ATTACHMENT OF MARINE MICROORGANISMS TO SURFACES

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by

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Summary

The attachment mechanisms of the main groups of marine microorganisms are similar. These similarities are particularly shown by the nature of the adhesive mucilages they produce. Marine bacteria, cyanobacteria and diatoms produce acidic polysaccharide mucilages. Macroalgal spores attach by the production of glycoprotein adhesives. The spores of several marine Ascomycete fungi attach by means of mucilaginous, fibrillar appendages, although little is known about their composition. Certain marine bacteria possess cell-surface structures such as stalks with holdfasts, flagella and spinae which may play an attachment role. Cyanobacteria possess pili and spinae which may serve the same purpose. The formation of proteinaceous conditioning films on substrata promotes bacterial adhesion. Polymeric polysaccharide materials and bacterial films also precondition surfaces for algal attachment. Substratum properties such as wettability and surface free energy can affect, and in some cases decrease, levels of bacterial and algal attachment.

Marine microbial adhesion is of importance in disease pathogenesis, biotic interactions, physical interactions and certain economic aspects. In disease pathogenesis, the attachment of *Aeromonas salmonicida* to fish cells by the 'A'-protein layer is important in the initiation of furunculosis. Some invertebrate diseases are also caused by attached microorganisms. The bacterium *Leucothrix mucor*, along with diatoms, cyanobacteria and protozoa can accumulate on the gills of shrimps, crabs and lobsters. This heavy infestation causes death by suffocation. *Vibrio cholerae* attaches to the oral region of planktonic copepods and the hindgut of the blue crab *Callinectes sapidus*. These observations may be important for the epidemiology of cholera in aqueous environments.

Microbial attachment may also be important in the establishment of symbiotic relationships with certain marine invertebrates.

Biotic interactions include the formation of primary microbial films on any new surface immersed in seawater. Extensive microbial epiphytic layers form on seaweeds and estuarine salt marsh grasses. Bacteria which attach to the heterocysts of the cyanobacterium *Anabaena* spp. in the freshwater environment could be involved in nitrogen fixation. The parasitic marine bacterium *Bdellovibrio bacteriovorus* attaches to host cell membranes by cell-surface fibres, which ultimately leads to cell lysis. Attached bacteria are responsible for the aggregation of particulate detritus, which eventually disaggregates due to protozoal activity. Bacteria utilize dissolved organic carbon for the formation of adhesive materials which cause detrital aggregation. The attachment of bacteria to certain phytoplankton, including diatoms, ultimately results in the formation of amorphous detrital aggregates.

Physical interactions include microbial attachment to sediments. Microbial attachment and adhesive production is important in the formation of microbial mats. Marine bacteria, cyanobacteria and diatoms are abundant in these mats. Microbial extracellular polymer material is responsible for lamination of mat layers and the structural integrity of the mats. Gelatinous mats are often formed in sediments from microalgal adhesive secretions. These mats are of importance in sediment stabilization. The adhesive mucilages of certain diatoms also reduce the resuspension of sediment particles. Microbial extracellular materials may also be utilized as a food source by benthic invertebrates.

One economic aspect of marine microfouling is metal corrosion. Corrosion can result from the formation of differential aeration cells

under a non-uniform film of attached microorganisms. Sulphate-reducing bacteria, which are often present in biofilms under anaerobic conditions corrode metals by cathodic depolarization. This process allows the formation of corrosive hydrogen sulphide and iron sulphide from sulphates. Some diatoms, such as *Amphora* spp., may inhibit corrosion by forming a uniform layer of adhesive mucilage over a metal surface.

A further economic aspect is the affect of microbial attachment on the development of antifouling techniques. Bacterial and diatom slime films are easily formed on cuprous oxide and organometallic antifouling paints. This makes them less effective. The effects of surface free energy and wettability of substrata on bacterial and algal adhesion could provide a further antifouling technique. Conditioning film formation can alter these substratum properties towards a biocompatible range where lower rates of microfouling occur. The incorporation of silicone elastomers in substrata also decreases bacterial and diatom attachment. The use of metabolic inhibitors or calcium chelating agents to remove bacterial and diatom films could be a further antifouling development.

Introduction

Microbial attachment is a widespread phenomenon which is of importance in several aspects of microbiology. These include the pathogenesis of microbial disease, industrial microbiology and various aspects of microbial ecology. This thesis examines the mechanisms and importance of microbial adhesion in the marine environment.

Bacterial attachment has long been recognized as an important initial step in the pathogenesis of human disease. For example, the attachment of *Vibrio cholerae* to the brush border of the intestinal epithelium is important in the initiation of the diarrhoeal disease cholera. Attachment of *Bordetella pertussis* to the respiratory tract is of importance in the infection whooping cough. Correlations have been made between the propensity of bacteria to cause infections *in vivo* with their ability to attach to the affected tissues *in vitro*. These studies have demonstrated a high degree of correlation between adherence and infection (Beachey, 1980).

There is much evidence to suggest that bacteria possess cell surface molecules which can bind in a specific fashion with complementary molecules on the surface of host tissue cells. The binding molecules on the bacteria are often called ligands, and those on host cells, receptors. The possible involvement of ligand-receptor interactions in adhesion is often referred to in this thesis. The specificity of the interaction can be demonstrated in three ways (Beachey, 1980). Inhibition of the interaction could occur by the addition of large excesses of "haptens" either identical to or resembling the native ligand or receptor. The bacteria or tissue cells could be enzymatically treated to abolish or alter

the specific surface structures involved in adhesion. Thirdly, the ligand or receptor could be blocked with specific antibodies directed against antigens composing these structures.

Microbial attachment and fouling in the marine environment has been recognized for most of this century. Lloyd (1930) made a bacteriological survey of the Clyde Sea Area over a one year period. Measurements were made of vertical and seasonal variations in bacterial populations. Vertical variation showed high bacterial numbers in the surface waters and a slight increase at the bottom deposits. Lloyd (1930) acknowledged that saphrophytic bacteria would tend to be attached to suspended organic particles of various origins. In seawater where there are no currents or vertical mixing, it is reasonable to assume that an accumulation of organic matter would occur at the surface. This would result in an increase in the accumulation of bacterial saphrophytes. Sediment deposits at the bottom also gave an increase in bacterial numbers.

Much of the pioneering work on marine fouling was carried out by Claude E. Zobell at the Scripps' Institution of Oceanography in S. California. Zobell and Allen (1933, 1935) made observations of the formation of microbial films on glass slides submerged in seawater for one to seven days. The slides were examined by light microscopy after staining. It took from 2-4 hrs. for microbial films to become fixed to the glass slides. All stages of bacterial development were observed, including individual and dividing cells, chains and microcolonies. Coccobacilli and slender rods were most numerous, whilst larger rods occurred less frequently. The majority of the attached bacteria were capsulated, although some developed a holdfast structure. These included Actinomycetes, consisting of small patches of slender mycelial threads and

possibly *Leptothrix* spp. producing larger, straighter, branched filaments. Certain bacterial forms produced a film of faintly staining material which extended beyond the cells. This could have been adhesive material. The other microscopic organisms that were mainly observed were diatoms. The common genera included *Grammatophora* spp., *Navicula* spp., *Licmophora* spp. and *Nitzschia* spp.. The diatoms help to make up the primary fouling film, although they are less abundant than bacteria. The formation of these films precedes the attachment of macroscopic fouling organisms, such as barnacles, hydroids, seaweeds and bryozoa. This was shown by submerging film-coated glass slides in seawater with sterile slides as controls. After one to five days' submergence, the film-covered slides had a greater number of attached macroorganisms than the sterile control slides suggesting that the film facilitates attachment by larger fouling organisms (Zobell & Allen 1933, 1935).

More recent studies have shown that any type of structural material becomes fouled when submerged in the biotic zone. Such materials include wood, metal, concrete, glass or plastic (Zobell, 1946).

Heukelekian and Heller (1940) showed that nutrient concentrations and solid surfaces affected the growth and attachment of bacteria. Cultures of *Escherichia coli* were inoculated into flasks containing glucose and peptone medium with concentrations ranging from 0.5 to 100 ppm.. 50g. of 4mm. glass beads were added to one series of flasks, whilst the other flasks were incubated without beads. *E. coli* failed to grow at a 0.5 ppm. concentration of glucose and peptone after 72 hrs. incubation without glass beads. Growth under these conditions was only slight at a nutrient concentration of 2.5 ppm.. However, there was considerable growth when glass beads were present. The effect of glass beads was noticeable up to

a 25 ppm. concentration of glucose and peptone. Beyond this concentration, the extent of bacterial growth was the same whether or not glass beads were present. The results showed that the limiting nutrient concentration is not a fixed value but depends on the amount of surface in contact with the growth medium. Food concentration was the limiting factor up to a glucose-peptone concentration of 25 ppm.. Increasing the surface-volume ratio by the addition of glass beads increased the concentration of limiting nutrient at the solid surface. This in turn increased the growth of *E. coli*. These results show that surfaces enable bacteria to develop in media which are otherwise too dilute for growth (Heukelekian and Heller, 1940).

Zobell (1943) also observed the beneficial effect of solid surfaces on marine bacterial attachment and growth, particularly in dilute nutrient solutions. Organic matter was adsorbed from seawater by glass surfaces. This was shown by the appearance of an irregularly stained film of material on glass slides immersed in seawater for several days. Biological evidence was also given for the adsorption of organic matter. Several pieces of thin-walled glass tubing were placed into 145 ml. glass-stoppered bottles containing seawater with bacteria. The glass tubing increased the area of solid surface in contact with the seawater. The control bottles did not contain any tubing. Both sets of bottles were incubated at either 0°C or 22°C, and oxygen levels in the seawater were measured. The bacteria multiplied more rapidly and consumed more oxygen in seawater incubated at 22°C. At both temperatures, however, more oxygen was consumed in the seawater which was exposed to the glass tubing. Plate counts of bacterial populations were taken in both sets of bottles at both temperatures. Increases in bacterial populations were observed, although

the plate counts at 22°C decreased sharply after ten days' incubation. The plate counts from the seawater containing the glass tubing were lower. This observation was explained by the abundance of periphytic or sessile bacteria attached to the glass. The greater oxygen consumption observed in the seawater with glass tubing may be caused by metabolic processes occurring during bacterial adhesion.

These observations show that bacterial attachment to surfaces is enhanced by the adsorption of organic matter. Such material predominates on surfaces in the early stages of submersion. Part of the organic matter may be in the form of attached bacterial cells (Zobell, 1943). However, although adsorbed nutrients may promote the multiplication of bacteria, they are not directly responsible for attachment.

Zobell (1943) also observed that some bacteria were attached to glass slides by the production of stalks. Other bacteria appeared to be in intimate contact with the surface. These physiologically active bacteria appeared to secrete a cementing substance which secured their attachment. Removal of the attached bacterial cells from the glass slides left a faintly staining film which had the shape and arrangement of the cells. The production of a film of staining material by other bacteria was also observed. Examination of glass slides after submergence in bacterial cultures for different periods showed that the film size increased with age. Microcolonies of bacteria were often seen on "islands" of this film. This suggests that bacterial attachment promotes the attachment of other bacteria.

Solid surfaces may reduce the diffusion of exoenzymes or hydrolysates from the bacterial cells. This could occur by the interstices at the contact point of the bacterial cell and surface acting as concentration

foci for exoenzymes or hydrolysates. The attachment of other bacteria or their development by cell division would form more interstitial or capillary spaces. These would further reduce the diffusion of materials from the cells, so promoting the assimilation of nutrients which must be hydrolyzed before ingestion (Zobell, 1943).

To summarise, then, the work of Zobell and Allen (1933, 1935) demonstrated the formation of primary films on submersed surfaces and possible attachment mechanisms of the main fouling microorganisms. The observations of Heukelekian and Heller (1940) and Zobell (1943) showed the importance of nutrient accumulation and organic film formation on solid surfaces in bacterial adhesion.

With this background, the thesis is divided into four main sections.
Section 1 discusses in detail and compares the attachment mechanisms of marine bacteria, algal spores and rhizoids, diatoms and fungi.
Section 2 considers microbial adhesion to living marine surfaces such as fish, marine invertebrates and marine plants. The importance of attachment in disease pathogenesis is discussed for fish and invertebrates. Examples of possible symbiotic association between microorganisms and invertebrates are included. There is also a chapter on attachment between marine microorganisms, which includes the interesting interaction of parasitic *Bdellovibrio bacteriovorus* with host bacteria.
Section 3 surveys microbial adhesion to non-living marine surfaces. This includes the attachment of microorganisms to sediment particles and their role in the formation of microbial mats and in sediment stabilization. The importance of attached sediment microorganisms in the nutrition of some benthic invertebrates is also discussed. This section also covers microbial adhesion to detritus, the role of microorganisms in the formation

of detrital aggregates and the degradation of faecal pellets.

Finally, Section 4 contains two chapters. The problem of metal corrosion caused by attached microfilms is firstly examined. This is a problem of great applied importance. The thesis ends with a general review of traditional and more recent antifouling and microfouling control methods.

SECTION 1.

ATTACHMENT MECHANISMS OF MARINE MICROORGANISMS.

Chapter 1: Adhesion mechanisms in marine bacteria.

(1.1) Physicochemical aspects of marine bacterial adhesion

This sub-chapter deals with the main events occurring during the initial stages of marine bacterial adhesion, before the deposition of organic polymers or production of stalks or holdfasts. Some of the literature published in this field describes it by a detailed mathematical approach; this is beyond the scope of this thesis.

There has frequently been uncertainty over whether bacterial adhesion is a passive, time-dependent process, or an active process requiring physiological activity (Fletcher, 1980). Zobell (1943) observed that marine bacterial attachment to glass surfaces was time-dependent. However, bacterial adhesion to surfaces can be very rapid, and so can be compared to a passive process of molecular adsorption. It should be possible, therefore, to treat bacteria as chemical species, and elute them off a substratum (Fletcher, 1980). Meadows (1964, 1965, 1966) found that marine bacteria were desorbed from sand grains by distilled water, glycerol and sucrose solutions. A second way in which bacterial attachment resembles chemical adsorption is by the influence of cations on the process, to be discussed in more detail later. Bacterial attachment also involves physiological activity, such as motility to make contact with the substratum, and synthesis and secretion of adhesive polymers. This makes it an active adhesion mechanism (Fletcher, 1980). Consequently, both active and passive bacterial adhesion can occur, although the initial stages closely resemble a passive process.

Ellwood *et al.* (1982) listed three main stages in microbial adhesion :-

(1) Deposition or adsorption of the microorganism on to a surface;

(2) Permanent attachment to the surface, often by production of polymeric materials;

(3) Colonization of the surface by growth of the organism.

Meadows (1966) observed that some strains of marine bacteria were not strongly attached to surfaces, as a moving bubble could dislodge them. Some strains, however, were completely immobile. Further observations (Meadows, 1970) showed three stages of marine bacterial attachment: states 1, 2 and 3. During state 1, bacteria attach at one cell pole, by a polar flagellum. In state 2, bacteria attach along their cell length showing slight Brownian movement, and in state 3 they attach this way showing no Brownian movement (Meadows, 1970). Marshall *et al.* (1971) found that sorption of two marine bacteria to surfaces involved an instantaneous reversible phase, and a time-dependent irreversible phase. During the reversible phase, bacteria are held weakly near the substratum surface, and are readily removed with 2.5% sodium chloride solution. These two phases resemble Meadows' (1966) observations of some bacteria not being strongly attached, and some being immobile. In addition, Meadows' (1970) state 2 resembles the reversible phase, while state 3 resembles the irreversible phase of adhesion. The reversible phase also resembles a passive adsorption mechanism (Fletcher, 1980).

In the process of particle or microbial adhesion there are both long-range and short-range attractive forces involved. Long-range forces include the London-van der Waals forces and electrostatic forces (Daniels, 1980; Rutter and Vincent, 1980; Tadros, 1980). Short-range forces are particularly important in aquatic systems at short separations between the microorganism and surface (Rutter and Vincent, 1984). They include chemical bonds such as hydrogen bonds, dipole interactions and hydrophobic

interactions (Rutter and Vincent, 1980; Tadros, 1980).

The role of London-van der Waals forces in particle adhesion was first proposed in the DLVO theory of Derjaguin, Landau, Verwey and Overbreek (Verwey and Overbreek, 1948). They are explained as being second-order forces between neutral atoms, attraction being due to polarization of one atom, by charge fluctuations in a second atom. These forces are assumed to be additive, so that each atom attracts all other atoms (Verwey and Overbreek, 1948). These forces operate in a similar way between a microorganism and substratum. This is because a microbial cell can be thought of as a macroscopic ion with a large number of electrically charged sites (Daniels, 1980). Although London-van der Waals forces are involved in the reversible stage of microbial adhesion, the total interaction resulting in deposition of a microbial cell comprises two opposing forces. These are the London-van der Waals forces, and a repulsive force resulting from the overlap of the electrical double layers surrounding cell and substratum (Verwey and Overbreek, 1948; Rutter and Vincent, 1980).

One layer of the electrical double layer is composed of the charges on the surface of the particles or cells. The second layer is composed of ions of opposite charge, found in the surrounding medium, which counter balance the particle charge (Verwey and Overbreek, 1948; Marshall, 1976; see p. 15). Formation of a compact layer of counter-ions at the particle or microbial cell surface is impeded by thermal agitation of the ions. This results in a loose association known as the Gouy-Chapman diffuse double layer (Marshall, 1976). According to this model, the potential initially drops rapidly with increasing distance from the particle, then more slowly. The distance over which the potential increases exponentially from bulk medium to the particle surface is the double-layer

thickness (1/x) (Marshall, 1976; see p. 15). A further model describing the electrical double layer is the Stern model. This model assumes that there is a layer of counter-ions held at the particle surface by forces sufficiently strong to overcome thermal agitation (Marshall, 1976; see p. 15).

In seawater, at a solid/liquid interface, the fixed layer is made up of ions adsorbed at the surface of a sand grain or bacterial cell. Opposite charged ions in seawater make up the diffuse layer, and together they constitute the electrical double layer in seawater (Meadows and Anderson, 1979).

Marshall *et al.* (1971) considered the effects of ionic strength on the electrical double layer. At low electrolyte concentrations, the layer was fairly thick; it was 200 Å at 2×10^{-4} M sodium chloride. At higher ionic concentrations, it was thinner (Marshall *et al.*, 1971). The thickness of the double layer at different ionic strengths in seawater, together with the London-van der Waals attractive forces, will affect reversible bacterial adhesion. This can be described graphically (Meadows and Anderson, 1979, see p. 15; Tadros, 1980; Rutter and Vincent, 1984). At low electrolyte concentrations, where the electrical double layer is thick, a large energy barrier prevents close contact of the bacterium with the substratum (Marshall, 1976; Meadows and Anderson, 1979; see p. 15 ; Tadros, 1980). In this case, there is strong repulsion between the bacterium and substratum. At high electrolyte concentrations, however, the repulsion energy is reduced, and a secondary attraction minimum exists (Marshall, 1976; Meadows and Anderson, 1979; see p. 15). The secondary minimum is defined as the point of minimum separation where a net attraction energy exists (Meadows and Anderson, 1979). Marshall (1976) proposed that marine

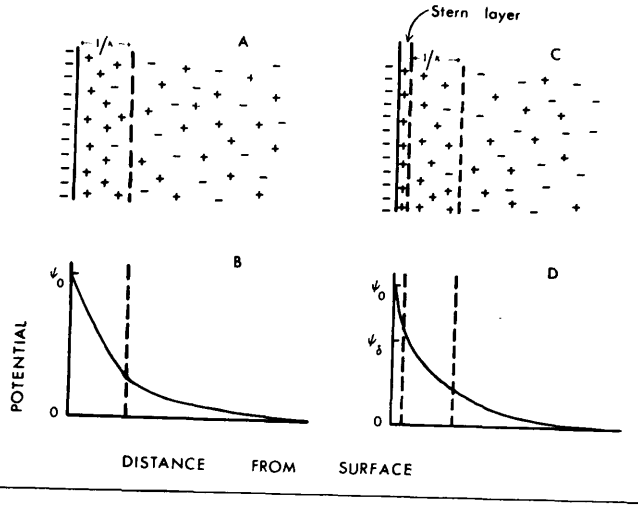


Fig. 1 Schematic representations of (A) the charge distribution and (B) the potential, in the Gouy–Chapman model of the diffuse double layer; (C) the charge distribution and (D) the potential in the Stern model

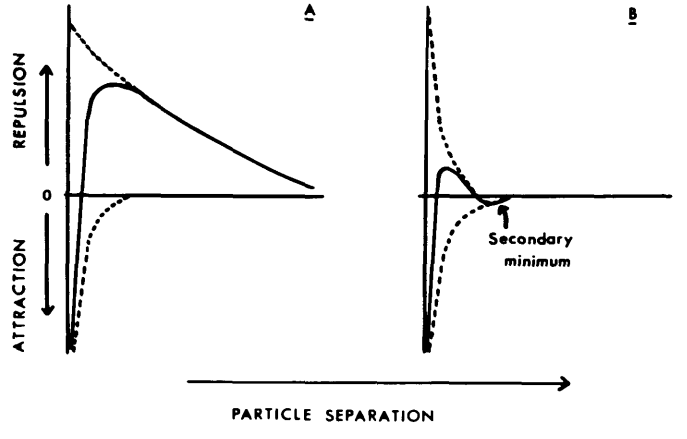


Fig. 2 Potential energy of interaction between two colloidal particles at: (A) low electrolyte concentrations where 1/X is large; (B) high electrolyte concentration where 1/X is small. Curves represent the London-van der Waals attraction and double layer repulsion energies (dotted lines) and the resultant energy (solid lines).

(Reproduced by permission from Marshall, 1976).

bacteria in Meadows' (1970) 'state 2' will be caught in the secondary attraction minimum. However, despite the attraction of bacteria to surfaces at high ionic strengths, the repulsion energy barrier ensures that bacteria remain a small, but finite distance from the substratum surface. Brownian motion of the cells is not enough to overcome the repulsion barrier (Marshall, 1976). The effects of a secondary attraction minimum on adhesion of *Achromobacter* sp. R8 was observed by Marshall *et al.* (1971). They found that the number of *Achromobacter* cells reversibly sorbed increased with increasing electrolyte concentration, as the thickness of the electrical double layer (1/x) decreased (see p. 18).

Gordon and Millero (1984) observed that attachment of an estuarine *Vibrio alginolyticus* strain to hydroxyapatite increased with increasing ionic strength of the medium. This is in accordance with the above observations of Marshall *et al.* (1971) and Meadows and Anderson (1979) of the decrease in electrical double layer size at high ionic concentrations.

An interesting theory was proposed by Ellwood *et al.* (1982) principally to explain accumulation of microorganisms at areas of high nutrient concentration on a surface (see Chap. 1.6). It could also be an alternative theory of bacterial adsorption to a surface. The theory is based on Mitchell's chemiosmotic hypothesis, whereby bacteria obtain energy by the translocation of protons outside the cell to generate a membrane potential. The resulting build-up of positive charge outside the bacterial cell causes protons to be pumped back into the cell by a proton motive force. This re-uptake of protons generates ATP synthesis.

The theory of Ellwood *et al.* (1982) states that localized proton concentrations at the bacterial cell surface will tend to increase in the restricted zone established when the cell interacts with a surface (see p.18).

As these protons will not diffuse so easily, the probability of their re-uptake into the bacterial cell will be increased. Consequently, a polarity across the cell will be established, which, together with ATP synthesis, can provide the driving force for adherence of the bacterial cell to the surface (see p. 18). Increased efficiency of proton re-uptake will provide the adsorbed bacterial cell with more energy to encourage growth and division. Additionally, proton uptake gradients could be shared amongst neighbouring bacterial cells. This could ultimately lead to the development of a microcolony or film of bacterial cells on a surface (Ellwood *et al.*, 1982; see p. 18).

Rutter and Vincent (1984) mentioned several factors which suggested that the physicochemistry of bacterial adhesion is difficult to explain. Firstly, the issue is complicated by the nature of the particles and substrata involved. Microorganisms such as bacteria are not "ideal" particles. They may be deformable on contact with a surface. Additionally, internal chemical reactions could lead to changes in molecular composition both in the interior and at the surface of the bacteria. Consequently, the theory and principles of particle deposition may not apply in practice to bacterial adhesion (Rutter and Vincent, 1984).

A further problem lies in the application of thermodynamic principles to bacterial adhesion. This is rooted in the question of whether bacterial adhesion to a surface occurs under equilibrium conditions (Rutter and Vincent, 1984). Additionally, the problem of whether the equilibrium state is maintained after adhesion is also important. Further chemical reactions may occur inside or at the surface of an attached microbial cell. If this occurs, the microorganisms cannot be in complete physicochemical equilibrium. Furthermore, equilibrium can only be

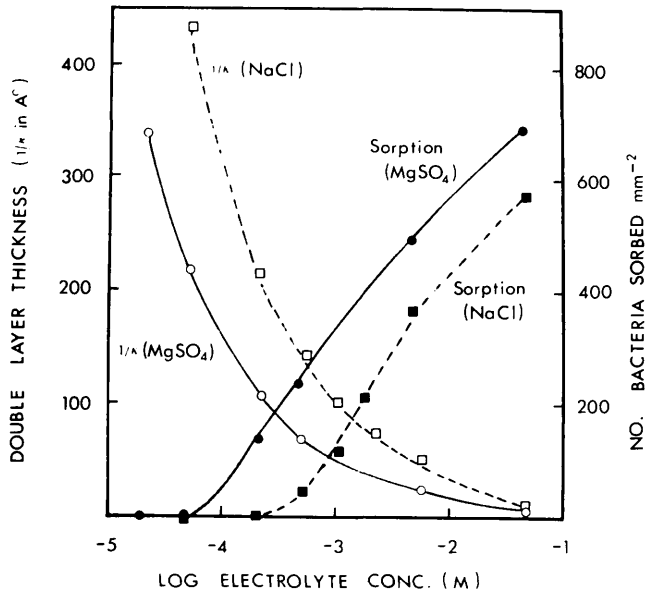


Fig. 3 Reversible sorption of *Achromobacter* sp. R8 and the theoretical double-layer thickness (1/X) in relation to electrolyte valency and concentration.

(Reproduced by permission from Marshall, 1976).

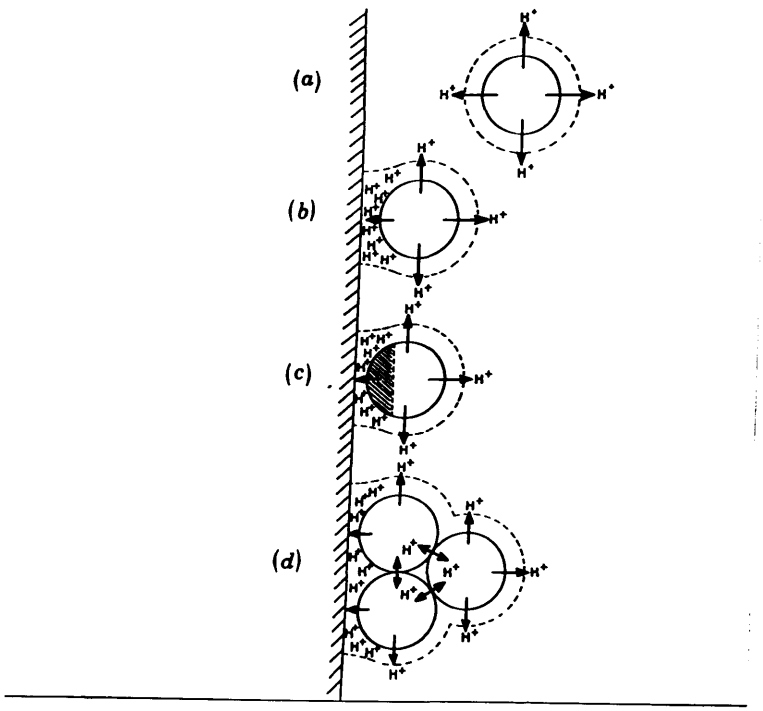


Fig. 4 Postulated chemiosmotic interactions of a bacterial cell at a surface. The diagram describes: (a) a cell in free suspension generating a proton gradient, (b) the interaction of the cell's domain with a surface, establishing a localised higher concentration of extruded protons, (c) localised Δp (proton motive force) and ATP synthesis leading to increased metabolic activity and polarity of the cell to drive adhering processes and (d) the establishment of a microcolony with sharing of proton gradients between cells.

(Reproduced by permission from Ellwood *et al.*, 1982)

achieved in a thermodynamically 'closed' system, where no exchange of energy or matter with the surroundings occurs (Rutter and Vincent, 1984). Many biological systems, including microbial adhesion, are 'open' systems, where equilibrium may never be reached.

Rutter and Vincent (1984) also feel that physical chemists and microbiologists should collaborate with their physicochemical theories of bacterial adhesion. By working with as well-defined and characterized systems as possible, they may be able to confirm many of the physicochemical theories discussed here.

This sub-chapter has shown the following points about physicochemical aspects of bacterial adhesion :-

(1) Bacterial adhesion to surfaces can be either a passive, time-dependent process or an active process requiring physiological activity. (Meadows, 1964; Fletcher, 1980).

(2) Sorption of marine bacteria to surfaces involves an instantaneous reversible phase and a time-dependent irreversible phase. (Marshall *et al.*, 1971).

(3) The two main opposing forces acting in the reversible phase of bacterial adhesion are the attractive London-van der Waals forces, and the electrical double layers' repulsive force. (Marshall, 1976; Rutter and Vincent, 1980).

(4) The electrical double layer, made up of oppositely charged ions, is affected by ionic concentrations. This in turn affects bacterial adhesion. (Marshall *et al.*, 1971).

(5) The thickness of the electrical double layer at different ionic strengths, together with the London-van der Waals forces, influences marine bacterial adhesion, as shown by energy curves. (Meadows and Anderson, 1979).

(6) The chemiosmotic hypothesis of energy generation may offer an alternative explanation of bacterial adsorption, and ultimate build-up of a microcolony on a substratum. (Ellwood *et al.*, 1982).

(1.2) Attachment by production of high molecular weight organic polymers and inorganic polymers

Extensive studies have demonstrated the involvement of high molecular weight organic polymers in the attachment of marine bacteria to solid surfaces. In addition, certain species produce inorganic materials which facilitate their adhesion.

Corpe (1970a, b, 1972, 1974) demonstrated, by histochemical staining, the production of an extracellular acidic polysaccharide involved in the attachment of a marine pseudomonad to glass surfaces immersed in seawater (p. 22). The polysaccharide was a polyanionic carbohydrate consisting of neutral sugars as well as uronic acid. The sugars were glucose, galactose and mannose, and pyruvic acid was also present. The marine sedimentary bacterium *Pseudomonas atlantica* produces an extracellular glycocalyx which possesses uronic acid substituents (Uhlinger and White, 1983).

A marine periphytic *Pseudomonas* strain NCMB 2021 was shown to produce two extracellular polysaccharides when grown in batch culture (Christensen *et al.*, 1985). Polysaccharide A was only produced during exponential growth and contained glucose, galactose, glucuronic acid and galacturonic acid. Polysaccharide B was released at the end of the exponential phase and in the stationary phase. It contained equimolar amounts of N-acetylglucosamine, 2-keto-3-deoxyoctulosonic acid and an unidentified 6-deoxyhexose (Christensen *et al.*, 1985). Histochemical staining and transmission electron micrographs (Jones et al 1969; Fletcher and Floodgate, 1973, 1976) showed acidic polysaccharide attaching marine bacteria to surfaces of glass slides. Fletcher and Floodgate (1973) suggested the formation of primary acidic polysaccharide which is involved in the initial reversible step of attachment (Meadows, 1970; Marshall *et al.*, 1971),

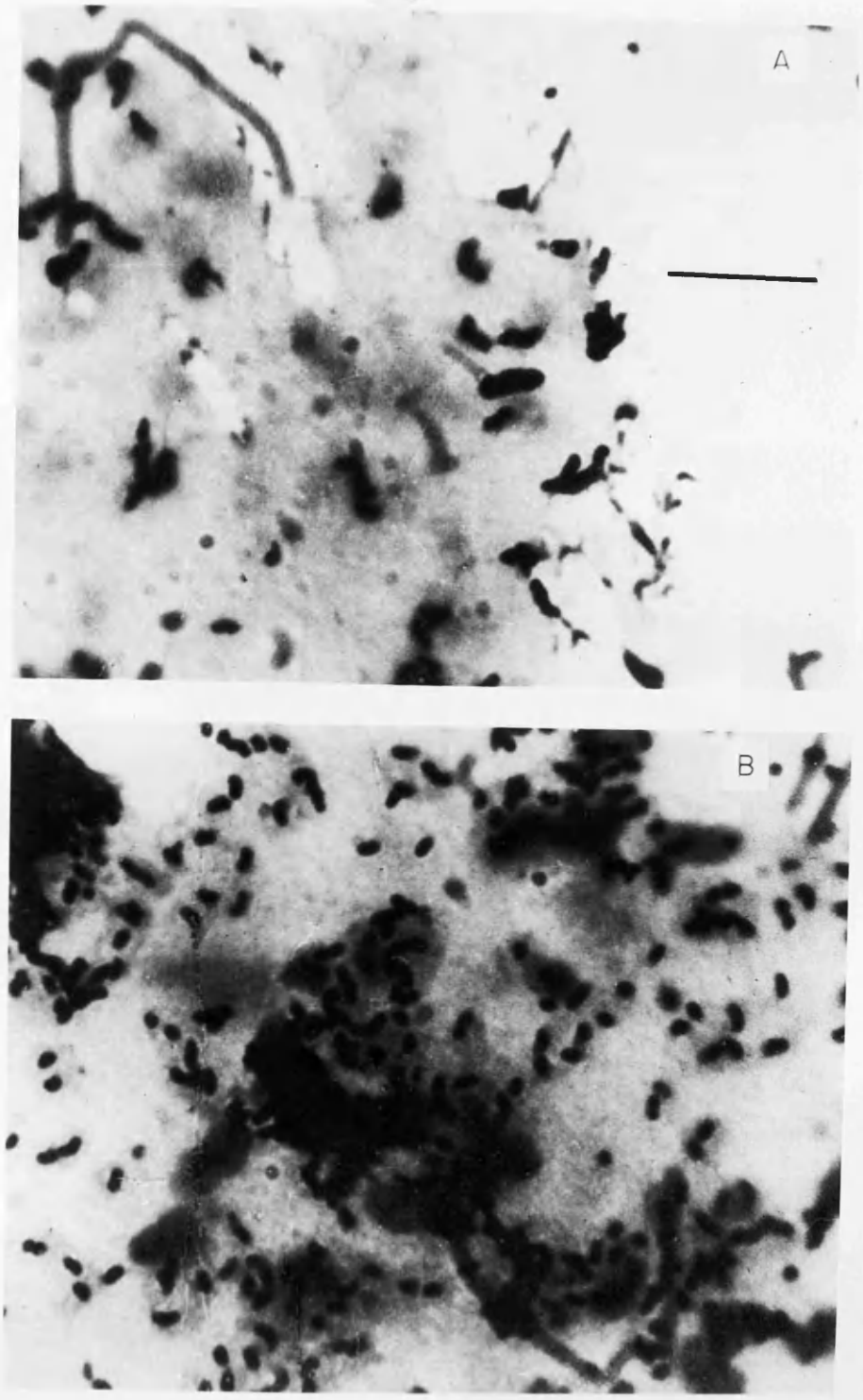


Fig. 5 Pure cultures of *Pseudomonas atlantica* growing as an attached film on the surface of a glass slide. The slide was suspended in a culture of artificial seawater containing 0.01% (w/v) Bacto-peptone. The scale shown is 5μm.

(Reproduced by permission from Corpe, 1970a).

followed by production of secondary acidic polysaccharide which mediates the final irreversible stage (see p. 24). Polysaccharide A isolated by Christensen *et al.* (1985) formed gels, so it may function as the secondary polysaccharide described by Fletcher and Floodgate (1973). The polysaccharide would be able to maintain a hydrated and mechanically stable matrix between the attached cells (Christensen *et al.*, 1985). However, Paul and Jeffrey (1985a) stated that it was unlikely that extracellular slimes and glycocalyces were involved in the initial adhesion process. Such materials are often loosely attached to cells and may decrease adhesiveness (Paul and Jeffrey, 1985a). It is more likely that physicochemical forces, such as London-van der Waals forces and electrostatic forces play a greater role in the initial stages of bacterial adhesion (see Chapter 1.1).

Observations of other marine bacteria have suggested the involvement of other types of adhesive. Electron microscopic observations of a gliding marine bacterium, *Flexibacter* BH3, revealed extracellular filamentous and amorphous material at some distance from the organisms (Humphrey *et al.*, 1979). In addition, vesicular material was present on the cell surface. This slime material exhibited properties characteristic of a linear colloid, so providing suitable conditions for temporary adhesion of *Flexibacter* BH3. The slime material was found to be a glycoprotein, consisting of glucose, fucose, galactose and protein, together with some uronic acid. The inability of the material to stain with ruthenium red indicated that it was not a highly acidic polysaccharide (Humphrey *et al.*, 1979). Production of vesicular, filamentous material from the surface of a marine *Flexibacter* spp. was previously demonstrated by Ridgway and Lewin (1973). Filament production occurred from goblet-

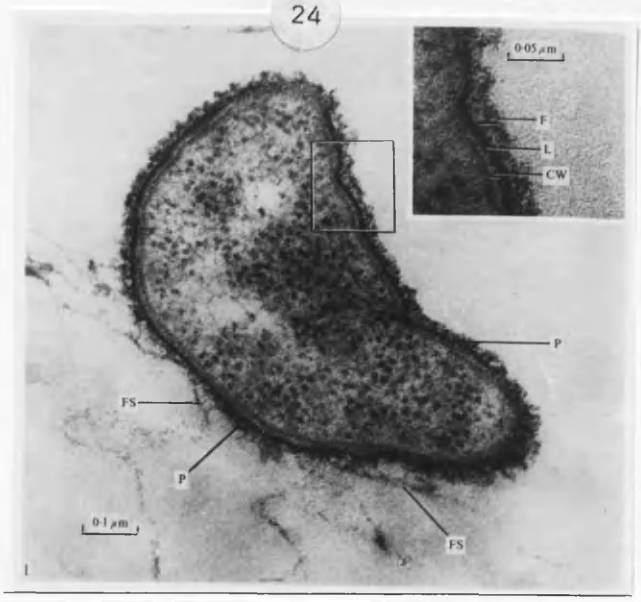


Fig. 6.1 A naturally attached bacterium treated with ruthenium red. The multilayered cell wall (CW) is surrounded by primary acidic polysaccharide (P) which appears to attach the bacterium to the filter surface (FS). Primary polysaccharide can be differentiated into a thin dense line on the wall surface (L) and an outer 'fringe' region (F).

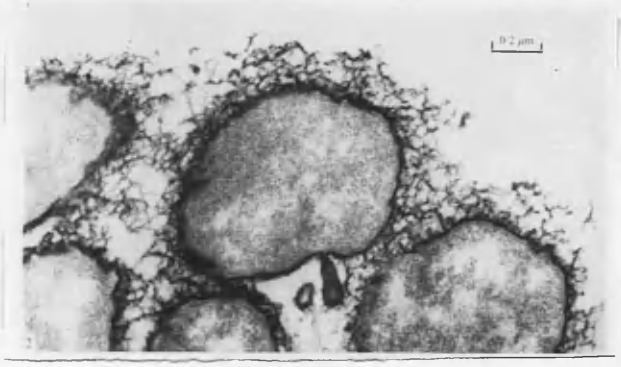


Fig. 6.2 A group of naturally attached bacteria treated with ruthenium red. Secondary acidic polysaccharide stretches around and between the organisms.

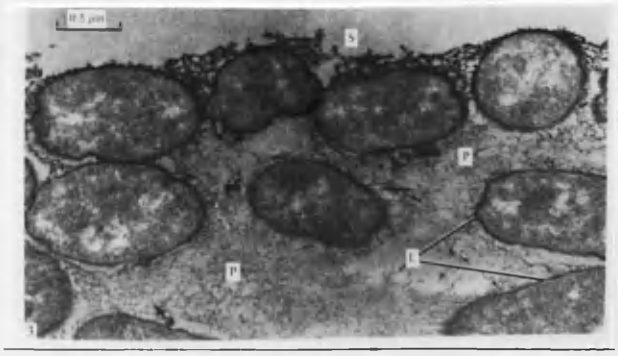


Fig. 6.3 A portion of a microcolony treated with ruthenium red. The bacteria are located in a mat of secondary acidic polysaccharide (P).

(Reproduced by permission from Fletcher and Floodgate, 1973).

shaped structures which were probably associated with the outermost layer of the cell wall. A large amount of negatively stained fibrillar material was associated with the surfaces of lysed *Flexibacter* cells. Ridgway and Lewin (1973) suggested that the fibres were aggregates of fine filaments arranged parallel to one another. Scanning electron microscopy indicated that aggregation of these filaments may play a role in attaching *Flexibacter* BH3 to the substratum or to other bacteria. The filaments consisted of lipid, protein, glucosamine, 2-keto,3-deoxyoctonic acid (KDO), phosphorous and pigment, further suggesting that they were derived from the outer membrane of the cell envelope (Ridgway and Lewin, 1973). This filamentous material may play a role in initial contact of *Flexibacter* with a surface, by providing a means of polymer bridging between the cell and surface (Humphrey *et al.*, 1979).

Studies of the effects of certain enzymes on attachment of marine bacteria have provided further information on the nature of adhesive material. Danielsson *et al.* (1977) found that pronase and trypsin released *Pseudomonas* spp. attached to glass slides submerged in seawater. Pronase released 70%, and trypsin, 50%, of attached cells after thirty minutes. After addition to the bacterial suspension, pronase and trypsin inhibited attachment of *Pseudomonas* strain NCMB 2021 to tissue culture dishes (Fletcher and Marshall, 1982). These results show that envelope proteins are probably involved in attachment of the marine *Pseudomonas* spp. to surfaces. Pronase, trypsin and chymotrypsin inhibited attachment of *Vibrio proteolytica* to polystyrene by over 97% (Paul and Jeffrey, 1985a). Removal of cells from polystyrene by pronase left material termed "footprints". The footprints were observed by scanning electron microscopy to be composed of adhesive material, and formed a rough outline

of the cell. Rifampin, an RNA-polymerase inhibitor, inhibited attachment of *V. proteolytica* to polystyrene, as did puromycin and chloramphenicol, both protein synthesis inhibitors (Paul, 1984). Both sets of results indicate that proteins are involved in attachment of *V. proteolytica* to polystyrene. Corpe (1974) found that all crude extracellular products of attaching marine bacteria contained protein. Protein could be incorporated in the polyanionic carbohydrates produced by these bacteria. However, lack of bacterial detachment after enzymic treatment does not necessarily indicate that proteins or polysaccharides are not involved in attachment. The particular configuration adopted by cell surface proteins interacting with the substratum and the closeness of the interaction may cause differences in the efficacy of degradative enzymes (McEldowney and Fletcher, 1986).

An attachment mechanism which is unique to aquatic bacteria, is adhesion by deposition of inorganic "cements". Sheathed bacteria of the *Sphaerotilus - Leptothrix* group are known to oxidize manganous compounds to give manganese dioxide precipitates (Silverman and Ehrlich, 1964; Corpe, 1970a; van Veen *et al.*, 1978). However, in some cases manganese ions can be adsorbed by cells or by a slimy extracellular sheath. *Sphaerotilus* cells use such structures for attachment to surfaces (van Veen *et al.*, 1978). When *Sphaerotilus discophorus* was grown in media containing manganese ions, the cultures became dark brown due to the formation and deposition of manganese oxide on the sheaths of the filaments (Johnson and Stokes, 1966). In addition, stalked, budding bacteria of the *Hyphomicrobium* type form manganese-rich deposits on the inner surfaces of pipelines (Tyler and Marshall, 1967). *Hyphomicrobium* strain ZV-580 has been shown, in electron micrographs, to form rosettes. The cells are

held together by a holdfast material which could be a manganous deposit (Conti and Hirsch, 1965).

Interesting observations of the deposition of iron and manganese oxides on the cell surfaces of budding *Pedomicrobium* bacteria were made by Ghiorse and Hirsch (1979). These authors found that when the *Pedomicrobium* strains were grown in the presence of iron or manganese, the corresponding oxides accumulated on their surfaces. Fine fibrils of electron dense material, presumably containing iron, were observed on thin sections of cells after seven days growth in iron-containing medium. The fibrils appeared to form a matrix surrounding the mother cells. Cells grown in the presence of an iron paper clip for twelve days accumulated a heavy coating of electron dense material on the surfaces of both mother cells and hyphae (Ghiorse and Hirsch, 1979). When the bacterial cells were grown in medium containing manganese, mother cells rapidly accumulated manganese oxide. Deposits around the mother cells appeared as branching, electron-dense filaments in thin sections. At higher magnifications, less heavily encrusted cells were surrounded by fine ribbons of electron-dense material, which was probably manganese oxide (Ghiorse and Hirsch, 1979). Ruthenium red staining of thin sections of cells revealed electron dense material, probably acidic polysaccharide, external to the outer layers of both *Pedomicrobium* strains. Extraction of the accumulated oxides followed by ruthenium red staining showed that polyanionic polymers previously deposited on the cells were associated with the metal oxides. The accumulation of positively charged, autooxidized iron hydroxides on the acidic polymers results in the association of iron oxide with the polyanionic polymer (Ghiorse and Hirsch, 1979). The mechanism of manganese oxide deposition is probably different. Manganese ions may

first adsorb to the polyanionic polymers, to be oxidized by an oxidizing factor(s) present at the *Pedomicrobium* cell surface or in the polymer matrix surrounding the cells. Once initiated, the accumulation of manganese ions on both polymers and newly formed oxides would continue. The manganese oxidizing factor may be an inducible protein, possibly an enzyme, present at the cell surface (Ghiorse and Hirsch, 1979).

Oxidation of ferrous ions is characteristic of the sheathed *Sphaerotilus - Leptothrix* group of bacteria (van Veen *et al.*, 1978). These bacteria have a tendancy to deposit large amounts of ferric iron in their sheaths. The amount of iron deposited in the sheaths of *Sphaerotilus discophorus* increased with increasing ferric ion concentration in the growth medium. Iron deposition was highest at the onset of the stationary growth phase. During the exponential phase, it was many times lower (van Veen *et al.*, 1978).

Iron and manganese accumulation also occurs in prosthecate marine bacteria, *Gallionella* spp., in the sheaths or stalks which they produce for attachment (Silverman and Ehrlich, 1964).

Summary

The main points reviewed in this sub-chapter together with the key cited papers, are :

(i) Certain marine bacteria, mainly *Pseudomonas* spp., attach to surfaces by production of extracellular acidic polysaccharides (Corpe, 1970a,b; Fletcher and Floodgate, 1973; Christensen *et al.*, 1985).

(ii) Other marine bacteria, such as gliding *Flexibacter* spp., attach by production of filamentous slime material consisting mainly of glycoprotein (Humphrey *et al.*, 1979).

(iii) Studies with proteolytic enzymes and antibiotics inhibiting RNA and protein synthesis suggest that cell envelope proteins may also be involved in adhesion of certain marine bacteria (Danielsson *et al.*, 1977; Paul, 1984; Paul and Jeffrey, 1985a).

(iv) Other bacteria, such as *Sphaerotilus - Leptothrix* spp. and *Pedomicrobium* spp. attach by production of inorganic materials, particularly iron and manganese oxides (van Veen *et al.*, 1978; Ghiorse and Hirsch, 1979).

(1.3) Attachment of marine bacteria by stalks, holdfasts and other surface structures

Certain species of marine bacteria produce stalks, holdfasts and other cell surface structures which allow attachment to solid surfaces. These periphytic bacteria normally colonize submersed surfaces after bacteria producing extracellular acidic polysaccharides have attached (Corpe, 1972, 1974).

Caulobacters, which are widespread in the ocean attach to solid surfaces by stalks (Poindexter, 1964, 1981; see p. 31). They also attach to one another and to other microbial cells to form rosettes (Poindexter, 1964, 1981). The rosettes are held together in the centre by holdfast material (Poindexter, 1964). Long-term attachment of *Caulobacter* cells occurs by this holdfast material at the tip of the stalk (Poindexter, 1981).

The stalked bacterium, *Asticcacaulis biprosthecum*, was shown to possess ruthenium red staining material over the entire cell surface (Umbreit and Pate, 1978). An accumulation of this material was present in the holdfast region. Mutant strains lacking holdfasts were still able to attach to wild type cells, forming rosettes. However, loss of the material resulted in a lack of ability to initiate attachment. This suggests a role for the material in attachment of *Asticcacaulis biprosthecum* (Umbreit and Pate, 1978).

Attachment of wild type strains to one another is explained by the polymer bridging theory (Harris and Mitchell, 1973). The holdfast material of one cell could stretch out and sorb to the holdfast region of another *Asticcacaulis* cell, forming a polymeric bridge. This eventually produces rosettes of cells. The staining nature of the holdfast material suggests

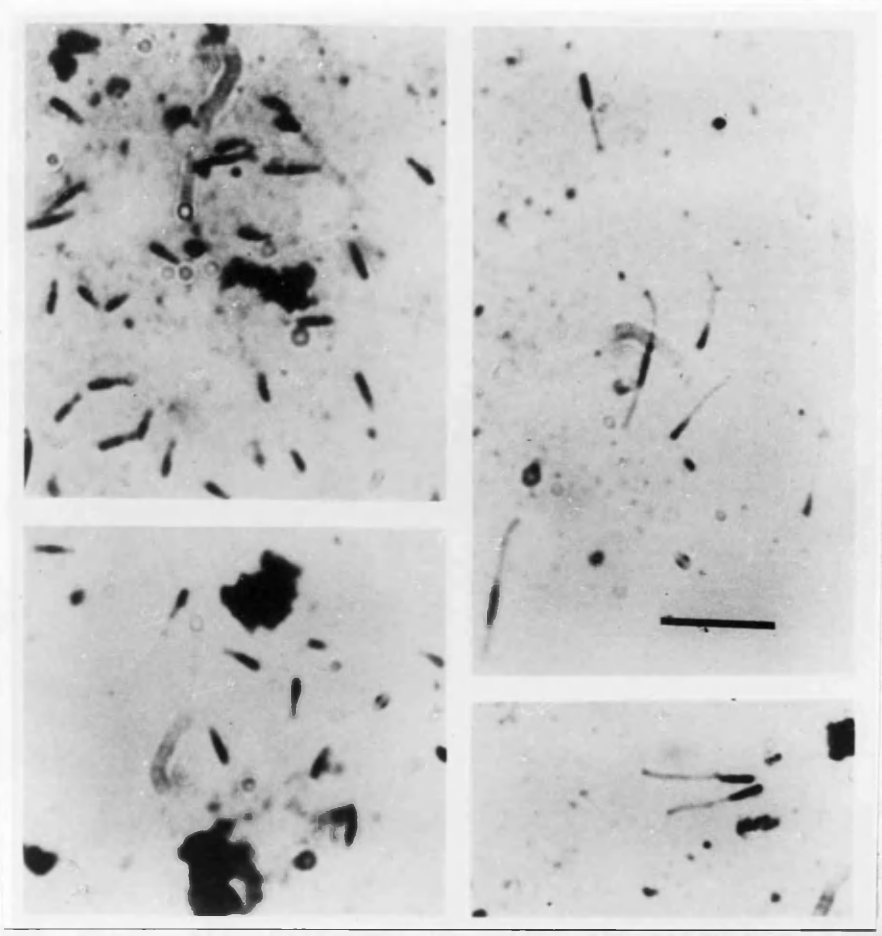


Fig. 7 Typical appearance of stalked bacteria believed to be *Caulobacter* spp. observed on glass slides submerged in seawater for 4-5 days. Stained with crystal violet. The bar represents 5μm.

(Reproduced by permission from Corpe, 1974).

that it consists of acidic polysaccharide. However, Poindexter (1964) found the material was neither periodate or trypsin sensitive. This suggests that it is not carbohydrate or protein. Similar densely staining material is seen at the distal region of the stalk in certain *Caulobacter* species (Poindexter and Cohen-Bazire, 1964). The greatest accumulation of material is in the centre of rosettes. Holdfast material is not secreted from the tip of the stalk in *Asticcacaulis* cells, but from the pole of the cell (Poindexter, 1981).

Pili have been observed on species of *Caulobacter* and *Asticcacaulis* (Umbreit and Pate, 1978; Poindexter, 1981). In *Caulobacter* spp. pili arise from the holdfast site and extend from cell to cell (Poindexter, 1981). Pili were also observed in large numbers at the centres of rosettes of *Asticcacaulis biprosthecum* (Umbreit and Pate, 1978). These pili may assist in the initial stages of *Caulobacter* or *Asticcacaulis* attachment.

Other marine bacteria are known to form stalk-like structures used in attachment. *Gallionella* spp. form flat, twisted stalks of ferric hydroxide which are different in origin and structure from *Caulobacter* stalks (Starr and Skerman, 1965). *Hyphomicrobium* and *Rhodomicrobium* spp. (see p. 33) form filamentous hyphal outgrowths which appear to be structurally related to *Caulobacter* stalks (Starr and Skerman, 1965). Manganese deposition from the hyphae allows firm attachment of these bacteria (see Chapter 1.2).

Bacterial flagella are normally used in locomotion. However, they are also sometimes involved in attachment. Variable flagellar organization may be ecologically important for bacteria alternating between solid supports or liquid surroundings (Scheffers *et al.*, 1976). Strains

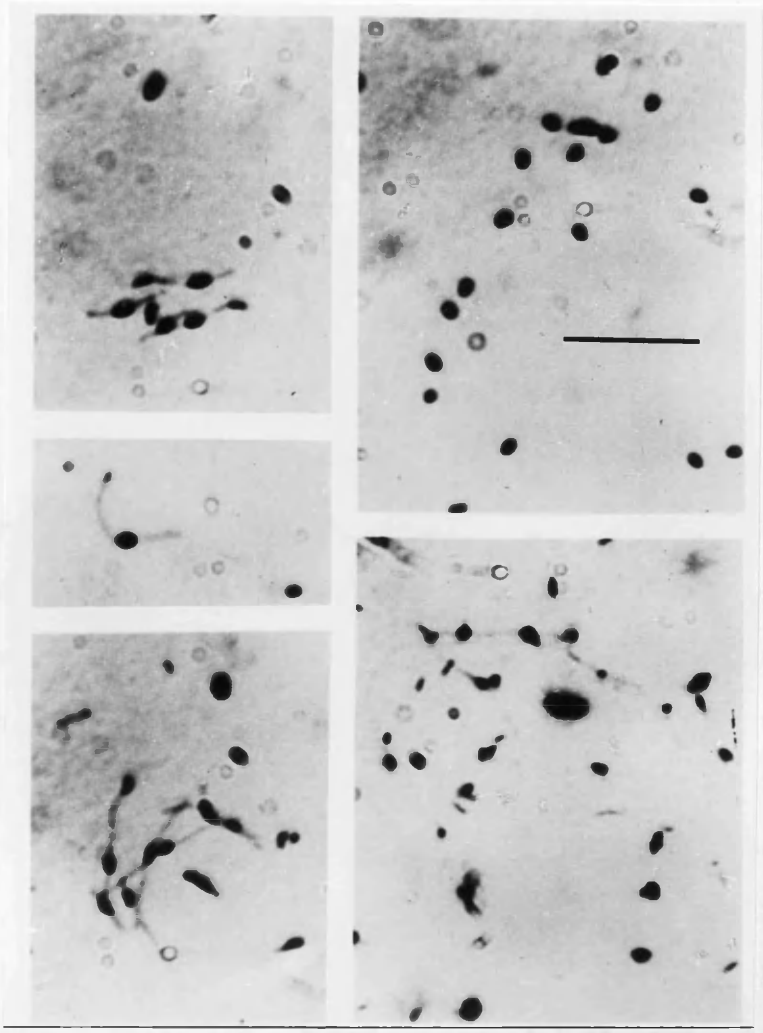


Fig. 8 Typical appearance of budding bacteria identified as *Hyphomicrobium* spp. attached to glass slides submerged in a marine aquarium for 5 days. Stained with crystal violet. The bar represents 5μm.

(Reproduced by permission from Corpe, 1974).

of *Vibrio parahaemolyticus* biotype 2 (*alginolyticus*) formed cells with a single sheathed polar flagellum in liquid medium. Cells with numerous unsheathed lateral flagella of shorter wavelength developed on agar and other solid supports. Adhesion to surfaces via flagella may be enhanced in cells with many flagella, especially if the flagella have a short wavelength. Momentary points of contact, together with slime excretions, may further enhance adhesion between flagella and the surface (Scheffers *et al.*, 1976). Similar observations were made for an epiphytic, marine *Vibrio* spp. isolated from submerged wooden pilings in tropical waters (Belas and Colwell, 1980). When grown on agar medium, this organism produced unsheathed peritrichous flagella, as well as a single sheathed polar flagellum and pili. Formation of peritrichous flagella may help the species to adhere to submerged aquatic surfaces (Belas and Colwell, 1980).

Meadows (1970) found that motile, gram negative marine bacteria attached to glass slides by their polar flagella. Cells attaching in this way tended to rotate. Three polarly flagellated marine bacteria, *Vibrio cholerae*, *Vibrio alginolyticus* and *Pseudomonas marina*, attached to glass surfaces at the pole of flagellar insertion (Sjoblad and Doetsch, 1982). After insertion, there was a brief period of bacterial rotation around the attachment axis. The bacteria did not attach to glass surfaces following deflagellation by blending. The results were interpreted in terms of short- and long-range attraction forces involved when particles of flagellar and bacterial dimensions approach a glass plate (see Chapter 1.1).

Pseudomonas marina rapidly aggregated when suspended in buffered artificial seawater (Sjoblad *et al.*, 1985). Light microscopy of stained preparations showed that flagella-flagella contact was responsible for this

aggregation. Aggregation did not occur if flagella were sheared off. This suggests a role for flagella in marine bacterial aggregation, a property already known for microbial cell surface polymers (Harris and Mitchell, 1973) and cyanobacterial sheaths (see Chapter 2).

Certain species of marine bacteria have been isolated which possess rigid, randomly arranged appendages on their cell surfaces. A bacterium was isolated from infusions of decaying marine algae in Nova Scotia which possessed such appendages, and did not possess a polar flagellum (McGregor-Shaw *et al.*, 1973). Similarly, a member of the *Pseudomonodaceae* family was isolated which possessed such appendages of about 70nm. diameter (Easterbrook *et al.*, 1973). These appendages are known as spinae (spines). They are normally expanded at the base, with a diameter of 120nm., and are attached to, but do not originate in, the cell wall. As they are not extensions of the cell wall, they are non-prosthecate appendages, and are "echinuliform" (spine-like) (McGregor-Shaw *et al.*, 1973). The number of spines on the bacterial cell surface varies from one to a maximum of fifteen, and they are randomly distributed (Easterbrook *et al.*, 1973 see p. 36). Spinae are composed of a subunit protein, spinin, which has a molecular weight of 37,000 daltons (Willison *et al.*, 1977). Striations are present on the surface of spinae (McGregor-Shaw *et al.*, 1973), and these represent their morphological subunits which are helically arranged (Easterbrook *et al.*, 1976). Spinae also occur on certain marine cyanobacteria (Perkins *et al.*, 1981; Sarokin and Carpenter, 1981). Cyanobacterial spinae can be conical (Easterbrook and Subba Rao, 1984), as well as being cylindrical. The function of bacterial spinae is not properly known, and several suggestions have been made.

Cyanobacterial spinae may act as a flotation aid (Perkins *et al.*,

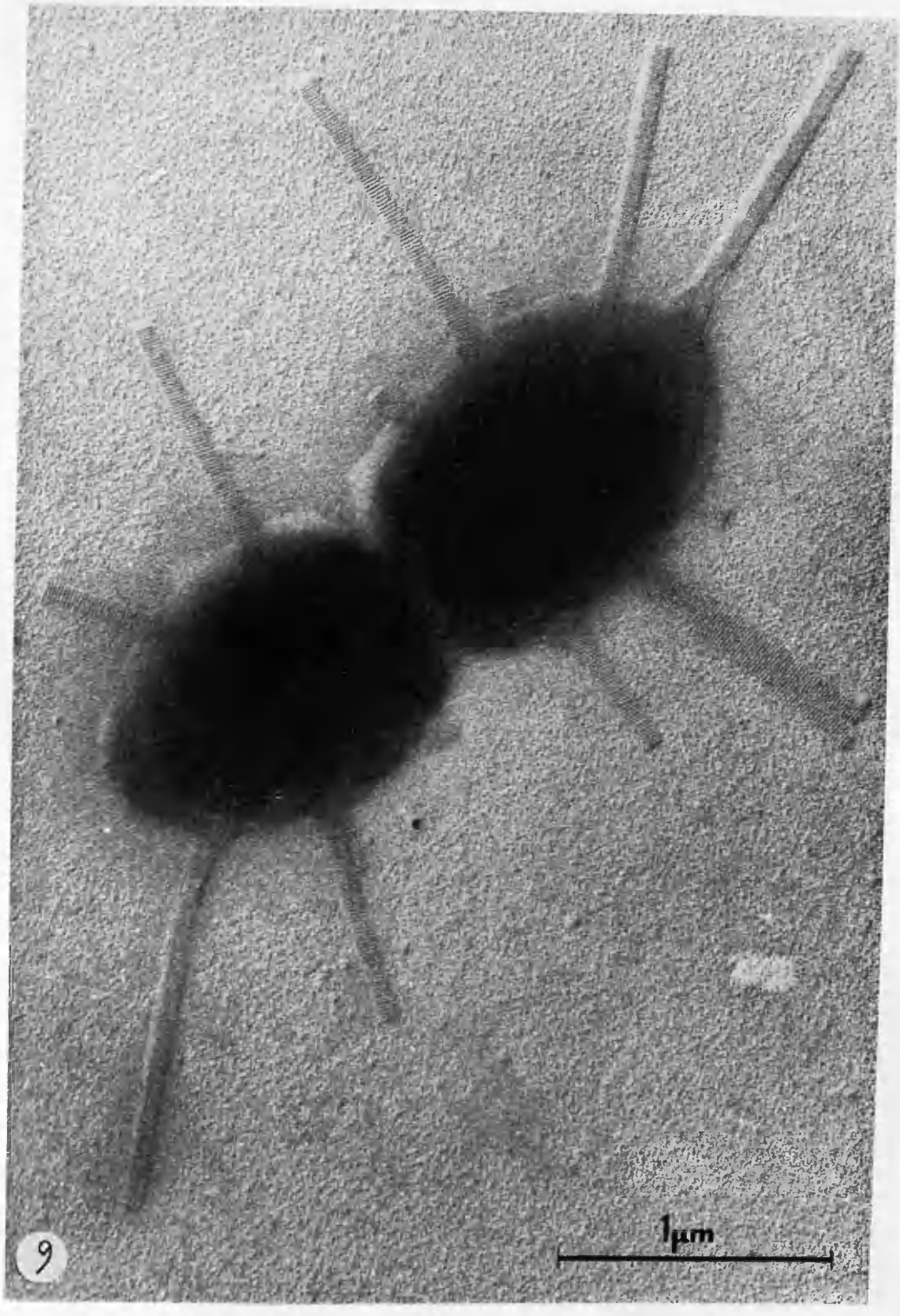


Fig. 9 Shadow-cast electron micrograph of a marine bacterium possessing cell-surface spinae.

(Reproduced by permission from Easterbrook *et al.*, 1973).

1981). Spined organisms tend to interact with each other. This could allow a population as a colony to take advantage of suitable areas of high nutrient concentration (Easterbrook and Sperker, 1982). Attachment has been suggested as a possible function of spinae (Easterbrook and Alexander, 1983). Spined cyanobacteria are surrounded by a mucilaginous capsule; the spinae do not protrude beyond the capsule boundary (Sarokin and Carpenter, 1981). Cyanobacterial capsules are thought to play a role in attachment (see Chapter 2). Spinae may be involved with the mucilaginous sheath in adhesion.

There is no direct evidence that spinae are involved in attachment of marine bacteria. Further experimental work could be done with spined bacteria. This would include both scanning and transmission electron microscopic observations of spined bacteria interacting with surfaces. Such observations could show whether spinae attach bacterial cells to substrata. Evidence of possible ligand-receptor interactions involving spinae could also be investigated. This would involve adding certain sugars to suspensions of spined marine bacteria and suitable substrata, such as seaweed or wood fragments. Association of bacteria with the substrates could be observed and enumerated by light microscopy. Any inhibition of bacterial association with the substrates could indicate binding of sugars to either the spinae or specific receptors. A further experiment would involve isolating the spinin protein from spinae removed from bacterial cell surfaces. Spinin could then be added to suspensions of spined bacteria and substrates, to see the effect of this protein on bacterial-substrate association. Proteolytic enzymes could be added to spinin, which would show whether bacterial attachment is further diminished.

The structural properties, chemical composition and dimensions of spinae are similar to bacterial pili. Pili of some cyanobacteria are known to be involved in adhesion (see Chapter 2), so that spinae may also serve this function.

Summary

1) (i) Certain marine bacteria, such as *Caulobacter* spp. and *Asticcacaulis biprosthecum*, produce stalks with holdfasts which allow attachment, Densely staining material is produced from the holdfasts, which could be involved in adhesion (Poindexter, 1964, 1981; Umbreit and Pate, 1978).

   (ii) Other bacterial species such as *Gallionella* spp. and *Hyphomicrobium* spp. produce stalks or hyphae depositing inorganic materials (Starr and Skerman, 1965).

2) Other bacteria, particularly marine *Pseudomonas* and *Vibrio* spp. attach by means of polar or peritrichous flagella (Belas and Colwell, 1980; Sjoblad and Doetsch, 1982).

3) Some marine bacteria, particularly *Pseudomonas* spp. and some cyanobacterial species, possess rigid spinae (Easterbrook *et al.*, 1973; McGregor-Shaw *et al.*, 1973; Sarokin and Carpenter, 1981). As well as serving several other functions, spinae may be involved in attachment.

(1.4). Importance of bacterial cell-surface hydrophobicity in adhesion.

Although electrostatic interactions and production of extracellular polymers or cellular appendages are important in marine bacterial adhesion, they are not the only factors involved. Bacterial cell surface hydrophobicity is also important. The bacterial cell surface is generally hydrophobic. At a solid-liquid interface bacteria tend to be rejected from the aqueous phase and attracted toward the solid surface (Marshall, 1976). Production of extracellular polymers or cell surface appendages then ensures firm adhesion.

Individual cells of *Flexibacter aurantiacus* and *Hyphomicrobium vulgare* orientated themselves perpendicularly at the interface in air/water, oil/water and solid/water systems (Marshall and Cruickshank, 1973; p. 40). If the cell surfaces were uniformly hydrophobic, they would lie horizontally at the interface. In the oil/water system the cells would then be immersed in the oil phase. However, the perpendicular orientation suggests that the more hydrophobic polar regions of the cells may be attracted to the non-aqueous phase. Rosette formation by both bacterial strains in the aqueous phase may also result from hydrophobic interactions between the cells (Marshall and Cruickshank, 1973).

The 'A'-protein layer of *Aeromonas salmonicida*, which causes furunculosis in fish, is hydrophobic (Parker and Munn, 1984, see also Chapter 5). The cell surfaces of *A. salmonicida* strains possessing this additional 'A'-layer were more hydrophobic than strains lacking it (Parker and Munn, 1984). The 'A'-protein is thought to be involved in *A. salmonicida* adhesion to fish tissues (see Chapter 5). The enhanced hydrophobicity caused by the 'A'-layer may be important in *A. salmonicida*

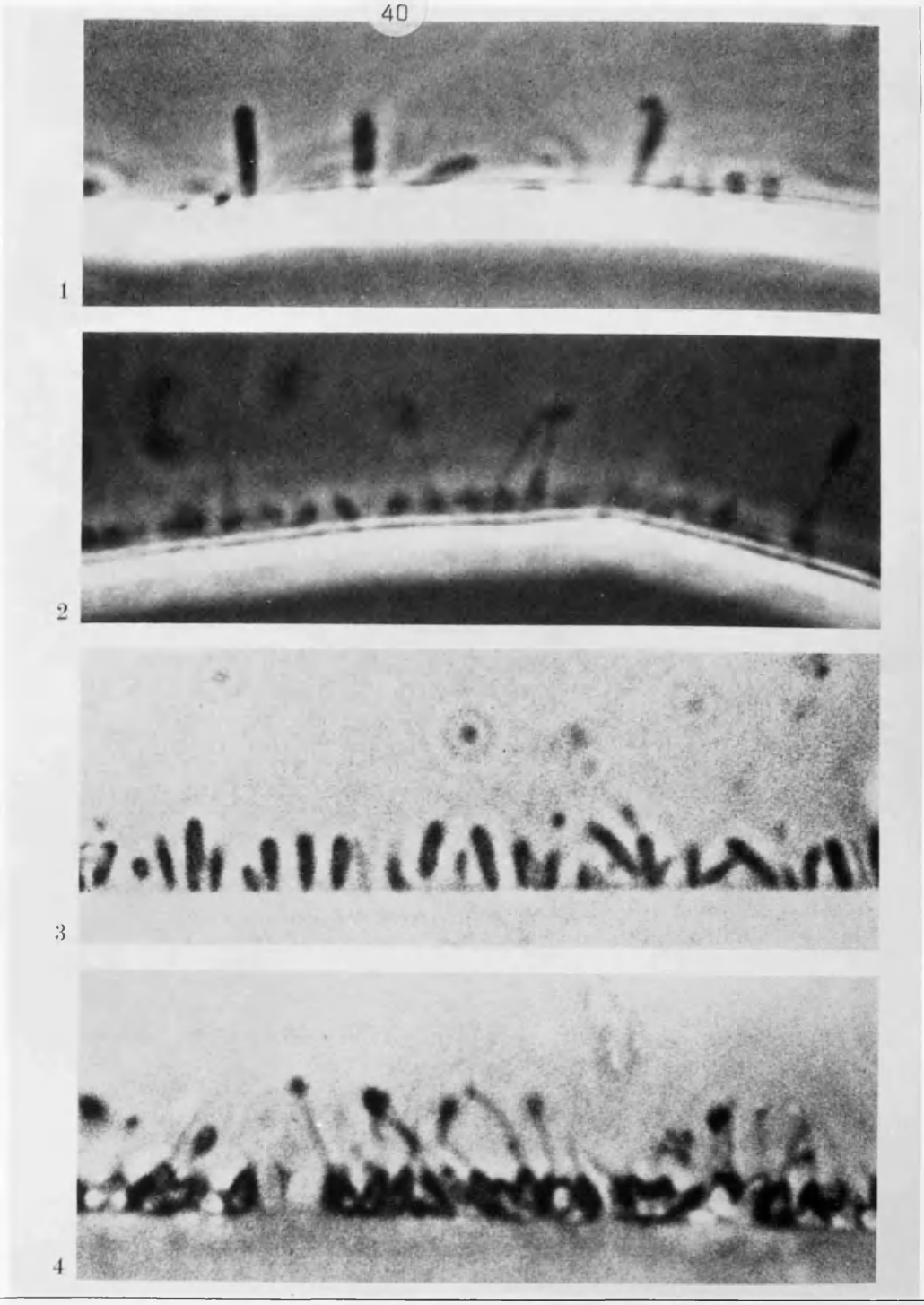


Fig. 10.1 Orientation of cells of *Flexibacter aurantiacus* CW-7 at an oil–water interface. Phase contrast (5000x).

