STUDIES ON SOME ASPECTS OF ANTIFOULING IN MARINE ENVIRONMENT

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STATEMENT

*As required under the ordinance No. 19.8, I state that the present thesis entitled "**Studies on some aspects of antifouling in marine environment**" is my original contribution and that the same has not been submitted elsewhere for award of any degree to any other University on any previous occasion to the best of my knowledge.*

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LIST OF ABBREVIATIONS

ATP = Adenosine triphosphate

A. P. = *Acacia pennata.*

B. A. = *Barringtonia acutangula.*

Cm = Centimetre

Chl. *a* = Chlorophyll *a*

C. S. = *Catunaregam spinosa.*

dm = Decimetre.

DNA = Deribose nucleic acid.

Ec_{50} = Effective concentration at which 50% inhibition.

Ec_{100} = Effective concentrations at which 100% inhibition.

G. G. = *Gnidia glauca.*

gm = Gram.

HPLC = High Performance Liquid Chromatography.

hrs = Hours.

IR = Infra Red.

Kg = Kilogram.

L = Litre.

ml = Millilitre.

mg = milligram.

μg = Microgram.

m = Metre.

mm = Millimetre.

M = Molar.

min = Minute.

nm = Nanometre.

ODS = Optical density.

OTEC = Ocean Thermal Energy Conversion.

PCHO = Particulate carbohydrates.

POC = Particulate organic carbon.

ppm = Parts per million.

PTLC = Preparative Thin Layer Chromatography.

PVC = Polyvinyl chloride.

RI = Refractive Index.

RNA = Rebose nucleic acid.

RPM = Rotation per minute.

S. L. = *Sapindus laurifolius*.

SPC = Self polishing co-polymer.

TBT = Tributyltin.

TLC = Thin layer chromatography.

CHAPTER 1

GENERAL INTRODUCTION

Growth of marine organisms and plants on man-made structure is termed as biofouling. However, this phenomenon in marine environment is a sequential process, wherein it starts at microlevel and ends at macrolevel fouling. The microlevel fouling begins immediately after the immersion of objects into the seawater. First, organic macromolecules from seawater get adsorbed on to the surfaces, followed by bacteria, protozoans and diatoms, thereby constituting the slime film (Zobell,1939, 1943, Daniel, 1955; Baier, 1972; Corpe, 1978; Loeb and Neihof, 1975; Baier, 1984; Mitchell and Kirchman, 1984). This is termed as microfouling. Thereafter, larvae of sedentary organisms invade onto the surface and start growing. This is called as macrofouling.

The adverse effects of fouling to the marine industries are well known. This was first noticed on the ship hull, when man started sailing in the sea. Micro as well as the macrofouling on ship hull cause increased frictional drag and enhance fuel consumption (Haderlie, 1984). It has been reported that the frictional resistance increases by 1/4% per day in temperate waters, whereas it increases by 1/2% in tropical waters (Anon, 1952). Fouling of the condenser tubes of power plants affects the heat transfer efficiency (Haderlie, 1984). Fouling of sonar domes and naval acoustic devices results in their malfunctioning (Ganti, 1987). In addition, a number of marine instruments such as wave rider buoy, data buoy, current meter, tide gauze and the sensors which are used for monitoring of environmental parameters are also found to be affected by the fouling settlement (Padilla and Moraoka, 1972). Offshore oil platforms and Ocean Thermal Energy Conversion (OTEC) plants are also adversely affected in various ways by the

fouling growth (Corpe, 1979; Mitchell and Benson, 1980). Fouling increases the effective diameter of the platform legs leading to increased hydrodynamic loading (Freeman, 1978; Heaf, 1981).

In addition to the above effects, fouling is also known to cause destruction of metallic surfaces by accelerating the corrosion rate (Edyvean et al., 1988). Uneven fouling settlement results in the heterogeneity over the metal surface, thereby leading to formation of anodic and cathodic sites (LaQue, 1975). Secondly, the calcareous growth may cause physical damage to the protective paint film by penetrating into the film and exposing the bare metal to marine environment leading to corrosion. It has also been reported that a thick mat of calcareous growth may protect metal against corrosion by virtue of acting as a physical barrier for the entry of oxygen at the metal surface (LaQue, 1975). On the other hand, algal growth can enhance the corrosion rate by supplying oxygen to the metal surface through photosynthetic action. Besides these, some of the metabolites, particularly organic and inorganic acids produced by algae and microfoulers, result in higher metallic corrosion (Miller, 1970; Hardy, 1981; Moss, 1981; Dexter, 1986).

Fouling is considered as a site specific phenomenon and hence the nature and extent of fouling varies from place to place (Anon, 1952). This is thought to be mainly due to variation of different environmental factors which govern the fouling assemblage under a particular set of environmental parameters. It has been reported that fouling activity is more intense in tropical environment than in temperate. This

variation was attributed to the less temperature variations in tropical waters than that in the temperate waters (Anon, 1952). A wide range of fluctuation in temperature restricts the breeding of fouling organisms. As far as Indian water is concerned, some foulers have been reported to be breeding almost throughout the year (Ganapati et al., 1958; Nair, 1967; Karande, 1968; Sawant, 1985; Anil and Wagh, 1988).

It has also been observed that the extent and nature of fouling vary with time, depth and distance from the shore (Bascon et al., 1976; Wolfson et al., 1979; Hardy, 1981; Kinsbury, 1981; Forteath et al., 1982). In temperate waters, maximum fouling intensity was observed at 30 m, below which it decreases drastically (Anon, 1952). Similarly, in tropical waters, especially at Bombay High area in the Arabian sea, intense fouling was observed at 22 m depth, below which it decreased drastically (Venugopalan, 1987).

Seasonal as well as annual variations in the extent and nature of fouling have been reported in the literature. Seasonal variations in the fouling arise from the inability of any given species to reproduce except under a limited range of conditions (Anon, 1952). On the other hand the cause of the annual variability of fouling is quite unknown.

There are numerous factors which determine the extent and nature of fouling at a given locality. The factors which determine the larval recruitment can be divided into two main groups. The first group includes the factors which determine the number of larvae coming into contact with the exposed surface, and the second group includes the

factors which control the attachment and growth of these larvae on to the surface. Some of the first group factors include movement of water, light penetration, water temperature, salinity, dissolved gases, nutrients, abundance of larvae etc., whereas the second group factors include type, texture, orientation and colour of the surfaces (Anon, 1952). The extent and nature of fouling also depends on the surface energy or hydrophobicity or degree of wettability of the given surface. It has been observed that paraffin, petrolatum and various waxes or greasy surfaces do not get fouled rapidly. The non-fouling action of these surfaces was attributed to the nonwettable nature or low surface energy. Subsequently, detailed studies on fouling of surfaces with low surface energy revealed that surfaces with low surface energies fouled less than the surfaces with high energies (Lindner, 1994). Some of these materials include coatings such as PTEF, silicon impregnated sulphur etc.

Exfoliation of surfaces has been found to affect fouling process. Corrosion and subsequent spalling of corrosion products from metal surface is considered as one of the exfoliation processes. In addition, numerous paints have been identified as exfoliating type. Thus, fouling organisms which attach on to these surfaces will be sloughed off because of the physical disintegration of the surface (Anon, 1952).

A vast range of methods has been suggested for controlling fouling process. The choice of preventive methods depends on the site and/or the structure to be protected. These methods can be classified as below.

1) Physical method

2) Electrolytical method

3) Ultrasonic and radiation method

4) Biological method

5) Chemical method

Physical method of combating fouling includes different techniques such as manual cleaning, hydraulic, compressed air, waterjets, sand blasting, high velocity ice particles etc. (Partridge 1981; Bain, 1981;Fisher et al., 1981; Fishcher et al., 1984).

A considerable amount of work could be done by hand tools. These include the divers knife, wire brushes, scrappers and chipping hammers. They offer virtually no hazard to divers. They are usually light in weight and small in size so they do not inhibit any difficulty to access to joints and crevice areas. This method is best suited for cleaning fixed offshore structures (Wilkins, 1981).

There are two types of hydraulically powered equipments viz., rotary brushes and rotary chippers. Rotary brushes do not damage the weld, and provide high cleaning standards. Rotary clipper which is comparatively a new device, provides only a lesser cleaning rate.

Hydraulically powered equipment does not need any physical effort from the operator and is less hazardous.

The compressed air needle gun has small chisel point "needles", which are impacted on the work surface in rapid succession. This tool cleans the surface well and does not pose problem for photographic observation. However, this method has some serious draw-backs. It is limited to shallow depths upto 30 m. Its performance drops off rapidly with increasing back pressure caused by the increasing depths. As regards to water jet, it is the most suitable technique for the under water cleaning especially the soft growth. However, corrosion products and hard shelled growth are very difficult to remove when they are well bonded to the metal surface. In such cases addition of abrasives to water jet, use of cavitating water jets and use of much higher jet pressure proved to be more effective. However, the draw-backs of the use of abrasives in water jet include damage to the surface beneath the fouling growth, small fatigue cracks in the structure and the difficulty in supplying abrasives to the working site at greater depths.

In the cavitation jet, the growth of vapour within a water jet is stimulated using an appropriate nozzle design. With proper adjustment of nozzle stand from the surface, the vapour cavities grow and then collapse in the high pressure stagnation region where they get impacts on the solid surface. Extremely high local stresses can be produced by application of pressure due to the collapsing of vapour cavities. This method appears to be fast and efficient, but it has some draw-backs. The nozzle stand distance must be closely controlled and this can make the

operation difficult. It has been found that the performance of a cavitation jet reduces with increasing depth.

High pressure water jet can be used for fairly extensive cleaning programs. A typical machine may have a 200 bhp prime mover with an output of around 14000 psi (9820 kg/cm^2.). Though expensive, this method can be used for removing soft fouling growth. The major draw back in this method is that the water pressure is so powerful that it can be fatal to the divers and also produces high noise, which may have an effect on the eardrums of the divers.

Dry sand blasting is used for cleaning ship's hull in dry dock. This method gives a well cleaned surface and is relatively fast (7-8 m^2/hr). However, this method is not widely used because of certain practical and economical reasons. It generates large amount of dust which creates bad working condition and poses danger to rubbing parts of ship as well as the dock machinery. However, the problem of dust is prevented in the wet sand blasting technique, where the machine is fed with sand and water simultaneously. The productivity of this method is 20% less than the dry sand-blasting. Bombarding of high velocity ice particles also works on the same principle as sand blasting. However, this does not cause surface pitting and hence has an advantage over sand blasting.

Electrical method includes placement of metal bars close to the structure to be cleaned for fouling. The metal bars are to be made as anodes and the structure as cathode in a electric circuit. When the current is impressed, large amount of hydrogen is liberated from the surface

under the corrosion products, breaking up rust and removing it from the surface. Thus, the fouling organisms along with the corrosion products will be removed. In another electrical method, the fouling can be prevented by generating electrolytic chlorine. In this method, platinum plate is made as anode and stainless steel as cathode. This electrolytic cells are mounted at close proximity to the surface to be protected from fouling in seawater. When the current is impressed, hypochlorite is generated, which prevents settlement of fouling larvae.

Recently ultrasonic and ultraviolet radiation methods have also been used for controlling fouling settlement (Plotner, 1968; Aras, 1980). Ultraviolet radiations interfere with the cellular components, causing the rupture of the cells.

The biological method of prevention of fouling includes release of marine organisms which feed on the sedentary organisms and algae on the surface to be cleaned. These organisms include crabs, star fishes, gastropods, some fishes etc. (Wahl, 1989). If these organisms are maintained in the vicinity of the structures to be protected, then, they can feed upon the fouling organisms resulting in the prevention of fouling. Secondly, bacterial film and soft bodied fouling organisms especially sponges, ascidians and algae once settled on the surface, will not allow the other forms to settle over them (Paul et. al., 1987; Porter and Targett, 1988; Maki et al., 1988 a, b; 1990). In view of this, if the under water structure is allowed to be settled with these forms, then the hard growth can be controlled. Some oil companies have attempted biological control of fouling growth, but attained very limited success.

This type of antifouling method has advantages over others as it is environmentally safe.

Besides all the methods described here, chemical method appears to be more effective in controlling fouling settlement, therefore it is used extensively. The method basically includes application of toxins or biocides to the marine fouling organisms. Application of biocides can be achieved in two ways. It can be added either directly into the environment (injectable biocides) or can be applied in the form of paint onto the surface to be protected from fouling.

Injectable biocides are advantageously used in places such as pipes of heat exchangers systems, flowing seawater systems etc. The most widely used biocide is hypochlorous acid. Other biocides include, aeroline, soluble copper salts, bromine, iodine, bromine chloride, ozone, chlorine dioxide and various trialkyl organometallic tin compounds (Fisher et al., 1981; Fishcher et al., 1984). Chlorine solution is very effective in controlling fouling inside pipes and conduits, which supplies seawater on board the ship or for cooling purposes in condensers of coastal installations. It has been demonstrated that a concentration of 10 parts per million in seawater is sufficient to kill any organisms, except sea anemones and mussels exposed for one hour per day. Residual chorine concentration as small as 0.25 ppm was reported to be effective in preventing the fouling growth (Anon, 1952).

Antifouling paints generally contain copper, mercury or arsenic compounds in various combinations. The action of these materials

depends on the toxicity of their ions and its solubility in seawater which in turn prevents fouling. It is the toxicity of these materials which acts as poison to the organisms and either repel their larvae at the time of attachment or kill them before they can secure footing permanently.

Use of copper as antifoulant on boats dates back to 1763. The first authentic record of a patent issued for the inclusion of a toxin in a paint to prevent fouling is in 16th century and since then many patents have been filed for antifouling formulations (Anon, 1952). During 18th century some of the paint formulations worked well, but failed to last for long exposure. This problem was solved to some extent in 19th century by the introduction of resins which can control the leaching rate of the toxins from the paint and can last longer (Anon, 1952). Among the toxins such as copper, mercury and silver, both copper and mercury proved to be very effective in preventing fouling settlement. Silver has not been extensively tested probably due to its high cost. There are contradictory reports on the ratings of the toxicity of copper and mercury, however, in general these two metals and their salts have consistently given good results in antifouling paints. In addition to the above compounds, salts of zinc , iron, arsenic and lead have been reported to be toxic to marine organisms or to their larvae (Anon, 1952). The concentration at which they are lethal varies with respect to the metal and the organisms. Zinc was found to be 1/5th as toxic as copper or mercury and the rest were even less toxic than zinc. The probable reason behind this could be that the metallic lead when exposed to seawater gets deposited with a very insoluble compound on its surface, thereby not allowing the bare metal to come in contact with the fouling organisms.

As regards arsenic, there are contradictory reports about its action on fouling organisms. The investigations carried out by many investigators on the effects of these metallic toxins on the fouling organisms did not clearly show the synergistic effect.

Besides toxins of heavy metals and other inorganic compounds, organic compounds were also investigated for their antifouling activity. Till date 91 organic compounds were reported to be studied for toxicity (Anon, 1952). Though most of the organic compounds were found to be toxic to fouling organisms, many of them could not give satisfactory results in paint coatings. They were found to be specific in their toxic action to certain organisms rather than general toxicity showed by the heavy metals.

The effectiveness of toxins is found to be directly related to the leaching rate of the toxic ingredient from the paint film. For instance, the effectiveness of toxins such as metallic copper and cuprous oxide when incorporated into the antifouling paints was found to be optimum at the leaching rate of 10 $\mu g/cm^2/day$ (Banfield, 1980). Similarly, Barnes, (1948) reported that about 2 $\mu g/cm^2/day$ leaching rate of mercury is adequate to prevent fouling. The leaching rate of toxins from the paint film depends upon factors such as their solubility, rate of dissolution and the way it is compounded in the paint matrix (Anon, 1952).

Generally antifouling paints can be grouped under two classes depending upon the mechanism of release of toxins into the seawater.

Class one include those paints from which toxin alone is released by diffusion through the paint matrix continuously while in contact with seawater whereas the class two include those paints from which the toxins are released along with the dissolution of the matrix. The matrix dissolution can be of inactive (self dissolution) or active, either by the bacterial action on the matrix or by the mechanical erosion. One of the new generation paints e.g. the self polishing copolymer (SPC) falls under this category of antifouling class of paints (Anon, 1952). Of these two classes of the antifouling paints, class two paints, i.e. matrix-soluble paints have higher utilization efficiencies than those of the class one i.e. continuous contact paints. The reasons are, better leaching of toxins to the surface and long lasting effectiveness.

The existing antifouling paints which are widely used, basically contain chemical biocides such as heavy metals and/or organometallic compounds. Among the various organometallic antifouling paints, self-polishing organotin copolymer formulations have the best toxin release rate characteristics and are capable of maintaining surface free from fouling up to five years (Christie and Dalley, 1987). However, in recent years it is evident that the Tributyltin (TBT) is a broad spectrum poison and harms many other forms of marine life besides the fouling organisms (Beaumont and Newman, 1986; Rexrode, 1987; Thain et al., 1987; Langston et al., 1990; Wester et al., 1990; Kelly et al., 1990 a, b). Reportedly, TBT was found to be responsible for high mortality of oysters larvae as well as deformation of shells of adult Oysters (Key et al., 1976; Alzieu et al., 1982; Champ and Lowenstein, 1986; Claisse and Alzieu, 1993) and dog Whelk snails (Bryan et. al., 1986). Accumulation of

butyltin in mussels tissue of *Salmon* sold in the market was reported by Short and Thrower (1986). Occurrence of imposex and effects on reproductive system of gastropods and Oysters have also been reported (Champ and Lowenstein, 1986; Bright and Ellis, 1989; Alvarez and Ellis, 1990; Ellis and Pattisina, 1990; Spence et al., 1990 a, b; Smith and Mc Veagh, 1991; Oehlmann et al., 1991; Stewart et al., 1992; Foale, 1993; Nias et al., 1993; Wilson et al., 1993). In general, the use of such chemical toxins in marine environment pose great threat to the marine biota (Vrijhof, 1985). For this reason, restrictions are imposed on the use of these compounds in most of the advanced countries. A complete ban on the use of organo-metallic compounds especially TBT now, operates in Japan, while in Britain its use for small boats are outlawed since, 1988 (Clare, 1995).

Due to the existing and the expected future restrictions on the use of TBT (Dalley, 1987) and probably other polluting antifouling compounds, there is a growing need for other methods of the prevention of fouling. One of the probable methods presently being investigated all over the world includes formulation of antifouling paint based on natural compounds, which are generally believed to be easily biodegradable, causing no harm to the marine environment. The work on this aspect was initiated elsewhere during 1980s. Till date numerous marine organisms and algae have been screened for search of biogenic compounds which can serve as antifouling agents (Sieburth and Conover, 1985; Katsuoka et al., 1990). Reports show that several species of marine invertebrates especially, sponges, coelenterates, bryozoans and ascidians were found to contain some compounds which

could prevent settlement of macrofoulers in laboratory (Targett et al.,1983; Bakus and Kawaguchi, 1984; Bandurraga and Fenical, 1985; Rittschof et. al., 1985, 1986; Gerehart et al., 1988; Wahl et al., 1989; Sears et al., 1990; Walls et al., 1993; Targett et al., 1994). The compounds include alkaloids, fatty acids (Goto et al., 1992), Terpenoids (Keifer et al., 1986; Sears et al., 1990) and bromopyrroles (Keifer et al., 1991). Though these groups of compounds have been identified, very few of them have been clarified with the structure and tried in the field.

In addition to marine organisms, terrestrial plants have also been screened for antifouling properties (Suzanne, 1993; Sawant et al., 1995). However, not much information is available on the compounds from terrestrial plants which could be used as antifouling agents. Plants are in fact miniature factories of nature for the production of food materials, essential oils, gums, resins, alkaloids, steroidal substances, glycosides and many other valuable compounds of daily use. The compounds synthesized by the plants are of such complexity and heterogeneity that human mind is left bewildered at the marvels and mysteries of plant processes. Due to this reason, the natural durability of plant species in marine environment is dependent on the nature and extent of these chemical constituents (Santhakumaran, 1994). A perusal of literature shows that certain quinones and sterens from some plants have been reported to prevent metamorphosis and growth of wood borer larvae (Bultman, 1976; Turner, 1976). Some of the plants are also being used since ages for intoxicating fish. Different parts of the plants, are crushed and the juice is allowed to mix with the water. The extract stupefies the fish and makes them float up, facilitating an easy

catch (Anon, 1961; Mitre, 1981). In view of this fact that extracts of some plants are being used locally for narcotizing and catching fishes, this work was taken up in order to find out whether the various local plants possess any antifouling compounds. The plants which have been investigated are *Catunaregam spinosa* (Rubeaceae), *Sapindus laurifolius* (Sapindaceae), *Gnidia glauca* (Thymelaceaceae), *Acacia pennata* (Leguminoceae) and *Barringtonia acutangula* (Lecythidaceae).

Different parts of these plants such as, fruits of *C. spinosa* and *S. laurifolius* and bark of *G. glauca*, *A. pennata* and *B. acutangula* have been used for isolating and identifying environmentally safe antifouling compounds.

CHAPTER 2

ANTIFOULING ASSAYS OF SOME PLANT EXTRACTS

2.1 INTRODUCTION

Bioassay is a tool by which any compound can be screened for biological activity, whereas, the term 'antifouling assay' refers to the assessment pertaining to antifouling. In this regard various criteria could be adopted for assessing antifouling action of any compound. These criteria are mortality, growth inhibition, attachment inhibition, narcotization, negative taxis etc. (Cooksey and Cooksey, 1991; Lindener, 1994). Perusal of literature shows that the most commonly used criteria in antifouling assays are the inhibition of growth and attachment. The researchers in the past have used growth inhibition as the criteria for bacterial and diatom assays, whereas, attachment inhibition was used for macrofoulers (Targett et al., 1983; Thomas and Robinson, 1986; Rittschof, et. al., 1992). In the present work both these assays have been made use of.

It has been reported that the term "Effective Concentration" has been used in antifouling assays to express the antifouling activity of any compound and had been abbreviated as "Ec" (French and Evans, 1988; Hunter and Evans, 1990; Rittschof et al., 1992). In the present study the term is used to express antifouling activity against the test organisms.

It is well known that organisms exhibit varying degree of resistivity towards different biocides. It is therefore, essential to quantify biocide which is required for inhibition of growth or settlement of organisms. It is also essential for the generalization of results that assays are

performed on wide range of organisms. Moreover, for quick results of bioassays the test organisms should be available in plenty and with ease. Their developmental stages should be short and one should be able to rear them in laboratory with minimum difficulty. This helps in screening any products without any difficulty. For these reasons most of the bioassay tests so far reported are based on bacteria, diatoms and invertebrate larvae of organisms which are readily available (Maureen et al., 1981; Standing et al., 1984; Thomas and Robinson, 1986; Rosell and Srivastava, 1987; Uchida et al., 1988; Szewzyk et al., 1991; Goto et al., 1992; Rittschof et al., 1992; Avelin Mary et al., 1994; Hadfield et al., 1994; Mokashe et al., 1994).

As regards the present work, bacteria, diatoms, barnacle larvae and mussels were used as test organisms. The bacterial species selected for the assays belong to genera *Chromobacterium*, *Alkaligenes*, *Moraxella*, *Arthrobacter*, *Vibrio*, *Pseudomonas* and *Flavobacterium*, whereas, the diatom species selected were of the genera *Navicula*, *Nitzschia* and *Amphora*. The choice of the bacterial and diatom species was based on their higher dominance in microfouling assemblages in Goa waters as reported by Sonak and Bhosle (1995) and Redekar (Personal communication). As regards sessile barnacle, *B. amphitrite*, which forms the major component of the fouling assemblage especially in Goa (Sawant, 1985; Anil and Wagh, 1988), has been used as test organism. This species is reported to be breeding throughout the year due to its high tolerance to wide fluctuation of salinity. Consequently rearing of its larvae in laboratory is comparatively easy. On the other hand, adult mussels were used in the present study as test

organism. The mussels used belong to the genus *Perna*. In order to have more credibility to the test conducted under laboratory condition, the field tests were carried out with all the five extracts.

2.2 MATERIALS AND METHODS

2.2.1 Preparation of plant extracts

Five terrestrial plants namely, *Catunaregam spinosa* (Thurb.) Tirvengadum (Class- Dicotyledonae; Family- Rubiaceae), *Sapindus laurifolius* Vahl, (Class- Dicotyledonae; Family- Sapindaceae), *Acacia pennata* (Linn.) Wield, (Class- Dicotyledonae; Family- Leguminoceae), *Gnidia glauca* (Fresen.) Gilg (Class- Dicotyledonae; Family- Thymelaeaceae), and *Barringtonia acutangula* (Linn.) Gaertn. (Class- Dicotyledonae; Family- Lecythidaceae) were selected for assessing their antifouling properties.

The choice of plants for the present study was based on the considerable use of these plants by local fishermen for fish capturing. Different parts of these plants such as, fruits of *Catunaregam spinosa* and *Sapindus laurifolius* and bark of *Acacia pennata*, *Gnidia glauca* and *Barringtonia acutangula* have been used. The plants species were identified following the latest literature available on the flora of the region (Almeida, 1990). The plant parts were collected from Canacona taluka of Goa. Fruits (ripped) and bark of above plants were cut into pieces and crushed. The crushed material was soaked in methanol

separately. After two weeks the solvent was drained off and the residue was subjected for re-extraction with an additional methanol. The methanolic extract was then filtered through whatman filter paper No.1 and concentrated under vacuum to dryness. The vacuum dried crude extracts were then used for assessing antifouling properties.

Before starting with the antifouling assays, the crude extracts were screened for bioactivity against the diatoms. For this purpose approximately 1 - 1.5 mg of the extracts were dissolved separately in 15 ml of Guillard F_2 medium followed by inoculation of diatoms. The extent of growth of the diatoms was observed after 3 days with the help of inverted microscope.

2.2.2. Laboratory assays

2.2.2.1 Assay using Bacterial strains

Seven fouling bacterial strains which are being maintained in the laboratory were used for the purpose. These strains were collected from biofilm developed on aluminium panels. They were isolated and purified on agar plates and maintained in the laboratory. These forms were *Chromobacterium* sp., *Alkaligens* sp., *Moraxella* sp., *Arthrobacter* sp., *Vibrio* sp., *Pseudomonas* sp. and *Flavobacterium* sp. Before taking them for experimental purpose, these species were revived on Zobell marine agar and then harvested in nutrient broth for 24 hrs. The harvested strains were then spread plated thickly on Zobell marine agar. Discs of dia. 7 mm were cut out from Whatman No.1 filter paper. A known quantity of the crude extract was dissolved in a known volume of

methanol to prepare a solution. The paper discs were soaked in the extract solution and dried at room temperature. These discs were weighed before and after adsorption of extract, so as to get the dry weight of the crude extract. The amount of adsorbed crude extract on the discs ranged between 800 to 1000 µg/disc. The discs containing crude extract were then placed over the agar plates seeded with bacterial cultures and incubated at room temperature for 24 hrs. In addition, discs without extract were also placed on agar in the same plate as experimental control. After incubation, the plates were examined visually for inhibition of bacterial growth around the discs. The zone of inhibition around the disc was measured from the edge of disc to the edge of bacteria inhibited zone and have been expressed in terms of millimetre (Rodina, 1972).

2.2.2.2. Assay using fouling diatoms

Four species of diatoms which form the major components of the microfouling assemblage in Goa waters were used for the purpose. The forms were collected from microfouling assemblage developed on glass panels suspended at Dona Paula, Goa. The microfouling assemblage from the panel was scraped gently with nylon brush in filtered seawater (Sharma et al., 1990). The cells were then spread plated on agar which was prepared in F_2 medium (55 gm in 1000 ml) (Yamasaki et al., 1988). The plates were incubated for 3-4 days at room temperature (28°C±2°C) with 12 hrs. light and 12 hrs. dark cycle. The algal colonies were observed under inverted microscope and the desired ones were removed aseptically from the plate and transferred in sterile Guillard F_2

medium. The composition of Guillard F_2 medium was as given in the annexure-I (Guillard and Ryther, 1962; Guillard, 1975).

The unialgal cultures were then revived in F_2 medium containing antibiotics (Penicillin G, 1 gm/L; Streptomycin sulphate, 0.2 gm/L and Chloromphenical, 0.025 gm/L). The antibiotically treated cells were further maintained in sterile F_2 medium and were used for bioassays (Bhosle et al., 1995). The cells of mid-log phase were used for the subsequent experimental purpose. The diatom species were *Navicula subinflata*, *N. crucicula*, *Amphora* sp. and *Nitzschia* sp.

A known weight of the crude extract was dissolved in F_2 medium to prepare stock solution, which was subsequently used for the preparation of serial dilutions (Thomas and Robinson, 1986). A series of Ehrlemeyer flasks (100 ml Capacity) containing gradually increasing concentrations of the individual crude extract were added with equal quantities of individual diatom species. The volume in each experimental flask was maintained to 15 ml. The grades of the succeeding concentrations were decided on the basis of pilot experiments which were conducted to find out the maximum concentration of the extract at which 100% inhibition of growth of diatoms occurs. The concentrations of the extracts used for the experimental purpose ranged between 0 to 500 µg/ml of dry weight of the extract. The flasks containing extracts and diatoms were incubated for 3 days at room temperature (28 °C±2° C) with 12 hrs. light and 12 hrs. dark cycle (Gotschalk and Alldredge, 1989). The flasks containing each concentration were maintained in triplicate. After incubation the adhered

cells were scraped using rubber policeman (Thomas and Robinson, 1986) and filtered on pre-ignited GF/C glass filter paper. Chlorophyll *a* (Chl. *a*) from these cells was extracted for overnight duration using 90% acetone and its quantity was estimated by using fluorescence spectrophotometer (Parsons et al., 1984; Sawant and Wagh, 1994; Sawant et al., 1995). The algal biomass has been expressed in terms of Chl. *a* concentration. From these biomass values, algal growth was calculated and the values have been expressed in terms of increase of Chl. *a* per unit area ($\mu g/cm^2$). From the biomass values expressed as Chl. *a*, percentage growth and growth inhibition in relation to the control were calculated by using the following equation:

$$\% \text{ Biomass growth} = \frac{(B - O) \times 100}{(A - O)} \quad \text{.....(i)}$$

Where, O = Inoculum cell biomass at zero hours,

A = Cell biomass in control flask after 72 hours.

B = Cell biomass in experimental flask after 72 hours.

Percentage Growth inhibition = 100 - (% Biomass growth)...(ii)

2.2.2.3. Assay using barnacle cyprid

For the mass culture of barnacle larvae in the laboratory, protocol described by Rittschof et al., (1984) was followed. About 8000 nauplii of

B. amphitrite were transferred in a perspex tank containing 8 litres of filtered (passed through 0.2 μm Millipore filter paper) aged seawater with antibiotics (Streptomycin, 36.5 mg/L and Penicillin - G, 21.9 mg/L). The culture was aerated in a controlled manner and maintained at room temperature (28°±2°C). The larvae were fed daily with 500 ml of diatom culture (*Skeletonema costatum*) having a density of 2 x 10^6 cells/ml. The larvae were checked daily for their progressive development. The cyprids were available on 5th or 6th day (Plate 2.6A). The cyprids so developed were sorted out and transferred in a bowl containing 50-100 millilitres of filtered seawater. Thereafter, they were stored in the dark at 6° C in the refrigerator for not more than three days, during which they were used for experimental purpose.

As regards the settlement assay of these cyprid larvae, protocol described by Rittschof et al., (1992) was followed. A known weight of the crude extract was dissolved in aged seawater which was filtered by passing through 0.2 μm Millipore filter paper to prepare stock solution. It was subsequently used for the preparation of serial dilutions. A series of glass beakers (50 ml capacity) containing crude extracts of gradually increasing concentrations (5, 10, 15, 20, 25, 50, 75, 100 and 200 μg/ml in seawater were added with 15 to 46 number of cyprids. The volume in each beaker was maintained to 30 ml. In addition, cyprids were also added into beakers containing only filtered seawater without extracts. They were used as control. Each concentration was studied in triplicate. All the beakers containing cyprids were kept at room temperature under dark. After 24 hrs, the unattached cyprids were flushed out, and thus

the attached and unattached cyprids were counted. The percentage of attached cyprids (Plate 2.6B) in the experimental beakers was calculated and expressed in relation to the percentage of attached cyprids in control beakers.

2.2.2.4. Assay using adult mussels

Due to certain practical limitations, adult mussels were used for the purpose. Mussels generally get themselves attached to any suitable surface by their byssus threads. Therefore, attachment of mussels by byssus threads was scored as attachment assay in the present study (Anon, 1952; Harada et al., 1984; Szewzyk et al., 1991). The forms of Mussels, *Perna viridis* of around 3 cm breadth and 6 cm length were collected during low tide from the rocks at Baga and Betul, Goa and utilized for these assays. The shells were cleaned for any adhered organisms and their byssal threads were cut off close to the shell (Szewzyk et al, 1991). They were then kept in 500 ml of aged filtered seawater containing increasing concentration of the crude extracts under study. The concentrations used for the studies were, 5, 10, 15, 20, 30, 40 and 50 µg/ml dry weight of the extracts. Six mussels were exposed to each concentration of extracts. In addition, mussels were also maintained into the beakers containing only seawater as experimental control. All the beakers were kept on a rotary shaker with 30 - 40 RPM for creating water movement as well as for facilitating diffusion of atmospheric oxygen. After 24 hrs the beakers were studied for attachment of mussels. The entire set of experiment was repeated for the reproducibility of the results obtained.

