# THE MICROBIOLOGICAL ASPECTS OF EARLY STAGES OF MARINE FOULING

by

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## Summary

In this study, the ecology of attached marine bacterial populations on materials of economic significance was considered. Specific aspects of the microbial film examined were its composition, construction, and dynamics of bacterial attachment.

Precise and practical methods for collecting data from the natural environment were developed; the construction of a novel sample holder was described together with a procedure for the isolation of attached bacteria from surfaces and the selection of an efficient 'non-selective' heterotrophic growth medium. Techniques for the enumeration of bacteria on surfaces were assessed and the use of an acridine orange staining method with UV-epiflourescence microscopy described.

Various substrates representing a range of surface energy values (wettability) were immersed in seawater for different lengths of time during summer and winter seasons. Bacterial densities and rates of attachment were found to be greater during the summer than the winter due to, and concomitant with, seasonal levels of bacterial number in seawater. The substrate characteristic wettability influenced dynamics during the first twelve hours of substrate immersion such that, with decreasing wettability, there was an increase in attachment density and rate.

It is suggested that microbial attachment may be effected by one of three processes: polymeric fibril synthesis, flagella cell/surface binding, and chemisorption. It is considered that the majority of adhesion events occur by chemisorption.

The microbial film was composed mainly of gram negative rods, predominantly from the genera *Pseudomonas* and *Alteromonas*, and to a lesser extent, by gram positive coryneforms. Composition was affected by the substrate; communities were found to consist largely of substrate non-specific and, to a lesser extent, substrate specific types.

Microbial film development, the product of an interaction between attached, attaching cells, and the surface, was found to be a population characteristic, independent of substrate and environmental factors.

FOULING : MICROBIAL FILMS : ATTACHMENT : SURFACES

*To my Wife, KAREN*

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*Yea, slimy things did crawl with legs*
*Upon the slimy sea.*

*The Rime of the Ancient Mariner, Pt.ii.*

**Samuel Taylor Coleridge**

# CHAPTER 1

## INTRODUCTION

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# INTRODUCTION

## 1.1 Introduction

Over the past decade, the study of microbial films in the marine environment has assumed a new significance. It is now not only of theoretical interest but of real practical value, since man has a technology which can exploit and use the sea on a scale hitherto unknown. Interest has largely been directed at the role of microbial films as deteriogens of submerged equipment such as underwater TV camera lenses (Plotner, 1968; Dyckman & Castelli, 1971), sonar domes (Phelps, Dennis & Power, 1977), and their implication in the algal (Mitchell & Young, 1972; Tosteson & Corpe, 1975) and crustacean (Young & Mitchell, 1973) fouling process of ships.

Much of the early work was observational, noting the density of bacteria immersed in seawater at world-wide locations (ZoBell & Allen, 1935; Wood, 1950; Skerman, 1956; O'Neil & Wilcox, 1971). More recently, greater emphasis has been placed on determining the mechanisms of microbial attachment (ZoBell, 1943); Marshall, Stout & Mitchell, 1971; Marshall, 1973; Fletcher & Floodgate, 1973); Fletcher, 1977). There is still, however, little information with regard to the natural ecology of the microbial film in the marine environment.

The aim of this work was to define the microbial film as an ecological unit within the marine environment and consider aspects of its development, composition, structure, and activity.

## 1.2 Marine Fouling

Surfaces immersed in seawater become 'fouled' after a short period of time by attachment of sedentary organisms including bateria, algae, and crustacea. The attachment and proliferation of these organisms on

structures of economic importance can render them either ineffective or reduce their efficiency considerably. The major maritime fouling problem is associated with the attachment of algae, particularly *Enteromorpha* and *Ectocarpus* species (Fletcher & Chamberlain, 1975) to ships and submerged structures. The general effect of fouling on ships is to increase the frictional resistance of the hull, resulting in increased fuel costs, which for a badly fouled vessel may mount to $92,500 per annum (Eglin, 1976). The cost of cleaning and repainting hulls with organotin antifouling paints is extremely expensive; for some 'super tankers' of 250,000 dwt., hull maintenance in dry-dock may be as much as £150,000 per week (Houghton, 1970). The overall cost of ship fouling to the U.K. has been put at £50 million per annum (Houghton, 1970), while for the U.S.A., it may be as high as $500 to $700 million per year (Liberatore, Dyckman, Montemarno, Cohn, 1972). Fouling of static structures is becoming an increasing problem, particularly on offshore oil rigs, where fouling organisms increase the structural loading, impair maintenance, and impede routine inspection programmes (Freeman, 1977).

Fouling of structures by micro-organisms is subtle and unlike the gross and chronic occlusions observed for algal fouling. The biodeteriogenic effect of microbial attachment has been observed on conduits in heat exchangers; growths can seriously reduce the efficiency of thermal transfer and also limit liquid throughput by frictional resistance (Norrman, Characklis & Bryers, 1977). In the marine environment, submerged electronic equipment is particularly susceptible to microbial films, and may impair the efficiency of sonar domes (Phelps *et al.*, 1977) and occlude lenses on underwater TV surveillance equipment (Dyckman & Castelli, 1971). Attached microbial growths have also been shown to accelerate the corrosion of steels (Lebedeva & Shtevneva, 1975), possibly

by the release of organic acids (Gerchakov, Marszalek, Roth & Udey, 1976).

The most significant aspect of microbial fouling is the implication that microbial films influence secondary colonisers such as algae and crustacae. Floodgate (1971) considers the development of the microbial film as the first stage of a successional sequence of fouling organisms. The microbial film can influence attachment of oyster larvae, *Crassostrea virginica* (Young & Mitchell, 1973), although it has been suggested (Crisp & Ryland, 1960) that it is not an absolute requirement. Algal zoospore response, however, is less clear, but it has been argued (Mitchell & Young, 1972) that attached microbial growths may have a significant influence.

## 1.3 Microbial Films

Microbial attachment to surfaces is an interfacial phenomenom of considerable significance in many aspects of microbial ecology (Marshall, 1976). Cell surface interactions resulting in attachment are characterised by cells, suspended in a liquid phase, adhering, passively or actively, to solid surfaces. The advantages of attachment depend largely on the particular environment; free living cells,immobilised on organic particulate matter, benefit from the nutrient status of the attachment locus (Jannasch & Pritchard, 1972), while attachment of *Salmonella* species to gut epithetial cells not only initiates invasion of the host cell (Smith, 1978), but also prevents its dislodgement from the gut lumen. Similarly, in the oral cavity, attachment of *Streptococcus mutans* to teeth, using a polysaccharide complex derived from sucrose, prevents its removal by saliva and swallowing (Gibbons & van Houte, 1975). Tooth decay, the result of organic acid release by *Str. mutans*, has a striking similarity to the process of metal corrosion by marine microbial films.

Microbial attachment and the development of stable microbial films have been studied in relation to fermentation systems (Atkinson & Fowler, 1974). Conventional fermentations may either be a batch or constant stirred tank process, but the efficiency of these methods may not always be high, and the kinetics of reaction can lead to a 'wash out' of the active organism. Immobilisation of bacteria as microbial films on tubes or on particles, however, ensures that a high throughput and efficient utilisation of substrate can be achieved. This process is utilised in waste water treatment where microbial films on trickle filter beds remove dissolved organic carbon from sewage effluent, so reducing its BOD value (Mack, Mack & Ackerson, 1975).