Fig. 10.2 Orientation of cells of *Hyphomicrobium vulgare* ZV-580 at an oil–water interface. Phase contrast (3500x).

Fig. 10.3 Thin section of an embedded araldite block showing the orientation of *F. aurantiacus* CW-7 at a solid–liquid interface. Crystal violet stain. (4200x).

Fig. 10.4 As in Fig. 10.3 but showing orientation of *H. vulgare* ZV–580 (3500x).

(Reproduced by permission from Marshall and Cruickshank, 1973).

adhesion and therefore in furunculosis.

The surfactant Triton X-100 inhibited attachment of ten marine bacterial isolates to polystyrene (Paul and Jeffrey, 1985b). In particular, attached *Vibrio proteolytica* cells were detached from polystyrene by Triton X-100, and detached cells formed clumps. This also occurred with *Alteromonas citrea*. Hydrophobic interactions between the cells may have caused this clumping (Paul and Jeffrey, 1985b). *V. proteolytica* cells were attached to silicone stopcock grease by their poles and sides in approximately equal numbers. Consequently, hydrophobic regions may be randomly distributed on the cell surface in *V. proteolytica*. Triton X-100 disrupted hydrophobic interactions between the cell surfaces and polystyrene (Paul and Jeffrey, 1985b).

Cell surface hydrophobicity is also involved in bacterial scavenging of nutrients accumulated at interfaces (see Chapter 1.6). Growth of microorganisms on long-chain hydrocarbon compounds by direct contact with them is a good example of the role of surface hydrophobicity in adherence. The gram-negative bacterium *Acinetobacter calcoaceticus*, originally isolated from aquatic oil spills, adhered strongly to hexadecane, octane and ethanol (Rosenberg *et al.*, 1980). It also attached to certain non-wettable surfaces such as polystyrene petri dishes and siliconized glass. Adhesion of *A. calcoaceticus* RAG-1 to hydrocarbons may, therefore, be via a general hydrophobic interaction. Cells harvested during the late exponential and stationary growth phases adhered strongly to the test hydrocarbons (Rosenberg *et al.*, 1980). An extracellular, non-dialysable emulsifying agent was produced by *A. calcoaceticus* RAG-1 when grown on hexadecane, ethanol or acetate media (Rosenberg *et al.*, 1979; Sar and Rosenberg, 1983). Strains which grew well on ethanol medium produced high

emulsifying activity (Sar and Rosenberg, 1983). Maximum amounts of emulsifier were produced during the stationary phase (Sar and Rosenberg, 1983), and production was parallel to growth during the exponential phase (Rosenberg *et al.*, 1979).

The emulsifier produced by *A. calcoaceticus* RAG-1 is known as "emulsan". It consists of D-galactosamine, an unidentified amino uronic acid, D-glucose, a fatty acid ester and protein (Zuckerberg *et al.*, 1979). The use of an emulsan-specific antibody preparation revealed that an emulsan-like antigen was a major component of the minicapsule enveloping exponential-phase *A. calcoaceticus* cells. Marked reduction of the capsule size in stationary-phase cells correlated with production of emulsifying activity (Pines *et al.*, 1983). A mutant strain, *A. calcoaceticus* MR-481, was isolated which had no affinity for three test hydrocarbons (Rosenberg and Rosenberg, 1981). Addition of emulsan, however, enabled the mutant to grow on hexadecane. This indicated that adherence of *A. calcoaceticus* is a critical factor in its growth on hydrocarbons, and that emulsan could be involved (Rosenberg and Rosenberg, 1981).

Late exponential-phase cells of *A. calcoaceticus* RAG-1 attached with high affinity to hexadecane and buccal epithelial cells (Rosenberg *et al.*, 1981). Hydrophobic interactions were thought to be involved in *A. calcoaceticus* adhesion to hexadecane and epithelial cells. As emulsan is mainly produced during the exponential and stationary growth phases, when *A. calcoaceticus* attaches well to hydrocarbons (Rosenberg *et al.*, 1980), it could be involved in these hydrophobic interactions.

Wild-type *A. calcoaceticus* RAG-1 cells were shown to possess thin fimbrae, of 3-5nm diameter, on the cell surface (Rosenberg *et al.*, 1982). The non-adherent mutant strain, *A. calcoaceticus* MR-481, lacked these

fimbrae. Prolonged incubation of *A. calcoaceticus* MR-481 in hexadecane medium produced large numbers of partial adherence revertant strains. Reappearance of thin fimbrae was observed in all such revertants (Rosenberg *et al.*, 1982). Adherence of RAG-1 cells to hexadecane was considerably reduced after shearing treatment. The thin fimbrae could be involved in *A. calcoaceticus* adhesion to hexadecane and other hydrophobic surfaces (Rosenberg *et al.*, 1982). Emulsan-deficient mutants of *A. calcoaceticus* RAG-1, which also lacked fimbrae, adhered strongly to hydrocarbons and regained the capacity to grow on them (Pines and Gutnick, 1984). This indicated that the cell surface of *A. calcoaceticus* RAG-1 contained additional hydrophobic sites normally masked by emulsan (Pines and Gutnick, 1984). When emulsan is released, these hydrophobic sites are exposed, so they could be involved in attachment to hydrocarbons.

Cell-surface hydrophobicity is also important in adhesion of benthic cyanobacteria (Shilo, 1982). Filaments of *Phormidium* spp. attached throughout their lengths to oil/water interfaces (Fattom and Shilo, 1984). This suggested that the hydrophobic sites were distributed along the entire cyanobacterial filament. Mechanical shearing demonstrated that hydrophobicity was confined to the outer surface layers (Fattom and Shilo, 1984). Mechanical shearing or treatment with chloramphenicol or proteolytic enzymes caused a shift from cell-surface hydrophobicity to hydrophilicity in *Phormidium* str. J-1 (Bar-Or *et al.*, 1985). Ultrastructural analysis showed the cells were enveloped by a double-layered minicapsule. The external surface of the minicapsule was composed of 40nm.-long beaded fibrils. The minicapsule possessed a rough surface in sheared *Phormidium* cells. The hydrophobic components could be anchored

relatively loosely in the cell wall, so that shearing dislodges them (Bar-Or *et al.*, 1985).

Phormidium str. J-1 also produced a polymeric, extracellular emulsifying agent, "emulcyan", particularly during its stationary growth phase. Its properties and structure were similar to the emulsan of *A. calcoaceticus* (Fattom and Shilo, 1985). Addition of emulcyan to *Phormidium* suspensions reduced hydrophobicity of the cell surfaces. Hydrophobic cells attached to hexadecane droplets became detached on emulcyan addition. As suggested for *A. calcoaceticus*, emulcyan could mask hydrophobic sites on the *Phormidium* cell envelope (Fattom and Shilo, 1985).

A number of substances accumulate at the air/sea interface. These substances are highly surface active, sparingly soluble, or have a specific gravity below that of seawater. Microorganisms and other particles often associate with these materials to form the surface microlayer (Parker and Barsom, 1970; Nörkrans, 1980). Lipids are the major constituents of the uppermost surface microlayer. Fatty acids, glycerides and phospholipids are the main lipid components (Odham *et al.*, 1978). A polysaccharide-protein complex is also found within the microlayer, which although essentially hydrophilic, sticks to the surface by means of some hydrophobic chains (Nörkrans, 1980). In general, high molecular weight, more hydrophobic compounds tend to accumulate nearest the surface (Parker and Barsom, 1970). Glass marbles contacting the air/sea interface accumulated three orders of magnitude more bacteria than those immersed below the interface (Di Salvo, 1973). In general, microorganisms in the uppermost 10μm. are 10-100 times more abundant than in underlying waters (Crow *et al.*, 1976; see Table 1 , p. 47).

The abundance of bacteria at the surface microlayer has been shown experimentally. Bacterial accumulation occurred at a lipid film spread at the interface of an aqueous saline subphase. In particular, the enrichment factor (no. of bacteria/ml in the surface microlayer relative to the subsurface water) for *Serratia marinorubra* was about 100 (Norkrans, 1980). Enrichment factors for *Serratia marinorubra* in a decalayer of oleic acid were approx. twice that in a monolayer (Norkrans and Sorensson, 1977; Odham *et al.*, 1978). *Pseudomonas halocrenaea*, however, attached in higher numbers to monolayers of stearic and palmitic acids than did *S. marinorubra* (Kjelleberg and Stenström, 1980). Enrichment factors for both *Serratia marcescens* and *S. marinorubra* increased during preparation of surface microlayer suspensions (Syzdek, 1982).

Pigmented *S. marcescens* attached to air bubbles in larger numbers than non-pigmented strains, probably because they were more hydrophobic (Blanchard and Syzdek, 1978). The enrichment factor was greater for pigmented *S. marcescens* cells than for nonpigmented cells at a monomolecular stearic acid film (Hermansson *et al.*, 1979). The pigment prodigiosin might increase surface hydrophobicity of pigmented *S. marcescens* cells. To test this, Rosenberg (1984) enriched for non-hydrophobic mutants of *S. marcescens*, to see if non-pigmented colonies were produced. Two mutant strains were produced. A non-pigmented mutant strain was isolated, which attached with intermediate affinity to hexadecane. In addition, a pigmented, non-hydrophobic strain was isolated which did not attach to hexadecane (Rosenberg, 1984). Hydrophobic interactions may be the main forces binding these *Serratia* cells to the surface microlayer. Studies have shown that a positive correlation exists between bacterial accumulation at the surface film and the average degree

of cell-surface hydrophobicity (Nörkrans, 1980; Dahlbäck *et al.*, 1981). Hydrophobic interactions are a major factor in microbial accumulation at the surface microlayer (Shilo, 1982). Some bacterioneuston produce extracellular fibrillar material (Young, 1978). This, together with surface hydrophobicity, may allow firmer adhesion of these microorganisms at the air/sea interface.

Summary

The main points discussed in this sub-chapter, together with the main cited references, are as follows.

1) Cell-surface hydrophobicity is of importance in marine bacterial adhesion, particularly at interfaces (Marshall and Cruickshank, 1973).
2) Specific cell surface components are involved in adhesion of bacteria and cyanobacteria to hydrophobic surfaces. Production of emulsan by *Acinetobacter calcoaceticus* during the exponential growth phase (Rosenberg *et al.*, 1979) correlated with its adhesion to hydrocarbons and other hydrophobic surfaces (Rosenberg *et al.*, 1980, 1981). *Phormidium* spp. produces an emulsifying agent "emulcyan" which may be involved in adhesion of this benthic cyanobacterium (Fattom and Shilo, 1984, 1985).
3) Bacterial cell-surface hydrophobicity is of ecological significance. This is shown by the accumulation of large numbers of bacteria at the surface microlayer of seawater (Nörkrans, 1980). High molecular weight, more hydrophobic compounds tend to accumulate near the surface (Odham *et al.*, 1978). A positive correlation exists between bacterial accumulation at the surface film and the degree of cell-surface hydrophobicity (Nörkrans, 1980).

TABLE 1

Concentration range of microorganisms in surface slicks and underlying waters.

Organisms	Surface slick (ml^{-1})	Surface slick (cm^{-2})	Subsurface (ml^{-1})	No samples
Bacteria	10^5-10^8	28-2.5x10^5	10^2-10^6	24
Yeasts	10^2-10^4	0-5	10^2	16
Moulds	10^3-10^4	0-28	10^2	16

(Taken from Crow *et al.*, 1976).

(1.5.) Influence of substratum properties on bacterial adhesion

Bacterial attachment to solid surfaces is known to be influenced by three components (Fletcher and Pringle, 1985). These are the bacterial surface, the liquid medium and the substratum. The earliest phases of adhesion in saline media are influenced by the surface chemistry, surface texture and surface charge of the solid substrate (Baier, 1972). This sub-chapter aims to discuss some of the main substratum properties which can influence marine bacterial adhesion.

The surface chemical composition of substrata immersed in seawater is known to influence microbial adhesion (Sechler and Gundersen, 1972). Metals such as aluminium, zinc and steel attracted a more heterogeneous bacterial population. Non-metals, wood and plexi-glass, however, exhibited a more stable bacterial community. Highest bacterial populations developed on wood panels. The surface electrical nature of the substrata was suggested as an important factor affecting bacterial adhesion in these experiments (Sechler and Gundersen, 1972). The initial physical and chemical changes occurring upon immersion of solid surfaces in seawater have been investigated by a number of authors. For example, Neihof and Loeb (1972) measured the surface electrical charge of different particles in seawater by electrophoresis. Some seawater samples were treated with ultraviolet light to destroy organic constituents. Particles of an anion exchange resin, germanium and quartz exhibited charges ranging from strongly positive to negative in irradiated seawater (Neihof and Loeb, 1972). Similar studies showed that platinum particles suspended in natural seawater became electronegative (Loeb and Neihof, 1977). These results suggest that all particles and surfaces immersed in seawater become

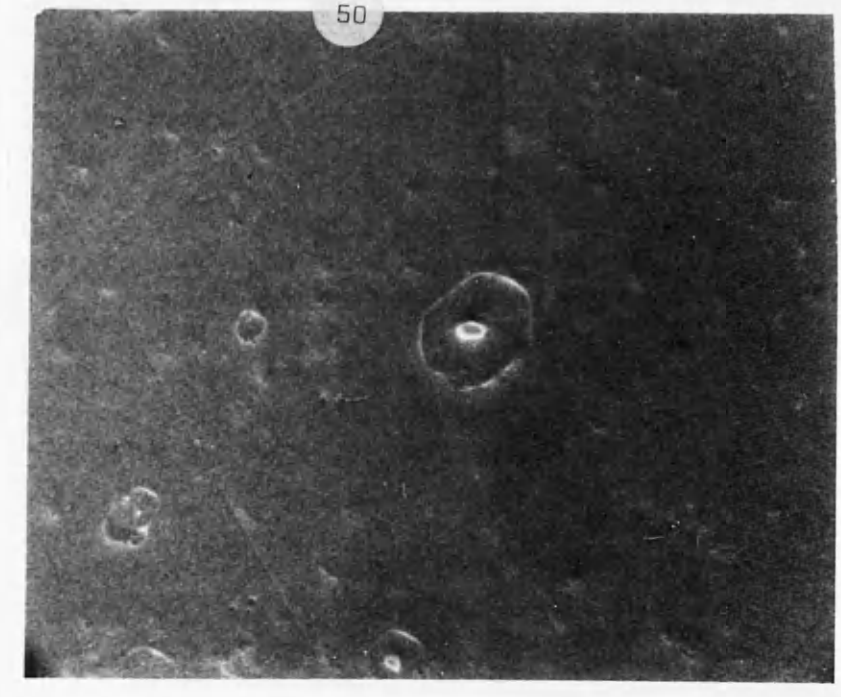
coated with dissolved electronegative, organic material known as a "conditioning film". Conditioning film formation has been observed on methylated germanium and germanium prisms (Baier, 1980, 1984), model heat exchanger surfaces (Baier, 1981), and various metals, including copper (Kristofferson *et al.*, 1982).

The constituents of the conditioning film must come from either seawater or the secretions of attached microorganisms. Zobell (1939) noted the accumulation of organic matter on surfaces submerged in seawater, and stated that it promoted attachment and development of bacteria. Harvey (1925, 1941) observed accumulation of bacteria in glass vessels containing seawater, and attributed it to adsorption of organic matter from seawater onto the glass surface. Baier (1984) showed that the conditioning film from seawater was composed of proteinaceous material. Other papers, however, showed the film to consist of glycoproteins and proteoglycans (Baier, 1972, 1981). This composition may arise due to secretion of protein-polysaccharide slimes by initially attaching microorganisms (Baier, 1972, 1984). Attached marine bacteria were embedded in a glycoproteinaceous matrix on germanium substrata exposed for two hours and two days to seawater cultures (Baier, 1980; see p.50,51).

Baier (1972) stated that a proteinaceous conditioning film modifies the initial surface condition of any substrate in seawater. He also said that this film is an obligatory precursor to biofilm formation.

Conditioning films are known to alter properties of substrata, such as wettability, critical surface tension and surface energy, all of which in turn effect bacterial adhesion. Before describing this, however, it is necessary to define these terms.

Wettability is a measure of how easily a liquid spreads on a solid



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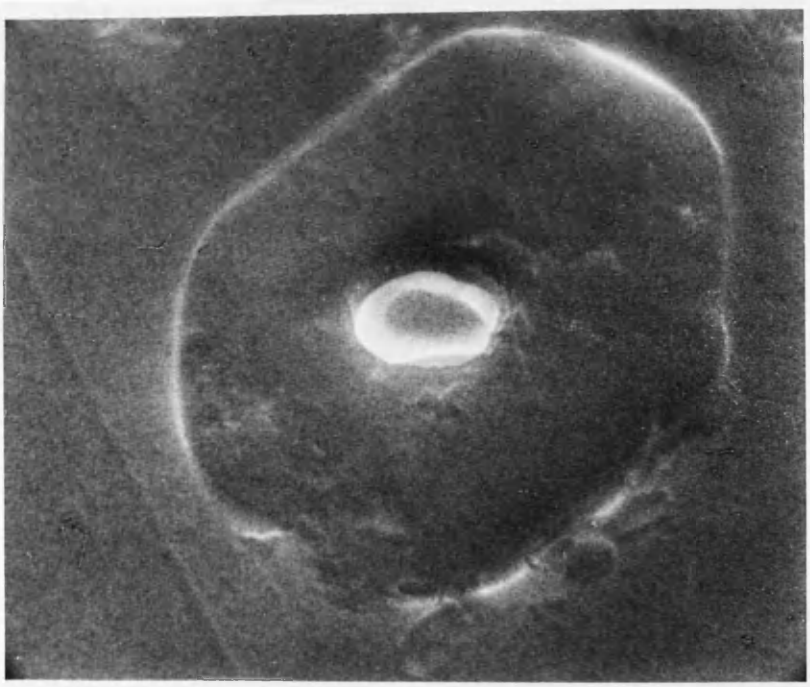

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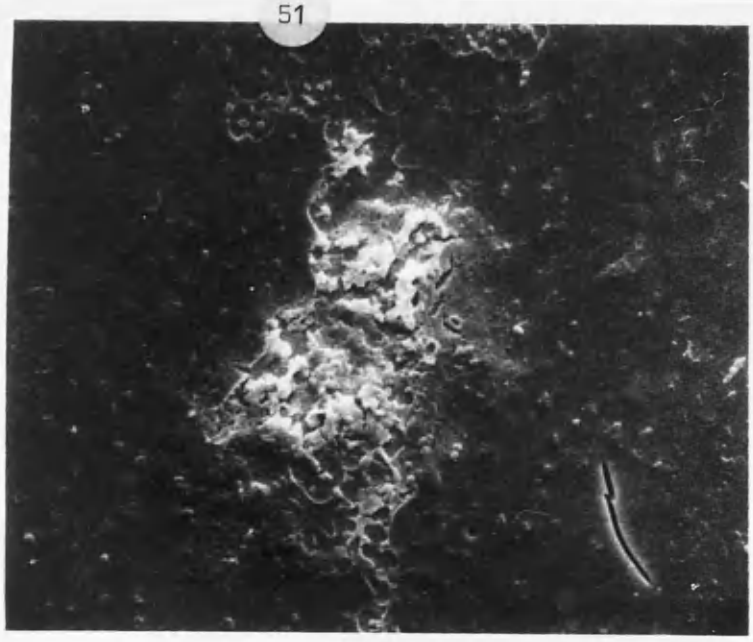
Fig. 11 Scanning electron micrographs of clean metallic (germanium) substrates after exposure for 2 hrs. to luminous bacterium (M_{43}) in artificial seawater and glucose at 28°C.

(Reproduced by permission from Baier, 1980).



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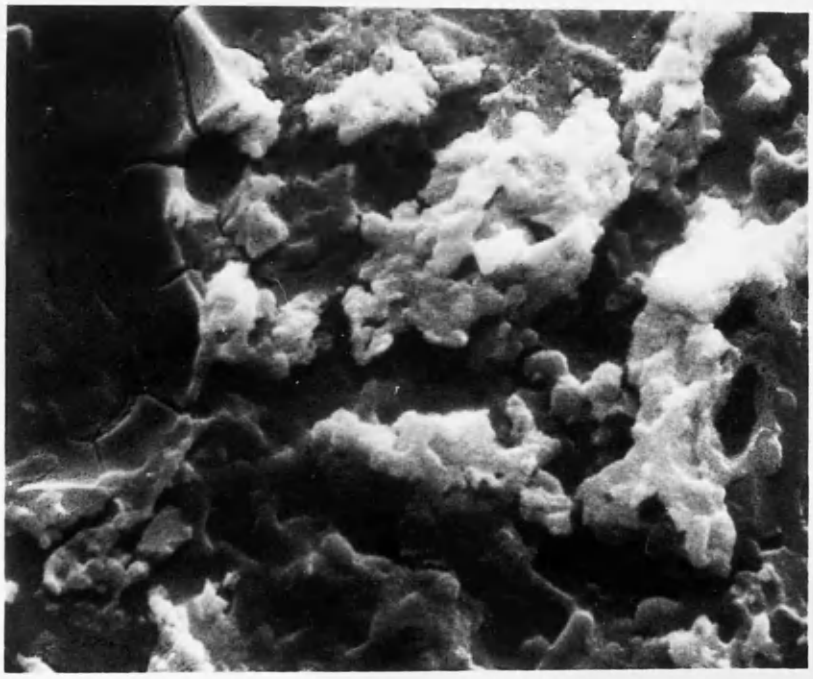

6000x

Fig. 12 Scanning electron micrographs of germanium substrates after exposure for 2 days to luminous bacterium (M_{43}) in artificial seawater and glucose at 28°C, showing the extent of conditioning film formation.

(Reproduced by permission from Baier, 1980).

surface. It is based on the value of the contact angle (θ) existing between a liquid drop and a solid surface (Young, 1805; Glasstone, 1940; Baier *et al.*, 1968; Baier, 1970). If $\theta = 0$, the liquid completely spreads over the surface, so the substrate is highly wettable. However, if $\theta \neq 0$, the liquid does not spread over the surface, so the substrate has low wettability (Glasstone, 1940; Baier *et al.*, 1968; Baier, 1970).

There is one disadvantage of using the contact angle as a method of measuring wettability. It is not reasonable to assume that a liquid is non-spreading when $\theta \neq 0$, as some liquid-to-solid adhesion is always found, and some spreading will occur (Baier *et al.*, 1968). Further problems arise over the use of water and water-miscible liquids for measuring contact angles. Variability arises in such measurements due to liquid penetration into or swelling of the substratum (Baier, 1980). In spite of these limitations, however, the contact angle method has been frequently used in the papers cited here. Surface tension of a solid (γ) is defined as the force acting at right angles to any line of 1 cm length on the surface (Glasstone, 1940). The critical surface tension of a solid surface is obtained from a graph of the cosine of the contact angle ($\cos \theta$) plotted against the liquid-vapour surface tension (γ_{LV}). The intercept of the extrapolated straight line plot with the $\cos \theta = 1$ axis gives the critical surface tension (see p. 53). Critical surface tension is characteristic of the particular surface, and can provide a ranking of materials according to their particular surface energies (Baier, 1970). Surface energy is numerically equal to surface tension (Glasstone, 1940). Surface energy of solids depends on the strength of intermolecular forces. Solids having strong intermolecular forces possess high surface energy values, and are known as "high-energy surfaces". Those solids with low

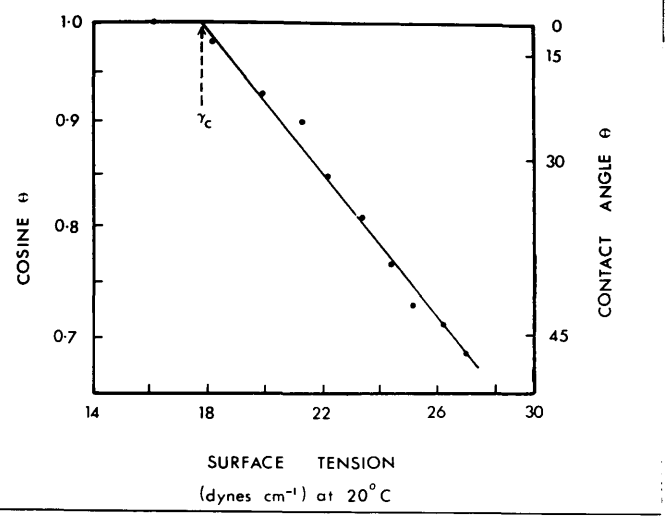

Fig. 13 Zisman plot showing wettability of polytetrafluoroethylene by the n-alkanes. The critical surface tension is given by the intercept of the horizontal line cos θ=1 with the extrapolated straight-line plot of cos θ against surface tension of the series of liquids.

(Reproduced by permission from Marshall, 1976).

melting points possess weaker intermolecular forces. Such solids have lower surface free energies, and are "low-energy" surfaces (Baier *et al.*, 1968). Liquids will generally spread well on solids of high surface energy, but may not spread on low surface energy solids (Baier, 1970).

Having defined wettability, critical surface tension and surface energy, the affects of conditioning film formation on some of these properties and on bacterial adhesion will now be discussed. Additionally, the affects of substratum hydration on surface tension, surface energy and bacterial adhesion will be discussed. The effects of alcohol adsorption on wettability and adhesion of a marine *Pseudomonas* spp. will also be looked at.

The affects of a conditioning film on critical surface tension, wettability and bacterial adhesion varies according to the substratum, organic concentration and bacterial species.

Formation of a conditioning film on platinum surfaces after immersion in seawater gave high contact angles for water and diiodomethane (Loeb and Neihof, 1977; see p. 57). These high contact angles indicate a less wettable, lower energy surface consistent with the presence of an organic film. Contact angles of glycerol, methylene iodide and polypropylene carbonate measured on gold and copper surfaces in seawater were not high (Kristofferson *et al.*, 1982). This indicated the formation of a thin organic film on these metal surfaces. Adsorption of such a thin film was observed. Initial contact angles of 90° and 29° were recorded for polystyrene petri dishes (PD) and tissue culture dishes (TCD), respectively. A 100 μg ml^{-1} concentration of bovine glycoprotein reduced the contact angle on the TCD substratum to 15°, making it completely wettable. The PD substratum became moderately wettable, with a contact

angle of 64° (Fletcher and Marshall, 1982).

McEldowney and Fletcher (1986) demonstrated the effects of conditioning film formation on substrate wettability and bacterial adhesion. Attachment of aquatic *Pseudomonas fluorescens* and *Chromobacterium* spp. grown in continuous culture to PD and TCD surfaces varied depending on the substratum, organism and dilution rate. Addition of yeast extract medium (PYE) either increased or decreased bacterial adhesion levels (see p. 58). PYE decreased the water contact angle values for PD and TCD substrata, indicating conditioning film formation (McEldowney and Fletcher, 1986). Formation of a conditioning film may explain the varied levels of bacterial adhesion in the presence of PYE. A reduction in interfacial free energy caused by a conditioning film would lower attachment, whilst polymer bridging and changes in substratum bonding capacity may increase attachment (McEldowney and Fletcher, 1986). Interfacial free energy is defined as the work required to enlarge the separation between two miscible or partially miscible liquids (Glasstone, 1940). The effects of PYE on bacterial adhesion differed between 5 and 60 mins. (p. 58). Increases in thickness of the conditioning films with time may account for these differences (McEldowney and Fletcher, 1986).

Some earlier studies have been made of how surface wettability and critical surface tension can affect bacterial adhesion.

Wood panels developed high microbial populations on immersion in seawater. This is related to the high polarity and wettability of wood (Sechler and Gundersen, 1972). Comparisons have been made of bacterial adhesion to surfaces differing in these properties. Dexter *et al.* (1975) and Dexter (1976) showed that substrata of high wettability such as glass had high numbers of attached marine bacteria. Substrata of low

wettability, such as polystyrene, had low attached bacterial numbers. In general, there was a decrease in bacterial adhesion with decreasing substrate wettability (Dexter *et al.*, 1975; Dexter, 1976). However, Fletcher and Loeb (1976, 1979), using a marine pseudomonad, obtained conflicting results. They found that this bacterium attached in high numbers to substrata of low energy and wettability. However, the pseudomonad attached in low numbers to high energy, high wettability substrata such as glass and mica (Fletcher and Loeb, 1979). Fletcher and Loeb (1979) suggested possible reasons for the differences in these results. One was the presence of different bacteria in the experiments which may possess different cell surface characteristics. Dexter *et al.* (1975) used seawater containing several bacterial species, whilst Fletcher and Loeb (1976, 1979) only used a marine pseudomonad. The presence of variable and undefined dissolved components may alter substrata properties and thus bacterial attachment through adsorption. Additionally, different types of interactions (e.g. electrostatic, hydrophobic) between the bacteria and substratum in the two experiments may produce different results (Fletcher and Loeb, 1979).

The surface free energy of a substratum is related to surface tension, and so can affect bacterial adhesion (see p. 59). Fletcher and Pringle (1985) compared levels of marine bacterial attachment to PD and TCD. These substrata differ in their surface free energy, having values of 28 and 73 mNm^{-1}, respectively. The bacteria differed in their ability to attach to the two polystyrene surfaces (see p. 59). For example, *Corynebacterium erythrogenes* and *Vibrio fisheri* attached in high numbers to both substrata. *Flavobacterium ulginosum* and *Pseudomonas* sp. NCMB 2021, however, only attached well to PD (Fletcher and Pringle, 1985). The

TABLE 2

Contact angles on known surfaces and on platinum exposed to seawater.

Sample	Contact angles with : diiodomethane	water
Clean platinum	< 10°	0°
Platinum after immersion in seawater from Chesapeake Bay	33°	45°
Nylon 2	30°	49°
Poly (methyl glutamate)	30°	49°
Polyethylene	52°	94°

(From Loeb & Neihof, 1977).

TABLE 3

Effect of nutrients on the attachment of *P. fluorescens* and *Chromobacterium* sp. to PD and TCD after growth in continuous culture at various dilution rates.

I_a (index of attachment) values were calculated as the ratio of the A_{590} ($\times 10^3$) of the test substratum to that of the relevant control substratum. I_a values of 1 were recorded for treatments whose 95% confidence limits of the mean (n = 8) overlapped with that of the controls.

Species	Dilution rate (h^{-2})	½ PYE				PYE			
		PD I_a		TCD I_a		PD I_a		TCD I_a	
		5 min	60 min	5 min	60 min	5 min	60 min	5 min	60 min
(a) *P. fluorescens*	0.05	1.3	1.76	1	2.4	1	1	1	1.9
	0.1	2.7	1	1.4	2.36	1	1	1.86	2.48
	0.15	1.22	1	1	1	1.67	0.55	1.42	1
	0.2	1	0.18	1	1	1	0.16	1.54	1
(b) *Chromobacterium* sp.	0.05	1.64	2.2	1	1	1	2.53	0.56	0.38
	0.1	1	1.89	1	1	1	1.46	0.72	0.54
	0.15	1	1.18	1	1.29	0.43	0.54	1	0.41
	0.2	0.74	0.75	0.86	1.21	0.33	0.6	0.66	0.86

(From McEldowney and Fletcher, 1986).

TABLE 4

Attachment of Marine Bacteria to Polystyrene Petri Dishes (PD) and Polystyrene Tissue Culture Dishes (TCD).

Bacterium	Number attached 100 μm^{-2} (± SEM)[1]	
	PD	TCD
Bacillus filicolonicus	0.7 (0.1)	1.8 (0.5)
Bacillus epiphytes	0.4 (0.1)	0.2 (0.1)
Bacillus pacificus	0.6 (0.2)	0.5 (0.2)
Micrococcus sp.	7.9 (1.6)	14.5 (0.8)
Flavobacterium uliginosum	15.6 (0.6)	1.0 (0.2)
Pseudomonas sp.	15.3 (0.7)	0.7 (0.1)
Corynebacterium erythrogenes	20.7 (1.7)	18.2 (1.1)
Vibrio fisheri	35.6 (3.7)	28.2 (2.5)

[1] SEM, Standard Error of the Mean.

(From : Fletcher and Pringle, 1985)

adhesion of bacteria to surfaces immersed in an aqueous environment is more related to solid/liquid interfacial free energies (Gerson and Scheer, 1980). The effects of interfacial free energies of five different plastic substrata on bacterial adhesion was studied by Gerson and Scheer (1980). There was a linear relationship between the density of adhesion of *Serratia marcescens* and the free energy of adhesion for four of the surfaces used. Free energy of adhesion, ΔG_a, is defined as the change in interfacial free energy which corresponds to the attachment process. These results confirm a direct relationship between the free energy of partition of bacteria between the solid surface and liquid phase and the free energy of adhesion (Gerson and Scheer, 1980).

The effects of water adsorption and of alcohol adsorption on some of these substrata properties and bacterial adhesion will now be discussed.

Adsorbed water is known to alter surface free energy and critical surface tension values of substrata. Surface energies of glass, silica, alumina and metals are decreased by an adsorbed water layer. One monolayer of adsorbed water converts these high-energy surfaces into low-energy surfaces (Baier *et al.*, 1968). As more than a monolayer of water is adsorbed onto a substratum, γ_c values decrease to those of a bulk water surface (approx. 22 dynes cm^{-1} at 20°C) (Baier *et al.*, 1968; Baier, 1970). Attachment of *Pseudomonas fluorescens* and an *Acinetobacter* spp. to hydrogel substrata decreased with increasing water content of the hydrogels (Pringle and Fletcher, 1986). Reduced attachment levels were found for all the hydrogels compared with polystyrene, polystyrene tissue culture dishes and sulphonated polystyrene substrata. Adsorbed water could affect adhesion by modifying the ionic microenvironment of the surface or by reducing the interfacial free energy of the solid-liquid interface. Water can also

sterically prevent close approach of the surfaces, so reducing the spontaneous adsorption of cells (Pringle and Fletcher, 1986). In order for adhesion to occur between hydrated bacterial and attachment surfaces, water must be displaced as the two surfaces move together (Fletcher and Pringle, 1985).

Adsorption of certain alcohols to substrata can also influence bacterial adhesion by altering substrata properties. Methanol, ethanol, propanol and butanol affected attachment of a marine *Pseudomonas* spp. to PD and TCD substrata (Fletcher, 1983). In particular, there was an increase in bacterial attachment to TCD at 1% butanol concentration, but not at 1.5% and 2.0% concentrations. Sessile drop and air bubble contact angles (θ_{SD} and θ_B, respectively) were measured on both substrata in the presence of the alcohols. Butanol at the above concentrations decreased the θ_{SD} amd θ_B values on PD. There was a progressive decrease in contact angle values on TCD with increasing butanol concentrations (Fletcher, 1983). This suggests that butanol adsorbs to the substrata. Adsorption of butanol to the PD surface may have prevented bacterial adhesion at 1.5% and 2.0% concentrations (Fletcher, 1983).

The remainder of this sub-chapter will discuss possible differences in bacterial adhesion mechanisms to hydrophobic and hydrophilic substrata, and an example of a material produced by *Serratia marcescens* which affects surface wettability.

The results of Dexter *et al.* (1975) and Fletcher and Loeb (1979), although conflicting, suggest that the degree of substratum hydrophobicity or hydrophilicity can affect marine bacteria attaching to hydrophobic substrata such as polystyrene, and low numbers attaching to hydrophilic substrata such as glass and mica (Fletcher and Loeb, 1979). As shown in

Table 4 (p. 59), with some species, bacterial adhesion to the hydrophilic TCD substratum and the more hydrophobic PD differed (Fletcher and Pringle, 1985). McEldowney and Fletcher (1986) also observed differences in bacterial adhesion levels to PD and TCD substrata. They suggested that there could be different adhesive interactions with these two substrata. The surfactant Triton X-100 inhibited attachment of *Vibrio proteolytica* to the hydrophobic substratum polystyrene by 99%. (Paul and Jeffrey, 1985a). However, it did not affect attachment of the bacterium to hydrophilic substrata such as glass or tissue culture dishes. In addition, mannose inhibited *V. proteolytica* adhesion to tissue culture dishes, but not to polystyrene. These results suggested the existence of separate mechanisms for adhesion of *V. proteolytica* to hydrophobic and hydrophilic substrata (Paul and Jeffrey, 1985a). This is also suggested by the other papers discussed here (Dexter *et al.*, 1975; Fletcher and Loeb, 1979; Fletcher and Pringle, 1985; McEldowney and Fletcher, 1986).

Serratia marcescens is a specific example of a marine bacterium producing material which alters substrata properties. The bacterium produced large amounts of a wetting agent when cultivated at 30°C (Matsuyama *et al.*, 1985). The contact angle of a *S. marcescens* suspension on a polystyrene surface was 26°C. The wetting agent was identified as an aminolipid similar to serratamolide. Cells of *S. marcescens* possessing such wetting activity spread spontaneously on a glass slide (Matsuyama *et al.*, 1985).

Microscopic examination of *S. marcescens* cells spread in this way showed red granular material surrounding the colourless bacterial cells. Further light and electron microscopic observations showed that the red pigment was present in vesicles in the bacterial cells (Matsuyama *et al.*,

1986). The vesicles had strong wetting activity. Prodigiosin, the red pigment present in *S. marcescens*, was isolated from these vesicles. Additionally, three lipids W1, W2 and W3 were isolated from *S. marcescens* strains possessing these vesicles. These lipids had strong wetting activity which was shown by small contact angles of dispersions of these materials on a polystyrene surface. In addition, the lipids lowered the surface tension of saline in which they were suspended on polystyrene. Chemical analysis showed that wetting agent W1 was serratamolide, whilst materials W2 and W3 were aminolipids, although their exact structure could not be obtained (Matsuyama *et al.*, 1986). Although prodigiosin was isolated from these vesicles, it did not impart wetting activity to *S. marcescens*.

The primary function of these aminolipid wetting agents is uncertain (Matsuyama *et al.*, 1986). However, it is possible that by increasing substrate wettability, these materials could facilitate adhesion of *S. marcescens* to submerged surfaces. These wetting materials would then be acting in a similar way to adsorbed organic conditioning films. Cell-surface hydrophobicity was mentioned as an important factor in *S. marcescens* adhesion at the air/water interface (see 1.4). This, together with increased wetting activity, could further facilitate *S. marcescens* adhesion in the marine environment.

SUMMARY

This sub-chapter has reviewed several aspects of the importance of substrata properties on adhesion of marine bacteria. The major points discussed, together with the key cited references are :-

(1). Surfaces immersed in seawater become coated with dissolved, electronegative organic constituents known as a conditioning film. This film is usually proteinaceous in nature (Baier, 1972, 1981, 1984).

(2). A conditioning film can affect marine bacterial adhesion by altering substrata properties such as wettability and critical surface tension (McEldowney and Fletcher, 1986).

(3) Properties such as surface wettability and critical surface tension can affect bacterial adhesion, the effects of which depend on the species or interactions between bacterium and substratum (Dexter *et al.*, 1975; Fletcher and Loeb, 1979).

(4) Surface free energy of a substratum affects bacterial adhesion (Fletcher and Pringle, 1985). Interfacial free energy at a solid/liquid interface can be important to attaching bacteria in the marine environment (Gerson and Scheer, 1980).

(5) (i) Substratum hydration can alter critical surface tension and surface free energy values and so affects bacterial adhesion (Pringle and Fletcher, 1986).

(ii) Butanol adsorption to certain substrata prevented adhesion of a marine *Pseudomonas* spp. (Fletcher, 1983).

(6) Separate adhesion mechanisms of marine bacteria to hydrophobic and hydrophilic substrata may exist (Paul and Jeffrey, 1985).

(7) *Serratia marcescens* strains produce lipid compounds with strong wetting activity from cellular vesicles. (Matsuyama *et al.*, 1986). These compounds could affect *S. marcescens* adhesion to submersed surfaces in the marine environment.

(1.6). Influence of nutrients and nutrient accumulation at interfaces on bacterial adhesion

An interface is the boundary between two phases in a heterogeneous system (Ellwood *et al.*, 1982). Interfaces are usually identified as liquid-liquid, air-liquid, air-solid and solid-liquid interfaces. The most commonly investigated type in the aqueous environment is the solid-liquid interface. Such interfaces act as areas of nutrient accumulation in the form of inorganic ions, lipids and other organic molecules (Marshall, 1980; Kjelleberg *et al.*, 1982). As the marine environment is largely oligotrophic (*i.e.*, nutrient deficient), interfaces allow bacteria to take advantage of higher nutrient levels. Irreversible bacterial adhesion at interfaces under starvation conditions is, therefore, common (Dawson *et al.*, 1981; Kjelleberg *et al.*, 1983; Kjelleberg and Hermansson, 1984).

Bacterial scavenging of nutrients located at interfaces appears to be related to the degree of irreversible binding of bacterial cells at interfaces (Kjelleberg *et al.*, 1983; Kefford and Marshall, 1984; Hermansson and Marshall, 1985). *Pseudomonas* sp. NCMB 2021 showed high irreversible adhesion to glass beads coated with stearic acid. This was coupled to a high respiration rate of surface-bound stearic acid (Hermansson and Marshall, 1985). In contrast, *Vibrio* sp. MH3 showed poor irreversible adhesion and a low total number of adhered cells. However, the species did show some respiration of the surface bound stearic acid, although this was half the rate of *Pseudomonas* NCMB 2021. Utilization of stearic acid by *Pseudomonas* NCMB 2021 was associated specifically with irreversible adhesion (Hermansson and Marshall, 1985). Two strains of *Leptospira*

biflexa serovar *patoc* displayed reversible and irreversible adhesion at a solid-liquid interface (Kefford and Marshall, 1984). Scavenging of nutrients localised at the interface occurred when this organism was reversibly and irreversibly bound. However, Hermansson and Marshall (1985) found that *Vibrio* MH3 was still able to scavenge stearic acid when reversibly attached. This suggests that irreversible adhesion is not absolutely essential for bacteria to benefit from interface-bound nutrients.

Surface hydrophobicity of the bacterial cell envelope is also involved in the scavenging of nutrients at interfaces (Kefford *et al.*, 1982; Kjelleberg *et al.*, 1983; Kjelleberg and Hermansson, 1984). *Leptospira biflexa* and a pigmented strain of *Serratia marcescens* displayed two different strategies of scavenging fatty acids which were related to their adhesion methods (Kefford *et al.*, 1982). *Leptospira biflexa* efficiently scavenged stearic acid from the surface in 24 hours. The pigmented, hydrophobic *Serratia* strain showed a faster rate of stearic acid removal than the hydrophilic, non-pigmented strain. The greater hydrophobicity of the pigmented *Serratia* strain allows closer interaction with the solid surface and more efficient scavenging of localised stearic acid. The differing rates of stearic acid uptake observed for *Leptospira biflexa* and pigmented *Serratia marcescens* suggests that two distinct scavenging strategies are used. The pigmented *Serratia* strains adhere irreversibly, so the adherent population benefits from the localised nutrients (Kefford *et al.*, 1982).

Starvation of *Spirillum* sp. strain 0114, *Vibrio* strain DW1 and a hydrophobic *Pseudomonas* sp. strain S9 gave increases in surface hydrophobicity. These in turn corresponded with high irreversible binding

to glass (Kjelleberg and Hermansson, 1984). Starvation of hydrophilic marine *Vibrio* sp. strain DW1 and hydrophobic *Pseudomonas* sp. strain S9 at a solid-liquid interface gave increases in irreversible binding (Kjelleberg *et al.*, 1983). This also seemed to be related to increased surface hydrophobicity and greater uptake of stearic acid localised at the interface (Kjelleberg *et al.*, 1983). Increased surface hydrophobicity may be the reason for the high irreversible adhesion of *Pseudomonas* NCMB 2021 compared with *Vibrio* MH3 (Hermansson and Marshall, 1985). Increases in cell surface hydrophobicity appear to lead to increases in irreversible binding of bacteria at nutrient-enriched interfaces (see Chapter 1.4).

Cell size reduction is a phenomenon frequently observed during starvation of copiotrophic marine bacteria at a solid-liquid interface. Copiotrophic bacteria are defined as those species requiring 100 to 1,000-fold concentrations of nutrients for growth on media containing only a few mg of organic carbon/l (Kjelleberg *et al.*, 1985). The copiotrophic marine *Vibrio* spp. DW1 became reduced in size at air-water and solid-water interfaces during starvation (Kjelleberg *et al.*, 1982). Twelve rod-shaped, hydrophilic marine bacteria decreased in size more rapidly at the solid surface than in the liquid phase (Humphrey *et al.*, 1983). Both the hydrophilic marine *Vibrio* spp. DW1 and hydrophobic *Pseudomonas* sp. S9 underwent continuous size reduction during starvation at a solid-liquid interface (Kjelleberg *et al.*, 1983). Marine bacteria grown in a medium with a high carbon concentration reduced in size after 24 h. starvation at a glass-seawater interface (Kjelleberg *et al.*, 1985; see p. 69). Size reduction of starved copiotrophic cells at interfaces may represent a survival strategy. This is because their surface area/volume ratio will increase allowing greater uptake of localised nutrients (Kjelleberg *et al.*,

1982). Size reduction of copiotrophic bacterial cells leads in turn to increases in irreversible binding at interfaces (Kjelleberg *et al.*, 1983; Kjelleberg and Hermansson, 1984; Kjelleberg *et al.*, 1985). Marine *Vibrio* sp. DW1 decreased rapidly in size within 5 h. of exposure to starvation conditions (Dawson *et al.*, 1981). Adhesion of these dwarf cells to siliconized glass surfaces was enhanced, and was accompanied by production of extracellular bridging polymer (Dawson *et al.*, 1981). Adhesion would allow greater uptake of localised nutrients, so enhancing bacterial survival in oligotrophic marine environments. Starvation of *Spirillum* strain 0114, *Vibrio* sp. DW1 and *S. marcescens* EF 190 at a glass-seawater interface gave increased surface roughness, shown by highly textured outer layers in electron micrographs (Kjelleberg and Hermansson, 1984). Reasons for the appearance of this roughness are unknown, but it could be the prelude to production of extracellular adhesive. Bleb formation at the surface of *Vibrio* DW1 during starvation occurred just before production of bridging polymer (Dawson *et al.*, 1981).

In contrast to these observations on copiotrophic marine bacteria, Kjelleberg *et al.* (1985) found that higher numbers of starving oligotrophic bacteria were found at a glass-seawater interface. Most of these oligotrophic cells were irreversibly bound. Reversibly attached aggregates were seen which presumably consisted of copiotrophic bacteria (Kjelleberg *et al.*, 1985). This conflicts with the observations that starving copiotrophic bacteria are the predominant colonizers of solid-liquid interfaces, whilst oligotrophic bacteria are more competitive in the liquid phase. Cell surface structures of oligotrophic bacteria may be different from those of copiotrophic bacteria. This may allow oligotrophic bacteria a higher ability to bind to surfaces under starvation

TABLE 5

Changes in bacterial cell size during starvation.

Media[1]		Time of starvation	Mean cell volume[2]
Original	Transferred to	(h)	(μm^3)
C	C	0	0.76 ± 0.22 (6)
		24	0.46 ± 0.15 (9)
B	B	0	0.83 ± 0.43 (7)
		24	0.73 ± 0.40 (7)
C	B	24	0.40 ± 0.10 (7)

[1] The growth media used differed in their concentrations of organic carbon. Medium B contained 50 mg. of organic C/l., whilst Medium C contained 500 mg. organic C/l.

[2] Figures in parantheses represent the number of bacterial isolates used in the measurements.

(Taken from Kjelleberg *et al.*, 1985).

conditions (Kjelleberg *et al.*, 1985).

The chemostat, by limiting the availability of specific essential nutrients in a liquid culture, often allows the effects of nutrient depletion on microbial activity to be tested. Circles of aluminium foil were placed into chemostats containing enrichment cultures of river water bacteria, which were nitrogen-limited or glucose-limited (Brown *et al.*, 1977). The aluminium surface taken from the chemostat supplied with nitrogen-limited medium was coated with polysaccharide material containing glucose. Very few bacteria were present. However, the surface taken from the chemostat with glucose-limited medium had a wide variety of bacterial types attached, but no surface polymer was present (Brown *et al.*, 1977). These results suggest that glucose-limited cultures result in a more diverse community of attached microorganisms that do not depend on extracellular polymer production for adhesion (Ellwood *et al.*, 1982). A possible explanation is that in the nitrogen-limited medium, any receptor sites for bacterial glucose-containing extracellular materials would be saturated, and not be available for attachment. Conversely, in the glucose-limited medium, a maximum number of bacterial polymer receptor sites would be available. Glucose could bind to aluminium as a molecular film. This polysaccharide layer could assist bacterial attachment (Brown *et al.*, 1977). In other words, under carbon-limited conditions there is an increased concentration of the limiting nutrient at the solid surface. Organisms with a high-affinity uptake system may recognise molecules of the limiting nutrient concentrated at a surface, so that interaction leading to adhesion could occur (Ellwood *et al.*, 1982).

However, this proposal does not consider whether there is a constant supply of the limiting substrate to the surface. In an attempt to answer

this, pure chemostat cultures of an estuarine pseudomonad were used to study kinetics of growth on an artificial surface (Ellwood *et al.*, 1982). Nitrogen-limited or carbon-limited cultures were again used. Glass slides were immersed at fixed time intervals of 5 h. and after 1-5 generation times. Examination of stained films and scanning electron micrographs showed that growth occurred as microcolonies that coalesced into relatively uniform films after five generations. At higher magnifications, fibrils connecting cell to cell and cell to surface were apparent. The fibrils were well developed in films from carbon-limited cultures, although they were also present in nitrogen-limited cultures (Ellwood *et al.*, 1982). Collisions between bacteria and the surface will be more frequent at high culture densities. This must in part account for the increased rate of surface film development. The microscopic evidence showed that large numbers of bacteria on the surface formed microcolonies. In turn, it seems likely that microcolony development is due to an increased rate of surface growth compared with the liquid phase (Ellwood *et al.*, 1982). This would arise if there was an increased concentration of the limiting nutrient at the solid-liquid interface.

Summary

1) Solid-liquid interfaces act as areas of nutrient accumulation in the marine environment (Marshall, 1980).

2) Bacterial scavenging of nutrients at interfaces is related to the degree of irreversible binding of cells at interfaces (Hermansson and Marshall, 1985) and to cell surface hydrophobicity (Kefford *et al.*, 1982; Kjelleberg *et al.*, 1983).

3) Copiotrophic marine bacteria frequently undergo cell size reduction during starvation at a solid-liquid interface (Kjelleberg *et al.*, 1983; 1985). This can lead to increases in irreversible adhesion, which could be a survival strategy (Dawson *et al.*, 1981).

4) Chemostat studies show how an increased concentration of a limiting nutrient at a solid-liquid interface can lead to increased bacterial adhesion (Ellwood *et al.*, 1982).

Chapter 2: Adhesion mechanisms in Cyanobacteria

This chapter describes the production of sheaths, cell surface pili and other appendages such as bristles and spikes in cyanobacteria. Although there is little direct evidence for the involvement of these structures in cyanobacterial adhesion, attempts will be made to justify this.

Many cyanobacteria synthesize a fibrous material, which although deposited outside the cell is always closely adherent to the outer membrane (Stanier and Cohen-Bazire, 1977; Drews and Weckesser, 1982). Aggregates of cyanobacterial cells are often formed in which groups of cells are enveloped by this material. Some cyanobacteria excrete slime or mucilage which becomes dispersed around them (Drews and Weckesser, 1982). It may make liquid cultures of cyanobacteria highly viscous or gelatinous (Stanier and Cohen-Bazire, 1977).

Ultrastructural studies of *Anabaena* spp. showed fine filaments of mucilaginous material radiating from the cell surface (Leak, 1967). This material stained with ruthenium red, suggesting that it was composed of mucopolysaccharides. Chemical analysis of the sheath material of *Anabaena cylindrica* showed that it consisted mainly of carbohydrate, with a small amount of amino compounds (Dunn and Wolk, 1970). The sugars present in the sheath material were glucose, mannose, galactose and xylose. Light microscopic observations of *Nostoc* spp. suggested that the sheath was similar to many bacterial capsules (Tuffery, 1969). Sheaths surrounding trichomes of *Nostoc* spp. stained intensely with Alcian blue, suggesting that the sheath was largely composed of polysaccharide. Electron microscopy showed a dense, striated sheath structure close to the cell, whilst a micro-fibrillar network was seen further out (Tuffery, 1969).

Nostoc spp. isolated from subaquatic habitats by Martin and Wyatt (1974) often possessed a slimy sheath. The pronounced sheath occurred mostly in cyanobacterial strains within the genera *Scytonemataceae, Stigonemataceae* and *Oscillatoraceae.* Filaments which had pronounced sheaths in liquid culture were adhesive and produced entangled masses. These were difficult to disrupt. Many euplanktonic *Anabaena* spp. and aquatic unicellular cyanobacteria such as *Microcystis* and *Aphanocapsa* spp. had cloud-like slimy sheaths (Martin and Wyatt, 1974). The branching blue-green bacterium *Fischerella ambigua* possesses a sheath continuous throughout the length of the filament (Thurston and Ingram, 1971). Numerous vesicles were associated with the regions of active sheath formation. These vesicles could be secreting the raw material from which the sheath is constructed (Thurston and Ingram, 1971). This may be similar to the involvement of vesicles in the secretion of adhesives in algal spores (see Chapter 3).

The colonial sheath of *Microcystis marginata* consists of a relatively smooth inner surface with densely packed, long fibrils on the outer surface (Kessel and Eloff, 1975). Shadow casting shows that the sheath surface appears as a very fine network of 50nm thick fibrils. Ruthenium red staining suggests that the *Microcystis marginata* sheath consists of mucopolysaccharides and pectates, similar to *Anabaena* spp. (Leak, 1967). The marine cyanobacterium *Agmenellum quadruplicatum* possesses an extracellular glycocalyx, which is shown by freeze-etching to consist of a network of small fibrils (Balkwill and Stevens, 1980; see p. 76). Ruthenium red staining again suggests that the glycocalyx is composed of acidic polysaccharides. Projections of glycocalyx material extend outwards at several points around *A. quadruplicatum* cells. (see p. 76). Freeze-etching shows that these projections are aggregates of closely

packed glycocalyx fibrils arranged in parallel. These projections probably bind adjacent *A. quadruplicatum* cells together. They could be areas of the glycocalyx which are stretched out by physical forces, and sorb to adjacent cells, as predicted by the polymer bridging theory of Harris and Mitchell (1973). The glycocalyx of *A. quadruplicatum* is probably involved in attachment to solid surfaces in the natural environment (Balkwill and Stevens, 1980).

The chemical composition and adhesive properties of cyanobacterial sheaths are similar to the extracellular polysaccharides produced by periphytic marine bacteria. These secretions appear to be involved in the adhesion of certain marine bacteria to surfaces (see Chapter 1.2). In the same way, sheath material may mediate cyanobacterial adhesion to surfaces in the aqueous environment.

There have been several reports on the occurrence of pili or surface fimbrae in cyanobacteria. However, cyanobacterial pili are poorly characterized. Lounatmaa *et al.* (1980) observed numerous projections penetrating the cell wall of the cyanobacterium *Synechocystis* sp. CB3. These projections were approx. 1µm in length, and negative staining showed them to consist of bundles of thin filaments (see p. 78,79). The filaments were morphologically similar to bacterial pili, although enzymic tests did not suggest that they were composed of protein. A survey of chroococcacean cyanobacteria showed piliation to be a common feature (Vaara, 1982). *Microcystis firma* and all *Synechocystis* strains which were studied produced pili, as did three out of twelve *Synechococcus* strains. Chroococcaean pili resembled those of heterotrophic bacteria, as they were long, virtually stiff, had an even width, and appeared to have a subunit structure. MacRae *et al.* (1977) found that all but three out of twenty-two strains of gliding cyanobacteria examined possessed polar fimbrae. In

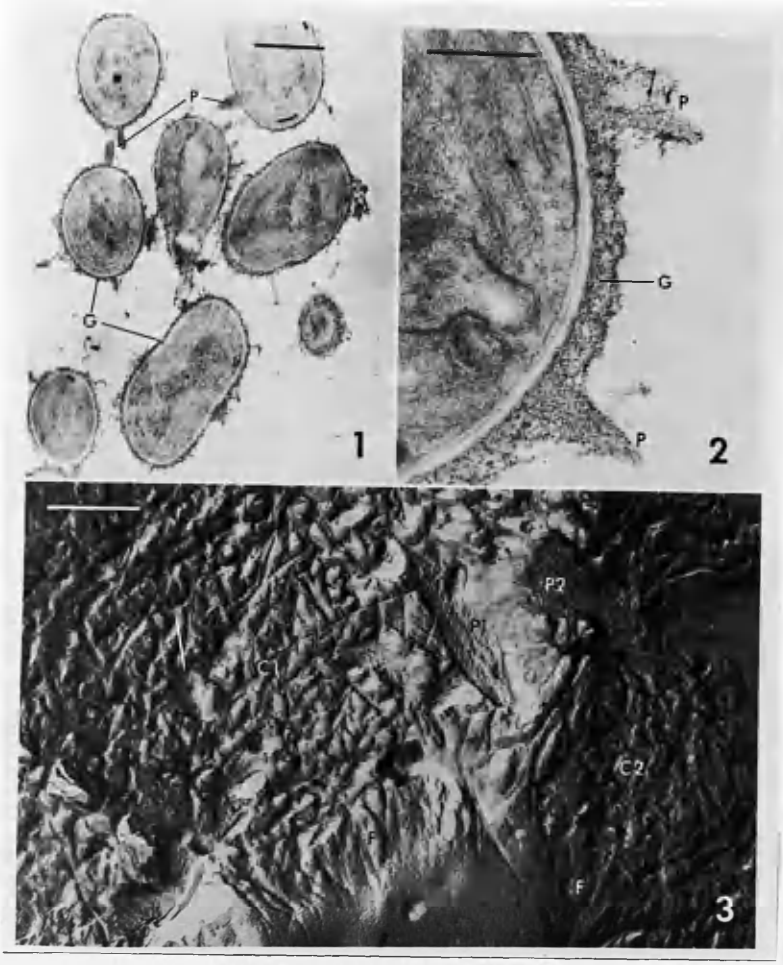

Fig. 14.1 Low Magnification electron micrograph of thin-sectioned *Agmenellum quadruplicatum* culture, showing glycocalyx (G) that surrounds each cell. Projections (P) of glycocalyx material extend from the surfaces of most cells. Ruthenium red procedure.
Bar = 1μm.

Fig. 14.2 High magnification electron micrograph of thin-sectioned *A. quadruplicatum* cell, showing details of glycocalyx (G) and projections (P). Ruthenium red procedure.
Bar = 0.25μm.

Fig. 14.3 Electron micrograph of frozen-etched *A. quadruplicatum* culture showing the glycocalyx surface of two adjacent cells (C1 and C2). Individual glycocalyx fibrils (F) are visible at various sites on the cell surfaces. Two glycocalyx projections (P1 and P2) are visible.
Bar = 0.25μm.

(Reproduced by permission from Balkwill and Stevens, 1980).

particular, *Chloroflexus aurantiacus* bore peritrichous fimbrae.

Further studies of *Synechocystis* str. CB3 showed that it was covered by a large number of pili with a diameter of approx. 6nm (Vaara *et al.*, 1984). The pili had a tendency to attach side by side to form bundles of several dozen pili (see p. 78). Isolation and purification of *Synechocystis* CB3 pili showed that they consisted mainly of hydrophobic amino acids, similar to other bacterial pili.

Dick and Stewart (1980) found that the *Nostoc* cyanobiont of the lichen *Peltigera canina* possessed discrete unbranched pili when grown on nitrogen-containing medium. These arose from the cyanobacterial cell surface in a peritrichous manner, and were up to 3μm long. The pili of this symbiotic cyanobacterium may serve as attachment organelles.

Pili of other bacterial species, particularly pathogenic bacteria, are known to play a role in their adhesion to tissue surfaces. The pili of the cyanobacterial species described here may be involved in their initial adhesion which would be further enhanced by mucilaginous sheath production. Further experimental work could show the possible involvement of pili in cyanobacterial adhesion. This would include comparing the attachment strengths of piliated cyanobacteria with cells of the same species from which pili were removed. Strength of attachment could be compared using a hosing technique similar to that described in Chapter 4 for fungal spores (see p. 139). Further scanning and transmission electron microscopic observations of piliated cyanobacteria interacting with artificial substrates could show the involvement of pili in adhesion.

The occurrence of spinae on the cell surfaces of certain marine bacteria was discussed in Chapter 1.3. Some reference has also been made to their occurrence on cyanobacteria (see p. 35,36). Marĕenko (1973)

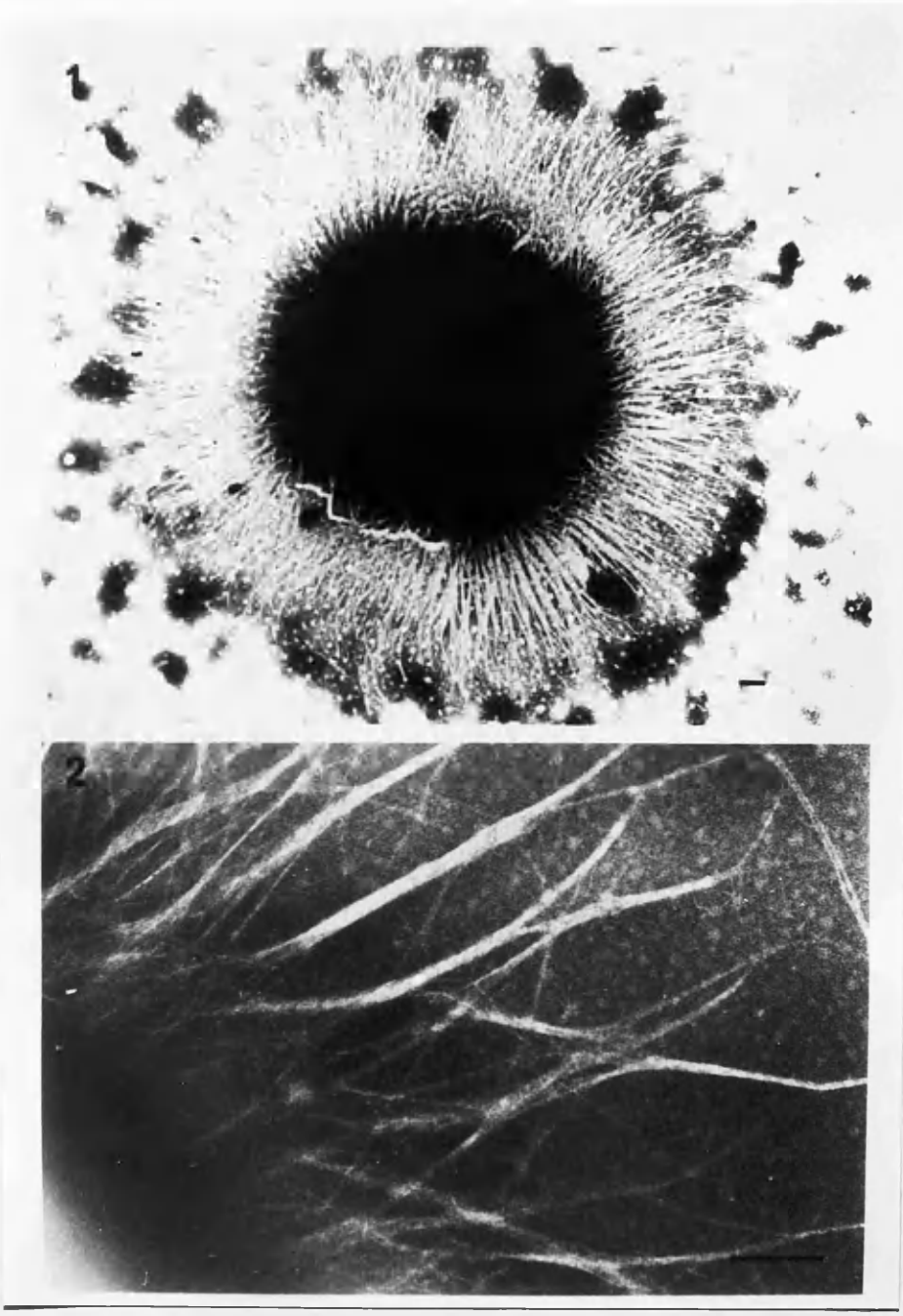

Fig. 15.1 *Synechocystis* CB3 cells abundantly covered by pili. Negative staining with 1% aqueous phosphotungstic acid, pH 6.5, was used in all the micrographs. Bar represents in this and all subsequent micrographs, 0.2μm.

Fig. 15.2 The *Synechocystis* CB3 pili attach side by side and form characteristic bundles. Due to the uneven length of the pilus filaments, individual filaments can be seen at the tips of the bundles.

(Reproduced by permission from Vaara *et al,* 1984).

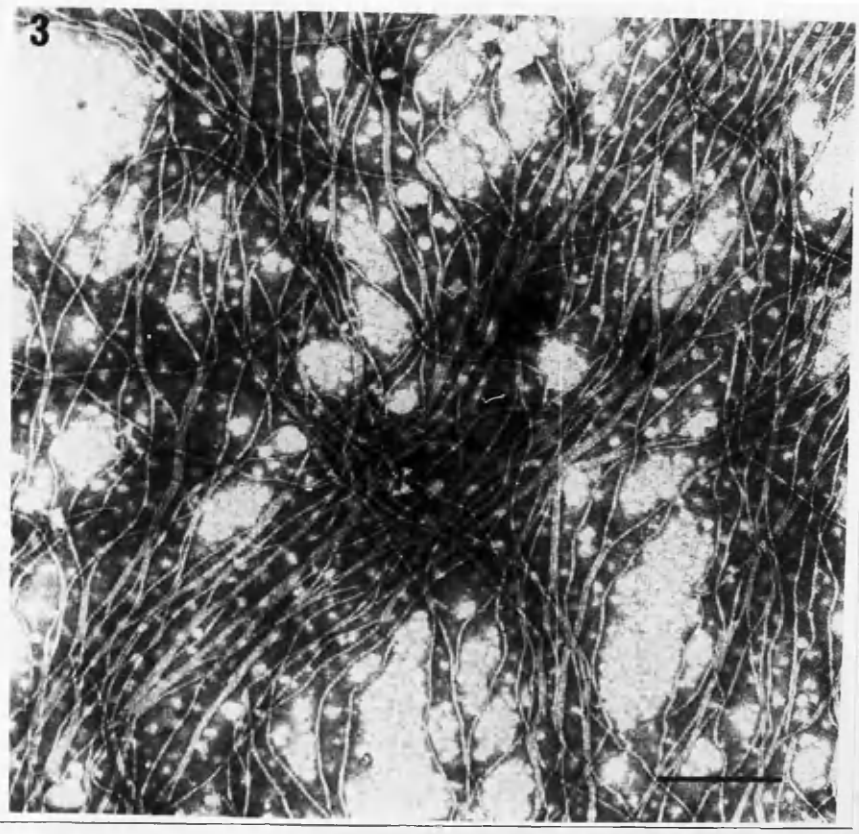

Fig. 15.3 Purified pili of *Synechocystis* CB3.

(Reproduced by permission from Vaara *et al,* 1984).

observed bristles or hair-like appendages on the cell surfaces of *Scenedesmus* species. Staining of the bristles suggested that they had a protein composition. The bristles had a morphological similarity to bacterial flagella, which are known to play a role in the adhesion of certain marine bacteria (see Chapter 1.3). Schnepf *et al.* (1980) investigated surface appendages in several chlorococcacean species of cyanobacteria. They found that bristles on the cell surfaces of *Scenedesmus armatus, Sc. opohiensis, Sc. subspicatus* and *Sc. nanus* had the same appearance as those described for other *Scenedesmus* species. They were also similar to the spiny bristles on *Polyhedriopsis spinulosa.* The bristles in this species were directly attached to the outer layer of the cell wall. *Micractinium pusillum* possessed asymmetrically distributed tapering appendages, which resembled the bristles of *Scenedesmus* and *Polyhedriopsis* spp. *Siderocystopsis fusca* possessed 8μm-long "spikes" which were attached directly to the cell wall. *Acanthosphaera zachariasi* also possessed between 20 and 40 spikes on the cell surface (Schnepf *et al.*, 1980).

Capitate surface appendages were seen on *Scenedesmus* str.16 using electron microscopy (Massalski and Trainor, 1971). They appeared as mushroom-like structures, with a straight or curved elongate stipe and an apical cap. Appendages on two adjacent *Scenedesmus* cells were frequently inter-connected and entangled. These long appendages may join adjacent cells causing colonial aggregations (Massalski and Trainor, 1971). If these appendages are involved in cell aggregation then they may also be involved in attachment of *Scenedesmus* spp. to surfaces.

These cell surface bristles and spinae may also facilitate cyanobacterial adhesion. They may allow initial settlement of

cyanobacterial cells, which would be enhanced by sheath production. Further experimental work could be done with cyanobacteria possessing these appendages to show their possible attachment role. Some of this work would be similar to that described in Chapter 1.3 (p. 37) for bacterial spinae. This would include the analysis and isolation of the main chemical components of these bristles, and a study of the effects of this material on cyanobacterial adhesion. This work would show whether protein is the main component, as suggested by Marčenko (1973) for *Scenedesmus* spp.

Summary

The main points discussed in this Chapter are :-

(1) Some cyanobacteria produce mucilaginous sheaths. Cytochemical analysis and electron microscopic work suggests that the sheaths are similar in structure and appearance to marine bacterial adhesives. Sheaths may be involved in adhesion of cyanobacteria to surfaces in aqueous environments (Tuffery, 1969; Martin and Wyatt, 1974; Kessel and Eloff, 1975; Balkwill and Stevens, 1980).

(2) Certain cyanobacteria have been shown by electron microscopy to possess pili or surface fimbrae. These include *Synechocystis* spp. The dimensions and chemical structure of some cyanobacterial pili are similar to other bacterial pili (Lounatmaa *et al.*, 1980; Vaara *et al.*, 1984). Pili could be involved in the initial attachment of cyanobacteria to surfaces.

(3) Other cyanobacteria, such as *Scenedesmus* spp. possess cell surface bristles or hair-like appendages (Marčenko, 1973; Schnepf *et al.*, 1980). Some of these appendages on adjacent cells can become entangled causing colonial aggregations (Massalski and Trainor,

1971). Like pili, these appendages may also be involved in initial cyanobacterial attachment.

Chapter 3 Adhesion mechanisms of marine algae.

Marine algae are major world-wide fouling organisms which occur on a wide range of immersed structures including ships, buoys and oil platforms (Fletcher *et al.*, 1984). The presence of algal growths on these structures can cause problems. Accumulation of algae on ships increases frictional resistance of the hull resulting in increased fuel consumption to keep at cruising speeds. Algal fouling on oil and gas platforms can increase structural loading and block safety inspections (Fletcher *et al.*, 1984). Firm attachment of algal spores is a necessary initial stage in the ultimate development of fouling macroalgae, and so is important in the understanding of algal fouling.

