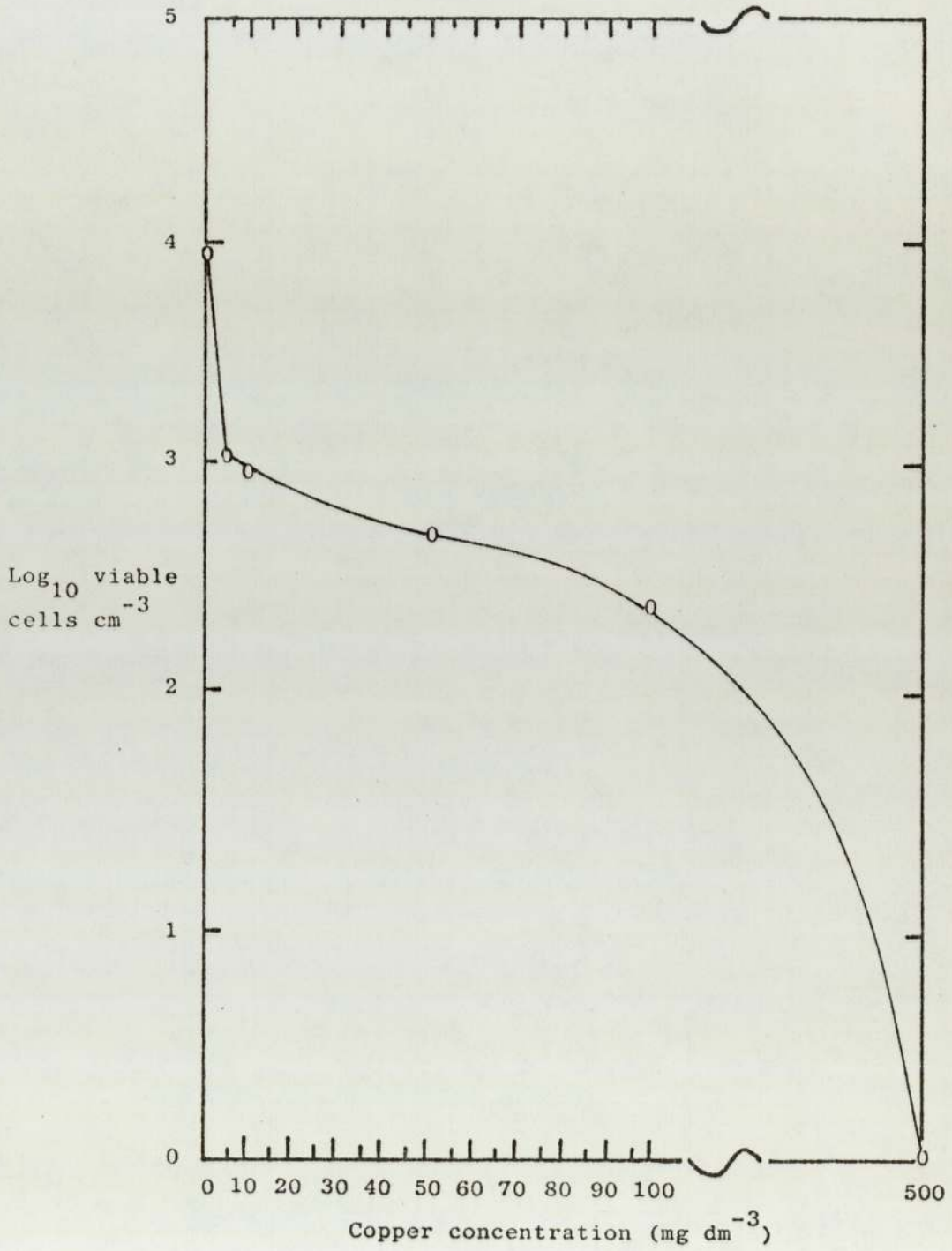


Figure 54: Effects of Cu^{2+} on bacterial isolate 1412SA2 (*Alteromonas* sp.) when grown in sea-water.



to note that this isolate appeared to discourage the settlement of Enteromorpha intestinalis zoospores. If this bacterial isolate is more sensitive to copper ions than the isolates which appear to encourage the settlement of Enteromorpha intestinalis, its effects could be negated by the use of copper-based antifoulant paints.

It appears that, in general, the bacterial isolates tested would be capable of colonising a surface coating with a copper-based antifoulant paint. The presence of a bacterial film could prevent the toxicant action of an antifoulant paint by affecting the leaching rate of copper, or copper chloride. This would be in addition to any effect that the bacterial films themselves might have upon the settlement of fouling organisms.

6.3 Effects of Cu^{2+} Upon the Growth of Selected Bacterial Isolates in Kylin's Modification of Schreiber's Solution (KSM)

Kylin's modification of Schreiber's solution is essentially sea water, with the addition of inorganic nitrogen (0.2g dm^{-3}) and phosphate (0.02g dm^{-3}) (Chapter 2, section 2.5.2).

KSM was used as the growth medium for Enteromorpha intestinalis zoospores in the experiments described in this thesis. Daniel and Chamberlain (1981) investigated the effects of cupric chloride upon marine diatoms from the genera Amphora and Navicula, and found that these diatoms

localised copper within polyphosphate bodies, or specialised "copper bodies".

Irving and Jones (1975) investigated the effects of cupric sulphate upon selected marine bacteria. In these experiments the cupric chloride was added to nutrient agar (Oxoid No. 2, code CM67) made up in sea water, and the results indicated that the minimum inhibitory concentration of cupric sulphate ranged from 274 to 1370mg dm^{-3}, for the unidentified bacterial strains tested. These values are higher than those found in the experiments described in section 6.2, where bacteria were grown in unsupplemented sea water; and considerably higher than the 20ppm Cu^{2+}, considered by Ramamoorthy and Kushner (1975) to be inhibitory to the growth of most microorganisms.

Data from Irving and Jones (1975) suggest that the composition of a medium can significantly increase the amount of Cu^{2+} required to inhibit the growth of marine bacteria. If this is the case, then the choice of medium could be a crucial factor in any experiments involving the interaction of marine bacteria, algal spores and copper compounds. The experiments described in this section were designed to investigate the response of selected marine bacteria to cupric chloride, when grown in KSM.

6.3.1 Materials and Method

The experimental conditions were identical to those

described in section 6.2.1, except that the basal medium was KSM (in place of unsupplemented sea water).

6.3.2 Results and Discussion

The results are presented in Figures 55 to 61, where each datum point represents the mean of five replicate plates. The coefficient of variation for each series of replicate plates was under 5%, this being an acceptable level of precision for colony counts (Jennison and Wadsworth, 1939).

In general the viable count decreases by one log cycle when 5mg dm^{-3} Cu^{2+} is present in the medium, and the results are similar to those seen with unsupplemented sea water.

The two exceptions are bacterial isolate 3011G1 (an unknown genus) and bacterial isolate 3011G4 (a Pseudomonas sp.). In the case of bacterial isolate 3011G1 there is less of a decrease in viable count than that observed with unsupplemented sea water; this could indicate that this isolate is less sensitive to copper when grown in KSM. An alternative explanation is that the additional nitrates and phosphates present in KSM decrease the availability of copper ions, but if this were the case then it could be expected that the other bacterial isolates would show similar results. This did not occur, and the other isolates showed a sensitivity to copper in KSM comparable to that seen in unsupplemented sea water.

Figure 55: Effects of Cu^{2+} on bacterial isolate 3011G1 (genus unknown) when grown in KSM.

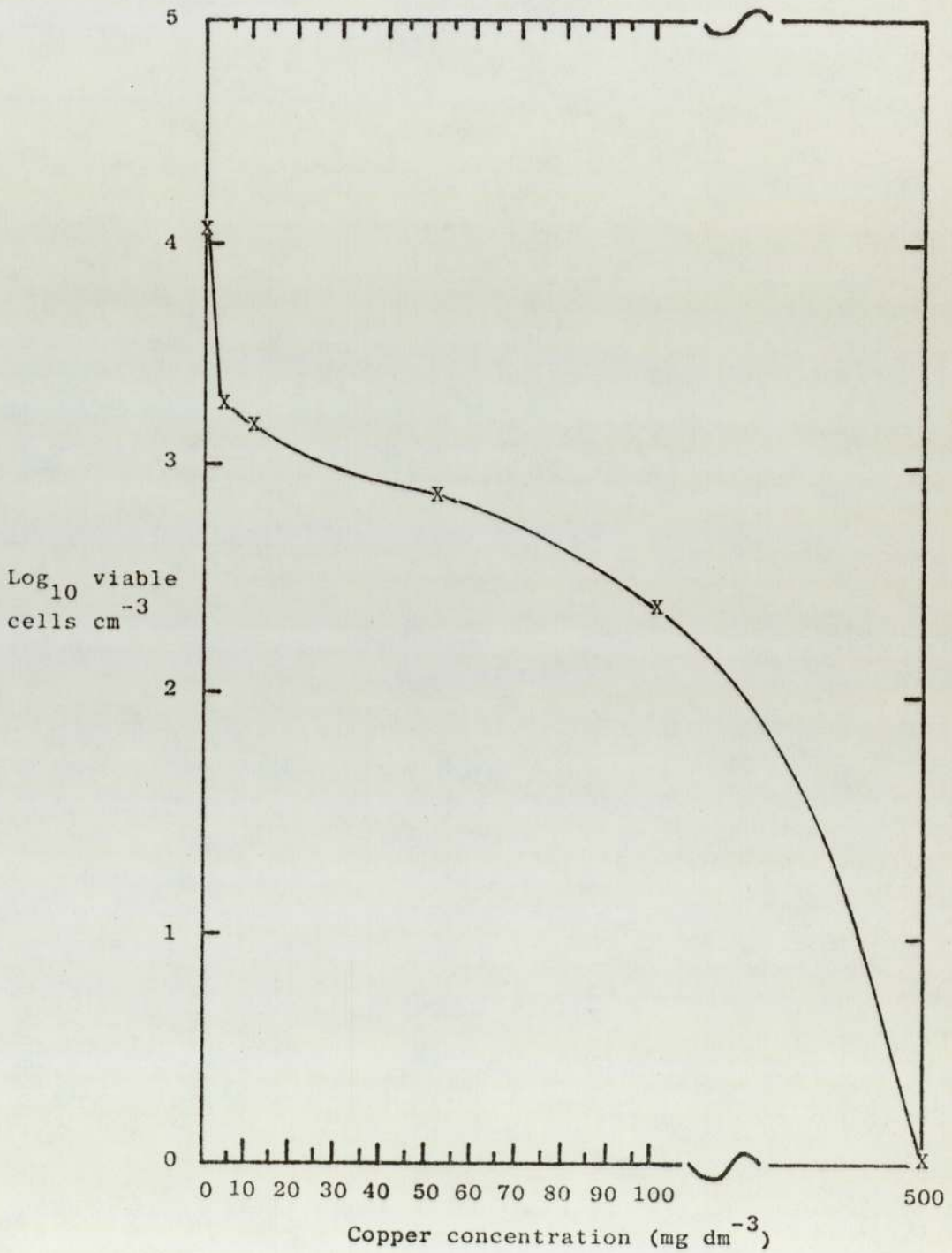


Figure 56: Effects of Cu^{2+} on bacterial isolate 3011G2 (*Pseudomonas* sp.) when grown in KSM.