2.2.3 Field trial

2.2.3.1. Assessment of microfouling

The crude extracts were mixed with nontoxic paint having alkyd binder. The concentration of the crude extract in the paint was maintained at 40% of the dry weight of the film, beyond which the adhesive property of the paint was found to be getting affected. A double coat of the mixture was applied on aluminium platelets of size 2 x 5 cm and allowed to dry at room temperature for 48 hours. The thickness of the film was measured with a statistical thickness gauze (Sheen Instruments Ltd., England). The thickness of the film so measured was found to vary between 200 to 300 μm. The painted platelets were then fixed on to a fibre glass frame with the help of PVC nuts and bolts and suspended in seawater at Dona Paula bay. Every day two platelets of each type were removed over a period of four days. They were transported to the laboratory in filtered seawater. In the laboratory the platelets were gently rinsed in filtered seawater to remove any loosely adhered diatom cells. The surface of the platelet was then scraped with nylon brush (Sharma et al., 1990) and the scraping was collected separately in a known volume of sterile seawater containing few drops of Lugol's solution. The diatom cells from this solution were subjected to sedimentation in a settling chamber (Hitchcock, 1982) and thereafter they were counted using haemocytometer (Thomas and Robinson, 1986). The diatom numbers from the extract coated platelets were then used for calculating percentage settlement, in relation to the control platelets by the following expression:

$$\% \text{ Settlement} = \frac{Y \times 100}{X}$$

Where, X = Number of diatom settled on control platelet

Y = Number of diatom settled on extract coated platelet.

2.2.3.2. Assessment of macrofouling

Aluminium panels admeasuring 5 x 10 cm were coated with paint mixture containing 40% of the crude extracts as above. The panels were suspended in estuarine seawater near Dona Paula, jetty Goa. The panels were examined visually at fortnightly intervals for the settlement of macrofoulers. The fouled panels were photographed and the organisms attached were scraped and weighed on balance. Four replicates of each extract were studied.

2.2.4. Analysis of Data

2.2.4.1. Probit method

For the sake of convenience, two different probit methods were adopted for the estimation of median effective concentration (Ec_{50} value) for the extracts against the different organisms. For the diatom assays graphic probit method was used (Anon, 1972). As per this method percentage values of diatom growth as well as growth inhibition were plotted on the one axis and the concentration of extract on the other. The intersecting point gives Ec_{50} value. On the other hand, a micro

computer FORTRAN programme for probit analysis has been used for the estimation of Ec_{50} values of the extracts against cyprids and mussels (Reddy et al., 1992). This programme helps to estimate Ec_{50} values with 95% confidence limit.

2.3. RESULTS

2.3.1. Laboratory assays

2.3.1.1. Assay using bacterial strains

Table 2.1 shows the results of the bacterial screening tests against the extracts. It was observed that out of the five, only three plant extracts exhibited antibacterial activity). These extracts were of the plants *C. spinosa*, *S. laurifolius* and *G. glauca*, the crude extracts of which were found to be active against all the seven species of bacteria. The inhibition zone around the discs were not very prominent, and ranged between 1 to 2 mm (Plate 2.1). Of the three extracts, that of *S. laurifolius* was found to be comparatively less active then the other two as was evident from the inhibition zone.

2.3.1.2. Assay using diatoms

Tables 2.2-2.6 indicate the extent of diatom growth against varying concentrations of the crude extracts under study. Decreasing trend was observed of the growth of diatoms with increased concentrations of extracts. The observations were made in triplicate for each concentration and the standard deviation was found to be ranging between ±0.002 to

±0.124 for all the extracts. The Ec_{100} values of each extract varied with the test species. The Ec_{100} values of *C. spinosa* against *N. subinflata*, *N. crucicula*, *Nitzschia* sp. and *Amphora* sp. were 50, 125, 50 and 200 µg/ml respectively (Tables 2.2a-2.2d) (Plate 2.2-2.5). For the extract of *S. laurifolius* the E c_{100} values were 100, 100, 100 and 250 µg/ml for *N. subinflata*, *N. crucicula*, *Nitzschia* sp. and *Amphora* sp. respectively (Tables 2.3a-2.3d). On the other hand, the Ec_{100} values of the extract of *G. glauca* were 200, 220, 150 and 300 µg/ml against *N. subinflata*, *N. crucicula*, *Nitzschia* sp. and *Amphora* sp. respectively (Tables 2.4a-2.4d). The Ec_{100} values of the extract of *A. pennata* against *N. subinflata*, *N. crucicula*, *Nitzschia* sp. and *Amphora* sp. were 50, 30, 150 and 200 µg/ml respectively (2.5a-2.5d). As regards the extract of *B. acutangula*, the Ec_{100} values were 80, 120, 150 and 200 µg/ml for *N. subinflata*, *N. crucicula*, *Nitzschia* sp. and *Amphora* sp. respectively (Tables 2.6a-2.6d). The extent of diatom percentage growth as well as growth inhibition against each concentration of the extract have been plotted against concentration of extract in figs. 2.1-2.5. Both the graphs are intersecting at a point where 50% of growth and growth inhibition has been observed. This concentration is termed as Ec_{50}. The Ec_{50} values of all the extracts under study are presented in table 2.19. The Ec_{50} values of the extract of *C. spinosa* against the diatom *N. subinflata*, *N. crucicula*, *Nitzschia* sp. and *Amphora* sp. were 40, 94.2, 25 and 130.7 µg/ml respectively. The Ec_{50} values of the extract of *S. laurifolius* for *N. subinflata*, *N. crucicula*, *Nitzschia* sp. and *Amphora* sp. were 74.5, 92.5, 62.2 and 152.6 µg/ml respectively. For the extract of *G. glauca* the estimated Ec_{50} values were found to be 51.4, 54.2, 116 and 134 µg/ml for *N. subinflata*, *N.*

crucicula, *Nitzschia* sp. and *Amphora* sp. respectively. Amongst the extracts under study, the Ec_{50} values of *A. pennata* were comparatively lower than the others. These values were 26.8, 8.9, 34.5 and 115.6 µg/ml for *N. subinflata*, *N. crucicula*, *Nitzschia* sp. and *Amphora* sp. respectively. The Ec_{50} values of the extract of *B. acutangula* for *N. subinflata*, *N. crucicula*, *Nitzschia* sp. and *Amphora* sp. were 27.4, 19, 48.8 and 125.3 µg/ml respectively. In general, the data pertaining to Ec_{50} values of all the extracts for different diatoms show that the extract of *A. pennata* appears to be the most active, whereas, the extract of *S. laurifolius* was found to be the least active one. Moreover, the Ec_{50} values also showed that the *Amphora* sp. was the most resistant diatom species against all the five extracts as compared to the other diatoms under study .

It was interesting to note that extracts of *C. spinosa*, *S. laurifolius* and *A. pennata* were found to promote growth of the diatoms, *N. subinflata* and *N. crucicula* well below their respective Ec_{50} values. A maximum of 99% increase of growth was observed of the diatom *N. crucicula* at 25 µg/ml concentration of the extract of *C. spinosa*.

2.3.1.3. Assay using Barnacle Cyprids

Tables 2.7a-2.11a represent the data on the extent of settlement of cyprids against increasing concentrations of the crude extracts under study. After 24 hrs. exposure of the larvae to the increasing concentrations of the crude extracts, it was observed that in control beakers about 59 to 76% settlement occurred on the glass surface. On

the contrary, the percentage settlement of cyprids in experimental beakers containing crude extract, varied with the plant species. As regards the effects of extract of *S. laurifolius* on cyprids, no appreciable change in the settlement was observed from 25 to 50 µg/ml of the extract. Moreover, the percentage settlement at 25 µg/ml was comparatively higher to the extent of 14% to that of the control . However, at 200 µg/ml concentration of the same extract, no settlement was observed. In case of the extract of *A. pennata* the percentage settlement of cyprid decreased gradually with increasing concentrations, resulting in 100% inhibition (Ec_{100}) at 200 µg/ml. Decreasing trend in settlement with increasing concentrations was also observed with the extract of *B. acutangula*. However, Ec_{100} value for this extract was found to be at 100 µg/ml concentration. On the other hand, increase in the percentage of settlement was observed at 25 µg/ml concentration of the extract of *C. spinosa*. But, subsequent increase of concentration of the extract resulted into 100% settlement inhibition of cyprids at 100 µg/ml. Amongst all the extracts under study, extract of *G. glauca* appeared to be quite effective at concentration as low as 25 µg/ml, at which 100% settlement inhibition was observed.

Tables 2.7b-2.11b show the results of probit analyses indicating Ec_{50} values with 95% confidence limit and related statistical parameters for the respective crude extract. All the Ec_{50} values of the plant extracts against cyprid are given in table 2.19. It may be seen from this table that the estimated Ec_{50} values of the extracts of *C. spinosa*, *S. laurifolius*, *G. glauca*, *A. pennata* and *B. acutangula* were 68.1, 91.3,

15.2, 78.8 and 35.8 µg/ml respectively. Amongst the plant extracts under study, extract of *G. glauca* appeared to be the most effective in controlling barnacle larval settlement under laboratory conditions. It was followed by *B. acutangula* and *C.spinosa.*

2.3.1.4. Assay using adult mussels

Tables 2.12a-2.16a present the data on the attachment of adult mussels at different concentrations of the crude extracts. The mussels exhibited 100% attachment in beakers containing plain seawater (control; plate 2.7a). On the other hand, 100% inhibition in attachment was observed at 20, 50, 20, 50 and 20 µg/ml concentrations of the extract of *C. spinosa*, *S. laurifolius*, *G. glauca*, *A. pennata* and *B. acutangula* respectively (plate 2.7b). Observations made on the attached byssus threads of the mussels revealed that the mussels in control beakers produced maximum number of byssus threads (< 50 Nos.), whereas, the mussels which were subjected to different concentrations of the extracts produced less number of byssus thread. The estimated Ec_{50} values of the different extracts for mussels have been presented in table 2.19. The Ec_{50} values were found to be 6, 24.4, 5, 27.3 and 5.9 µg/ml for *C. spinosa*, *S. laurifolius*, *G. glauca*, *A. pennata* and *B. acutangula* respectively. Furthermore, it has been observed that the mussels which were unable to attach in seawater containing Ec_{100} dose, were found to attach when transferred in plain seawater. The attachment in this case was found to be 60 to 80%.

2.3.2. Field trial

2.3.2.1. Assessment of microfouling

In general, the numbers of diatoms on paint film containing crude extract were found to be increasing with exposure period (Table 2.17). However, the number of diatoms settled on extract coated platelets were comparatively less than the platelets coated with only paint. The density of diatoms on the control platelet was 32 in number per cm^2 on the first day, which increased to 177 per cm^2 on the fourth day. On the other hand the diatom settlement on experimental platelets (coated with extracts) for the corresponding periods ranged between 5-65, 13-71, 4-32, 18-22 and 15-28 for *C. spinosa*, *S. laurifolius*, *G. glauca*, *A. pennata* and *B. acutangula* respectively. The diatoms which were encountered on the platelets, belonged to genera *Navicula*, *Nitzschia*, *Synedra*, *Pleurosigma*, *Rhizosolenia*, *Amphora* and *Licmophora*. Amongst these the forms belonging to *Navicula* were the most dominant.

The diatom numbers from the extract coated platelets were calculated on the basis of percentage settlement in relation to the diatom number on control platelets. These values were observed to be 15.6, 40.6 12.5, 56 and 46.8% for the extracts of *C. spinosa*, *S. laurifolius*, *G. glauca*, *A. pennata* and *B. acutangula* respectively for 24 hours exposure period. For 96 hours exposure period, the percentage settlement recorded was 36.7, 40, 18, 12.4, and 15.8 for the extracts of *C. spinosa*, *S. laurifolius*, *G. glauca*, *A. pennata* and *B. acutangula* respectively. Though the diatom number on extract coated platelets increased with increased exposure period, no significant difference in the relative

percentage of settlement was evident in case of the extracts of *C. spinosa*, *S. laurifolius* and *G. glauca* for 24 and 96 hours exposure periods. On the other hand, for the extracts of *A. pennata* and *B. acutangula*, the relative percentage settlement decreased with the increased exposure period.

2.3.2.2. Assessment of macrofouling

Periodical visual observations made at the panels surfaces coated with and without extracts revealed that hydroids were the first invader on the control panels after four days of exposure. It was followed by settlement of barnacles after 15 days (plate 2.8A). In contrast to this the panels coated with the extracts were found to remain free from fouling till 3rd observation (45 days). However, at subsequent observation (60 days) it was found that the same panels were fouled by few macrofoulers comprising of barnacles, encrusting bryozoans and oysters (plate 2.8B-F). The biomass of the fouling assemblage collected on various panels is presented in Table 2.18. It is evident that the average biomass on control panels was 56 gm/dm^2, whereas, these values were 6.8, 5.6, 12.2, 15.3 and 24.6 gm/dm^2 for panels coated with extracts of *C. spinosa*, *S. laurifolius*, *G. glauca*, *A. pennata* and *B. acutangula*, respectively.

2.4 DISCUSSIONS

Different criteria could be used for assessing antifouling effect of a given compound on test organism. These criteria include, growth

inhibition, motility inhibition, non-attachment, mortality, negative taxis, repulsion etc. (Cooksey and Cooksey, 1991; Lindener, 1994). For instance, if a given compound interferes with growth processes of fouling organism, it can lead to inhibition of growth. This phenomena will have adverse effects on the fouling biomass build-up. In the present, work growth inhibition and non-attachment criteria were used for assessing antifouling action of the extracts. The first criterion was used for bacteria and diatoms, whereas, the latter was deployed in case of barnacles and mussels.

The results on the bioassays of the crude extracts against seven species of fouling bacteria suggest that, only three extracts were found to be active. They were of the plants *C. spinosa*, *S. laurifolius* and *G. glauca*. Furthermore, it was observed that the extract of *S. laurifolius* exhibited comparatively lower activity as evident from the zone of inhibition (1 mm). The amount of extracts of these three plants which was required for bacterial inhibition ranged between 800 - 1000 µg/disc. Therefore, it could be assumed that the compounds which prevented the growth of the bacterial species may be mild in action or its diffusivity in agar may be low, resulting in narrow zone of inhibition.

A perusal of the published literature revealed that little is known about the antibacterial activity of the plants under study. However, Mishra et. al.(1979a,b) have reported on the antibacterial activity of an oily extract from fruits of *C. spinosa* against four bacterial species. Moreover, the bacterial strains used by them for assays were non-marine, hence there is little scope for comparison of the present results.

Number of measures have been suggested for expressing diatom cell biomass in antifouling assays. These measures include cell count, chlorophyll *a*, etc. (Maureen et al., 1981; Targett et al., 1983; Thomas and Robinson, 1986; Targett et al., 1994). Of these, cell count provides more reliable data than other measures. However, counting of diatom cells especially of fouling forms were found to be impractical due to the clumping of cells (Thomas and Robinson, 1986; French and Evans, 1988). Hence, chlorophyll *a* concentration was used for the purpose in the present work. The measure is thought to be most convenient (French and Evans, 1988).

From the present data it is evident that all the five extracts exhibited fairly considerable inhibition of growth of the fouling diatom species under study. The plant extracts thus appear to possess antifouling properties against fouling diatoms. It is likely that the active compound present in the crude extracts may be interfering with some of the enzymes leading to inhibition of cell growth (Lewin, 1955; Lehninger, 1970). The present technique deals with the cell growth assay instead of settlement assay. Very few workers have used this techniques (Thomas and Robinson, 1986; French and Evans, 1988; Hunter and Evans, 1990; Sawant and Wagh, 1994; Sawant et al., 1995) for screening antifouling compounds. However, this method is the most convenient one because, mass culturing and maintenance of diatoms in laboratory is easy and more convenient, which ultimately facilitates screening process.

In the present investigation, growth enhancement was evident in the diatoms of genus *Navicula* at concentrations ranging between 10-75, 20-40 and 5-50 µg/ml for the extracts of *C. spinosa*, *S. laurifolius* and *A. pennata* respectively. These concentrations were found to be far below their respective Ec_{50} values. These results suggest that the extracts probably contain both growth stimulator/promoter as well as growth inhibitor depending upon its concentration used. Similar properties have also been reported in the past by Baslow (1969), Burkholder (1973), Standing et al., (1984), Vitelina et al., (1991) and Avelin Mary et al., (1994) in the case of extracts from marine organisms. They have stated that growth stimulating/promoting and growth inhibiting properties of a given extract could be due to presence of two different type of compounds. According to Standing et al., (1984), growth stimulators/promoters are believed to be of high molecular weight substances which readily get adsorbed on to the surfaces, whereas, growth inhibitors are believed to be of low molecular substances. In the present investigation only the diatom species belonging to genus *Navicula* were found to respond to the growth promoting activity. These differences in the response of the diatoms under study probably suggest that the compound which is responsible for growth promotion may be specific for the forms of *Navicula* sp.

There are several simple methods to treat the data derived from a test series for the estimation of Ec_{50} values. These methods are probit and logit. The probit method plots a integrated curve whereas, the logit method adopts logistic curve for estimation of Ec_{50} values (Reddy at al., 1992; Krishna and Reddy, 1993). These methods logically interpret that

the organism that grows or attaches at a given dose of extract, would also have grown or attached at any lower dose. Conversely, an organism that failed to grow or attach at certain dose would have also not grown or attached at any other higher dose. Thus the information from any one group can be added to that of the other group in the range of doses tested. This information helps to estimate 50% growth and growth inhibition or 50% settlement and settlement inhibition and is termed as "Effective Concentration 50" (Ec_{50}). The estimated Ec_{50} values of the extracts against the diatoms, helps to decide the degree of activity of the extract.

From the table 2.19 it could be seen that amongst the extracts under study, the extract of *A. pennata* appears to be the most active one against all the diatom species tested, followed by *B. acutangula*. Comparison of both Ec_{100} and Ec_{50} values of each extract helps to hypothesize that greater is the difference between these two values higher is the efficiency and vice versa. According to the data in figures 2.1 to 2.5, the efficiency of the extracts varied with the test organisms. In general, amongst the extracts, the Ec_{100} and Ec_{50} values of *S. laurifolius* are found to be closer to each other, which suggests that the extract possesses low efficiency against the diatoms. Similar results were also observed in case of *C. spinosa* against *N. subinflata*, *N. crucicula* and *Amphora* sp.

Invertebrate larvae especially barnacle have been widely used in the past as target organism for screening natural products in laboratory (Maureen et al., 1981; Branscomb and Rittschof, 1984;

Rittschof et al., 1984; Standing et al., 1984; Keifer et al., 1986; Gerhart et al., 1988; Sears et al., 1990; Roberts et al., 1991; Szewzyk et al., 1991; Rittschof et al., 1992; Avelin et al., 1994). The main reason behind this, was the ease of maintenance and management of larvae in the laboratory. Barnacle larvae pass through number of developmental stages before settlement. In view of this if a compound can interfere with the developmental process of any one of the stage, the settlement can be prevented. The same strategy has been adopted in the present investigation wherein, the cyprid stage which is a settling stage of the barnacle was used for fouling settlement assay. The estimated Ec_{50} values of the extracts are presented in table 2.19. These values were found to range between 15.2 to 91.3 μg/ml, and are comparable with the Ec_{50} values reported by DeNys et al., (1995) for the extracts of marine organisms. The crude extracts from marine invertebrates and fractions thereof, have been reported to have Ec_{50} values in the range of 1 to 100 μg/ml. Similarly, the comparison of the Ec_{50} values of the extracts and inorganic copper, suggest that the activity of the plant extracts is 25 times less than that of the activity of inorganic copper against cyprid. The difference in the degree of activity of these two could be explained on the basis that natural compounds are thought to be non-toxic, whereas, inorganic copper is toxic to cyprid. In the present observations it was found that the cyprid larvae which were exposed to the Ec_{100} dose were normal in terms of their appearance, behaviour. and motility. These observations suggest that the extracts probably function by some mechanism other than toxic one. Similar opinion was also expressed by Williams et al., (1983). According to them the natural

compound/inhibitor probably interferes with chemoreception of cyprid in a fashion similar to supressants of oyster drill chemotaxis. Morse, (1984) has also opined that inhibitor may interfere with chemoreception of cyprid in the same way similar to lectin inhibition of larval settlement in the case of *Haliotis refescens*. However, this needs further studies for confirmation.

Adult mussels attach themselves to any substratum with the help of their byssus threads. Therefore, they can be used for quick attachment assay tests (Anon, 1952; Harada, 1984). However, one drawback in this method is that it requires large amount of extract. This difficulty was not encountered in the present investigation as the crude extracts were available in sufficient quantity. The results were very encouraging, wherein, 100% inhibition of attachment was observed at 20 µg/ml concentration of each of *C. spinosa, G. glauca* and *B. acutangula*, whereas, it was 50 µg/ml each *of S. laurifolius* and *A. pennata*. Subsequently, it was observed that these mussels which were subjected to the Ec_{100} doses, were found to revive and attach when transferred into plain seawater. These observations suggest that the action of the extracts on the mussels may be reversible.

The results of the field trials in the present studies indicated clear difference in the pattern of settlement of diatoms on control and experimental sets of panels. In general, though the numbers of diatoms were found to increase on both the sets of panels with increase of exposure period, their number was considerably low on the experimental

panels than that on control ones. The percentage inhibition of diatoms on experimental panels was found to be ranging between 35.5 to 87.5%, which suggests positive antifouling action of the crude extracts against the diatoms fouling.

The dense settlement of macrofoulers on control and no settlement on experimental panels till 45 days suggests that the extracts were solely responsible for the antifouling action. However, the subsequent observation at the end of 60 days revealed spares settlement of macrofoulers on experimental panels. This suggests that the paint film might have lost its antifouling action. It could be presumed that due to high aqueous solubility, the extracts might have leached off the paint surface with very fast rate leading to shortening of duration of antifouling action. These results confirm the antifouling activity of the five plant extracts in the field too.

In conclusion, the laboratory bioassay and the field studies of the plant extracts suggest that these extracts do indicate the presence of bioactive compound which can be used as antifouling agent if isolated and structurally identified. Although these extracts exhibited antifouling activity on broad spectrum of organisms, there was clear variation in their effectiveness on different fouling organisms. In some cases extracts which were most effective against one organism showed low activity against other organism. The nonconsistency in the degree of activity of the extracts probably suggesting species specificity, which could be explained on the bases of natural resistivity of organisms. The organisms possess varying degree of resistivity towards any biocide. For instance

diatoms, especially forms of genus *Amphora* have been reported to be most resistant towards highly toxic antifouling agent such as Tributyltine and Dibutyltine (Thomas and Robinson, 1986). The stiff resistance towards any toxins by the diatoms was thought to be probably due to development of "sheath" of exopolysaccharides around their cells, which in turn protects the organism against any toxins (Decho, 1990). The difference in the degree of activity of the different extracts under study against a single culture could be due to various reasons. They could be stated as, the difference in type and nature of the active compound, synergism of compounds in the crude extract etc. It has also been stated in this context that the soil conditions where in the plant species grow may have a bearing on the nature of bioactive metabolites (Santhakumaran, 1994).

The results of field trials of the extracts coating are the indicative of the antifouling activities of the plant extracts in natural environment. The bioassay and the field trials of the crude extracts form the scientific information on the activity of plant extracts which may lead to isolation of antifouling compound. Thus, this information may lead to develop environment friendly antifouling technology if the chemical nature of the most active compound is found out and a analog is prepared.

ANNEXURE - I

Composition of Guillard F_2 medium

(Concentrations in 1 litres of aged seawater)

Nutrients	
$NaNO_3$	75 mg
$NaH_2PO_4.H_2O$	5 mg
$Na_2SiO_3.9H_2O$	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
$ZnSo_4.7H_2O$	0.022 mg
$CoCl_2.6H_2O$	0.01 mg
$MnCl_2.4H_2O$	0.18 mg
$Na_2MoO_4.2H_2O$	0.006 mg
Vitamins	
Thiamine. HCl	0.1 mg
Biotin	0.5 µg
B12	0.5 µg

Table 2.1 Degree of inhibition (mm) of fouling bacteria species around the discs impregnated with the plant extracts.

Plant extract	*C. spinosa*	*S. laurifolius*	*G. glauca*	*A. pennata*	*B. acutangula*
Chromobacterium sp.	2	1	2	N	N
Alkaligenes sp.	1	1	2	N	N
Moraxella sp.	2	1	2	N	N
Arthrobacter sp.	1	1	2	N	N
Vibrio sp.	2	1	2	N	N
Pseudomonas sp.	2	1	2	N	N
Flavobacterium sp.	2	1	2	N	N

N = Not active

Table 2.2a Growth of *N. subinflata* after 72 hrs. exposure to different concentrations of *C. spinosa*

Crude extract (µg/ml)	Diatom growth Chl *a* (µg/cm^2)	Growth (%)	Inhibition (%)
0	0.10 (±0.008)	100.0	0.0
10	0.12 (±0.008)	116.2	-16.0
20	0.14 (±0.004)	135.2	-35.2
30	0.12 (±0.009)	116.2	-16.2
40	0.05 (±0.005)	51.4	48.6
50	0.00 -	0.0	100.0

Table 2.2b Growth of *N. crucicula* after 72 hrs. exposure to different concentrations of *C. spinosa*

Crude extract (µg/ml)	Diatom growth Chl *a* (µg/cm^2)	Growth (%)	Inhibition (%)
0	0.43 (±0.008)	100.0	0.0
25	0.86 (±0.012)	199.0	-99.0
50	0.81 (±0.008)	187.5	-87.5
75	0.67 (±0.016)	155.3	-55.3
100	0.09 (±0.009)	21.6	78.4
125	0.00 -	0.0	100.0

Figures in brackets indicate standard deviation of three replicate values

Table 2.2c Growth of *Nitzschia* sp. after 72 hrs. exposure to different concentrations of *C. spinosa*

Crude extract (µg/ml)	Diatom growth Chl *a* (µg/cm^2)	Growth (%)	Inhibition (%)
0	2.51 (±0.074)	100.0	0.0
10	2.29 (±0.049)	91.4	8.6
20	1.76 (±0.052)	70.3	29.7
30	0.79 (±0.032)	31.7	68.3
40	0.18 (±0.028)	7.4	92.6
50	0.00 -	0.0	100.0

Table 2.2d Growth of *Amphora* sp. after 72 hrs. exposure to different concentrations of *C. spinosa*

Crude extract (µg/ml)	Diatom growth Chl *a* (µg/cm^2)	Growth (%)	Inhibition (%)
0	1.41 (±0.081)	100.0	0.0
40	1.17 (±0.047)	83.5	16.5
80	1.04 (±0.049)	74.3	25.7
120	0.87 (±0.056)	62.2	37.8
160	0.24 (±0.047)	17.5	82.5
200	0.00 -	0.0	100.0

Figures in brackets indicate standard deviation of three replicate values

Table 2.3a Growth of *N. subinflata* after 72 hrs. exposure to different concentrations of *S. laurifolius*

Crude extract (µg/ml)	Diatom growth Chl *a* (µg/cm^2)	Growt h (%)	Inhibition (%)
0	0.49 (±0.012)	100.0	0.0
20	0.53 (±0.008)	108.1	-8.1
40	0.47 (±0.008)	95.9	4.1
60	0.37 (±0.009)	75.5	24.5
80	0.003 (±0.002)	0.6	99.4
100	0.00 -	0.0	100.0

Table 2.3b Growth of *N. crucicula* after 72 hrs. exposure to different concentrations of *S. laurifolius*

Crude extract (µg/ml)	Diatom growth Chl *a* (µg/cm^2)	Growt h (%)	Inhibition (%)
0	0.38 (±0.008)	100.0	0.0
20	0.44 (±0.008)	115.7	-15.7
40	0.45 (±0.008)	118.4	-18.4
60	0.36 (±0.012)	94.7	5.3
80	0.31 (±0.004)	81.5	18.5
100	0.00 -	0.0	100.0

Figures in brackets indicate standard deviation of three replicate values

Table 2.3c Growth of *Nitzschia* sp. after 72 hrs. exposure to different concentrations of *S. laurifolius*

Crude extract (µg/ml)	Diatom growth Chl *a* (µg/cm^2)	Growth (%)	Inhibition (%)
0	2.57 (±0.057)	100.0	0.0
10	1.83 (±0.016)	71.4	28.6
20	1.63 (±0.016)	63.6	36.4
30	1.39 (±0.016)	54.2	45.8
40	0.20 (±0.008)	7.9	92.1
50	0.00 -	0.0	100.0

Table 2.3d Growth of *Amphora* sp. after 72 hrs. exposure to different concentrations of *S. laurifolius*

Crude extract (µg/ml)	Diatom growth Chl *a* (µg/cm^2)	Growth (%)	Inhibition (%)
0	1.38 (±0.033)	100.0	0.0
50	1.18 (±0.012)	85.5	14.5
100	1.14 (±0.029)	82.6	17.4
150	0.75 (±0.024)	54.3	45.7
200	0.07 (±0.002)	5.0	95.0
250	0.00 -	0.0	100.0

Figures in brackets indicate standard deviation of three replicate values

Table 2.4a Growth of *N. subinflata* after 72 hrs. exposure to different concentrations of *G. glauca*.

Crude extract (µg/ml)	Diatom growth Chl *a* (µg/cm^2)	Growth (%)	Inhibition (%)
0	0.47 (±0.012)	100.0	0.0
40	0.28 (±0.032)	59.7	40.3
80	0.12 (±0.02)	25.8	74.2
120	0.08 (±0.016)	17.3	82.7
160	0.02 (±0.009)	5.9	94.1
200	0.00 -	0.0	100

Table 2.4b Growth of *N. crucicula* after 72 hrs. exposure to different concentrations of *G. glauca*

Crude extract (µg/ml)	Diatom growth Chl *a* (µg/cm^2)	Growth (%)	Inhibition (%)
0	0.54 (±0.012)	100.0	0.0
40	0.33 (±0.016)	61.1	38.9
80	0.15 (±0.016)	27.7	72.3
120	0.11 (±0.012)	20.9	79.1
160	0.08 (±0.009)	14.8	85.2
200	0.06 (±0.008)	6.0	94.0
220	0.00 -	0.0	100.0

Figures in brackets indicate standard deviation of three replicate values

Table 2.4c Growth of *Nitzschia* sp. after 72 hrs. exposure to different concentrations of *G. glauca*

Crude extract (µg/ml)	Diatom growth Chl *a* (µg/cm^2)	Growth (%)	Inhibition (%)
0	1.45 (±0.041)	100.0	0.0
25	1.35 (±0.016)	93.1	6.9
50	1.22 (±0.033)	84.1	15.9
75	1.01 (±0.016)	69.7	30.3
100	0.91 (±0.016)	62.8	37.2
125	0.62 (±0.033)	42.8	57.2
150	0.00 -	0.0	100.0

Table 2.4d Growth of *Amphora* sp. after 72 hrs. exposure to different concentrations of *G. glauca*

Crude extract (µg/ml)	Diatom growth Chl *a* (µg/cm^2)	Growth (%)	Inhibition (%)
0	1.14 (±0.024)	100.0	0.0
50	1.07 (±0.074)	83.1	16.9
100	0.73 (±0.032)	62.9	37.1
150	0.52 (±0.024)	44.8	55.2
200	0.36 (±0.009)	31.0	69.0
250	0.15 (±0.016)	12.9	87.1
300	0.00 -	0.0	100.0

Figures in brackets indicate standard deviation of three replicate values

Table 2.5a Growth of *N. subinflata* after 72 hrs. exposure to different concentrations of *A. pennata*

Crude extract (µg/ml)	Diatom growth Chl *a* (µg/cm^2)	Growth (%)	Inhibition (%)
0	0.37 (±0.020)	100.0	0.0
10	0.51 (±0.012)	139.0	-39.0
20	0.40 (±0.008)	108.0	-8.0
30	0.09 (±0.012)	24.7	75.3
40	0.06 (±0.008)	18.2	81.8
50	0.00 -	0.0	100.0

Table 2.5b Growth of *N. crucicula* after 72 hrs. exposure to different concentrations of *A. pennata*

Crude extract (µg/ml)	Diatom growth Chl *a* (µg/cm^2)	Growth (%)	Inhibition (%)
0	0.92 (±0.054)	100.0	0.0
5	1.37 (±0.016)	148.1	-48.1
10	0.29 (±0.043)	31.3	68.7
15	0.22 (±0.020)	23.7	76.3
20	0.07 (±0.025)	7.5	92.5
25	0.02 (±0.021)	2.1	97.9
30	0.00 -	0.0	100.0

Figures in brackets indicate standard deviation of three replicate values

Table 2.5c Growth of *Nitzschia* sp. after 72 hrs. exposure to different concentrations of *A. pennata*

Crude extract (µg/ml)	Diatom growth Chl *a* (µg/cm^2)	Growth (%)	Inhibition (%)
0	1.22 (±0.061)	100.0	0.0
25	0.76 (±0.050)	62.4	37.6
50	0.37 (±0.016)	30.6	69.4
75	0.18 (±0.008)	15.1	84.9
100	0.09 (±0.016)	7.8	92.2
125	0.03 (±0.012)	2.9	97.1
150	0.00 -	0.0	100.0

Table 2.5d Growth of *Amphora* sp. after 72 hrs. exposure to different concentrations of *A. pennata*

Crude extract (µg/ml)	Diatom growth Chl *a* (µg/cm^2)	Growth (%)	Inhibition (%)
0	1.16 (±0.081)	100.0	0.0
25	1.20 (±0.009)	103.4	-3.4
50	1.21 (±0.012)	104.3	-4.3
75	1.02 (±0.124)	87.9	12.1
100	0.91 (±0.017)	78.9	21.1
125	0.36 (±0.081)	31.0	69.0
150	0.32 (±0.047)	27.5	72.5
175	0.12 (±0.047)	10.3	89.7
200	0.00 -	0.0	100.0

Figures in brackets indicate standard deviation of three replicate values

Table 2.6a Growth of *N. subinflata* after 72 hrs. exposure to different concentrations of *B. acatangula*

Crude extract (µg/ml)	Diatom growth Chl *a* (µg/cm^2)	Growth (%)	Inhibition (%)
0	0.36 (±0.012)	100.0	0.0
20	0.22 (±0.016)	61.1	38.9
40	0.11 (±0.008)	30.5	69.5
60	0.03 (±0.009)	10.0	90.5
80	0.00 -	0.0	100.0

Table 2.6b Growth of *N. crucicula* after 72 hrs. exposure to different concentrations of *B. acutangula*

Crude extract (µg/ml)	Diatom growth Chl *a* (µg/cm^2)	Growth (%)	Inhibition (%)
0	0.99 (±0.009)	100.0	0.0
20	0.46 (±0.058)	46.4	53.6
40	0.29 (±0.006)	29.2	70.8
60	0.25 (±0.020)	25.2	74.8
80	0.21 (±0.026)	21.2	78.8
100	0.11 (±0.016)	11.1	88.9
125	0.00 -	0.0	100.0

Figures in brackets indicate standard deviation of three replicate values

Table 2.6c Growth of *Nitzschia* sp. after 72 hrs. exposure to different concentrations of *B. acatangula*

Crude extract (µg/ml)	Diatom growth Chl *a* (µg/cm^2)	Growth (%)	Inhibition (%)
0	1.26 (±0.033)	100.0	0.0
25	1.12 (±0.033)	88.1	11.9
50	0.59 (±0.024)	47.1	52.9
75	0.42 (±0.024)	33.7	66.3
100	0.37 (±0.016)	29.8	70.2
125	0.07 (±0.016)	6.1	93.9
150	0.00 -	0.0	100.0

Table 2.6d Growth of *Amphora* sp. after 72 hrs. exposure to different concentrations of *B. acatangula*

Crude extract (µg/ml)	Diatom growth Chl *a* (µg/cm^2)	Growth (%)	Inhibition (%)
0	1.20 (±0.032)	100.0	0.0
25	1.21 (±0.049)	100.8	-0.8
50	1.22 (±0.025)	101.6	-1.6
75	1.13 (±0.016)	94.2	5.8
100	1.03 (±0.049)	85.9	14.1
150	1.33 (±0.028)	27.8	72.2
175	0.06 (±0.025)	5.4	94.6
200	0.00 -	0.0	100.0

Figures in brackets indicate standard deviation of three replicate values

Table 2.7a Results of the settlement assay of barnacle cyprids against the extract of *C. spinosa*.