Microscopic diatoms, together with bacteria, fungi and protozoa, are important components of the initial primary film community (Jones *et al.*, 1983). Formation of the primary film allows settlement of macroscopic fouling organisms such as seaweeds, barnacles and mussels. Formation of diatom films on metal surfaces has been shown to inhibit corrosion (see Chapter 11).

This chapter considers adhesion mechanisms of algal spores and primary rhizoids. Attachment mechanisms of several diatom species are also discussed. Although the major fouling seaweeds are macrofouling organisms, it was considered useful to discuss their initial stages of adhesion, as these stages have similarities with marine bacterial adhesion. Some recent antifouling methods are based on certain factors which affect algal spore and rhizoid adhesion (see Chapter 12).

(3.1). Adhesion mechanisms of algal spores and initial stages of rhizoid adhesion.

(3.1.1). Adhesion of algal spores

Marine algal dissemination involves a dispersal phase in which spores are released into the surrounding seawater, followed by an attachment phase in which the spores attach to a new substratum (Jones *et al.*, 1983). Spore attachment is divided into stages of initial and permanent attachment. Initial attachment of red algal spores is probably achieved by the copious mucilage envelope surrounding spores after expulsion from the sporangium (Chamberlain, 1976; Boney, 1981; Jones *et al.*, 1983). This mucilage is a fibrillar polysaccharide formed by the Golgi apparatus during spore differentiation within the sporangium, and is sticky in texture (Chamberlain, 1976; Boney, 1981; Jones *et al.*, 1983). Similar mucilaginous material surrounds differentiating green and brown algal spores. However, it does not remain around the spores following their release into seawater. Motile green and brown algal spores initially attach to substrata by their flagella (Jones *et al.*, 1983; see p. 90).

Following the initial phase, more permanent attachment of algal spores occurs by the release of an adhesive material. This process appears to occur in all the major algal fouling groups, and involves the Golgi apparatus. The spore adhesive appears to be reticulate and fibrillar by S.E.M.. The adhesive is a continuous sheet near the spore which becomes perforated in the outer regions, eventually forming a fibrous meshwork at the periphery (Chamberlain, 1976; Jones *et al.*, 1983; see p. 85).

The next part of this chapter discusses in more detail the permanent attachment of certain algal spores. Representative species from the main algal families, the Chlorophyceae (green algae), Rhodophyceae (red algae)

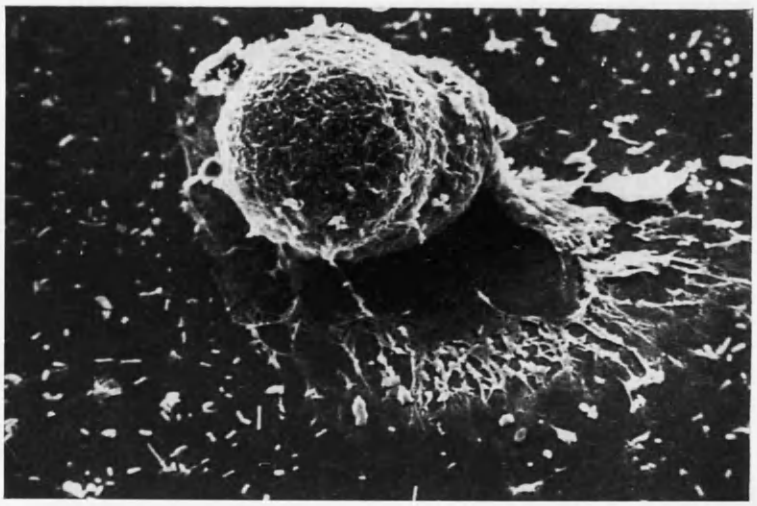

Fig. 16 A settled algal spore with secreted adhesive pad. The adhesive material becomes more reticulate at the periphery. (Mag. x3420).

(Reproduced by permission from Chamberlain, 1976).

and Phaeophyceae (brown algae), are discussed.

(3.1.1.1). Chlorophyceae

Swimming zoospores of *Enteromorpha intestinalis* can be seen, by transmission electron microscopy, to possess several types of cytoplasmic vesicles. Large vesicles, of diameter 450-650 Å, are present in the anterior region of settling zoospores. These vesicles, together with other smaller vesicles, are derived from the Golgi apparatus. Zoospores settled for 1 hour appear to have lost these vesicles, and fibrillar material attaches them to the substratum (Evans and Christie, 1970; Christie, 1972). The vesicles can occasionally be seen in the fibrillar material. This observation, together with the complete absence of these vesicles from settled *E. intestinalis* zoospores, suggests that the vesicles may be involved in the early stages of attachment. Additionally, the plasmalemma of settled zoospores appears convoluted. The vesicles may cause this by coalescing with the plasmalemma, and extruding their contents by reverse pinocytosis (Evans and Christie, 1970).

Further evidence of the adhesive function of these zoospore vesicles came from biochemical studies. Addition of the enzymes trypsin and pronase to suspensions of swimming *E. intestinalis* zoospores completely digested the anterior vesicles contents. The external fibrillar material was completely absent from settled zoospores incubated in trypsin (Evans and Christie, 1970; Christie, 1972). Zoospores were, additionally, found to attach only weakly in the presence of the enzyme α-amylase. These results suggested that proteinaceous material is present in the anterior vesicles of settling zoospores. The adhesive material secreted by *E. intestinalis* zoospores appears to be a glycoprotein. These observations

further suggest that the adhesive is secreted from the anterior vesicles. A carbohydrate moiety, produced from the Golgi apparatus, may also be present in the zoospore vesicles (Evans and Christie, 1970; Christie, 1972).

Further cytochemical staining of the anterior vesicles of *E. intestinalis* zoospores confirmed that carbohydrate was present as well as protein (Callow and Evans, 1974). Autoradiographic experiments using ^{3}H-leucine showed that material synthesized in the endoplasmic reticulum moved into the Golgi apparatus. From these, the labelled material passed into anterior vesicles, the contents of which passed through the plasmalemma to form the attachment material (Callow and Evans, 1974; see p. 88).

Settled zygotes of *Ulva mutabilis* produced a ruthenium red-staining adhesive material. Treatment of attached zygotes with pronase and α-amylase enzymes removed this material (Bråten, 1975). This suggests that the spore adhesive is a glycoprotein, similar to *E. intestinalis* zoospores. Large numbers of electron dense vesicles were present in the cytoplasm of free-swimming and newly settled zygotes. These vesicles were secreted during the settling of *U. mutabilis* spores, and they may be involved in secretion of the adhesive material. However, the ruthenium red stain did not penetrate into the cells. As a result, it was not possible to stain the vesicles to see if they represented the origin of the ruthenium red-positive material (Bråten, 1975). Further autoradiographic work, as performed by Callow and Evans (1974), could show the role of the spore vesicles of *Ulva mutabilis* in secretion of adhesive. Additionally, further enzymic work, such as that described by Evans and Christie (1970), could show any similarities between the vesicle contents and spore adhesive of *Ulva mutabilis*.

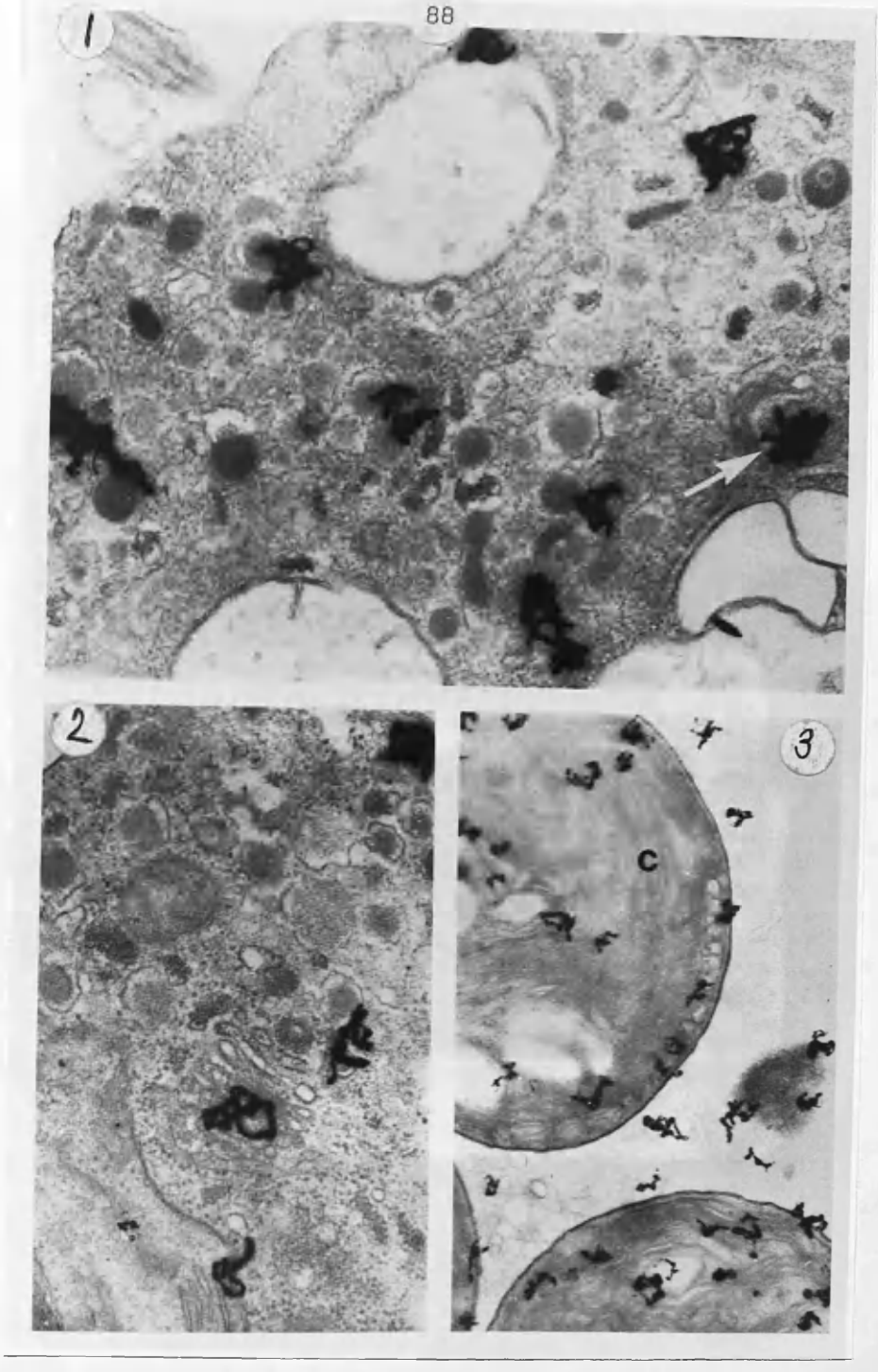

Fig. 17.1 and 17.2
Autoradiographs of anterior region of swimming zoospore of *Enteromorpha intestinalis* after 30 mins. incubation in ^{3}H-leucine, showing the association of silver grains with a Golgi body (arrow) and vesicles.
(Mag. x30000).

Fig. 17.3 Autoradiographs of zoospores incubated in ^{3}H-leucine for 30 mins. and allowed to settle for 1 hour. Silver grains are mainly associated with the chloroplast (C) and external fibrillar adhesive.
(Mag. x15000).

(Reproduced by permission from Callow and Evans, 1974).

(3.1.1.2). Rhodophyceae

Permanent attachment of red algal spores occurs by a similar mechanism to that of green algae. During attachment of tetraspores of *Ceramium* spp., large, dense-cored vesicles are produced in the cytoplasm from the Golgi apparatus. These vesicles remain in the cytoplasm until initial surface contact by the spore, and are believed to secrete the spore adhesive (Chamberlain, 1976; Chamberlain and Evans, 1981). Histochemical staining suggests that the spore adhesive is largely polysaccharide in nature. However, enzymic experiments show that α-amylase and pronase enzymes detach settled *Ceramium* spores, suggesting that the adhesive is a polysaccharide-protein complex (Chamberlain, 1976; Chamberlain and Evans, 1981).

Attachment of spores of *Polysiphonia* spp. occurs by a similar process. Once again, the Golgi apparatus is involved in the production of dense cored vesicles with swirled contents. The vesicle contents are secreted as the spore adhesive during the settlement of the spores (Fletcher, 1979). Few details have been given, however, of the chemical composition of the spore adhesive of *Polysiphonia* spp. Further experimental work on this would be useful, as it would allow the adhesive composition of *Polysiphonia* spores to be compared with *Ceramium* spp. and other algae.

(3.1.1.3). Phaeophyceae

Zoospores of the filamentous brown alga *Ectocarpus* spp. also contain electron transparent vesicles during their settlement to substrata. These vesicles are also produced by the Golgi apparatus (Baker and Evans, 1973). Fibrous adhesive material is produced upon zoospore settlement and this again coincides with disappearance of the vesicles. Cytochemical

staining also suggests that the spore adhesive is initially located in the vesicles. The adhesive material is mainly composed of polysaccharide, although a protein moiety may also be produced from the Golgi apparatus (Baker and Evans, 1973). The composition and production of the *Ectocarpus* zoospore adhesive are very similar to that which occurs in *Enteromorpha intestinalis* and *Ceramium* spp.

Studies have been made of the life history and attachment mechanisms of the fouling alga *Giffordia granulosa* in the Solent. Zoospores of *G. granulosa* attached by the production of a fibrillar, mucilaginous adhesive material (Fletcher, 1981; see p. 91). Electron transparent vesicles were observed discharging their contents into the zoospore cell wall. However, there was no evidence of these vesicles discharging the extracellular adhesive of the zoospores (Fletcher, 1981). Autoradiographic work and cytochemical staining could show the involvement of vesicles in production of zoospore adhesive. Additionally, work to reveal the composition of *G. granulosa* adhesive, such as enzymic detachment of zoospores, would be useful.

Released zoospores of the brown alga *Chorda tomentosa* possess a long anterior flagellum which consists of a tightly coiled terminal region and a rigid lower section (Toth, 1976). A settling zoospore initially contacts a substratum by the tightly coiled region of the flagellum. This part of the flagellum may be coated with a sticky protein, which allows zoospore attachment. Following this initial attachment, the *C. tomentosa* zoospore draws itself to the substratum by withdrawing the flagellum. Immediately after settlement, numerous vesicles containing fibrillar material are present in the cytoplasm of the zoospores. These vesicles also discharge their contents producing a cushion of fibrous adhesive material for the

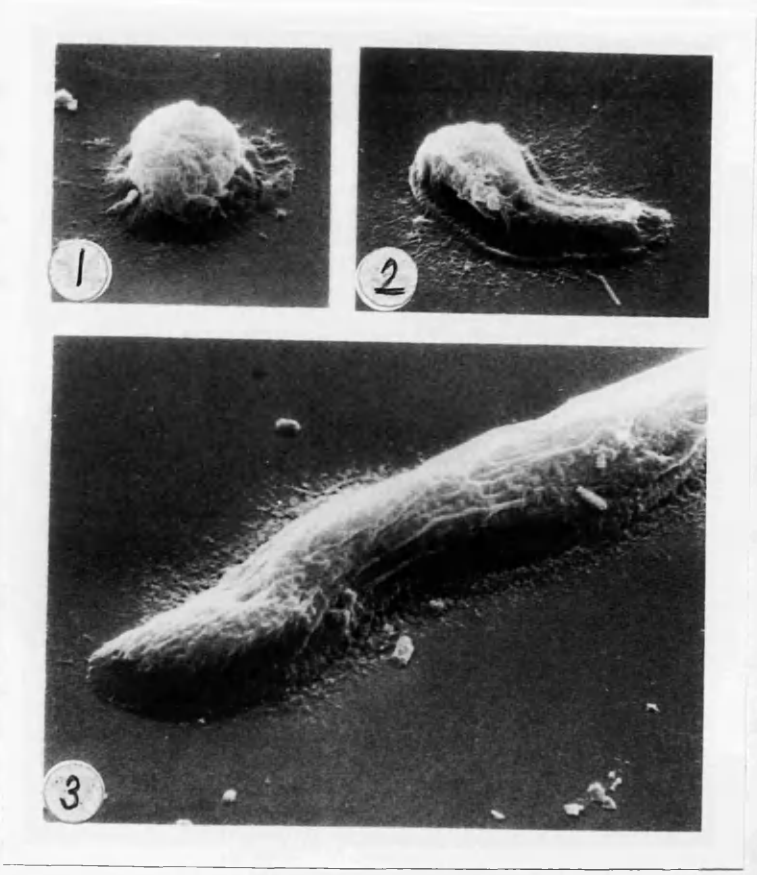

Fig. 18.1 Scanning electron micrograph (S.E.M.) showing settled zoospore of *Giffordia granulosa* with released adhesive material. (Mag. x4000).

Fig. 18.2 and 18.3
S.E.M. showing development and attachment of primary rhizoids of *G. granulosa.* Note hemispherical shape and peripheral rhizoid adhesive in fig. 18.3.
Mags: 18.2 x3350
18.3 x4650

(Reproduced by permission from Fletcher, 1981).

settled zoospores (Toth, 1976). The initial adhesion of *Chorda tomentosa* zoospores occurs by a different mechanism from the production of an initial mucilage layer in spores of the red alga *Ceramium* spp.

(3.1.1.4). Strength of algal spore adhesion

Attached algal spores have to contend with a surrounding mass of seawater which is in constant motion. They must remain strongly attached if they are to successfully germinate and develop into a macroscopic plant. Studies have been made of how strongly certain algal spores attach to substrata.

Charters *et al.* (1973) used a "waterbroom" apparatus to measure the attachment strength of spores of *Agardhiella tenera, Cryptopleura violacea* and *Gracilariopsis sjoestedtii.* The "waterbroom" produces water motion, similar to wave surge, over the surface of a submerged substrate in a laboratory aquarium. Strength of spore attachment was measured by a spore survival ratio, $N_A:N_B$, where N_A = the number of attached spores remaining after the waterbroom test, and N_B = the numbers attached before the test (Charters *et al.*, 1973). The spore survival ratio of *Agardhiella tenera* improved with time, whilst that of *Cryptopleura violacea* decreased. The ratio for *Gracilariopsis sjoestedtii* spores increased with time up to 10 hrs after settlement. After this period, however, the survival ratio decreased, reaching an asymptotic limit after 20 hrs. The spores of *G. sjoestedtii* were found to attach firmly, resisting removal by shear forces nearly 100 times their weight (Charters *et al.*, 1973).

A "water tunnel" was used by Jones *et al.* (1983) to study the strength of attachment of *Ceramium rubrum* spores and germlings (see p. 99). Spore attachment strength is measured in the water tunnel by the water velocity required to separate spores from a substratum. Three main stages of

attached *C. rubrum* spores were used. These were recently settled spores, settled spores with released adhesive and germinating spores. The effect of calcium on spore attachment was also examined, by growing the stages in calcium deficient seawater. After exposure of recently settled *C. rubrum* spores for up to 2 hours in the water tunnel, 30% of the spores remained attached. They were held to the substrate by a combination of physical/chemical forces and the peripheral mucilage layer (Jones *et al.*, 1983). Spores exposed for between 2 and 9 hours had produced adhesive mucilage. Consequently, there was an increase to 55% in the number remaining attached. After 9 hours exposure spore germination was observed, and the number of germlings remaining attached increased to 80%. The absence of calcium from seawater had little effect during the early stages of spore attachment (Jones *et al.*, 1983). However, post germination attachment stages were markedly affected (see p. 99).

The results of Charters *et al.* (1973) and Jones *et al.* (1983) confirm that in the initial stages of algal spore adhesion spores attach to substrata mainly by chemical bonding. Both sets of results also suggest that spore adhesion involves wetting the surfaces of both spore and substratum by a liquid adhesive, before it changes phase to a solid adhesive (Charters *et al.*, 1973). The strong attachment of *Gracilariopsis sjoestedtii* spores, as well as the large numbers of *Ceramium rubrum* spores remaining attached in the water tunnel, further supports formation of a solid adhesive.

It would be interesting to perform further experiments of the type described by Charters *et al.* (1973) and Jones *et al.* (1983) to compare attachment strengths of other algal spores. Such work could be done with spores of *Enteromorpha intestinalis, Ectocarpus* spp., *Giffordia granulosa*

and *Polysiphonia* spp. This work would show whether spores from different algal families, which attach by the same mechanism, possess similar attachment strengths. It would also allow comparison with strength of adhesion of marine fungal spores through appendages and mucilage production (see Chapter 4).

(3.1.2). Adhesion of primary rhizoids

When an algal spore has attached successfully to a substratum, spore germination and rhizoid formation occurs under suitable environmental conditions. Primary rhizoids develop directly from the initial germ tube. In some algae, however, rhizoid production occurs after spore cell division has taken place and they emerge from the basal rhizoid initial cell (Jones *et al.*, 1983). A characteristic feature of all rhizoids is their adhesive property. Rhizoid attachment results from secretion of a peripheral, mucilaginous adhesive material. The mucilaginous adhesive is usually secreted from the rhizoid tip and spreads out across the substratum. Scanning electron microscopy shows that the adhesive is reticulate and fibrillar (Fletcher, 1976; Jones *et al.*, 1983). Vesicles produced from the Golgi apparatus are believed to be involved in the production of rhizoid adhesive material. Histochemical work has indicated that rhizoid adhesive is composed of complex sulphated polysaccharides (Jones *et al.*, 1983).

The next section will describe in more detail primary rhizoid adhesion in species from the *Chlorophyceae, Rhodophyceae* and *Phaeophyceae.*

(3.1.2.1). *Chlorophyceae*

Spores of the species *Blidingia marginata* undergo divisions to form a small rosette-shaped disc of cells. This basal disc is composed of large

numbers of small intertwined rhizoidal filaments. The rhizoids are covered by a fibrous matrix which connects to adjacent filaments helping to attach them together (Fletcher, 1976). The basal disc is probably the main attachment structure for this species.

Rhizoidal filaments of *Enteromorpha intestinalis* also cohere to form a basal disc. The constituent filaments in the disc are enveloped in mucilage, which makes the disc a strong attachment structure. Individual rhizoidal filaments also extend from the basal disc across the substratum (Fletcher, 1976; see p. 96).

Germinating spores of *Ulothrix flacca* produce an erect filament which is attached by the original spore adhesive. The base of the filament attaches by the pointed tip of a downwardly extending germ tube. A small circle of mucilage is present at the point of contact of the filament. This mucilage flows out over the substratum and allows attachment of the rhizoids (Fletcher, 1976).

Attached zygotes of *Ulva mutabilis* develop a pear shape, and the pointed end of the cell initiates formation of the primary rhizoid (Bråten, 1975). The developing rhizoid is surrounded by a homogeneous substance which makes intimate contact with the substratum. This material is the rhizoid adhesive. It appears as a fibrous coating around the rhizoid cells on fixation with osmium tetroxide. However, no electron dense vesicles which may secrete the rhizoid adhesive are seen in the cytoplasm of *Ulva mutabilis* rhizoids. The enzymes hyaluronidase and α-amylase do not affect the rhizoid adhesive material. However histochemical staining of the adhesive suggests that it contains mainly proteins. Consequently, the composition of the *Ulva mutabilis* rhizoid adhesive is different from the zygote adhesive (Bråten, 1975).

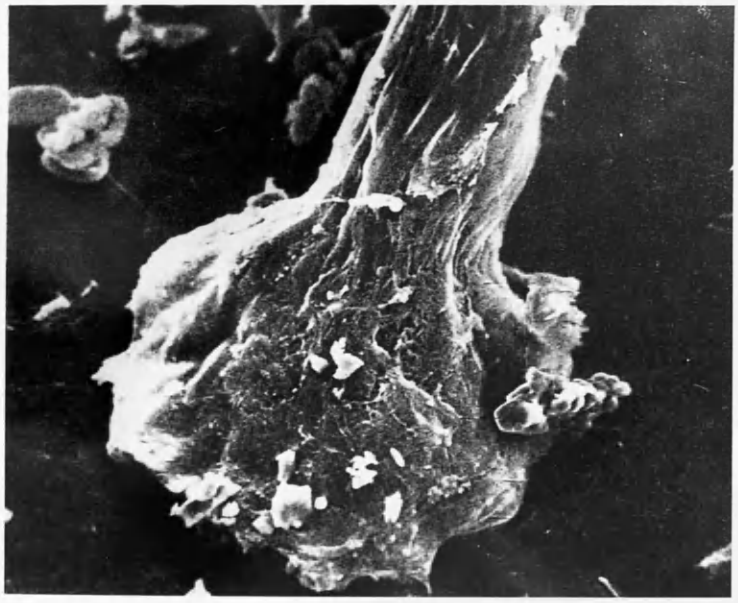

Fig. 19 S.E.M. of young basal disc of *Enteromorpha intestinalis.* (Mag. x9090).

(Reproduced by permission from Fletcher, 1976).

(3.1.2.2). *Rhodophyceae*

A small bulbous germ tube is produced at the base of the germinating rhizoidal cell in *Polysiphonia* spp. This germ tube has a rounded mucilaginous terminal region which ultimately develops into a terminal disc-like structure upon further rhizoid growth (Fletcher, 1976, 1979). *Polysiphonia urceolata* characteristically produces a four-lobed attachment disc from which mucilage spreads over the substrate, allowing firm attachment (Fletcher, 1976).

Polysiphonia lanosa also produces a mucilaginous attachment disc during rhizoid development on the surface of *Ascophyllum nodosum*. Large numbers of electron-dense vesicles with fibrous content are present in the peripheral rhizoid region. These vesicles appear to be discharging their contents at the cell surface, and this coincides with the appearance of toluidine-blue staining material in the mucilaginous disc (Rawlence and Taylor, 1972). This observation suggests the involvement of the vesicles in secretion of mucilaginous material in the attachment discs. However, further experimental work using cytochemical stains, enzyme treatment and autoradiographic studies of adhesive production are needed to confirm the role of the vesicles.

The type of substrate influences the formation of holdfast discs in rhizoids of *Polysiphonia* spp. The discs are formed on plastic petri dishes, but not on glass coverslips (Fletcher, 1976). These observations suggest that rhizoid attachment in *Polysiphonia* spp. is affected by the nature of the substratum, an observation shown to occur in other algae. (see p. 102). Rhizoid attachment in *Ceramium rubrum* is very similar to *Polysiphonia* spp.. Following spore germination, a multicellular rhizoid filament develops which produces a terminal disc. Additional thinner

rhizoids are then produced which spread over the substrate, although they do not produce discs (Fletcher, 1976).

(3.1.2.3). *Phaeophyceae*

Settled spores of *Ectocarpus fasciculatus* produce lateral branches which continue to expand, forming an elaborate network over the substrate. The actively growing apices of the filamentous rhizoids produce a mucilaginous material. This material spreads out over the substrate, allowing firm attachment of the growing rhizoidal filaments (Fletcher, 1976).

The developing germ tubes and primary rhizoidal filaments of *Giffordia granulosa* maintain close surface contact. This contact is achieved through frequent lateral adherence of the filaments, and by secretion of surrounding fibrillar adhesive (Fletcher, 1981; see p. 91). Transmission electron microscopic examination of the growing apex of the *Giffordia granulosa* rhizoids shows large, electron-transparent vesicles containing a reticulate microfibrillar material. These vesicles are derived from Golgi bodies situated slightly further back from the rhizoid apex (Fletcher, 1981). It is possible that they may be involved in the secretion from the rhizoid apex of adhesive material surrounding rhizoids. Further experimental work, such as autoradiographic studies is needed of any passage of rhizoid adhesive from the vesicles. Cytochemical and enzymic studies of the vesicle contents and rhizoid adhesive are also necessary.

These descriptions of the main algal groups show that most rhizoids in their initial stages of development attach by production of mucilaginous material from the rhizoid apex or the formation of a mucilaginous disc. However, little is known about the origin of rhizoid adhesive, particularly in the growing apex. Some transmission electron microscopic studies of

rhizoid attachment, such as *Polysiphonia lanosa* (Rawlence and Taylor, 1972) and *Giffordia granulosa* (Fletcher, 1981), have shown the possible involvement of cytoplasmic vesicles in production of adhesive material. However, it has also been suggested that these vesicles may be secreting material into the rhizoid cell wall. The adhesive may, therefore, be excess gelatinous cell wall which has exuded from the rhizoid tip (Fletcher, 1976). Further transmission electron microscopic studies of the tip regions of developing rhizoids in a wide range of algae are needed. This, together with autoradiographic, cytochemical and enzymic work, may show whether rhizoid adhesive production from vesicles occurs generally in algae.

Calcium has been shown to affect rhizoid attachment (Jones *et al.*, 1983). Growth of *Ceramium rubrum* germlings in seawater deficient in calcium resulted in only 40% of the germlings remaining attached after five days compared with 100% attachment in normal seawater. Calcium may increase the cohesive strength of the *Ceramium rubrum* rhizoid adhesive by forming divalent cationic bridges linking negatively charged sites (Jones *et al.*, 1983). The lack of rhizoid attachment observed in the absence of calcium suggests that calcium removal from a fouling film, such as by a chelating agent, could be a useful antifouling mechanism (see Chapter 12).

(3.1.3). Some physicochemical aspects of algal adhesion and effects of substratum properties on attachment of algal spores and rhizoids.

Before algal spores even settle onto a substratum by production of organic adhesives, they are influenced by physical and chemical forces similar to those occurring in bacterial adhesion (see Chapter 1.1).

However, very few studies have been conducted on the physicochemistry of algal spore adhesion. Substratum properties such as surface free energy or the formation of conditioning films can, as with marine bacteria (see Chapter 1.5), influence attachment of algal spores and rhizoids. This may lead to the development of new antifouling techniques.

The initial stages in the adhesion of the unicellular green alga *Chlorella* spp. to glass surfaces in ionic solutions was studied by Nordin *et al.* (1967). The principal mechanisms governing *Chlorella* adhesion were electrostatic interaction between the electrical double layers and specific surface interactions resulting from surface heterogeneity and ion adsorption. Sodium chloride concentration in the suspending medium affected *Chlorella* attachment. The *Chlorella* cells attached to glass more readily at higher sodium chloride concentrations (Nordin *et al.*, 1967). At high electrolyte concentrations, the thickness of the electrical double layer surrounding the cells decreases, as occurs in bacterial adhesion (see Chapter 1.1; see p. 14). Consequently, the algal cells are held in a secondary attraction minimum. Therefore, forces other than electrostatic interactions, such as London-van der Waals forces, must operate in attachment. An increase in ferric chloride concentration also increased *Chlorella* adhesion (Nordin *et al.*, 1967). Other forces must also attract the algal cells to glass in the presence of ferric chloride.

Little other work has been reported on the physicochemistry of algal adhesion. Further work, such as the effects of electrolytes on attachment of spores from a wide range of algal species, is needed. Such work could show whether algal spore adhesion, like bacterial adhesion, involves an instantaneous reversible phase and time-dependent irreversible phase.

Some studies on the affects of substratum properties on algal spore

and rhizoid attachment will now be discussed.

Adhesion of *Chlorella vulgaris* to glass tubes was enhanced by non-diffusable materials isolated from *Chlorella* adhesive exudate, marine bacterial cultures, natural seawater and fouled marine surfaces (Tosteson and Corpe, 1975). Cell aggregation was observed when a *Chlorella* exudate concentration of 0.2ng/cell was used. The materials which were most active at enhancing *Chlorella vulgaris* adhesion were the adhesive polymer isolated from the bacterium *Pseudomonas atlantica* and seawater-insoluble material isolated from wooden panels exposed to seawater. Active polymer materials isolated from several sources were chromatographed on DEAE-cellulose (DEAE - Diethylamino-ethyl Cellulose, an anionic exchanger). The major fraction eluted contained both carbohydrate and protein. Chromatographed polymeric material enhanced *Chlorella vulgaris* adhesion more than unchromatographed material. Adhesion-enhancing materials from bacteria and other marine sources may function as "inducers" of adhesive polymer synthesis by *Chlorella vulgaris* or could stabilize the secreted adhesive. These materials could also substitute for the adhesive material synthesized by *Chlorella vulgaris* (Tosteson and Corpe, 1975).

In addition to bacterial exudates affecting algal adhesion, periphytic marine bacteria precondition surfaces prior to colonization by marine algae. Thomas and Allsopp (1983) found that some marine bacteria, when present in thin films on glass surfaces, encouraged settlement and growth of *Enteromorpha* germlings. Other bacteria discouraged settlement of the algal spores. An unidentified marine pseudomonad, isolate 01, increased *Enteromorpha* adhesion. 110 algal germlings per 47mm^2 were found on glass coverslips in the presence of this bacterium. Another bacterial isolate, 03, had the opposite effect. In this case, 7 germlings per 47mm^2 were

found on glass coverslips coated with the bacterium (Thomas and Allsopp, 1983). These results indicate that certain marine bacteria can alter substratum properties to allow attachment of algae.

These results of both Tosteson and Corpe (1975) and Thomas and Allsopp (1983) show that polymeric materials and bacterial films can condition a surface for algal attachment. This is similar to the formation of a proteinaceous conditioning film which modifies a substratum allowing attachment of marine bacteria (see Chapter 1.5, see p.49). It would be interesting to carry out further experimental work on the effects of proteinaceous materials on attachment of spores and rhizoids of other algae.

The results of Thomas and Allsopp (1983) also suggested that certain bacterial films inhibit settlement of algal spores. This observation may have applications in antifouling technology. This would occur by thin bacterial films grown upon antifouling paints preventing attachment of *Enteromorpha* spp. and other fouling algae. Further work studying the affect of bacterial films on attachment of a wider range of algal spores and rhizoids is needed to show whether an effective antifouling technique can be devised.

Substratum properties such as surface free energy, known to affect bacterial attachment (see Chapter 1.5), have been shown to influence attachment of algal rhizoids. Glass slides, which were coated with silane-based compounds with surface free energies ranging from less than 20mN/m to over 70mN/m, were used to study attachment of *Enteromorpha intestinalis* rhizoids (Fletcher and Baier, 1984). Considerable differences were observed in the development of the basal attachment rhizoids of this alga on the different surface types. A decrease in

surface free energy resulted in an increase in the growth and outward spread of the rhizoids (Fletcher and Baier, 1984; see p. 105). Long, outwardly spreading rhizoidal filaments were produced by *E. intestinalis* on glass slides with low surface free energies. The alga developed a compact, discoid rhizoidal base with a smaller diameter on silane coatings with high surface free energy (see p. 105). Short, branched and tightly adjoined rhizoidal filaments were produced on such surfaces. There was a significant difference observed in the attachment strengths of the rhizoid bases on these glass slides. The disc-like attachment base produced on the high-energy surfaces was strongly adherent and difficult to remove by gentle brushing. However, the filamentous rhizoid base produced on the low-energy surfaces was more loosely attached and easily removed (Fletcher and Baier, 1984). Similar observations were made on *Ulva lactuca* rhizoids (Fletcher *et al.*, 1984).

The effects of surface free energy on the attachment of rhizoids of some brown and red algae has also been investigated. The same silane coatings on glass slides with surface free energies from 20mN/m to 70mN/m were used. Differences were observed in the development of primary rhizoids of *Giffordia granulosa* on each surface (Fletcher *et al.*, 1984). The average diameter of the rhizoid base produced on the glass slide with intermediate surface energy of 30mN/m was smaller than those rhizoids that developed on the other surfaces. The appearance of the rhizoids on this glass surface, in addition, was different from those produced on the other surfaces. The rhizoids were shorter and comprised short, closely adherent cells. Additionally, the rhizoids on this surface were very firmly attached and could not be removed by gentle brushing (Fletcher *et al.*, 1984).

Development of rhizoids of the red algae *Bangia atropurpurea* was also influenced by the surface energies of the silane-coated glass slides. Several rhizoidal filaments were produced on the high energy surfaces, and these were closely adherent and disc-like in appearance. Single rhizoidal filaments were formed on low energy surfaces. The rhizoids which developed on the high energy glass surfaces were more firmly attached than those on the other surfaces (Fletcher *et al.*, 1984).

Polysiphonia spp. formed two types of attachment system, depending on the surface energy of the glass slide. Discoid attachment systems developed on the silane-coated slides with intermediate surface energies of 20-30mN/m. The formation of attachment discs on the glass reduced the horizontal spread of the rhizoid attachment system. Filamentous rhizoids, however, developed on the high surface energy slides. There was little difference between the strengths of attachment of filamentous and discoid rhizoid systems (Fletcher *et al.*, 1984).

The surface energy properties of the substrata therefore exert an influence on two main features of algal rhizoid development. They influence the outward spread of the rhizoids and modify the degree of surface contact. This causes development of the compact, discoid attachment structures and the long rhizoidal filaments (Fletcher *et al.*, 1984). In most of the algae investigated, with the exception of *Polysiphonia* spp., the short discoid bases were firmly attached, whilst the long rhizoidal filaments were more weakly attached. The surface energy properties of the substrata may have affected the attachment of the algal spores, which will ultimately influence rhizoid attachment. In addition, surface energy may influence the adhesive quality of the rhizoids, either by affecting the attachment properties of the cell wall material or the

TABLE 6

Effect of surface energy on attachment in *Enteromorpha intestinalis*

Surface type	Measured critical surface tension (mN/m).	Algal base (diameter in μm)
(1) RFGDT* - water stored.	> 70	69
(2) RFGDT - lab equilibrated.	30-40	75
(3) Chloroprophyltrichlorosilane.	30	125
(4) Dichlorodimethylsilane.	20-30	143
(5) Fluorosilane	> 20	195

* RFGDT - Radio Frequency Glow Discharge Treated glass slides.
'water stored' - glass slides stored in boiled 3x distilled water.
'lab. equilibrated' - glass slides allowed to equilibrate in a laboratory "white room" and then packaged in tissue.

(Taken from Fletcher and Baier, 1984).

rhizoid adhesive material or both (Fletcher *et al.*, 1984).

Silane coatings of low energy are least favourable for the development of effective rhizoidal attachment systems. Rhizoids of *Enteromorpha intestinalis, Ulva lactuca* and *Bangia atropurpurea,* in particular, were loosely attached on the low energy silane surfaces (Fletcher and Baier, 1984; Fletcher *et al.*, 1984). These observations could allow the development of an antifouling technique based on substratum properties (see Chapter 12; p. 334). Algae would only attach weakly to low energy silane surfaces, for example, and so would be easily dislodged. Further experiments on attachment of a wider range of fouling algae to low surface energy silane coated substrates could be done.

(3.2). Adhesion mechanisms of diatoms

Diatoms, along with marine bacteria, are amongst the earliest colonizers of surfaces submerged in seawater. They make up the primary slime film along with adhesive secretions and various organic and inorganic particles (Jones *et al.*, 1983). Diatom attachment to substrata occurs by the production of mucilage which is morphologically elaborated to form particular attachment structures.

An early observation of a possible diatom adhesion mechanism was a gelatinous capsule surrounding *Navicula pelliculosa* cells (Lewin, 1955). Capsule formation occurred when the diatom grew in culture medium deficient in certain elements, such as silicon, nitrogen or phosphorous. The gelatinous nature of the capsule, which is similar to some pathogenic bacterial capsules, suggests that it may have an attachment role. However, other functions were suggested, such as a flotation device or as a protection mechanism against aquatic herbivores (Lewin, 1955).

(3.2.1). Methods of diatom attachment

There are four main methods of diatom attachment to surfaces, which can be grouped as follows.

Group 1 - Production of basal or unipolar adhesive pads

This group includes *Cocconeis scutellum*, which produces a peripheral pad of adhesive mucilage (Chamberlain, 1976; Jones *et al.*, 1983; see p.108). *Synedra affinis* secretes adhesive material through pore fields situated in the corner of the valves (Jones *et al.*, 1983).

Group 2 - Formation of mucilaginous stalks.

Achnanthes subsessilis cells produce either simple or compound branched stalks which attach them to substrata. The stalk extends from the apical region of the raphe valve (The raphe is an opening in the lower diatom valve from which cytoplasmic or adhesive contents can pass). The stalk material is believed to be secreted from Golgi-derived vesicles observed near the raphe opening (Blunn and Evans, 1981). A cup-shaped collar is found at the apical region. The stalk spreads out over the substratum at the point of attachment. *Achnanthes subsessilis* frequently grows on antifouling paint surfaces, and the stalk raises the cell above the toxic boundary layer (Blunn and Evans, 1981; see Chapter 12). *Licmophora flabellata* also produces a mucilaginous stalk which attaches to the substratum by a basal mucilage pad. This pad consists of an irregularly perforated sheet of mucilage with a fibrous appearance. The mucilage is probably secreted through pores in the diatom frustule (Chamberlain, 1976; Jones *et al.*, 1983; see p. 108).

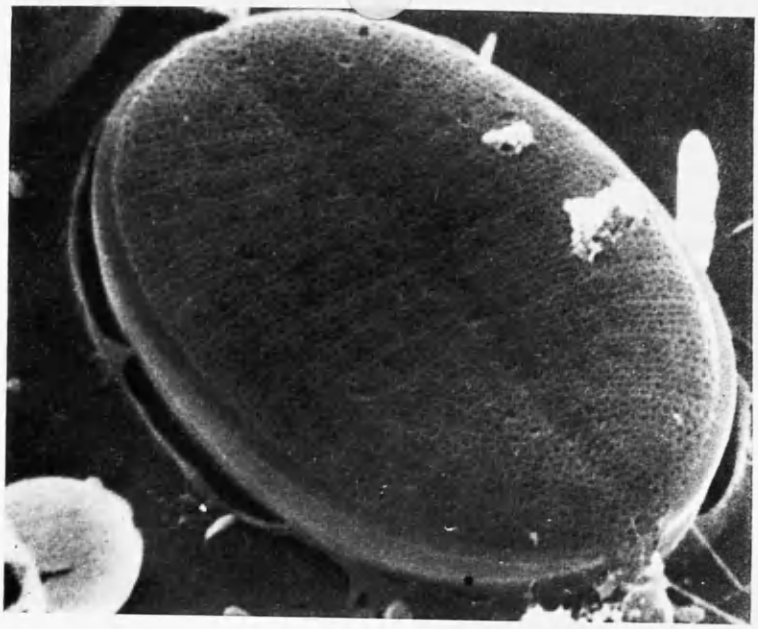

Fig. 20 *Cocconeis scutellum* with mucilaginous adhesive around the periphery of the lower valve. (Mag. x15000).

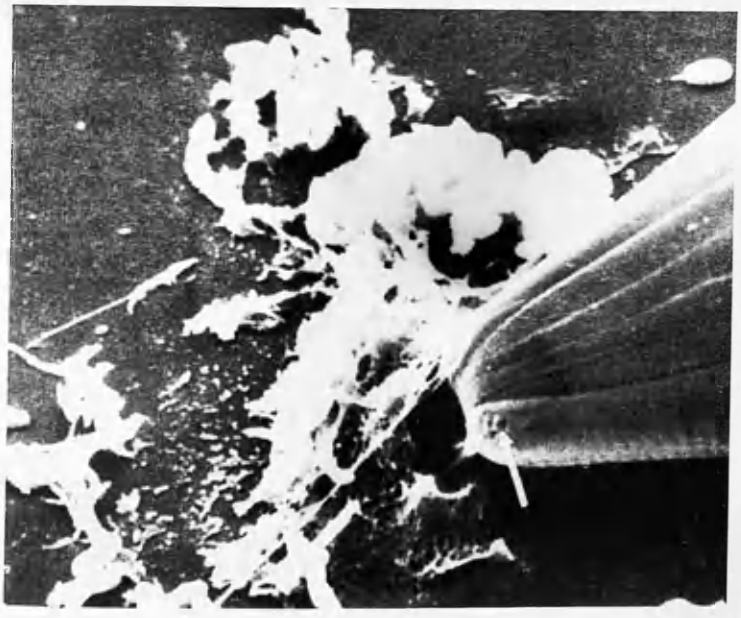

Fig. 21 *Licmophora flabellata*. The basal region with mucilage pad showing irregular perforations and frustule pores (arrowed). (Mag. x17250).

(Reproduced by permission from Chamberlain, 1976).

Group 3 - Formation of mucilaginous tubes

Diatoms such as *Navicula grevellei* and *Berkeleya rutilans* form groups of cells which are enveloped by a tube of mucilage. The colonies are usually flat and closely attached to the substratum by the mucilage (Chamberlain, 1976; Jones *et al.*, 1983).

Group 4 - Mucilage production from single cells

Amphora spp., particularly *Amphora veneta*, colonize submerged metal test panels coated with copper oxide antifouling paint (Daniel *et al.*, 1980; see also Chapter 12). The diatoms attach to the test panels by the production of copious amonts of mucilage, which unites the cells into a cohesive mass. Observations of attached *Amphora veneta* cells by scanning electron microscopy suggests that the adhesive mucilage is produced from the lower diatom valve, particularly the polar regions. Detached *Amphora veneta* cells leave a characteristic "footprint" pattern of mucilage complementing the underside of the cell (Daniel *et al.*, 1980; see p. 110). Distinct lines of adhesive, indicative of the position of the raphes, are located along the longitudinal axis of the adhesive "footprint" (see p.110). These observations suggest that mucilage secretion is associated with the polar, raphe and valve margin areas of the cells. Transmission electron microscopic studies have indicated the presence of large numbers of vesicles in close proximity to the raphe fissures. Fibrous material is often observed extruding from these fissures, and is believed to be the mucilaginous adhesive of *Amphora veneta* (Daniel *et al.*, 1980). The adhesive material may be produced from these vesicles. Further autoradiographic studies of the passage of the adhesive or cytochemical staining of the vesicles and secreted material could show this.

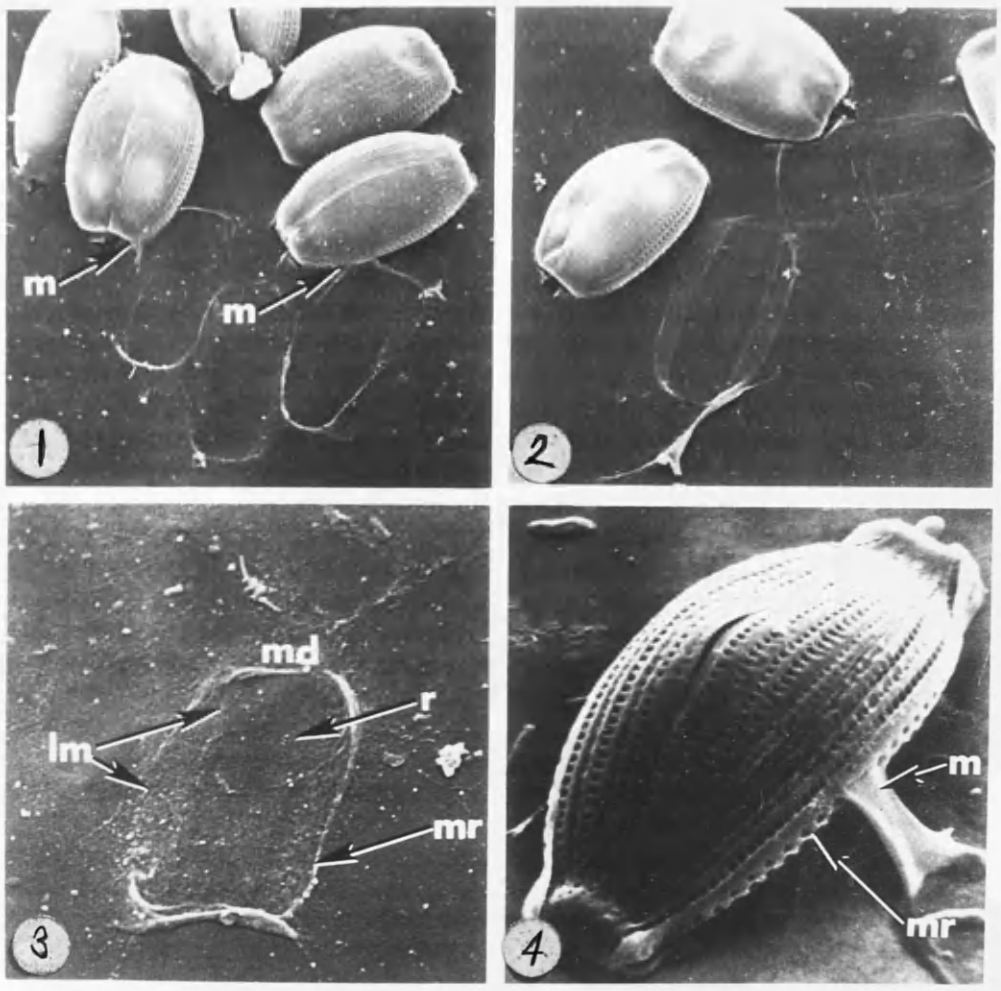

Fig. 22 S.E.M. of cultured *Amphora cells* on "Thermanox" coverslips.

22.1 Recently attached cells. Mucilage (m) is located around the peripheral margins of the cells. (mag. x2000).

22.2 Showing cells and respective mucilaginous moulds, complementing the underside of the cell. (Mag. x2000).

22.3 An adhesive 'mould' (md) remaining after removal of the cell. The mould is characterised by distinct lines of mucilage (lm) in the region of the raphes (r) and margin (mr). (Mag. x3360).

22.4 Shows the sub-lunate shape of the cell and adhesive material (m) extruding from the margin (m). (Mag. x3360).

(Reproduced by permission from Daniel *et al*, 1980).

(3.2.2). Cytochemistry of diatom adhesives

Early work suggested that diatom adhesive mucilage was "pectoid" in nature (Jones *et al.*, 1983). The capsule surrounding *Navicula pelliculosa* cells was shown to be a polyuronide, consisting solely of glucuronic acid (Lewin, 1955). More recent studies have shown that diatom extracellular mucilage consists of high molecular weight polysaccharides, often with protein and sulphated substituents (Jones *et al.*, 1983; Daniel *et al.*, 1987). Examples of the cytochemistry of diatom adhesives from the four main groups will now be discussed.

Group 1

The mucilage produced in the pad of *Cocconeis scutellum* consists predominantly of carboxylated polysaccharide with little sulphate. However, the adhesives of *Grammatophora marina* and *Synedra affinis* contain larger amounts of sulphate with smaller quantities of carboxyl components (Daniel *et al.*, 1987).

Group 2

The stalk produced by *Achnanthes longipes* consists of anionic polysaccharides with a high concentration of uronic acids in the peripheral layers and a sulphated core (Daniel *et al.*, 1987). The adhesive stalk of *Achnanthes subsessilis* has a similar composition (Blunn and Evans, 1981). *Licmophora flabellata* stalks also consist largely of anionic polysaccharides. Sulphated polysaccharide was also found in longitudinal striations of the stalk. The attachment pad formed when the stalks contact the substratum consists of carboxylated polysaccharides in both *A. longipes* and *L. flabellata* (Daniel *et al.*, 1987).

Group 3

The mucilage tubes of *Navicula delognei* consist predominantly of sulphated polysaccharides with small amounts of carboxyl groups. Intact tubes of *Berkeleya rutilans* also contain large amounts of sulphated polysaccharide with some protein (Daniel *et al.*, 1987). These findings confirm the observations of Lewin (1958), who found a protein content of 30% in the mucilage tubes.

Group 4

The mucilaginous adhesive surrounding cells of *Amphora veneta* also consists of a carboxylated polysaccharide with uronic acid and sulphate substituents. No protein was detected in the mucilage (Daniel *et al.*, 1980). The adhesive composition of *Amphora coffeaeformis* and *Amphora turgida* is identical to *A. veneta.* In addition, application of the Calcium Red stain to the adhesive of *A. veneta* shows a weakly positive reaction. This suggests that calcium is present in the mucilage (Daniel *et al.*, 1987).

These observations suggest that diatom adhesives are very similar to marine bacterial extracellular polymers, in consisting largely of acidic polysaccharides (see Chapter 1.2). Differences exist between the composition of diatom adhesive mucilages and thos of macroalgal spores, which are mostly protein-polysaccharide complexes (Daniel *et al.*, 1987). However, the attachment mechanisms are similar in some cases. The presence of vesicles which may secrete the mucilaginous stalk of *Achnanthes subsessilis* or the enveloping mucilage of *Amphora veneta* show similarities with algal spore adhesion.

Table 7 (p. 114) summarises the attachment mechanisms of some diatoms

from the four groups, together with their adhesive compositions.

(3.2.3). Other aspects of diatom attachment

Studies have been made of the attachment strengths of certain diatoms using a radial flow growth chamber (Pyne *et al.*, 1984). In this apparatus, test perspex discs on which the particular diatoms have been grown are subject to water flow under different conditions of shear stress. Water is pumped from a reservoir through an inlet pipe and flows radially across the test disc between two parallel metallic discs. At constant flow, the water velocity and surface shear stress decreases radially from the centre of the test disc (Milne and Callow, 1985). Consequently, the radius of a zone of cell detaachment on the test disc is used as a measure of the strength of cell attachment. The diatoms used in this study were *Licmophora flabellata, Achnanthes longipes* and *Amphora coffeaeformis.* They were allowed to settle on the perspex discs for varying periods from 1 hr. to 120 hrs. There was a general increase in the force needed to detach all three diatom species with an increase in settlement time. After 120 hrs. incubation, *Achnanthes longipes* cells on the outer part of the perspex disc were attached by a stalk and pad of mucilage. However, only cells with a stalk were attached in the inner region of the disc after this time (Pyne *et al.*, 1984). Similar observations were made for the stalked diatom *Licmophora flabellata.* Both diatoms remained attached at a high shear stress of $4N/m^2$ after 120 hrs. *Amphora coffeaeformis,* which produces copious amounts of mucilage, did not attach so strongly. After 8 hrs. incubation, the diatom remained attached at a shear stress of 2.9 N/m^2, but a monolayer of cells was easily removed after 120 hrs. incubation. These results suggest that strength of diatom attachment is interspecific and dependent on the attachment method. Stalked diatoms,

TABLE 7

DIATOM ATTACHMENT MECHANISMS

AND CYTOCHEMISTRY OF MUCILAGE

SPECIES	ATTACHMENT MECHANISM.	HISTOCHEMICAL NATURE OF MUCILAGE	REFERENCES
Cocconeis scutellum	Production of basal mucilage pad.	Carboxylated polysaccharide.	Chamberlain (1976); Daniel *et al.*(1987)
Achnanthes subsessilis	Production of extracellular, mucilaginous stalk.	Anionic polysaccharide with uronic acids in peripheral region and sulphated core.	Blunn & Evans (1981)
Licmophora flabellata	Production of stalk.	Anionic polysaccharide with substituents.	Chamberlain (1976); Daniel *et al.*(1987)
Berkeleya rutilans	Diatom colonies surrounded by mucilage tube.	Sulphated polysaccharide with protein.	Lewin (1958); Daniel *et al.*(1987)
Navicula delognei	Formation of mucilage tube.	Carboxylated, sulphated polysaccharide.	Daniel *et al.*(1987)
Amphora veneta	Encapsulating mucilage produced from raphe fissures.	Carboxylated, polysaccharide with sulphate and uronic acid substituents.	Daniel *et al.*(1980)

such as *Achnanthes longipes* and *L. flabellata*, attach more strongly at high shear stresses than mucilage-producing *Amphora coffeaeformis*. This could result from the greater flexibility of the stalks, allowing the diatoms to be orientated with the water flow and reduce drag (Pyne *et al.*, 1984). The attachment strength of spores of *Gracilariopsis sjoestedtii* is lower than these diatoms. The spores of this alga were found to resist shear stresses of $2N/m^2$ after 24 hours settlement (Charters *et al.*, 1973). However, Jones *et al.* (1983) found that greater shear stresses were required to remove *Ceramium rubrum* spores after 60 hrs. settlement than the above three diatoms. It would be useful to carry out further experimental work with other species of diatoms using the radial flow growth chamber. This work would further show whether diatom attachment strength is related to mode of adhesion, and whether shear stress could be an effective microfouling control method (see Chapter 12).

Adhesion of *Amphora coffeaeformis* to glass surfaces increased in the presence of calcium ions (Ca^{2+}) (Cooksey, 1981; Cooksey *et al.*, 1984). Strontium ions (Sr^{2+}) also promoted adhesion, although a higher concentration of strontium was required. This suggests that calcium is necessary for adhesion of *A. coffeaeformis*, an observation which is further suggested by the Calcium Red staining of *Amphora* spp. mucilage (Daniel *et al.*, 1987). Calcium could stabilize the structure of the acidic polysaccharide mucilage. However, it is not properly known whether calcium acts inside or outside the diatom. Adhesion of *A. coffeaeformis* was inhibited by cycloheximide and by the compounds CCCP (carbonyl cyanide 3-chlorophenyl hydrazone) and D-600. Cycloheximide inhibits protein synthesis and CCCP inhibits both photosynthesis and oxidative phosphorylation, thereby preventing ATP synthesis. Consequently, adhesion

of *Amphora coffeaeformis* must be an active process, requiring metabolic activity. Inhibition of protein synthesis by cycloheximide is unlikely to be important in preventing adhesion, as there is no protein in the diatom's adhesive mucilage. However, cycloheximide may interfere with calcium membrane transport. The drug D-600 inhibits *A. coffeaeformis* adhesion in this way (Cooksey, 1981; Cooksey *et al.*, 1984).

The use of metabolic inhibitors or compounds which prevent calcium transport could provide a means of preventing fouling by *Amphora* spp. Additionally, the use of calcium chelating agents which could remove a diatom fouling film could provide a further anti-fouling method (Cooksey, 1981; Cooksey *et al.*, 1984; see Chapter 12).

(3.3). Summary of Chapter

The main aspects of macroalgal and diatom adhesion discussed in this chapter, together with the key cited references, are as follows.

1) Permanent attachment of spores from the three main algal families occurs by a similar mechanism. This involves the secretion of adhesive material by reverse pinocytosis from vesicles which are derived from the Golgi apparatus (Evans and Christie, 1970; Baker and Evans, 1973; Chamberlain and Evans, 1981).
2) The extracellular adhesive of spores of *Enteromorpha intestinalis, Ulva mutabilis* and *Ceramium rubrum* has a polysaccharide-protein composition (Evans and Christie, 1970; Bråten, 1975; Chamberlain and Evans, 1981). *Ectocarpus* spp. spores produce a mainly polysaccharide adhesive (Baker and Evans, 1973).

3) Algal spores attach with great strength to substrata. This is shown by spores of *Gracilariopsis sjoestedtii* withstanding shear forces 100 times their weight (Charters *et al.*, 1973) and 80% of *Ceramium rubrum* spores withstanding shear stress after 9 hrs. settlement (Jones *et al.*, 1983).

4) Primary rhizoids of algae have adhesive properties. Some algae, such as *Blidingia marginata, Enteromorpha intestinalis* and *Polysiphonia* spp. form basal, mucilaginous discs allowing attachment (Fletcher, 1976). Other species, such as *Ulothrix flacca, Ectocarpus fasciculatus* and *Giffordia granulosa* produce filamentous rhizoids which are attached by mucilage flowing over the substrate. The adhesive is produced from the rhizoid tip in these species (Fletcher, 1976).

5) (i) Adhesion of unicellular *Chlorella vulgaris* to glass surfaces was particularly enhanced by adhesive polymer of *Pseudomonas atlantica* and seawater-insoluble material isolated from submerged wooden panels (Tosteson and Corpe, 1975). This suggests that polymeric materials condition a surface for algal attachment as occurs in marine bacterial attachment.

(ii) Periphytic marine bacterial films also appear to precondition surfaces prior to colonization by marine algae. Some marine bacteria encouraged settlement of *Enteromorpha* spp. germlings, whilst other bacteria discouraged this (Thomas and Allsopp, 1983).

6) Surface free energy of substrata influences attachment of algal rhizoids. Silane-coated glass slides with surface free energies ranging from 20 to over 70mN/m affected attachment of *E. intestinalis, Giffordia granulosa, Bangia atropurpurea* and *Polysiphonia* spp.

rhizoids. Filamentous rhizoids which formed on low-energy surfaces were weakly attached, whilst discoid attachment structures formed on high-energy surfaces were more strongly attached. These observations suggest that surface free energy properties of substrata could be developed as an anti-fouling technique (Fletcher and Baier, 1984; Fletcher *et al.*, 1984).

7) Diatoms attach to surfaces by the production of mucilage which is morphologically elaborated to form attachment structures. These are basal adhesive pads, mucilaginous stalks and tubes, and mucilage which encapsulates single cells (Chamberlain, 1976; Daniel *et al.*, 1983).

8) The adhesive material of most diatoms consists of acidic polysaccharide with sulphate and uronic acid substituents. Diatom adhesives are similar in composition to extracellular polymers of marine bacteria (Jones *et al.*, 1983; Daniel *et al.*, 1987).

9) (i) The strength of diatom attachment, as measured by the radial flow growth chamber, is dependent on the attachment method. Stalked diatoms, such as *Achnanthes longipes* and *Licmophora flabellata* attach more strongly after 120 hrs. than mucilage-producing *Amphora coffeaeformis* (Pyne *et al.*, 1984).

(ii) Calcium increases adhesion of *Amphora coffeaeformis* to glass. Cycloheximide and the metabolic inhibitors CCCP and D-600 inhibit adhesion of this diatom. The use of metabolic inhibitors or compounds which interfere with calcium transport could provide an anti-fouling method for *Amphora* spp. (Cooksey, 1981).

Chapter 4 - Adhesion mechanisms in marine fungi

Marine fungi are frequently found as part of the primary slime film which forms on surfaces immersed in seawater. They play an important role in the biodeterioration of wood materials in seawater.

Successful adhesion of the fungal spore to the wood surface is an important initial stage in the biodeterioration process. Firm adhesion allows spore germination which results in hyphal and mycelial formation. This will ultimately lead to fungal penetration of the wood and production of substances which will degrade the wood structure. This chapter discusses in detail the methods which some marine fungi have adopted to allow adhesion. There is also a discussion of possible anchoring mechanisms in thraustochytrids, marine coccoid fungi.

Many species of marine fungi, classified within the families of Ascomycetes, Basidiomycetes and Fungi Imperfecti, possess spores with appendages. These appendages may have several functions. (Jones, 1972; Kohlmeyer and Kohlmeyer, 1979).

(1) They may enlarge the spore surface, so minimizing the settling rate in seawater which helps to keep the spores afloat;

(2) They may help spores to catch unorganised eddy diffusion currents;

(3) They may help to entangle and attach the spores to suitable substrates.

Spore attachment appears to be a likely function of these appendages. Certain aquatic fungi produce tetraradiate spores which have four appendages. A tetraradiate shape is known to increase the impaction efficiency of aquatic fungal spores (Kohlmeyer and

Kohlmeyer, 1979). Some appendages may only be involved in the initial stages of spore adhesion, while others allow firmer attachment. This will be further discussed later in the chapter.

The Ascomycetes, particularly the family Halosphaeriaceae show the greatest variability of appendages. Their ascospore appendages are thorn-, spine-, cap- or fibrelike, and have a tough, gelatinous or mucilaginous nature (Kohlmeyer and Kohlmeyer, 1979). Consequently, the appendages are dry or sticky, which enhances their ability to attach to substrata. Ascospore appendages develop in different ways. Before discussing this, however, it is necessary to define the terms endospore, mesospore and epispore. These are the three inner layers of the fungal spore, the epispore surrounding the mesospore which in turn surrounds the endospore (Kirk, 1976; see p. 123). An exosporic layer usually surrounds the epispore. In some fungal species a mucilaginous sheath derived from the epispore surrounds the ascospore and appendages (Kirk, 1976; Kohlmeyer and Kohlmeyer, 1979). The endospore is usually only prominent during germination, when it is continuous with the germ tube (Kirk, 1976).

Ascospore appendages have been studied cytochemically in some marine ascomycetes. They are usually chitinized membranous or non-chitinized, mucilaginous processes and are exosporic (Kirk, 1976; Kohlmeyer and Kohlmeyer, 1979). The presence of chitin would make the appendages rigid and tough. Further details of the cytochemistry of ascospore appendages of specific Ascomycetes will be given later.

Fungal spore appendages may serve to enhance the attachment response of the spore by increasing the surface area available for physicochemical

forces (Rees and Jones, 1984). The physicochemical factors which are involved are similar to those in the initial stages of bacterial adhesion (see Chapter 1.1). They include forces of electrical attraction and repulsion, chemical forces, and physical forces such as London-van der Waals forces. Charges of the substratum and spore and surface energy of the substratum are also important factors (for a definition of surface free energy, see Chapter 1.5). The physicochemical forces will act regardless of the "stickiness" of the appendages (Rees and Jones, 1984).

In some fungal species, spore adhesion resembles an active process (see Chapter 1.1). This is because attachment consists of two phases, a reversible phase followed by an irreversible phase of firm attachment resulting from mucilage production from appendages. However, there are few further details about the physicochemical factors involved in spore adhesion in specific marine fungi. The initial stages of spore adhesion as described by Rees and Jones (1984), however, appear to resemble those in bacterial adhesion (see Chapter 1.1) and algal spore adhesion (see Chapter 3).

The next part of this chapter will discuss the types of appendages produced by spores of several marine fungal spores, and their roles in adhesion. The fungi described are mainly Ascomycetes.

(4.1). Spore appendage types of marine fungi and their roles in adhesion.

Johnson (1980) divides marine Ascomycetes into three groups, according to their spore appendage morphology and method of formation :-

Group (1.) Appendages formed by direct outgrowth or exudation from an ascospore wall;

Group (2.) Appendages formed by fragmentation or elaboration of an outer wall layer of the ascospore;

Group (3.) Appendages formed by a combination of methods in groups (1) and (2).

These groupings will be used to discuss representative marine fungal species possessing these spore appendage types. The formation and morphology of the spore appendages will be discussed in detail. This is considered necessary in order to emphasise the possible role that the appendages may play in spore adhesion.

Group (1) includes Ascomycete species in the genera *Torpedospora* and *Halosphaeria*. *Amylocarpus encephaloides* possesses ascospores with 10-20 spine-like appendages radiating from the surface (Jones and Moss, 1978; Johnson, 1980; see p. 123). These appendages arise as extensions of the mesospore. There were no observations of slime production from these appendages (Jones and Moss, 1978). These appendages, therefore, may not be involved in firm attachment of *A. encephaloides* spores. They may, however, be involved in initial entrapment of the spores to a substrate. *Halosphaeria appendiculata* possesses four equatorial and two polar appendages (Jones and Moss, 1978; Johnson, 1980). ('Polar' appendages are

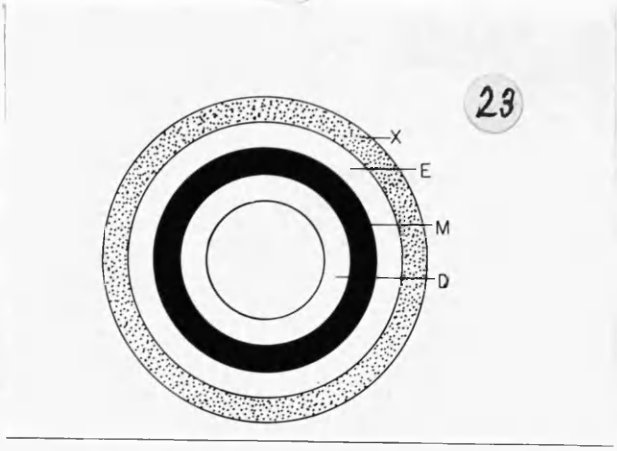

Fig. 23 Schematic representation of laminated wall of germinating ascospore. D: endospore; E: epispore; M: mesospore; X: exosporic layer.

(Reproduced by permission from Kirk, 1976)

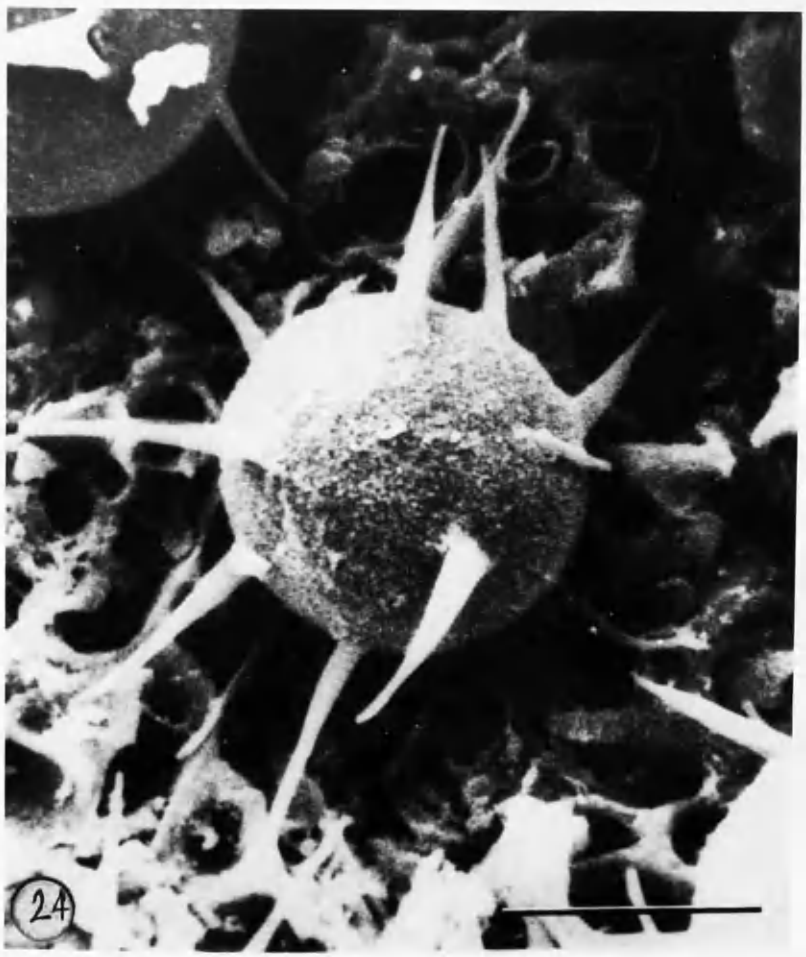

Fig. 24 Unicellular ascospore of *Amylocarpus encephaloides* on wood, with many spine-like radiating appendages. Bar represents 5μm.

(Reproduced by permission from Jones and Moss, 1978)

those structures found at the 'top' and 'bottom' of a spore as viewed in electron micrographs. 'Equatorial' appendages are found in the central part of the spore). These appendages appear to have a spoon-shaped structure, and seem sticky when viewed under the light microscope (Rees and Jones, 1984). The spore appendages of *H. appendiculata* possess chitin, which makes them rigid (Kirk, 1976). This rigid nature of the appendages, together with their stickiness, probably allows firm attachment of *H. appendiculata* spores. The spore appendages of *Halosphaeria trullifera* differ from *H. appendiculata* in structure and development. The appendages are initially adpressed to the spore wall, and are composed of an amorphous material. However, upon exposure to seawater, the appendages uncoil to form long, thread-like polar appendages (Jones *et al.*, 1983). Spores of *H. trullifera* do not possess equatorial appendages, whilst these are present on *H. appendiculata* spores. A further difference arises in the thread-like nature of *H. trullifera* spore appendages, compared with the spoon-shaped appendages of *H. appendiculata.* However, the long, thread-like and tightly coiled nature of *Halosphaeria trullifera* ascospore appendages probably aids attachment to substrata.

