SEAWEED EXUDATES AND SETTLEMENT BEHAVIOUR OF SEDENTARY ORGANISMS

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MEERA JAN ABRAHAM

DEPARTMENT OF MARINE BIOLOGY, MICROBIOLOGY AND BIOCHEMISTRY
SCHOOL OF MARINE SCIENCES
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
COCHIN - 682 016

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To my loving husband

CERTIFICATE

This is to certify that the thesis entitled **"Seaweed exudates and settlement behaviour of sedentary organisms"**, is an authentic record of the research work carried out by Ms. Meera Jan Abraham, under my scientific supervision and guidance in the School of Marine Sciences, Cochin University of Science and Technology, in partial fulfilment of the requirements for the degree of Doctor of Philosophy of the Cochin University of Science and Technology and that no part thereof has been presented before for the award of any other degree, diploma or associateship in any University.

Prof. Dr. N.RAVINDRANATHA MENON
Director
School of Marine Sciences
Cochin University of Science and Technology
Cochin - 682 016.

Cochin - 16.
May 2001

DECLARATION

I hereby declare that this thesis entitled **"Seaweed exudates and settlement behaviour of sedentary organisms"**, is a genuine record of the research work done by me under the scientific supervision of **Prof. Dr. N. Ravindranatha Menon,** Director, School of Marine Sciences, Cochin University of Science and Technology, Cochin and that this has not previously formed the basis of the award of any degree, diploma or associateship in any University.

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May 2001

Meera Jan

Meera Jan Abraham.

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PREFACE

The problems caused by the settlement of undesirable substances and organisms on man made surfaces both stationary and movable, immersed in the seas has attracted the attention of physicists, chemists, biologists, engineers and planners world over. The attention so drawn is due to multifarious reasons, ranging from simple aspects of scientific enquiry to the huge financial losses caused for maintenance of submercibles. Various aspects can be brought in between the above two generalities. The interests from the part of marine biologists has started with simple enquiries of finding out the animals and plants that cause biofouling and the microbes that worsen the situation by 'eating' into the metalic surfaces or shredding wooden surfaces as the case may be. Design of an antifouling surface based exclusively on chemical defences would be directed towards killing either the settling spores, larvae or the settled adults. So much so such chemicals will be designed to kill. In this respect these chemicals very much resemble the pesticides and the insecticides introduced into the terrestrial realm. Unlike terrestrial situations, the efficacy of antifouling surfaces immersed into such a complex chemical solution like the seawater depended mainly on the leaching capability of such substances. This particular aspect can be compensated only by increasing the longevity of retention on the substrata of chemical substance which face rigorous physical, chemical and biological effects while such antifouling coated surfaces remains at sea. Study of the antifouling capabilities of natural biological substrata which remain unfouled marked the beginning of the study of bioactive compounds and settling behaviour of larvae of sedentary organisms. The common examples to natural

surfaces which remain unfouled are macroalgae, sedentary crustaceans, lophophorates, and the major vertebrates which are inhabitants of the sea. Scientific enquiries led to research on bioactive compounds that causes avoidance reaction to mortality of larvae. Being bioactive and organic in nature such substances would be prone to biodegradation, nullifying the toxic effects. Constant production on biological surfaces which have to be protected from settlement, makes this process a truly biological phenomenon involving time, energy and complexities of biological maintenance. Identification of bioactive compounds followed and now this has led to even the establishment of research groups involved in identifying pharmaceutical and medicines from the marine environment. The natural antifouling strategies including mechanical, physical, chemical and biological aspects require the attention of scientists with distinct multidisciplinary research approach.

The present investigation has looked exclusively into the aspect of the biological phenomenon of settling behaviour by two serious fouling offenders encountered in the tropical seas mainly on the hulls of ships and stationary structures in the harbours. The cue to study the behaviour was adopted from the observations so far made by scientists on the epizoic growth of these organisms on the surfaces of algal fronds of variegated shape, texture, size etc. The results do indicate that there are sufficient qualities of bioactive substances produced by plants occupying the lowest categories in organic evolution and curiously enough these substances have withstood the test of time.

GENERAL INTRODUCTION

The marine environment provides support to a diverse assemblage of flora and fauna. The unwanted accumulation of organisms that is detrimental to the functioning of underwater devices, waterfront structures, ship hulls, and boats is grouped under the term "biofoulers." The control of biofouling is of particular concern to modern marine engineering and shipping operations. Uncontrolled settlement and adhesion of marine invertebrates to the hulls of ships increase frictional drag, with a corresponding decrease in speed, manoeuvrability and fuel efficiency. Biofouling also affects offshore oil mining, coastal power generation, marine electronics, mariculture, marine construction and naval operations.

The problem of successfully combating the settlement of marine organisms is of considerable magnitude. From the number of preventive measures adopted from time to time, a periodic coating of paint possessing antifouling properties seem to be the only accepted method. In the last two decades, a wide range of organotin copolymer toxicants was developed and with the use of certain formulations, suppression of fouling was obtained for up to seven years. However, these organotin compounds have brought about considerable environmental and health problems.

Environmental problems posed by metal based antifouling coatings, in particular those containing organotins, have led to legislation curbing their use and a concomitant increase in interest in the development of nontoxic alternatives. One of the main avenues being pursued is the isolation of marine natural products that

inhibit settlement of potential epibionts on the host organism and accordingly could be incorporated into coatings to inhibit biofouling.

This concept had its genesis in the field observation that despite being perpetually exposed to a great variety of spores and invertebrate larvae, some animals that lack appendages and plants manage to keep their body surface clean from fouling. This investigation will concentrate on this option, highlighting the effects of seaweed secondary metabolites on the settlement of larvae of two ubiquitous foulers, the barnacle *Balanus amphitrite communis* and the polychaete *Hydroides elegans.*

The use of marine bioactive substances as natural antifouling agents has the added advantage of employment of nontoxic substances that should have no adverse environmental impact, a factor of considerable significance in the context of current concerns for safeguarding the environmental purity of seawater. An enhanced understanding of the natural functions, effects, and mechanisms of action of secondary metabolites would provide a biologically - based rationale for the productive development of products based on marine natural products. Research in the area of marine natural products has, in the recent past, grown geometrically and spread geographically unfolding some fascinating phenomena at the interface between biology and chemistry.

In seeking to develop non toxic, non polluting antifouling applications, we have made tremendous advances by examining and understanding the mechanisms by which sessile and sedentary organisms mitigate the effects of the continuous biofouling pressure that they face in their natural habitats. The key to their success is the diversity of their antifouling arsenal. These organisms take advantage of

mechanical, physical, and chemical antifouling mechanisms that are then integrated to have a composite effect on the fouling community.

Marine algae are an extensive and prolific source of secondary metabolites. Algal secondary metabolites may be active against a number of biological factors simultaneously, thereby increasing the adaptive benefit of secondary metabolite production. Because many seaweeds are conspicuously free of epibionts an antifouling role has been suggested for the unusual secondary metabolites isolated from them. Unfortunately, only in a few instances has experimental evidence been provided to support these claims. Whether chemical substances diffusing from the surfaces of seaweeds or the chemical constitution of the surface itself plays a role as chemical defences against biofoulers in the marine environment need to be investigated.

Most marine organisms have a highly specialized larval settlement stage. Chemical cues that induce settlement provide a mechanism to assure larval recruitment into a suitable habitat. The settlement stage is a prime target for management strategies because it is a key and vulnerable step in the colonization process.

To date, most of the applications for marine natural products, and often the natural products themselves, have been found by conducting large scale screening programs. Since these programs are not based on a compelling biological rationale, they are often costly and inefficient. Many inactive organisms are screened and many active compounds are not detected because they degrade during collection, storage and extraction, or because they are not tested against appropriate target organisms.

The biotechnological application of chemicals from living organisms is widespread and of commercial significance. Natural products from a wealth of terrestrial organisms as well as synthesized analogs have provided a broad and firm basis for the agrochemical and pharmaceutical sectors of the chemical industries of the world. Historical interest in natural products of terrestrial organisms contrasts with considerably less interest in organisms that live in the vast seas of the world, despite the fact that the sea harbours a tremendous diversity of life and, at the level of phyla, a much greater diversity than the terrestrial realm.

For most marine natural products there is little knowledge of their natural function in marine systems or of their behaviour in a wide array of biological assays. A perusal of the literature revealed that no concerted effort has so far been made in the tropics to delineate the antifouling properties of seaweeds. With the above broad aspects in mind, the present work was planned and carried out. The protocols included experiments to study effects of seaweed secondary metabolites on survival and settlement of larvae of various biofoulers.

REVIEW OF LITERATURE

Fouling is one of the most important problems in the proper development of marine technological advancement. The term "fouling" originally described those "aufwuchs" communities that develop on man-made structures in the sea and that usually interfere with human interests. Fouling pressure of variable intensities is omnipresent in the sea. Materials submerged in seawater experience a series of discrete chemical and biological events which eventually result in the formation of a complex layer of attached organisms known as biofouling. Biofouling can be further divided into microfouling and macrofouling phenomena. Microfouling includes initial events that result in biofilm formation, while macrofouling describes the settlement and development of macroscopic fouling species. The establishment of a fouling community is initiated by the development of a primary film comprising of bacteria, fungal spores, diatoms, and colloidal organic matter. In the second phase, there is the establishment of macrofoulers, which in Indian waters usually consists of colonial ciliates, hydroids, barnacles, tubicolous polychaetes, bryozoans, mussels, oysters and compound ascidians. Generally the fouling pressure decreases with latitude, distance from shore, distance from natural hard bottom communities, and depth (Richmond and Seed, 1991). The tropical marine environment is less cyclical regarding larval and spore availability and recruitment occurs at a more or less constant level resulting in the continuous accumulation of fouling material.

The adsorption of biopolymers onto a surface (Lewin, 1984), the attraction and adhesion of bacteria to that surface, and their subsequent multiplication and exopolymer production leads to the formation of biofilms (Lappin – Scot and

Costerton, 1989). Morphological studies have indicated that the microfouling biofilm is initiated by attachment of rod-shaped bacteria to surfaces (Marshall *et al.*, 1971). These organisms are first reversibly bound to the surface and then may form an extracellular polymer that irreversibly binds them to the surface. The initial colonizers are followed by bacteria with more complex shapes and then by micro eukaryotes such as diatoms and other algae. This film is believed to influence the settlement of larvae of marine fouling organisms (Henschell and Cook, 1990). Morse (1984) pointed out that, the natural inducers of larval recruitment or fouling for a number of invertebrates are often surface associated molecules. Attached bacteria modify the chemistry of a surface through the production of extracellular polymers and the hydrolysis of these polymers (White, 1984), making available a vast array of molecules for recognition by competent larvae examining the suitability of a surface for attachment.

Crisp and Ryland (1960) reported that a biofilm can either promote or reduce settlement rates depending on the organism being considered. Walker *et al.* (1985) found that marine sponges without a biofilm exhibit greater antimicrobial properties than those with biofouled surfaces.

The biofilm can stimulate settlement in hydroids (Freeman and Ridgway, 1987), polychaetes (Kirchman *et al.*, 1982a), bivalves (Fitt *et al.*,1990), barnacles (Le Tourneux and Bourget, 1988; Maki *et al.*, 1990), bryozoans (Keough and Raimondi, 1995) and echinoderms (Pearce and Scheibling, 1991). According to Floodgate (1971), the bacterial film may help secondary fouling in several ways. It may simply provide a site for mechanical attachment. The presence of the film on a surface can darken the surface and reduce the amount of light reflected from it and there by help settlement of fouling larvae since majority of them are photonegative at the time of settlement (Thorson, 1950; Cameron, 1986). Moreover, the film may

act as a source of food for the attached larvae, or concentrate the nutrients for developing plants. Further, the film may help in cementing the attached organisms onto the surface. Finally, the film may cause changes favourable for fouling in the actual surface itself. The presence of a biofilm is not required to ensure settlement in all fouling organisms, in fact, biofilms may actually inhibit the settlement of some marine invertebrate larvae (Todd and Keough, 1994; Keough and Raimondii, 1995).

Most benthic marine invertebrates produce planktonic larvae that must locate a suitable substratum to get settled. Metamorphically competent pelagic larvae delay metamorphosis until they encounter a suitable substratum (Strathman,1978). The process of settlement may be preceded by attachment, exploration and fixation. Larvae are able to make repeated and active attempts to find a suitable place for development and growth.

The significance of settlement and recruitment has been recognised only recently by some ecologists (Connell, 1985; Gaines and Roughgarden, 1985; Sutherland, 1990; Menge, 1991); although it had been acknowledged for decades by others (Thorson,1950, 1966; Scheltema, 1974; Lewis, 1976; Kendall *et al.*, 1982; Crisp, 1984).

Mitchell and Kirchman (1984) suggested that the larvae of fouling invertebrates produce proteins called lectins, which in turn bind specifically to extracellular bacterial polysaccharides. This property of lectins enable the larvae of fouling invertebrates to respond to cues provided by bacteria on marine surfaces. Morse (1984) expressed that the recognition of recruiting surfaces by the larval forms is primarily controlled by biochemical inducers and cues. These biochemical inducers belong to a family of simple biochemical substances, all structurally

homologous to the amino acid-γ aminobutyric acid (GABA). These act as potent neurotransmitters in many species.

Settlement and metamorphosis constitute, arguably, the most complex period in the life history of marine invertebrate larvae. Reversible contact with the substratum, exploratory behaviour, orientation and metamorphosis are all part of the process of settlement. Chemical cues that induce settlement provide a mechanism to ensure larval recruitment into a suitable habitat.

Chemical cues that influence the induction of larval settlement may be present in the water column, but are more commonly associated with the substratum. Specific biochemical triggers are required for induction of larval settlement, attachment and metamorphosis (Mitchell *et al.*, 1982; Morse, 1982).

Crisp (1984) referred fixation as the irreversible cementing process which terminates all further exploratory missions of the larval forms. However, before the permanent settlement of the larvae, there is another phase during which temporary adhesion takes place to enable the exploration of surface by the larvae. During the exploratory phase, the attaching larval forms of the fouling organisms explore a large area of the substratum in order to evaluate their acceptability. During the period of exploration the larvae have to maintain their hold on the substratum against water turbulence. If a larva finds the site unacceptable, it should be able to leave the substratum, get back to the water column and be carried by water currents towards new sites (Linder, 1984). This phase is controlled by pre-settlement adhesives which differ from animal to animal. This phase is followed by the post settlement adhesion and secondary adhesion during adulthood. Two types of adhesive mechanisms, in general, are observed; a suction apparatus and a secreted sticky mucous substance (Linder, 1984).

Larval settlement and metamorphosis can be positively or negatively affected by the presence of catecholamines and choline compounds (Pawlik, 1990). This imply the existence of specific neuronal receptor within larvae that use pathways similar to those found in vertebrate systems. It appears that the choline and catecholamine derivatives act directly on endogenous receptors of the larval nervous system or act as precursors in the synthesis and release of an important neurotransmitter (Coon *et al.*, 1990b; Pawlik, 1990).

Burke (1983) suggested a neuronal control mechanism over the onset of metamorphosis. Neural signals are dependent on the maintenance of an electrical potential across a cell membrane and thus, any ionic gradient that might change the action potential could, through a "cascade" affect larval metamorohosis (Yool *et al.*, 1986). Specific biochemical triggers are required for induction of larval settlement, attachment, and metamorphosis (Mitchell *et al.*, 1982; Morse, 1982). These required biochemical inducers may be macromolecular conjugates of amino acid – derived neurotransmitter analogs (Morse, 1982).

Settlement of barnacle and polychaete larvae can be chemically influenced through a variety of mechanisms. Identification of pure compounds that affect larval barnacle settlement (Gerhart *et al.*, 1988; Tegtmeyer and Rittschof, 1989), could provide the molecular probes necessary to study pathways of mediation.

There is an ever increasing evidence to show that the settlement and attachment of fouling larvae involve a series of complex processes and therefore, depend on a number of factors. This makes them a feeble link in the lengthy process of biofouling and renders them vulnerable at points where attachment can be concentrated. Therefore, the study of the larvae of such fouling species in general,

their structure and settlement pattern assume great importance (Crisp, 1984; Cameron, 1986; Hadfield, 1986).

The settlement, attachment and metamorphosis of the planktonic larvae of many species are subject to stringent control by defined, exogenous as well as endogenous biochemical factors and mechanisms. This suggest that the operative biochemical control sites may be especially useful targets for a new generation of effective and specific counter measures to control marine fouling.

Hydrographic parameters, including pollution, in a locality decides the presence or absence of a species in an area and thereby in a fouling community (Santhakumaran and Sneli, 1984). Environmental factors that influence larval settlement fall into physical, biological and chemical categories (Butman, 1987; Hadfield, 1986). Physical factors that might have an impact on larvae in the water column include water flow, light, gravity, temperature, salinity and pressure (Barile *et al.*, 1994; Dineen and Hines, 1994). Physical characteristics of substratum such as boundary flow (Wethey *et al* ., 1988; Mullineaux and Butman, 1991), surface texture and contour (Walters and Wethy, 1991; Raimondi, 1990), colour (James and Underwood, 1994), sediment size and percent cover (Bourget *et al.*, 1994; Maida *et al.*, 1994) can affect larvae as they leave the water column.

High temperature favours faster growth and maturity, shorter larval life and therefore, continued breeding and settlement in tropical seas. Low salinity retards breeding and growth rate. So very less fouling occurs during the monsoon months in those areas prone to appreciable fresh water incursion. But these physical characters are less specific in their control over larval settlement responses than biological and chemical cues (Le Tourneux and Bourget, 1988; Harrold *et al.*, 1991).

About 65 species of macrofoulers are known from the Cochin harbour area (Purushotham and Rao, 1971; Menon and Nair, 1972) Quantity wise maximum fouling occurs during the post-monsoon period and quality wise during the pre-monsoon season. Least settlement occurs during the active monsoon periods along the west coast of India in general and harbour areas in particular.

The major organising factors influencing the development of fouling communities are recruitment of species onto a surface, competition between resident organisms and disturbance by predation or environmental factors. Fluctuations in densities of larvae and spores can play a very important role in the developing fouling communities.

The microscopic fouling forms found in Indian waters are bacteria, diatoms, fungi, protozoans and rotifers. The macroscopic forms are sponges, coelenterates, flatworms, bryozoans, tube worms, amphipods, barnacles molluscs and ascidians in addition to some planarians, nemertines, polychaetes, isopods, decapods, gastropods and pisces. Barnacles, tubeworms, bryozoans and mussels are by far the most important with regard to surface coverage, volume and weight in the fouling communities of the tropical waters, whereas simple and compound ascidians along with hydroids, barnacles and tube worms form a major bulk of fouling community of tropical open seas.

With increasing use of seawater as a medium of transportation, communication, energy generation, oil and mineral exploitation and as an industrial fluid, the problems associated with the maintenance of marine structures and machinery are growing. The development of microfilm or macrofouling on immersed structures or on marine machinery has now been recognised as one of the major problems and is receiving increasing attention all over the world.

Fouling is unsightly and it shorten the service expectancy of immersed structures and make their operation expensive, less dependable and even hazardous. A marine structure designed to last for several decades fails within a span of a few years. Marine machinery components fabricated to provide a trouble free performance develop problems within months of their installation. A well researched ship hull structure, designed to reduce the influence of the hydrodynamic forces and thereby to achieve highest cost efficiency performance, proves a futile exercise. All these frequently reported failures occur because the marine environment supports a variety of sedentary life on man made structures.

The destructional potential of fouling is known since man began using wooden boats. Material degradation may be favoured by fouling. The corrosion promoting role of foulers consists of creating favourable conditions at the metal surface (low oxygen tension, extreme pH values, high H_2S concentrations) or by directly attaching on the metal. Relini (1988) provided a brief list of marine structures needing protection against biofouling. It includes ship and submarine hulls, seawater intakes and pipe line systems, condensers, OTEC and offshore rigs, sonar domes, navigational buoys, moored oceanographic instruments and fishing gear.

The best known fouling caused problem is the increase of frictional drag. Even biofilms are reported to increase friction on a ship hull by 80% (Lewis, 1994). The hull of a ship which is painstakingly designed and made to have clean and streamlined contour so as to reduce the frictional resistance to its minimum, gets roughened due to marine growth. The resultant problems include increased fuel consumption, loss of speed, decrease in range, increased noise production and vibrations etc. which affects the sonic equipments. In addition, water intake tubes associated with fire-fighting systems, sea chest etc, get clogged due to calcareous

growth inside the pipelines. Haderlie (1984) while reviewing the effects of macrofouling has mentioned that the increased frictional drag has been estimated to cost an annual loss of 150 million US $ in terms of increased fuel bill to US Navy alone in 1975 and the figure must be much higher by now.

In India, the loss due to increased hull roughness alone is estimated to cost more than Rs. 100 crores. It excludes cost of antifouling measures, labour charges involved in the application of paints, drydocking charges etc.

A badly fouled ship may have its speed reduced to 30%, and many fouling species have been transported on the hulls of ships to locations all over the world. Fouling may shorten the service expectancy of immersed structures and make their operation expensive, less dependable or even hazardous. For offshore platforms, excessive fouling causes increase in hydrodynamic loading, masking of underwater diver reference points, and greater velocity for localized corrosion. Platforms are usually fouled by barnacles, oysters, mussels and kelps (Moss *et al.,* 1981; Hardy, 1981). Underwater cleaning of these huge structures is very costly. Stuller (1987) reports that diver operated jet-cleaning of a single platform may cost as much as $100,000 per platform. Offshore platforms in the tropical and sub-tropical waters might require cleaning more frequently and each cleaning operation per platform lasts for about 20-25 days. Since offshore oil platforms are designed for a life span of 20-30 years, antifouling paints with short time span cannot be used. About 8% of the life – time cost of a platform is expended on antifouling measures (Haderlie, 1984), both in design and subsequent periodic cleaning. This fact, assumes great significance in the economy of a developing country such as India, which makes massive investment in the field of offshore oil production.

Biofouling is a problem generally common to power plant cooling systems. An increase in condenser back pressure due to fouling in a 250 MW (e) plant could cost a utility about $ 250,000 back in 1987. In 1989, fouling by the Asiatic clam alone cost the United States over $ 1 billion (Strauss, 1989). Some of the conditions enhancing organism's growth inside cooling conduits are a continuous supply of water rich in oxygen and food, reduction in density of predators, lack of competition from algal communities due to absence of light and reduction in silt deposition. Biofouling causes reduction in heat transfer, blockage of intake gates and tubes, mechanical damage, pump vibration, changes in water quality that sometimes render the water more aggressively corrosive, that leads to expensive shutdowns.

The plant maintenance units usually resolve to chlorination and back flushing of heated seawater. However, these methods have their own limitations since these could pose pollution as well as operational hazards.

Fouling in the splash or intertidal zone of steel structures may serve as a sponge and effectively retain seawater, even during low tide conditions. The retained seawater may aggravate corrosion through a continuous supply of abundant oxygen and chloride, thus leading to excessive corrosivity (Pipe, 1979). The effects of this fouling may be noticed either by the disruption of the protective coating or by the removal of the stable anti-corrosion film.

Formation of small "pockets" of metabolic wastes, decomposition products, etc., and consumption of oxygen during the respiratory activity of barnacles within a fouling layer lead to a very nonuniform environment in terms of ionic distribution and concentration of dissolved oxygen. Not infrequently, the dead-water pocket between the base of the barnacle and the metallic surface may set up galvanic

cells resulting from the growth of anaerobic bacteria and other microorganisms. This is turn, leads to a corrosion current.

The weight of foulers occurring on floating objects such as boats, rafts, floats, moorings etc. may eventually exceed buoyancy, resulting in sinking of these objects (Lewis, 1994). The added crust on originally flexible structures makes them not only heavier, but also more rigid and brittle, increasing the risk of breakage. Fouling of aquaculture nets increase their weight and decrease water exchange through the meshes, causing a deterioration of water quality within the cages (Hodson and Burke, 1994; Lewis, 1994). The insulation effect of fouling impairs the functioning of immersed heat exchangers, reverse osmosis membranes (Ridgway *et al.,* 1985) and electronic or optical probes.

The performance of sonar domes which is of strategic importance in the naval ships is degraded due to fouling. Haderlie (1984) stated that maintenance of antifouling coatings on the sonar domes is a difficult task since these coatings do not adhere to surfaces radiating acoustic signals.

The Ocean Thermal Energy Conversion (OTEC) plants also come under the realm of biofouling menace. In this case, however, the concern over microfouling in the heat exchangers is greater than the effects of macrofouling since microfouling brings down the efficiency of heat exchangers considerably.

The oceanographic instruments that are moored in the marine environment are affected to a considerable extent. The fouling on the rotors of current meters as well as the sensors used for collection of data on parameters like temperature, salinity, water current may lead to the collection of erraneous data. The fouling related costs of marine industry are composed of antifouling protection measures,

increased operational costs, use of more resistant and usually more expensive materials, replacement of fouling damaged parts, restoration after fouling caused accidents. It is quite clear that a proper fouling control strategy is vital for many industries where seawater comes into contact with material surfaces.

Fouling can be kept at a tolerable minimum by periodic renewal of the surface, inducing unstable physicochemical conditions at the surface, mechanical cleaning, impeding adhesion, repelling potential settlers or intoxication.

Natural products or simple derivatives of these were used for protection against foulers, before the birth of the modern chemical industry. Tar, pitch or other bituminous materials, waxes, seed oils, lime, cashew nut shell oil and rubbers (Santakumaran and Rao 1988; De 1989; Foster 1994) have been explored and are still often employed. They do not provide sufficient protection, and have to be combined with other methods in the borer and fouler rich waters of western Indian coast.

The control of biofouling is of particular concern to modern marine engineering and shipping operations. A great deal of time and effort is expended each year to protect the ship's hulls and other submerged surfaces from fouling. The fuel crisis and the increase in the number of supertankers and the lack of adequate dry-docking facilities have been instrumental for the increased tempo of investigations for developing more effective technological solutions to the problem of marine fouling during the past 30 years. The problem of marine fouling has assumed a new dimension with the setting up of coastal industries and power plants. Paint containing antifoulants is the usual counter measure against fouling. Antifoulants prevent attachment of fouling organisms or kill them before they grow to a significant size.

Types of modern antifouling compositions are described by Bowmer and Ferrari (1989) and Foster (1994). The most widely used antifouling paints are tributyltin self polishing compositions (TBT-SPCs) . In this system, organotin is covalently bound to an acrylic polymer matrix. Hydrolization of the bonding releases the antifouling agent and solubilizes the remaining acryl polymer revealing a fresh layer of paint. By this method, a more constant leaching rate and a continuous renewal of the substratum surface are achieved. To boost the antifouling activity of organotin, additional agents like cuprous oxide, cuprous thiocyanate, tributyltin fluoride may be mixed into the composition (De, 1989). This ingenious concoction provides almost complete antifouling protection for years, saves billions of dollars yearly, and helps "reduce the green house effect" by reducing yearly fuel consumption by 7.2 million tons (Collinson and Grant, 1994).

The most effective, long term solution to marine biofouling, the self-polishing (ablative) copolymer organotin coatings – is unfortunately also the most toxic. Tributyltin, probably the most toxic substance ever released willingly into the oceans, is known to cause considerable damage to natural populations of molluscs and other organisms. Such chemicals also have the ability to concentrate in higher organisms through food chains. The toxicity of organotins and the associated environmental problems posed by their use in the marine environment have been well documented. As a result, regulations curtailing the use of organotins have been introduced in a number of countries.

The broad spectrum activity of TBT against most prokaryotes and eukaryotes is based on its inhibition of ATP synthesis. Toxicity of TBT to diverse non-target organisms has been found at exceptionally low levels (40 ng Sn/l for bivalves, 20 ng Sn/l for gastropods, 0.6 to 15 μg Sn/l for fishes). In the presence of

other toxic compounds leaching from antifouling paints or other sources, toxicity levels of TBT may be expected to be much lower still, especially with regard to the observed synergism between TBT and copper uptake rates.

Replacing TBT or copper of standard antifouling paints by other biocides like chromated copper-arsenate or antibiotics is a doubtful and possibly a dangerous undertaking. The application of nontoxic marine natural products which are capable of inhibiting the fouling of ships and other structures may potentially provide a solution to this sensitive environmental issue. Environmental concerns about the use of toxic antifoulants have led to increased interest in the development of non toxic, or non polluting alternatives (Clare *et al.*, 1992: Clare, 1995). Numerous authors have recommended to screen for natural antifoulants in marine organisms and incorporate these in appropriate paint matrices to replace the discredited TBT, copper, aresenic, etc. (Goto *et al.*, 1992; Foster, 1994; Holmstroem and Kjellberg, 1994). Use of nontoxic or limited toxicity antifoulants or environmentally benign antifoulants is of considerable interest (Target *et al.*, 1983; Standing *et al.*, 1984; Rittschof *et al.*, 1985; Keifer *et al.*, 1986; Gerhart *et al.*, 1988; Sears *et al.*, 1990).

In seeking to develop nontoxic, non polluting antifouling applications, tremendous advances were made by examining and understanding the mechanisms by which sessile and sedentary organisms mitigate the effects of the continuous biofouling pressure that they face in their natural habitats. The diversity of their antifouling arsenal is the key to their success.

Epibiosis on basibionts produce a great number of detrimental effects. Epibionts producing calcareous tissues or structures are denser than seawater. Their settlement and growth increase the weight of the living substratum and decreases the buoyancy. If the loss of buoyancy is not compensated, the basibiont

will sink and possibly meet intolerable conditions with regard to light water flow, oxygen tension and the like. Bacterial enzymes, oxygen deficiency, highly alkaline or extremely acidic pH, accumulation of toxic wastes etc. are likely to chemically attack many basibiont's surfaces.

In wave-swept habitats, algae and other sessile upright "aufuwuchs" organisms depend on flexibility and toughness for survival (Denny, 1988; Gaylord *et al.*, 1994). Any impairing of flexibility by calcareous, encrusting epibionts could be detrimental, increasing brittleness and risk of breakage. Many "aufuwuchs" community increase siltation that may lead to anoxic conditions on a basibiont's surface and contribute to the insulating effect. Water exchange near the basibiont's surface may be severely reduced by epibiosis (Wahl, 1997).

Antiepibiosis is the multitude of defence systems developed by marine organisms to protect themselves or parts of themselves against any "aufuwuchs". The ability of many sessile marine organisms to maintain an essentially clean surface in the face of competition for space by epibionts is well documented (Davis *et al.*, 1989; Wahl, 1989; Clare *et al.*, 1992; Paul, 1992; Holmstorm and Kjelleberg, 1994). Often these natural antifouling mechanisms involve a combination of physical and chemical defences (Davis *et. al.*, 1989). Many settler species discriminate between diverse available substrata. Rejected surfaces may be simply unattractive or actively repellent. Cases of nontoxic repellent activity of natural compounds towards larval settlement have been reported (Maki *et al.*, 1988).

Natural antiepibiosis (antifouling) by chemical activities have been suggested for numerous marine organisms (Bakus *et al.*, 1986; Davis *et al.*, 1989; Paul, 1992). Marine algae have yielded a wealth of noval compounds and only in a few instances antifouling role of these secondary metabolites have been provided.

Algae produce antilarval and antibacterial compounds. Seaweeds are particularly susceptible to fouling because they are sessile and are restricted to the photic zone where conditions for the growth of fouling organisms are optimal. Fouling can significantly harm seaweeds. While some seaweeds are heavily fouled, other species in the same habitat are rarely epiphytized, indicating the presence of antifouling mechanisms.

Surface mucus production is extremely widespread among marine animals and algae, (Fontaine, 1964; Ducklow and Mitchell, 1979; Krupp, 1984; Dyrynda, 1986; Thorp *et al.*, 1991). Many algae slough mucus cuticles (Sieburth and Tootle, 1981; Moss 1982; Martinez and Correa, 1993) or epidermis layers (Filion – Myklebust and Norton, 1986). Numerous organisms are known to create non neutral pH conditions at their surfaces. Baker and Orr (1986) describe highly alkaline leaf surfaces in aquatic plants and suggest that this may be an antiepibiosis adaptation.

The allelochemical effects of marine natural products have been substantiated in a wide variety of ecological situations (Bakus *et al.*, 1986). The active metabolites encompass virtually all classes of chemicals (Bakus *et al.*, 1986). Increasingly, their role in the regulation of antifouling and fouling by organisms in the marine environment is being discerned. Studies of carefully chosen systems will allow us to understand the effect and importance of naturally produced antifouling and fouling chemicals in the marine environment. It is essential to recognize that each active compound need not have only one ecological function, nor is it necessary that a given compound affect two organisms in the same manner.

A large number of compounds with antibacterial, antiviral, antifungal and pharmacological properties were isolated from micro and macroalgae, analyzed

and tested for medical purposes in the last few years. Several bioactive compounds such as bromophenols (Pedersen and Da Silva, 1973), Cyanobacterin (Mason *et al.*, 1982; Gleason and Baxa, 1986), hapalindoles (Moore *et al.*, 1987), fischerellin (Gross *et al.*, 1991), cyanobacterin LU-1 (Gromov *et al.*, 1991), galactosoyl – diacylglycerols (Murakami *et al.*, 1991), tjipanazoles (Bonjouklian *et al.*, 1991), have either antibacterial, antialgal, antifungal or antiprotozoan effects. Macrofoulers were not tested.

The bioactive compounds such as terpenes/carbohydrates, goniodomin (Sharma *et al.*, 1968), fatty acids/chlorophylls (Uchida *et al.*, 1988) and α-linolenic acid (Ohta *et al.*, 1994) isolated from special species of Dinophyceae and Chlorophyceae have inhibitory effects on the growth of either bacteria or algae.

Macroalgae like Phaeophyceae, Chlorophyceae, Conjugatophyceae and Characeae also produce bioactive compounds with antibacterial, antialgal, antiprotozoan and antimacrofouling effects. *Laminaria digitata* produce bioactive compounds having antimacrofouling effect on *Spirorbis inornatus* and also exhibiting anti-bacterial effects (Al-Ogily and Knight – Jones, 1977). *Costaria costatum* and *Undaria pinnatifida* produce glycerols which have an anti-macrofouling effect on *Mytilus edulis* (Katsuoka *et al.*, 1990).

Unsaturated fatty acids produced by *Laminaria saccharina* (Rosell and Srivastava, 1987) terpenoids produced by *Caulerpa ashmeadii* (Paul *et al.*, 1987), benzene, diethylether and petroleum ether extracts of *Ulva reticulata* (Charles and Sivalingam, 1994) showed antibacterial effects. Dithiolantrithian produced by *Chara globularis* (Wium – Andersen *et al.*, 1982) showed antialgal effect. Antiprotozoan effect was shown by tannin produced by *Spirogyra* sp. (Misra and Sinha, 1979).

The special bioactive compounds isolated from micro-and macroalgae were tested predominantly with regard to antibacterial and antialgal activities. Effects on the growth of macrofoulers were investigated only in a few cases. Halogenated furanones from the red alga *Delisea pulchra* showed activity in antifouling screens against barnacle cyprids, macrolagal germlings and a marine bacterium at concentration levels in the nannogram range (de Nys *et al.*, 1995). Extracts of the brown alga *Dictyota menstrualis*, which contain diterpene alcohols, inhibited the settlement and development of the common fouling bryozoan *Bugula neritina* (Schmitt *et al.* 1995). A sulphated phenolic metabolite, p-coumaric acid sulphate isolated from the eelgrass *Zostera marina* has been shown to have potent antifouling activity, preventing the attachment of marine bacteria, epiphytic algae, and invertebrates on submerged surfaces (Todd *et al.*, 1993).

A variety of sessile marine invertebrates contain secondary metabolites affecting the settlement of fouling organisms. It is known that representatives of Porifera, Cnridaria and Tunicata are rarely overgrown by epiphytic organisms. They frequently produce high concentrations of bioactive compounds with potent antifouling activities (Willemsen, 1991). A series of chemical compounds like lactones (Porter and Targett, 1988), fatty acids (Goto *et al.*,1992), bromopyrroles (Keifer *et al.*, 1991), peptides, steroids, homarine (Targett *et al.*, 1983), saponins and diterpenoids (Gerhart *et al.*, 1988) have been isolated from several species of Porifera, Cnidaria, Tunicata and Mollusca with antibacterial and antialgal activities which prevent the settlement of macrofouling organisms. Potent antimacrofouling activity against the blue mussel *Mytilus edulis* and the barnacle *Balanus amphitrite* was found in extracts of several sponges (*Lissodendoryx isodictyalis, Phyllospongia papyracea, Agelas conifera* and corals (*Leptogorgia virgulata, L.. setacea*). Bioassay directed purification of *Leptogorgia virgulata* extracts has

led to the identification of two diterpenoid hydrocarbons, pukalide and epoxypukalide, as antifouling agents (Gerhart *et al.*, 1988). Standing *et al.* (1984) showed that octocorals contain compounds that both stimulate and inhibit settlement of larval barnacles. Extracts of the Atlantic sea pansy *Renilla reniformis* were found to contain three new antifouling diterpenes: renilla foulins A(1), B(2) and C(3) (Keifer *et al.*, 1986).

The natural products juncellin, renillafoulin, and pukalide are potent natural product antifoulants that prevent settlement of barnacle larvae in laboratory assays. These natural products were characterized by low toxicity and high settlement inhibition (Rittschof *et al.*, 1994). Indian Ocean is a potentially rich source of bioactive compounds. Methylene chloride extracts from the gorgonian octocorals, *Solenocaulon tortuosum, Suberogorgia suberosa, Echinogorgia complexa, Juncella juncea,* and alcyoracean octocoral *Spongodes* sp. prevent larval settlement of *Balanus amphitrite* (Vitalina *et al.*, 1991 a).

Exudation of bioactive metabolites into the surrounding water is known for the Pacific soft corals *Sinularia flexibilis* and *Sarcophyton crassocaule* (Coll *et al.*, 1982). Other studies have noted that tissue fluids of gorgonians can be supersaturated with respect to cembranolides (Papastephanou and Anderson, 1982). The release of metabolites from sponges has been demonstrated in both laboratory and field trials (Young and Chia, 1981; Thompson, 1985; Walker *et al.*, 1985).

Metabolites with specific antifouling activity have been isolated from *Aplysina fistularis* (Thomson , 1985; Walker *et al.*, 1985). The *Aplysina* metabolites, aerothionin and homoaerothionin, inhibited metamorphosis of gastropod veliger larvae and caused behavioural modifications in invertebrate adults tested. Both

metabolites were exuded into the water with rates of exudation of the order of 0.7 ng / min/g dry weight.

It must be possible to develop an environmentally safe antifouling system by using antifouling compounds and applying them in antifouling paints. (Bakus *et al.*, 1986; Wahl, 1989). There have been a few attempts (Seiburth and Conover, 1965 and Standing *et al.*, 1984; Keifer *et al.*, 1986; Rittschof *et al.*, 1986;) but no applicable successes have been reported to date. The discovery and identification of compounds with biological effects specific to targeted marine fouling organisms (Avelin *et al.*, 1991; Vitalina *et al.*, 1991 a, b) and the knowledge of the effects on metamorphosis of compounds with known biochemical mechanisms, provide the basis for understanding the pathways and processes involved in settlement of marine invertebrate larvae. It is through this understanding that nontoxic or limited toxicity antifouling control can be realized (Rittschof *et al.*, 1991).

Determination of the action mechanisms of natural product antifoulants (Targett *et al.*, 1983; Standing *et al.*, 1984; Rittschof *et al.*, 1984; Keifer *et al.*, 1986; Gerhart *et al.*,1988) should provide additional flexibility for attempts at biological control. These compounds many of which are as effective or more effective than broad – spectrum, toxic antibiotics, show promise (Rittschof *et al.*, 1991).

There are several practical constraints to consider with respect to the application and implementation of chemical antifouling strategies. First, it is important to recognize that sustained release of organic biofouling inhibitors at a rate of ($\leq 5\mu g/cm^2$/day is necessary to achieve desirable coating lifetimes (Weisman *et al.*, 1992). This constraint sets minimum threshold activity levels for potentially useful metabolites.

The problem associated with natural antifoulants is that these compounds are only available in limited quantities and mass production from the natural sources is difficult. Commercial analogues of natural products could be a useful solution to this problem (Rittschof *et al.*, 1992, 1994). Moreover, the compounds are often structurally very complex and synthetic production of the entire molecule is uneconomical. Nevertheless, it is possible to identify the active sites of the molecules and develop structural analogues that can be tested for antifouling properties (Targett and Stockaj, 1994). In today's environmentally conscious world, the search for natural antifoulants and their analogues represents a laudable scientific pursuit. It is unlikely that there will be a single nontoxic, non polluting natural metabolite that will be active against the broad specturm of biofouling taxa. From the outset, consideration should be given to multiple component mixtures that act synergistically.

The sessile and sedentary organisms take advantage of mechanical, physical and chemical antifouling mechanisms that are then integrated to have a composite effect on the fouling community. In developing new antifouling applications, we should increasingly seek integrated approaches to mimic nature.

CHAPTER 1

TECHNIQUES EMPLOYED TO REAR THE TEST ORGANISMS *HYDROIDES ELEGANS* (POLYCHAETA) AND *BALANUS AMPHITRITE COMMUNIS* (CIRRIPEDE)

1.1 INTRODUCTION

Biofouling on man-made surfaces in the marine environment is controlled primarily by the antifouling paints having broad spectrum toxicity . The key to nontoxic approaches to antifouling is an intimate knowledge of the target organisms. In many marine invertebrates of diverse phylogenetic affinity, reproduction results in the production of a planktonic larval phase. Larvae of marine benthic organisms are important ontogenic link which decides the pattern of survival, abundance and distribution of adults. High fecundity, which is needed to offset mortality in species with long-lived pelagic larvae, would be expected to give rise to wide genetic variability.

Larval dissemination is necessary for the survival of many species with transient habitats. The process of attachment is the cardinal factor which controls survival of the adults. The site selection for attachment which is so essential to the survival of larvae has empowered them with remarkable powers of discrimination, their sensory equipment reaching a peak at the time of exploration and settlement (Raimondi, 1990).

Signal molecules acting as inducers of larval settlement, metamorphosis and differentiation have been shown to play a significant role in structuring the

distribution, interactions of organisms, and controlling recruitment, in the marine environment (Morse, 1991). Chemical cues in the ambient water play crucial role in the control of spawning and metamorphosis.

The planktonic larval phase with ephemeral existence can be an important developmental period fueled by exogenous (planktotrophic alecithal) or endogenous (lecithotrophic) nutrient sources leading to competence (Burke, 1983; Pechenik, 1990). Such larvae form excellent experimental material for various methods of testing including behaviour at the point of settlement. The fact that planktotrophic larvae remain competent for extended periods (Pechenik,1987) helps in preparing effective experimental protocols to analyse pre-settlement behaviour. Biochemical factors which control transition from the pelagic larval to the benthic juvenile stage can be studied experimentally with the aid of larval culture and settlement bioassays. Precise knowledge of the timing of the transition from the larval to the settled stage is critical to the experimental approach. If the expected settlement of a population can be predicted, the impact of a second variable can also be assessed. The ideal experimental test is one which enables detection of either facilitation or induction or inhibition of settlement. The development of nontoxic or non-polluting additives that can be formulated in practical antifouling coatings require assays involving target organisms. Acorn barnacles and serpulid polychaetes are major target organisms for antifouling technology. Systematic study of pre-settlement behaviour and settlement behaviour of a target species should provide the basis for the most effective level of prevention.

In recent years considerable progress has been made in rearing planktotrophic larvae. The advances in larval rearing rely largely upon the techniques

developed to improve the reliability of selected microalgal and diatom cultures on which the larvae are fed (Crisp ,1984).

If the larvae are maintained in synchronous batch culture with careful attention to time and culture conditions, the percentage of the population that will metamorphose can be predicted. The use of two types of larvae in assays provides information about the stability and toxicity of the compounds being tested. The cyprid larvae of *Balanus amphitrite* and the trochophore larvae of *Hydroides elegans* can be cultured or obtained in sufficient numbers for useful testing.

1.2 MATERIALS AND METHODS

1.2.1. Selection of test organism - *Balanus amphitrite communis*

1.2.1.1 Distribution

Barnacles are marine, sessile cirripedes. There are more than 600 species, worldwide, and many are colourful. Twenty two species of barnacles are reported from the Indian Ocean (Avelin and Sarojini, 1997). Of these seven species of barnacles including *Balanus amphitrite communis,* are frequently encountered on test panels and harbour installations. *Balanus amphitrite* is one of the most successful fouling organisms in Indian harbours (Purushotham and Rao, 1971). It is an important cirripede macrofouler in the Cochin Back waters. Barnacle settlement in Cochin harbour is very much pronounced during the post monsoon period (Meenakumari and Nair, 1984).

1.2.1.2 Significance as a fouler

Barnacles merit detailed investigation as they cause serious problems to submerged surfaces due to their surface coverage, volume and weight.

Moreover, they are highly resistant to the various toxic chemicals used in antifouling paints. Compared to other animals, barnacles are better equipped to colonize many man made structures placed in the sea than other animals. Practical experience confirm that they are the dominant forms of animal fouling . The most important economic consequence of fouling by barnacles is the increase in cost of fuel to run ships. There are also considerable economic losses when they settle inside pipes conveying seawater. Barnacles are also implicated in the process of corrosion. Micro environments beneath barnacle attachments provide conditions suitable for rapid growth of sulphate reducing bacteria, which play a significant role in the corrosion process.

With respect to biofouling control, the study of larval settlement is a study of the responses of whole animal (Rittschof *et al.*, 1991). Intervention of the metabolic pathways involved in metamorphosis of barnacle larvae is a potential means of biofouling control. Identification of pure compounds that affect larval barnacle settlement (Gerhart *et al.*, 1988; Tegtmeyer and Rittschof ,1989) could provide the molecular probes necessary to study pathways of mediation.

1.2.1.3 Life cycle

Balanus amphitrite amphitrite Darwin, reproduce as frequently as once in a week (Holm, 1990) . The *Balanus amphitrite* larvae are used in basic studies of larval settlement and metamorphosis (Clare *et al.*, 1992) and in the development and assessment of antifouling technology (Roberts *et al.*, 1991) . The synchronous mass culture of cyprid larvae is essential to conduct successful settlement bioassays.

1.2.1.3.1 Reproduction and Development

Most barnacles are hermaphrodites and carry out cross fertilization. During the incubation period, ovigerous lamellae, are transferred to the base of the

mantle cavity where they are retained throughout the period of embryonic development. Development is relatively synchronous within a brood, but hatching and release may be linked to feeding or moulting of the adult, possibly mediated by attaching substance produced by adult tissue. A single gravid individual may release over 13,000 larvae .

The life cycle of this cirripede crustracean is composed of seven free - swimming larval stages and a shelled adult stage, permanently attached to a substrate. The eggs produced within the shell of the adult, hatch out as the first naupliar stage and develop through five other stages by periodical moulting of its cuticle, each stage being larger and more complex than the preceding one. The stage VI nauplius, then, metamorphoses into the last larval stage which is the cyprid. This is a significant change in form and function into the settling stage of the life cycle. The cyprid stage is free swimming until it settles on a substrate and then itself metamorphoses to develop into a young adult barnacle.

1.2.1.3.2 Identification of the larval stages

Nauplii

The nauplius larvae represent the hatching stage and can be easily recognized by its triangular shield - shaped carapace (Fig. 1.1). The first stage nauplii hatch within the parental mantle cavity and are released. The dorsal surface of the nauplius is composed almost entirely of a unitary carapace shield or cephalic shield with a pair of prominent fronto-lateral horns and a posterior dorsal thoracic spine. Ventrally, there are three pairs of jointed appendages - the antennules, the antennae, and the mandibles. A pair of unjointed filiform appendages, extend anteriorily between the antennules. A prominent labrum lies between the antennae and overhangs the mouth. The posterioventral " post naupliar region" consists of

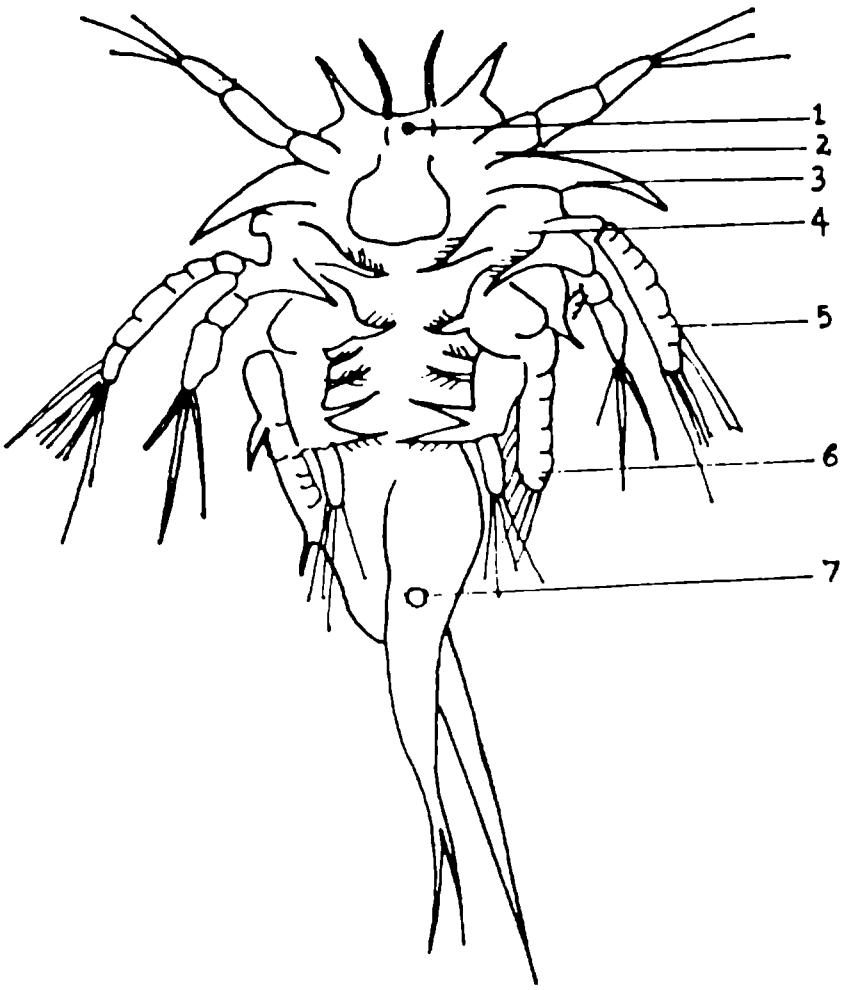


Fig. 1.1.
Morphological features of nauplius larvae of *Balanus amphitrite*
1 - naupliar eye 2 - first limb 3 - anterior horn
4 - gnathobase 5 - second limb 6 - third limb
7 - anus.

the abdominal process, which terminates in the furcal spines. The ventral surface between the mouth and post naupliar region has a complex array of small processes referred to as the setose region.

Stage I: The first naupliar stage of a healthy organism is brief. Frontal horns of the stage I nauplius are folded backwards (Fig. 1.2). It is nonfeeding with a blocked foregut and survives on the remnants of glycoprotein and lipid yolk reserves in the anterior midgut before moulting in to stage II. This ecdysis occurs after each of the six naupliar stages. Nauplii are photopositive and swim upto the surface waters thus avoiding benthic filter feeders.

Stage II : The animal assumes the gross structure that is retained to the sixth naupliar stage. Stage II is characterized by the prominent frontolateral horns, the carapace, three pairs of swimming appendages, the abdominal process with furca, and the extended caudal process (Fig. 1.2).

Stage III : During this larval stage the organism increases in size and the appendages, comprising of three pairs of antennules, antennae, and mandibles become elongated. The margin of frontolateral horns are arched with two small lateral spines (Fig. 1.2).

Stage IV: Animal grows in size, the caudal process is delimited from the carapace while the posterior edge of the carapace forms two spines (Fig. 1.2). These spines persist to the fifth and sixth naupliar stages. The abdominal process enlarges and gets tucked in so that it is not evident from dorsal or ventral views.

Stage V: Growth continues and the enlarged abdominal process becomes segmented.

Stage VI : The abdomen enlarges and in the latter phases the appendages become so well delineated that they show movement. Also in the latter phase a pair of

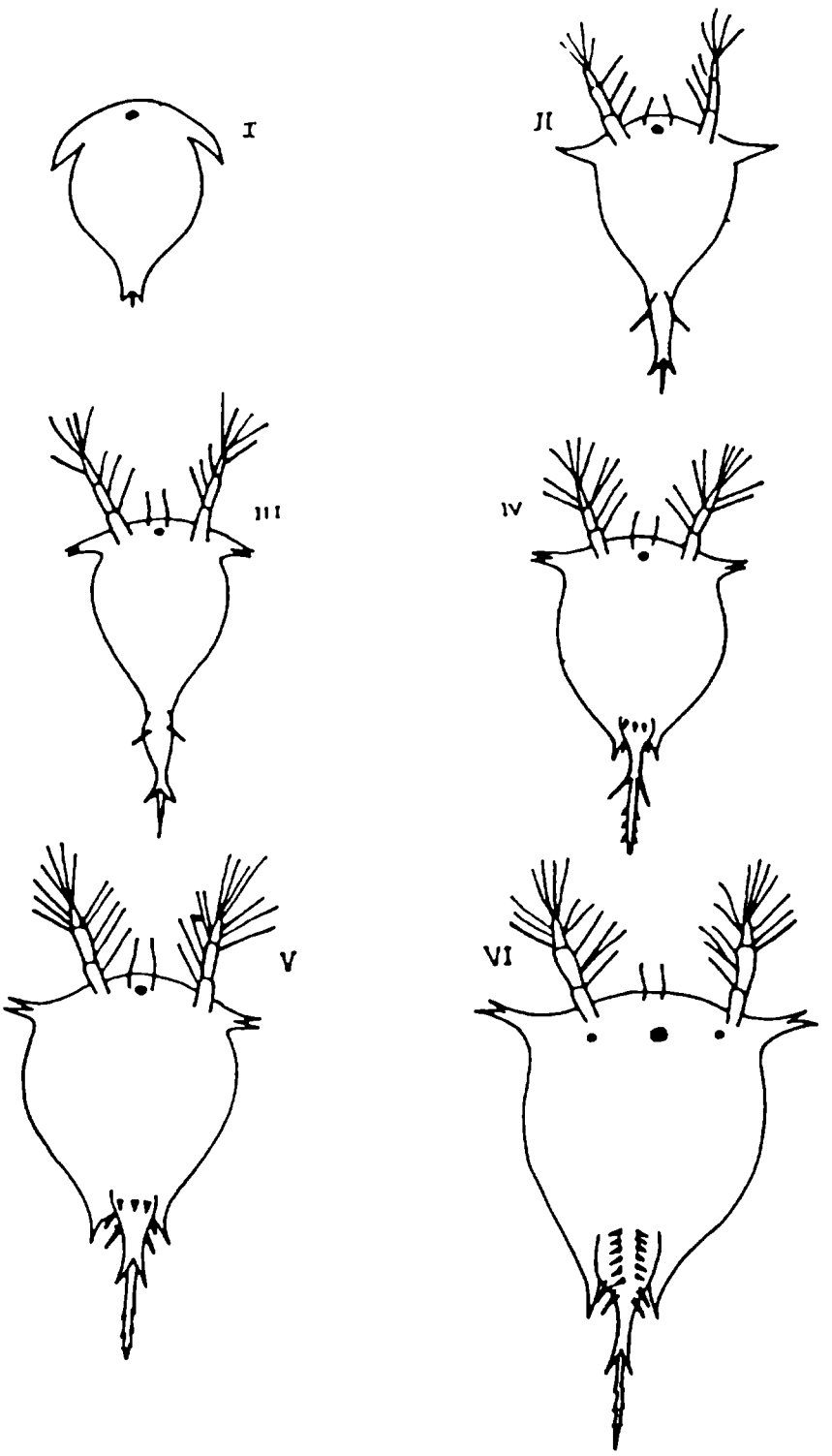


Fig. 1.2
Morphological features of *Balanus amphitrite* nauplii at each stage.

I = pear shape, frontolateral horns folded
II = plain tip
III = split tip, one preaxial seta
IV = posterior - shield margin and spines; two preaxial setae
V = three preaxial setae
VI = compound eye

compound eyes are formed in addition to the already present median naupliar eye spot (Fig. 1.2). The organism then metamorphoses to a cyprid, the process being retrogressive in nature.

Cyprid

The cyprid is a unique larval stage, its sole function being to select a site for settlement. It can survive for several weeks on reserves of triglycerides contained in oil cells which accumulate during the naupliar stages (Holland and Walker, 1975). The cyprid has a bivalved carapace with an anterior and posterior mantle cavity (Fig. 1.3). The anterior one encloses the prominent antennules with their attachment discs which are used in walking, while the posterior one encloses the thoracic appendages which are used in swimming and are later modified to form the adult feeding organs - the cirri. The cyprid, like most final stage larvae, is more richly endowed with sense organs than at any stage in the life history.

Cyprid sense organs

The barnacle represents the peak of evolution of sessile animals and its larvae therefore provide the best illustration of the sensory adaptations and pattern of behaviour during settlement. Settlement behaviour of the barnacle cyprid stage has been studied extensively.

The median naupliar eye persists in the cyprid and the adult stages, but the compound eyes, formed during the last naupliar stage, are present during the cyprid stage only - presumably they are used to orient the cyprid more precisely to light during settlement (Barnes *et al.*, 1951). During settlement the cyprid larvae is negatively phototropic, and the eye may be used to locate shaded areas which however is not correct as barnacles are found to settle abundantly in the lighted

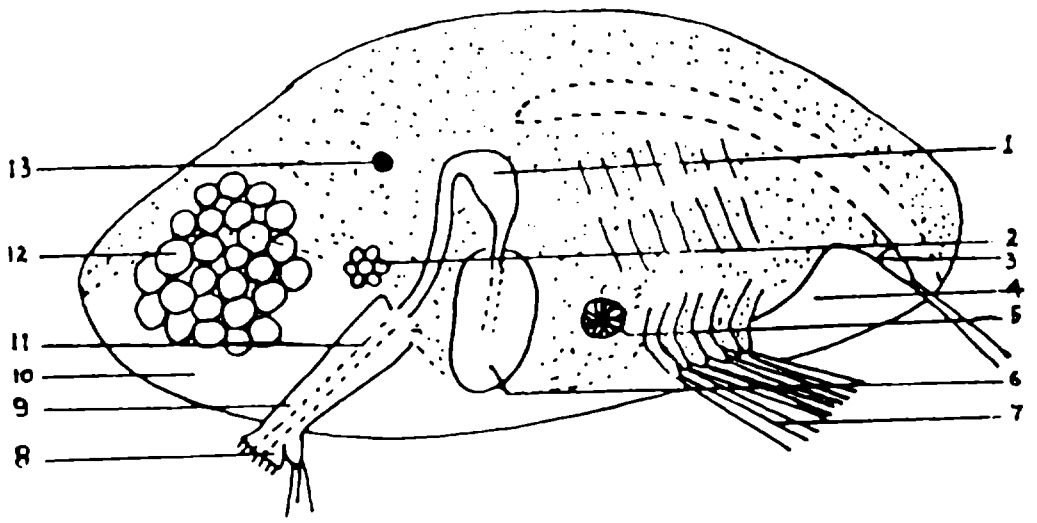


Fig. 1.3
Anatomy of the cyprid.

1 - musicular sac
2 - compound eye
3 - caudal appendage
4 - posterior mantle cavity
5 - adductor muscle
6 - cement gland
7 - thoraiic appendage
8 - adhesive disc
9 - antennule
10 - anterior mantle cavity
11 - cement duct
12 - oil cell
13 - naupliar eye

zone of the intertidal and subtidal realm. The surface of the carapace is provided with numerous small sensory setae, which possibly detect water flow. Tactile setae extend from the terminal point of each antennule. The long caudal appendages appear to be used to determine contour, for the cyprid can sometimes be seen attached by the anterior end with the caudal appendages describing an arc in the manner of a pair of dividers. Crisp and Barnes (1954) has described the great appreciation of surface contour by the cyprid. On the ventral side of the cyprid, there are two sensory filaments, possibly containing pressure or inertial receptors (Walker, 1974). Most spectacular of all cyprid structures, are the attachment discs in the penultimate segment of the antennule (Nott and Foster, 1969). These organs are not only used for locomotion, but are believed to be responsible for the recognition of specific molecular structures on surfaces. At metamorphosis all these sense organs disappear with the sole exception of the naupliar eye.

1.2.1.3.3 Habitat selection

Field observations and experiments on barnacles as well as laboratory choice experiments showed that barnacle cyprids are strongly gregarious (Crisp, 1974). For barnacles, that are obligate cross fertilizers, potential partners need to be within the range of the extensible penis of the 'acting male' for fertilization to occur (Anderson, 1994). As the adult is sessile, proximity is achieved at the cypris stage through gregarious settlement behaviour. Chemical cues evoking barnacle larvae to settle and metamorphose are produced by juvenile and adult nonspecific (Crisp and Meadows, 1962; Crisp, 1990). Barnacle cyprids are rugophilic (Yule and Walker, 1984), settle more densely in pits and grooves (Crisp and Barnes, 1954) and in the shade.