Con. of extract (µg/ml)	Number settled	Number unsettled	Percentage settlement	Relative percentage	Percentage inhibition
Control	14	7	66	-	-
25	16	4	80	121	-
50	14	10	58	87	13
100	8	18	30	45	55
200	0	27	0	0	100

Table 2.7b Results of the probit analysis of the above data.

	Log Ec_{50}	Ec_{50}	SE	UL	LL
Values	2.83	68.13	2.91	73.85	62.41

Ec_{50} = Effective concentration at 50% inhibition
SE = Standard error
UP = Upper limit
LP = Lower limit

Table 2.8a Results of the settlement assay of barnacle cyprids against the extract of *S. laurifolius*.

Con. of extract (µg/ml)	Number settled	Number unsettled	Percentage settlement	Relative percentage	Percentage inhibition
Control	16	11	59	-	-
25	22	8	73	123	-
50	15	12	55	93	7
100	6	15	28	47	53
200	0	28	0	0	100

Table 2.8b Results of the probit analysis of the above data.

	Log Ec_{50}	Ec_{50}	SE	UL	LL
Values	2.96	91.31	7.07	105.18	77.43

Ec_{50} = Effective concentration at 50% inhibition
SE = Standard error
UP = Upper limit
LP = Lower limit

Table 2.9a Results of the settlement assay of barnacle cyprids against the extract of *G. glauca*.

Con. of extract (µg/ml)	Number settled	Number unsettled	Percentage settlement	Relative percentage	Percentage inhibition
Control	16	11	59	-	-
5	15	11	57	96	4
10	12	10	54	91	9
15	13	21	38	64	36
20	4	36	10	16	86
25	0	24	0	0	100

Table 2.9b Results of the probit analysis of the above data.

	Log Ec_{50}	Ec_{50}	SE	UL	LL
Values	2.18	15.25	0.86	16.96	13.55

Ec_{50} = Effective concentration at 50% inhibition
SE = Standard error
UP = Upper limit
LP = Lower limit

Table 2.10a Results of the settlement assay of barnacle cyprids against the extract of *A. pennata*.

Con. of extract (µg/ml)	Number settled	Number unsettled	Percentage settlement	Relative percentage	Percentage inhibition
Control	20	26	76	-	-
25	24	17	58	76	24
50	19	8	70	92	8
75	15	19	44	57	43
100	4	20	16	21	79
200	0	28	0	0	100

Table 2.10b Results of the probit analysis of the above data.

	Log Ec_{50}	Ec_{50}	SE	UL	LL
Values	2.89	78.83	12.82	103.96	53.70

Ec_{50} = Effective concentration at 50% inhibition
SE = Standard error
UP = Upper limit
LP = Lower limit

Table 2.11a Results of the settlement assay of barnacle cyprids against the extract of *B. acutangula*.

Con. of extract (µg/ml)	Number settled	Number unsettled	Percentage settlement	Relative percentage	Percentage inhibition
Control	12	5	70	-	-
25	9	6	60	85	15
50	3	27	10	14	86
75	2	23	8	11	89
100	0	18	0	0	100

Table 2.11b Results of the probit analysis of the above data.

	Log Ec_{50}	Ec_{50}	SE	UL	LL
Values	2.55	35.80	3.57	42.80	28.81

Ec_{50} = Effective concentration at 50% inhibition
SE = Standard error
UP = Upper limit
LP = Lower limit

Table 2.12a Results of the settlement assay of mussels against the extract of *C. spinosa*.

Con. of extract (µg/ml)	Number settled	Number unsettled	Percentage settlement	Percentage inhibition
Control	12	0	100	0
5	7	5	58	42
10	3	9	25	75
15	2	10	16	84
20	0	12	0	100

Table 2.12b Results of the probit analysis of the above data.

	Log Ec_{50}	Ec_{50}	SE	UL	LL
Values	1.77	6.00	1.24	8.44	3.56

Ec_{50} = Effective concentration at 50% inhibition
SE = Standard error
UP = Upper limit
LP = Lower limit

Table 2.13a Results of the settlement assay of mussels against the extract of *S. laurifolius.*

Con. of extract (µg/ml)	Number settled	Number unsettled	Percentage settlement	Percentage inhibition
Control	12	0	100	0
10	12	0	100	0
20	7	5	59	41
30	5	7	42	58
40	3	9	25	75
50	3	12	0	100

Table 2.13b Results of the probit analysis of the above data.

	Log Ec_{50}	Ec_{50}	SE	UL	LL
Values	2.38	24.49	3.13	30.64	18.34

Ec_{50} = Effective concentration at 50% inhibition
SE = Standard error
UP = Upper limit
LP = Lower limit

Table 2.14a Results of the settlement assay of mussels against the extract of *G. glauca*..

Con. of extract (µg/ml)	Number settled	Number unsettled	Percentage settlement	Percentage inhibition
Control	12	0	100	0
5	6	6	50	50
10	2	10	17	83
15	1	11	8	92
20	0	12	0	100

Table 2.14b Results of the probit analysis of the above data.

	Log Ec_{50}	Ec_{50}	SE	UL	LL
Values	1.70	5.09	1.17	7.38	2.80

Ec_{50} = Effective concentration at 50% inhibition
SE = Standard error
UP = Upper limit
LP = Lower limit

Table 2.15a Results of the settlement assay of mussels against the extract of *A. pennata*..

Con. of extract (μg/ml)	Number settled	Number unsettled	Percentage settlement	Percentage inhibition
Control	12	0	100	0
10	12	0	100	0
20	8	4	67	33
30	8	4	67	33
40	2	10	17	83
50	0	12	0	100

Table 2.15b Results of the probit analysis of the above data.

	Log Ec_{50}	Ec_{50}	SE	UL	LL
Values	2.44	27.32	2.53	32.79	22.84

Ec_{50} = Effective concentration at 50% inhibition
SE = Standard error
UP = Upper limit
LP = Lower limit

Table 2.16a Results of the settlement assay of mussels against the extract of *B. acutangula.*

Con. of extract (µg/ml)	Number settled	Number unsettled	Percentage settlement	Percentage inhibition
Control	12	0	100	0
5	7	5	58	42
10	3	9	25	75
15	1	11	8	92
20	0	12	0	100

Table 16b Results of the probit analysis of the above data.

	Log Ec_{50}	Ec_{50}	SE	UL	LL
Values	1.77	5.93	1.09	8.07	3.76

Ec_{50} = Effective concentration at 50% inhibition
SE = Standard error
UP = Upper limit
LP = Lower limit

Table 2.17a Temporal variations in the extent of settlement of diatoms (cells/dm^2) on extract coated platelets exposed to marine waters.

Extracts	24 hrs.	48 hrs.	96 hrs.
Control	32 (±4)	32 (±4)	32 (±4)
C. spinosa	5 (±2)	5 (±2)	5 (±2)
S. laurifolius	13 (±3)	13 (±3)	13 (±3)
G. glauca	4 (±1)	4 (±1)	4 (±1)
A. pennata	18 (±3)	18 (±3)	18 (±3)
B. acutangula	15 (±3)	15 (±3)	15 (±3)

Table 2.17b Temporal variations in the percentage settlement of diatoms on extract coated platelets against the control (without extract) .

Extracts	24 hrs.	48 hrs.	96 hrs.
C. spinosa	15.6	33.8	36.7
S. laurifolius	40.6	64.5	40.0
G. glauca	12.5	29.0	18.0
A. pennata	56.0	45.1	12.4
B. acutangula	46.8	32.2	15.8

Table 2.18 Extent of macrofouling on panels coated with and without the plant extracts.

Coating	Biomass (g/dm^2)	Inhibition (%)
Control	56.0 (± 4.0)	-
C. spinosa	6.8 (± 0.8)	87
S. laurifolius	5.6 (± 0.4)	90
G. gluca	12.2 (± 1.6)	78
A. pennata	15.3 (± 2.1)	72
B. acutangula	24.6 (± 3.2)	56

Figures in brackets indicate standard deviation of the three replicate values.

Table 2.19 Estimated Ec_{50} values of the plant extracts against diffferent test organisms.

Plant extract	*C. spinosa*	*S. laurifolius*	*G. glauca*	*A. pennata*	*B. acutangula*
N. subinflata	40.0	74.5	51.4	26.8	27.4
N. crucicula	94.2	92.5	54.2	8.9	19.0
Nitzschia sp.	25.0	62.2	116.0	34.5	48.8
Amphora sp.	130.7	152.6	134.0	115.6	125.3
Cyprid	68.1	91.3	15.2	78.8	35.8
Mussel	6.0	24.4	5.0	27.3	5.9

Fig. 2.1 Estimation of Ec_{50} values of C. spinosa against the fouling diatoms.

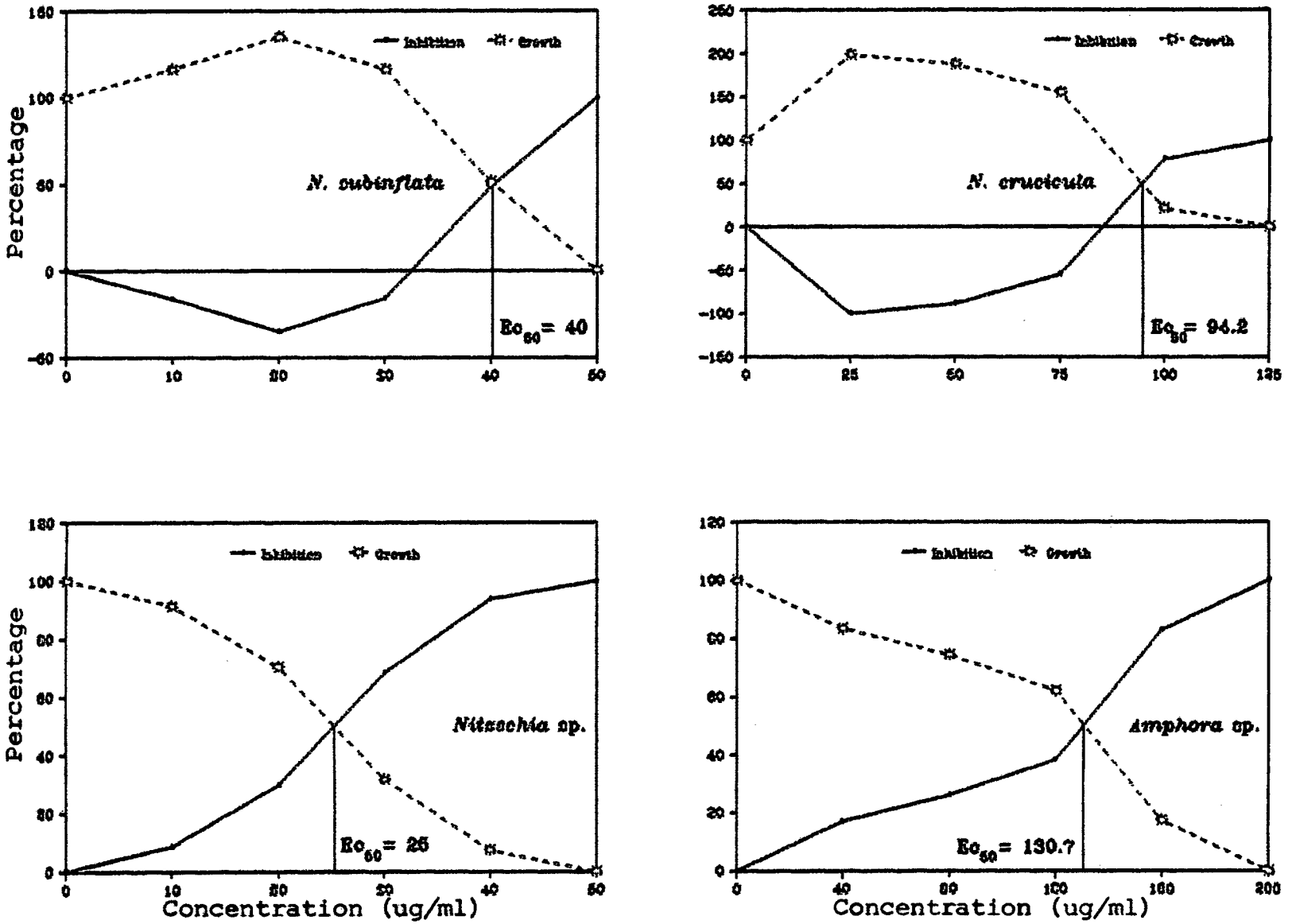


Fig. 2.2 Estimation of Ec_{50} values of S. laurifolius against the fouling diatoms.

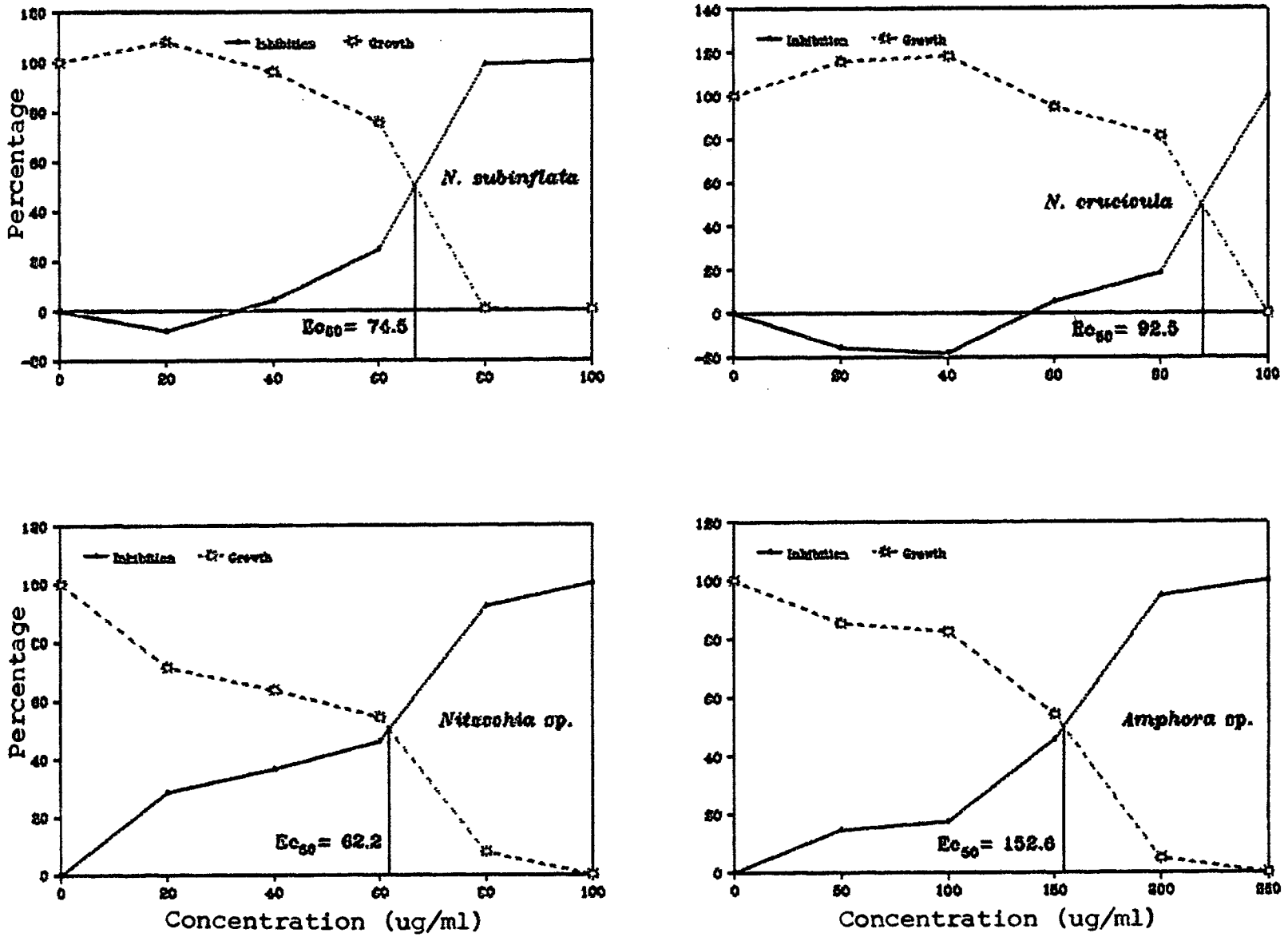


Fig. 2.3 Estimation of Ec_{50} values of G. glauca against the fouling diatoms.

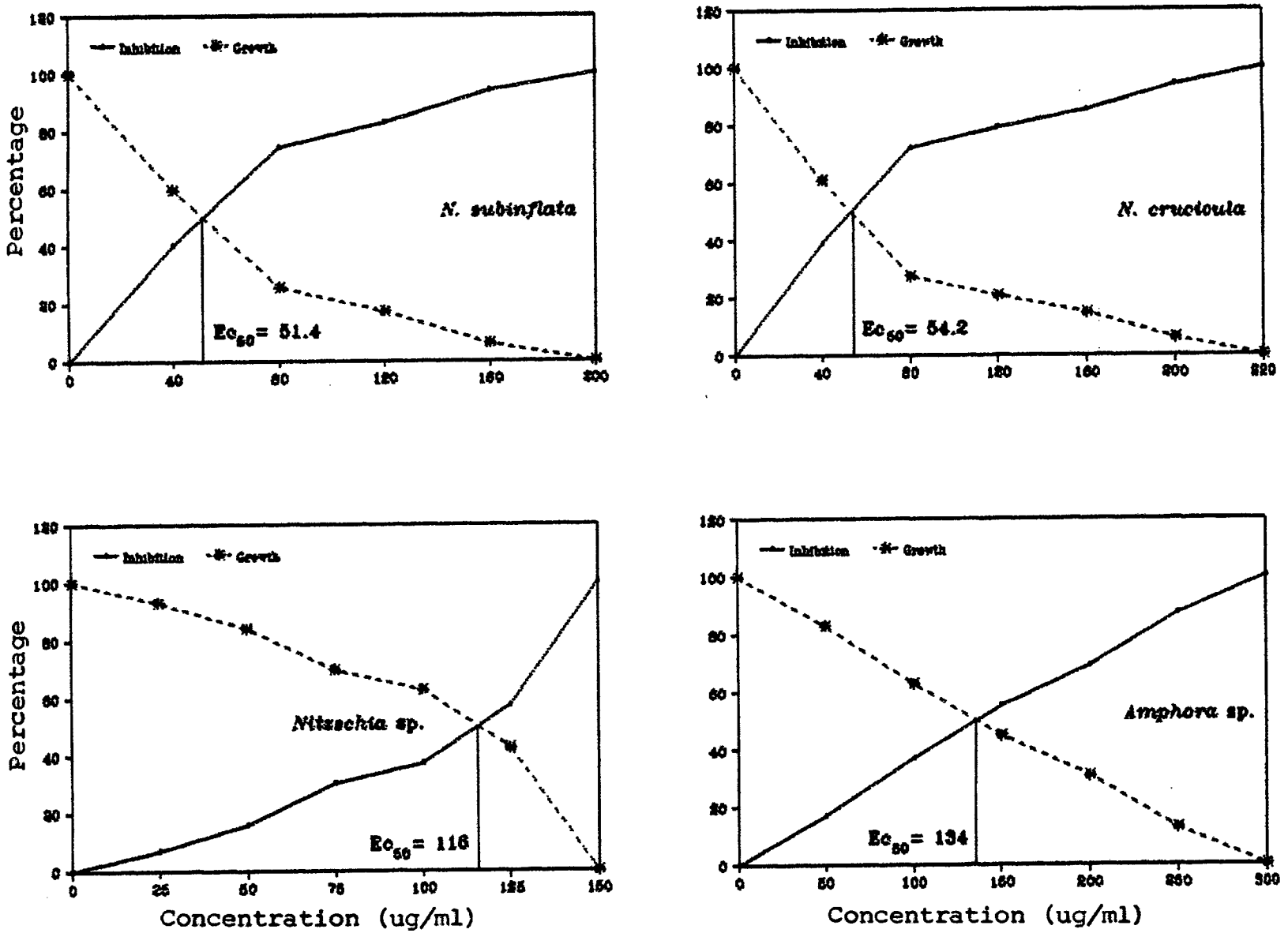


Fig. 2.5 Estimation of Ec_{50} values of B. acutangula against the fouling diatoms.

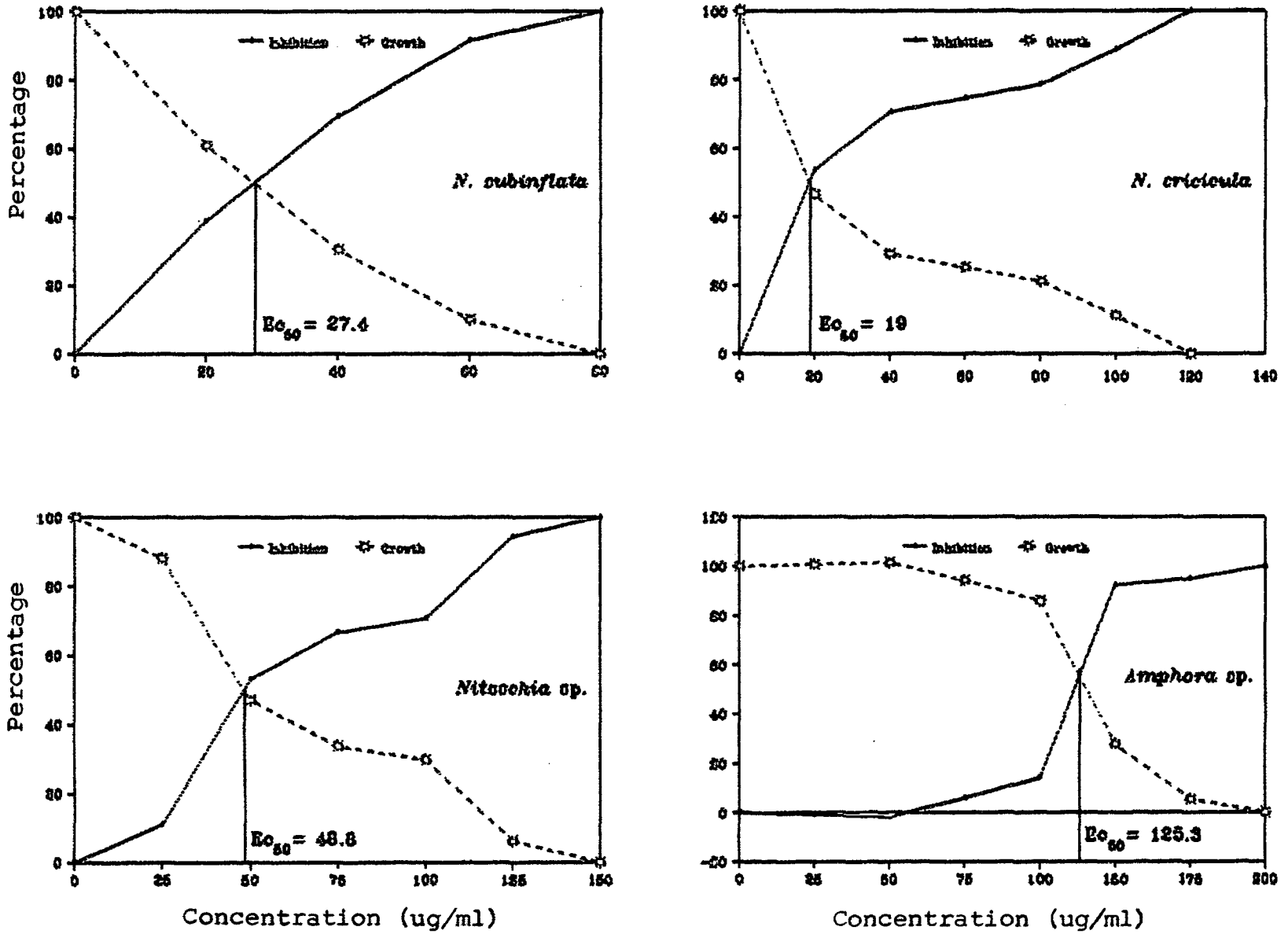
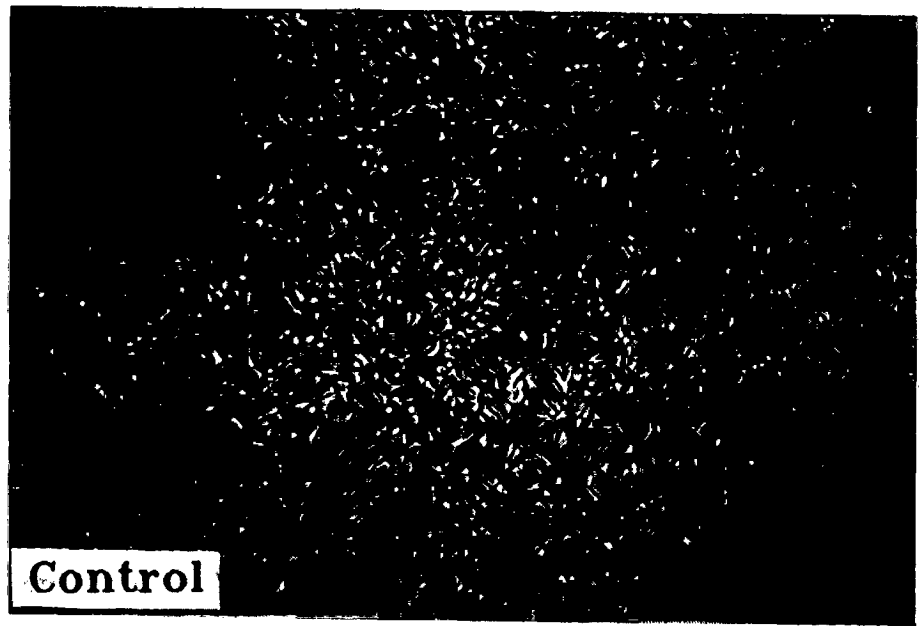
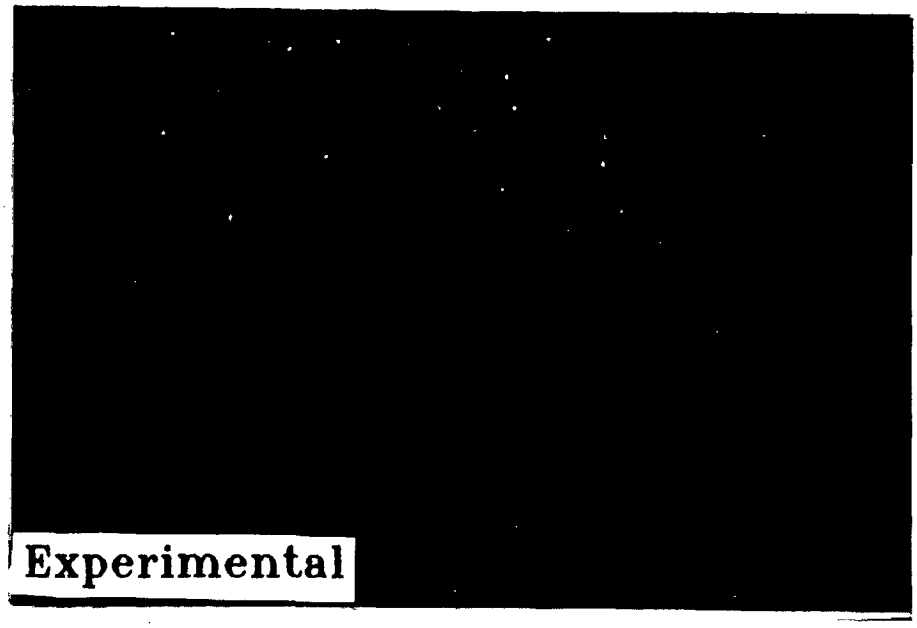


Plate - 2.1

Bacterial growth inhibition around the discs by the extract of *G. glauca*.