Cucullospora mangrovei is a new species isolated from mangrove wood in the Seychelles (Hyde and Jones, 1986). The ascospore appendages of this species are initially composed of tightly coiled filaments enclosed within a membrane. On exposure to seawater these appendages uncoil to form sticky polar filamentous threads (Hyde and Jones, 1986). These are similar to the spore appendages of *Halosphaeria trullifera* (Jones *et al.*, 1983). The long, sticky nature of the *Cucullospora mangrovei* spore appendages probably aids their entrapment and firm attachment to wood surfaces.

Torpedospora radiata possesses three spine-like, radiating appendages at the ascospore apex (Jones and Moss, 1978; Johnson, 1980). The appendages are composed of numerous, electron-dense fibrils which can be seen by transmission electron microscopy (Johnson, 1980). As these appendages swell in seawater, they become more fibrillar, developing a "tuft-like" appearance (Jones and Moss, 1978; see p.126). The fibrillar nature of the appendages increases the surface area available for contact with a substratum. This probably ensures attachment of *T. radiata* spores. Spore appendages in a wood isolate of *Torpedospora ambispinosa* were quite different from *T. radiata* (Jones, 1985). *T. ambispinosa* possesses subterminal, radiating appendages at either end of the spore. Spore appendages in this species are rigid, straight or curved, and do not seem to be fibrillar as in *Torpedospora radiata* (Jones, 1985). The rigid nature of the spore appendages of *T. ambispinosa* probably allows their involvement in the initial stages of spore entrapment.

Group (2.). Appendages formed by fragmentation of an outer ascospore wall layer.

The sheath surrounding ascospores of *Halosphaeria mediosetigera* undergoes fragmentation to form three equatorial appendages and two small, apical, cap-like appendages (Moss and Jones, 1977; Jones and Moss, 1978; Johnson, 1980). The equatorial appendages are initially spirally arranged around the central septum of the ascospore. The apical appendages are formed by inversion of the sheath remnants (Moss and Jones, 1977; Jones and Moss, 1978). Both polar and equatorial appendages are smooth and do not become fibrillar. There is no microscopic evidence of mucilage production

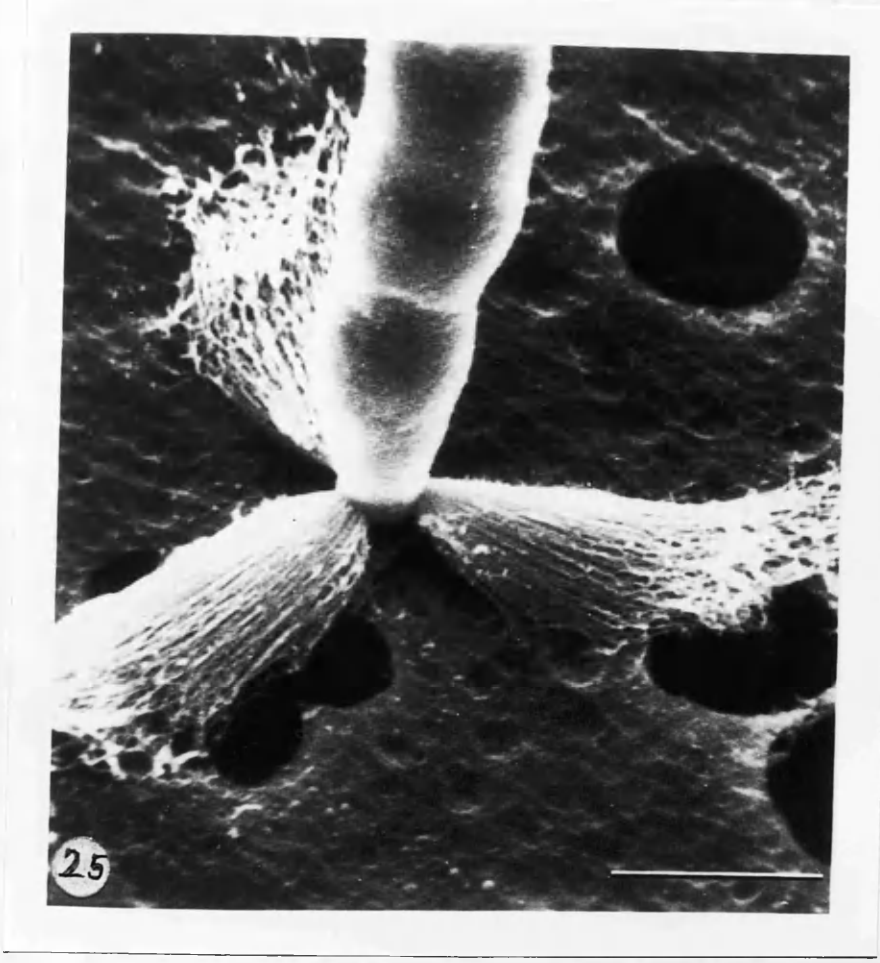

Fig. 25 Ascospore of *Torpedospora radiata* showing polar appendages which become fibrillar with age.
Bar represents 5μm.

(Reproduced by permission from Jones and Moss, 1978)

and stickiness in the spore appendages of *H. mediosetigera*. However, the presence of several equatorial and polar appendages on *H. mediosetigera* spores probably ensures their initial entrapment.

Ascospore appendages of *Corollospora comata* are formed in a similar way. Two sets of appendages develop from the sheath. Polar, hair-like, flexuous appendages are formed by fibrillation of the sheath surrounding the two apical cells of the spore (Jones and Moss, 1978; Johnson, 1980). Similar tuft-like equatorial appendages develop from the sheath surrounding the two central spore cells. Appendage fibres are electron-dense structures. The equatorial appendage tufts are themselves frequently bordered by electron-dense material (Johnson, 1980). This may be mucilaginous material produced by the equatorial appendages which would allow firmer spore attachment. The tuft-like nature of the *Corollospora comata* spore appendages is similar to those of *Torpedospora radiata* (Jones and Moss, 1978).

Five *Remispora* species possess spore appendages which are very similar. The appendages in all four species are composed of an amorphous, electron-transparent and a fibrous, electron-dense component (Johnson, 1980). *Remispora maritima* spores possess wing-like polar appendages which appear to attach spores firmly to substrata (Jones and Moss, 1978; Rees and Jones, 1984; see p.128). *Remispora pilleata* possesses similar wing-like polar appendages at either end of the spore (Jones and Moss, 1978). These polar appendages form an extensive attachment zone along the length of the spore (Rees and Jones, 1984). A *Remispora "hamata"* -type fungus (a previously undescribed species similar in morphology to *R. hamata*) possesses tufts of fibrillar material at either end of the spores.

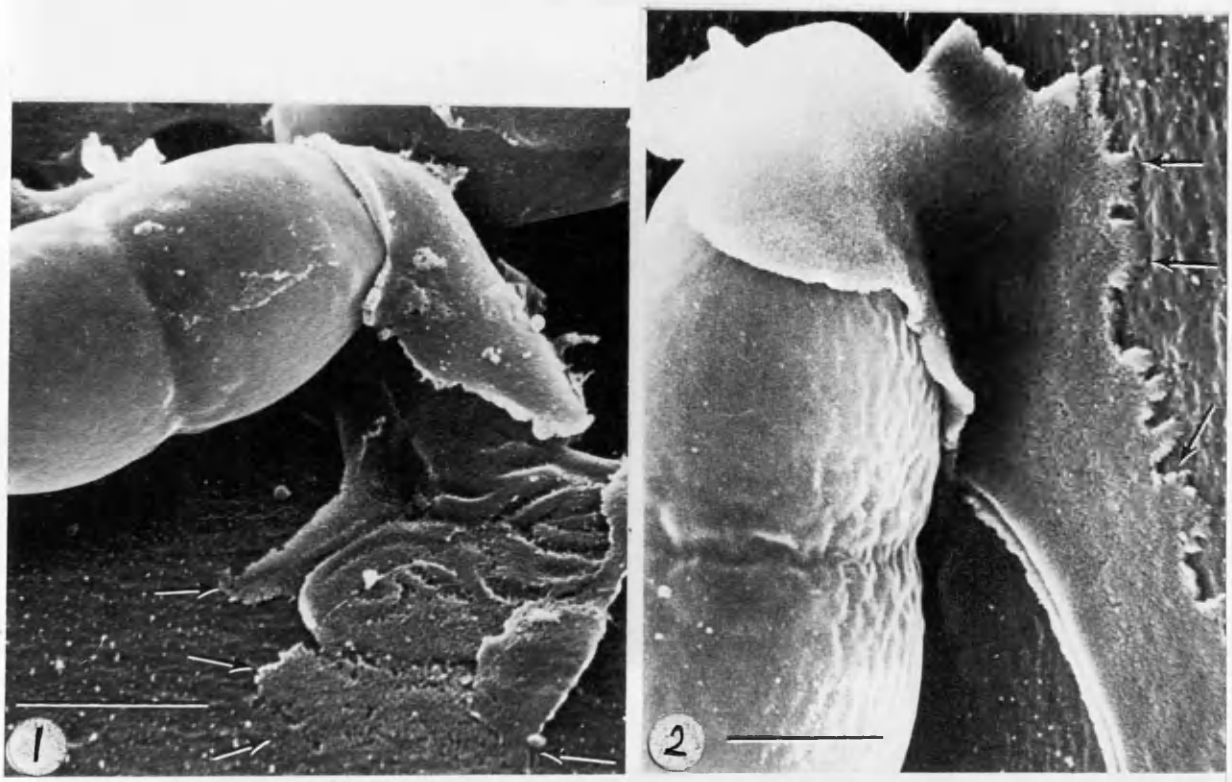

Fig. 26 (1 and 2)

Ascospore of *Remispora maritima* showing adhesion of polar appendage to the surface (arrowed).

Bar lines: In fig. 26.1 represent 10μm;
In fig. 26.2 represent 5μm.

(Reproduced by permission from Rees and Jones, 1984)

These tufts allow attachment of the spores to substrata (Rees and Jones, 1984). *Remispora galerita*, a more recently isolated species, also possesses polar appendages formed from a fragmented sheath. These appendages are composed of fine fibrillar elements which appear cobweb-like in electron micrographs (Jones, 1985). As with the other *Remispora* species, the fibrillar nature of the polar appendages in *R. galerita* probably allows firm spore attachment.

Haligena salina, a new species isolated from wood in the U.S.A., possesses spore appendages which show similarities and differences to those of *Remispora maritima* (Farrani and Jones, 1986). As with *R. maritima*, *Haligena salina* spores possess polar appendages which are amorphous in nature, and appear to secure spore attachment (Farrani and Jones, 1986; see p. 130). However, these appendages develop as outgrowths of the ascospore wall, and not by fragmentation of a sheath as occurs in *Remispora maritima*. In addition, although the spore appendages of *Haligena salina* appear to have a fibrillar structure (see Fig.27.3, p. 130), the fibrillar material appears unorganized compared to the spore appendages of *R. maritima* (Farrani and Jones, 1986).

Groenhiella bivestia, isolated from wood buried in sand dunes, possesses ascospores with polar and equatorial appendages (Koch *et al.*, 1983). The appendages are amorphous in appearance, and are similar to those of *Remispora* spp. However, spores of *Remispora* spp. lack equatorial appendages.

Carbosphaerella leptosphaeroides ascospores are initially surrounded by a reticulate, net-like sheath. This sheath fragments to form long, flexuous, fibrillar strands which surround the spore (Jones and Moss, 1978;

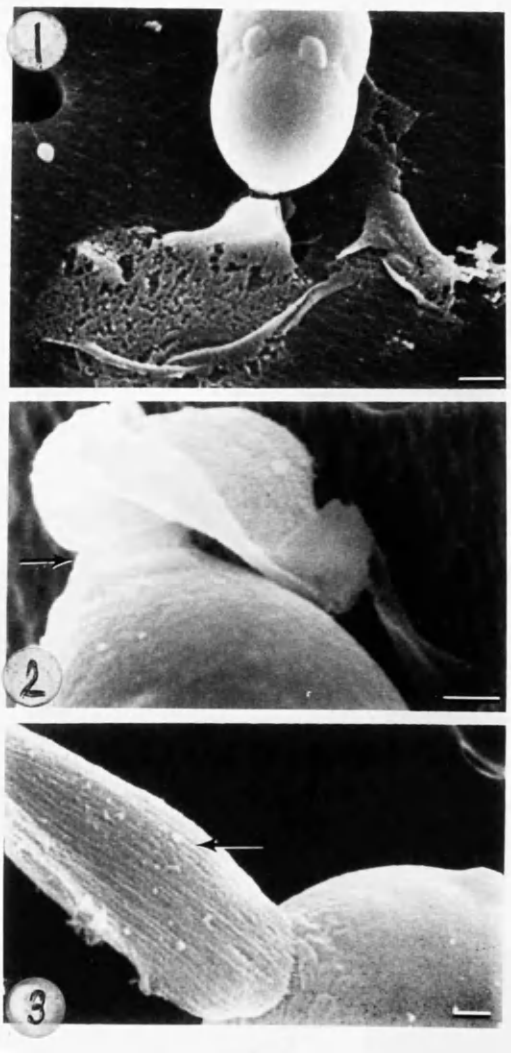

Fig 27.1 Ascospore of *Haligena salina* showing amorphous nature of polar appendage.
Bar represents 5μm.

Fig. 27.2 Appendage attachment to protrusion from *H. salina* spore (arrowed).
Bar represents 1μm.

Fig. 27.3 Appendage (arrowed), showing fibrillar structure.
Bar represents 0.5μm.

(Reproduced by permission from Farrant and Jones, 1986)

Johnson, 1980). The polar ends of the spore, however, do not become fibrillar, but retain the reticulate appearance of the sheath. Amorphous material is associated with the fibrillar appendages, which could be mucilaginous material aiding spore attachment (Johnson, 1980).

The ascospore appendages of *Crinigera maritima* develop in a similar way to those of *C. leptosphaerioides.* The sheath enveloping the ascospores of *Crinigera maritima* is composed of closely aggregated fibrillar material. When ascospores are immersed in seawater, the outer layer of the sheath swells, becomes more diffuse, and breaks up to form ball-like structures (Jones *et al.*, 1980; see p. 132). This leaves an inner layer of spirally arranged strands in the spores. These strands in turn uncoil to form a large number of radiating appendages (see p.132). These appendages frequently terminate in the ball-like structures derived from the outer sheath (Jones *et al.*, 1980; see p. 132). The ball-like structures become more fibrillar with time and appear to attach *C. maritima* spores to the substratum (Jones *et al.*, 1980; Rees and Jones, 1984, see p. 132). This development of aggregated ball-like structures from the *C. maritima* spore sheath is similar to the fibrillation of the sheath in *C. leptosphaerioides.* However, the compact, ball-like structures produced on *Crinigera maritima* spores probably secures firmer attachment than the fibrillar appendages of *C. leptosphaerioides* spores.

Spore appendages of *Halosarpheia retorquens* initially form compact, tightly coiled polar structures. These uncoil to form extremely long, thread-like polar appendages which increase the spore surface area in contact with the substratum. These appendages ensure firm attachment of *Halosarpheia retorquens* spores to substrata (Rees and Jones, 1984). The

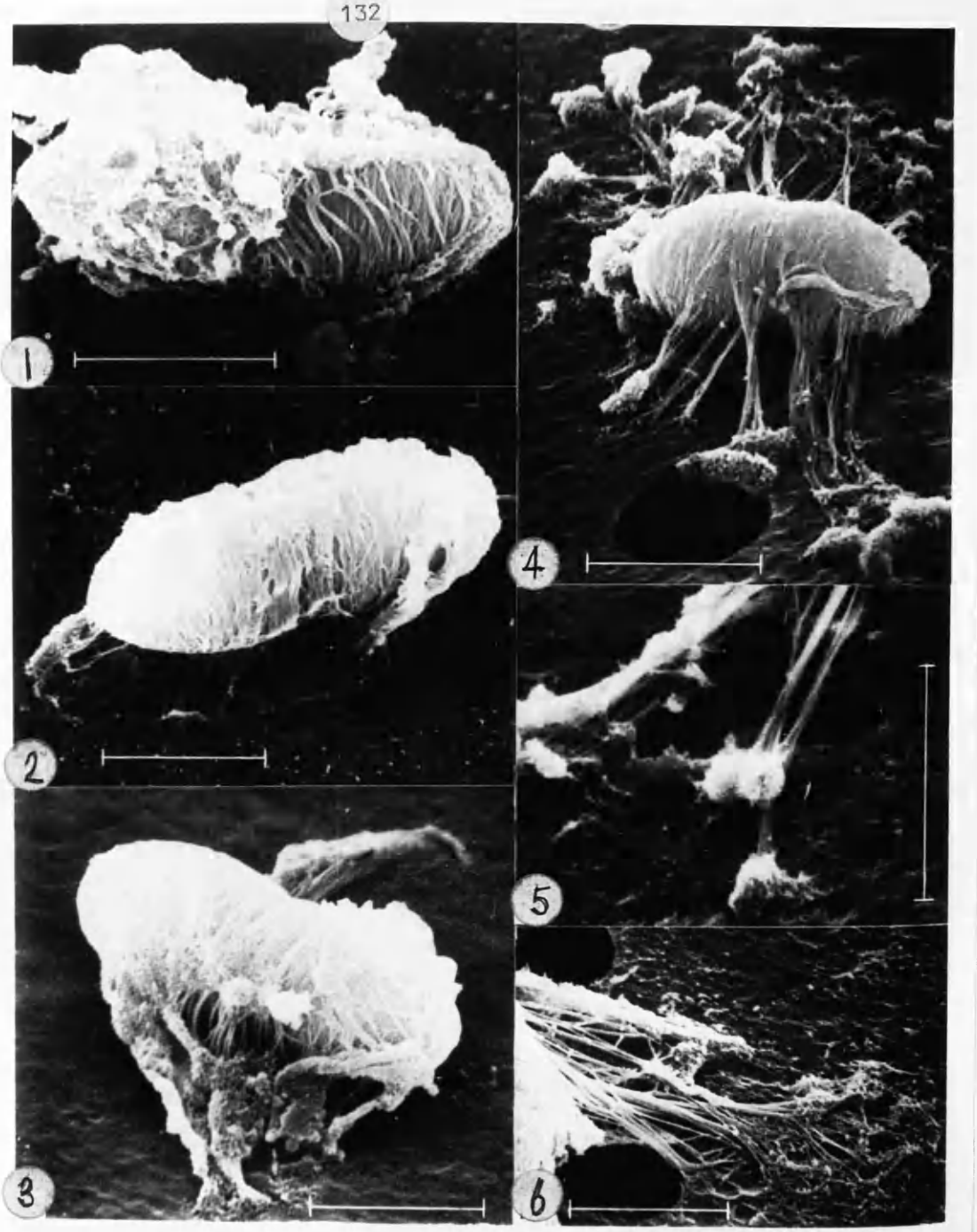

Fig. 28 SEMs of *Crinigera maritima* spores. Bar line represents 5μm in all micrographs.

Fig. 28.1-28.3
Progressive separation of the outer sheath revealing the strands of the inner layer.

Fig. 28.4 Mature ascospore showing uncoiled appendages terminating in ball-like structures.

Fig. 28.5 Terminal ball-like structures.

Fig. 28.6 Fibrillar terminal portion of the appendages derived from the ball-like structures attaching the spores to the substratum.

(Reproduced by permission from Jones *et al*, 1980)

formation of these thread-like polar appendages in *H. retorquens* spores is similar to the uncoiling of the sheath observed in *Crinigera maritima* ascospores.

Group (3.) Appendages formed by a combination of fragmentation and exudation from the ascospore wall

Ascospores of *Corollospora martima* are initially surrounded by a sheath which, at maturity, splits longitudinally from the septum region to the spore tips. The strips formed in this way recurve and detach to form paired sheet-like equatorial appendages (Jones and Moss, 1978; Johnson, 1980). A primary, spine-like polar appendage is also initially present on *C. maritima* spores. The sheath region surrounding this appendage also splits and inverts to form a trumpet-like polar appendage. These secondary appendages are smooth and do not become fibrillar (Jones and Moss, 1978; Johnson, 1980).

Appendages of spores of *Chaetosphaeria chaetosa* develop in a similar way to those of *Corollospora maritima*. Ascospores of *Ch. chaetosa* are also surrounded by a sheath within which fragmentation lines develop. Individual sheath segments peel away from the spore wall and recurve to form equatorial appendages (Jones *et al.*, 1983). Incomplete fragmentation of the sheath at the polar spore regions results in formation of cap-like appendages. There are, however, slight differences in spore appendage development between the two species. The sheath in *C. maritima* spores separates along preformed cleavage lines to form equatorial appendages. However, in *Ch. chaetosa*, the appendages are irregular in morphology and are not formed along predetermined cleavage lines. Additionally, the

polar appendages of *Chaetosphaeria chaetosa* are not spine-like as in *C. maritima* (Jones *et al.*, 1983). The presence of polar and several equatorial appendages on spores of both species probably secures initial spore entrapment. However, there is no evidence of mucilage production from the spore appendages of both fungi which would allow firm attachment.

Ascospores of *Ceriosporopsis tubulifera* are surrounded by an appendage which forms a ring around the septum and a tube containing mucilage at each end (Johnson, 1980). This appendage appears in electron micrographs, as an expanded, sponge-like sheath composed of electron-dense reticulated fibres close to the epispore. These are surrounded by a continuous electron-dense fibrous layer. At the spore apex, the reticulated appendage fibres form a fibrous collar that extends a short length along the tube (Johnson, 1980). An electron-dense, amorphous, mucilaginous material is present within this tube. This mucilage may exude from the tube, providing a means for firm attachment of *Ceriosporopsis tubulifera* spores to substrata (Johnson, 1980).

Ceriosporopsis halima ascospores are surrounded by a pair of unit membranes, the outer of which becomes the spore outer membrane, the inner becoming the spore plasmalemma (Lutley and Wilson, 1972). The entire spore is in turn surrounded by a mucilaginous sheath. Polar spore appendages develop from apical caps of the epispore and are contained within the spore outer membrane. The appendages are composed of an electron-transparent matrix supported by an electron-dense internal skeleton of fibrils (Lutley and Wilson, 1972). Both the spore sheath and apical appendages in *C. halima* appear to be composed of polysaccharides (Kirk, 1976). When the spores are released into seawater, the

mucilaginous sheath surrounding them expands and swells. The appendages stretch out but remain intact. The appendage tips become soft and sticky, and the whole appendage structures are drawn out into long tendrils. These tendrils are formed on contact with any solid object, and probably ensure firm attachment of *Ceriosporopsis halima* spores to substrata (Lutley and Wilson, 1972).

Spores of *Ceriosporopsis circumvestita* possess disc-shaped polar and equatorial appendages. These appendages differ considerably from those of *Ceriosporopsis tubulifera* and *C. halima*. However, the disc-shaped polar appendages form adhesive pads which appear to aid firm attachment of *Ceriosporopsis circumvestita* spores to substrata (Rees and Jones, 1984).

Kohlmeyeriella tubulata possesses single-celled, sigmoid-shaped ascospores. Each end of the spore extends into a tube-like spine appendage (Johnson, 1980; Rees and Jones, 1984; see p. 136). At maturity, the tip of the appendage spine produces a mucilage droplet. This mucilage droplet forms a disc or pad-like structure on contact of the spore with a surface (Rees and Jones, 1984; see p. 136). These disc-like structures probably ensure firm attachment of *K. tubulata* spores, as considerable force is required to dislodge them (Rees and Jones, 1984).

Nimbospora effusa and *Nimbospora bipolaris* are two species of filamentous Ascomycetes isolated from the Seychelles (Hyde and Jones, 1985). The ascospores of both species are surrounded by an elongated sheath. This sheath is drawn out at the spore apices, and forms an appendage in both species. In addition, *N. effusa* and *N. bipolaris* spores possess equatorial, lateral appendages at the spore septa. The equatorial appendages in *N. bipolaris* spores are longer than those of *N. effusa*.

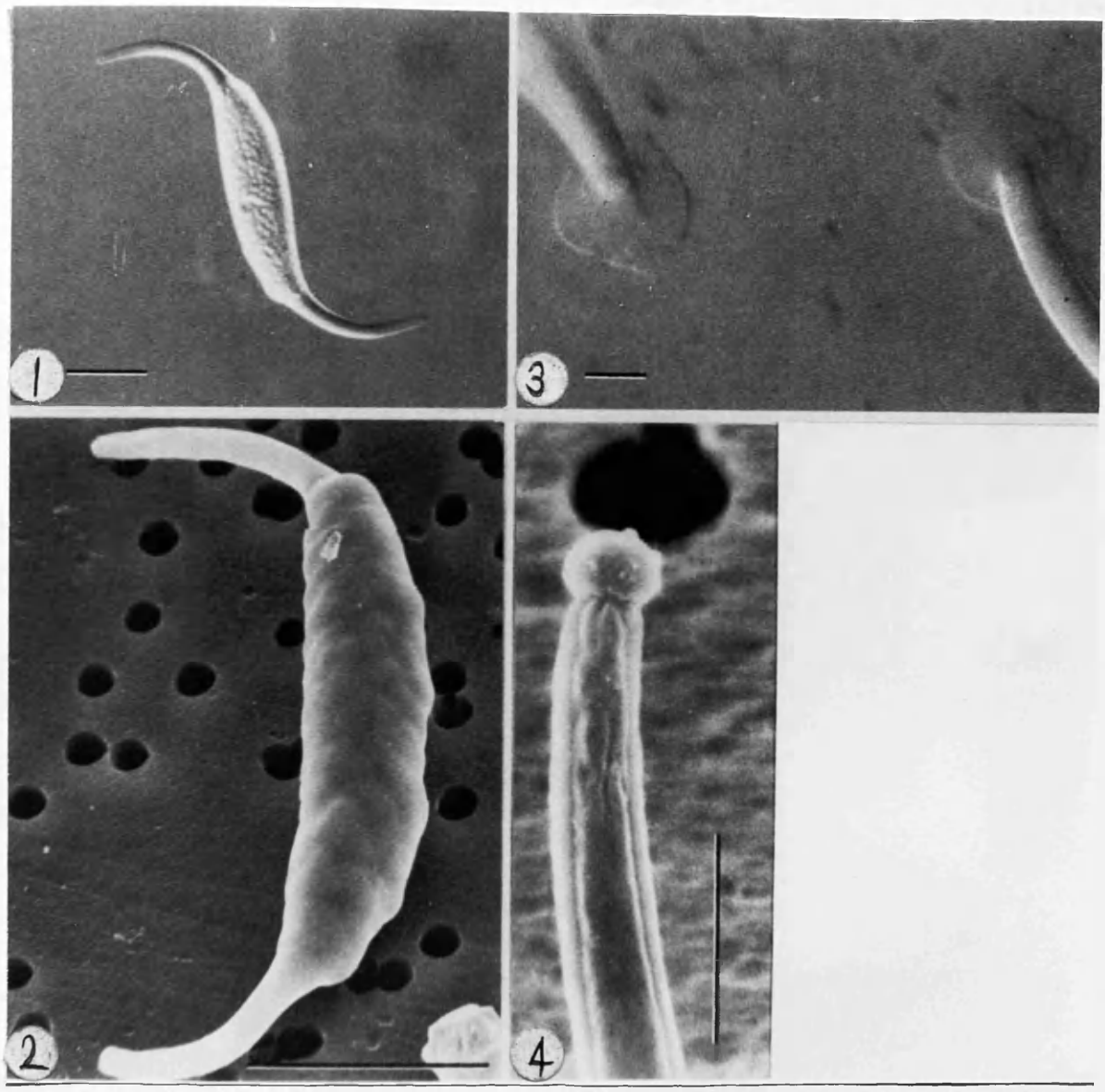

Fig. 29 *Kohlmeyeriella tubulata* spores.

Fig. 29.1 & 29.2
Light microscope and SEMs showing the primary polar appendages.
Bar lines: Fig. 29.1 represents 10μm
Fig. 29.2 represents 40μm.

Fig. 29.3 Shows attachment discs. Bar represents 2μm.

Fig. 29.4 Polar appendage with a drop of extruded mucilage.
Bar represents 5μm.

(Reproduced by permission from Rees and Jones, 1984)

These appendages become more fibrillar and mucilaginous in appearance in seawater. The outer sheath-like appendage aids firm attachment of spores of both *N. bipolaris* and *N. effusa* to substrata. The sticky equatorial appendages may also assist in adhesion of spores of both species (Hyde and Jones, 1985).

(4.2). Spore appendages of some other marine fungi.

Ascospores of *Lulworthia medusa* possess conoid polar appendages from which a drop of mucilage is released at maturity. The mucilage drop is initially surrounded by a membrane-like structure which ruptures to release the mucilage. When the spore contacts the substratum, the mucilage forms a pad-like structure (Rees and Jones, 1984; see p. 138). This pad structure provides firm attachment, and the spores anchored at both ends form a 'U'-shape. When the spores of *Lulworthia medusa* are firmly anchored, the mucilage spreads out along the substratum to form a thin adhesive layer (Rees and Jones, 1984; see p. 138). Cytochemical studies of the spore mucilage of *Lulworthia medusa* have shown it to be a mucoprotein or neutral polysaccharide resembling dextran (Kirk, 1976).

Spores of *Lulworthia purpurea* also release drops of mucilage from the end chambers. The mucilage is also surrounded by a thin membrane which ruptures when the spore contacts a substratum (Jones, 1985). As with *L. medusa*, this mucilage ensures firm attachment of *L. purpurea* spores to substrata.

The basidiomycete *Nia vibrissa* frequently causes a white rot-type decay of wood in seawater. Basidiospores of *Nia vibrissa* were ovoid or ellipsoidal with a single terminal appendage and four sub-equatorial

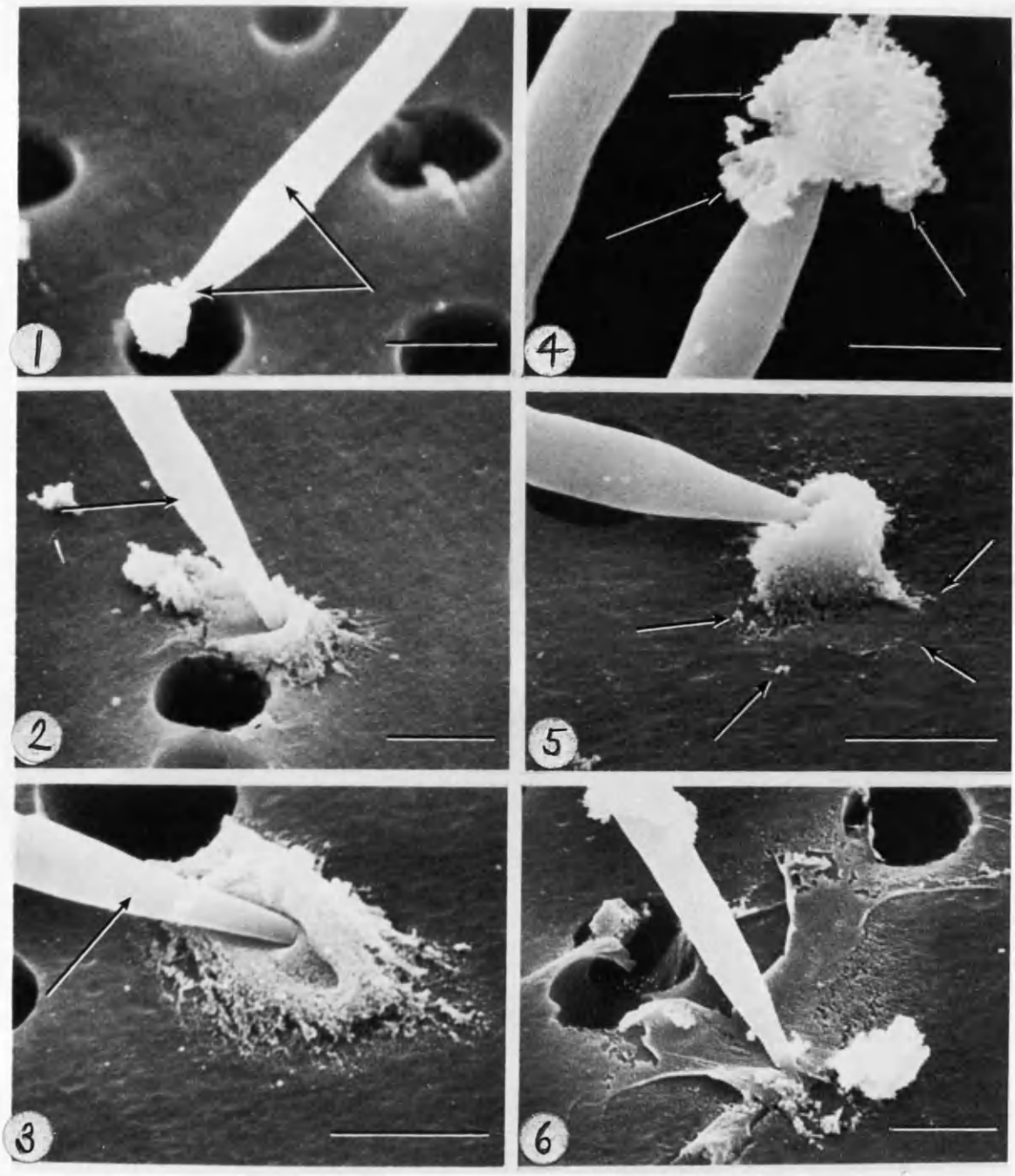

Fig. 30 SEMs of *Lulworthia medusa* spores.

Fig. 30.1 Drop of mucilage released from the conoid appendage (arrowed).

Fig. 30.2 &30.3 Ascospore attached to polycarbonate membrane by disc of mucilage. Conoid appendage arrowed.

Fig. 30.4 Drop of mucilage with enveloping membrane arrowed.

Fig. 30.5 Mucilage (arrowed), from appendage spreading out on to the polycarbonate membrane.

Fig. 30.6 Spore settled on to polycarbonate membrane for 1 hour, showing mucilage forming a thin layer over the surface.
All bar lines represent 5μm.

(Reproduced by permission from Rees and Jones, 1984)

appendages (Leightley and Eaton, 1979). The spore appendages all have slightly inflated and sticky tips. The appendages were frequently observed entangling the *Nia vibrissa* basidiospores within wood crevices (Leightley and Eaton, 1979; Rees and Jones, 1984). The inflated appendage tips probably ensure firmer attachment of basidiospores to the wood surface. Basidiospores of *Nia vibrissa* were embedded in mucilage within the basidiocarps. This mucilage probably imparts stickiness to the inflated appendage tips and ensures firm attachment of basidiospores to wood (Leightley and Eaton, 1979).

(4.3). Strength of fungal spore attachment and discussion of role of appendages in adhesion

Rees and Jones (1984) carried out quantitative studies on the strength of spore attachment in several marine fungi. Measurements were made of the percentage of spores of particular species which remained attached to wood after exposure to a water jet at a specific pressure (hosing). Spores were allowed to settle for periods of 0, 0.5, 1, 3, 6 and 24 hours before being subjected to hosing. With *Halosphaeriopsis mediosetigera*, *Remispora maritima* and *Nia vibrissa*, the number of spores remaining attached increased up to 24 hours. A similar pattern was observed with *Groenhiella bivestia* spores up to 3 hours after settlement. Following this period, there was a reduction in the number of spores of this species remaining attached (Rees and Jones, 1984). The number of *Halosphaeriopsis mediosetigera* spores remaining attached increased linearly, reaching 76% after the 24 hour period. After three hours, 45-50% of *Remispora maritima* spores remained attached. However, spores of *Amylocarpus encephaloides* did not attach so firmly. After 30 minutes, the quantity of spores

attached fell from 40% to 30%, and remained at this value until the end of the 24 hour period (Rees and Jones, 1984).

Rees and Jones' (1984) experiments have implications for the role of appendages in marine fungal spore attachment. In some species, the appendages allow firm, irreversible spore adhesion. In other species, the appendages may only be involved in initial stages of spore entrapment. This will now be discussed with reference to some of the fungal species previously described. Further experimental work which could prove whether or not appendages are involved in firm spore adhesion will also be discussed.

In the case of *Amylocarpus encephaloides* spores, the results of the hosing experiments of Rees and Jones (1984) indicate that the spore appendages may not be involved in firm spore adhesion. There is no evidence, in electron micrographs of mucilage production from the appendages which would ensure firm adhesion (Jones and Moss, 1978). The spine-like nature of the appendages of *Amylocarpus encephaloides* spores probably aids in flotation of the spores to a substrate and in their initial entrapment. Firmer adhesion of *Amylocarpus encephaloides* occurs upon production of hyphae (Rees and Jones, 1984; see p. 144). Sedimentation experiments could be carried out with *Amylocarpus encephaloides* spores to study the effect of the appendages on spore settlement. This could be done by measuring the speed of spore movement through a seawater column onto wood particles. Similar measurements could be made for *A. encephaloides* spores from which appendages were removed. The hosing method of Rees and Jones (1984) could be used to compare the strength of adhesion of appendaged and non-appendaged *A. encephaloides*

spores.

There are other fungal species for which there is little direct evidence that appendages are involved in firm spore adhesion. Hosing experiments could be used to demonstrate strength of spore attachment in these species. In each case, control experiments using spores from which appendages were removed would also be carried out. Hosing experiments could be done with the fibrillar appendaged spores of *Torpedospora radiata*. This technique could also be used for *Halosphaeria mediosetigera*, *Corollospora maritima* and *Chaetosphaeria chaetosa*.

There are also some fungal species described here in which further detailed electron microscopic work could show involvement of appendages in spore adhesion. Both scanning and transmission electron microscopic techniques could be used. Such work could be done with spores of *Corollospora comata*. Electron-dense material was associated with the equatorial appendages of *C. comata* spores (Johnson, 1980). Electron microscopic studies would show the possible involvement of the equatorial appendages, and the electron-dense material in securing spore adhesion. Similar work could be done with the fibrillar strands of *Carbosphaerella leptosphaerioides* ascospores which also have amorphous material associated with them (Jones and Moss, 1978; Johnson, 1980). Electron microscopic work could also show whether the mucilage produced from the "tube-like" appendage surrounding *Ceriosporopsis tubulifera* spores is involved in their adhesion (Johnson, 1980). Spores of *Groenhiella bivestia* were shown by Rees and Jones (1984) not to attach so firmly after 3 hours. Electron microscopic work could show how firmly spores of this species attach with polar and equatorial appendages. This technique could also show the extent

of involvement of the sticky equatorial appendages in attachment of *Nimbospora bipolaris* and *N. effusa* spores (Hyde and Jones, 1985).

Further experimental work which could be done with some of these fungi is cytochemical analysis of the appendages or material produced by them. Such work could indicate the chemical nature of the spore appendages and of any mucilage produced. The results of these cytochemical analyses could in turn indicate whether material produced from the appendages resembles extracellular adhesives of bacteria (see Chapter 1.2) or algal spores and diatoms (see Chapter 3). These results, together with the electron microscopical observations, could further show the role of appendages and their secretions in firm spore adhesion. Cytochemical work could be used for spores of *Corollospora comata*, *Carbosphaerella leptosphaerioides*, *Ceriosporopsis tubulifera*, *Nimbospora bipolaris* and *N. effusa*.

There is strong evidence, however, particularly from electron micrographs, that the spore appendages of all other fungal species discussed here are involved in firm adhesion. This direct involvement of spore appendages and mucilage secretions has been shown for *Remispora maritima* (Rees and Jones, 1984), *Haligena salina* (Farrani and Jones, 1986), *Crinigera maritima* (Jones *et al.*, 1980), *Kohlmeyeriella tubulata* and *Lulworthia medusa* (Rees and Jones, 1984). Although the electron microscopic evidence of firm spore adhesion is conclusive it would be useful to do further experimental work with these species. For example, hosing experiments using spores of these species would demonstrate the firmness of adhesion. Additionally, cytochemical analysis of mucilage material produced from spore appendages of these species would be useful. Such work would show whether the chemical composition of spore mucilage

from these species resembles the long-chain, "sticky" polysaccharide dextran produced from *Lulworthia medusa* spores (Kirk, 1976).

(4.4). Post-germination adhesion mechanisms in marine fungi.

Attachment of fungal hyphae following spore germination has been observed in several marine fungi. There is again firm evidence of hyphal adhesion in some species but not in others.

Well-defined, extracellular sheaths were observed surrounding hyphae of *Amylocarpus encaphaloides* (see p. 144) and *Orbimyces spectabilis* immediately after spore germination. Extracellular material was also observed enveloping spores and germ tubes of *Corollospora maritima* (Rees and Jones, 1984; see p. 144). The sheaths were present along the whole length of the hyphae, with the exception of the hyphal tips in *Corollospora maritima.* The hyphal extracellular material appears to ensure firm adhesion of these fungi to a substratum (Rees and Jones, 1984; see p.144). In *Amylocarpus encephaloides,* the hyphal material allows firmer adhesion than the spine-like appendages (see p. 144). The observations of Rees and Jones (1984) again show how electron microscopy can be used to confirm an adhesion mechanism in certain marine fungi.

Light microscopic examination of cultured mycelia of the filamentous marine fungus *Leptosphaeria albopunctata* suspended in India ink showed the presence of capsules (Szaniszlo *et al.*, 1968). These capsules became dissociated from the fungal mycelia during centrifugation. Precipitation of the supernatant of centrifuged *L. albopunctata* cultures with ethanol

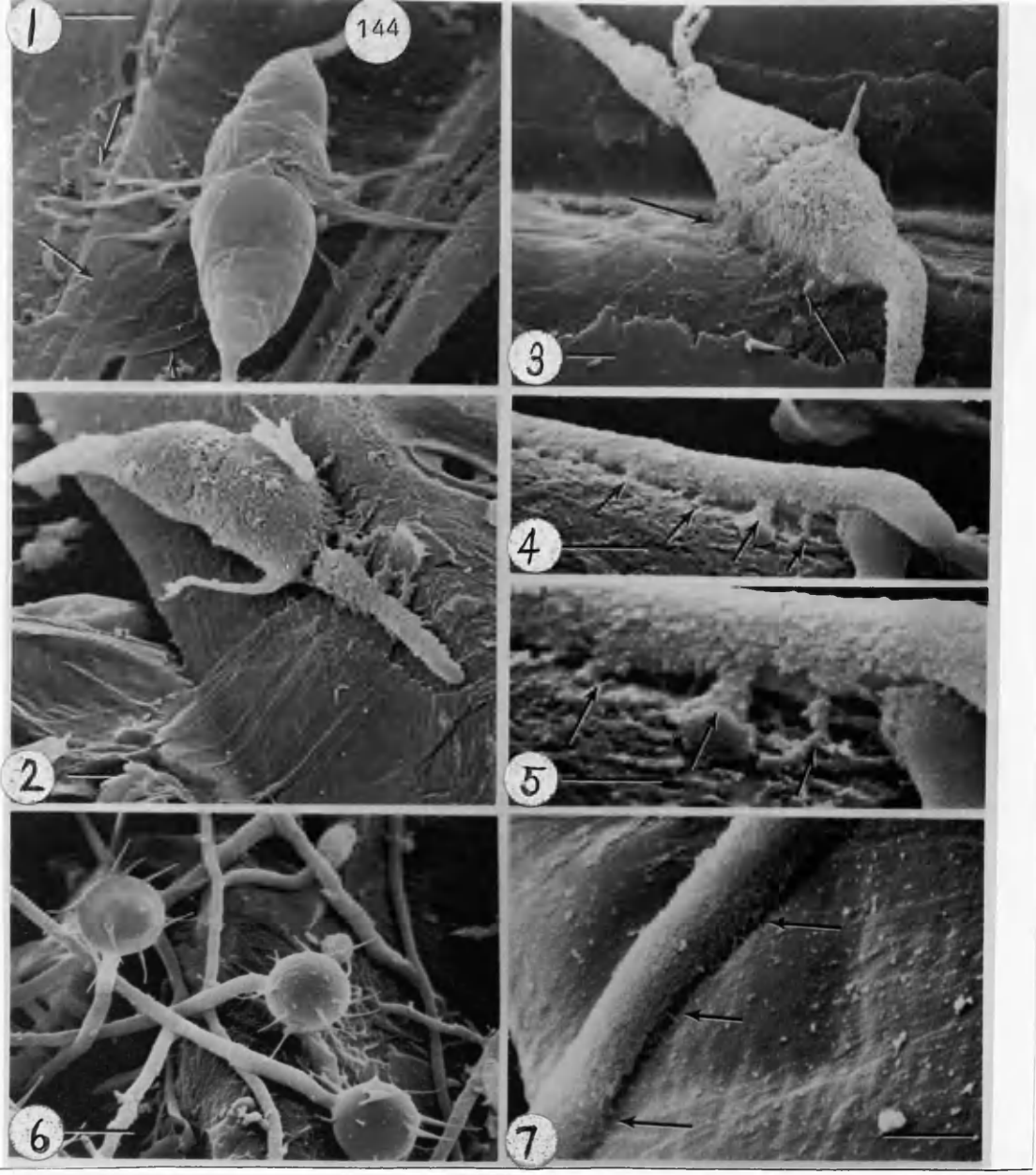

Fig. 31 SEMs of hyphal adhesion.

Fig. 31.1 Ascospore of *Corollospora maritima* settled on wood for 48 hours. Equatorial appendages (arrowed) attach the spore to the surface. Bar represents 2μm.

Fig. 31.2 Ascospore of *C. maritima* settled for 48 hours onto wood germinating with extracellular material enshrouding the spore and germ tubes. . Bar represents 2μm.

Fig.31.3 Ascospore settled onto wood for 24 hours, showing spore and germ tube covered with extracellular material and forming an attachment pad (arrowed).
Bar represents 2μm.

Figs 31.4 & 31.5
Hypha of *C. maritima*, after 48 hours, on wood ensheathed in extracellular material which forms numerous attachment points (arrowed).
Bar: in fig. 31.4 represents 2μm, in 31.5 represents 1μm.

Fig. 31.6 Ascospores of *Amylocarpus encephaloides*, settled onto wood for 48 hours, germinating and attaching the spores to the surface. Bar represents 4μm.

Fig. 31.7 Hypha of *A. encephaloides* after 42 hours settlement. Dense fibres of extracellular material form attachment regions to the wood surface (arrowed).
Bar represents 1μm.

(Reproduced by permission from Rees and Jones, 1984)

yielded polysaccharide material containing large amounts of glucose and smaller amounts of mannose. The hyphal capsular material is composed of this polysaccharide. High amounts of capsular polysaccharide were produced when $MgCL_2$ or $CaCl_2$ were added to the seawater medium (Szaniszlo *et al.*, 1968). The capsular material appears to resemble some bacterial capsules as well as the hyphal sheaths observed on *Amylocarpus encephaloides* and *Corollospora maritima*. The polysaccharide nature of the capsules surrounding *L. albopunctata* hyphae suggests that they may secure hyphal adhesion. However, Szaniszlo *et al.* (1968) did not provide any direct evidence of this.

A similar mucoid sheath was observed, by light microscopy, surrounding hyphae of *Lulworthia medusa* (Davidson, 1973). The sheath resembled the hyphal capsule of *Leptosphaeria albopunctata* when it was unstained. Additionally, the *Lulworthia medusa* hyphal sheath stains for mucin, suggesting that it is largely polysaccharide in nature. The mycelium of *L. medusa* adhered to the sides of the culture flask when grown in natural or artificial seawater media (Davidson, 1973). This observation suggests that the hyphal sheath of *Lulworthia medusa* may be involved in adhesion of this species. However, Davidson (1973) did not give any direct evidence.

The electron microscopic observations of *Amylocarpus encephaloides*, *Orbimyces spectabilis* and *Corollospora maritima* clearly showed the involvement of the hyphal sheaths in post-germination adhesion (Rees and Jones, 1984). There is little direct evidence, however, for involvement of the capsular sheaths in hyphal adhesion of *Leptosphaeria albopunctata* and *Lulworthia medusa*. Further light and electron microscopic observations of the hyphal sheaths of both species should be done. As the

sheaths are largely polysaccharide in nature, they should be easily stained for light microscopy. Light microscopic observations may indicate the possible involvement of the capsular sheaths in hyphal adhesion of both fungi. Scanning and transmission electron microscopy would conclusively show whether the hyphal sheaths are involved in secondary adhesion. In addition, hyphal capsule production in both *L. albopunctata* and *Lulworthia medusa* only occurred in seawater growth media, not in the natural environment. It would be interesting to know whether hyphal sheaths are also formed by these fungi in the marine environment.

Further cytochemical analysis of hyphal mucilage produced by *Amylocarpus encephaloides*, *Orbimyces spectabilis* and *Corollospora maritima* could also be done. This would allow comparisons with the capsular material produced by *L. albopunctata* and *Lulworthia medusa*, as well as adhesive material of bacteria, algal spores and diatoms. These results would in turn show any similarities between fungal hyphal mucilage and adhesives of other marine microorganisms. Conclusions could then be made about the role of hyphal mucilage in secondary adhesion of marine fungi.

Transmission electron microscopic observations of the tip regions of mucilage-producing fungal hyphae could also be done. This work would show whether vesicles producing mucilage material are present, as occurs during primary rhizoid adhesion in marine algae (see Chapter 3, p. 94). Vesicle production has not yet been shown to occur at the tips of marine fungal hyphae.

(4.5). Possible adhesion mechanisms in Thraustochytrids.

Thraustochytrids are marine coccoid fungi. They are often thought to

be "lower" marine fungi, which include eukaryotic organisms in the Myxomycota and Zygomycotina families. The family Thraustochytriaceae is made up of thraustochytrids and labyrinthulids (Perkins, 1976). Thraustochytrids are characterised by the formation of ectoplasmic nets from zoospores (see p. 148), which are produced from specialized organelles called sagenogenetosomes (Perkins, 1973, 1976). Thraustochytrids have been observed associated with sand particles (see Chapter 9, p.251).

The ectoplasmic nets produced by thraustochytrids are interesting because they may be involved in zoospore adhesion.

Labyrinthula algeriensis produced a coarse ectoplasmic network on formvar rafts in seawater (Perkins, 1973). Cells of *L. algeriensis* were embedded in the larger net elements. *Thraustochytrium motivum* and *Schizochytrium aggregatum* formed smaller ectoplasmic networks, which were produced from one side of the cells. Perkins (1973) acknowledged that ectoplasmic nets serve to anchor thraustochytrid cells.

Ectoplasmic net material is similar in appearance to bacterial extracellular polysaccharide material and algal spore adhesives (see p.148). This observation alone does not conclusively prove the involvement of ectoplasmic material in adhesion. However, if there were similarities in composition between ectoplasmic net material and other microbial adhesives, this would imply an attachment role.

Bremer (1976) showed that zoospores of *Thraustochytrium kinnei* attached to the exoskeleton of the brine shrimp *Artemia salina* by production of extracellular adhesive material. This material appears to be produced from *T. kinnei* zoospores before the development of the

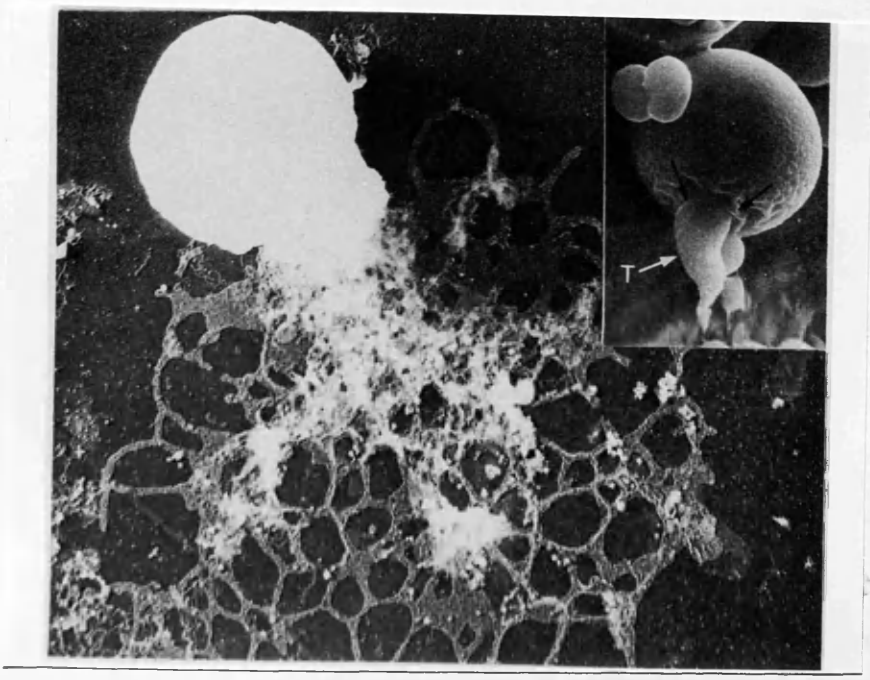

Fig. 32 Electron micrograph of thraustochytrid zoospore (T), producing ectoplasmic net material.

(Reproduced by permission from Perkins, 1976)

ectoplasmic net system. It is possible that this adhesive material may allow initial anchorage of *T. kinnei* zoospores to a substrate (Bremer, 1976). Zoospore adhesion may then be further strengthened by production of ectoplasmic net material.

Experimental work is needed to demonstrate the role of ectoplasmic net material in adhesion. Hosing experiments of the type described by Rees and Jones (1984) could be done. These experiments would involve exposing settled thraustochytrid zoospores which had produced ectoplasmic net material to a water jet. A wide range of thraustochytrid species could be treated in this way. This work, as for fungal spores, would demonstrate the strength of thraustochytrid zoospore adhesion.

Electron microscopic observations of settling and settled thraustochytrid zoospores would be interesting. In particular, transmission electron micrographs could be made at specific time intervals before and after thraustochytrid zoospore adhesion. The resulting observations would indicate whether extracellular material is produced prior to ectoplasmic net material as was observed in *T. kinnei* zoospores (Bremer, 1976). The observations would in turn indicate the extent to which ectoplasmic nets secure adhesion of zoospores. By sectioning thraustochytrid zoospores and substrata, transmission electron microscopy would accurately distinguish between involvement of initial extracellular material and ectoplasmic nets.

Cytochemical studies could also be made of the ectoplasmic material, as was suggested for fungal spore mucilage and hyphal sheaths (see p. 142). Little work of this kind has been reported.

The interesting observations of Miller and Jones (1983) suggest that attached thraustochytrids may be involved in seaweed decomposition.

Miller and Jones (1983) observed that thraustochytrid propagules were associated with the seaweed *Fucus serratus*. The thraustochytrids were initially present in low numbers on the seaweed surface. However, as the amount of dissolved carbohydrate in the seawater increased, the numbers of thraustochytrids increased. Thraustochytrids continued to increase in number until 16 days after the start of the experiment. There was then a decrease in the numbers recovered from the seaweed surface, and an increase in the numbers of other higher marine fungi (Miller and Jones 1983). An increasing cover of the algal surface with coccal and filamentous bacteria during the experiment was observed by scanning electron microscopy. Immature thraustochytrid zoospores were also observed on the seaweed, and some ectoplasmic net material was present. The shape of the *Fucus serratus* epithelial cells could be seen in the first few days, but had visibly deteriorated by the end of the experiment. High numbers of zoospores were observed on sections of decaying algal thalli (Miller and Jones, 1983). Further experiments studying the effects of thraustochytrids on growth of axenic algal cultures are needed. Additionally, biochemical studies of enzymes which thraustochytrids may produce on seaweed surfaces are needed.

The observations of Miller and Jones (1983) suggest that thraustochytrids need a microorganism layer before they will attach to macroalgae. The microorganism layer may condition the seaweed surface in some way. This can be compared with algal spores of *Chlorella* spp. and *Enteromorpha* spp. depending on a bacterial "conditioning layer" for

attachment to substrata (see Chapter 3, p.101). The thraustochytrids may also depend on release of nutrients from the microorganisms (Miller and Jones, 1983). Experiments looking at the attachment and growth of several thraustochytrid species on bacterial "conditioning" films should be done. A seawater microbial film, to which thraustochytrids had been added, could be radioactively labelled with ^{14}C-isotopes. After a suitable incubation period, ^{14}C levels within the thraustochytrids could be measured. This may indicate possible transfer of nutrients from the bacterial film to the thraustochytrids.

(4.6). Summary

(1) Many marine fungi possess spores with appendages. These appendages may serve several functions, including attachment of spores to substrata (Jones, 1972; Kohlmeyer and Kohlmeyer, 1979).

(2) Ascospores of marine Ascomycetes tend to form appendages in three different ways, as discussed by Johnson (1980).

(3) Ascospore appendages of some Ascomycetes may not allow firm adhesion, although they may serve initial entrapment. Such species include *Amylocarpus encephaloides* (Jones and Moss, 1978), *Halosphaeria mediosetigera* (Moss and Jones, 1977) and *Groenhiella bivestia* (Rees and Jones, 1984). Ascospore appendages of other Ascomycetes, however, produce mucilage which secures firm adhesion. This occurs in *Remispora maritima* (Rees and Jones, 1984), *Crinigera maritima* (Jones *et al.*, 1980) and *Lulworthia medusa* (Rees and Jones, 1984).

(4) Some marine fungi have been shown to produce extracellular, mucilaginous hyphal sheaths. These sheaths appear to secure hyphal adhesion following spore germination. Firm hyphal adhesion by sheaths has been shown to occur in *Amylocarpus encephaloides* and *Corollospora maritima* (Rees and Jones, 1984). Hyphal sheaths were also observed in *Leptosphaeria albopunctata* (Szaniszlo et al., 1968) and *Lulworthia medusa* (Davidson, 1973). However there was no direct evidence for involvement of the sheaths in hyphal adhesion.

(5) (i) Zoospores of thraustochytrids produce ectoplasmic net material which could anchor them to substrata (Perkins, 1973; 1976).

(ii) *Thraustochytrium kinnei* zoospores, on attaching to the brine shrimp *Artemia salina*, produce extracellular adhesive material prior to ectoplasmic material (Bremer, 1976). Further experimental work with other thraustochytrids is needed to distinguish between involvement of ectoplasmic material and any extracellular adhesives in zoospore adhesion.

(iii) Thraustochytrid zoospores have been observed on the surface of the seaweed *Fucus serratus*. They appear to be present on the seaweed after the development of a microorganism layer (Miller and Jones, 1983). Thraustochytrids may be involved in seaweed decomposition, as large numbers have been found on decaying *F. serratus* thalli. Thraustochytrids may depend on nutrient supply from the microorganism layer for attachment to *F. serratus* (Miller and Jones, 1983).

Chapter 5 - Attachment of marine microorganisms to fish tissues

Attachment of a microorganism to host surfaces is an essential prerequisite in the initiation of an infection. In the same way, adhesion of pathogenic microorganisms to fish tissues is essential for their successful entry and proliferation, and ultimately, in the occurrence of fish diseases (Munro, 1982).

This chapter describes the importance of microbial adhesion in fish diseases.

A bacterial fish pathogen which has been extensively studied is *Aeromonas salmonicida*. This bacterium causes a systemic disease called furunculosis, and other skin diseases, in salmonid fishes. Several workers have observed an additional surface layer on highly virulent strains of *A. salmonicida* (Udey and Fryer, 1978; Hubbert and Brain, 1980; Trust *et al.*, 1980; Hamilton *et al.*, 1981; Trust *et al.*, 1983). Strains of *A. salmonicida* which aggregated in liquid culture possessed this external layer, whilst non-aggregating strains did not (Udey and Fryer, 1978). Trust *et al.* (1980) observed an additional surface layer external to the outer membrane on aggregating strains of *A. salmonicida*.

Ultrathin sections of virulent strains of *A. salmonicidia* subsp. *achromogenes* isolated from ulcer lesions in roach and grown in axenic culture revealed an additional layer external to the cell envelope (Hubbert and Brain, 1980). *A. salmonicida* strain 1107/1B isolated from ulcers in Australian goldfish also possessed an extra layer beyond the outer membrane of approximately 10nm. thickness (Hamilton *et al.*, 1981; see p. 156). The bacteria aggregated when suspended in isotonic saline. Electron

microscopy showed that aggregating bacteria were joined by contact of the extra layers (Hamilton *et al.*, 1981; see p. 156). This additional layer may play an important role in the attachment of A. *salmonicida* to either the slime layer or skin of the fish.

Udey and Fryer (1978) showed that aggregating strains of A. *salmonicida* attached to human, rabbit and fish white cells as well as to fish intestinal mucosa cells. They also showed that A. *salmonicida* strain SS-70, an aggregating strain, adhered markedly to chinook salmon embryo cells. However, A. *salmonicida* SS-70-Sm^{d}-REV, a streptomycin sensitive, non-aggregating revertant mutant did not adhere so markedly to these cells (Udey and Fryer, 1978). This suggested that the additional layer present on aggregating strains of A. *salmonicida* facilitated its adhesion to fish tissue cells. This provides a mechanism whereby the bacterium could deliver aggressins to the fish (Udey and Fryer, 1978).

Electron microscopic studies of aggregating cells of A. *salmonicida* showed that the additional layer had a periodic staining pattern, and consisted of subunits with a tetragonal repeat pattern (Udey and Fryer, 1978; Hubbert and Brain, 1980; Trust *et al.*, 1980; Kay *et al.*, 1981). Ultrathin transverse sections of bacteria with this additional layer confirmed that it had a subunit arrangement with centre to centre spacing of 7.5nm. Some bacterial cells exhibited more than one subunit layer (Hubbert and Brain, 1980). Electrophoretic analysis using SDS-PAGE of four aggregating strains of A. *salmonicida* showed a common protein which was not present in smooth, non-aggregating strains. This protein layer was associated with the outer membrane (Trust *et al.*, 1980). Virulent strains of A. *salmonicida* were again shown by SDS-PAGE to contain a 49k

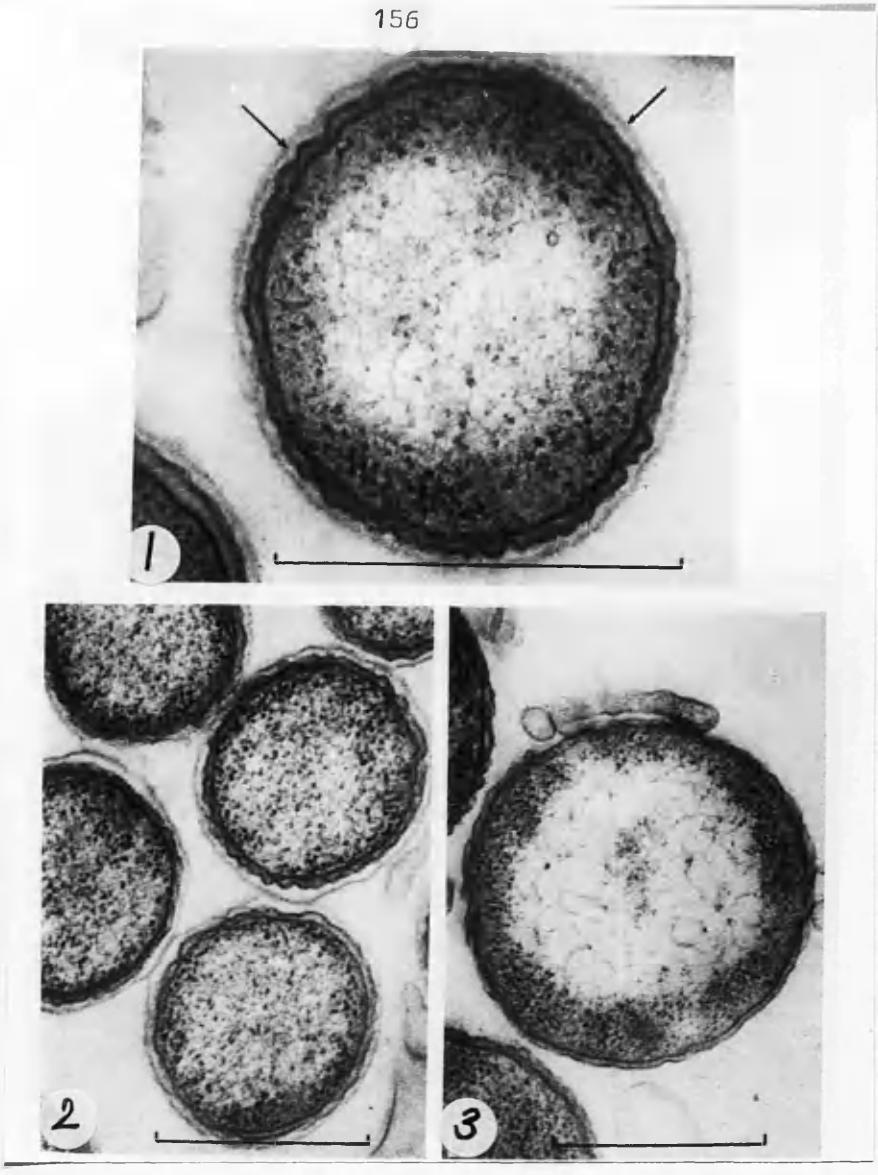

Fig. 33 Electron micrographs of *Aeromonas salmonicida*. Bar lines represent 0.5μm in all micrographs.

Fig. 33.1 *A. salmonicida* str. 1107/1B showing the extra surface layer (arrowed).

Fig. 33.2 *A. salmonicida* str. 1107/1B suspended in isotonic saline, showing the zones of cell aggregation.

Fig. 33.3 Cells from aberrant colonies grown with 0.25% (w/v) LiCl and suspended in growth medium. Note the absence of the extra layer and the blebs of outer membrane.

(Reproduced by permission from Hamilton *et al*, 1981)

dalton protein, known as the 'A'-protein (Kay *et al.*, 1981). The 'A'-protein was shown to be a major component of outer membrane fractions of virulent *A. salmonicida* strains. Growth of virulent strains of *A. salmonicida* at a higher-than-optimal temperature resulted in loss of the A-layer and attenuation of virulence (Ishiguro *et al.*, 1981). This again suggests a connection between the A-layer and virulence of *A. salmonicida*.

The amino acid composition of the 'A' protein has been determined, and has shown it to be extremely hydrophobic (Kay *et al.*, 1981; Evenberg and Lugtenberg, 1982; Phipps *et al.*, 1983; Parker and Munn, 1984). The protein was insoluble in water, which is further proof of its hydrophobicity (Evenberg and Lugtenberg, 1982). Strains of *A. salmonicida* possessing the A-layer dramatically increased in cell surface hydrophobicity. This was shown by 80% of such cells binding to octyl and phenyl sepharose gels and bacterial aggregation in 0.01M ammonium sulphate (Trust *et al.*, 1983; Parker and Munn, 1984). Strains lacking the A-layer, however, adsorbed poorly to both hydrophobic gels and only aggregated in 1.2-1.5M ammonium sulphate solutions. *A. salmonicida* strains possessing the A-layer also displayed enhanced association with both trout and mouse macrophages (Trust *et al.*, 1983). The increased hydrophobicity of the cell surface in these strains is likely to be important in this enhanced association. The increase in hydrophobicity may be caused by the physical masking by the A-layer of many of the lipopolysaccharide 'O' side chains (Trust *et al.*, 1983).

Studies by Chart *et al.*, (1984) suggested that some lipopolysaccharide O-side chains penetrated the A-layer on *A. salmonicida* strains and were exposed on the cell surface. This was shown by anti-lipopolysaccharide

polyclonal antibodies and anti-O-polysaccharide monoclonal antibodies reacting with the lipopolysaccharide on both fixed and unfixed cells possessing the A-layer (Chart *et al.*, 1984). It was confirmed by using bacteriophage 55R-1, which uses the lipopolysaccharide of *A. salmonicida* as a receptor. Bacteriophage 55R-1 adsorbed to *A. salmonicida* strain A451 which possesses the A-layer, confirming the exposure of lipopolysaccharide (Chart *et al.*, 1984). Non-aggregating strains of *A. salmonicida* were found to possess cellular appendages which may be lipopolysaccharide extrusions, again serving as bacteriophage receptors (Udey and Fryer, 1978). Lipopolysaccharides of Gram-negative bacteria are thought to play a role in bacterial adherence. It is possible that an additional function of the exposed lipopolysaccharides of *A. salmonicida* is in the initial attachment of this bacterium to tissue surfaces. Further experimental work which could show this would involve isolating exposed 'O'-side chains from *A. salmonicida* strains. These 'O'-chains, or structural analogues of them, could be added to assays of *A. salmonicida* strains with the A-layer attaching to fish tissue cells. Any inhibition of attachment by the 'O'-chains may indicate involvement of the exposed lipopolysaccharides in *A. salmonicida* adhesion. No inhibition of attachment may confirm that the A-layer is principally involved in adhesion.

Aeromonas salmonicida has also been isolated from Atlantic salmon with severe fin rot disease (Schneider and Nicholson, 1980). Electron microscopy of fins with severe rot revealed numerous Gram-negative, rod-shaped bacteria with glycocalyx fibres extending from them. This again suggests the role of glycocalyx in mediating adhesion of infective bacteria to fish tissue surfaces (Schneider and Nicholson, 1980).

Red-sore disease causes heavy mortality among species of fish having sport or commercial value. The bacterium *Aeromonas hydrophila* appears to be intimately associated with the peritrich ciliate *Epistylis* spp. in causing this disease. Scanning electron micrographs of red-sore disease lesions revealed that *Epistylis* attachment appeared as a spreading of stalk fibres over the substrate (Hazen *et al.*, 1978). Large numbers of rod-shaped bacteria were clearly adhering to the stalk of *Epistylis* spp.. Transverse and longitudinal sections of *Epistylis* spp. stalks revealed a fuzzy material, which may have been a mucilaginous coat with attachment qualities for the associated bacteria (Hazen *et al.*, 1978). *A. hydrophila* could be the primary invader in red-sore disease, inducing the lesions, whilst *Epistylis* spp. appears to be a secondary, benign ectocommensal (Hazen *et al.*, 1978).