The chemical basis of gregarious settlement behaviour in barnacles has long been attributed to the detection of an adult glycoprotein called arthropodin by a tactile chemical sense (Crisp and Meadows, 1962; Gabbott and Larman, 1987; Clare and Matsumura, 2000). More recently, evidence has been presented to support the existence of an additional waterborne cue during the settlement of cyprid larvae of the barnacle *Balanus amphitrite*, cypris settlement (Rittschof, 1985). Barnacle water borne settlement pheromone (BWSP) (Clare and Matsumura, 2000), is a 3 - 5 k Da peptide with a basic carboxy terminus and a neutral, or basic, amino terminus.

The duration of the exploratory phase of the life cycle varies between species. To avoid being dislodged by shear forces whilst exploring a substratum, the cyprid employs a temporary adhesive, which is secreted by unicellular antennulary glands (Nott and Foster, 1969). This temporary adhesive acts as a settlement pheromone (Walker and Yule, 1984) raises the possibility that the adhesive and settlement inducing protein complex (SPIC) are related (Clare and Matsmura, 2000).

Exploratory behaviour (Crisp, 1984) is complex and poorly understood, but at some point the cyprid commit itself to permanent fixation to the substratum by secreting a cement from the paired cement glands (Walker, 1971; Okano et al., 1996, 1998). A pair of kidney shaped cement glands, ventrolaterally positioned, is the location of cement storage and secretion (Walker, 1971). Histochemical and electron microscopic studies in *Balanus balanoides* (Walker, 1971) have shown that, when attachment occurs, cement in each gland is secreted into a median collecting duct transported via a cement duct and released onto the surface of the antennule attachment disc. The muscular sac, located at the proximal portion of the cement duct, is believed to help expel cement by pumping action. As the cyprid

has finite energy reserves (Walker, 1995), settlement is eventually compromised (Satuito *et al.,* 1996) and there may be adverse effects on post-metamorphic development (Pechenik *et al.*, 1993).

A hypothetical sequence of larval behaviour has been illustrated by Crisp (1984) for a barnacle cyprid. At the time of settlement, the larva responds sequentially to light, current, presence of conspecifics, surface contour, surface hardness, and proximity of conspecifics. If a physical or biological condition is unacceptable to the larva, it returns to its previous position in the sequence and continues its exploratory behaviour.

In common with other arthropods, metamorphosis of the cypris larva into the juvenile barnacle is under endocrine control. At least two hormones are thought to be involved, 20 hydroxyecdysone (20E) and methyl farnesoate (MF). Under normal physiological conditions, a reduction in MF levels presumably allows an altered pattern of gene expression essential to metamorphosis. Such gene expression may in part be modulated via protein kinase C activation.

1.2.1.3.4 Cement Secretion

For barnacles which are gregarious in nature, the signal transduction pathway for settlement inducing protein complex (SIPC) effects permanent attachment through secretion of the cyprid cement. There is now good evidence to suggest that dopamine modulates the exocytotic release of cement from the cement glands into the median - collecting duct (Okano *et al.,* 1996). It is not clear what causes the transport and release of cement from the collecting duct to the exterior via the attachment disc, although the involvement of a muscular sac and a valve has been postulated (Nott and Foster, 1969).

The settled cyprid undergoes extensive changes to become a permanently attached barnacle (photograph 1.1). The basal membrane or calcareous plate attached to the substratum by a layer of adhesive cement. Walls of calcareous plates enclose the viscera and thoracic appendages. Testes develop in the tissues around the gut, and ovaries develop in the connective tissue lining the mantle cavity.

1.2.2 Culture of larvae of *Balanus amphitrite communis*

Rittschof *et al.* (1984, 1992) have proved that cultures of larvae of *Balanus amphitrite* can be maintained throughout the year. Studies on the developmental stages of several barnacle species have been conducted by Costlow and Bookhout (1957), Barnes and Costlow (1961), and Rittschof *et al.* (1984, 1992). Pioneering work on fouling using barnacle larvae was set forth by Freiberger and Cologer (1966). A few rearing techniques have been described by Karandae and Thomas (1971) for the barnacles of Indian waters.

1.2.2.1 Factors affecting larval development

Development and survival of larval populations reared in the laboratory are affected by fluctuations in the temperature of the culture medium. Experiments have shown that the development of larvae of *Eliminus modestus* and *Balanus balanoides* is dependent on temperature. At lower temperatures slower development and size increase of the larvae results (Crisp, 1962). Tolerance to temperature fluctuations was found to be species specific. High mortality was recorded at temperatures above 23°C for *E. modestus* and 28°C for *B. amphitrite.*

Light is necessary for the normal larval development. Larvae reared under dark conditions appear to be retarded. Development time for cyprid larvae

of *Elminus modestus* reared at 16°C in dark was found to be 17 days while those reared at constant illumination took 12 days for development. A cycle of 15 hours of light (500 lux) to 9 hours darkness is necessary for the normal growth of the larvae of *Balanus amphitrite* (Rittschof *et al.*, 1992). The influence of temperature was found to be greatest on the second instar.

1.2.2.2 Water treatment

Water quality is a major concern in the successful culture of larval barnacles. The sea water used for experimental work was collected from Kannamali area in the Arabian sea 15 kms off Cochin. The water was stored in total darkness for about a week and the particulate fractions were allowed to settle. The sea water used for the larval culture was filtered using fibre glass filter, containing glass wool and activated charcoal. The coarse filtered seawater was allowed to stand without aeration, in dark storage containers at room temperature, for seven days.

The seawater used for the culture was sanitized using recommended antibiotics 21.9 mg/ml of Sodium Penicillin G and 36.5 mg/ml of Streptomycin Sulphate base were the antibiotics (Rittschof., 1992). Both antibiotics were made up in the same stock solution.

1.2.2.3 Culture of food organism - *Skeletonema costatum*

In the marine environment, phytoplankton, chiefly diatoms, dinoflagellates and flagellates are of primary importance in the aquatic food chain, forming the primary trophic level and serving as food for larger animals. Availability of live diatoms and flagellates in the laboratory is a prerequisite to maintain a culture of larvae of important macrofouling organisms like the barnacle *Balanus amphitrite.* Morse (1963) compared the food values of different algae for a number of barnacle

species and concluded that *Skeletonema costatum* is the best suited live algal feed for the larvae of *Elminius modestus.*

Cultures of *Skeletonema* past their prime as food sources show clumping, decreased chain size, smaller cell size and can actually trap larvae with their sticky exudates. This diatom is an ideal food source of barnacle nauplii as it gives easily recognizable indications of its quality (Rittschof, 1992). Light intensity was carefully controlled to ensure the quality of the diatom culture. Autoclaved sea water containing f/2 (Guillard, 1975) nutrient with 30‰ salinity was used for the diatom culture.

1.2.2.4 f/2 Medium Constituents in one litre seawater

Major nutrients:

$NaNO_3$	75 mg.
$NaH_2PO_4.H_2O$	5 mg.
$Na_2Sio_3.H_2O$	15.30 mg.

Trace metals:

Na_2 EDTA	4.36 mg.
$Fecl_3.6H_2O$	3.15 mg.
$CuSO_4.5H_2O$	0.01 mg.
$Zn\ SO_4.H_2O$	0.022 mg.
$CoCl_2.7H_2O$	0.01 mg.
$MnCl_2.4H_2O$	0.18 µg
$Na_2\ Mo\ O_4.2H_2O$	0.006 mg.

Vitamins

Thiamin HCl	0.1 mg
Biotin	0.5 mg
B_{12}	0.5 mg

1.2.2.5 Brood stock collection, larval release and culture

Terra - cotta panels of dimensions 22.5 x 22.5 cm were hung at sub-tidal levels in the Cochin backwaters at a pier located in the Cochin harbour. The barnacles that settled on the panels were allowed to grow for a period of 60 days. After this duration, the panels were transported to the laboratory and cleaned thoroughly off detrital matter and silt settled on them. The adult barnacles were carefully removed without damaging the basal plate and were gently crushed and placed in a sterile beaker. Millipore filtered sea water was added so as to immerse the crushed barnacles and the water level was kept around 20cm above the crushed cirripedes. The nauplii released from the brood pouches were allowed to concentrate at the top of the beaker by directing a strong beam of light. The larvae were collected using a pasteur pipette in a 1 L beaker containing 500 ml filtered seawater. These larvae were used for rearing. To determine the density of nauplii in the container, two 1 ml aliquotes of stirred larvae were killed using freshwater and counted using a dissection microscope. The nauplii were cultured at an initial density of one per ml in 2 L of aged, seawater containing streptomycin and penicillin at 36.5 mg/ml and 21.9 mg/ ml respectively. The culture was aerated gently, covered and incubated at a constant temperature (28°C) and light regime of 15:9 light (intensity, 500 lux) and dark. Larvae were fed with 125 ml of *Skeletonema costatum* at a density of 2 x 10^6/ml/day. After four days the culture was monitored for cyprids.

1.2.2.6 Collection of Cyprids

Cyprids can be visually differentiated from nauplii by observing their movement. Nauplii move very fast by the opening and closing of antennule while cyprids show jerky dart-like movements. After around 50% of the larvae metamorphosed to cyprids, the culture was filtered through a 300, 230 and 169 μm filter in series to separate cyprids from nauplii. The following precautions were taken while separating the cyprid larvae.

1. Cyprids and nauplii collected on the sides of the glass beaker were periodically rinsed down.
2. After the siphoning of water, the beaker and the siphon were rinsed thoroughly and allowed to pass through the filter to collect the available cyprid larvae on the sides of the beaker and the siphon.
3. Filters were rinsed clear of debris. This process was done quickly to avoid injury to the cyprids due to desiccation. Each filter was inverted over a bowl and the larvae were rinsed out using filtered seawater.

Nauplii were retained by the 300 μm screen and the cyprids were collected either in the 230 μm or 169 μm filter. Nauplii on the 300 μm filter was returned to the glass beaker and kept at 28°C for further development. Cyprids were put in a bowl containing 500 ml of filtered sea water and stored in the dark at 6°C until used. The containers were covered to reduce evaporation. At room temperature, cyprids quickly attach and metamorphose in the culture bowl. It was found out by trial and error that at 6°C the cyprid larvae did not derive any change in their morphology. Cyprids which were 3 days old were used in the settlement inhibition assays (Rittschof *et al.*,1984, 1992). The cyprids were transferred to 6°C within half an hour.

1.2.3 Selection of test organism - *Hydroides elegans* (Haswell)

1.2.3.1 Distribution

Hydroides elegans (Haswell) is an ubiquitous serpulid polychaete with a cosmopolitan distribution (Chandramohan *et al.*, 1997). It is one of the dominant species of biofouling communities in the Arabian Sea, attaching itself to hard substrata. It is a dominant fouler in the Cochin backwaters and shows an advanced rate of settlement during post monsoon months (Meenakumari and Nair, 1994).

1.2.3.2 Significance as a fouler

Rapid and dense colonization of submerged surfaces, natural and experimental, by *Hydroides elegans* has been recorded in India (Sasikumar *et al.*, 1989). In many locations, 100% coverage of this organisms is found on unprotected submerged surfaces, such as the hulls of ships and intake seawater conduits of coastal factories and power plants that use seawater for cooling purpose.

The accumulation of these serpulids in great masses on the bottom and propellers of ship may be more troublesome than barnacles in certain localities. Such coverage creates drag on ships, increase in fuel consumption, hull corrosion and reduction in the cruising speed and manoeuvrability (Fischer, *et al.*,1984). They may also cause blockage of water front installations and are found to be highly tolerant to chlorine. These encrustations are the result of settlement of larvae of *Hydroides elegans.* So the most effective way to prevent fouling by *H. elegans,* is to prevent larvae from settling on the surface. Development of any antifouling strategy demands a clear understanding of the biology, larval behaviour and toxicity aspects of *Hydroides* sp. among which *Hydroides elegans* is the most common serpulid encountered in tropical waters.

1.2.3.3 Life Cycle

Chandramohan and Aruna (1994) have described the biology of serpulid worms . Collar setae of *Hydroides elegans* have a finely toothed blade and long basal fin that has one or two large teeth as well as a few smaller ones. It is a strictly sexual, dioecious polychaete with external fertilization. Fertilization occurs in sea water within 1 to 2 hours of release of gametes and the fertilized eggs hatch into typical trochophore stages in 10 to 14 hours (Wisely, 1958). The planktotrophic trochophore attains metamorphic competence in six days. The settlement process is preceded by the secretion of a mucoid adhesive from the posterior end of the larvae with which it attaches itself to the substratum. The calcareous tube of this species is cylindrical, faintly wrinkled and fragile.

1.2.3.4 The trochophore

The trochophore larva has a hemispherical episphere and a tapered hyposphere, the two being separated by the prototroch. An apical tuft of several cilia, feeding cilia, a metatroch and a neurotroch are present (Fig. 1.4). The feeding cilia extend in a band around the larva and beat towards the mouth, the metatroch is post oral in position and the neurotroch extend ventrally towards the posterior end. The mouth is followed by a short stomodaeum that dilate into a large stomach and narrow out into an intestine opening posteriorly through the anus. A tuft of cilia project from alongside the anus, which lay dorsally; the anal vesicle has a prominent nucleus is usually traversed by one or two threadlike structures. At this stage only the right eyespot is present.

In the presence of copious food the trochophore gains size rapidly during the initial 48h, total length approaching *ca* 210 μ and breadth 165 μ. Notable

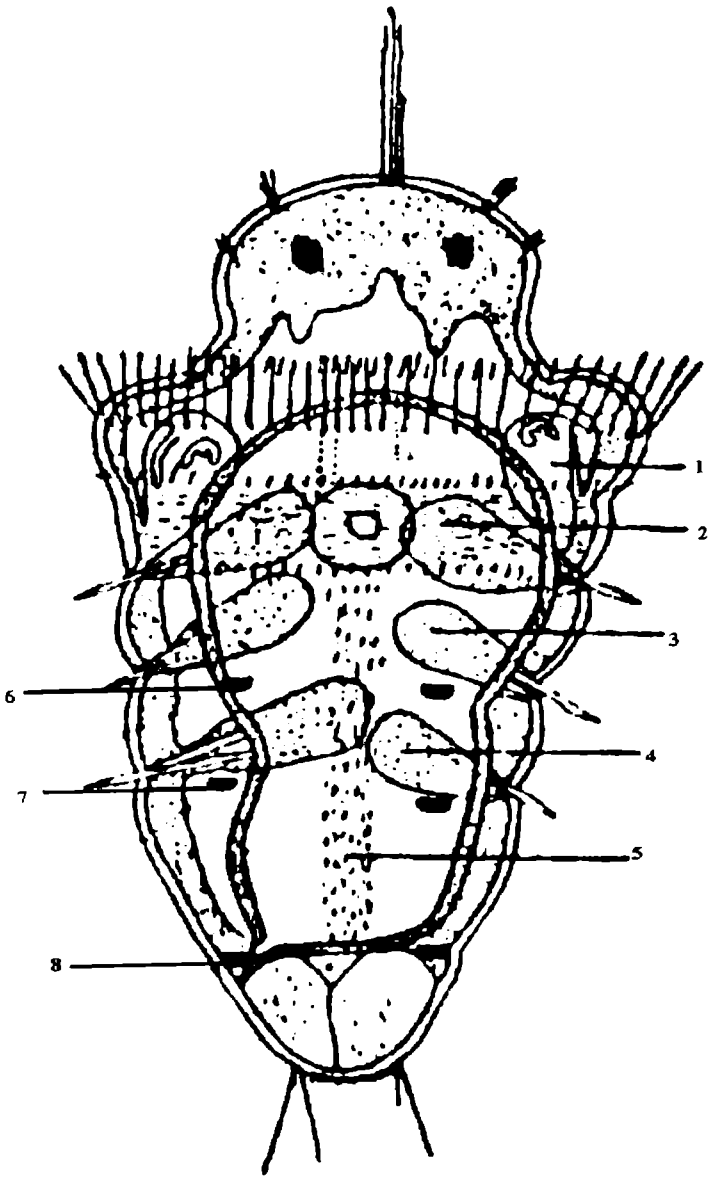


Fig. 1.4
Morphological features of trochophore larvae of *Hydoides elegans*.

1 - collar	2 - 4 - chaetal sacs
5 - neurotroch	6 - 8 - uncini

changes at this stage include the addition of the left eyespot and the differentiation of the prototroch into three rings, viz. 1) a ring of short cilia anteriorly 2) a ring of long (the original) cilia on the middle of the prototroch region, and 3) a ring of short cilia in the depression between the prototroch region and the prospective first setigerous segment.

The planktotrophic trochophore larvae attain metamorphic competence in six days; then the movements become sluggish. The head become spatulate and cilia appear on each side of the head in the vicinity of the eyes. Considerable differentiation and increase of length occurs in the trunk region. Three setigerous segments appear simultaneously, each of which possesses a pair of chaetal sacs. Each sac has two setae, the larger ones of the first pair already bearing the conical processes, characteristic of the genera *Hydroides* and *Serpula*. Uncini are absent behind the first pair of chaetal sacs but are present between the second and the third. At the posterior end there is a pair of uncini which do not appear to be accompanied by setae; they lay laterally to the anterior boundary of the anal vesicle.

Substratum selection by serpulids is a specific and not a random process (Hadfield, 1994). The pre-settlement larvae of *Hydroides elegans* spend much of their time swimming across submerged surfaces, repeatedly contacting the surfaces with the apical - tuft region.

Metamorphically competent larvae prefer biologically filmed surfaces to clean ones in a nearly absolute manner (Photograph 1.2). Additionally they distinguish quantitatively more densely filmed surfaces (Hadfield *et al.*, 1994).

Holm *et al.* (1998) studied the signal transduction pathways involved in settlement of *Hydroides elegans* . No evidence was obtained to substantiate the involvement of G protein - linked receptors, or a phosphotidylinositol (PI) signalling

pathway. Results were also inconsistent with regard to adenyl cyclase (AC)/ cAMP signalling, although the phosphodiesterase inhibitor, isobutyl methylxanthine, whose action raises intracellular cAMP levels (Clare *et al.,* 1995) induced metamorphosis (Bryan *et al.,* 1997; Holm *et al.,* 1998; Pechenik and Qian, 1998). Instead, the involvement of lectin binding receptors, ligandgated ion channels (Hadfield, 1998; Holm *et al.,* 1998), or transmembrane enzyme receptors (Hadfield, 1998) has been postulated. Optimum salinity is a requisite for the settlement, growth and breeding of this species. At metamorphosis, the settled larva undergoes several transformations in which the prototroch and metatroch are lost; the larva sticks to the substratum and is no longer able to swim. Branchial rudiments appear and subsequently a tube is formed. The secretion of the tube is a continuous process and the growth of the tube is by the addition of calcified rings glued to the already existing tube.

1.2.3.5 Factors affecting the development of *H. elegans* larvae.

Effect of salinity, temperature and food concentration on early development of the polychaete *Hydroides elegans* (Haswell) were examined in the laboratory by Qiu and Qian (1997). Temperature had no effect on survivorship within the experimental range (15 - 30°C) but low temperature led to longer duration of development. Temperature, does not seem to be a limiting factor for early development and settlement of *H. elegans*. Low salinity and low food concentration reduced survivorship, settlement and lengthened the duration of development.

1.2.4 Culture of Larvae of *Hydroides elegans*

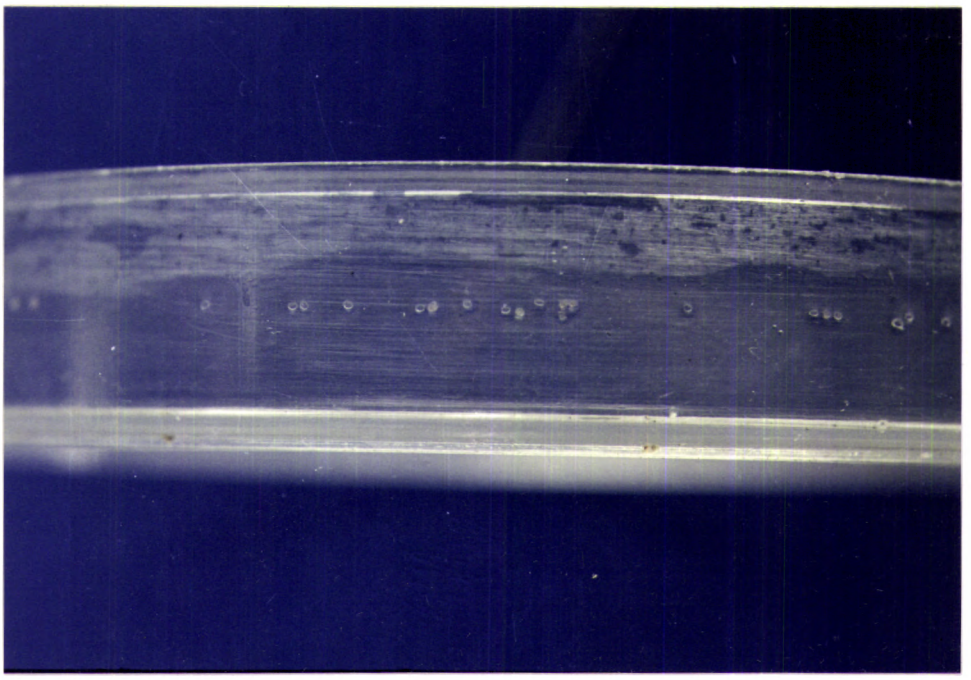
Terra cotta panels of dimensions 22.5 cm x 22.5 cm were suspended at sub-tidal levels in the cochin harbour. The polychaetes that settled on the panels were allowed to grow for a period of 60 days. After this duration the panels were

transported to the laboratory and cleaned thoroughly. Adult animals were carefully removed from the panel.

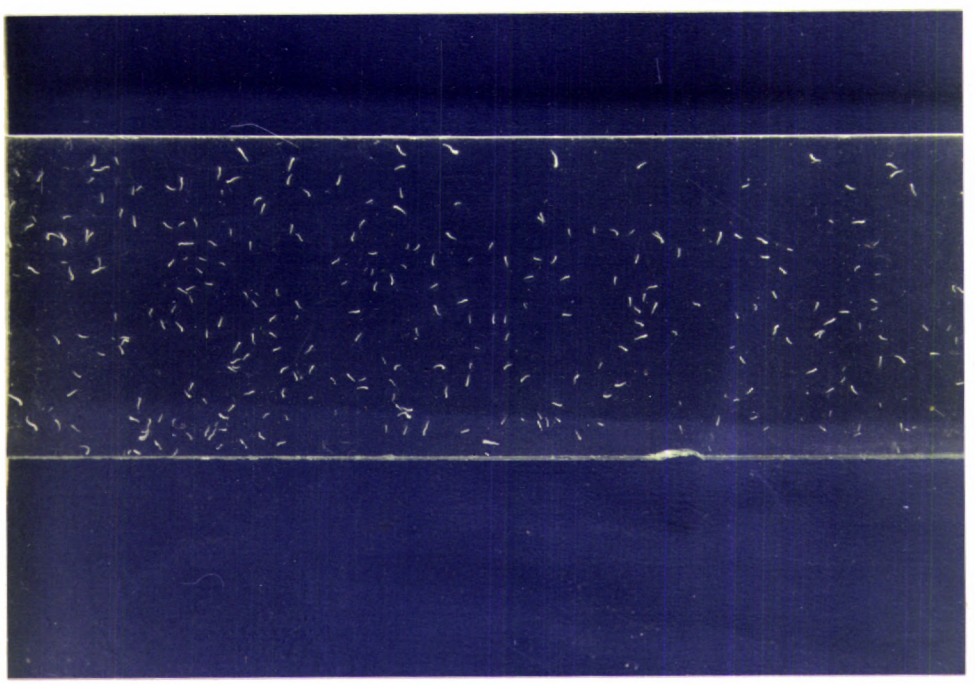
Hydroides elegans larvae were reared to metamorphic competence in six days using methods described by Hadfield *et al.*, (1994). The polychaete tubes containing paired male and female worms were gently broke open and were put into finger bowls containing aged, filtered seawater (salinity 30‰). Both males and females released gametes into the seawater where fertilization rapidly occurred. After an hour when all the eggs had settled to the bottom of the finger bowl, they were transferred to 2 L beakers containing aged filtered seawater, and the temperature of the sea water was maintained at 28°C throughout the development. *Isochrysis galbana* was used as the food source (Hadfield *et al.*, 1994). As the young trochophore larvae disperse fairly evenly in the sea water, they could be then decanted off to another glass beaker and separated from unfertilized or abnormally developed. The trochophore larvae were cultured at an initial density of 5 to 10 larvae per ml. Every 24h, 50ml of uni-algal culture of *Isochrysis galbana* (6 x 10^5 cells/ml) was given as food. The fully developed (six day old) larvae showed a tendency to remain near the bottom of the containers. They moved sluggishly and glided slowly at the bottom of the container. The fully mature trochophore larvae which showed pre settlement behaviour were used in the settlement bioassays.

1.3 RESULT

Nauplii of *Balanus amphitrite communis* fed on *Skeletonema costatum* metamorphosed into cyprids on the fourth day when cultured in the laboratory at 28°C. The trochophore larvae of *Hydroides elegans* attained metamorphic competence in six days in the laboratory. The phytoplankton *Isochrysis galbana* served as food for the trochophore larvae.



Photograph 1.1
Settlement pattern of cyprid larvae of *Balanus amphitrite* on the petridish.



Photograph 1.2
Settlement pattern of trochophore larvae of *Hydroides elegans* on biofilmed glass slide

1.4 DISCUSSION

Barnacle nauplii and mass cultured settlement stage larvae were used in quantitative laboratory assays of toxicity and barnacle settlement inhibition. Larvae can be cultured predictably on *Skeletonema costatum*. This diatom is an excellent food when raised in Tris - free Guillard medium (Guillard, 1975) at 19°C and with illumination of approximately 100-200 μ E.cm^{-2} 5^{-1} (Rittschof *et al.*, 1992). Too rapid a growth rate of the diatom results in a culture that will not support the larvae. Barnacle larvae that are cultured on the correct food develop to cyprids in 4 days and behave and settle predictably with respect to time (Rittschof *et al.*, 1984). Predictability is lost if the salinity changes more than three parts per thousand of the optimum (Rittschof *et al.*, 1992). When artificial seawater is employed, calcium ion concentration shall maintained (Rittschof *et al.*, 1986).

During the development rearing technique, supplementary food and its concentrations were found to be important factors in growth rate and survival. The diatom, *Skeletonema costatum* concentration used in rearing vessels were relatively high, 5x10^{5}cells/ml seawater. Using this concentration, the larvae invariably have a full gut. Morse (1963) showed that the growth rate of barnacle larvae is dependent on the amount of food present in the water. Freiberger and Cologer (1966) successfully used a concentration of approximately 2 x 10^5 cells/ml of the diatom *Cyclotella nana for rearing larvae of Balanus eburneus, Balanus amphitrite, Balanus trigonus,* and *Balanus imprivisus*. Rittschof *et al.*, (1992) used a 2 x 10^6 cells/ml of diatom *Skeletonema costatum* for rearing larvae of *Balanus amphitrite, Balanus amphitrite amphitrite* and *Balanus variegatus.*

It was found in agreement with Rittschof *et al.*, (1992) that for the normal development of the larvae of *Balanus amphitrite communis* a cycle of 15 hours of light and 9 hours of darkness is necessary.

Balanus amphitrite is an excellent model organism for studies of antifouling compounds because of its rapid larval development, the ease of raising synchronous mass culture, and the predictable settlement in static conditions (Branscomb and Rittschof, 1984). The model is relevant because the adult animal is an important fouling species. The larvae of other balanoid barnacles and *Elminius modestus* as well as those of a chthamalid barnacle, *Chthamalus fragilis* (Rittschof *et al.*, 1992) have been cultured . None is comparable to *Balanus amphitrite* with respect to ease of culture and laboratory experimentation. The other balanoid barnacles tested were less synchronous in culture and were much more variable in settlement tests. The culture of *Chthamalus fragilis* is slow and inconsistent and successful settlement of this barnacle has not been achieved in the laboratory.

Being a tropical / sub-tropical barnacle, development from egg to the cyprid stage of *Balanus amphitrite* is comparatively rapid (Rittschof *et al.*, 1984). When cultured in the laboratory at 28°C, an environmentally realistic temperature for this species, and fed on *Skeletonema costatum* Greville, larval development typically takes 4 to 5 days.

Hydroides elegans provides a very good model system for biological fouling control studies due to the following reasons. It is cosmopolitan in its distribution, creates hazardous impact on man made objects put in the ocean, and its larvae are easy to rear in the laboratory. Density of the phytoplankton *Isochrysis galbana* in the culture containers determine the duration of the development of larvae of *Hydroides elegans* (Qiu and Qian 1997). Light has no influence in the development of the larvae. In the marine environment, larvae of *Hydroides elegans*

rapidly settle and metamorphose in response to acceptable surface bound bio-organic films (Harder, T. and Qian, P.Y. 1999). The observation that the pre-settlement larvae searched for cues from the bio film for settlement is in agreement with Hadfield *et al.* (1994). By transferring metamorphically competent larvae to glass beaker devoid of biofilm we can delay settlement of the pre-settlement stage of trochophore. Young trochophores are not positively phototropic. They remain evenly dispersed in containers eventhough eyespot is present. Beakers containing abnormally heavy densities of larvae shows a slight positive phototropism during the fifth day.

CHAPTER 2

SETTLEMENT PATTERN OF THE LARVAE OF *HYDROIDES ELEGANS* (POLYCHAETA) AND *BALANUS AMPHITRITE COMMUNIS* (CIRRIPEDE) IN THE PRESENCE OF SECONDARY METABOLITES OF SEAWEEDS

2.1 INTRODUCTION

Environmental concerns about the use of toxic antifoulants have led to increased interest in the development of nontoxic or nonpolluting alternatives (Clare *et al.*, 1992; Clare, 1995). A promising avenue of approach to this problem has been the study of the mechanisms by which selected sessile and sedentary marine organisms, which are persistent in their habitats and continuously subjected to biofouling pressure, mitigate the growth of biofoulers on their surfaces (Wahl, 1989; Paul, 1992).

Many of the macroalgae and seagrasses share the characteristic of sessile and sedentary animals that enhance the likelihood of the presence of chemical antifouling strategies, ie., they are perennial and/or persistant for extended periods in their habitats and negatively impacted by biofoulers.

The negative effects of epiphytes are shading the anchor-species, impeding gas and nutrient exchange and thereby decreasing its growth rate and increasing drag on the fronds (Sand - Jensen, 1977; Silberstein *et al.*, 1986). Moreover, heavy encrustation of kelp blades by bryozoans can lead to loss of the

blades to predatory fishes, which can not get the animal prey without taking the algae.

Some algae, including *Ulva, Enteromorpha*, and *Cladophora*, may avoid epiphytism simply because of their very rapid growth and their changes in pH at the thallus surface caused by a rapid metabolic rate (Hartog, 1972). Many macrophytes deter epiphytes either through periodic sloughing off of their body surfaces or by the production of antibiotic chemicals.

Marine algae are an extensive and prolific source of secondary metabolites. More than 600 natural products have been isolated from seaweeds, and majority of these have come from tropical algae (Faulkner, 1984a, 1986, 1987, 1988). In general, these compounds occur in relatively low concentrations, ranging from 0.2% to 2% by dry weight (Paul and Fenical, 1986; Hay and Fenical, 1988). Secondary metabolites contain derivatives of simple phenolics, such as coumarins of green algae (Hay and Fenical, 1988), halogenated phenols in red algae (Fenical, 1975), and phlorotannins in brown algae (Ragan and Glombitza, 1986). Phlorotannins may be halogenated or sulphated (Ragan and Glombitza, 1986).

Majority of the macroalgal compounds are terpenoids, especially sesqui- and diterpenoids. Acetogenins, including unusual fatty acids, constitute another common class of seaweed secondary metabolites. Most of the remaining metabolites result from mixed biosynthesis and are often composed of terpenoid and aromatic portions. The greatest variety of secondary metabolites is probably found among the red algae (Rhodophyta), in which all classes of compounds are represented and many metabolites are halogenated (Fenical, 1975; Faulkner, 1984a, 1986, 1987, 1988)

Most of these compounds are bioactive and have been extensively studied using laboratory and pharmacological assays (Faulkner, 1984, 1986; Paul and Fenical, 1987). However, their natural functions under ecologically realistic conditions have been investigated only recently (Steinberg, 1985, 1988; Targett *et al.*, 1986; Hay *et al.*, 1987a, b, 1988a, b; Paul ,1987; Paul *et al.*, 1987; Paul and Van Alstyne, 1988; Hay and Fenical, 1988; Van Alstyne, 1988).

An enhanced understanding of the natural functions, effects, and mechanisms of action of seaweed secondary metabolites would provide a biologically - based rationale for the productive development of antifouling compounds based on marine natural products.

Many possible defensive functions for algal secondary metabolites have been proposed, including antimicrobial, antifouling, and antiherbivore. The role of these compounds as defence against herbivores is best known. Studies have clearly shown that many seaweed natural products function as feeding deterrents (Hay and Fenical, 1988); however, many compounds may also have other roles or may function simultaneously as defences against pathogens, fouling organisms, and herbivores thereby increasing the adaptive value of these metabolites. Some algal secondary metabolites do show antimicrobial (Almodovar, 1964; Caccamese *et al.*, 1980; Hodgson, 1984) or antifouling (Seiburth and Conover, 1965; McLachlan and Craigie, 1966; Al-Ogily and Knight-Jones, 1977) effects. Algae produce antilarval and antibacterial compounds.

Several specific metabolites have been identified with antifouling activity. Halogenated furanones from the red alga *Delisea pulchra* showed activity in antifouling screens against barnacle cyprids, macroalgal germlings and a marine

bacterium at concentration levels in the nannogram range (de Nys *et al.,* 1995) Phlorotannins and some other algal products, such as stypotriol and stypoldione from *Stypopodium zonale* and lansol from *Neorhodomela* larix which appear to be released from the algae act as antifoulants, perhaps under stress (Hay *et al.*, 1988b).

Other marine plants have also shown evidence of antifouling strategies. Extracts of the brown algae *Dictyota menstrualis* which contain diterpene alcohols, inhibited the settlement and development of the common fouling bryozoans *Bugula neritina* (Schmitt *et al.*, 1995). It is interesting to note that dictyols are produced by numerous brown algae in the order Dictyotales (Faulkner, 1991) . Algae that produce these compounds are often very abundant in a wide variety of divergent habitats in both temperate and tropical latitudes.

Phlorotannins from brown algae have long been implicated in antifouling (Sieburth and Conover, 1965; Valiela and Buchsbaum, 1991) and the brominated phenol, lansol, exuded by red alga *Rhodemela larix* may act to control biofoulers (Phillips and Towers, 1982 a,b).

A sulphated phenolic metabolite, p-coumaric acid sulphate isolated from the eelgrass *Zostrea marina* has been shown to have potent antifouling activity, preventing the attachment of marine bacteria, epiphytic algae, and invertebrates on submerged surfaces (Todd *et al.*, 1993). Comparisons of sulphated and nonsulphated derivatives suggest that the presence of the sulphate ester is necessary for the antifouling activity. Other allelopathic effects of algal secondary metabolites have also been demonstrated (de Nys *et al.*, 1991).

2.2 MATERIALS AND METHODS

2.2.1 Collection of seaweeds

The seaweeds used in the study were collected from the intertidal rocky areas of Kovalam and Tuticorin by handpicking, diving and snorkelling.

Kovalam: Kovalam (8°22'N; 76 ° 57'E) is situated on the south-west coast of India. It has a typical rocky shore extending fairly to a large area, consisting of hard granite rock formations. This coast is enriched with thick algal vegetation.

Tuticorin: Tuticorin (9°5 'N; 78° 12 'E) is located on the south-east coast of India. The shore is mainly comprised of sand, interposed with rocky formation in certain areas. Rocky areas harboured seaweed vegetation.

2.2.2 Processing of seaweeds

The collected seaweeds were washed thoroughly to eliminate extraneous matters and air dried. Then seaweeds were dried in the oven at 40 ±2°C for 2 hours and ground to powder.

2.2.2.1. Preparation of extract

Seaweed secondary metabolites were extracted applying protocols for extraction of antifoulants as per Rittschof *et al.* (1992) .Powdered seaweed was soaked in HPLC grade methanol. The methanol extraction was done with the minimum amount of methanol required to completely immerse the material being extracted. The material was extracted for one day, with occasional agitation. The extract was decanted and filtered through Whatman No.1 filter paper. The residue

was re extracted for an additional day and the extracts combined to give a crude methanol extract. This was concentrated to dryness using rotary evaporator. When sodium chloride precipitated, the methanol extract was redissolved in required volumes of 80% methylene chloride: 20% methanol to leave a white sodium chloride precipitate. The sodium chloride was removed by filtration, rinsing with methylene chloride/ methanol mixture.

The methylene chloride/ methanol solution was dried in a rotary vacuum evaporator. The dried crude extracts were kept in refrigerator until further use.

2.2.2.2 Fractionation

The crude methanol extract was diluted with half its volume of water, giving a 2:1 methanol: water solution. This aqueous methanol solution was extracted three times in a separating funnel with 1/3 volumes of methylene chloride. Each extract was filtered through Whatman No.1 filter paper and the extracts were then combined and concentrated in a rotary vacuum evaporator.

2.2.2.3 Preparation of Test substances

Assays testing effects of solvents showed that cyprid settlement was inhibited by small amounts of organic solvents. (Rittschof *et al.*, 1985) Therefore, samples for bioassay were dried under vacuum to remove solvents.

In order to maximize dispersion, substances in organic solvents were spread in a thin film over the bottom of a glass stock container. In general, only enough substance to generate sufficient solution for the planned dilution series was added to the container. After the solvent has been removed under vacuum, filtered

seawater was added and mixed thoroughly with a Pasteur pipette for 2 min in every 30 minutes. After 2h, serial dilutions were made from the stock and the biological tests were initiated.

2.2.3. Settlement Assay with Cyprid larvae of *Balanus amphitrite communis*.

Barnacle settlement assays were patterned as per Rittschof *et al.* (1992). The effects of seaweed secondary metabolites on settlement of barnacle larvae were tested using three days old cyprids of *Balanus amphitrite communis.* Seaweed extracts were screened at a concentration of 1000 mg. ml^{-1} for activity in barnacle settlement assays. In an assay, 25 to 30 cyprids were pipetted into six control and six experimental dishes (polystyrene petridishes), each containing 20 ml of solution with minimal dilution. Covered petridishes were incubated for 22 h in a controlled environment of 28 ° C. Assays were always initiated at 11 a.m. After incubation, the dishes were observed for signs of toxicity. Cyprids were scored as dead if they did not move or its, appendages were extended. Unattached larvae were rinsed with deionized water and transferred to another petridish. Permanently attached and free larvae were counted with the aid of a dissecting microscope. When appropriate, a distinction was made between attached and metamorphosed cyprids. Frequencies of barnacles permanently attached and not permanently attached in filtered seawater without additions (controls) were tested against frequencies of larvae in the same two categories in seawater with additions. Comparison was done by t-test. Bioassays using crude methanol extract which showed significant inhibition ($P<0.05$) when compared with settlement frequencies in seawater without addition to seawater containing test substance were subjected to more detailed assays using methanol and methylene chloride fractions. Such assays were designed to

determine the amount of secondary metabolites required for 50% inhibition of settlement. (effective concentration corresponding to 50% maximal response = EC_{50}). Dilution series and controls for EC_{50} assays were run in six replicate in sterile polystyrene petridishes.

2.2.4 Toxicity Assays using *Balanus amphitrite communis* nauplii.

The naupliar toxicity of the extract was evaluated as per the method of Rittschof *et al.* (1992). Studies have shown that nauplii and cyprids exhibit similar responses to many toxicants. Continuous swimming is characteristic of nauplii and so the inability of nauplii to stay in the water column was scored as the toxic response (Rittshof *et al.*, 1992). Nauplii were collected 2 hours prior to the commencement of toxicity assay. A series of dilutions of the seaweed extracts were made in aged filtered seawater. Seawater without added test substance served as the control. 5ml aliquots of each dilution was pipetted into test tubes . For the assay $\approx$ 100 µl of seawater containing 20 -30 numbers of stage II nauplii were added. Nauplii were not counted at the time of addition. After 24 hours of incubation at room temperature ($28^{\circ}C$) assay results were quantified . First, the test tubes were held up in the light and the part of the dilution series in which the nauplii had stop swimming was determined. All tubes with nauplii present in the water column were counted to determine the number of live and dead, using a chambered petridish. Non swimming larvae were regarded as dead and the data were expressed as a 24 h LC_{50} with a 95% confidence interval. Eight concentrations, each with six replicates were tested. Care was taken to use larvae from the same batch of nauplii..

2.2.5. Settlement assays with trochophore larvae of *Hydroides elegans*

The Hadfield *et al.*, method (1994) was used as the guideline for designing the experiment . Crude methanol extracts of seaweeds were screened at a concentration of 1000 $\mu g\ m^{-1}$ in the *Hydroides elegans* trochophore settlement assays. In the assay, about 150 metamorphically competent (six days old) trochophore larvae were pipetted into control and experimental dishes containing 25 ml of solution and a biofilmed glass slide. Biofilmed glass slides were prepared by immersing glass slides in seawater for three days. The biofilmed glass slide provided the larvae a positive settlement cue (Hadfield *et al.*, 1994). Five replicates of polystyrene petridishes were utilized for both control and experimental dishes. Dishes were covered and incubated for 24 hours after adding 1 ml of the phytoplankton, *Isochrysis galbana* as food. Permanently attached and free larvae were counted with the help of a dissecting microscope. When appropriate, a distinction was made between attached and metamorphosed larvae. The total number of larvae which had settled was computed as a percentage of the total number of larvae introduced and compared with that of the percentage of the total number settled in control dishes. Crude methanol extract that showed significant settlement inhibition were subjected to more detailed assays. Such assays were designed to determine the amount of polar and non polar extracts required for 50% inhibition of settlement (effective concentration corresponding to 50% maximal response = EC_{50}). Required concentrations of methanol and methylene chloride extracts were prepared and the settlement bioassays were conducted as described above. Five replicates of each of the eight concentrations were tested using larvae from the same batch. Controls were invariably run along with experiments.

In order to determine the survivorship *of Hydroides elegans* larvae in the presence of the sea weed extracts, percentage of survivors in experimental dishes were compared with that of control after seven days (Walters *et al.*,1996).

2.2.6 Statistical analysis

Concentrations of test agents giving 50% inhibition of settlement (the effective concentration for 50% inhibition or EC_{50} and LC_{50} were calculated by the computer software SPSS, based on probit technique of Finney (1971). Non swimming larvae were regarded as dead in the naupliar toxicity assay and the data were expressed as 24 h LC_{50} with a 95% confidence interval. *Hydroides elegans* larval survivorship assays of the larvae and juveniles were expressed as 7 day LC_{50}. Regression equation were worked out taking logarithm of the concentration as independent variable (x) and percentage of larval survival as the dependent variable (p). All experiments were conducted in triplicate.

2.3 RESULTS

The present study has dealt with the changes in larval survival and settlement rate of sedentary organisms such as *Balanus amphitrite communis* and *Hydroides elegans* in relation to the presence of different concentrations of seaweed secondary metabolites in the medium. The results are presented in figures 2.1 to 2.24 and tables 2.1 to 2.112.

Certain antifouling agents produce LC_{50} values that are similar in magnitude to EC_{50} values obtained in settlement inhibition assays. This evidence suggest that the antifouling properties of the molecules are a consequence of their toxicity. For other seaweed secondary metabolites, however, the toxicity assay LC_{50} values are several orders of magnitude higher than the settlement inhibition EC_{50}.

TABLE 2.1

Effect of methanol accommodated secondary metabolites of *Caulerpa scalpelliformis* on the survival of larvae of *Balanus amphitrite.*

Concentration (μ g/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	100.0	0.0
100	83.3	8.8
1000	66.7	11.1

TABLE 2.2

Effect of methanol accommodated secondary metabolites of *Caulerpa scalpelliformis* on the settlement of larvae of *Balanus amphitrite.*

Concentration (μg/ml)	% settlement	S.E.M.
Control	60.4	11.5
0.0001	59.3	11.6
0.001	58.5	11.6
0.01	56.5	11.7
0.1	55.6	11.7
1	53.9	11.8
10	53.9	11.8
100	51.9	11.8
1000	51.7	11.8

TABLE 2.3

Effect of methylene chloride accommodated secondary metabolites of *Caulerpa scalpelliformis* on the survival of larvae of *Balanus amphitrite*.

Concentration (μ g/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	94.8	5.2
100	29.4	10.7
1000	0.0	0.0

TABLE 2.4

Effect of methylene chloride accommodated secondary metabolites of *Caulerpa scalpelliformis* on the settlement of larvae of *Balanus amphitrite*.

Concentration (μg/ml)	% settlement	S.E.M.
Control	65.0	11.2
0.0001	23.0	9.9
0.001	16.7	8.8
0.01	13.3	8.0
0.1	13.3	8.0
1	13.3	8.0
10	13.3	8.0
100	5.0	5.1
1000	0.0	0.0

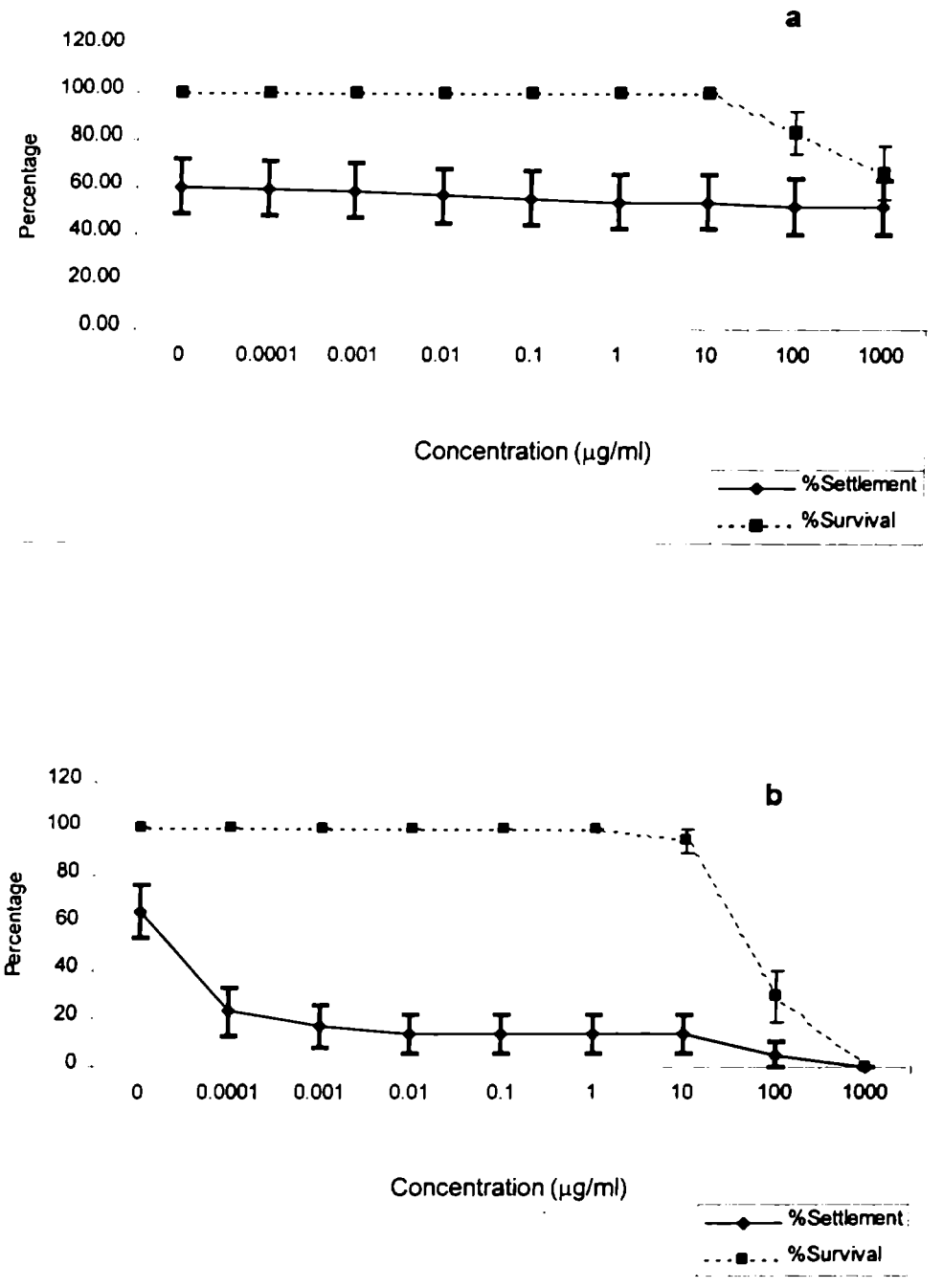


Fig. 2.1

Effect of secondary metabolites of *Caulerpa scalpelliformis* accommodated in (a) methanol, (b) methylene chloride on the survival and settlement of larvae of *Balanus amphitrite*

TABLE 2.5

Effect of methanol accommodated secondary metabolites of *Chaetomorpha linoides* on the survival of larvae of *Balanus amphitrite*.

Concentration (μ g/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	100.0	0.0
100	100.0	0.0
1000	0.0	0.0

TABLE 2.6

Effect of methanol accommodated secondary metabolites of *Chaetomorpha linoides* on the settlement of larvae of *Balanus amphitrite*.

Concentration (μg/ml)	% settlement	S.E.M.
Control	60.0	11.6
0.0001	60.0	11.6
0.001	57.0	11.7
0.01	55.0	11.7
0.1	53.7	11.8
1	53.7	11.8
10	53.0	11.8
100	52.0	11.8
1000	22.2	9.8

TABLE 2.7

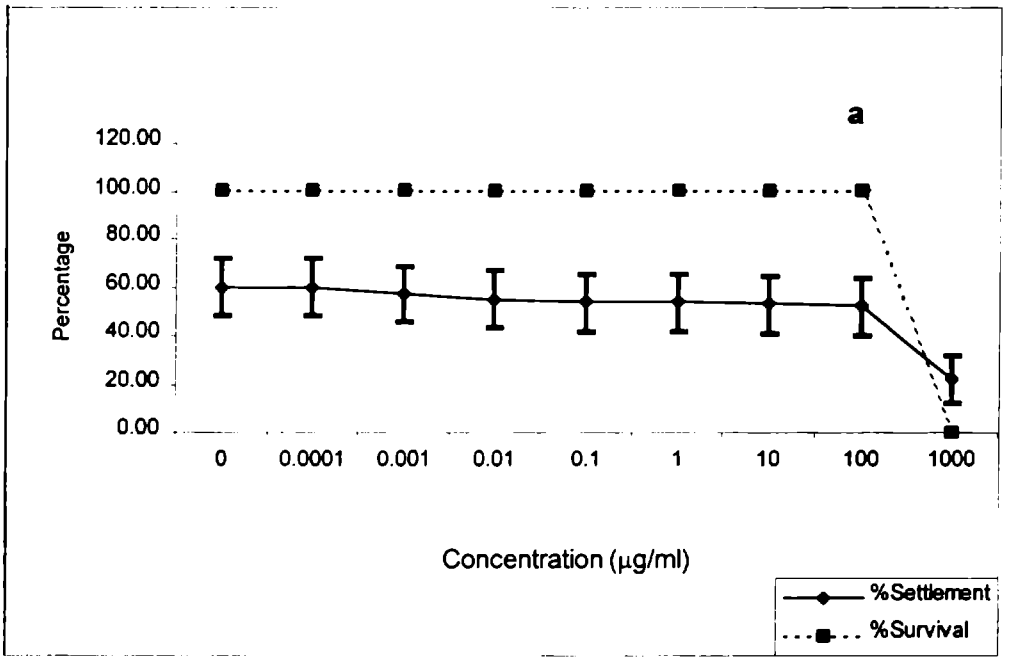
Effect of methylene chloride accommodated secondary metabolites of *Chaetomorpha linoides* on the survival of larvae of *Balanus amphitrite.*

Concentration (μg/ml)	% survival	S.E.M.
Control	100.00	0.0
0.0001	100.00	0.0
0.001	100.00	0.0
0.01	100.00	0.0
0.1	100.00	0.0
1	100.00	0.0
10	88.15	7.6
100	10.00	7.1
1000	0.00	0.0

TABLE 2.8

Effect of methylene chloride accommodated secondary metabolites of *Chaetomorpha linoides* on the settlement of larvae of *Balanus amphitrite.*

Concentration (μg/ml)	% settlement	S.E.M.
Control	62.0	11.4
0.0001	10.0	7.1
0.001	0.0	0.0
0.01	0.0	0.0
0.1	0.0	0.0
1	0.0	0.0
10	0.0	0.0
100	0.0	0.0
1000	0.0	0.0



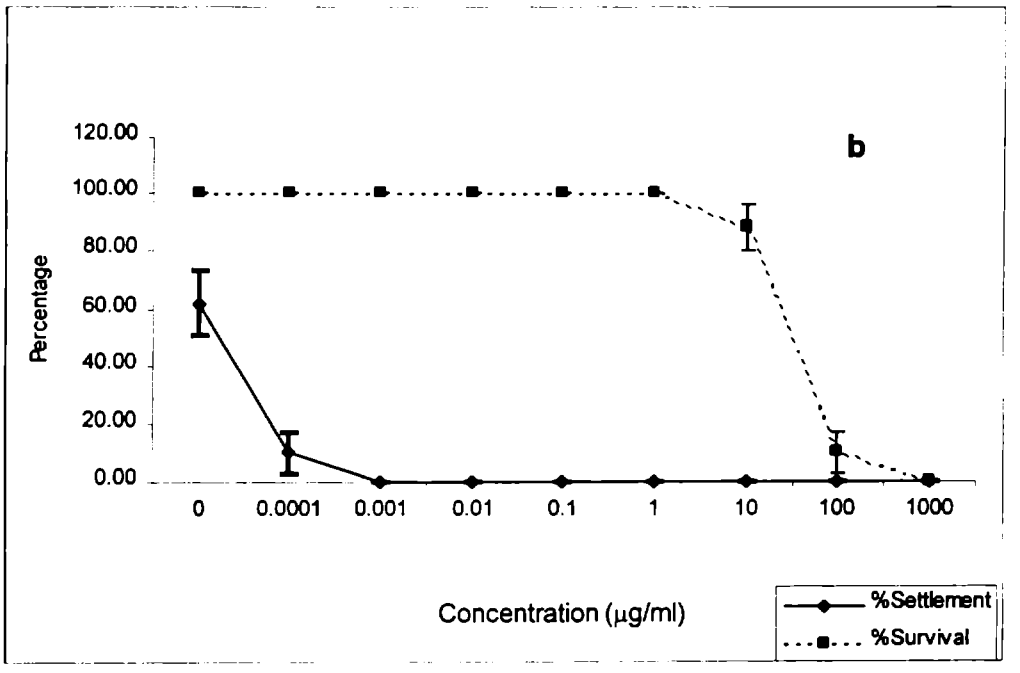


Fig. 2.2

Effect of secondary metabolites of *Chaetomorpha linoides* accommodated in (a) methanol, (b) methylene chloride on the survival and settlement of larvae of *Balanus amphitrite*.

TABLE 2.9

Effect of methanol accommodated secondary metabolites of *Gracilaria corticata* on the survival of larvae of *Balanus amphitrite*.

Concentration (μg/ml)	% survival	S.E.M.
Control	100.00	0.0
0.0001	100.00	0.0
0.001	100.00	0.0
0.01	100.00	0.0
0.1	100.00	0.0
1	100.00	0.0
10	100.00	0.0
100	85.00	8.4
1000	0.00	0.0

TABLE 2.10

Effect of methanol accommodated secondary metabolites of *Gracilaria corticata* on the settlement of larvae of *Balanus amphitrite*.

Concentration (μg/ml)	% settlement	S.E.M.
Control	65.0	11.2
0.0001	55.0	11.7
0.001	50.0	11.8
0.01	47.0	11.8
0.1	43.0	11.7
1	40.9	11.6
10	40.0	11.6
100	37.0	11.4
1000	0.0	0.0

TABLE 2.11

Effect of methylene chloride accommodated secondary metabolites of *Gracilaria corticata* on the survival of larvae of *Balanus amphitrite*.

Concentration (μ g/ml)	% survival	S.E.M.
Control	89.3	7.3
0.0001	70.9	10.7
0.001	62.0	11.4
0.01	62.4	11.4
0.1	55.6	11.7
1	56.5	11.7
10	40.0	11.6
100	35.9	11.3
1000	0.0	0.0

TABLE 2.12

Effect of methylene chloride accommodated secondary metabolites of *Gracilaria corticata* on the settlement of larvae of *Balanus amphitrite*.

Concentration (μg/ml)	% settlement	S.E.M.
Control	63.0	11.4
0.0001	41.7	11.6
0.001	30.0	10.8
0.01	18.0	9.1
0.1	11.3	7.5
1	11.3	7.5
10	0.0	0.0
100	0.0	0.0
1000	0.0	0.0

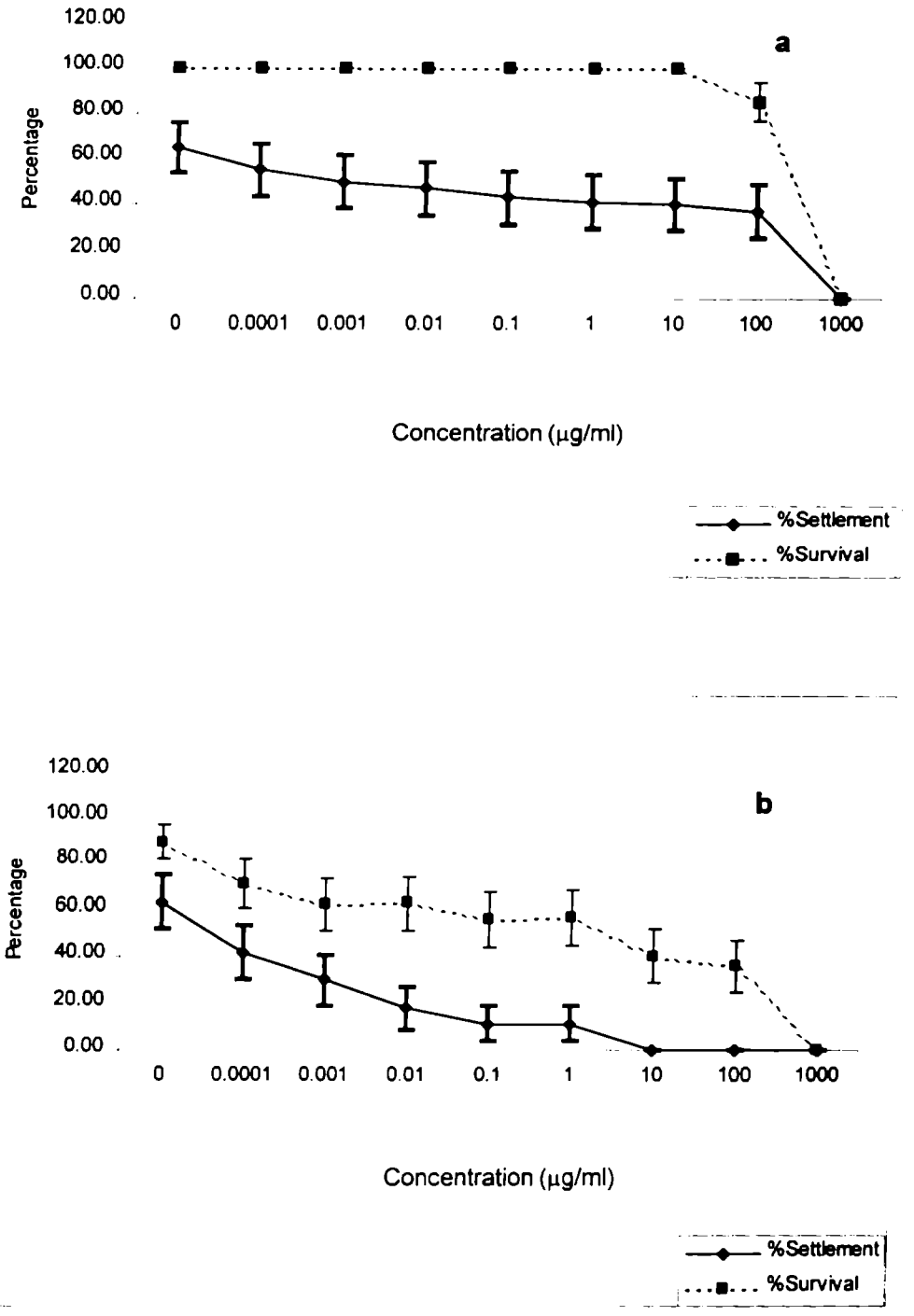


Fig. 2.3

Effect of secondary metabolites of *Gracilaria corticata* accommodated in (a) methanol, (b) methylene chloride on the survival and settlement of larvae of *Balanus amphitrite*

TABLE 2.13

Effect of methanol accommodated secondary metabolites of *Gracilaria lichenoides* on the survival of larvae of *Balanus amphitrite*.

Concentration (μg/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	93.2	6.0
10	86.7	8.0
100	0.0	0.0
1000	0.0	0.0

TABLE 2.14

Effect of methanol accommodated secondary metabolites of *Gracilaria lichenoides* on the settlement of larvae of *Balanus amphitrite*.