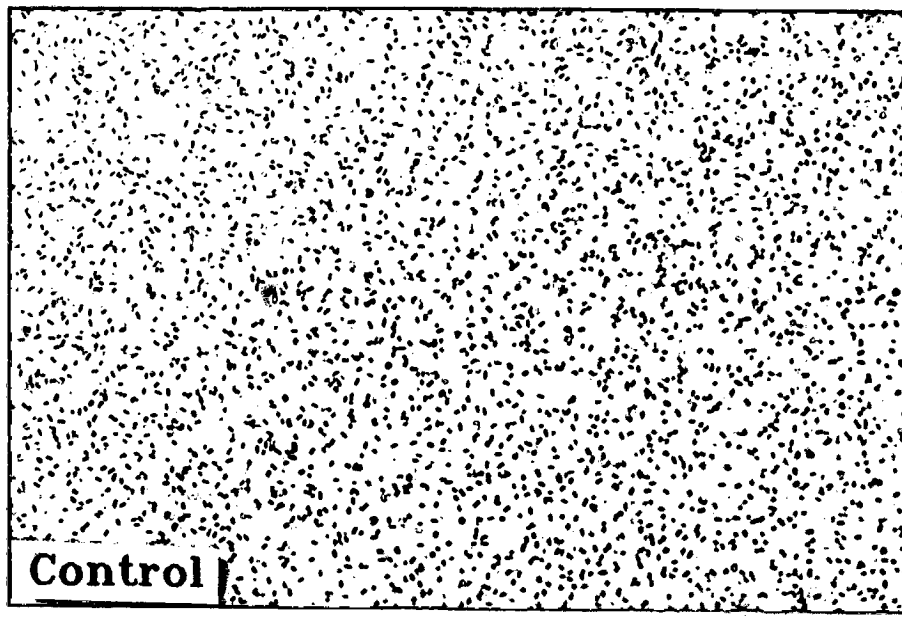
Plate - 2.2

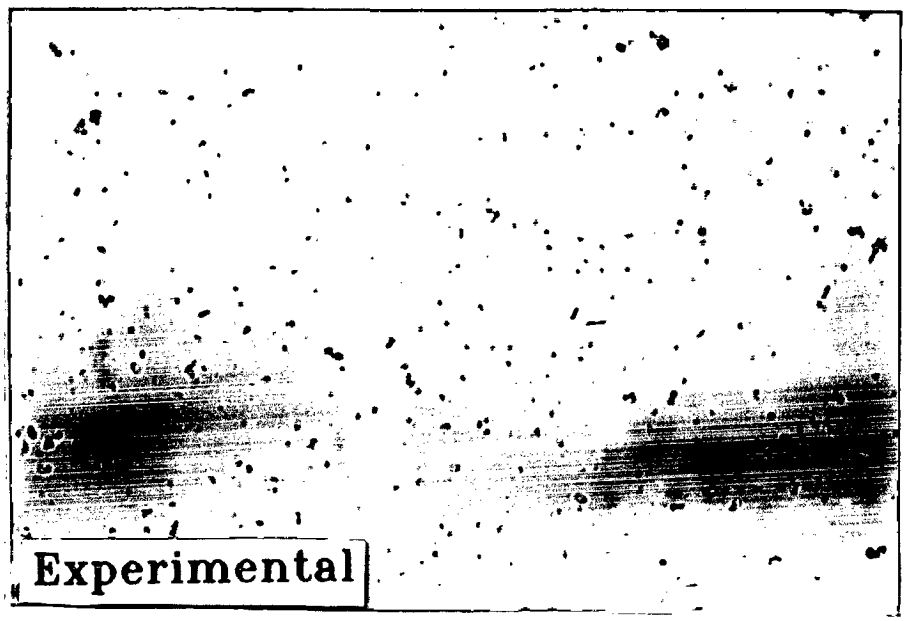




Photomicrographs showing the extent of growth of *N. subinflata* cells treated without (Control) and with the extract of *A. pennata* (Experimental)

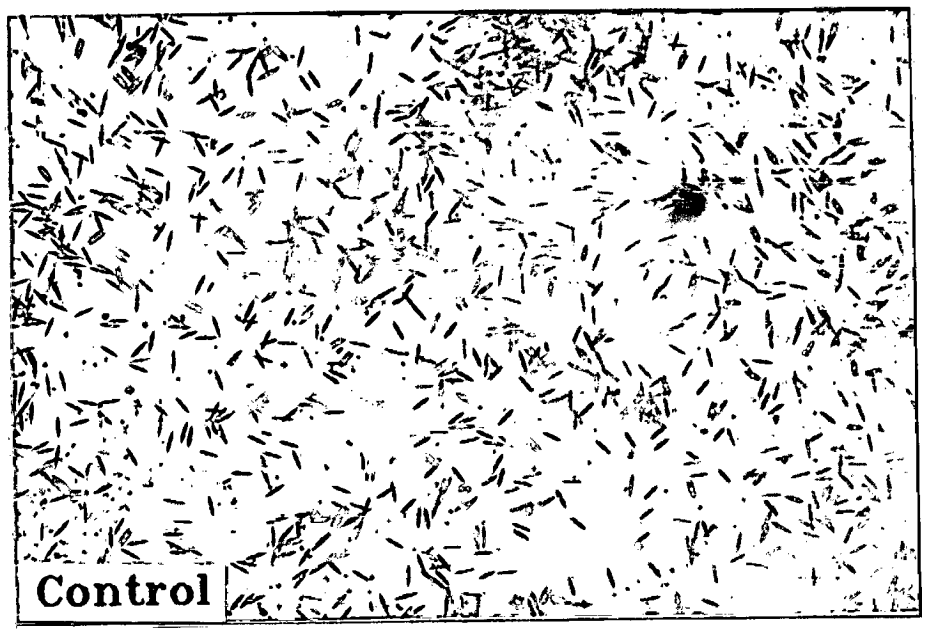
Plate - 2.3

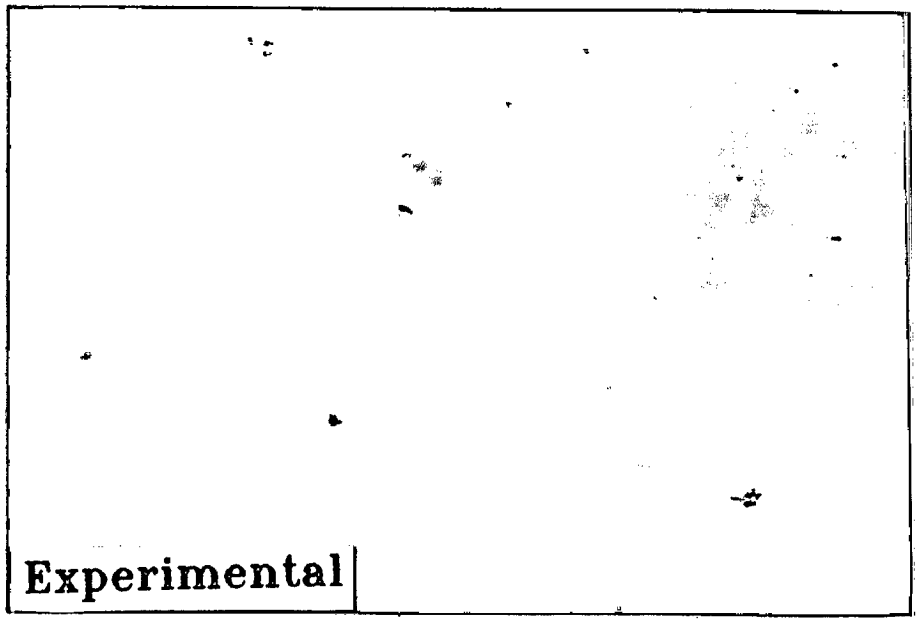




Photomicrographs showing the extent of growth of *N. crucicula* cells treated without (Control) and with the extract of *A. pennata* (Experimental)

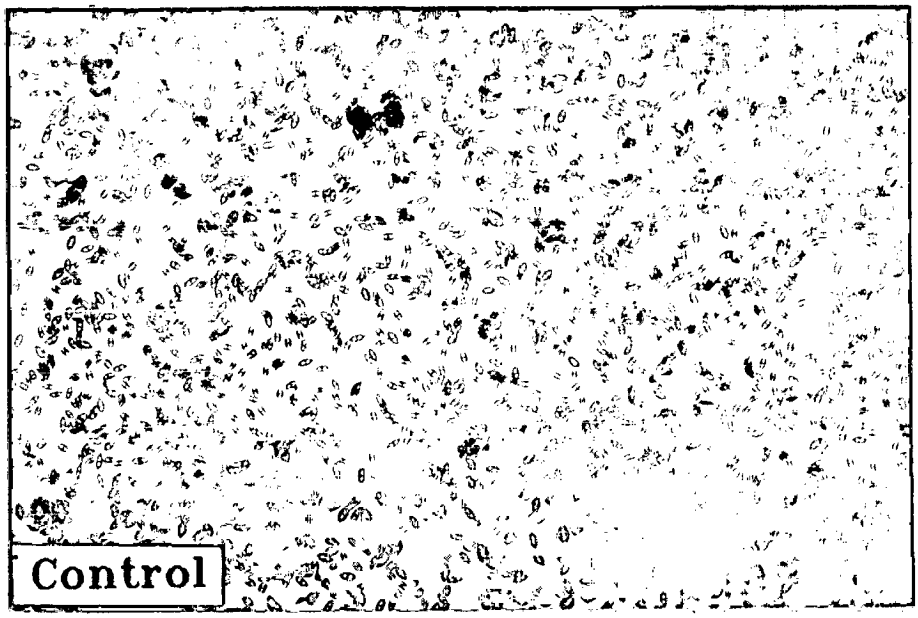
Plate - 2.4

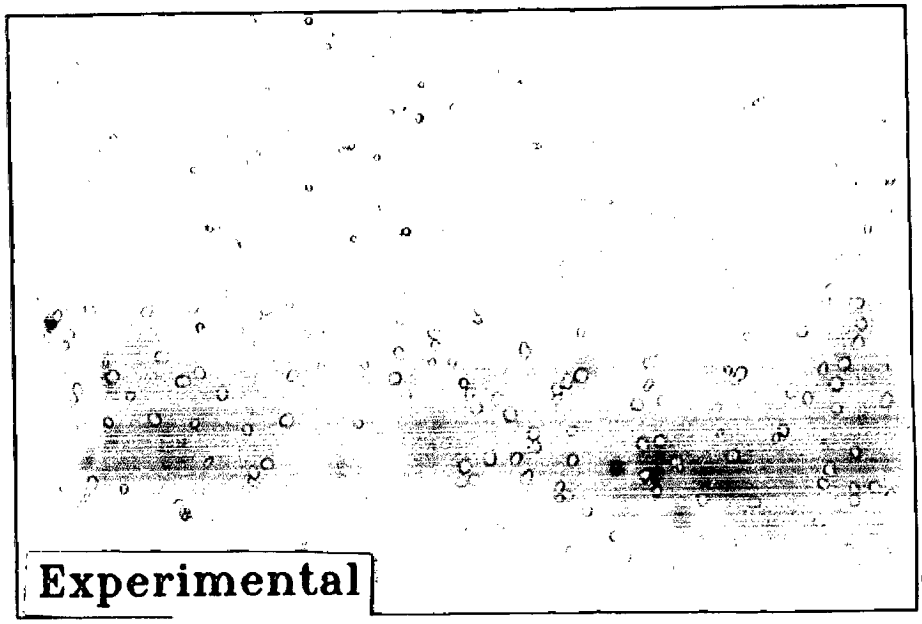




Photomicrographs showing the extent of growth of *Nitzschia* sp. cells treated with (Control) and with the extract of *A. pennata* (Experimental)

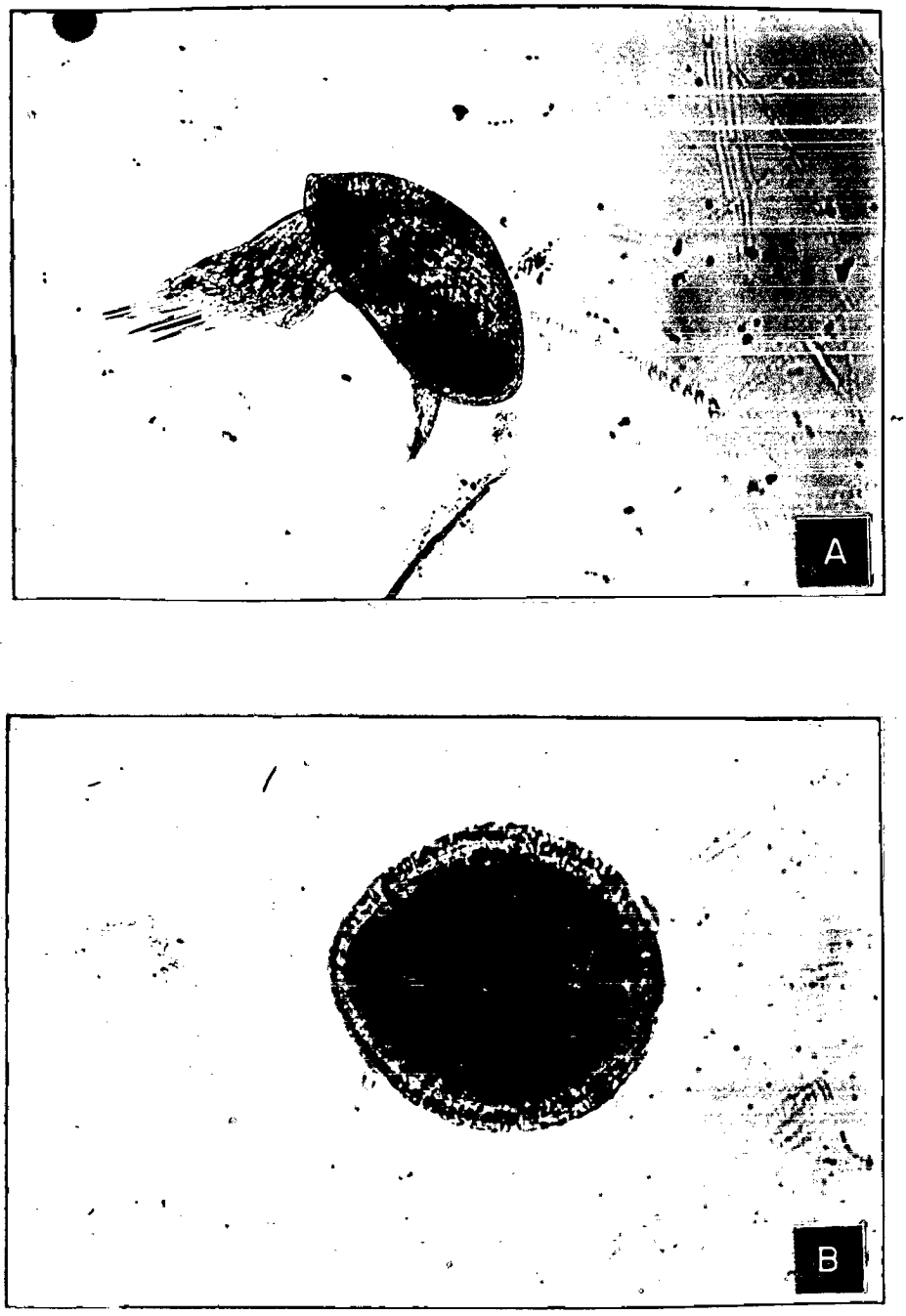
Plate - 2.5





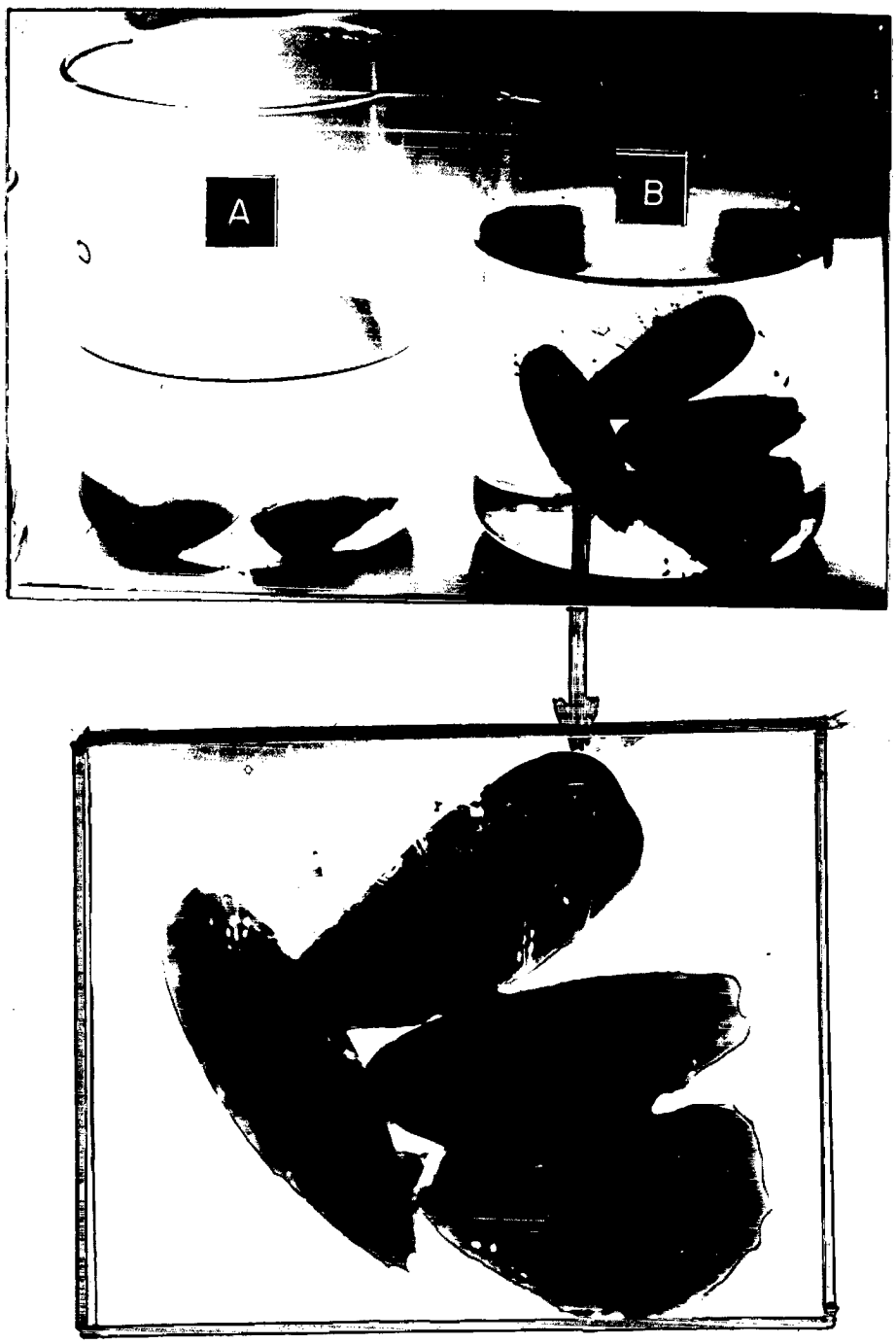
Photomicrographs showing the extent of growth of *Amphora* sp.cells treated without (Control) and with the extract of *A. pennata* (Experimental)

Plate - 2.6



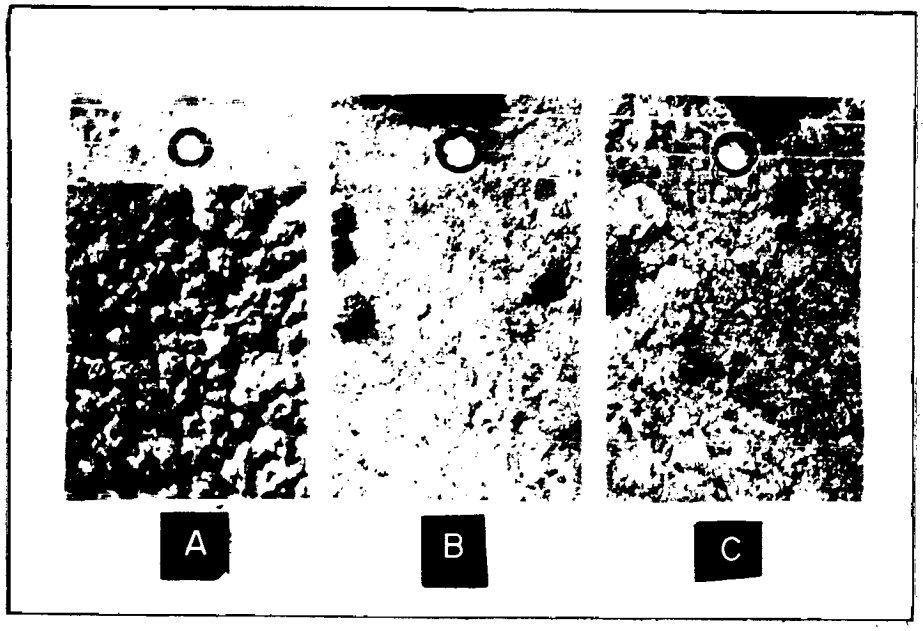
Cyprid of Barnacle *B. amphitrite*, before attachment (A) and Barnacle after shedding off cyprid shell (B).

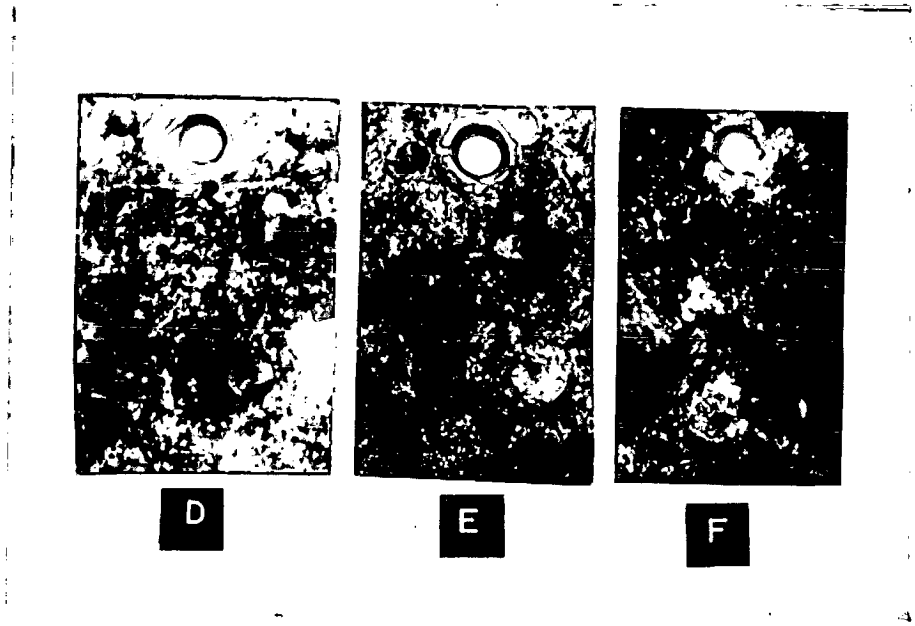
Plate - 2.7



Extent of attachment of mussels treated with (A) and without (B) extract of *A. pennata*.

Plate - 2.8





Results of the field test (after 45 days) of paint coatings, without (A) and with the extracts of *C. spinosa* (B), *S. laurifolius* (C), *G. glauca* (D), *A. pennata* (E) and *B. acutangula* (F).

CHAPTER 3

EFFECT OF THE PLANT EXTRACTS ON FOULERS

3.1 INTRODUCTION

The chemical toxins which are widely used in antifouling paints are believed to disrupt physiological activities of the fouling organisms. Millner and Evans, (1981) and Callow and Evans, (1981) have shown that triphenyltin chloride inhibits phosphorylation in chloroplasts isolated from the macroalgae *Enteromorpha intestinalis* and H^+ $C0_3^-$ fixation in *Achnanthes subsessilis*. A variety of toxic effects on phytoplankton have been described earlier. Copper which is the toxic component of a antifouling paint reportedly inhibits growth by preventing cell division, affects process of photosynthesis as well as of silicate uptake (Harrison et al., 1977, Thomas et al., 1980, Rueter et al., 1981). In some cases nitrate uptake and assimilation and amino acid synthesis are reported to have been affected (Harrison et al., 1977; Fisher et al., 1981). A considerable portion of the research done on the toxic effects of copper ions on foulers includes the investigation on algal species which are very sensitive to copper (Goering et al., 1977, Harrison et al., 1977).

Unlike the chemical toxins, natural toxins are unique compounds which possess some common properties, irrespective of source. One such common characteristic is that they exert a pronounced effect on the metabolism and biological functions of the organisms with just a minute quantity (Anthony, 1988). Any such natural toxin may serve as one of the antifoulants if incorporated in paint matrix.

Though several natural compounds have been identified as antifouling agent their mechanism of action on fouling organisms is not

yet clearly known. However, some workers have suggested that the antifouling function of natural compounds is by interfering with chemoreception in a fashion similar to suppressant of oyster drill chemotaxis, glucose in the case of settlement of *Junua brasiliensis* larvae or lectin inhibition of larval settlement in the case of *Haliotis refescens* (Kirchman et al., 1982; Williams et al., 1983; Morse, 1984). But, these hypothesis need confirmation. Garshkov and his colleagues (1982) have reported on the inhibition of Na, K-ATPase and Ca, Mg-ATPase activity by marine triterpene and steroidal glycosides. It is believed that natural antifouling agents generally suppress physiological activities in fouling organisms. Therefore, investigations on the effects of sublethal and lethal levels of antifouling agents on the physiology, morphology and biochemistry of tolerant diatoms are key to understanding of the antifouling capabilities of these agents.

In view of this, in the present work an effort has been made to study the effect of the active plant extracts on the physiology of fouling organisms. For this purpose a fouling diatom *N. subinflata* was used as a model organism, so as to understand the possible mechanism of growth inhibition of diatom.

3.2 MATERIALS AND METHODS

3.2.1 Nutrient uptake

Temporal variations in the uptake of nitrate and silicate by the diatom cells treated without and with extract of Ec_{100} dose were assessed.

Two sets of 100 ml capacity Ehrlemeyer flasks containing 15 ml of F_2 medium were taken for experimental purpose. These flasks were then inoculated with equal quantity of mid-log phase *N. subinflata* culture. To one of the sets effective concentration (Ec_{100}) of the crude extract was added and the other was maintained as control without addition of extract. Both the sets of flasks were incubated at room temperature with 12 hrs. dark and 12 hrs. light cycle. At the end of each incubation period, three flasks each from control as well as of experimental set were taken for estimation of nutrients and cell biomass. The incubation periods were 6, 12, 24, 48 and 72 hours. The algal cell biomass was estimated and expressed in terms of chlorophyll *a* concentration as described earlier.

3.2.1.1 Nitrate:

Aliquot of the culture medium was suitably diluted with distilled water and used for estimation of nitrate concentration. The procedure described by Parsons et al., (1984) was adopted for the purpose. The sample was added with 1 ml ammonium chloride solution and passed through amalgamated cadmium column for reduction of nitrate into nitrite. The reduced nitrite was then estimated by allowing the sample to react with 0.5 ml each of sulfanilamide and N-(1-naphthyl)-ethylenediamine dihydrochloride.

The extinction of red coloured azo-dye which was formed after the addition of the reagents was measured after 2 hours of reaction and compared with standard nitrate solution which was also treated in the

same manner as that of the sample. The nitrate concentration in the medium was calculated by using the expression,

µg-at N/Litre = E x F,

where F = calibration factor,

E = observed extinction of the sample

3.2.1.2. Silicate

Likewise nitrate, the aliquot of the culture medium was diluted suitably and used for estimation of silicate. Procedure described by Parsons et al., (1984) was adopted for the analysis. To 25 ml of the diluted sample of the growth medium 10 ml of molybdate solution (4 g ammonium molybdate +12 ml 2N HCl and the volume made to 500 ml with distilled water) was added and mixed. After 10 minutes, 15 ml of reducing reagent was added to the sample and allowed to stand for 2 - 3 hours. The blue coloured complex was measured at 810 nm on spectrophotometer. The extinction was compared with standard silicate solution and the concentration was calculated using the following expression:

µg-at Si/Litre = E x F,

where F = calibration factor,

E = observed extinction of the sample

3.2.2. Estimation of Cell contents

A known quantity of bacteria-free mid-log phase algal cells were aseptically inoculated in a series of Ehrlemeyer flasks (1 litre capacity) containing 100 ml of F_2 medium. To these flasks effective concentrations (Ec_{100}) of the plant extracts were added and incubated in the same manner as above for the periods of 6, 12, 24, 48 and 72 hours. After the above periods, 3 flasks of each extracts were taken for observation. The supernatant from these flasks were discarded and the attached cells were scrapped with soft nylon brush in a known volume of 1 M. NaCl solution (Read and Costerton, 1987; Sharma et al., 1990; Bhosle et al., 1995). The scraping was divided into five equal parts and each part was filtered separately on pre-ignited GF/C glass micro filters. The filters containing algal cells were then separately used for the estimation of chlorophyll *a*, particulate organic carbon, protein, total cell carbohydrates, and carbohydrate fractions. In addition, control experiment was also run in the same manner.

3.2.2.1 Chlorophyll a

Chlorophyll *a* from the diatom cells was extracted for overnight duration using 90% acetone. The fluorescence intensity of the extract was measured with the help of spectrofluorometer (Hitachi, Model F-2000) by exciting the sample at 430 nm and reading the fluorescence intensity at 670 nm. The fluorescence intensity of the sample was compared with chlorophyll *a* standard and the concentration was

calculated. The chlorophyll *a* concentration has been expressed in terms of per unit area of attachment (dm^2).

3.2.2.2 Particulate organic carbon

Particulate organic carbon contents of the diatom cells was estimated by wet oxidation method (Parson et al., 1984). The algal cells which were retained on GF/C glass filter paper were placed in glass test tube and then dispensed with 1 ml of phosphoric acid. It was followed by addition of 1 ml distilled water. The contents of the test tube were heated at 100 - 110°C for 30 minutes in the water bath. Thereafter, appropriate volume (5 - 10 ml) of sulphuric acid-dichromate and distilled water were added to the test tubes. The contents after mixing, were heated again for 60 minutes. The mixture was cooled and the volume was made to suitable quantities by adding distilled water (25 - 50 ml). An aliquot of this solution was centrifuged and the extinction of the sample was measured against the blank solution at 440 nm. The resulting extinction for the absorbance of trivalent chromium was corrected by the expression,

$$E = 1.1E_f$$

where, E_f is the observed extinction.

The concentration of particulate organic carbon was calculated by using the following expression:

$$\mu g\ C/dm^2 = E \times F \times v/A$$

where, F = calibration factor of standard,

v = volume of oxidant used.

A = Total area (in decimeter) to which cells were attached.

3.2.2.3 Total Cell Carbohydrate (PCHO)

The total cell carbohydrate content was estimated following the procedure described by Dubois et al., (1956). The algal cells which were retained on the filter paper were subjected to extraction for overnight duration with 1 ml of 80% sulphuric acid. To this extract 2 ml of 2% phenol solution was added. It was followed by addition of 5 ml concentrated sulphuric acid. The extinction of this solution was measured with the help of spectrophotometer at 490 nm against distilled water as blank. The intensity of the absorbance by the sample was compared with absorbance by glucose solution which was used as standard. The concentration of total carbohydrate was calculated and expressed as:

$$PCHO\ \mu g/dm^2 = Fx\ E / A$$

where F = calibration factor obtained from standard.

E = observed absorbance of the sample.

A = Total area (in decimetres) to which cells were attached.

3.2.2.4 Carbohydrate fractions

The procedure described by Haug et al., (1973) and used by Bhosle and Dhople, (1988) was adopted for separation of carbohydrate fractions. The filter paper containing the diatom cells was cut into pieces and placed in a centrifuge tube. To that, 5 ml of 0.1 N. sulphuric acid was added and kept in boiling water bath for 10 minutes. After cooling the contents, it was centrifuged and the extract was decanted. The residue was subjected to re-extraction for second time as above and both the extracts were pooled together. The residue was then subjected to extraction twice with 5 ml of 0.1 N. sodium hydroxide in the same manner as that of extraction with suphuric acid. The acidic as well as the alkaline extracts were used for estimation of carbohydrate contents by using phenol-sulphuric acid method as described earlier. The remaining residue was also subjected for estimation of carbohydrate concentration in the same manner as described in 3.2.2.3.

Thus the acid soluble, alkali soluble and residual fractions of carbohydrate were estimated and the concentration has been expressed on per unit area basis as above.

3.2.2.5 Protein

Estimation of cell protein was done by following the method described by Lowry et al., (1951). The algal cells along with the filters were homogenized with 4 ml 0.5 N. sodium hydroxide in a test tube and then heated at 80°c in a water bath for 30 minutes. The test tubes were then cooled and added with 0.2 ml of 0.5N. hydrochloric acid. The neutralized mixture was centrifuged and 1 ml of the supernatant was added with 2.5 ml of mixed reagent (2% $CuSO_4$. $5H_2O$, 4% K-sodium tartarate, 3% Na_2CO_3 in 0.1 N. NaOH in the ratio 1:1:48). and 0.5 ml Folins phenol reagent. After 30 minutes the extinction of the sample was measured against distilled water as blank at 750 nm with the help of spectrophotometer. The absorbance values of the samples were compared with the absorbance values of protein standard (Albumin) and the concentration of protein in sample was calculated using the following formulae:

Protein $\mu g/dm^2 = E \times F/A$

where, E = Observed extinction of the sample.

F = Calibration factor

A = Total area (in decimetres) to which cells were attached.

3.2.3 Intracellular nitrate assimilation

Two sets each of 10 Ehrlemeyer flasks (100 ml capacity) containing 20 ml F_2 medium were inoculated with equal quantity of diatom cells of *N. subinflata*. To one of the sets effective concentration (Ec_{100}) of the plant extract was added and the other set was left as a control. Both these sets were incubated as mentioned earlier. Three flasks from each set were removed at a time interval of 6, 12, 24, 48 and 72 hrs. for analysis. The supernatant was decanted and the attached cells were scrapped with soft brush in a known volume of 1 M. NaCl solution. The scrapping was divided into two equal parts. The diatom cells along with the filter were homogenized in a glass homogenizer. The homozinized sample was then washed down in a centrifuge tube with distilled water. It was then centrifuged at 6000 RPM for 10 minutes. The supernatant was collected in 50 ml measuring cylinder and the volume was adjusted to 50 ml with distilled water. The nitrate concentration in this was then estimated as mentioned above.

The second part of the scrapping, containing cells was filtered and the cell chlorophyll *a* was estimated as described earlier. The observations were carried out in triplicate. The intracellular inorganic nitrate concentrations in the diatom cells have been expressed in terms of per unit chlorophyll *a* values.

3.3 RESULTS

The data on variations of the nutrient uptake by the diatom cells for different exposure periods in medium, treated with and without plant extracts has been presented in tables 3.1-3.2. It has been observed that the diatom cells in plain medium gradually utilized both the types of nutrients from the medium. The silicate uptake by these cells was found to be as low as 0.38% at the initial exposure period of 6 hrs. which increased to 89.74% at the end of 72 hours. Similarly, the nitrate uptake by these cells was found to be 0.25% at 6 hrs. exposure period, which increased to 46.1% at the end of 72 hrs. On the other hand, it may be seen from the tables 3.1 and 3.2, that the diatom cells which were treated with the plant extracts appear to have utilized negligible amount of both the type of nutrients from the medium.

The intracellular inorganic nitrate content of the untreated diatom cells increased with increasing period of exposure, whereas, no appreciable increase in the intracellular nitrate was evident in the diatom cells treated with extracts. The extent of intracellular nitrate when calculated on per unit chlorophyll *a* basis exhibited different ratios for both the types of cells (Figs 3.2-3.6). Nitrate/chlorophyll *a* ratios for the untreated cells were found to vary from 0.18 to 0.25. Contrary to this, the ratio for the cells treated with extracts, the values ranged between 0.05 to 0.19. This trend was almost identical for all the extracts under study. The coefficient of variations of triplicate observations ranged between 3-8 % for control and 3-18% for the experimental sets.

The data on variations of cell contents of the diatoms treated with and without extracts for different duration has been presented in Tables 3.3-3.6. It could be seen from the data that the values of particulate organic carbon (POC), chlorophyll *a*, protein and carbohydrates of the untreated cells, exhibited increasing trend in their concentrations with increased exposure period. The concentrations of POC in untreated cells were found to vary between 453.1 $\mu g/dm^2$ at 6 hrs. to 2461.8 $\mu g/dm^2$ at 72 hrs. Chlorophyll *a* values of these cells ranged between 14.0 $\mu g/dm^2$ at 6 hrs. to 63.1 $\mu g/dm^2$ at the end of 72 hrs. As regards protein content of the untreated cells, its concentrations were found to be 155.3 $\mu g/dm^2$ at 6 hrs., which increased to 1770.3 $\mu g/dm^2$ at the end of 72 hrs. Similarly, it was observed that the concentrations of carbohydrate in these cells increased from 314.3 $\mu g/dm^2$ at 6 hrs. to 1086.7 $\mu g/dm^2$ at the end of 72 hrs. exposure period. However, these values when calculated and expressed on the basis of per unit chlorophyll *a*, exhibited very marginal increase in the concentrations of carbohydrate at the end of 72 hrs of exposure period (Tables 3.7-3.9).