Vibriosis is a disease of fish caused by the organism *Vibrio anguillarum* which can often be fatal (Horne, 1982). The pathogenesis of vibrio infections such as cholera in mammals, is primarily one of gut infection. *V. anguillarum* bacteria were shown to adhere to sections of rainbow trout gut (Horne and Baxendale, 1983). Bacteria consistently attached in greater numbers to the mid and upper gut regions, followed by the oesophagus, stomach and lower gut of rainbow trout (Horne and Baxendale, 1983). The numbers of adherent vibrios fell during the first two days, probably as a result of host clearance mechanisms. This suggested that the fish intestine is important in slower developing infections of *V. anguillarum*. However, the mechanism of adhesion of *V. anguillarum* to fish tissues is not known. Krovacek *et al.* (1987) investigated the adhesion of marine environmental and clinical isolates of

V. anguillarum and *A. hydrophila* to rainbow trout liver and chinook salmon embryo tissue cells. Adsorption of these bacteria to glass slides coated with mucus from rainbow trout body surfaces was also studied. Two of the marine *V. anguillarum* strains attached strongly to the fish cells, with over 100 bacteria attached per cell (Krovacek *et al.*, 1987). Only one strain of *A. hydrophila* isolated from fish was highly adhesive. Human isolates of this bacterium were poor binders. Treatment of both species with the enzymes papain, trypsin and pepsin decreased their adhesion to fish tissue cells. The same bacterial strains attached strongly to glass slides coated with mucus, whilst they did not bind to uncoated slides. Treatment with heat and proteolytic enzymes also decreased adhesion to mucus. These results suggest that cell-surface adhesins are involved in the attachment of *V. anguillarum* and *A. hydrophila* to fish tissue culture cells. In particular, the effects of the proteolytic enzymes and heat on bacterial adhesion suggest that proteinaceous adhesins could be involved. Adhesion may also involve specific receptors on the fish cells. The adsorption of bacteria to mucus from rainbow trout demonstrates another property of fish-associated bacteria. The mucus may also possess receptors necessary for adsorption. In addition, the presence of such mucus material may enhance the accumulation of *V. anguillarum* and *A. hydrophila* in the vicinity of marine fish. Successful attachment to fish skin mucus may allow *V. anguillarum* to produce a cytotoxin which penetrates the skin through the mucus layer (Krovacek *et al.*, 1987).

The epidermis of the sea horse *Hippocampus kuda* is characterized by flame cone cells. These cells protrude 20-40μm. above the epidermal surface, and are covered by a mucous cap. Histochemically, the flame cone

cell cap is a neutral mucopolysaccharide-protein complex (Bereiter-Hahn *et al.*, 1980). Scanning electron microscopy showed that epiphytic microorganisms were associated with the mucous flame cone cell caps, but not with the epidermal surfaces between them. Threads of algae or bacteria interconnected several mucous caps. The concave side of the sea horse's prehensile tail was covered by a confluent layer of epiphytes. Bacterial adhesion is known to increase with decreasing negative charge and increasing hydrophobicity of the cell surface (see Chapter 1.4). This is supported by the observation that bacteria and other microorganisms settled extensively on neutral glycoproteins of the flame cell mucous caps. The epiphytic growth of microorganisms is ecologically significant, in that it may offer protection against cnidarian nematocysts in coral environments (Bereiter-Hahn *et al.*, 1980). This example shows how epiphytic microbial growth can be beneficial to sea horses rather than causing disease.

Summary

The main points discussed in this chapter are as follows.

(1.). Attachment of *Aeromonas salmonicida* to fish tissues is important in furunculosis and fin rot disease. Adhesion of this bacterium appears to be mediated by a layer external to the outer membrane, the 'A'-protein (Udey and Fryer, 1978; Hubbert and Brain, 1980; Hamilton *et al.*, 1981). This protein is hydrophobic in nature (Kay *et al.*, 1981; Evenberg and Lugtenberg, 1982; Parker and Munn, 1984), and this may be important in the enhanced association of *A. salmonicida* with trout macrophages (Trust *et al.*, 1983).

(2.). Red-sore disease is characterized by attachment of the ciliate *Epistylis* spp., although it may be initiated by *Aeromonas hydrophila*. This bacterium appears to be intimately associated by mucilage with the ciliate's stalk. *A. hydrophila* could be the primary invader in red-sore disease, whilst *Epistylis* spp. may be a secondary, benign ectocommensal (Hazen *et al.*, 1978).

(3.). Adhesion of *Vibrio anguillarum* to fish intestine is important in vibriosis (Horne and Baxendale, 1983). Attachment of *V. anguillarum* to fish tissue cells and mucus-coated glass slides was decreased by proteolytic enzymes and heat. This suggests that proteinaceous adhesins and receptors in the fish mucus may be involved in *V. anguillarum* attachment (Krovacek *et al.*, 1987).

(4.). Flame cone cell caps of the sea horse *Hippocampus kuda* are coated by a neutral glycoprotein complex. Extensive microbial adhesion and growth occurred on these cells. Such epiphytic growth could protect the sea horse against predation (Bereiter-Hahn *et al.*, 1980).

Chapter 6 - Microbial attachment to marine plants

Macroscopic marine plants play an important role in the production of organic matter in inshore waters. Periphytic microorganisms which attach to them may be an important part of this production (Sieburth *et al.*, 1974). In addition, seaweeds are grazed by snails and gammarid amphipods. Microbial enrichment of these plants may be an important factor in their nutritional value (Sieburth *et al.*, 1974).

(6.1). Microbial attachment to seaweeds

A great variety of microbial epiphytes occur on seaweed surfaces. The brown alga *Ascophyllum nodosum* supported microcolonies of diatoms, yeasts and the filamentous bacterium *Leucothrix mucor* during winter (Sieburth *et al.*, 1974; Sieburth, 1975; see p.164). Cundell *et al.*(1977) observed differences in the microbial populations on the holdfast, internodal regions of the stipe and the apical tips of *Ascophyllum nodosum*. A lawn of rod-shaped bacteria covered the surface above the holdfast, and *L. mucor* projected from the surface. Pennate diatoms and filamentous cyanobacteria covered the internodal region. Near the apical region, the surface was mainly colonized by yeast microcolonies. However, the apical tips of *A. nodosum* were completely devoid of adherent microorganisms. Tannin secretion by *A. nodosum* in this region probably caused this (Cundell *et al.*, 1977). Graze marks from the marine snails *Littorina littorea* and *Littorina obtusata* were seen on the stipe. The exposed tissue was colonized by a diverse population of bacteria, including a number of curved and pointed rods, together with a possible dinoflagellate (Cundell *et al.*, 1977). The red alga *Polysiphonia lanosa*, an epiphyte on *A. nodosum*, supported a dense epiflora during the summer. Filaments of *L. mucor*, yeasts and pennate diatoms were present (Sieburth *et al.*, 1974; p. 166).

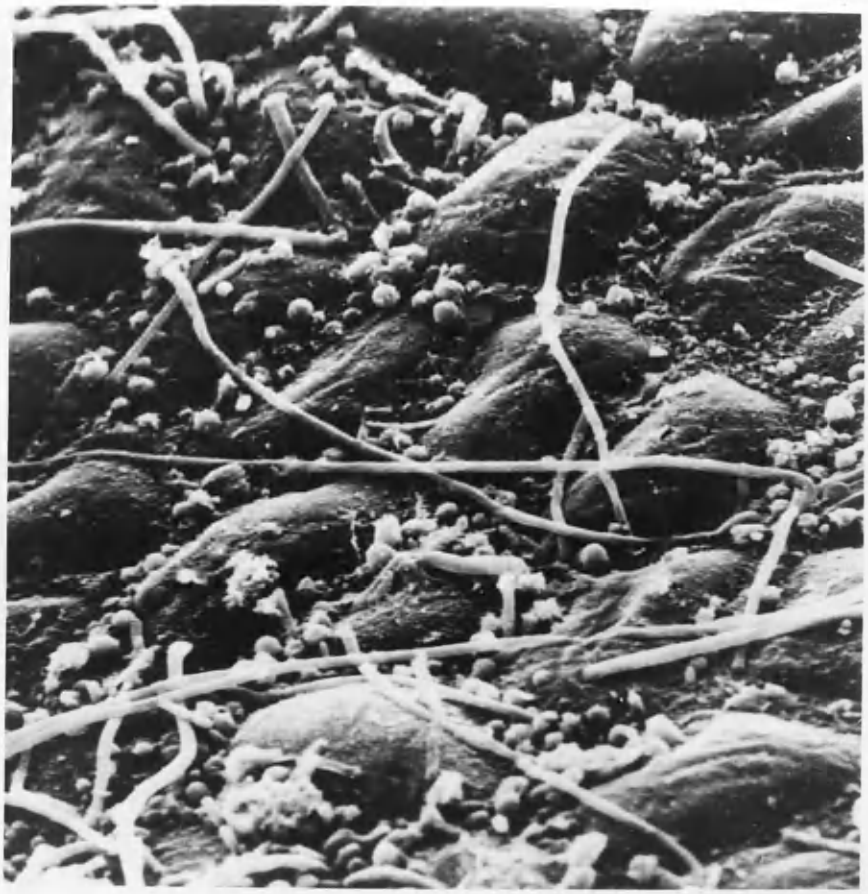

Fig. 34 The brown alga *Ascophyllum nodosum* showing light fouling by coccoid bacteria, yeasts and *Leucothrix mucor*, possibly due to tannin production.
Mag.: x6420

(Reproduced by permission from Sieburth, 1975)

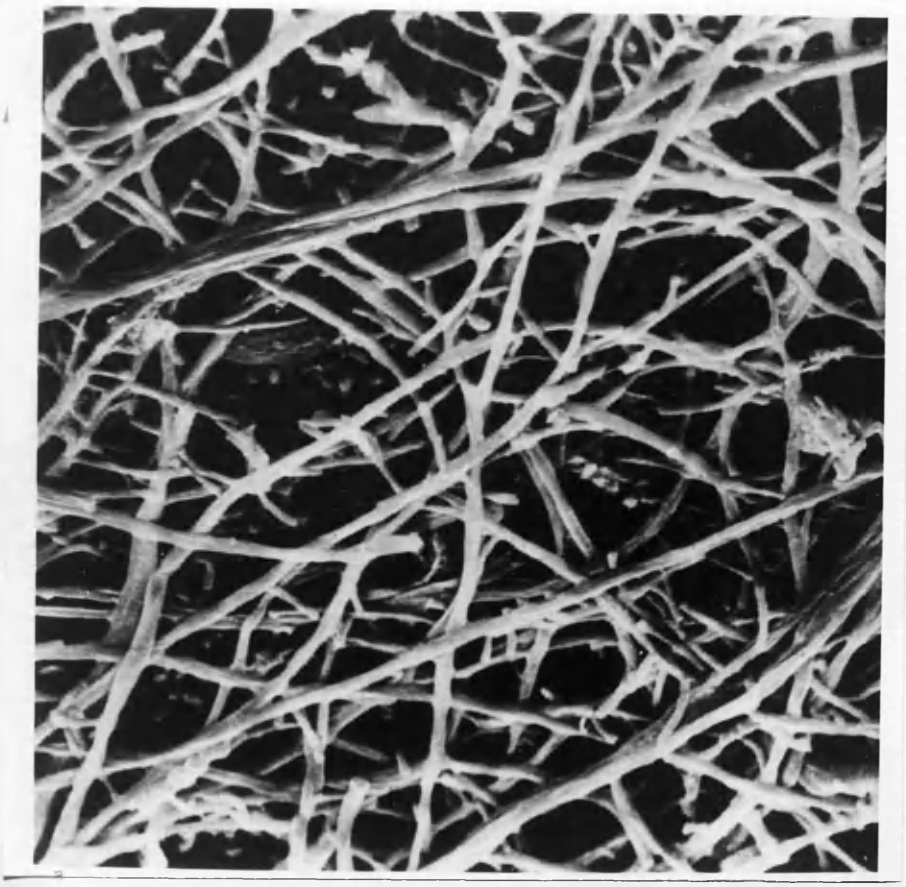

Fig. 35 *Ascophyllum nodosum*, showing heavy colonisation by filaments of *Leucothrix mucor*. Mag.: x5370.

(Reproduced by permission from Sieburth, 1975)

Fig. 37 Filaments of *Leucothrix mucor* on the surface of *Polysiphonia lanosa*. Mag.: x4900.

(Reproduced by permission from Sieburth, 1975)

L. mucor is a common microbial epiphyte of algae, which provide a suitable substratum for its attachment (Brock, 1966). It attaches particularly well to the red alga *Bangia fuscopurpurea* from which it may obtain nutrients (Bland and Brock, 1973).

Yeasts of the genus *Candida* were observed in high numbers on nine seaweed species isolated from Narrangansett Bay (Seshadri and Sieburth, 1975). Patches of pseudomycelia characteristic of *Candida* spp. were seen on the seaweed surfaces. Budding vegetative cells and bud scars were also seen, together with well-developed pseudomycelia with attached blastospores. *Candida* yeasts may also utilize nutrients released from the seaweed surfaces. *Leucothrix mucor* and pennate diatoms were again the predominant epiphytes of these seaweed species. *Candida* yeasts appeared sporadically as single cells or microcolonies in the surface areas examined (Seshadri and Sieburth, 1975).

Microorganisms associated with the surfaces of seaweeds and sea grasses are involved in the degradation of particulate debris and released mucilage (Linley *et al.*, 1981; see also Chap. 10). There was a clear succession of microorganisms which colonized mucilage released from the kelps *Ecklonia maxima* and *Laminaria pallida*. Rod-shaped and coccoid bacteria colonized the mucilage first. These were subsequently replaced by flagellates together with some diatoms and ciliates (Linley *et al.*, 1981). Adhesion of these colonizing microorganisms is essential in order for mucilage degradation to occur.

(6.2). Microbial attachment to salt marsh grasses.

Estuarine salt marsh grasses show markedly different patterns of microbial colonization from seaweeds. The fungus *Sphaerulina pedicellata*

colonizes the shaded and moist internodal areas of *Spartina alterniflora* (Gessner *et al.*, 1972; Sieburth *et al.*, 1974). Mycelia of this fungus possess hyphopodial appendages which serve for attachment (see p. 170). Ascocarps and ascospores characteristic of this species developed, and there was some evidence of mucilage production from ascospores, which could also be involved in adhesion (see Chap. 4 ; see also p. 171). When *S. alterniflora* falls and is decomposed, bacteria and diatoms become associated with it (Gessner *et al.*, 1972; Sieburth *et al.*, 1974). The eelgrass *Zostera marina* was initially colonized by the diatom *Cocconeis scutellum*, which formed a unialgal mat (Sieburth and Thomas, 1973; Sieburth *et al.*, 1974; see p. 172). Other pennate diatoms, such as *Navicula* and *Amphora* spp. settled on this crust, together with cyanobacteria, *L. mucor* and fungi (Sieburth and Thomas, 1973; Sieburth, 1975; see p.173). The crust that eventually formed could equal or exceed the biomass of *Zostera marina*. Heavy colonization like this would probably interfere with nutrient adsorption and photosynthetic activity. However, an advantage of this heavy microfouling is that it could offer protection for the eelgrass from grazing omnivores and herbivores (Sieburth and Thomas, 1973; Sieburth *et al.*, 1974).

Diatom colonization was prominent on the estuarine grass *Ruppia maritima* (Ferreira and Seeliger, 1985). A unialgal layer of *Cocconeis placentula* formed initially at the base of the leaf in a similar way to the colonization of *Z. marina*. Dense mucilage production by this diatom allowed firm adhesion to the host epidermis. *Synedra fasciculata* attached to the layer of *Cocconeis* cells by the production of mucus pads. A secondary, selective colonization by *Synedra fasiculata* also occurred over detritus, broken frustules and living *Cocconeis* cells (Ferreira and

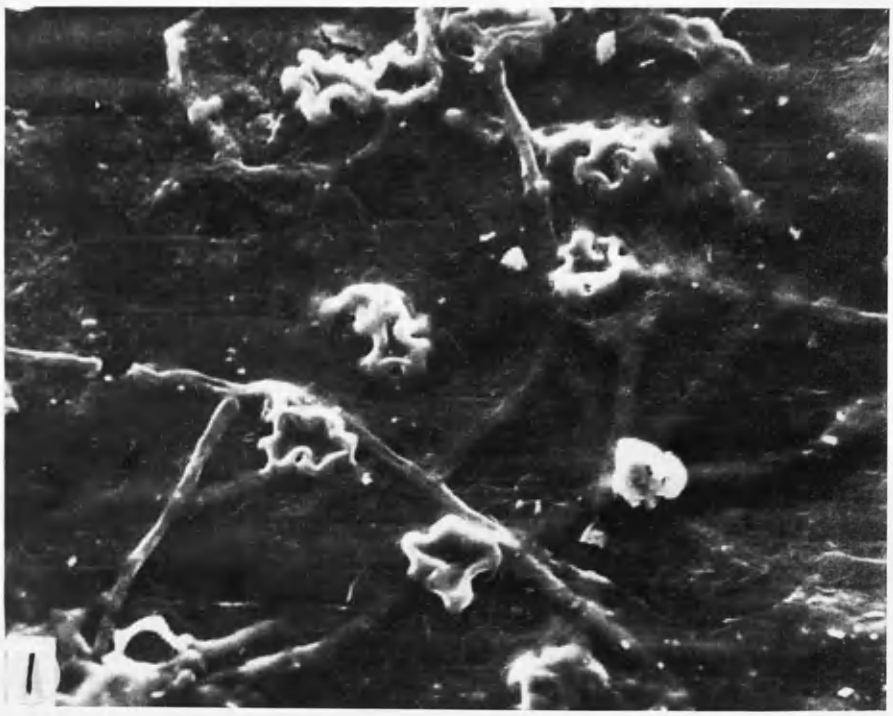

Fig. 38.1 and 38.2

SEMs of hyphopodia of the fungus *Sphaerulina pedicellata* allowing its attachment to the internodes of *Spartina alterniflora*.

(Reproduced by permission from Gessner *et al*, 1972)

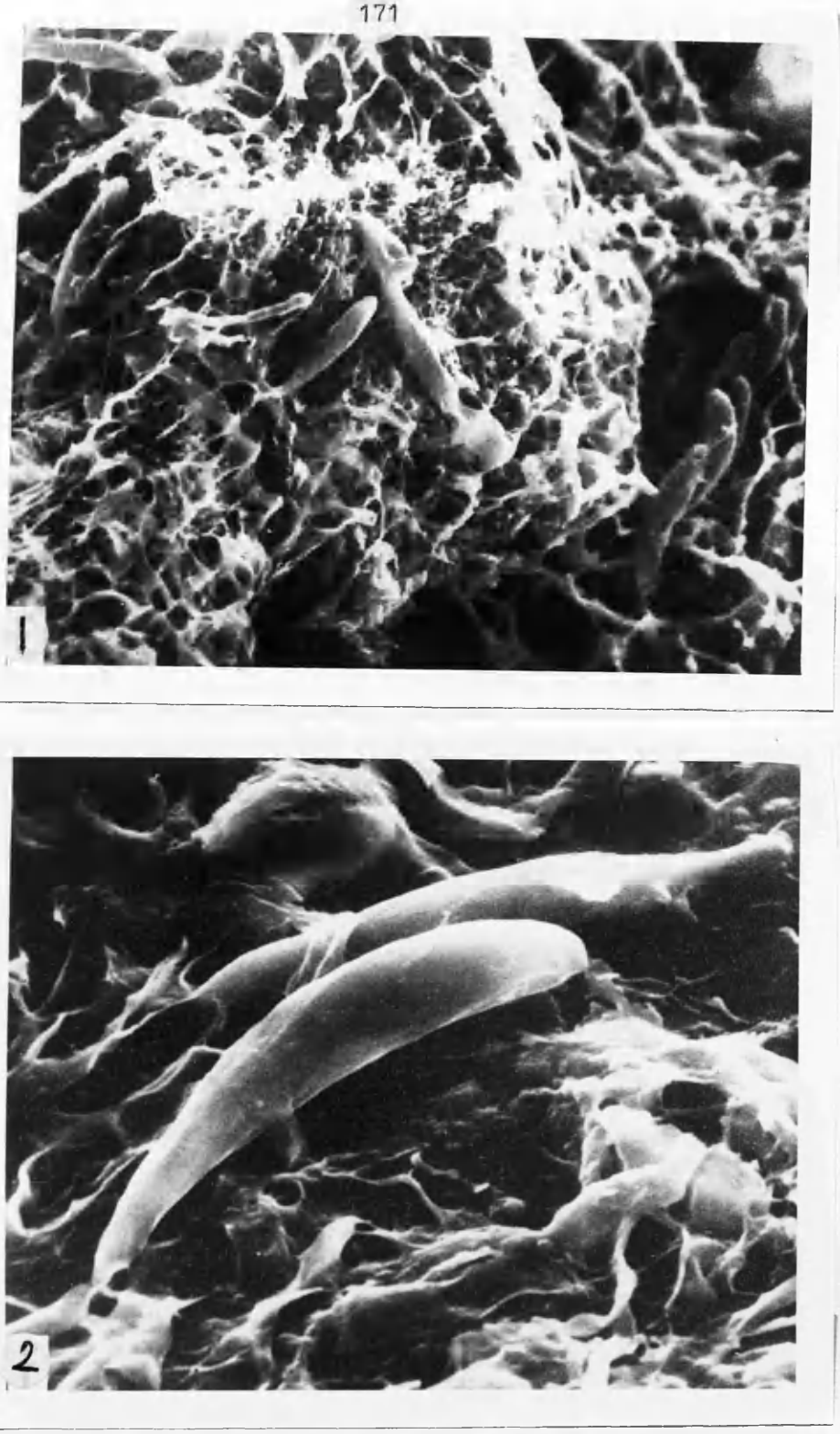

Fig. 39.1 and 39.2

SEMs of ascospores of *Sphaerulina pedicellata* in the internodal regions of *Spartina alterniflora*, showing evidence of possible mucilage production.

(Reproduced by permission from Gessner *et al*, 1972)

Fig. 40 Unialgal layer of the diatom *Cocconeis scutellum* on the surface of the eelgrass *Zostera marina*. Mag.: x540.

(Reproduced by permission from Sieburth, 1975)

Fig. 41 Multispecies diatom crust, together with epibiotic bacteria, on the surface of *Zostera marina*. Mag.: x300

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Seeliger, 1985). The formation of dense *Synedra* populations in the median leaf region permitted attachment of *Amphora* and *Nitzschia* spp.. During the final stage of colonization the entire apex of the leaf was covered. Filamentous diatoms such as *Melosira* and *Biddulphia* spp. were found among the upright epiphytes in this region (Ferreira and Seeliger, 1985).

(6.3). Ecological aspects of microbial adhesion and control of epiphytic populations

As well as their beneficial roles for the plants, microbial epiphytes of seaweeds and sea grasses can also be involved in plant decomposition. Cellulolytic bacteria were isolated from newly emerged and decomposing leaves of *Potamogeton pectinatus* from a brackish lake (Robb *et al.*, 1979). A rough-walled bacterium, which showed strongest cellulase enzyme production, was attached by a stalk. A cover of smooth-walled bacilli were seen attached by their axes to a leaf in the early stages of decomposition. The bacilli were surrounded by a sheet of fimbriate mucilaginous material. Microscopic examination of older decomposing plant material showed extensive damage to the epidermis, with some destruction of the outer cellulose cell walls. Short bacilli, surrounded by fimbriate material, were seen adhering to the inner walls of the damaged epidermal cells (Robb *et al.*, 1979). The fimbriate material and holdfasts produced by these bacteria, apart from allowing adhesion, may be involved in localization of cellulolytic enzymes. These bacteria are highly likely to be responsible for tissue injury and subsequent destruction of *Potamogeton pectinatus* leaves (Robb *et al.*, 1979). Further experimental work could be done to isolate these cellulolytic bacteria and study their role in the decomposition of *P. pectinatus* leaves. This could involve observations of the extent of decomposition in liquid culture, and measurements of levels

of cellulase activity. The effects of the detachment of these bacteria, through enzymic action, on the extent of leaf decomposition could also be studied.

Marine plants have mechanisms of controlling epiphytic microbial populations. Production of antimicrobial compounds, such as the tannin produced by *Ascophyllum nodosum* is one means of control. Peeling or sloughing of the algal cuticle occurs in certain seaweeds. This removes the epiphytic fouling layer leaving a clean algal surface. Cuticular sloughing occurs in *A. nodosum* and *Chondrus crispus* (Sieburth, 1975; Sieburth and Tootle, 1981; see p. 177). Peeling of the extensive *Cocconeis scutellum* layer on *Zostera marina* has also been observed (Sieburth, 1975; see p.176). Although cuticular or epidermal sloughing can clear an epiphytic layer, the newly exposed algal surface usually becomes rapidly re-colonized. A more artificial means of controlling epiphytic diatom populations on seaweeds is by using enzymes (Booth, 1981). Short-term exposure to the proteolytic enzymes actinidin and pepsin removed the stalked diatoms *Synedra tabulata* and *Licmophora* spp. from their algal hosts. However, *Cocconeis scutellum*, *Gomphonema pseudexigium* and *Achnanthes brevpipes* were more resistant to detachment (Booth, 1981). These enzymes may act by breaking down the acidic polysaccharides secreted by the diatoms (see Chapter 3.2). Enzyme mediated separation occurs at the stalk of *Licmophora* spp. and *Synedra tabulata* (Booth, 1981).

Fig. 42 Diatom crust peeling from the surface of eelgrass *Zostera marina*. Mag.: x220

(Reproduced by permission from Sieburth, 1975)

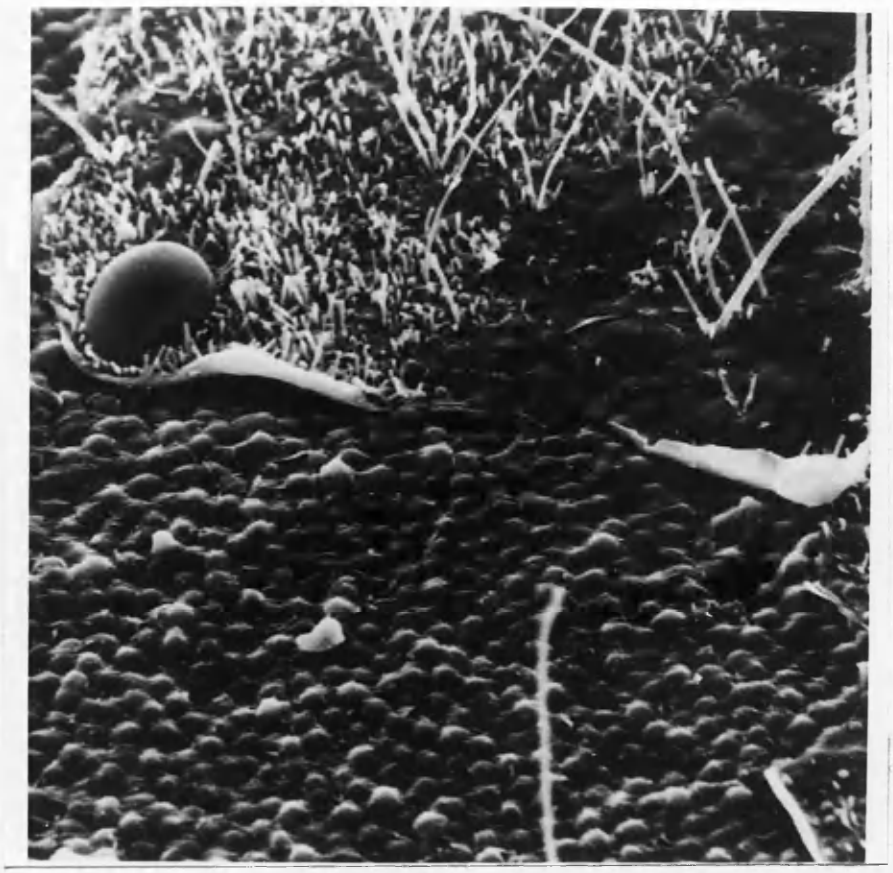

Fig. 43 Removal of the fouling layer consisting of bacterial rods, filaments and diatoms, during cuticular sloughing in *Chondrus crispus*. Mag.: x2170

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Summary

The main points shown in this chapter are as follows :

1.) Marine plants can accumulate dense and varied populations of microbial epiphytes. These can include bacteria, cyanobacteria, diatoms, and yeasts (Sieburth *et al.*, 1974; Sieburth, 1975; Cundell *et al.*, 1977; Sieburth and Tootle, 1981; Ferreira and Seeliger, 1985). *Leucothrix mucor* is a common epiphyte, particularly of red algae (Bland and Brock, 1973).

2.) (i.). Epiphytic microorganisms are involved in the degradation of particulate debris and mucilage released from seaweeds and salt marsh grasses, so contributing to the formation of detritus (Linley *et al.*, 1981).

(ii.). A heavy microfouling layer, such as the crust which forms on *Zostera marina*, may protect the plants from grazing omnivores and herbivores (Sieburth and Thomas, 1973).

(iii.). Some epiphytic bacteria may be involved in the decomposition of marine plants. Cellulolytic bacteria attached by fimbriate material to damaged epidermal cells of *Potamogeton pectinatus* (Robb *et al.*, 1979). This showed that bacterial adhesion is necessary for cellulose breakdown.

3.) Marine plants have methods of controlling epiphytic microorganisms. Production of antimicrobial compounds, which occurs in *Ascophyllum nodosum*, is one method (Cundell *et al.*, 1977). Peeling or sloughing of the cuticular layer also removes the fouling layer (Sieburth, 1975; Sieburth and Tootle, 1981).

Chapter 7 - Attachment between marine microorganisms.

(7.1). Bacterial attachment to cyanobacteria and other phytoplankton.

Several studies have shown that certain marine bacteria undergo chemotactic attraction towards marine phytoplankton. This led to the use of the term "phycosphere", which is the zone surrounding phytoplankton cells created by their production of extracellular products, which chemotactically attract bacteria. The phycosphere is ecologically important to bacteria, as it represents a source of organic nutrients and photosynthetically derived organic carbon. In turn, the bacteria surrounding the algae may themselves produce nutrients which benefit the phytoplankton. Only Gram-negative bacteria are attracted to phytoplankton. This may be because the algae produce inhibitors specific for Gram-positive bacteria (Jones, 1982).

The first part of this Chapter examines mechanisms of bacterial attachment to cyanobacteria and other phytoplankton which result in such associations. Chapter 7.2 discusses in detail a specific example of association between the parasitic marine bacterium *Bdellovibrio* spp. and other host bacteria.

(7.1.1.). Bacterial attachment to cyanobacteria and its ecological significance.

Bacterial association with cyanobacteria seem to produce symbiotic benefits for both microorganisms. Most of the associations discussed here were observed in the freshwater environment. It should be reasonable to assume that such associations also occur between bacteria and particular cyanobacteria in the marine environment. This is further suggested by

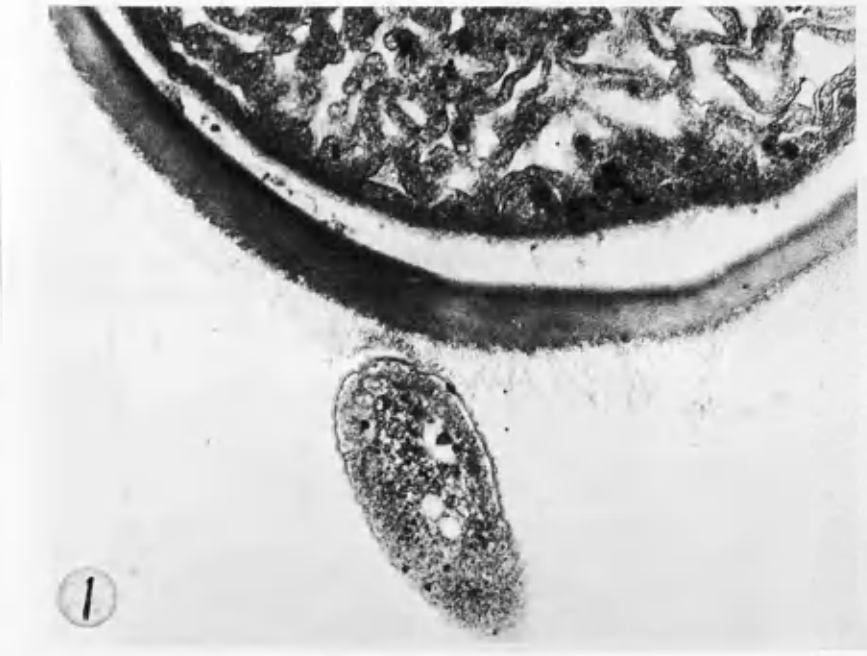
observations of bacteria associated with the mucilage sheath of blue-green bacteria in the Baltic Sea (Rheinheimer, 1985).

Bacteria were associated with the heterocysts of *Anabaena circinalis* and *Aphanizomenon flos-aquae* in two lakes (Paerl, 1976 ; see p. 182). In particular, extensive bacterial colonization of heterocysts of *Anabaena circinalis* was observed. The bacteria were observed in large numbers on the polar regions of the heterocysts. Autoradiographic examination showed that the attached bacteria on both species readily assimilated glucose, acetate and alanine. The accumulation of bacteria at the polar regions of the heterocysts suggests that this is a major site of nutrient secretion by the cyanobacteria. A successional pattern in bacterial colonization of heterocysts was observed in Clear Lake, U.S.A.. During late spring, *Aphanizomenon flos-aquae* heterocysts were mainly colonized, while heterocysts of *Anabaena circinalis* were not. During the summer months, bacterial attachment to *A. circinalis* heterocysts was more common. Bacterial colonization of both cyanobacterial species was highest at the times of year when heterocyst frequency was high. As heterocysts are sites of nitrogen fixation in cyanobacteria, these observations suggest a possible bacterial association with this process (Paerl, 1976).

Two marine bacterial isolates, *Pseudomonas* sp. SL10 and *Zoogloea* sp. SL20 also attached to heterocysts of *Anabaena* spp., and showed a high degree of selectivity (Lupton and Marshall, 1981). *Pseudomonas* sp. SL10 attached in greater numbers to heterocysts of *Anabaena flos-aquae* and *Anabaena azollae* than to *Anabaena cylindrica* and *Anabaena oscillarioides* heterocysts. *Zoogloea* sp. SL20 attached more to heterocysts of *A. cylindrica* and *A. oscillarioides.* This selectivity may reflect the distribution and abundance of binding sites on the heterocysts of different

cyanobacteria. *Pseudomonas* sp. SL10 attached perpendicularly to the heterocysts, with the polar end of the bacterium attached to the outer fibrous covering of the heterocysts. The polar orientation caused this bacterium to attach as a monolayer. *Zoogloea* sp. SL20 orientated in a random manner around the heterocysts, and some cells were attached by production of extracellular fibrils. *Zoogloea* cells were agglutinated to each other and formed a multilayer, with bacterial cells enmeshed in a matrix of extracellular polysaccharide fibrils (Lupton and Marshall, 1981). *Pseudomonas* sp. SL10 and *Enterobacter aerogenes* produced a greater stimulation of growth of *A. cylindrica* than *Zoogloea* sp. SL20. However, *Zoogloea* sp. SL20 promoted acetylene reduction, which is a measure of nitrogen fixation, by the cyanobacterium. Both bacteria promoted acetylene reduction under oxygenated conditions, although by a smaller amount than under anaerobic conditions. These observations suggest that bacteria attached to cyanobacterial heterocysts are involved in nitrogen fixation (Lupton and Marshall, 1981).

Other transmission electron microscopic studies have shown that bacteria attach to heterocysts of *Anabaena* spp. by production of fibrillar material (Paerl, 1980; see p. 182). Further evidence of the nitrogen fixation role comes from more observations of bacteria associated with heterocysts of *Anabaena* spp. in a freshwater lake. Axenic cultures of *A. oscillarioides* were allowed to develop heterocysts by omission of nitrogen. Bacteria added to the cultures were exclusively located on the heterocysts, and were mainly attached to the polar regions (Paerl, 1978). Attached bacteria were also observed in the mucus layers surrounding *Anabaena spiroides*. The use of the redox indicator NBT formazan showed blue darkening in the heterocyst regions and in the bacteria associated with



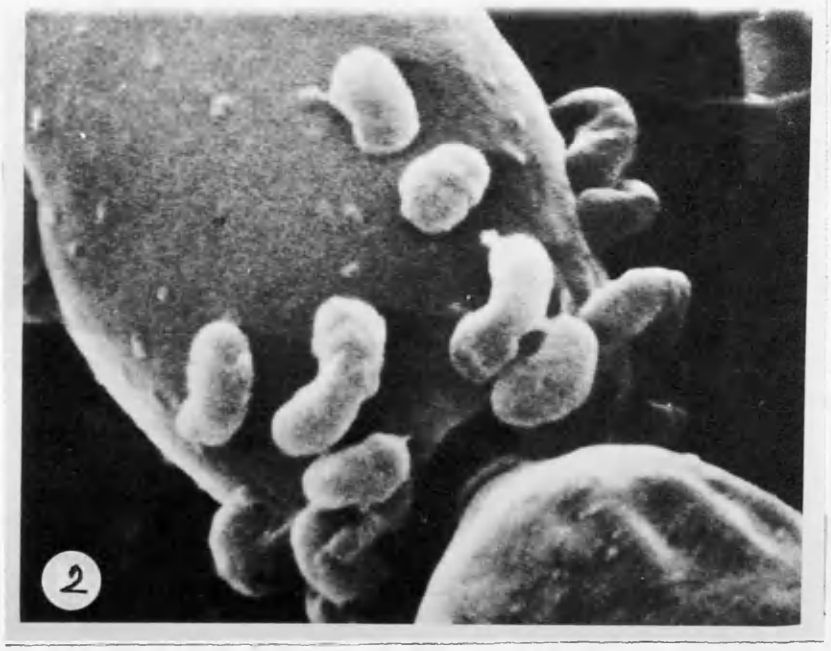


Fig. 44.1 Transmission electron micrograph (TEM) of bacteria attached to a heterocyst of *Anabaena* spp., showing some evidence of the production of fibrillar material from bacterial and heterocyst surfaces. Mag. x40,500.

Fig. 44.2 SEM of bacteria attached to a heterocyst of *Anabaena* spp. Mag. x12,625.

(Reproduced by permission from Paerl, 1980).

them. Steep gradients of NBT formazan deposition occurred in the polar regions of the heterocysts. This suggests the existence of reductive microzones in the heterocyst polar regions. The attached bacteria could remove oxygen, thereby protecting the oxygen-sensitive nitrogenase enzyme at the heterocyst poles (Paerl, 1978, 1980; Jones, 1982). Acetylene reduction by heterocyst-attached bacteria also suggests their role in nitrogen fixation by *Anabaena* spp. (Paerl, 1978).

Autoradiographic experiments using ^{3}H-serine showed that heterocystic bacteria were able to utilize serine, alanine, protein hydrolysate, glycine and glucose. More ^{3}H-serine labelling was found in the bacteria colonizing the heterocysts than in bacteria embedded in the mucilage layers. Consequently, a symbiotic association exists whereby bacteria are nourished by cyanobacterial extracellular products and the attached bacteria protect the heterocystous nitrogenase system from oxygen (Paerl, 1978; Jones, 1982).

There is a need for such studies to be performed with cyanobacteria in the marine environment.

Aggregates of *Zoogloea* spp. were associated with the mucilage of both *Anabaena flos-aquae* and *Microcystis aeruginosa*. The population density of bacteria in the mucilage was 2.6×10^{11} cells/ml. (Caldwell and Caldwell, 1978). When the *Zoogloea* spp. was grown with culture filtrates of *Anabaena* spp., it produced tough colonies on agar growth media. Soft colonies were produced when the *Zoogloea* spp. was grown without *Anabaena* spp. filtrates. This bacterium may therefore utilize an extracellular carbohydrate from the cyanobacterium in order to produce its own mucilage. Some of the extracellular carbohydrate products of *Anabaena* spp. were

adsorbed to suspensions of *Zoogloea* spp. Consequently, the cyanobacterium must be responsible for the production of mucilage precursors.

In addition, aggregates were formed when *Zoogloea* sp. was added to a liquid culture of *Anabaena* spp. Other bacteria which were isolated from the planktonic environment did not form aggregates with *Anabaena* spp. This suggests that *Zoogloea* spp. depends on the cyanobacterium for growth in liquid culture (Caldwell and Caldwell, 1978). There is a possibility of nutrient cycling between *Anabaena flos-aquae* and *Zoogloea* spp. because of the high numbers of bacteria in the cyanobacterial mucilage. Further autoradiographic work within the mucilage could show nutrient transfer to *Zoogloea* spp.. *Anabaena flos-aquae* may also receive nutrients from the bacterium for its own growth, and this could be shown by autoradiographic work.

(7.1.2). Bacterial attachment to diatoms and other phytoplankton.

There is conflicting evidence in the literature over bacterial association with diatoms. Bacteria were loosely associated with *Coscinodiscus concinnus* and a low attached bacteria/diatom cell ratio was observed during a diatom bloom period in the Clyde Estuary (Droop and Elson, 1966). Some diatoms produce an acidic microzone which repels attaching bacteria (Rheinheimer, 1985).

However, a rod-shaped bacterium was observed, by DAPI staining, attached to the frustule of the diatom *Thalassiosira* sp. (Coleman, 1980). (DAPI - 4'6-diamidino-2-phenylindole, a fluorescent DNA stain). Other rod-shaped bacteria producing polymeric fibrils were attached to *Thalassiosira* sp. (Rheinheimer, 1985). Bacterial attachment to pennate diatoms was also observed by Sieburth (1975) (see p. 185).

An abundant and diverse bacterial community was found in brine

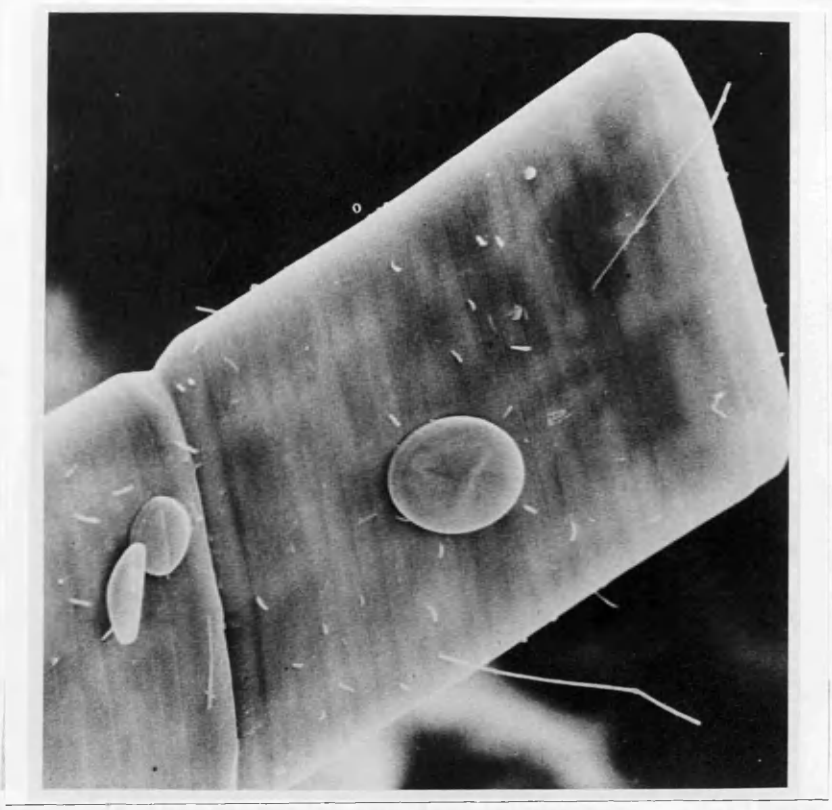


Fig. 45 SEM showing bacterial attachment to a pennate diatom. Mag. x1,780.

(Reproduced by permission from Sieburth, 1975).

channels of sea ice and at the ice-seawater interface in Antarctica. Approximately 70% of the ice bacteria were free-living, whereas 30% were attached to either living algal cells or detritus (Sullivan and Palmisano, 1984). Epibacteria were associated with the diatom *Amphiprora* spp.. Scanning electron microscopy showed a diversity of epibacteria, including cocci, rods, straight and branching filamentous cells and prosthecate forms. The stalked bacterium resembled *Prosthecobacter fusiformis* as it possessed a well-defined holdfast on the stalked end of both symmetrical daughter cells. Some of the epibacteria colonizing *Amphiprora* spp. produced exocellular polymeric substances (EPS) which may aid their attachment (Sullivan and Palmisano, 1984). A filamentous rod-shaped bacterium also showed cell surface modification at one pole. Transmission electron microscopy showed that the pole of this bacterium was surrounded by an EPS layer which was embedded in the diatom wall. This layer probably anchors the bacterium to *Amphiprora* spp. (Sullivan and Palmisano, 1984).

Evidence of possible symbiotic relationships between sea ice algae and bacteria comes from a positive correlation between bacterial concentration and chlorophyll 'a' at the bottom of the ice. The close physical association observed between epibacteria and *Amphiprora* spp. suggests that a symbiotic relationship exists between them. However, there is no evidence for this. *Amphiprora* spp. may excrete a large portion of its photosynthate, which could be utilized by the epibacteria. Metabolic activities of the attached bacteria could provide vitamins and growth factors for the diatom. In addition, the bacteria could recycle waste organic nitrogen compounds to inorganic nutrients, such as nitrate, nitrite and ammonium compounds (Sullivan and Palmisano, 1984). Further

autoradiographic work using ^{14}C could show transfer of photosynthetic products from *Amphiprora* spp. to epibacteria in the sea ice. Transfer of nitrogenous compounds to the diatom could be shown by ^{3}H-thymidine labelling. This work could be extended to other bacterial-algal associations in sea ice.

Kogure *et al.* (1982) studied bacterial attachment to *Skeletonema costatum* in natural seawater and in cultures. Most of the diatom cells were free of bacteria when they were collected from Otsuchi Bay, Japan. When cultured in a flask, however, diatom cells began to harbour bacteria. *Flavobacterium* sp. T-8D began to attach to *S. costatum* after 1-2 days incubation. There was an increase in the numbers of this attached bacterium after 5-6 days. *Flavobacterium* spp. attached perpendicularly, forming "clusters" near the edge of the diatom cells. "Cluster" formation may arise by the bacterium altering the physicochemical or biological conditions around it when attached to *S. costatum.* This may make the surface more favourable for the subsequent attachment of other bacteria (Kogure *et al.*, 1982). Further incubation caused coagulation of heavily bacteria-colonized diatom cells, resulting in formation of amorphous detrital particles. Rapid bacterial colonization must occur in order for detritus formation from phytoplankton to take place (see Chapter 10). *Flavobacterium* spp. T-8D may become the dominant attached bacterium on decaying *S. costatum* cells (Kogure *et al.*, 1982).

Albright *et al.* (1986) carried out an investigation into the factors affecting bacterial attachment to particles, including phytoplankton, in Howe Sound, Canada, a coastal temperate fjord. Silt and salinity levels in the fjord seawater did not appear to influence bacterial attachment. However, the percent attached bacteria was related to both chlorophyll 'a'

concentrations and primary productivity. During periods of high primary productivity, the percentage of attached bacteria was low, whilst during periods of low, increasing and declining primary productivity, attachment was high (Albright *et al.*, 1986). A significant portion of the euphotic zone bacteria became attached at the time primary production increased. The physiological condition of the phytoplankton, the major nutrient source, was the main factor affecting bacterial attachment. A similar pattern of bacterial attachment was observed when three phytoplankton, *Prorocentrum minimum, Dunaliella tertiolecta* and *Skeletonema costatum*, were grown in batch culture. The sequence of events which occurred during the association of bacteria, with *Dunaliella tertiolecta* in culture were followed (Albright *et al.*, 1986). During growth of this alga, bacterial cells were both free and attached. Attached bacteria appeared to be enmeshed in a sheetlike organic matrix. When this alga entered the stationary growth phase, bacterial numbers in the culture increased. Many bacteria accumulated near, but not on, the algal cells as aggregated masses of cells within a translucent mesh of organic material (Albright *et al.*, 1986). Late in the stationary phase, most of the culture was composed of a mass of amorphous detritus-like material containing bacteria. The bacteria were not attached to the algae at that time. This sequence of events also occurred in cultures of *Prorocentrum minimum* and *Skeletonoma costatum* (Albright *et al.*, 1986). These observations further show that bacterial attachment to phytoplankton causes decomposition of the algal cells and ultimate formation of detrital material.

The changes in attached bacterial numbers during phytoplankton growth was similar in both batch culture and seawater (Albright *et al.*, 1986). The portion of attached bacteria increased at about the same time that

phytoplankton growth started. Whilst the algae were growing, attached bacterial numbers decreased. After cessation of growth, a high number of bacteria became attached. The low numbers of attached bacteria observed during phytoplankton growth may partly be due to algal antibiotic production (Albright *et al.*, 1986). Formation of an amorphous, organic matrix observed after cessation of *D. tertiolecta* growth may result from bacteria utilizing nutrients released by this alga. The synthesis of glycocalyx material by the bacteria produces an attachment matrix. The bacteria may be using the algal cell wall and other less soluble materials as substrates for synthesis of glycocalyx (Albright *et al.*, 1986).

It would be useful to do further experimental work using ^{14}C-isotopes to trace the possible transfer of nutrients from *D. tertiolecta* to the bacteria. Such autoradiographic work could show the incorporation of nutrients into bacterial attachment material. This work could also be done with *Skeletonema costatum* and *Prorocentrum minimum*.

Cystodinium bataviense is a dinoflagellate which colonizes the epineustonic region of seawater. Its cell wall is often colonized by bacteria (Timpano and Pfiester, 1985). These bacteria may affect the surface/volume ratio of the dinoflagellate and the wettability of its hydrophobic cell wall. This in turn could affect the stability and attachment of *C. bataviense* in the epineuston (Timpano and Pfiester, 1985).

Further work could be done to investigate possible hydrophobicity changes in *C. bataviense* cells possessing attached bacteria. This could be done by measuring cell hydrophobicity using octyl sepharose beads. Control measurements would be made using dinoflagellates from which bacteria were removed. Accumulation of *C. bataviense* cells possessing attached bacteria at the surface microlayer could be observed using

seawater samples and light microscopy. If the bacteria increased the surface hydrophobicity of the dinoflagellate cells, there would be an accumulation of them at the surface microlayer in the epineuston (see Chapter 1.4).

Imam *et al.* (1984) looked at the specificity of the interaction of bacterial cells with the cell surface of the microalga *Chlorella vulgaris.*

Three bacterial strains were used. These included *Micrococcus* sp. ASB1, a bacterium known to be closely associated with *C. vulgaris* in the natural environment. The other two bacteria were *Escherichia coli* str. NAS, and *E. coli* str. NAS-OTEC, which was isolated from Ocean Thermal Energy Conversion equipment (Imam *et al.*, 1984). Cell surface antigens isolated from these bacteria, which were of high molecular weight, enhanced the adhesion of *C. vulgaris* to glass surfaces (Imam *et al.*, 1984). Consequently, these antigens are referred to as "Adhesion-enhancing" (AE) molecules. Antigens were also isolated from the surface of *C. vulgaris* cells.

The effects of certain sugars on the adhesion of *C. vulgaris* to glass surfaces in the absence and presence of AE antigens was examined (Imam *et al.*, 1984). Three sugars, L-fucose, α L-rhamnose and D-glucosamine, when tested alone, significantly enhanced the adhesion of *C. vulgaris.* However, AE antigens isolated from *E. coli*-NAS and NAS-OTEC strains inhibited *C. vulgaris* adhesion in the presence of D-mannose (Imam *et al.*, 1984). The effects of five other sugars on the AE antigens were also measured. No individual sugar inhibited the activities of all the AE antigens. Consequently, although all the AE antigens enhanced *C. vulgaris* adhesion, their activities were inhibited by different sugars. These observations suggest that attachment between *C. vulgaris* and its associated

bacteria is mediated by lectin-like macromolecules on the surfaces of both cells (Imam *et al.*, 1984).

The association of the bacterial strains with *C. vulgaris* in the absence of all four AE antigens was observed by epifluorescence microscopy. Significantly more *C. vulgaris* cells were colonized by *Micrococcus* sp. ASB1 cells than by the *E. coli* NAS and NAS-OTEC strains (Imam *et al.*, 1984; see p. 192). This was observed when the three bacterial strains were individually suspended with *C. vulgaris*, and when all three bacteria were mixed with the alga. The number of sites on the *Micrococcus* sp. ASB1 cells available for binding to *C. vulgaris* must be higher than for both *E. coli* strains (Imam *et al.*, 1984).

The association between the bacteria and *C. vulgaris* was also observed in the presence of the AE antigens. This was done firstly to show whether the AE antigens produced by different bacterial strains interact with distinct receptors on the *Chlorella* cell surface. It was also done to show if the AE antigens affect the surface interactions of bacterial cells with *C. vulgaris* in mixed suspension (Imam *et al.*, 1984). In the presence of ASB1 antigen, significantly more *C. vulgaris* cells were colonized by *Micrococcus* sp. ASB1 than by *E. coli* NAS-OTEC. However, in the presence of the NAS-OTEC antigen, significantly more *C. vulgaris* cells were colonized by *E. coli* NAS-OTEC than by *Micrococcus* sp. ASB1 (Imam *et al.*, 1984; see p. 192). The ASB1 antigen also enhanced aggregation of *C. vulgaris* cells. This suggests that both the *Micrococcus* sp. ASB1 cell surface and the *Chlorella* surface interact with this antigen (Imam *et al.*, 1984). The NAS-OTEC antigen enhanced the aggregation of *E. coli* NAS-OTEC cells, suggesting that the bacterial cell surfaces were affected by this antigen.

TABLE 8 Percentage of *Chlorella vulgaris* cells colonized by bacterial strains.

Suspension	Antigen[1]	Strain	Percentage of colonized *C. vulgaris* ± SE[2] (no. of duplicate assays).
Individual	None (control)	ASB1	74.6 ± 0.40 (20)
		NAS-OTEC	35.6 ± 0.13 (20)
		NAS-*E.coli*	24.6 ± 0.10 (20)
Mixed	None (control)	ASB1	56.1 ± 0.50 (15)
		NAS-OTEC	30.0 ± 0.15 (15)
		NAS-*E.coli*	20.1 ± 0.20 (15)
	ASB1	ASB1	94.8 ± 1.80 (15)
		NAS-OTEC	33.5 ± 0.25 (15)
	NAS-OTEC	ASB1	49.5 ± 0.44 (20)
		NAS-OTEC	52.0 ± 0.42 (20)
	NAS-OTEC	NAS-OTEC	36.5 ± 0.50 (15)
		NAS-*E.coli*	18.5 ± 0.10 (15)

[1] Antigen concentration, 10^{-5} µg/ml.

[2] SE, Standard error of the mean.

(Taken from Imam *et al.*, 1984).

These results indicate that the ASB1 AE antigens are distinct from the NAS-OTEC antigens in both their inter- and intraspecific aggregations (Imam *et al.*, 1984). In general, the AE antigens isolated from the four microbial sources used in this study are distinct in their interactions with the *C. vulgaris* cell surface. They are also distinct to the bacterial surfaces which the *Chlorella* cells interact with in mixed culture (Imam *et al.*, 1984).

The specificity of microbial cell interactions observed by Imam *et al.* (1984) could provide an alternative explanation of other such interactions discussed here. It could be a further reason as to why certain bacteria attach to the heterocysts of certain cyanobacteria in the freshwater environment. Specificity of interactions could also explain why bacteria attach to certain diatoms but not to others. Further work of the type carried out by Imam *et al.* (1984) could be done with other microbial interactions. Extraction and investigation of possible antigens involved in bacterial attachment to cyanobacterial heterocysts or phytoplankton could be done. The possible involvement of lectins in binding could be shown by addition of sugars to these microbial interactions.

(7.1.3). Summary

(1.). (i.). Bacteria are attached to the heterocysts of *Anabaena* spp. in the freshwater environment (Paerl, 1976, 1978). Staining by redox indicators (Paerl, 1978) and evidence of acetylene reduction in the heterocyst regions (Paerl, 1978; Lupton and Marshall, 1981) suggests bacterial involvement in nitrogen fixation by *Anabaena* spp.. Heterocystic bacteria were also shown to utilize nutrients from *Anabaena* spp., so that a

symbiotic relationship could exist (Paerl, 1978).

(ii.). *Zoogloea* spp. were associated with the mucilage surrounding *Anabaena flos-aquae*. The bacterium produced tough colonies on agar media when grown in culture filtrates of *A. flos-aquae*. *Zoogloea* spp. may utilize an extracellular carbohydrate from the cyanobacterium, and nutrient cycling may occur between the microorganisms (Caldwell and Caldwell, 1978).

(2.). (i.). Conflicting evidence exists over bacterial attachment to diatoms. Some authors report extensive bacterial attachment to diatoms, such as *Thalassiosira* spp. (Rheinheimer, 1985) whilst others report weak or little association (Droop and Elson, 1966).

(ii.). Bacteria were attached to the diatom *Amphiprora* spp. in Antarctic sea ice (Sullivan and Palmisano, 1984). Some of the bacteria produced exocellular polymeric substances which may aid their attachment. A positive correlation between bacterial and chlorophyll 'a' concentration in the sea ice suggests existence of a symbiotic relationship between bacteria and the diatoms (Sullivan and Palmisano, 1984).

(3.). (i.). *Flavobacterium* sp. attached to *Skeletonema costatum* in seawater cultures. The bacterium attached perpendicularly, forming "clusters" on the diatom. Further incubation caused coagulation of heavily bacteria-colonized diatom cells, resulting in formation of amorphous detrital particles (Kogure *et al.*, 1982).

(ii.). Bacterial attachment to phytoplankton in a Canadian coastal temperate fjord was inversely related to primary productivity. The physiological condition of the phytoplankton

also affected bacterial attachment (Albright *et al.*, 1986). During growth of *Dunaliella tertiolecta* in batch culture, attached bacteria were enmeshed in an organic matrix. Late in the stationary phase, the culture was mainly composed of amorphous detritus-like material containing bacteria. This sequence of events also occurred in cultures of *Prorocentrum minimum* and *S. costatum* (Albright *et al.*, 1986).

(4.). The dinoflagellate *Cystodinium bataviense* usually colonizes the epineuston. Bacteria often colonize its cell wall, and they may affect the hydrophobicity of the cell wall. This could affect the stability and attachment of *C. bataviense* in the epineuston (Timpano and Pfiester, 1985).

(5.). Specific surface interactions occurred between the microalga *Chlorella vulgaris* and three associated bacteria, *Micrococcus* sp. ASB1, *E. coli* strains NAS and NAS-OTEC (Imam *et al.*, 1984). The effects of "adhesion-enhancing" antigens isolated from the bacterial cells on *C. vulgaris* adhesion was inhibited by different sugars. This suggests that associations between the bacteria and *C. vulgaris* are mediated by lectin-receptor interactions. The AE antigens isolated from the bacterial strains were distinct in their interactions with the *C. vulgaris* cell surface. They were also distinct to the bacterial surfaces which the *Chlorella* cells interact with in mixed culture (Imam *et al.*, 1984).

(7.2). Attachment of *Bdellovibrio bacteriovorus* to host bacterial cells

Bdellovibrio spp. are a group of parasitic bacteria which are found in the marine environment, in soil and sewage. They were first discovered in 1962 in the course of experiments designed to isolate bacteriophages from soil (Stolp and Starr, 1963). They attack host bacteria in a similar way to bacteriophages, by attaching to and lysing the bacterial cells. Plaques are produced on an agar overlay consisting of *Bdellovibrio* spp. and host bacteria, in the same way as for bacteriophages (Stolp and Starr, 1963; Scherff *et al.*, 1966).

Bdellovibrio bacteriovorus is the parasitic species which has been most extensively studied. It is a Gram-negative, vibrio-shaped organism, with a diameter of 0.3μm and possesses a single polar flagellum of about 50mμ diameter (Stolp and Starr, 1963).

Extensive work has been performed on the parasitic life cycle of *Bdellovibrio bacteriovorus*. However, this sub-chapter will be concerned only with the initial stage of the process, namely the attachment of the *Bdellovibrio* cell to its host bacterium.

Attachment of *Bdellovibrio bacteriovorus* to its host cell is initially a reversible process (Stolp and Starr, 1963). The *Bdellovibrio* cell can become detached from one host cell and become attached to another cell. The initial interaction of *Bdellovibrio* with its host can be described as a "recognition of prey", which is probably mediated by chemotaxis (Starr and Seidler, 1971; Starr and Huang, 1972). A violent collision occurs on impact between the highly motile *Bdellovibrio* cell and its host cell. This often results in the host cell being moved for a distance equivalent to several cell lengths (Stolp and Starr, 1963; Starr and Baigent, 1966).

When the *Bdellovibrio* is firmly attached to its host cell it often rotates about its own long axis, with speeds of up to 100 revolutions per second (Starr and Seidler, 1971). This is similar to an "arm-in-socket" type of motion and suggests a strong surface binding between *Bdellovibrio* and its host (Starr and Baigent, 1966).

The mechanism by which *Bdellovibrio bacteriovorus* attaches to its host cell is not properly understood, although some evidence has been reported. Abram *et al.* (1974) did a transmission electron microscopic study of the penetration of *B. bacteriovorus* into host cells. They showed that the penetration pole of the *Bdellovibrio* cell was firmly associated with the cytoplasmic membrane of the host cell. They found that this firm contact persisted throughout the penetration process and at the end of this phase of the infection. Abram *et al.* (1974) also showed that electron opaque material, which was seen as fine strands, was present in the interspace between the two unit membranes of the *Bdellovibrio* cell and host cell. This material was seen to extend from the *Bdellovibrio* cytoplasmic membrane through its cell wall and interspace to the host cell membrane. Abram *et al.* (1974) suggested that these linearly oriented fine strands probably mediated the association between *B. bacteriovorus* and the host cell.

Similar fibres have been reported in other papers. Transmission electron micrographs of the infection of *Erwinia amylovora, Pseudomonas tabaci* and *P. phaeseolicola* by *B. bacteriovorus* clearly showed fibres attaching the anterior pole of the *Bdellovibrio* cell to the host cell membrane (Starr and Baigent, 1966). Further electron micrograph studies of the penetration of *B. bacteriovorus* into two strains of *E. coli* (Burnham *et al.*, 1968), showed several rigid fibres emerging from a "holdfast" structure present at the *Bdellovibrio* anterior pole. These fibres may be

involved in attachment to the *E. coli* cytoplasmic membrane. Abram and Davis (1970) also observed fibres emerging from the anterior end of *B. bacteriovorus*. They also suggested that these structures may support a firm connection between the parasite and host cell. Negatively stained, shadow cast electron micrograph studies of the attachment of *B. bacteriovorus* to *E. coli* and *Pseudomonas putida* (Abram and Shilo, 1967) showed several rigid straight fibres emerging from the aflagellated pole of the *Bdellovibrio* cell. Similar rigid, spike-like filaments have been observed at the anterior pole of *Bdellovibrio bacteriovorus* by other authors (Shilo, 1969; Stolp, 1973), and have also been suggested to allow attachment to the host cell.

Starr and Seidler (1971) suggested that these fibre-like structures may be pili, which form sporadically at the cell anterior. They suggested that these structures may have a direct function in the attachment process (Starr and Seidler, 1971). Scherff *et al.* (1966) suggested the existence of an "infection cushion" which attaches the cell wall of *B. bacteriovorus* to the host's cell wall. Starr and Huang (1972) suggested that the filaments may be artifacts formed during the electron microscopic fixation and staining. This is unlikely, however, due to the number of similar reports of fibres on the anterior cell pole of *B. bacteriovorus*.

Cells of the cyanobacterium *Microcystis aeruginosa* were observed to be lysed by *Bdellovibrio*-like bacteria (Caiola and Pellegrini, 1984). Transmission electron micrographs showed that both the cyanobacterium and extracellular bacteria were surrounded by glycocalyx material. The glycocalyx probably acts as a bridge between the *Bdellovibrio* bacterium and its prey. In addition, the penetrated bacteria were mainly localized

between the cell wall and cytoplasmic membrane of *Microcystis* cells. Tubular structures were observed attaching the bacterial outer membrane to the cyanobacterial plasmalemma (Caiola and Pellegrini, 1984). Owing to their dimensions, they could not be identified as pili, fimbrae or flagella. It is possible that these structures could be the fibres previously observed on the *Bdellovibrio* anterior pole.

Further evidence that the *Bdellovibrio* cell associates with the host cell's cytoplasmic membrane has come from electron microscopic studies (Snellen and Starr, 1974). They described localized damage to the cytoplasmic membrane of *Spirillum serpens* VHL. This damage took the form of a "scar" or "blister" which was either adjacent or fairly close to the *Bdellovibrio* cell (see p. 200). The scar may be the site of attachment of the *Bdellovibrio* cell. However, it was uncertain whether this structure was a response of *Spirillum* to the presence or activity of the *Bdellovibrio* cell or if *Bdellovibrio* itself was directly responsible for its formation. They concluded that *Bdellovibrio* may inflict some localised damage to a host cell's cytoplasmic membrane whilst leaving the rest of the membrane intact (Snellen and Starr, 1974). Extensive "blebbing" or deformation, in the form of wrinkling, of the substrate cell wall next to the *Bdellovibrio* attachment site, was also reported for *S. serpens* VHL (Snellen and Starr, 1976).

During intraperiplasmic growth of *B. bacteriovorus* 109J in cells of *Salmonella typhimurium* and *E. coli*, the substrate cell surfaces became more hydrophobic (Cover and Rittenberg, 1984). At least two sites on the *Bdellovibrio* cell surface bound to octyl Sepharose beads, one of these being the cell pole opposite the flagellum which is the attachment site. The binding may be via a hydrophobic interaction between the *Bdellovibrio*

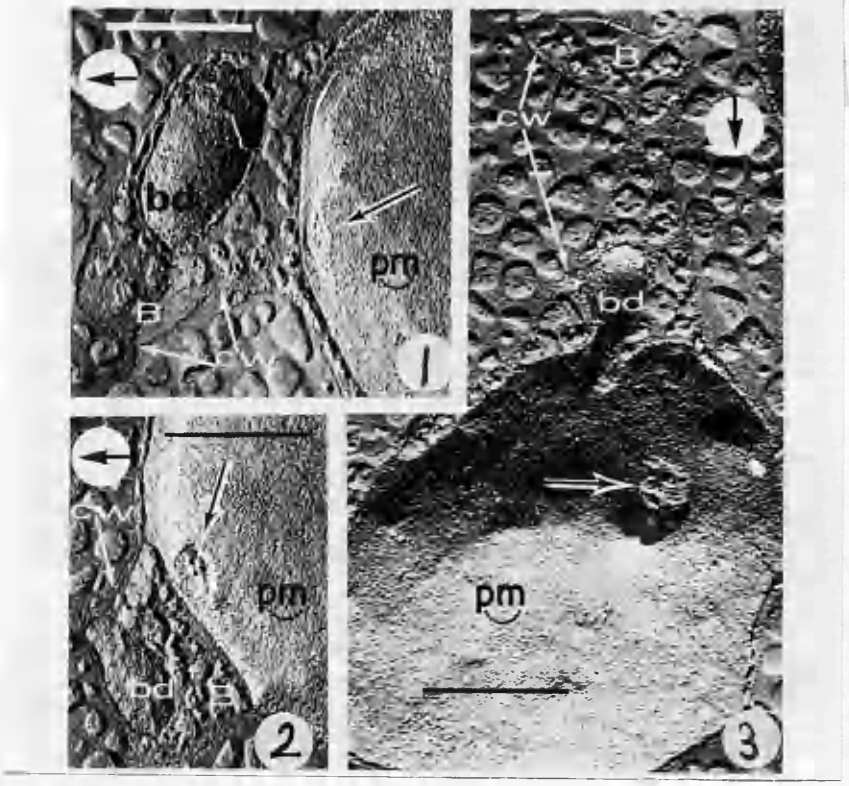


Fig. 46 Interaction of *Bdellovibrio bacteriovorus* 109D with *Spirillum serpens* VHL.

Fig. 46.1 A glutaraldehyde-fixed freeze-etched preparation of Spirillum serpens VHL 40 mins. after association with *Bdellovibrio bacteriovorus* has begun. The prominent bleb (B) on the cell wall (cw), is shown to contain a bdellovibrio (bd). Note the blister site (arrow) on the plasma membrane (pm) adjacent to the bleb.

Fig. 46.2 & 46.3 Glutaraldehyde-fixed preparations similar to 46.1. The bdellovibrio (bd) in 46.3 extends below the substrate level and very likely is in contact with the blister site on the plasma membrane (pm) of *S. serpens* VHL.

(Reproduced by permission from Snellen and Starr, 1974).

cell surface and the host's cytoplasmic membrane (Cover and Rittenberg, 1984).

Investigations have been carried out on the nature of a receptor molecule for *Bdellovibrio* in the envelope of the host cell. Varon and Shilo (1969) studied the attachment of *B. bacteriovorus* to wild type and mutant strains of *Salmonella* spp. and *E. coli*. Mutant strains had deficiencies in the structure of the cell envelope lipopolysaccharide. Host bacteria lacking the O-specific side chains but containing a complete "rough" core were better receptors than wild type or "smooth" strains containing complete cores and outer repeating units. Absence of specific sugars from the "rough" core or R-antigen of the host cell, reduced the numbers of attached *Bdellovibrio* cells (Varon and Shilo, 1969). Houston *et al.* (1974) found that as increasing amounts of the R-antigen were used in an assay, there was greater inhibition of attachment of *B. bacteriovorus* to *S. typhimurium*. This suggests that the receptors for *Bdellovibrio* are in the host cell's R-antigen (Houston *et al.*, 1974). However, a masking of receptors or steric hindrance of attachment may also explain the decrease in *Bdellovibrio* attachment (Varon and Shilo, 1980).

Chemeris *et al.* (1984) carried out investigations into carbohydrate-lectin interaction during *Bdellovibrio* attachment. Sugars capable of binding to lectins and so blocking the *Bdellovibrio* - host cell contact, were added to the host-parasite mixtures. The sugars which inhibited attachment of *B. bacteriovorus* IBFM B-608 to *Erwinia carotovora* were mannose, glucose, arabinose, lactose, sucrose and rhamnose. When *B. bacteriovorus* 109 was combined with *E. coli* the inhibitory sugars were mannose, glucose and galactose. Both sets of host-parasite cells were treated with concanavalin A (con A) to clarify which of the partners was

the carrier of carbohydrate receptors (Chemeris *et al.*, 1984). Treatment of *B. bacteriovorus* 109 with con A had no effect on the interaction with *E. coli* B. Con A blocks the mannose receptors on the surfaces of *E. coli* cells, so preventing *Bdellovibrio* attachment (Chemeris *et al.*, 1984). Blocking of mannose and glucose-containing receptors in the cells of *Erwinia carotovora* had no effect on *Bdellovibrio* attachment. This is due to the presence of free host cell receptors in *E. carotovora* containing arabinose, lactose, sucrose and rhamnose (Chemeris *et al.*, 1984). Modification of the polysaccharide layer of *E. carotovora* with sodium periodate slowed down the interaction with *Bdellovibrio.* The presence of lectins on the *Bdellovibrio* cells for which carbohydrates in the host cells act as receptors appears to determine the specificity of interaction (Chemeris *et al.*, 1984).

On a molecular level, Thomashow and Rittenberg (1978) found that during the initial stages of intraperiplasmic growth of *B. bacteriovorus* on *E. coli*, peptidoglycan of *E. coli* becomes acylated with long-chain fatty acids. The fatty acids, some from the murein lipoprotein in the *E. coli* envelope, were also covalently linked to the peptidoglycan of the developing bdelloplast (Thomashow and Rittenberg, 1978). Ruby and Rittenberg (1984) found that an early event in the infective cycle of *B. bacteriovorus* 109J was the attachment of diaminopimelic acid to the peptidoglycan of its prey. This process occurs over a range of prey genera, so that it is a general facet of the *Bdellovibrio* attack process.