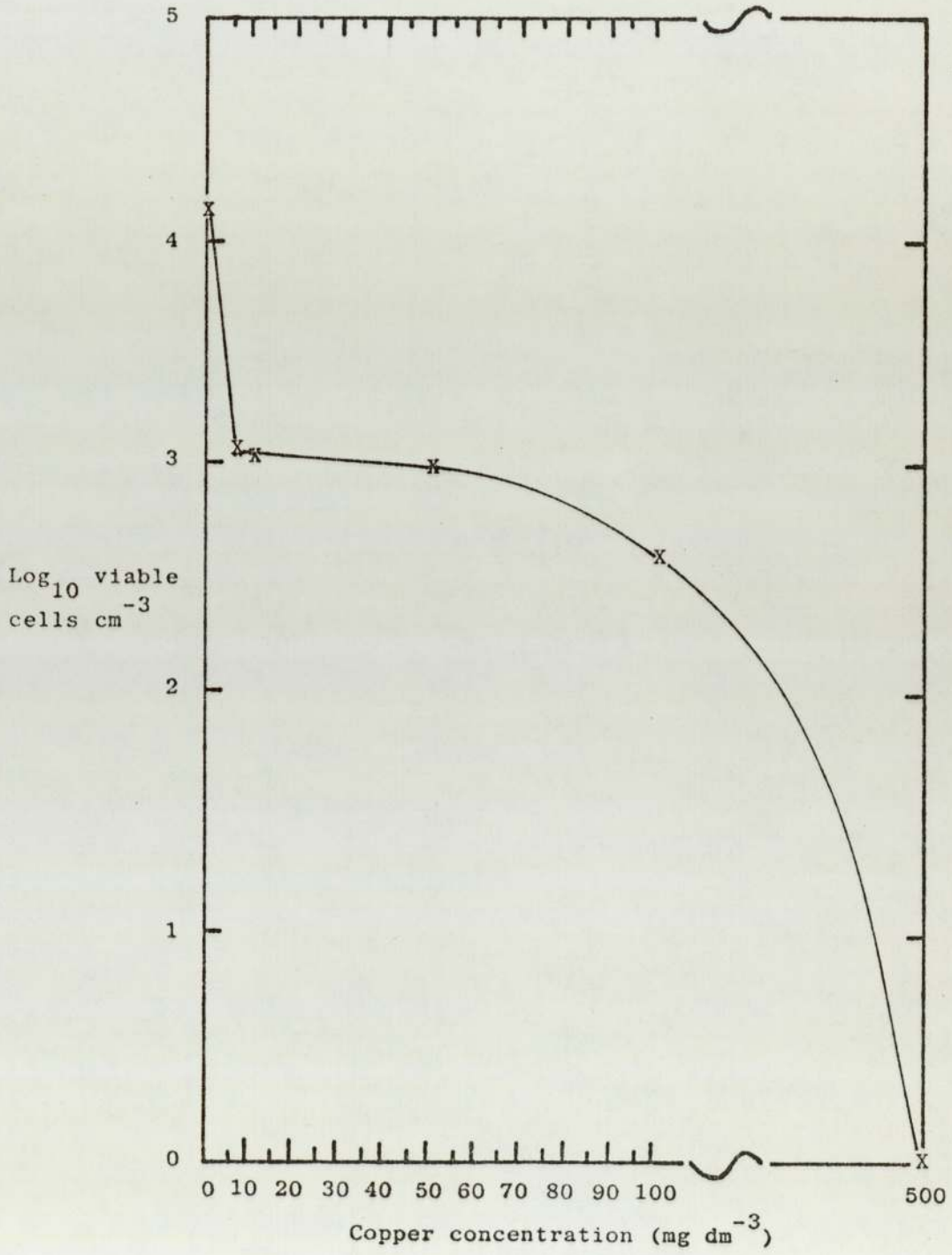


Figure 57: Effects of Cu^{2+} on bacterial isolate 3011G4 (*Pseudomonas* sp.) when grown in KSM.

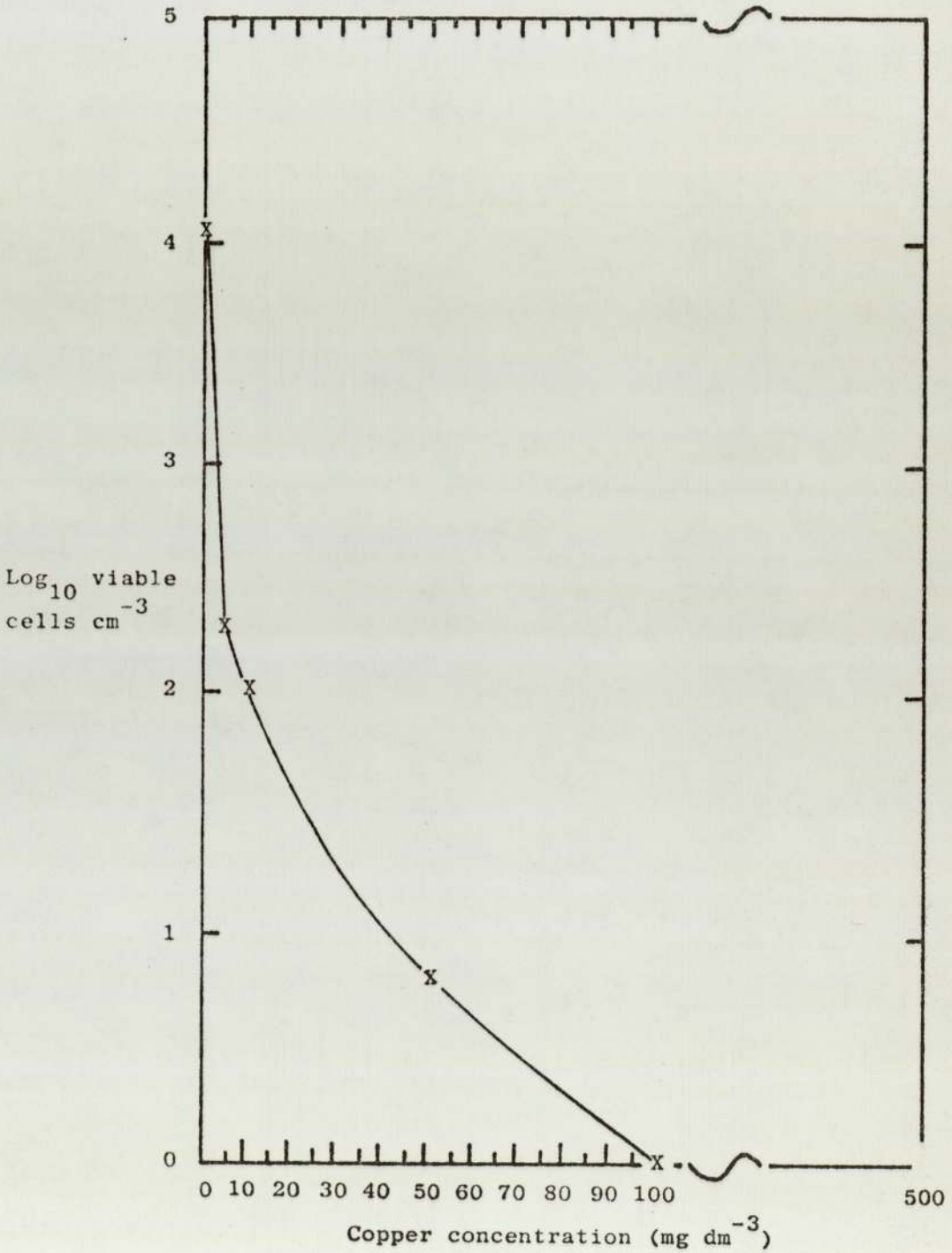


Figure 58: Effects of Cu^{2+} on bacterial isolate 712P2 (*Pseudomonas/Alteromonas* group) when grown in KSM.

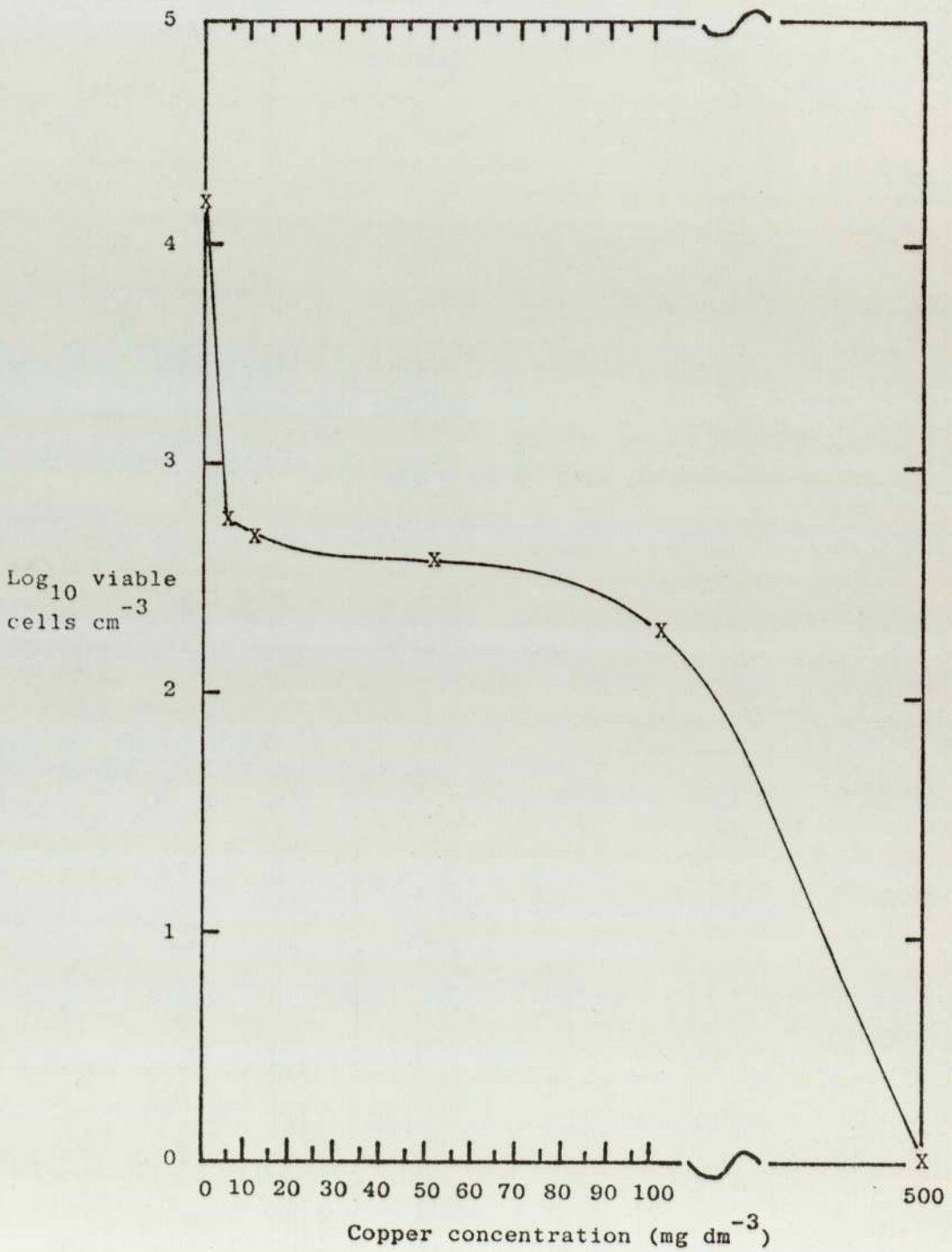


Figure 59: Effects of Cu^{2+} on bacterial isolate 712P4 (*Coryneform* sp.) when grown in KSM