Concentration (μg/ml)	% settlement	S.E.M.
Control	60.9	11.5
0.0001	26.9	10.5
0.001	26.9	10.5
0.01	26.9	10.5
0.1	26.9	10.5
1	26.9	10.5
10	3.9	4.6
100	3.9	4.6
1000	0.0	0.0

TABLE 2.15

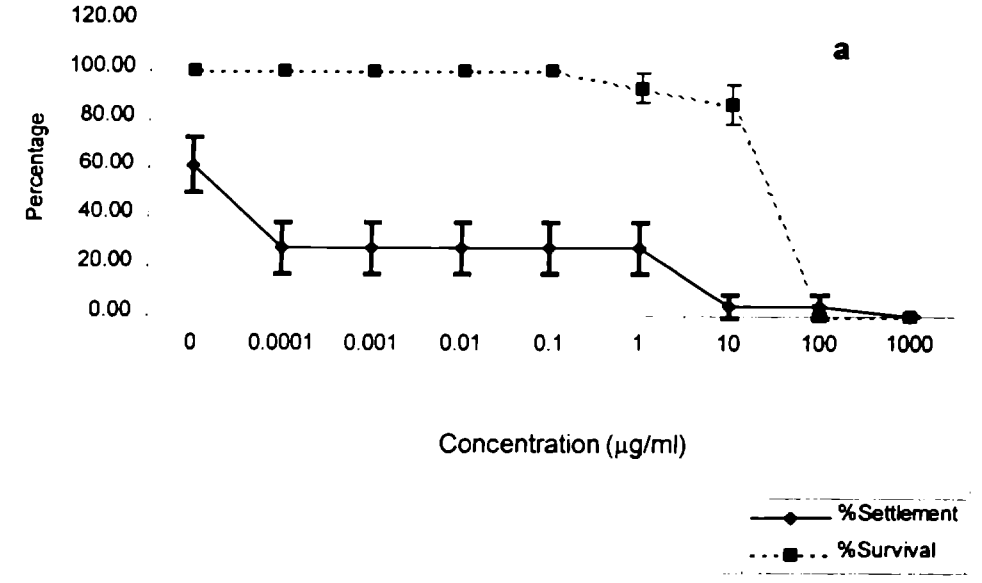
Effect of methylene chloride accommodated secondary metabolites of *Gracilaria lichenoides* on the survival of larvae of *Balanus amphitrite*.

Concentration (μ g/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	96.1	4.6
0.1	96.0	4.7
1	91.7	6.5
10	83.3	8.8
100	0.0	0.0
1000	0.0	0.0

TABLE 2.16

Effect of methylene chloride accommodated secondary metabolites of *Gracilaria lichenoides* on the settlement of larvae of *Balanus amphitrite*.

Concentration (μg/ml)	% settlement	S.E.M.
Control	65.9	11.2
0.0001	10.0	7.1
0.001	5.0	5.1
0.01	3.3	4.2
0.1	3.3	4.2
1	0.0	0.0
10	0.0	0.0
100	0.0	0.0
1000	0.0	0.0



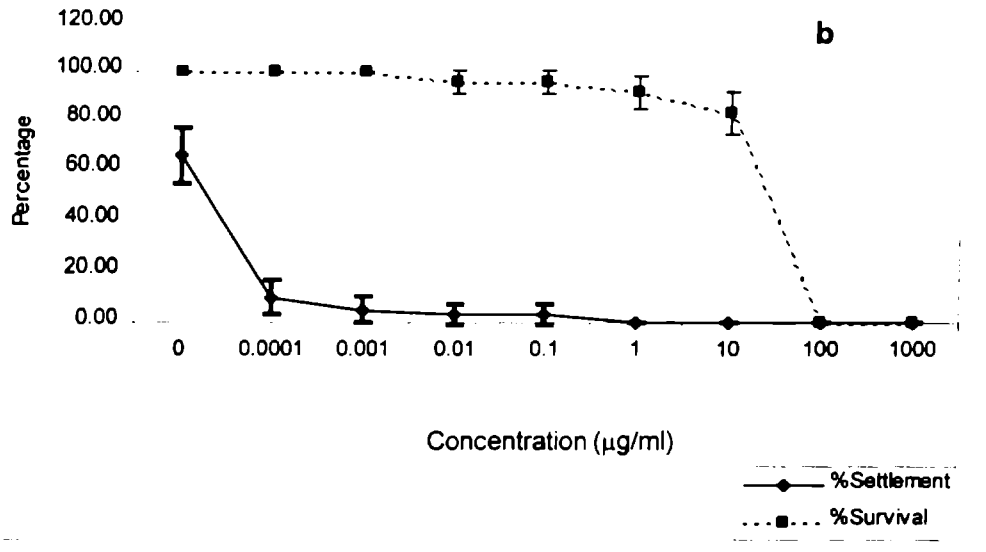


Fig. 2.4

Effect of secondary metabolites of *Gracilaria lichenoides* accommodated in (a) methanol, (b) methylene chloride on the survival and settlement of larvae of *Balanus amphitrite.*

TABLE 2.17

Effect of methanol accommodated secondary metabolites of *Padina australis* on the survival of larvae of *Balanus amphitrite*.

Concentration (μ g/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	100.0	0.0
100	36.3	11.3
1000	0.0	0.0

TABLE 2.18

Effect of methanol accommodated secondary metabolites of *Padina australis* on the settlement of larvae of *Balanus amphitrite*.

Concentration (μg/ml)	% settlement	S.E.M.
Control	66.7	11.1
0.0001	68.7	10.9
0.001	68.7	10.9
0.01	68.7	10.9
0.1	66.7	11.1
1	53.7	11.8
10	53.7	11.8
100	0.0	0.0
1000	0.0	0.0

TABLE 2.19

Effect of methylene chloride accommodated secondary metabolites of *Padina australis* on the survival of larvae of *Balanus amphitrite.*

Concentration (μ g/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	100.0	0.0
100	100.0	0.0
1000	6.7	6.4

TABLE 2.20

Effect of methylene chloride accommodated secondary metabolites of *Padina australis* on the settlement of larvae of *Balanus amphitrite.*

Concentration (μg/ml)	% settlement	S.E.M.
Control	60.0	11.6
0.0001	33.3	11.1
0.001	20.9	9.6
0.01	18.7	9.2
0.1	12.6	7.8
1	12.6	7.8
10	11.7	7.6
100	11.7	7.6
1000	11.7	7.6

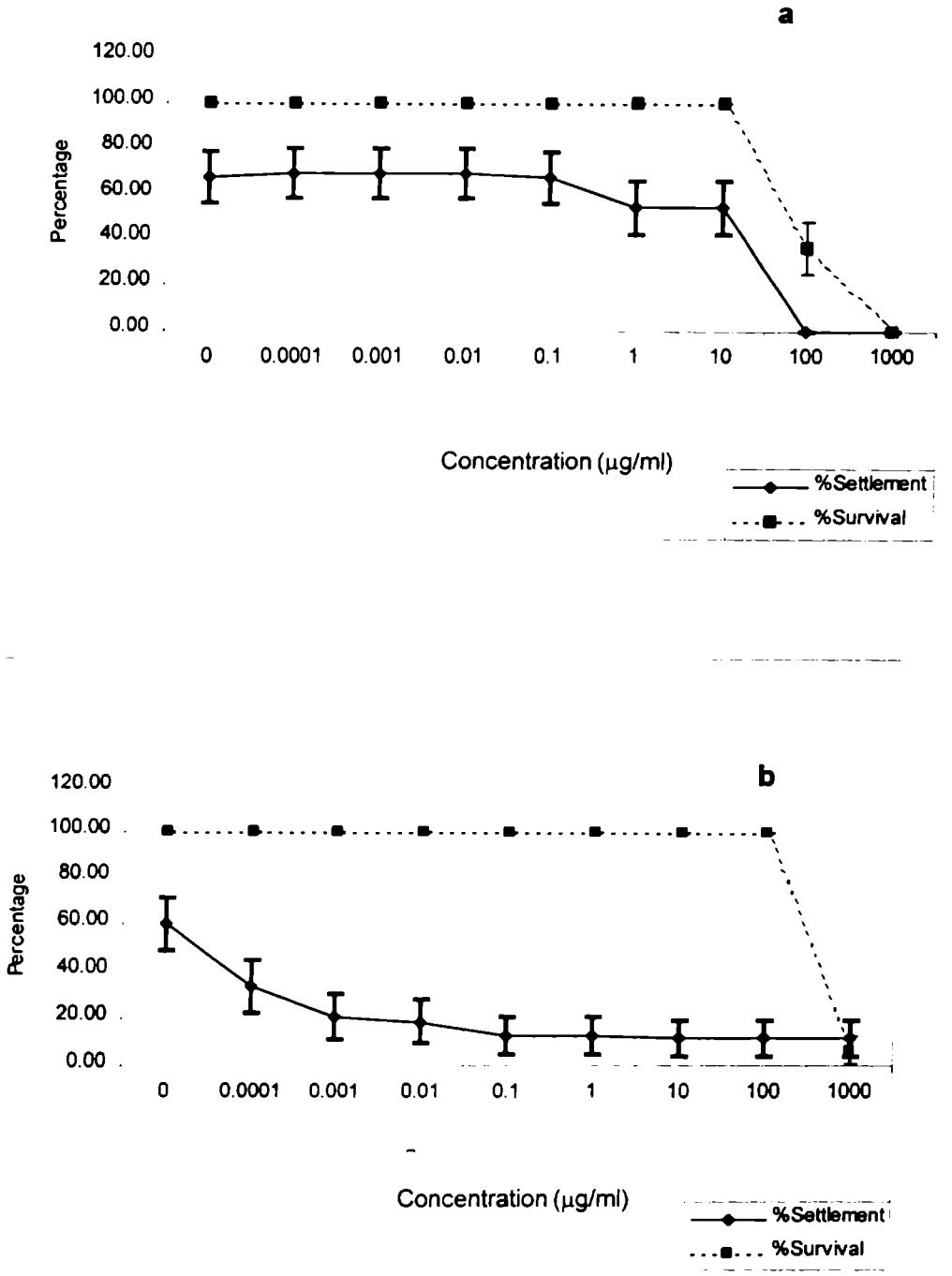


Fig. 2.5

Effect of secondary metabolites of *Padina australis* accommodated in (a) methanol, (b) methylene chloride on the survival and settlement of larvae of *Balanus amphitrite*.

TABLE 2.21

Effect of methanol accommodated secondary metabolites of *Padina tetrastromatica* on the survival of larvae of *Balanus amphitrite*.

Concentration (μg/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	100.0	0.0
100	100.0	0.0
1000	14.3	8.2

TABLE 2.22

Effect of methanol accommodated secondary metabolites of *Padina tetrastromatica* on the settlement of larvae of *Balanus amphitrite*.

Concentration (μg/ml)	% settlement	S.E.M.
Control	67.0	11.1
0.0001	34.1	11.2
0.001	33.3	11.1
0.01	28.5	10.6
0.1	16.7	8.8
1	16.7	8.8
10	16.7	8.8
100	13.3	8.0
1000	0.0	0.0

TABLE 2.23

Effect of methylene chloride accommodated secondary metabolites of *Padina tetrastromatica* on the survival of larvae of *Balanus amphitrite*.

Concentration (μ g/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	86.5	8.1
0.001	73.5	10.4
0.01	59.6	11.6
0.1	54.6	11.7
1	50.9	11.8
10	41.5	11.6
100	16.7	8.8
1000	0.0	0.0

TABLE 2.24

Effect of methylene chloride accommodated secondary metabolites of *Padina tetrastromatica* on the settlement of larvae of *Balanus amphitrite*.

Concentration (μg/ml)	% settlement	S.E.M.
Control	67.0	11.1
0.0001	40.7	11.6
0.001	40.7	11.6
0.01	29.1	10.7
0.1	17.4	8.9
1	13.0	7.9
10	0.0	0.0
100	0.0	0.0
1000	0.0	0.0

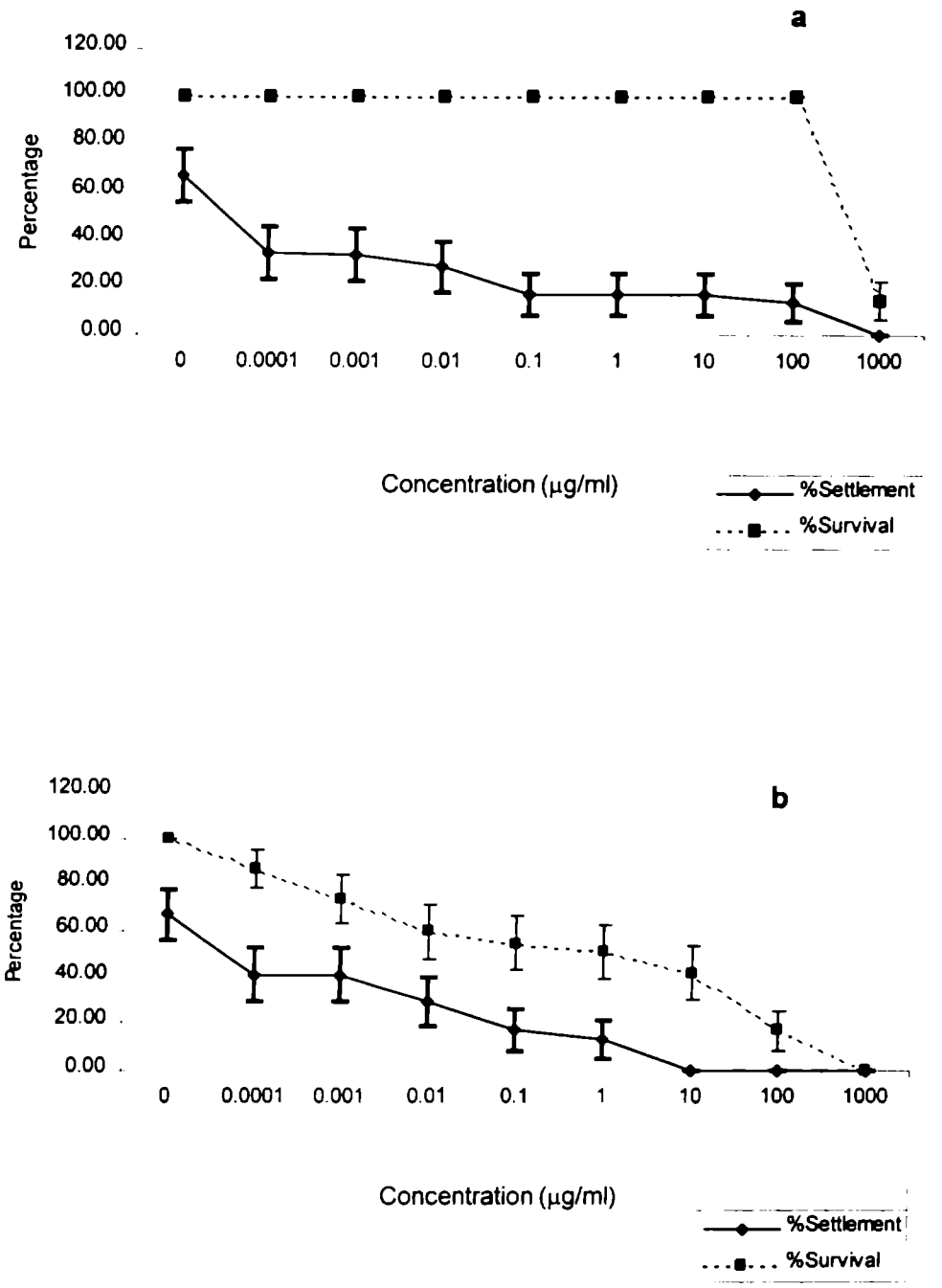


Fig. 2.6

Effect of secondary metabolites of *Padina tetrastromatica* accommodated in (a) methanol, (b) methylene chloride on the survival and settlement of larvae of *Balanus amphitrite*.

TABLE 2.25

Effect of methanol accommodated secondary metabolites of *Sargassum wightii* on the survival of larvae of *Balanus amphitrite.*

Concentration (μ g/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	90.9	6.8
0.1	80.0	9.4
1	75.0	10.2
10	50.0	11.8
100	25.0	10.2
1000	9.3	6.8

TABLE 2.26

Effect of methanol accommodated secondary metabolites of *Sargassum wightii* on the settlement of larvae of *Balanus amphitrite.*

Concentration (μg/ml)	% settlement	S.E.M.
Control	66.1	11.2
0.0001	62.6	11.4
0.001	62.6	11.4
0.01	62.8	11.4
0.1	62.6	11.4
1	50.0	11.8
10	22.0	9.8
100	14.3	8.2
1000	0.0	0.0

TABLE 2.27

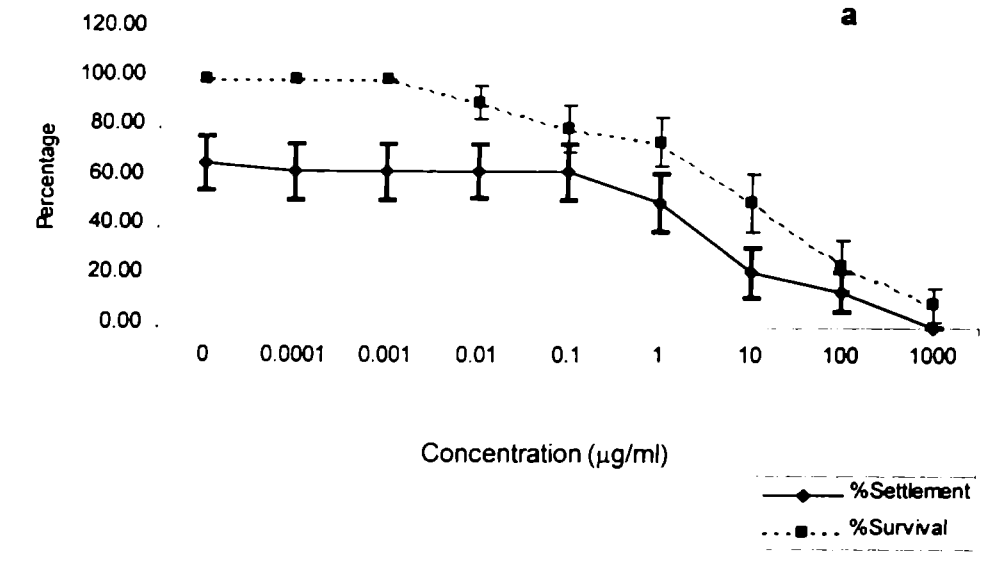
Effect of methylene chloride accommodated secondary metabolites of *Sargassum wightii* larvae of on the survival of larvae of *Balanus amphitrite.*

Concentration (μ g/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	34.1	11.2
100	0.0	0.0
1000	0.0	0.0

TABLE 2.28

Effect of methylene chloride accommodated secondary metabolites of *Sargassum wightii* on the settlement of larvae of *Balanus amphitrite.*

Concentration (μg/ml)	% settlement	S.E.M.
Control	60.0	11.6
0.0001	5.2	5.2
0.001	3.3	4.2
0.01	3.3	4.2
0.1	3.3	4.2
1	3.3	4.2
10	0.0	0.0
100	0.0	0.0
1000	0.0	0.0



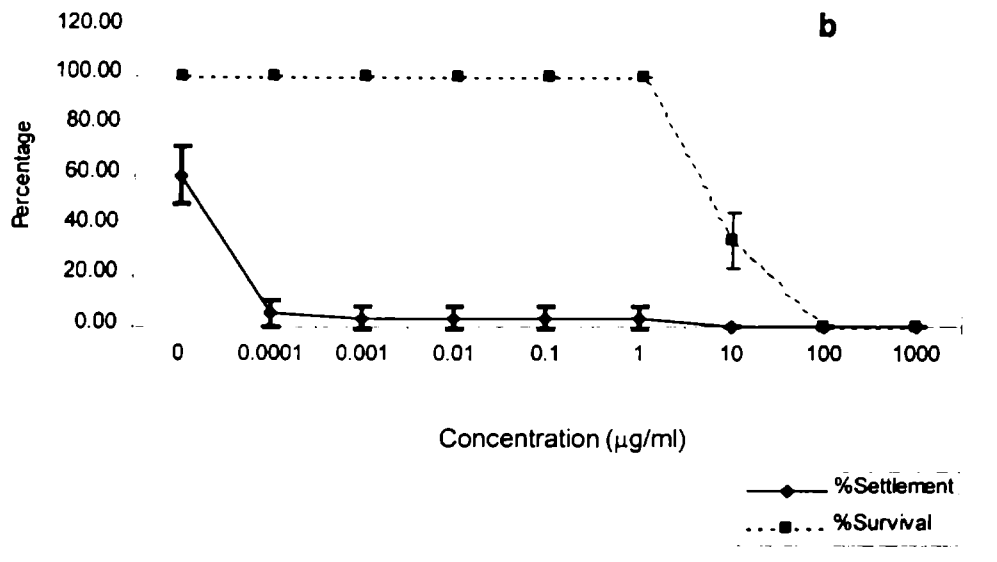


Fig. 2.7

Effect of secondary metabolites of *Sargassum wightii* accommodated in (a) methanol, (b) methylene chloride on the survival and settlement of larvae of *Balanus amphitrite*.

TABLE 2.29

Effect of methanol accommodated secondary metabolites of *Spathoglossum sp.* on the survival of larvae of *Balanus amphitrite*.

Concentration (μ g/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	100.0	0.0
100	58.0	11.6
1000	10.0	7.1

TABLE 2.30

Effect of methanol accommodated secondary metabolites of *Spathoglossum sp.* on the settlement of larvae of *Balanus amphitrite*.

Concentration (μg/ml)	% settlement	S.E.M.
Control	83.0	8.9
0.0001	81.1	9.2
0.001	81.1	9.2
0.01	80.9	9.3
0.1	76.3	10.0
1	71.5	10.6
10	66.7	11.1
100	33.3	11.1
1000	4.8	5.0

TABLE 2.31

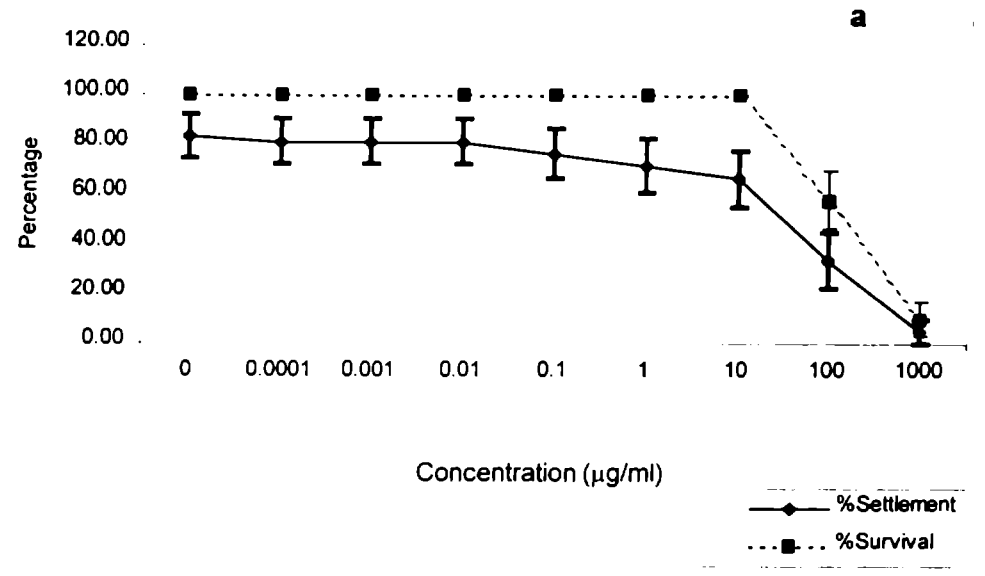
Effect of methylene chloride accommodated secondary metabolites of *Spathoglossum sp.* on the survival of larvae of *Balanus amphitrite.*

Concentration (μg/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	93.7	5.7
100	25.0	10.2
1000	10.0	7.1

TABLE 2.32

Effect of methylene chloride accommodated secondary metabolites of *Spathoglossum sp.* on the settlement of larvae of *Balanus amphitrite.*

Concentration (μg/ml)	% settlement	S.E.M.
Control	60.0	11.6
0.0001	50.0	11.8
0.001	50.0	11.8
0.01	50.0	11.8
0.1	50.0	11.8
1	50.0	11.8
10	50.0	11.8
100	3.3	4.2
1000	0.0	0.0



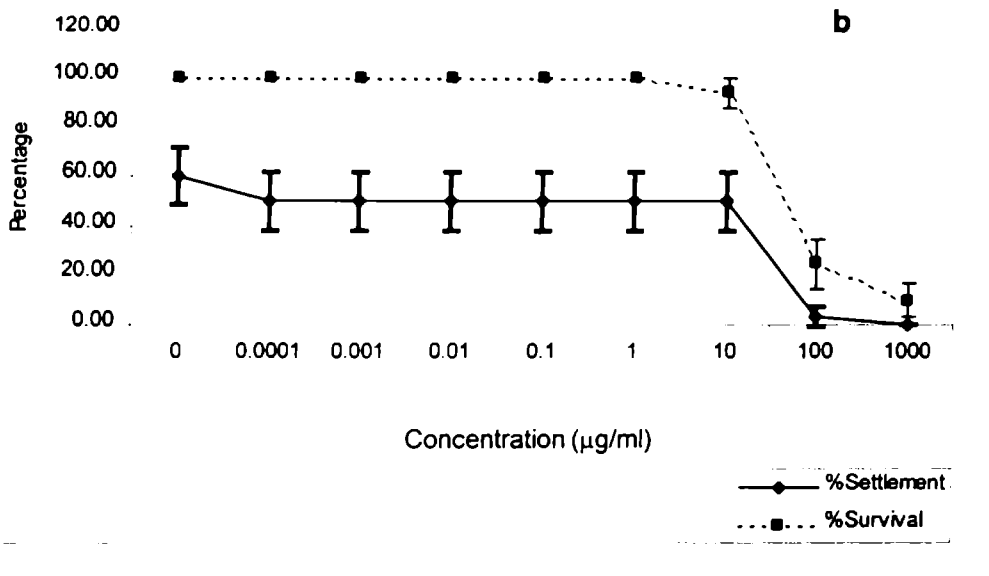


Fig. 2.8

Effect of secondary metabolites of *Spathaglossum* sp. accommodated in (a) methanol, (b) methylene chloride on the survival and settlement of larvae of *Balanus amphitrite*.

TABLE 2.33

Effect of methanol accommodated secondary metabolites of *Spyridia filamentosa* on the survival of larvae of *Balanus amphitrite*.

Concentration (μg/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	100.0	0.0
100	91.9	6.5
1000	0.0	0.0

TABLE 2.34

Effect of methanol accommodated secondary metabolites of *Spyridia filamentosa* on the settlement of larvae of *Balanus amphitrite*.

Concentration (μg/ml)	% settlement	S.E.M.
Control	66.0	11.1
0.0001	21.0	9.6
0.001	17.0	8.6
0.01	13.0	7.9
0.1	11.0	7.1
1	0.0	0.0
10	0.0	0.0
100	0.0	0.0
1000	0.0	0.0

TABLE 2.35

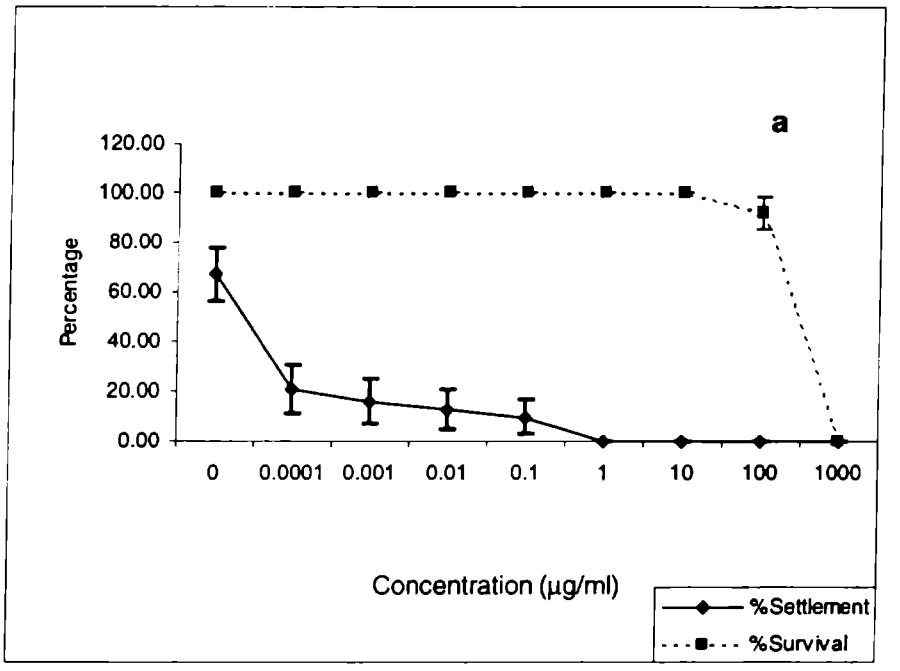
Effect of methylene chloride accommodated secondary metabolites of *Spyridia filamentosa* on the survival of larvae of *Balanus amphitrite.*

Concentration (μg/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	85.0	8.4
10	75.0	10.2
100	15.7	8.6
1000	0.0	0.0

TABLE 2.36

Effect of methylene chloride accommodated secondary metabolites of *Spyridia filamentosa* on the settlement of larvae of *Balanus amphitrite.*

Concentration (μg/ml)	% settlement	S.E.M.
Control	60.0	11.6
0.0001	31.1	10.9
0.001	30.0	10.8
0.01	29.1	10.7
0.1	25.0	10.2
1	20.0	9.4
10	20.0	9.4
100	0.0	0.0
1000	0.0	0.0



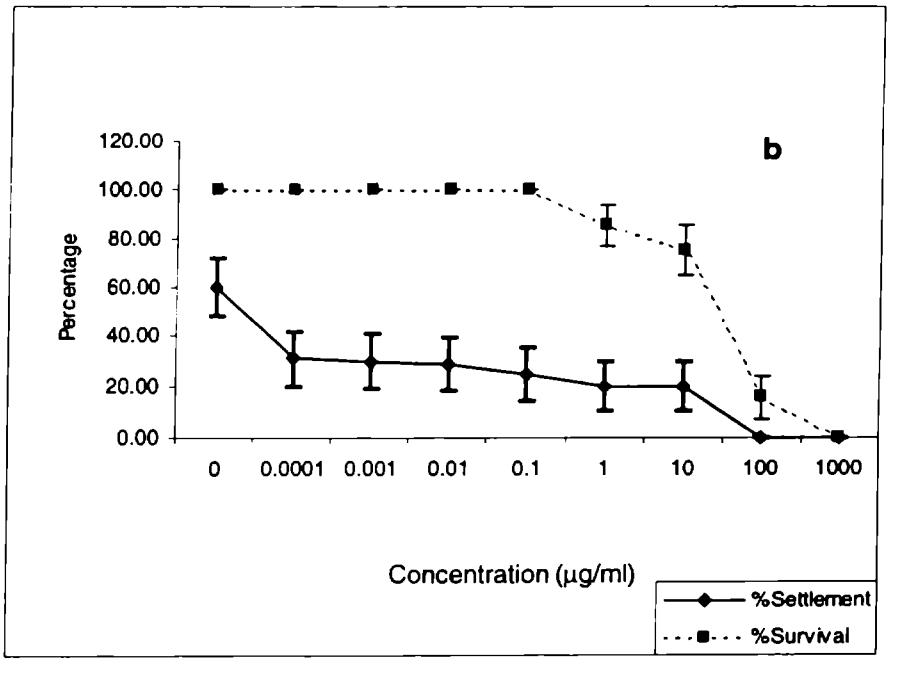


Fig. 2.9

Effect of secondary metabolites of *Spyridia filamentosa* accommodated in (a) methanol, (b) methylene chloride on the survival and settlement of larvae of *Balanus amphitrite.*

TABLE 2.37

Effect of methanol accommodated secondary metabolites of *Stoechospermum marginatum* on the survival of larvae of *Balanus amphitrite.*

Concentration (μ g/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	100.0	0.0
100	0.0	0.0
1000	0.0	0.0

TABLE 2.38

Effect of methanol accommodated secondary metabolites of *Stoechospermum marginatum* on the settlement of larvae of *Balanus amphitrite.*

Concentration (μg/ml)	% settlement	S.E.M.
Control	60.0	11.6
0.0001	57.2	11.7
0.001	57.2	11.7
0.01	57.2	11.7
0.1	57.2	11.7
1	57.2	11.7
10	50.0	11.8
100	0.0	0.0
1000	0.0	0.0

TABLE 2.39

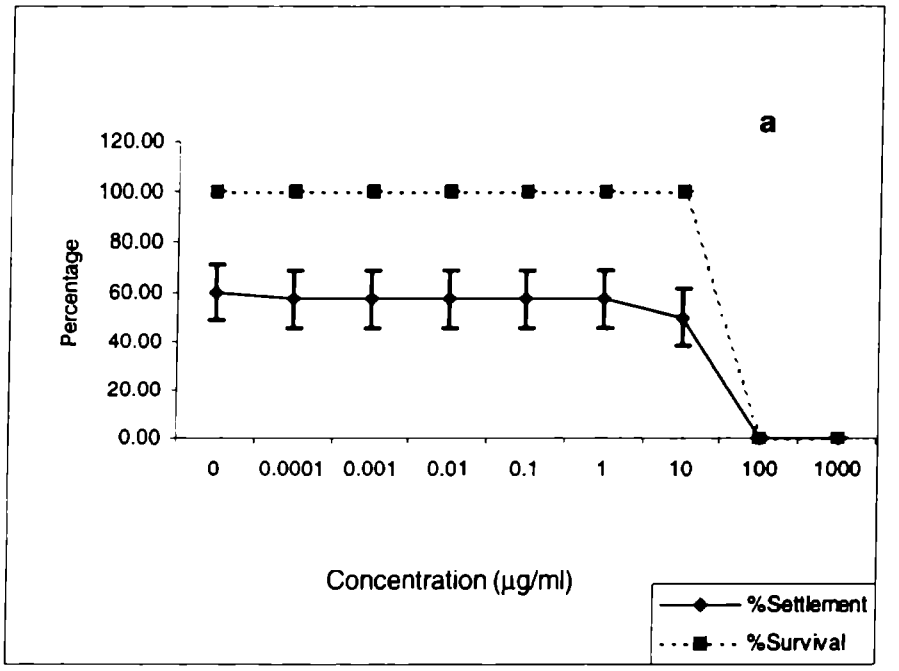
Effect of methylene chloride accommodated secondary metabolites of *Stoechospermum marginatum* on the survival of larvae of *Balanus amphitrite.*

Concentration (μ g/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	90.9	6.8
100	0.0	0.0
1000	0.0	0.0

TABLE 2.40

Effect of methylene chloride accommodated secondary metabolites of *Stoechospermum marginatum* on the settlement of larvae of *Balanus amphitrite.*

Concentration (μg/ml)	% settlement	S.E.M.
Control	63.3	11.4
0.0001	31.5	11.0
0.001	30.9	10.9
0.01	30.0	10.8
0.1	23.3	10.0
1	10.0	7.1
10	3.3	4.2
100	0.0	0.0
1000	0.0	0.0



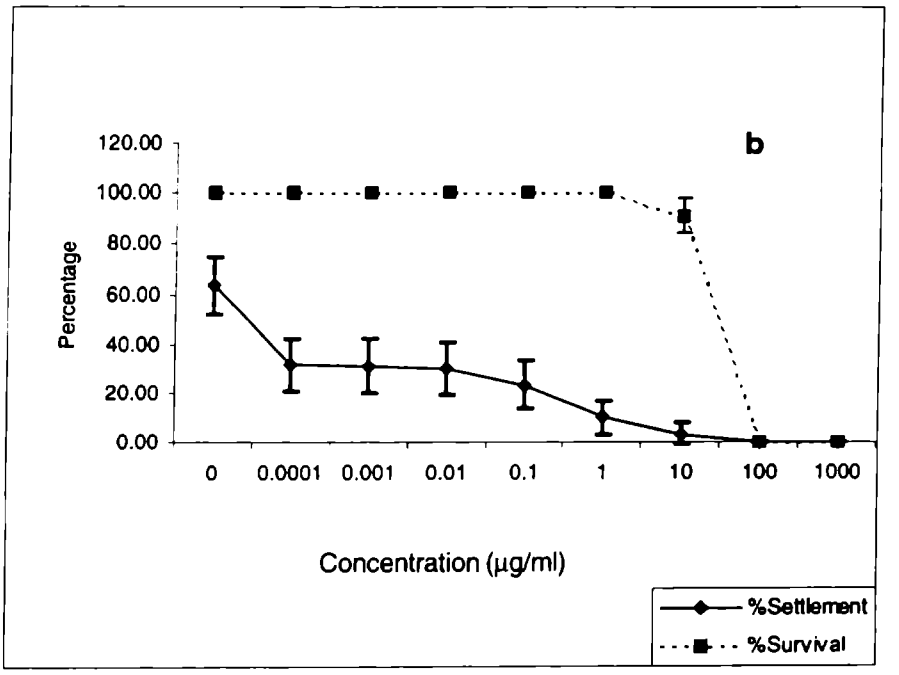


Fig. 2.10

Effect of secondary metabolites of *Stoechospermum marginatum* accommodated in (a) methanol, (b) methylene chloride on the survival and settlement of larvae of *Balanus amphitrite.*

TABLE 2.41

Effect of methanol accommodated secondary metabolites of *Turbinaria ornata* on the survival of larvae of *Balanus amphitrite*.

Concentration (μ g/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	100.0	0.0
100	100.0	0.0
1000	0.0	0.0

TABLE 2.42

Effect of methanol accommodated secondary metabolites of *Turbinaria ornata* on the settlement of larvae of *Balanus amphitrite*.

Concentration (μg/ml)	% settlement	S.E.M.
Control	60.0	11.6
0.0001	55.9	11.7
0.001	52.4	11.8
0.01	50.9	11.8
0.1	50.0	11.8
1	10.0	7.1
10	10.0	7.1
100	10.0	7.1
1000	0.0	0.0

TABLE 2.43

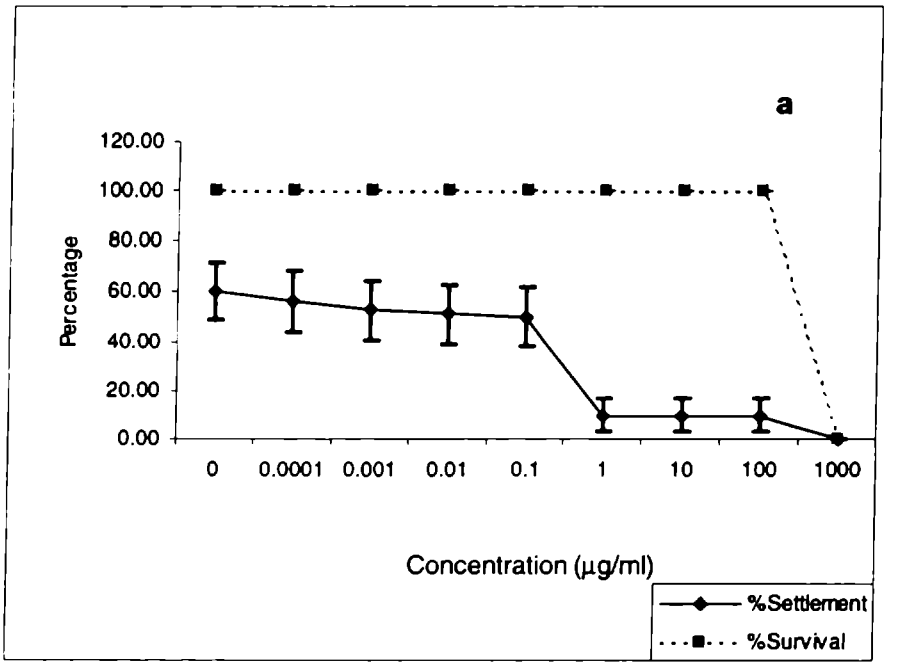
Effect of methylene chloride accommodated secondary metabolites of *Turbinaria ornata* on the survival of larvae of *Balanus amphitrite.*

Concentration (μg/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	83.3	8.8
100	0.0	0.0
1000	0.0	0.0

TABLE 2.44

Effect of methylene chloride accommodated secondary metabolites of *Turbinaria ornata* on the settlement of larvae of *Balanus amphitrite.*

Concentration (μg/ml)	% settlement	S.E.M.
Control	60.0	11.6
0.0001	20.0	9.4
0.001	16.7	8.8
0.01	14.3	8.2
0.1	12.0	7.7
1	7.2	6.1
10	7.2	6.1
100	0.0	0.0
1000	0.0	0.0



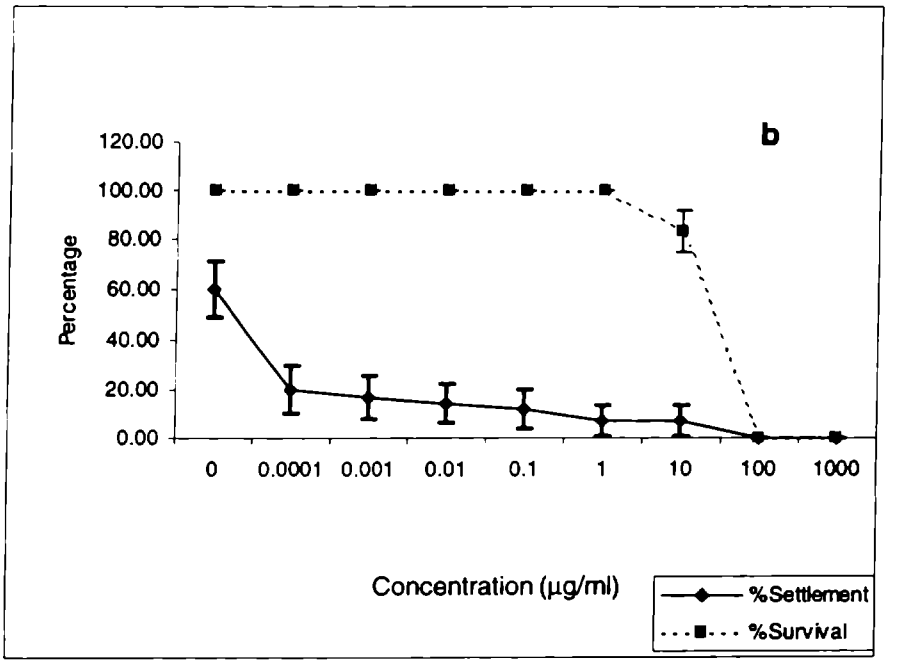


Fig. 2.11

Effect of secondary metabolites of *Turbinaria ornata* accommodated in (a) methanol, (b) methylene chloride on the survival and settlement of larvae of *Balanus amphitrite.*

TABLE 2.45

Effect of methanol accommodated secondary metabolites of *Ulva lactuca* on the survival of larvae of *Balanus amphitrite*.

Concentration (µ g/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	100.0	0.0
100	100.0	0.0
1000	0.0	0.0

TABLE 2.46

Effect of methanol accommodated secondary metabolites of *Ulva lactuca* on the settlement of larvae of *Balanus amphitrite*.

Concentration (µg/ml)	% settlement	S.E.M.
Control	60.0	11.6
0.0001	7.8	6.3
0.001	6.5	5.8
0.01	3.3	4.2
0.1	0.0	0.0
1	0.0	0.0
10	0.0	0.0
100	0.0	0.0
1000	0.0	0.0

TABLE 2.47

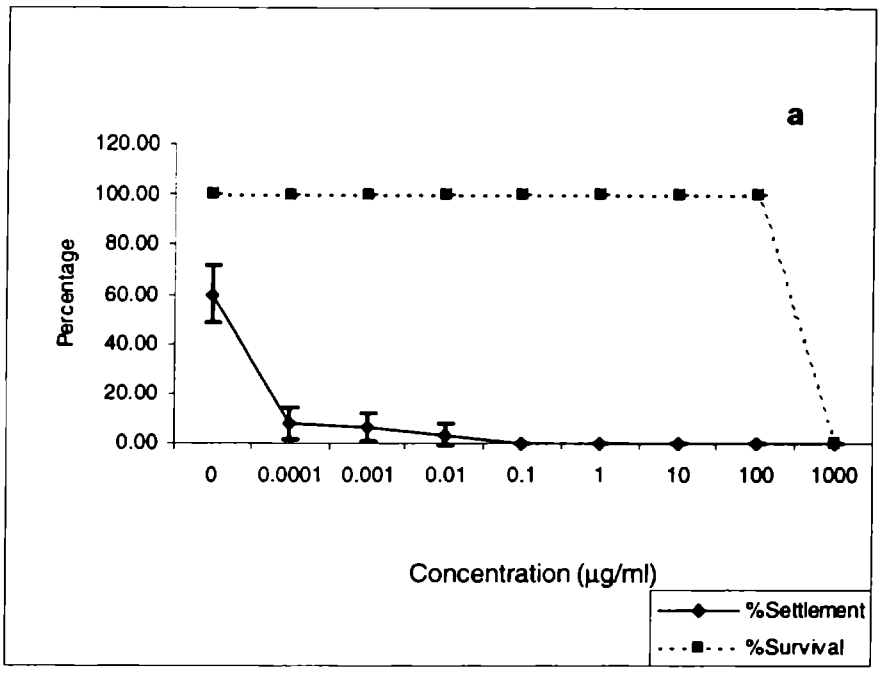
Effect of methylene chloride accommodated secondary metabolites of *Ulva lactuca* on the survival of larvae of *Balanus amphitrite.*

Concentration (µg/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	80.0	9.4
100	65.4	11.2
1000	0.0	0.0

TABLE 2.48

Effect of methylene chloride accommodated secondary metabolites of *Ulva lactuca* on the settlement of larvae of *Balanus amphitrite.*

Concentration (µg/ml)	% settlement	S.E.M.
Control	60.9	11.5
0.0001	47.0	11.8
0.001	34.8	11.2
0.01	5.9	5.6
0.1	5.9	5.6
1	5.9	5.6
10	5.9	5.6
100	0.0	0.0
1000	0.0	0.0



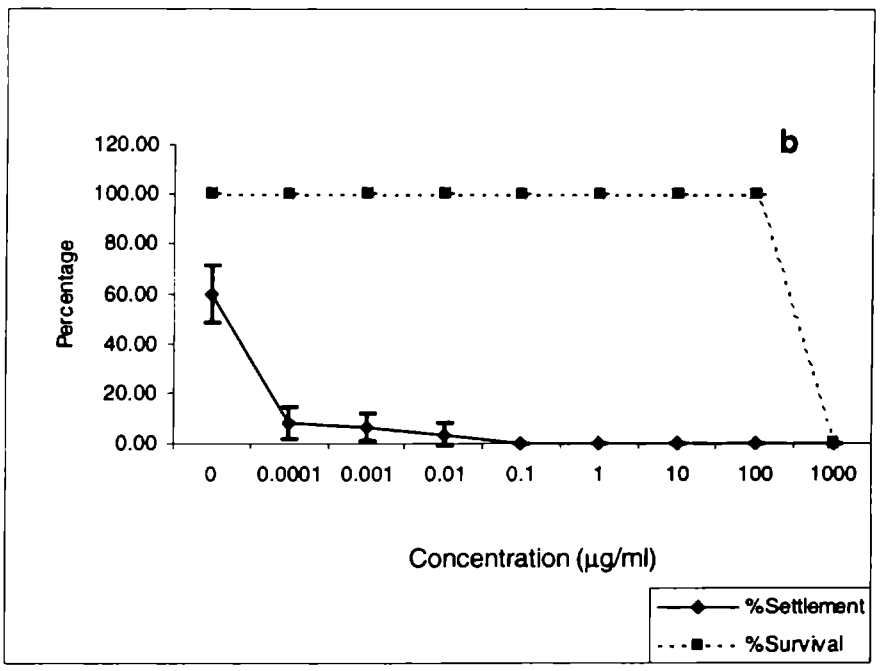


Fig. 2.12

Effect of secondary metabolites of *Ulva lactuca* accommodated in (a) methanol, (b) methylene chloride on the survival and settlement of larvae of *Balanus amphitrite.*

TABLE 2.49

Regression analysis on the effect of methanol accommodated secondary metabolites of the seaweeds on the survival of larvae of *Balanus amphitrite*.

Seaweeds	Regression equation	F ratio	Probability	DF
Caulerpa scalpelliformis	Y = 91.87 + -3.77 X	7.5	0.0340	6
Chaetomorpha linoides	Y = 83.33 + -8.33 X	3.0	0.1340	6
Gracilaria corticata	Y = 81.01 + -9.23 X	4.3	0.0837	6
Gracilaria lichenoides	Y = 65.06 + -14.84 X	11.3	0.0152	6
Padina australis	Y = 73.48 + -12.13 X	8.2	0.0288	6
Padina tetrastromatica	Y = 85.71 + -7.15 X	3.0	0.1340	6
Sargassum wightii	Y = 59.50 + -13.55 X	70.3	0.0002	6
Spathoglossum sp.	Y = 78.49 + -10.00 X	7.2	0.0361	6
Spyridia filamentosa	Y = 82.10 + -8.81 X	3.7	0.1043	6
Stoechospermum marginatum	Y = 67.86 + -14.29 X	8.0	0.0300	6
Turbinaria ornata	Y = 83.33 + -8.33 X	3.0	0.1340	6
Ulva lactuca	Y = 83.3 + -8.33 X	3.0	0.1340	6

TABLE 2.50

Regression analysis on the effect of methanol accommodated secondary metabolites of the seaweeds on the rate of settlement of larvae of *Balanus amphitrite*.

Seaweeds	Regression equation	F ratio	Probability	DF
Caulerpa scalpelliformis	Y = 54.57 + -1.14 X	208.3	6.93E - 06	6
Chaetomorpha linoides	Y = 49.07 + -3.52 X	6.8	0.0406	6
Gracilaria corticata	Y = 36.31 + - 5.63 X	12.2	0.0130	6
Gracilaria lichenoides	Y = 15.54 + - 4.42 X	16.9	0.0062	6
Padina australis	Y = 42.27 + -10.50 X	16.6	0.0065	6
Padina tetrastromatica	Y = 17.68 + - 4.45 X	53.6	0.0033	6
Sargassum wightii	Y = 37.26 + - 9.70X	31.6	0.0014	6
Spathoglossum sp.	Y = 57.08 + -9.77 X	16.2	0.0070	6
Spyridia filamentosa	Y = 6.06 + -3.35 X	43.0	0.0006	6
Stoechospermum marginatum	Y = 37.80 + -8.43 X	10.2	0.0189	6
Turbinaria ornata	Y = 25.35 + - 9.12 X	34.0	0.0011	6
Ulva lactuca	Y = 1.62 + -1.15 X	17.5	0.0058	6

TABLE 2.51

Regression analysis on the effect of methylene chloride accommodated secondary metabolites of the seaweeds on the survival of larvae of *Balanus amphitrite*.

Seaweeds	Regression equation	F ratio	Probability	DF
Caulerpa scalpelliformis	Y= 71.67+ - 12.72 X	9.4	0.0222	6
Chaetomorpha linoides	Y= 67.71+ - 14.11 X	10.5	0.0177	6
Gracilaria corticata	Y= 43.79 + - 8.25 X	24.1	0.0027	6
Gracilaria lichenoides	Y= 63.48 + - 14.79 X	12.5	0.0122	6
Padina australis	Y=84.45 + - 7.78 X	3.0	0.1314	6
Padina tetrastromatica	Y= 42.28 + - 11.28 X	106.6	4.83 E-05	6
Sargassum wightii	Y= 58.44 + - 16.64 X	18.0	0.0054	6
Spathoglossum sp.	Y= 72.49 + - 12.19 X	9.7	0.0207	6
Spyridia filamentosa	Y= 64.76 + - 14.42 X	17.9	0.0055	6
Stoechospermum marginatum	Y=66.56 + -14.61 X	9.5	0.0215	6
Turbinaria ornata	Y= 65.48 + - 14.88 X	10.9	0.0163	6
Ulva lactuca	Y= 75.12+ - 11.11 X	9.1	0.0236	6

TABLE 2.52

Regression analysis on the effect of methylene chloride accommodated secondary metabolites of the seaweeds on the rate of settlement of larvae of *Balanus amphitrite*.

Seaweeds	Regression equation	F ratio	Probability	DF
Caulerpa scalpelliformis	Y= 10.94 + - 2.61 X	30.7	0.0015	6
Chaetomorpha linoides	Y= 0.8333 + -0.8333 X	3.0	0.134	6
Gracilaria corticata	Y= 11.08 + -5.90 X	49.3	0.0004	6
Gracilaria lichenoides	Y= 2.06 + -1.29 X	22.4	0.0032	6
Padina australis	Y= 15.34 + -2.61 X	14.0	0.0096	6
Padina tetrastromatica	Y= 14.16 + -6.91 X	81.8	0.0001	6
Sargassum wightii	Y= 1.94 + -0.75 X	29.1	0.0017	6
Spathoglossum sp.	Y= 34.44 + -6.94 X	8.2	0.0287	6
Spyridia filamentosa	Y= 17.02 + -4.76 X	32.6	0.0013	6
Stoechospermum marginatum	Y= 13.35 + -5.58 X	68.1	0.0002	6
Turbinaria ornata	Y= 8.19 + -2.97 X	193.2	8.63E-06	6
Ulva lactuca	Y= 10.20 + -5.99 X	13.6	0.0102	6

TABLE 2.53

Probit analysis on the effect of methanol accommodated secondary metabolites of the seaweeds on the survival of larvae of *Balanus amphitrite*.

Seaweeds	Regression equation	LC 50 (µg/ml)	95% confidence limit	X^2	Proba-bility	DF
Caulerpa scalpelliformis	P= - 2.21 + 0.72 X	1195.25	186.63 -1538636908.8	273.69	0.000	6
Cheaetomorpha linoides	P= -5.08 + 2.1 X	266.4	100.74 - 743	194.03	0.000	6
Gracilaria corticata	p= - 5.03 + 2.19 X	196.7	124.39 - 319.54	69.76	0.000	6
Gracilaria lichenoides	P= -2.34 + 1.88 X	17.4	5.25 - 83.16	328.53	0.000	6
Padina australis	P= -4.82 + 2.57 X	74.9	68.23 - 81.91	7.96	0.241	6
Padina tetrastromatica	P= -5.14 + 1.98 X	397.9	222.47 - 722.23	89.11	0.000	6
Sargassum wightii	P= -0.47 + 0.56 X	7.0	3.35 - 15.33	50.34	0.000	6
Spathoglossum sp.	P= -3.87 + 1.76 X	156.4	121.11 - 201.60	20.36	0.002	6
Spyridia filamentosa	P= -5.06 + 2.15 X	225.4	120.42 - 439.21	112.45	0.000	6
Stoechospermum marginatum	P= -4.50 + 3.04 X	30.4	19.40 - 48.13	74.82	0.000	6
Turbinaria ornata	P= -5.08 + 2.1 X	266.4	100.74 - 743	194.03	0.000	6
Ulva lactuca	P= -5.08 + 2.1 X	266.4	100.74 - 742.99	194.03	0.000	6

TABLE 2.54

Probit analysis on the effect of methanol accommodated secondary metabolites of the seaweeds on the rate of settlement of larvae of *Balanus amphitrite.*

Seaweeds	Regression equation	LC 50 (μg/ml)	95% confidence limit	X^2	Proba-bility	DF
Caulerpa scalpelliformis	P= -0.12 + 0.03 X	9513.16	110.73 - 13763997286	0.354	0.999	6
Cheaetomorpha linoides	P= 0.12 + 0.09 X	0.539	.0 - 322560743074	104.11	1.000	6
Gracilaria corticata	P= -0.37 + 0.16 X	0.0039	1.79E -14 - 0.3146	221.99	0.000	6
Gracilaria lichenoides	P= 1.12 + 0.2 X	<0.00045	0.0 - 1.38 E -25	197.87	0.000	6
Padina australis	P= 0.2499 + 0.3043 X	0.1509	0.00039 - 32.78	552.12	0.000	6
Padina tetrastromatica	P= 1.0 + 0.17 X	<0.00001	1.38 E - 11 - 0.0001	72.42	0.000	6
Sargassum wightii	P= 0.4 + 0.28 X	0.0396	0.0007 - 0.7065	265.79	0.000	6
Spathoglossum sp.	p= -0.21 + 0.29 X	5.56	0.2333 - 2661.95	341.85	0.000	6
Spyridia filamentosa	p= 1.98 + 0.33 X	<0.00002	1.86 E - 10 - 0.00002	65.92	0.000	6
Stoechospermum marginatum	P= 0.37 + 0.24 X	0.0304	2.70 E - 16 - 2.03	578.86	0.000	6
Turbinaria ornata	P= - 0.83 + 0.31 X	0.00231	0.00003 - 0.02494	211.59	0.000	6
Ulva lactuca	P= 2.83 + 0.39 X	.>9.50 E - 12	9.50E -12 - 0.0	17.27	0.008	6

TABLE 2.55

Probit analysis on the effect of methylene chloride accommodated secondary metabolites of the seaweeds on the survival of larvae of *Balanus amphitrite*.

Seaweeds	Regression equation	LC 50 (μg/ml)	95% confidence limit	X^2	Proba-bility	DF
Caulerpa scalpelliformis	P= -3.90 + 2.23 X	55.78	50.62 - 61.45	1.81	0.937	6
Cheaetomorpha linoides	P= -3.66 + 2.47 X	30.4	27.66 - 33.31	0.132	1.0	6
Gracilaria corticata	P= 0.26 + 0.26 X	0.1	0.0029 - 1.97	221.59	0.000	6
Gracilaria lichenoides	P= -1.12 + 1.03 X	12.3	Nil	1396.56	0.000	6
Padina australis	P= -5.11 + 2.04X	323.4	154.96 - 680.31	129.996	0.000	6
Padina tetrastromatica	P= 0.25 + 0.34 X	0.2	0.0259 - 1.25	154.96	0.000	6
Sargassum wightii	P= -3.13 + 3.57 X	7.6	6.76 - 8.19	0.684	0.995	6
Spathoglossum sp.	P= -2.65 + 1.45 X	66.4	32.86 - 130.25	107.94	0.000	6
Spyridia filamentosa	P= -1.45 + 1.18 X	16.9	8.14 - 36.45	107.93	0.000	6
Stoechospermum marginatum	P= -4.40 + 3.17X	24.4	20.29 - 29.94	16.47	0.011	6
Turbinaria ornata	P= -4.30 + 3.29X	20.3	18.60 - 22.40	6.77	0.342	6
Ulva lactuca	P= -2.59 + 1.35 X	83.3	33.48 - 240.42	177.17	0.000	6

TABLE 2.56

Probit analysis on the effect of methylene chloride accommodated secondary metabolites of the seaweeds on the rate of settlement of larvae of *Balanus amphitrite*.

Seaweeds	Regression equation	LC 50 (μg/ml)	95% confidence limit	X^2	Proba-bility	DF
Caulerpa scalpelliformis	P= -1.29 + 0.14 X	5.50E-10	5.73 E - 26 - 0.0	62.37	0.0	6
Cheaetomorpha linoides	P= 5.06 + 0.95 X	<0.00001	0. 000 - 0.00001	8.41	0.209	6
Gracilaria corticata	P= 1.61 + 0.36 X	0.00004	0.000 - 0.0002	59.54	0.0	6
Gracilaria lichenoides	P= 2.49 + 0.31 X	7.65 E - 09	3.06 E -13 - 0.0	19.76	0.003	6
Padina australis	P= 1.05 + 0.10 X	9.16 E - 11	2.002 E - 23 - 0.000	35.83	0.000	6
Padina tetrastromatica	P= 1.42 + 0.39 X	0.00011	0.0000 - 0.00074	89.37	0.000	6
Sargassum wightii	P= 2.21 + 0.16 X	3.13 E - 14	1.30 E - 55 - 5.28 E -09	31.81	0.000	6
Spathoglossum sp.	P= 0.45 + 0.20 X	0.0058	Nil	498.74	0.000	6
Spyridia filamentosa	P= 1.05 + 0.20 X	<0.0006	1.27 E - 16 - 0.0006	165.8	0.000	6
Stoechospermum marginatum	P= 1.34 + 0.29 X	0.00002	0.000 - 0.00035	106.37	0.000	6
Turbinaria ornata	P= 1.54 + 0.20 X	>8.46 E - 14	8.46 E - 14 - 0.000	48.24	0.000	6
Ulva lactuca	P= 1.71 + 0.39 X	0.00004	0.00 - 0.00039	128.87	0.000	6

TABLE 2.57

Effect of methanol accommodated secondary metabolites *of Caulerpa scalpelliformis* on the survival of larvae of *Hydroides elegans*.

Concentration (µg/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	100.0	0.0
100	100.0	0.0
* 1000	0.0	0.0

TABLE 2.58

Effect of methanol accommodated secondary metabolites of *Caulerpa scalpelliformis* on the settlement of larvae of *Hydroides elegans*.