As regards to the diatoms treated with individual extract, it exhibited different pattern in the concentration of cell contents as compared to that of untreated cells. The POC values were 219.8 $\mu g/dm^2$ for 6 hrs. and 1088.4 $\mu g/dm^2$ for the exposure period of 72 hrs. The values of protein concentrations for these cells at the end of 6 hrs. and 72 hrs. were 87.1 $\mu g/dm^2$ and 1063.8 $\mu g/dm^2$ respectively. Contrary to this, the chlorophyll values were found to decrease in these cells with increase of exposure period. As regards carbohydrate content of these cells, it was

found to increase marginally at the end of 48 hours and decreased at the end of 72 hrs of exposure period. However, the concentration of these parameters when calculated and expressed in terms of per unit chlorophyll *a* basis, increasing trend was observed in all the parameters at the end of 72 hrs. A maximum of 16 to 44 fold increase in the concentration of these parameters was observed at the end of 72 hrs. of exposure period.

The data on the concentrations of different sugar fractions such as acid soluble, alkali soluble and residual have been presented in figures 3.7-3.11. It is seen from these figures that the extent of metabolism of these three sugars is different in extract treated and untreated cells. The concentration of these sugars was found to be higher in the untreated cells than in the treated cells. Though the difference in the concentrations of individual sugar in both the types of cells is marginal at 6 hrs., the difference was quite high at the end of 72 hours exposure period. The concentration of these sugars in untreated diatom cells exhibited gradual increase in the concentration with increase of exposure period. On the other hand, no appreciable change in the concentration of these sugars was observed for increasing exposure period in the cells treated with extracts. Amongst the three sugar fractions, acid soluble fraction was higher and ranged between 24 $\mu g/dm^2$ at 6 hours to 1002 $\mu g/dm^2$ at 72 hours exposure periods in untreated cells. The alkali soluble fraction in these cells for the corresponding period ranged between 3 $\mu g/dm^2$ and 140 $\mu g/dm^2$. The residual fraction of the sugars in these cells for 6 hrs. and 72 hrs. ranged between 4 $\mu g/dm^2$ and 313 $\mu g/dm^2$

respectively. As regards the extent of concentrations of the acid soluble sugar in treated cells, it ranged between 5 $\mu g/dm^2$ at 6 hours to 52 $\mu g/dm^2$ at 72 hours of exposure. The alkali soluble fraction in these cells varied from 0.6 $\mu g/dm^2$ at 6 hours to 40.2 $\mu g/dm^2$ at 72 hours exposure. The residual fraction content in these cells ranged between 6.2 $\mu g/dm^2$ at 6 hrs. to 27.4 $\mu g/dm^2$ at 72 hrs. exposure.

3.4 DISCUSSIONS

Despite the considerable efforts for the search of environmentally safe antifouling compounds, little is known about their mechanism of action on fouling organisms. In light of this, the present work is aimed at finding out the antifouling effect of the plant extracts under study on some of the diatoms from the fouling community. It has been observed that, there has been a considerable effect of the extracts on the metabolic activities of the treated cells as compared to the untreated cells. The uptake of essential nutrients such as, silicate and nitrate in untreated diatoms is related to exposure period. Uptake of nutrients is the removal of the nutrients from the surrounding medium by the diatoms *N. subinflata*. These cells appeared to be utilizing silicate at higher rate (89%) as compared with the nitrate uptake rate (<50%) at the end of 72 hrs. of exposure period. The higher uptake rate of the silicate from the medium suggests that the nutrient might have been used by the untreated alga for building the cell wall. Contrary to this, the cells which were treated with the extracts appeared to have utilized negligible amount of both the nutrients. This suggests that the cell bound enzyme

which is responsible for transporting the nutrients through the cell wall, might have been affected. This could be one of the reasons, due to which the growth of extract treated cells was inhibited as evident from the chlorophyll *a* data. The present results on the silicate as well as nitrate uptake by the treated cells are in agreement with the results obtained by others with metal toxins (Goering et al., 1977; Harrison, 1977; Thomas and Robinson, 1986).

It has been reported that copper interferes with silicate metabolism in diatoms (Goering et al., 1977; Morel et al., 1978). Furthermore, it has been found that diatom cells respond to toxic levels of metals such as cobalt, copper, mercury, lead and selenium by depressed cell division rates and increased cell size (Shrift, 1959; Nuzzi, 1972, Erickson, 1972; Blankenship and Wilbur, 1975; Davies, 1976; Bentley-Mowat and Reid, 1977; Foster, 1977; Rivkin, 1979). Increase of cell size was attributed to the uncoupling of photosynthesis from division, i.e. they continue to photosynthesize, but were unable to divide (Nicholas and Gary, 1981).

It has been reported that uncoupling of photosynthesis from cell division and consequent cell enlargement may result from nitrogen or silicon limitation as well as from metallic toxins, whereas, nitrogen uptake appears to remain unaffected in *Asterionella japonica* cells which were treated with metallic toxin (Harrison et al., 1977). Furthermore, it has been reported by Harrison et al., (1977) that silicon per cell does not increase at a pace equal to other factors such as dry weight, volume, nitrogen, chlorophyll etc. It therefore, appears that

copper interferes either directly or indirectly with silicon uptake in *A japonica*. This interference may lead to reduced rate of cell division as the each diatom cell has to regulate its silicon uptake for frustule formation and consequently the cell division.

Silicic acid uptake in diatoms as examined with *Navicula pelliculosa* appears to be regulated by sulfhydryl (-SH) groups on the cell surface (Lewin, 1954) . Silicon uptake declined in the presence of -SH binders like iodoacetamide or cadmium chloride. Mercury, copper and silver are very reactive with -SH groups (Lehninger, 1970) and can inhibit enzymes (i.e. some ATPase) whose active sites contain them. It could be possible that copper, or other toxic heavy metal may bind to -SH groups and interfere with a number of associated metabolic pathways one or more of which are essential for maintaining normal cell division rates.

It is believed that silicate uptake from the growth medium is apparently limited to the cell wall formation stage of the division cycle and once taken up it is rapidly incorporated into the growing shell (Coombs & Volcani, 1968). Furthermore, it is believed that diatoms do not accumulate and store silica during the early stage of the cycle for use later in cell wall formation (Coombs & Volcani, 1968). The actual mechanism whereby $Si(OH)_4$ is taken into the cell, transported across it and polymerized in the SiO_2 deposition process is not known. The process is aerobic, temperature dependent and occurs only in living cells (Coombs and Volcani, 1968). It is likely that water-soluble sulfhydryl group in the cell membrane or enzymes are involved in $Si(OH)_4$ uptake (Lewin, 1955). Because of the implication of cell bound enzymes and

sulfhydryl groups with $Si(OH)_4$, uptake, copper is believed to inhibit diatom growth by inhibiting $Si(OH)_4$ uptake (Goering et al., 1977). From the present results it could be assumed that similar mechanism may be taking place, which might have resulted in inhibition of silicate uptake in diatoms treated with the plant extracts. Exposure of diatoms to the extracts under study does not appear to be lethal. If it were, one would expect drastic increase in the release of silicate from shells, unless the extracts act as retardants to dissolution (Goering et. al., 1977).

The results on the intracellular inorganic nitrate stock of the extract treated and untreated diatom cells suggest that relatively low inorganic nitrate reserve was maintained by the extract treated cells as compared to the untreated cells. These results substantiate the data on nitrate uptake from the medium. It could be presumed that the initial internal nitrate reserve might have been utilized by the treated cells at very slow pace for survival, but failed to multiply. It could also be assumed that the synthesis of the enzyme which is responsible for nitrate reduction (NR) might have been either affected or its activity might have been reduced (Harrison et al., 1977). These results suggest that the transfer of inorganic nitrate from the surrounding medium into the cell was affected, which might have resulted in low amount of intracellular inorganic nitrate.

It has been established that when inorganic nitrogen is taken up by the phytoplankter cells, it is not only assimilated primarily into protein, but also assimilated into DNA, RNA, pigments and nitrogen containing lipids (Syrett, 1953; Thomas and Krauss, 1955; Krey, 1958;

Reisner et al., 1960; Lui and Roels, 1972; Conover, 1975a,b; Picard, 1976; Wheeler, 1977; Rhee, 1978; Maske, 1979). Consequently, the assimilation of inorganic nitrogen into the above metabolites inside the cell, leads to cell growth. However, protein-N can constitute as little as 10% (Conover, 1975) and as much as 95% (Thomas and Krauss, 1955) of the total cellular nitrogen. The remaining cellular nitrogen consists of the assimilated nitrogen compounds mentioned above, and unassimilated nitrogen compounds such as nitrate ammonium, and aminoacids (Thomas and Krauss, 1955, Eppley and Coats-worth, 1968; Lui and Roels, 1972; Conover, 1975, Malone et al., 1975; Collos and Slawyk, 1976,1977; Picard, 1976; Wheeler, 1977; Kahn and Swift, 1978; Rhee, 1978; Demanche et al., 1979; Maske, 1979). The nitrogen assimilation in a diatom cell is represented schematically in figure 3.1.

The temporal variations in the cell contents, such as chlorophyll *a*, protein, particulate organic carbon and carbohydrate in the diatoms treated with the extracts and the untreated ones revealed that marginal increase in the concentration of cell protein and POC was observed, whereas, in the chlorophyll *a* concentration slight decline was observed. The carbohydrate concentration in these cells did not exhibit appreciable change. Contrary to this, the untreated diatom cells exhibited increase of all the above parameters with respect to increase of exposure period. The values of these parameters when calculated and expressed in relation to per unit value of the chlorophyll *a* , suggest that the values of these parameters are a function of chlorophyll *a* content. On the other hand the cells treated with extracts, exhibited increase of these parameters per unit of chlorophyll *a*. In the untreated

diatom cells the production of POC, protein and carbohydrate appears to have maintained almost constant ratios for increasing exposure periods. These results suggest that production and metabolism of these parameters are well balanced in untreated cells. Contrary to this, the cells which were treated with extracts, exhibited increasing trend in the ratios for above parameters at the end of 72 hrs. (Tables 3.7-3.9). These results suggest that the process of production of these parameters was faster than that of metabolism, resulting in accumulation of these parameters in the cells. It is well known that the extent of cell contents of any diatom cell is controlled by the availability of nutrients. It has been reportedly found that normal diatom cells produce higher amount of carbohydrates, protein and POC under nutrient stresses. It therefore, could be inferred that though enough quantities of nutrients were available for the growth of treated cells, they could not utilize these nutrients, resulting in stressed condition which might have triggered the production of these parameters.

Carbohydrates are common storage and structural compounds in organisms. The production and chemical composition of these compounds in micro-organism may be influenced by several factors including nutrient availability in the growth medium and the growth phase of the organism. Cellular carbohydrates from diatoms are divided into two groups such as, storage and structural carbohydrates (Handa, 1969); Haug and Myklestad, 1976). The structural carbohydrates can be further divided into dilute alkali soluble and residual carbohydrates (Haug et al., 1973). Thus the storage and structural

carbohydrates of the diatoms under study were isolated by sequential extraction of algal cells with dilute acid and dilute alkali respectively.

Differences in the relative concentrations of carbohydrate fractions were observed in both, untreated as well as treated cells. The results indicated that acid-soluble carbohydrate in these cells comprised the major component, whereas, the dilute alkali-soluble and residual carbohydrates were the minor components of the total cellular carbohydrate. It has been reported by Myklestad, (1977), that acid-soluble fraction of the cellular carbohydrate contains mainly β-1,3, glucan. Furthermore, it has been opined that β-1,3, glucan fraction is more likely to be associated with living material only (Myklestad, 1977). Therefore, the presence of higher amount of acid-soluble fraction in both the type of cells in the present work, indicates that these cells may contain living material. From these results it can be inferred that the plant extracts under study were nontoxic.

Several studies have indicated that diatoms produce higher amount of carbohydrates and lower amount of protein during nitrate depleted conditions (Antia et al., 1963). On the other hand protein content increases during exponential stage of diatoms. The present results on the cell carbohydrates and protein of the diatom species do not agree well with the results reported earlier. These differences in the results could not be explained and it need further studies. However, the diatoms used in the present study belong to fouling community, whereas, the one used in the earlier studies belong to non-fouling community. It is therefore, thought that the differences in the type of diatoms used in the

two studies may be one of the reasons for differences in the results. According to the present results the cell carbohydrates and protein contents of the extract treated cells increased with the increase of exposure period.

It is believed that a high protein-carbohydrate ratio generally indicates rapid growing population, whereas, low protein-carbohydrate ratio shows nutrient stressed conditions in the diatom cells. Contrary to this, in the present investigation increase of the protein-carbohydrate ratios have been observed in both the type of cells for 72 hrs., exposure period. However, the values of ratio were marginally higher for treated cells than that of untreated cells (Table 3.10). The difference of protein-carbohydrate ratio especially at 72 hrs., cannot be explained and needs further studies. However, it is likely that the extract treated cells failed to produce cell carbohydrates resulting in the increase of protein-carbohydrate ratio. Chlorophyll *a* contents of these cells also supplement this fact.

The data on the carbohydrate fractions in the extract treated diatom cells indicated that the carbohydrate reserve decreased considerably as compared to the carbohydrates of untreated cells. These results lead to infer that the extract treated cells might have utilized their carbohydrate reserve for overcoming the stress caused by the extracts. It has been reported in the past that the diatom cells can detoxify the effect of any toxicant by producing extracellular carbohydrate, which binds the toxic ions to it (Daniel and Chamberlain, 1981). Similarly the extract treated cells might have also used its carbohydrate reserve possibly for the same purpose, resulting in the

decrease of carbohydrate reserve. It is also likely that these cells might have used its carbohydrate reserve for conversion into either biochemical constituents such as protein and lipids (Handa, 1969), which is evident from higher protein carbohydrate ratios in the present investigation.

It is evident from the present investigation that the plant extracts of *C. spinosa*, *S. laurifolius*, *A. pennata*, *G. glauca* and *B. acutangula* interfered with the metabolic activities of the fouling diatoms. It appears that the influence of these plant extracts on the physiological processes of the diatoms leads to inhibition of growth, which in turn can lead to antifouling.

Table 3.1 Temporal variations in silicate uptake (%) by *N. subinflata*, exposed to Ec_{100} concentrations of the plant extracts.

Time (Hrs.)	Control	*C. spinosa*	*S. laurifolius*	*G. glauca*	*A. pennata*	*B. acutangula*
6	0.38	0.10	0.21	0.71	0.97	0.54
12	9.24	0.88	0.03	0.15	0.38	0.38
24	11.44	0.71	0.08	0.12	0.54	0.18
48	53.82	0.24	0.88	0.46	1.00	0.07
72	89.74	0.13	0.54	0.11	0.54	0.71

Table 3.2 Temporal variations in nitrate uptake (%) by *N. subinflata*, exposed to Ec_{100} concentrations of the plant extracts.

Time (Hrs.)	Control	*C. spinosa*	*S. laurifolius*	*G. glauca*	*A. pennata*	*B. acutangula*
6	0.00	0.25	0.00	0.00	0.00	0.00
12	15.30	1.20	2.40	1.80	1.13	1.24
24	21.50	1.90	2.72	1.58	2.53	1.71
48	34.49	2.63	2.24	2.26	2.46	2.36
72	46.13	1.93	2.46	2.98	2.02	3.59

Table 3.3 Temporal variations in the P.O.C. content (µg/dm^2) of *N. subinflata*, exposed to Ec_{100} concentration of the plant extracts.

Time (Hrs.)	Control	*C. spinosa*	*S. laurifolius*	*G. glauca*	*A. pennata*	*B. acutangula*
6	453.1 (±28.3)	261.5 (±22.5)	219.8 (±27.6)	309.9 (±44.2)	311.8 (±25.9)	220.7 (±21.8)
12	741.6 (±79.8)	494.1 (±30.7)	803.4 (±83.5)	524.1 (±66.7)	405.2 (±48.6)	366.0 (±30.4)
24	1173.3 (±66.2)	413.8 (±45.3)	971.5 (±67.8)	784.3 (±82.6)	622.4 (±71.3)	641.5 (±53.5)
48	1691.3 (±84.8)	388.8 (±60.4)	884.5 (±92.3)	422.8 (±58.3)	548.6 (±32.4)	868.3 (±39.1)
72	2461.8 (±101.4)	494.5 (±51.8)	720.4 (±47.8)	482.0 (±28.2.8)	606.9 (±67.8)	1088.4 (±89.2)

Figures in brackets indicate standard deviation of three replicate values

Table 3.4 Temporal variations in the chlorophyll *a* content (µg/dm^2) of *N. subinflata*, exposed to Ec_{100} concentration of the plant extracts.

Time (Hrs.)	Control	*C. spinosa*	*S. laurifolius*	*G. glauca*	*A. pennata*	*B .acutangula*
6	14.0 (±1.0)	6.9 (±0.3)	9.2 (±0.4)	12.6 (±1.5)	9.2 (±0.7)	7.8 (±0.4)
12	23.3 (±1.6)	17.1 (±0.8)	15.6 (±0.8)	16.8 (±1.2)	13.6 (±1.1)	14.7 (±1.2)
24	31.2 (±1.5)	12.1 (±1.2)	13.4 (±1.0)	11.5 (±1.6)	11.4 (±0.8)	13.2 (±0.8)
48	53.0 (±2.1)	3.0 (±0.2)	5.8 (±0.3)	2.7 (±0.6)	6.5 (±0.7)	8.1 (±0.5)
72	63.1 (±1.8)	1.4 (±0.2)	2.4 (±0.2)	1.2 (±0.4)	2.1 (±0.3)	3.0 (±0.2)

Figures in brackets indicate standard deviation of three replicate values

Table 3.5 Temporal variations in the protein content (µg/dm^2) of *N. subinflata*, exposed to Ec_{100} concentration of the plant extracts.

Time (Hrs.)	Control	*C. spinosa*	*S. laurifolius*	*G. glauca*	*A. pennata*	*B. acutangula*
6	155.3 (±11.2)	128.3 (±17.4)	120.5 (±21.4)	148.6 (±20.1)	114.0 (±13.6)	87.1 (±1.8)
12	313.6 (±15.0)	229.1 (±36.8)	402.4 (±51.3)	193.2 (±8.7)	208.0 (±27.5)	180.8 (±17.3)
24	856.1 (±8.6)	410.1 (±52.7)	564.1 (±80.5)	385.2 (±15.8)	487.9 (±38.8)	208.5 (±20.7)
48	1438.3 (±20.1)	370.2 (±62.7)	386.2 (±62.0)	311.5 (±30.2)	581.7 (±41.3)	471.4 (±24.8)
72	1770.3 (±25.4)	473.2 (±48.1)	690.9 (±42.3)	509.7 (±28.8)	656.8 (±28.9)	1063.8 (±36.9)

Figures in brackets indicate standard deviation of three replicate values

Table 3.6 Temporal variations in the carbohydrate content (µg/dm^2) of *N. subinflata*, exposed to Ec_{100} concentration of the plant extracts.

Time (Hrs.)	Control	*C. spinosa*	*S. laurifolius*	*G. glauca*	*A. pennata*	*B. acutangula*
6	314.3 (±32.7)	134.5 (±27.6)	112.2 (±12.9)	209.1 (±28.2)	108.5 (±13.7)	76.4 (±12.6)
12	483.4 (±38.4)	294.1 (±48.3)	524.1 (±89.3)	292.3 (±24.7)	171.3 (±27.4)	127.8 (±17.1)
24	437.9 (±48.6)	150.0 (±18.9)	737.0 (±64.2)	526.7 (±72.1)	215.4 (±30.6)	294.3 (±28.8)
48	736.4 (±84.3)	120.6 (±12.7)	488.9 (±70.1)	216.5 (±31.5)	417.9 (±62.0)	519.2 (±57.3)
72	1086.7 (±112.8)	57.5 (±8.1)	276.4 (±22.8)	129.7 (±27.2)	194.4 (±31.8)	294.6 (±49.0)

Figures in brackets indicate standard deviation of three replicate values

Table 3.7 Temporal variations in the ratios of POC-Chl *a* of *N. subbinflata* exposed to Ec $_{100}$ concentration of the plant extracts.

Time (Hrs.)	Control	*C. spinosa*	*S. laurifolius*	*G. glauca*	*A .pennata*	*B. acutangula*
6	32.2	37.9	23.9	24.6	33.9	28.3
12	31.7	28.9	51.5	31.2	29.8	24.9
24	37.6	34.2	72.5	68.2	54.6	48.6
48	31.9	129.6	152.5	156.4	84.4	107.2
72	38.9	341.1	300.2	401.7	289.0	1088.4

Table 3.8 Temporal variations in the ratios of protein - chl *a* of *N. subinflata* exposed to Ec_{100} concentration of the plant extracts.

Time (Hrs.)	Control	*C. spinosa*	*S. laurifolius*	*G. glauca*	*A. pennata*	*B. acutangula*
6	11.0	18.6	13.1	11.8	12.4	8.6
12	13.4	13.4	25.8	11.5	15.3	12.3
24	27.4	33.9	42.1	33.5	42.8	15.8
48	27.1	123.4	66.6	115.4	89.5	58.2
72	28.0	326.4	287.9	424.8	312.8	354.6

Table 3.9 Temporal variations in the ratios of carbohydrate-chl *a* of *N. subinflata* exposed to Ec_{100} concentration of the plant extracts.

Time (Hrs.)	Control	*C. spinosa*	*S. laurifolius*	*G. glauca*	*A. pennata*	*B. acutangula*
6	22.3	19.5	12.2	16.6	11.8	9.8
12	20.7	17.2	33.6	17.4	12.6	8.7
24	14.0	12.4	55.0	45.8	18.9	22.3
48	13.8	40.2	84.3	80.2	64.3	64.1
72	17.2	83.4	115.2	108.1	92.6	98.2

Table 3.10 Temporal variations in the ratios of protein -carbohydrate of *N. sunbinflata* exposed to Ec_{100} concentration of the plant extracts.

Time (Hrs.)	Control	*C. spinosa*	*S. laurifolius*	*G. glauca*	*A. pennata*	*B. acutangula*
6	0.5	0.9	1.1	0.7	1.0	1.1
12	0.6	0.8	0.7	0.6	1.2	1.4
24	1.9	2.7	0.7	0.7	2.2	0.7
48	1.9	3.0	0.8	1.4	1.4	0.9
72	1.6	3.9	2.5	3.3	3.4	3.6

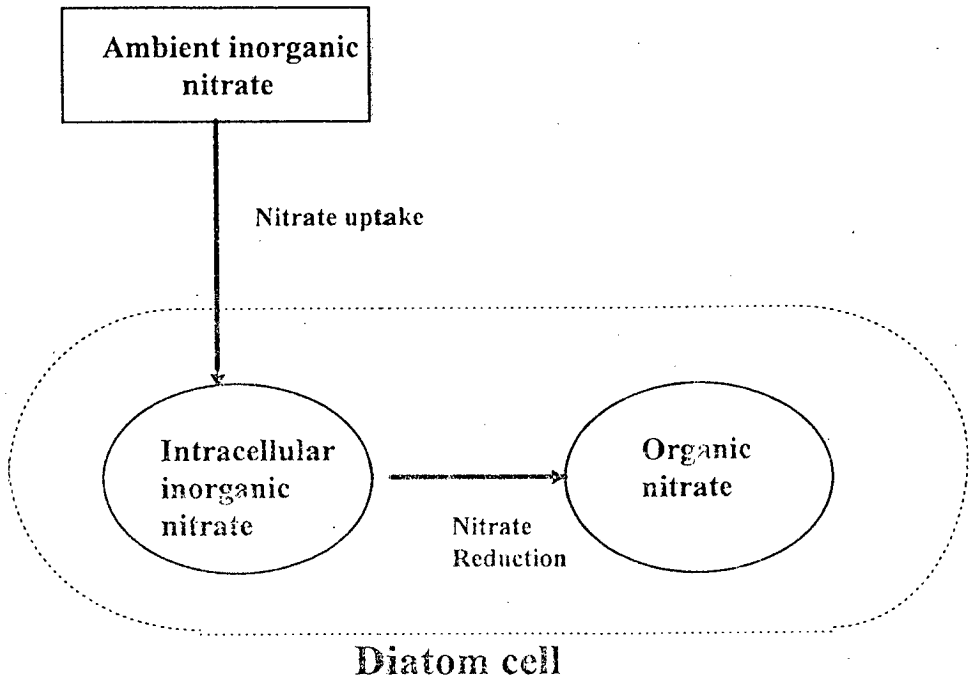


Fig. 3.1 Schematic representation of nitrogen assimilation in the diatom *N. subinflata* cell. (Modified from DeManche et. al., 1979).

Fig. 3.2 Temporal variations in the ratios of intracellular nitrate/chlorophyll *a* of *N. subinflata* treated without and with the extract of *C. spinosa*.

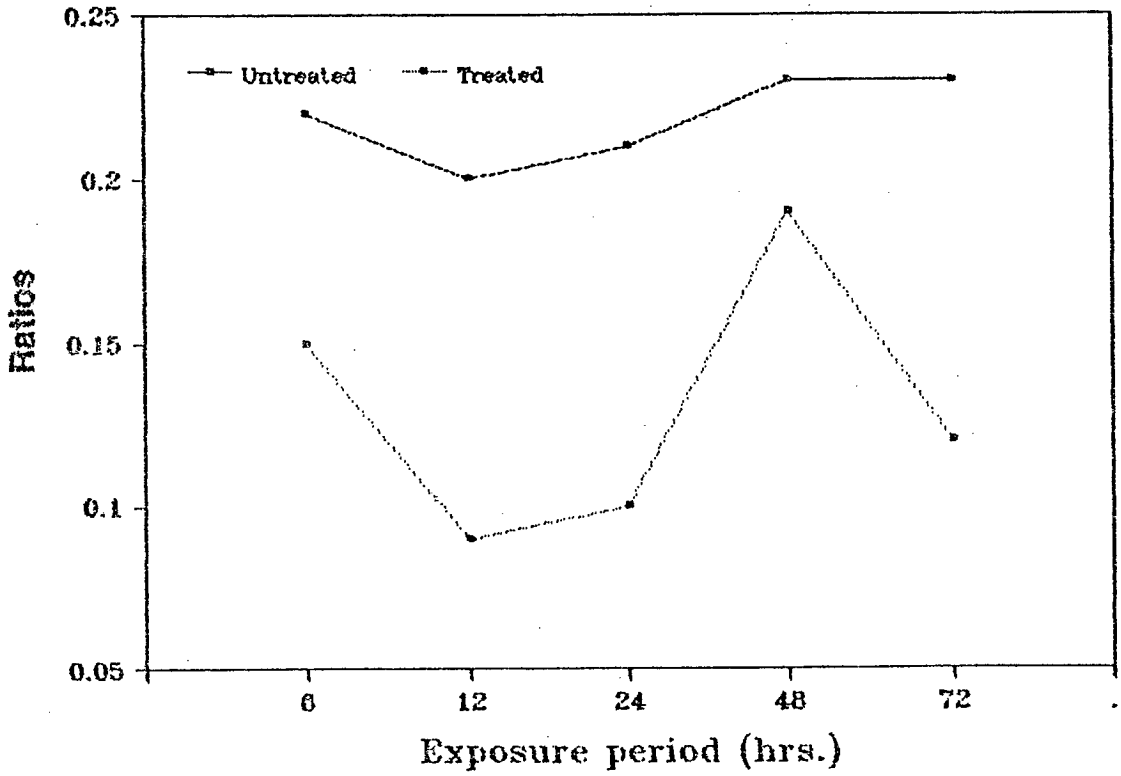


Fig. 3.3 Temporal variations in the ratios of intracellular nitrate/chlorophyll *a* of *N. subinflata* treated without and with the extract of *S. laurifolius*.

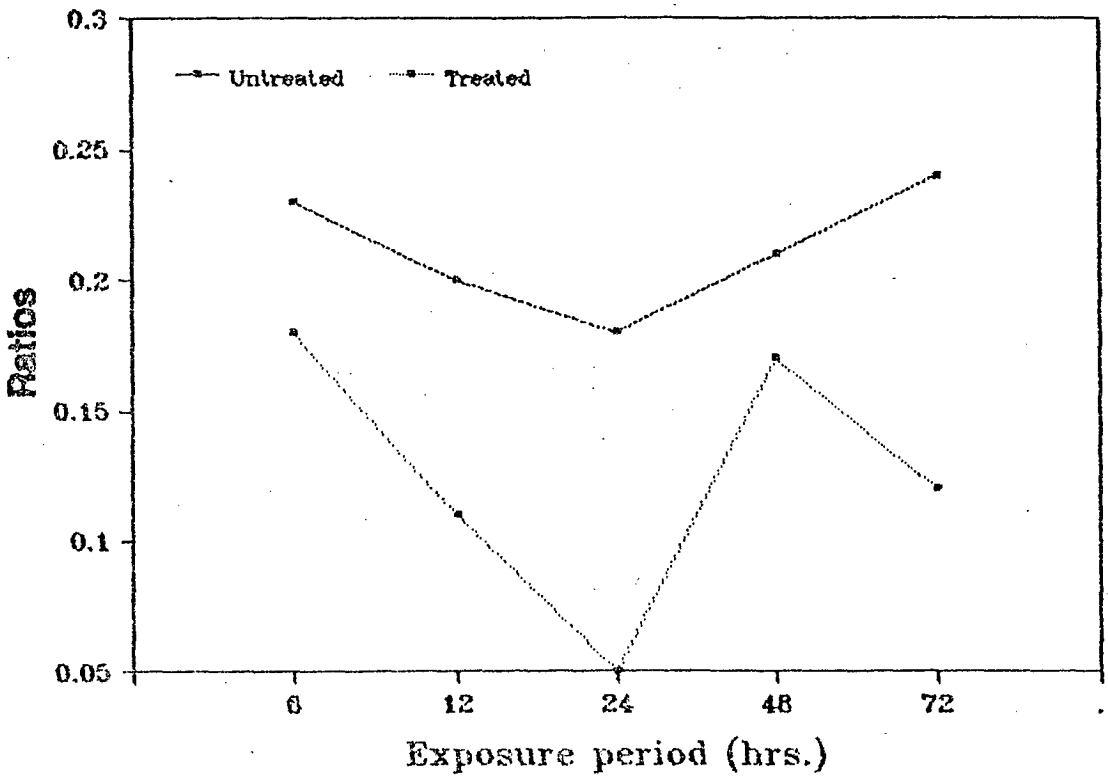


Fig. 3.4 Temporal variations in the ratios of intracellular nitrate/chlorophyll *a* of *N. subinflata* treated without and with the extract of *G. glauca*.

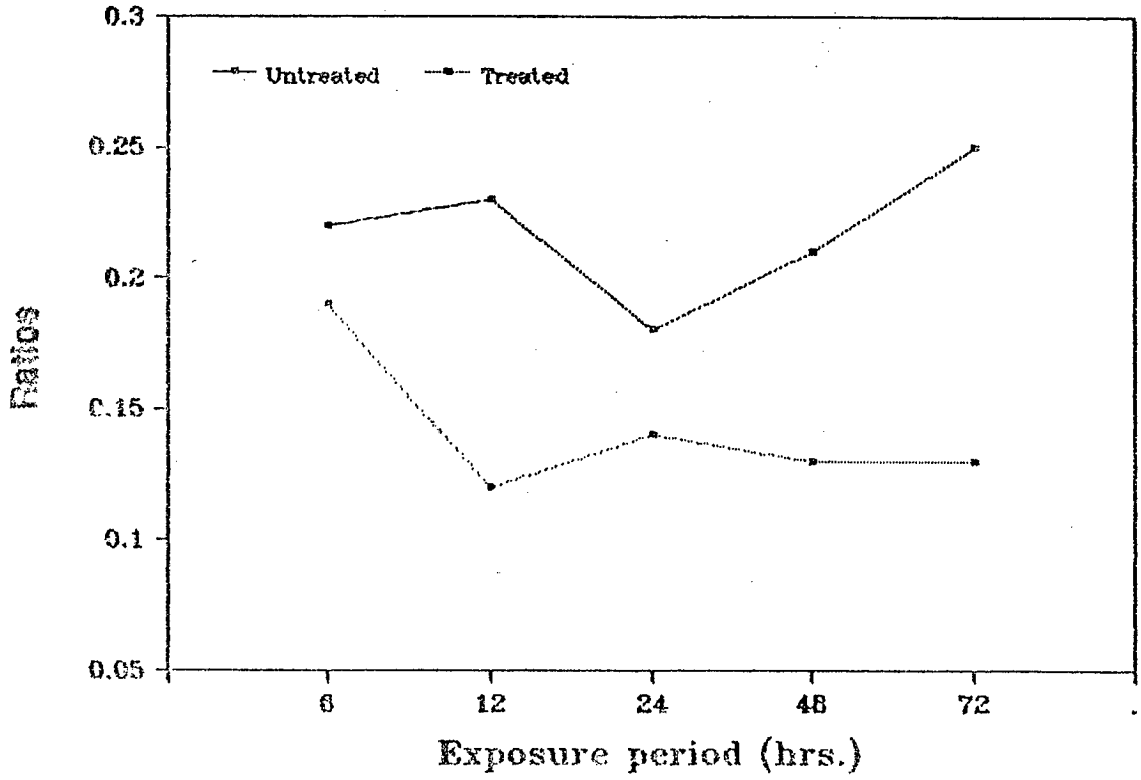


Fig. 3.5 Temporal variations in the ratios of intracellular nitrate/chlorophyll *a* of *N. subinflata* treated without and with the extract of *A. pennata*.