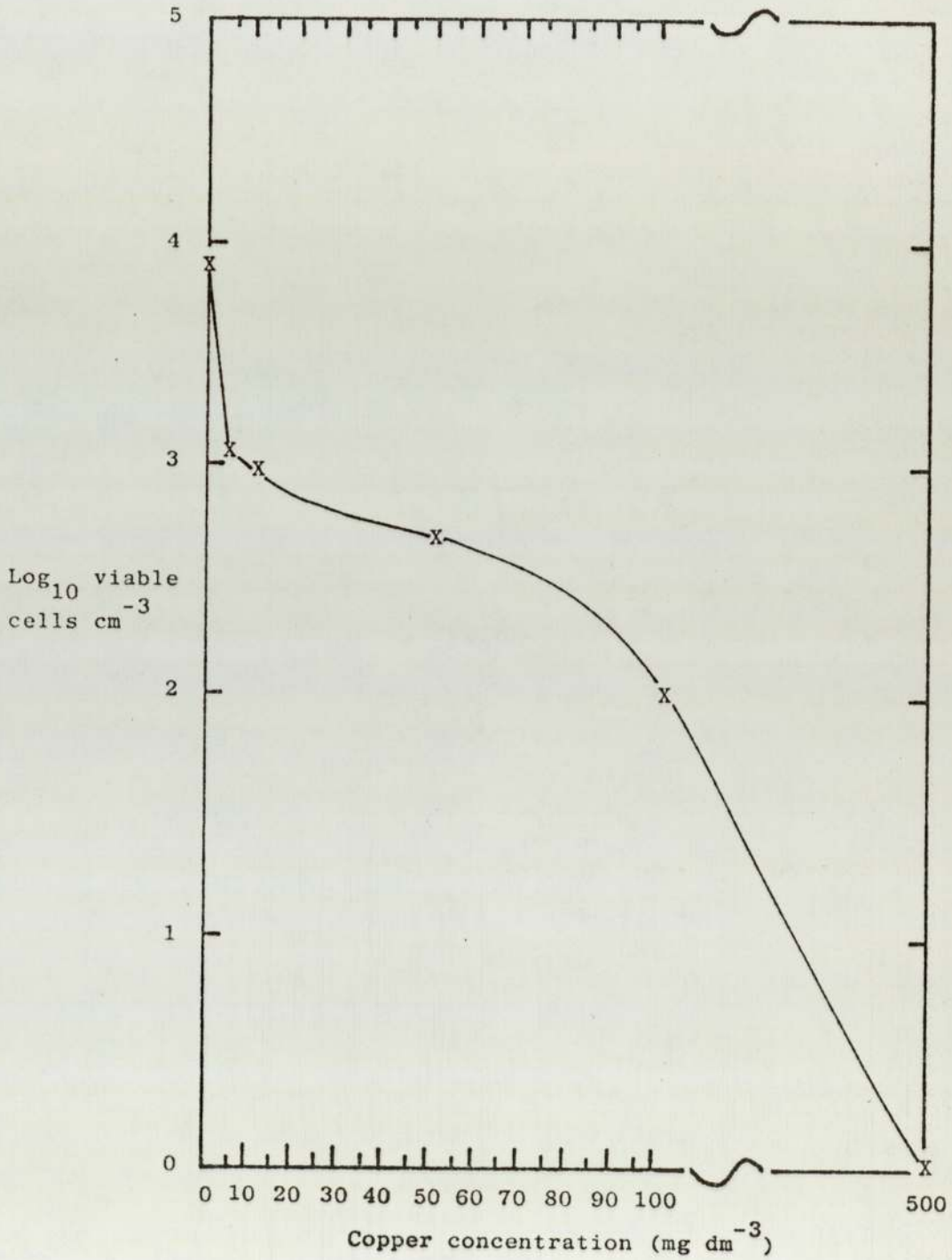


Figure 60: Effects of Cu^{2+} on bacterial isolate 1512RA4 (*Pseudomonas/Alteromonas* group) when grown in KSM.

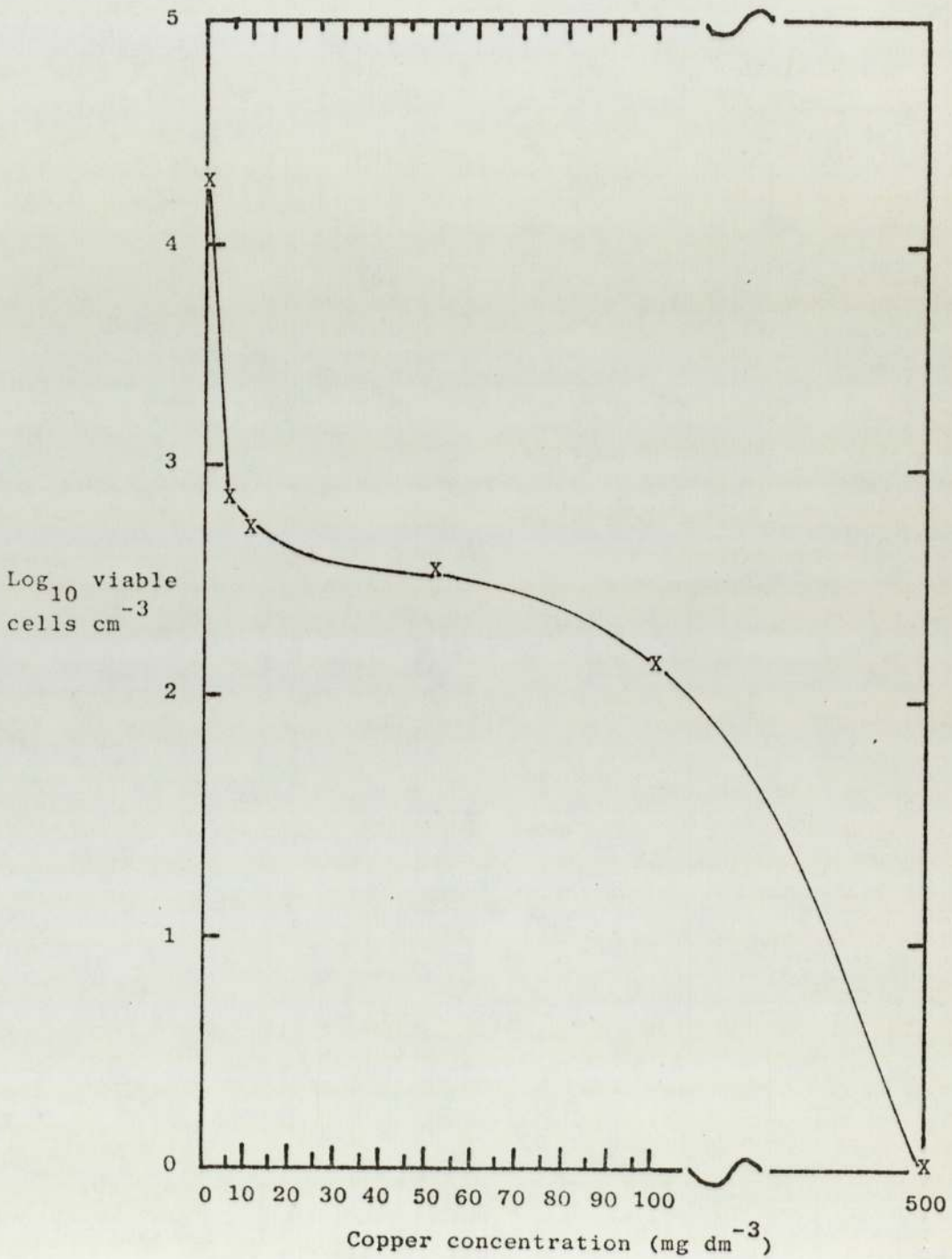
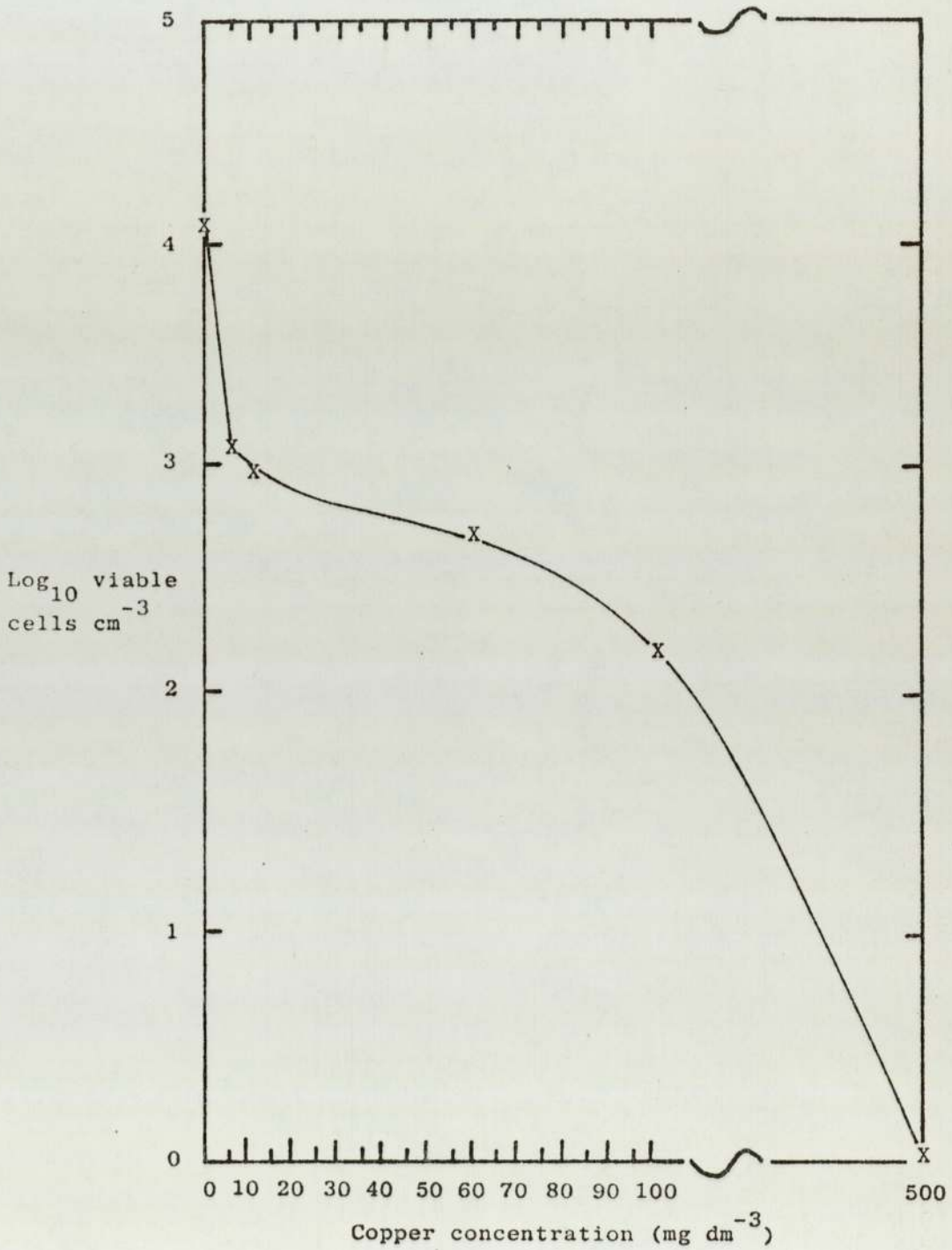


Figure 61: Effects of Cu^{2+} on bacterial isolate 1412SA2 (*Alteromonas* sp.) when grown in KSM.



Bacterial isolate 3011G4 appeared to be more sensitive to Cu^{2+} when grown in KSM, with no viable cells being detected by the plating method used when copper was present in the KSM at levels of 100mg dm^{-3}. Figure 62 shows the effects of copper on bacterial isolate 3011G4, in both sea water and KSM. The greater sensitivity to copper shown by isolate 3011G4 when grown in nitrate and phosphate supplemented sea water could be an important factor if experiments were conducted upon the interaction of this bacterial isolate with algal spores, in the presence of copper compounds.