Concentration (µg/ml)	% settlement	S.E.M.
Control	93.0	6.6
0.0001	81.0	10.1
0.001	73.0	11.5
0.01	68.0	12.0
0.1	60.0	12.7
1	54.2	12.9
10	52.4	12.9
100	41.6	12.7
1000	0.0	0.0

TABLE 2.59

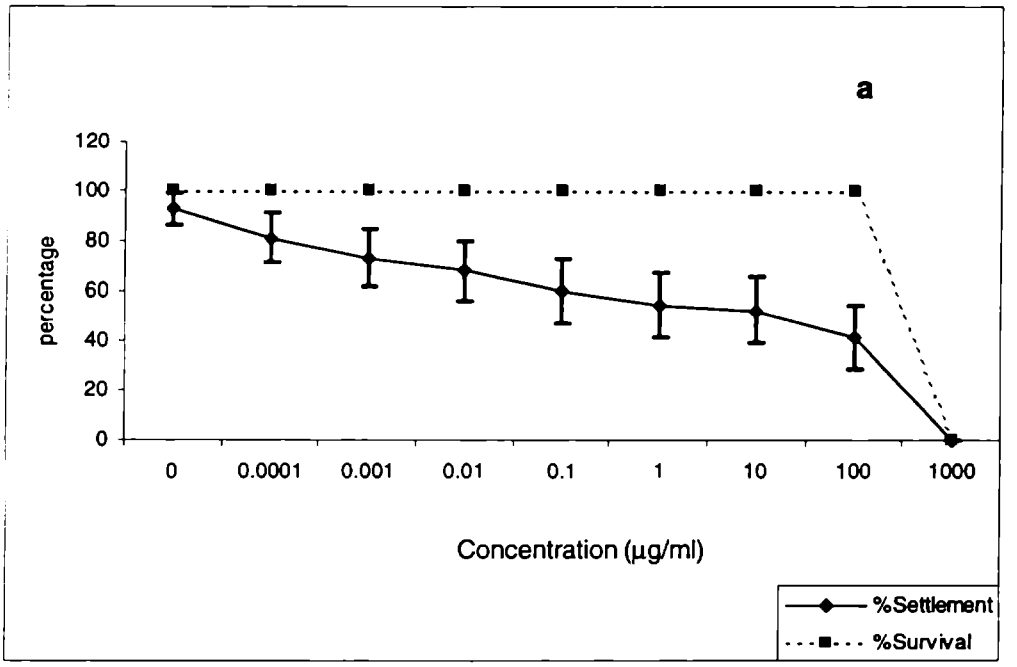
Effect of methylene chloride accommodated secondary metabolites of *Caulerpa scalpelliformis* on the survival of larvae of *Hydroides elegans*.

Concentration (µg/ml)	% survival	S.E.M.
Control	80.0	10.3
0.0001	23.0	10.9
0.001	22.0	10.7
0.01	22.0	10.7
0.1	15.3	9.3
1	13.3	8.8
10	13.3	8.8
100	5.0	5.6
1000	0.0	0.0

TABLE 2.60

Effect of methylene chloride accommodated secondary metabolites of *Caulerpa scalpelliformis* on the settlement of larvae of *Hydroides elegans*.

Concentration (µg/ml)	% settlement	S.E.M.
Control	90.0	7.8
0.0001	22.0	10.7
0.001	20.0	10.3
0.01	18.7	10.1
0.1	13.0	8.7
1	9.0	7.4
10	5.0	5.6
100	0.8	2.2
1000	0.0	0.0



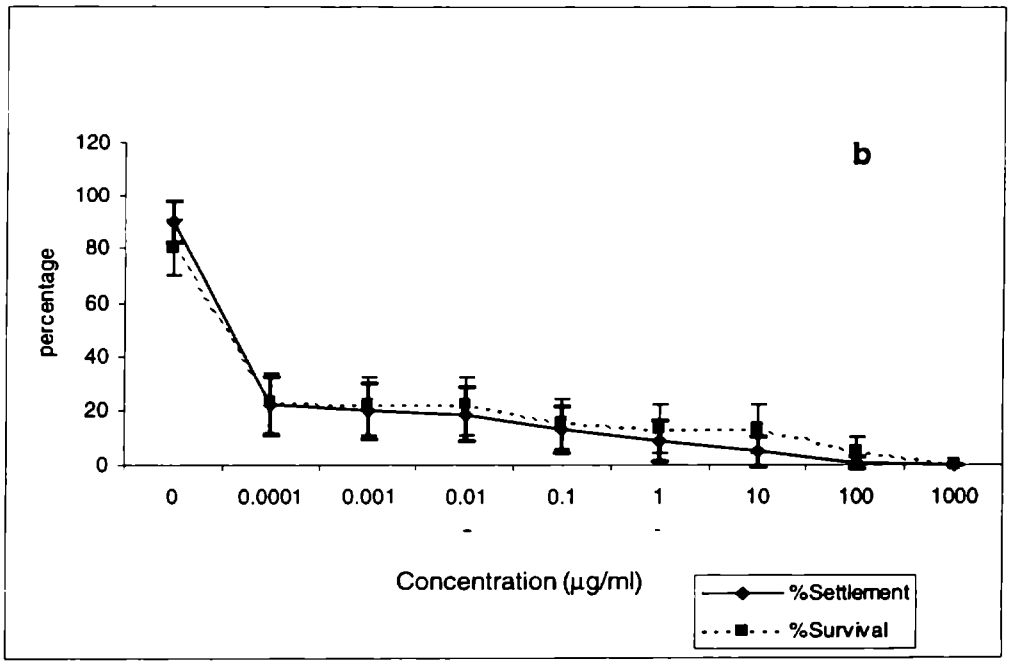


Fig. 2.13

Effect of secondary metabolites of *Caulerpa scalpelliformis* accommodated in (a) methanol, (b) methylene chloride on the survival and settlement of larvae of *Hydroides elegans*.

TABLE 2.61

Effect of methanol accommodated secondary metabolites of *Chaetomorpha linoides* on the survival of larvae of *Hydroides elegans*.

Concentration (µg/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	100.0	0.0
100	100.0	0.0
1000	0.0	0.0

TABLE 2.62

Effect of methanol accommodated secondary metabolites of *Chateomorpha linoides* on the settlement of larvae of *f Hydroides elegans*.

Concentration (µg/ml)	% settlement	S.E.M.
Control	89.0	8.1
0.0001	87.0	8.7
0.001	85.0	9.2
0.01	85.0	9.2
0.1	82.0	9.9
1	78.0	10.7
10	66.7	12.2
100	57.1	12.8
1000	0.0	0.0

TABLE 2.63

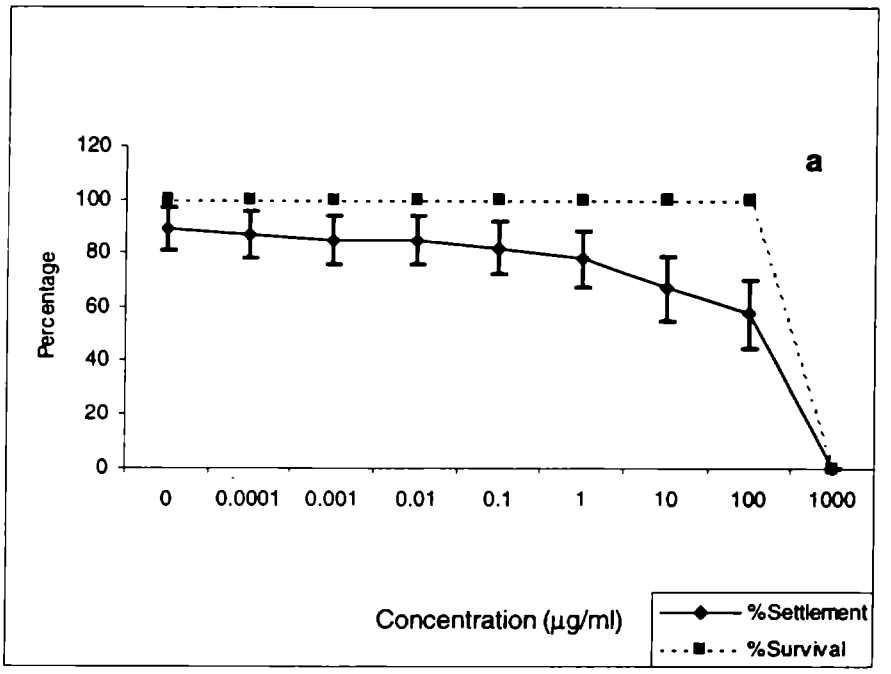
Effect of methylene chloride accommodated secondary metabolites of *Chaetomorpha linoides* on the survival of larvae of *Hydroides elegans*.

Concentration (µg/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	100.0	0.0
100	100.0	0.0
1000	0.0	0.0

TABLE 2.64

Effect of methylene chloride accommodated secondary metabolites of *Chaetomorpha linoides* on the settlement of larvae of *Hydroides elegans*.

Concentration (µg/ml)	% settlement	S.E.M.
Control	80.0	10.3
0.0001	16.7	9.6
0.001	18.6	10.0
0.01	20.9	10.5
0.1	10.4	7.9
1	13.7	8.9
10	11.8	8.3
100	5.2	5.8
1000	0.0	0.0



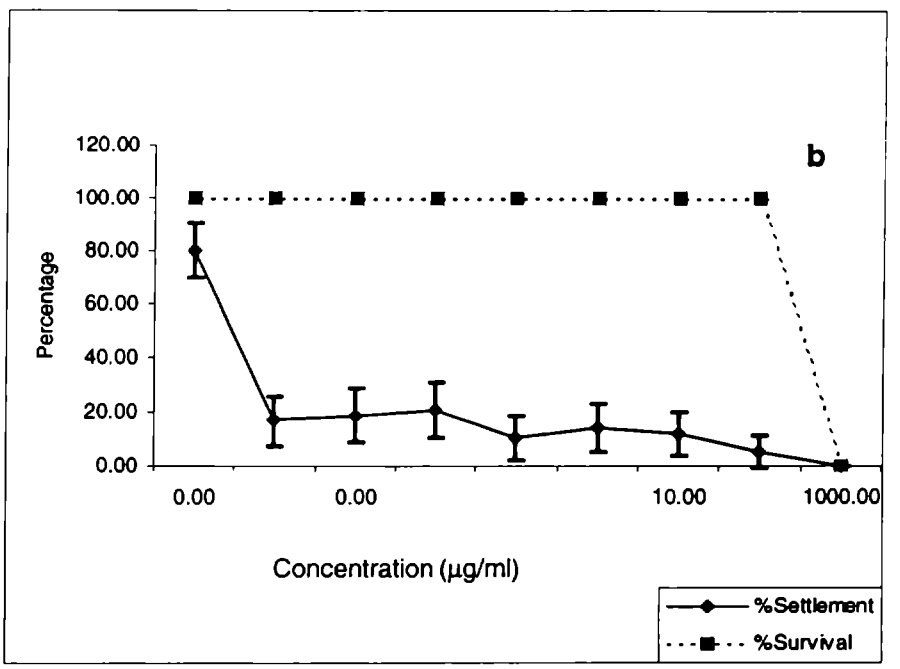


Fig. 2.14

Effect of secondary metabolites of *Chaetomorpha linoides* accommodated in (a) methanol, (b) methylene chloride on the survival and settlement of larvae of *Hydroides elegans.*

TABLE 2.65

Effect of methanol accommodated secondary metabolites of *Gracilaria corticata* on the survival of larvae of *Hydroides elegans.*

Concentration (µg/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	100.0	0.0
100	100.0	0.0
1000	0.0	0.0

TABLE 2.66

Effect of methanol accommodated secondary metabolites of *Gracilaria corticata* on the settlement of larvae of *Hydroides elegans.*

Concentration (µg/ml)	% settlement	S.E.M.
Control	86.3	8.9
0.0001	86.0	9.0
0.001	76.0	11.0
0.01	73.0	11.5
0.1	68.0	12.0
1	69.0	11.9
10	69.0	12.1
100	67.0	12.1
1000	0.0	0.0

TABLE 2.67

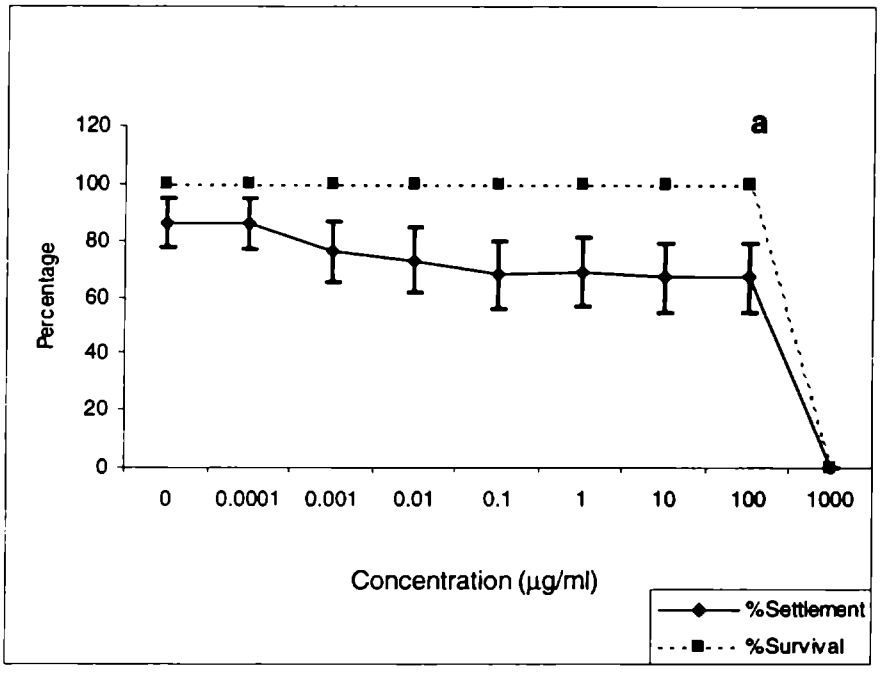
Effect of methylene chloride accommodated secondary metabolites of *Gracilaria corticata* on the survival of larvae of *Hydroides elegans*.

Concentration (µg/ml)	% survival	S.E.M.
Control		
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	60.0	12.7
100	0.0	0.0
1000	0.0	0.0

TABLE 2.68

Effect of methylene chloride accommodated secondary metabolites of *Gracilaria corticata* on the settlement of larvae of *Hydroides elegans*.

Concentration (µg/ml)	% settlement	S.E.M.
Control	78.0	10.7
0.0001	33.0	12.1
0.001	33.0	12.1
0.01	32.0	12.0
0.1	27.0	11.5
1	21.9	10.7
10	21.9	10.7
100	0.0	0.0
1000	0.0	0.0



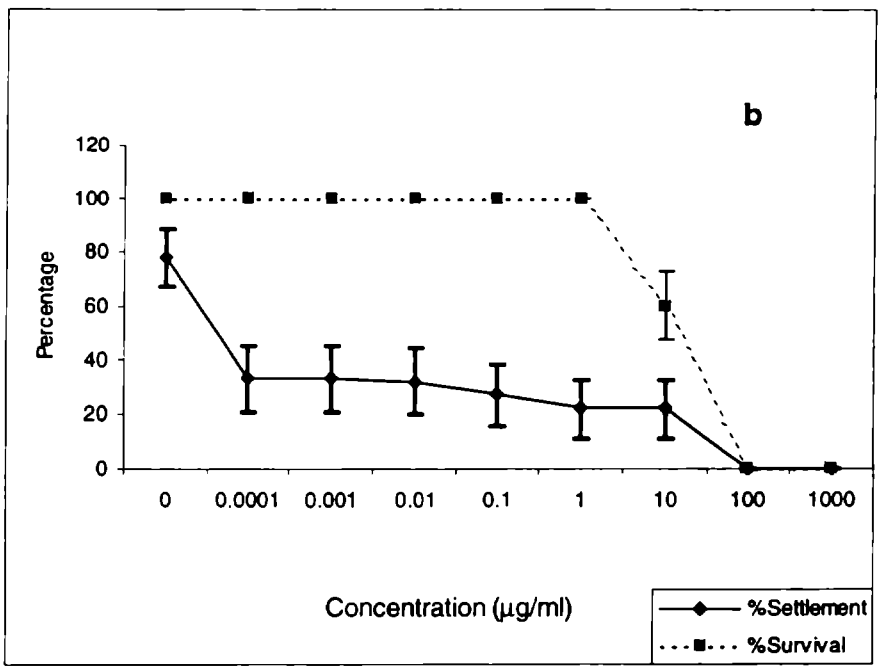


Fig. 2.15

Effect of secondary metabolites of *Gracilaria corticata* accommodated in (a) methanol, (b) methylene chloride on the survival and settlement of larvae of *Hydroides elegans.*

TABLE 2.69

Effect of methanol accommodated secondary metabolites of *Gracilaria lichenoides* on the survival of larvae of *Hydroides elegans.*

Concentration (µg/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	86.0	9.0
0.001	85.0	9.2
0.01	84.2	9.4
0.1	84.2	9.4
1	84.2	9.4
10	71.4	11.7
100	0.0	0.0
1000	0.0	0.0

TABLE 2.70

Effect of methanol accommodated secondary metabolites of *Gracilaria lichenoides* on the settlement of larvae of *Hydroides elegans.*

Concentration (µg/ml)	% settlement	S.E.M.
Control	70.0	11.8
0.0001	60.0	12.7
0.001	60.0	12.7
0.01	56.0	12.8
0.1	52.0	12.9
1	50.0	12.9
10	47.2	12.9
100	0.0	0.0
1000	0.0	0.0

TABLE 2.71

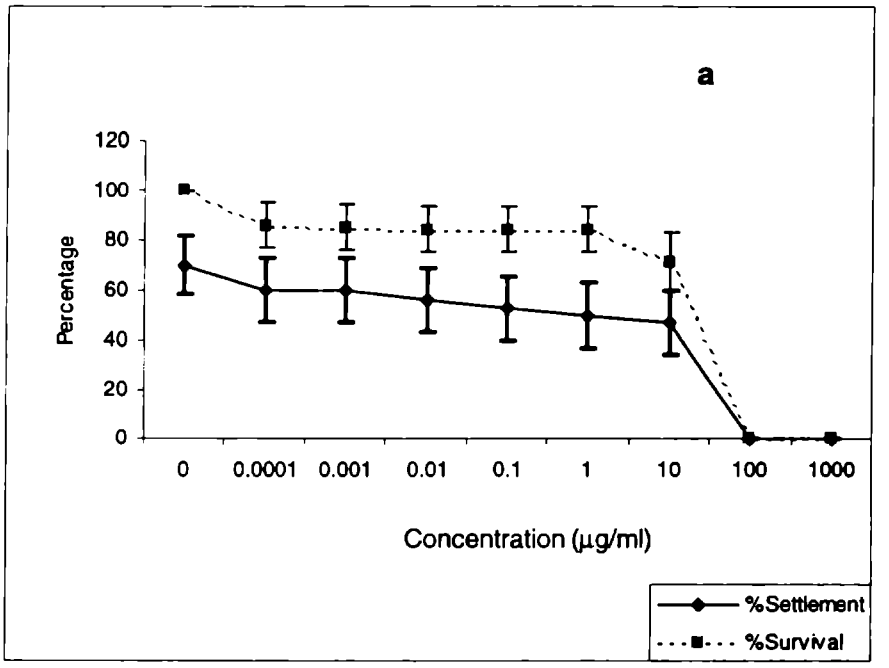
Effect of methylene chloride accommodated secondary metabolites of *Gracilaria lichenoides* on the survival of larvae of *Hydroides elegans.*

Concentration (µg/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	66.7	12.2
10	12.4	8.5
100	0.0	0.0
1000	0.0	0.0

TABLE 2.72

Effect of methylene chloride accommodated secondary metabolites of *Gracilaria lichenoides* on the settlement of larvae of *Hydroides elegans.*

Concentration (µg/ml)	% settlement	S.E.M.
0	91.0	7.4
0.0001	13.3	8.8
0.001	6.7	6.4
0.01	6.7	6.4
0.1	0.0	0.0
1	0.0	0.0
10	0.0	0.0
100	0.0	0.0
1000	0.0	0.0



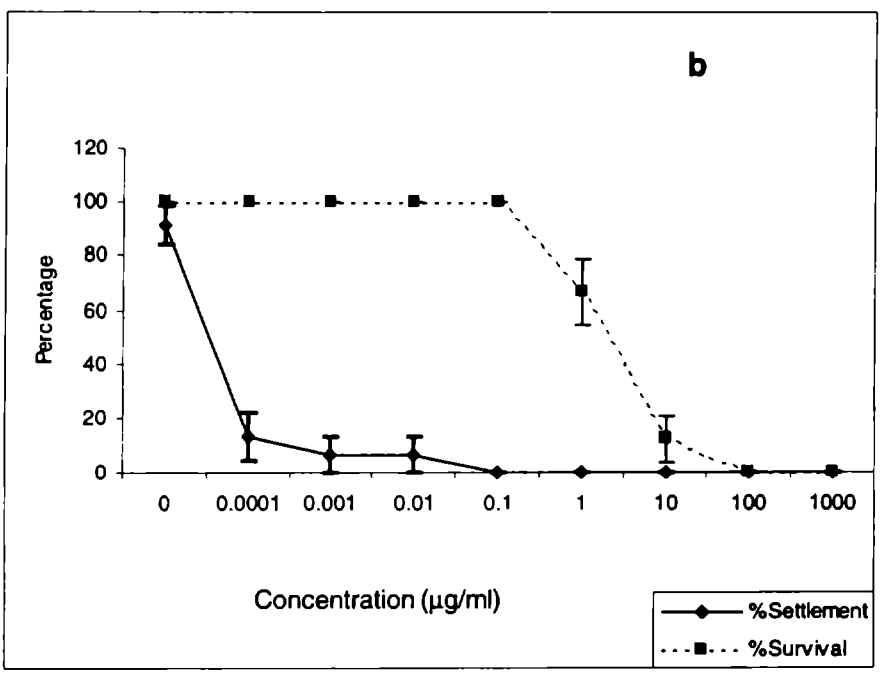


Fig. 2.16

Effect of secondary metabolites of *Gracilaria lichenoides* accommodated in (a) methanol, (b) methylene chloride on the survival and settlement of larvae of *Hydroides elegans.*

TABLE 2.73

Effect of methanol accommodated secondary metabolites of *Padina australis* on the survival of larvae of *Hydroides elegans*.

Concentration (µg/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	100.0	0.0
100	100.0	0.0
1000	0.0	0.0

TABLE 2.74

Effect of methanol accommodated secondary metabolites of *Padina australis* on the settlement of larvae of *Hydroides elegans*.

Concentration (µg/ml)	% settlement	S.E.M.
Control	86.0	9.0
0.0001	80.0	10.3
0.001	78.3	10.6
0.01	75.0	11.2
0.1	73.3	11.4
1	62.0	12.5
10	50.0	12.9
100	0.0	0.0
1000	0.0	0.0

TABLE 2.75

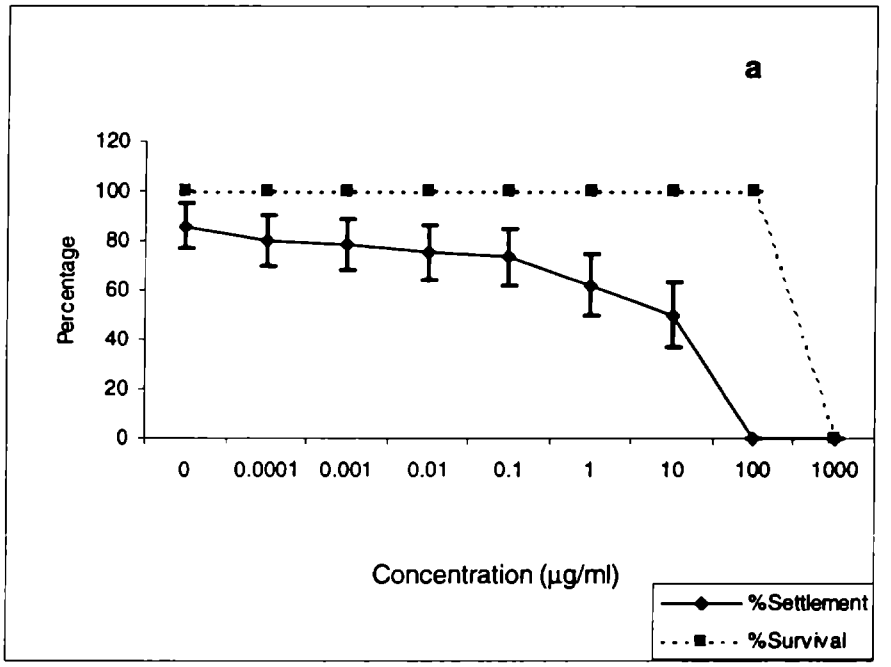
Effect of methylene chloride accommodated secondary metabolites of *Padina australis* on the survival of larvae of *Hydroides elegans.*

Concentration (µg/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	89.0	8.1
1	79.0	10.5
10	0.0	0.0
100	0.0	0.0
1000	0.0	0.0

TABLE 2.76

Effect of methylene chloride accommodated secondary metabolites of *Padina australis* on the settlement of larvae of *Hydroides elegans.*

Concentration (µg/ml)	% settlement	S.E.M.
Control	75.0	11.2
0.0001	15.0	9.2
0.001	13.0	8.7
0.01	7.8	6.9
0.1	3.0	4.4
1	0.0	0.0
10	0.0	0.0
100	0.0	0.0
1000	0.0	0.0



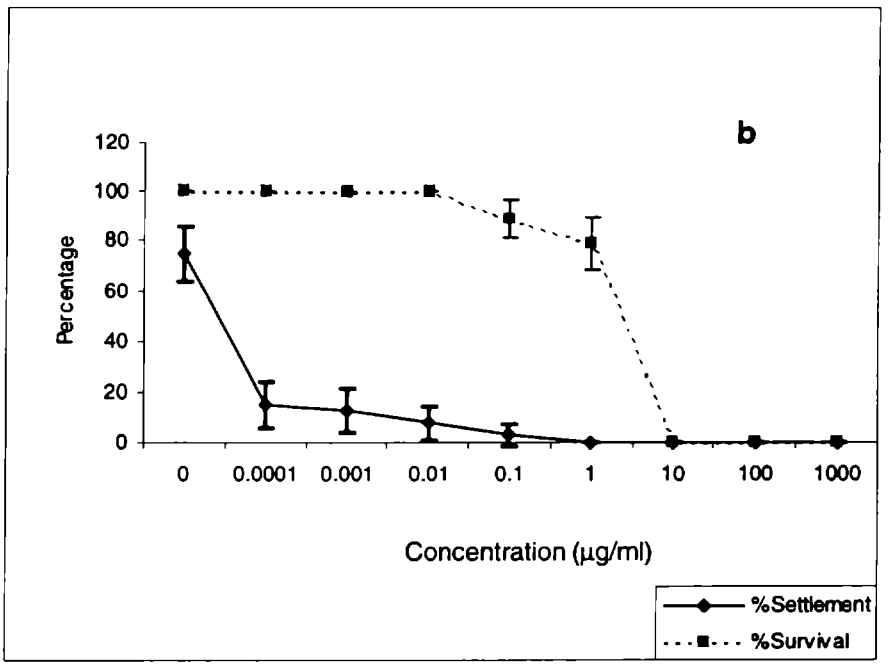


Fig. 2.17

Effect of secondary metabolites of *Padina australis* accommodated in (a) methanol, (b) methylene chloride on the survival and settlement of larvae of *Hydroides elegans.*

TABLE 2.77

Effect of methanol accommodated secondary metabolites of *Padina tetrastromatica* on the survival of larvae of *Hydroides elegans.*

Concentration (µg/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	100.0	0.0
100	100.0	0.0
1000	0.0	0.0

TABLE 2.78

Effect of methanol accommodated secondary metabolites of *Padina tetrastromatica* on the settlement of larvae of *Hydroides elegans.*

Concentration (µg/ml)	% settlement	S.E.M.
Control	53.0	12.5
0.0001	8.3	7.1
0.001	8.3	7.1
0.01	8.3	7.1
0.1	8.3	7.1
1	8.3	7.1
10	18.8	10.1
100	21.4	10.6
1000	0.0	0.0

TABLE 2.79

Effect of methylene chloride accommodated secondary metabolites of *Padina tetrastromatica* on the survival of larvae of *Hydroides elegans*.

Concentration (µg/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	100.0	0.0
100	0.0	0.0
1000	0.0	0.0

TABLE 2.80

Effect of methylene chloride accommodated secondary metabolites of *Padina tetrastromatica* on the settlement of larvae of *Hydroides elegans*.

Concentration (µg/ml)	% settlement	S.E.M.
Control	74.8	11.2
0.0001	16.7	9.6
0.001	16.7	9.6
0.01	6.2	6.2
0.1	24.0	11.0
1	35.5	12.4
10	25.7	11.3
100	0.0	0.0
1000	0.0	0.0

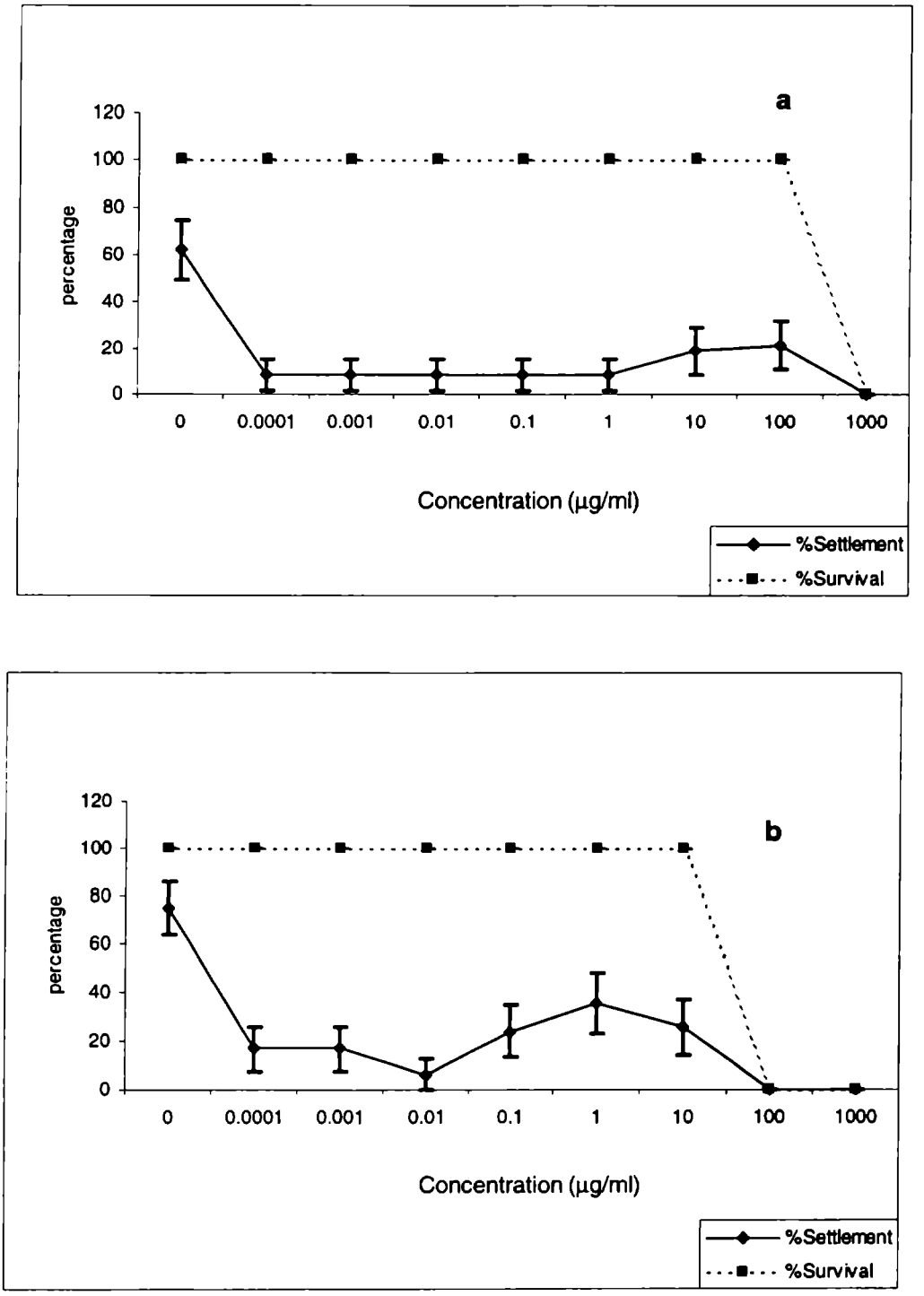


Fig. 2.18

Effect of secondary metabolites of *Padina tetrastromatica* accommodated in (a) methanol, (b) methylene chloride on the survival and settlement of larvae of *Hydroides elegans.*

TABLE 2.81

Effect of methanol accommodated secondary metabolites of *Sargassum whightii* on the survival of larvae of *Hydroides elegans*.

Concentration (µg/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	89.0	8.1
1	78.0	10.7
10	57.0	12.8
100	38.0	12.5
1000	15.0	9.2

TABLE 2.82

Effect of methanol accommodated secondary metabolites of *Sargassi whightii* on the settlement of larvae of *Hydroides elegans*.

Concentration (µg/ml)	% settlement	S.E.M.
Control	92.0	7.0
0.0001	91.0	7.4
0.001	91.0	7.4
0.01	91.0	7.4
0.1	84.0	9.5
1	79.0	10.5
10	49.0	12.9
100	27.0	11.5
1000	10.0	7.8

TABLE 2.83

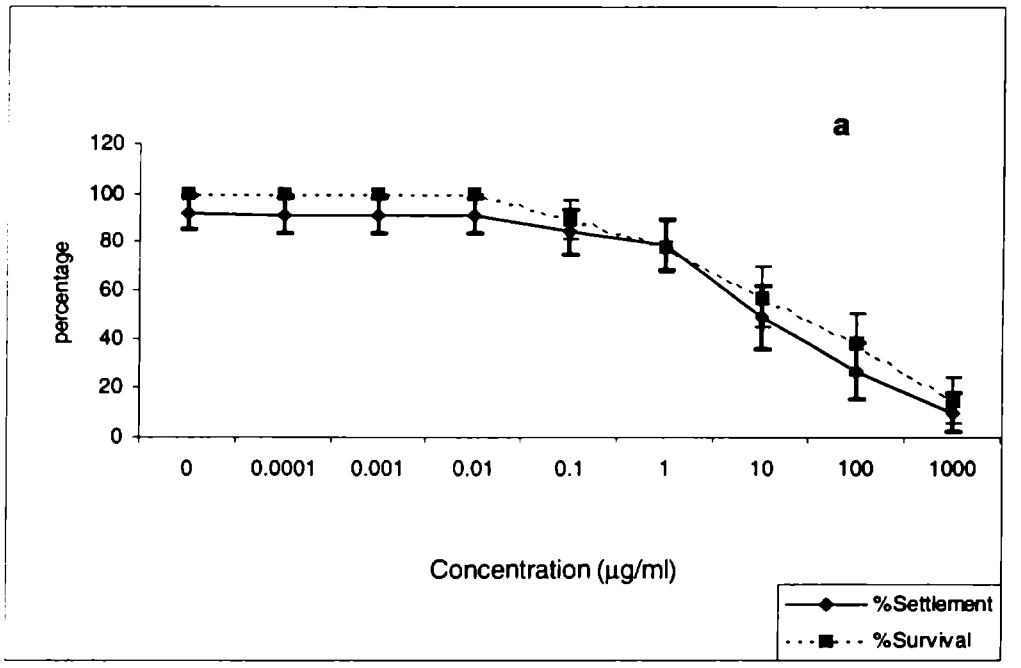
Effect of methylene chloride accommodated secondary metabolites of *Sargassum whightii* on the survival of larvae of *Hydroides elegans.*

Concentration (µg/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	100.0	0.0
100	36.4	12.4
1000	0.0	0.0

TABLE 2.84

Effect of methylene chloride accommodated secondary metabolites of *Sargassum whightii* on the settlement of larvae of *Hydroides elegans.*

Concentration (µg/ml)	% settlement	S.E.M.
Control	60.0	12.7
0.0001	30.8	11.9
0.001	29.0	11.7
0.01	28.6	11.7
0.1	28.6	11.7
1	21.4	10.6
10	15.4	9.3
100	0.0	0.0
1000	0.0	0.0



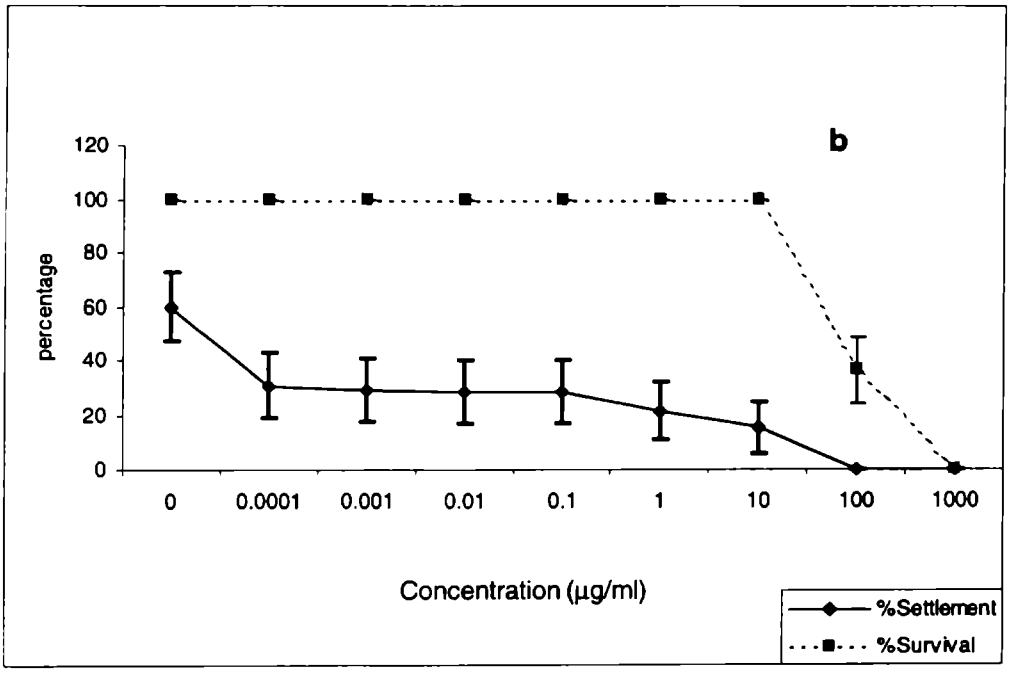


Fig. 2.19

Effect of secondary metabolites of *Sargassum wightii* accommodated in (a) methanol, (b) methylene chloride on the survival and settlement of larvae of *Hydroides elegans*.

TABLE 2.85

Effect of methanol accommodated secondary metabolites of *Spathoglossum sp.* on the survival of larvae of *Hydroides elegans.*

Concentration (µg/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	100.0	0.0
100	100.0	0.0
1000	0.0	0.0

TABLE 2.86

Effect of methanol accommodated secondary metabolites of *Spathoglossum sp.* on the settlement of larvae of *Hydroides elegans.*

Concentration (µg/ml)	% settlement	S.E.M.
Control	88.0	8.4
0.0001	88.0	8.4
0.001	88.0	8.4
0.01	80.0	10.3
0.1	73.0	11.5
1	63.0	12.5
10	52.0	12.9
100	41.0	12.7
1000	0.0	0.0

TABLE 2.87

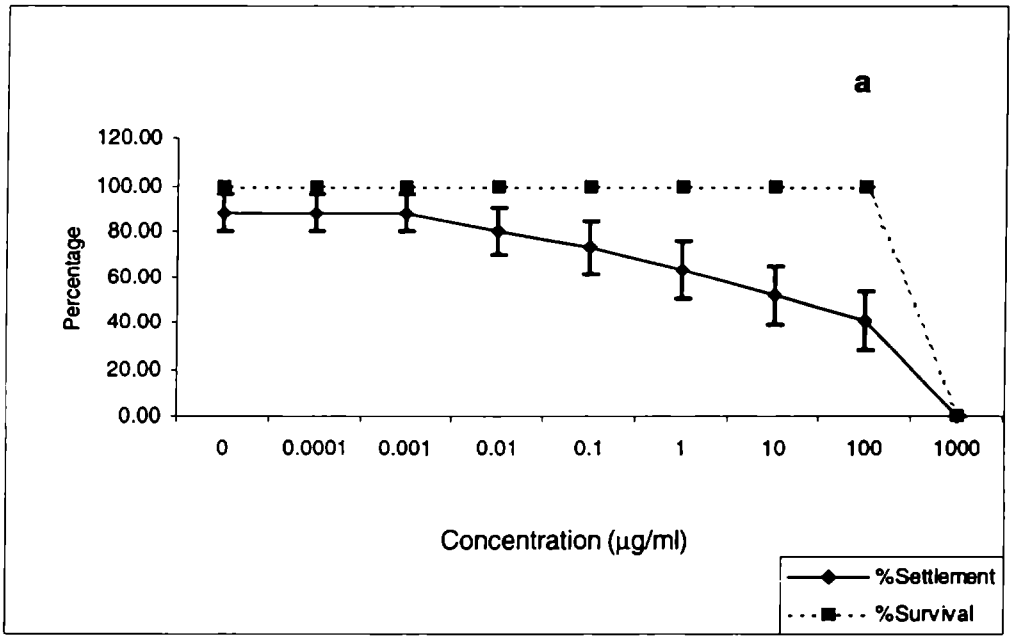
Effect of methylene chloride accommodated secondary metabolites of *Spathoglossum sp.* on the survival of larvae of *Hydroides elegans.*

Concentration (µg/ml)	% survival	S.E.M.
Control	100.0	0
0	100.0	0
0.0001	100.0	0
0.001	100.0	0
0.01	100.0	0
0.1	100.0	0
1	100.0	0
100	100.0	0
1000	0.0	0

TABLE 2.88

Effect of methylene chloride accommodated secondary metabolites of *Spathoglossum sp.* on the survival of larvae of *Hydroides elegans.*

Concentration (µg/ml)	% settlement	S.E.M.
Control	81.0	10.1
0.0001	80.0	10.3
0.001	69.0	11.9
0.01	61.0	12.6
0.1	55.0	12.8
1	50.0	12.9
10	41.6	12.7
100	0.0	0.0
1000	0.0	0.0



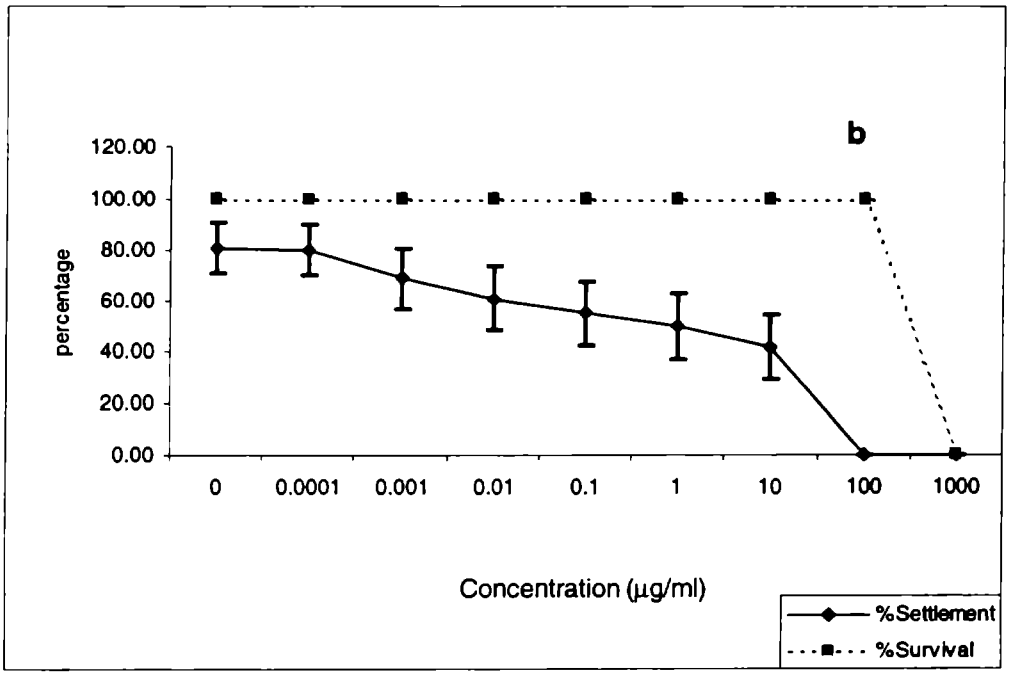


Fig. 2.20

Effect of secondary metabolites of *Spathoglossum* species accommodated in a) methanol, (b) methylene chloride on the survival and settlement of larvae of *Hydroides elegans.*

TABLE 2.89

Effect of methanol accommodated secondary metabolites of *Spyridia filamentosa* on the survival of larvae of *Hydroides elegans*.

Concentration (µg/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	100.0	0.0
100	90.9	7.4
1000	0.0	0.0

TABLE 2.90

Effect of methanol accommodated secondary metabolites of *Spyridia filamentosa* on the survival of larvae of *Hydroides elegans*.

Concentration (µg/ml)	% settlement	S.E.M.
Contro	60.0	12.7
0.0001	17.0	9.7
0.001	14.3	9.0
0.01	11.4	8.2
0.1	8.6	7.2
1	0.0	0.0
10	0.0	0.0
100	0.0	0.0
1000	0.0	0.0

TABLE 2.91

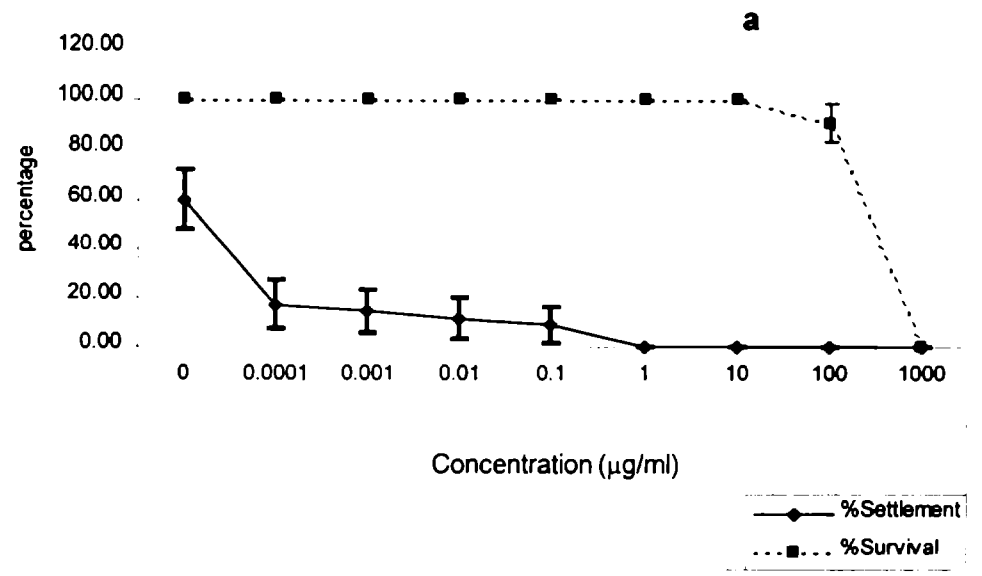
Effect of methyelene chloride accommodated secondary metabolites of *Spyridia filamentosa* on the survival of larvae of *Hydroides elegans*.

Concentration (µg/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	70.0	11.8
100	0.0	0.0
1000	0.0	0.0

TABLE 2.92

Effect of methylene chloride accommodated secondary metabolites of *Spyridia filamentosa* on the settlement of larvae of *Hydroides elegans*.

Concentration (µg/ml)	% settlement	S.E.M.
Control	90.0	7.8
0.0001	12.5	8.5
0.001	12.5	8.5
0.01	8.3	7.1
0.1	8.3	7.1
1	4.2	5.2
10	0.0	0.0
100	0.0	0.0
1000	0.0	0.0



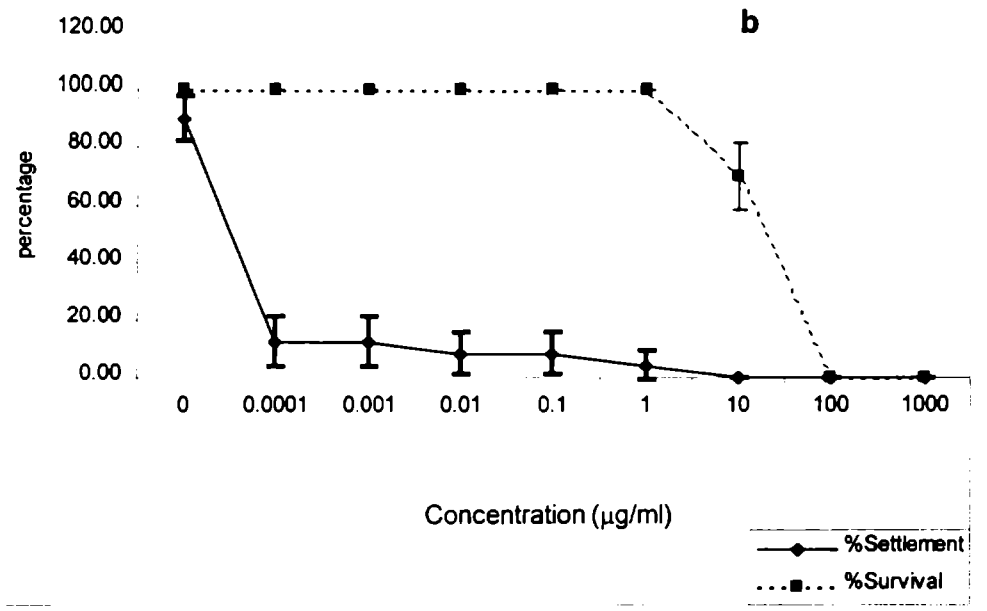


Fig. 2.21

Effect of secondary metabolites of *Spyridia filamentosa* accommodated in (a) methanol, (b) methylene chloride on the survival and settlement of larvae of *Hydroides elegans*.

TABLE 2.93

Effect of methanol accommodated secondary metabolites of *Stoechospermum marginatum* on the survival of *larvae of Hydroides elegans.*

Concentration (µg/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	100.0	0.0
100	0.0	0.0
1000	0.0	0.0

TABLE 2.94

Effect of methanol accommodated secondary metabolites of *Stoechospermum marginatum* on the settlement of larvae of *Hydroides elegans.*

Concentration (µg/ml)	% settlement	S.E.M.
Control	85.0	9.2
0.0001	80.0	10.3
0.001	80.0	10.3
0.01	80.0	10.3
0.1	80.0	10.3
1	80.0	10.3
10	80.0	10.3
100	0.0	0.0
1000	0.0	0.0

TABLE 2.95

Effect of methylene chloride accommodated secondary metabolites of *Stoechospermum marginatum* on the survival of *larvae of Hydroides elegans.*

Concentration (µg/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	0.0	0.0
100	0.0	0.0
1000	0.0	0.0

TABLE 2.96

Effect of methylene chloride accommodated secondary metabolites of *Stoechospermum marginatum* on the settlement of larvae of *Hydroides elegans.*

Concentration (µg/ml)	% settlement	S.E.M.
Control	90.0	7.8
0.0001	38.0	12.5
0.001	33.0	12.1
0.01	26.0	11.3
0.1	12.0	8.4
1	5.0	5.6
10	0.0	0.0
100	0.0	0.0
1000	0.0	0.0

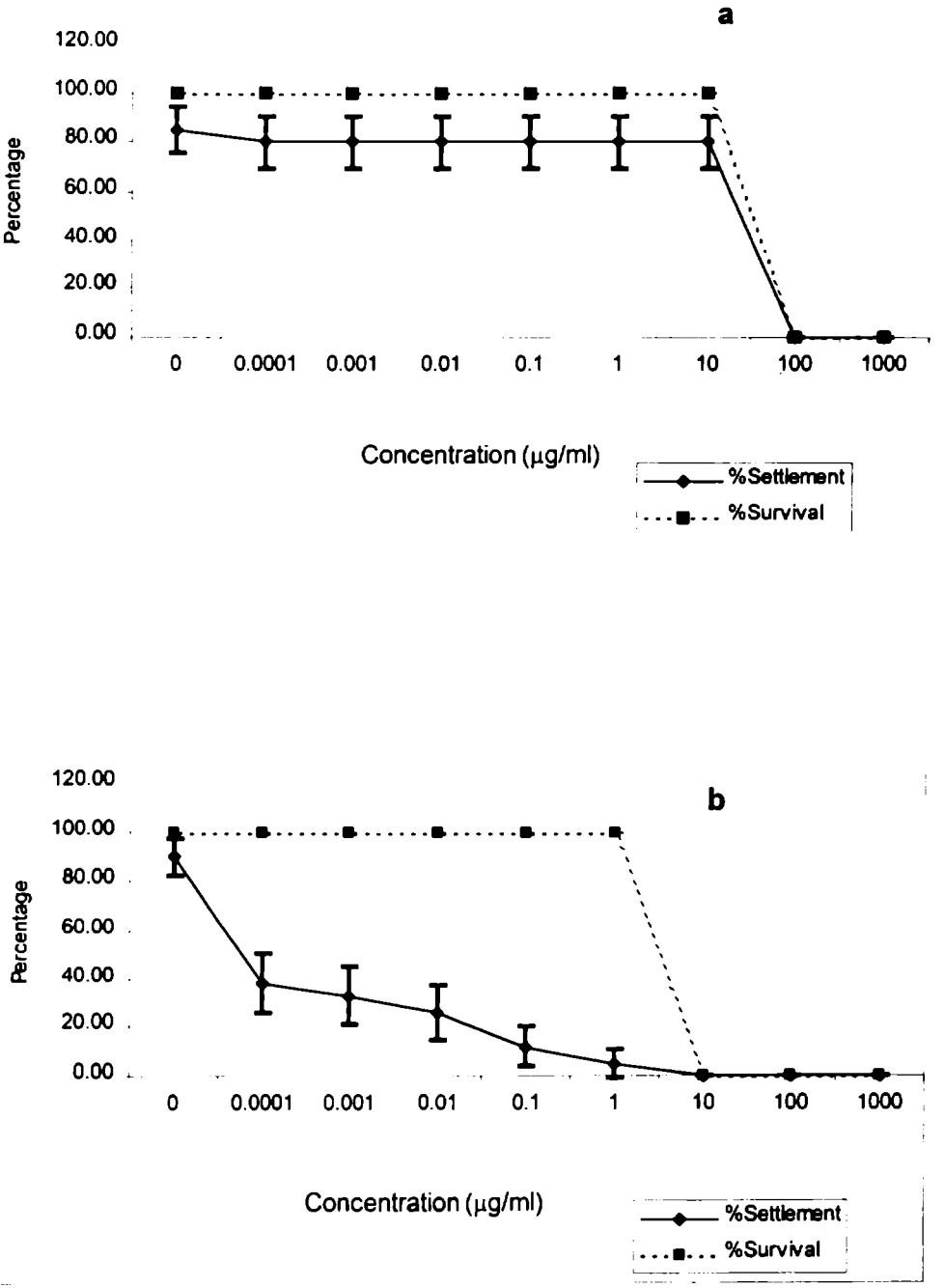


Fig. 2.22

Effect of secondary metabolites of *Stoechospermum marginatum* accommodated in (a) methanol, (b) methylene chloride on the survival and settlement of larvae of *Hydroides elegans.*

TABLE 2.97

Effect of methanol accommodated secondary metabolites of *Turbina ornata* on the survival of larvae of *Hydroides elegans*.

Concentration (μg/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	0.0	0.0
100	0.0	0.0
1000	0.0	0.0

TABLE 2.98

Effect of methanol accomodated secondary metabolites of *Turbinaria ornata* on the settlement of larvae of *Hydroides elegans*.

Concentration (μg/ml)	% settlement	S.E.M.
Control	67.0	12.1
0.0001	53.9	12.9
0.001	53.9	12.9
0.01	33.3	12.2
0.1	33.3	12.2
1	33.3	12.2
10	0.0	0.0
100	0.0	0.0
1000	0.0	0.0

TABLE 2.99

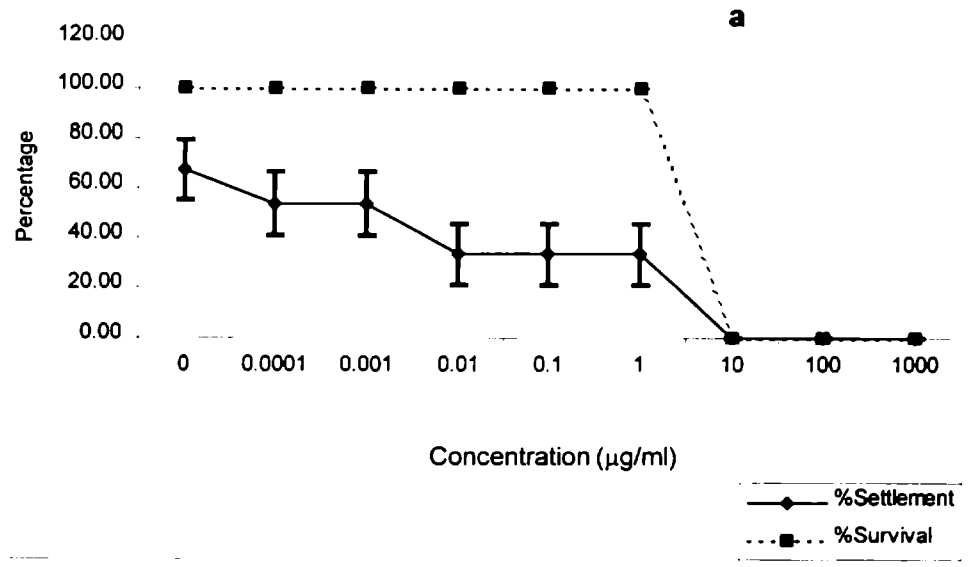
Effect of methylene chloride accomodated secondary metabolites of *Turbinaria ornata* on the survival of larvae of *Hydroides elegans*.

Concentration (μg/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	63.0	12.5
100	31.6	12.0
1000	0.0	0.0

TABLE 2.100

Effect of methylene chloride accommodated secondary metabolites of *Turbinaria ornata* on the settlement of larvae of *Hydroides elegans*.

Concentration (μg/ml)	% settlement	S.E.M.
Control	72.0	11.6
0.0001	12.0	8.4
0.001	16.7	9.6
0.01	20.0	10.3
0.1	13.6	8.9
1	7.7	6.9
10	6.7	6.4
100	11.7	8.3
1000	0.0	0.0



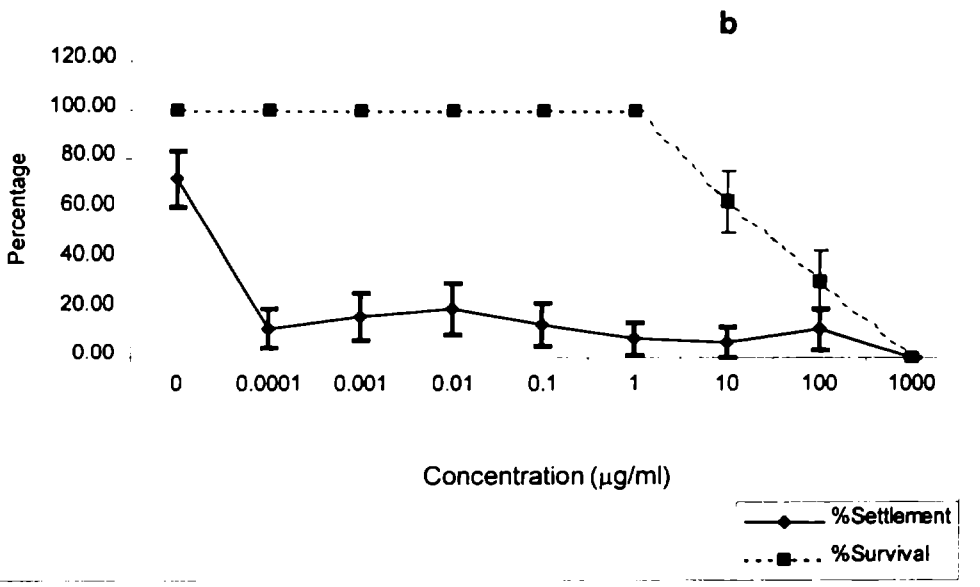


Fig. 2.23

Effect of secondary metabolites of *Turbinaria ornata* accommodated in (a) methanol, (b) methylene chloride on the survival and settlement of larvae of *Hydroides elegans*.