Summary

1) *Bdellovibrio bacteriovorus* is a parasitic bacterium which occurs in the marine environment. *Bdellovibrio* multiplies in, and eventually lyses, its host cells (Stolp and Starr, 1963; Scherff *et al.*, 1966).

2) (i.). *B. bacteriovorus* appears to associate firmly with the host cell's cytoplasmic membrane during penetration. Fibres linking the *Bdellovibrio* anterior cell pole to the host's cytoplasmic membrane have been observed (Starr and Baigent, 1966; Burnham *et al.*, 1968; Abram and Davis, 1970; Abram *et al.*, 1974).

(ii.). Localized damage, in the form of a "scar" or "blister" was observed on the cytoplasmic membrane of *Spirillum serpens* during association with *Bdellovibrio* (Snellen and Starr, 1974). This could be the site of attachment of the *Bdellovibrio* cell.

3) (i.). *Bdellovibrio* appears to bind to sugars in the "rough" core of the outer cell envelope lipopolysaccharide of Gram-negative bacteria (Varon and Shilo, 1969). This was further shown by the inhibition of *B. bacteriovorus* attachment to *S. typhimurium* on addition of R-antigen (Houston *et al.*, 1974).

(ii.). Sugar inhibition tests have suggested that lectins on the *Bdellovibrio* cell may bind to carbohydrate receptors in the cell envelope of certain host cells during the interaction (Chemeris *et al.*, 1984).

Chapter 8 Microbial adhesion to marine invertebrates

The adhesion of microorganisms to certain marine invertebrates, as with fish tissues, can be an important initial stage in causation of invertebrate diseases. Additionally, the association of bacteria pathogenic to man with some invertebrates can spread human diseases. There are, however, some cases where microbial adhesion to invertebrates can be a symbiotic association, where both microorganism and invertebrate benefit. This quite extensive chapter considers microbial association with several invertebrate species, in an attempt to illustrate these points.

(8.1.). Wood-boring isopods

The exoskeleton of the marine wood-boring isopod *Limnoria tripunctata* is extensively colonized by microorganisms (Sleeter *et al.*, 1978; Boyle and Mitchell, 1981, 1984; Zachary *et al.*, 1983). A diverse population of rod-shaped, stalked and coccal bacteria are present on the outer surfaces of both *Limnoria tripunctata* and *Limnoria lignorum*. In particular, the pleopods and telson of these isopods are densely colonized (Sleeter *et al.*, 1978). However, there are few colonizing bacteria on the mandibular apparatus and head surfaces of these isopods. These are closely pressed against the wood surface during boring (Sleeter *et al.*, 1978). Many rod-shaped bacteria seen on the pleopods and telson produce large amounts of extracellular polymer (Boyle and Mitchell, 1981, 1984). *Limnoria* spp. frequently groom their appendages by passing them over the mouth parts; this could result in ingestion of bacteria. Consequently, attached bacteria on these appendages may represent a food source for *Limnoria* spp.

(Boyle and Mitchell, 1981). Bacteria attached to the pleopods may utilize waste products and respiratory metabolites passed across the pleopod surfaces (Sleeter *et al.*, 1978; Boyle and Mitchell, 1981). The association of bacteria with *Limnoria* spp. may be symbiotic, as both the isopods and bacteria appear to benefit.

Bacteria have been readily observed in the digestive tract of *L. tripunctata* feeding on creosote-treated wooden pilings (Zachary and Colwell, 1979; Zachary *et al.*, 1983). This resident microflora was closely associated with the gut lining, and was separated by a peritrophic membrane from other ingested microorganisms. The bacteria attached to the gut lining by production of extracellular polysaccharide material (Zachary and Colwell, 1979). Some of these ingested bacteria are lysed and so serve as an additional nitrogen source for *L. tripunctata*. Additionally, the isopod may benefit from bacterial detoxification of the creosote hydrocarbons, so allowing greater boring activity into creosoted wood (Zachary *et al.*, 1983). The creosote hydrocarbons could also provide nutrition for the bacteria, so that this could also be a symbiotic association.

However, species of *L. tripunctata* and *L. lignorum* exist which have a digestive tract free of attached microorganisms (Boyle and Mitchell, 1978; Sleeter *et al.*, 1978). Zachary *et al.* (1983) found that isopods on non-creosoted wood did not possess a gut microflora. Possible reasons for this are discussed later in this chapter (see p.225).

(8.2.) Crustacea - (1) Copepods

Most of the examples given here discuss the role of attached microorganisms in causing crustacean diseases.

Vibrio parahaemolyticus was found to adsorb to chitin particles and copepods from estuarine water suspensions (Kaneko and Colwell, 1975). The adsorption mechanism of *V. parahaemolyticus* to both surfaces may be electrostatic. Bacterial adsorption to copepods was less than to chitin particles. This may arise due to the wax layer on the copepod exoskeleton surface affecting the electrostatic interaction. Adsorption of *V. parahaemolyticus* to chitin is important in establishing an ecological niche for this bacterium (Kaneko and Colwell, 1975).

The human pathogen *Vibrio cholerae* associated in high numbers with calcium carbonate particles, but not with chitin (MacDonnell *et al.*, 1984). Strains of *V. cholerae* isolated from Chesapeake Bay and Bangladesh waters multiplied to high numbers when grown in association with live copepods (Huq *et al.*, 1983; 1984b). The bacterium was seen to attach in high numbers to the oral region of the copepods and the egg sac surfaces (Huq *et al.*, 1983, 1984b; see p. 207). Low attachment values of *V. cholerae* to dead copepods is backed up by the observations of low association with chitin by MacDonnell *et al.* (1984). Live copepods may excrete chemical attractant compounds specific for *V. cholerae* (Huq *et al.*, 1983). Attachment of *V. cholerae* to the copepod oral region suggests that the bacteria may act as a food source. *V. cholerae* may multiply when taken into the copepod gut, and subsequently be released back into the aquatic environment via faecal material. Attachment to the female copepod egg sac may also disseminate *V. cholerae*, as most planktonic copepods spawn fertilised eggs freely into the water (Huq *et al.*, 1983, 1984b). This association between *V. cholerae* and live planktonic copepods has implications for the epidemiology of cholera in endemic and non-endemic

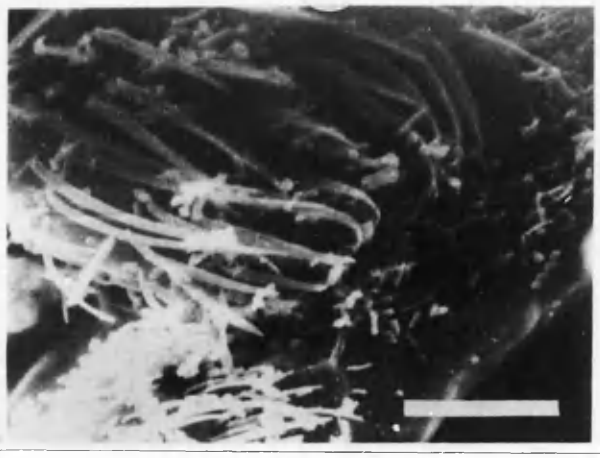


Fig. 47 Colonisation of a copepod oral region after incubation for 36 hours in Patuxent River water and *Vibrio cholerae* CA 401. Bar represents 10μm.

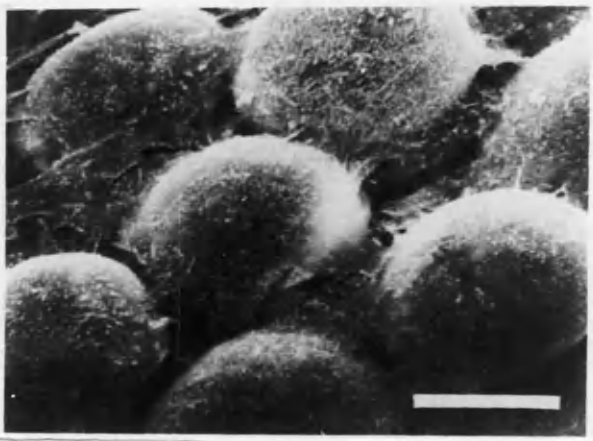


Fig. 48 Colonisation of a copepod egg sac after incubation for 36 hours in Patuxent River water and *Vibrio cholerae* CA 401. Bar represents 50μm.

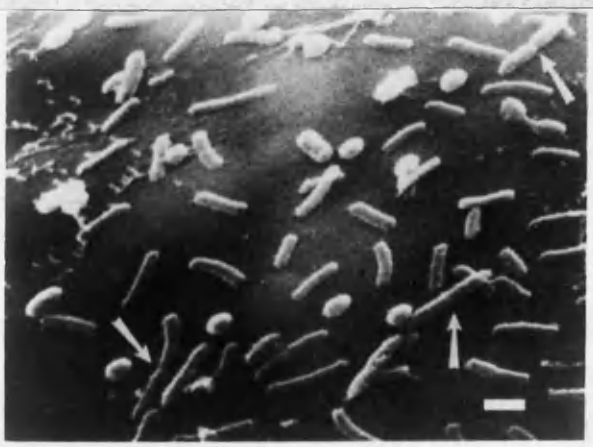


Fig. 49 Attachment of *Vibrio cholerae* CA 401 to a copepod egg sac surface and the presence of dividing cells (arrows). Bar represents 1μm.

(Reproduced by permission from Huq *et al.*, 1984b).

global areas. There is an epidemic of cholera in Bangladesh every year, commencing around September or October (Huq *et al.*, 1983, 1984b). Zooplankton populations in Bangladesh waters increase significantly during this time. This increase is usually followed by the appearance of epidemic cholera in Bangladesh (Huq *et al.*, 1983, 1984b). The observed colonisation of copepods by *V. cholerae* may well be an important means of spreading cholera in such locations.

However, although these observations show the role of planktonic copepods in dissemination of cholera in the aquatic environment, they do not show that this applies for other zooplankton. Huq *et al.* (1983, 1984b) only used planktonic copepods isolated from Chesapeake Bay (U.S.A.) and Bangladesh in their studies. *V. cholerae* may not attach so readily to other zooplankton which may dominate planktonic copepod populations in other parts of the World. Consequently, although the spread of cholera through planktonic copepods is probably important in Bangladesh, this should not be assumed to apply in general.

The observed association between *V. cholerae* and live copepods also has ecological significance. *Vibrio* spp. are known to produce active chitinase enzymes. Bacteria such as *V. cholerae* and *V. parahaemolyticus* could, therefore, be involved in the degradation of dead copepods in aquatic ecosystems (Huq *et al.*, 1983).

The effects of water temperature, salinity and pH on attachment of *V. cholerae* serovar 01 to planktonic copepods was studied by Huq *et al.* (1984a). Maximum attachment of the bacterium to copepods was observed to occur at 30°C, with significantly less attachment occurring at 5 and 15°C. Increases in salinity from 5 to 15% gradually increased *V. cholerae*

attachment. Most of the copepod surfaces were colonized at a salinity of 15‰. An alkaline pH of 8.5 was optimal for attachment and multiplication of *V. cholerae* (Huq *et al.*, 1984a). As observed by Huq *et al.* (1983), the highest concentration of attached bacterial cells was seen in the oral region. These results also have implications for cholera epidemiology in Bangladesh. The water temperature in Bangladesh during the zooplankton bloom often exceeds 25°C. This temperature allows extensive colonization of copepods by *V. cholerae*, despite the low salinities in much of the Bangladesh delta regions. A temperature range of 25-30°C gave the largest increase in numbers of *V. cholerae* attaching to copepods (Huq *et al.*, 1984a). The conditions existing in Bangladesh just after the monsoon season are ideal for extensive attachment of *V. cholerae* to copepods, and consequently for the spread of cholera (Huq *et al.*, 1984a). However, these particular conditions of pH, salinity and temperature may not be so suitable for *V. cholerae* attachment in other aquatic environments, or to other crustacea (see p. 221). Consequently, although these conditions may allow the spread of cholera in Bangladesh, they cannot be assumed to be generally suitable.

Some of these factors also affected *V. parahaemolyticus* adsorption to chitin and copepods (Kaneko and Colwell, 1975). Maximum adsorption in sodium chloride solution was found at 17‰ salinity. A pH range of 4-8 gave high *V. parahaemolyticus* adsorption, whilst 50% adsorption occurred at pH 10 (Kaneko and Colwell, 1975). This suggests that a more acidic environment favours adsorption of *V. parahaemolyticus* to copepods. These results also suggest that other pathogenic bacteria favour different environmental conditions from *V. cholerae* for adhesion to copepods and other crustacea.

Bacterial colonization of other copepods has been observed in some recent publications. There has been no taxonomic identification of the fouling species, although filamentous bacteria are frequent.

Extensive bacterial colonization of the copepod *Acartia clausi* was observed by Nagasawa *et al.* (1985b). In particular, the joints of segments and legs and indented parts of the copepod body surface were densely covered by bacteria. The attached bacteria included short rods, long and slender rods and stalked bacteria. Some attached rod-shaped bacteria were surrounded by extracellular material (Nagasawa *et al.*, 1985b). In addition, a branch-like filamentous growth, possibly of the bacterium *Leucothrix mucor* or cyanobacteria, covered parts of the copepod surface. The presence of abundant copepods with associated bacteria is ecologically important for marine food chains, as copepods are important food sources for carnivores (Nagasawa *et al.*, 1985b).

Extensive bacterial colonization of normal and abnormal specimens of the chaetognath *Sagitta crassa* was observed by Nagasawa *et al.* (1985a). Three main types of periphyte were observed on the chaetognath body surfaces : branch-like growths, filamentous bacteria and protruberances. Abnormal chaetognaths were also colonized by large numbers of bacteria. Large colonies of bacteria were seen in some cases, and there was evidence of extracellular polymer production which would attach bacteria to tissues (Nagasawa *et al.*, 1985a). Bacterial colonization was also observed in the muscles. The musculature of the body wall looked as if it had degenerated. Consequently, chaetognaths became knotty and flabby. Some chaetognaths had damage to their heads; extensive bacterial colonization was found on these

specimens. Attached bacteria thus appear to be agents of two different types of deformity in *Sagitta crassa* (Nagasawa *et al.*, 1985a).

Bacteria are also involved in two different 'X'-diseases of *Sagitta crassa* (Nagasawa and Nemoto, 1984). One of these diseases again results in an abnormal and grotesque appearance of the chaetognath. The other 'X'-disease involves a bacterial attack on the ciliary sense organs of *Sagitta crassa* and *Sagitta helenae*. The sense organs become heavily infested with bacteria, and some ciliary hairs are covered by bacterial slime (Nagasawa and Nemoto, 1984).

Leucothrix mucor frequently attaches to living marine surfaces, including seaweeds (see Chapter 6), and several invertebrate species. Appendages and eggs of benthic marine crustacea are often populated with *L. mucor*. Eggs of the rock crab *Cancer irroratus* possess a dense fungal-like growth between them which is *L. mucor* (Johnson *et al.*, 1971). The planktonic copepods *Acartia clausi* and *Pseudocalanus minutus* develop growths of *L. mucor* on their appendages in aquaria without antibiotics. Eggs of the copepod *Pseudocalanus americanus* also develop dense filamentous growth of *L. mucor* (Johnson *et al.*, 1971). Treatment with penicillin and streptomycin reduces the numbers of *L. mucor* filaments attaching to the crustacea. In some crustacea, the bacterium acts as a food source (Johnson *et al.*, 1971).

L. mucor frequently fouls the gills of shrimps, crabs and lobsters, and is usually accompanied by other epiphytes. Fouling of gills of these crustacea follows a similar sequence, with *L. mucor* and cyanobacterial attachment causing diatoms and detritus to become entangled. Stalked protozoa also attach to the crustacean gills or cuticle. This heavy

accumulation of microbial epiphytes on the gills is usually fatal. This next part discusses fouling of shrimps, crabs and lobsters by *L. mucor* and other microorganisms, and also gives some fouling control methods.

(8.2.). (2) Shrimps

The chemoreceptor setae of the brown shrimp *Crangon crangon* are infested by *L. mucor* filaments. Multifilament rosettes, characteristic of this epiphyte, can be seen arising from a holdfast structure on the setae (Shelton *et al.*, 1975). The association did not appear to be pathogenic in this case, as *Crangon crangon* could graze on the attached *L. mucor* filaments and obtain nutrients.

Certain species of shrimps, such as brown shrimp (*Penaeus aztecus* and white shrimp (*Penaeus setiferus*) develop filamentous gill disease. *Leucothrix mucor* has frequently been isolated from gills and pleopods of shrimps with this disease (Lightner *et al.*, 1975; Lightner, 1977). Filaments of *L. mucor* become entangled in the shrimp gills and trap detritus, filamentous blue-green bacteria and diatoms. This causes a green to dark brown discoloration of the gills. Death of shrimps with this disease usually results from anoxia. This is due to heavy accumulation of filamentous bacteria on the gills (Lightner *et al.*, 1975). *L. mucor* also appears to infest the brine shrimp *Artemia salina.* It attaches by a holdfast to the gills, swimming appendages and antennae of the shrimp. However, the bacterial filaments do not penetrate into the cuticle (Solangi *et al.*, 1979).

The peritrich ciliate *Zoothamnion* spp. also causes gill disease in penaeid shrimp (Lightner *et al.*, 1975). The protozoan attaches to the

shrimp's cuticle by a stalk, which terminates in a circular plate of 9-12μm in diameter (Foster *et al.*, 1978; see p.214). This basal disc adheres to the cuticle without penetrating it or the underlying tissue. As with filamentous gill disease, death results from anoxia due to the presence of numerous colonies of *Zoothamnion* spp. on the shrimps (Lightner *et al.*, 1975; Foster *et al.*, 1978).

Chemotherapy has been useful in the treatment of *L. mucor* infestations of crustacea. The commercially available algacide Cutrine-Plus was effective against filamentous gill disease of penaeid shrimp (Lightner and Supplee, 1976). Cutrine-Plus was administered to infested shrimps using both static and "flow-through" techniques. In 24hr "flow-through" treatments, a 0.1 ppm. concentration of Cutrine-Plus reduced *L. mucor* infestation of *Penaeus californiensis.* A 0.5 ppm Cutrine-Plus concentration over 4hr static treatments was equally effective (Lightner and Supplee, 1976; see p.217). Solangi *et al.* (1979) found that treatment with 100 ppm. of terramycin over a 2-day period effectively reduced *L. mucor* infestation of *Artemia salina.* The routine use of chemotherapeutic agents, such as Cutrine-Plus, in high density crustacean culture seems to be essential in controlling microbial diseases.

(8.2.). (3) Lobsters and Crabs

Certain cultured lobsters are extensively fouled by *L. mucor,* particularly on the carapace (Sieburth, 1975; see p.215,216). The epibiont frequently occurs on the cultured American lobster *Homarus americanus* (Nilson *et al.*, 1975; Fisher *et al.*, 1978). Certain filamentous cyanobacteria, such as *Oscillatoria* spp. and *Anabaena* spp. also attach to

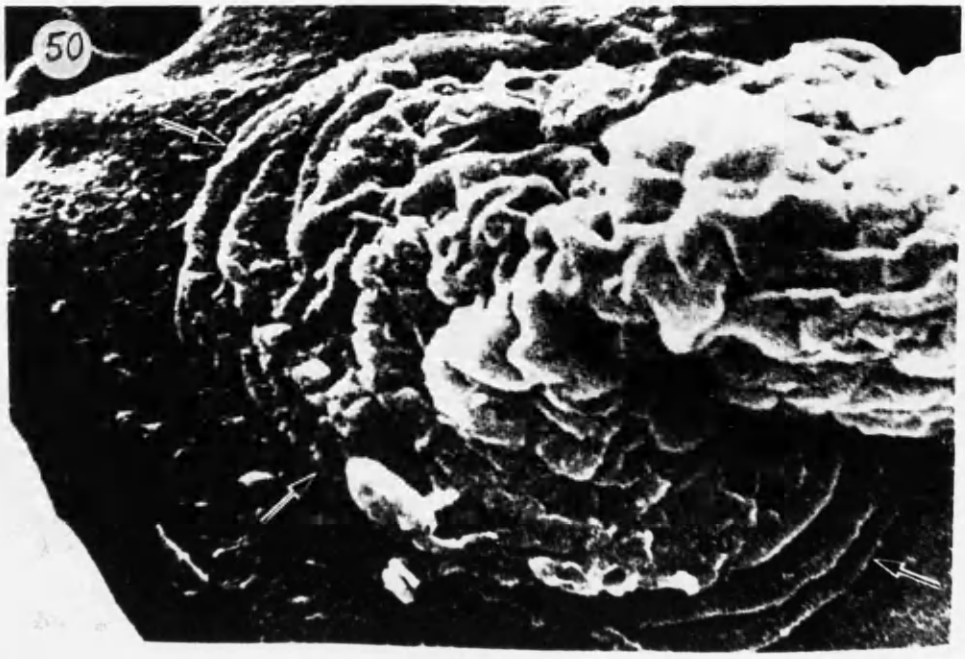


Fig. 50 SEM showing the attachment of *Zoothamnion* spp. to the cuticle of penaeid shrimp by a stalk terminating in a basal disc. Arrows denote the edge of the attachment plate on the cuticle. Mag. x11,000.

(Reproduced by permission from Foster *et al.*, 1978).

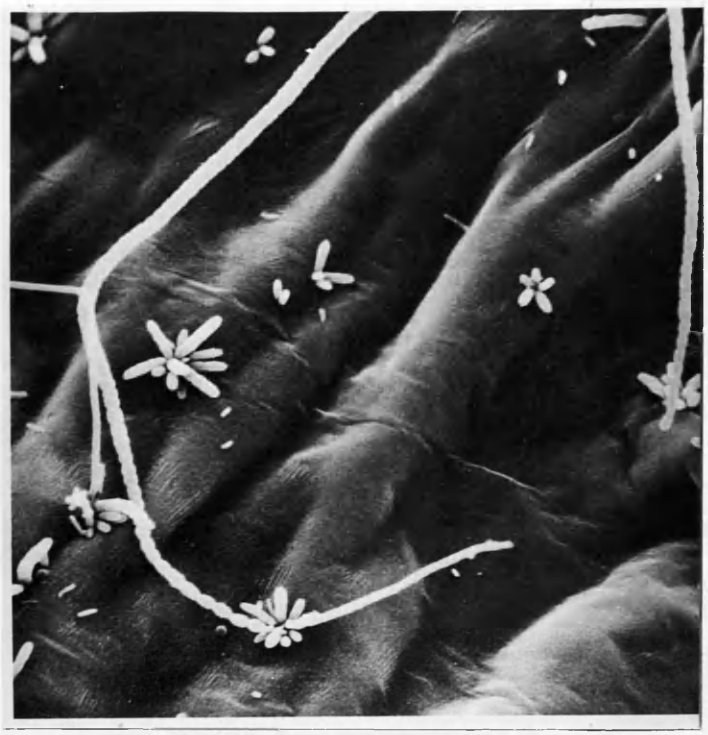


Fig. 51 Carapace of three-day-old lobster, showing different stages in the life cycle of *Leucothrix mucor.* Mag. x2,600.

Fig. 52 Carapace of three-day-old lobster, showing microcolonies of *Leucothrix mucor* and rod-shaped bacteria. Mag. x2,770.

(Reproduced by permission from Sieburth, 1975).

TABLE 9

Results of 4-hr. static treatments using 0.5 ppm copper as Cutrine-Plus as a chemotherapeutic for filamentous gill disease of shrimps.

Time period	No. examined	Average filamentous infestation of gills
Sept. 8-15	6	0.33
Sept. 16-30	6	0.66
Oct. 1-15	6	0.33
Oct. 16-31	6	0.33
Nov. 1-17	9	0.11
		$\bar{x}$ = 0.40

(from Lightner and Supplee, 1976)

the gills of this lobster. Stalked protozoans such as *Vorticella* spp. also occur heavily on egg or larval stages. Severe diatom accumulations which spread rapidly over the lobster gills can restrict metabolic exchange across the gill membranes. This causes mortality in juvenile stages of the lobster. This massive infestation of gill tissues by filamentous microorganisms kills lobsters (Nilson *et al.*, 1975; Fisher *et al.*, 1978). The antibiotics streptomycin and neomycin are effective in treatment of *L. mucor* infestations. Malachite green inhibits growth of bacterial and algal epibionts (Fisher *et al.*, 1978). Heavy microbial fouling was observed on eggs of the Dungeness crab *Cancer magister* (Fisher and Wickham, 1976). Egg mortalities were higher as the fouling increased. These were higher in the more developed eggs. Once again, prominent fouling microorganisms included filamentous cyanobacteria such as *Oscillatoria* spp. and filamentous *Leucothrix* spp. Stalked protozoans were also observed in heavily fouled egg samples. Again, the filamentous microorganisms trapped detrital material, which increased the overall fouling of the eggs (Fisher and Wickham, 1976). As with microbial fouling of shrimp and lobster gills, egg mortalities are caused by anoxia. The reproductive capacity of the Dungeness crab must be affected by epibiotic fouling of the eggs (Fisher and Wickham, 1976).

Gills of the rock crab *Cancer irroratus* were also extensively fouled by bacteria, peritrich and suctorian ciliates and diatoms (Bodammer and Sawyer, 1981). The authors did not identify many species of fouling microorganisms. However, *L. mucor* appeared to attach to the gill epicuticle by lightly staining holdfast material. Other filamentous bacteria were enmeshed in extracellular polysaccharide material on the

gill surface. Some bacteria, possibly *Caulobacter* spp., attached to the gill by holdfast structures embedded in amorphous material. Certain attached filamentous bacteria showed narrow hyphal-like extensions resembling *Rhodomicrobium* spp. or *Hyphomicrobium* spp. (Bodammer and Sawyer, 1981). Naviculoid diatoms were found associated with the extracellular material produced by fouling bacteria. A substratum, such as the crab gills, enriched with a bacterial polysaccharide slime layer, may attract fouling diatoms (see Chapter 3). As with shrimp and lobster gills, fouling bacteria and diatoms attracted dense detrital material between the gill lamellae. This extensive fouling would also greatly impair respiration in the rock crab. However, these epibionts may also serve as a food source for the grazing protozoa which were present.

From this account, it can be seen that there are similarities in epibiotic microbial fouling of gills of shrimps, lobsters and crabs. This also applies to Dungeness crab eggs (Fisher and Wickham, 1976).

The similarities in fouling sequence in these invertebrates suggests that there may be similarities in the substratum nature, that is, the gill surfaces. The presence of bacterial polysaccharide slime may attract fouling diatoms to the gills, as suggested by Bodammer and Sawyer (1981). An accumulation of microorganisms producing extracellular slime would easily trap detrital material. This would result in the heavy fouling observed on the gills. Certain antibiotics appear to effectively alleviate this fouling. However, further research on the effectiveness of antibiotics and other anti-fouling agents on invertebrate gill fouling is needed.

Examples are known where pathogenic microorganisms, usually present in

discharged sewage, associate with crab tissues. This can result in the spread of human disease. These will now be described.

Several potentially pathogenic bacteria were isolated from edible crabs collected near Kodiak Island in Alaska (Faghri *et al.*, 1984). The bacteria isolated included *Yersinia enterocolitica*, *Klebsiella pneumoniae* and *Staphylococcus* spp. The presence of these bacteria may have resulted from faecal contamination of the crab tissues with sewage. Examination of the rock crab shells by scanning electron microscopy revealed many cracks and fissures. Bacteria and fungi were attached within these fissures. Many attached coccoid-shaped bacteria appeared to be growing through the crab shells (Faghri *et al.*, 1984). Chitinase-producing microorganisms, many of which were coccoid-shaped, could cause breaks in the shell surface. These openings through the shell provide one route by which microorganisms could reach and contaminate underlying muscle tissues. This in turn could result in food poisoning outbreaks from consumption of these crabs (Faghri *et al.*, 1984).

These observations raise questions over the safety of crabs collected from sea regions that receive untreated sewage effluent. They show that potential human pathogens in seawater can readily accumulate in crab tissues. The levels of contaminating, pathogenic microorganisms in the crab tissues must be measured by adequate microbiological tests. These tests could measure the levels of pathogenic bacteria before possible consumption of the crabs.

Eleven sporadic incidents of cholera occurred in Louisiana during 1978. The disease was probably spread by the blue crab *Callinectes*

sapidus. *Vibrio cholerae* must be associated with the blue crab for this to occur. Dietrich *et al.* (1984) studied the effects of certain environmental factors on attachment of *V. cholerae* to blue crab shell. The organism adhered more readily at a temperature of 35°C than at 25°C. This compares with maximum attachment of the bacterium to copepods occurring at 30°C (Huq *et al.*, 1984a; see p.209). A sodium chloride concentration in seawater of 1.5 to 2.0% was optimum for bacterial adherence (see p. 222). Acidic to neutral pH values were more suitable for *V. cholerae* attachment to blue crab shell (Dietrich *et al.*, 1984). This bacterium did not associate well with chitin compared to calcium carbonate particles (MacDonnell *et al.*, 1984; see p.206). Approximately the same number of *V. cholerae* cells attached to blue crab shell with some chitin extracted as attached to crude chitin (Dietrich *et al.*, 1984). This suggested that other components of the crab shell, such as calcium carbonate, influence *V. cholerae* adhesion.

Dietrich *et al's* (1984) results show differences from those of Huq *et al.* (1984a). An alkaline pH value was optimal for *V. cholerae* attachment to copepods (Huq *et al.*, 1984a). This differs from the acidic to neutral pH values which allowed higher *V. cholerae* adhesion to blue crab shell (Dietrich *et al.*, 1984). A salinity of 15% was optimal for *V. cholerae* adhesion to copepods (Huq *et al.*, 1984a). This is higher than the 1.5 to 2.0% salinity range which gave high bacterial adhesion to blue crab shell (Dietrich *et al.*, 1984). These observations support the suggestion (see p. 209), that different environmental conditions may be suitable for adhesion of *V. cholerae* to different crustacea.

Vibrio cholerae was also observed to attach to the gut wall of

TABLE 10

Adherence of *Vibrio cholerae* to Crab Shells as a function of salt concentration after 3-hrs. incubation at 35°C.

Salt Concentration	Cell counts per gram of shell[a,b].
0%	5.0×10^7
0.5%	8.6×10^7
1.0%	1.7×10^8
1.5%	1.9×10^8
2.0%	2.2×10^8
2.5%	1.3×10^8
3.0%	1.1×10^8
3.5%	4.2×10^7

[a] Initial inoculum : 1.4×10^7 cells/g of shell.

[b] Mean of two trials.

(from Dietrich *et al.*, 1984).

Callinectes sapidus (Huq *et al.*, 1986). Examination of the crab guts showed that the bacterium attached in greater numbers to the hindgut than the midgut. Huq *et al.* (1983, 1984b) showed that *V. cholerae* attached in high numbers to the oral region and egg sacs of planktonic copepods (see p. 207). These observations, together with those of Huq *et al.* (1986), suggest that crustacea are important reservoirs of *V. cholerae* in the aquatic environment. These findings are also significant for the transmission and epidemiology of cholera in aqueous environments. The observations of Huq *et al.* (1986) further emphasize the importance of adequate microbiological tests of seafood to detect levels of human pathogens.

(8.3.). Absence or presence of an intestinal microflora in certain invertebrates

Certain invertebrates, such as the oyster *Crassostrea gigas* and some wood boring isopods, do not possess an intestinal microflora. However, others such as the large deep-sea isopod *Bathynomus giganteus* do. Reasons for these differences are not fully known. Some examples will now be discussed.

An attached microflora was absent from the epithelial surfaces of the mantle cavity and alimentary tract of the adult oyster *Crassostrea gigas* (Garland *et al.*, 1982). The external shell surface of this oyster, however, was colonized by various diatoms and bacteria. Some anatomical and physiological aspects of suspension-feeding oysters could explain these results. The alimentary tract of *Crassostrea gigas* contains a ciliated epithelium and mucous layer. Some microorganisms can become enmeshed in the mucous layer. However, the rapid pumping of the ciliated epithelium will clear any settled microorganisms from the digestive tract. Release

of digestive enzymes from the digestive tract and crystalline style of the oyster may also inhibit microbial growth (Garland *et al.*, 1982).

In contrast to these observations, *Cristispira* spp. spirochaetes were associated with the crystalline style of *Crassostrea virginica* (Tall and Nauman, 1981). *Cristispira* spp. were not observed on the styles of *Crassostrea gigas* by Garland *et al.* (1982). The style of *Crassostrea virginica* consists of a spongy inner layer covered by a smoother outer layer. *Cristispira* spp. were associated with both the inner and outer layers of the style. Some spirochaetes were observed emerging through and adhering to the style surface by blunt-tipped ends (Tall and Nauman, 1981). The presence of *Cristispira* spp. spirochaetes inside the style suggests that they may obtain nutrients from the style.

Digestive tracts of the wood-boring isopods *Limnoria tripunctata* and *L. lignorum* are free of attached microorganisms. This was observed by Boyle and Mitchell (1978) and Sleeter *et al.* (1978) using entire digestive tracts. Faecal pellets within the isopod digestive tracts are sparsely colonized by bacteria.

There was also a lack of surface microorganisms associated with the Arctic amphipod *Boeckosimus affinis* (Atlas *et al.*, 1982). As with the wood-boring isopods, no microorganisms were associated with the lining of the foregut, midgut and hindgut. However, some microorganisms were observed colonizing food particles within the amphipod's midgut. Low microbial populations were attached to faecal matter in the hindgut. There are possible reasons for the lack of a surface microflora. These amphipods burrow in sediment, and so become covered with sediment particles

and microorganisms. However, when they move into seawater in search of food, these sediment particles and microorganisms may be removed during swimming. The amphipods used in this work (Atlas *et al.*, 1982) were captured on bait suspended in the water column. Consequently, the swimming period prior to capture could remove any attached microorganisms from the amphipod intestine.

On the other hand, the large deep-sea isopod *Bathynomus giganteus* possessed a diverse and dense digestive tract microflora (Boyle and Mitchell, 1982). Anterior sections of the isopod intestine contained a mixed microflora occurring in close association with the gut lining. Large numbers of bacteria and other microorganisms were seen associated with food material filling the gut lumen. Some stalked bacteria were seen, together with filamentous material which was probably bacterial extracellular polysaccharide (Boyle and Mitchell, 1982). This material would allow bacterial adhesion. There was a distinctive microflora in the digestive tract area posterior to the midgut sphincter. The population consisted almost entirely of a large, rod-shaped bacterium. These bacterial cells measured approx. 1.9 x 8.5μm. They were firmly attached to the intestinal lining, and were associated with a large quantity of mucus-like material (Boyle and Mitchell, 1982).

Wood-boring isopods, oysters and an Arctic amphipod appear to have digestive tracts which contrast with the tract of *Bathynomus giganteus*. The production of anti-microbial agents or digestive enzymes within these invertebrate groups could be possible reasons for the absence of a gut microflora. However, no such substances have been isolated from their guts. Certain colonizing bacteria may become resistant to such substances

a ciliated epithelium or by the passage of seawater through the gut are unlikely to occur in *Limnoria tripunctata*. Conditions such as pH, temperature and nutrient availability within the digestive tract of *Bathynomus giganteus* may be more suitable for extensive microbial colonization.

(8.4.). Bacterial associations with octopuses and squids

Skin ulcers developed on the octopuses *Octopus joubini* and *O. briareus* when they were reared in high density groups (Hanlon *et al.*, 1984). The ulcers first affected the skin epidermis of the octopuses, before penetrating down through the dermis and underlying muscle tissues. These ulcers were usually fatal if untreated. Bacteria were found in moderate to high densities on diseased octopus skin epidermis. Numerous bacteria were embedded in a mesh of polymeric fibres when the ulcers penetrated the muscle tissues. These fibres may have been extracellular polysaccharide material, which would aid bacterial adhesion to the diseased tissue. The bacterial mat in the ulcers was similar in appearance to the floc produced by *Vibrio alginolyticus* in liquid culture. Five bacterial species were isolated from octopus ulcers, including *V. alginolyticus*, *Vibrio parahaemolyticus* and *Pseudomonas stutzeri*. The ulcers were successfully treated with the antibacterial agent nufurpirinol. This further suggests that skin ulcer formation was a bacterial disease, with few other microorganisms involved (Hanlon *et al.*, 1984).

The accessory nidamental gland of the female squid *Loligo pealei* is thought to play a secretory role in the reproductive system. The tubules of the accessory gland contain a dense population of rod- and coccoid-

shaped bacteria (Bloodgood, 1977). There was some evidence in electron micrographs of extracellular polymeric fibrils being produced by these bacteria. This material would aid their adhesion in the nidamental gland. The accessory glands of sexually mature squids contain a mixture of red, white and yellow tubules. In each case, the colours of tubules were due to the colours of the bacterial populations present. This was particularly true of the red coloured tubules. Repeated washings of cell pellets of the red-pigmented colonizing bacteria did not remove the colour (Bloodgood, 1977). The red coloration of the squid nidamental gland makes it sexually attractive. Consequently, the red pigmented bacteria within the tubules are in turn making the squid sexually attractive.

This example shows that bacteria colonizing a marine invertebrate serve a commensalistic function. The red pigmented bacteria in the accessory nidamental gland tubules make the squid more sexually attractive. Consequently, *Loligo pealei* benefits, in reproductive terms, from these red pigmented bacteria. There are other examples of possible symbiotic relationships resulting from microbial associations with marine invertebrates. The existence of these relationships have not all been proved experimentally, but have been mainly derived from electron microscope observations. Some of these will now be discussed.

(8.5.) Role of microbial adhesion in symbiosis with marine invertebrates

Most of these examples relate to microbial associations with deposit-feeding and deep-sea invertebrates. However, an interesting example of symbiosis between a sponge and marine bacterium is also mentioned.

The gutless marine oligochaete *Phallodrilus leukodermatus* possesses a narrow subcuticular space between the epidermis and cuticle. This subcuticular space contained numerous aggregations of rod-shaped bacteria (Giere, 1981). Some of these bacterial cells were close to the oligochaete's cuticle. The cells would probably aggregate by production of extracellular polymeric material, although large amounts were not observed (Giere, 1981). As these bacteria appear to be well integrated in the anatomy of *Phallodrilus leukodermatus*, a symbiotic relationship seems possible. The bacteria could act as nutritional intermediaries for the oligochaete. They could take up dissolved organic compounds for their own use, whilst producing substances needed for metabolism of *Phallodrilus leukodermatus* (Giere, 1981). This relationship may not be symbiotic, however, as none of the bacteria were attached to the oligochaete tissues. Closer association between the bacteria and oligochaete would allow nutrient exchange to occur more easily.

Another marine oligochaete, *Tubificoides benedii* found in a sulphide-rich sediment habitat, was colonized in the posterior region by filamentous epibacteria (Dubilier, 1986). The bacteria were embedded in the oligochaete cuticle, although they did not penetrate into the epidermis. Collection sites of densely colonized oligochaetes smelt strongly of hydrogen sulphide. This suggested that the epibacteria could be filamentous sulphur-oxidizing bacteria such as *Leucothrix* spp. There was no evidence of the colonization being pathogenic. The bacteria were never observed in the vicinity of cuticle lesions. There was no change in the physical condition and behaviour of colonized worms (Dubilier, 1986). These two observations suggest that this bacterium is unlikely to be an ectoparasite of *Tubificoides benedii*. The presence of bacterial

colonization on the posterior end of the oligochaete suggests existence of an interaction. *T. benedii* is usually oriented in the sediment with its tail sticking out. This could benefit the colonizing bacteria by giving them an aerobic environment. An influx of essential nutrients, such as oxygen, sulphate and low molecular weight organics, from seawater alongside the oligochaete, may also benefit the bacteria. This interaction, therefore, appears to be commensalistic (Dubilier, 1986). Possible benefits for *T. benedii* are not fully understood. Further investigations are needed to show whether the association is a symbiotic one.

A previously undescribed archaeogastropod limpet was found on hydrothermal vents in the N.E. Pacific. Dense aggregations of filamentous bacteria were observed on the epithelium of the limpet gill (de Burgh and Singla, 1984). Densely-staining material was observed in the space between the bacterial cell walls and the gill epithelial cell membrane. This was probably bacterial extracellular polymeric material which would allow adhesion of the bacterial cells to the limpet gill. Bacteria similar in appearance to those on the gill were also observed in transverse sections of the limpet's gut (de Burgh and Singla, 1984). These observations suggest the existence of a mutualistic relationship. The filamentous bacteria on the gill surface may benefit from the exchange of essential nutrients across the gill. The limpet could benefit from the release of dissolved organic molecules as by-products of bacterial metabolism. These substances would be absorbed by the gills. Endocytosis of bacteria into the gill tissues was also observed. The bacteria were seen to pass into lysosomes, in which they were digested (de Burgh and Singla, 1984). Digestion of the endocytosed bacteria would also

benefit the limpet. A further benefit for the limpet may arise from digestion of bacteria seen in the gut. It is uncertain how these bacteria are taken into the gut, although they could be orally ingested (de Burgh and Singla, 1984). Further electron microscopic investigations of possible bacterial colonization of the limpet's oral region are needed to study possible ingestion.

Individuals of the deposit-feeding echinoid *Echinocardium cordatum* possessed irregular to round-shaped nodules in their intestinal caeca (de Ridder *et al.*, 1985). The nodules consisted of a central detrital core surrounded by coats of actively growing bacteria and empty bacterial sheaths. Four main types of bacteria were observed around the core. The included chains of large rod-shaped bacteria which formed the main coat of the nodules and rosette-forming bacteria. The large rod-shaped bacteria possessed sheaths which contained ferric oxide and some polysaccharide material. The sheaths probably help bacterial adhesion in the nodule's core. The bacterial rosettes were attached to chains of large, unsheathed bacteria (de Ridder *et al.*, 1985). A symbiotic relationship could exist between the bacteria around the core and the echinoid, although it has not been experimentally proven. Some hypotheses have been suggested which will now be discussed.

The central detrital core in the nodules may provide energy for the colonizing bacteria and the echinoid (de Ridder *et al.*, 1985). Experimental work which could prove this would include isolating the *Leptothrix*-like bacteria and core detrital material to carry out bacterial growth and metabolism experiments. The bacteria and detrital material could firstly be separated by a technique such as ultracentrifugation.

Following this, they would be grown in a medium containing the detrital material alone. A control experiment using a medium containing detrital material together with additional, essential growth supplements would also be needed. Comparisons would then be made of bacterial metabolism in each of the media, by, for example, bacterial protein measurements and lipid content assays. This would show whether the *Leptothrix* spp.-like bacteria are utilizing the detrital material. A further experiment could show whether the echinoid, as well as the sheathed bacteria, are obtaining energy from the detrital core. This would involve measuring ATP levels in isolated *E. cordatum* caecal tissue which contains nodules. Similar control measurements would be made in echinoid caecal tissue from which nodules were extracted. However, the disadvantage of this method is that any increased ATP levels in caecal tissue may not necessarily indicate utilization of core organic material. Increased ATP levels may result from aerobic respiration or lipid metabolism in the caecal tissues. An alternative method would involve ^{14}C-labelling of the nodule core organic material. After a few hours incubation, caecal tissue would be isolated and ^{14}C-levels measured. The presence of ^{14}C in surrounding caecal tissue would indicate possible assimilation of detrital material into carbohydrate or lipid. A control experiment would be set up by ^{14}C-labelling *E. cordatum* caecal tissues from which nodules were removed.

Further evidence of possible symbiosis comes from previous observations of high quantities of ferric phosphate in the echinoid's intestinal connective tissue. The investigation by de Ridder *et al.* (1985) confirmed the presence of oxidized iron deposits in the echinoid's tissue. *E. cordatum* may benefit from bacterial iron oxidation preventing reduction of intra-digestive sediment to form hydrogen sulphide in the

hindpart of the gut (de Ridder *et al.*, 1985). However, this theory fails to allow for the possibility that iron oxidation may provide the necessary anaerobic conditions in the hindgut for reduction of sediment sulphate to hydrogen sulphide (see Chapter 11). An experiment which could show this would involve removing hindgut tissue from echinoids containing nodules, and ligaturing it at either end to keep it closed. The hindgut tissue could then be placed on a small iron sheet to show iron sulphide formation. The formation of a black precipitate on the iron sheet, representing iron sulphide, would indicate hydrogen sulphide formation within the hindgut. A control experiment would involve using hindgut tissue from echinoids whose nodules were removed.

Deposits of ferric iron were also found in the sheaths of the rod-shaped bacteria in the nodule coats. The bacteria may benefit from iron oxidation for the following reasons. *Leptothrix*- spp. bacteria produce sheaths of inorganic materials, containing iron or manganese oxides which facilitate their adhesion to surfaces (see Chapter 1.2, p. 26). Formation of further ferric iron within the bacterial sheaths would allow production of more adhesive material. This, in turn, may either allow firmer adhesion in the nodules or greater numbers of bacteria to attach. Experiments to test these possibilities would involve isolating nodule bacteria and culturing them in a medium containing iron. A control experiment would involve growing the bacteria in a medium without iron. Attachment of the cultured bacteria to glass slides could then be observed by light microscopy. These observations would show whether oxidation of iron in the medium leads to higher numbers of *Leptothrix* spp.-like bacteria attaching. Increased firmness of adhesion could be tested by subjecting

the glass slide to shear such as that produced by a water jet. If the nodule bacteria benefit more from iron oxidation than does the echinoid, then the relationship could be regarded as being commensalistic rather than symbiotic. The matter clearly needs further investigation.

A further interesting example of possible symbiosis arose from the isolation and characterization of a lectin from the marine sponge *Halichondria panicea* (Muller *et al.*, 1981). Bacteria of the species *Pseudomonas insolita* were also isolated from this sponge. The lectin consisted of 80.7% protein and 14% carbohydrate. Addition of the lectin to a culture of *P. insolita* caused rapid growth. Other lectins, such as concanavalin A, did not exhibit a growth-promoting effect. The lectin isolated from the sponge did not affect growth of *P. insolita* isolated from six other species of marine sponges.

These observations led Muller *et al* (1981) to suggest the possibility of a lectin-mediated symbiotic relationship existing between the sponge and *P. insolita.* However, there is a weakness in this suggestion. Muller *et al.* (1981) only showed the benefits of this lectin to the bacterium. They did not demonstrate that *Halichondria panicea* benefits. Further experimental work would be required to show this.

There is another interesting aspect of this work. Certain lectins are known to be involved in a re-aggregation of sponge cells (see refs. in Muller *et al.*, 1981). If cells of *P. insolita* are added to a suspension of dissociated *H. panicea* cells, then the bacteria may bind to sponge cell lectins. The bacteria would be able to bind to lectin molecules on the surfaces of several sponge cells. This would ultimately lead to clumping of the separated *H. panicea* cells, which would in turn result in sponge

cell re-aggregation. This process is similar to viral haemagglutination, where viruses bind to red blood cells by haemagglutinins present in the virion. This results in clumping of the red blood cells. However, it is also possible that bacteria binding to sponge cell lectins may block any interaction between the sponge cells leading to re-aggregation.

Muller *et al.* (1981) provided further evidence which suggested that there is an interaction between *P. insolita* and the sponge cell lectin. A polysaccharide-containing fraction was isolated from the cell envelope of *P. insolita*. Addition of the polysaccharide fraction to a suspension of lectin and the bacteria abolished the growth promoted by the lectin. The polysaccharide may bind to receptor molecules on the *P. insolita* cell surface which themselves bind to the sponge cell lectin. The polysaccharide may therefore prevent the bacterium binding to the lectin. Muller *et al.* (1981) suggested that the *H. panicea* lectin may trigger bacterial nucleic acid synthesis after binding to the cell wall. Hence, the polysaccharide material, by blocking the bacterium-lectin interaction, may diminish growth of *P. insolita* by preventing bacterial protein synthesis.

There is further experimental work which could be done to show that an interaction occurs between *P. insolita* and the sponge cell lectin. These would include ^{14}C-labelling experiments. ^{14}C isotopes would be added to a suspension of the lectin and *P. insolita*. They would label any polysaccharide-containing material on the bacterial cell surface which may bind to the lectin, as well as labelling the carbohydrate material in the lectin. Autoradiographs would show any bacterium-lectin interaction. Other work would involve use of enzymes which could inhibit any

interaction. Enzymes, such as amylases, lipases or proteinases, could be added at specific concentrations to suspensions of the lectin and bacterium. If the enzymes block the interaction, then bacterial growth will cease. The enzymes might completely digest or alter the bacterial cell surface component which binds to the lectin or the lectin itself. Either process would certainly block the bacterium-sponge lectin interaction. Further work could involve addition of certain sugars, such as L-fucose, D-galactose or D-galacturonic acid, which could be lectin or bacterial receptor analogues, to bacterium-lectin suspensions. As with the enzyme work, any block in the interaction would be shown by diminished bacterial growth. The sugars would block the interaction either by binding to the chemically related binding site on the sponge lectin, or to the bacterial cell surface component. Further experimental work, using antibodies targeted against specific antigens on the bacterial cell surface or the sponge lectin, could ultimately isolate the specific components involved in the interaction. The antibodies would either bind to bacterial cell surface antigens or to antigens in the sponge lectin, so again blocking the interaction.

Muller *et al.* (1981) also showed that erythrocytes bound to *H. panicea* cells to form rosettes. Rosette formation was inhibited by 80% upon addition of D-galacturonic acid to the erythrocyte-sponge cell suspension. These observations suggest that lectin molecules on the sponge cell surfaces interact with the erythrocytes resulting in rosette formation. It should therefore be possible, in the same way, to set up *P. insolita* - *H. panicea* suspensions. Bacteria binding to sponge cells could then be observed by light or phase-contrast microscopy. These observations could

be further enhanced by, for example, acridine orange straining. This would further show that an interaction occurs between lectin molecules on the sponge cells and *P. insolita* cells. This in turn could allow further sugar or enzyme or antibody inhibition tests to further study the interaction mechanism.

Overall summary of Chapter

This extensive Chapter has discussed several important ecological aspects of microbial attachment to marine invertebrates. The major points shown, together with the key cited papers, are as follows.

1) Microorganisms frequently extensively colonize the pleopods and telson of wood-boring isopods, such as *Limnoria tripunctata* (Sleeter *et al.*, 1978; Boyle and Mitchell, 1981). These microorganisms may act as a food source for the isopods. Bacteria are also found associated with the gut-lining of isopods feeding on creosote-treated wood (Zachary *et al.*, 1983).

2) *Vibrio cholerae* attaches in high numbers to the oral region and egg sacs of planktonic copepods (Huq *et al.*, 1983,1984a,b). The pathogen may subsequently be released back into the aquatic environment. An increase in zooplankton populations is usually followed by a cholera epidemic in Bangladesh. Attachment of *V. cholerae* copepods is affected by salinity, water temperature and pH (Huq *et al.*, 1984a).

3) Extensive bacterial colonization of other copepods, such as *Acartia clausi*, has also been observed (Nagasawa *et al.*, 1985b). Some attached bacteria produced extracellular material; filamentous *Leucothrix mucor* were also attached to the copepod surface.

4) (i) *Leucothrix mucor* frequently attaches to gill tissues of shrimps, lobsters and crabs (Lightner *et al.*, 1975; Nilson *et al.*, 1975; Bodammer and Sawyer, 1981). Attachment of this bacterium to gill tissues is usually accompanied by attachment of diatoms, cyanobacteria and detritus. Stalked protozoa such as *Zoothamnion* spp. (Foster *et al.*, 1978) and *Vorticella* spp. (Fisher *et al.*, 1978) also attach to gills of these invertebrates. This heavy accumulation of microbial epiphytes on these invertebrate gills causes death by suffocation.

(ii) Chemotherapy can control *L. mucor* and other microbial infestations of these invertebrate gills. The algacide Cutrine-Plus was effective at controlling *L. mucor* infestation of penaied shrimp gills (Lightner and Supplee, 1976). Antibiotics, such as terramycin, streptomycin and neomycin are also effective at controlling *L. mucor* infestations of shrimps and lobsters (Fisher *et al.*, 1978; Solangi *et al.*, 1979).

5) *Vibrio cholerae* attaches to the shell of the blue crab *Callinectes sapidus*. Attachment was also affected by temperature, pH and salinity values (Dietrich *et al.*, 1984). This bacterium also attaches to the gut wall, particularly the hindgut, of the blue crab (Huq *et al.*, 1986). These findings are also important for the transmission and epidemiology of cholera in the aqueous environment.

6) (i) Certain marine invertebrates appear to lack an attached gut microflora. These include the oyster *Crassostrea gigas* (Garland *et al.*, 1982), wood-boring isopod *Limnoria tripunctata* (Boyle and Mitchell, 1978) and the amphipod *Boeckosimus affinis* (Atlas *et al.*, 1982). Production of anti-microbial agents within the guts or removal of attached microorganisms by seawater could be possible reasons for absence of a microflora.

(ii) In contrast, the large deep-sea isopod *Bathynomus giganteus* possesses a rich and diverse gut microflora (Boyle and Mitchell, 1982). The tract area posterior to the midgut sphincter contained a large rod-shaped bacterial morphotype which was firmly attached to the gut lining. Conditions within this isopod's digestive tract may be

more suitable for microbial colonization.

7) Fatal skin ulcers developed on the octopuses *Octopus joubini* and *O. briareus* when they were reared in high density groups. Bacteria isolated from the diseased tissue included pathogenic *Vibrio* spp. Numerous bacteria were embedded in a mesh of polymeric fibres, probably extracellular adhesive, within the ulcer tissue (Hanlon *et al.*, 1984).

8) The tubules of the accessory nidamental gland of the squid *Loligo pealei* contain a large population of rod- and coccoid-shaped bacteria. The accessory glands of these squids contain red pigmented tubules. The colour was due to the red pigmented bacteria present. The red colouration of the squid nidamental gland makes it sexually attractive. Consequently, the red pigmented bacteria in the tubules are making *L. pealei* sexually attractive. These pigmented bacteria are serving a commensalistic function for the squid (Bloodgood, 1977).

9) Several examples are known of microbial associations with marine invertebrates which lead to possible symbiotic relationships. The existence of these relationships have not all been proved experimentally. Further experimental work is required to prove the hypotheses.

(i) The marine oligochaete *Tubificoides benedii*, found in a sulphide-rich sediment habitat, was colonized by filamentous epibacteria in the posterior region (Dubilier, 1986). There was no evidence of the colonization being pathogenic. The existence of an aerobic environment, together with an influx of essential nutrients from

seawater, would benefit the bacteria. This appears to be a commensalistic relationship.

(ii) Dense aggregations of filamentous bacteria were observed on the gill epithelium of an archaeogastropod limpet (de Burgh and Singla, 1984). Bacteria similar to those on the gill were also observed in the limpet's gut. The bacteria on the gill surface may benefit from the exchange of essential nutrients across the gill. The limpet, in turn, could benefit from the release of dissolved organic molecules as by-products of bacterial metabolism. Bacteria were also observed to be endocytosed into lysosomes in the gill tissues. Subsequent digestion of the bacteria would also benefit the limpet (de Burgh and Singla, 1984).

(iii) Individuals of the deposit-feeding echinoid *Echinocardium cordatum* possess irregular to round-shaped nodules in their intestinal caeca. These nodules consisted of a central detrital core surrounded by coats of actively growing bacteria (de Ridder *et al.*, 1985). The bacteria included *Leptothrix* spp.-like rod-shaped bacteria possessing ferric oxide sheaths. The central detrital core in the nodules may provide energy for the echinoid and the bacteria. Further experimental work is needed to prove this. High quantities of oxidized iron were found in the echinoid's intestinal connective tissue. *E cordatum* may benefit from bacterial iron oxidation preventing reduction of intradigestive sediment to form hydrogen sulphide in the gut hindpart. The *Leptothrix*-like bacteria could themselves benefit from iron oxidation producing more adhesive material in their sheaths. This could either allow firmer adhesion

or greater bacterial numbers to attach in the nodules.

10) A lectin containing protein and carbohydrate was isolated from the marine sponge *Halichondria panicea.* This lectin caused rapid growth of *Pseudomonas insolita,* which was isolated from the sponge (Muller *et al.,* 1981). This suggested the existence of a symbiotic relationship, although only the bacterium appeared to benefit from it. *P. insolita* may be involved in re-aggregation of dissociated sponge cells by binding to lectin molecules on several sponge cells. Addition of a polysaccharide-containing fraction from the *P. insolita* cell envelope to a bacteria-lectin suspension abolished the bacterial growth-promoting effect. This suggested that the bacterial polysaccharide may block the interaction between *P. insolita* and the sponge cell lectin (Muller *et al.,* 1981). Further experimental work is needed to study the mechanism of this interaction, and to reveal any benefits which *H. panicea* as well as *P. insolita* may receive from this association.

SECTION 3.

ATTACHMENT OF MICROORGANISMS TO NON-LIVING MARINE SURFACES

Chapter 9 Microbial attachment to sediment particles

(9.1.). Attachment of marine microorganisms and role in formation of microbial mats and sediment stability

This chapter considers observed mechanisms, and the ecological importance of, microbial attachment to sediments. The first part describes observations of marine microbial attachment and its importance in the weathering of sediment particles and microbial mat formation. The second part discusses the importance of the attached sediment microflora in the nutrition of benthic invertebrates.

(9.1.1.). Observations of marine microbial attachment to sediment particles

Waksmaan and Vartiovaara (1938) made some of the earliest observations of microbial adsorption to marine sediments. They found that marine muds exerted an adsorptive effect upon bacteria. They added marine mud samples to flasks containing liquid bacterial cultures, which were then shaken. The bacterial populations in the supernatant liquid decreased over 21 hrs., indicating adsorption by the muds. Sand, however, had little adsorptive action upon either mixed or pure cultures of bacteria.

Meadows and Anderson (1968) made light microscopic observations of the distribution, types and abundance of microorganisms attached to littoral and sublittoral sand grains. Attached microorganisms were often present as colonies, consisting of 5-150 cells per colony. Many colonies contained one microbial species. Some colonies were surrounded by staining material, whilst others were unstained. The staining surrounding the colonies may represent microbial extracellular adhesive material

(Meadows and Anderson, 1968). Stained colonies were predominantly found in hollows on the sand grains. Large areas of the grain surface in between the colonies were completely bare. These observations suggest that microbial distribution may be related to the microtopography of the sand grain surface. Microorganisms appear to preferentially colonize sheltered parts of the grains. Coccoid bacteria, diatoms and blue-green bacteria were the main microorganisms observed on grains from the sediment surface. Meadows and Anderson (1968) also performed abrasion experiments to study the effects of agitation of the sand grains on the microbial distribution. Grains in flasks were shaken daily over a four week period, whilst control flasks were left unshaken. At the end of this period, some microbial colonies had appeared on the open surfaces of sand grains from the non-shake flasks but not from the shake flasks. However, microorganisms were still attached in the sheltered parts of grains from the shaken flasks. This shows that abrasion must be an environmental factor limiting attached microorganisms to hollows on sand grains (Meadows and Anderson, 1968). Abrasion may explain the lack of bacterial adsorption by sand grains added to shaken liquid bacterial cultures observed by Waksmaan and Vartiovaara (1938).

Wiese and Rheinheimer (1978) made similar observations of microbial colonization of quartz grains using scanning electron microscopy. They also observed a correlation between the site and density of bacterial colonization and the microtopography of individual sand grains. Quartz grains with a medium or weak degree of roundness showed the highest microbial population density. The highest density of colonizing bacteria was found in the vicinity of detrital depositions. These were also mainly observed in sheltered regions of the quartz grain, such as surface

fissures, conchoidal breakage sites and slight indentations. Diatoms were also attached in these sites (Wiese and Rheinheimer, 1978). Rod-shaped bacteria were attached to exposed parts of the grains which were free of detritus. Coccoid bacteria were observed in microcolonies of 5-20 cells. large colonies of 150 cells, such as those seen by Meadows and Anderson (1968), were rarely observed. Cocci were often attached to diatom frustules. Disc-shaped bacteria were also firmly attached to the grain surface. They produced protrusions which appeared to be polymeric threads excreted by the cells. However, the diameter of some of these appendages was so small that they could have been fimbrae. Bacteria which attached by polymer production were often associated with detrital matter. Filamentous, net-shaped polymeric structures were often observed. Cocci were often "tied" to these nets without having direct contact with the grain surface. This indirect mode of attachment allows these bacteria to use the major part of their cell surfaces to absorb nutrients from the interstitial water.

Observations of microbial colonization of silicate sand grains differing in microtopography also showed differences in biomass and community structure after eight weeks exposure to running seawater (Nickels *et al.*, 1981a). Scanning electron microscopy showed that a diverse microflora was sheltered in surface irregularities on some sand grains. Rod-shaped bacteria and diatoms were observed on these grains. Biomass measurements showed that phospholipid content, which was a measure of bacterial biomass, increased as the silicate grain shape became more irregular. Quantities of α-linoleic fatty acids, which was a measurement of algal populations, increased progressively like the bacteria. Increasing smoothness of the silicate grain surface resulted in a marked

decrease in bacterial and microeukaryotic populations. This was shown by a decrease in the total microbial biomass measured as phospholipid and extractable palmitic acid. Sand particles with rounded edges but with cracks and crevices on the grain surfaces had higher microeukaryotic populations than on the smooth glass beads. This was shown by increased amounts of polyenoic fatty acids.

Nickels *et al.* (1981a) also investigated the effects of location on microbial colonization of angular sand grains. Sand grains were incubated at the surface of a running seawater microcosm or taken from the sea bottom at 32m.. The angular sand grains incubated at the surface in flowing seawater showed a different microbial population than those taken from the sea floor. Sand grains from the running seawater microcosm had higher amounts of cyclopropane fatty acid-containing bacteria than grains from the sea floor. Microeukaryotes were found in greater amounts on sand grains from the sea bottom. Surface abrasion, which would tend to occur more often on the bottom sediment than in the running seawater tanks, probably would not have caused the community changes. This is mainly because the bottom sediment particles supported the richest microbial assemblies. Bacterial grazing by benthic invertebrates may have altered the community structure giving higher microeukaryotic populations (Nickels *et al.*, 1981a). However, the bacterial communities present on surface sand grains in the microcosms exposed to running seawater may not occur in the natural environment. Surface abrasion caused by tidal and wave action could alter the bacterial populations on the sand grains more than in the seawater microcosms. Bacterial populations in the natural environment may be much lower than those shown by Nickels *et al.* (1981a).

De Flaun and Mayer (1983) conducted scanning electron microscope

observations of microbial colonization of feldspar and clay grains from intertidal sediments. They also found that microtopography of feldspar grain surfaces influenced colonization by microbiota. Bacterial colonization was particularly observed in surface fissures, crevices, cleavages or ledges and concave abrasions. Bacteria colonizing feldspar grains attached by mucilage secretions. The mucilage was initially secreted as a holdfast, which became more intricate, webbed and fibrous until entire bacterial colonies were covered by mucopolysaccharide. Diatoms were also attached by mucilage produced from the periphery of their frustules. These mucilaginous webs tended to collect fine clay particles. These were either caught in bacterial fibrous webs or embedded in mucus surrounding diatoms. However, bacteria did not colonize clay particles which were smaller than 10μm.. The main reason for this is that the clay grains have smooth surfaces, with no shallow depressions favourable for bacterial colonization. In addition, clay particles will not provide protection against resuspension by shear forces unless they are larger than the bacteria.

De Flaun and Mayer (1983) also followed changes in the surface area of the sediment particles over a one year period. They found that particle surface areas increased from February to a peak in April. There was then a decline through the spring and summer until a further increase in the autumn. Bacterial populations on the sediment particles also fluctuated seasonally. Bacterial numbers and the extent of mucus coating development gradually increased from February to June, and there was a rapid increase into July. Bacterial populations appeared to follow increases in particle surface area during the first part of the year. However, the strong association of clay grains with bacterial exudates suggests that the

relationship of bacteria to surface area may partly be due to the bacteria. This can be explained as follows. Clay accumulation is important to the total sediment surface area. The accumulation of clays in these intertidal sediments requires biological incorporation of particles in bacterial extracellular polymers. Consequently, the relationship between bacteria and surface area may be caused by the extent of bacterial attachment and polymer secretion controlling clay incorporation (De Flaun and Mayer, 1983).

The extensive development of microbial mucus coatings observed during the spring and summer suggests that they may also be a significant source of nutrition for deposit feeders (De Flaun and Mayer, 1983; see Chapter 9.2).

The measurement of the biomass of sediment microflora by biochemical methods (Nickels *et al.* 1981a) and direct observation by scanning electron microscopy have several advantages and disadvantages. Some of these will now be discussed and compared.

One disadvantage of the biomass measurements which were used by Nickels *et al.* (1981a) was the use of phospholipid levels as a measure of bacterial populations. Phospholipids are also present in the cytoplasmic membranes of other attached microeukaryotes, such as green algae, diatoms and cyanobacteria. Hence they may over-estimate bacterial populations. In addition, the measurements of Nickels *et al.* (1981a) may include dead as well as living microbial cells. It is difficult using this method to distinguish between such dead cells and living microbial cells which could be associated with detritus in sheltered parts of the sand grain. However, an advantage of biomass measurements is that they can give an overall indication of the effects of environmental factors on attached

microbial populations.

The use of scanning electron microscopy has the advantage of allowing direct observation of attached microorganisms and their modes of adhesion. It may also be easier to distinguish between living and dead microbial cells. Disadvantages of scanning electron microscopy include the detachment of loosely attached or non-attached cells during the preparation of samples. Microbial extracellular polymers may shrink during the alcohol dehydration step, causing significant distortion in its appearance (Nicholson *et al.*, 1987).

Tufail (1985, 1987) made further observations of microbial attachment to intertidal sand grains which were enriched in photosynthetic and heterotrophic growth media. Sand grains from both growth media showed rich microbial growth. Grains from both enrichment media showed microbial growth on flat, rough and hollow regions (Tufail, 1985). This is in contrast to previous observations (Meadows and Anderson, 1968; Wiese and Rheinheimer, 1978). Tufail (1985) suggested that microbial growth may develop on flat surfaces when high levels of nutrients and low levels of sediment disturbance occur. This would occur in sheltered depositional environments such as estuaries and muddy shores.

Amphora spp. and the cyanobacterium *Schizothrix* spp. often formed single species aggregates on sand grains from the photosynthetic enrichment medium (Tufail, 1985 ;see p. 249). They were often also associated with each other as a double species aggregate. *Schizothrix* spp. formed an intricate network of filaments enveloping *Amphora* spp. (Tufail, 1985, 1987; see p. 249). The physiological basis for this association is not known. It may be a symbiotic relationship, although there is no evidence for this. Further work to show possible nutrient transfer between the microorganisms

Fig. 53.1 SEM showing mixed colony of *Amphora* spp. (diatom) and *Schizothrix* (cyanobacterium) on a sand grain. Mag x1100.

Fig. 53.2 A monospecific colony of *Amphora* spp. on a sand grain. Mag. x1100.

(Reproduced by permission from Tufail, 1985)

would be needed. Cells of *Amphora* spp. often formed clumps resembling segments of an orange which were found in crevices. Bacteria forming stellate colonies were also observed on sand grains from the photosynthetic medium. These bacteria had a beaded structure with pointed ends similar to the epiphyte *Leucothrix mucor* (Tufail, 1985; see Chapters 6 and 8).

Clusters of coccoid bacteria and scattered growth of bacilli were observed on sand grains grown in the heterotrophic enrichment medium. Coccoid cells formed dense and almost spherical colonies surrounded by a peripheral growth-free zone. This implied that some bacterial inhibition of the growth of adjacent microbial colonies was occurring. Bacilli were sometimes aggregated around what may have been detrital organic material. If this material was organic, then this observation further indicates the importance of the accumulation of organic matter on surfaces in bacterial adhesion (Zobell, 1943; Tufail, 1985).

Further observations were made by Tufail (1987) using sediment cores incubated with photosynthetic or heterotrophic media in the light and dark (PL, PD and HL, HD). The sediments incubated in the light (PL, HL) were designed to be comparable to intertidal and inshore surface sediments where there is high light intensity. The cores incubated in the dark (PD, HD) represented subsurface sediments in the same environments and also surface sediments below the euphotic zone (Tufail, 1987).

The observations of PL sand grains were similar to those made by Tufail (1985). More microbial growth was observed on subangular than on subrounded grains. This confirms the observations of Wiese and Rheinheimer (1978). Clumps of three to seven grains were sometimes bound together by dense growth of filamentous cyanobacteria and pennate diatoms. Sporangia of *Thraustochytrium* spp. (see Chapter 4) also occurred on PL sand

grains. The sporangium appeared as a thick-walled, semi-globular structure with a corrugated surface. Thraustochytrids occurred amongst diatoms, bacteria and detritus or near cyanobacterial filaments. They were also found on flat, bare grain surfaces (Tufail, 1987). There was no sign, however, of ectoplasmic net material produced from the thraustochytrid sporangia (see Chapter 4).

Microbial growth occurred mainly in crevices and depressions on PD sand grains. Blue-green bacteria and diatoms were very rare. Bacterial rods, cocci, a few filaments and detrital aggregates were present (Tufail, 1987). Clumping of sand grains rarely occurred. Long fungal-like hyphae were attached to individual grains. They also stretched from one point to another on the same grain surface or to an adjacent grain.

The HL sand grains had a similar appearance to those observed by Tufail (1985). Microbial mats were common and contained rods, cocci, filaments and detrital aggregates. Filamentous bacteria sometimes formed a characteristic network amongst other bacteria and detritus (Tufail, 1987). *Caulobacters* were also occasionally seen. Similar coccoid bacterial colonies to those observed by Tufail (1985) were seen. Some cocci formed microcolonies of 15 to 50 cells connected by polymer strands.

Observations of HD sand grains showed that dense microbial growth occurred more on subrounded than on subangular grains. Cocci and rods formed thick, irregular mats (Tufail, 1987). Other recognisable bacteria including *Caulobacter* spp. and *Flexibacter* spp. were also seen. Some spirochaetes were observed in close association with other bacteria and detrital aggregates. Fine threadlike strands, probably adhesive polymer, were observed bridging between bacterial cells and detritus.

These observations show how sediment enrichment cores simulating

different environments can produce different microbial communities. They also demonstrate how ecological stress factors can influence the development of different microbial communities.

There were some interesting aspects of the populations that developed in these enrichment cores. Sand grains from the PL cores showed a large growth of photosynthetic microorganisms such as diatoms and blue-green bacteria. These would develop in highly illuminated sheltered sediments occurring intertidally or in the immediate subtidal range (Tufail, 1987). In addition, the occurrence of thraustochytrids on PL sand grains is interesting. Such observations show that thraustochytrid sporangia from marine sediments can be grown under controlled enrichment conditions similar to their natural environment.

The microbial populations on the PD grains were similar to those on the HL grains. Heterotrophic bacteria were present on the PD grains, although not in such large numbers as in the HL and HD sediments. Their appearance on the PD grains suggests that the abundance of heterotrophic bacteria is not affected by light (Tufail, 1987). The HL and HD cores contained a wide range of morphological types and high numbers of heterotrophic bacteria. This shows the effects of high levels of nutrients, as would occur near sewage outlets, on bacterial numbers.