The attachment of bacteria to surfaces immersed in seawater leads to the development of complex biocoenoses of potential influence in the marine environment. The attached communities may develop on a variety of substrates; the microbiota are termed periphytic when attached to, but not penetrating, inert surfaces; on animals, epizoic; on plants, epiphytic; on stones, epilithic; and on sand, epipsammic (ZoBell, 1972).

One of the earliest quantitative studies of marine microbial films was made by ZoBell & Allen (1935) where bacterial densities of $4.65 \times 10^4$ to $7.02 \times 10^5$ cells/$cm^2$ on glass slides immersed for 24 hours were recorded. Wood (1950), however, observed that in some localities, namely Australia, microbial film composition was dominated by diatoms. Skerman (1956), working off the New Zealand coast, found similar attachment patterns, with microbial films dominated either by diatoms or bacteria. Latterly, microbial films have been reported to consist predominantly of bacteria, though most work has been in U.S.A. coastal waters (O'Neil & Wilcox, 1971; Corpe, 1974), and the Adriatic

(Cviic, 1953). The microbial films are composed of a wide range of bacterial types, including the genera *Pseudomonas*, *Vibrio*, *Spirillum*, *Arthrobacter*, *Corynebacterium*, (Floodgate, 1971), *Flavobacterium*, *Bacillus* and *Micrococcus* (O'Neil & Wilcox, 1971); some attached bacteria, *Caulobacter* and *Hyphobacterium*, have a specialised mode of attachment- the holdfast (Corpe, 1973). The predominant type of organism is the gram negative rod. Attachment of marine bacteria to surfaces other than glass have been made using metals (Sechler & Gundersen, 1973); plastics (Dexter, Sullivan, Williams, Watson, 1975); wood (Cundell & Mitchell, 1977); and on sand grains (Meadows & Anderson, 1968).

## 1.4 Microbial Attachment

Microbial attachment is the result of a bacterial/surface interaction involving surface, bacterial, and environmental factors. In dilute nutrient conditions where growth is limited, microbial activity can be promoted by increasing the surface area (Heukelekian & Heller, 1940). ZoBell (1943) suggested that bacteria attach in order to utilise surface adsorbed organic material (Loeb & Neihof, 1977), and it has been shown, using natural populations derived from river water, that attached bacteria are metabolically more active than free living types (Hendricks, 1974). Following adhesion, detritus, a nutrient source, collects in cell surface interstices and accelerates attached bacterial growth and microbial film development.

Bacterial adhesion is achieved by extra-cellular polymers binding the cell to the surface. Polymers may either cover the cell as a discrete layer (Fletcher & Floodgate, 1973), or exist as a coat of polymeric fibrils, termed the glycocalyx (Costerton, Geesey & Cheng, 1978). The polymers have been characterised as polyanionic carbohydrates

containing uronic acid and neutral sugars (Corpe, 1974). A polysaccharide exopolymer produced by *Pseudomonas atlantica* (Humm), isolated from surfaces, contained the neutral sugars, glucose, galactose, mannose, the acidic carbohydrate galacturonic acid, and pyruvic acid (Corpe, 1970). The ratio of neutral to acid carbohydrates varies with bacterial type but ranges from 1:1 to 1:3 ; the anionic element of the polymer confers on the cell a net negative charge (Corpe, 1973). Amino acids have also been detected in the polymer, which suggests that it may be a glycoprotein (Corpe, 1974). Two layers of acidic polymer on surface bound cells have been described (Fletcher & Floodgate, 1973) ; the first layer is discrete and believed to be involved in initial attachement ; the second layer is more extensive and requires calcium and magnesium ions to stabilise it (Fletcher & Floodgate, 1976). In some instances, copious quantities of polymers are produced by attached microbial communities in alpine streams (Geesey, Richardson, Yeomans, Irvin & Costerton, 1977). The population is immersed in the polymer matrix which affords physical protection to the micro-colonies.

Attachment has been described as a two-stage process : the reversible phase and irreversible phase (Marshall, Stout, Mitchell, 1971). During the reversible stage, cells may collide, as a random event, with the surface, or actively seek it as a chemotactic response to adsorbed nutrients (Young & Mitchell, 1973 A); cells are held at a short distance away from the surface by an equilibrium of van der Waal attractive forces and double ion repulsion layers which surround the cell and surface. Irreversible attachment is achieved by the synthesis of extracellular polymeric fibrils which bind the cell to the surface. Attachment may be influenced by the substrate characteristic of wetting, which is an index of substrate surface energy (Zisman, 1972). Dexter *et al.* (1975) have shown that microbial attachment to surfaces in the marine environment is

greater on wettable (high surface energy) substrates - a pattern similar to blood and cell tissue systems (Baier, 1970). Other work (Fletcher & Loeb, 1976), however, using a marine pseudomonad isolated from surfaces, has shown a reverse trend with greater bacterial densities on less wettable (low surface energy) substrates.

## 1.5 Investigation Objective

Marine microbial films have been examined from two broad view points: firstly, observational studies, which have noted the development of microbial films in seawater and, secondly, mechanistic studies using selected organisms, have attempted to determine the details of microbial attachment. The aim of this work is to integrate observational studies of microbial films in the natural environment with mechanistic descriptions of microbial attachment, and develop a rationale of microbial film development, structure, and activity in the marine environment.

Microbial parameters in the natural environment were measured using techniques developed to ensure accuracy of population estimates, sampling and isolation of bacteria from the attached community.

Aspects of microbial film ecology considered were the dynamics of microbial attachment on various substrates, and the factors which influenced density, rate, and distribution of attached bateria during summer and winter periods.

The structure of attached communities was also examined; components of the microbial film were identified, and the specificity of attached populations for particular substrates assessed.

# CHAPTER 2

## SAMPLING ISOLATION AND ENUMERATION METHODS FOR BACTERIA ON SURFACES

# SAMPLING ISOLATION AND ENUMERATION METHODS FOR BACTERIA ON SURFACES

## 2.1 Introduction

The scope of this work is to redefine and extend the concept of the microbial film as a habitat. To achieve this aim, consideration was given to the techniques used to obtain data, for on the basis of information gathered in the field, interpretations of the size, scope, and function of a particular habitat are made. To determine these parameters using mensural techniques which are inaccurate or inappropriate, can only lead to a misleading description. This is particularly true of the microbial film. Much of the early work was descriptive, providing information useful in defining the microbial film *per se* (ZoBell, 1972). However, a point has been reached when an approximate description of the habitat cannot be expected to form the basis of a dynamic conceptual scheme. To provide the necessary detail, new techniques (which also reflect accuracy) were required.

The emphasis of data collection was centred on descriptive parameters of the population. This direction was chosen because it provides information about individuals in the community and their collective interactions as the autochthon which enables a composite but unified description of the habitat to be constructed. The foundation of such an approach is in the accuracy and efficiency of sampling the habitat. Two sampling devices were designed, and are described in this Chapter. Concomitantly, a suitable isolation procedure for bacteria in a microbial film and a culture medium which would express the largest number and variation of bacteria present was developed. Lastly, an enumeration process was devised which would detect bacteria on any flat surface and so enable precise population estimates to be made.