TABLE 2.101

Effect of methanol accommodated secondary metabolites of *Ulva lactuca* on the survival of larvae of *Hydroides elegans.*

Concentration (µ g/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	100.0	0.0
100	100.0	0.0
1000	0.0	0.0

TABLE 2.102

Effect of methanol accommodated secondary metabolites of *Ulva lactuca* on the settlement of larvae of *Hydroides elegans.*

Concentration (µg/ml)	% settlement	S.E.M.
Control	53.5	12.9
0.0001	51.0	12.9
0.001	61.0	12.6
0.01	35.1	12.3
0.1	49.0	12.9
1	69.7	11.9
10	0.0	0.0
100	0.0	0.0
1000	0.0	0.0

TABLE 2.103

Effect of methylene chloride accommodated secondary metabolites of *Ulva lactuca* on the survival of larvae of *Hydroides elegans.*

Concentration (µg/ml)	% survival	S.E.M.
Control	100.0	0.0
0.0001	100.0	0.0
0.001	100.0	0.0
0.01	100.0	0.0
0.1	100.0	0.0
1	100.0	0.0
10	0.0	0.0
100	0.0	0.0
1000	0.0	0.0

TABLE 2.104

Effect of methylene chloride accommodated secondary metabolites of *Ulva lactuca* on the settlement of larvae of *Hydroides elegans.*

Concentration (µg/ml)	% settlement	S.E.M.
Control	93.0	6.6
0.0001	85.7	9.0
0.001	85.7	9.0
0.01	75.0	11.2
0.1	70.0	11.8
1	53.9	12.9
10	0.0	0.0
100	0.0	0.0
1000	0.0	0.0

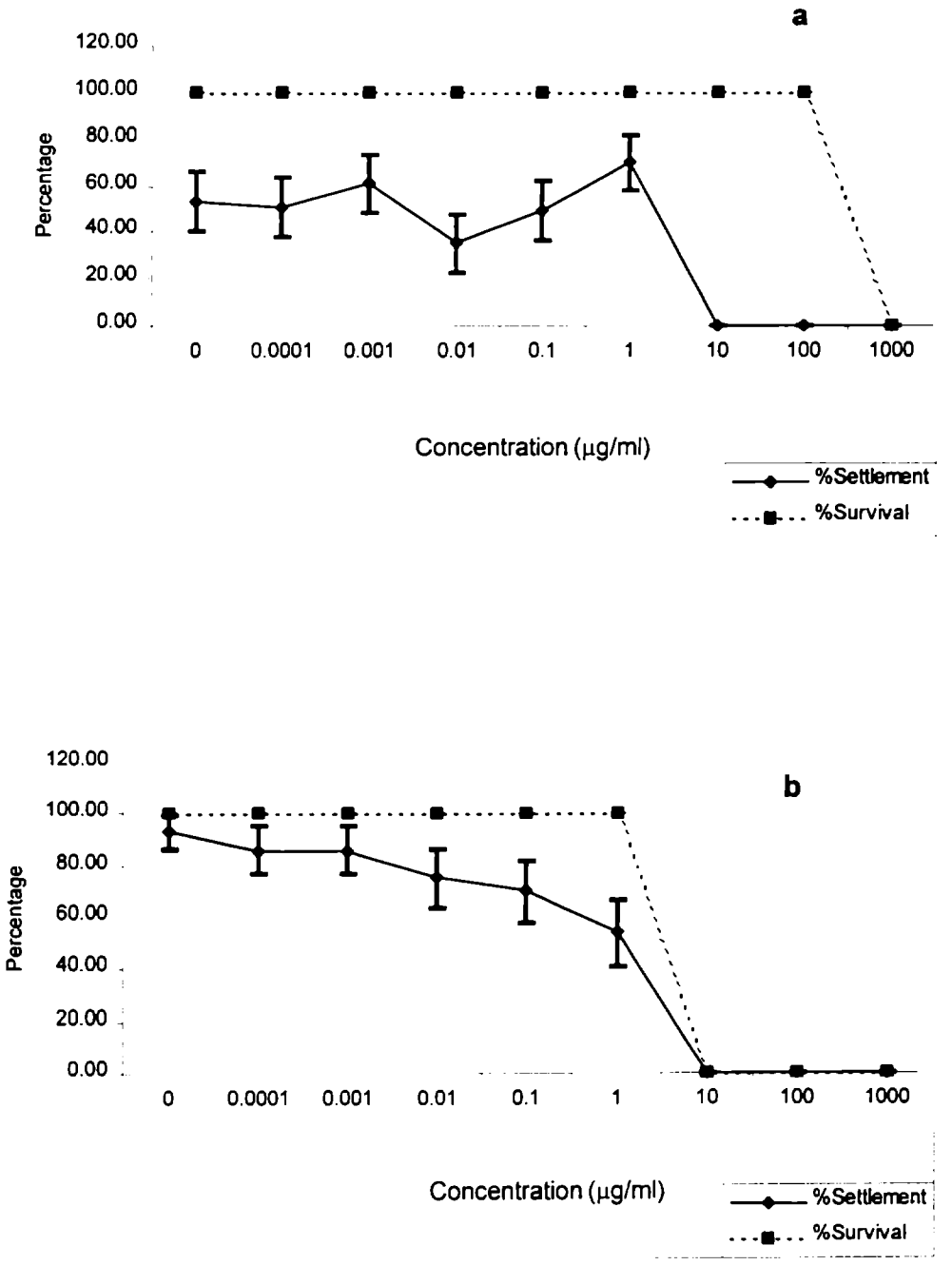


Fig. 2.24

Effect of secondary metabolites of *Ulva lactuca* accommodated in (a) methanol, (b) methylene chloride on the survival and settlement of larvae of *Hydroides elegans*.

TABLE 2.105

Regression analysis on the effect of methanol accommodated secondary metabolites of the seaweeds on the survival of larvae of *Hydroides elegans*.

Seaweeds	Regression equation	F ratio	Probability	DF
Caulerpa scalpelliformis	Y= 83.33 + - 8.33 X	3.0	0.1340	6
Cheaetomorpha linoides	Y= 83.33 + - 8.33 X	3.0	0.1340	6
Gracilaria corticata	Y= 83.33 + - 8.33 X	3.0	0.1340	6
Gracilaria Lichenoides	Y= 55.55 + -12.68 X	11.2	0.0154	6
Padina australis	Y= 83.33 + - 8.33 X	3.0	0.1340	6
Padina tetrastromatica	Y= 83.33 + - 8.33 X	3.0	0.1340	6
Sargassum vvightii	Y= 65.90 + - 12.44 X	48.4	0.0004	6
Spathoglossum sp.	Y= 83.33 + - 8.33 X	3.0	0.1340	6
Spyridia filamentosa	Y= 81.93 + - 8.87 X	3.8	0.1009	6
Stoechospermum marginatum	Y= 67.86 + - 14.29 X	8.0	0.0300	6
Turbinaria ornata	Y= 53.57 + - 17.86 X	15.0	0.0082	6
Ulva lactuca	Y= 83.33 + - 8.33 X	3.0	0.1340	6

TABLE 2.106

Regression analysis on the effect of methanol accommodated secondary metabolites of the seaweeds on the rate of settlement of larvae of *Hydroides elegans*

Seaweeds	Regression equation	F ratio	Probability	DF
Caulerpa scalpelliformis	Y= 49.16 + -9.24 X	26.8	0.0021	6
Cheaetomorpha linoides	Y= 69.79 + -9.61 X	11.1	0.0158	6
Gracilaria corticata	Y= 59.29 + - 7.91 X	7.1	0.0377	6
Gracilaria lichenoides	Y= 36.32 + - 8.92 X	16.3	0.0068	6
Padina australis	Y= 46.15 + -12.36 X	24.7	0.0025	6
Padina tetrastromatica	Y= 10.45 + 0.4611 X	0.2	0.6931	6
Sargassum wightii	Y= 59.18 + 12.12 X	32.4	0.0013	6
Spathoglossum sp.	Y= 54.99 + -11.25 X	37.5	0.0009	6
Spyridia filamentosa	Y= 5.03 + -2.78 X	43.8	0.0006	6
Stoechospermum marginatum	Y= 54.29 + - 11.43 X	8.0	0.0300	6
Turbinaria ornata	Y= 21.52 + -8.89 X	46.7	0.0005	6
Ulva lactuca	Y= 28.77 + - 8.89 X	7.5	0.0340	6

TABLE 2.107

Regression analysis on the effect of methylene chloride accommodated secondary metabolites of the seaweeds on the survival of larvae of *Hydroides elegans.*

Seaweeds	Regression equation	F ratio	Probability	DF
Caulerpa scalpelliformis	Y= 12.61 + - 3.26 X	64.7	0.0002	6
Cheaetomorpha linoides	Y= 83.33 + - 8.33 X	3.0	0.1340	6
Gracilaria corticata	Y= 62.14 + - 15.71 X	15.5	0.0077	6
Gracilaria lichenoides	Y= 50.98 + - 17.81 X	30.9	0.0014	6
Padina australis	Y= 49.51 + - 17.98 X	25.4	0.0024	6
Padina tetrastromatica	Y= 67.86 + - 14.29 X	8.0	0.03	6
Sargassum wightii	Y= 73.48 + -12.12 X	8.2	0.0289	6
Spathoglossum sp.	Y= 83.33 + - 8.33 X	3.0	0.1340	6
Spyridia filamentosa	Y= 63.571 + -15.36 X	13.6	0.0103	6
Stoechospermum marginatum	Y= 53.57 + - 17.86 X	15.0	0.0082	6
Turbinaria ornata	Y= 67.45 + -13.73 X	16.6	0.0065	6
Ulva lactuca	Y= 53.57 + - 17.86 X	15.0	0.0082	6

TABLE 2.108

Regression analysis on the effect of methylene chloride accommodated secondary metabolites of the seaweeds on the rate of settlement of larvae of *Hydroides elegans*.

Seaweeds	Regression equation	F ratio	Probability	DF
Caulerpa scalpelliformis	Y= 9.29 + -3.52 X	256.6	3.76E-06	6
Cheaetomorpha linoides	Y= 10.93 + -2.47 X	18.3	0.0052	6
Gracilaria corticata	Y= 18.52 + -5.14 X	30.3	0.0015	6
Gracilaria lichenoides	Y= 2.46 + -1.75X	15.5	0.0077	6
Padina australis	Y= 3.67 + - 2.[illegible] X	29.8	0.0016	6
Padina tetrastromatica	Y= 14.82 + -1.55 X	0.6	0.4758	6
Sargassum wightii	Y= 16.79 + - 4.84X	33.6	0.0012	6
Spathoglossum sp.	Y= 38.82 + - 11.53X	50.5	0.0004	6
Spyridia filamentosa	Y= 4.67 + - 2.13 X	76.0	0.0001	6
Stoechospermum marginatum	Y= 11.18 + - 6.14 X	56.5	0.0003	6
Turbinaria ornata	Y= 10.12 + - 1.84X	6.6	0.0427	6
Ulva lactuca	Y= 38.73 + - 15.12X	41.6	0.0007	6

Table 2.109

Probit analysis on the effect of methanol accommodated secondary metabolites of the seaweeds on the survival of larvae of *Hydroides elegans.*

Seaweeds	Regression equation	LC 50 (µg/ml)	95% confidence limit	X^2	Proba-bility	DF
Caulerpa scalpelliformis	P= -5.08 + 2.1 X	266.36	100.74 - 743	808.5	0.000	6
Cheaetomorpha linoides	P= -5.08 + 2.10 X	266.36	100.74 - 743	808.5	0.000	6
Gracilaria corticata	P= -5.08 + 2.10 X	266.36	100.74 - 743	808.5	0.000	6
Gracilaria lichenoides	P= -0.19 + 0.41 X	2.89	0.0304 - 133861.87	3798.0	0.000	6
Padina australis	P= -5.08 + 2.1 X	266.36	100.74 - 743	808.5	0.000	6
Padina tetrastromatica	P= -5.08 + 2.1 X	266.36	100.74 - 743	808.5	0.000	6
Sargassum wightii	P= -0.82 + 0.61 X	21.93	13.00 - 38.641	112.9	0.000	6
Spathoglossum sp.	P= -5.08 + 2.1 X	266.36	100.74 - 743	808.5	0.000	6
Spyridia filamentosa	P= -5.05 + 2.16 X	221.28	121.44 - 418.97	440.0	0.000	6
Stoechospermum marginatum	P= -4.50 + 3.04 X	30.41	19.40 - 48.13	311.8	0.000	6
Turbinaria ornata	p = - 2.31 + 4.56 X	3.21	2.60 - 3.95	51.4	0.000	6
Ulva lactuca	P = -5.08 + 2.10 X	266.36	100.74 - 743	808.5	0.000	6

TABLE 2.110

Probit analysis on the effect of methanol accommodated secondary metabolites of the seaweeds on the rate of settlement of larvae of *Hydroides elegans*.

Seaweeds	Regression equation	LC 50 (μg/ml)	95% confidence limit	X^2	Proba-bility	DF
Caulerpa scalpelliformis	P= 0.02 + 0.26 X	0.801	0.0364 - 38.94	1009.3	0.000	6
Cheaetomorpha linoides	P= -0.39 + 0.3 X	21.05	0.6185 - 585428.59	1887.1	0.000	6
Gracilaria corticata	P= -0.26 + 0.23 X	14.58	0.11 - 8.95 E + 19	2083.4	0.000	6
Gracilaria lichenoides	P= -0.42 + 0.26 X	0.0249	0.0000 - 2.216	1940.0	0.000	6
Padina australis	P= - 0.99 + 0.20 X	0.00001	1.57 E-15 - 0.0011	768.9	0.000	6
Padina tetrastromatica	P= 1.26 +- 0.03 X	1.63 E + 47	Nil	693.5	0.000	6
Sargassum wightii	P= -0.34 + 0.41 X	6.81	1.02 - 92.12	914.0	0.000	6
Spathoglossum sp.	P= -0.17 + 0.35 X	3.05	0.35 - 57.62	931.4	0.000	6
Spyridia filamentosa	P= 2.03 + 0.31 X	<0.00001	8.89E-12 - 0.00001	251.0	0.000	6
Stoechospermum marginatum	P= -0.13 + 0.34 X	2.36	0.0033 - 7.33 E + 16	4267.1	0.000	6
Turbinaria ornata	P= 1.03 + 0.35 X	0.0011	0.0000 - 0.0144	1151.6	0.000	6
Ulva lactuca	P= 0.69 + 0.29 X	0.0042	NIL	3960.1	0.000	6

Table 2.111

Probit analysis on the effect of methylene chloride accommodated secondary metabolites of the seaweeds on the survival of larvae of *Hydroides elegans*.

Seaweeds	Regression equation	LC 50 (µg/ml)	95% confidence limit	X^2	Proba-bility	DF
Caulerpa scalpelliformis	P= 1.22 + 0.16 X	>0.00001	2.67E -16 - 0.00001	245.5	0.000	6
Cheaetomorpha linoides	P= -5.08 + 2.10 X	266.36	100.74 - 743	808.5	0.000	6
Gracilaria corticata	P= - 3.60 + 3.31 X	12.24	11.78 - 12.76	5.2	0.523	6
Gracilaria lichenoides	P= -0.53 + 1.78 X	1.99	1.65 - 2.40	48.6	0.000	6
Padina australis	P= - 0.20 + 1.61 X	1.34	0.4933 - 4.345	980.6	0.000	6
Padina tetrastromatica	P= -4.50 + 3.04 X	30.41	19.40 - 48.13	311.8	0.000	6
Sargassum wightii	P= -4.82 + 2.57 X	74.95	65.48 - 85.31	33.1	0.000	6
Spathoglossum sp.	P= -5.08 + 2.1 X	266.36	100.74 - 743	808.5	0.000	6
Spyridia filamentosa	P= - 3.86 + 3.28 X	15.05	13.98 - 16.38	12.3	0.056	6
Stoechospermum marginatum	P= -2.31 + 4.56 X	3.21	2.60 - 3.95	51.4	0.000	6
Turbinaria ornata	P= -1.97 + 1.36 X	28.19	15.96 - 49.78	310.0	0.000	6
Ulva lactuca	P= -2.31 + 4.56 X	3.21	2.60 - 3.95	51.4	0.000	6

Table 2.112

Probit analysis on the effect of methylene chloride accommodated secondary metabolites of the seaweeds on the rate of settlement of larvae of *Hydroides elegans.*

Seaweeds	Regression equation	LC 50 (μg/ml)	95% confidence limit	X^2	Proba-bility	DF
Caulerpa scalpelliformis	P= 1.49 + 0.22 X	>0.00001	9.91 E - 11 - 0.00001	164.9	0.000	6
Cheaetomorpha linoides	P= 1.29 + 0.13 X	2.28 E -10	4.57E - 36 - 0.0000	305.6	0.000	6
Gracilaria corticata	P= 0.99 + 0.20 X	0.00001	1.57 E -15 - 0.0011	768.9	0.000	6
Gracilaria lichenoides	P= 2.69 + 0.41 X	>0.00001	4.35 E-11 - 0.00001	138.2	0.000	6
Padina australis	P= 2.34 + 0.36 X	<2.44E-09	2.44E-09 - 0.0000	96.7	0.000	6
Padina tetrastromatica	P= 1.06 + 0.07 X	8.45 E -16	Nil	2039.6	0.000	6
Sargassum wightii	P= 1.07 + 0.20 X	0.00001	4.82 E-15 - 0.00054	666.2	0.000	6
Spathoglossum sp.	P= 0.37 + 0.35 X	0.0871	0.0037 - 1.32	1310.9	0.000	6
Spyridia filamentosa	P= 1.91 + 0.24 X	8.25E -09	2.55E -15 - 0.0000	183.0	0.000	6
Stoechospermum marginatum	P= 1.67 + 0.39 X	0.00005	0.0000 - 0.00025	212.2	0.000	6
Turbinaria ornata	P= 1.31 + 0.10 X	2.70 E - 13	Nil	399.4	0.000	6
Ulva lactuca	P= 0.49 + 0.55 X	0.1286	0.01 - 1.80	1923.4	0.000	6

This pattern in the data indicates that molecules of this class inhibit settlement via non toxic mechanisms.

2.3.1 Toxicity assays using barnacle nauplii

Observations on the swimming activity of *nauplii* at the termination of the assays indicated that seaweed exudates with metabolites of all seaweeds except *that of Gracilaria corticata* and *Padina tetrastromatica* were toxic at the maximum concentration (1000 µg/ml) tested. Toxicity of metabolites was found to be concentration dependent. Methylene chloride accommodated metabolites of *Gracilaria corticata* and *Padina tetrastromatica* were toxic to the nauplii even at 0.0001 µg/ml, the lowest concentration tested (Table 2.11, 2.23, Fig.2.3 b, 2.6 b).

Regression equation were worked out taking logarithm of the concentration as independent variable (x) and percentage of larval survival as the dependent variable (p).

In cases of seaweeds tested except *Chaetomorpha linoides*, *Gracilaria corticata, Padina tetrastromatica, Spyridia filamentosa, Turbinaria ornata, Ulva lactuca* (Table 2.49) and all others except the seaweed *Padina australis* (Table 2.51) the regression equation fitted showed that the results obtained were highly significant. This shows that a linear relationship exists between concentration of methylene chloride accomodated secondary metabolites of seaweeds and percentage of *Balanus amphitrite* larval survival, in the case of majority of the seaweeds, tested.

LC_{50} values of the metabolites varied between 0.1 µg/ml and 323.4 µg/ml for the methylene chloride extract of *Gracilaria corticata and Padina australis respectively* (Table 2.55) and between (Table 2.53) 7.0 µg/ml and 1195.25 µg/ml for methanol extract of *Sargassum wightii* and *Caulerpa*

scalpelliformis respectively. The methylene chloride accommodated secondary metabolites of all seaweeds tested except that of *Caulerpa scalpelliformis, Chaetomorpha linoides Sargassum wightii* and *Turbinaria ornata* the probit equation fitted showed that the results obtained was highly significant as indicated by X^2 statistics value (Table 2.55). This shows that a linear relationship exists between concentration of methylene chloride accommodated secondary metabolites of seaweeds and percentage survival of larvae of *Balanus amphitrite* in the case of majority of the seaweeds tested.

2.3.2 Inhibition of barnacle cyprid settlement

Methylene chloride accommodated secondary metabolites of seaweeds significantly inhibited settlement of cyprid larvae of *Balanus amphitrite* (Fig. 2.1, 2.12). The metabolites from all the seaweeds except *Padina australis* (Table 2.20, Fig. 2.5 b) completely inhibited settlement at the highest test concentration (1000 µg/ml) and several methylene chloride fractions were still significantly inhibitory at 0.0001 µg/ml , the lowest concentration tested. The metabolites in the methylene chloride extract of *Sargassum wightii* inhibited settlement by approximately 94.81% at 0.0001 µg/ml (Table 2.28, Fig.2.7b). Metabolites in the methanol extract of seaweeds were comparatively less active.

Regression and probit equations were worked out taking logarithm of the concentration as independent variable (X) and percentage of cyprid settlement as the dependent variable (p).

Among the seaweeds tested, except for the seaweed *Chaetomorpha linoides,* the regression equation fitted showed that the results of settlement prevention was highly significant as evidenced by the F ratio (Table 2.50, 2.52).

This shows that a linear relationship exists between concentration of methanol and methylene chloride accomodated secondary metabolites of seaweeds and settlement of cyprid larvae of *Balanus amphitrite*, for majority of the seaweeds tested.

EC_{50} values of the metabolites in the methanol extract (Table 2.54) and methylene chloride extract (Table 2.56) varied between >9.50 E-12 µg/ml (methanol extract of *Ulva lactuca)* and 9513.16 µg/ml (methanol extract of *Caulerpa scalpelliformis*) and between 3.13 E−14 µg/ml (methylene chloride extract of *Sargassum wightii*) and 0.0059 µg/ml (methylene chloride extract of *Spathoglossum* sp.).

Among the seaweeds tested except for the seaweeds *Caulerpa scalpelliformis* (Table 2.54) and *Chaetomorpha linoides* (Table 2.54, 2.56) for all others the probit equation fitted shows that the results obtained ie. settlement inhibition was highly significant as X^2 statistics value indicates. This shows that a linear relationship exists between concentration of methanol and methylene chloride accommodated secondary metabolites of seaweeds and percentage settlement of cyprid larvae of *Balanus amphitrite* for majority of the seaweeds tested.

2.3.3 Hydroides elegans larval survivorship

Studies on the percentage of survivors at the termination of the assays after 7 days indicated that metabolites in the methylene chloride fractions of all seaweeds were toxic at the maximum concentration (1000 µg/ml) tested. Metabolites in the methylene chloride extract of *Caulerpa scalpelliformis* were toxic to the larvae even at 0.0001 µg/ml, the lowest concentration tested.

Among the seaweeds tested for the seaweeds *Gracilaria lichenoides, Sargassum wightii, Stoechospermum marginatum* and *Turbinaria ornata* the regression equation fitted shows that the results obtained that is toxicity was highly significant. This shows that a linear relationship exists between concentration of methanol accommodated secondary metabolites of these seaweeds and survival of trochophore larvae of *Hydroides elegans* (Table 2.105). Among the seaweeds tested except for the seaweeds *Chaetomorpha linoides* and *Spathoglossm* sp. for all others the regression equation fitted showed that the results obtained that is toxicity was highly significant as evidenced by the F ratio (Table 2.107). This shows that a linear relationship exists between concentration of methylene chloride accommodated secondary metabolites of seaweeds and survival of trochophore larvae of *Hydroides elegans* for majority of the species tested.

LC_{50} values of the methanol accommodated (Table 2.109) and methylene chloride accommodated (Table 2.111) secondary metabolites varied between 2.89 µg/ml (methanol extract of *Gracilaria lichenoides*) and 266.36 µg/ml (methanol extract of several seaweeds) and between >0.00001 µg/ml (methylene chloride extract of *Caulerpa scalpelliformis*) and 266.36 µg/ml (methylene chloride extracts of *Spathoglossum* sp. and *Chaetomorpha linoides*)

Among the seaweeds tested the probit equation fitted shows that the results obtained that is toxicity was highly significant for all the seaweeds except for the seaweeds *Gracilaria corticata* and *Spyridia filamentosa* as evidenced by the X^2 statistics value (Table 2.109, 2.111). This shows that a linear relationship exists between concentration of methanol and methylene chloride accomodated secondary

metabolites of seaweeds and the percentage of survival of larvae of *Hydroides elegans* for majority of the seaweeds tested.

2.3.4 Inhibition of settlement of the trochophore larvae of *Hydroides elegans*

Settlement of trochophore larvae was significantly inhibited by seaweed metabolites (Fig. 2.13 to 2.24). Metabolites in the methylene chloride extracts of all seaweeds except *Spathoglossum* sp. completely inhibited trochophore settlement at 1000 µg/ml, being the highest concentration tested.

Seaweed metabolites had significantly different effects on *Hydroides elegans* trochophore larvae in the settlement assays. Methylene chloride fraction of *Padina australis* showed presence of active metabolites, inhibiting settlement by 85% at 0.0001 µg/ml (Table 2.76, Fig. 2.17). Methylene chloride extracts of *Spathoglossum sp.* and *Ulva lactuca* contained less active metabolites, inhibiting settlement by 20% and 14.3% respectively at 0.0001 µg/ml, the lowest concentration tested (Table 2.88, 2.104, Fig. 2.20b, 2.24b). Among the seaweeds tested except for the seaweed *Padina tetrastromatica* for all the others the regression equation fitted showed that the results obtained was highly significant as the F ratio indicate (Table 2.106, 2.108). This shows that a linear relationship exists between concentration of methanol and methylene chloride accommodated secondary metabolites of seaweeds and percentage settlement of trochophore larvae of *Hydroides elegans* for majority of the seaweeds tested.

EC_{50} values of the metabolites in the methanol extract (Table 2.110) and methylene chloride extract (Table 2.112) varied between <0.00001 µg/ml (methanol extract of *Spyridia filamentosa*) and 1.63 E + 47 µg/ml (methanol extract of *Padina*

tetrastromatica) and between 8.45 E -16 µg/ml (methylene chloride extract of *Padina tetrastromatica*) and 0.1286 µg/ml (methylene chloride extract of *Ulva lactuca*).

The probit equation fitted shows that the result obtained in the case of settlement prevention was highly significant for all the seaweeds as X^2 statistics value indicates (Table 2.110, 2.112). This shows that a linear relationship exists between concentration of methanol and methylene chloride accommodated of secondary metabolites of all the seaweeds and settlement of trochophore larvae of *Hydroides elegans,* for the seaweeds tested.

2.4 DISCUSSION

Exogenous biochemical control of larval settlement is a well documented phenomenon (Morse, 1984, Burke, 1984; Morse, 1988 a, Morse 1988 b; Pawlik and Faulkner, 1988, Rittschof *et al.,* 1988; Targett, 1988).

The action mechanism of the compounds from seaweeds can be generated when compounds are identified and structures determined. Many of the effective compounds are not toxic even at a concentration exceeding thousand times excess of the settlement EC_{50}.

The seaweeds examined here contained compounds with wide variations in biological activity. Methylene chloride fractions effectively inhibited barnacle and polychaete larval settlement at very low concentrations. At these concentrations, the inhibitors do not kill nauplii and trochophore larvae. Thus compounds that act as inhibitors of larvae of crustaceans and polychaetes appear to be common in seaweeds.

EC_{50} values of these metabolites in the barnacle cyprid assays compare favourably to previously reported values for antifoulants extracted from natural

sources or commercial antifoulants such as copper or tributyl tin. For example, crude extracts from marine invertebrates by Willemsen (1994) and fractions thereof, have EC_{50} values in the range 1-100 mg. ml^{-1}. The only previous report of natural antifoulants with EC_{50} values in cyprid assays comparable to those of the present study are that of *Delisea pulchra* metabolites (De Nys *et al.*, 1995) and the "renillafoulins" extracted from the sea pansy *Renilla reniformis* (Keifer and Rinehart, 1986; Rittschof *et al.*, 1986), and pukalide and epoxypukalide isolated from the gorgonian octocoral *Leptogorgia virgulata* (Gerhart *et al.*, 1988). EC_{50} value of copper are in the range of 100's of ng per ml. Thus the activity of seaweed metabolites against barnacle and polychaete larvae is comparable to or greater than most other previously described natural antifoulants, and is in an order of magnitude lower than that of copper. Toxicity of inorganic and organic toxicants as well as that of biomolecules depend on the rapidity with which 'decay' takes place as well as the rate at which they become bio available. Therefore a direct comparison of toxicity of the above substances on a quantity scale should be done with due caution.

In barnacle settlement assays, settlement in the controls is governed by timing of the mass cultured larvae so that 30 to 60% of the individuals will settle in the control condition. This is the most sensitive time point in the assay and enables identification of inhibitory compounds. A comparison of LC_{50} and EC_{50} data provides the relationship between toxicity and settlement inhibition. Compounds that are known to be toxic (copper and tributyl-tin) have similar LC_{50} and EC_{50} values. In contrast, compounds such as the renillafoulins, natural products from the octocoral *Renilla reniformis* (Keifer *et al.*, 1986; Rittschof *et al.*, 1986) have a low EC_{50} and an LC_{50} that is high both for barnacle larvae and for human cell culture (Keifer *et al.*, 1986). Thus, even if the nature of a compound is unknown, these assays provide

insights into their mechanism of action. Compounds of low or negligible toxicity that inhibit settlement are believed to be potential and a more environmentally acceptable solution to fouling. However in laboratory assays non-toxic antifoulants appear to be more variable than toxic agents in their ability to inhibit settlement of barnacle larvae where multiple batches are considered (Rittschoff *et al.*, 1992). This is due to the potential for variability in genetic make up, physiological condition, and sensory perception between batches. The variability in effectiveness of non toxic antifoulants may, perhaps, be overcome by employing mixtures of several compounds that could cover a range of antifoulant mechanisms. The LC_{50} value indicates that the metabolites in the methylene chloride fractions are more toxic to nauplii than in the methanol fraction. Based upon EC_{50} values of the barnacle and polychaete larval settlement, metabolites in the methylene chloride fractions were found to be more potent than those from methanol fractions, except in the case of *Ulva lactuca*. This suggests that some of the bioactive agents from seaweeds are relatively non polar compounds. Further isolation and purification of these metabolites may provide better understanding of the chemical defences of seaweeds.

Swimming larvae of sedentary marine animals require a chemical signal from the environment before they can metamorphose and settle. In certain cases it is necessary for the larvae to actually touch the substrata for the induction of metamorphosis; the basis for this contact dependence is chemical recognition of a signal molecule, uniquely available at the surface of the substratum by the larvae. Barnacle larvae are stimulated to attach and metamorphose in the presence of settlement factor proteins and glycoproteins adsorbed to surfaces, but not in solution (Rittschof *et al.*, 1984). The larvae also adhere more strongly to surfaces that have been treated with settlement factor (Yule and Crisp, 1983). Barnacle larvae may

not settle on surfaces to which they cannot adhere strongly. The presence of specific inhibitory molecules in the exopolymer adsorbed to surfaces inhibited settlement of barnacle larvae (Rittschof *et al.*, 1985).

Molecular events can mediate invertebrate larval settlement by at least four pathways. The characteristics of a surface can affect settlement most simply by interaction with a larval adhesive (Yule and Walker, 1984, 1985). Alternatively, the forces that are generated between the surface and the larvae can either be sensed by the larvae or can effectively trap them . Diffusible molecules associated with the surface can act either through several receptor pathways (Morse, 1984, 1988) by binding with external larval receptors (Tarpido – Rosenthal and Morse, 1985, 1986 a,b) or by entering the larvae and altering its metabolism (Baloun and Morse, 1985, Rittschof *et al.*, 1986).

Determination of the action mechanism of natural product antifoulants (Targett *et al.*, 1983, Standing *et al.*, 1984, Rittschof et al., 1984, Keifer *et al.*, 1986, Gerhart *et al.*, 1988) should provide additional flexibility for attempts at biological control. These compounds, many of which are as effective or more effective than broad spectrum, toxic antifoulants are highly promising.

Chemical mediation of larval settlement can be affected through a variety of pathways. These include pathways involved in the mechanism of larval adhesion (Baier, 1984; Yule and Walker ,1984) and behaviour. Larval settlement, adhesion, and metamorphosis in a number of species are controlled by a molecular reaction pathway called the Trigger pathway. Through this pathway, chemical signals associated with immersed surfaces activate a specific group of chemosensory receptors on the larval epithelium (Morse, 1984). This, in turn, activates a cascade of receptor – regulated signal transducers leading to a cyclic AMP – dependent

ionic depolirization of the chemosensory membrane, thus causing excitatory firings of sensory neurons that initiate settlement, adhesion, and metamorphosis (Morse *et al.*, 1980a; Baloun and Morse, 1984).

The activation of the signal transducers identified in the Trigger pathway is sufficient to turn on the genetically programmed sequence of behavioural and developmental processes that had been arrested in the larval stage, resulting in settlement, attachment, metamorphosis and the growth (Morse et al., 1979, 1980b; Morse, 1984, 1985). Interrupting any step of this process – the original binding, the enzyme cascade, the depolarization, or the nervous system activation by seaweed metabolites will halt the metamorphosis.

Very similar signal molecules, receptors, and mechanisms of signal transduction, control the settlement and metamorphosis of larvae of a number of species. Metamorphosis of undesirable fouling organisms can be checked by simply blocking steps in the transduction of the metamorphic signal, using seaweed metabolites, thereby avoiding highly toxic substances, presently employed. The chemosensory receptors that recognize surface – associated inducers of settlement, are highly specific and sensitive. They are capable of detecting surface – associated signals at very low concentrations. It is possible that seaweed metabolites inhibit the chemosensory receptors and prevent larval settlement.

Cyclic AMP and/or membrane depolarization are involved in the signal transduction controlling metamorphosis of larvae, including bryozoans, coelenterates, echinoderms, molluscs , annelids (fouling and cementing worms) and crustaceans (barnacles) (Yool *et al.*, 1986) . Inhibition of membrane depolarization or cyclic AMP by seaweed metabolites may inhibit metamorphosis. Both the co-operativity and the up regulation occur at a post receptor level of signal transduction

or information processing, rather than through allosteric activation of the chemosensory receptor itself (Trapido – Rosenthal and Morse, 1986 a,b).

The results of the settlement study of barnacles and polychaetes (Fig. 2.1 - 2.24) reveal a declension in the number of settlement as the concentration of seaweed metabolites in the medium increased. This could be due to the following factors.

The functional significance of the Amplifier pathway is that it provides a mechanism by which a second class of chemical signals in the water column may regulate the sensitivity of the larvae to surface associated signals, and thus regulate their propensity to settle. This process may be disrupted by the metabolites in the medium.

Selective inhibition of signal transducers that act in the Trigger pathway do, in fact, inhibit larval settlement and metamorphosis in a number of species (Baloun and Morse, 1984; Morse, 1984). Inhibition of signal transducers may be the mechanism by which seaweed metabolites prevent settlement and metamorphosis.

Second messenger systems, including cAMP and calmodulin, are involved in the metamorphosis of diverse groups of larvae such as molluscs (Morse, 1984, 1988) and crustaceans (Rittschof *et al.*, 1986). Intervention of these pathways by seaweed metabolites may be another means of antifouling activity.

The discovery and identification of compounds with biological effects specific to targeted marine fouling organisms (Avelin *et al.*, 1991; Vitalina *et al.*, 1991a, b) and the knowledge of the effects on metamorphosis of compounds with known biochemical mechanisms, provide the basis for understanding the pathways

and processes involved in the settlement of marine invertebrate larvae. It is through this understanding that nontoxic or limited toxicity antifouling control can be realized.

Biofilms are an important step in the biofouling process (Holmstorm and Kjellerberg, 1994). It was noticed that the presence of a biofilm was essential for the settlement of the trochophore larvae of *Hydroides elegans*. According to Lau, *et al.* (1997) brown algal phlorotannins as well as two related compounds, tannic acid and phloroglucinol were inhibitory to some of the bacterial species that induce high rates of *Hydroides elegans* larval settlement. Thus the seaweed metabolites inhibit *Hydroides elegans* larval settlement by the regulation of the growth of micro foulers, which inturn affect larval settlement or may target them directly. The metabolites from several seaweeds tested exhibited broad spectrum activity against fouling organisms with strong effects against representatives from the two major groups of fouling organisms viz., cirripedes and polychaetes. Since any antifouling coating must work against the range of fouling organisms which occur in the field, such broad spectrum activities are important. The breadth of activity of most other natural antifoulants is not clear, since previously published works have focused on the activities of compounds against single species of fouling organisms, or a taxonomically restricted group. In some instances, tests against both invertebrate larvae and bacteria have been done (Todd *et al.*, 1993). *Delisea pulchra* metabolites have been tested against representatives of the invertebrates, algae and bacteria (de Nys *et al.*, 1995).

Caulerpa scalpelliformis metabolites in the methylene chloride fraction inhibited settlement of *Hydroides elegans* polychaete in a toxic way (Table 2.59, 2.60, Fig. 2.13b) while in the case of *Balanus amphitrite* it inhibited settlement in a non toxic way (Table 2.3, 2.4, Fig. 2.1b).

In some instances metabolites which were most effective against a particular organism were less effective against others. Methylene chlordie accommodated secondary metabolites of *Sargassum wightii* and *Padina tetrastromatica* were highly effective against larvae of barnacle and polychaete respectively. Variation in the test procedures of different assays may account for some of the variation in the effects of these metabolites on different test organisms. Extent of adsorption of metabolites from solution to the substratum could not be quantified.

Seaweed metabolites which differ only slightly at a single functional group have very different effects against marine herbivores (de Nyes *et al.*, 1995) and structurally similar amino acid derived neurotransmitters vary greatly in their effects on the settlement or metamorphosis of invertebrate larvae (Pawlik, 1990). The pharmacological literature also contain many examples of differences in the activity of structurally related compounds (Hawkins *et al.*, 1990).

According to de Nyes *et. al.* (1995), the variation in the effects of structurally similar *Delisea pulchra* metabolites suggests that the metabolites are not simply functioning as broad spectrum toxins against marine organisms. They appear to be species specific. In the case of barnacle assay, even when larvae remained live and active, their settlement were inhibited throughout the period of exposure. The toxicity of many of these compounds against barnacle larvae appears low.

It would be desirable to link specific variation in the structure of seaweed secondary metabolites with variation in activity against different fouling organisms. The lactone moiety which is present in *Delisea pulchra* metabolites is emerging as a characteristic feature of many marine natural products with antifouling properties

(Keifer *et al.*, 1986; Coll *et al.*, 1987). Holmstorm and Kjelleberg (1994) also discuss lactones as a common component of natural antifoulants.

The power of the laboratory assays is in the rapid, highly sensitive screening of potential antifouling compounds for toxicity and antifouling effectiveness. Their weakness is in the lack of precision with respect to solution concentrations. In general the assays can distinguish at best a two fold change in solution concentration. This level of sensitivity enables comparable results with wide ranges of larval species. Should the researcher need to know exact solution concentrations techniques should be modified and radioactive tracers employed. Bioassay directed purification of natural product antifoulants (Rittschof *et al.*, 1984; Standing *et al.*, 1984; Keifer *et al.*, 1986; Gerhart *et al.*, 1988; Sears *et al.*, 1990), can be used as a screening tool to search for commercially viable analogues to the complex natural products. These assays have been used to describe relationship between physicochemical properties of surfaces, microbial films, and barnacle settlement (Maki *et al.*, 1989, 1990. Studies to correlate results of laboratory assays with effects in the field (Branscomb and Rittschof, 1984; Rittschof, 1985; Rittschof and Costlow, 1989 a,b; Roberts *et al.*, 1991) indicate fair agreement. Trends of settlement observed in the laboratory may be more substantial when tested in the field (Rittschof and Costlow, 1989a).

From the commercial perspective of developing novel antifouling coatings, the secondary metabolites from seaweeds have a number of useful characteristics. First, they are very active against a range of fouling organisms. Secondly, their activity appears at least in part to be due to chemical signalling rather than simple toxicity. Thirdly, the variation in activity suggests that particular metabolites may be able to be used for specific situations. Thus, in situations

were polychaetes are the primary fouling organisms, metabolites which have high activities against polychaetes but low activities against cirripedes may be appropriate, thus minimizing effects on non-target organisms. This logic also suggests that broad spectrum antifouling coatings are likely to contain a mixture of these compounds, each of which will be most effective against a particular suite of fouling organisms. Fourthly, because of the variation in chemical properties such as polarity of the metabolites, there is both a range of potentially useful polymers which could be used as carriers for the compounds, and a range of possible means of presenting the compounds. Finally any commercial antifouling product must be able to be produced in large quantities. Several of the sea weed *Delisea pulchra* compounds, and closely related structures, have been synthesized in high yields (Jefford *et al.*, 1989).

The tests described here, and field studies also indicate that these metabolites are important in the natural ecology of seaweeds studied. *Balanus amphitrite* and *Hydroides elegans* are the common foulers in the areas where sea weeds occurs but they were absent on the seaweeds. These observations and the results highlight the link between basic field ecology and the development of novel antifouling coatings from marine organisms.

CHAPTER 3

WATERBORNE CHEMICAL COMPOUNDS IN MACROALGAE : POSITIVE AND NEGATIVE CUES FOR LARVAL SETTLEMENT

3.1 INTRODUCTION

External biochemical cues are very important in the settlement, attachment and metamorphosis of the planktonic larval stages of fouling and other sessile marine invertebrates (Morse, 1984; Rittschof, 1985). These cues may function by stimulating or inhibiting one or more of the above steps in the metamorphosis process.

It is maintained that due to tiny larval brains and poor memories, it is environmental cues rather than larval choice that determines larval settlement. The importance of settlement cues appears to vary with the species; some may be relatively non-specific, but the highly localised distributions of many adult invertebrates can ultimately be traced to patterns established at the time of larval settlement. Chemical signals, environmental and organisimic are likely to be important for species that form aggregations, those associated with heterospecific organisms, and those that settle preferentially on a surface film of microorganisms.

An antifouling compound may deter potential settlers either in the water column (waterborne cues) (Coll *et al.*, 1982; Thompson, 1985; Hadfield, 1984 and Tamburri *et al.*, 1992) or on their encounter with the surface (surface cues). (Crisp and Meadows, 1962, 1963; Morse *et al.*, 1980; Targett *et al.*, 1983; Standing *et al.*, 1984; Rittschof *et al.*, 1985, 1986; and Pawlik, 1986).

According to Morse *et al.* (1979) and Pawlik (1990) settlement can be stimulated by water - soluble neuroactive agents. Crisp and Meadows (1962) recognised that the presence of a water-soluble factor might be able to alter the behaviour of a cyprid. Rittschof (1985) presented the first direct evidence of a waterborne pheromone. Using a 'cypris chromatography assay' (Rittschof *et al.*, 1984), seawater conditioned by the presence of adult *Semibalanus balanoides* was found to induce temporary attachment of *Balanus amphitrite* cyprids. Subsequent work on synthetic peptides led to the hypothesis that the waterborne phermone is a small peptide with a basic carboxy-terminus and a neutral, or basic, amino terminus (Tegtmeyer and Rittschof, 1989; Brown *et al.*, 1998; Rittschof *et al.*, 1998).

For over two decades, Hadfield and co-workers have studied the larval settlement of the nudibranch mollusc *Phestilla sibogae* an obligate predator of hard corals of genus *Porites* (Hadfield and Karlson, 1969; Hadfield, 1977, 1984; Hadfield and Scheuer, 1985; Hadfield and Pennington, 1990). Larvae of the nudibranch settled in response to a waterborne inducer released from *Porites compressa* or *Porietes lobata* or in response to seawater containing an aqueous coral extract. Young and Chia (1981) demonstrated a delay in the settlement of the bryozoan *Bugula pacifica* in response to sea water conditioned by the presence of the ascidian *Diplosoma macdonaldi*.

The lamina, stipes and holdfasts of the various macroalgae found in both temperate and tropical areas provide large surface area for the settlement of a variety of sessile invertebrate larvae (Dawson, 1966; Littler *et al.*, 1987). Coverage of an alga's lamina or stipes by sessile invertebrates may depress photosynthesis and increase the alga's chances of dislodgement (Davis *et al.*,

1989). Oswald *et al.* (1984) documented that photosynthesis was significantly lowered when *Fucus serratus* fronds were heavily fouled by encrusting bryozoans mainly *Electra pilosa* and *Membranipora membranaceae*. Likewise, biological fouling reduced the growth rate and increased blade loss in the kelp *Macrocystis pyrifera* (Woollacott and North, 1971; Dixon *et al.*, 1981).

Seaweeds produce a diverse array of secondary metabolites (Faulkner, 1984b; Paul and Hay, 1986), that deter biofoulers. It is possible that seaweeds produce an assortment of secondary metabolites because different compounds deter different biofoulers. This may be especially true for tropical seaweeds exposed to a diverse assemblage of herbivores. However, the defensive value of a compound is a specific function of compound structure and the fouling species settling on the plant. The function of compounds cannot be predicted by structural class alone. The spatial and temporal distributions of secondary metabolites within cells, within plants and between plants often vary in ways that are adaptive. Individual plants or plant portions that are at greatest risk are often best defended. Metabolite types and concentrations vary within and among plants in a population, with the age of plants and among conspecific populations of seaweeds. (Hay and Fenical, 1988).

Natural exudation of allelochemicals has been proposed as a possible explanation for ecological interactions involving seaweeds and reduced larval recruitment within seaweed dominated assemblage (Waltres *et al.*, 1996). According to Gschwend *et al.* (1985) five species of temperate macroalgae released volatile halogenated organic compounds into surrounding water column at rates of nannograms to micrograms of each compound per gram of dry algae per day. The continuous release of toxic or deterrent waterborne compounds by macroalgae may significantly increase their fitness if the chemicals reduce biological

fouling by sessile invertebrates. The same metabolite may show pronounced differences in their effects even on closely related species of biofoulers. Walters *et al.* (1996) have reported continuous release of compounds into the ambient medium by Hawaiian macroalgae that prevented larval settlement of *Hydroides elegans* and *Bugula neritina*. However, the chemical compositions, release rates and biological effects of these compounds are yet to be elucidated.

Predictions about the toxic effects of particular secondary metabolites based on their chemical structures or results of pharmacological assays may be difficult. It is now widely accepted that field and laboratory bioassays with biofoulers form an integral part of a comprehensive approach to examine these ecological interactions.

3.2 MATERIALS AND METHODS

The aim of the present investigation was to examine the effect of secondary metabolites released into ambient medium (Waterborne compounds) by seaweeds, on the larval settlement of the ubiquitous foulers, the tube worm *Hydroides elegans* and the barnacle *Balanus amphitrite communis*. Toxicity and longevity of such compounds were also analysed.

3.2.1. Collection of seaweeds

Seaweeds for laboratory biological assays were collected from the rocky coast of Kovalam(8° 22' N ; 76° 57'E) by handpicking, diving and snorkelling. They were selected on the basis of the survey on recruitment of sessile invertebrates on them. The seaweeds were brought to the laboratory in plastic bags containing seawater collected *in situ*.

3.2.2. Test Seaweeds

The seaweeds used for bioassays included three members of the phaeophyta (*Padina tetrastroamatica, Sargassum wightii and Sargassum myriocystum*), two members of the chlorophyta (*Caulerpa peltata, Chaetomorpha sp.*) four members of the Rhodophyta (*Gelidium pusillum, Gracilaria corticata, Gracilaria foliferca and Spyridia filamentosa*).

Brood stock collection, larval release and culture of larvae of *Balanus amphitrite* are given in Chapter 1 section 1.2.25. Culture of larvae of *Hydrides elegans* is given Chapter 1, section 1.2.4.

3.2.3 Laboratory conditioning of seaweeds

Seaweeds were acclimated in large fibre glass tanks. Well aerated seawater of 30 $\pm$ 2‰ salinity was used for maintaining the seaweeds. The seaweeds were allowed to acclimate for a day under laboratory conditions before being used for experiments.

3.2.4 Preparation of seaweed conditioned water

Walters *et al.* (1996) was followed for the preparation of seaweed conditioned water.

Healthy seaweeds belonging to different species were selected and acclimated under controlled conditions for 24 hour. They were placed individually in 500 ml sterilized glass beakers containing 0.45 μm - millipore filtered seawater. The number of intact and undamaged fronds of seaweeds placed in each beaker ranged from one for larger ones and many for smaller ones (Table 3.1) These beakers containing seaweeds were kept at room temperature for 16 h in the laboratory without

aeration. During this time, all algae were subjected to 8h light and 8h dark regime with overhead fluorescent room lighting. Lastly, seaweeds were removed from each beaker, blotted dry and wet weights taken. The seaweed conditioned water was filtered using a 0.45 μm millipore filter and volume recorded. Seaweed biomass : conditioned water was calculated for each seaweed. Seaweed conditioned water used within ten minutes after the seaweeds were removed are referred to as "immediate - use water".

3.2.5 Preparation of test dishes

Settlement bioassays were conducted in triplicates using metamorphically competent trochophore larvae of *Hydroides elegans* and cyprid larvae of *Balanus amphitrite communis.* Conditioned water of each seaweed species was taken as the test substance. Seaweed conditioned millipore filtered (0.45 μm) seawater was used as the test media and normal filtered aged seawater served as controls. Seaweed conditioned water and controls for settlement assays were run in five replicates (20 ml each) in sterile polystyrene petridishes (85 x 15 mm).

3.2.6 Survival and Settlement assays with trochophore larvae of *Hydroides elegans* employing immediate use water

Trochophore settlement assays were patterned after Walters *et al* . (1996). The effects of seaweed conditioned water (immediate - use water) on survival and settlement of larvae of *Hydroides elegans* were tested using metamorphically competent trochophore larvae.

In the settlement bioassay, approximately 150 metamorphically competent (six day old) trochophore larvae of *Hydroides elegans* were added into control and test dishes with minimal dilution. To each test dish, 1ml culture of

Isochrysis galbana (6 x 10^5 cells/ml) and a biofilmed glass slide (75 x 25 mm) were added. The biofilmed glass slides were prepared by immersing glass slides in seawater for three days. Hadfield *et al.* (1994) opined that presence of biofilm provide the larvae a positive settlement cue. *Isochrysis galbana* was used as food, since this phytoplankton caused fastest larval growth rates (Hadfied *et al.*, 1994). The petridishes were covered to prevent evaporation. Light was not excluded from the test dishes.

Permanently settled and unsettled larvae were counted with the help of a dissecting microscope after 24 h and rate of settlement (percentage) computed.

In order to determine the survivorship of *Hydroides elegans* larvae in the presence of seaweed conditioned water, a comparison was made between the percentage of the total number of survivors in the experimental dishes to the percentage of the total number of survivors in control dishes after seven days (Walters *et al* ., 1996).

3.2.7 Settlement assay with cyprid larvae of *Balanus amptitrite communis.*

The effects of seaweed conditioned water (immediate - use water) on settlement of barnacle cyprid larvae were tested using three days old cyprids of *Balanus amphitrite communis*. In an assay, 25 to 30 cyprids were pipetted into each of the six control and six experimental dishes containing 20 m l of solution with minimal dilution. Covered petridishes were incubated for 22 h in a controlled environment of 28° C. After 22 h unattached larvae were rinsed with deionized water and transferred into another petridish. Permanently attached and free larvae were counted with the aid of a dissecting microscope.

3.2.8 Toxicity Assays using nauplii of *Balanus amphitrite communis*

The naupliar toxicity of the seaweed conditioned water was evaluated as per the method of Rittsch of *et al*. (1992). Continuous swimming is characteristic of nauplii and so the inability of nauplii to stay in the water column was scored as the toxic response (Rittschof *et al*., 1992). Nauplii were collected 2 hours prior to the commencement of toxicity assay. 5 ml aliquots seaweed conditioned water (immediate - use water) was pipetted in into test tubes. For the assay $\approx$ 100 µl. of seawater containing 20 to 30 numbers of stage II nauplii were added. Nauplii were not counted at the time of introduction. After 24 hours of incubation at room temperature (28°C), assay results were quantified. First, the test tubes were held up in the light and the seaweed conditioned water was observed for mortality. All tubes with nauplii in the water column were counted to determine the number of live and dead using a chambered petridish. Non swimming larvae were regarded as dead. Six replicates of control containing 0.45 µm millipore filtered seawater and six replicates of seaweed conditioned water (immediate- use water) were tested. Care was taken to use larvae from the same batch of nauplii.

3.2.9 Bioassays employing "aged water"

To determine if any of the aged seaweed conditioned water continued to influence larvae of *Hydroides elegans* or *Balanus amphitrite communis* over time, all seaweed conditioned water were re-tested after keeping at room temperature (28°c) for 24 h (hereafter referred to as "aged water"). The same protocols were used as described above.

3.2.10 Statistical analysis

To determine the toxicity of the seaweed - conditioned water, t - tests were employed. The means of the total number of survivors in algae - conditioned water were compared to the means of the total number of survivors in control dishes of filtered seawater at the end of each trial.

The results of the three trials were combined for each seaweed treatment because there were no significant differences in mortality, between trials ($P > 0.2$). For all seaweed conditioned water, differences in the rate of settlement relative to the rate of settlement in control dishes were determined.

In the settlement assays employing trochophore larvae of *Hydroides elegans*, the ratio between the number of larvae that settled within a day to the total number of larvae added to each seaweed - conditioned water was compared to the proportion of larvae that settled during the same time - period in control dishes. Similarly, in the case of *Balanus amphitrite communis* larvae, the number of larvae that settled within a day to the total number of larvae added, was compared with each seaweed conditioned water and control for each trial. The same comparisons were run with the aged algae - conditioned water. All data were analysed by students 't' test.

3.3 RESULT

It is apparent from the results obtained from the laboratory studies that the waterborne chemical compounds of seaweeds affect the rate of settlement of the larvae of both *Hydroides elegans* and *Balanus amphitrite communis.* The results are presented in table 3.1 to 3.7 and figures 3.1 to 3.20.

3.3.1 Survival and settlement of larvae of *Balanus amphitrite communis.*

Balanus amphitrite larval response to seaweed exudates showed similarity in all the trials. All the conditioned water containing seaweed exudates were lethal, and the number of settlers were significantly reduced when conditioned - water was tested immediately or after 24 hr (Table 3.2). Immediate use water containing exudates from *Gracilaria corticata, Gracilaria folifera, Sargassum myriocystum, Sargassum wightii* and *Spyridia filamentosa* killed all barnacle nauplii within minutes in all trials.

Water conditioned with *Gracilaria corticata, Caulerpa peltata, Sargassum wightii, Sargassum myriocystum, Gelidium pusillum, Spiridia filamentosa* and *Gracilaria folifera* caused 100% settlement inhibition in cyprid larvae in both immediate use and aged water trials (Table 3.4, 3.5).

Water conditioned with *Padina tetrastromatica* and *Chaetomorpha* sp. were much less effective in delaying settlement of cyprid larvae than water conditioned with all other species of seaweeds considered in these trials (Table 3.4, 3.5).

3.3.2 Survival and settlement of larvae of *Hydroides elegans*

Variations were found in the response of the trochophore larvae of *Hydroides elegans* exposed to different seaweed conditioned water. Except exudates from two macroalgae, *viz. Chaetomorpha* sp. and *Gracilaria corticata* all other seaweed exudates were significantly lethal to trochophore larvae of *Hydroides elegans* (Table 3.3). All the trochophore larvae stopped moving and

TABLE 3.1

The details of the algal biomass (g) in relation to the volume of sea water (ml) employed for the experiment.

Seaweeds	*Hydroides elegans*		*Balanus amphitrite*	
	Mean	**S.E**	**Mean**	**S.E**
Caulerpa peltata	0.2616	± 0.01	0.2496	± 0.006
Chaetomorpha species	0.1913	± 0.003	0.1948	± 0.004
Gelidium pusillum	0.2044	± 0.002	0.2073	± 0.002
Gracilaria corticarta	0.1709	± 0.01	0.1774	± 0.009
Gracilaria folifera	0.1578	± 0.007	0.1513	± 0.005
Padina tetrastromatica	0.0806	± 0.003	0.0792	± 0.001
Sargassum myriocystum	0.1299	± 0.003	0.1229	± 0.004
Sargassum wightii	0.1854	± 0.017	0.1787	± 0.019
Spyridia filamentosa	0.1045	± 0.003	0.1141	± 0.005

TABLE 3.2

Effect of waterborne chemical compounds of seaweeds accommodated in seaweed conditioned water on the percentage survival of larvae of *Balanus amphitrite*

Seaweed	Immediate use water		Aged Water	
	Mean	**S.E**	**Mean**	**S.E**
Caulerpa peltata	0.017	± 0.31**	0.02	± 0.33**
Chaetomorpha species	40.18	± 11.56**	51.00	± 11.78**
Gelidium pusillum	3.00	± 4.02**	9.00	± 6.75**
Gracilaria corticarta	0.00	± 0.00**	9.00	± 6.75 **
Gracilaria folifera	0.00	± 0.00**	2.00	± 3.2**
Padina tetrastromatica	96.65	± 4.24**	98.00	± 3.3*
Sargassum myriocystum	0.00	± 0.00**	0.00	± 0.00**
Sargassum wightii	0.00	± 0.00**	0.00	± 0.00**
Spyridia filamentosa	0.00	± 0.00**	0.00	± 0.00**

** p>0.01

* p>0.05

TABLE 3.3

Effect of waterborne chemical compounds of seaweeds accommodated in seaweed conditioned water on the percentage survival of larvae of *Hydroides elegans*.

Seaweed	Immediate use water		Aged Water	
	Mean	**S.E**	**Mean**	**S.E**
Caulerpa peltata	0.00	± 0.00**	2.00	± 3.3**
Chaetomorpha species	100.00	± 0.00	100.00	± 0.00
Gelidium pusillum	7.00	± 6.01**	10.00	± 7.07**
Gracilaria corticarta	100.00	± 0.00	100.00	± 0.00
Gracilaria folifera	10.00	± 7.07**	12.00	± 7.66**
Padina tetrastromatica	21.14	± 9.62**	35.00	± 0.00**
Sargassum myriocystum	0.00	± 0.00**	0.00	± 0.00**
Sargassum wightii	0.00	± 0.00**	0.00	± 0.00**
Spyridia filamentosa	0.00	± 0.00**	0.00	± 0.00**

** $p>0.01$

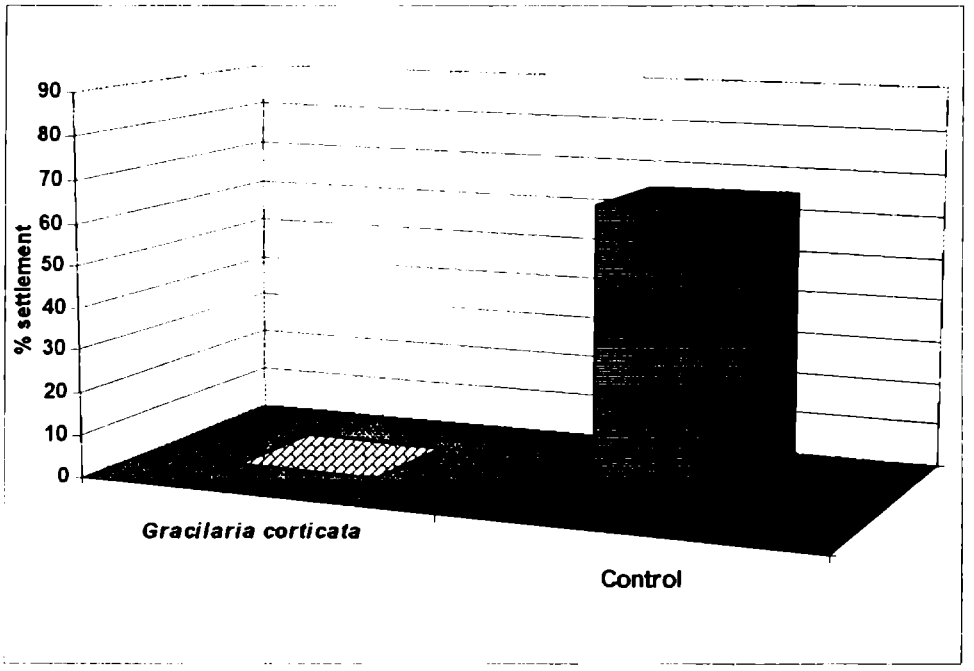
TABLE 3.4

Effect of waterborne chemical compounds of seaweeds accommodated in seaweed conditioned (immediate use) water on the settlement of larvae of *Balanus amphitrite*.