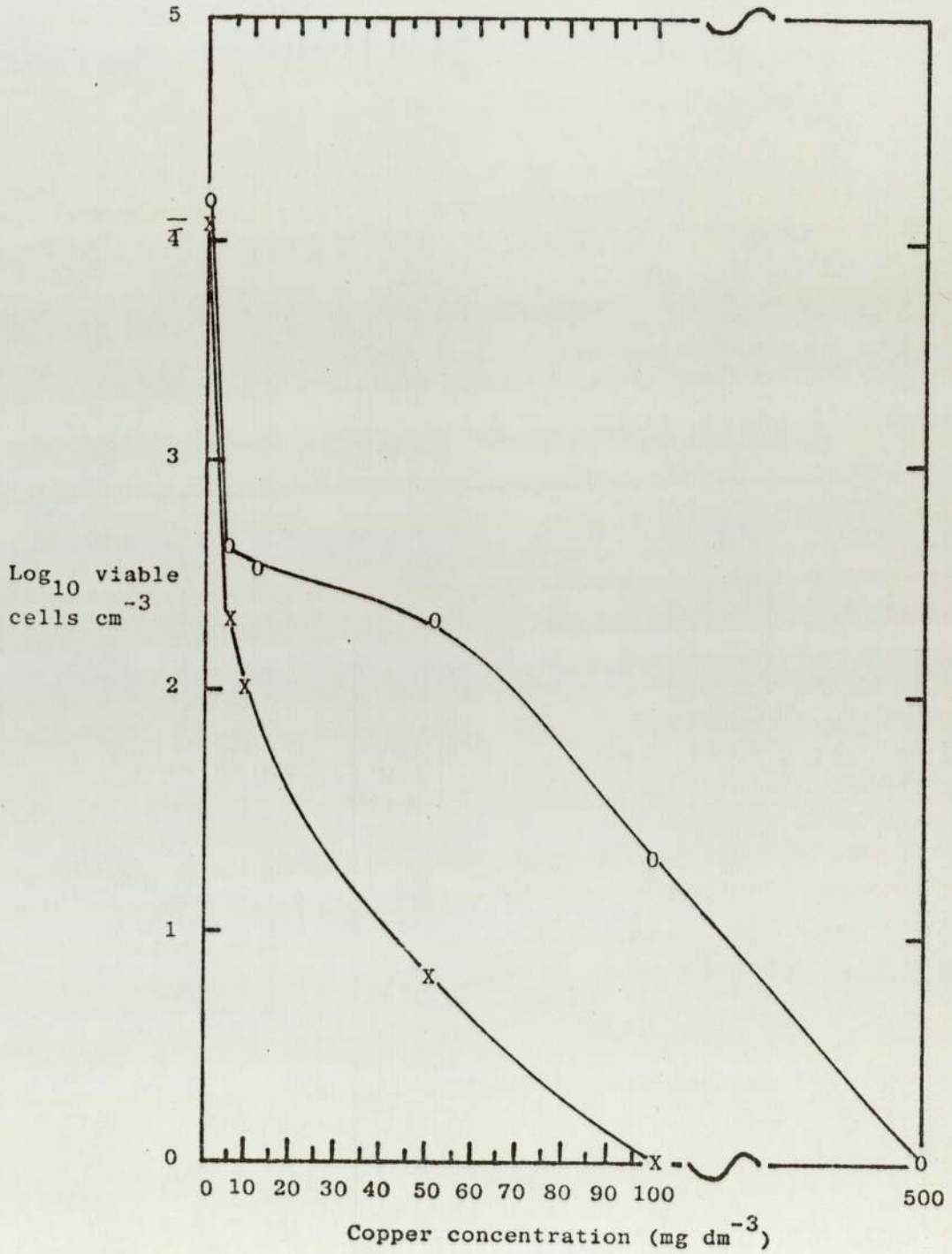
6.4 Chapter Conclusions

The bacterial isolates tested were sensitive to cupric chloride when this salt was present at about 5mg dm^{-3} (with respect to Cu^{2+}). This sensitivity to copper was observed in sea water, and in sea water modified by the addition of 0.2g dm^{-3} of inorganic nitrogen and 0.02g dm^{-3} of phosphate (Kylin's Modification of Schreiber's Solution).

Most of the bacterial isolates tested showed a complete loss of viability when the amount of cupric ion reached 500mg dm^{-3} an exception being bacterial isolate 3011G4 (a *Pseudomonas* sp.) which was killed by 100mg dm^{-3} cupric chloride (with respect to Cu^{2+}).

These results have implications for the control of *Enteromorpha intestinalis*, as the presence of copper compounds in antifouling paints could prevent the growth of bacterial

Figure 62: Effects of Cu^{2+} on bacterial isolate 3011G4 (*Pseudomonas* sp.) when grown in sea-water or in KSM.



isolate 3011G4, which has been shown (see Chapter 4) to discourage the settlement and/or subsequent growth of Enteromorpha intestinalis.

Bacterial isolate 3011G4 showed an increased sensitivity to cupric ions when grown in KSM, and this could be an important factor if this bacterial isolate were to be used in experiments with Enteromorpha intestinalis in the presence of copper compounds.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS, AND AREAS FOR FUTURE WORK

7.1 General Discussion and Conclusions.

7.2 Final Conclusions and Areas for Further Work.

7.1 General Discussion and Conclusions.

Species of the genus Enteromorpha are recognised as commonly encountered fouling organisms (Houghton et al., 1972), with Enteromorpha intestinalis constituting a particular problem (Christie, 1972; Fletcher and Chamberlain, 1975). The economic losses resulting from marine fouling are high (Christie and Hearson, 1968; Gitlitz, 1980), and are particularly high for military vessels (Fisher et al., 1975). In recent years a new area for concern has arisen regarding offshore oil installations (Hardy, 1981), and Enteromorpha spp. have been found to modify iron oxide scales, present on mild steel, with implications for increased corrosion rates (Edyvean and Terry, 1983).

The most widely practiced method of fouling control is the incorporation of toxic components into paint formulations (Saroyan, 1969), but the traditional copper-based antifouling paints are largely ineffective against marine algae (Evans, 1981). Organotin compounds are highly effective in the prevention of animal fouling (Houghton, 1978), and in the prevention of some algal fouling, including fouling by some species of Enteromorpha (Fletcher and Chamberlain, 1975). There is, however, some debate about the environmental impact of organotin compounds, and the hazard to workers involved in the application of antifouling paints containing these compounds. Vizgirda (1972); Smith (1980) and Blinden and Chapman (1982), all state that organotin compounds are unlikely to cause any long-term environmental problems as they are broken down to non-toxic inorganic tin compounds.

Smith (1980) adds that there had been no reports of serious toxic effects among workers who followed the manufacturer's instructions during the application of paints containing organotin compounds. The view that organotin compounds do not present an environmental hazard is not supported by Monaghan et al., (1980), and these workers consider that as organotin compounds can be released ungraded from antifouling paint matrices, they may represent a potential environmental problem. This view is supported by the work of Barug (1981), who found that microbial degradation of bis (tributyltin) oxide did not occur in sea-water when this organotin compound was the only carbon source. Muir (1977) considers organotin compounds to be potentially harmful by skin absorption, and can represent a hazard to workers. Considering these environmental and safety factors, it is desirable to find alternatives to the toxic compounds frequently used as the active ingredients of antifouling paints.

Along with the possible environmental effects and the potential health hazards, an equally, and perhaps more important, consideration from an owner's point of view, is the effectiveness of present-day antifouling coatings in the prevention of marine fouling. Phillip (1972) stated that the practical limit for the effectiveness of copper-based antifouling paints, under normal operating conditions, on a ship's hull, is two years. The contact leaching type of antifouling paint has, according to Evans (1981), an even shorter in-service life of only 15 months. De la Court

(1980) states that a leaching rate of 4µg cm^{-2} day^{-1} of tributyltinfluoride is required to kill barnacle larvae, and 9µg cm^{-2} day^{-1} to kill algal spores. De la Court considers that these leaching rates are a great disadvantage to the formulation of long-life antifouling paints, which are required to bridge the two-and-a-half year interval, that is common between the dry dockings, of a ship for routine maintenance.

This dissatisfaction with the toxicant approach to the control of marine fouling organisms has led to investigations of other methods for the prevention of biofouling. Skinner (1972) discussed the role of the inland laboratory in the study of marine fouling, and concluded that the inland laboratory did have a useful role in such studies. The work described in this thesis was carried out at an inland site, and whilst this presented some problems regarding the maintenance of Enteromorpha intestinalis in a state suitable for zoospore production, these problems were overcome by the construction of a controlled environmental system. The main problem encountered in growing marine algae at a site other than a coastal one, is the provision of an adequate supply of sea-water, maintained in such a way that algal growth and reproduction occurs. In her research, Skinner (1972) did not attempt to keep mature Enteromorpha plants for any extended time, but utilised them for the preparation of a spore suspension after overnight drying of the sexually mature Enteromorpha plants. Whilst this procedure is adequate, when relatively small amounts of algal material are being used, it

is unsatisfactory when a constant supply of fertile material is required. Skinner further suggested that by keeping marine algae in the laboratory, fertile material can be kept in a fertile condition throughout the year; even if the reproductive cycle is normally seasonal.

Van den Hoek et al., (1979) states that the growth of Enteromorpha species is subject to seasonal variations, with an "explosive" period of growth during June and July. Jones and Dent (1970) point out that the use of artificial sea-water medium can lead to the cultured species reacting in a different manner than it would in its natural habitat. The controlled environmental system described in Chapter 2 was based upon a recirculating natural sea-water system, and proved effective in maintaining Enteromorpha intestinalis in a viable state for spore production throughout the year. Fritsch (1961) states that members of the genus Enteromorpha regularly undergo an alternation of generations between a diploid asexual and a haploid sexual generation. Zoospores only occur in species of Enteromorpha which undergo alternation of generations (Bliding, 1964) and originate in diploid plants by meiosis (Bliding, 1964). In Enteromorpha intestinalis zoospores may be distinguished from sexual gametes, as the former are tetra-flagellate, whilst the latter are bi-flagellate (Bliding, 1964). Bliding further states that the diploid plants that produce zoospores may be differentiated from the haploid plants that produce sexual gametes by the colour of the fertile portion of the thalus; haploid male plants having orange-yellow fertile regions,

haploid female plants green-yellow fertile regions and diploid zoospore plants darker greenish-yellow fertile regions (Bliding, 1964). Evans and Christie (1970) state that zoospores show a negative phototactic response, and may be separated from gametes by exposure to light, the zoospores congregating away from the light when tubes containing them were exposed to uni-directional illumination. However Fritsch (1961) states that a negative phototactic response is also seen in gametes immediately after fusion. Fused Enteromorpha gametes (zygotes) appear very much like zoospores in morphology, in that they appear tetra-flagellate because of the fusion of the two bi-flagellate gametes.

The method of Bliding (1964) was used in this study for the selection of zoospore-producing Enteromorpha intestinalis plants, and appeared to work well, as no bi-flagellate gametes were observed when spore suspensions were examined microscopically. Enteromorpha intestinalis zoospores provide a good experimental tool for the study of settlement processes in this species, as by using zoospores, some of the problems encountered with male and female gametes are avoided. Bliding (1964) notes that delayed copulation may occur between different species of Enteromorpha, with the formation of hybrid zygotes. These hybrid zygotes cannot develop into mature plants, and their growth is halted after a few cells have developed. According to Bliding (1964) delayed copulation and the formation of hybrid zygotes has not been observed between Enteromorpha intestinalis and other Enteromorpha species, but it has been observed between

Enteromorpha intestinalis and Ulva lactuca. Since Ulva lactuca inhabits a similar habitat to Enteromorpha intestinalis, small pieces of Ulva lactuca thalus can easily be present in samples of Enteromorpha intestinalis collected from the intertidal zone.

Delayed copulation and hybrid zygote formation, with the resultant inability of the hybrid plants to develop, could have led to erroneous results, in that the failure of Enteromorpha intestinalis zygotes to develop could have been ascribed mistakenly to the bacterial film under investigation.

This problem was overcome by using zoospore plants, as Enteromorpha intestinalis zoospores germinate directly into male or female plants, without zygote formation (Bliding, 1964). The use of opaque attachment substrates was ruled out, due to the technical difficulties inherent in the enumeration of organisms on opaque surfaces. When opaque attachment substrates are used, epifluorescence microscopy is the best method for the enumeration of viable organisms (Duddridge, 1981), but this requires a substantial amount of preparative work, and there is the risk that the staining procedure could produce errors due to the loss of Enteromorpha intestinalis germlings during the staining procedure. Apart from this consideration the use of glass attachment substrates allowed Enteromorpha intestinalis germling development to be recorded photographically.

Experiments were conducted to select a suitable medium,

and Kylin's modification of Schreiber's solution (Bliding, 1964) was selected. This choice was based upon the good results obtained when Enteromorpha intestinalis plants were grown in this medium, and the hypothesis that this minimally fortified medium would allow the growth of Enteromorpha intestinalis to occur under conditions similar to those found in its natural habitat.