## 2.2 Sampling Apparatus

The types of apparatus used for immersing samples are numerous. Most of them have been designed around the glass microscope slide, the traditional substrate of choice. As many of the studies were short term, aimed at determining the nature of the microbial film, equipment was not complex or designed to hold large numbers of samples. Few of the early workers described their sample carriers, except ZoBell and Allen (1935), whose design, although practical on the small scale, became cumbersome when enlarged. More recently, the review by Sladeckova (1962) describes many different designs of sample carriers, but none of them were able to meet the requirements for this investigation. The limitations were the number of samples held, ease of use, mooring, strength, constructional materials, and designs which occluded the samples.

The following criteria were used in the design of a sample holder. It was to be made from a cheap, non-toxic, non-corrodable material, able to contain a large number of samples readily accessible, able to withstand heavy seas and long periods of immersion. Two sample carriers were constructed; the first was used for general sampling, and the second for short-term sampling (less than 12 hours). The advantage of the second design was the ease of access to samples *in situ*.

### 2.2.1 Design

The general sampling apparatus was constructed out of high pressure PVC tubing. It consisted of two parallel tubes set into a cross-bar of PVC tubing and secured with two small brass bolts. The top of the carrier was set into a similar cross-bar which could be removed to gain access to the samples. A groove was machined along the length of each parallel upright and aligned so that samples could be slotted

into grooves and stacked on edge (Fig.1). The device had a carrying capacity of 40 samples, cut to the dimensions of a standard microscope slide. Materials other than glass were shaped with rounded corners to ensure their smooth insertion and removal. The sample holder was moored by threading a length of polypropylene cord down the parallel tubes and joined above the top bar by a shackle, to which a mooring rope or buoy could be fastened. To provide some stability, a weight was attached to the bottom of the sample holder.

The second type of slide carrier was constructed from 19 mm PVC tubing and 90$^{o}$ push-fit plumbing connections (Bartol Ltd.), to form a rectangle. Ten triangular A4 plastic slide binders were suspended equidistantly along the length of the carrier and supported on polypropylene stubs (Fig.2). Ten samples could be inserted into each binder to give a total capacity of 100 samples. The frame was weighted and fastened by four cords,attached to each corner. The carrier was moored just under the surface of the water.

## 2.3 The Isolation of Bacteria from Surfaces

The isolation of bacteria from surfaces has involved some form of mechanical removal such as swabbing (O'Neil & Wilcox, 1971; Corpe, 1974), or relied on direct physical contact between the substrate and a nutrient surface such as agar (ZoBell & Allen, 1935). Irrespective of method, these techniques ignore the likelihood of not only sampling the surface flora but also other unwanted sections of the habitat such as bacteria held by physico-chemical interactions at the surface but not attached to it — reversibly sorbed — (Marshall *et al.*, 1971), and bacteria uninvolved with the surface but held in the film of water adhering to the sample on its removal from the sea. The free living

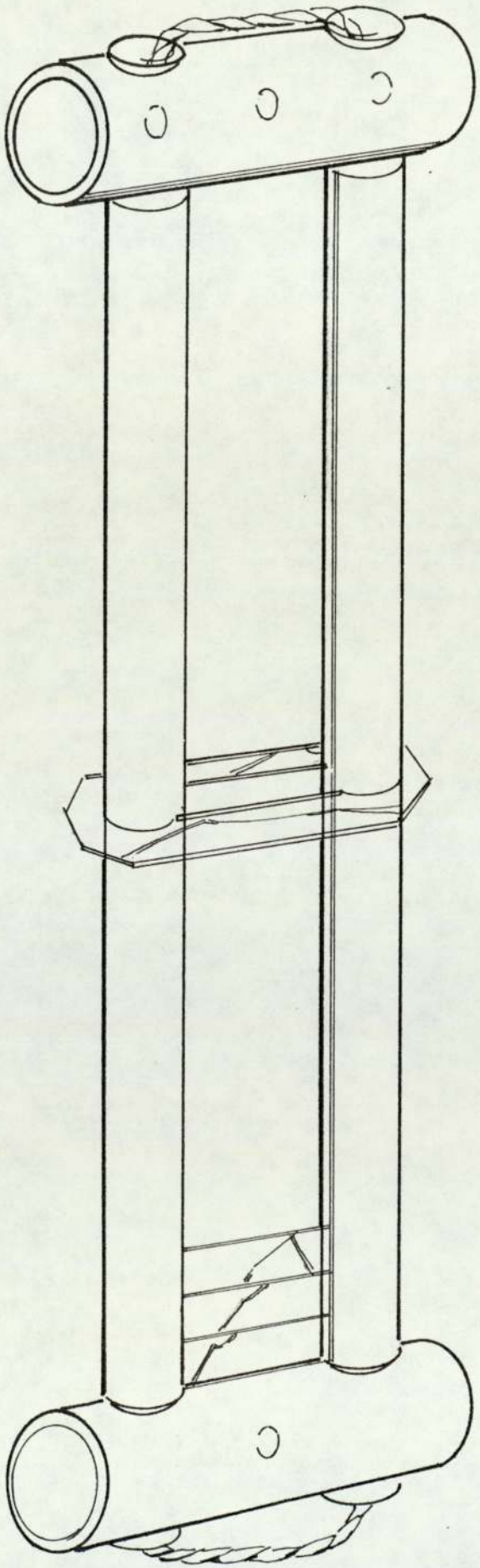


Fig. 1 40 slide sample holder fitted with central stiffener.