Seaweeds	Percentage Settlement	
	Control	Test
	Mean S.E.P.	Mean S.E.P.
Gracilaria corticata	67.17 ± 11.07	0.00 ± 0.00*
Padina tetrastromatica	67.15 ± 11.07	5.82 ± 5.52*
Caulerpa peltata	66.42 ± 11.13	0.00 ± 0.00*
Sargassum wightii	66.30 ± 11.14	0.00 ± 0.00*
Sargassum myriocystum	66.37 ± 11.14	0.00 ± 0.00*
Gelidium pusillum	67.87 ± 11.01	0.00 ± 0.00*
Spyridia filamentosa	67.77 ± 11.02	0.00 ± 0.00*
Gracilaria folifera	65.93 ± 11.17	0.00 ± 0.00*
Chaetomorpha sp.	65.93 ± 11.17	29.12 ± 10.71*

* $P<0.01$

S.E.P. - Standard Error of Proportion



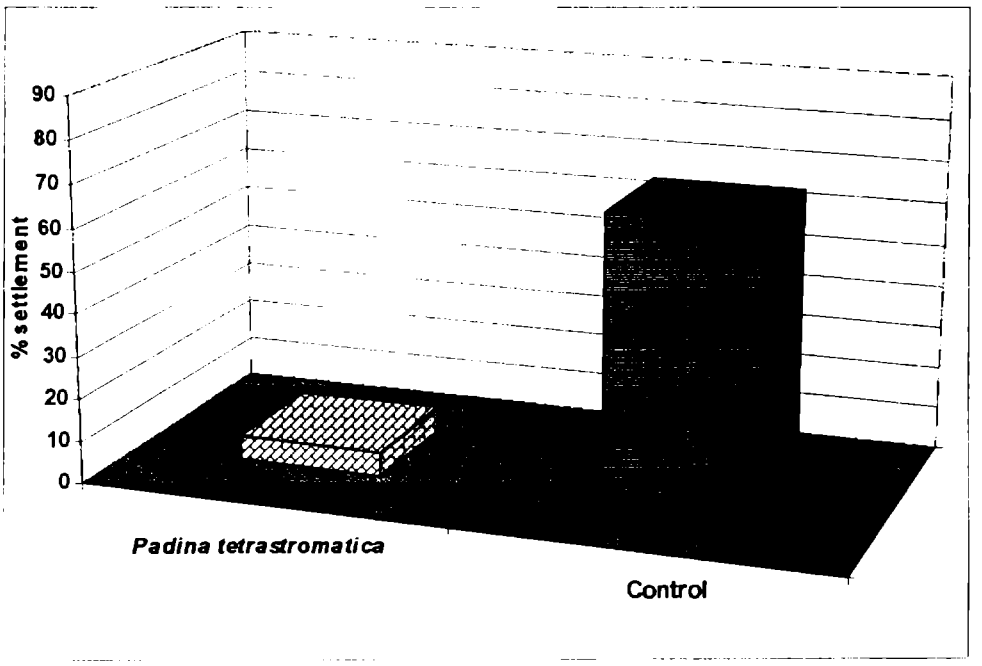
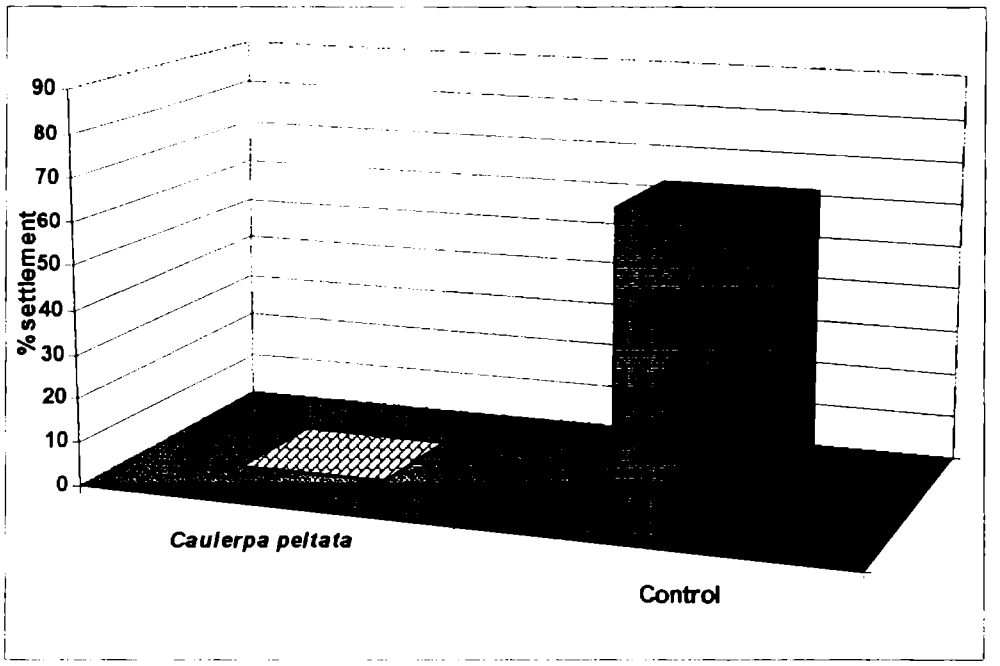


Fig.3.1

Effect of waterborne chemical compounds accommodated in immediate use, seaweed conditioned water on the rate of settlement of larvae of *Balanus amphitrite*.



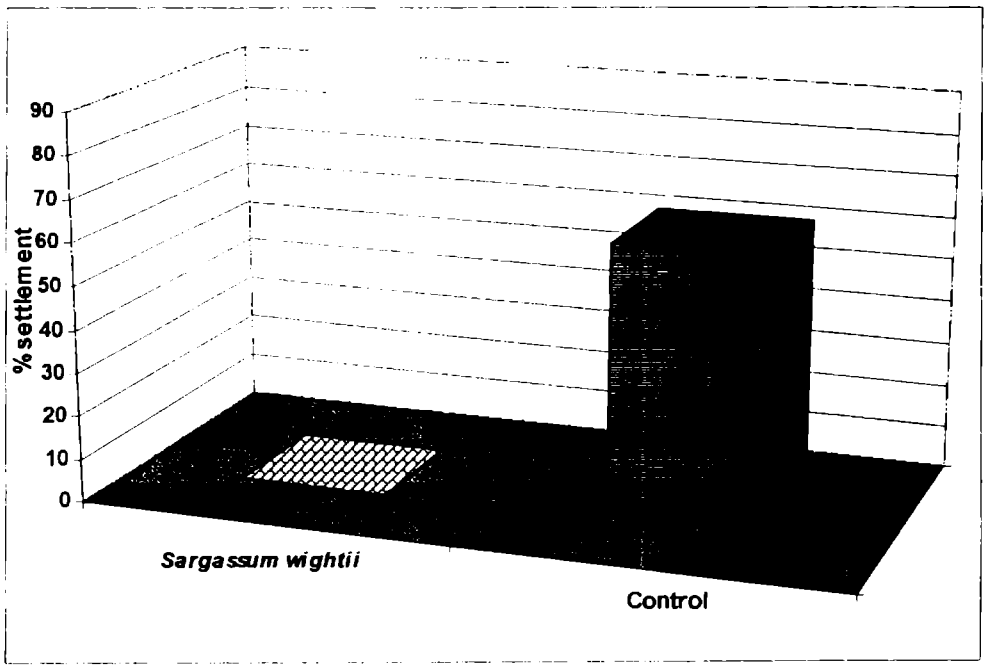


Fig.3.2

Effect of waterborne chemical compounds accommodated in immediate use, seaweed conditioned water on the rate of settlement of larvae of *Balanus amphitrite.*

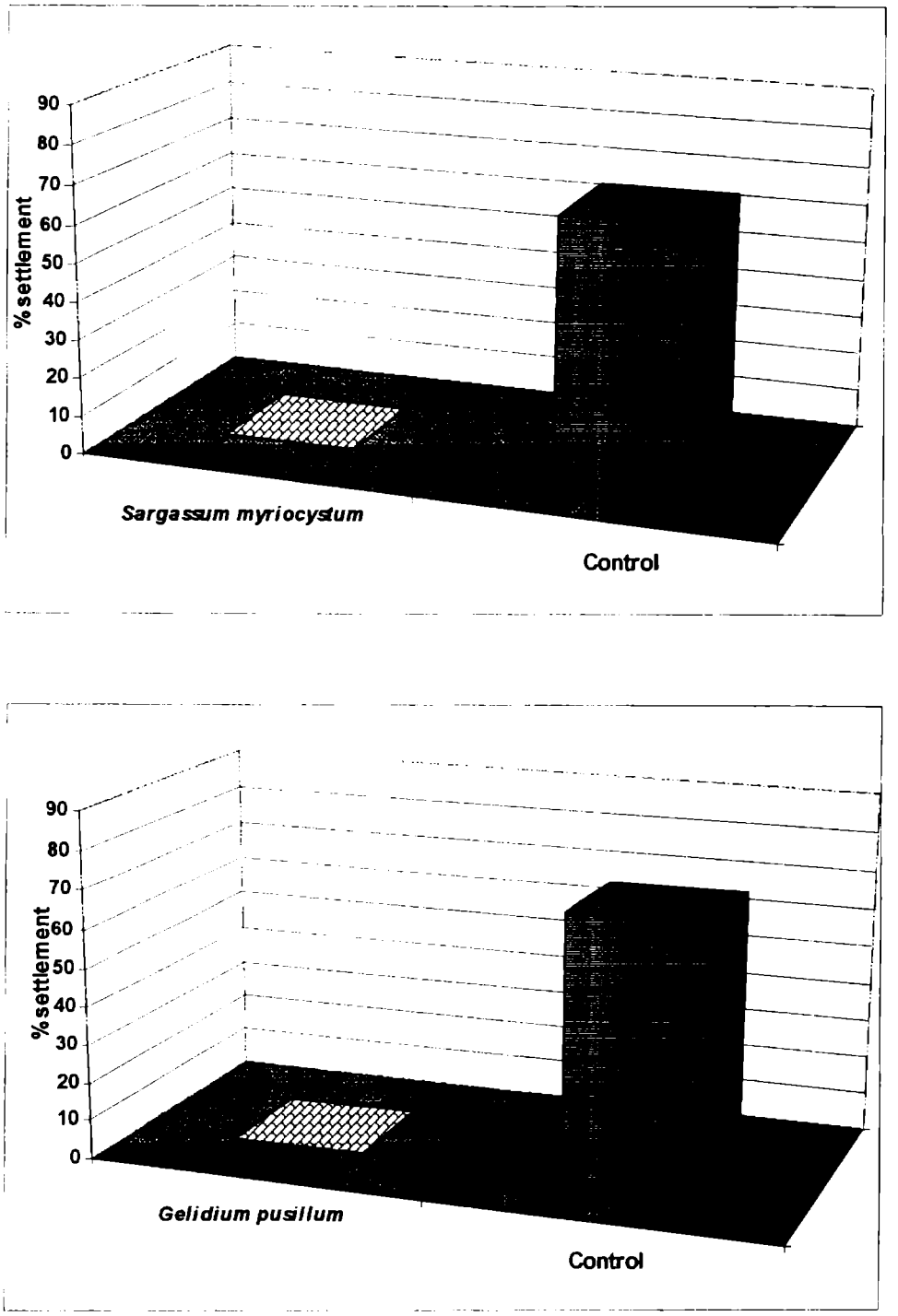
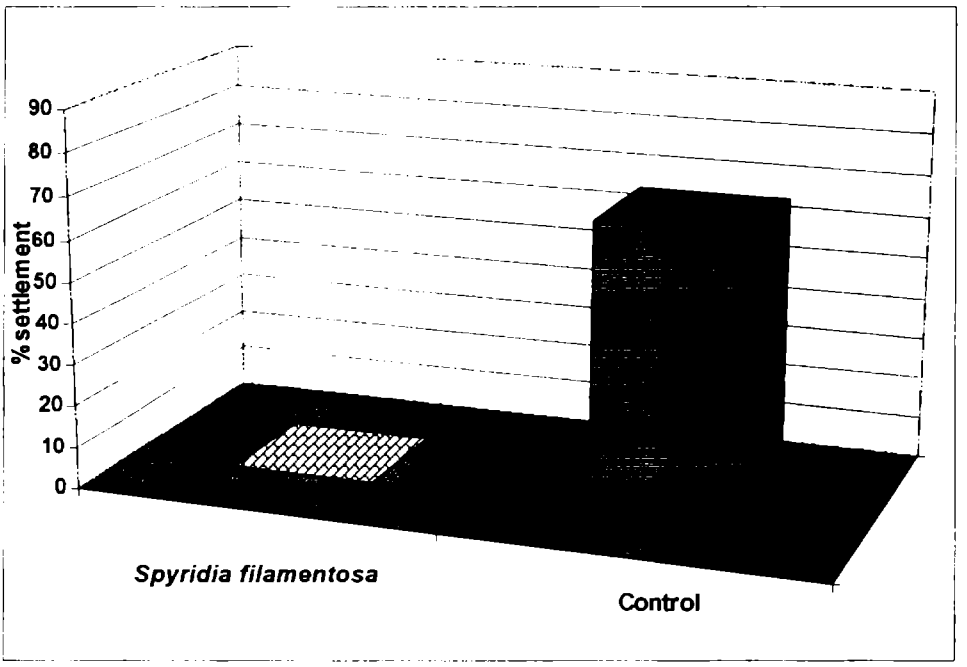


Fig. 3.3

Effect of waterborne chemical compounds accommodated in immediate use, seaweed conditioned water on the rate of settlement of larvae of *Balanus amphitrite.*



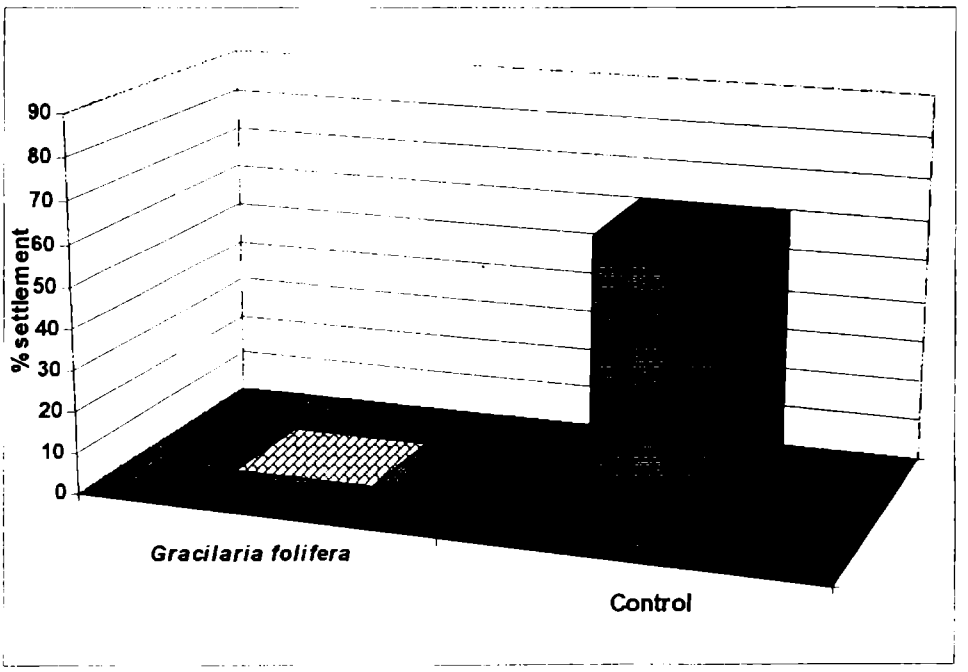


Fig.3.4

Effect of waterborne chemical compounds accommodated in immediate use, seaweed conditioned water on the rate of settlement of larvae of *Balanus amphitrite.*

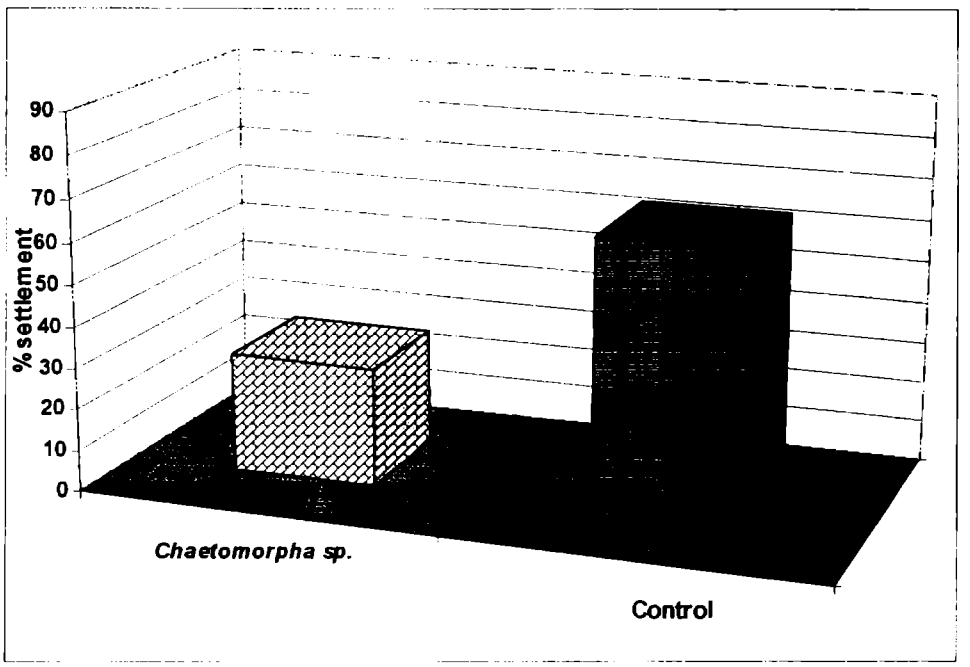


Fig.3.5

Effect of waterborne chemical compounds accommodated in immediate use, seaweed conditioned water on the rate of settlement of larvae of *Balanus amphitrite*.

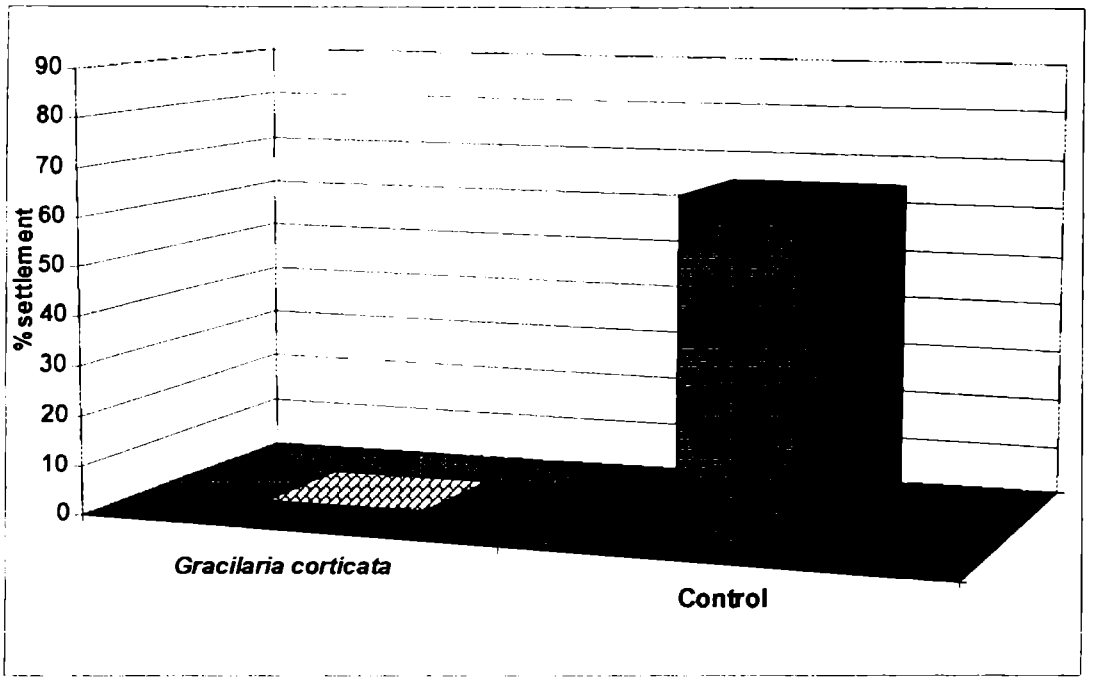
TABLE 3.5

Effect of waterborne chemical compounds of seaweeds accommodated in seaweed conditioned (aged) water on the settlement of larvae of *Balanus amphitrite.*

Seaweeds	Percentage Settlement	
	Control	Test
	Mean S.E.P.	Mean S.E.P.
Gracilaria corticata	66.18 ± 11.15	0.00 ± 0.00*
Padina tetrastromatica	66.19 ± 11.15	8.91 ± 6.71*
Caulerpa peltata	67.84 ± 11.01	0.00 ± 0.00*
Sargassum wightii	67.68 ± 11.02	0.00 ± 0.00*
Sargassum myriocystum	67.23 ± 11.06	0.00 ± 0.00*
Gelidium pusillum	65.4 ± 11.21	0.00 ± 0.00*
Spyridia filamentosa	67.19 ± 11.07	0.00 ± 0.00*
Gracilaria folifera	63.93 ± 11.32	0.00 ± 0.00*
Chaetomorpha sp.	66.19 ± 11.15	29.08 ± 10.7*

* $P<0.01$

S.E.P. - Standard Error of Proportion



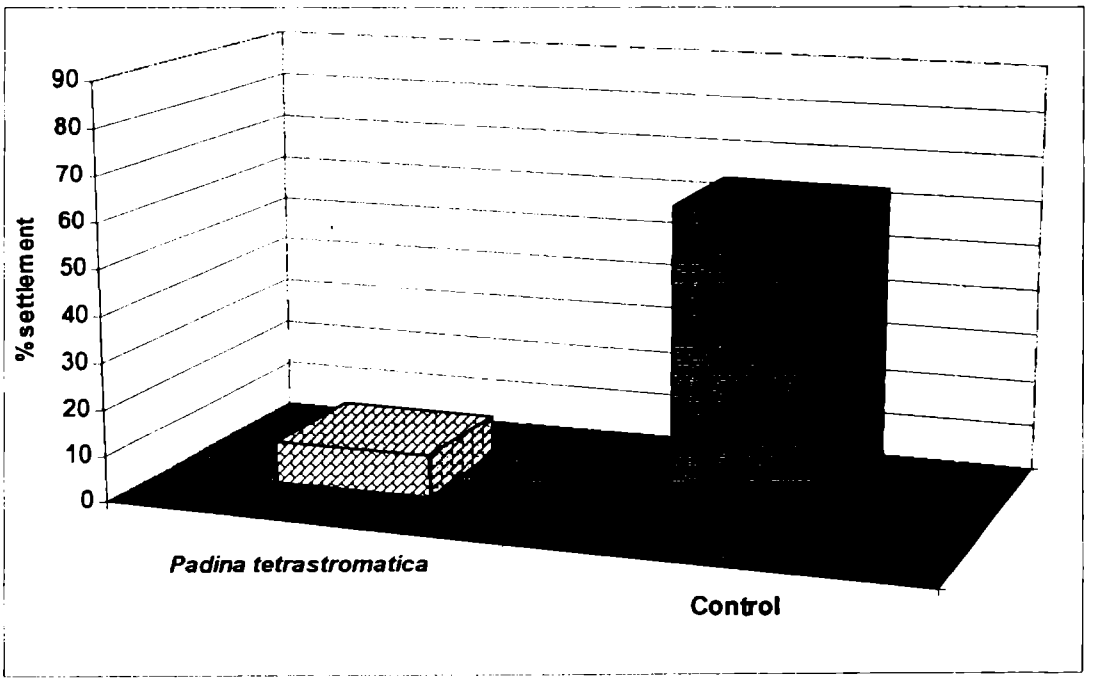
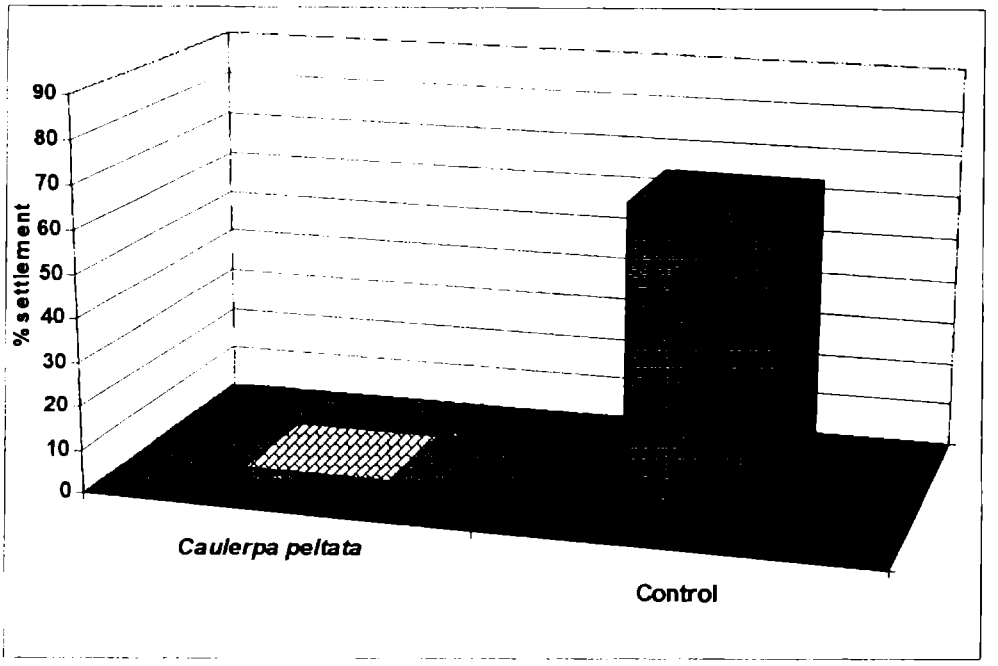


Fig.3.6

Effect of waterborne chemical compounds accommodated in aged, seaweed conditioned water on the rate of settlement of larvae of *Balanus amphitrite.*



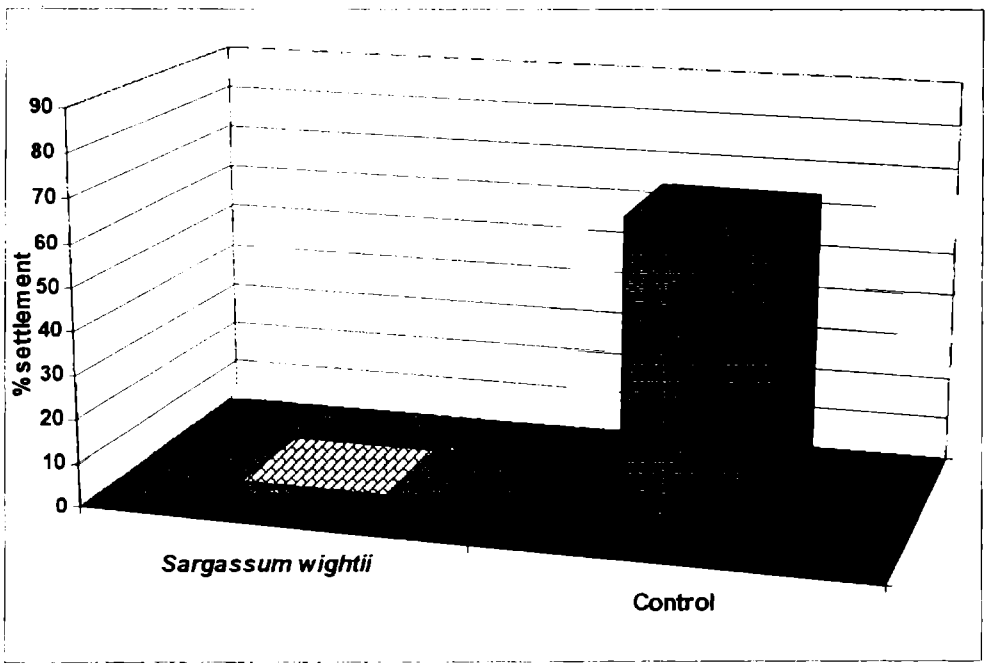
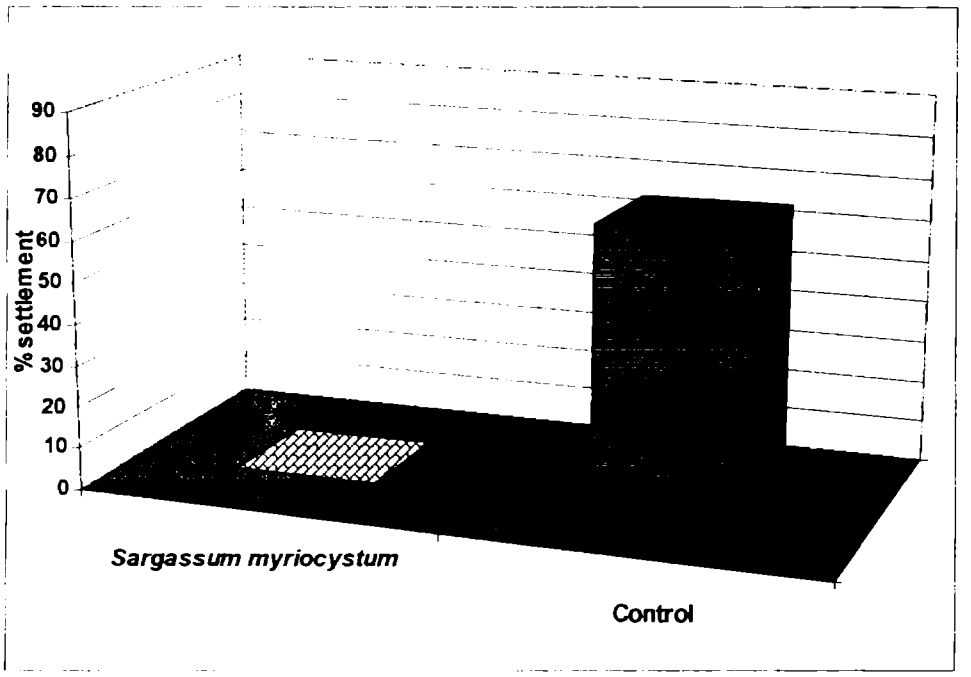


Fig. 3.7

Effect of waterborne chemical compounds accommodated in aged, seaweed conditioned water on the rate of settlement of larvae of *Balanus amphitrite.*



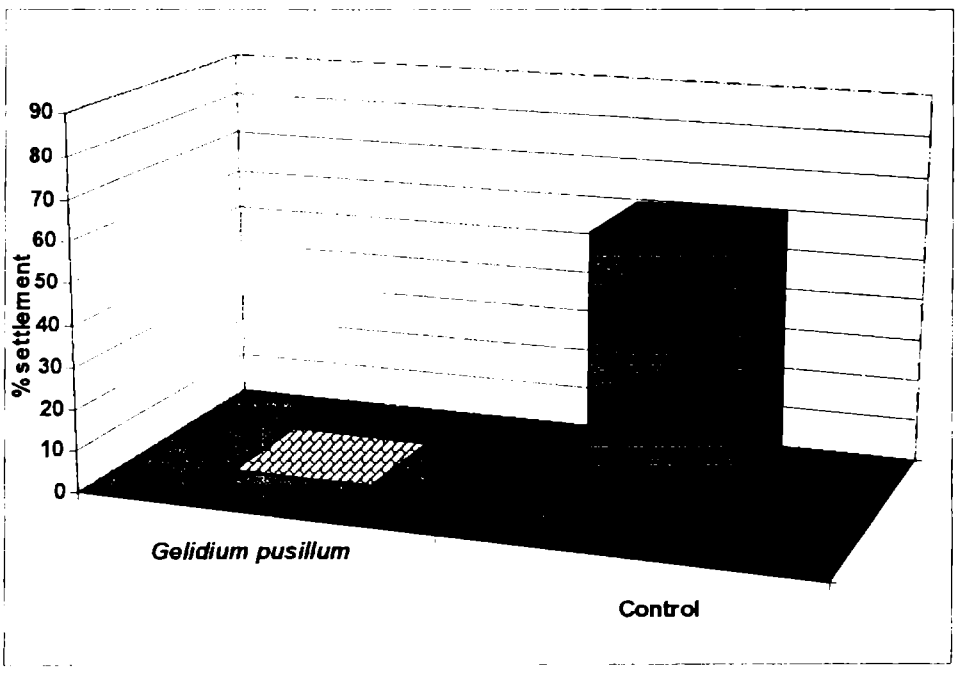
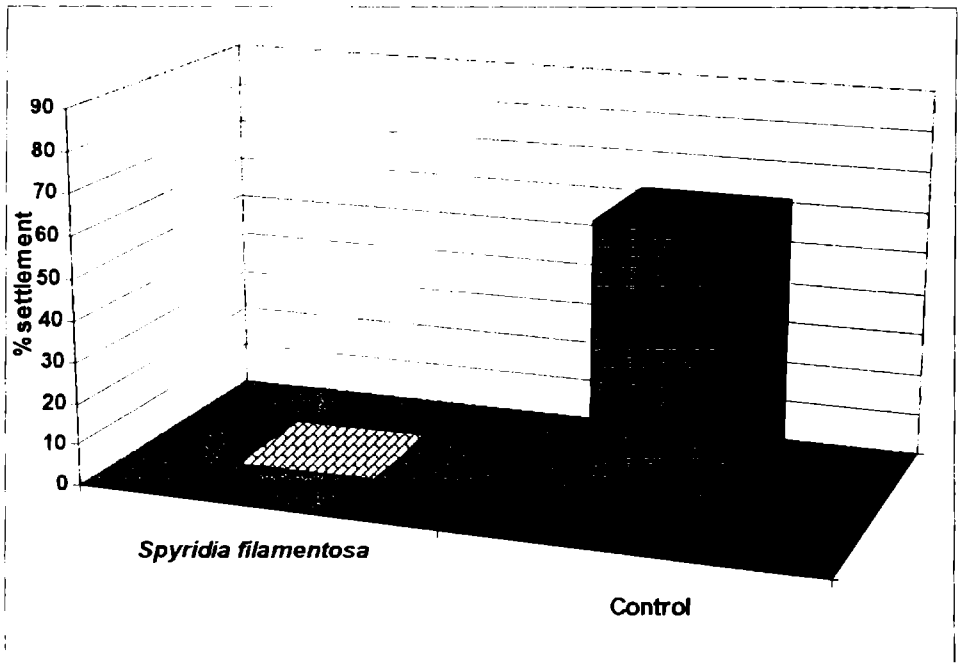


Fig. 3.8

Effect of waterborne chemical compounds accommodated in aged, seaweed conditioned water on the rate of settlement of larvae of *Balanus amphitrite.*



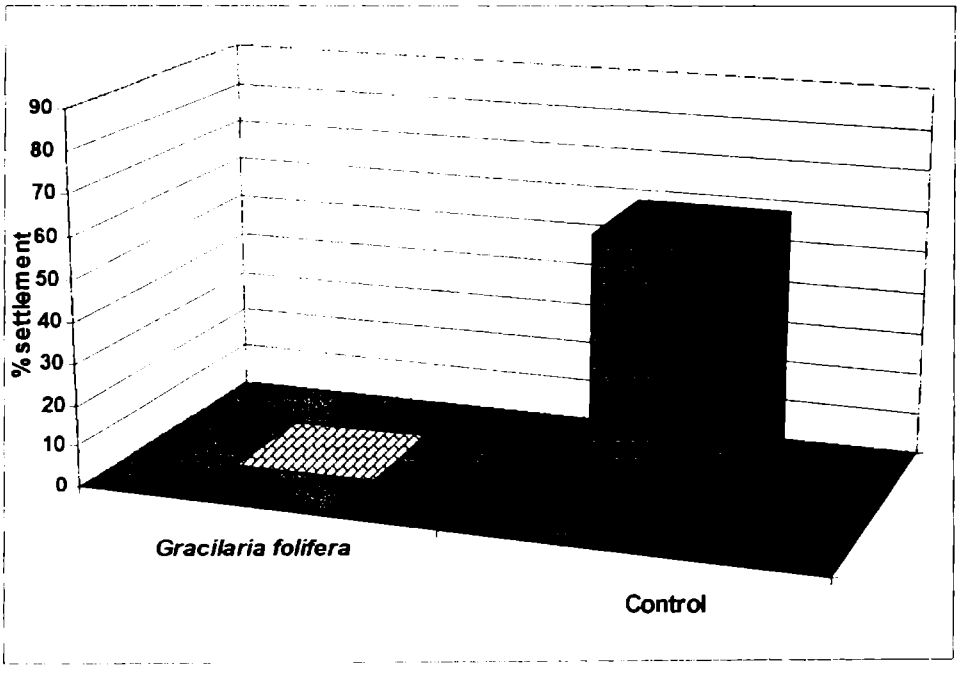


Fig.3.9

Effect of waterborne chemical compounds accommodated in aged, *seaweed* conditioned water on the rate of settlement of larvae of *Balanus amphitrite*.

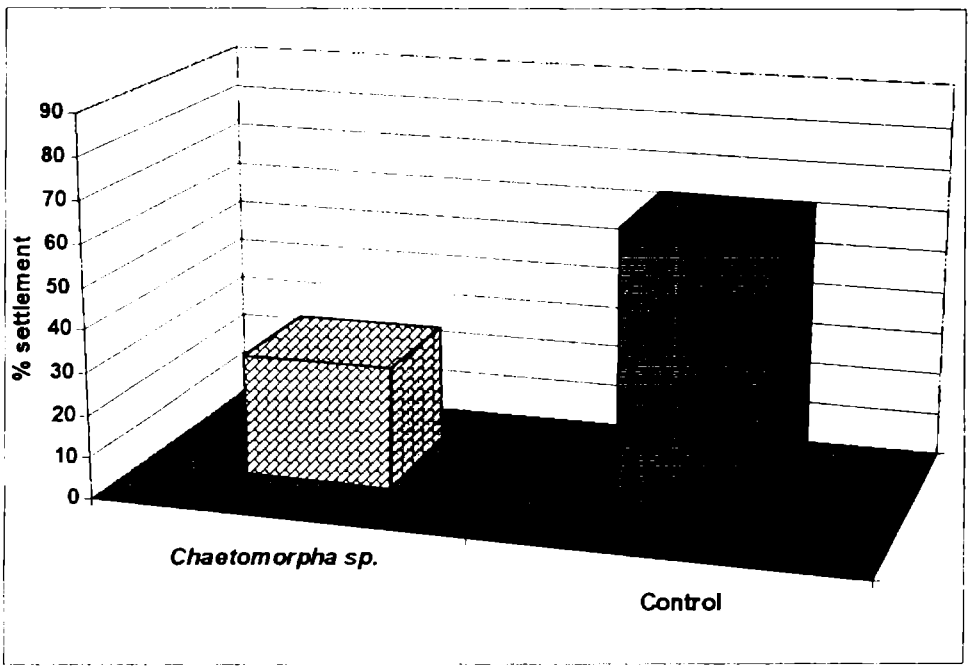


Fig. 3.10

Effect of waterborne chemical compounds accommodated in aged, *seaweed* conditioned water on the rate of settlement of larvae of *Balanus amphitrite*.

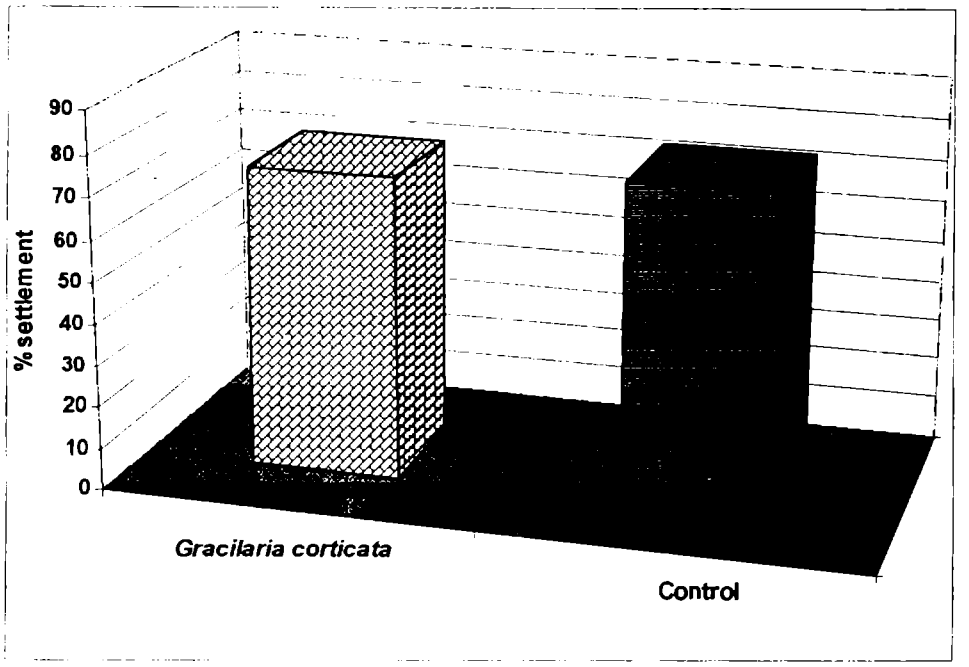
TABLE 3.6

Effect of waterborne chemical compounds of seaweeds accommodated in seaweed conditioned (immediate use) water on the settlement of larvae of *Hydroides elegans*.

Seaweeds	Percentage Settlement	
	Control	Test
	Mean S.E.P.	Mean S.E.P.
Gracilaria corticata	76.04 ± 10.06	72.78 ± 10.49
Padina tetrastromatica	79.39 ± 9.53	19.88 ± 9.41*
Caulerpa peltata	77.71 ± 9.81	0.00 ± 0.00*
Sargassum wightii	77.24 ± 9.88	0.00 ± 0.00*
Sargassum myriocystum	81.85 ± 9.08	0.00 ± 0.00*
Gelidium pusillum	78.00 ± 9.76	0.00 ± 0.00*
Spyridia filamentosa	77.98 ± 9.77	0.00 ± 0.00*
Gracilaria folifera	78.04 ± 9.76	0.00 ± 0.00*
Chaetomorpha sp.	78.11 ± 9.75	80.41 ± 9.55

* $P<0.01$

S.E.P. - Standard Error of Proportion



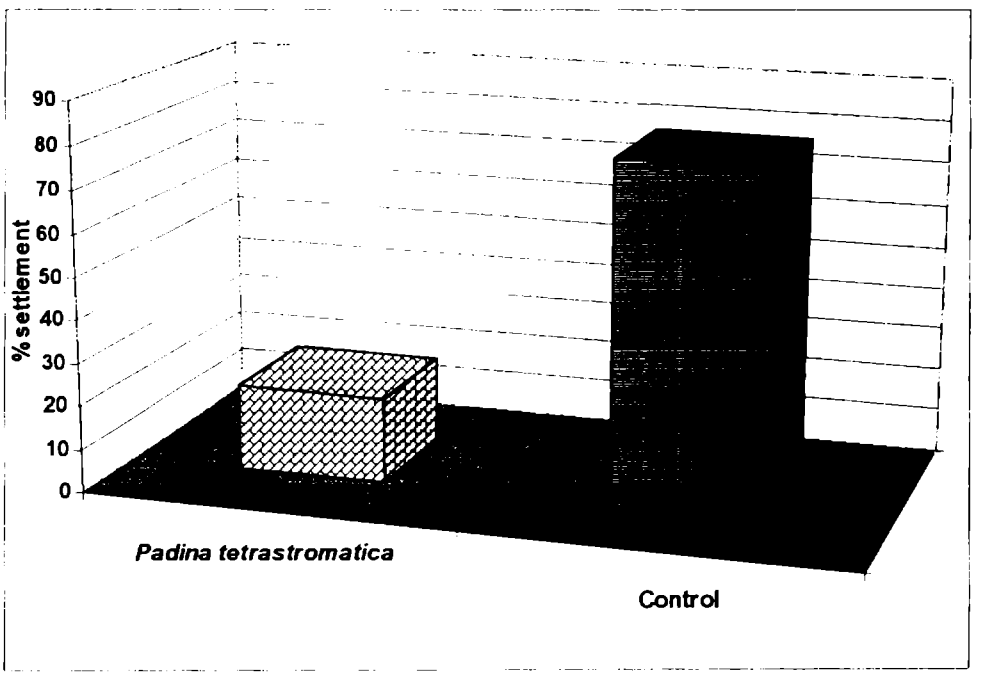
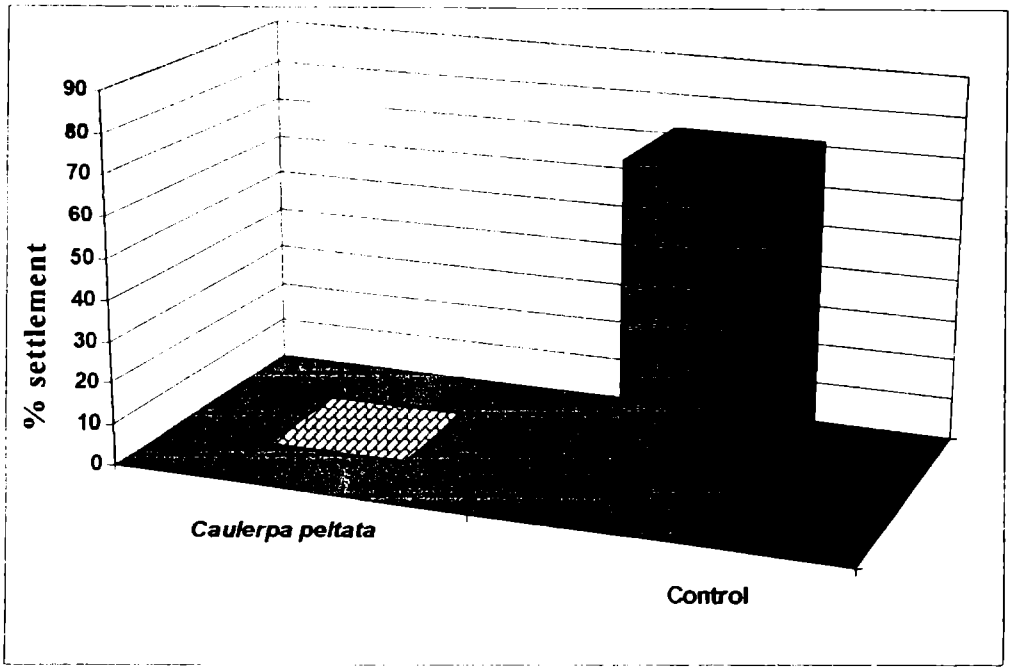


Fig. 3.11

Effect of waterborne chemical compounds accommodated in immediate use, seaweed conditioned water on the rate of settlement of larvae of *Hydroides elegans*.



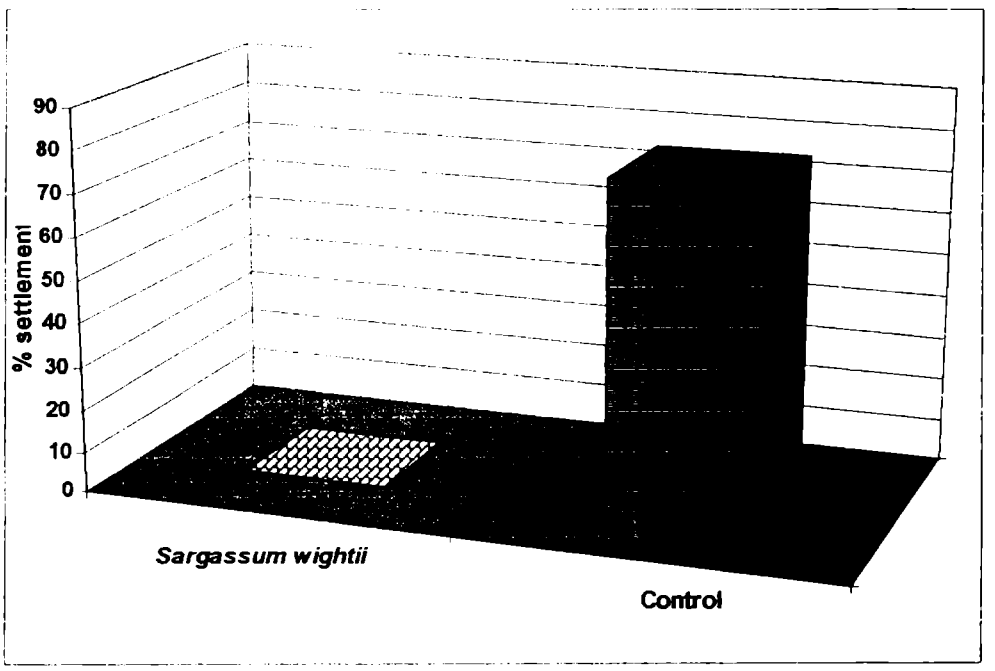
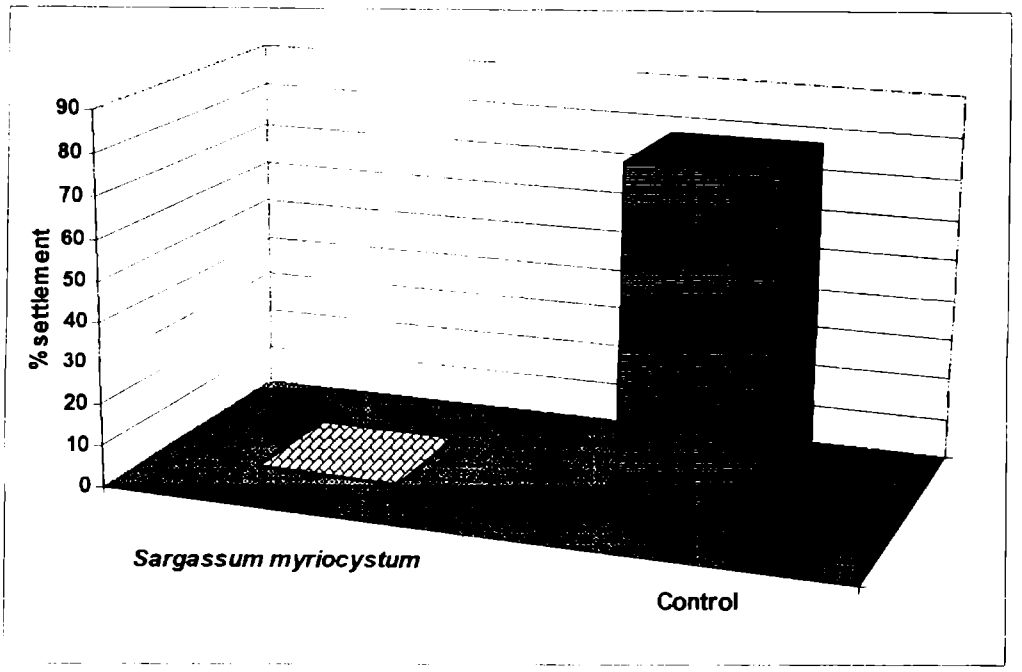


Fig. 3.12

Effect of waterborne chemical compounds accommodated in immediate use, seaweed conditioned water on the rate of settlement of larvae of *Hydroides elegans.*



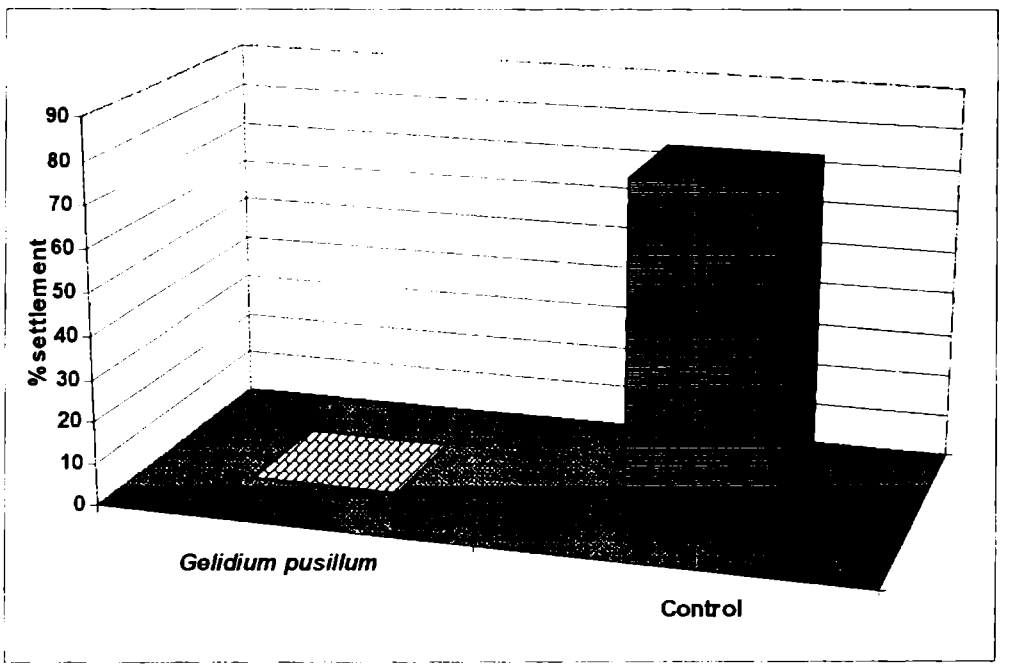
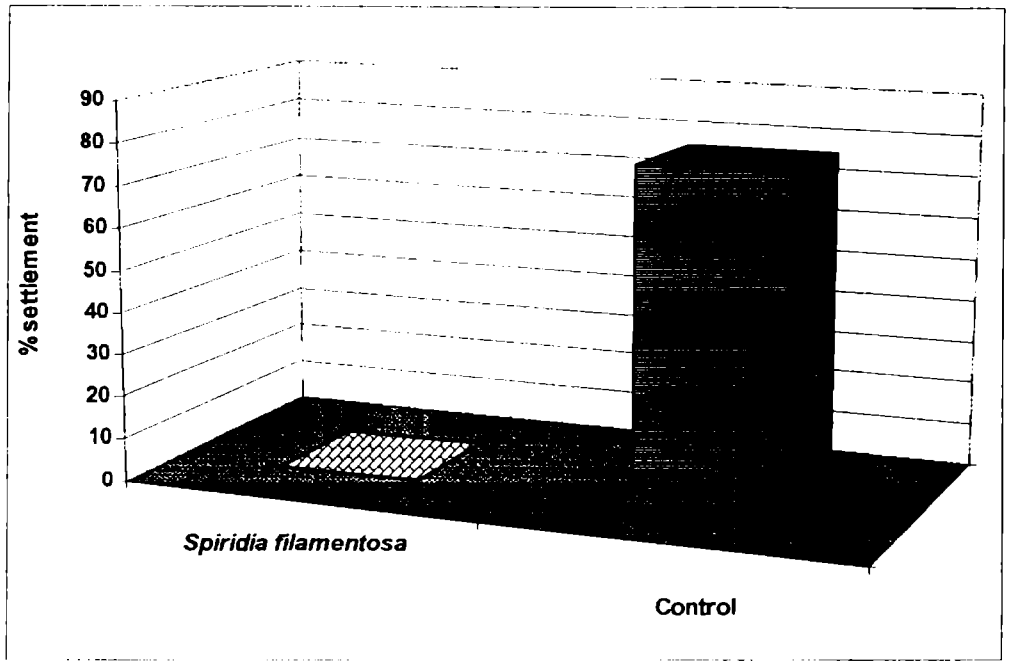


Fig. 3.13

Effect of waterborne chemical compounds accommodated in immediate use, seaweed conditioned water on the rate of settlement of larvae of *Hydroides elegans.*



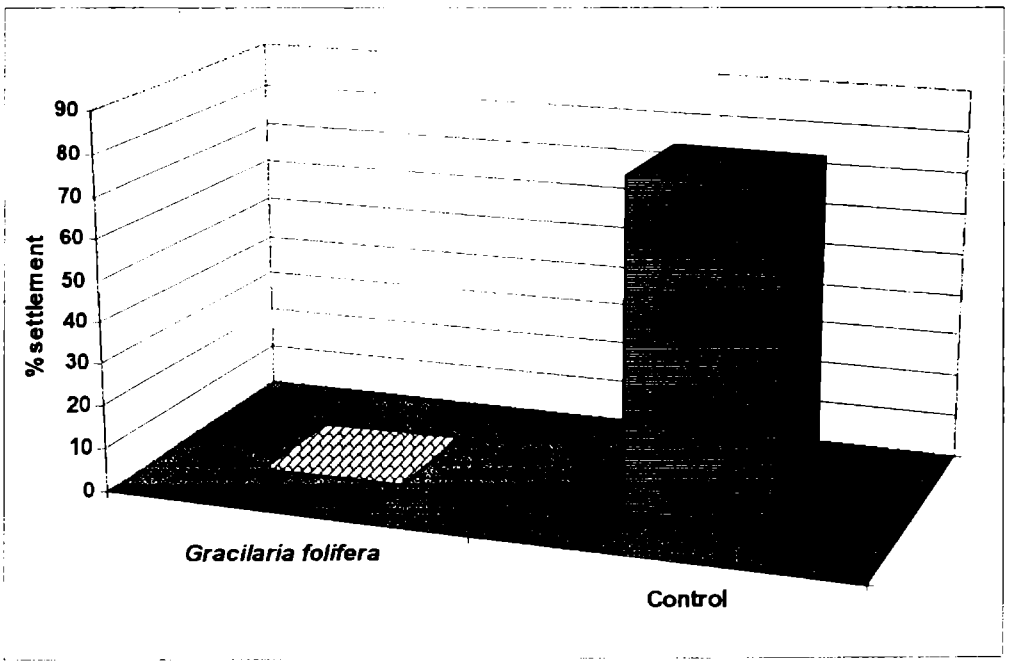


Fig. 3.14

Effect of waterborne chemical compounds accomodated in immediate use, seaweed conditioned water on the rate of settlement of larvae of *Hydroides elegans.*

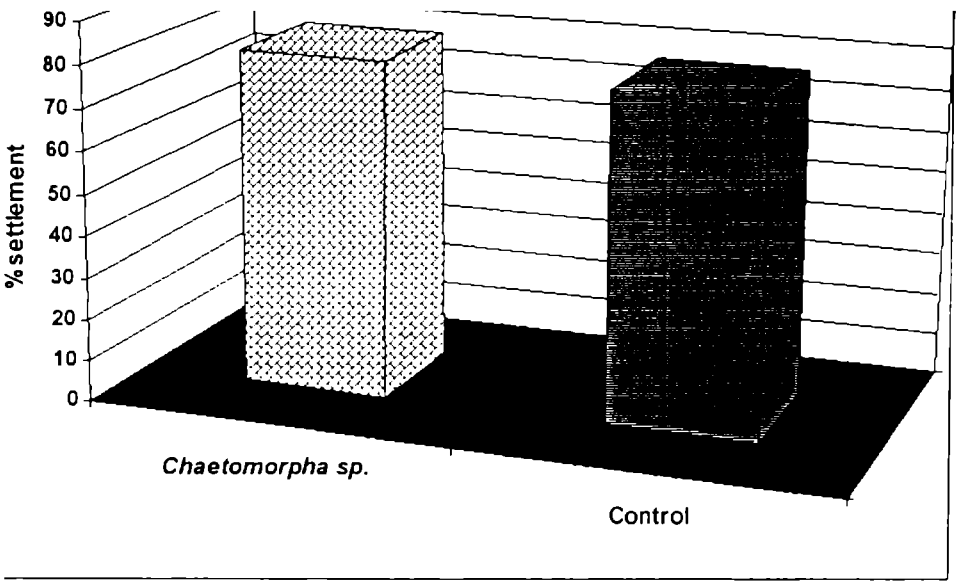


Fig. 3.15

Effect of waterborne chemical compounds accommodated in immediate use, seaweed conditioned water on the rate of settlement of larvae of *Hydroides elegans*.

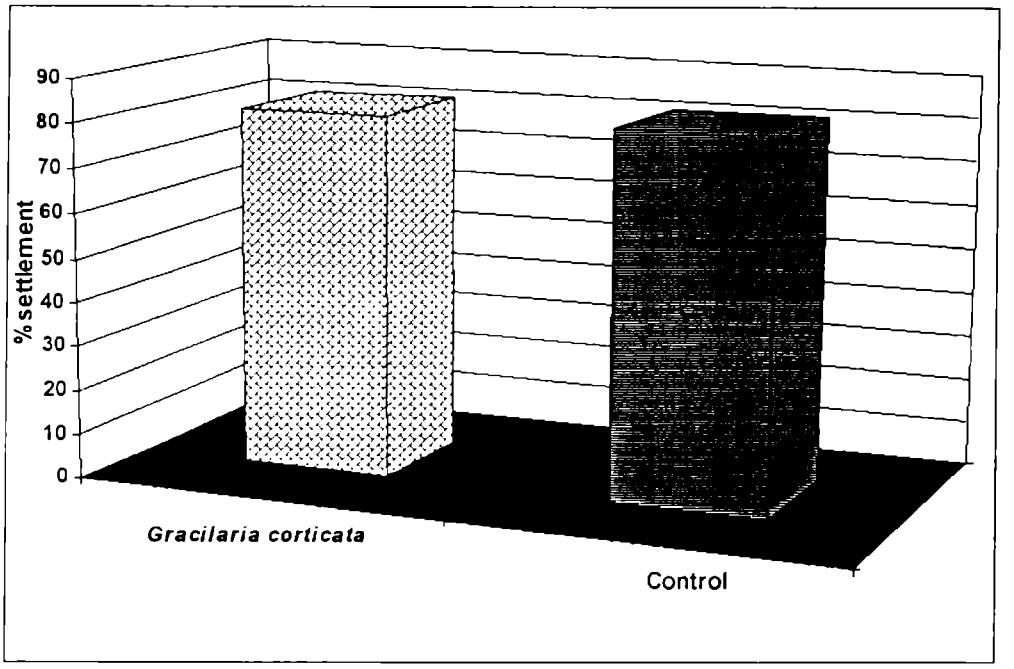
TABLE 3.7

Effect of waterborne chemical compounds of seaweeds accommodated in seaweed conditioned (aged) water on the settlement of larvae of *Hydroides elegans*.