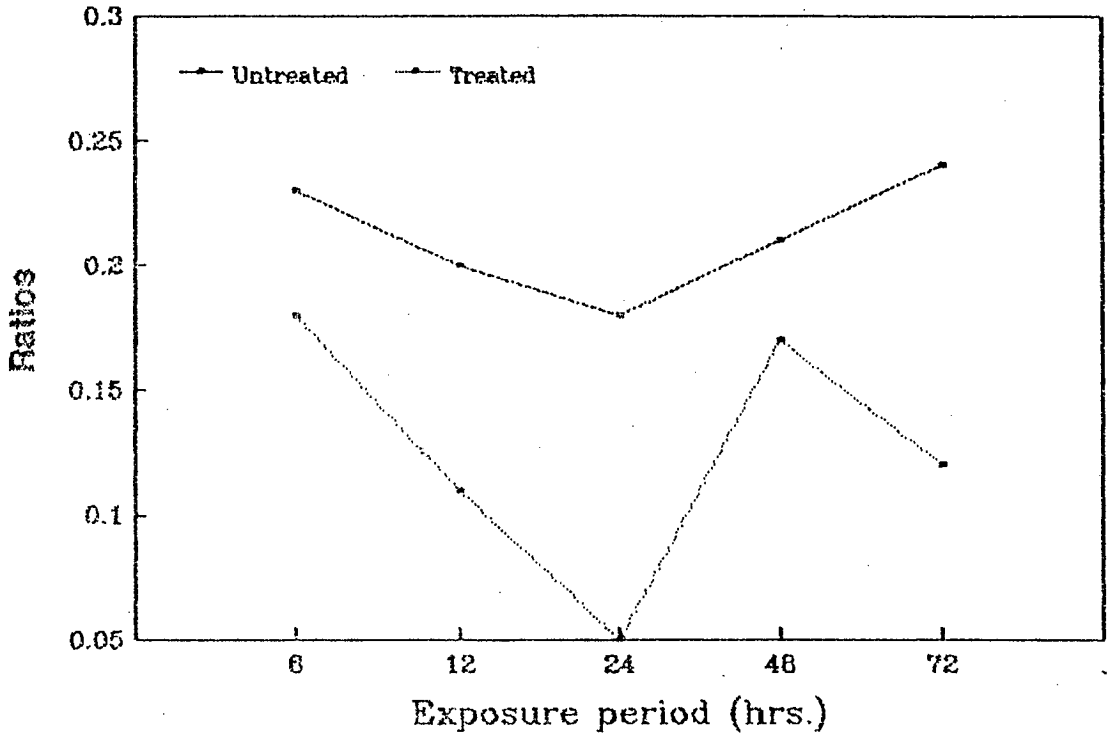


Fig. 3.6 Temporal variations in the ratios of intracellular nitrate/chlorophyll *a* of *N. subinflata* treated without and with the extract of *B. acutangula*.

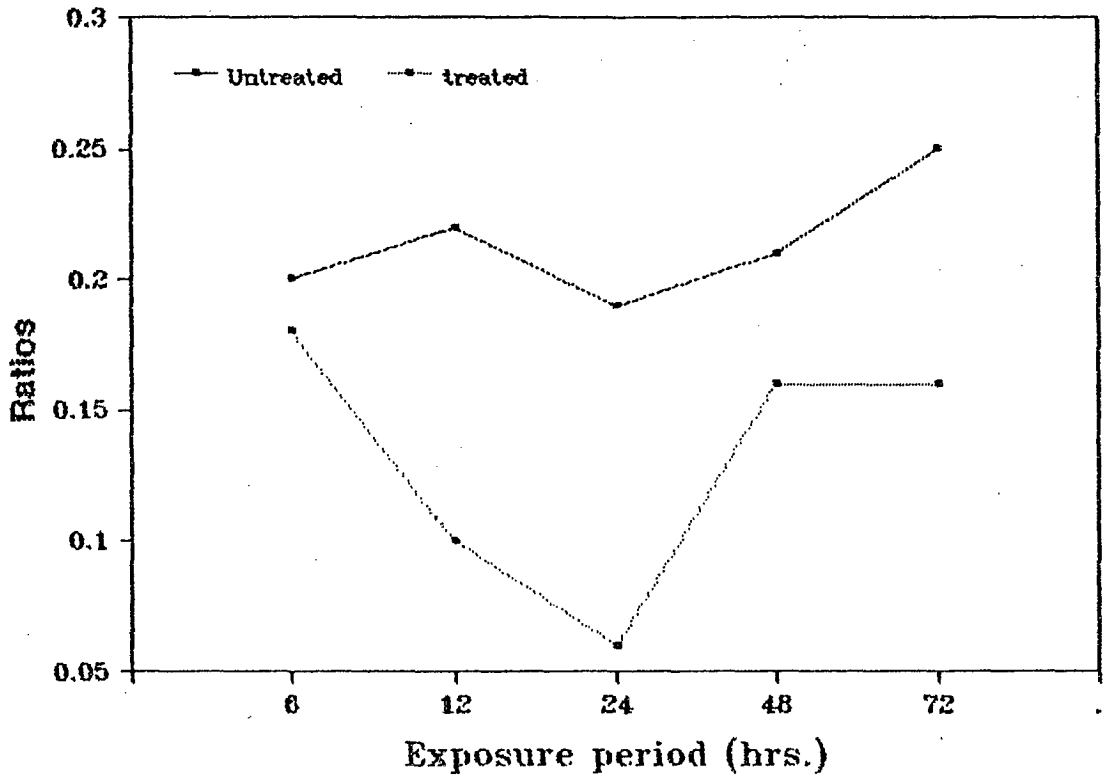


Fig. 3.7 Temporal variations in the concentration of sugar fractions *of N. subinflata* cells, treated without and with the extract of *C. spinosa*.

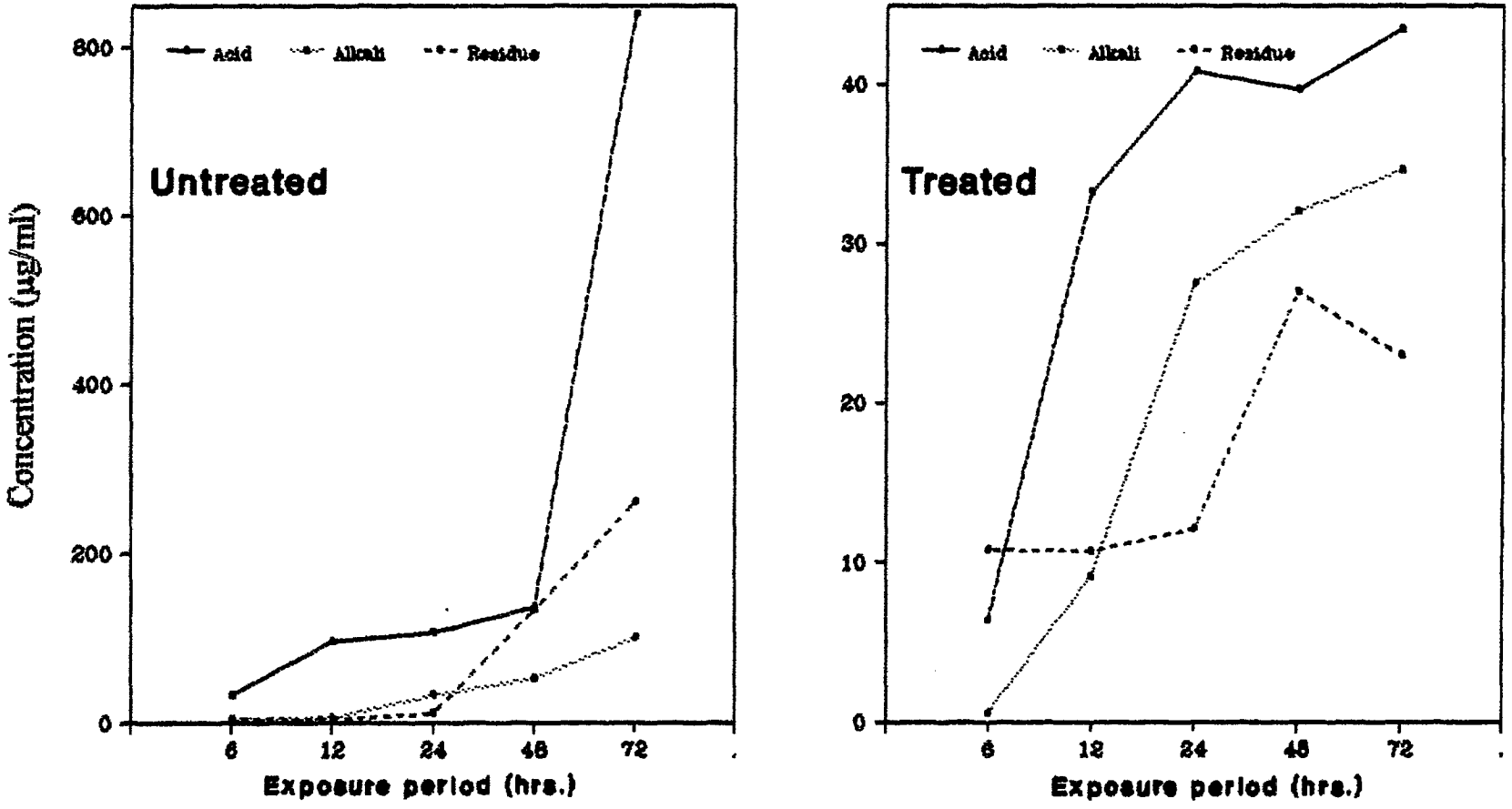


Fig. 3.8 Temporal variations in the concentration of sugar fractions of *N. subinflata* cells, treated without and with the extract of *S. laurifolius*.

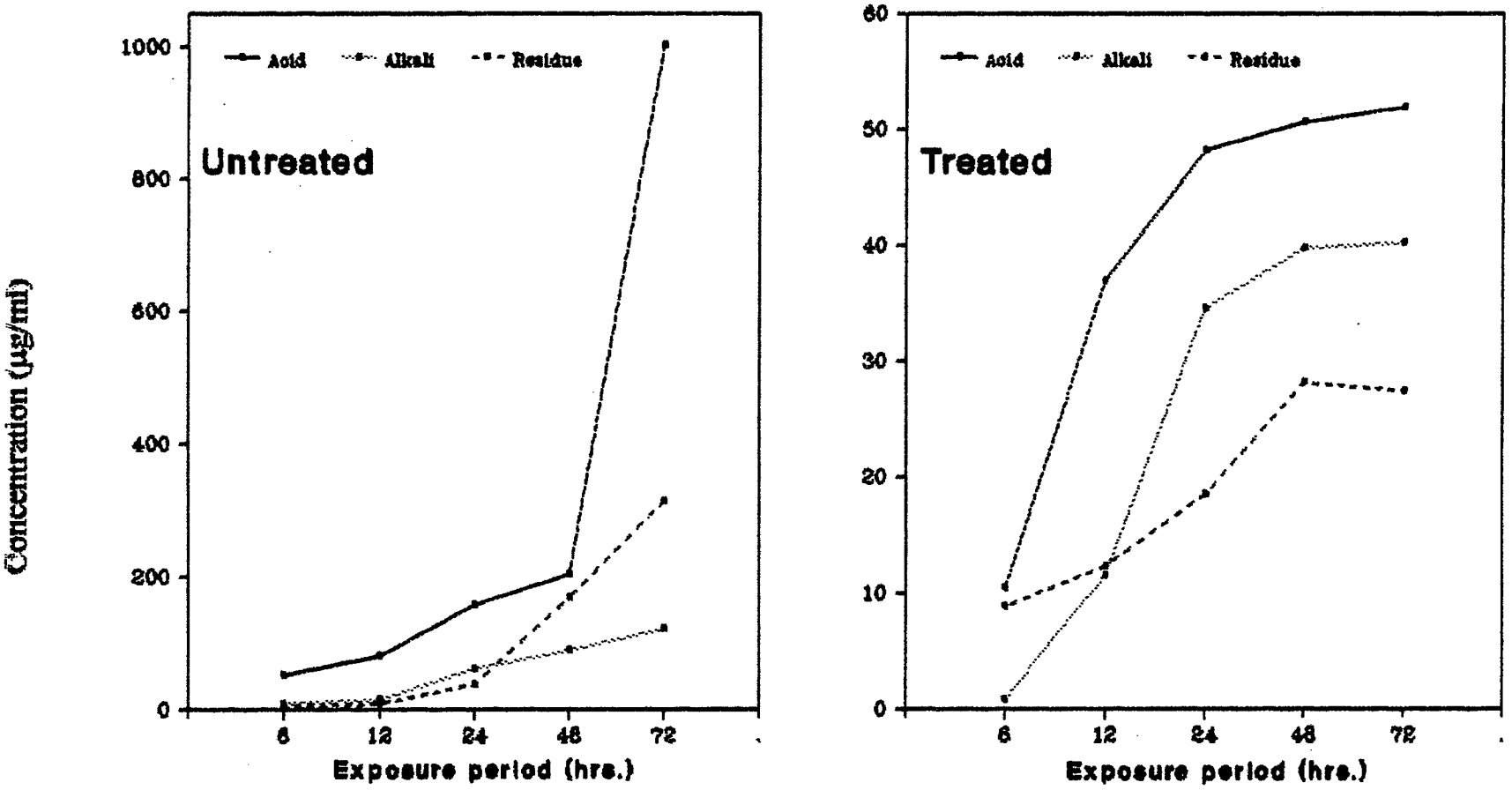


Fig. 3.9 Temporal variations in the concentration of sugar fractions *of N. subinflata* cells, treated without and with the extract of *G. glauca*.

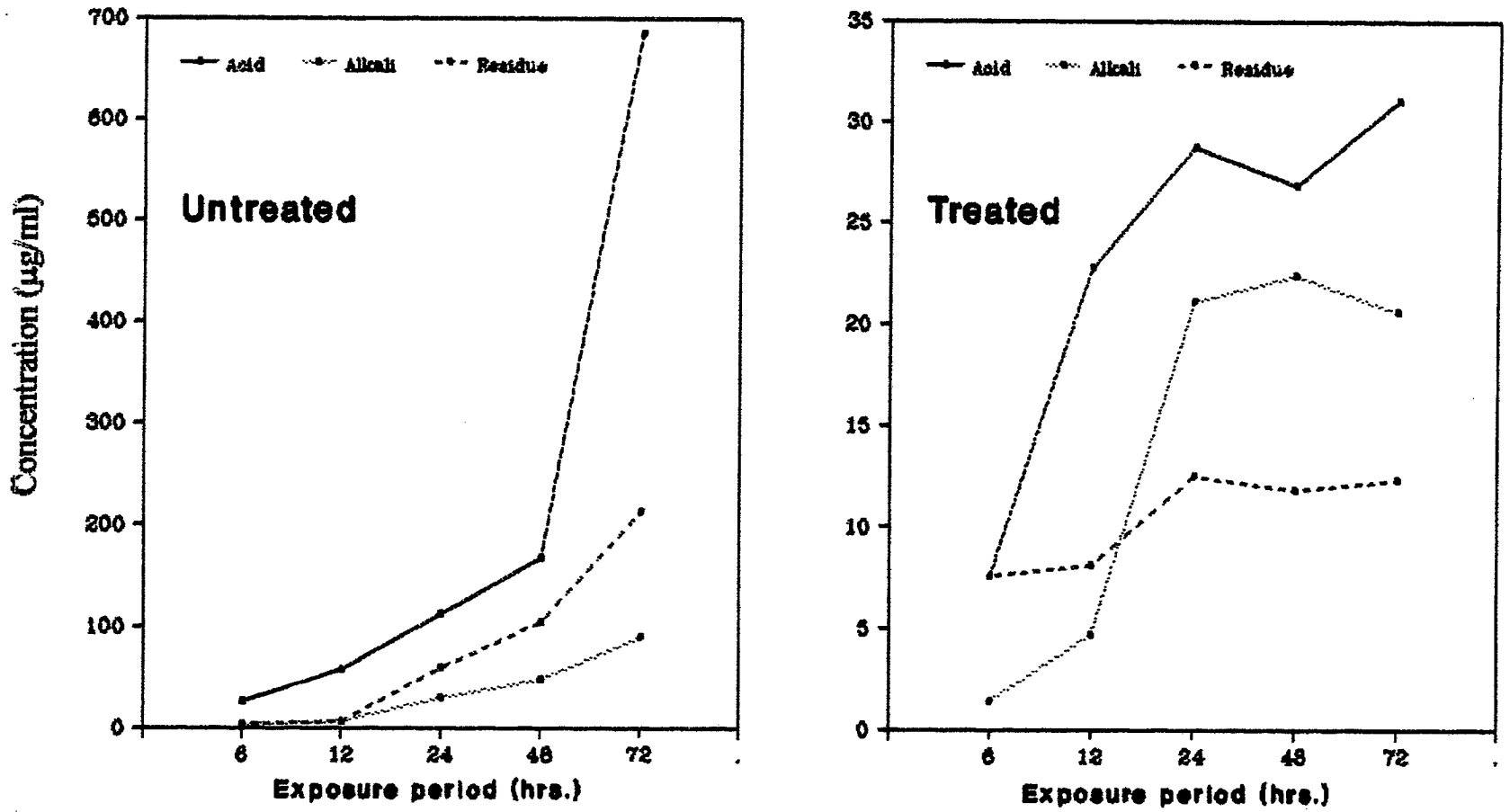


Fig. 3.10 Temporal variations in the concentration of sugar fractions of *N. subinflata* cells, treated without and with the extract of *A. pennata*.

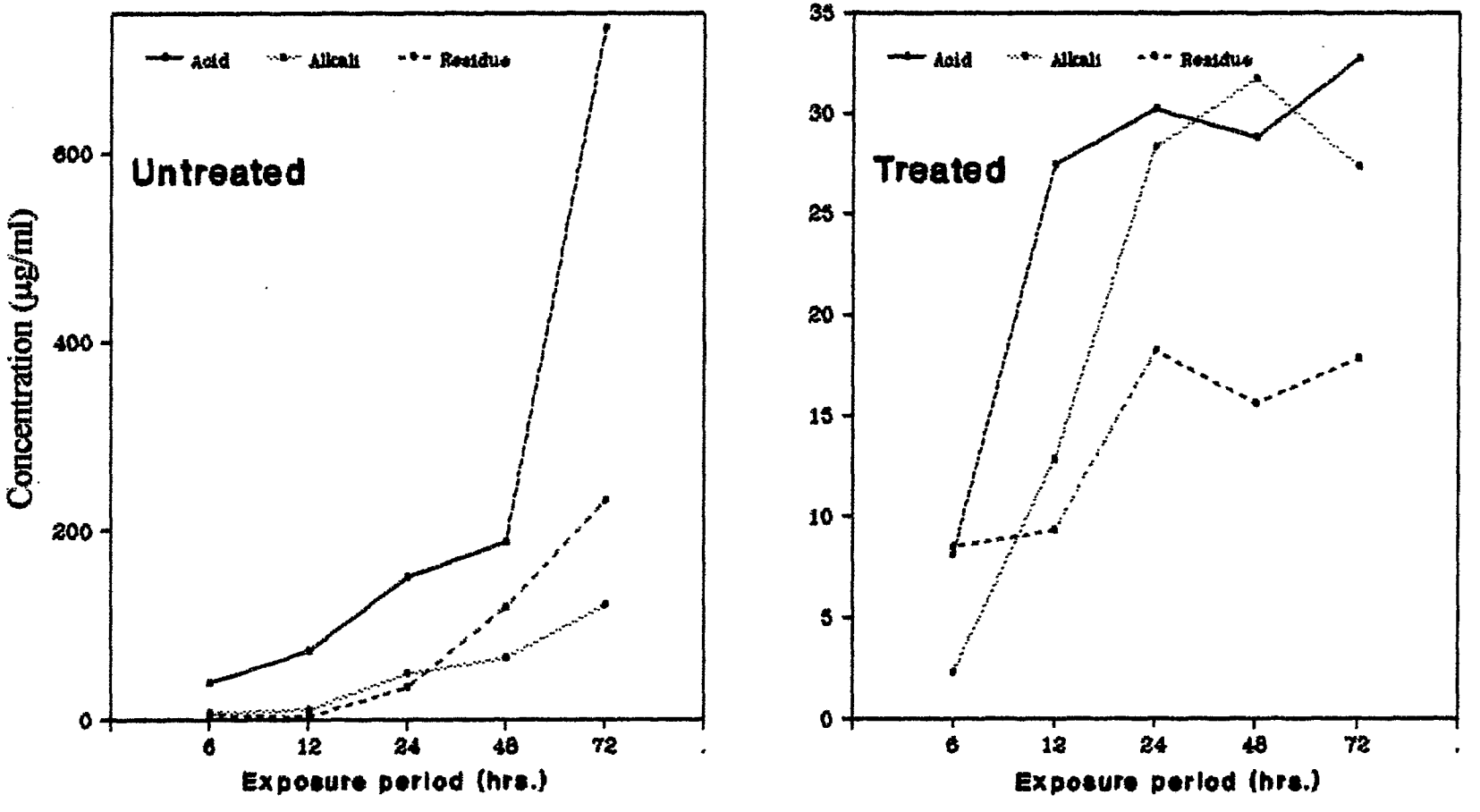
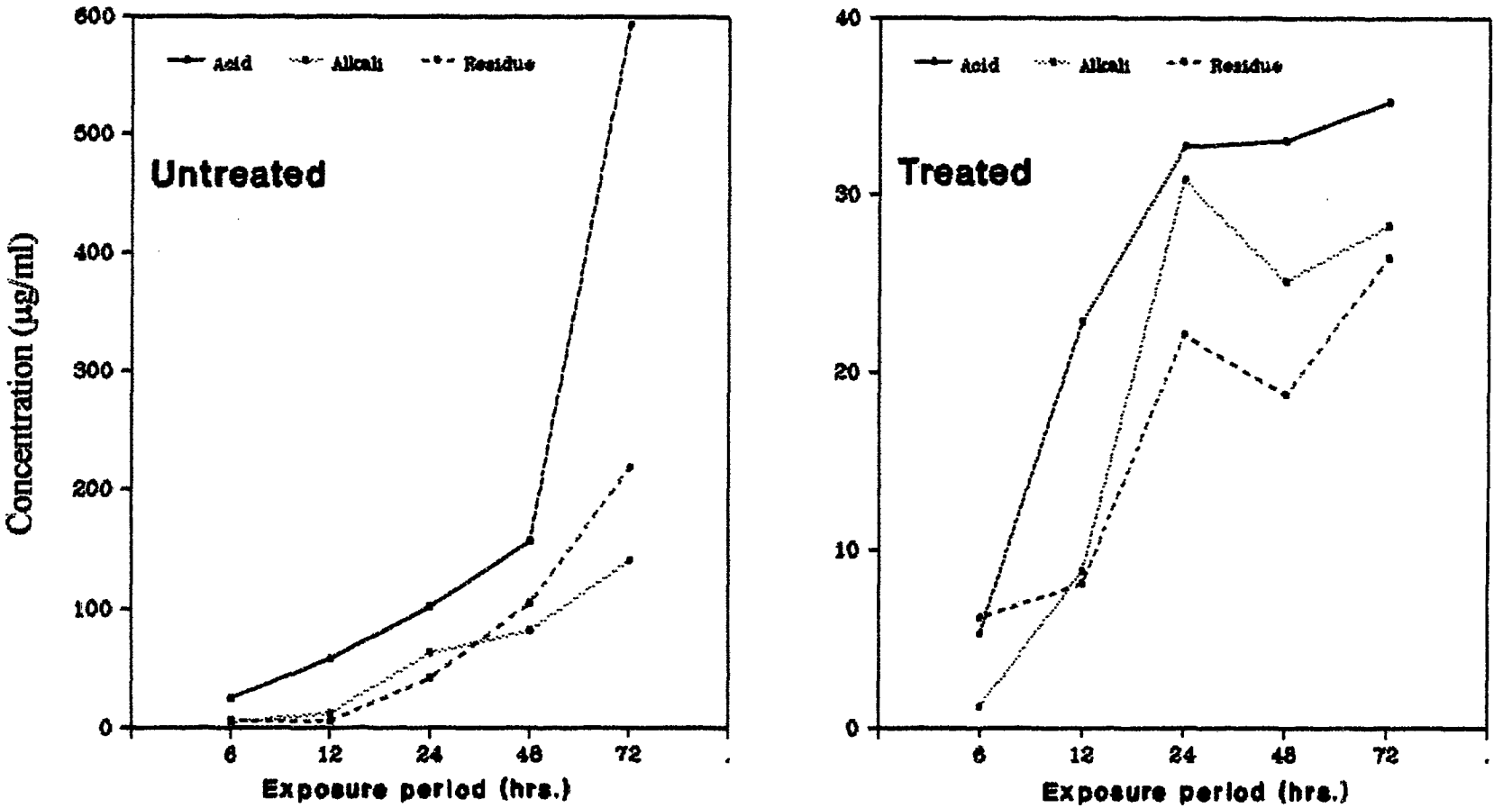


Fig. 3.11 Temporal variations in the concentration of sugar fractions of *N. subinflata* cells, treated without and with the extract of *B. acutangula*.



CHAPTER 4

ISOLATION OF ANTIFOULING COMPOUNDS AND STUDY OF THEIR PARTIAL STRUCTURE

4.1 Introduction

Chemical separation and purification of natural products from a given crude extract is a difficult task due to the structural complexity of these compounds. This involves series of procedures such as extraction, fractionation, and finally purification using modern chromatographic techniques. However, all these steps are to be followed by bioassay with appropriate cultures of organisms. Therefore, the process is referred as bioassay guided separation of compound. The chromatographic techniques are, column chromatography, Preparative Thin Layer Chromatography (PTLC) and High Performance Liquid Chromatography (HPLC).

Among these, ordinary column chromatography, developed more than a century ago by Russian scientist, Tseratt, deserves special mention. In this process, compounds are applied on top of a column packed with a suitable adsorbent and is eluted with solvents which flow downwards by gravity flow. Of course, this is a slow process, requiring large volumes of solvents and yielding only crude separation of compounds. Despite its severe shortcomings, this method is still widely used by chemists and biologists all over the world. Presently a modified version of this method, Viz.," Flash Column Chromatography" is in practice. It uses much smaller column bed particles (Stationary phase) and elution is aided by applying pressure on the top of the column or vacuum at the bottom. This has cut down the time required for chromatography and yields much better resolution.

As regards the Thin Layer Chromatography (TLC), the principle is similar to that of the column chromatography. But in this case, the mobile phase moves upward due to capillary action on a thin layer of adsorbent coated on a glass plate. The compounds are thus subjected to the forces of adsorption (compound verses adsorbents) and capillary of the solution (solubility in the mobile phase), which results in the separation of compounds. Moreover, it is basically an analytical tool, which provides information pertaining to the quality of isolated compound. The nature of isolated compound can also be determined by running appropriate standard simultaneously. On the other hand High Performance Liquid Chromatography (HPLC) analyses compound qualitatively as well as quantitatively. It is at present the final and ultimate tool in chromatography technique for separation and purification of compounds.

Contrary to this, the Preparative Thin Layer Chromatography (PTLC) is basically qualitative TLC. The main difference between analytical TLC and PTLC is that, in analytical TLC, the thickness of the adsorbent is around 0.25 mm, whereas, in case of PTLC, it is 1-2 mm thick. It has certain advantages over column chromatography. The distinct advantages of PTLC are, the use of small amount of solvent, ease of estimating compounds, direct detection on the layer, easy removal of separated zones from the plate and the ability to run reference compounds simultaneously under identical conditions to assist in locating the desired material etc. It is a simple, inexpensive, faster method and provides better resolution for the separation of natural compounds (Friad and Sherma, 1982). However, one disadvantage of

this technique is the lack of a satisfactory method for the permanent recording of results (Wagner et al, 1984). Generally, analyses with this technique are being recorded simply by verbal description of the chromatogram, by schematic drawing, or by idealised coloured diagrams, Rf values etc.. All these methods are clearly makeshift solutions to the problems of documentation. In view of the advantages of the technique, preparative Thin-layer chromatography (PTLC) technique was adopted in the present work for separation and isolation of the natural compounds from the crude extracts of the plants under study. The purity of the active fractions, thus isolated from PTLC were checked by analytical TLC and reverse phase HPLC.

4.2 Materials and Methods

4.2.1 Fractionation by solvent-solvent partitioning

A Known quantity of vacuum dried methanol extract was redissolved in sufficient quantity of aqueous methanol (90%) to obtain a clear solution. The above aqueous methanol solution was initially extracted with hexane for three times by shaking in a separating funnel and collecting the hexane fraction. The aqueous methanol solution was then extracted with chloroform for three times as before and the chloroform fraction was collected. All the three fractions of the chloroforms were combined. The left out portion of the solution is the

aqueous fraction. All the three fractions namely, hexane, chloroform and aqueous were tested for biological activity using fouling diatom, *N. subinflata*. For this purpose residue of each fractions ranging from 15 - 20 mg weight was dissolved in 15 ml F2 medium. To this, known quantity of diatom cells were added. The chlorophyll *a* concentration was assessed after three days incubation period at room temperature with 12 h light and 12 h dark cycle. The chlorophyll *a* concentration of the fraction treated cells was compared with the chlorophyll *a* of the diatom cells in the control flask. Based on these results the fractions which exhibited positive results were subjected for isolation of compounds using preparative TLC technique.

4.2.2 Preparative TLC

Before proceeding with separation of compounds by preparative TLC, the biologically active fractions were subjected to analytical TLC in order to decide the appropriate solvent system. The plate with the spots was developed sequentially in solvents of increasing polarity. The binary solvent systems comprising of Hexane and chloroform, or chloroform and methanol were used for the purpose.

As regards the preparative Thin-layer chromatography (PTLC), glass plates admeasuring 20 x 20 cm were coated with silica gel G (1 - 7 mm). They were dried at room temperature for 5 - 6 hours and activated in oven at 110^0 C for almost the same period. Approximately 5 - 10 mg of sample (fractions) was dissolved in 1-2 ml methanol and applied over the activated silica layer as a narrow streak across the plate. The solvent from the streak was allowed to evaporate. The plate

was then developed in chloroform : methanol (70 : 30) binary mobile phase in a rectangular tank. After the development, the plate was removed from the tank and the solvents were allowed to evaporate at room temperature. The developed plate was then kept in iodine chamber for detection of bands. Each band was marked by outlining with a pointed object. The marked bands containing the materials were scraped off the plate and collected separately in conical flasks. The compound from the scraped silica was recovered by adding appropriate volume of ethanol followed by shaking and filtration. The solute was thus extracted thrice and the extracts were combined. The solutes recovered from each of the bands were then subjected to biological activity screening by using fouling diatom *N. subinflata* as the test organism. Depending on the results, the active and non-active compounds were separated. The active compounds were checked for their purity by TLC spotting and further purified on silica gel column.

4.2.3 High Performance Liquid Chromatography (HPLC)

Small amount of the isolated compounds, after dissolving in appropriate volume of methanol were injected into the HPLC column (ODS column. Mobile phase, methanol 100%; flow rate, 1 ml/min.). The progress of the chromatography was monitored using RI detector.

4.2.4 Screening of purified compounds against foulers

The purified compounds were subjected to quantitative bioassay screening. For this purpose diatom *N. subinflata* and cyprid

larvae of *B. amphitrite* were used as test organisms. The dried compound was weighed on analytical microbalance and stock solution in F_2 medium was prepared. Increasing concentrations of the compound were added separately to a series of 50 ml capacity conical flasks containing 15 ml Guillard F_2 medium. To each beaker a known quantity of culture of diatom *N. subinflata* was inoculated and incubated for 3 days under 12 h light : 12 h dark cycle. The concentrations of the compound used for the purpose were 0, 0.5, 1, 5, 10, 20 and 50 µg/ml. After 3 days, the diatom cells were scraped and filtered on glass microfiber filters (GF/C). The filters containing algal cells were then subjected to chlorophyll *a* extraction with 90% acetone for overnight duration and estimated spectrofluorometrically (Parsons et al,1984, Sawant and Wagh,1994, Sawant et. al.,1995). As regards the bioassay with cyprid, to the glass beakers (50 ml) containing 15 ml of filtered seawater increasing concentrations of the compounds were added separately. The concentrations selected for the test were 0.1, 1, 10 and 20 µg/ml. Each beaker was then inoculated with 15 - 20 cyprids which were maintained in the laboratory. The attached and unattached cyprids were recorded and the Ec_{50} values were estimated using probit analysis method.

4.2.5 Partial structure of the active compounds/fractions

The broad structural pattern of the active compounds/fractions were studied using Infra Red absorption spectroscopy (IR) method. For this purpose, the isolated compounds (solid) were separately mixed with KBr (sample:KBr ratio is 10:90) using mortar and pestle. This mixture was loaded into sampler and IR spectrum was recorded.

4.3 RESULTS

Figures 4.1-4.5 show the protocol of the separation and identification of biologically active compounds from the plant extracts. Each of the crude extracts was subjected to partitioning with solvents of increasing polarity. This process of partitioning yielded three fractions, namely hexane, chloroform and aqueous. Each of these fractions were screened for biological activity. Of the 15 fractions which were resulted from five crude extracts, only 10 fractions exhibited potent activity against the fouling diatom, *N. subinflata*. The active fractions were aqueous and chloroform fractions of all the five crude extracts under study. The water and the chloroform fractions of all the five extracts were subjected to separation of compounds by preparative TLC with silica gel as stationary phase. Before starting with the separation of compounds. TLC spotting was done in order to choose an appropriate solvent system for separation of most of the compounds on TLC plate. After developing the TLC spots in a series of polar solvent system, it was observed that a binary solvent system, comprising of chloroform and methanol in the ratio 70:30 was most appropriate for optimum separation. Thereafter, all the 10 active fractions of the crude extracts were subjected to separation on TLC plate by using this binary solvent system. The zone at the top of the plate (solvent front) represented less polar compounds and numbered as 1 whereas, the one at the bottom of the plate represented more polar compound. These PTLC fractions when subjected to biological activity assay, showed that one or two fractions of each extract, except the aqueous fraction of *G. glauca* were active.