The bacteria used in this study were selected from a culture collection of marine bacteria, isolated by Carson between December, 1976 and July, 1978 (Carson, 1980). Seventeen of these bacterial isolates were chosen for the production of bacterial films on glass attachment substrates. The growth curves presented in Chapter 3 (Figures 3 to 19) show that, in general, the bacteria reached the plateau phase of growth within two hours of adding a 10^7 inoculum to K S M. The increase in cell numbers did not exceed two log cycles, which suggests some limiting factor to bacterial growth in this medium, probably the low level of available carbon in the minimally fortified KSM.

In the experiments described in Chapter 4, Enteromorpha intestinalis zoospores were inoculated onto thin films of the seventeen bacterial isolates shown in Table 5 (Chapter 3, section 3.2), and the numbers of Enteromorpha intestinalis germlings counted by optical microscopy. Two growth forms of Enteromorpha intestinalis germlings were observed, single filaments and multi-filament, rhizoidal, clumps. Multi-filament clumps have been observed in Enteromorpha linza

by Fries (1975), who considered that this was the result of growth in axenic culture. It is possible that the minimally enriched KSM in which the germlings were grown could have lacked the co-factors for normal growth, such as vitamins, but the germlings were not in axenic culture and the medium was similar to the sea-water in which Enteromorpha intestinalis normally grows. An alternative explanation for the formation of multi-filament clumps is that there is a general tendency for Enteromorpha intestinalis zoospores to aggregate. Elliott (1971) states that the contagious (clumped) distributions are frequently seen in nature, and it is possible that settled Enteromorpha intestinalis zoospores secrete a chemical which attracts other zoospores, resulting in the formation of clumps of Enteromorpha intestinalis germlings.

Mitchell (1972) cites the chemical sperm attractant, produced by fungi of the genus Allomyces, the aggregation of amoeboid cells in species of the slime mould Dictyostelium mediated by cyclic adenosine monophosphate and the 'female' substance produced by species of the green alga, Volvox, which induces gonidia to develop into sperm packets.

With regard to the interaction between Enteromorpha intestinalis zoospores and bacterial films, the results indicate that 10 of the bacterial isolates had no effect upon the settlement and/or subsequent growth of Enteromorpha intestinalis zoospores, when compared with Enteromorpha intestinalis germlings settled on glass attachment substrates

not pre-treated by the growth of bacterial films.

Significantly more Enteromorpha intestinalis zoospores settled on thin films of bacterial isolate 3011G1 (genus unknown), when compared with glass attachment substrates not pre-treated with a bacterial film, and this was observed for all growth forms of Enteromorpha intestinalis (filaments, clumps and filaments plus clumps). Bacterial isolates 3011G2 (a Pseudomonas species), 1512RA4 (a member of the Pseudomonas/ Alteromonas group) and 1412SA2 (an Alteromonas species) also encouraged zoospore settlement.

Three bacterial isolates discouraged the settlement of Enteromorpha intestinalis zoospores, these were bacterial isolates 3011G4 (a Pseudomonas species), 712P2 (a member of the Pseudomonas/Alteromonas group) and 712P4 (a Coryneform species). Neither the type of attachment substrate from which the bacteria were originally isolated, nor the time of isolation, seemed to influence the ability of an isolate to encourage, discourage or have no effect upon, the settlement of Enteromorpha intestinalis zoospores.

When frequency distributions were drawn for the distribution of Enteromorpha intestinalis germlings settled on their attachment substrates, the distributions were positively skewed, with a large number of fields containing no germlings, this suggests a contagious distribution (Elliott, 1971), but did not conform to the most common contagious distribution, the negative binomial (Elliott, 1971).

From the experiments described in Chapter 5, it appears that the factor(s) influencing the settlement of Enteromorpha intestinalis zoospores resides in the bacterial cell wall or the extracellular polymers produced by these film-forming bacteria. Kirchman and Mitchell (1983), suggest that a lectin binding mechanism occurs in larvae of Janua (Dexiospira) brasiliensis, in which the larvae have lectins on their surfaces that bind to the carbohydrate moieties of bacterial extracellular polymers. Lectins are widely distributed in the plant kingdom (Lis and Sharon, 1973), and a similar mechanism could operate in Enteromorpha intestinalis.

The use of copper compounds in antifouling paints has been discussed in the opening paragraphs of this chapter. Copper is an essential trace element in man (Prasad, 1979), and its mamalian toxicity is low (Todd, 1967). It is, however, highly toxic to a variety of marine and fresh water organisms, including crustacea and mollusca (Scott and Major, 1972); fish (Cairns et al., 1981); rotifers (Buikema et al., 1977); diatoms (Daniel and Chamberlain, 1981) and unicellular algae (Wong and Beaver, 1980). Many species of algae are, however, resistant to the effects of currous compounds, and only respond to cupric ions (Sunda and Guillard, 1976; Anderson and Morel, 1978; Jackson and Morgan, 1978; Morel et al., 1978). Gavis et al., (1981), suggests that cupric ions act as a toxin by binding to the cell surface and reducing the negative surface charge of the cell. The principle copper compound employed in antifouling paints is cuprous oxide (Kronstine, 1975), which dissolves in sea-

water to form cuprous chloride (De la Court and De Vries, 1973). The insensitivity of marine fouling algae to copper-based antifouling paints can be explained by the relatively low toxicity of cuprous salts to algae. The resistance to copper compounds is not uniform within a genus, and copper sensitive and copper tolerant strains of Enteromorpha (Goodman et al., 1976) and Ectocarpus (Russell and Morris, 1970) are known. Hall et al., (1979) provides evidence that an exclusion mechanism is operating in Ectocarpus siliculosus, in which changes in the cell membrane, and intracellular changes, lead to copper tolerance. In a later paper, Hall (1981) suggests that copper immobilisation in Ectocarpus siliculosus may be the underlying mechanism, rather than changes in enzyme systems.

Of the bacteria used in this study which were found to have a significant effect upon the settlement of Enteromorpha intestinalis, all the bacterial isolates were reduced in number by one log cycle (90%) when grown in sea-water or KSM containing copper in a concentration of 5mg dm^{-3} (copper present as the cupric, Cu^{2+}, ion). It is not surprising that all the bacteria strains investigated showed a resistance to copper, as resistance to heavy metals is known to be carried on plasmids (Olson et al., 1979) and these plasmids can be transferred between bacteria, especially those of the genus Pseudomonas and related genera (Hayakawa et al., 1975). Baldry and Dean (1980) grew bacteria in a medium containing 5mg dm^{-3}, and found that the uptake of copper was between 0.2% and 0.5% (of the dry-cell weight), and suggests that an

exclusion mechanism operates in the strains studied, including strains of Pseudomonas.

The commonly achieved leaching rate from copper based antifouling paints, as stated by Mesich and Huff (1973), of 10 g $cm^{-2}day^{-1}$ is much lower than 5mg dm^{-3}, that was toxic to the bacteria used in the experiments described in this thesis. If these bacterial isolates are representative of the bacteria which colonise marine antifouling paints, then it is probable that bacterial growth is unaffected by the level of copper released by conventional antifouling paints.

Bacterial isolate 3011G4 (a Pseudomonas species), which discouraged the settlement of Enteromorpha intestinalis zoospores, was particularly sensitive to copper. This could have implications for the control of Enteromorpha intestinalis as copper-based antifouling paints could prevent the growth of this bacterial strain, thus negating any beneficial effect which this bacteria could produce by discouraging the settlement of Enteromorpha intestinalis.

7.2 Final Conclusions and Areas for Further Work

The work presented in this thesis indicates that marine bacteria have an effect upon the settlement and/or subsequent growth of Enteromorpha intestinalis. Some bacterial isolates were shown to encourage the settlement of Enteromorpha intestinalis, whilst other bacterial isolates were shown to discourage the settlement of this alga.

The experiments described in Chapter 5 suggest that the bacterial factor that effects the settlement of Enteromorpha intestinalis resides in the bacterial cell wall, or the extracellular polymer produced by these bacterial isolates. Cell-free bacterial extracts had little effect upon the incidence of settlement of Enteromorpha intestinalis, and this indicates that inhibiting the metabolism of primary film-forming bacteria may have little effect upon the settlement of Enteromorpha intestinalis.

Cupric ions were shown to be toxic to many of the bacterial isolates used in this study, but only at levels that are not commonly achieved with present-day antifouling paints. A bacterial isolate which discouraged the settlement of Enteromorpha intestinalis proved to be particularly sensitive to the effects of cupric ions, and it is suggested that the presence of copper salts could discourage the growth of this organism.

Although this work has shown that marine bacteria do effect the settlement of Enteromorpha intestinalis, there are several areas for further work to determine the usefulness of bacteria in retarding the growth of Enteromorpha intestinalis. Future areas of research could be:

i) The isolation of bacteria from ship's hulls, and the assessment of such bacteria for their effects upon the growth of Enteromorpha spp.

ii) The investigation of the precise mechanism by which bacteria influence the settlement of

Enteromorpha intestinalis. This could involve a study of lectin-mediated bacterial/algal interactions.

iii) An assessment of the effects of antifouling paint formulations on the interaction between Enteromorpha intestinalis and marine bacteria.

Should these areas of research be pursued it is possible that an alternative to the present toxic methods of control of Enteromorpha spp. could be developed.

APPENDIX 1

STATISTICAL METHODS

Arithmetic mean, $\bar{x}$ (Elliott, 1971):-

$$\bar{x} = \sum \frac{x}{n}$$

Variance, s^2 (Elliott, 1971):-

$$s^2 = \frac{\sum(x^2) - \bar{x} . \sum x}{n - 1}$$

Standard Deviation, s (Elliott, 1971):-

$$s = \sqrt{s^2}$$

Coefficient of Variation, cv (Elliott, 1971):-

$$cv = s . \frac{100}{\bar{x}}$$

Calculation of 95% Confidence Limits for a Small Sample ($n < 30$) from a Poisson Series (Elliott, 1971):-

$$\bar{x} - t . \sqrt{\frac{\bar{x}}{n}} \quad \text{to} \quad \bar{x} + t . \sqrt{\frac{\bar{x}}{n}}$$

with n - 1 degrees of freedom.

Chi-squared Test (Variance to Mean Ratio) for Agreement with a Poisson Series; Small Sample ($n < 31$), (Elliott, 1971):-

$$\chi^2 = \frac{(s^2 . n - 1)}{\bar{x}}$$

with n - 1 degrees of freedom.

Statistic U, the Difference Between the Sample Estimate of Variance and the Expected Variance in a Negative Binomial Distribution, (Elliott, 1971):-

The negative binomial distribution has two parameters, the arithmetic mean ($\bar{x}$) and an exponent function, k. A rough estimate of k was obtained as follows:-

$$\hat{k} = \frac{\bar{x}^2 - (s^2 / n)}{s^2 - \bar{x}}$$

This rough estimate of k was substituted into the maximum likelihood equation (Elliott, 1971), and the equation solved by iteration, to produce a better estimation of k:-

$$n \, \mathrm{Log}_e \left(1 + \frac{\bar{x}}{\hat{k}}\right) = \sum \frac{A(x)}{\hat{k} + \bar{x}}$$

The statistic U could then be calculated from the equation:-

$$U = s^2 - \left(\bar{x} + \frac{\bar{x}^2}{\hat{k}}\right)$$

The statistic U has an expected value of zero for perfect agreement with a negative binomial, but agreement is accepted if the value of U differs from zero by less than its standard error.

The standard error of U was calculated by reference to Figure 10 in Elliott, 1971.

Mann-Whitney U-test, (Elliott, 1971):-

As the non-parametric alternative to the t-test, the Mann-Whitney U-test has a power-efficiency of not less than 86%,

and can be between 90% and 96% for normally distributed data. The Mann-Whitney U-test is a test of rank order, in which the counts are replaced by rank values in a single sequence. The following procedure was adopted:-

1) The counts (sample units) in the experimental and control groups were arranged in single array from lowest to highest.

2) A rank was substituted for each count. If any counts were equal, they were given the average of tied ranks.

3) The ranks were totalled for the experimental (R_1) and control groups (R_2), a check was made to ensure that:-

$$R_1 + R_2 = \frac{(n_1+n_2).(n_1+n_2+1)}{2}$$

(where n_1 and n_2 = number of sampling units in experiment and control groups respectively).