Seaweeds	Percentage Settlement	
	Control	Test
	Mean S.E.P.	Mean S.E.P.
Gracilaria corticata	81.86 ± 9.08	81.61 ± 9.13
Padina tetrastromatica	75.85 ± 10.09	38.52 ± 11.47*
Caulerpa peltata	78.15 ± 9.74	0.00 ± 0.00*
Sargassum wightii	77.09 ± 9.91	0.00 ± 0.00*
Sargassum myriocystum	81.89 ± 9.08	0.00 ± 0.00*
Gelidium pusillum	85.41 ± 8.32	0.00 ± 0.00*
Spyridia filamentosa	81.85 ± 9.08	0.00 ± 0.00*
Gracilaria folifera	80.37 ± 9.36	0.00 ± 0.00*
Chaetomorpha sp.	80.66 ± 9.31	81.45 ± 9.16

* $P<0.01$

S.E.P. - Standard Error of Proportion



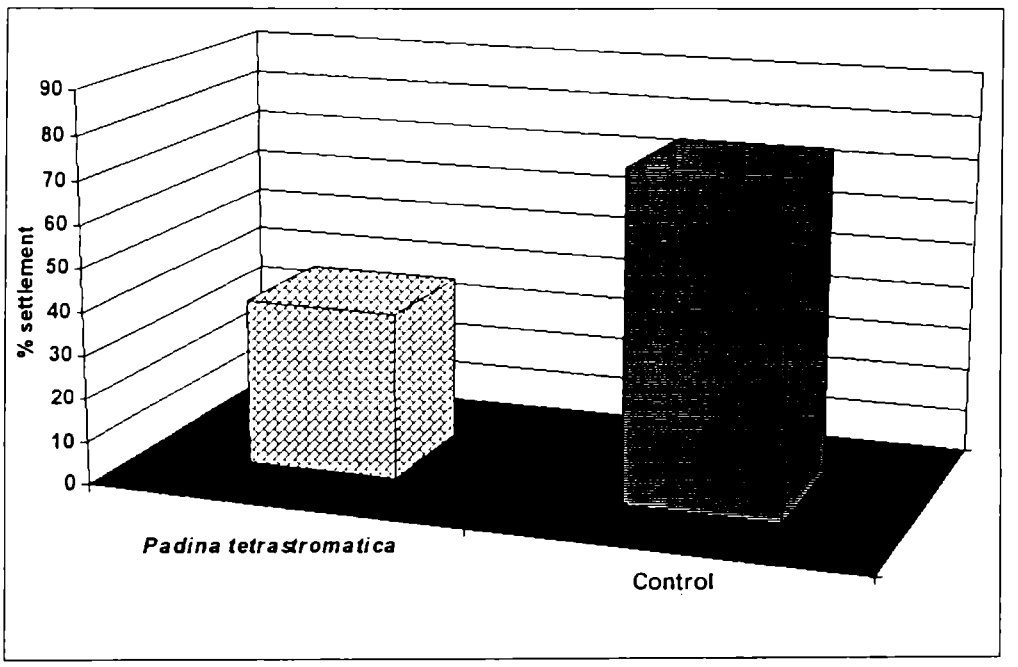
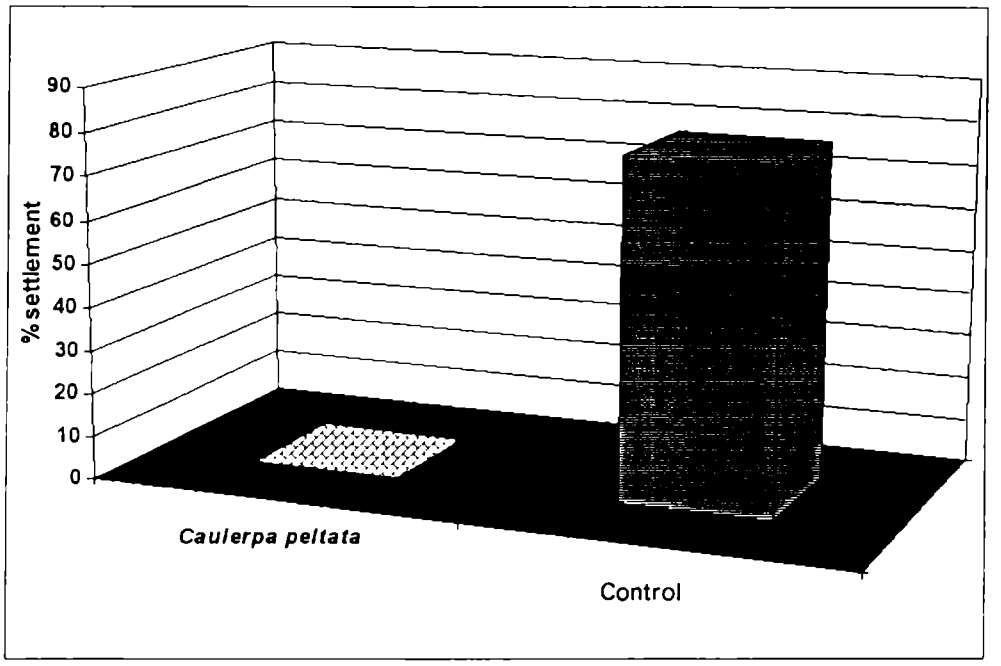


Fig. 3.16

Effect of waterborne chemical compounds accommodated in aged, *seaweed* conditioned water on the rate of settlement of larvae of *Hydroides elegans*.



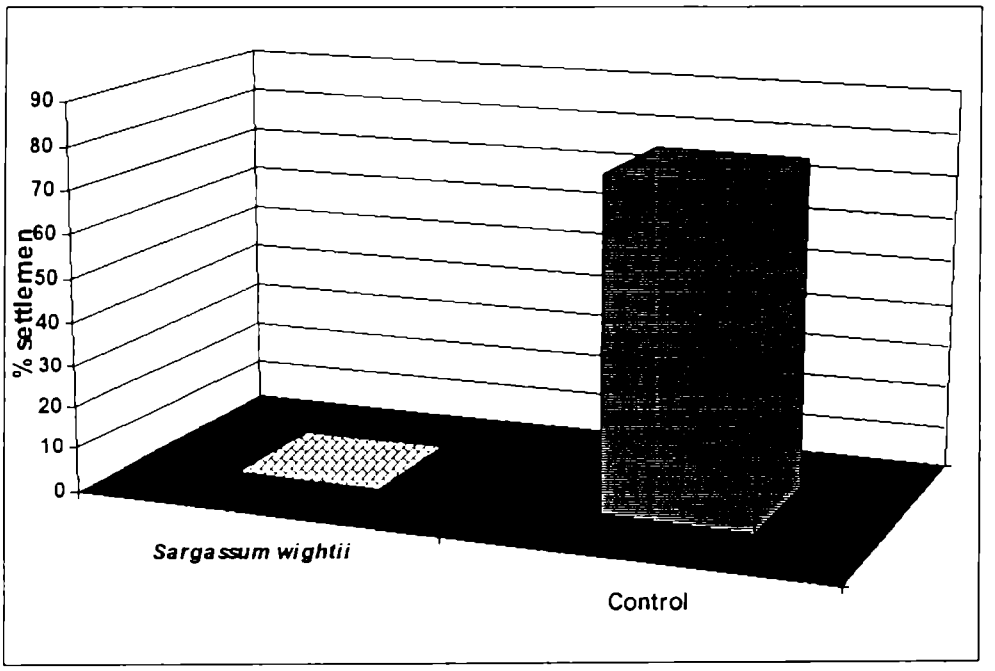
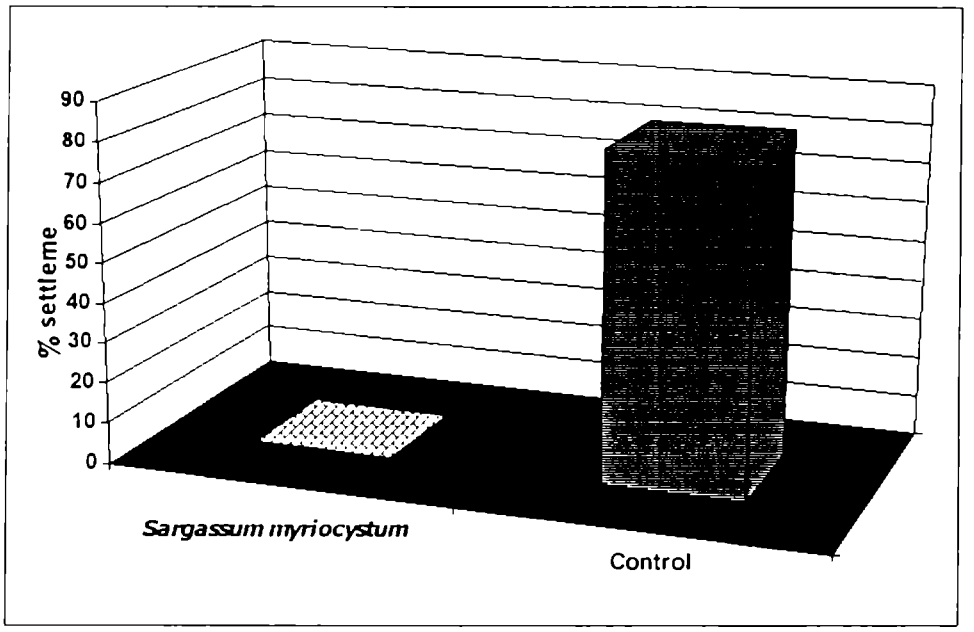


Fig. 3.17

Effect of waterborne chemical compounds accommodated in aged, *seaweed* conditioned water on the rate of settlement of larvae of *Hydroides elegans*.



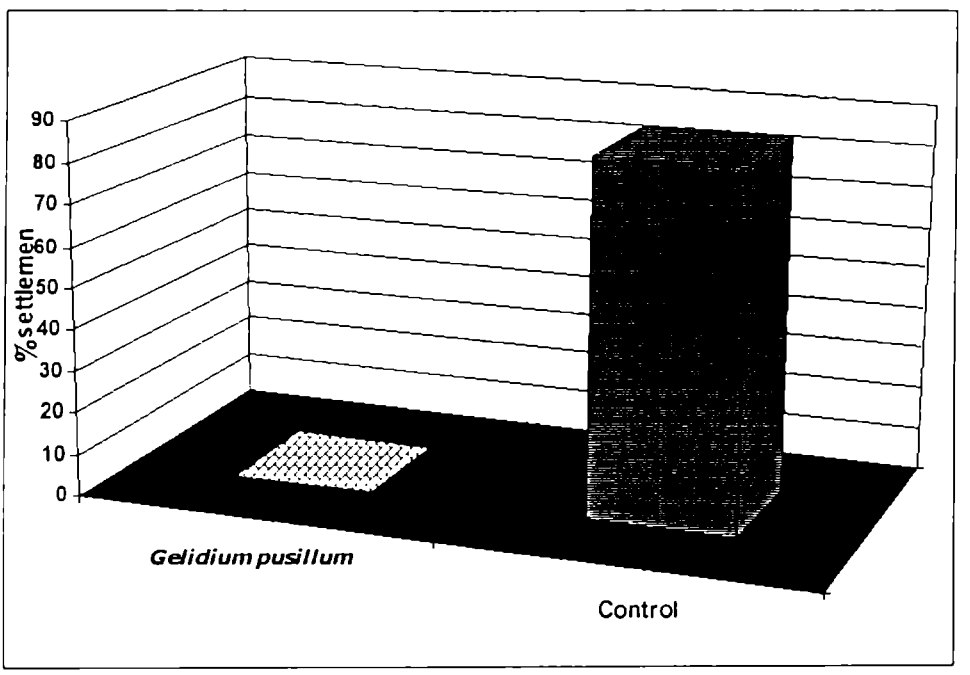
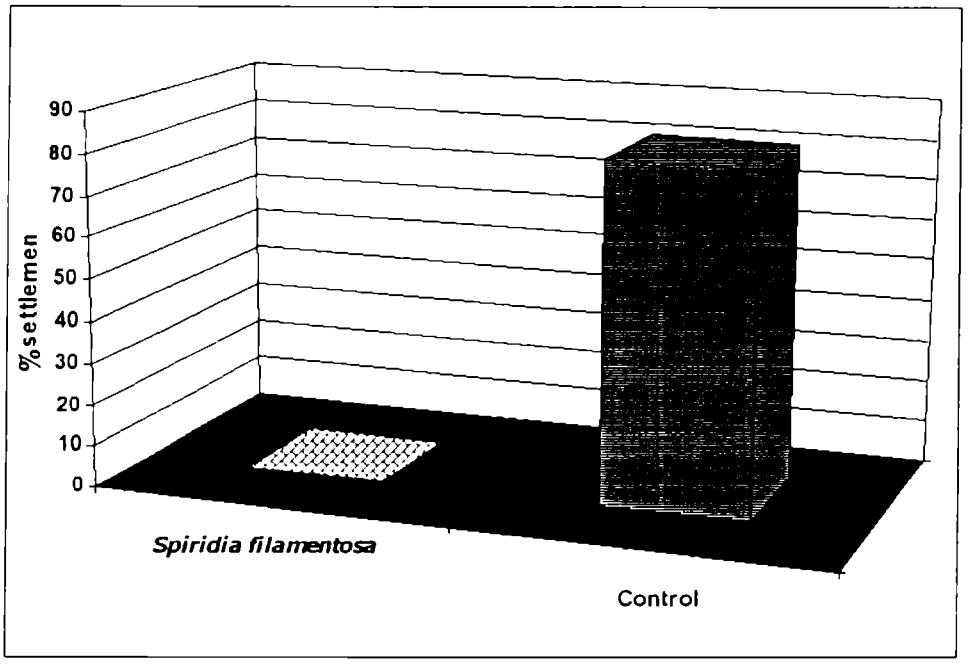


Fig. 3. 18

Effect of waterborne chemical compounds accommodated in aged, *seaweed* conditioned water on the rate of settlement of larvae of *Hydroides elegans*.



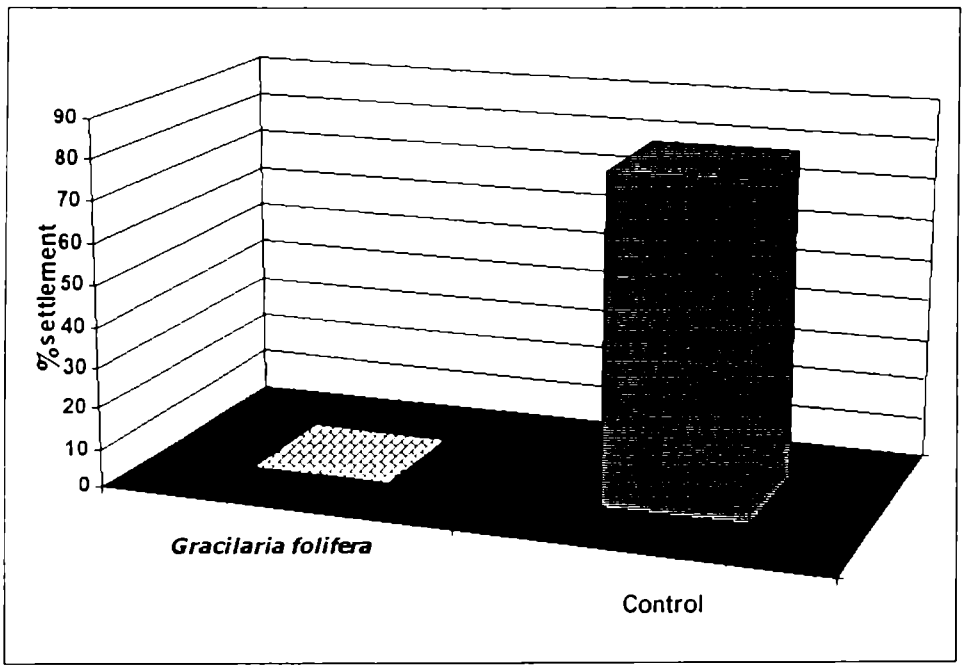


Fig. 3. 19

Effect of waterborne chemical compounds accommodated in aged, seaweed conditioned water on the rate of settlement of larvae of *Hydroides elegans*.

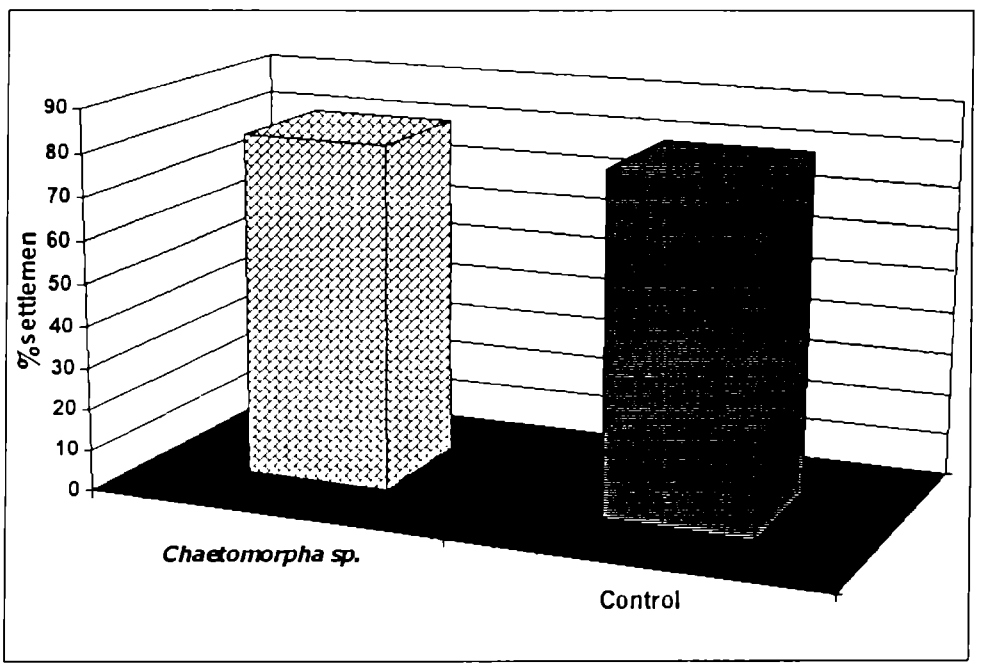


Fig. 3. 20

Effect of waterborne chemical compounds accommodated in aged, *seaweed* conditioned water on the rate of settlement of larvae of *Hydroides elegans*.

sank to the bottom of the petridishes within minutes of contacting these exudates. The same rapid toxic response occurred when the trochophore larvae were tested in aged seawater from these species (Table 3.3).

Trochophore larval settlement of *Hydroides elegans* was significantly delayed in both immediate use and aged seaweed conditioned water trials using *Padina tetrastromatica* ($P < 0.01$) (Table 3.6, 3.7).

3.4 DISCUSSION

The bioassays were designed to determine the influence of high levels of waterborne compounds exuded from seaweeds on the rate of settlement and survival of the larvae of ubiquitous foulers *Hydroides elegans* and *Balanus amphitrite communis*. High levels of metabolites were expected in these assays due to accumulation of these metabolites in small volumes of seawater for 16h and due to disturbance during seaweed handling. In the field, such concentrations may only exist in the first few mm of water immediately surrounding the seaweed. In areas where wave action is less, active compounds that remain effective over time will be dispersed to a great extend. The cue for settling of oyster larvae is a soluble peptide. Such a waterborn settling cue only exists in effective concentrations within a few mm of the bottom, but larvae sinking within the bottom boundary layer can detect this cue and settle.

According to Morse and Morse (1984) all foliose red algae contained water-soluble inducers, although the intact forms of these algae were inactive. A gentle brushing of the surface of *Lithothamnium sp.* released appreciable quantities of inducing substances: this mild disruption partially released surface inducers and phycobiliproteins into solution. Giraud and Cabioch (1976) opined that the sloughing

of surface layers and active secretion observed for most crustose species may be the mechanism by which inducers are made available to the larvae at crustose red algal surfaces.

The rate of exudation by various seaweeds under natural conditions remain a matter of debate. Some experiments showed that exudation was 30-40% of net assimilation in seaweeds (Khailov and Burlakova, 1969; Sieburth, 1969). Other experiments showed it to be much less, ranging from a small percentage down to less than 1%, except under stress (Carlson and Carlson, 1984).

The waterborne compounds affect metamorphosis without prior attachment and vice versa (Yamamoto *et al.*, 1997) or affect the whole process of settlement. Following the reasoning of Leitz (1998), it is proposed that compounds which affect attachment only, or the entire settlement process, are acting proximal to the pheromone receptors.

Ion channels and depolarisation of externally accessible receptors have been widely implicated in larval settlement (Baloun and Morse, 1984; Pawlik, 1990; Freeman, 1993; Pearce and Scheibling, 1994; Leitz, 1997). The chemoreceptors for settlement cues can be regarded as extensions of the nervous system. According to Baloun and Morse (1984), the signal molecules effect induction of larval recruitment by interacting with stereochemically specific receptors on externally accessible excitatory cells of larval nervous system.

According to Williams *et al.* (1989), secondary metabolites increase the producer's fitness by acting on receptors in competing organisms. These compounds often carry reactive groups such as aldehyde, acetate, alcohol or halogens.

The responsiveness of the 'Trigger Pathway' (Morse, 1988, P. 454) of larval recruitment can be regulated and greatly enhanced by a second pathway called the 'Amplifier pathway' (Morse, 1988, P. 454) . Water-borne chemical signals in the dissolved organic material activate the Amplifier pathway by interaction with a separate group of receptors on the larvae (Trapido- Rosenthal and Morse, 1985, 1986 a, b). These receptors, in turn, activate membrane - associated molecular transducers consisting of a specialized G-protein and a protein kinase C, causing the phosphorylation of a specific target protein. This internal phosphorylation covalently modifies an essential element of the Trigger pathway, priming the larvae, and greatly enhancing their sensitivity to induction of settlement and metamorphosis in response to the surface-associated inducing signal molecules (Trapido-Rosenthal and Morse, 1985 ; 1986 a, b; Baxter and Morse, 1987).

The differences in the responses shown by the larvae of *Hydroides elegans* and *Balanus amphitrite communis* to the waterborne compounds cannot be attributed to differences in the seaweed biomass : water volume as there were no significant differences in the above values ($p > 0.2$) (Table 3.1).

Many chemically rich seaweeds probably resist fouling pressure because of the toxic or deterrent secondary metabolites they produce or because of their combined chemical and morphological defences. It is not clear what chemical features determine the degree of deterrency or toxicity of a particular secondary metabolite. Toxicity and deterrency are not intrinsic properties of any compound; they result from physiological interactions between a metabolite and its consumer. The same metabolite may show pronounced differences in its effects even on closely related species of biofoulers.

Different population of the same species of seaweed may accumulate significantly different metabolites (Mynderse and Faulkner,1978). The majority of seaweed secondary metabolites appear to be produced by species that are predominantly tropical or subtropical in distribution. The temperate representatives appear to contain less defensive compounds than do their tropical counter parts. Caulerpalean greens produce toxic compounds, and many species are also calcified (Paul and Fenical, 1986). Among the red algae, notable toxin producers include *Halymenia, Laurencia* and many others (Hay and Fenical, 1988). In tropical and warm temperate oceans, brown algae in the order *Dictyotales* produce complex mixtures of terpenoids, acetogenins, and terpenoid aromatic compounds (Faulkner, 1986).

Biosynthesis, storage, and turnover of secondary metabolites of seaweeds might vary according to the types of defences resorted to such as constitutional, inducible or activated defences. The turnover rates of different secondary metabolites are important considerations in estimating the cost of a particular defence, but these have not been measured in seaweeds. The information about the physiological mechanisms of toxicity of secondary metabolites is very few. Pharmacological assays have shown that some marine secondary metabolites function as neurotoxins or haemotoxins in laboratory animals. The seaweed metabolite, helimedatrial has diverse and potential biological activity including inhibition of cell division.

Brown algae in the genus *Dysmarestia* concentrate sulphuric acid upto 18% of its dry mass and these plants act as "acid brooms" that prohibit urchins from entering their area (Anderson and Velimirov, 1982). Macroalgae contain

derivatives of simple phenolics, such as coumarins of green algae (Hay and Fenical, 1988), halogenated phenols in red algae (Fenical, 1975) and phlorotannins in brown algae (Ragan and Glombitza, 1986). In brown algae, most of the phlorotannins are contained in physodes and these phlorotannins do tend to be released into the environment (Ragan and Glombitza, 1986). One of the ways in which phenolics produce a toxic effect is by precipitating proteins (Keen and Evans, 1988). They are readily exuded and are abundant in the fucus 'juice' that are released when *Fucus* is kept in plastic bags (Ragan and Glombitza, 1986). Phenolic exudates also have allelopathic effects in the tide pools, where exudates such as *Petalonia* and *Laminaria* inhibit ephemerals such as *Chaetomorpha sp.*

Ragan and Glombitza (1986) have suggested that the production of broad-spectrum, weak polyphenolics, which might last 100 days, is more economical than production of high-activity molecules that are short lived. Turnover rates, rates of release, and levels of energy required for maintenance of the deterrents vary markedly among species (Hay and Fenical, 1988).

Water conditioned with *Sargassum myrioicystum, Sargassum wightii* and *Spyridia filamentosa* killed virtually all larvae of both *Hydroides elegans* and *Balanus amphitrite communis*. Change in the rate of settlement of at least one of the type of larvae was noticed in all the algae conditioned water. Cyprid larvae of *Balanus amphitrite* never settled at rates greater than control. Water conditioned with *Chaetomorpha* sp. increased the settlement rate of trochophore larvae of *Hydroides elegans* when compared with control, although the rate of increase was not statistically significant. Different responses of the larval stages of *Hydroides elegans* and *Balanus amphitrite communis* may be attributed to a number of causes. Larvae of the two invertebrates must have responded to seperate substances in the potentially heterogeneous mixtures of compounds released into

the water by the seaweeds. Water conditioned with *Chaetmorpha* sp. caused delayed settlement of *Balanus amphitrite* larvae (Fig. 3.5) but exhibited neutral effect on the rate of settlement of larvae of *Hydroides elegans* (Fig 3.15). Different thresholds of response to a particular compound or class of compounds may explain cases where one larval type responded positively or negatively to the algae-conditioned water while the other larval type had no response. Elevation of external K^+ has been shown to induce larval settlement across a wide range of phyla, although exceptions have been noted (Todd *et al.*, 1991 ; Woollacott and Hadfield, 1996; Hadfield, 1998). Inhibition of settlement under the above conditions has only been reported for one species, *Balanus amphitrite* (Rittschof *et al.*, 1986b).

Mechanical damage associated with grazing or physical disturbance increases the production of protective compounds. Paul and Van Alstyne (1988) have documented increased internal concentrations of specific secondary metabolities in *Halimeda* sp. with result to plant damage. Although the tropical green seaweeds do not increase their production of secondary metabolities in response to damage, several species of *Halimeda* and *Udotea* can rapidly convert a less toxic and deterrent terpenoid metabolite to a more deterrent compound. This process, which has been termed 'activation' (Paul and Van Alstyne, 1988), occurs within seconds of tissue injury and appears to be enzymatically mediated.

In the temperate alga, *Fucus distichus* (Van Alstyne, 1988) levels of polyphenolic compounds increased in response to mechanical damage. Similar experiments with green algae of the genera *Halimeda, Udotea* and *Caulerpa* did not result in any change in levels of chemical defences in response to mechanical injury for periods ranging from several hours to several weeks (Van Alstyne, 1988). These species appeared to produce consistently high levels of secondary metabolites.

Populations of *Halimeda* from habitats in which herbivory is intense tend to contain higher levels of the more potent deterrent halimedatrial than do populations from areas of low herbivory (Paul and Van Alstyne, 1988). Other green algae such as *Penicillus, Udotea, Rhipocephalus,* and *Caulerpa* also often produce higher concentrations or different types of secondary metabolites in populations from herbivore - rich reef habitats than in populations from herbivore - poor areas such as reef flats or seagrass beds (Paul and Fenical, 1986, 1987; Paul *et al.*, 1987b). The reason behind these variations in concentrations and types of secondary metabolites may result from either herbivore-induced chemical defences, localized selection resulting in high levels of defence or other factors related to herbivory. From this it can be concluded that seaweed population under continuous fouling pressure, may develop secondary metabolites having antifouling activity.

The data presented here demonstrate that larvae respond to waterborne compounds from tropical macroalge in different ways. Some compounds are toxic while others provide, negative, positive or neutral cues for settlement. In laboratory bioassays, larvae of *Hydroides elegans* and *Balanus comphitrite communis* responded to algae - conditioned water in a species - specific manner.

CHAPTER 4

IN SITU STUDIES ON THE SETTLEMENT OF BIOFOULERS EMPLOYING SEAWEED EXTRACT COATED TEST PANELS

4.1 INTRODUCTION

Many ocean - dwelling animals reproduce to yield millions of tiny planktonic larvae that are dispersed by drifting and swimming for varying durations. For many species, the success of their larvae in settling from the plankton and metamorphosing to the adult depends on the ability of the larvae to detect and recognize specific chemical signals in the marine environment. Recognition of these chemical signals, often associated with unique niches or habitats in the marine environment, ensures that the larvae are induced to settle and metamorphose in environments especially suited for their development and growth.

The development of marine biological fouling communities is regulated by physical, biochemical and ecological factors, which act by controlling the expression of genetically programmed behavioural and developmental processes intrinsic to the fouling organisms. Hydrodynamics will control the number of larvae encountering a substrate, and indirectly control larval settlement, as long as the larvae have a high probability of accepting the substrate, once they come into contact with it.

Larvae of many marine benthic organisms appear to discriminate between settlement sites. Chemical properties are among the most important cues used by larvae actively selecting habitat sites (Pawlik, 1986; Jensen and Morse, 1990). Detection of environmental conditions by many invertebrate larvae has been

shown to involve the recognition of very specific chemical cues associated with the surfaces on which the organisms settle or attach (Crisp, 1974, 1984; Scheltema, 1974; Hadfield, 1986; Burke, 1983; Rittschof and Bonaventura, 1986).

There is very strong evidence for active choice of substratum by barnacles. Gregarious settlement (Knight - Jones ,1953) is apparently mediated by proteins adsorbed on the substratum (Crisp and Meadows, 1962; Larman and Gabbott, 1975; Larman *et al.*, 1982). The larvae from different taxa respond uniquely to surfaces. This implies that surface chemistry plays a role in the distribution of adults (Strathmann *et al.*, 1981).

Competition for space among benthic marine organisms living on hard substrata is generally believed to be intense, and the production of allelopathic chemicals is a suggested mechanism by which established organisms deter the settlement of colonising larvae (Good body, 1961). The creation of unfavourable or toxic conditions at or immediately above an organism's surface is certainly a widespread antifouling adaptation in the marine environment. Antifouling defence by means of surface-bound or exuded secondary metabolites appears to be quite a common phenomenon (Stoecker, 1980; Bakus *et al.*, 1986).

Many seaweeds have no obvious physical defences against larval settlement and yet remain free of epibionts, suggesting that they are chemically defended against fouling. Many chemically rich seaweeds probably pérsist in habitats rich in foulers because of the toxic or deterrent secondary metabolites they produce or because of their combined chemical and morphological defences. The most important chemical defence mechanism is mediated by secondary metabolites which include terpenes, aromatic compounds, acetogenins, amino acid derived substances and phlorotannin polyphenolics (Faulkner, 1984b, 1986). The sea has proven to be a rich source of natural products; over 1400 have been isolated from

marine invertebrates and algae within the last decade (Faulkner, 1984b,1986, 1987) and many of these are biologically active. The ecological role (if any) of many of these compounds is completely unknown. More than 600 unique structually different and biologically active compounds have been isolated and identified from marine algae (Faulkner, 1984b, 1986).

Marine ecosystems are usually diverse, defenses which are effective against one organism may not be effective against another. Indeed, it is appropriate to consider if one compound can fulfil more than one defensive role.

In shaping the patterns of distribution and abundance of individuals, chemical ecology has important implications for the structure and dynamics of algal and invertebrate communities. (Faulkner, 1987; Littler *et al.*, 1986; 1987). The fouling of living surfaces represents a means of invading a community in which spatial resources are extremely limited. Hence chemical and physical antifouling defenses may determine which epibionts may invade a community and when they may do so. Several reviews address the direct effects of seaweed chemical defenses (Paul and Fenical, 1987; Hay and Fenical, 1988; Hay *et al.*, 1988b; Duffy and Hay, 1990; Hay, 1991; Hay and Steinberg, 1992). Association between sedentary invertebrates and seaweeds appear to be largely dependent on chemical cues received by the larvae as they settle. Nevertheless, bio-activity by chemicals in marine organisms has usually been assessed by testing a single concentration, either of the active metabolites (Thompson *et al.*, 1985) or the different chemical fractions (Wahl and Banaigs, 1991) or the crude extract (Uriz *et al.*, 1991, 1992).

Frequently, several active substances are contained in different chemical fractions of a species in such a way that the total active response can be a simple addition of the individual toxicities or the result of a synergestic effect. Consequently,

testing the crude extract has been considered an acceptable way to take into account the species activity as a whole, even though it also contains substances other than bioactive metabolities.

The behaviour of animals in the laboratory does not always reflect accurately the behaviour of animals in the field where other variables produce, uncontrollable, unpredictable, interactive effects. Although the induction of settlement and metamorphosis of larvae of many species of marine invertebrates has been shown in the laboratory to depend upon very specific external cues, the actual contribution of these factors to settlement in the field is poorly understood. For instance, Butman *et al.* (1988) demonstrated that larvae of *Capitella* sp. and of *Mercenaria mercenaria,* when in still water, metamorphosed in response to cues from organic-rich muddy sediments or clean glass beads, respectively, but only *Capitella* exhibited enhanced metamorphosis in response to the same materials in moving water. Cumo (1985) demonstrated that larvae of some species do respond to the chemical nature of substratum during settlement and metamorphosis. It is well known that compounds that alter surface chemistry have the potential to modulate settlement (Rittschof and Costlow, 1989a; Roberts *et al.*, 1991; Gerhart *et al.*, 1993). Therefore, assays were conducted employing coated panels.

Experiments performed in still sea water on laboratory benches do not accurately reflect the environmental conditions faced by most larvae. Greater effort should be devoted to testing compound under more realistic flow regimes, ie., in flumes or in the field, perhaps with a concomitant effort toward tracking larval movements (Levin, 1990).

The use of potentially biodegradable organic compounds and marine natural products as possible antifoulants for inhibition of microbial, algal and invertebrate components of the biofouling community is highly promising. It is important to develop new antifouling coatings to deliver such inhibitors at sustained, constant rates to obtain a long coating life.

Antifouling assays were conducted in the field to test the effectiveness of an extract or secondary metabolites against natural populations of biofoulers within a community. Results of field assays with extracts and isolated metabolites in different biofouling communities should indicate whether the effectiveness of seaweed chemical defenses varies among habitats.

4.2 MATERIALS AND METHODS

4..2.1 Collection of seaweeds

The seaweeds selected for the study were collected from the intertidal rocky areas of Kovalam by handpicking, diving and snorkeling. They are *Ulva fasciata, Caulerpa peltata*, *Gelidium pusillum, Gracilaria corticata, Padina tetrastromatica*, *Sargassum myriocystum* and *Sargassum wightii.*

4.2.2 Processing of seaweeds

After washing and shade drying, the collected seaweeds were oven dried at a temperature of 40 $\pm$ 2°C for 2 hours and ground to powder.

4.2.2.1 Preparation of extract

Seaweed secondary metabolites were extracted applying protocols for extraction of antifoulants as per Rittschof *et al.* (1992). 5gms of powdered seaweed

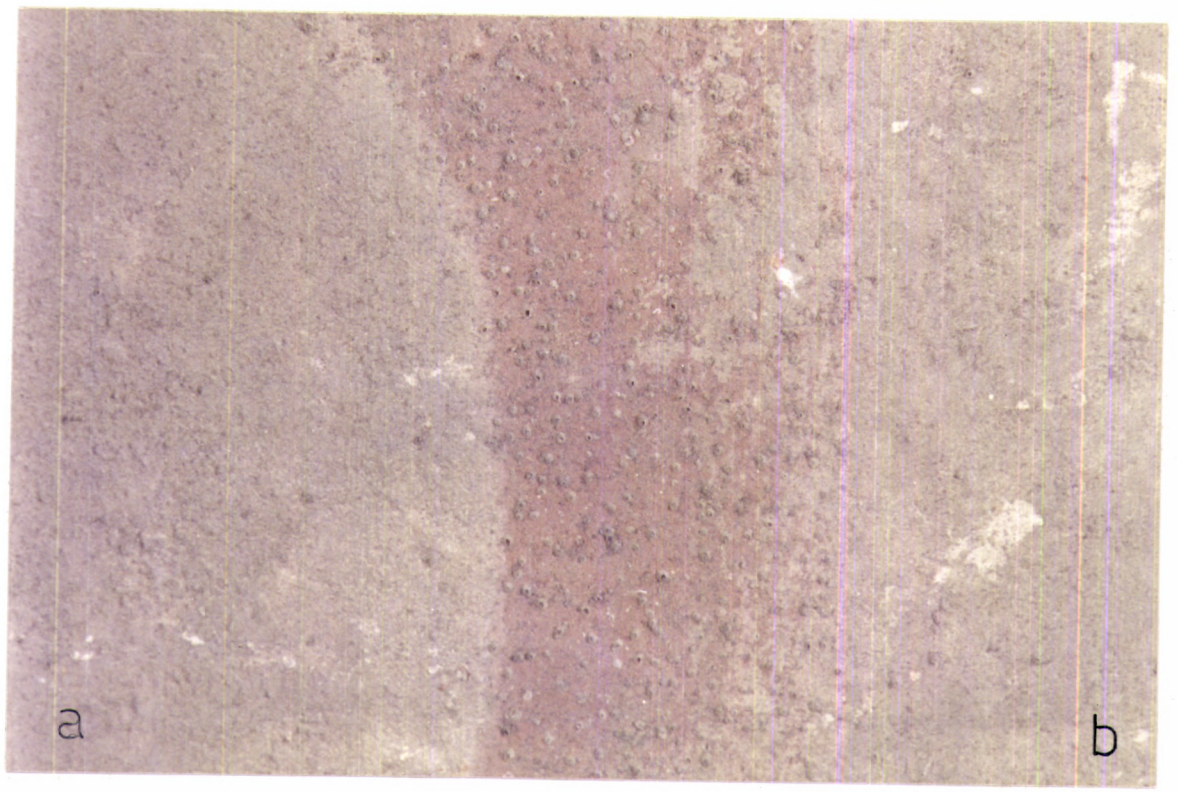
dissolved in minimum amount of methanol was allowed to stand for a day with occassional stirring and the extract was filtered through whatman no. 1 filter paper and the residue was re-extracted. The crude extract was concentrated using rotary evaporator.

4.2.2.2. **Preparation of test panel**

Experiment was designed as per Wright (1991). Terra cotta test panels half coated with binder + extract and the other half coated with binder in plain solvent were employed to study the effect of methanol accommodated secondary metabolites and the settlement pattern of biofoulers. Panels prepared as above were suspended at approximately 1m depth in the jetty area of Cochin University of Science and Technology in the Cochin harbour. Six replicates of test panels were used. Settlement on the panels were compared seven days after immersion. Macrofouling organisms selected for the evaluation of antifouling properties of methanol accommodated secondary metabolites of seaweeds include hydroids, tube worms, bryozoans, barnacles and bivalves. All these organisms are early settlers in the community and are therefore, regarded as better suited to reflect the effects of the compounds on settlement behaviour.

4.3. RESULT

The results of the *in situ* studies on the settlement of biofoulers employing seaweed extract coated test panels are presented in tables 4.1 to 4.7, figures 4.1 to 4.7 and photograph 4.1. Settlement test results of these crude extracts suggest that the search for natural antifouling is promising, especially if considering the antifouling effect magnification by purification of these extracts and the possible discovery of new molecules or functional groups.



Photograph 4.1
Test panels showing the settlement pattern of biofoulers on a) extract + binder and b) binder alone.

TABLE 4.1

Effect of crude methanol accommodated secondary metabolities of *Ulva fasciata* on the settlement of larvae of different biofoulers.

Biofoulers	Number of biofoulers settled	
	Control	Test
	Mean S.D.	Mean S.D
Barnacles	40.8 ± 10.5	1.6 ± 1.6***
Bryozoans	42.6 ± 4.8	4.6 ± 1.0***
Bivalves	69.8 ± 7.1	6.4 ± 1.0***
Tube worms	67.6 ± 3.3	3.2 ± 1.2***
Hydroids (% coverage)	11.3 ± 2.4	5.0 ± 3.8**

*** $P < 0.01$

** $P < 0.05$

TABLE 4.2

Effect of crude methanol accommodated secondary metabolities of *Caulerpa peltata* on the settlement of larvae of different biofoulers.

Biofoulers	Number of biofoulers settled	
	Control	Test
	Mean S.D.	Mean S.D
Barnacles	16.2 ± 2.3	3.4 ± 2.4***
Bryozoans	48.6 ± 6.6	13.0 ± 2.2***
Bivalves	68.4 ± 5.3	4.0 ± 1.4***
Tube worms	32.6 ± 4.5	10.4 ± 1.6***
Hydroids (% coverage)	5.0 ± 1.5	10.7 ± 3.9**

*** $P < 0.01$

** $P < 0.05$

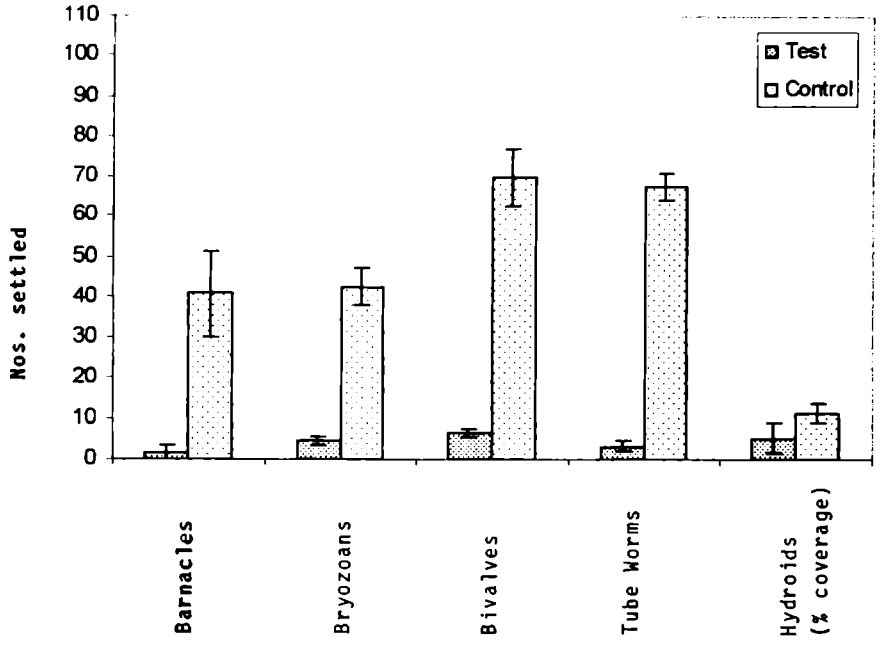


Fig. 4.1

Effect of crude methanol accommodated secondary metabolites of *Ulva fasciata* on the settlement of different biofoulers.

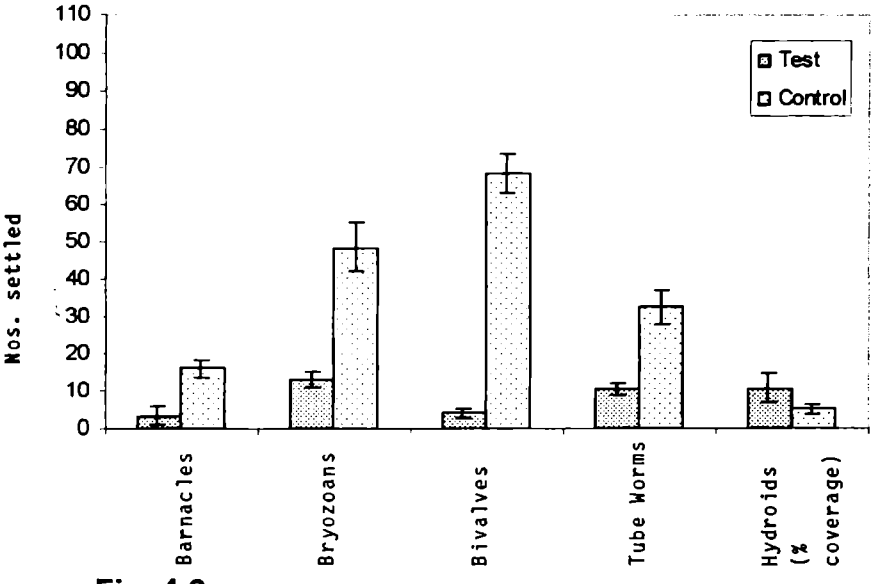


Fig. 4.2

Effect of crude methanol accommodated secondary metabolites of *Caulerpa peltata* on the settlement of different biofoulers.

TABLE 4.3

Effect of crude methanol accommodated secondary metabolities of *Gelidium pusillum* on the settlement of larvae of different biofoulers.

Biofoulers	Number of biofoulers settled	
	Control	Test
	Mean S.D.	Mean S.D
Barnacles	28.8 ± 5.3	13.4 ± 3.6***
Bryozoans	34.2 ± 6.0	3.0 ± 1.4***
Bivalves	28.0 ± 6.4	4.0 ± 2.1***
Tube worms	42.4 ± 5.7	4.0 ± 1.4***
Hydroids (% coverage)	21.9 ± 7.4	9.4 ± 6.6***

*** $P < 0.01$

TABLE 4.4

Effect of crude methanol accommodated secondary metabolities of *Gracilaria corticata* on the settlement of larvae of different biofoulers.

Biofoulers	Number of biofoulers settled	
	Control	Test
	Mean S.D.	Mean S.D
Barnacles	13.8 ± 2.8	1.2 ± 1.2***
Bryozoans	13.8 ± 2.6	10.6 ± 2.3*
Bivalves	51.6 ± 9.5	7.6 ± 1.5***
Tube worms	35.8 ± 2.6	15.4 ± 1.9***
Hydroids (% coverage)	15.0 ± 1.3	6.3 ± 4.0***

*** $P < 0.01$

* $P < 0.2$

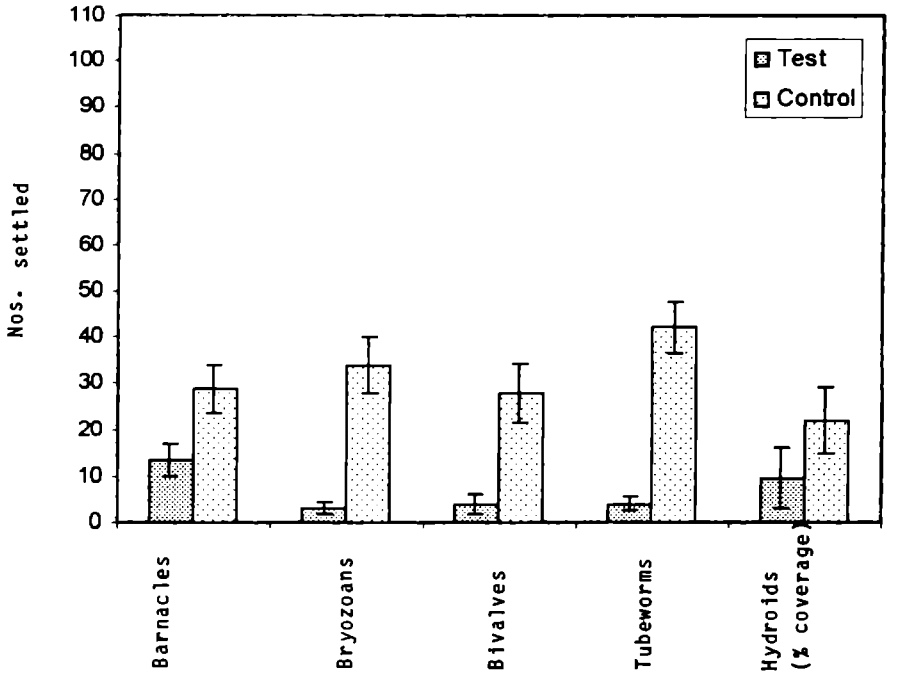


Fig.4.3

Effect of crude methanol accommodated secondary metabolites of *Gelidium pusillum* on the settlement of different biofoulers.

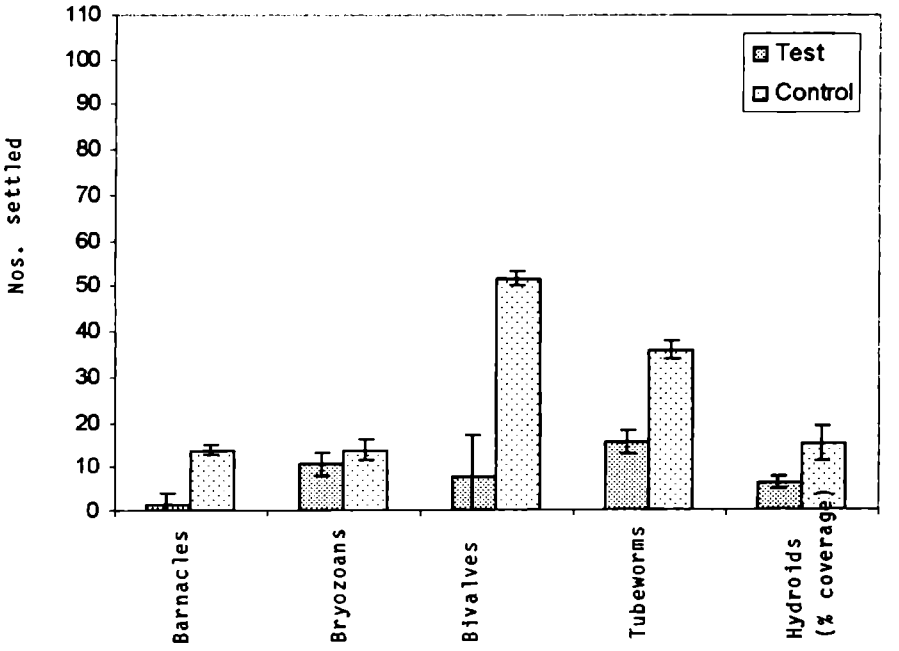


Fig.4.4

Effect of crude methanol accommodated secondary metabolites of *Gracilaria corticata* on the settlement of different biofoulers

TABLE 4.5

Effect of crude methanol accommodated secondary metabolities of *Padina tetrastromatica* on the settlement of larvae of different biofoulers.

Biofoulers	Number of biofoulers settled	
	Control	Test
	Mean S.D.	Mean S.D
Barnacles	16.8 ± 2.4	15.8 ± 2.8
Bryozoans	13.8 ± 1.3	13.0 ± 0.9
Bivalves	10.8 ± 0.7	16.6 ± 1.0***
Tube worms	16.4 ± 2.2	16.8 ± 3.4
Hydroids (% coverage)	14.8 ± 3.6	3.8 ± 1.3***

*** $P < 0.01$

TABLE 4.6

Effect of crude methanol accommodated secondary metabolities of *Sargassum myriocystum* on the settlement of larvae of different biofoulers.

Biofoulers	Number of biofoulers settled	
	Control	Test
	Mean S.D.	Mean S.D
Barnacles	9.4 ± 2.01	5.2 ± 0.4***
Bryozoans	36.2 ± 5.6	1.0 ± 0.6***
Bivalves	27.4 ± 1.9	14.8 ± 1.7***
Tube worms	28.0 ± 2.6	1.4 ± 1.2***
Hydroids (% coverage)	38.81 ± 6.2	27.0 ± 2.8***

*** $P < 0.01$

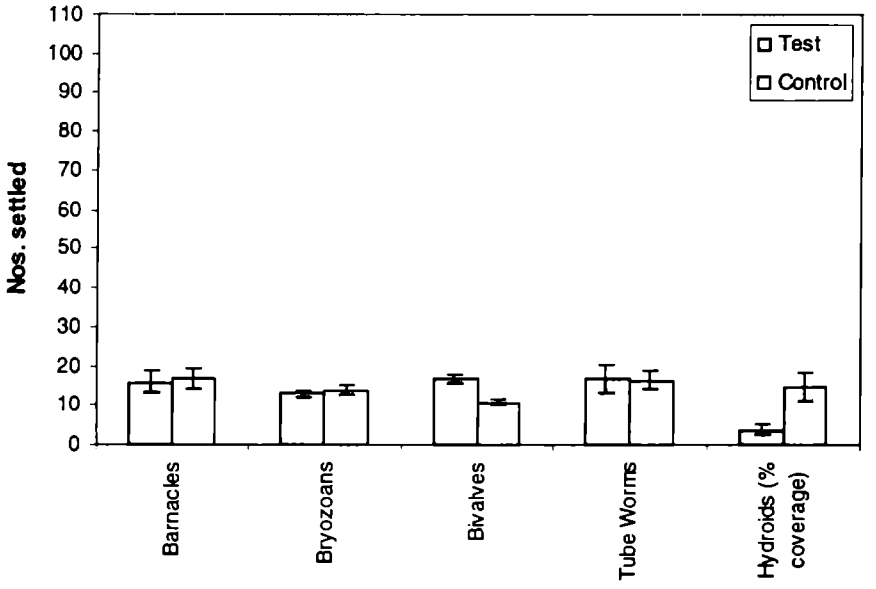


Fig. 4.5

Effect of crude methanol accommodated secondary metabolites of *Padina tetrastomatica* on the settlement of different biofoulers

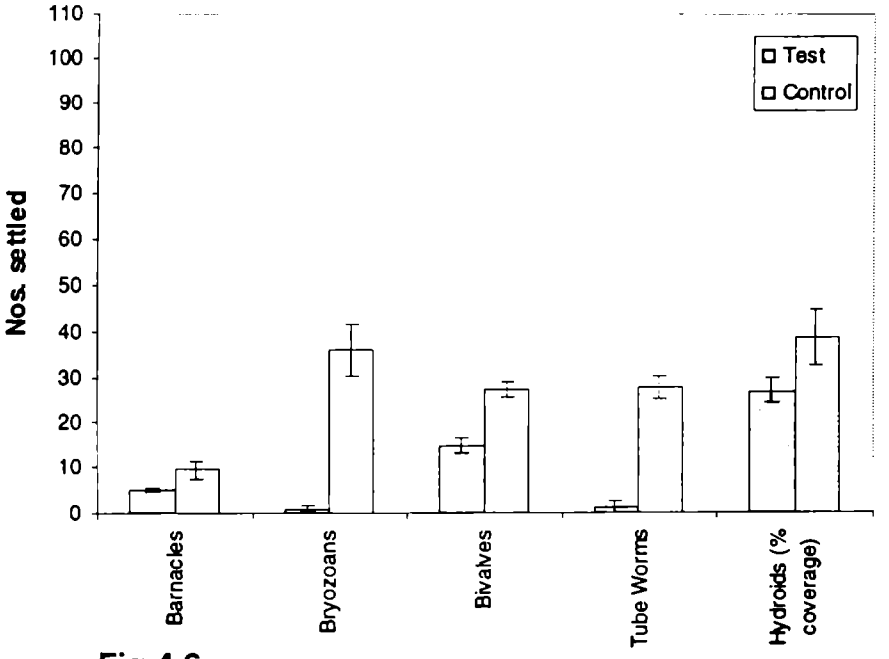


Fig.4.6

Effect of crude methanol accommodated secondary metabolites of *Sargassum myriocystum* on the settlement of different biofoulers.

TABLE 4.7

Effect of crude methanol accommodated secondary metabolities of *Sargassum wightii* on the settlement of larvae of different biofoulers.

Biofoulers	Number of biofoulers settled	
	Control	Test
	Mean S.D.	Mean S.D
Barnacles	33.6 ± 4.0	9.0 ± 0.9***
Bryozoans	37.6 ± 7.4	34.6 ± 7.2
Bivalves	91.8 ± 4.5	44.4 ± 5.3***
Tube worms	92.8 ± 6.0	18.2 ± 1.6***
Hydroids (% coverage)	8.31 ± 1.2	4.6 ± 1.0***

*** $P < 0.01$

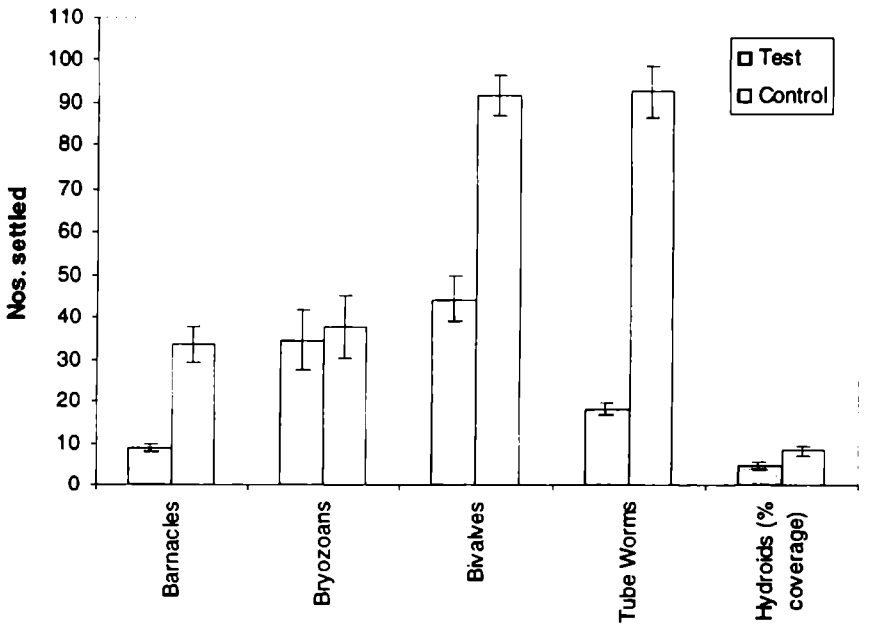


Fig. 4.7

Effect of crude methanol accommodated sencondary metabolites of *Sargassum wightii* on the settlement of different biofoulers

In the present investigation, the effect of seaweed secondary metabolites accommodated in crude methanol on the settlement pattern of larvae of barnacles were studied. Settlement on binder was used as the control. Settlement analysis showed that presence of secondary metabolites of all seaweeds except *Padina tetrastromatica* (Table 4.5, Fig. 4.5) on test panels significantly ($p<0.01$) inhibited the settlement of cyprid larvae of barnacles, when compared to settlement on control panel. The secondary metabolites of the seaweed *Ulva fasciata* was proved to be a potent barnacle settlement inhibitant (Table 4.1, Fig. 4.1).

Secondary metabolites of *Sargassum myriocystum* and *Gelidium pusillum* accommodated in the crude methanol extract significantly ($p<0.01$) inhibited settlement of larvae of barnacles, bivalves, tube worms and hydroids.

Hydroid settlement was significantly inhibited by the presence of secondary metabolites of *Padina tetrastromatica* on the test panels (Table 4.5, Fig. 4.5). While the presence of crude methanol extract of *Padina tetrastromatica* on the test panels increased the settlement of bivalves it couldnot inhibit the settlement of barnacles, bryozoans and tube worms. Secondary metabolites of *Ulva fasciata* showed a significant antifouling activity against barnacles, bryozoans, bivalves and tube worms ($P<0.01$) (Table 4.1, Fig. 4.1). *Caulerpa peltata* and *Gracilaria corticata* (Table 4.2, 4.4, Fig. 4.2, 4.4) also exhibited antifouling activity against majority of the biofoulers tested.

Sargassum wightii didnot show antifouling activity against bryozoans (Table 4.7, Fig. 4.7) while *Sargassum myriocystum* significantly ($p<0.01$) inhibited settlement of larvae of bryozoans (Table 4.6, Fig. 4.6).

4.4 DISCUSSION

The present study employing seaweed extract coated panels, strongly supports the involvement of secondary metabolites of seaweeds in the prevention

of larval settlement. Clearly, chemically mediated interactions between biofoulers and marine plants are complex and depend on the responses of different species of biofoulers and the types and concentrations of secondary metabolites produced by different populations of seaweeds. Chemical variation occurs widely in tropical seaweeds and it is not known how biological and physical environmental factors causes this variation. Variability in seaweed chemistry could result from previous grazing history, from physical environmental factors such as light and nutrients, from interactions with competitors or pathogens, from genetic differences among algal populations, or as a result of localized selection.

It is inappropriate to consider chemical mechanisms of antifouling defence in isolation, as many organisms posses physical antifouling defences, many of which may act in concert with chemical defences. Chemical compounds may also interact synergistically. Indeed, the true effectiveness of antifouling defences may only be determined when the overall antifouling "strategy" of an organism has been assembled (Targett, 1988). The presence of antifouling defences across a broad range of non-motile taxa argues strongly for biofouling as a potent selective pressure, moulding the defenses of these plants and animals.

Although it is difficult to establish a cause-effect relationship in field studies because of numerous physical, chemical and biological factors that could influence the biological response, in most cases, a decline was noted in the rate of settlement in the presence of secondary metabolites of seaweeds.

Evidence suggests that chemically induced metamorphosis of many species of marine invertebrate larvae is under control of the larval nervous system with specific sensory receptors acting, at least in part, to interface the nervous system with the environment (Morse et al., 1979, 1980a, b; Burke, 1983, 1984; Baloun and

Morse, 1984; Morse, 1984, 1985; Morse and Morse, 1984; Trapido - Rosenthal and Morse, 1985, 1986a, b; Baxter and Morse, 1987).