These are PTLC fractions number 3 and 4 of chloroform extract as well as fractions 3 and 7 of aqueous extract of *C. spinosa*. However, fraction 3 of aqueous extract was mildly active as compared to fraction number 7 of the same extract. Therefore, the fraction number 3 was not processed further. Further, the PTLC fractions bearing number 3 and 4 of the chloroform extract of *C. spinosa* when examined on TLC spot, exhibited identical TLC pattern and hence have been combined and processed further. Similarly, two PTLC fractions each of chloroform and aqueous extracts of *S. laurifolius* exhibited activity against *N. subinflata*. However, PTLC fractions numbering 2 and 3 of the aqueous extract exhibited very mild activity at the concentration range of 1 - 1.5 mg/ml. Therefore, both these fractions were not taken up for further processing. On the other hand , the PTLC fraction numbers 3 and 4 of the chloroform fraction of *S. laurifolius* exhibited strong activity. These two fractions after purification on column, exhibited identical TLC pattern. Therefore, these fractions were combined and processed further. As regards the crude extract of plant *G. glauca*, the PTLC fraction 1 of chloroform extract was found to be active whereas, none of the seven PTLC fractions of the aqueous extract were found to be active. In case of *A. pennata*, the PTLC fraction number 2 of the chloroform and fraction number 4 of the aqueous extract exhibited activity. These fractions after purification on column, exhibited identical TLC patterns. Therefore, both the fractions were combined and processed further. The PTLC fraction one each of chloroform and aqueous extracts of *B. acutangula* exhibited activity against *N. subinflata*. These fractions were PTLC fraction number 7 of the chloroform extract and the PTLC fraction number 4 of the aqueous extract. However, these active fractions were

found to have identical Rf values after purification on column. Therefore, these fractions were combined and processed further. Thus, total six fractions were found to be active. These fractions were, two from the plant *C. spinosa* whereas, one each from *S. laurifolius*, *G. glauca*, *A. pennata* and *B. acutangula*.

The chromatograms and Infra Red absorption spectra (IR) of the isolated compounds are given in figures 4.6-4.12. The chromatogram and IR spectra of the compound isolated from chloroform fraction of *C. spinosa* shows that it contains two components, which got separated on HPLC column at 8.01 and 8.81 min respectively. The relative composition of these compounds were 35% and 65%. The IR spectrum of this compound shows absorption at 3300, 2950, 1690, 1610, 1385 and 1075 cm^{-1}, which represent the functional groups as hydroxyl (-OH), aliphatic (-CH), carbonyl (C=C-C=O), vinyl (C=C), methyl ($-CH_3$) and ether, hydroxyl (C-O) respectively. Based on IR spectrum, it may be inferred that the compound may be branched aliphatic, hydroxyl bearing β- unsaturated acid or ketone or an aldehyde.

The chromatogram of the compound separated from aqueous fraction of the plant, *C. spinosa* shows two compounds which were separated at 8.07 and 8.77 min (Fig.4.6 & 4.8). Their relative concentrations were 7% and 68% respectively. The IR spectrum was found to be almost identical as that of TLC fraction of chloroform extract of the same plant. Therefore, the compounds of both the extracts appear to be the same.

The chromatogram and IR spectral data of the active fraction of the chloroform extract of the plant *S. laurifolius* shows two compounds which got separated at 7.97 and 9.13 and their relative concentrations were 62% and 37% respectively. The IR spectral data of this fraction showed absorption at 3500, 2927, 1754, 1564, 1453, 1360, 1380 and 1080 cm^{-1}, which represent hydroxyl (-OH), aliphatic (-CH), γ-lactone, vinyl (C=C), methylen ($-CH_2$), methyl ($-CH_3$) and ether, hydroxyl groups (-C-OH) respectively (Table 4.1). From this it could be inferred that the active compound may be unsaturated aliphatic hydroxyl bearing γ-lactone.

As regards the chromatographic data of the active fraction of the extract of *A. pennata*, two clear peaks of 54 and 45% area were observed. It shows that it is a mixture of two. The IR peaks are also very broad. The only major peak of IR is at 2918 cm^{-1} whereas, all other peaks are minor and have been observed at 1650, 1550 and 1380 cm^{-1}. This compound may be relatively less polar, as there are no strong absorptions due to carbonyl hydroxyl groups. Perhaps this might be a long chain of aliphatic compound.

The chromatogram of the active fraction of *G. glauca* shows only one peak, which got separated at 12.7. The IR spectral data of this fraction showed absorbance at 2923, 1640, 1567, 1463 and 1373 cm^{-1}. The peaks at these values indicated the presence of aliphatic, amide, aromatic, methylene and methyle groups. However, the peak at 3436 cm^{-1} appears to be due to interference of moisture

It is evident from figure 4.6, that the active fraction of the extract of *B. acutangula* is a mixture of two compounds with their relative composition being 69 and 31%. The presence of impurities in the compound is reflected in the IR spectra which show broadening of the peaks (Fig.4.11). Strong IR peaks at 3750, 1700, 1616, 1100 - 1050 cm^{-1} indicate its polyhydroxy nature. The peak at 1700 cm^{-1} indicates the presence of carbonyl group. The peak at 1616 cm^{-1} indicates the presence of C=C bonds (Unsaturated). There is no strong absorption in the region at 2900 cm^{-1}. This and the presence of peak at 1616 cm^{-1} indicates that the compound is aromatic and not aliphatic.

All these active fractions of PTLC which tentatively represent individual compound were screened quantitatively for biological activity using the fouling diatom *N. subinflata* and cyprids of *B. amphitrite* as test organisms. The results indicated that except for the PTLC fraction 1 of chloroform extract of *G. glauca* and the PTLC fraction 3 of both chloroform as well as aqueous extract of *C. spinosa*, all other active fractions of rest of the plants exhibited activity (Ec_{100}) at 50 µg/ml concentration. The estimated Ec_{50} values for these fractions were 5, 6.7, 10, 0.9, 16.5 and 9.8 µg/ml for chloroform extract of *C. spinosa*, aqueous extract of *C. spinosa*, extract of *S. laurifolius*, *G. glauca*, *A. pennata* and *B. acutangula* respectively (Table. 4.2). Amongst these fractions, the compound which was isolated from *G. glauca* appears to be the most active. As regards the bioassay of these fractions against the cyprids, fractions of *G. glauca* and *C. spinosa* exhibited higher activity with Ec_{50} values being 0. 5µg/ml each whereas, the active fraction of *A.*

pennata was found to have Ec_{50} at 10.2 µg/ml, which is the highest value as compared with the others (Table. 4.3).

4.4 DISCUSSIONS

Chromatographic techniques are widely used for separation and isolation of natural compounds. These techniques are Preparative Thin Layer Chromatography (PTLC), column chromatography and High Performance Liquid Chromatography (HPLC). However, preparative TLC is widely used for separation and isolation of natural products of biological interest (Avelin et al, 1994 a & b; Wagner et al, 1984). The reason behind this is that, it is faster, economical in terms of sample as well as solvent utility, easy to estimate parameters, direct detection on the layer, easy removal of separated zones from the plates etc. More importantly, it enhances the speed of screening of compounds for biological activity. Because of these reasons the plant extracts under study were subjected to separation and identification of compound by using preparative TLC technique.

Prior to PTLC, the methanol extracts were fractionated by solvent-solvent partitioning into three main fractions namely hexane, chloroform and aqueous. These fractions contained compounds in the increasing order of polarity from hexane to aqueous fraction. Amongst these, the chloroform and aqueous fractions of all the plants under study exhibited activity against the diatom *N. subinflata*. The active fractions were then subjected for separation of compounds using PTLC with silica gel as stationary phase. Of the many solvent systems used, a

binary solvent system composing of 70% chloroform and 30% methanol provided best separation of compounds for all the active compounds. The separated PTLC fractions when screened for biological activity against the fouling diatom *N. subinflata*, exhibited that 1 or 2 fractions were active, except for the fractions of aqueous extract of *G. glauca*. This shows that the aqueous extract of *G. glauca* which was initially active before separation by PTLC, lost its activity after separation, suggesting synergism of compound. The active PTLC fractions were found to exhibit varying degree of activity. The compounds isolated from *S. laurifolius*, *A. pennata* and *B. acutangula* were found to be less effective, whereas, that of *C. spinosa* and *G. glauca* were observed to be more active. In general, compound from *G. glauca* appears to be the most effective and was found to inhibit 100% growth (Ec_{100}) of the diatom *N. subinflata* at a concentration of 5 µg/ml. However, this compound needs to be investigated further as regards to its chemistry as well as mode of action on fouling organisms.

All other fractions except PTLC fraction 1 of *G. glauca* and fraction 3 and 4 of chloroform and aqueous extract of *C. spinosa*, exhibited 100% growth inhibition of the diatom under study at 50 µg/ml concentrations. These results suggest that the degree of activity of the isolated compounds of plants *S. laurifolius*, *A. pennata* and *B. acutangula* was no way different from that of their respective crude extract. The activity of the crude extracts of these plants on the same diatom species ranged between 50 - 100 µg/ml. This leads to the inference that the compounds in question might have undergone changes during the process of isolation or it might have been oxidised due to

exposure to atmospheric oxygen. This type of degradation in natural compounds is normally observed due to removal of natural antioxidant which acts as preservative for such compound (Parameswaran - personal communication). On the other hand the compounds of the PTLC fraction 1 of chloroform extract of *G. glauca* and PTLC fraction 3 and 4 of chloroform extract of *C. spinosa* exhibited activity at concentration below 20 μg/ml.

Literature survey regarding the chemistry of these plants shows, that plants *C. spinosa*, *S. larifolius*, *G. glauca* and *B. acutangula* have been worked out earlier (Mishra et. al, 1979a,b; Saharia and Sheshadri, 1980; Bhandari et. al., 1980; Bhandari and Rastogi, 1980; 1981; Chavan and Nikam, 1982; Yadava and Bhatnagar, 1987; Kasai et. al., 1988; Sotheeswar et. al, 1989 ; Dubois et. al, 1990; Mandal et. al., 1994). Of these, former two have been extensively worked out. Contrary to this, hardly any information is available on the chemistry of the plant *A. pennata*. Number of compounds have been reported by various workers from the fruits of *C. spinosa*. These compounds are Beta-D-galactopyronosyl (1 -> 3) - oleanolic acid, saponins, Randianin etc (Saharia and Sheshadri, 1980; Ansari and Khan, 1981; Sotheeswar, 1989; Dubois et. al., 1990). Fruits of *S. laurifolius* have been reported to contain saponins and trifolioside II, whereas, plant *G. glauca* contains Erioside, eriocephaloside, Syringaresinol, Syringin, Genkwanin and Glycosides (Bhandari et. al., 1980; Bhandari and Rastogi, 1981; Bhandari et. al., 1981; Bhargava, 1988; Kasai et. al., 1988). As regards the chemical constituents of *B. acutangula*,

Triterpenoid glycoside, Tangulic and Acutangulic acids have been reported (Anjaneyulu et. al., 1978).

Though number of reports are available on the chemistry of some of these plants under study, little is known about the application of these plant extracts for their chemical constituents. Majumdar (1984) has reported on the herbals preparation containing *S. laurifolius* as one of the constituents for the purpose of curing R-arthritis and Rheumatism. Aqueous extracts of the pericarp of fruits of *S. laurifolius* have been reported to contain an active compound which can prolong the 'anagen' stage of hair growth (Anand et. al., 1980). A saponin rich fraction isolated from fruits of *S. laurifolius* has been reported to impair fertility in albino rat (Bhargava, 1988). Yadava and Bhatnagar (1987), have reported on the pesticidal property of *S. laurifolius*. Two new triterpene - saponins isolated from *C. spinosa* have been reported to possess haemolytic, molluscicidal and immunostimulating properties (Dubois et. al.,1990). Essential oil obtained from the seeds of *C. spinosa* is reported to show antibacterial activity against *Escherichia coli*, *Protens valgaris*, *Pseudomonas aeruginosa* and *Staphylococcus pyogens* as well as many fungal cultures (Mishra et. al., 1979a,b). The same oil is also reported to have anthelmintic activity against earthworm and tapeworms (Mishra et. al., 1979a, b). Chavan and Nikam (1982) have reported on the larvicidal activity of *G. glauca*. Although, various properties of some of these plants under study have been reported in the past, they have not been investigated for the antifouling aspects. Therefore, this could be the first report of its kind on the antifouling properties of these plants.

In the present work the compounds which were isolated from the extracts of plants have been found to contain various functional groups. These groups include, -OH, -CH, -CO, -C=C, -C-OH, $-CH_2$, $-CH_3$, -COOH γ-lactone etc., which points to the presence of unsaturated aliphatic or aromatic compounds.

Bioassays which were carried out with the crude extracts of *C. spinosa*, *S. laurifolius* and *A. pennata* on both the species of *Navicula*, exhibited stimulatory activity at lower concentrations. Contrary to this, no such activity was observed in the compounds of the same plants after isolation and purification, which clearly suggests that the compound which is responsible for stimulation of growth might have been separated out during the process of isolation. These results further substantiate the hypothesis that the crude extract contained two different compounds, one being as a stimulant and the other as inhibitor. According to Standing et al (1984) the inhibitors are of low molecular substances whereas, stimulator or inducer are high molecular compounds. However, his work is based on the compounds from marine organisms.

Though the effective concentrations (Ec_{100}) of the isolated compounds have been determined by bioassays, this does not give the concentration of compound in the seawater due to certain practical difficulties . This difficulty arises in case of the low polar compounds, which are normally less soluble in seawater. At the same time the lower the solubility of a compound in seawater, the longevity of the life of

the compound. In such cases it would be appropriate to determine the leaching of compound in the seawater from the surface of the experimental substratum.

It has been reported earlier that natural toxins so far known belong to wide range of classes. They are, lactone, furans, glycosides, fatty acids, terpenoids, terpins, steroid, sterols, alkaloids etc.(Goto et. al., 1992; Mizobuchi et. al., 1993; Keifer, et.al., 1986; Sears et. al., 1990; Davis, 199; Keifer, et. al., 1991). Moreover, the partial structural study of the present active principals also shows the presence of lactone as one of the functional groups in the compound isolated from the extract of *S. laurifolius*.

As regards the present work, the active compounds have been partially studied for their structure. Consequently, these compounds could not be placed in any of the above classes of compounds. However, the antifouling property of the compound of the plant *S. laurifolius* could be attributed to the presence of lacton group. The antifouling property of other compounds under study may be attributed to one or more functional groups present in them. Moreover, further studies on the structure and bioactivity of these compounds will help to identify the actual compound or the functional group which is responsible for antifouling activity.

Table 4.1 IR spectral data showing peak values and their chemical groups.

Extract	Peak Value (cm^{-1})	Chemical Group	Nomenclature
C.S. (1)	3300	OH	Hydroxyl
	2950	CH	Aliphatic
	1690	C=C-C=O	Conjugated Carbonyl
	1610	C=C	Vinyl
	1385	CH_3	Methyl
	1075	C-OH	Ether, Hydroxyl
C.S. (2)	3367	OH	Hydroxyl
	2918	CH	Aliphatic
	1696	C=O	Ketone
	1611	C=C	Aromatic Group
	1460	CH_2	Methylene
	1380	CH_3	Methyl
	1075	C-OH	Ether, Hydroxyl
S.L.	3500	OH	Hydroxyl
	2927	CH	Aliphatic
	1754		γ-Lactone
	1564	C=C	Vinyl
	1453	CH_2	Methylene
	1360-1380	CH_3	Methyl
	1080	C-OH	Ether, Hydroxyl
A.P.	2918	CH	Aliphatic
	1650	CONH	Amide
	1550	C=C	Vinyl
	1380	CH_3	Methyl
B.A.	3750	OH	Hydroxyl
	1700	C=O	Ketone, Aldehyde
	1616	C=C	Aromatic
	1100-1050	C-OH, C-O-C	Ether, Hydroxyl
G.G.	2923	CH	Aliphatic
	1640	CONH	Amide
	1567	C=C	Vinyl
	1463	CH_2	Methylene
	1375	CH_3	Methyl

Table 4.2 Ec_{50} values of the isolated compounds against *N. subinflata*.

Compound/ Fraction	Ec $_{100}$ (μg/ml)	Ec $_{50}$ (μg/ml)
C. spinosa (1)	20	5
C. spinosa (2)	20	6.7
S. laurifolius	50	10
G. glauca	10	0.9
A. pennata	50	16.5
B. acutangula	50	9.8

Table 4.3 Ec_{50} values of the isolated compounds against barnacle cyprids.

Compound/ Fraction	Ec $_{50}$ (μg/ml)
C. spinosa (1)	0.5
C. spinosa (2)	0.6
S. laurifolius	6.4
G. glauca	0.5
A. pennata	10.2
B. acutangula	7.8

Fig. 4.1 Flow chart of the bioassay guided separation of the antifouling compounds from methanolic extract of *C. spinosa*.

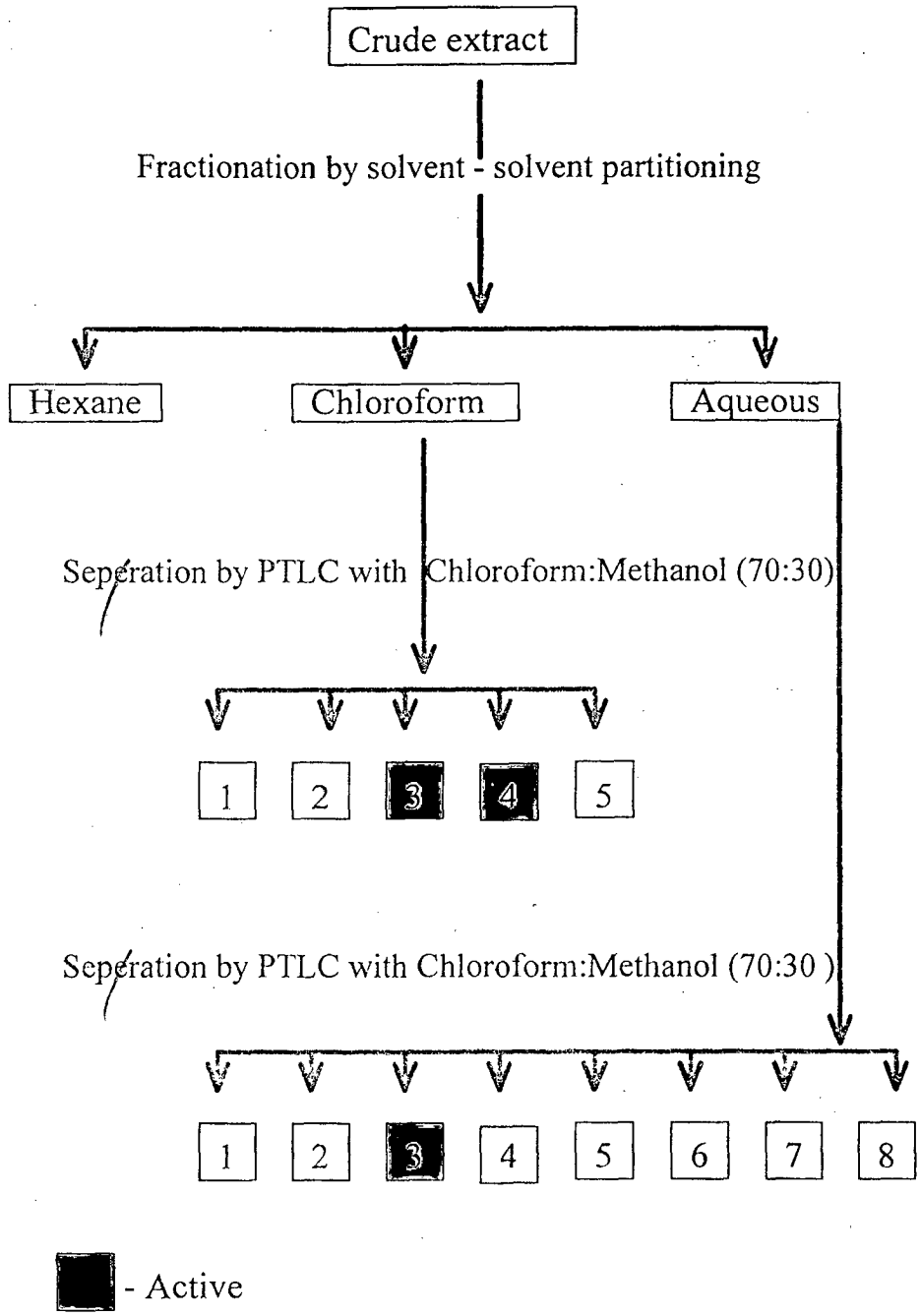


Fig. 4.2 Flow chart of the bioassay guided separation of the antifouling compounds from methanolic extract of *S. laurifolius*.

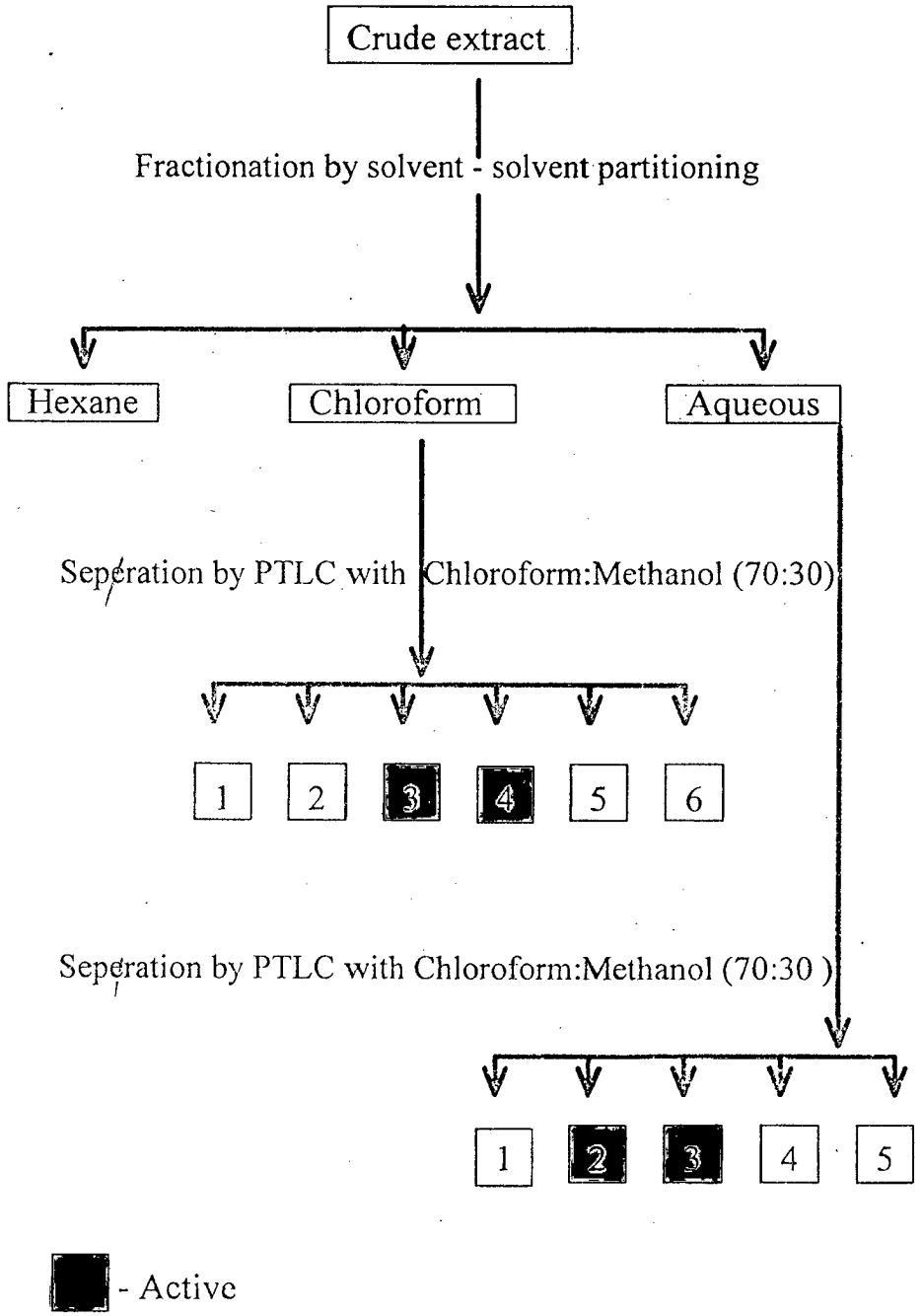


Fig. 4.3 Flow chart of the bioassay guided separation of the antifouling compounds from methanolic extract of *G. glauca*.

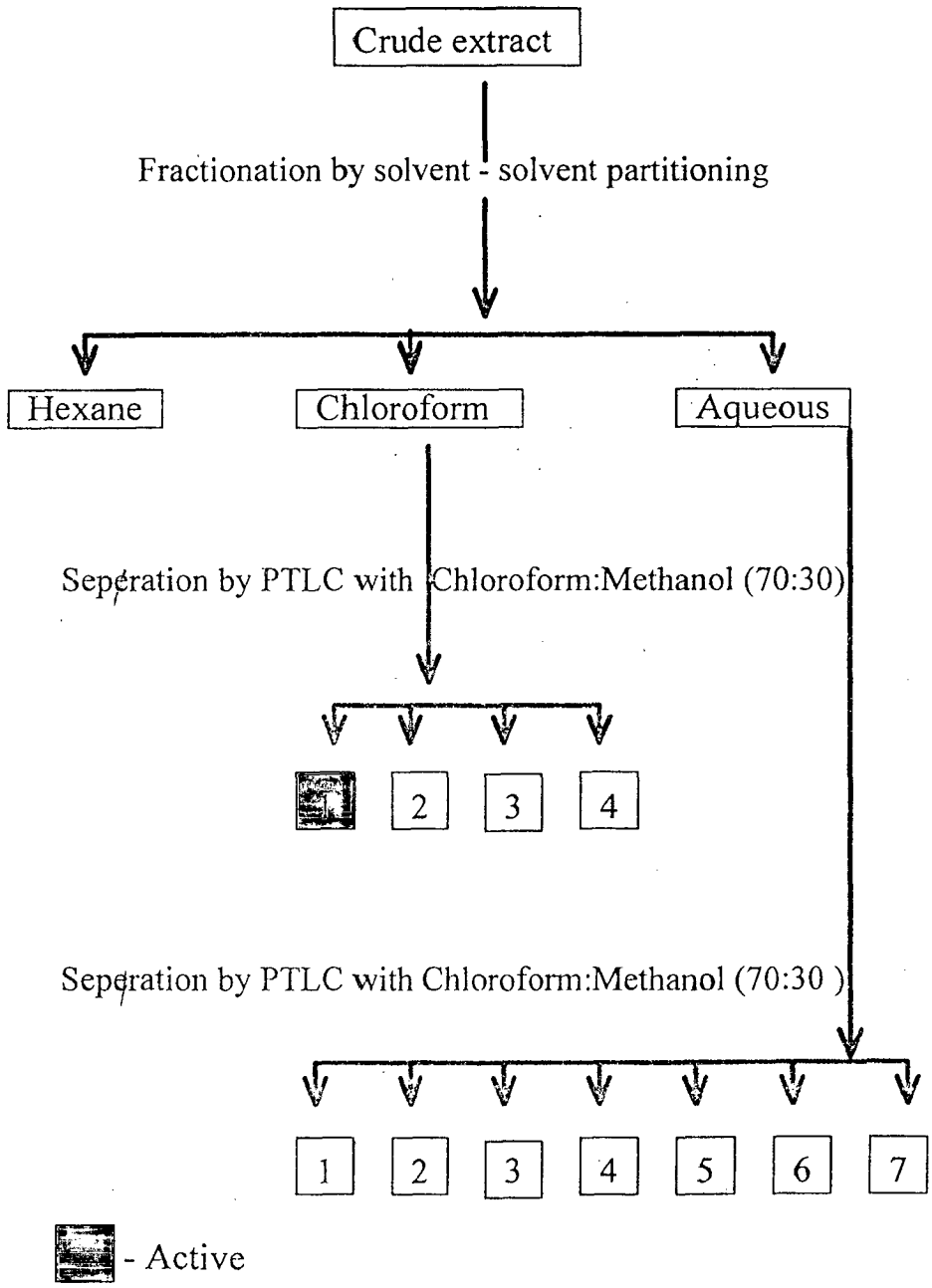


Fig. 4.4 Flow chart of the bioassay guided separation of the antifouling compounds from methanolic extract of *A. pennata*.

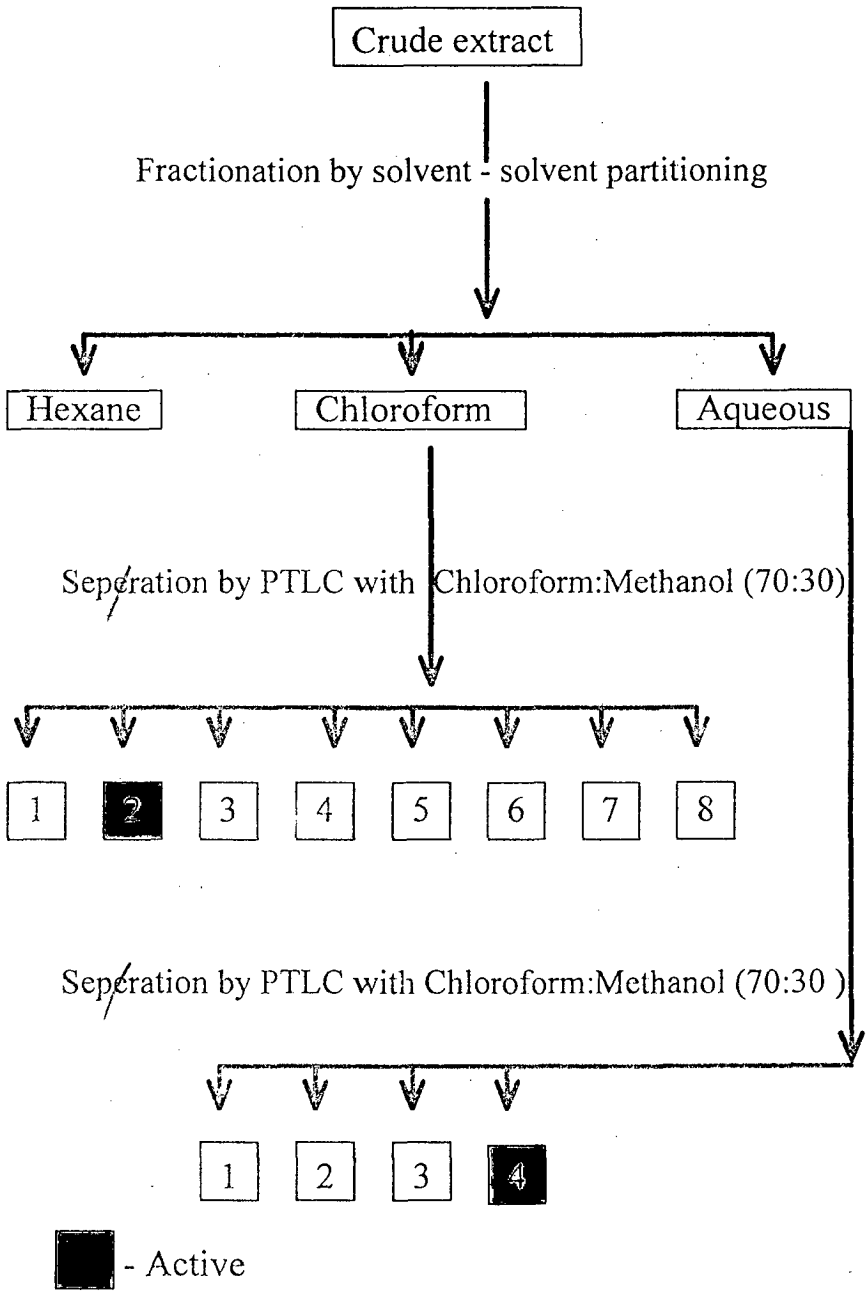
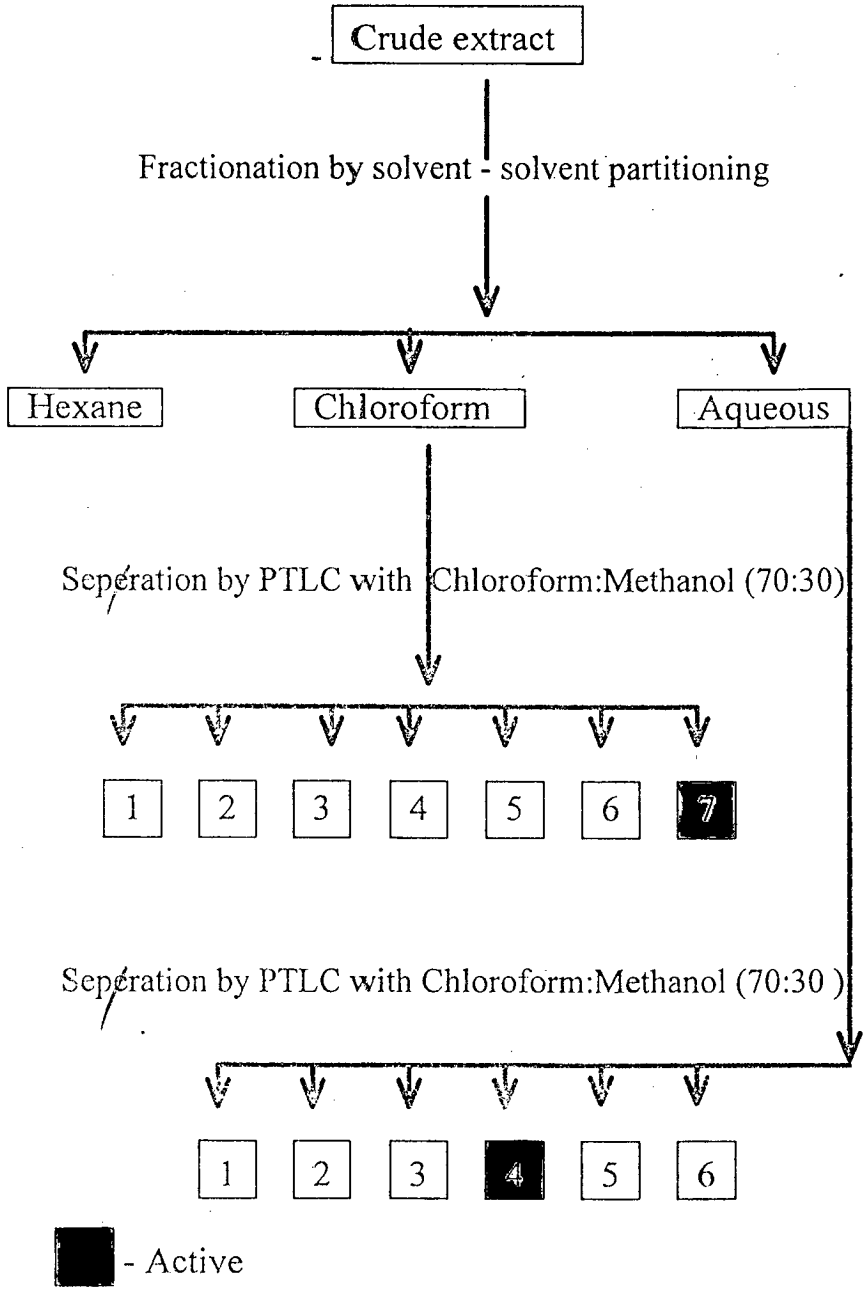


Fig. 4.5 Flow chart of the bioassay guided separation of the antifouling compounds from methanolic extract of *B. acutangula*.