4) The test statistics, U_1 and U_2 were then calculated:-

$$U_1 = n_1.n_2 + \frac{n_2.(n_2+1)}{2} - R_2$$

$$U_1 = n_1.n_2 + \frac{n_1.(n_1+1)}{2} - R_1$$

5) The smaller of the two values, U_1 and U_2, was selected, and reference made to tables of Mann-Whitney U (Pearson and Hartley, 1966). If the calculated value of Mann-Whitney U was equal to, or less than, the tabulated value of the Mann-Whitney U statistic at an appropriate level of

significance (ie. P = 0.05; P = 0.02; P = 0.01 for a two-tailed test), then there was a significant difference between the experimental and the control groups.

t-Test to Test the Differences Between the Means of Two Small Samples - Population Variances not Assumed to be Equal, (Parker, 1979):-

$$T = \frac{\bar{x}_1 - \bar{x}_2}{(s_1^2/n_1 + s_2^2/n_2)}$$

With f degrees of freedom, where f is given by:-

$$1/f = \frac{u^2}{(n_1 - 1) + (1 - u)^2/(n_2 - 1)}$$

and u by:-

$$\frac{s_1^2/n_1}{(s_1^2/n_1) + (s_2^2/n_2)}$$

If the calculated value of t is greater than the tabulated value of t, then the results are significant at a given probability level (ie. P = 0.05; P = 0.02; P = 0.01; P = 0.001 for a two-tailed test).

Experimental and control groups were compared by calculating t for each bacteria isolate and its matched control, and referring to tabulated values of t in Pearson and Hartley, 1966.

SYMBOLS AND TERMS USED IN STATISTICAL METHODS

Mathematical Symbols

$<$	Less than
$\wedge$	"Hat"; indicates an estimate of a term
+	Plus
-	Minus
/ or ——	Divided by
.	Multiplied by
=	Equal to
$\sqrt{}$	Square root
( )	Brackets.

Greek Symbols

Σ	Sigma; sum of
χ^2	Chi-squared.

Latin Symbols

A	Total sample area
cv	Coefficient of variation
f	degrees of freedom
1/f	Reciprocal of f
k	Estimate of exponent in negative binomial equations
Log_e	Natural, Napierian, logarithms
n	Total number of sampling units
n_1	Total number of sampling units in experimental group
n_2	Total number of sampling units in control group
P	Probability or level of significance

R_1	Sum of ranks in experimental group
R_2	Sum of ranks in control group
s	Standard deviation of sample
s_1^2	Variance of experimental group
s_2^2	Variance of control group
t	Student's t-statistic
U	Difference between observed and expected variance in negative binomial
U	Test statistic in Mann-Whitney U-test
U_1	Mann-Whitney U-statistic for experimental group
U_2	Mann-Whitney U-statistic for control group
u	Population mean
x	Sampling unit
$\bar{x}$	Arithmetic mean
$\bar{x}_1$	Arithmetic mean of experimental group
$\bar{x}_2$	Arithmetic mean of control group.

REFERENCES

ACOCK, G.P. (1966). The protection of ships' bottoms - A Review. British Corrosion Journal, 1 : 129-133.

ANDERSON, D.M. and MOREL, F.M.M. (1978). Copper sensitivity of Gonyaulax tamarensis. Limnology and Oceanography, 23 : 283-295.

APPELBEE, J.F. and KINGSTON, P.F. (1981). The development and trials of an antifouling system involving the controlled release of copper. Proceedings of the 13th Offshore Technology Conference, 4 : 385-389.

BAINBRIDGE, R., EVANS, C.G. and RACKMAN, O. (1965). Light as an Ecological Factor. Oxford University Press, 452 pp.

BALDRY, M.G.C. and DEAN, A.C.R. (1980). Copper accumulation by bacteria, moulds and yeasts. Microbios, 29 : 7-14.

BANFIELD, T.A. (1974). Paint technology in the marine environment part 2. Paint Manufacture, 44: (6) : 22-25.

BARUG, D. (1981). Microbial degradation of bis (tributyltin) oxide. Chemosphere, 10 : (10) : 1145-1154.

BERK, S.G., MITCHELL, R., BOBBIE, R.J., NICKELS, J.S. and WHITE, D.C. (1981). Microfouling on metal surfaces exposed to seawater. International Biodeterioration Bulletin, 17 : (2) : 29-37 (addendum in I.B.B., 17 : (4) : 141).

BLANKLEY, W.F. (1973). Toxic and inhibitory materials associated with culturing. A Handbook of Psychological Methods: Culture Methods and Growth Measurements Chapter 14. (Ed. Stein, J.R.). Cambridge University Press.

BLIDING, C. (1964). A critical survey of European taxa in Ulvales. Part 1. Capsosiphon, Percursaria, Blidingia, Enteromorpha. Opera Botanica 8 : (3) : 159pp.

BLUNDEN, S.J. and CHAPMAN, A.H. (1982). The environmental degradation of organotin compounds - A review. Environmental Technology Letters, 3 : 267-272.

BONOTTO, S. (1976). Cultivation of plants. 4.2. Multicellular plants. Marine Ecology, Volume III, Cultivation, part 1 (Ed. Kinne, O.). Chichester, John Wiley and Sons, 467-529

BUIKEMA, A.L., SEE, C.L. and CAIRNS, J. Jr. (1977). Rotifer sensitivity to combinations of inorganic water pollutants. Virginia Water Resources Research Center, Bulletin 92. Virginia Polytechnic Institute and State University Blacksburg, Virginia 24060, U.S.A., 44 pp.

CAIRNS, J. Jr., THOMPSON, K.W. and HENDRICKS, A.C. (1981). Effects of fluctuating, sublethal applications of heavy metal solutions upon the gill ventilatory response of Bluegills (Lepomis macrochirus). United States Environmental Protection Agency, EPA-600/S3-81-003, 4 pp.

CALLOW, M.E., EVANS, L.V. and CHRISTIE, A.O. (1976). The biology of slime films. Part 2. Shipping World and Shipbuilder, November 1976 : 949-951.

CARMIGNANI, G.M. and BENNETT, J.P. (1976). Leaching of plastics used in closed aquaculture systems. Aquaculture, 7 : 89-91.

CARSON, J. (1980). The Microbiological Aspects of Marine Fouling. Thesis submitted to the University of Aston, March 1980, 210 pp.

CARSON, J. and ALLSOPP, D. (1983). Composition of fouling bacterial films on submerged materials. Biodeterioration 5. (Eds. Oxley, T.A., Barry, S.), Chichester, John Wiley and Sons, 291-303.

CASTELLI, V.J. (1977). Organo-metallic polymer (OMP) anti-fouling coatings: A status report. Seventeenth Annual Marine Coatings Conference, Biloxi, Mississippi, U.S.A., March 16-18, 7 pp.

CHANDLER, H.E. (1979). Corrosion-biofouling relationship of metals in sea water. Metal Progress, June 1979 : 47-53.

CHAPMAN, A.R.O. (1973). Methods for macroscopic algae. A Handbook of Phycological Methods: Culture Methods and Growth Measurements, Chapter 5. (Ed. Stein, J.R.). Cambridge University Press.

CHARACKLIS, W.G. (1981). Fouling biofilm development: A process analysis. Biotechnology and Bioengineering, 23 : 1923-1960.

CHARACKLIS, W.G., NIMMONS, M.J. and PICOLOGLOU, B.F. (1981). Influence of fouling biofilms on heat transfer. Heat Transfer Engineering, 3 : (1) : 23-37.

CHRISTIE, A.O. (1972). Spore settlement in relation to fouling by Enteromorpha. In: Proceedings of the Third International Congress on Marine Corrosion and Fouling. (Eds. Acker, R.F., Brown, B.F., Depalma, J.R. and Iverson, W.P.). National Bureau of Standards, Gaithersburg, Maryland, U.S.A. 674-681.

CHRISTIE, A.O. and EVANS, L.V. (1975). A new look at marine fouling. Part 1. Shipping World and Shipbuilder, October 1975 : 953-955.

CHRISTIE, A.O., EVANS, L.V. and CALLOW, M.E. (1975). A new look at marine fouling. Part 2. Shipping World and Shipbuilder, November 1975 : 1043-1062.

CHRISTIE, A.O., EVANS, L.V. and SHAW, M. (1970). Studies on the ship-fouling algae Enteromorpha II. The effect of certain enzymes on the adhesion of zoospores. Annals of Botany 34 : 467-482.

CHRISTIE, A.O. and HEARSON, A. (1968). Marine Biology (fouling). Journal of the Oil and Colour Chemists Association, 51 : (2) : 180-182.

COLWELL, R.R., BELAS, M.R., ZACHARY, A., AUSTIN, B. and ALLEN, D. (1980). Attachment of microorganisms to surfaces in the aquatic environment. Developments in Industrial Microbiology, 21 : 169-178.

CORPE, W.A. (1970). An acid polysaccharide produced by a primary film-forming marine bacterium. Developments in Industrial Microbiology, 11 : 402-412.

COSTELLO, J.A. (1969). The corrosion of metals by micro-organisms a literature survey. International Biodeterioration Bulletin, 5 : (3) : 110-118.

CRISP, D.J. (1974). Factors influencing the settlement of marine invertebrate larvae. Chemoreception in Marine Organisms. (Eds. Grant, P.T., Mackie, A.M.). Academic Press, London and New York, 1977-265.

CUNDELL, A.M. and MITCHELL, R. (1977). Microbial succession on a wooden surface exposed to the sea. International Biodeterioration Bulletin, 13 : (3) : 67-73.

DAFT, M.J., McCORD, S.B. and STEWART, W.D.P. (1975). Ecological studies on algal-lysing bacteria in fresh water. Freshwater Biology, 5 : 577-596.

DANIEL, G.F., CHAMBERLAIN, A.H.L. and JONES, E.B.G. (1980). Ultrastructural observations on the marine fouling diatom Amphora. Helgoländer Meeresunters, 34 : 123-149.

DANIEL, G.F. and CHAMBERLAIN, A.H.L. (1981). Copper immobilization in fouling diatoms. Botanica Marina, 24 : 229-234.

DANIEL, W.W. (1978). Biostatistics: A Foundation for Analysis in the Health Sciences. Chichester, John Wiley and Sons, 504 pp.

de la COURT, F.H., de VRIES, H.J. (1973). The leaching mechanism of cuprous oxide from antifouling paints. Journal of the Oil and Colour Chemists Association, 56 : 388-395.

de la COURT, F.H. (1980). The value of tributyltinfluoride as a toxicant in antifouling formulations. Journal of the Oil Chemistry Association, 63 : 465-473.

DEKKER, T.T. and HAPPE, J. (1981). Advanced epoxy coatings for new buildings and maintenance of offshore and other structures. Offshore Inspection Repair and Maintenance Conference. Day 2. 'The Corrosion Problem' 11th February, 1981 : 10 pp.

DEMPSEY, M.J. (1981). Colonisation of antifouling paints by marine bacteria. Botanica Marina, 24 : 185-191.

DUDDRIDGE, J.E. (1981). Some techniques commonly used in the study of biological films. Conference on Progress in the Prevention of Fouling in Industrial Plant. Nottingham University, 1-3 April, 54-67.

DYER, D.L. and RICHARDSON, D.E. (1962). Materials of construction in algal culture. Applied Microbiology, 10 : 129-132.

EDYVEAN, H.G.J. and TERRY, L.A. (1983). Polarization studies of 500 steel in cultures of marine algae. International Biodeterioration Bulletin, 19 : (1) : 1-11.

ELLIOTT, J.M. (1971). Some Methods for the Statistical Analysis of Samples of Benthic Invertebrates. Published by the Freshwater Biological Association; Scientific Publication No. 25, 157 pp.

ENGELHART, J.E. (1975). A review of toxicology and environmental impact of organotin antifoulants - guidelines for safe handling in chemical and paint manufacturin and paint removal. Fifteenth Annual Marine Coatings Conference of the National Paint and Coatings Association, Feb. 7th, 1-9.

EVANS, L.V. (1981). Marine algae and fouling: a review, with particular reference to ship-fouling. Botanica Marina, 24 : 167-171.