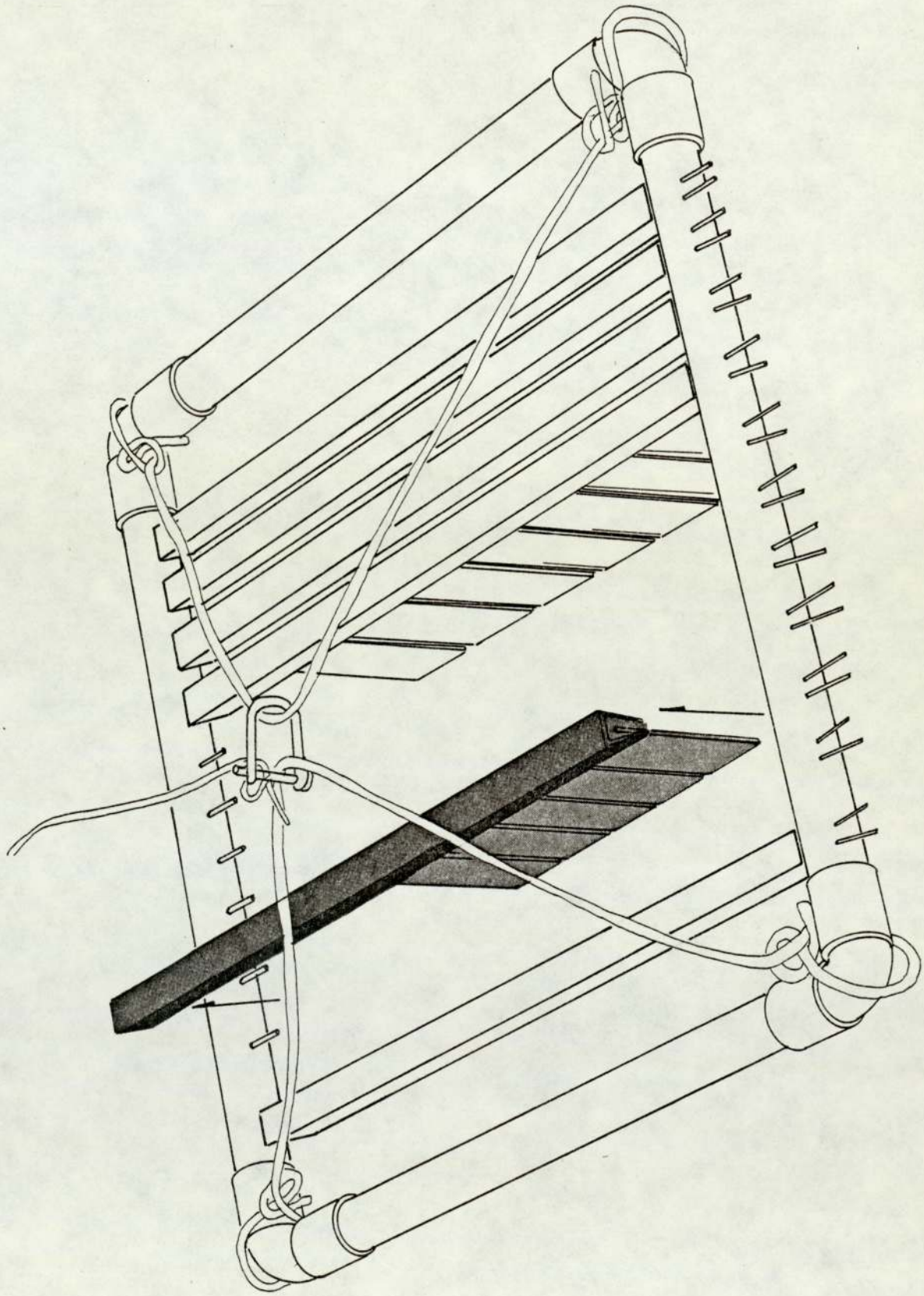


Fig. 2 Short term immersion,ready access sample holder.

bacteria may either be organisms incapable of attachment (ZoBell & Allen, 1935), or types which, although able to adhere, cannot do so because of a physico-chemical incompatibility preventing the cell entering the sequence of sorption events leading to attachment. As the habitat contains a number of dissimilar groups of bacteria, it is necessary to ensure the selectivity of the isolation procedure, otherwise incorrect conclusions may be drawn from information based on groups of organisms assumed to be representative of the habitat.

Corpe (1973, 1974) rinsed samples in unspecified volumes of sterile seawater, presumably to remove unwanted and unattached bacteria from the surface. Marshall *et al.* (1971) have shown that rinsing removes 'reversibly sorbed' bacteria, while Corpe (1974 A) has demonstrated that firmly attached bacteria are not dislodged by such treatment. The object of this study was to devise and test a reproducible technique which would remove all extraneous unattached bacteria from a sample and allow the isolation of only attached bacteria.

### 2.3.1 Equipment

The removal of unattached bacteria was effected by rinsing samples with sterile seawater under controlled reproducible conditions. The apparatus (Figs.3, 4, & 5) consisted of a constant-head one-litre aspirator, gravity fed by a ten-litre aspirator reservoir. The outlets had ground-glass joints sealed with silicone rubber glue (Dow Corning Ltd.), and the aspirators were vented via cotton-wool plugged thistle tubes. The washing device itself consisted of a glass tube (33 mm dia. x 175 mm long) in the top of which a removable slotted T-piece was mounted which locates and distributes sterile seawater over the sample. The sample was supported on a Y-shaped glass rest, which could be raised or lowered to facilitate its alignment. Each section of the apparatus was

Fig. 3 Assembled sample washing equipment.

Fig. 4 Constant head aspirator and washing tube.

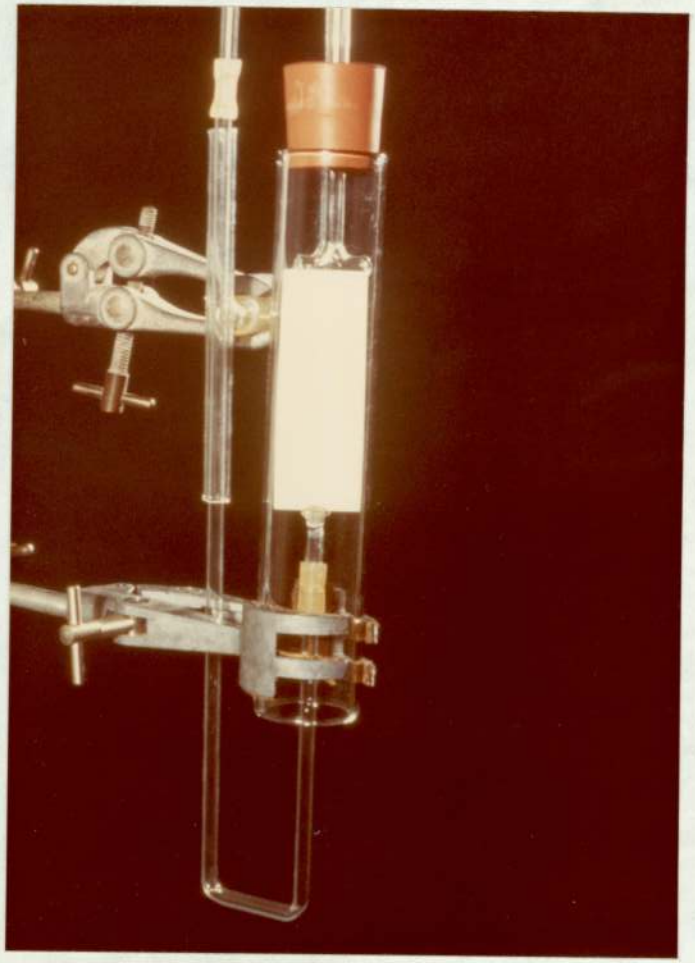


Fig. 5 Washing tube with "T" bar and "Y" rest.

steam sterilized at 121°C for twenty minutes, assembled and connected aseptically with silicone rubber tubing.

During operation, the flow of sterile seawater from the reservoir was regulated to maintain a 750 ml capacity in the constant head.

### 2.3.2 Test Procedure

The efficiency of the equipment was tested by determining the volume of rinsing water required to remove a known concentration of bacteria held but not attached to a sample. A tracer organism *Serratia (Enterobacteriaceae)* was chosen; it is used as a marker in faecal pollution studies because of its ease in handling and distinctive bright red colonies detectable at very low concentrations (Ormerod, 1964).

*Preparation of a Standard Suspension* :

A standard suspension of *Serratia* in the logarithmic phase of growth was prepared by :

(i) Suspending three colonies of *S.marcescens* grown on nutrient agar (Oxoid Ltd.) at 30°C for 72 hours in 1 ml of phosphate buffered saline (PBS).

(ii) Inoculating 5 ml of nutrient broth with 0.1 ml of suspension and incubating at 30°C for 17 hours.

(iii) Inoculating 40 ml of nutrient broth in a 100 ml erlenmyer flask with 0.1 ml of standard inoculum (ii, above) and incubating statically at 30°C.

This culture was sampled at hourly intervals to determine the growth curve of the organism under the conditions stated. Estimates of cell concentration were by direct counts, using a Helber chamber. The procedure was replicated five times. The growth of *S.marcescens* over

the period of incubation was logarithmic ($r = 0.9978$, $p = 0.01$) (Fig.6). As $r$ is greater than $p = 0.01$, a degree of reproducibility is indicated in the preparation of the standard inocula and suspensions.