Microbial attachment is also of importance in the weathering of sediment particles. Frankel (1977) showed how this occurred in biotite and hornblende grains from estuarine sands. Attached microorganisms were concentrated in depressed areas of hornblende grains. Diatoms, cyanobacteria and suctorian ciliates were particularly abundant in cleavage regions on hornblende grains. Microorganisms penetrated the grains via fractures and cleavage planes. Further "quarrying" by the microorganisms

resulted in short cleavage fragments breaking off, which reduced the size of the grains. A similar process occurred in biotite grains. Bacteria were the main colonizers of the flat and smooth grain surfaces. The largest number of colonizing microorganisms were again found in grain fractures and cleavages. Weathering of biotite grains occurred in a similar way to hornblende. Growth and reproduction by colonizing microorganisms caused openings to develop, which ultimately resulted in the grains splitting apart (Frankel, 1977). Weathering can increase the surface area of sediment particles available for microbial colonization. An increase in the numbers of attached microorganisms could increase sediment stability. This would result from microbial extracellular secretions binding sediment particles together (see sub-Chapter 9.1.2.).

Rades-Rohkohl *et al.* (1978) observed the attachment of *Brevibacterium* sp. S and *Bacillus* sp. U and W to quartz surfaces using TEM, SEM and phase-contrast microscopy. They assessed microbial weathering of the quartz surface. The three bacterial strains were chosen because they had the following properties (Rades-Rohkohl *et al.*, 1978):-

1.) They were all originally isolated from a quartzitic surface ;
2.) They differed in their ability to produce surface polymer ;
3.) They showed differences in their tendancy to adhere.

After eight days' incubation, *Bacillus* sp. U and *Brevibacterium* sp. S covered nearly the entire quartz surface. This could result from a gradual accumulation of nutrients on quartz (Rades-Rohkohl *et al.*, 1978).

The three bacteria showed different mechanisms of adhesion. Molecular attraction forces probably allowed the attachment of

Brevibacterium sp. S. There was no evidence for the production of adhesive polymer and fimbrae or flagella were not present.

Bacillus sp. U was attached by copious amounts of adhesive polymer. It was difficult to see whether the entire polymer capsule mediated bacterial attachment or only specific polymer threads. *Bacillus* sp. W showed little tendency to adhere. This bacterium seemed to attach partly by polymer production and partly by adhesive forces.

Rades-Rohkohl *et al.* (1978) used a replica technique to observe hollows on the quartz surface which may have been caused by microbial weathering. The technique involved placing thin cellulose acetate replicating tape over the entire quartz surface, including any attached bacterial colonies. The tape was then removed and turned upside down and coated with gold. It was then turned back and the cellulose acetate was dissolved with acetone. The resulting mould showed the upper surface of attached bacterial colonies and any hollows on the quartz surface. It was also possible to see the part of the bacterial colonies which had been in contact with the quartz (Rades-Rohkohl *et al.*, 1978). Some hollows were observed close to colonies of *Bacillus* sp. U. These hollows may have resulted from erosion, or been areas of bacterial contact with the quartz, which were similar in appearance. However, processes such as repeated drying and wetting during the preparation of materials for S.E.M. may have caused interaction of bacterial polymers with quartz.

In interpreting these observations, it should be remembered that quartzite may not be the natural habitat of these bacteria (Rades-Rohkohl *et al.*, 1978). It would be interesting to undertake further studies of these bacteria interacting with other sediment minerals such as feldspar. This may show whether such bacterial interactions are unique to quartz, or

whether certain minerals are more prone to bacterial weathering than others.

(9.1.2). Formation of microbial mats and role of these and microbial polymeric secretions in sediment stability.

Microbial mat formation is known to be important in binding together sediment particles and providing sediment stability. Production of microbial adhesive polymers often occurs during mat formation and is also of importance in stabilization.

Stal *et al.* (1985) made observations of vertically stratified microbial communities in the upper intertidal zones of the North Sea island Mellum. Growth and development of the cyanobacterial mat was followed over three years. Initial colonization was by *Oscillatoria* spp. *Microcoleus chthonoplastes* then became the dominant organism, and was sometimes mixed with *Oscillatoria* spp.. *M. chthonoplastes* formed a tough, coherent mat structure largely through the production of a mucilaginous sheath. Cyanobacteria occurred in the top layer of the mat, covering a layer of purple sulphur bacteria and sulphate-reducing bacteria (Stal *et al.*, 1985).

The appearance of *Oscillatoria* spp. in the mat was correlated with nitrogenase activity. This indicates that nitrogen fixation remains an important ecological factor even after establishment of the mat system (Stal *et al.*, 1985). In addition, cyanobacteria in microbial mats need to glide in the direction of light so as to find optimal growth conditions. Experiments showed that light penetrated through 5mm. of the sand above the microbial mat. Light penetrated better through wet sand than dry sand. This would tend to occur in the natural environment. Artificial mats of

M. chthonoplastes were buried under 3mm. of sand in the laboratory and exposed to light. The cyanobacteria were found at the surface within 2 or 3 h.

Nicholson *et al.* (1987) made interesting observations of a flat, laminated microbial mat at Great Sippewissett Marsh, Cape Cod, Massachusetts. They studied the microzonation of phototrophic bacteria in the mat using a combination of light microscopy, scanning and transmission electron microscopy. The mat was approx. 1 cm. thick and was located in sandy intertidal sediments of the marsh. It consisted of four to five distinctly coloured layers, which were named as follows :

1) Layer 1 on the surface was yellowish-brown in colour and was named the "Gold Layer";
2) Layer 2 below that was dark green to bluish green in colour, and was named the "Upper Green Layer";
3) Layer 3 was the "Pink Layer";
4) Layer 4 was the "Peach Layer" because of its salmon-orange hue,
5) Layer 5 was the "Lower Green Layer" which was very thin, of 1 mm. thickness, or was non-existent in places.

Extensive production of microbial extracellular polymers was observed in all the layers. This material was responsible for cell attachment to sand grains, for lamination of layers and the structural integrity of the mat.

Filamentous cyanobacteria and diatoms were the predominant microorganisms in the uppermost Gold Layer. Small coccoid bacteria were observed singly or in clusters. Cyanobacteria which were present included *Lyngbya aestaurii*, some heterocystous and filamentous

cyanobacteria and *Phormidium* spp.. Diatoms, particularly *Navicula* spp. were particularly abundant. Areas of the mat and marsh which appeared brown on the surface contained large numbers of diatoms producing much extracellular polymer. This adhesive material bound sediment particles together, so contributing to sediment stability.

The Upper Green Layer was similar to the Gold Layer in that the main photosynthetic organisms present were filamentous cyanobacteria and diatoms. Clusters of coccoid purple sulphur bacteria, *Thiocapsa* spp., were also present in smaller amounts. The diversity of cyanobacteria in this layer was more limited. Filaments of *Oscillatoria* spp. dominated this layer and probably imparted its blue-green colour. *Phormidium* spp. were also observed. Many diatoms were dead or undergoing degradation. The sediment particles were also coated and held together by the abundant microorganisms and their extracellular polymers. Intertwining of the sediment particles by filamentous microbial adhesive together with their coating of bacterial slime resulted in good cohesion of this layer.

In the Pink Layer, various photosynthetic purple sulphur bacteria were present in abundance, forming a "coccoid mat layer". Such bacteria which could be distinguished included *Amoebobacter roseum*, *Thiocapsa* spp. and *Chromatium* spp.. Large clumps of coccoid bacterial cells were held together by extracellular polymers and/or a film-like sheath. Single coccoid cells were often attached to these larger clumps or to sediment particle surfaces. Long chains of cyanobacterial cells, possibly *Nostoc* or *Nodularia* spp. were directly attached to the sediment particles. A large amount of microbial polymeric material was present. This probably accounted for the high degree of cohesion of both this layer and the Peach Layer below. The purple sulphur bacteria contributed greatly to the

binding of sediment by copious production of extracellular polymer.

The Peach Layer was dominated by small to medium-sized coccoid cells which possessed internal sulphur globules. These were identified as being the purple sulphur bacterium *Thiocapsa pfennigii.* "Asterisk-shaped" cells which were probably the green sulphur bacterium *Prosthecochloris aestaurii* were also present. Some filamentous bacteria were also observed. Coccoid cells in this layer formed looser sheets of cells than in the Pink Layer. Filamentous bacteria were often intertwined amongst coccoid cells. The cohesion of sediment particles in this layer was comparable to the Pink and Upper Green Layers. Sediment stability was again imparted by bacterial polymeric material, which often formed filamentous strands.

The small prosthecate green sulphur bacterium *Prostheocochloris aestaurii* was conspicuous in the very thin Lower Green Layer. This bacterium possessed numerous knobby cell surface projections. *Thiocapsa pfennigii* and other purple sulphur bacteria also occurred in this layer. Small coccoid cells were frequently observed. Cells of *P. aestaurii* occurred in large or small irregular masses surrounded by large amounts of extracellular polymeric material. Some of this was dried into long, uneven strands. Sediment cohesion in this layer was much less than in the above layers.

Although microbial extracellular polymers gave good cohesion to each of the mat layers, some differences existed between them. This was shown by differential separation of the Pink, Peach, Upper Green and Gold Layers occurring when any shear force was applied.

The overall appearance of the microbial populations in each mat layer was not only due to their cellular morphologies, but also to their general habits of growth. This included the degree of cell clumping and the

degree of binding of cells to sediment particles by filamentous microorganisms and adhesive polymers.

It should be remembered that this study by Nicholson *et al.* (1987) represented the mat community at the height of its development in the summer. Seasonal changes occurred, and markedly changed the mat's structure. These changes may be more drastic for these portions of the mat closely adjacent to sand dunes. Prevailing onshore winds and tidal inundation would tend to bury the mat. Observations during the winter indicated that the mat was periodically buried by sand but remained colourful for some weeks. However, it began to decompose when the temperature rose above freezing. Decomposition of the Upper Green Layer was most marked with the development of black sulphide-rich sediments. Periodic burial of a mat near a sand dune resulted in the formation of layers of distinctively banded sediment. Iron sulphide precipitation took place in these layers. Sediment stability would probably not be so high in the decomposing mat. It would be interesting to do further measurements of the cohesion of the mat during decomposition. Further electron microscopic observations of the extent of microbial adhesive production in decomposing mats could show the extent of sediment stability.

Gelatinous material was observed binding together calcarenite grains to form a coherent mat on the Bahamas sea floor (Bathurst, 1967). The subtidal mat was of a pale brown or green colour, and had a fibrous appearance under the microscope. It contained many microorganisms, including filamentous bacteria, diatoms and numerous cyanobacteria including *Oscillatoria* spp.. The colour of the mat was largely derived from the motile diatoms that were on the upper surfaces of the calcarenite grains. The gelatinous material is probably derived from microbial

adhesive secretions, mainly from diatoms and cyanobacteria. The gelatinous nature of the mat binds surface calcarenite grains together, making a hydrodynamically stable sediment. However, the mat was rapidly destroyed if buried. This suggests a similar pattern of decomposition to that observed by Nicholson *et al.* (1987) after the burial of the mat at Great Sippewissett Marsh.

Mucilaginous material was also observed on and within the interstices between sand grains in estuarine sand flats in Connecticut (Frankel and Mead, 1973). This matrix also probably results from mucilaginous adhesive secretions of the microorganisms. Bacteria, diatoms, cyanobacteria, filamentous algae, peritrichs and suctorian ciliates were abundant in this matrix. Chemical analysis showed that the mat was largely composed of pectic carbohydrate with small amounts of protein. This compares favourably with microalgal adhesive secretions. Microscopic examination showed that each mucilage clump was an aggregate. It was composed of many small subclumps of clear to cloudy material which enveloped mineral clumps, skeletal parts and small living organisms. The mucilage consisted of viscid, viscous and elastic substances. It acted not only as a grain binder but also as an elastic cushion between the grains. The algal mat complex represented only the upper part of an extensive biogenic sediment stabilizing system. Below the mat, stability was rendered by the accumulation of adhesive secretions produced by microorganisms attached to the grains and within the interstices.

Mucilage-producing diatoms were cultured in flasks containing a variety of sediments (Holland *et al.*, 1974). The sediments used included sand, mixtures of sands, silts and clays, sand and kaolin, and kaolin itself. The diatoms were effective sediment stabilizers, in that they

significantly reduced resuspension and retarded laminar flow of sediments. The ability of diatoms to reduce sediment resuspension was related to their production of adhesive mucilage. This could be associated with the production of mucilaginous tubes or stalks (see Chapter 3.2). Non-mucilage secreting diatoms were not so effective at stabilizing sediments. The diatoms *Cylindrotheca closterium, Hantzschia amphioxys* and *Navicula directa* significantly reduced resuspension in cultures containing silts and clays or clay alone. These diatoms also produced mucilage in control flasks which did not contain sediment. The mucilage formed a film on the walls of the flasks. When these flasks were severely agitated, pieces of mucilage broke away from the walls. They formed flakes that consisted of large numbers of cells bound by a common mucilage.

The diatom mucilage promoted adhesion between sediment particles by filling interstitial voids. This in turn will decrease particle resuspension and increase sediment stability. Stabilization could also be extended to deeper layers of the sediment, due to the vertical migration of benthic diatoms (Holland *et al.*, 1974).

Further experimental work of this kind should be done with other mucilage-producing diatoms. These would include *Amphora* spp., *Licmophora* spp. and *Achnanthes* spp. (see Chapter 3.2). Sheath-producing cyanobacteria, such as *Oscillatoria* spp. which have frequently been observed in mucilaginous mats, should also be studied. These studies would further show the effects of other adhesive microorganisms on sediment stability.

Summary

The key points discussed in this sub-chapter are as follows :

1.) (i.). Observations of microbial attachment to sand grains suggests that microbial distribution is related to the microtopography of the grain surface (Meadows and Anderson, 1968; Wiese and Rheinheimer, 1978; Nickels *et al.*, 1981a). Rod-shaped bacteria, coccoid bacterial colonies and diatoms were often attached to sheltered regions of the grains.

(ii.). Agitation of sand grains limited attached microorganisms to hollow regions on the grain surface (Meadows and Anderson, 1968). Nickels *et al.* (1981a) showed that location of silicate grains affects the attached microbial populations. Grains incubated at the surface of a seawater microcosm had higher bacterial populations than those from the sea bottom, which were high in microeukaryotes.

2.) Microtopography of feldspar grain surfaces also influenced bacterial colonization (De Flaun and Mayer, 1983). Bacteria attached to feldspar grains by mucilage production which ultimately covered entire colonies. Clay particles tended to collect in the mucilaginous webs. Bacterial populations and mucilage production tended to follow increases in feldspar grain surface area. The accumulation of clay particles in bacterial polymers suggests that bacterial attachment and polymer secretion may control sediment surface area.

3.) Observations of microbial attachment and growth to sand grains incubated in photosynthetic or heterotrophic growth media were made by Tufail (1985, 1987). Photosynthetic microorganisms, such as *Amphora* spp. and cyanobacteria, tended to attach to grains from the photosynthetic enrichment medium. Thraustochytrids were mainly

observed on grains from the heterotrophic growth medium. The observations showed how sediment enrichment simulating different environment produce different microbial communities.

4.) (i.). Microorganisms attached to biotite and hornblende grains can cause weathering. Attached diatoms, cyanobacteria and ciliates were particularly abundant in cleavage regions (Frankel, 1977). Further penetration by these microorganisms into cleavage planes and fractures may cause grain fragments to break off.

(ii.). Rades-Rohkohl *et al.* (1978) observed that three bacterial strains, *Brevibacterium* sp. S, *Bacillus* spp. U and W, attached in different ways to quartz. A replica technique showed hollows on the quartz surface close to bacterial colonies. These may have been sites of bacterial erosion.

5.) (i.). Stal *et al.* (1985) followed the growth and development of a cyanobacterial mat in an upper intertidal zone. *Oscillatoria* spp. was the initial colonizing organism, and its appearance was correlated with nitrogenase activity. *Microcoleus chthonplastes* then became the dominant organism, and formed a tough coherent mat structure.

(ii.) Nicholson *et al.* (1987) made observations of a laminated microbial mat in Cape Cod, Massachusetts. The mat was approx. 1 cm. thick and consisted of four to five distinctly coloured layers. Extensive production of microbial extracellular polymers was observed in all layers. This material was responsible for cell attachment to sand grains, lamination of mat layers and structural integrity of the mat. Seasonal changes markedly altered the mat's structure and stability.

6.) (i.). Gelatinous material was observed binding together

calcarenite grains on the Bahamas sea floor to form a coherent mat (Bathurst, 1967). It contained many microorganisms, and its gelatinous nature was probably derived from microbial adhesive secretions. The gelatinous mat produced a hydrodynamically stable sediment.

(ii.). Mucilaginous material was also observed on and within the interstices between sand grains in estuarine sand flats (Frankel and Mead, 1973). Chemical analysis showed the mat's composition to be similar to microalgal adhesive secretions. The mucilage acted as a grain binder and an elastic cushion.

7.) The diatoms *Cylindrotheca closterium, Hantzschia amphioxys* and *Navicula directa* significantly reduced resuspension and retarded laminar flow of a variety of sediments. The ability of the diatoms to stabilize sediments was related to their production of adhesive mucilage (Holland *et al.*, 1974).

(9.2). Importance of attached sediment microflora in the nutrition of benthic invertebrates

The utilization of microorganisms attached to sediment particles by various species of benthic invertebrates has generated an extensive literature. Other studies have indicated the importance of microbial extracellular adhesive materials as alternative food sources for these invertebrates. This sub-chapter will review the importance of both these factors.

Hargrave (1970) compared the relative importance of benthic bacteria, algae and non-living organic matter as food for the common freshwater amphipod *Hyallela azteca*. In preliminary experiments, *H. azteca* rapidly converted samples of surface sediment into faecal pellets. Their contents consisted of aggregates of diatom frustules, green algal and cyanobacterial chains which had been attached to the sediment particles (Hargrave, 1970). Certain bacteria, such as *Pseudomonas* spp. and the diatom *Navicula* spp. were efficiently assimilated. Measurements of sediment protein levels suggested that bacteria were consumed more often by *H. azteca* than the rest of the attached organic matters.

Fenchel *et al.* (1975) compared the particle size selection of the prosobranch *Hydrobia ulvae* with that of the amphipod *Corophium volutator*. Qualitative differences in the diets of these invertebrates were explained by their particle size selection. Diatoms played a larger role in the diet of *H. ulvae*, whilst *C. volutator* fed mainly on bacteria (Fenchel *et al.*, 1975). Feeding experiments using ^{14}C-labelled microorganisms showed that *C. volutator* only utilized bacteria adsorbed to sand particles in the size range of 4-63 μm.. This explained why clay and silt particles were

necessary in this sediment for efficient feeding of *C. volutator*. However, *H. ulvae* only utilized microorganisms attached to larger sediment particles in the size range 200-300 µm.. There was also evidence that this prosobranch utilized free, unattached bacteria on the seawater surface film by using secreted mucus (Fenchel *et al.*, 1975).

Kofoed (1975) studied the benthic nutrition of *Hydrobia ventrosa*. The assimilation efficiencies of seven diatom species, two cyanobacteria and one bacterial species were compared using a ^{14}C-radiotracer technique. The diatoms were assimilated at an efficiency of 60-70% (see p. 267). This suggested that attached diatoms are an important energy source for *H. ventrosa*. The bacteria were assimilated with 75% efficiency, whilst the cyanobacteria were less well utilized. Attached and clumped bacteria were also an important food source. Single, unattached bacterial cells were below the normal size range for utilization by *H. ventrosa*.

Biochemical measurements of microbial cellular components have been used as a means of observing microbial colonization of silicate grains (see Chapter 9.1). Such methods can also be used to observe benthic microbial consumption. Findlay and White (1983) used biochemical analysis to study the effects of the sand dollar *Mellita quinquiesperforata* on a benthic microbial community. Several lipid components of the microbiota decreased during feeding. There was a 45% reduction in the community microbial biomass measured as lipid phosphate. Movement of *M. quinquiesperforata* through the field sediments decreased the amounts of muramic acid, which was also used as a measure of microbial biomass. Analysis of the gut contents showed that the sand dollar fed mainly on diatoms, foraminifera, dinoflagellates, and other non-photosynthetic microeukaryotes. *M. quinquiesperforata* also utilized bacteria attached to silt and clay

TABLE 11

The assimilation efficiency of *Hydrobia ventrosa* measured by the "total consumption radiotracer" method. (Number of experiments in parantheses).

Food	% Assimilation efficiency	
	Range	Mean
Chroococcus sp. (1)	8	8
Oscillatoria sp. (5)	48-53.	49.
Nitzschia angularis (6)	60-70.	64.
Cylindrotheca fusiformis (2)	64-66.	65.
Nitzschia sp. (3)	68-70.	69.
Amphora sp. (1).	61.	61.
Diatom sp. 30d (1)	74.	74.
Diatom sp. A2 (2)	63-70.	67.
Diatom sp. 500 (2)	66-69.	67.

(Taken from Kofoed, 1975).

particles in the sediments.

Possible disadvantages of using biochemical assays to measure microbial colonization of sediment particles were discussed in Chapter 9.1 (see p. 247). There are also some disadvantages in using this method to study microbial utilization. One disadvantage is that reductions in the levels of certain compounds, such as lipid phosphate, may not completely reflect microbial consumption. Microorganisms may become detached from sand particles by movements of invertebrates. In addition, measurements of changes in the community microbial biomass (Findlay and White, 1983) may include unattached as well as attached microorganisms. Consequently, this may not give such accurate results. The use of muramic acid levels as a measure of microbial biomass (Findlay and White, 1983) is also inaccurate. Muramic acid is a component of bacterial cell walls and is not present in other microorganisms. It would therefore be a more accurate measure of bacterial levels, and not of other microeukaryotes which formed a large part of the sand dollar's diet (Findlay and White, 1983).

The ^{14}C-measurements used by Fenchel *et al.* (1975) are probably a more accurate method. This technique provides a direct means of observing the passage of attached microorganisms into invertebrates. The ^{14}C-method does not rely on measuring changes in the levels of certain microbial cell components. However, as with the biochemical assays, this method does not take account of the detachment of microorganisms.

Robertson and Newell (1982) compared the abilities of three species of fiddler crab to remove particle-bound bacteria from sandy sediments. The crab species were *Uca pugilator, Uca pugnax* and *Uca minax*. *Uca pugilator* removed over 60% of bacteria bound to noningested particles, whilst *U. pugnax* removed half as many bacteria. *Uca minax*, however, did not remove

any bacteria (Robertson and Newell, 1982). These results showed that *U. pugilator* was more efficient at utilizing bacteria bound to and dispersed among large uningested sand grains than were *U. pugnax* and *U. minax.* This in turn explains why the latter two species predominate in muddy rather than sandy sediments.

The subtidal gelatinous mat which binds together calcarenite grains in the Bahamas (Bathurst, 1967; see Chapter 9.1) may be an important food source. Invertebrates which browse on the sand floor may not be able to survive without this mat, which may occupy an essential position in the local food chain.

Cammen (1980) looked at the carbon resources available to the deposit feeding polychaete *Nereis succinea,* and suggested that the species might obtain some organic carbon from microbial cellular debris or mucopolysaccharides in the sediment. This organic carbon fraction may be more readily available to consumers than the remainder of the organic carbon in the sediment.

Hobbie and Lee (1980) discussed these views of benthic nutrition in more detail. They suggested that sediment microbes were not abundant enough to nourish a non-selective feeder. This led to their hypothesis that microbial extracellular mucopolysaccharides are more abundant than the microbes themselves, and may provide the majority of food for many benthic invertebrates. The major evidence for this came from studies of bacterial attachment to particles and the accumulation of microbial slimes where water moves past surfaces.

The source of the mucopolysaccharide could be the dissolved organic matter in the surrounding seawater. Attached microorganisms are particularly effective at removing dissolved organic matter from solution

and transforming it into particulate organic matter in the form of mucopolysaccharide (see Chapter 10). The apparent resistance of the bacterial mucopolysaccharide material to microbial enzymes does not rule out its use as food by benthic invertebrates. As an example, some nematodes are able to produce mucopolysaccharide hydrolases from ingested bacteria (Hobbie and Lee, 1980).

Moriarty and Hayward (1982) did transmission electron micrograph studies of the bacteria present in the surface aerobic layer and a deeper anaerobic layer of sediments. Many bacterial cells, especially those in colonies, were surrounded by extensive slime layers. These authors suggested that the slime layers and bacterial cell envelopes would be trophically important to benthic animals. Bacterial biosynthetic products would represent a larger proportion of utilizable organic matter than the bacterial biomass.

Moriarty (1982) studied the utilization of organic carbon and nitrogen by the holothurians *Holothuria atva* and *Stichopus chloronotus* in sediments of the Great Barrier Reef. Transmission electron micrographs showed that many of the sediment bacteria were embedded in slime layers and mucus. Mucus from bacterial slime and that produced by other organisms were probably the main sources of detritus in the reef sediments. Some of this material was readily digested by the holothurians.

These observations of Moriarty (1982) are the only studies discussed that have substantiated Hobbie and Lee's (1980) hypothesis that benthic invertebrates may utilize microbial extracellular materials. Further experimental work should be done. This would involve radioactive labelling, using ^{14}C or ^{3}H-isotopes, of sediment samples containing microbial mucilage. Uptake of such material by benthic invertebrates

could then be followed. Biochemical measurements of the utilization of mucilage components could also be used. This method would probably be more accurate than when used to measure the utilization of attached sediment microflora (Findlay and White, 1983; see p. 266). Measurements of enzymic activity occurring during invertebrate feeding could also be done.

Summary

(1.). Studies have shown that microorganisms attached to sediment particles are an important food source for benthic deposit-feeding invertebrates. This has been shown for several invertebrates, including *Hyallela azteca* (Hargrave, 1970), *Corophium volutator* and *Hydrobia ulvae* (Fenchel *et al.*, 1975), *Hydrobia ventrosa* (Kofoed, 1975) and *Mellita quinquiesperforata* (Findlay and White, 1983).

(2.). Extracellular mucopolysaccharide material produced by attached microorganisms could act as an alternative food source. It may provide a richer source of organic nutrients than the microbial cells (Bathurst, 1967; Cammen, 1980; Hobbie and Lee, 1980; Moriarty, 1982; Moriarty and Hayward, 1982).

Chapter 10 Microbial adhesion to detritus.

This chapter firstly considers the attachment of marine microorganisms to particulate detritus and the formation of detrital aggregates from dissolved detritus. The ecological importance of microbial adhesion and aggregation in the decomposition and recycling of detrital material is also considered. The second part considers microbial adhesion to a specific type of particulate detritus, copepod faecal pellets.

(10.1). Microbial adhesion to particulate detritus and role of dissolved detritus in aggregation.

Detritus has had several definitions in the literature. One definition (Fenchel and Jørgensen, 1977) has described it as "non-predatory losses of organic carbon from any trophic level (including egestion, excretion, secretion, etc.) or inputs from sources external to the ecosystem that enter and cycle in the system". Detritus is usually found in two forms in the marine environment, particulate and dissolved.

Particulate detritus usually constitutes the largest part of suspended particles in natural waters. It is found in concentrations between 0.02 and 6mg./l. in oceanic waters (Fenchel and Jørgensen, 1977). Particulate detritus comes from several sources. Animals which have avoided predators eventually contribute to the pool of dead particulate organic material. Decomposing chitin from crustacean exoskeletons also forms particulate material (see Chapter 8), as do microbial cell fragments, such as diatom frustules. Faecal pellets produced by herbivorous or detritivorous animals also contribute significantly to particulate detritus. Broken down tissue of dead leaves, roots, stems or thallus of macrophytes can also form a considerable part of suspended material. The macrophytes

in the sea which form such particulate detritus include seagrasses, mangroves and macroalgae (Fenchel and Jørgensen, 1977).

Dissolved detritus is also derived from several sources. Healthy and growing macrophytes secrete a part of their assimilated carbon as dissolved organic matter. Active bacteria also produce dissolved organic carbon through the decomposition of particulate material (see p. 280). However, the main source of dissolved organic matter in seawater comes from primary producers. Between 10 and 30% of all primary production enters the pool of dissolved organic matter. This dissolved material is rendered available to higher levels of the food chain as particulate organic matter mainly through bacterial activities (see p. 277). The utilization of dissolved detritus is embodied in the term "detritus food chain". This is defined as "any route by which chemical energy contained within detrital organic carbon becomes available to the biota" (Fenchel and Jørgensen, 1977).

Microscopic observations have shown that the microbial communities associated with detritus are complex but with a relatively constant composition. Most microbial activity is associated with the surface of individual particles. Between 2 and 15 bacterial cells per 100μm^2 of surface area are often found, depending on the nature of the particle surface. The bacterial flora is diverse and contains rods, cocci, filamentous and mycelial forms. Other microorganisms which are found on detritus include diatoms and unicellular cyanobacteria (Fenchel and Jørgensen, 1977).

Paerl (1973, 1974, 1975) made scanning electron microscopic observations of microbial attachment to particulate detritus obtained from Lake Tahoe, California and from the Pacific Ocean. Scanning electron microscopy of detrital samples from various depths in Lake Tahoe showed

vertical changes in microbial attachment. At relatively shallow depths of 20m., there was a close association between bacterial and fungal cells and pieces of detrital material (Paerl, 1973). Gram-negative, rod shaped bacteria with polar flagella, which may have been *Pseudomonas* spp., were predominant. Cocci were also observed, and filamentous bacteria and fungi were tightly attached. Extensive fungal and bacterial "webbing", caused by production of adhesive material, was observed in near-surface samples. Bacterial cells were surrounded by fine adhesive webs which kept them tightly anchored to detrital particles. Luxuriant webs surrounding small cocci and rods were seen, and filamentous bacteria were embedded in networks of fibrillar material (Paerl, 1974, 1975). There was a decrease in microbial attachment with increasing depth in Lake Tahoe. At a depth of 75m., detrital particles were larger and smoother and showed less attached microorganisms. Fungal attachment was absent at depths greater than 75m.. Below 150m. depth there were few attached bacteria on detritus, although attached remains of microbial cell walls and stalks were observed. Extensive microbial attachment was only noticed to diatoms, which showed signs of partial decomposition. Between 150m. and 400m., there was little change in the extent of microbial association. At these depths, detritus was composed of aggregated pieces of mineral and organic material which were compacted into smooth pellets with little microbial attachment. At 440m., where the sample was taken approx. 20cm. above the lake bottom, compacted pellets showed a slight increase in attached microorganisms. However, microbial growth was far less at this depth than it was on near-surface detritus (Paerl, 1973). Bare detrital surfaces were more common in deeper water, with cellular remains scattered on, as well as within, particles. In the same way, fibrillar networks, which were well defined

above 100m., were either absent or partially destroyed in deep water. Fragmentation of detritus occurred wherever there was destruction of fibrillar material.

The general appearance of attached microbial growths on detritus from both marine and freshwater environments was similar. However, the marine samples possessed a more heterogeneous composition of bacteria, diatoms, dinoflagellates and green algae (Paerl, 1974). Near-surface particles were composed of diatom frustules and the remains of algal cells coated with mucoid material and bacteria. Both rod and coccal forms of bacteria were present. Single cells were often seen on particles with strings of secreted material behind them. Stalked bacteria were often attached to particles which also supported small rods (Paerl, 1975). There was also a decrease in microbial attachment observed in deeper water. However, attached growth was not completely absent. Marine samples collected at 300m. depth contained clusters of small coccoid cells attached to detritus. It was difficult to observe fibrillar structures associated with these cells. The occurrence of clumping suggested that the cells were adhesive (Paerl, 1974).

Paerl (1973) also conducted a separate study using fresh detritus collected from the mouth of a tributary, Ward Creek. The detritus consisted mainly of the remains of attached periphytic algae. The detrital samples were divided equally among two sets of sealed 150ml. dialysis bags. The bags allowed free diffusion of nutrients and metabolic waste products, although they were impermeable to detritus and associated microorganisms. One set of dialysis bags contained sterile detritus and a 50ml. inoculum of lake water collected from 20m. depth which contained live microorganisms. The other had a similar content but was sterilized by

immersion in 0.001M mercuric chloride for 12 hrs. after sealing. Both sets of bags were incubated at a depth of 20m. in Lake Tahoe. A sequence of light microscopic pictures of the contents of both sets of bags were made. Aggregation of small detrital particles into large particles was observed over three days in the bags containing live microorganisms. The sterilized bags showed a slight aggregation of particles. The particle size was only 10% of that in the unsterilized bags. Aggregation of detrital particles was largely caused by microbial adhesion and S.E.M. observations showed that particles were trapped together by web-like adhesive structures produced by bacteria and fungi.

Paerl (1974) carried out autoradiographic studies of the uptake of ^{3}H-labelled glucose and acetate in detrital aggregates in both marine and freshwater systems. Initial incubation showed dissolved organic material to be confined to filamentous bacteria. At a depth of 10m. and after 24 hrs. incubation, dispersion of the label to sections of detritus adjacent to bacterial cells occurred. Dense patches of radioactivity surrounded bacterial filaments which were embedded in detritus. After 48 and 70 hrs. incubation, dense areas of radioactivity appeared within and around detrital aggregates. These areas were mostly centred around bacteria. Scanning electron micrographs in Lake Tahoe showed that after 24 hrs., bacteria and associated fibrillar networks were present in aggregates consisting of fragments of the diatom *Cyclotella* spp.. The uptake of both substrates by attached microorganisms was significantly higher in the euphotic than in the aphotic zone, as was the extent of detrital aggregation. This may be due to the high amount of dissolved organic matter there. Extracellular production or leakage of low molecular weight dissolved organic carbon (DOC) in this zone presents one source of

substrates for bacteria, while lysis and partial decay of cellular materials also supply DOC. Paerl's (1974) results show that DOC is essential for biologically mediated detrital aggregation. In deeper water, microbial death decreased detrital aggregation levels. The autoradiographs showed that dissolved organic materials which were assimilated by bacteria were linked to the formation of capsular materials. This process is important in building detrital aggregates. A favourable combination of substrates and physical growth conditions is necessary for the formation of capsular and fibrillar materials from DOC in both environments.

These observations also show that particles serve as growth-conducive surfaces in both marine and freshwater environments. Extensive microbial attachment occurred even in oligotrophic waters. This may be explained by the tendancy for bacteria to remain near nutrient sources in oligotrophic environments (see Chapter 1.6). Particulate material may act as a suitable source of nutrients. Adsorption of nutrients leading to the formation of "microlayers" of concentrated nutrients may occur in dilute aquatic systems (Paerl, 1975). This may offer an attractive growth site for microorganisms capable of attaching to particles.

Biddanda (1986) made transmission electron microscopic observations of laboratory-produced marine aggregates. Bacterial extracellular polysaccharide processes were responsible for the formation of aggregates. They caused inter-bacterial bridging which enabled the bacteria to attach to each other, resulting in the formation of macroaggregates. The same material was responsible for bacterial attachment to particles of decomposing seaweed. This resulted in the formation of bacterio-seaweed particle aggregates. The electron opaque and fibrillar appearance of the

bacterial extracellular material resembled that observed by Fletcher and Floodgate (1973) (see Chapter 1.2). These observations show the importance of microbial extracellular adhesive in the ecology of detritus and macroaggregate synthesis. Microorganisms invest considerable energy into the production and maintenance of extracellular structures that aid them in attachment. This may be an adaptation to allow utilization of nutrients occurring at a solid/liquid interface, such as a particle surface (Biddanda, 1986). The aggregation of bacteria and their attachment to detrital particles allows easier utilization of them by grazer organisms. Both selective and non-selective feeders may derive significant amounts of energy from bacterial aggregates and bacteria in detritus. This is similar to the utilization of microbial extracellular materials by benthic invertebrates (see Chapter 9.2). The transfer of microbial macroaggregates directly to the macro food chains may also occur. This may be a major pathway for the flow of carbon and energy to the higher trophic levels in aquatic ecosystems (Biddanda, 1986).

Elevated counts of bacteria were found in the surface microlayers of two salt marshes (Harvey and Young, 1980). At both sites, the concentration of bacteria in the surface microlayer was linearly related to the surface concentration of particulate material. A significant concentration of bacteria were attached to particles in the surface microlayer of both marshes. Bacterial populations in the subsurface water were largely planktonic. Both plate count and total count methods were used to measure bacterial populations. There was some variability between the results obtained using these methods. The numbers of plate count bacteria were several orders of magnitude lower than the total counts in all samples (Harvey and Young, 1980). The total count method alone may

have been more accurate. This is because it gives a measurement of the total bacterial population associated with particulate material rather than a selected population. Large bacterial counts in the surface microlayers were, however, recorded using both methods. The relationship between the surface enrichment of plate counts and total bacterial counts in relation to particle concentration was investigated graphically. The slope of the line relating surface enrichment of plate count bacteria was significantly steeper. This observation suggests that as the enrichment of particles increases, there is a greater influence on the surface enrichment of plate count bacteria (Harvey and Young, 1980). The ratio of plate counts to total counts in the surface microlayers and the percentage of particle-bound bacteria for each sample were also illustrated graphically. The ratios were highest in samples with the highest proportion of particle-bound cells. This further suggests that particle association positively influences the numbers of bacteria which grow on plates. In addition, as a higher concentration of surface-collected bacteria were particle-bound, this suggests that particles influence bacterial activity in the microlayer environment. The higher concentration factors observed for plate count bacteria suggests that the metabolically active population is greater in the surface layers. The greater increase observed in the enrichment of plate count bacteria with increasing particle surface enrichment further suggests that particulates may promote bacterial metabolic activity. The particulates in the microlayers may also be a resource of nutrients for the bacterioneuston (Harvey and Young, 1980).

Biddanda and Pomeroy (1988) made a series of observations of the microbial degradation of three phytoplankton species in seawater cultures. The phytoplankton studied were the cyanobacterium *Synechococcus* spp., the

flagellate *Dunaliella* spp. and the pennate diatom *Cylindrotheca fusiformis*. The sequence of events occurring during decomposition were followed at various time intervals over a sixteen-day period using epifluorescence microscopy and SEM. A similar and well-defined pattern of microbial succession occurred in all three species (see Albright *et al.* (1986), Chapter 7.1). The successional pattern was closely followed in *Cylindrotheca fusiformis*, as the stages could be clearly identified. During the first few days of incubation, an increasing proportion of rod-shaped bacteria became associated with the phytoplankton. These were then replaced by a mixed assemblage of bacteria including cocci, rods, spirilla and filamentous forms. Aggregation of bacteria and the phytoplankton detritus occurred after four days. This was mediated by the production of bacterial extracellular mucopolysaccharides. A mixed assemblage of bacterivorous protozoa also appeared at this time, including flagellates, ciliates and amoeboid forms. These protozoa kept the bacterial numbers in check by consumption. The appearance of the protozoan community was accompanied by changes in morphology of aggregates. Disaggregation occurred due to the consumption of bacteria and their associated attachment structures. At the end of the sixteen-day period, the aggregates had broken up and largely disappeared. The aggregation and disaggregation events are important to the functioning of marine food chains (Biddanda and Pomeroy, 1988). The microbe-rich aggregated phase is of food value to metazoans. Disaggregation of the phytoplankton detritus may be of nutrient value to the primary producers. Such bacterial and microzooplankton activity may establish a microzone of enriched nutrients in and around the detrital aggregate. Microorganisms may optimize their position within these nutrient fields, leading to the formation of

"microbial clusters" in the vicinity of aggregates. This microenvironment around degrading detritus is known as the "detritosphere" (Biddanda and Pomeroy, 1988). It is similar to the 'phycosphere' concept describing the environment of bacteria associated with live phytoplankton (see Chapter 7.1).

Biddanda and Pomeroy (1988) also proposed a model of detrital aggregate dynamics based on their observations of particle aggregation and disaggregation. The model is driven by the inputs from primary and secondary producers. It proposes that regardless of the form of organic input, particulate matter is aggregated by bacteria and subsequently degraded by them, together with the actions of protozoa. The organic material could be mucus, faecal pellets, dead animals or degrading phytoplankton. Consequently, the fate of detritus in the water column can be seen as aggregation-disaggregation sequences in time and space. This model is generally consistent since loss of detritus by degradation or sinking would be compensated by primary and secondary production inputs or resuspension from the benthos.

Morrison *et al.* (1977) made observations of successional changes in microfloral populations on plant litter, made up of *Quercus virginica* leaves, in a semi-tropical estuary. Changes in hydrolytic, respiratory, catabolic and lipid biosynthetic activities were used as a measure of successional changes in microbial populations. SEM was also used to observe the dorsal and ventral surfaces of *Quercus virginica* leaves over a six-week period. The dorsal surface was colonized slowly and in patches. Newly fallen leaves, which were not yet exposed to estuarine water, had a sparse distribution of debris particles. Most of these particles were unidentifiable even at high magnifications. After two weeks, the dorsal

surface was covered by patches of microorganisms and debris, although much of it remained uncolonized. Several bacterial forms were seen during the early stages of colonization. These included debris-covered organisms attached by filaments, bacilli with distinct attachment appendages and coccoid forms. A few filamentous bacteria were also present which could have been *Leucothrix mucor*. By the fourth week, the surface was more densely colonized, and filamentous fungi were in abundance. The dorsal surface was not fully colonized until the fifth and sixth weeks. The ventral leaf surface was also rapidly colonized by debris. Bacterial forms were present, but were often barely visible among the mass of debris. Other microorganisms were commonly observed, including intact and fragmented diatoms, algal or fungal filaments and spirochaete-like organisms. As with the dorsal surface, fungal filaments became more abundant in the later stages of submersion. Dense filamentous fungal mats, which were probably responsible for macroscopic white patches on the leaf surface were observed after four or five weeks.

Biochemical measurements of microbial succession showed high levels of muramic acid in the early stages of colonization (Morrison *et al.*, 1977). This was indicative of high bacterial populations, which were the initial colonizers. With longer submersion time in the estuary, ATP levels on the leaves rose, whilst muramic acid levels decreased. The increase in ATP levels corresponded to increased colonization by fungi, algae and other complex organisms not containing muramic acid. These biochemical changes agreed with the SEM observations. However, the use of ATP to measure levels of algae or fungi may not be accurate. Such measurements could also include ATP levels in attached bacteria which were still present. Measurements of certain lipid or fatty acid levels could have been a more

accurate measure of algal or fungal populations (see Chapter 9.1) at later stages in the succession.

The observations of Morrison *et al.* (1977) did not show any protozoal colonization of *Quercus virginica* leaves later in the succession. The absolute microbial population sizes occurring on detritus depends on a number of factors, including nutrient levels in the surrounding water (Fenchel and Jørgensen, 1977).

(10.2). Microbial attachment to copepod faecal pellets

Faecal pellets are an example of particulate detritus (see p. 272). This part concentrates on bacterial attachment to, and their role in, decomposition of, copepod faecal pellets.

Turner (1979) analysed faecal pellets produced by the copepod *Pontella meadi* over 14 days of aging at 5°C and 22°C. Initial decreases in carbon and nitrogen levels occurred at 22°C, followed by fluctuating values for both. These fluctuations may reflect the contents of microorganisms colonizing the pellets. SEM examination showed that fresh pellets were surrounded by intact peritrophic membranes. These became colonized by rod-shaped bacteria within a few hours. After 3-11 days of aging at 22°C, the membranes were biodegraded, and stalked ciliates were attached to the pellets. By the end of 35 days' aging at 22°C, pellet fragmentation had reached an advanced state. At a temperature of 5°C, however, pellet carbon levels were unchanged over 14 days. SEM observations showed that microbial attachment to the pellets was greatly reduced at this temperature. After 35 days aging, the pellets and their peritrophic membranes were still relatively intact. These observations suggest that when faecal pellets descend to the cooler waters of the deep sea, they

remain virtually intact. Zooplankton faecal pellets with their attached microbiota may then be a significant source of nutrition for abyssal detritivores (Turner, 1979).

Gowing and Silver (1983) examined bacterial populations on the surface and interior of copepod faecal pellets. Some pellets were collected from the upper 50m. of seawater using particle interceptor traps. Faecal pellets were also produced by swimming crabs, *Pleuroncodes planipes*, when they were placed in fixative solutions in a shipboard laboratory. Both sets of pellets were examined by SEM and TEM. The contents of the larger faecal pellets collected by the sediment traps were identical to those produced by *Pleuroncodes planipes*. They consisted predominantly of amorphous material, including diatom fragments, crustacean cuticle pieces, *Chlorella*-like cells, dinoflagellates and several bacterial types. There were noticeable differences in the surface bacterial populations on incubated field and laboratory pellets. After 7h. incubation, bacterial populations on pellets collected in the laboratory were much higher than those on field-collected pellets. Some of these bacteria could have been obtained from contact with the glass vials in which the pellets were incubated (Gowing and Silver, 1983). The inner surfaces of the glass vials were examined by SEM in an attempt to show this. These examinations showed that the walls of vials incubated for 48h. had a bacterial population of 1.4×10^{24} bacteria mm^{-2}. The population increased substantially in vials incubated for longer periods. These results suggest that the vial walls could be a source of the bacteria found on the pellet exteriors. There may be other reasons. Bacteria could have been selectively removed from faecal pellets collected in the sea but not from those incubated in the laboratory. Bacteria may have been more loosely

attached to pellets collected in the sea. However, examination by SEM and TEM showed no differences in the surface condition of laboratory and field pellets. Bacterial populations on pellets from the sea may have been substantially reduced by metazoan grazing. Such grazers were excluded from shipboard incubations. The metazoans must be very efficient grazers if they do remove bacteria from pellet surfaces in the sea (Gowing and Silver, 1983).

Faecal pellets collected from the sea contained a higher internal bacterial population than those produced in the laboratory. The low bacterial population in the laboratory pellets could be caused by factors which alter bacterial numbers or the environment within the pellets. These factors could include the use of antibiotics in the laboratory. The high bacterial populations inside faecal pellets collected from the sea suggests that pellet decomposition occurs from the interior. Bacteria inside the faecal pellets could arise as either enteric or ingested species.

Whatever their origins, the bacterial populations would be introduced into the pellets at the same time that they are produced by the zooplankton. Any increase in these populations could only occur through their own growth. Decomposition on the faecal pellet surfaces would be an aerobic process, as long as they remained in oxygenated waters. The microhabitats of pellet interiors may differ considerably from those on the surface. Contents of fresh pellets would already have low oxygen levels at the time of defecation. Further internal decomposition by bacteria would continue to deplete oxygen. Surface bacterial populations could also further reduce oxygen levels in the microzone of the pellet boundary. Only microaerophilic or anaerobic bacteria would be able to decompose the

pellets at these low oxygen levels (Gowing and Silver, 1983). Further experimental work to study the role of internal bacteria in faecal pellet decomposition should be done. This could include measurements of carbon dioxide production from pellets using ^{14}C-labelling (see p. 287). Bacteria from the interior of the pellets could be isolated and taxonomically identified. Studies could then be made of the decomposition of *Pleuroncodes planipes* faecal pellets under anaerobic or microaerophilic conditions. Biochemical studies on the decomposition pathways, using enzymic or metabolic inhibitors would be interesting. Similar work could also be done with bacteria on the pellet surfaces.

Jacobsen and Azam (1984) studied the colonization of faecal pellets of *Calanus pacificus* by bacteria during a four day decomposition experiment. Bacterial populations were estimated by acridine orange staining. Initial colonization was very rapid. Bacteria occupied 27% of the available area of the pellets after 24 h.. The bacterial concentration after one day was 1×10^5 cells per faecal pellet. The numbers declined to 1×10^4 cells per pellet after four days. Bacterial attachment to sinking and non-sinking faecal pellets was compared. The sinking pellets had a higher initial rate of colonization than those which were in a container. However, the enhanced initial colonization rate of the sinking pellets did not further increase the bacterial numbers after one or more days. The same bacterial densities were found on sinking and non-sinking pellets after 24 h.. The exact mechanism of attachment of bacteria to the sinking faecal pellets is not known. Observations of bacteria attached to fluorescent-labelled pellets suggested that the peritrophic membrane may allow attachment. The high bacterial populations on the stationary pellets in containers suggests that surface receptors may be present

(Jacobsen and Azam, 1984). Further experimental work, such as TEM observations of bacterial colonization could be done. Studies of sugar inhibition of bacterial attachment could also be done to show possible ligand-receptor interactions (see Chapter 7.1 and 8).

Measurements of bacterial mineralization of faecal pellet carbon and bacterial growth rates were also made. Faecal pellets were incubated in a 1 μm. filtrate of bacteria in seawater or a 35 μm. filtrate of bacteria and microzooplankton. Bacterial mineralization of ^{14}C-carbon to carbon dioxide in the pellets with the 35 μm. filtrate including zooplankton doubled the rate of mineralization. However, the results suggest that bacteria alone are not responsible for the complete remineralization of faecal pellet carbon. If the estimates made under laboratory conditions were correct, complete pellet decomposition by bacteria would require approx. 50 days at 18°C. However, the remineralization results may be inaccurate. This is because the release of dissolved ^{14}C-label and the presence of particulate label in seawater may make up as much of the lost label as the carbon dioxide evolved. Some of the particulate label may arise from portions of the faecal pellet breaking off. Bacteria may also utilize the dissolved ^{14}C-label. These labelled bacteria could become detached from the pellets (Jacobsen and Azam, 1984).

The growth rates of bacteria associated with the faecal pellets were estimated by measuring ^{3}H-thymidine incorporation. Measurements were made using faecal pellets incubated in the 1 μm. or 35 μm. filtrates. The specific growth rate of free bacteria in the water was greater than attached bacteria in both filtrates. This observation does not support those that suggest that attached bacteria are metabolically more active than free-living cells (Harvey and Young, 1980; see p.279). However, the

growth rate of attached bacteria could be underestimated if the faecal pellet was acting as a "baby machine". A "baby machine" is defined as a site of bacterial growth where the progeny do not remain at that site (Jacobsen and Azam, 1984). Detachment of bacteria from the pellet could cause this, which in turn could significantly increase the production estimates of free-living bacteria. Bacterial detachment tends to occur with the initial colonizers of faecal pellets. It may not occur in the latter stages of pellet decomposition.

Colonizing bacteria were also found on the inside of the faecal pellets within a few hours. This suggests that a similar pattern of colonization occurs to that observed by Gowing and Silver (1983) for *Pleuroncodes planipes* faecal pellets. Decomposition of *Calanus pacificus* pellets may occur in a similar way to those of *P. planipes*. The number of bacteria associated with young *C. pacificus* pellets was a minimal estimate. It was difficult to accurately quantify bacteria within the faecal pellet. Further experimental work, such as comparing the internal and surface bacterial populations of the pellets should be done, along with some biochemical work. The results of these laboratory studies need to be confirmed in natural samples. In addition, they need confirmation in relation to faecal pellet size and abundance (Jacobsen and Azam, 1984). The observations which have been discussed suggest that free-living bacteria colonize freshly egested copepod faecal pellets and are involved in their decomposition.

Summary

The main points discussed in this chapter are as follows :

1. Detritus usually occurs in two forms in the marine environment as particulate and dissolved detritus. A diverse bacterial flora together with diatoms and cyanobacteria often colonize detritus (Fenchel and Jørgensen, 1977).

2. (i.). SEM observations of microbial attachment to particulate detritus in freshwater and marine environments showed vertical changes. Microbial attachment was common in near-surface samples, with extensive production of extracellular adhesive keeping them tightly anchored to particles. There was a decrease in microbial attachment with increasing depth (Paerl, 1973, 1974, 1975).

   (ii.). Autoradiographic studies on detrital aggregates over a 70 hr. period showed dense patches of radioactivity centred around bacteria in the aggregates. These observations suggested that the bacteria utilize dissolved organic carbon for the formation of adhesive materials which result in detrital aggregation (Paerl, 1974).

   (iii.). Bacterial extracellular polysaccharide was responsible for bacterial attachment to decomposing seaweed particles, and in the formation of bacterio-seaweed aggregates (Biddanda, 1986). Aggregation of bacteria and their attachment to detrital particles may allow grazer organisms to utilize them more easily.

3. High bacterial populations were found in the surface microlayers of two salt marshes. A higher enrichment of surface particles gave an increase in the numbers of plate count bacteria. These observations suggest that particles influence bacterial activity, and that the

metabolically active population is highest in the surface microlayer (Harvey and Young, 1980).

4. (i.). Observations of the microbial degradation of the diatom *Cylindrotheca fusiformis* and two other phytoplankton showed a common successional sequence. Bacteria were initially associated with the phytoplankton, causing detrital aggregation by the production of adhesive. Protozoa appeared after four days, reducing the bacterial numbers by consumption. This protozoal activity caused disaggregation of the detrital aggregates. These observations suggest that the fate of detritus in the water column can be seen as aggregation-disaggregation sequences in time and space (Biddanda and Pomeroy, 1988).

(ii.). Successional changes in microfloral populations were similar on the dorsal and ventral surfaces of *Quercus virginica* leaves in a semi-tropical estuary. Several bacterial forms were present, together with diatoms, and algal or fungal filaments. Dense filamentous fungal mats were later observed on both leaf surfaces. Changes in muramic acid and ATP levels appeared to follow observed changes in microbial populations. Protozoa were not observed (Morrison *et al.*, 1977).

5. (i.). Attached marine bacteria appear to be involved in faecal pellet decomposition. Faecal pellets of *Pontella meadii* incubated at 22°C became heavily colonized by bacteria and stalked ciliates. Pellet fragmentation reached an advanced state after 35 days aging at 22°C. Microbial attachment was greatly reduced at 5°C, however, and the pellets remained intact after 35 days (Turner, 1979).

(ii.). Comparisons were made of surface and internal bacterial

populations of faecal pellets collected from seawater and those produced by *Pleuroncodes planipes* crabs in the laboratory (Gowing and Silver, 1983). A higher internal bacterial population was found in the pellets collected from the sea. These observations suggest that faecal pellet decomposition occurs from the interior. The microhabitats of pellet interiors may differ from the surface. Microaerophilic or anaerobic bacteria would only be able to decompose the pellets at the low oxygen levels which would exist (Gowing and Silver, 1983).

(iii.). Bacteria initially rapidly colonized faecal pellets of *Calanus pacificus.* The same bacterial densities were found on sinking and non-sinking pellets after 24 h.. Measurements of bacterial mineralization of faecal pellet carbon suggests that bacteria alone are not responsible for it's complete remineralization. The specific growth rate of free bacteria in the surrounding water was greater than for attached bacteria. The growth rate of attached bacteria could be underestimated if the pellet was acting as a "baby machine". Colonizing bacteria were also found on the inside of the pellets, suggesting a similar decomposition pattern to those of *P. planipes* (Jacobsen and Azam, 1984).

SECTION 4

AN ECONOMIC PROBLEM CAUSED BY MARINE MICROFOULING AND METHODS OF PREVENTION AND CONTROL OF MICROFOULING

Chapter 11 Corrosion of metals by marine microfilms

Corrosion is best defined as the destructive attack of a metal by reaction with its environment (Iverson, 1974). It is frequently thought of as "rusting". This term, however, applies to the corrosion of iron or iron-based alloys which results in the formation of corrosion products such as hydrous ferric oxides (Iverson, 1974). In an electrolytic solution, such as seawater, a metal undergoes an anodic, or oxidation, reaction (Gerchakov and Udey, 1984) :-

$$M \longrightarrow M^{n+} + ne^- \qquad (1.)$$

A cathodic, or reduction, reaction must occur simultaneously to accept these electrons. Water can act as an electron acceptor with the evolution of hydrogen (Gerchakov and Udey, 1984):-

$$nH_2O + ne^- \longrightarrow \frac{n}{2}H_2 + nOH^- \qquad (2.)$$

Alternatively, oxygen may also serve as an electron acceptor (Iverson, 1974; Gerchakov and Udey, 1984):-

$$\frac{n}{4}O_2 + \frac{n}{2}H_2O + ne^- \longrightarrow nOH^- \qquad (3.)$$

Reaction (3) usually occurs in solutions of neutral or alkaline pH. In reaction (2), the hydrogen which forms accumulates on the metal surface. Removal of hydrogen by bacterial action causes reactions (1) and (2) to move to the right, resulting in corrosion of the metal (Iverson, 1974). In this case, the metal corrodes by cathodic depolarization, to be discussed later (see p. 301).

Seawater is a mildly alkaline solution of various salts, of which sodium chloride is predominant. Seawater can become corrosive depending

on the concentrations of salts present (Godard, 1979).

Microbial films forming on metal surfaces in seawater may cause corrosion in various ways. Corrosion occurs by the microorganisms being in close contact with the metal surface through attachment (La Que, 1975; Gerchakov and Udey, 1984). One corrosion mechanism is by the production of organic acids. Organic acids which are involved in the reactions of glycolysis and the citric acid cycle are particularly effective at corroding copper (Staffeldt and Calderon, 1967). These acids cause much corrosion at gas/liquid interfaces and under submerged conditions.

A further mechanism may be the reduction in oxygen concentration which occurs under respiring microbial colonies on the metal surface (Iverson, 1974). This is thought to cause the formation of differential aeration cells, where areas under the microbial colonies are depleted of oxygen relative to the surrounding areas (La Que, 1975; Gerchakov and Udey, 1984). Such differential aeration cells result particularly from non-uniform microbial colonization on the metal (Gerchakov and Udey, 1984). A potential difference is formed as a result of the lower oxygen concentration underneath the microbial colonies and higher oxygen concentration adjacent to them (Iverson, 1974).

This in turn will give rise to corrosion currents. Pitting corrosion results from sustained differential aeration cells (Godard, 1979; Gerchakov and Udey, 1984). On the other hand, a heavy microbial film on a metal surface can suppress a corrosion reaction which is controlled by access of oxygen to cathodic surfaces (La Que, 1972).

Various microalgae, particularly diatoms, as well as cyanobacteria, attach readily to submersed metals (see Chapters 3 and 12). Terry and Edyvean (1981) made observations of microalgal attachment to unprotected

steel, cathodically protected steel, and steel coated with antifouling paint. Diatoms and cyanobacteria attached by copious mucilage production to all the steel samples (Terry and Edyvean, 1981, see p.295 ; Edyvean and Terry, 1983b). Graphs of pH changes under such an algal film showed pH falls during the night (Terry and Edyvean, 1981). Increased acidity may increase the corrosion rate, and could give rise to corrosion cells (Edyvean and Terry, 1983b). Edyvean and Terry (1983a) studied the polarization characteristics of 50D structural steel in cultures of marine algae. Polarization can be defined as follows (McDonnell *et al.*, 1984). When a metal is placed in a solution, it establishes a corrosion potential, E_{corr}. At E_{corr}, the oxidation-reduction reactions are balanced, so the current is zero, and the metal is polarized. If an external negative voltage is applied, the metal will obtain a potential more negative than E_{corr}, and the cathodic (reduction) reaction will predominate. In this case, the metal becomes cathodically depolarized. However, if a positive voltage is applied, the anodic (oxidation) reaction will predominate, and the metal becomes anodically depolarized (McDonnell *et al.*, 1984).

In the studies of Edyvean and Terry (1983a), corrosion was enhanced by anodic depolarization under cultures of the cyanobacterium *Oscillatoria* spp. (see p. 296). A primary film of bacteria and diatoms on 50D steel produced some cathodic depolarization (see p. 297). (50D steel is defined as carbon-manganese steel conforming to British Standard 4360, which is designated 50D). Corrosion enhancement by *Oscillatoria* spp. may be caused by the organism itself, or by associated bacteria. However, under a thick, mucilaginous mat of *Oscillatoria* spp., anodic polarization occurred which resulted in a decrease in corrosion. This protection may have been caused by the closely adherent mucilage preventing oxygen

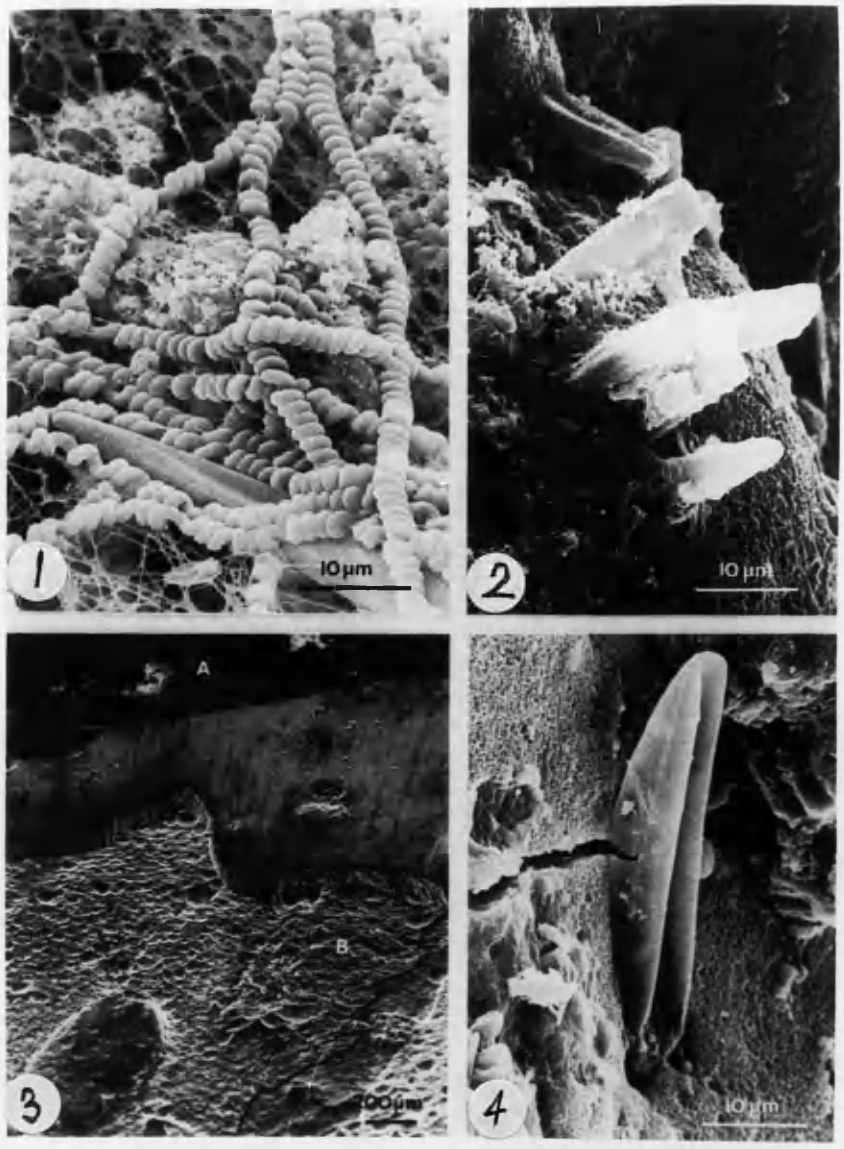


Fig. 54 Corrosion of mild steel by attached microorganisms

Fig. 54.1 S.E.M. showing rust surface of unprotected mild steel exposed to flowing seawater for 105 days. The surface is covered by a mucilage-bound mat of filamentous cyanobacteria and a pennate diatom.

Fig. 54.2 S.E.M. showing the penetration of the epoxy coal tar paint surface by pennate diatoms grown in static laboratory culture after 100 days.

Fig. 54.3 S.E.M. showing the layered structure of the scale formed on mild steel cathodically protected using a zinc alloy sacrificial anode, after 100 days. A, upper surface of scale; B, under surface showing an imprint pattern of the steel surface.

Fig. 54.4 Pennate diatom after 100 days in static laboratory culture attached by one end to the scale formed on cathodically protected mild steel. The attachment is so secure, that the cracking of the scale during S.E.M. preparation also resulted in the cracking of the diatom itself.

(Reproduced by permission from Terry and Edyvean, 1981)

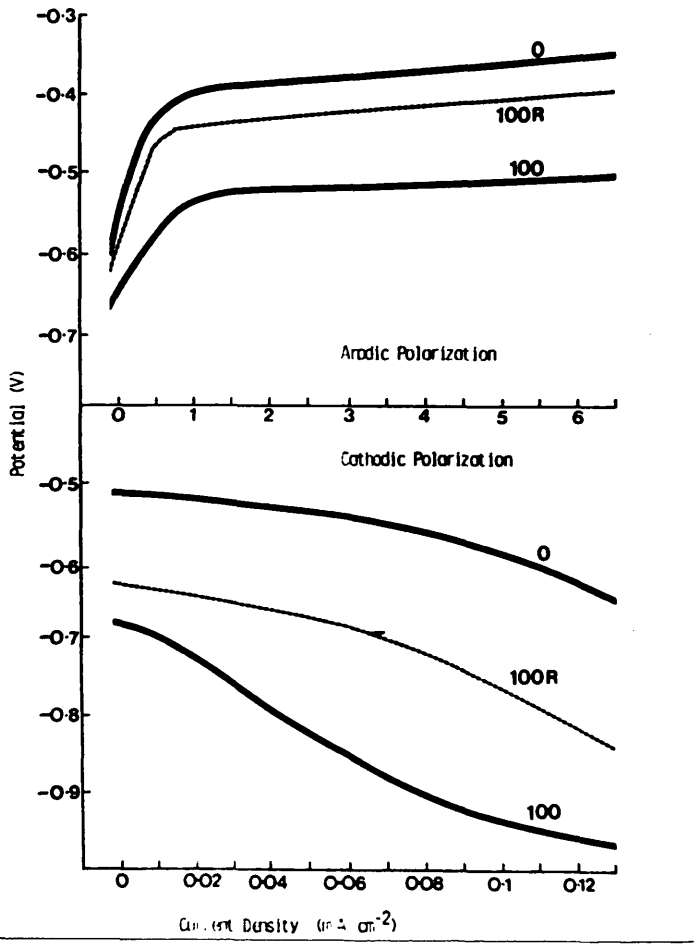


Fig. 55 Polarisation curves for a 50D steel electrode exposed to the cyanobacterium *Oscillatoria* spp. cultured in enriched seawater medium. After 1 hour (O) (control); after 100 days (100); after 100 days then repickled (100R).

(Reproduced by permission from Edyvean and Terry, 1983a)

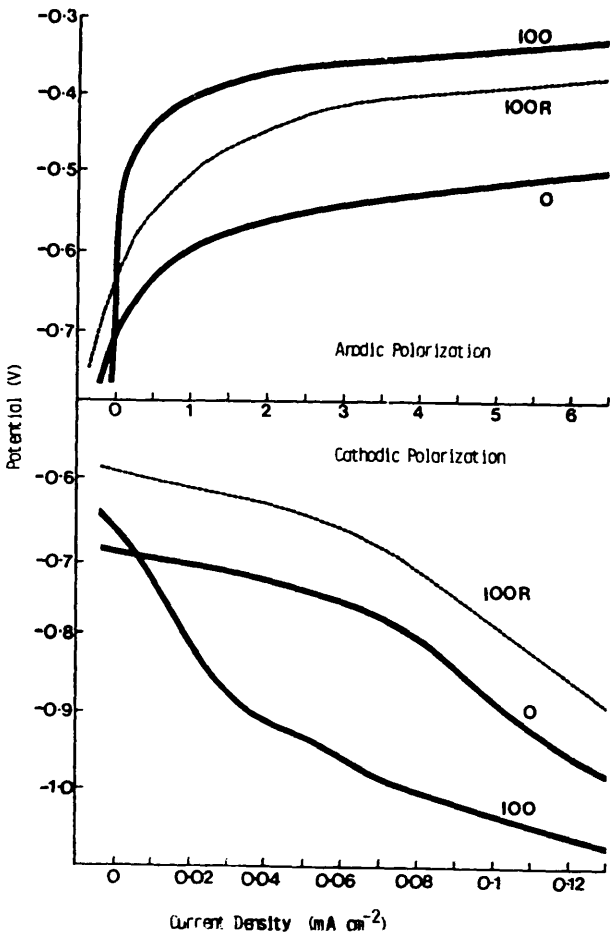


Fig. 56 Polarisation curves for 50D steel in seawater exposed to primary film formation (bacteria and diatoms). After 1 hour (0) (control); after 100 days (100); after 100 days then repickled (100R).

(Reproduced by permission from Edyvean and Terry, 1983a)

diffusion to the steel surface, hence smothering the corrosion reaction (Edyvean and Terry, 1983a).

Comparisons have been made by McDonnell *et al.* (1984) of the attachment patterns, polarization and amount of corrosion produced by the diatoms *Amphora* spp. and *Achnanthes* spp. on 50D steel surfaces. Diatom cultures were added to stubs of 50D steel which were observed by SEM. Rust formation was observed with both diatoms. However, the corroding metal surfaces showed different appearances with the two species. After two weeks the stubs with *Achnanthes* spp. showed abundant rust formation, while those with *Amphora* spp. had little loose corrosion material but a more dense adherent layer. Polarization measurements initially showed no significant differences in the cathodic polarization curves of both steel samples. After four weeks, the *Achnanthes* spp.-treated steel showed greater cathodic depolarization than the steel with *Amphora* spp.. After 20 weeks, the *Amphora* spp.-treated steel continued to show less cathodic depolarization, whilst *Achnanthes* spp. seemed to cause most corrosion. These observations can be explained by the nature of the adhesive mucilage produced by the diatoms which is thought to act as a barrier to oxygen diffusion. Cells of *Amphora* spp. produce mucilage pads which can ultimately cover the entire metal surface, producing a uniform mucilage layer (see Chapter 3.2). This layer would act as an effective corrosion barrier. On the other hand, *Achnanthes* spp. would form an irregular and incomplete mucilage cover over the metal surface due to the production of stalks (see Chapter 3.2 also). This non-uniform mucilage layer may accelerate corrosion.

The polarization results obtained with these diatoms differ from the anodic polarization observed by Edyvean and Terry (1983a) under a thick *Oscillatoria* spp. mat. Such dissimilarities may result from the variable

effects of different organisms. In addition, the time scale of these events may also be important (McDonnell *et al.*, 1984). The longer term effects of microalgae on corrosion would depend upon interactions with other microorganisms such as bacteria, which are often found in close association with diatoms in the natural environment (see Chapter 7.1).

Experimental evidence has shown that photosynthetic and non-photosynthetic bacteria can corrode metals by a hydrogenase enzyme system (Mara and Williams, 1971, 1972). This system enables the organisms to depolarize the cathodic areas of a metal surface by the removal of hydrogen. This enhances corrosion by cathodic depolarization. Cyanobacteria as well as photosynthetic and non-photosynthetic bacteria also oxidize hydrogen by a nitrate-reducing enzyme system (nitrate reductase). Coupling of hydrogenase and nitrate reductase systems provides a corrosion mechanism which may be widespread in natural environments (Mara and Williams, 1971, 1972).

Analysis of algal holdfasts on 50D steel surfaces revealed high amounts of sulphur. Sulphate-reducing bacteria, together with green and blue-green algae can reduce sulphur and sulphates to hydrogen sulphide and iron sulphide which accelerates corrosion (Edyvean and Terry, 1983b). Corrosion was enhanced by cathodic depolarization in a suspension of decomposing *Enteromorpha* spp. under anaerobic conditions (see p. 300). This occurred through hydrogen removal by sulphate-reducing bacteria (Edyvean and Terry, 1983a).