EVANS, L.V. and CHRISTIE, A.O. (1970). Studies on the ship-fouling algae Enteromorpha 1. Aspects of the fine-structure and biochemistry of swimming and newly settled zoospores. Annals of Botany, 34 : 451-466.

FAVALI, M.A., BARBIERI, N. and BASSI, M. (1978). A green alga growing on a plastic film used to protect archaeological remains. International Biodeterioration Bulletin, 14 : 3 : 89-93.

FISHER, E.E., BIRNBAUM, L.S., DEPALMA, J., MURAOKA, J.S., HING DEAR, and WOOD, F.G. (1975). Survey Report: Navy Biological Fouling and Biodeterioration, Naval Undersea Center San Diego, California 92132, Report No. NUC TP 456, 30 pp.

FISHER, R.A., THORNTON, H.G. and MACKENZIE, W.A. (1922). The accuracy of the plating method of estimating the density of bacterial populations. Annals of Applied Biology 9 : 325-359.

FLETCHER, M. and FLOODGATE, G.D. (1973). An acid electron-microscopic demonstration of an acidic polysaccharide involved in the adhesion of a marine bacterium to solid surfaces. Journal of General Microbiology, 74 : 325-334.

FLETCHER, R.L. and CHAMBERLAIN, A.H.L. (1975). Marine fouling algae. Microbial Aspects of the Deterioration of Materials. (Eds. Lovelock, D.W., Gilbert, R.J.), London and New York, Academic Press : 59-81.

FRIES, L. (1975). Some observations on the morphology of Enteromorpha Linza (L.) J. Ag. and Enteromorpha compressa (L) Grev. in axenic culture. Botanica Marina, 18 : (4) : 251-253.

FRITSCH, F.E. (1961). The Structure and Reproduction of the Algae. Volume 1. Cambridge University Press, 791 pp.

FRY, W.G. (1975). Raft fouling in the Menai Strait, 1963-1971. Hydrobiologia, 47 : 527-558.

GAVIS, J., GUILLARD, R.R.L. and WOODWARD, B.L. (1981). Cupric ion activity and the growth of phytoplankton clones isolated from different marine environments. Journal of Marine Research, 39 : (2) : 315-333.

GILPIN-BROWN, J.B. (1970). Applications for plastic piping in marine laboratories. Plastic Pipes Symposium, Southampton, England, 14-16 September. 290-301.

GITLITZ, M. (1980). Recent developments in marine anti-foulants. 20th Annual Marine Offshore and Inland Waterways Conference, March 26, New Orleans, LA. U.S.A. 23 pp.

GHANEM, N.A. and EL-MALEK, M.M.A. (1978). Antifouling coatings - part 1. Preparation and testing of antifouling coatings based on tributyltin fluoride. Corrosion Control by Coating Meeting : (1) : 391-398.

GOODMAN, C., NEWALL, M. and RUSSELL, G. (1976). Rapid screening for copper tolerance in ship-fouling algae. International Biodeterioration Bulletin, 12 : (3) : 81-83.

GOVINDJEE and GOVINDJEE R. (1974). The primary events of photosynthesis. Scientific American, 231 : (6) : 68-82.

HAACK, T.K. and McFETERS, G.A. (1982). Nutritional relationships among microorganisms in an epilithic community. Microbial Ecology, 8 : 115-126.

HALL, A. (1980). Heavy metal co-tolerance in a Copper-tolerant population of the marine fouling alga, Ectocarpus siliculosus (Dillw.) Lyngbye. New Phytology, 85 : 73-78.

HALL, A. (1981). Copper accumulation in Copper-tolerant and non-tolerant populations of the marine fouling algae, Ectocarpus siliculosus (Dillw.) Lyngbye. Botanica Marina, 24 : 223-228.

HALL, A., FIELDING, A.H. and BUTLER, M. (1979). Mechanisms of copper tolerance in the marine fouling alga Ectocarpus siliculosus - evidence for an exclusion mechanism. Marine Biology, 54 : 195-199.

HANSMANN, E. (1973). Pigment analysis. Handbook of Phycological Methods: Culture Methods and Growth Measurements, Chapter 23. (Ed. Stein, J.R.), Cambridge University Press.

HANSON, C.H. and BELL, J. (1976). Sub-tidal and inter-tidal marine fouling on artificial substrata in northern Puget Sound, Washington. Fishery Bulletin, 74 : (2) : 377-385.

HARDY, F.G. (1981). Fouling on North Sea platforms. Botanica Marina, 24 : 173-176.

HAYAKAWA, K., KUSAKA, I. and FUKUI, S. (1975). Resistance to Mercuric Chloride in Pseudomonas K-62. Agr. Biol. Chem., 39 : (11) : 2171-2179.

HOUGHTON, D.R. (1968). Mechanisms of marine fouling. Proceedings of the 1st International Biodeterioration Symposium. (Eds. Walters, A.H. and Elphick, J.J.), London, Elsevier Press, 55-61.

HOUGHTON, D.R. (1970). Foul play on the ship's bottom. New Scientist, 48 : (728) : 383-385.

HOUGHTON, D.R., PEARMAN, I. and TIERNEY, D. (1972). The effect of water velocity on the settlement of swarmers of Enteromorpha spp. In: Proceedings of the Third International Congress on Marine Corrosion and Fouling. (Eds. Acker, R.F., Brown, B.F., DePalma, J.R. and Iverson, W.P.). National Bureau of Standards, Gaithersburg, Maryland, U.S.A. 682-690.

HOUGHTON, D.R. (1978). Marine fouling and offshore structures. Ocean Management, 4 : 347-352.

HUGHES, D.E., WIMPENNY, J.W.T. and LLOYD, D. (1971). The disintegration of microorganisms. Methods in Microbiology. Volume 5B. Chapter 1. (Eds. Norris, J.R. and Ribbons, D.W.). London and New York, Academic Press.

IRVINE, J. and JONES, E.B.G. (1975). The effect of a Copper-Chrome-Arsenate preservative and its constituents on the growth of aquatic microorganisms. Journal of the Institute of Wood Science, 7 : (1) : 5 pp.

ISAACSON, W. (1983). The winds of reform. Time, March 7, 1983. 12-30.

JACKSON, G.A. and MORGAN, J. (1978). Trace metal-chelator interactions and phytoplankton growth in seawater media: Theoretical analysis and comparison with reported observations. Limnology and Oceanography, 23 : 268-282.

JENNISON, M.W. and WADSWORTH, G.P. (1939). Evaluation of the errors involved in estimating bacterial numbers by the plating method. Journal of Bacteriology, 39 : 389-397.

JOHNSON, P.T. (1968). A new medium for the maintenance of marine bacteria. Journal of Invertebrate Pathology, 11 : 144.

JONES, E.B.G., TURNER, R.D., FURTADO, S.E.J. and KUHNE, H. (1976). Marine biodeteriogenic organisms. I. Lignicolous fungi and bacteria and the wood boring mollusca and crustacea. International Biodeterioration Bulletin, 12 : (4) : 120-134.

JONES, J.G. (1979). A Guide to Methods for Estimating Microbial Numbers and Biomass in Fresh Water. Freshwater Biological Association; Scientific Publication No. 39, 112 pp.

JONES, W.E. and DENT, E.S. (1970). Culture of marine algae using a re-circulating sea water system. Helgolander wiss. Meeresunters, 20 : 70-78.

KARANDE, A.A., GAONKAR, S.N. VISWANATHAN, R. and SRIRAMAN, A.K. (1982). Bioassay of antifoulant Chlorine. Indian Journal of Marine Sciences, 11 : 177-179.

KINNE, O. (1976). Cultivation of marine organisms: water-quality management and technology. Marine Ecology, Volume III, Cultivation, Part 1. (Ed. Kinne, O.). Chichester, John Wiley and Sons, 19-300.

KIRCHMAN, D., GRAHAM, S., REISH, D. and MITCHELL, R. (1981). Bacteria induce settlement and metamorphosis of Janua (Dexiospira) brasiliensis (Grube). Journal of Experimental Marine Biology and Ecology, 56 : 135-163.

KIRCHMAN, D. and MITCHELL, R. (1983). Biochemical interactions between micro organisms and marine fouling invertebrates. Biodeterioration 5. (Eds. Oxley, T.A., Barry, S.). Chichester, John Wiley and Sons. 281-290.

KRETSCHMER, J.R., SMITH, A.P. and STREETS, B.C. (1980). A technique for prevention or removal of biofouling from surfaces exposed to the marine environment. Proceedings of the 12th Offshore Technology Conference, 4 : 39-43.

KRONSTEIN, M. (1975). Mechanism of organo metal toxicants in controlled-release antifouling paints. Acs. Division of ORPL., Philadelphia, U.S.A., 169th Meeting : 274-281.

KRONSTEIN, M. and DENNINGER, C.R. (1976). Cuprous oxide antifoulants. Modern Paint and Coatings, 66 : (10) : 29-36.

LIS, H. and SHARON, N. (1973). The biochemistry of plant lectins (phytoheamagglutins). Annual Review of Biochemistry, 42 : 541-574.

LOWRY, T.M. and CAVELL, A.C. (1939). Intermediate Chemistry. MacMillan and Company, London, 876 pp.

LUND, J.W.G., KIPLING, G. and le GREN, E.D. (1958). The inverted microscope method of estimating algal numbers and the statistical basis of estimations by counting. Hydrobiologia, 11 : 143-170.

LUTHER, G. (1976). Bewuchsuntersuchungen auf naturste insubstraten im gezeitenbereich des Nordsylter Wattenmeeres : Algen. (Fouling studies on natural-stone substrates in the Tidal zone of the North Sylt Wadden Sea : Algae). Helgolander wiss. Meeresunters, 28 : 318-351.

McLACHLAN, J. (1973). Growth media-marine. A Handbook of Phycological Methods: Culture Methods and Growth Measurements, Chapter 2 (Ed. Stein, J.R.). Cambridge University Press.

MARSHALL, K.C. (1976). Interfaces in Microbial Ecology. Cambridge, Massachusetts, Harvard University Press. 152 pp.

MARSHALL, K.C. (1981). Bacterial behaviour at solid surfaces - a prelude to microbial fouling. Fouling of Heat Transfer Equipment. (Eds. Somerscales, E.F.C. and Knudsen, J.G.). Hemisphere Publishing Corporation. 305-312.

MARSHALL, K.C., STOUT, R. and MITCHELL, R. (1971b). Mechanism of the initial events in the sorption of marine bacteria to surfaces. Journal of General Microbiology, 68 : 337-348.

MARZALEK, D.S., GERCHAKOV, S.M. and UDEY, L.R. (1979). Influence of substrate composition on marine microfouling. Applied and Environmental Microbiology, 38 : (5) : pp. 987-995.

MESICH, F.G. and HUFF, K. (1973). A program to determine the feasibility of a unique approach to non-polluting antifouling coatings for sonar domes. Radian Corporation, Austin, Texas, U.S.A. 53 pp.

MILNE, A. (1973). Problems in anti-fouling paint. Tanker and Bulk Carrier, 20 : 22-24.

MITCHELL, R. (1972). Bacterial chemotaxis, marine fouling and pollution. Naval Research Reviews, 25 : (11) : 1-6.

MITCHELL, R. and BENSON, P. (1981). Control of marine biofouling in heat exchanger systems. MTS Journal, 15 : (4) : 11-20.

MONAGHAN, G.P., O'BRIEN, E.J. Jr., REUST, H. and GOOD, M.L. (1980). Current status of the chemical speciation of organotin toxicants in antifoulants. Developments in Industrial Microbiology, 21 : 211-215.

MONTEMARANO, J.A. and COHEN, S.A. (1976). Antifouling glass-reinforced composite materials. Naval Ship Research and Development Center, Materials Department, Annapolis, Report MAT-75-33, 27 pp.

MOORE, H.B. and GRAY, M.K. (1970). A pilot model of a salinity control system for running sea water. University of Miami, Institute of Marine Science Special Report, ML 70073, 20 pp.

MOREL, N.M.L., KEUTER, J.G. and MOREL, F.M.M. (1978). Copper toxicity to Skeletonema costatum (Bacillariophyceae). Journal of Phycology, 14 : 43-48.