*Estimation of Direct and Viable Count Efficiency*

From the data obtained using the standard procedure, a culture incubated for five hours would have a cell concentration of approximately $1.42 \times 10^7$ cells/ml, as estimated by direct counts. The test, however, requires a known concentration of viable cells/ml. Viable counts are, however, of an order lower than direct counts, because it is an inefficient estimator of cell number.

The ratio difference between direct and viable counts was therefore calculated so that a suspension could be made of known concentration with respect to viable cells.

Five replicate Erlenmyer cultures were prepared from standard inocula and incubated statically at $30^{\circ}C$ for five hours. The cell count was then estimated, using direct count and viable count (spread plate) methods. The mean ($\bar{x} = 5$) ratio difference between the direct and viable count was 8.847 times for the growth and culture procedure stated.

*Efficiency Test of the Washing Tube* :

A logarithmic culture of *S.marcescens* was prepared, using the standard procedure outlined. After five hours incubation, a culture was diluted using the mean ratio difference coefficient of 8.847 to give a final concentration of $10^3$ cells/ml$^{-1}$. This concentration represents the likely number of viable cells/ml$^{-1}$ in seawater (Section 2.4, and Floodgate, 1964) at the Menai Straits (Section 3.4), the sampling site during this study. Replicate spread plates were inoculated with the dilution to provide a retrospective estimation of the cell concentration in the dilution.

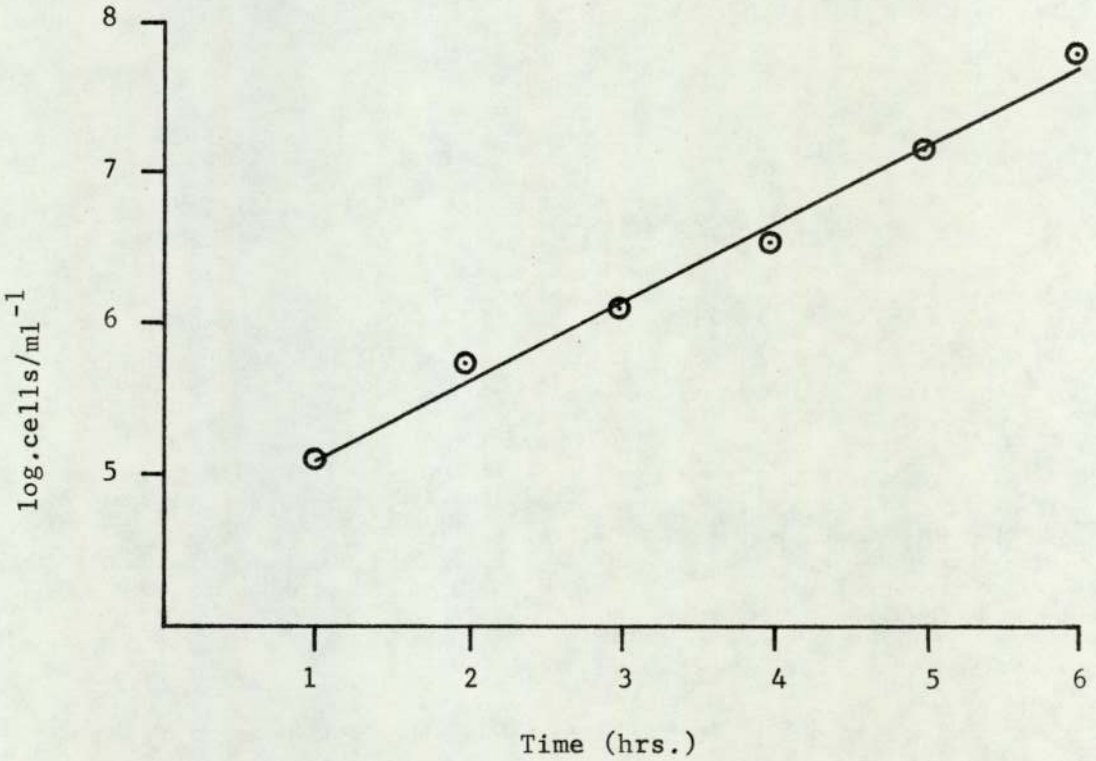



Fig. 6 Growth curve of S.marcescens prepared from a standard inoculum. r = 0.9978

The washing apparatus was prepared as described, with PBS. A sterile, chromic acid cleaned slide was dipped in the suspension and rinsed in volumes of PBS. The effluent was collected and membrane filtered to assay the number of cells removed during rinsing. The slide, once washed, was placed in nutrient broth and incubated at 30°C for seven days to determine if all the cells had been removed by the process. The procedure was replicated six times to determine reproducibility. The volumes analysed and the results obtained are summarised in Table 1 and Fig.7. The volume of liquid adhering to a sample was approximately 0.1 ml, and suggests that the majority of cells was removed during the early stages of rinsing. It was found, however, that rinsing with one and two litres of PBS did not remove all the cells of *S.marcescens* from the sample. Consequently, the rinsing volume was increased to 3 litres, which removed all the cells associated with the sample when checked by the sterility test. The concentration of cells in the test suspension was in the right order of magnitude and representative of the concentration of bacteria in seawater. The accuracy of the counts was gauged by the percentage standard deviation (% $\delta_n$, see Appendix I), and although some exceeded the 5% accuracy limit (Jennison & Wadsworth, 1939), they were below 10% — the maximum deviation acceptable for biological data (Southwood, 1971).

### 2.3.3 Discussion

The volume required to remove all the cells was considerably larger than expected, but it appears that, irrespective of bacterial concentration, the majority of cells were removed with the initial volume of rinsing fluid (Fig.7), and it is the remaining quantity which removes the more recalcitrant or inaccessible cells still held at the surface. It is likely that, because the initial rinsing volume is small, the

TABLE 1 : Results of washing tube efficiency test. For each replicate experiment the initial cell concentration and cell count of effluent wash volumes is given.

| n | Total wash vol./ml | Proportions of wash volume analysed | | | | | | | | Retrospective viable count cells/ml$^{-1}$ | % $\delta_n$ * |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 50 | 100 | 150 | 200 | 400 | 600 | 800 | 1000 | | |
| 1 | 1000 | 27[1] | 7 | 1 | 0 | 0 | 0 | 0 | 0 | 938 | 4.6 |
| 2 | 2000 | 91 | 4 | 2 | 0 | 0 | 0 | 0 | 0 | 1126 | 4.2 |
| 3 | 3000 | 73 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 630 | 5.6 |
| 4 | 3000 | 35 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 224 | 9.5 |
| 5 | 3000 | 65 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 528 | 6.2 |
| 6 | 3000 | 107 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 742 | 5.2 |
| 7 | 3000 | 56 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 426 | 2.7 |

* Percent standard deviation of cell count.