As reviewed by Morse (1985), several cases of chemically induced settlement and metamorphosis of marine invertebrate larvae involve neurotransmitter - like inducers. Although the involvement of many neurotransmitter candidates has been proposed for the process of attachment and metamorphosis in a range of marine invertebrate larvae, including the red abalone *Haliotis rufescens* (Morse *et al.*, 1979), the Pacific oyster *Crassostrea gigas* (Coon and Bonar, 1985; Beiras and Widdows, 1995), the Japanese scallop *Patinopecten yessoensis* (Kingzett *et al.*, 1990), the serpulid polychaetes *Hydroides ezoensis*, *Pomatoleios kraussii* and *Ficopomatus enigmaticus* (*Okamoto et al.*, 1995), and the barnacle *Balanus amphitrite* (Kon-ya and Endo, 1995), most of these studies fail to specify the site of action.

To induce a biological response, a compound must interact with a receptor binding site. A receptor can have varying degrees of specificity, and there can be multiple receptors that bind a molecular species. Little is known about the physiology of marine biofoulers with regard to the way the secondary metabolites are detected or metabolized. It is likely that many marine biofoulers detect algal secondary metabolites by contact, because many compounds are sequestered in membrane - bound vesicles in cells and are not exuded into seawater.

The apical tuft has been implicated as the organ utilized when "sensing" the substratum prior to settling in larvae as diverse as polychaetes, chitons and gastropods (Baxter and Morse, 1992). The characteristics of a surface can affect settlement most simply by interaction with a larval adhesive (Yule and Walker, 1984, 1985). Alternatively, the forces that are generated between the surface and the larvae

can either be sensed by the larvae or can effectively trap them. Diffusible molecules associated with the surface can act either through several receptor pathways (Morse, 1984, 1988) by binding with external larval receptors (Trapido-Rosenthal and Morse, 1985, 1986a, b) or by entering the larval body and altering its metabolism (Baloun and Morse, 1985, Rittschof *et al.*, 1986a).

Studies have clearly demonstrated that many secondary metabolites produced by tropical seaweeds function as chemical defences against biofoulers in habitats where fouling is intense. Some compounds appear to be broadly active against a variety of biofoulers, while others may prevent settlement of only a few species or none at all. Compounds that vary only slightly in their chemical structures may have very different toxic or antifouling effects. The same metabolite may differ in its effects on even related species of biofoulers. Given this variability in the responses of biofoulers to seaweed chemical defences, it is not surprising that a great deal of chemical variation occurs in the secondary metabolites produced by seaweeds. Metabolite types and concentrations vary within and among plants in a population of seaweeds. Variation in the types and concentrations of secondary metabolites may result from increased production of compounds due to increased settlement over time (induction), from rapid conversion of compounds from less active to more active metabolites when plants are injured (activation), or from localized selection.

Even though algal secondary metabolites may function as defences against biofoulers, they may have evolved for other reasons, such as resistance to pathogens or competitors. Many tropical algae are evolutionarily older than the macrofoulers. Fossils of calcareous green algae are known from the Cambrian (500 million years ago).

Caulerpa racemosa produces sesquiterpenoid metabolites in relatively high concentrations. The algae of class chlorophyta produce a number of related groups of sesquiterpeniods and diterpenoid compounds of great biological activity (Paul and Fenical, 1986, 1987; Hay *et al.*, 1988b). The order Caulerpales has received much attention and their chemistry has been studied well (Paul and Fenical, 1986, 1987; Paul and Van Alstyne, 1988; Hay *et al.*, 1988). Paul and Fenical (1986) found caulerpenyne and caulerpin as the major compounds present in the organic extract of *Caulerpa* sp. The secondary metabolite caulerpenyne exhibit bioactive property (Paul and Fenical, 1986; Targett *et al.*, 1986), while caulerpin was not reported to have deterrent effect on herbivores. Caulerpenyne has been shown to be antimicrobial, anti-neoplastic and feeding deterrent (Hodgson, 1984). Paul and Fenical (1985) isolated caulerpenyne from nine species of *Caulerpa* which includes *C. racemosa* also. The presence of caulerpenyne in the *Caulerpa racemosa* could be the reason for the observed antifouling property.

In the red algae laurinterol and debromolaurinterol, having antimicrobial activity is present. Fenical (1975) and Faulkner (1984a) observed that the families of Bonnemaisoniaceae, Plocamiaceae, Rhizophyllidaceae and Rhodomeliaceae were found to be rich in biologically active compounds ranging in structure from simple aliphatic haloketones and brominated phenols to more complex monoterpenes, sesquiterpenes and diterpenes.

Generally phaeophytes have been very successful in their use of defensive mechanisms. These brown algae produce phlorotannins (polyphenolics) and these compounds function like terrestrial tannins (Steinberg, 1985; Van Alstyne 1988). Faulkner (1986) observed that the brown algae in the Dictyotales produce complex mixtures of terpenoids, acetogenins and terpenoid aromatic compounds. Norris

and Fenical (1982) stated that *Padina jamaicensis* and *P. vickersiae* contain no unusual secondary metabolites and noticed the absence of biological activity. The absence of secondary metabolites in these algae can explain the absence of antifouling activity against barnacles, bryozoans, bivalves and tube worms of *Padina tetrastromatica* in the present study. Norris and Fenical (1982) suggested polyphenolics as the main proposed defence in *Sargassum polyceratium* and they also observed that the extract of this plant showed antibacterial and antilarval activities. In the present study *Sargassum myriocystum* and *Sargassum wightii* showed different levels of antifouling activity. Terrestrial studies depict that tannins may often function as cell toxins rather than as digestibility reducers. This may be the possible reason for the high antifouling nature of the brown algae tested. The different levels of deterrence exhibited by different species of *Sargassum* plants may be due to the variation in polyphenolic concentration among them. According to plant apparency theory (Feeny, 1976; Rhoades and Cates, 1976) phenolic compounds are dose dependent in defence that are characteristic of apparent plants. Later Steinberg (1985, 1988) and Van Alstyne (1988) also noticed that the degree of deterrence is related to the phenolic concentration.

A major concern is that this study does not address the long term stability of these compounds. If the goal of the paint formulator is to achieve a coating with a service lifetime of several years, then the release rate must be controllable over time. Field studies suggest that behavioural responses at the time of substratum selection may be important for many species (Strathmann and Branscomb, 1979; Woodin, 1985; Raimondi, 1991) and short-term flume experiments of initial settlement confirm the importance of larval behaviour at settlement in flow (Butman *et al.,* 1988; Grassle and Butman, 1989; Mullineaux and Butman, 1991). As larvae age, they may deplete their energy reserves with increasing time in the plankton

and become less selective. Both barnacles and bryozoans show effects of physiological ageing on settlement. In the settle or die situation both kinds of larvae settle if they are in a position to attach. Barnacles settle upon the surface in the settle or die situation while bryozoans will settle either on the surface or upon the surface film at the air-water interface.

Individual larvae may also differ in their response to a given stimulus. In behaviour assays, bryozoan and barnacle larvae respond in a species specific way to chemically defined surfaces. Microscopic observations indicated that there are at least three mechanisms that alter the measured response. Responses can result from (1) larval behaviour, (2) repulsion, or (3) trapping of the larvae. The local hydrodynamic environment can also affect the ability of larvae to respond to stimuli. The combination of all these processes means that settlement is unlikely to be predictable. Even if we know the distribution of potential cues or deterrents, we may not be able to predict the subsequent colonization of surfaces with much accuracy, except under unusual circumstances, such as extremely high settlement rates of individual fouling species. Larvae that are physically trapped do not metamorphose unless they get attached.

Natural product settlement inhibitors appear to work by alteration of the settlement surface (Rittschof *et al.*, 1985). It is possible that in addition to altering surface characteristics, natural product inhibitors also interfere with some other aspect of the physiology of the organism as is the case with barnacles and bryozoans with picrotoxin, barnacles with Dibuteryl cAMP and diatoms with Homarine (Targett *et al.*, 1983). Natural product addition can result in 100% inhibition of settlement without trapping larvae.

Bacteria can produce chemicals which act as cues that stimulate or inhibit further settlement and that bacteria can modify the characteristics of a surface to enhance or inhibit further settlement. Bacterial influence on the settlement and metamorphosis of macrofoulers has been attributed to the exopolymer which acts as an adhesive bridge in irreversibly binding bacteria to the surfaces. Films of some microorganisms may encourage while those of other may discourage the settlement of the same invertebrate larvae (Meadows and Williams, 1963), and the metabolic activity of the micro-organisms may also influence the settlement. The reaction of marine invertebrate larvae to bacterial films and diatom slimes raises an important question whether chemical substances eminating from the surfaces of living organisms or the chemical constitution of the surface itself play any part in habitat selection. If a recognition of complex chemical substances could reinforce the appreciation of the physical attributes of the substratum and its environment, then a much more precise and definite choice could be made.

Partially purified inducers of associative settlement have been described for species in several phyla. Experimental surfaces treated with extracts of fucoid brown algae elicit the settlement of epibiotic bryozoans, bivalves and spirorbid polychaetes. Larvae of the bryozoan *Alcyonidium polyoum*, an epibiont on *Fucus serratus*, were induced to settle on surfaces treated with aqueous extracts of the alga (Crisp and Williams, 1960). Extracts of two other fucoids, *F. vesiculosus* and *Ascophyllum nodosum*, also stimulated settlement. Similar responses were observed for larvae of another bryozoan, *Flustrellidra hispida* (Crisp and Williams, 1960). Kiseleva (1966) noted that settlement of the bivalve *Brachyodontes lineatus* was stimulated by aqueous extracts of *Cystoseira barbata*. Extracts of *Fucus serratus* similarly promoted settlement of the epibiotic polychaete *Spirorbis borealis* (William, 1964). Experimental plates treated with aqueous extracts of *Lithothamnion*

polymorphum stimulated high levels of settlement of the spirorbid, *Spirorbis rupestris* (Gee, 1965).

Further studies are required to determine the exact nature of the biosynthesis of secondary metabolites and the mechanisms by which they inhibit settlement. This leads to the conclusion that there is considerable scope in the natural product approach to non toxic antifouling development and that structural analogues offer an alternative to the large - scale collection of source organisms. Overall, the current study provides some tantalizing evidence that secondary matabolites accommodated in the crude methanolic extracts of seaweeds are important inhibitors of larval settlement. A more effective solution may require a coating that more, closely mimics nature by combining chemical repellency with a physical mechanism of antifouling such as a foul release or self-polishing capability (Clare *et al.*, 1992; Targett and Stochaj, 1994; van Altena and Butler, 1994).

CHAPTER 5

BRYOZOANS ASSOCIATED WITH SEAWEEDS

5.1 INTRODUCTION

Bryozoans are a group of animals which inhabit both fresh water and marine ecosystems. Bryozoans comprise about 5,000 living species and about 16,000 fossil species. They normally occur prolifically below tide marks. Marine bryozoans exhibit a greater degree of abundance and diversity from the shore down to the oceanic regions going to a depth of about 8000 meters.

These animals are found to colonise free surfaces of submerged rocks and similar hard substrata. There are instances where 30 species were found on the surface of a single bivalve shell or as much as 90 species in one dredge haul. This shows the greatest abundance and diversity of this group. They are very prominent fouling organisms settling on the hulls of ships, submerged portions, directional buoys, harbour structures etc. Their quick growth results in rapid spreading of colonies on the hulls of ships, offer safe substratum for the settlement of other sedentary organisms.

Bryozoans are widely distributed in the Indian waters. To understand the fouling biology of bryozoans, it is essential to know about species composition, substrate preferences and relative growth of these animals. Since taxonomy is mainly based on the highly calcified zooecia, even dry material offer excellent specimens for taxonomic investigations. The historical introduction gives an idea

of the extensive literature available on recent bryozoa. However, information on the taxonomy of bryozoans of Indian waters after the classical work of Menon and Nair (1967) is rather limited. The present account probably could help in adding to the already known information of this group in Indian waters. The specimens were collected from the seaweeds.

5.2 REVIEW OF LITERATURE

Bryozoa (Polyzoa) is a distinct phylum of colonial aquatic sessile either free living or commensal invertebrates. Thompson (1830) gave the term Polyzoa and Ehrenberg (1831) the term Bryozoa. Nitsche (1869) divided bryozoa into two groups Entoprocta and Ectoprocta. Ectoprocta is now generally recognised as equivalent to Polyzoa as an alternative name of the phylum, with Entoprocta as another phylum (Hyman, 1959).

Works on the systematics of this group are numerous. Most important studies on the Bryozoans of Indian waters are those of Thornely (1905,1907), Annandale (1906, 1907a,b,c, 1908a, 1911a,b and 1912) etc. Menon and Nair (1967) reported 70 species from the south west and south east coasts of India, of which three species, one subspecies, one variety and three forms assigned to three genera were new to science. Menon and Nair (1970) described a new species *Schizoporella cochinensis* sp. novo. from the Cochin backwaters.

Three species of the genus *Tremogasterina* were also described by Menon and Nair in the same year from Indian waters. Menon and Nair (1972b) described six species of *Bugula* Oken collected from the intertidal zone of the south west coast of India. In the same year Menon described 12 species of Ctenostomatous bryozoans from Indian waters. Nine species of the genus *Scrupocellaria* Van Beneden were described by Menon (1972a) from south west

and south east coasts of India. Of these, six species were described for the first time. Nine bryozoan species belonging to the genera *Parasmittina* were also described by Menon (1972c). Nair (1973) observed the fouling characters of four bryozoan species of Cochin Harbour area. Twenty species of polyzoans belonging to the division Malacostega occurring in the Indian waters are described and illustrated by Menon and Nair (1975). Of these two species *Electra crustulenta* sub species *bogii* are described as new.

Six intertidal bryozoan species of Godavari Estuary was reported by Rao and Ganapati (1975). Seven encrusting Cheilostomatous bryozoan species were reported from the highly polluted coastal waters of Bombay by Swami and Karande (1987). Ravindran *et al.* (1990) reported eight fouling bryozoan species in the offshore waters of Bombay high. Nair (1991) reported 15 species of bryozoans from the Vellar estuary, Tamil Nadu and six species from the mangroves of Pichavaram.

5.3 MATERIALS AND METHODS

The material for the taxonomic studies was mainly obtained from collections made from the intertidal and sub-tidal regions of south west coast of India. Bryozoans associated with seaweeds in the Kovalam beach, Trivandrum were utilised for the study.

The measurements of zooecium given are averages of the length and breadth of about six fully grown and normal zooecia measured with the help of an eyepiece micrometer. Temporary slides for the examination of the chitinous parts (opercula, avicularian mandibles etc.) were prepared by gently crushing few zooecia and adding glycerine before placing coverslips over the material. This procedure

yielded fairly good mounts of the chitinous parts for study. 'Skeletal' parts of the cheilostome bryozoans were cleaned by sodium hypochlorite, which removes all the organic materials from the animal colony.

5.4 LIST OF SPECIES DESCRIBED

ORDER *CHEILOSTOMATA* Busk

SUB-ORDER *ANASCA* Levinsen

DIVISION *MALACOSTEGA* Levinsen

FAMILY *MEMBRANIPORIDAE* Busk

GENUS *ELECTRA* Lamx

1. *Electra bengalensis* (Stoliczka)
2. *Electra indica* (Menon and Nair)
3. *Electra sp.* (Menon and Nair)

DIVISION *COELOSTEGA* Levinsen

FAMILY *THALAMOPORELLIDAE* Levinsen

GENUS *THALAMOPORELLA* Hincks

4. *Thalamoporella rozierii* (Audouin)

SUB-ORDER *CTENOSTOMATA* Busk

DIVISION *STOLONIFERA* Ehlers

GROUP *VESICULARINA* Waters

FAMILY *VESICULARIIDAE* Johnston

GENUS *BOWERBANKIA* Farre

5. *Bowerbankia imbricata*

ORDER *CYCLOSTOMATA* Busk

DIVISION *ARTICULATA* Busk

CAMPTOSTEGA Borg

FAMILY *CRISIIDAE* Johnston

GENUS *CRISIA* Lamouroux

6. *Crisia eburnea*

5.5 DESCRIPTION OF SPECIES

POLYZOA THOMPSON, 1830

BRYOZOA EHRENBERG, 1831

PHYLUM *ECTOPROCTA* NITSCHE, 1869

CLASS *GYMNOLAEMATA* ALLMAN, 1856

ORDER *CHEILOSTOMATA* BUSK, 1852

SUB-ORDER *ANASCA* LEVINSEN, 1909

Cheilostomata without a compensation sac possessing an external frontal membrane.

DIVISION *MALACOSTEGA* Levinsen, 1902

Frontal membrane retained in its original primitive condition. No conspicuous differentiation between the frontal membrane and the operculum.

FAMILY *MEMBRANIPORIDAE* Busk, 1854

Frontal membrane fills the aperture, an opesia occupying the same area of the frontal membrane or reduced by the development of a cryptocyst. Encrusting in habit. Ovicells are not found in any cheitostome. Known to possess a

cyphonautes larva, or in which the occurrence of this larva is probable. Hence Harmer thinks that the presence of cyphonautes larva is of great taxonomic importance.

GENUS *ELECTRA* Lamouroux, 1816

1816. *Electra* Lamouroux, "Histoire des Polypier Coralligenes Flexibles," p.120.

1898. '*Membranipora pilose*' group Waters, *J. Linn. soc. (Zool).*, 26, pp. 660-665.

1926. *Electra* Harmer, *Siboga Exped.*, 28b, p.206.

1950. *Electra* Osburn, *Rep. Allan Hancock Pacific Exped.*, 14, I, p.35.

Colonies encrusting, zooecia pear shaped or rectangular. Proximal gymnocyst may be evanescent distally. Frontal membrane covers an opesium of the same extent. Opesia over arched by marginal spines, sometimes only one spine on the proximal side of the opesia. Cryptocyst wanting or barely indicated. No avicularia, ovicells, or pore chambers.

1. ***Electra bengalensis* (Stoliczka), 1869**

(Fig.5.1).

1869. *Membranipora bengalensis* Stoliczka, *J. Asiat. Soc. Bengal* 38, 2, p.55.

1907. *Membranipora bengalensis* Thornely, *Rec. Indian Mus.*, 1, p.186, fig. 4.

1950. *Electra anomala* Osburn, *Rep. Allan Hancock Pacific Exped.*, 14, 1, pp.36-37, pl.3, fig. 6.

1975. *Electra bengalensis* Menon and Nair, *J. mar. biol. Ass. India* 17, 3, pp.561, fig. 2.

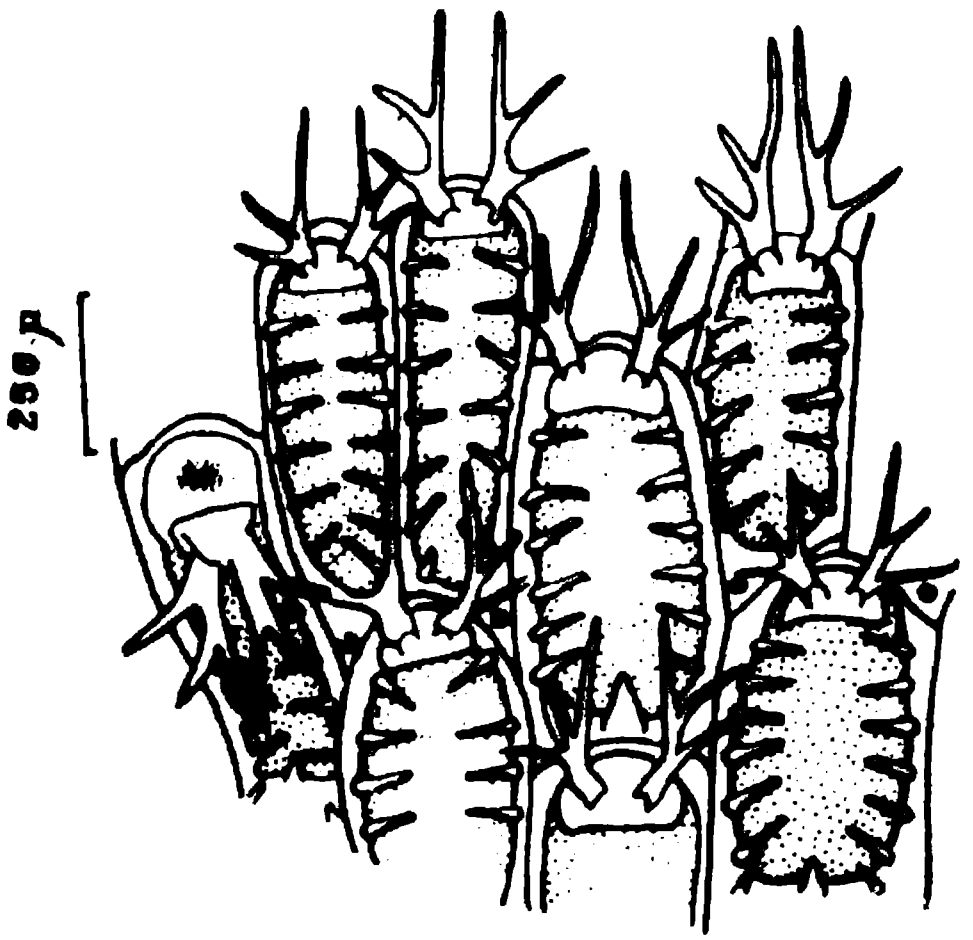


Fig.5.1.
Electra bengalensis - portion of a colony showing the details of zooecia.

Occurrence

Collected from seaweeds in the environs of Kovalam.

Material

Preserved in alcohol

Measurements

Zooecium

Length - 630 μ

Breadth - 295 μ

Salient features

Encrusting, colonies white in colour reaching large dimensions. Zooecia delicate, rectangular with adjacent rows arranged in alternate fashion and separated by distinct grooves. Gymnocyst, moderately developed proximally. Opesia elongate oval with a very vestigial proximal cryptocyst. Three spines present at distal end, one above median line of operculam, the other two lateral. Six to eight pairs of basally jointed rarely branching slender spines overarch the frontal membrane. Operculum large, quite distinct from the frontal membrane, chitinised at border. A pair of long forked (once, twice or thrice) spines ornament the operculum.

Remarks:

This form agrees in all but one respect with *Electra anomala* figured and described by Osburn (1950). Only difference noticed is in the nature of the opercular spines. Osburn noticed in this specimens opercular spines with a single bifurcation but in the present material the opercular spines of some zooids have undergone branching of the second or even third order. Simple spines have also been noticed.

In live specimens with the opercula open the spines which they bear are seen parallel to the frontal membrane, a fact which testifies to the flexibility of the spines (Menon and Nair, 1975).

Osburn (1950) has made the following observation: 'from the middle of the front surface of the operculum arises a pair of very elongate, bifurcate chitinous spines which extend far over the base of the distal zooecium, this anomalous condition is without parallel. This anomalous condition, together with the spinous adornment of the marginal region, are the characters which give specific distinction for this species. Stoliczka (1869) who described *M. bengalensis* noticed the same type of opercular spines arising from the lower lip. However, this observation was corrected subsequently by Thornely (1907) who stated that the spines arise from the operculum and when the operculum is opened fully the spines occupy the peculiar position which confused Stoliczka. In numerous colonies of this species examined during the present study spines of the operculum were invariably present in all zooecia. The absence of marginal spines is a usual occurrence and this observation agrees well with those of Thornely (1907) and Osburn (1950). Osburn has observed numerous colonies possessing zooecia with and without marginal spines. So it is evident that Osburn was in fact examining specimens of *E. bengalensis,* for *E. anomala* does not vary in any character from *E. bengalensis.* Osburn seems to have overlooked Stoliczka's and Thornely's papers.

Previous records from Indian waters

Stoliczka (1869) recorded this species from Bengal and Thornely (1907) reported it from Snod Island; Arabian sea (Menon and Nair, 1967).

Distribution:

The only record of this species from a locality outside Indian waters is that of Osburn (1950), who recorded it from Balboa, Pacific Coast of Panama. This species frequently attached on to glass and wooden panels used for the study of fouling in the Cochin Harbour during the pre monsoon period when the salinity in this brackish environment attained that of the adjoining sea (Menon and Nair, 1975).

2. ***Electra indica* (Menon and Nair), 1975**

(Fig. 5.2)

1975. *Electra indica* sp.novo. Menon and Nair, *J. mar. biol. Ass. India* 17, 3, pp.562, fig. 26.

Occurrence

Colonies encrusting on algal fronds were collected from Kovalam.

Material

Preserved in alcohol

Measurements

Zooecium

Length - 440 μ

Breadth - 275 μ

Salient features:

Zoaria encrusting on algal fronds. Zooecia of uniform shape uncalcified, basal wall. Opesia oval, gymnocyst well developed proximally, provided with well defined pores. Three tubercles occupy zooecial margin, of which one occupies

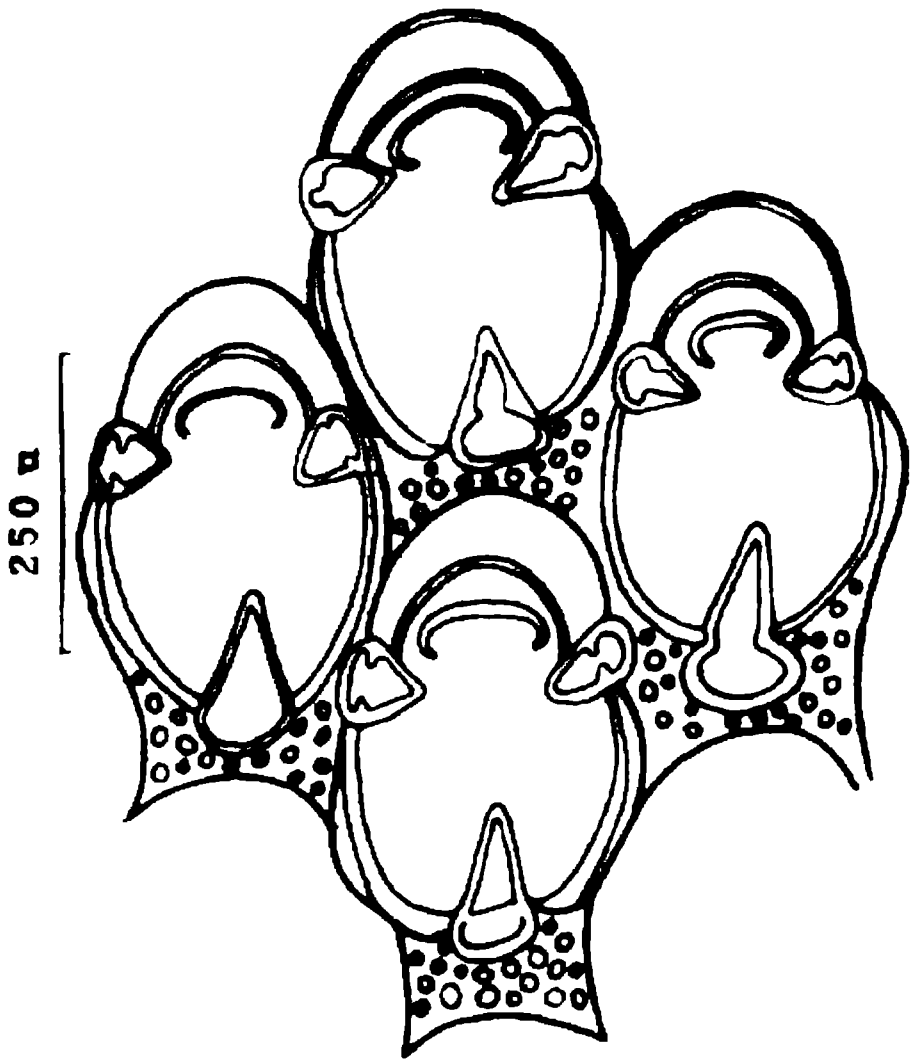


Fig.5.2

Electra indica - portion of a colony showing the details of zooecia.

proximal median point and the other two the distal region on either side of the operculum. Median tubercles in young zooecia long and spinous. All these tubercles connected with a transparent membrane which has two lateral flanks and distal extensions of this membrane forming a hood - like structure. Cryptocyst present but not tuberculate.

Remarks

The hood like extension at the distal end of the zooecium is not a forerunner of the ovicell since it is developed even in immature zooecia. The ancestrula is single. The salient features of the present form are the swollen distal part of the zooecium, the large pores of the gymnocyst and the well developed tubercles. From a study of the available literature it seems that the Genus *Electra* established by Lamouroux in 1816, based on the form *Flustra verticillata* Solander, now includes about 14 recent species and varieties. The only species which *E. indica* resembles is *Electra biscuta* Osburn (1950). Osburn, in *E.biscuta* noticed that the gymnocyst is usually very limited in extent, but can occupy one fourth or more of the zooecial length, the cryptocyst is smooth or slightly granulated, there is a strong arching of the distal walls on the dorsal side and spines of three kinds: (1) a set occupying the lateral sides of the operculum overarching the opesia, when fully developed; (2) two stout conical distal spines opposite the distal end of the operculum; and (3) a transverse series of short and stout conical spines projecting forward in a row proximal to the opesia on the gymnocyst. The number of the last kind of spines varied from 1 to 5. Osburn was doubtful about the generic status of his species, but he wrote 'the absence of ooecia and avicularia and the presence of a gymnocyst, mural spines and thin lateral walls without dietellae, suggest the genus *Electra* though there is little resemblance in appearance to any other of that

genus. "An attempt to treat the form under consideration close to *E. biscuta* is vitiated by the presence of pores on the gymnocyst and the lesser development of the tubercles in *E. indica*. Regarding the presence of a hood like structure, Osburn's remark that "the distal wall is strongly arched forward on the dorsal side" suggests a lesser developed hood-like structure in *E. biscuta*. In certain colonies of *E. indica* proximal gymnocystal tubercles have attained a spinous nature as a result of their elongation and tapering. It seems that this difference between the tubercles is the result of growth and of age. Based on the differences noticed the present form is *Electra indica* (Menon and Nair, 1975). It can be defined as follows. Encrusting zooecia more or less of uniform shape with a swollen distal portion. Opesia oval, well developed proximal gymnocyst porous. Three tubercles on the zooecial margins one proximal and two disto - lateral connected by a transparent membrane. Cryptocyst smooth. Ovicells absent.

3. **Electra sp.**

(Fig. 5.3)

1975. *Electra* sp. Menon and Nair, *J. Mar.biol.Ass.India* 17, 3, pp.563 fig. 2k.

Occurrence

A single colony encrusting on an algal frond was collected from Kovalam.

Material

Preserved in alcohol.

Measurements

Zooecium

Length - 490 μ

Breadth - 250 μ

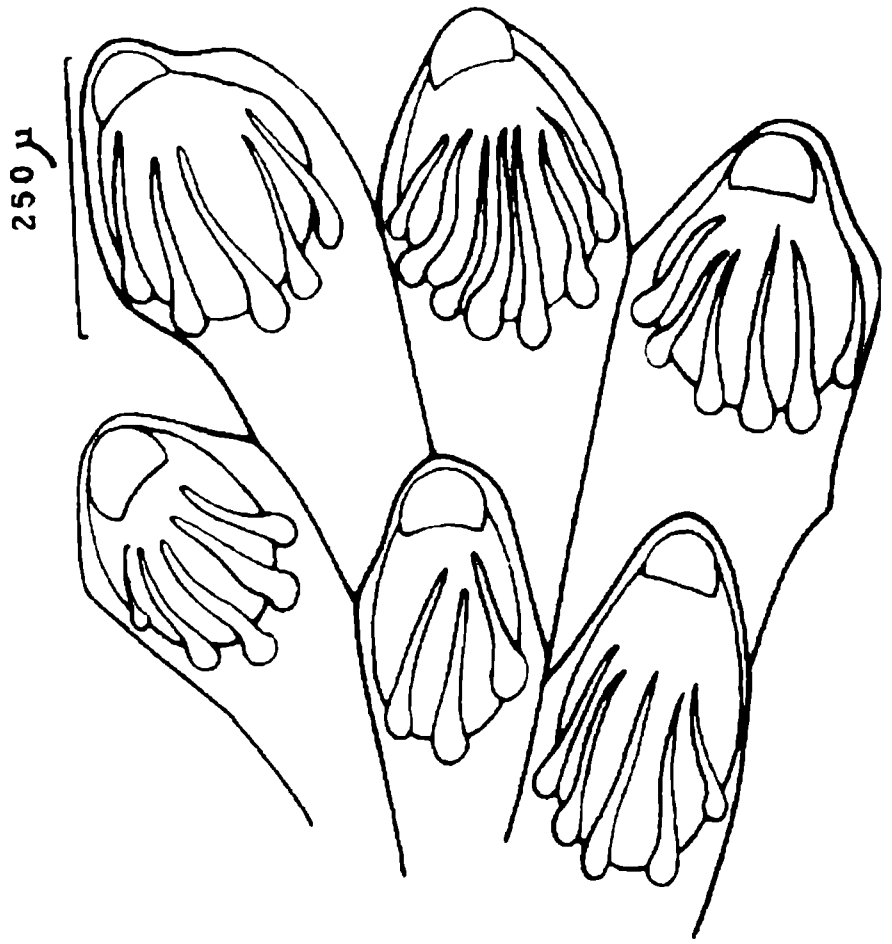


Fig.5.3

Electra sp. - portion of a colony showing the details of zooecia.

Salient features

Encrusting. Zooecia pear-shaped. Opesia oval. spines present, spine number varies from 3 to 7, elongated, arranged along the proximal and proximo-lateral portions of the extensive gymnocyst, over arching the opesia. Operculum membraniporine. Avicularia and ovicells absent.

Remarks

A perusal of the literature shows that though the shape of the opesium resembles that of *Electra biscuta* Osburn, in the very peculiar arrangement of the marginal spines the present form differs from all other species of *Electra* hitherto described. The spines are less calcified than in other species and are so fragile, that they may be easily lost during handling. Nevertheless, the well developed gymnocyst and the marginal spines doubtlessly support the present generic placing.

DIVISION *COELOSTEGA* Levinsen*, 1909

Raised zooecial margins surround an aperture which is larger than the opesia, owing to the development of a cryptocyst. Cryptocyst frequently with a descending portion which unites near its distal ends with the vertical or basal walls. The horizontal cryptocyst distally possesses in many zooecia a median process which forms the inner margin of the two lateral opesiules, through which the depressor muscles pass to their insertion in the frontal membrane. Marginal and oral spines wanting.

Levinsen (1909) has used COILOSTEGA but Harmer (1926) prefers to use COELOSTEGA

FAMILY *THALAMOPORELLIDAE* Levinsen, 1909

Unique among *Cheilostomata* in possessing numerous free, calcareous spicules in the body cavity. Median process highly developed and the closed opesiules are asymmetrical. The opesia, situated near the distal end is much reduced. Large, rounded or acute vicarious avicularia present. Ovicells are bivalvular when present.

GENUS *THALAMOPORELLA* Hincks, 1887

1887. *Thalamoporella* Hincks, Ann. Mag. nat. Hist., 5, 19, p.164.

1909. *Thalamoporella* Levinsen, Publ. Calsberg Fund., p.178.

1926. Thalamoporella Harmer, *Siboga Exped.*, 28b, p. 289.

1950. *Thalamoporella* Osburn, *Rep. Allan Hancock Pacific Exped.*, 14, 1, p.110.

Zooecia with a depressed porous cryptocyst, perforated by two asymmetrical opesiules which are rounded and not slit like. Opesia and orifice practically coextensive. Opesiules meeting either the basal or the lateral walls, their median walls assisting in the formation of the characteristic polypide tube, which terminates beneath the orifice. Internal spicules present in the form of compasses or calipers or both. Avicularia vicarious, rounded or pointed type. Ovicells which are not known in all the species very globose and prominent, the cavity between the ectooecium and entooecium divided by a median suture.

4. ***Thalamoporella rozierii* (Audouin), 1826**

(Fig. 5.4)

1826. *Flustra?rozieri* Audouin, *Hist. Nat.*, 1, 4, p.225.

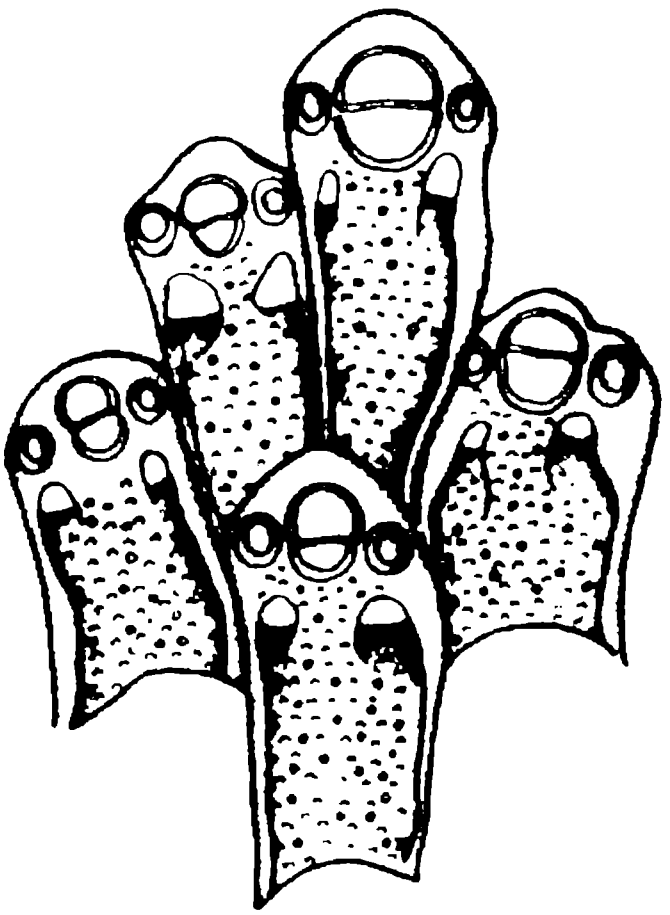


Fig. 5.4

Thalamoporella rozierri – portion of a colony showing the details of zooecia.

1880. *Steganoporella smittii* Hincks, *History British Marine Polyzoa*, p.178, pl.24, figs. 5 & 6.

1887. *Thalamoporella smittii* Hincks, *J. Linn. soc. Zool.*, 21, p.123.

1907. *Thalamoporella smittii* Thornely, *Rec. Indian Mus.*, 1, 13, p.187.

1909. *Thalamoporella rozieri* Waters, *J. Linn. soc. (Zool).*, 31, p.123.

1921. *Thalamoporella rozieri* Robertson, *Rec. Indian Mus.*, 22, p.52.

1926. *Thalamporella rozieri* Harmer, *Siboga Exped.*, 28b, p.292-295, pl.19, figs. 3-13.

Occurrence

Six colonies were collected incrusting on seaweed fronds from Kovalam south west coast of India.

Material

Preserved in alcohol.

Measurements

Zooecium

Length - 560 μ

Breadth - 330 μ

Salient features

Incrusting. Zooecia elongated and rectangular arranged alternatively separated by raised, thick calcareous margins. Opesia terminal, broad, with large

proximal sinus. Opesiules asymmetrical placed distally. Operculum with rounded distal margin, the proximal region with a complete bar. Cryptocyst granulated and slopes distally, the proximal portions bearing small pores. The median process of the cryptocyst forms the polypide tube, the lateral walls of which form those of the two opesiules. Large adoral tubercles present. Ovicells large and bilobate, with a median septum. The elongated opening of the ovicell occupies the proximal half. Spicules in the form of calipers and compasses, and also a third widely 'V' shaped type present.

Remarks

All the species described by Harmer (1926) under the genus *Thalamoporella* Hincks possess avicularia. In the key provided for the species of *Thalamoporella* collected during Siboga Expedition, Harmer (1926) has pointed out the presence of avicularia as an important character. It is particularly noteworthy that the present form does not possess vicarious avicularia. However, Hincks (1880) has distinguished three forms of this species based on the presence or absence of avicularia, tuberosities and ovicells. Those forms with the tuberosities and the ovicells are grouped under the 'form' *'Thalamoporella rozieri'*, those with avicularia and tuberosities under the form *Thalamoporella gothica* and a third form *Thalamoporella indica* with avicularia and ovicells. According to Hincks (1880) the common type is form *Thalamoporella rozieri* with which the present form agrees well since it possesses ooecia and tuberosities but no avicularia. Ovicells are typically bilobate in the present specimens. The opening for the escape of embryo is very large. The view that ovicells are modified adoral tuberosities (Harmer, 1926) agrees in the present case since in zooecia with ovicells, adoral tuberosities are absent and all the zooecia without ovicells have tuberosities. In addition the ovicells are found to develop as two valvular extensions from the adoral region uniting in the

middle forming the calcareous bar characteristic of bivalvate ovicells. It is probable that these valvular structures are modified tuberosities as suggested by Harmer (1926). Tuberosities in the present specimens are large and are with two walls, an outer and an inner with a space in between. In young zooecia the tuberosities are found in the form of two rounded projections opened at the tip and continuous with the wall of the orifice. As growth proceeds the area between the orifice and the tuberosities increases separating the former from the latter. In addition to the two types of internal spicules namely the calipers and the compasses in the developing zooids a third type of spicules is discernible which is widely 'V' shaped. These are found in clusters in the developing zooids together with the other two types of spicules. These probably represent a third type.

Previous records from Indian Waters

Pedro shoal Indian Ocean (?) (Thornely, 1907).

Distribution

Cornwall, England (Hincks, 1880); Egypt, Red Sea (Audouin, 1826); Mergui archipelago, Burma (Hincks, 1887); Makassar strait, South West celebes,Sulu Archipelago, Banda Sea (Harmer, 1926); Indian Ocean (Robertson, 1921); Cape Verde Is. (Waters, 1909).

SUB - ORDER *CTENOSTOMATA* Busk, 1852

Chitinous zoaria, encrusting, erect, stolonate or burrowing. Aperture simple, closed by inversion of tentacle sheath, or retraction of polypide. Specialised apertures present in some genera, where they may be bilabiate produced or operculate. No avicularia or true external ovicells. Kenozooecia in the form of stolons in Stolonifera and as spines in *Carnosa*.

DIVISION *STOLONIFERA* Ehlers, 1876

"Zoaria with delicate creeping stolons, with occasional points of expansion where a diaphragm occurs and either stolonal branches or zooecia may arise. A gizzard may or may not be present." (Osburn, 1953).

FAMILY *VESICULARIIDAE* Johnston, 1838

"Zooecia contracted below, not closely united to the stem at the base, deciduous, destitute of membraneous area. Zoarium repent or erect." (Hincks, 1880).

GENUS *BOWERBANKIA* Farre, 1837

1837. *Bowerbankia* Farre, *Phil. Trans. Roy. Soc., London,* 127, 2, p.391, Pls. 20-27.

1880. *Bowerbankia* Hincks, *History British Marine Polyzoa*, p.518.

1915. *Bowerbankia* Harmer, *Siboga Exped.*, 28a, p.70.

1953. *Bowerbankia* Osburn, *Rep. Allan Hancock Pacific Exped.*, 14, 3, p.743.

"Zooecia arising irregularly from an erect or creeping axis, commonly in definite groups. Tentacles 8-10. Gizzard present" Harmer (1915).

5. ***Bowerbankia imbricata* (Adams), 1800**

(Fig.5.5)

1800. *Sertularia imbricata* Adams, *Trans. Linn. Soc. London.* 5, pp.11, pl.2.

1900. *Bowerbankia imbricata* Robertson, *Proc. Wash. Acad. Sci.* 2, pp.331, pls.19-21.

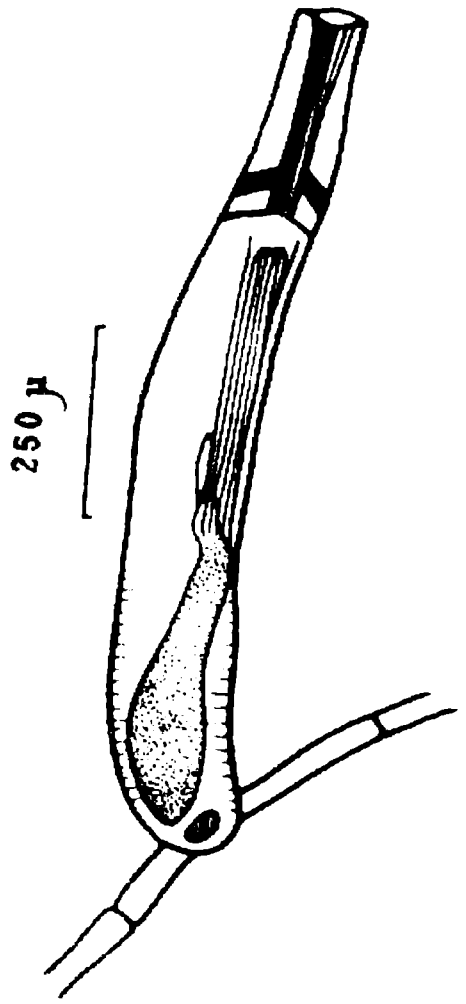


Fig.5.5

Bowerbankia imbricata - portion of a colony showing the details of zooecia.

1847. *Bowerbankia imbricata* Johnston, *A History of the British Zoophytes*, pp.377, pl.L22, fig. 5-6.

1880. *Bowerbankia imbricata* Hincks, *A History of the British Marine Polyzoa*, pp.519, pl.L23, f. 1-2.

1930. *Bowerbankia imbricata* Borg, *Tierwelt Deutschl. u. angrenz. Meeresteile*, pp.101, fig. 127.

Occurrence

Five colonies incrusting on the seaweeds were collected from the sub-tidal region at Kovalam in Trivandrum.

Material

Kept dry

Measurements

Zooecium

Length - 1160 μ

Breadth - 220 μ

Salient features

The zoaria are in the form of prostrate and free-growing stolons, which are usually covered with small, spaced groups of zooids. The stolon is thick, usually more than half the largest width of the zooid (diameter 0.13 to 0.23 mm), branched, and divided by transverse walls located at varying distances in the internode. The zooids are transparent, cylindrical, medium sized (length 1.00 to 1.50mm, width 0.25 to 0.30mm), and usually located in paired groups before the diaphragm 8 to 12 in each place; but sometimes the zooids are found in the internodes in less

specific groups and, rarely, are present singly. The orifice on the distal end of the zooid is rectangular. The gizzard is in the form of a spherical structure (diameter 0.10mm), and continuously lined with very keratinized cells at the distal end. The number of tentacles is 14, but Hincks (1880) reported 10.

The species lives on *Fucus,* hydroids, Bryozoa, and stones in the belt of ebb and flow and the upper sublittoral zone, at a depth of 0 to 53m, on a bed of stone and sand, in a salt concentration of 32.27 to 33.22%.

Distribution

Barents sea in the waters of Finmark (Nordgaard, 1905, 1918), White sea (Gostilovskaya, 1957), in the coastal waters of Western Norway (Nordgaard, 1912a, 1918), Skagerrack and Kattegat (Levinsen, 1894), North Sea (Borg, 1930), Great Britain (Hincks, 1880), Northern France (Joliet, 1877), and the Mediterranean Sea (Calvet, 1902).

ORDER *CYCLOSTOMATA* Busk, 1852

DIVISION *ARTICULATA* Busk, 1859

=

CAMPTOSTEGA Borg, 1926

"Primary zooid erect, separated by chitinous joint from the proancestrula, zoarium jointed, rhizoids present. Body wall a gymnocyst, vestibular sphincter present, brood chamber a gonozooid, moderately dilated in its middle part, polypide of gonozooid degenerating before having been full grown" Borg (1944).

FAMILY *CRISIDIAE* Johnston, 1838

Erect and jointed zoarium, with zooecia arranged in single series or alternating in two series. Ovicell a gonozooid, with a zooeciostome.

GENUS *CRISIA* Lamouroux, 1812

1915. *Crisia* Harmer, *Siboga Exped.*, 28a, p.96.

1953. *Crisia* Osburn, *Rep. Allan Hancock Pacific Exped.*, 14, 3, pp.678-679.

Long internodes. Zooecia arranged symmetrically into two alternating series. The projecting peristome giving the edges a serrated appearance. Gonozooids usually placed in the median line between the zooid rows.

6. ***Crisia eburnea* (Linnaeus) 1758**

(Fig.5.6)

1865. *Crisia eburnea* sine *cornibus* Smitt, *Ofvers. Kongl. Vetensk. Akad. Forh.*, 23, pp.117, t.16, figs. 7, 10-11.

1891. *Crisia eburnea* Harmer, *Quart. Journ. Micr. Sci.*, N.S., 32, pp.131, 154, pl.12, fig. 6.

1930. *Crisia eburnea* Borg, *Tierwelt Deutschl. u. angrenz. Meeresteile*, 17, pp.40, figs. 14-15.

Occurrence

Three colonies were collected epiphytic on seaweed from Kovalam in Trivandrum.

Material

Preserved in alcohol.

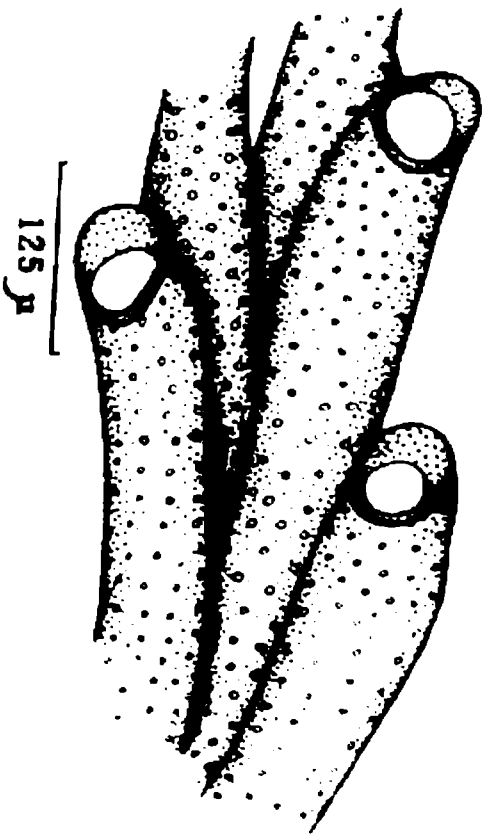


Fig. 5.6

Crisia eburnea – portion of a colony showing the details of zooecia.

Measurements

Zooecium

Length - 360 μ

Breadth - 60 μ

Salient features

Delicate zoarium. Projecting peristomes give the branches a serrated appearance. Zooecia long and adnate, the peristome placed obliquely directing forwards. No ovicell noticed.

The zoaria are ramose and from 1 to 3 cm in height. The branches are usually bent inward, particularly in their distal part, due to which the zoarium acquires a typical agminate form. The branches consist of more or less short internodes. The majority of sterile internodes usually consist of 5 to 7 zooids, the fertile internodes of 8 to 12. The branches usually originate from the first zooid on both sides, rarely from the second. The articulations are brown in colour, turning pale toward the distal end of the branches. The basis rami is short, adjoining the distal half of the zooid. The zooids in the internode are arranged in alternate rows, which are more or less bent and adjoin almost throughout their entire length; their distal part is bent upward, opens through a round orifice, and often has a denticle on the outer corner. The gonozooid is situated in the proximal part of the internode, and more frequently pre-empts the place of the 2nd, rarely the 3rd, zooid. The gonozooid is pyriform in shape, and the oeciostome located on its distal end in the form of a well-developed and slightly bent tube, is wider at the base, tapering gradually toward the round or transversely oval orifice, and directed toward the frontal side and upward.

The species lives on algae, shells, and stones, at a depth of 0 to 235m, more frequently from 0 to 100m, in a salt concentration from normal to a very pronounced freshwater state in estuaries or rivers.

Remarks

The close similarity shown by the present form with the figures and descriptions of *Crisia eburnea* given by Harmer (1891) and Borg (1930) made it possible to assign the form under consideration to *Crisia eburnea*. The width of the internode is considerable because of the extension of the proximal ends of the zooecia along the median sides of the preceding zooecia.

Distribution

Barents Sea (Smitt, 1865, 1879b; Bidenkap, 1897, 1900; Anderson, 1902; Norman, 1903a; Waters, 1904; Kluge, 1908a, 1915; Nordgaard, 1923. Kluge, 1928; Gostilovoskaya, 1957, Kara Sea (Smitt, 1879; Levinsen, 1887), Arichipelago of the Canadian Islands (Verrill, 1879a, 1879b), Western Greenland (Smitt, 1868; Kluge, 1908b; Levinsen, 1914; Osburn, 1919), Labrador (Hincks, 1887; Packard, 1868), Gulf of St. Lawrence (Whiteaves, 1901), and Yan-Maien Island (Lorenz, 1886).

In the boreal region, this species is widely distributed along the eastern coast of the Atlantic Ocean from northern Norway to the Mediterranean Sea (Smitt, 1865; Ortmann, 1894; Levinsen, 1894; Joliet, 1877; Hincks, 1880; Calvet, 1902; Nordgaard, 1918).

5.6 THE SETTLEMENT OF BRYOZOAN LARVAE ON ALGAE

A number of species of bryozoa grow on algae, ranging from *Electra pilosa* (L.), an abundant species found on all types of substrates including many

species of algae, to others such as *Membranipora membranacea* (L.) and *Amathia lendigera* (L.) which are found only on one or two algae.

The number of species found in the intertidal zone is rather small, although some of them, such as *Flustrellidra hispida* (Fabricius), may be abundant. Most of these species grow as tufts or incrustations on the commoner algae, and the following associations are characteristic of British shores: ***Crisia eburnea* (L.):** often quite abundant in the infra-littoral fringe on a small red algae, notably *Phyllophora membranifolia.* ***Membranipora membranacea***: typical of the infra-littoral fringe on the fronds of *Laminaria digitata* and *L. hyperborea,* occasionally on *L. saccharina, Saccorhiza polyschides* and *Fucus serratus.* ***Celleporella hyalina* (L.):** on more exposed shores found in small amounts on a number of algae, especially in *Laminaria* holdfasts, but in certain sheltered localities it forms heavy incrustations on the fronds of *L. saccharina.* ***Alcyonidium polyoum* (Hassal), *A. hirsutum* (Fleming)** and ***Flustrellidra hispida***: all three species are found abundantly only on *Fucus serratus.* ***Bowerbankia imbricata* (Adams)**: an abundant species on *Ascophyllum nodosum.* ***Amathia lendigera:*** abundant on *Halidrys siliquosa.* ***Valkeria uva* (L)**: the small creeping variety (*f. uva*) grows on a number of substrates, but the luxuriant tufts of *f. cuscuta* have only been found on *Halidrys siliquosa.*

5.7 FACTORS INFLUENCING CHOICE OF ALGAL SUBSTRATE

Surface contour

In the choice experiment using the algae, *Pelvetia* and *Gigartina* (Ryland, 1959) having channelled fronds, the concave surface was greatly preferred by the bryozoan larvae. The frond of *Laminaria saccharina* is characteristically crinkled, and it is very noticeable that the young colonies of *Celleporella* are located in the

concavities. If the *Laminaria* frond is held up to the light, it can be seen that the pattern of settlement on one surface is exactly complementary to that on the other, in each case closely following the surface contour.

Definite advantages would seem to be obtained by settlement in concavities or in the channelled surface of a frond. The newly settled larva would receive protection from rubbing against rocks and other algae and from abrasion by sand. The latter factor may be important in the case of *Celleporella*, for *L. saccharina* may be found attached to stones on an otherwise sandy shore. Similar behaviour has been recorded during the settlement of other invertebrate larvae, such as barnacle cyprids (Crisp and Barnes, 1954) where it was also shown to be a direct tactile response to the shape of the surface.

It was previously suggested (Ryland, 1959) that in choice experiments settlement on *L. saccharina* would be higher if crinkled rather than flat pieces of frond were used. Later experiments have not supported this view: they suggest rather that contour is of subsidiary importance to surface texture. It seems probable that not all pieces of thallus of any given alga are equally favourable for settlement, and that certain discrepancies between experiments on the choice of algae are to be explained in this way. Providing that the texture is suitable, the larva will explore to find a groove or depression for permanent attachment.

Surface texture appears more important than contour as a factor influencing the choice made by larvae between algal substrates, although the physical and/or chemical factors responsible for the observed differences in attractiveness of algae are largely unknown. However, it is evident that the nature of the surface alters with age, and that this influences favourability. The presence of mucus has an adverse effect on settlement. Once the actual substrate has been chosen, the larvae respond to surface contour and, if possible, select a groove or concavity as a site for fixation.

Age of the frond

To find out the influence of frond age on the settlement of bryozoan larvae on *Fucus serratus,* larvae of *Alcyonidium polyoum* and *Flustrellidra hispida* were offered pieces of *Fucus serratus* thallus of equal area cut from four different regions. These were frond tips, the region just below the tips ('sub-tips'), and pieces from the centre and the base of the frond, the latter being largely composed of stipe. The results clearly showed for *A. polyoum* the tips of the fronds are the most favourable for settlement, and the older the thallus the less favourable it is. The results for *Flustrellidra*, although based on a smaller number of larvae, did not show this trend, and only the base of the frond appears unfavourable. These results appeared to reflect a difference in the ecology of these species, and may help to explain how the two coexist on the same algal substrate.

Mucus

Mucus probably plays an important part in determining settlement preferences. Cut surfaces of thick, older *Laminaria* fronds exude large quantities of mucus, but the exudation is much less from young, thin fronds.

It has been suggested that in the brackish waters of the Baltic, the heavy settlement of epiphytic animals - bryozoans, serpulids and tunicates - on the *Laminaria* fronds is partly due to a reduced mucus content in the thalli (Bock, 1954). *Alcyonidium hirsutum* larvae showed preferences for non-fruiting rather than fertile frond tips of *Fucus serratus*. As tufts of paraphyses may be present on non-fruiting fronds, where they do not depress settlement, as well as on fruiting tips, it seems most likely that it is the mucus produced by the conceptacles which accounts for the reduced favourability of fruiting tips.

SUMMARY

The thesis deals with different aspects connected with the seaweed secondary metabolites and settlement behaviour of biofoulers. Information has been gathered and presented on the present status of research in the field based on a thorough review of recent literature. It is seen from the literature that no concerted effort has been made in tropics to delineate the antifouling properties of seaweeds.

Environmental concerns with respect to the use of biocides as the active components of antifouling paints have led to an increase in effort to develop nontoxic alternatives. In recent years, a concerted effort has been directed at isolating and screening natural products from candidate species for a number of functions, including antifouling efficacy. While natural products and analogs that exhibit broad spectrum activity at nontoxic concentrations have been identified, little is known about their mechanism of action. Such information could potentially be of use in screening for nontoxic antifoulants and in allowing the more rational design of analogs for testing.

Larvae of marine benthic organisms are an important ontogenic link which decides the pattern of survival, abundance and distribution of adults. With respect to biofouling control, the study of larval settlement is a study of responses of whole animal. The process of attachment is the cardinal factor which controls survival of the adults.

General introduction explains the relevance and scope of the investigation. A review of the characteristic features of biofouling and research efforts on natural antifouling compounds is presented in the review of literature.

The chapter on the rearing techniques of the test organisms is a useful addition to the information available on the rearing methods of *Hydroides elegans* (Haswell) and *Balanus amphitrite communis* (Darwin). Barnacle nauplii and mass cultured settlement stage larvae are used in quantitative laboratory assays of toxicity and barnacle settlement inhibition. Larvae can be cultured predictably on *Skeletonema costatum*. The trochophore larvae of *Hydroides elegans* attained metamorphic competence in six days in the laboratory.

The chapter on the settlement pattern of the larvae deals with the settlement pattern of *Hydroides elegans* (Polychaeta) and *Balanus amphitrite communis* (cirripede) in the presence of secondary metabolites of seaweeds. The seaweeds examined contained secondary metabolites with wide variations in biological activity. The activity of these metabolites against barnacle and polychaete larvae is comparable to or greater than most other previously described natural antifoulants. Most of the seaweed secondary metabolites tested inhibit settlement via nontoxic mechanisms.

Water borne chemical compounds: Positive and negative cues is the subject matter for the third chapter. Survival and settlement rates of larvae of *Balanus amphitrite communis* and *Hydroides elegans* in algae conditioned water tested immediately after the seaweeds were removed from the water (immediate - use water) and after 24 hrs. (aged water) was tested. The bioassays were designed to determine the influence of high levels of waterborne compounds exuded from seaweeds. Water conditioned with *Sargassum myriocystum*, *Sargassum wightii* and *Spyridia filamentosa* killed larvae of both *Hydroides elegans* and *Balanus amphitrite communis* at a significant rate. Change in the rate of settlement of at least one of the type of larvae was noticed in all the algae conditioned water.

The chapter dealing with *in situ* studies on settlement of biofoulers employing seaweed extract coated test panels gives information on the aspect. that compounds that alter surface chemistry have the potential to modulate settlement. Antifouling assays conducted in the field, test the effectiveness of an extract or secondary metabolites against natural populations of biofoulers within a community. Effect of secondary metabolites accommodated in the crude methanol extract of seaweeds on settlement of biofoulers was investigated using test panels coated with binder and crude methanol extract of seaweeds. The current study provides some tantalizing evidence that many secondary metabolites produced by tropical seaweeds are important inhibitors of larval settlement.

Chapter on bryozoans deals with taxonomic and ecological aspects of bryozoans associated with seaweeds. This section of the thesis presents the list and description of bryozoans collected from seaweeds in the Kovalam area. A total number of six species belonging to the genera *Electra, Thalamoporella, Bowerbankia* and *Crisia* are described.

A list of references which have been consulted during the course of the study has been presented at the end of the thesis.

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