MORETON, B.B. (1982). Marine industry applications for corrosion and biofouling resistant Copper alloys. Metallurgie, 22 : (2) : 47-52.

MORGENSTERN, E.A. (1978). Advances in antifouling technology. Eighteenth Annual Marine Coatings Conference, Monterey, California, March 22-24. 11 pp.

MUIR, G.D. (editor) (1977). Hazards in the Chemical Laboratory 2nd edition, London. The Chemical Society, 473 pp.

NEWTON, L. (1931). A Handbook of the British Seaweeds. London, The Trustees of the British Museum, 492 pp.

NICKELS, J.S., PARKER, J.H., BOBBIE, R.J., MARTZ, R.F., LOTT, D.F., BENSON, P.H. and WHITE, D.C. (1981). Effect of cleaning with flow-driven brushes on the biomass and community composition of the marine microfouling film on aluminium and titanium surfaces. International Biodeterioration Bulletin, 17 : (3) : 87-94.

OLSON, B.H., BARKAY, T. and COLWELL, R.R. (1979). Role of plasmids in mercury transformation by bacteria isolated from the aquatic environment. Applied and Environmental Microbiology, 38 : (3) : 478-485.

ORDNANCE SURVEY, SHEET 159 (1974). Swansea and the Gower. Showing part of the National Park. Southampton, The Director General of the Ordnance Survey.

ORDNANCE SURVEY, SHEET 196 (1974). The Solent. Showing part of the New Forest. Southampton, The Director General of the Ordnance Survey.

ORDNANCE SURVEY, SHEET 201 (1974). Plymouth and Launceston. Showing part of the National Park. Southampton, The Director General of the Ordnance Survey.

OVERMARS, H.G.J., de la COURT, F.H. and HZENBERG, J.F.A. (1980). Synthesis and application of polymer-bound biocides with antifouling properties. 5th International Congress on Marine Corrosion and Fouling : 99-112.

OVERNELL, (1975). The effect of heavy metals on photo-synthesis and loss of cell potassium in two species of marine algae, Dunaliella tertiolecta and Phaedactylum tricornutum. Marine Biology, 29 : 99-103.

PARKER, R.E. (1979). Introductory Statistics for Biology. (Ed. Edward Arnold), 122 pp.

PARTINGTON, A. and JENNINGS, F.J. (1971). Growing Enteromorpha in laboratories. Journal of Applied Ecology, 8 : 269-270.

PEARSON, E.S. and HARTLEY, H.O. (Editors), (1966). Biometrika Tables for Statisticians, Volume I. 3rd Edition. Cambridge University Press, 264 pp.

PEARSON, E.S. and HARTLEY, H.O. (Editors), (1972). Biometrika Tables for Statisticians, Volume II. Cambridge University Press, 386 pp.

PERSOONE, G. and de PAUW, N. (1968). Pollution in the harbour of Ostend (Belgium), biological and hydrographical consequences. Helgolander wiss. Meeresunders, 17 : 302-320.

PETRIE, A. (1978). Lecture Notes on Medical Statistics. Blackwell Scientific Publications, 194 pp.

PHILLIP, A.T. (1972). Underwater Marine Coatings Part 1: Modern Trends in Marine Antifouling Paint Research. Defence Printing Establishment, Brunswick, Victoria, 3056, Australia. 55 pp.

PHILLIPS, C.R. (1952). Relative resistance of bacterial spores and vegetative bacteria to disinfectants. Bacteriological Reviews 16 : 135-138.

PIPE, A. (1978). The fouling of fixed structures. Seminar on Marine Fouling, London, U.K., 23 April, 8 pp.

PIPE, A. (1979a). North Sea fouling organisms and their potential effects on the corrosion of North Sea structures. Marine Corrosion on Offshore Structures. A symposium sponsored by the Society of Chemical Industry's Aberdeen section. 13-14 September : 13-22.

POSTGATE, J.R. (1969). Viable counts and viability. Methods in Microbiology. Volume 1, Chapter 18. (Eds. Norris, J.R. and Ribbons, D.W.). London and New York, Academic Press.

PRASAD, A.S. (1979). Trace elements: biochemical and clinical effects of Zinc and Copper. American Journal of Hematology, 6 : 77-87.

PRITCHARD, A.M. (1981). Fouling in heat exchange plant - a British view. Fouling in Heat Exchange Equipment, 20th National Heat Transfer Conference, (Eds. Chenoweth, J.M. and IMPAGLIAZZO, M.). 105 pp.

PROVASOLI, L., McLAUGHLIN, J.J.A. and DROOP, M.R. (1957). The development of artificial media for marine algae. Arch Mikrobiol., 25 : 392-428.

RALPH, R., GOODMAN, K. and PICKEN, G. (1979). Marine growth corrosion problems: the need for a central information unit. Marine Corrosion on Offshore Structures. A symposium sponsored by the Society of Chemical Industry's Aberdeen section, 13-14 September, 62-67.

RALPH, R. and TROAKE, R.P. (1980). Marine growth on North Sea oil and gas platforms. Proceedings of the 12th Offshore Conference, 4 : 49-52.

RAMAMOORTHY, S. and KUSHNER, D.J. (1975). Binding of Mercuric and other heavy metal ions by microbial growth media. Microbial Ecology, 2 : 162-176.

RAYNER, S.M. (1975). The Natural History of Teredinid Molluscs and Other Marine Wood Borers in Papu, New Guinea. The International Research Group on Wood Preservation, Working Group IV, Preservation of Wood in the Marine Environment. Office of Forests, Forest Research Centre, Papua, New Guinea. Document No. IRG/WP/410, 75 pp.

REITSMA, W.A.A. (1981). At last predictability in offshore painting projects - pretreatment and coating for underwater and wet surfaces. The Corrosion Problem, Offshore Inspection, Repair and Maintenance Conference, day 2, 11th February, 19 pp.

RUSSEL, G. (1971). Algae as fouling organisms. Marine Borers, Fungi and Fouling Organisms of Wood. (Eds. Jones, E.B. G. and Eltringham, S.K.). Organisation for Economic Co-operation and Development, Paris, 125-134.

RUSSELL, G. and MORRIS, O.P. (1970). Copper tolerance in the marine fouling alga Ectocarpus siliculosus. Nature 228 : 288-289.

SAENKO, G.N., KORYAKOVA, M.D., MAKIENKO, V.F. and DOBROSMYSLOVA, I.G. (1976). Concentration of polyvalent metals by seaweeds in Vostok Bay, Sea of Japan. Marine Biology, 34 : 169-176.

SAROYAN, J.R. (1969). Marine Biology in antifouling paints. Journal of Paint Technology, 41 : (531) : 285-303.

SCHREIBER, E. (1927). Die reinkultur von marinen phytoplankton und deren bedeutung fur die erforschung der produktionsfahigkeit des meerwassers. Wiss. Meeresunters., Abt. Helgoland, 16 : 1-34.

SCOTT, K.R. (1972). Temperature control system for recirculation fish-holding facilities. Journal of the Fish Research Board, Canada, 29 : 1071-1074.

SCOTT, D.M. and MAJOR, C.W. (1972). The effect of copper (II) on survival, respiration and heart rate in the common blue mussel, Mytilus edulis. Biological Bulletin, 143 : 679-688.

SEELIGER, U. and CORDAZZO, C. (1982). Field and experimental evaluation of Enteromorpha sp. as a quali-quantitative monitoring organism for Copper and Mercury in estuaries. Environmental Pollution, 29 : 3 : 197-206.

SKINNER, C.E. (1972). The inland laboratory - it's role in the study of marine fouling. Biodeterioration of Materials, Volume 2, (Eds. Walters, A.M. and Hueck Van Der Plas, E.H.) Allied Science, 1972 : 456-463.

SMITH, C.A. (1980). Long-life paint systems. Shipbuilding and Marine Engineering International, 103 : (1239) : 255-258.

SOROKIN, C. (1973). Dry weight, packed cell volume and optical density. Handbook of Phycological Methods: Culture Methods and Growth Measurements, Chapter 21. (Ed. Stein, J.R.). Cambridge University Press.

SOUTHWELL, C.R., BUILTMAN, J.D. and HUMMER, C.W. Jr. (1974). Influence of Marine Organisms on the Life of Structural Steels in Seawater. Naval Research Laboratory, Washington DC, U.S.A. Report Number 7672, 19 pp.

SPITSYN, V.I., STREKALOV, P.V., BALAKHOVSKII, O.A. BUKOV, K.G., TITOVA, T.K., MIKHAILOVSKII, Yu. N., KAPLIN, Yu.M., SHIROKOLOBOV, V.N. and SHCHERBININA, G.I. (1982). Biological fouling and corrosion properties of Technetium-99 metal and certain constructional metals in seawater. Protection of Metals, 17 : (6) : 522-528.

SPOTTE, S.H. (1971). Biological filtration. Fish and Invertebrate Culture, Wiley Interscience, New York, 210 pp.

SUNDA, W.G. and GUILLARD, R.R.L. (1976). The relationship between cupric ion activity and the toxicity of copper to phytoplankton. Journal of Marine Research, 34 : 511-529.

SUSSMAN, S. and WARD, W.J. (1977). Microbiological control with chlorine dioxide helps save energy. Materials Performance, 16 : (7) : 24-28.

TAYLOR, G.E. and EVANS, L.V. (1976). The biology of slime films. Part 1. Shipping World and Shipbuilder, October 1976 : 857-858.

TENORE, K.R. and HUGUENIN, J.E. (1973). A flowing experimental system with filtered and temperature-regulated seawater. Chesapeake Science, 14 : 280-282.

THOMAS, R.W.S.P. and ALLSOPP, D. (1983). The effects of certain periphytic marine bacteria upon the settlement and growth of Enteromorpha, a fouling alga. In: Biodeterioration 5(Eds. Oxley, T.A. and Barry, S.). Chichester, John Wiley and Sons : 348-357.

THORN LIGHTING (1978). Comprehensive Catalogue, 1978. 205-209.

TODD, R.G. (editor) (1967). Extra Pharmacopia Martindale. London. Pharmaceutical Press.

TURNER, R.D. (1971). Australian shipworms. Australian Natural History, 17 : (4) : 139-146.

Van BAALEN, C. and EDWARDS, P. (1973). Light-temperature gradient plate. A Handbook of Phycological Methods: Culture Methods and Growth Measurements, Chapter 17. (Ed. Stein, J.R.). Cambridge University Press.

Van den HOEK, C., ADMIRAAL, W., COLIJN, F. and de JONG, V.N. (1979). The role of algae and seagrasses in the ecosystem of the Wadden Sea: a review. Flora and Vegetation of the Wadden Sea, Chapter 2. (Ed. Wolff, W.H.J.), Rotterdam, A.A. BALKEMA.

VAN LONDEN, A.M., JOHNSEN, S. and GOVERS, G.J. (1975). The case of long-life anti-foulings. Journal of Paint Technology, 47 : (600) : 62-68.

VIZGIRDA, R.J. (1972). Fighting marine fouling. Paint and Varnish Production, 62 : (12) : 25-28.

WONG, S.L. and BEAVER, J.L. (1980). Algal bioassays to determine toxicity of metal mixtures. Hydrobiologia, 74 : 199-208.

YAMAGUCHI, M. (1975). Growth and reproductive cycles of the marine fouling ascidians Ciona intestinalis, Styela plicata, Botrylloides violaceus, and Leptoclinum mitsukurii at Aburatsubo-Moroiso Inlet (central Japan). Marine Biology, 29 : 253-259.

YOSHIDA, M. (1980). Heavy-duty antifouling coatings. Shipbuilding and Marine Engineering International, 103 : (1239) : 258-260.

YOUNG, L.Y. (1978). Bacterioneuston examined with critical point drying and transmission electron microscopy. Microbial Ecology, 4 : 267-277.

YOUNG, L.Y. and MITCHELL, R. (1973). The role of microorganisms in marine fouling. International Biodeterioration Bulletin, 9 : (4) : 105-109.

ZOBELL, C.E. and ALLEN, E.C. (1935). The significance of marine bacteria in the fouling of submerged surfaces. Journal of Bacteriology, 29 : 230-251.