1 Viable count on nutrient agar

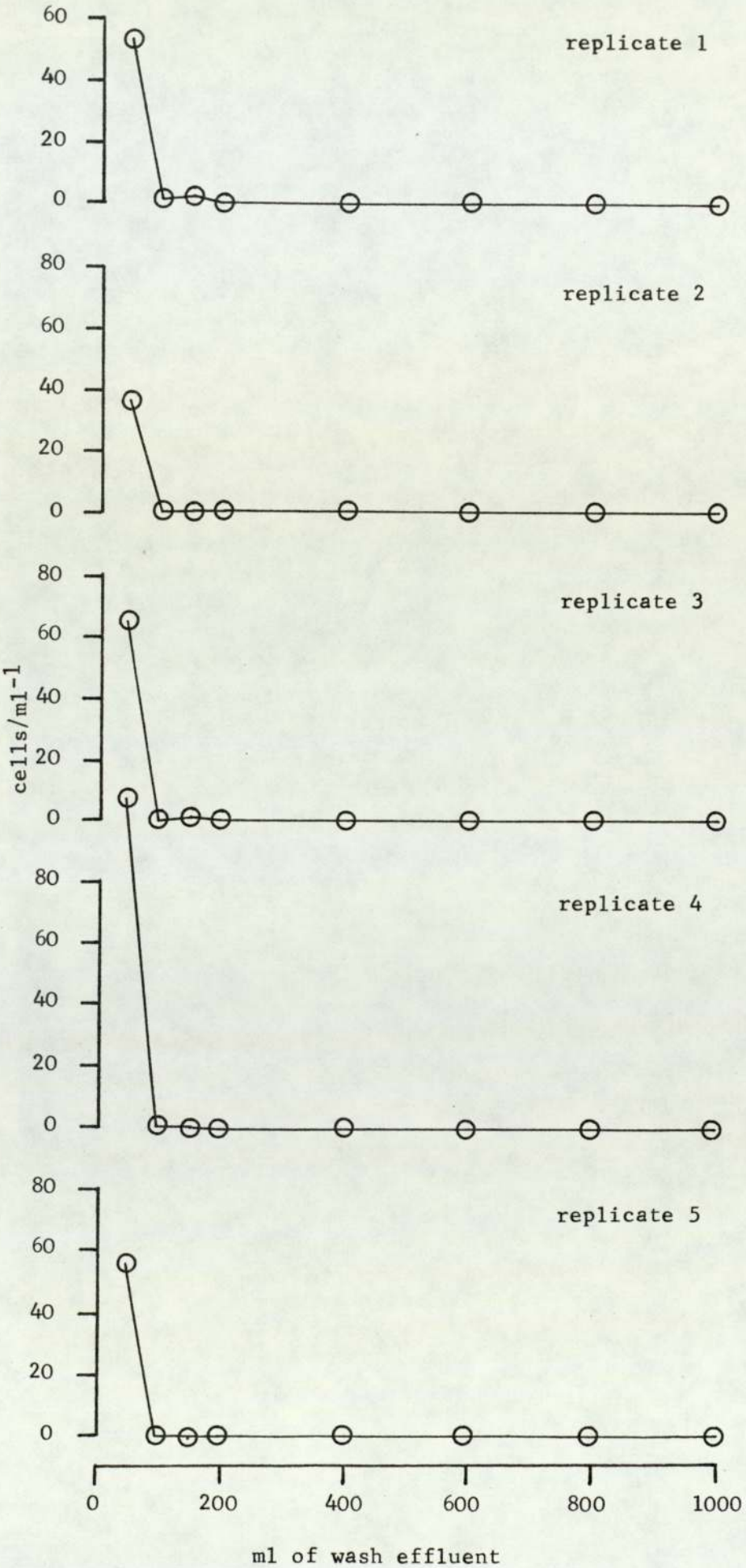



Fig. 7 Removal by rinsing, of unattached bacteria from 5 replicate slides.

quantity used by Corpe (1974) to rinse slides could not, for practical purposes, have been much smaller and therefore would have removed most of the allochthonous bacteria from the sample. It must be pointed out that this form of rinsing can at best be only perfunctory because, as demonstrated, larger volumes are required to remove all the cells; consequently, no reliability can be placed in the precise origin of cells isolated in this manner.

In conclusion, the method for rinsing samples to remove allochthonous bacteria has been demonstrated to be effective and, to allow for variables which might be encountered in the field, a volume of 4 litres per sample was used.

### 2.3.4 Isolation Procedure

Isolation from surfaces has been of the indirect (swabbing) or direct (agar contact) method, as indicated in Section 2.3. Both forms of isolation must be regarded as inefficient as they do not consider the form and structure of the habitat. Corpe (1974 A) has demonstrated that attached bacteria are not readily removed from a surface. It is unlikely that a representative cross-section of a community will be obtained if a surface, particularly with a low cell density, is swabbed or scraped. This has been shown in sampling studies of poultry carcasses. In a situation with a similar mechanical limitation, swabbing was found to be an inefficient method of sampling the surface flora (Cox, Mercuri, Thomson, Chew, 1976).

The disadvantages of the indirect method result from the firm adhesion of attached cells. If the substrate to be sampled is touched on to a nutrient surface, few of the attached bacteria will be dislodged. On the other hand, if it is left in place on the nutrient surface, overcrowding and prolific growth by vigorous types will give a distorted

representation of the sampled community.

Inevitably, any isolation procedure is selective. The compromise which must be achieved has to reduce to an acceptable level the selectiveness of the process. Sampling studies of beef carcasses have shown that tissue samples are more efficient and less selective than swab isolation methods (Lazarus, Abu-Bakar, West & Oblinger, 1977). The effectiveness is due to the whole community being presented for isolation in a state where spatial and surface factors have been reduced. To minimise the selectiveness of isolation, samples in this study were grown in liquid culture. This ensured that spatial and surface limitations were kept to a minimum. The advantages of this system are that all of the community is provided with the opportunity of expression (within the constraints of the nutrient medium), and selection is not biased by mechanical inefficiency.

## 2.4 Isolation and Enumeration Media

A number of media have been used to isolate and enumerate bacteria in seawater. ZoBell's formulation 2216 (1941) and 2216 E (Oppenheimer & ZoBell, 1952) are the most frequently used media, and ones from which many others have been derived. The peptone-yeast extract group can be designated a 'non-selective' heterotrophic medium, able to isolate a wide range of organisms.

The efficiency of media in this group has been assessed in a variety of situations, but with conflicting conclusions. Floodgate (1964) using permutations of peptone and yeast extract, found the optimum results at the Menai Straits (Section 3.4.1) were obtained using 0.3% peptone. Goulder (1976), however, comparing 2216 and Anderson's medium (Anderson, 1962) in estuarine water, found the latter formulation more

efficient; but 2216 E modified with carbohydrates was found to be superior to other media when tested off the Finnish coast (Vaatanen, 1977). Conversely, Chaina (1968), using Lemco seawater agar and 2216 in the North Sea, determined the former more efficient but 2216 was able to isolate a wider range of organisms. A version of 2216 E (Johnson, 1968) has been reported satisfactory (Collins, Jones, Hendrie, Shewan, Wyn-Williams & Rhodes, 1973), while Ashby and Rhodes-Roberts (1976) found no statistical difference in counting efficiency between 2216, 2216 E, and Johnson's marine agar. These paradoxical reports are to be expected; the sea is not homogenous. It would be unrealistic to presume that any one medium will give consistent results in different localities and for different analyses. The conclusion to be made is that a range of media must be tested to determine efficiency and considered with regard to data requirements.

Concomitantly with the choice of an efficient medium for determining microbial numbers is the premise that the medium will isolate a wide range of microbial types from the environment. The purpose of this section was to test a number of media in the peptone-yeast extract group to determine the most suitable non-selective medium appropriate for the sampling site at the Menai Straits (Section 3.4).