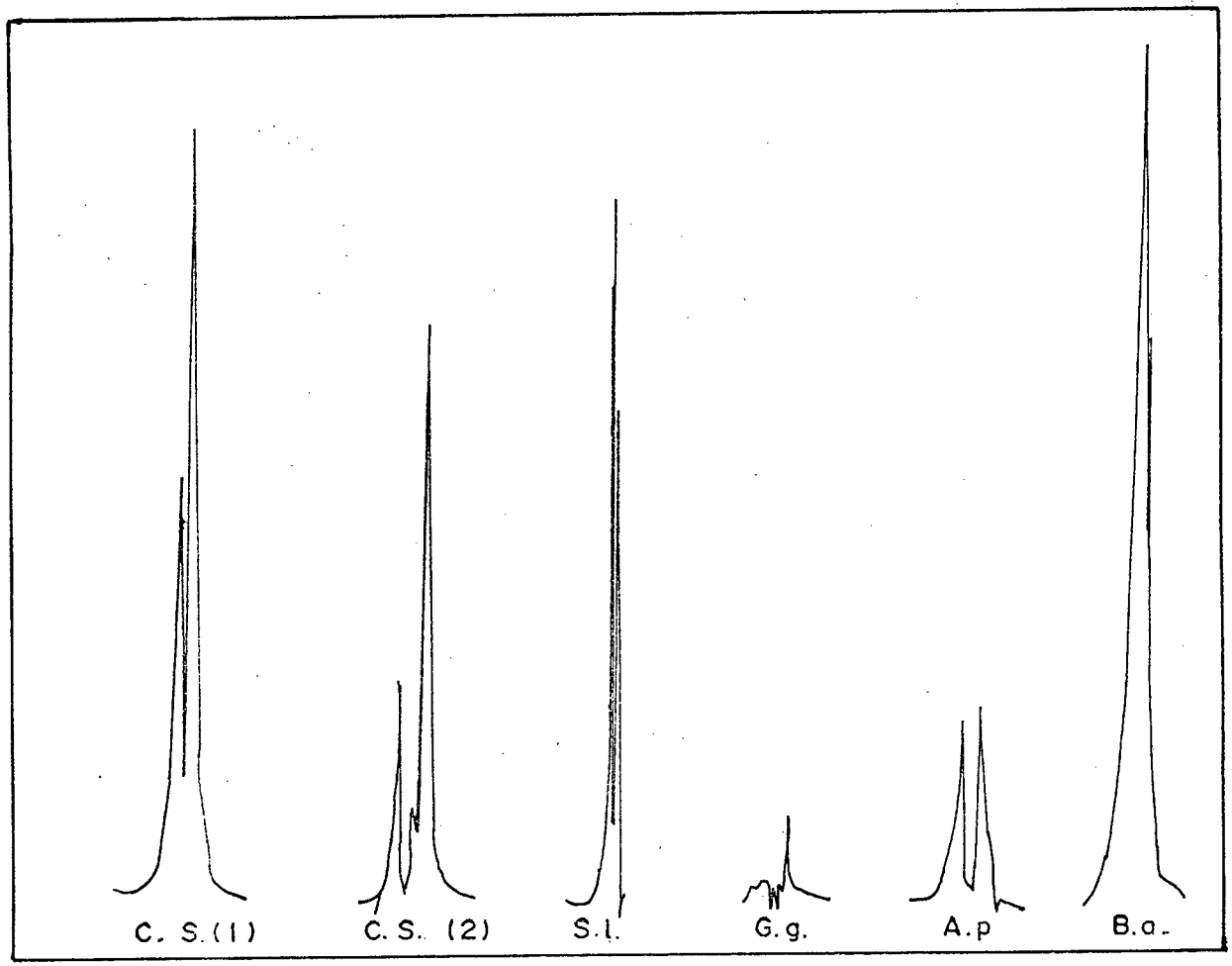


Fig. 4.6 Chromatograms of the active compounds isolated from the plants extracts.

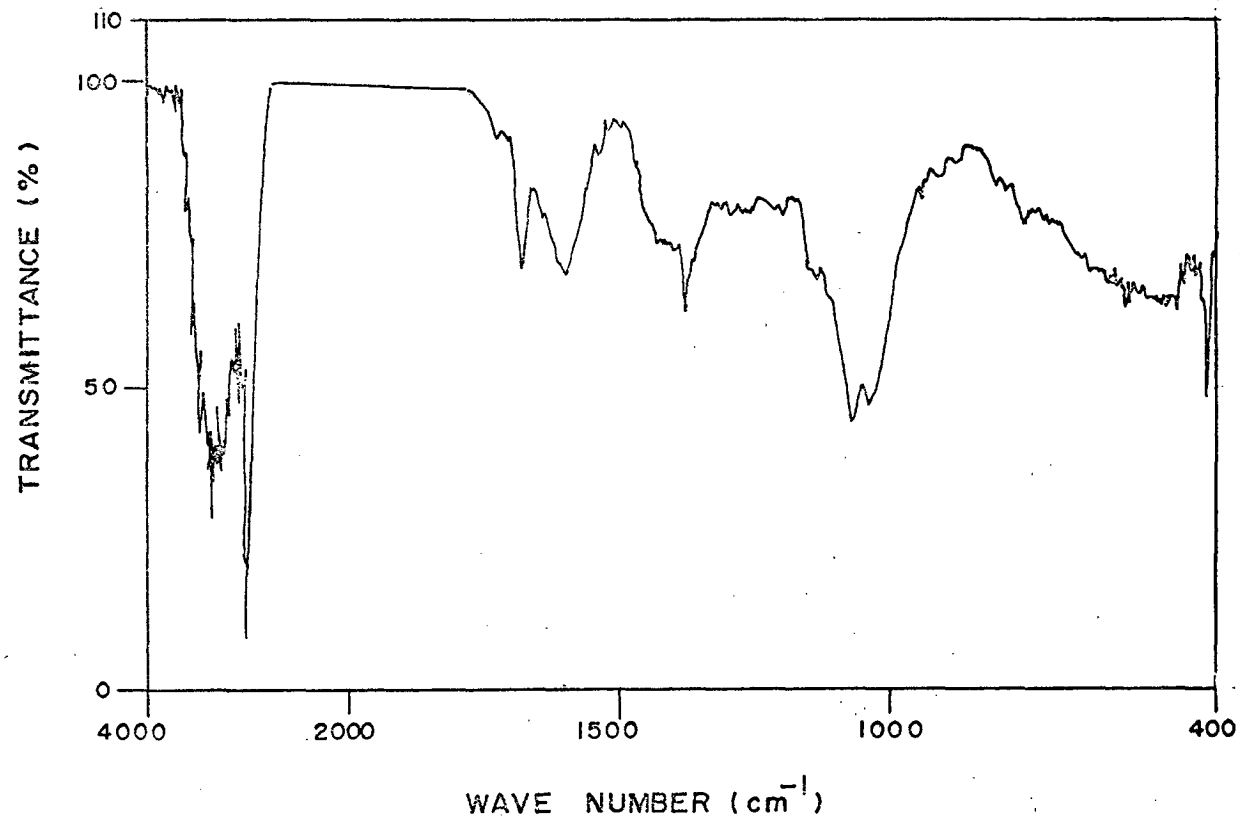


Fig. 4.7. IR SPECTRUM OF THE ACTIVE COMPOUND (1) OF C. spinosa.

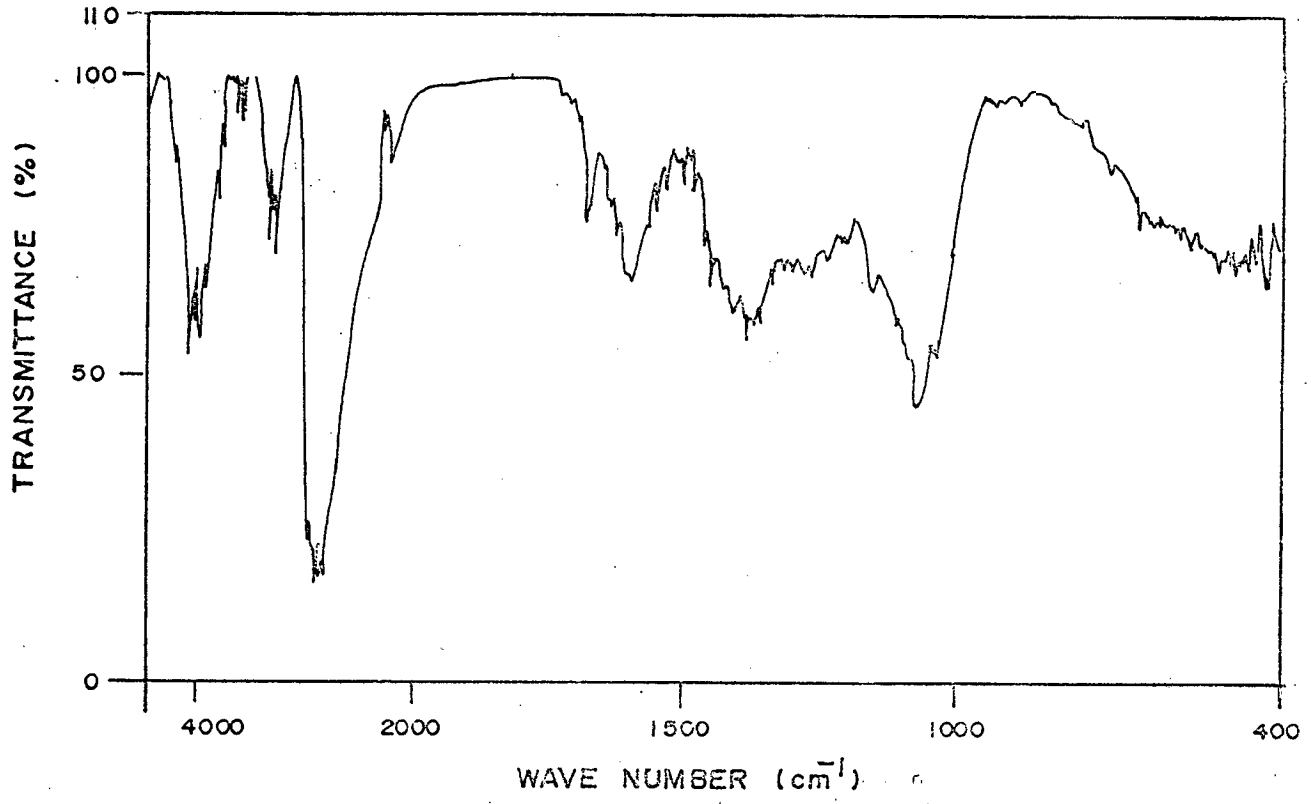


Fig. 4-8. IR SPECTRUM OF THE ACTIVE COMPOUND (2) OF C. spinosa.

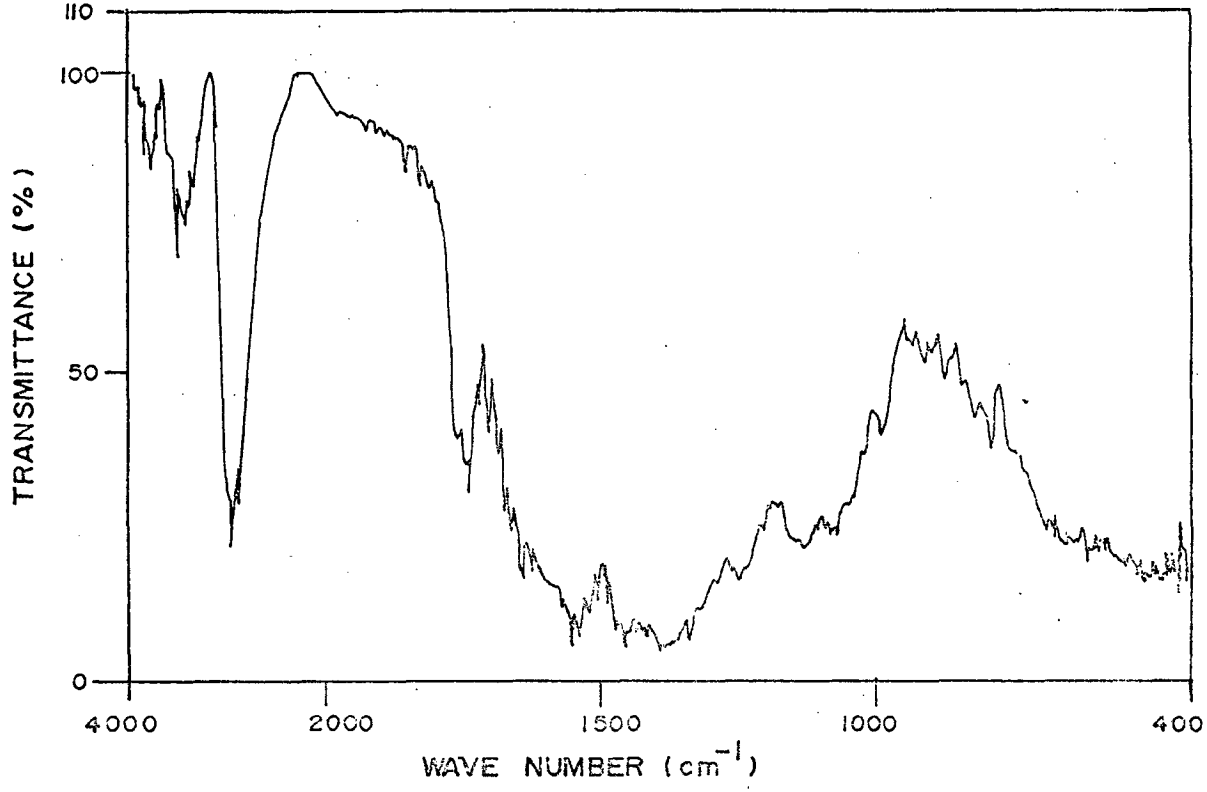


Fig. 4.9. IR SPECTRUM OF THE ACTIVE COMPOUND OF *S. laurifolius*.

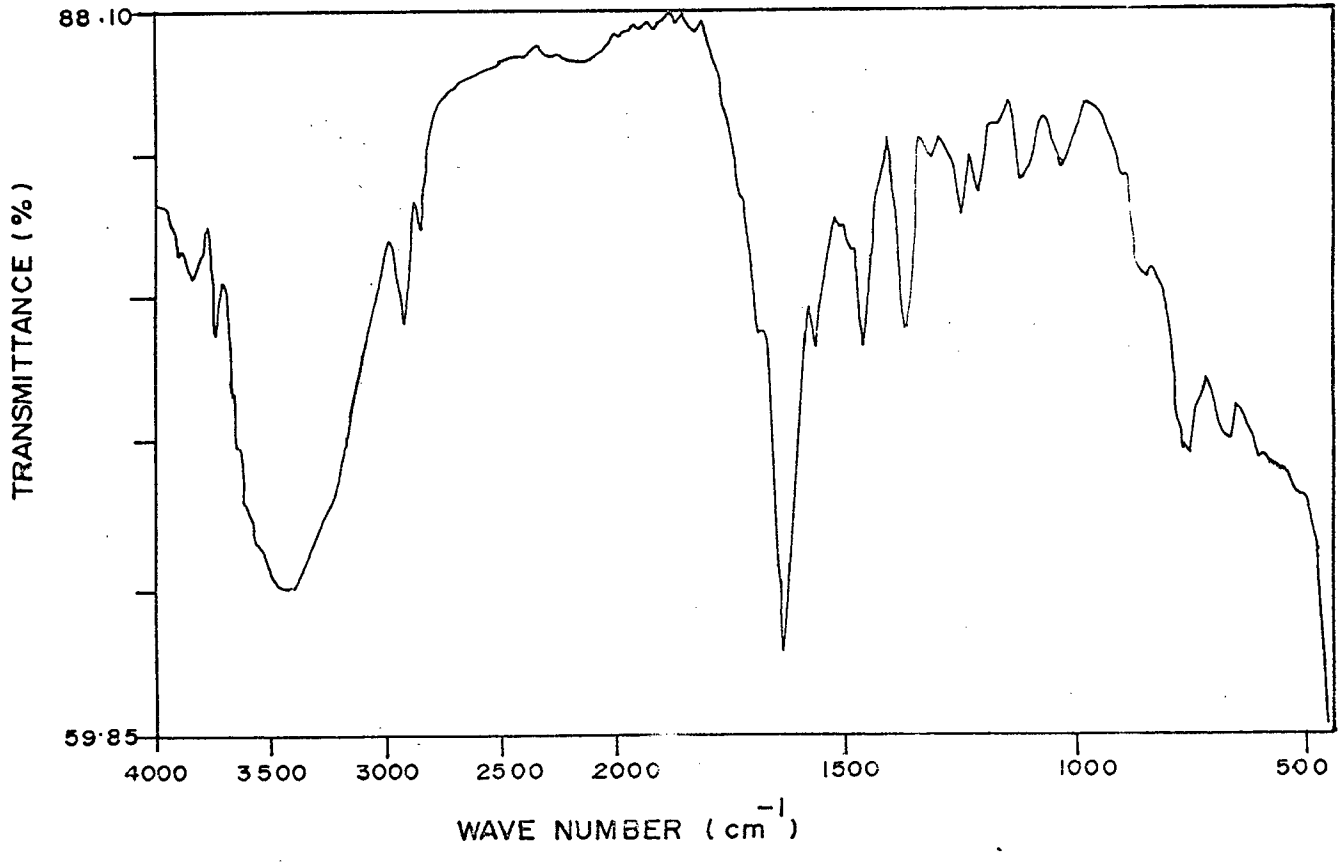


Fig. 4·10. IR SPECTRUM OF THE ACTIVE COMPOUND OF G. glauca.

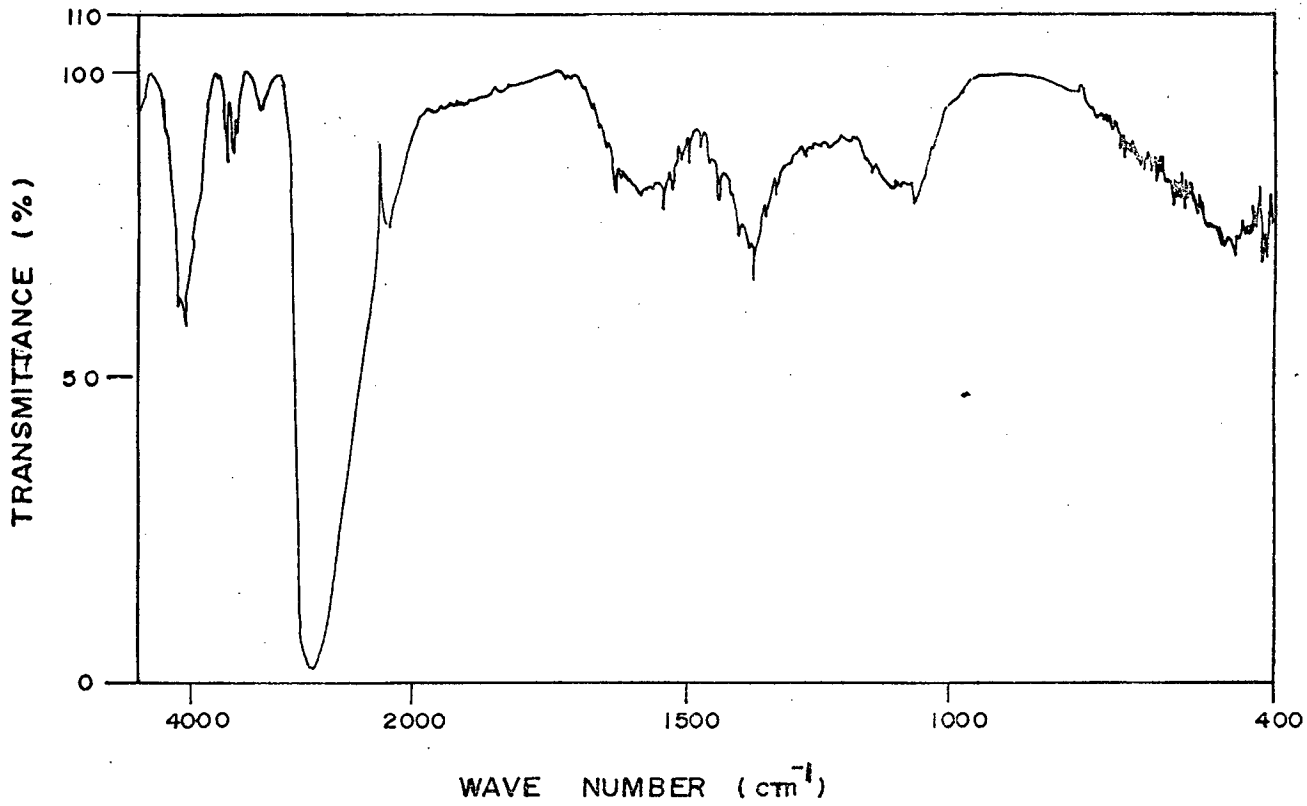


Fig. 4-11. IR SPECTRUM OF THE ACTIVE COMPOUND OF A. pennata.

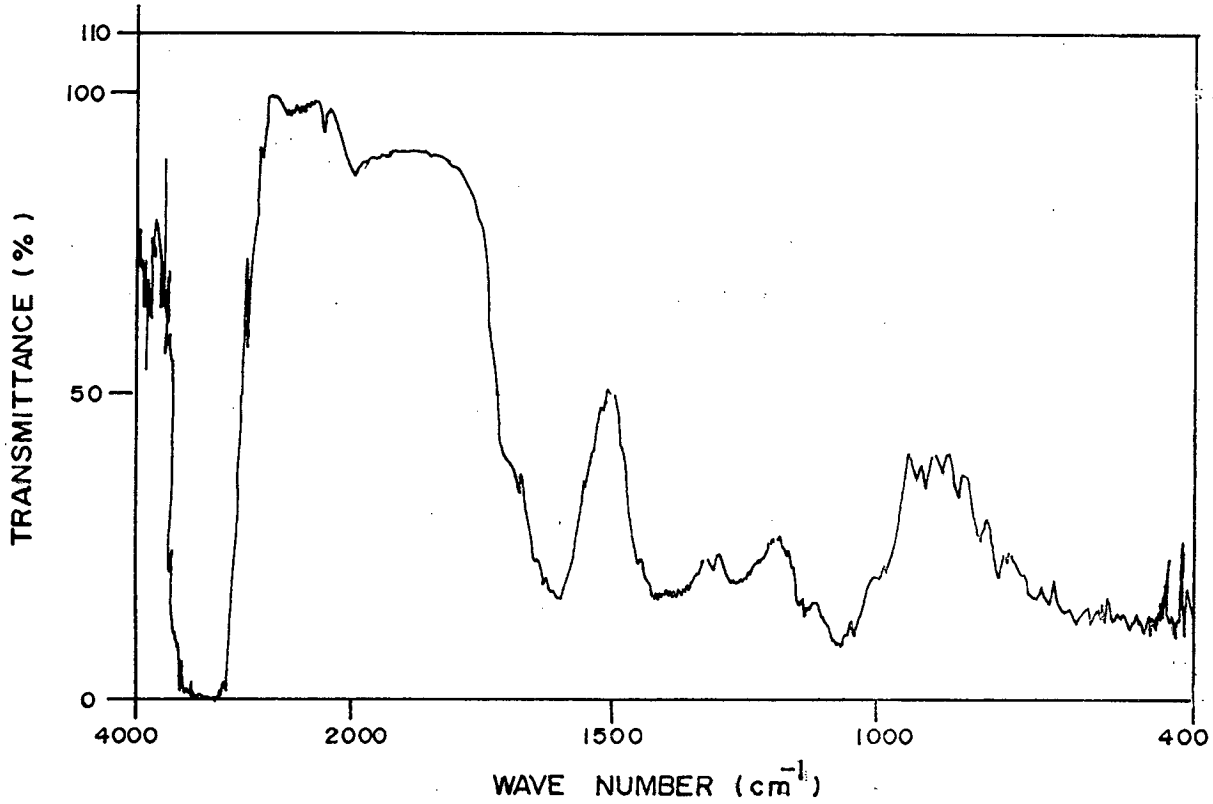


Fig. 4·12. IR SPECTRUM OF THE ACTIVE COMPOUND OF B. acutangula.

CHAPTER 5

SUMMARY AND CONCLUSIONS

The Biofouling is the process in which some marine organisms get attached to man-made structures. The resultant effect of biofouling is the heavy loss to economy. For controlling this process, various methods are in practice. These methods include, physical, Chemical and biological ones. Amongst these, the chemical methods are commonly used, in which antifouling paints with a toxic component of a chemical are used. However, the toxins which have been used so far in the antifouling paints have been found to be harmful not only to biofoulers, but also to entire ecosystem. As a result the use of toxins, such as organo-metallic compounds have been legislatively banned in number of countries from western Europe as well as in other industrialised countries such as, Japan, U.S., Canada and Australia. In view of these restrictions, copper compounds have re-emerged as the main active ingredients of antifouling coatings in recent years. Copper based mixture works well for the short term and the best formulation can last at the most for three years (Clare, 1995). It also affects the organisms other than the fouling organisms like that of organo-metallic compounds. Consequently, it is felt that the copper based antifouling biocide may also cause environmental problems in the long run.

Due to these constraints, there is a growing need for other environmentally friendly antifouling toxin for the prevention of fouling. One of the methods which is presently being investigated includes formulation of antifouling paint based on natural compounds, which are believed to be easily biodegradable, thereby causing no harm to the marine environment. The work on this aspect was initiated during 1980s

in United states. For the search of biogenic compounds which can serve as antifouling toxins, numerous marine organisms have been screened. Reports show that several species of marine invertebrates especially, sponges, coelenterates, bryozoans and ascidians were found to contain certain compounds which could prevent settlement of macrofoulers under laboratory conditions (Targett et. al., 1983; Bakus and Kawaguchi, 1984; Rittschof et. al., 1985; 1986; Wahl, 1989; Gerehart et .al., 1988; Sears et. al., 1990; Targett et. al., 1983; Walls et. al., 1993). The compounds include, alkaloids, fatty acids, terpenoids and bromopyrroles (Keifer et al., 1986; Sears et. al., 1990; Goto et. al., 1992). Though these groups of compounds have been identified, the structure of very few of them have been understood and tried in the field.

Furthermore, though a great deal of attention is being paid to the marine organisms, no attention seems to have been paid to terrestrial plants for this purpose. In light of the information that extracts of some plants are being used by local fishermans for narcotizing and catching the estuarine fishes, this work was taken up, in order to find out whether certain local plants possess antifouling compounds (Mitre, 1981). The plants which have been investigated are, *C. spinosa* (Family- Rubiaceae), *S. laurifolius* (Family- Sapindaceae), *G. glauca* (Family- Thymelaeaceae), *A. pennata* (Family- Leguminoceae), and *B. acutangula* (Family- Lecythidaceae). Different parts of these plants such as, fruits of *C. spinosa* and *S. laurifolius*, and the bark of *G. glauca*, *A. pennata* and *B. acutangula* have been tried for this purpose. Therefore, laboratory assays is the only viable tool by which rapid screening of antifoulant is possible. For this reason, the extracts of these plants have

been screened against fouling bacteria, diatoms, barnacles and mussels. The dominance in fouling assemblage in Goa waters justify the choice of these organisms for the present work (Sawant, 1985; Anil and Wagh, 1988; Sonak and Bhosle, 1995, Redekar- personal communication).

The experimental data on the bioassays under laboratory conditions have been analysed using probit methods in order to arrive at median effective dose (Ec_{50}) (Anon, 1972; Reddy et. al., 1992). The results on antifouling assay suggested that crude extracts of *C. spinosa*, *S. laurifolius* and *G. glauca* were active against all the seven species of bacteria which were tested. As regards the bioassay with the diatom species, the estimated Ec_{50} values of the extract of the plants under study varied with the type of plant and the diatom species. In general the Ec_{50} values of the extracts against the diatoms ranged between 8.9 to 152.6 µg/ml. Amongst the various diatoms studied *Amphora* appeared to be the most resistive species against all the plant extracts. The differences in the activity of these plants extracts could be due to two reasons. Firstly, the resistivity of the organisms varies with each organism. It is due to their characteristic defence mechanisms. These mechanisms could be either chemical or physical in nature. Secondly, the nature of the toxicant also may contribute to the difference in the toxicity. The degree of toxicity especially of natural compounds is believed to be dependent on the type and position of functional group in the molecule. These reasons explain the observations on the effectiveness of the crude extracts under study.

Interestingly at lower concentration of the crude extracts, both the species of *Navicula* under study were found to show promotion in the extent of growth. This suggests that the crude extracts probably contain both growth promoting/stimulating as well as growth inhibiting components. Similar observations have also been reported for the natural products of marine organisms by Standing et al (1984), and Avelin et al (1994). However, in the present investigation except the species of *Navicula*, the other forms did not exhibit any growth promotion at lower concentration. The difference in the response of different species to the extracts, probably may be different in different species. According to Standing et al (1984), inhibitors are of low molecular substances whereas, the stimulants/inducers or growth promoters are of high-molecular substances. The inducers have been reported to be responsible for the gregarious settlement of barnacles (Standing et al., 1984).

Assays on the settlement of barnacle cyprids show some differences in their response to the extracts under study. The estimated Ec_{50} values of the extracts against the cyprids ranged between 15.2 to 91.3 µg/ml. Extract of *G. glauca* appeared to be the most active product. As regards the activity of the extracts against mussels, extracts of *G. glauca* was found to be the most active one with Ec_{50} value being 6.6 µg/ml, whereas, *A. pennata* exhibited low activity with Ec_{50} value being 33.4 µg/ml. The Ec_{100} and Ec_{50} values of all the extracts for all the test organisms have been compared and their efficiencies have been discussed.

For the field trials, the crude extracts were mixed with non-toxic paint having Alkyd binder in different proportions and exposed in seawater. They were examined visually for microfoulers and macrofoulers. The results were encouraging for the short exposure period, suggesting antifouling action of the extracts under study.

It has been stated by Anthony (1988) that natural toxins exert pronounced effect on the metabolism and biological functions of the animals with just a minute quantity. The mechanism of action of natural toxins on foulers appears to be somewhat different from that of chemical toxins. The natural toxins are believed to interfere temporarily with the enzymatic activity of the organism, leading to inhibition of growth or settlement. Furthermore, natural toxins are believed to be more biodegradable than the heavy metal toxins, thus restricting their toxicity both spatially and temporally. In the present investigation also it has been observed that the compounds have interfered with the metabolic processes of the test organism, resulting in growth or settlement inhibition. The compounds were found to interfere adversely with the essential nutrients uptake in diatoms. Furthermor, it has been observed that the carbohydrates metabolism has been affected considerably in the diatom cells. The resultant effects of all these might have caused inhibition of cell growth.

Natural toxins belong to wide classes of compounds, such as lactons, furans, glycosides, tepenoids, terpins, steroids, sterols, fatty acids, alkaloids etc. Their toxicity is believed to be dependent on the type

and position of functional group. In the present work the compounds which were isolated from the extracts of plants have been found to contain various functional groups such as -OH, -CH, -CONH, -CO, -C=C, -C-OH, $-CH_2$, $-CH_3$, -COOH, γ-lactons etc.. It in turn, points to the presence of unsaturated aliphatic or aromatic compounds.

From the present work, it may be concluded that terrestrial plants are the good source for finding out antifouling compounds. Secondly, the compounds from the plants under study temporarily interfere with some of the physiological activities of fouling organisms and thus lead to antifouling activity. In view of this, it seems that these compounds can serve as environment friendly antifouling components in paint.

Any natural antifouling compounds once identified, it needs to be developed into proper antifouling technology. The technological aspect includes, preparation of analog, development of control release system, etc. In light of this, the present work is the first step towards the development of antifouling technology. It also suggest that natural antifouling agent could be tapped from terrestrial plants, which are available in plenty.

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