Sulphate-reducing bacteria are frequently found within bacterial slime films, or biofilms, in the aquatic environment. Even in aerobic environments, biofilms can create ideal anaerobic conditions for the growth of sulphate-reducing bacteria. This gives rise to sulphide production and

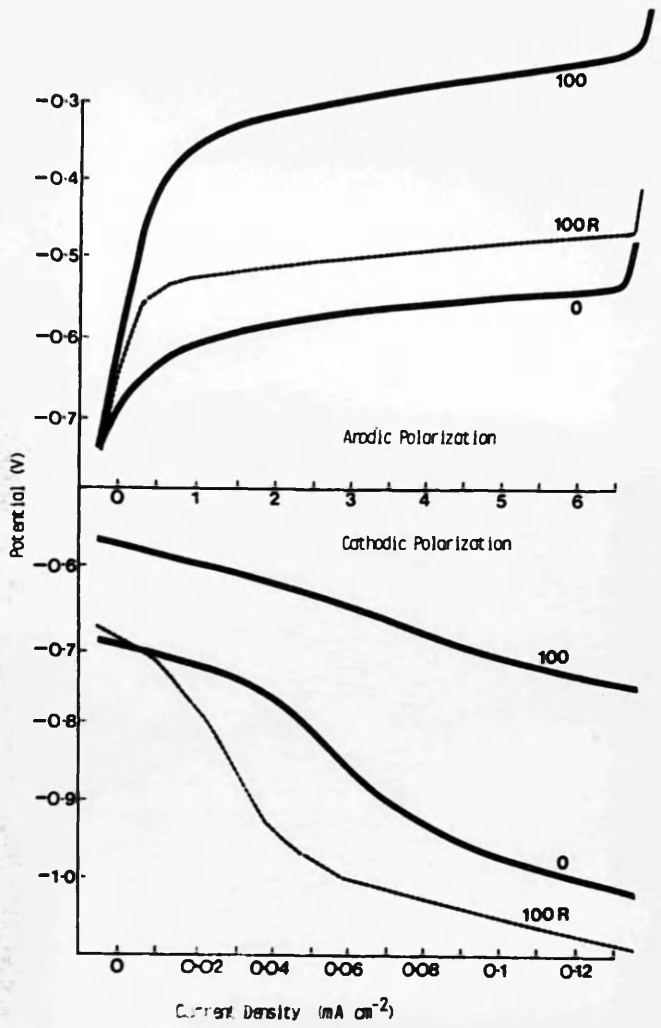


Fig. 57 Polarisation curves for 50D steel exposed to putrefying *Enteromorpha* material. After 1 hour (0) (control); after 100 days (100); after 100 days then repickled (100R).

(Reproduced by permission from Edyvean and Terry, 1983a)

corrosion (Hamilton, 1985).

These bacteria, such as *Desulphovibrio desulphuricans*, cause corrosion by hydrogen sulphide production from sulphates (La Que, 1975; Hamilton, 1985). The corrosion occurs by cathodic depolarization, which can be represented by the following equations (La Que, 1975):-

$$4Fe \longrightarrow 4Fe^{2+} + 8e^- \qquad (1.)$$

$$8e^- + 4H_2O + SO^{2-}_4 \xrightarrow[SRB]{} S^{2-} + 8OH^- \qquad (2.)$$

Combining these two equations gives :

$$4Fe + 4H_2O + SO^{2-}_4 \longrightarrow 3Fe(OH)_2 + FeS + 2OH^- \qquad (3.)$$

The reduction of hydrogen ions, shown in Eq. 2, is the only possible cathodic reaction in oxygen-free neutral solutions. The theory of von Wolzogen Kuhr and van der Vlugt suggests that bacteria depolarize the cathode by the removal of hydrogen atoms from the cathodic surface, as in Eq. 2 (La Que, 1975). This would drive the equilibrium of Eq. 3 to the right, with subsequent formation of iron sulphide which would cause corrosion. Removal of hydrogen atoms depends on the action of the enzyme dehydrogenase. This bacterial enzyme catalyses the overall reaction of cathodic depolarization (La Que, 1975; Hamilton, 1985) :-

$$SO^{2-}_4 + 8H \longrightarrow S^{2-} + 4H_2O \qquad (4.).$$

Direct experimental evidence for this reaction was obtained by Iverson (1966), using benzyl viologen as an electron acceptor in place of sulphate. Benzyl viologen is a dye which is colourless when oxidized and violet when reduced. A culture of *Desulphovibrio desulphuricans* was used together

with a steel coupon. After incubation, a violet area of reduced benzyl viologen was observed underneath the part of the coupon (test plate) over the area previously covered with cells. This showed that hydrogen, formed at the iron surface, was removed by *D. desulphuricans* to reduce benzyl viologen (Iverson, 1966). However, Hamilton (1985) suggested that hydrogenase-dependent cathodic depolarization was an experimental artifact resulting from using benzyl viologen to 'simplify' the system. Using electrochemical theory, it was concluded that the cathodic reactant is hydrogen sulphide, which is reduced according to the equation (Hamilton, 1985) :-

$$H_2S + e^- \rightleftharpoons HS^- + \frac{1}{2}H_2 \qquad (5.).$$

However, hydrogenase may have a secondary role through removal of the hydrogen generated in Eq. 5, with further generation of hydrogen sulphide. Indeed, hydrogen sulphide is a common factor in many areas where severe corrosion conditions exist (Munger, 1978). It reacts rapidly with iron to form iron sulphide, which in turn reacts with oxygen forming iron oxide, commonly known as "rust". Iron sulphide can itself remove hydrogen on a metal surface, causing cathodic depolarization. This increases the anodic reaction giving increased corrosion at the anode (Munger, 1978).

Iverson (1972) found that addition of iron (ferrous, Fe^{2+}) ions to a *Desulphovibrio*-free culture filtrate to remove sulphide ions increased the corrosion of mild steel. A black precipitate formed on the steel surface upon the addition of iron ions. This was thought to be iron sulphide. When the black precipitate was removed the resulting filtrate was still highly corrosive which suggested that the corrosive agent was water soluble. The high corrosion rates obtained suggest that the corrosive

agent removes electrons directly from the metal rather than through the utilization of hydrogen (Iverson, 1972). The corrosive agent has so far not been characterized further than being a volatile phosphorus compound (Hamilton, 1985).

The presence of ferrous iron was shown to affect the cathodic depolarization of mild steel in semi-continuous cultures of sulphate-reducing bacteria (Booth *et al.*, 1969). In the presence of a minimum concentration of ferrous ions, cathodic depolarization was transient, and a protective film of iron sulphide was formed on the steel surface. The organisms were not able to utilize hydrogen from a film of ferrous sulphide. However, in the presence of high ferrous iron concentrations much cathodic depolarization occurred. This was due to ferrous ions combining with the sulphide produced by the bacteria, thus preventing protective film formation and increasing corrosion. The presence of high ferrous ion concentrations even allowed a hydrogenase-negative organism such as *Desulphotomaculum orientis* to cause vigorous cathodic depolarization (Booth *et al.*, 1969). This observation reinforces Hamilton's (1985) suggestion that hydrogen sulphide may be the main electron acceptor in cathodic depolarization, with hydrogenase playing a secondary role.

As mentioned previously (p. 293), biofilms on a metal surface can give rise to differential aeration cells causing non-uniform corrosion. This process can occur with films of sulphate-reducing bacteria. Hardy and Brown (1984) investigated the influence of sulphate-reducing bacteria and the effects of aeration on the corrosion of mild steel. Corrosion rates of steel determined by weight loss measurements, were low in anaerobic cultures (see p. 305). However, the exposure of steel to

aerobic conditions by air sparging increased corrosion, resulting in pitting and perforation of the steel. A black precipitate, presumably of iron sulphide, developed on the steel surface during incubation. Mounds or tubercles of precipitate were randomly distributed across the steel surface. The areas of steel underneath these tubercles corresponded to the sites of pitting corrosion, shown by air sparging (Hardy and Brown, 1984). These observations again show how areas under microbial colonies on a model surface can act as differential aeration cells, so increasing localised corrosion.

Pitting potentials of steel became more active in seawater containing sulphate-reducing bacteria (Salvarezza and Videla, 1980). This was also found for steel in seawater containing sulphide ions. This again shows that sulphate-reducing bacteria corrode localized areas of steel by cathodic depolarization causing pitting corrosion.

Thomas *et al.* (1987) compared the effects of microbially produced and abiological hydrogen sulphide (H_2S) on the corrosion of two high strength steels. Biologically produced hydrogen sulphide was obtained from the decomposition of the algae *Enteromorpha* spp., *Porphyra* spp. and *Pelvetia* spp. in an enclosed seawater environment. The seawater containing bacterially produced hydrogen sulphide was transferred to corrosion fatigue test chambers. Corrosion measurements were made using 13mm. thick compact tension specimens obtained from steel grades RQT 501 and RQT 701. The results showed that the crack growth rates for RQT 701 steel in seawater containing biologically generated hydrogen sulphide increased with increasing H_2S concentrations. Crack growth rates became similar at higher H_2S levels of 204, 370 and 477 ppm.. The corrosion fatigue results obtained for RQT 501 steel exposed to biologically produced H_2S were

TABLE 12

Weight Losses from Mild Steel Foils in Batch Cultures of Sulphate-Reducing Bacteria and in Sterile Media.

Treatment and Time	Mean Weight Loss (mg) per Foil ± Standard Deviation
Aerated sterile culture (100h)	205
Anaerobic culture of SRB (5 days)	1.73 ± 0.39
Air-sparged (5h) culture of SRB (5 days)	8.17 ± 1.3

(from Hardy and Brown, 1984).

similar. However, higher levels of abiologically produced H_2S had a much greater effect on the crack growth rates of RQT 501 steel than biologically produced H_2S. The reason for the lack of crack growth enhancement with biologically produced H_2S is not properly known. However, it appeared that when there was a detectable increase in sulphide ions, there was no sulphide available at the crack tip to enhance hydrogen entry. The formation of a bacterial slime layer on the steel surfaces could provide a barrier to the transport of sulphide ions to the metal. An interspecies transfer of ions between bacteria could also make much of the measured H_2S unavailable to the steel. In contrast, all of the artificially added H_2S could contribute to the corrosion fatigue process (Thomas *et al.*, 1987).

A specific example of metal corrosion related to microfouling on mild steel coupons was shown with a ferric (Fe^{3+})-reducing pseudomonad isolated from crude oil (Obuekwe *et al.*, 1981a,b). In the absence of the bacterium, a dense amorphous crystalline coat formed on the steel surface (see p.307,309). However, in the presence of the pseudomonad the crystalline coating was removed, exposing the metal to the atmosphere (see p. 308). Bacterial colonization was mediated by the production of fibrous, exopolysaccharide material over the steel surface (Obuekwe *et al.*, 1981a, b). The steel remained passive in the absence of the organism; the crystalline surface coat formed a protective coating. However, in the presence of the bacterium, intense anodic depolarization of the steel occurred (Obuekwe *et al.*, 1981a; see p. 310). The pseudomonad (*Pseudomonas* isolate 200) reduces ferric [Fe(III)] iron to soluble ferrous [Fe(II)] iron, which removed the protective coating so exposing the steel to the atmosphere and enhancing corrosion (Obuekwe *et al.*, 1981a, b). Addition of nitrate inhibited corrosion as a result of the formation of

Fig. 58 S.E.M. of mild steel coupon submerged in uninoculated (control) medium for 48 hours. The coupon was first rinsed in distilled water and dehydrated in a regime of increasing ethanol concentration - 30% to 100%. The micrograph shows the formation of a protective, densely packed, crystalline surface covering in the absence of the iron reducing bacterium.

(Reproduced by permission from Obuekwe *et al.* 1981a)

Fig. 59 S.E.M. of mild steel coupon submerged in medium containing culture of isolate #200 (iron reducing bacterium) for 42 hours. The micrograph shows extensive removal of the protective, dense, crystalline surface covering in the presence of the bacterium. Mag. x245.

(Reproduced by permission from Obuekwe *et al.* 1981a)

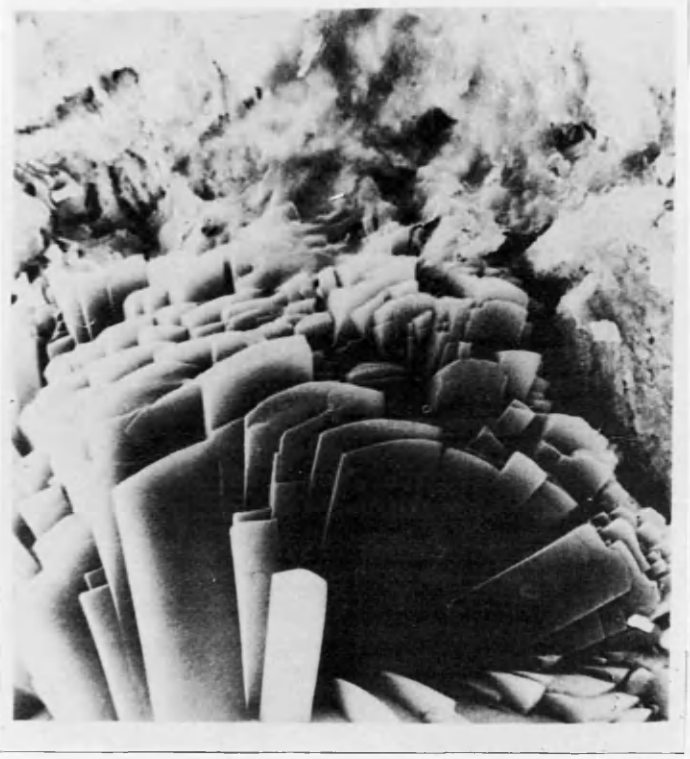


Fig. 60 Closer view of the protective, densely packed, crystalline covering formed on submerged metal in the absence of the iron reducing bacterium. Such a covering will pose a protective barrier between the metal and its corrosive environment. Mag. x1225.

(Reproduced by permission from Obuekwe *et al.* 1981a)

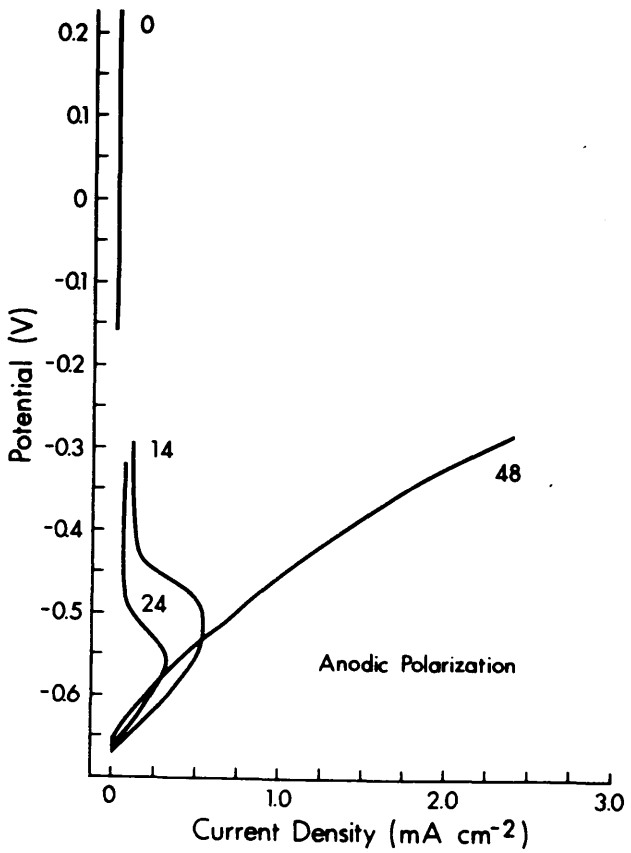


Fig. 61 Anodic polarisation of mild steel in medium containing 0.7g/l sodium nitrite and inoculated in isolate #200 (iron reducing bacterium). (0, 14, 24 and 48 denote incubation time - hours at 25^{0}C ±2^{0}C).

(Reproduced by permission from Obuekwe *et al.* 1981a)

nitrite and protective ferric oxide layers by the bacterium which prevented the corrosion of the steel specimen (Obuekwe *et al.*, 1981a). Additionally, corrosion of the steel may also be due to the formation of differential aeration cells under the exopolysaccharide deposited by the pseudomonad (Obuekwe *et al.*, 1981a).

Summary

This chapter has brought out the following main points.

(1.). The corrosion of metals in seawater can be caused by marine microorganisms which are attached to the metal surface (Obuekwe *et al.*, 1981a, b; Gerchakov and Udey, 1984).

(2.). One corrosion mechanism is the formation of differential aeration cells under a non-uniform film of attached microorganisms (La Que, 1975; Gerchakov and Udey, 1984).

(3.). (i.). Certain attached microorganisms, such as bacteria and diatoms and the cyanobacterium *Oscillatoria* spp. can corrode metals by depolarization of the anodic or cathodic areas of the metal surface (Edyvean and Terry, 1983a).

(ii.). Diatoms of *Amphora* spp. appear to inhibit corrosion by the production of a uniform layer of adhesive mucilage over the metal surface (McDonnell *et al.*, 1984).

(4.). (i.). Sulphate-reducing bacteria, which can be present in biofilms under anaerobic conditions, can corrode metals by the production of hydrogen sulphide and iron sulphide formation from sulphates by cathodic depolarization (Iverson, 1966; La Que, 1975; Munger, 1978; Hamilton, 1985).

(ii.). Artificially produced hydrogen sulphide had a greater effect on the corrosion fatigue of steel than hydrogen sulphide produced biologically from the decomposition of seaweeds (Thomas *et al.*, 1987).

(iii.). Photosynthetic and non-photosynthetic bacteria and cyanobacteria can also corrode metals by cathodic depolarization, through hydrogenase and nitrate reductase enzyme systems (Mara and Williams, 1971, 1972).

Chapter 12 Antifouling and microfouling control methods

As this thesis has concentrated mainly on adhesion mechanisms of marine microorganisms and the consequences of their attachment to living and non-living surfaces, it was felt necessary to end it by a discussion of methods of preventing attachment. Microfouling control methods, in particular, are seen as being important in controlling macrofouling by seaweeds and fouling invertebrates (Fischer *et al.*, 1984). This could be of importance for bulk carrier ships and oil tankers where algal fouling and its removal can be a problem.

There is no single solution to the problem of marine fouling. Antifouling systems which have worked well in similar circumstances have sometimes failed. Although 'traditional' antifouling techniques such as the use of toxic paints or chlorination may prove useful in some cases, they do not represent a universal solution. As no individual antifouling method may be completely effective, broad-spectrum treatments need to be tried. These methods use general energy inputs to remove attached microorganisms, and include mechanical scrubbing, heat and ultraviolet light (Fischer *et al.*, 1984). Antifouling techniques can also be based on an understanding of the attachment mechanisms of microorganisms and of factors affecting their attachment.

This chapter discusses traditional and more advanced chemical fouling control methods and non-chemical antifouling methods. More recent developments and possible future antifouling techniques are also discussed.

(12.1). Chemical fouling control methods

Marine microfouling can be most economically controlled by chemical methods (Fischer *et al.*, 1984). The use of cuprous oxide antifouling paints is one of the most traditional methods (Meadows and Anderson, 1979). These paints work by dissolving in seawater and releasing toxic copper ions from the metal surface, a process called leaching. A boundary layer of high toxin concentration is created at the paint surface which poisons settling organisms (Evans, 1981; Fischer *et al.*, 1984). The leaching rate, which influences the thickness of the toxic boundary layer, depends on the temperature, pH and salinity of the seawater and on the water flow rate over the surface.

Two main types of cuprous oxide antifouling paints have been widely used. They are separated by the mechanism of toxin release into the seawater. In soluble matrix paints, the toxin particles are distributed in a binder based on compounds called rosins. Rosins are resinous organic acids which dissolve slowly in the slightly alkaline seawater, so exposing the toxic paint particles, which themselves dissolve. Degradation of rosins is also accelerated by slime-forming marine bacteria which foul the paint surfaces (see p. 317) (Evans, 1981; Fischer *et al.*, 1984).

Contact leaching paints have a higher copper oxide content, and the particles are closely packed. As particles at the surface dissolve, deeper ones become exposed (Evans, 1981).

Cuprous oxide antifouling paints are still widely used in most situations nowadays. However, during the 1960s, they were not performing efficiently, particularly in the control of algal fouling on tankers. This led to the introduction of organometallic biocides in antifouling compositions by the end of the 1960s (Evans, 1981).

The use of tributyltin and triphenyltin compounds gave early promise of fouling control on steel, aluminium and wood test panels (Evans, 1970). Vinyl-based organotin paints were more effective in preventing fouling on aluminium boats.

There are two main types of organometallic paints now used. Type I are coatings which incorporate organotin compounds into the composition. These would include tri-n-butyltin fluoride and tripropyltin fluoride used as plasticizers and pigments. Type II are coatings based on film-forming resins containing a chemically bound organotin moiety (Fischer *et al.*, 1984). They further separate into Type IIA and Type IIB coatings. Type IIB have been successfully used commercially and are used in co-polymer antifouling paints. These consist of an organotin residue, such as tributyltin, incorporated into methacrylate-organotin copolymers. In seawater, the organotin residue and the polymer dissolve from the surface and are released together, so exposing more toxicant (Evans, 1981; Fischer *et al.*, 1984).

Toxicity experiments with *Enteromorpha* spp. showed that the organotin toxin should be present early during settlement to prevent spore attachment. Settled spores of *Enteromorpha* spp. were more resistant to triphenyltin chloride than swimming spores (Christie, 1972). There was a progressive increase in LD^{50} value (the concentration of triphenyltin chloride needed to kill 50% of *Enteromorpha* spp. spores) with increasing time after spore settlement. This resistance to toxic action was thought to be due partly to synthesis of a cell wall around the settled spore. The cell wall rapidly thickened, so giving considerable protection to the spore from the organotin toxin (Christie, 1972).

The effects of triphenyltin chloride on attachment and growth of the stalked diatom *Achnanthes subsessilis* has also been studied (Callow and Evans, 1981). The microscopic appearance of diatom cells after five days incubation with triphenyltin chloride was recorded. A diffuse clump of cells with long mucilage stalks was observed at a toxin concentration of 50$\mu molm^{-3}$. At a concentration of 1$mmolm^{-3}$, the diatom cells were dead (Callow and Evans, 1981). The effects of triphenyltin chloride on $H^{14}CO^{-}_{3}$ (^{14}C-labelled bicarbonate) fixation, which is a measure of CO_2 uptake, was also investigated. A greater inhibition of $H^{14}CO^{-}_{3}$ fixation was observed after 1-2 hrs. incubation of diatoms with triphenyltin chloride than after five days incubation at the same toxin concentration. These observations suggest that *Achnanthes subsessilis* cells can become more resistant to organotin paints after a period of time. The increased length of stalks observed at a toxin concentration of 50$\mu molm^{-3}$ suggests that triphenyltin chloride may stimulate stalk production. As stalk formation proceeds on the paint surface, the diatom cells will move out of the highly toxic boundary layer into a region of lower toxicity (Callow and Evans, 1981).

These observations suggest that certain organometallic paints may not be so effective at controlling microfouling, particularly by algae. There have been several other detailed studies of microfouling of traditional antifouling paints which have brought their effectiveness into doubt. Some of these studies will now be discussed.

The formation of microfouling slimes on antifouling paint surfaces makes a considerable contribution to the frictional resistance of moving ships. In addition, their presence restricts the outward diffusion of

toxin, which enables spores of macrofouling algae to settle (Evans, 1981). Bacteria and diatom slime films developed on cuprous oxide paints within five days. The quantity of copper in these slimes was 0.1-0.3g. per 100g., which was insufficient to poison the larvae of other settling organisms. These observations suggest that fouling microorganisms are resistant to copper in antifouling paints (Dolgopolskaya and Gurevich, 1968).

Diatoms, predominantly *Amphora* spp. were the main fouling microorganisms found on copper oxide antifouling paint specimens from Australian naval vessels (Bishop *et al.*, 1974). Bacterial slimes were also observed. Biodegradation of the paint material by attached bacteria can occur, which increases the rate of release of copper ions. Large numbers of *Amphora* spp. were observed on the paint surfaces, producing layers varying in thickness from 2-30 μm. These layers formed a barrier to the diffusion of toxic material from the coating, and affected the frictional resistance of the ships. The effectiveness of the antifouling paint must be reduced because of the rapid release of toxic material by bacteria and diffusion being hindered by diatoms (Bishop *et al.*, 1974).

Further observations of microfouling of copper-containing paints showed a wide range of diatom species to be present. A dense bacterial film developed after two months exposure of paint-coated aluminium surfaces to seawater (Robinson *et al.*, 1985). After four months, dense colonies of the diatoms *Amphora coffeaeformis, Nitzschia ovalis* and *Amphiprora paludosa* were present. Two other diatoms, *Stauroneis constricta* and *Achnanthes brevipes*, had settled after five months (Robinson *et al.*, 1985). There was evidence of sloughing of the slime film as it approached a thickness of

100μm. This left sparsely settled areas which were recolonized by the same diatom species. *Nitzschia ovalis* was the predominant species in the slime films, representing 94% of the population.

Amphora spp. was the most common fouling organism observed on cuprous oxide antifouling paints by Callow (1986). This diatom often forms thick, mucilaginous slime films on such paint surfaces (Daniel *et al.*, 1980; see p. 319 ; see also Chapter 3). Transmission electron microscopic examination of *Amphora* cells from such films showed dense inclusion bodies in the cytoplasm. The elemental composition of these inclusion bodies was analysed using an energy dispersive spectrometer. Copper was one of the elements present, together with silicon, phosphorous, sulphur and calcium (Daniel *et al.*, 1980). In a further investigation of these inclusion bodies, *Amphora* cells isolated from toxic coatings were cultured and treated with 0,0.75 and 1.5 ppm. of copper chloride. Diatoms were treated this way for one week, and then examined by transmission electron microscopy. *Amphora* cells treated with 0.75 ppm. of copper chloride contained at least five inclusion granules per cell (Daniel *et al.*, 1980). These granules were usually located within or protruding into large cell vacuoles (see p. 320). Elemental analysis showed that this contained copper, sulphur, silicon and aluminium. The presence of copper suggests that the granule may allow *Amphora* spp. to immobilize copper ions from toxic paints. This could allow *Amphora* spp. to become resistant to cuprous oxide paints. The extracellular, anionic polysaccharide mucilage produced by this diatom could also bind copper ions (Daniel *et al.*, 1980).

Microfouling has also been observed on organometallic paints (Robinson *et al.*, 1985; Callow, 1986). A dense bacterial layer developed

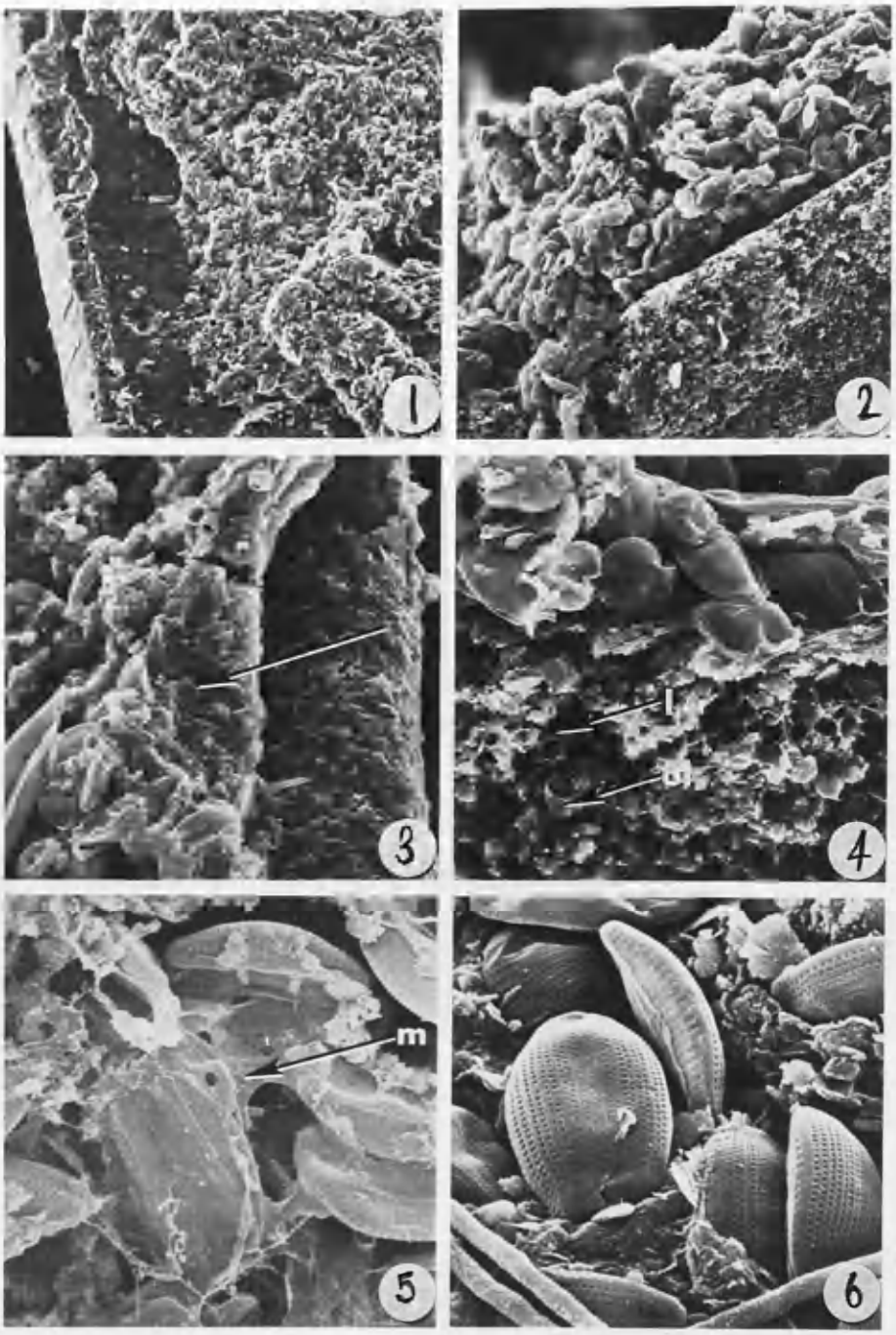


Fig. 62 (1-6) S.E.M. of paint fragments from test panels immersed in seawater.

Fig. 62.1 Shows heavy settlement of *Amphora* spp. on a slow-leaching cuprous oxide paint. Mag. x280

Fig 62.2 Cross-section of cuprous oxide paint showing thickness of *Amphora* film in comparison with paint. Mag. x800

Fig. 62.3 Shows peeling from the surface of a primary film. Note the amorphous underside of the film (arrowed) which is probably bacterial in origin. Mag. x1520

Fig. 62.4 Cross section of a cuprous oxide paint with *Amphora* cells adpressed to the surface. The leached (l) and unleached (ul) layers of the paint are apparent. Mag. x1760

Fig. 62.5 Showing the underside of an *Amphora* film. Note the intricate meshwork and copious mucilage (m) involved in holding the film together. Mag. x2600

Fig. 62.6 High power micrograph showing *A. veneta,* one of the most prominent diatom foulers found in this study. Mag. x3000

(Reproduced by permission from Daniel *et al.* 1980)

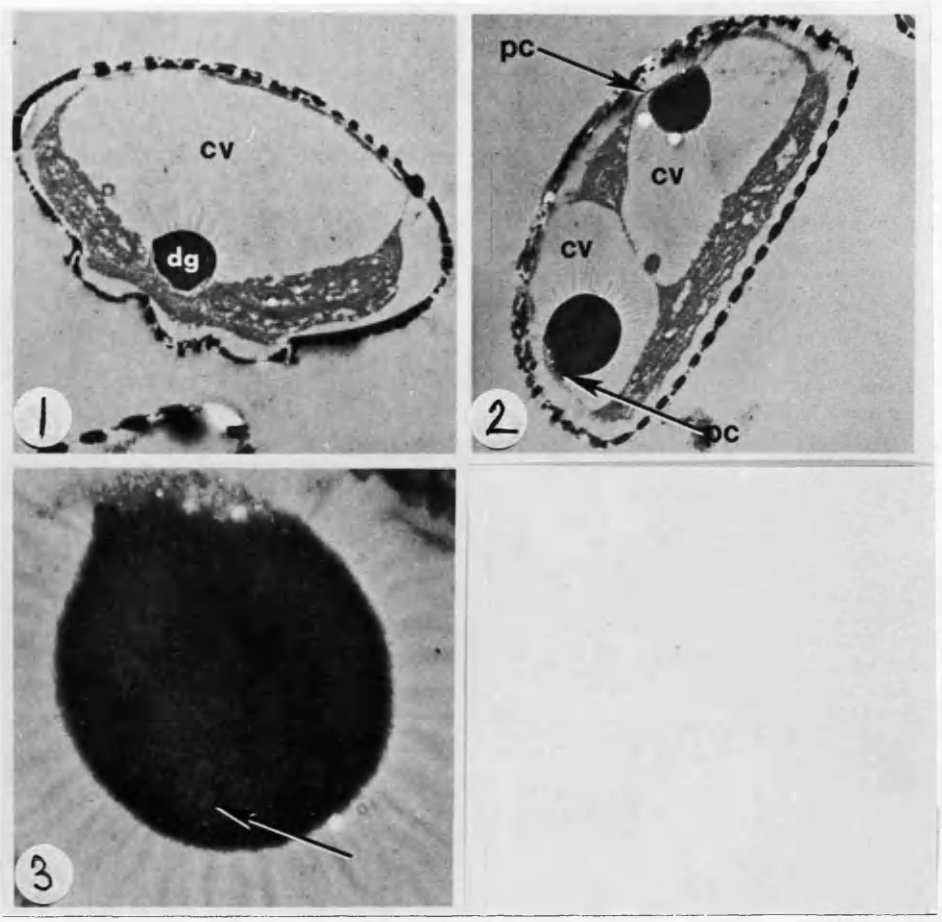


Fig. 63 (1-3) *Amphora* cells treated with varying concentrations of copper chloride ($CuCl_2$).

Fig. 63.1 Transverse section of an *Amphora* cell treated with 0.75 ppm $CuCl_2$ for one week. A dense granule (dg) is observed protruding into the cell vacuole. Mag. x11200

Fig. 63.2 Amphora cells (oblique transverse section) treated as above. Two large granules are visible within the cell vacuoles (cv) and appear attached to peripheral cytoplasm (pc). Mag. x8160

Fig. 63.3 High power of an intracellular granule. Lighter regions (arrows) may be observed within the structure. Mag. x37850

(Reproduced by permission from Daniel *et al.* 1980)

on aluminium surfaces coated with tributyltin fluoride after one month's submersion in seawater. After a further month, the surface was covered by a single layer of ten species of pennate diatoms (Robinson *et al.*, 1985). *Amphora coffeaeformis* and *Nitzschia ovalis* were amongst the diatoms present on tributyltin fluoride. There was a greater diversity of fouling species on this paint surface than on cuprous oxide paint (Robinson *et al.*, 1985).

Achnanthes spp. was a major fouling organism on traditional paints containing tributyltin or organoarsenical compounds. The diatom was particularly found on coastal vessels (Callow, 1986).

The extent of microfouling of self-polishing copolymer (SPC) paints used on large ocean-going vessels was also investigated by Callow (1986). Self-polishing copolymer paints are composed of a copolymer formed between tributyltin methacrylate and methyl methacrylate, which incorporates other biocides. The copolymer hydrolyses in seawater releasing tributyltin (Callow, 1986). The SPC paints used in this study contained either cuprous oxide or cuprous thiocyanate. *Amphora* spp., *Amphiprora* spp., *Navicula* spp. *Stauroneis* spp. and *Achnanthes* spp. were observed on SPC paints from large ocean-going vessels (Callow, 1986). *Amphora palludosa* and *A. coffeaeformis* were observed in large numbers on aluminium panels coated with SPC paints (Robinson *et al.*, 1985). Differences were found in the composition of diatom slime films on a Very Large Crude Carrier after six months lay-up and also after cleaning and three months "in-service". After lay-up, the composition was *Stauroneis* spp. 80%, *Navicula* spp. 10% and *Amphora* spp. 10%; cyanobacteria were also present. After cleaning and three months trading, the slime composition was *Amphora* spp. 80%, *Stauroneis* spp. 10% and *Navicula* spp. 10% (Callow, 1986). This shows

that, even after a period of cleaning to remove fouling growths the same diatoms attach to the ship's surface, although in different amounts.

Fouling by macroalgae, particularly *Enteromorpha* spp. and *Ectocarpus* spp., was observed on both soluble matrix and contact leaching cuprous oxide paints. They were also found as the dominant fouling organisms on SPC-coated vessels which had been laid up for 12-24 months (Callow, 1986). This observation further suggests that formation of diatom slime films on such surfaces allows settlement of algal spores, which ultimately leads to macrofouling by other organisms.

These observations raise doubt about the overall effectiveness of 'traditional' cuprous oxide and organometallic antifouling paints. The quality of the fouling microflora, rather than the quantity, is an important factor indicating the potential effectiveness of a paint. A higher number of attached microalgal species may reduce toxicity in the long term (Robinson *et al.*, 1985). Microorganisms may have developed methods of resistance to such toxic paints over a long period of time. In addition, triphenyltin paints have recently been found to be highly toxic in the marine environment, and their use has been discontinued.

Although traditional antifouling paints are still widely used, their disadvantages may result in more use of alternative chemical fouling control methods (Fischer *et al.*, 1984). Some of these will now be discussed.

(12.2). Advanced and Alternative Chemical Control Methods

Flocculants are non-toxic additives which can remove fouling growths from surfaces. Acrylate additives, which are cationic flocculants, are

often used with chlorination to remove slime films from heat exchangers. They improve the efficacy of chlorine in removal of fouling deposits (Fischer *et al.*, 1984). The calcium chelating agent EGTA removed a biofilm from the walls of a recycle tube reactor. This probably occurred by removal of calcium from the biofilm by EGTA, which may suggest that calcium is essential for its structural integrity (Turakhia *et al.*, 1983). Suspended solids in the effluent after EGTA treatment were mainly composed of carbohydrates. This suggests that the biofilm material removed by EGTA consists largely of bacterial adhesive material. Further evidence came from the addition of EGTA to biofilm samples suspended in growth media. Disaggregation of the bioflocs, and presumably extracellular polymeric material, was observed (Turakhia *et al.*, 1983).

Chelating agents could be used to remove diatom fouling films, particularly films of *Amphora* spp.. Calcium was present in the mucilage of *Amphora coffeaeformis*, and was essential for adhesion of this diatom to glass (see Chapter 3.2). Further research is needed on the effects of chelating agents, such as EGTA, on the removal of bacterial and algal fouling films.

Young and Mitchell (1973) studied the effects of certain toxic chemicals on the attraction of certain bacteria. They found that bacteria avoided the area adjacent to a capillary mouth when such chemicals were present. Chloroform, ethanol, benzene and toluene repelled over 90% of the test bacteria from the capillary. These observations suggest that certain toxic chemicals evoke a negative chemotactic response by bacteria (Young and Mitchell, 1973).

Further studies were made using certain chemical compounds incorporated into non-toxic paints on submersed metal surfaces (Chet *et al.*, 1975; Chet and Mitchell, 1976). The most effective compounds to cause negative bacterial chemotaxis were acrylamide, tetramethylethylene diamine, indole, tannic acid, benzoic acid and thiourea. The bacterial population on untreated panels after 12 days immersion was 5 x 10^{12} cells/cm^2. However, the population on panels treated with benzoic and tannic acids was $10^6/cm^2$ after the same period (Chet *et al.*, 1975; Chet and Mitchell, 1976). The amounts of slime film developing on panels coated with repellant paints and untreated panels was compared gravimetrically and by chemical analysis. The slime which developed on panels coated with acrylamide, tannic and benzoic acids was of lower weight than on untreated panels (Chet *et al.*, 1975; Chet and Mitchell, 1976; see p. 325). In addition, algal growth on the metal panels was inhibited by tannic, benzoic and humic acids, phenylthiourea and thiosalicylic acid (Chet and Mitchell, 1976).

These results show that certain non-toxic organic compounds can be used in repellant paints to prevent microbial film formation on surfaces. These compounds work by preventing marine bacteria and other microorganisms from approaching them. Repellant paints could be used to replace conventional, toxic antifouling paints. They have the advantage of not being so hazardous in the marine environment as conventional paints (Chet *et al.*, 1975; Chet and Mitchell, 1976). Further research on the effects of a wider range of non-toxic compounds on the settlement of marine microorganisms is needed. The use of repellant paints has so far not been shown to be practical on a large scale (Fischer *et al.*, 1984).

TABLE 13

Effect of repellants on the production of bacterial slime on Surfaces 40 days after immersion in the sea.

Repellants	Amount of slime ($\mu g/cm^2$)
Control	3,450
Phenylthiourea	2,400
N,N,N',N'-tetramethylethylenediamine	1,630
Acrylamide	620
Tannic acid	210
Benzoic acid	190

(Taken from Chet *et al.*, 1975).

The use of chlorine and its derivatives has been effective in preventing microbial settlement (Meadows and Anderson, 1979). The usual method is the injection of gaseous chlorine. Electrolytic generation of chlorine has also been used, particularly in closed systems such as ships' condensers. In these structures, chlorine is effective against slime-forming bacteria and diatoms (Meadows and Anderson, 1979).

Production of chlorine by the electrolytic hypochlorination of seawater has been developed in recent years. This technique has been used in the U.S.A. to study biofouling control using an ocean thermal current sensor (Smith and Kretschmer, 1984). Electrolytic hypochlorination involves making the surface to be protected become the anode for chlorine generation by the electrolysis of seawater. The anode was formed by depositing a thin platinum film on the exterior of the thermal current sensor (Smith and Kretschmer, 1984). Biofouling control on the thermal current sensor and on glass slides containing platinum films were studied using continuous and intermittent hypochlorination. Continuous hypochlorination at a current density of 5μA/cm² over six months adequately controlled fouling on both the thermal current sensor and glass slides. A current density of 10μA/cm² or greater completely removed all fouling organisms from both surfaces (Smith and Kretschmer, 1984).

Intermittent hypochlorination was also carried out at different current densities during active cycle times of 15, 30 and 60 mins. over 8 and 24 hrs. After six months of exposure, no significant biofouling had formed on any of the test slides. Examination by light microscopy showed scattered colonies of algae on the slides exposed to a current density of

25μA/cm² (Smith and Kretschmer, 1984). Intermittent current densities of 125 and 250 μA/cm² removed all biofouling from the slides. A submerged thermal current sensor was also subject to intermittent hypochlorination over a four month period at a current density of 80μA/cm² for 30 min. every 24 hrs.. After this period, the thermal sensor did not possess so many attached algae as the control sensor, which was not chlorinated (Smith and Kretschmer, 1984).

In order to further show the effectiveness of chlorine, a thermal current sensor was allowed to accumulate biofouling for one month. The sensor was then chlorinated at a current density of 500 μA/cm², and was observed continuously. No biofouling was observed on the sensor's surface by the light microscope after 24 hrs. hypochlorination (Smith and Kretschmer, 1984). Additional tests were performed on the sensor after two and three months of biofouling accumulation. The current densities used during chlorination were 500 and 1000 μA/cm², respectively. In both cases, 24 hrs. was required to completely remove biofouling growth (Smith and Kretschmer, 1984).

These observations demonstrate that electrolytic hypochlorination is a practical, non-toxic method of controlling biofouling. This technique could be used to control fouling in large-scale heat exchangers and on other large surface areas (Smith and Kretschmer, 1984). However, it is uncertain whether this technique would be practical on ship's hulls. Further research is needed to show whether electrolytic hypochlorination is as effective as antifouling paints in controlling accumulated fouling on such large surface areas as ship's hulls.

(12.3). Other non-chemical microfouling control methods

Certain antifouling methods have been developed which do not depend on the use of toxic or non-toxic chemical methods. These methods tend to use physical principles, such as scrubbing or irradiation or temperature changes to control fouling.

Scrubbing is one of the oldest fouling control methods known. It is a broad-spectrum technique which uses mechanical energy to remove fouling growths (Fischer *et al.*, 1984). Scrubbing of ship's hulls reduces the drag or damage caused by marine fouling. Scrubbing is also justified economically, due to the fuel penalty suffered by ships whose hulls have fouling growths (Fischer *et al.*, 1984).

Three main scrubbing techniques are used for exterior surfaces, such as ship's hulls. These include rotary-powered brushes, which can be mounted on an underwater cart designed to travel along the hull. Cavitating jets use high pressure and imploding cavitating bubbles to rapidly clean surfaces (Fischer *et al.*, 1984). Large, boat-mounted brushes up to 20 ft. long are used for scrubbing the waterlines of ships. All of these methods, however, must work by removing the fouling growths without damaging the underlying antifouling paint. Such damage could lead to severe corrosion problems which may result in serious structural damage (Fischer *et al.*, 1984).

Mechanical cleaning systems have also been used for certain nonship devices, such as the optical surfaces of submerged instruments. Scrubbing is also used to clean the surfaces of OTEC (Ocean Thermal Energy Conversion) systems (Fischer *et al.*, 1984). Microbial slime films

frequently develop on OTEC equipment, and must be completely removed so as to prevent losses in performance (Fischer *et al.*, 1984).

Scrubbing methods are also used to clean interior surfaces, such as piping systems and heat exchangers. Rods and brushes can be used to remove fouling communities from such surfaces (Fischer *et al.*, 1984). Sponge rubber balls are often used which are slightly larger than the internal diameter of the fouled tube or pipe. They are usually inserted upstream of the area of the pipe or tube to be cleaned, and are retrieved downstream (Fischer *et al.*, 1984). Although this technique has shown potential in fouling control, it has also caused tube corrosion and erosion. Another interior cleaning system uses flow-driven brushes. These brushes are often used in marine heat exchangers (Fischer *et al.*, 1984). The system works by having permanently attached catching baskets at either end of the tubing to be cleaned. The brush is contained in one of these baskets. During operation, the flow of seawater to the tube is reversed, and the brush is driven to the opposite end. As it moves, the brush cleans the tubing and is ultimately caught in the other basket. When the seawater flow is returned to normal, the brush cleans the tubing in the opposite direction (Fischer *et al.*, 1984).

Caron and Sieburth (1981) investigated the use of a circular brush assembly to remove microfouling communities from fibre-glass reinforced plastic surfaces. The surfaces were immersed in an estuarine environment over a 14-day period. Before cleaning, diatoms dominated the fouling population, covering 90% of the plastic surfaces. A bacterial film which had developed after three days submersion covered 12% of the surface (Caron and Sieburth, 1981). The main diatoms present were *Nitzschia* spp.. A twice-weekly brushing removed most of the diatoms. Stalked bacteria and

those possessing pili or fimbrae were easily removed by the circular brush. A bacterial film consisting mainly of bacteria surrounded by extracellular secretions was left on the plastic surface. Certain protozoa, such as amoebae and ciliates, which had a strong affinity for the substratum, were removed (Caron and Sieburth, 1981). The smooth nature of the fibre-glass surface probably allows a high efficiency of removal of microorganisms by brushing. Periodic removal of the faster accumulating diatoms, whilst leaving a bacterial film, seems to prevent further succession in the fouling film (Caron and Sieburth, 1981).

Nickels *et al.* (1981b) observed that aluminium and titanium surfaces exposed to rapidly flowing seawater quickly developed fouling communities. The effects of manual brushing using a stiff-bristle nylon brush on removal of fouling microorganisms was investigated. Three cycles of free fouling and manual brushing increased the rate of fouling on both surfaces (Nickels *et al.*, 1981b). The rate of fouling was greater on the titanium surface than on aluminium, although titanium was more readily cleaned. The microfouling community which re-formed on aluminium after cleaning was enriched in bacteria containing short-branched fatty acids. Progressive colonization of aluminium by bacteria producing web-like adhesive was observed by scanning electron microscopy (Nickels *et al.*, 1981b). Following cleaning, the microfouling community on titanium contained a diverse population, including microeukaryotes. There was an increase in bacteria containing short-branched fatty acids on titanium as cleaning continued (Nickels *et al.*, 1981b).

The results of Nickels *et al.* (1981b) indicate that certain scrubbing techniques may not be so effective as others in removing microfouling growths from surfaces. The scrubbing device must itself be protected from

marine fouling, as failure to do so will reduce its effectiveness. Rotating-brush devices which are used to clean the heat-exchanger surfaces of OTEC equipment often become fouled with microorganisms (Fischer *et al.*, 1984). Fouling of the nylon brush after cleaning may have caused the accelerated fouling of aluminium and titanium surfaces observed by Nickels *et al.* (1981b). The results of Caron and Sieburth (1981) suggest that more frequent brushing of surfaces may control microfouling more effectively. Scrubbing could be used in combination with a technique such as chlorination. This might well be particularly effective in controlling fouling on interior surfaces, such as the tubes of heat exchangers.

Optical methods, such as the use of ultraviolet light, have been shown to be completely effective in repelling all forms of microfouling and macrofouling (Fischer *et al.*, 1984). Di Salvo and Cobet (1974) investigated the use of ultraviolet radiation to control microfouling on quartz underwater windows submerged in an estuarine environment. Ultraviolet light was administered to the quartz windows from three different positions : exterior to the window, from directly behind the window (regarded as 'internal' irradiation) and from the edge of the window (Di Salvo and Cobet, 1974). Preliminary results using internal irradiation produced clean, unwettable quartz windows. This was compared with the non-irradiated controls which were heavily obscured by slime and macroorganisms. Further work using internal irradiation showed that the threshold for fouling control was 10$\mu W/cm^2$ of ultraviolet light (Di Salvo and Cobet, 1974). Below this irradiation level, the fouling sequence occurred slowly resulting in the development of wettable films. Above this level, however, slime formation was prevented and only minor amounts of microscopic particulate matter attached to the quartz windows.

External irradiation was not found to be so effective as the internal methods. Almost two orders of magnitude more external ultraviolet energy was needed to achieve the same level of fouling control (Di Salvo and Cobet, 1974). Ultraviolet radiation administered from the edge of the quartz windows over six weeks produced a generally nonwettable surface with poorly developed microcolonies. A gradient of ultraviolet energy was also delivered to the quartz window from the internal position. This was done by half covering a window on the inside with silver foil. The gradient of ultraviolet energy began at the border of the foil and proceeded towards the darkened end of the window (Di Salvo and Cobet, 1974). The bacterial count on the quartz surface decreased with increasing ultraviolet irradiation. Towards the non-irradiated end of the window, the population of stalked protozoans increased markedly, although there was a decrease in bacterial numbers.

These results suggest that low-power ultraviolet lamps can effectively prevent fouling of glass surfaces in an estuarine environment. The greater effectiveness of ultraviolet light applied from the internal position may occur because particle shading effects are avoided (Di Salvo and Cobet, 1974). Bacteria and other fouling organisms require intimate physical contact when attaching to the substratum. Shading out of externally applied ultraviolet radiation by surrounding detrital particles could allow microbial adhesion to occur. However, shading out of ultraviolet light applied from directly behind the quartz window is unlikely to occur. Di Salvo and Cobet (1974) put forward a unifying biological principle for fouling control based on the laws of thermodynamics. They stated that a higher level of stress energy, such as

ultraviolet radiation, is required to arrest succession in a high entropy, rapid turnover fouling microsystem.

The results of Di Salvo and Cobet (1974) also suggest that ultraviolet radiation could control fouling on submerged optical instruments. At present, this method appears to be suitable for closed systems only (Fischer *et al.*, 1984). Further research is needed to show the effectiveness of use of ultraviolet radiation on normal surfaces.

Thermal-control methods are based on temperature changes affecting the growth of fouling microorganisms. If the temperature is raised 40°C above the freezing point of water it goes well above the optimal growth temperature for nearly all microorganisms (Fischer *et al.*, 1984). As the temperature continues to increase, fewer organisms are able to grow. Significantly higher temperatures are required to prevent growth of slime-forming microorganisms. High temperatures have been found to be effective at keeping optical surfaces free of fouling for three month periods. However, this technique is energy intensive and can only be justified for small, extremely critical surfaces (Fischer *et al.*, 1984). Further work would be needed to show the effectiveness of thermal methods on larger, external surfaces. This method, however, may be one of the more environmentally acceptable fouling control methods currently available (Fischer *et al.*, 1984).

The control of fouling by osmotic methods has also been suggested. The attachment of most common fouling microorganisms is inhibited by salinity changes (Meadows, 1965; Fischer *et al.*, 1984). This method makes use of the lower salinity of freshwater rather than higher salinity waters. However, the diversion of ships to freshwater areas to control fouling is

not economically feasible. A ready source of freshwater must be available in order for osmotic-control methods to be economically effective (Fischer *et al.*, 1984).

(12.4). Control of microfouling by substratum properties and possible future developments in antifouling technology

Substratum properties are known to affect adhesion of marine bacteria (see Chapter 1.5) and algal rhizoids (see Chapter 3.1). The use of surface chemical properties as a means of preventing microfouling has been recognized for many years (Fischer *et al.*, 1984).

Baier (1972) proposed an approach to antifouling based on the formation of a proteinaceous conditioning film on surfaces immersed in seawater (see Chapter 1.5). He suggested that the adsorbed protein film should be maintained in a form similar to the three-dimensional conformational state assumed in solution. If this occurred, cellular elements would arrive at a passive surface and not accumulate (Baier, 1972).

Goupil *et al.* (1973) suggested three theoretical "routes" for achieving resistance to biological fouling using substratum chemical properties :-

(1) Adjustment of the initial surface properties of a substratum to form a "biocompatible range" which prevents fouling. This could be achieved by using selected materials which create a properly organized "watery" surface, such as hydrophilic polymers.

(2) Creation of ablative or exfoliating surfaces from which initially attached fouling masses could be spontaneously shed. This could be done by the use of surfactant-doped polymers or exfoliating additives such as drag-reducing polymers or sparingly soluble polysaccharides.

(3) Formation of "natural" fouling-resistant layers, which could be types of fouling organisms whose initial attachment would discourage subsequent colonization by other organisms. This could be done by the formation of a film of algal spores or bacteria (see Chap. 3.1.1.3) which would resist layer thickening.

Studies were made of the effects of the formation of a conditioning film on surface properties, such as critical surface tension, of certain substrates. The substrates included polyvinylchloride, FEP Teflon, cellulose acetate and polyvinyl fluoride. In all cases, it was found that acquisition of a conditioning film altered the critical surface tension of these substrates towards a common middle ground. This in turn suggests that specific outermost chemical arrays on certain substrata might minimize the attraction and adhesion of microorganisms. Surface arrays of closely packed methyl groups were found to be particularly fouling resistant (Goupil *et al.*, 1973).

Surface wettability and critical surface tension are known to influence, and in some cases to decrease, marine bacterial adhesion (see Chapter 1.5). Surface free energy has also been shown to affect attachment of algal rhizoids (see Chapter 3). This suggests that these substratum properties could inhibit fouling by certain microorganisms. Most substrata immersed in seawater possess a "biocompatible range" of

critical surface tension within which lower rates of microfouling occur (Goupil *et al.*, 1973; Fischer *et al.*, 1984). The biocompatible range for most substrata occurs within critical surface tension values of 22-25 dynes/cm. (Fischer *et al.*, 1984). Formation of a conditioning film on substrata was thought to modify the critical surface tension values towards the biocompatible range (Goupil *et al.*, 1973). It was also proposed that the first effect of surface wettability was to influence the nature or rate of accumulation of the conditioning film. In addition, as the conditioning film influences bacterial attachment, then wettability will also indirectly affect attachment. Studies have shown, however, that microbial fouling does increase with time on surfaces in the biocompatible range, although at a lower rate (Fischer *et al.*, 1984).

Studies with substrata incorporating silicone elastomer compounds have indicated a further method of controlling bacterial and diatom fouling (Milne and Callow, 1985; Callow *et al.*, 1986). The structure of silicone elastomers is based on a backbone of repeating silicate (-Si-O-) units. The silicone polymers can be modified by the addition of other organic compounds, such as phenyl methyl silicone fluid (Milne and Callow, 1985; Callow *et al.*, 1986).

A comparison was made of bacterial and diatom adhesion to glass, polytetrafluoroethylene (PTFE) and silicone elastomers (Milne and Callow, 1985). There were 80% fewer bacteria attached to the silicone elastomers than either to glass or PTFE. Silicone elastomers also reduced the number of attached diatoms by 69% compared with glass. The numbers of bacteria and diatoms attaching to the silicone elastomers was further reduced by the inclusion of 5% phenyl methyl silicone fluid (PMS) (Milne and Callow, 1985;

see p.338).

Further studies of the detachment of *Amphora* spp. from silicone elastomers were made using a radial flow growth chamber (Milne and Callow, 1985; see Chapter 3.2 for a description of the principles of the radial flow growth chamber). The radial zone of diatom detachment was greater on the silicone elastomers compared with glass or PTFE. The addition of 5% PMS to the silicone elastomers further increased the zone of *Amphora* spp. detachment. There was no change over 48 h. in the shear force required for cell detachment from the silicone elastomers with 5% PMS (Milne and Callow, 1985).

Further work was then performed on the effects of three groups of silicone elastomers on the attachment of *Amphora coffeaeformis* (Callow *et al.*, 1986). The three groups of elastomers used were :-

1) Room-temperature vulcanizing silicone elastomers, referred to as RTV(i) - (iv);
2) Platinum-cured silicone elastomers, known as PC(i) - (iii);
3) Moisture-cured acetyl-tipped silicone elastomers, referred to as MC(i) - (iv).

Measurements were made of chlorophyll 'a' content in the *Amphora coffeaeformis* films. The results showed the lowest biomass to occur on the RTV silicone elastomers, particularly RTV (i) (Callow *et al.*, 1986). This also occurred when ATP measurements were used as an indicator of cell biomass. Consequently, the RTV (i) silicone elastomer caused the greatest reduction in adhesion of *A. coffeaeformis*. Addition of PMS to RTV (i) did not influence the adhesion of *Amphora* spp. (Callow *et al.*, 1986).

TABLE 14

Number of bacteria attached to a range of surfaces after 2h.

Surface	Number of cells/μm^2
Glass	22.3 ± 1.8
PTFE[1]	23.3 ± 0.5
SE[2]	4.3 ± 0.5
SE + 5% PMS[3]	2.8 ± 0.2

TABLE 15

Number of cells of *Amphora* attached to a range of surfaces after 16h.

Surface	Number of cells/mm^2	Reduction compared to glass (%).
Glass	427.6 ± 7.8	
SE[2]	130.6 ± 4.3	69
SE + 5% PMS[3]	102.3 ± 3.4	76

[1] PTFE - Polytetrafluorethylene

[2] SE - Silicone elastomer

[3] PMS - Phenyl methyl silicone fluid.

(Taken from Milne and Callow, 1985).

However, visual fouling differences were observed on glass slides coated with the RTV (i) elastomer with and without addition of PMS. The slides were immersed in seawater for periods of 3 months or longer. After 16 weeks, slides coated with RTV (i) possessed a mixed diatom slime, whilst those with PMS added were free of any organisms (Callow *et al.*, 1986). The addition of PMS also improved the antifouling performance of other silicone elastomers on slides immersed for 16 weeks. Polymer RTV (ii) had an approximately 60% and 20% cover of slime and macroalgae, respectively. After the addition of PMS, the slime cover was reduced to 20% and there was no macroalgal attachment. The polymer MC(i) possessed approx. 80% slime and 5% macroalgal cover after 16 weeks immersion. PMS reduced the slime cover to 30% and completely removed the macroalgae (Callow *et al.*, 1986). These observations suggest that addition of PMS to silicone elastomers is effective at removing fouling macroalgae.

Differences were apparent in the quantitative observations of Milne and Callow (1985) and Callow *et al.* (1986) on the effects of PMS addition on diatom adhesion. It is uncertain whether these differences are due to cell variability or variability between the batches of RTV polymer used. Variability would have existed between replicates treated with any one silicone elastomer. The techniques used did not allow small differences in diatom adhesion caused by this variability to be determined (Callow *et al.*, 1986). In addition, these quantitative measurements do not take account of surface shear forces. The glass slides coated with silicone elastomers and immersed in seawater for 16 weeks were exposed to shear stress caused by tidal flow (Callow *et al.*, 1986). This could remove more attached organisms with or without PMS addition. Consequently, these visual observations showed more fouling removal than the biomass

measurements (Callow *et al.*, 1986). Shear stress would also contribute to the enhanced detachment of *Amphora* spp. observed in the radial flow growth chamber (Milne and Callow, 1985).

Paul (1984) studied the effects of certain antibiotics and metabolic inhibitors on the attachment of estuarine *Vibrio proteolytica* to polystyrene. Some of these results indicated the involvement of proteins in the attachment of *V. proteolytica* (see Chapter 1.2). The observations could also have implications for antifouling methods. Some antibiotics, such as ampicillin, oxacillin and streptomycin, inhibited *V. proteolytica* adhesion after growth at the 25% minimal inhibitory concentration (MIC) (Paul, 1984). These antibiotics inhibit bacterial cell wall synthesis, and caused deformation of cell morphology. Other antibiotics inhibited attachment when administered simultaneously with the substratum. These included dinitrophenol, chloramphenicol, puromycin and cephalothin (Paul, 1984). In these cases, a greater concentration of antibiotic was required to inhibit attachment than to inhibit growth. This may be caused by an adhesive moiety present on the bacterial cell surface. Elevated concentrations of the antibiotics may be required to prevent further synthesis of this material (Paul, 1984). In addition, certain metabolic uncouplers also inhibited bacterial attachment. These included inhibitors of oxidative phosphorylation such as valinomycin, DNP (2,4 dinitrophenol) and azide. Elevated concentrations of these compounds were also required to inhibit attachment (Paul, 1984).

These observations of the inhibition of *V. proteolytica* adhesion by certain antibiotics suggests that this could be a further antifouling development. The use of antibiotics suggests a further way of using the

substratum to influence bacterial adhesion. This is particularly shown by the antibiotics which inhibited attachment when simultaneously administered with the substratum (Paul, 1984). Further work is needed to show the effectiveness of antibiotics in inhibiting attachment of other fouling marine bacteria. In addition, work on the ease of incorporating antibiotics into antifouling paints would be needed. A problem with using antibiotics in antifouling is that certain marine bacteria may develop resistance to them. Research would also be needed into this problem, and ways of overcoming it.

Antibiotics are less likely to affect the attachment of algal spores and diatoms. The different structures of algal and diatom cell walls and the lack of protein in algal adhesives (see Chapter 3) may make antibiotics less effective. However, metabolic uncouplers which inhibited *V. proteolytica* adhesion (Paul, 1984), may be effective. Certain metabolic inhibitors, such as DNP and azide, removed films of *Amphora coffeaeformis* (see Chapter 3.2). Further work is also needed on the effectiveness of metabolic uncouplers in preventing algal fouling.

The control of marine microfouling by substrata physicochemical properties is a major development in antifouling technology. More research is needed on the effects of a wide range of substrata of differing properties on the attachment of various microorganisms. The difficulty in this work is finding individual substrata whose properties decrease the attachment of all fouling bacteria, algae, fungi and protozoa.

The use of silicone elastomers (Milne and Callow, 1985; Callow *et al.*, 1986) is an encouraging development. In particular, the results obtained

with RTV silicone elastomers suggests that they are worth studying further. However, their physical properties of poor abrasion resistance and tear strength limit the range of possible applications (Callow *et al.*, 1986). Silicone elastomers may be best applied in situations where these characteristics are not important. These include in aquaculture or on offshore structures and piping systems (Callow *et al.*, 1986). The use of substrata properties to control fouling is a non-hazardous, non-polluting technique. There may be a tendancy in the future to move away from using the 'traditional' antifouling paints. Both cuprous oxide and organometallic paints have been found to be less effective as microorganisms develop resistance to them. In addition, some organometallic paints are known to be toxic in the marine environment (see p. 322).

Other chemical antifouling techniques such as the use of repellant paints or flocculants (Fischer *et al.*, 1984) are worthy of further study. In particular, repellants appear to offer a non-toxic, non-polluting way of preventing microbial adhesion. However, at present the long-term effectiveness of these methods is much less than conventional toxic paints. In addition, these methods have not yet been shown to be practical on the large scale (Fischer *et al.*, 1984).

Overall, as environmental pollution is recognised as a major global problem, more use may be made of non-polluting antifouling methods.

(12.5). Summary

It is not possible to cover all aspects of antifouling technology in this thesis. However, this chapter has discussed several traditional

antifouling methods as well as some more recent developments. The main points discussed in this chapter, together with the key cited references, are as follows.

1) (i) Cuprous oxide and organometallic paints have been, and are still used as traditional antifouling paints. These paints were effective in preventing fouling by marine bacteria, diatoms and macrofouling algae such as *Enteromorpha* spp. (Evans, 1981; Fischer *et al.*, 1984).

(ii) Further studies showed that bacterial and diatom slime films were easily formed on copper oxide antifouling paints (Bishop *et al.*, 1974; Daniel *et al.*, 1980). *Amphora* spp. were found to possess inclusion bodies which may immobilize copper ions from toxic paints (Daniel *et al.*, 1980). Fouling by a wide range of diatoms, such as *Amphora* spp., *Nitzschia ovalis*, *Achnanthes* spp. and *Navicula* spp. was observed on tributyltin-containing paints (Robinson *et al.*, 1985; Callow, 1986). These observations suggested that traditional antifouling paints may not be so effective.

2) (i) Flocculants, such as calcium chelating agents, could detach biofilms or diatom films from surfaces by the removal of calcium (Turakhia *et al.*, 1983; Fischer *et al.*, 1984).

(ii) Certain non-toxic organic compounds can be used in repellant paints to prevent microbial film formation. These compounds cause negative chemotaxis to occur in bacteria and other microorganisms approaching them. Acrylamide, tannic and benzoic

acids were most effective at reducing bacterial populations on treated panels (Chet *et al.*, 1975; Chet and Mitchell, 1976).

3) Electrolytic generation of chlorine is particularly effective at controlling fouling in closed systems such as ships' condensers. Intermittent hypochlorination was effective at removing biofouling from an ocean thermal current sensor. Two to three months biofouling growth on a thermal current sensor was completely removed within 24 hrs. by intermittent hypochlorination (Smith and Kretschmer, 1984).

4) (i) Scrubbing is one of the oldest fouling control methods known. It can be used for cleaning both interior and exterior surfaces. Rods, brushes, sponge rubber balls and flow-driven brushes are often used for interior surfaces (Fischer *et al.*, 1984).

(ii) A twice-weekly brushing of fibre-glass reinforced plastic surfaces was effective at preventing succession in a microbial fouling film (Caron and Sieburth, 1981). However, three cycles of manual brushing increased the rate of microfouling on both aluminium and titanium surfaces (Nickels *et al.*, 1981b). This suggests that certain scrubbing techniques may not be so effective as others.

5) (i) Ultraviolet radiation administered from directly behind a quartz underwater window was particularly effective at controlling an estuarine microfouling sequence (Di Salvo and Cobet, 1974). Radiation administered exterior to the quartz

window did not control microfouling so well as the 'internal' method. Delivery of a gradient of ultraviolet radiation to the quartz window showed that the bacterial population decreased with increasing irradiation (Di Salvo and Cobet, 1974).

(ii) Temperature increases which affect the growth of fouling microorganisms could be used to prevent fouling. Significantly high temperatures are needed to prevent growth of slime-forming microorganisms. Thermal-control methods could be one of the most environmentally acceptable methods available (Fischer *et al.*, 1984).

6) (i) A proteinaceous conditioning film formed on surfaces immersed in seawater could prevent microbial adhesion by maintaining a three-dimensional conformational state (Baier, 1972).

(ii) Conditioning film formation can alter substratum wettability and critical surface tension values towards a biocompatible range. Lower rates of microfouling occur within this range (Goupil *et al.*, 1973; Fischer *et al.*, 1984).

(iii) Silicone elastomers incorporated in substrata were effective in controlling bacterial and diatom fouling (Milne and Callow, 1985; Callow *et al.*, 1986). Room-temperature vulcanizing silicone elastomers caused a large reduction in attachment of *Amphora coffeaeformis*. Addition of phenyl methyl silicone fluid (PMS) further reduced bacterial and diatom

attachment. PMS addition to other silicone elastomers was also effective at removing fouling macroalgae (Milne and Callow, 1985; Callow *et al.*, 1986).

(iv) Certain antibiotics, such as ampicillin and streptomycin, which prevent bacterial cell wall synthesis, inhibited attachment of *Vibrio proteolytica* to polystyrene (Paul, 1984). Other antibiotics inhibited bacterial attachment when administered simultaneously with the substratum. The inhibition of bacterial attachment by antibiotics could be a further antifouling development. Metabolic inhibitors such as DNP and azide also inhibited *V. proteolytica* attachment (Paul, 1984). These compounds could prevent algal as well as bacterial attachment.

(v) The control of marine microfouling by substratum physicochemical properties could be further developed in the future. It offers a non-hazardous, non-polluting technique. Further research is needed on the effects of a wide range of substrata on the attachment of various microorganisms.

APPENDIX OF SELECTED TERMS AND ABBREVIATIONS

Prosthecae/prosthecate - Cell organelles, usually localised protruberances of the bacterial cell wall and membrane, which occur at one or more sites on the cell surface (Chapter 1.3).

Chlorococcacean cyanobacteria - An order of cyanobacteria having non-motile unicellular and colonial vegetative stages, the colonial ones with no vegetative cell division (Chapter 2).

Silane compounds - A group of compounds based on the silicon atom, with organic and halogen substituents present (Chapter 3).

R-antigen - Also known as the 'rough' core or R-core of the lipopolysaccharide molecule found in the outer membrane of the cell envelope of Gram-negative bacteria. It is a short chain of sugars, which include 2-keto, 3-deoxyoctonic acid (KDO) and heptose (Chapter 7.2).

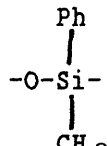
'O'-side chain - A long chain of sugars which are linked to the R-core in the lipopolysaccharide molecule. It is much longer than the R-core, being composed of many repeating tetra- or pentasaccharide units. Confers strain-specific antigenic properties on certain Gram-negative bacteria (Chapters 5 and 7.2).

Murein lipoprotein - A molecule found in the cell envelope of certain enteric bacteria, such as *E. coli*. The lipoprotein is covalently linked to the peptidoglycan layer of the cell envelope. It extends outward from this layer, thus serving as a bridge from the peptidoglycan layer to the outer membrane of the cell envelope (Chapter 7.2).

Algacide - A chemotherapeutic agent which is toxic to algae (Chapter 8).

Silicone elastomers - Silicone polymer compounds which are based on a backbone of repeating [-Si-O-] units with the non-backbone valencies of the silicon attached to saturated organic radicals (Chapter 12).

PMS - Phenyl methyl silicone fluid, a silicone polymer based on the repeating unit :-



(Chapter 12)

SDS-PAGE - Sodium dodecyl sulphate polyacrylamide gel electrophoresis (Chapter 5).

ATP - Adenosine triphosphate (Chapter 8).

SEM - Scanning electron microscopy (Chapter 9 and other Chapters).

TEM - Transmission electron microscopy (Chapter 9 and other Chapters).

EGTA - Ethylene glycol-bis (β-aminoethyl ether)-N,N-tetraacetic acid (Chapter 12).

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