### 2.4.1 Materials and Methods

The efficiency of the media was tested, using viable counts. Although this method is recognised as inaccurate (Lewin, 1974), it is still a useful index of microbial number and comparator of microbial populations. The spread plate technique was used to determine the counts, as it is the most appropriate for marine studies (Buck & Cleverdon, 1960), and the most effective (Clark, 1967). The media from the peptone-yeast extract group used were :

(i) Peptone-Yeast Extract with synthetic seawater (MacLeod, Onofrey & Norris, 1954) (PYAR)

(ii) Peptone-Yeast Extract with seawater (PY)

(iii) ZoBell's 2216 E Agar (ZMA)

(iv) Johnson's Marine Agar (JMA)

(v) Anderson's Medium (AMA)

(vi) Nutrient Agar (Oxoid) modified with seawater (NA)

The formulations are given in Appendix II, and where appropriate prepared with natural seawater obtained from the Menai Straits. The media were steam sterilized at 121°C for 15 minutes and the pH adjusted after autoclaving to 7.4 - 7.6. Samples were collected in sterile universal bottles from surface water at the sampling site, and processed within 30 minutes. Sea temperatures ranged from 15°C to 17°C. Ten replicate plates were prepared from undiluted samples with a 0.1 ml inoculum delivered from a disposable tip, fixed volume pipette (Labpipette Jencons Ltd.). Sampling was repeated on five consecutive days. The plates were incubated at 15°C for twenty-one days, following the recommendations of Floodgate (1964).

### 2.4.2 Results

The media, on the basis of the results, can be placed into two groups. The first contained the more efficient media, including PYAR, JMA, and ZMA; the second group contained the less efficient media, PY, AMA, and NA. The results are summarised in Table 2.

Plate counts are a discontinuous variable and are not normally distributed (Ashby, Rhodes-Roberts, 1976; Goulder, 1976; Kaper, Mills, Colwell, 1978). In order that confidence limits could be calculated, the distribution species were determined using the chi-squared variance

TABLE 2 : Comparative efficiency results of viable count media.

| | PYAR | | PY | | ZMA | | JMA | | AMA | | NA | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Day | cells/ml$^{-1}$ | % $\delta_n$ | cells/ml$^{-1}$ | % $\delta_n$ | cells/ml$^{-1}$ | % $\delta_n$ | cells/ml$^{-1}$ | % $\delta_n$ | cells/ml$^{-1}$ | % $\delta_n$ | cells/ml$^{-1}$ | % $\delta_n$ |
| 1 | 547 | 4.28 | 631 | 3.98 | 983 | 1.09 | 1108 | 3.01 | 664 | 1.65 | 733 [1] | 1.64 |
| 2 | 1218 | 0.86 | 872 | 1.21 | 1266 | 0.83 | 1642 | 0.64 | 1015 | 1.06 | 801 | 1.34 |
| 3 | 1120 | 2.99 | 765 | 1.38 | 1349 | 0.78 | 1533 | 2.56 | 1025 | 3.12 | 465 [2] | 2.47 |
| 4 | 1163 | 0.91 | 817 | 3.50 | 1268 | 2.81 | 1465 | 0.72 | 1011 | 3.15 | 434 [3] | 4.79 |
| 5 | 1201 | 2.89 | 869 | 3.39 | 1066 | 0.99 | 1506 | 0.69 | 991 | 1.08 | 587 [4] | 1.87 |

1 = 7n 2 = 8n 3 = 5n 4 = 9n n = no. of replicates.

For NA, reduced no. of replicates (< 10) due to confluent growth

to mean ratio test (Elliott, 1971). The precision and, hence, confidence which can be placed in the cell counts was determined using the percentage standard deviation $\%\delta_n$ (Jennison & Wadsworth, 1939), where an index of reliability is accepted if the percent standard deviation (see Appendix I) does not exceed 5% of the mean. All the counts were within this limit, and indicate the reliability of their estimation.

For inter- and intra-experimental comparisons of skewed distributions, data can be transformed and analysed by a parametric test. Alternatively, an equivalent non-parametric test may be used which, although it has a marginally reduced efficiency, outweighs the disadvantages of tedious transformation of data and complex calculations of a parametric test (Elliott, 1971).

The significance of efficiency differences between media was therefore determined using the Kruskal-Wallis test (see Appendix I). This is the non-parametric equivalent to an analysis of variance where the null hypothesis (*Ho*) states that there is no difference in mean levels between samples. The K value was calculated, and the null hypothesis was rejected at the 1% level ($p < 0.01$), indicating a significant difference in counting efficiency between media. The null hypothesis was also tested on the high counting efficiency group of PYAR, ZMA, and JMA. The *Ho* was also rejected at the 1% level ($p < 0.01$), indicating a significant difference between media. It was concluded that Johnson's marine agar was the most efficient enumeration and isolation medium.

### 2.4.3 Discussion

The major conclusions which can be drawn from this experiment is that it enforces the argument that a battery of media must be tested for the particular sampling area under examination. This is highlighted by

Vaatanen (1977) and Goulder (1976) who indicated that the media tested and the conclusions drawn were only relevant to the area investigated. Similarly, Floodgate (1964) concluded that the optimum formulation for the Menai Straits was 0.3% peptone, and that additions of iron and yeast extract had no effect on the counting efficiency. This he concluded was due to local seawater having sufficiently high concentrations of iron to preclude a supplement.

The most interesting result was the division of the media into two categories, based on the counting efficiency. The basis of this distinction may lie in the inhibitory action of copper and its relative concentration in the media. Floodgate (1964) observed that $Cu^{++}$ present in peptones (10 p.p.m.) and naturally in seawater (10 - 24 $\mu g/l^{-1}$) (Collier, 1970) were approaching levels which were growth inhibitory, particularly to gram negative rods. Bacteria are inhibited by concentrations of $Cu^{++}$ as low as 64 $\mu g/l^{-1}$ and are damaged by yet lower concentrations (Kushner, 1971). PYAR, PY, and AMA media had a yeast extract concentration of 0.2%, 0.2%, and 0.25%, respectively, equivalent to 10.8 $\mu g/l^{-1}$ and 13.5 $\mu g/l^{-1}$ of $Cu^{++}$. ZMA and JMA , conversely, had a yeast extract concentration of 0.1% equivalent to only 5.4 $\mu g/l^{-1}$ of $Cu^{++}$. It is possible that the poor efficiency of AMA and PY media was due to the inhibitory concentration of $Cu^{++}$ contributed by the yeast extract and natural seawater. This was not observed in PYAR because although it had a high level of yeast extract, the $Cu^{++}$ concentration did not reach the inhibitory threshold because synthetic seawater (MacLeod *et al.*, 1954) was substituted for natural seawater. Significantly, Goulder (1976) found Anderson's medium to be the most efficient for estuarine situations, but using synthetic seawater (MacLeod *et al.*, 1954) at half concentration. The inhibitory level of copper was therefore

not reached and the stimulatory effects on the yeast extract realised. The poor performance of the nutrient agar (NA) was probably due to inhibitory $Cu^{++}$ levels a combination of two factors; high peptone concentration and natural seawater.

In conclusion :

(i) the most efficient enumeration and, hence, isolation medium for the Menai Straits was found to be Johnson's marine agar;

(ii) the choice of a suitable medium should be determined from a battery of test media to take account of local variables;

## 2.5 Enumeration of Bacteria on Surfaces

Estimates of cell density on substrates by direct counts provide information on the distribution and rates of bacterial attachment. Traditionally, counting methods have used glass as the test substrate and crystal violet as the cell stain (Henrici, 1933; ZoBell & Allen, 1935; Wood, 1950; Skerman, 1956; O'Neil & Wilcox, 1971; Corpe, 1973,1974). In practice, there are a number of limitations associated with this method. The major problem is differentiating bacteria from detritus which has led to a dichotomy in counting procedures; either all the particles are regarded as cells (Bott & Brock, 1970), or counts are made by subjective identification. The latter method, if used without bias, can only distinguish cells with an identifiable morphology such as a rod. Clearly, this narrow interpretation will under-estimate the true size of the population. An objective estimation, however, cannot be achieved because of bacterial pleomorphism (Winogradsky, 1937), an expression of nutrient status (Novitsky & Morita, 1976), and environmental factors (Henning, 1975).

Another limitation of direct counts by light microscopy is the type of substrate which can be considered, and is confined to those which are transparent, e.g. glass and acrylics. In studying only a restrictive range of substrates, a distorted impression of attachment, distribution, and density can be created. To surmount this problem, an overlayer technique, using a plastic film, has been developed (Margalef, 1946; Sechler & Gundersen, 1971). The plastic film is peeled off, removing the underlying bacteria, which are then stained in the conventional manner. The efficiency of this method is questionable. It has been demonstrated that attached bacteria are not readily removed (Corpe, 1974 A) and, coupled with this, the dilemma of differentiation would still exist. This procedure can only be regarded as qualitative.

Alternative methods of estimating cell density have been devised using Limulus amebocyte lysate (Dexter *et al.*, 1975; Watson, Novitsky, Quinby & Valois, 1977), and scanning electron microscopy (SEM). The former method, although a sensitive indicator of bacterial biomass, can only detect gram negative cells and gives no indication of spatial distribution. SEM techniques enable a very detailed survey to be made, but it is difficult to analyse significantly large areas (Weise & Rheinheimer, 1978) and prepare and manufacture substrates in a form which can be examined.

An accurate and sensitive enumeration procedure has been developed for soil and aquatic biomass estimations, using epifluorescence microscopy. It has been successfully used to count bacteria in soil samples (Strugger, 1948; Trolldenier, 1973),on wheat roots (Vuurde & Elenbaas, 1978), on stones (Batoosingh & Anthony, 1971), and in water samples (Francisco, Mah & Rabin, 1973; Fliermans & Schmidt, 1975). The combination of optics and fluorochrome stain enables not only a critical differentiation between

bacteria and detritus to be made, but also the examination of opaque surfaces. The ability to differentiate is a function of the fluorochrome's specificity for a particular binding site in or on the cell. Fluorescein iso-thiocyanate (FITC) has an affinity for protein (Cherry, McKinney, Emmel, Spillane, Herbert, Pittman, 1969), acridine orange (AO) for DNA and RNA (Dutta, 1968), and auramine for protein (Conrad, Heitz, Brand, 1960), and mycolic acid (Mote, Muhm and Gigstad, 1975).

It was considered that the application of epifluorescence microscopy as an enumeration technique for bacteria in microbial films would enable accurate measurements of cell density and precise estimates of bacterial distributions to be made. In order to determine the most suitable fluorochrome, concentration and contact time of four stains were tested against pure cultures and microbial films developed in seawater. The effectiveness of a decolourising procedure (Zimmerman & Meyer-Reil, 1974) to enhance microbial fluorescence was also tested.

### 2.5.1 Materials and Methods

Four fluorochromes were tested: acridine orange, auramine-o, rhodamine, and fluorescein. The concentration of each stain tested was :

| | | |
|---|---|---|
| (i) | 1:100 | (10 $g/l^{-1}$) |
| (Ii) | 1:1000 | ( 1 $g/l^{-1}$) |
| (iii) | 1:10,000 | (0.1 $g/l^{-1}$) |
| (iv) | 1:100,000 | (0.01 $g/l^{-1}$) |
| (v) | 1:1,000,000 | (0.001 $g/l^{-1}$) |

The contact times used for each concentration and for each stain were: 1, 3, 5, 10, and 15 minutes. It has been suggested that the spectral emission of acridine orange after UV excitation is linked to

concentration and pH (Trolldenier, 1973). Consequently, each concentration of acridine orange was prepared in a range of phosphate buffers from pH 6 to 8 in 0.5 increments. All solutions were filtered through a 0.22 μm membrane.

Pure culture bacteria were isolated from a microbial film and smears prepared on glass slides. Microbial films were obtained by immersing glass slides for one week in seawater at the sampling site in the Menai Straits (Section 3.4). The slides were stained unfixed, rinsed in filtered water, and air-dried.

The microscope and ancillaries included: a Zeiss 'standard' microscope, adapted with a IV Fl epiflourescence condenser, UV from a HBO 50 W mercury vapour lamp, filter combination for blue light excitation, KP 490, KP 500 exciter filter, FT 510 chromatic diffractor, LP 528 barrier filter. Magnification was by a X 100 Neofluar objective and X 12.5 occular. Photography was with a C 35 Zeiss camer, X 0.5 viewfinder prism and Zeiss/Ikon Ikophot M light meter. Film used was high-speed Ektachrome 200 ASA uprated to 400 ASA.

### 2.5.2 Results

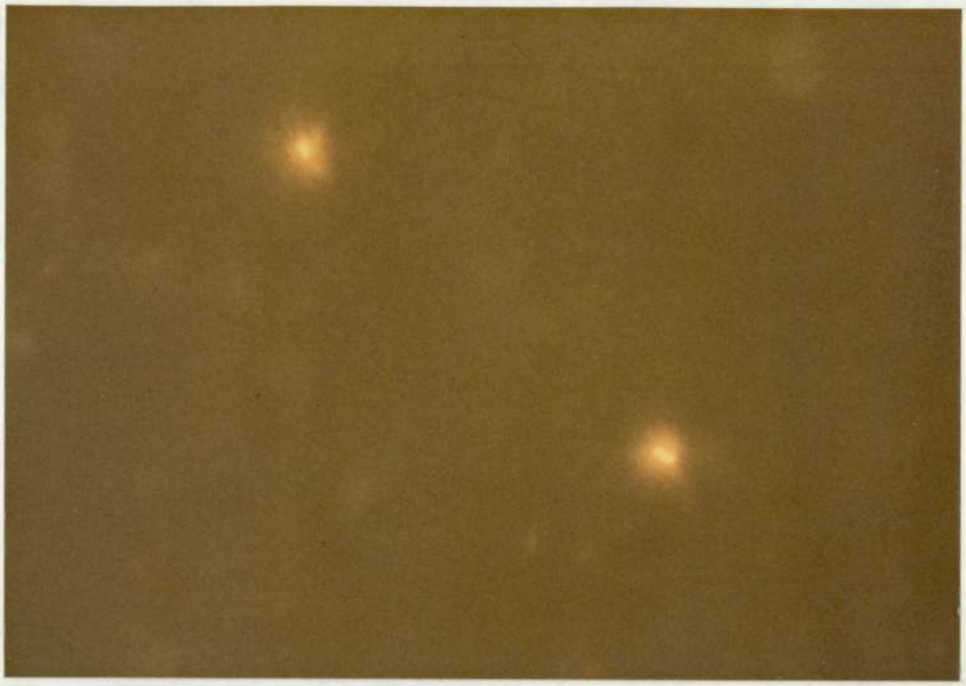
Auramine and acridine orange (pH 6.5) both at a concentration of 1:10,000 and a contact time of three minutes, fluoresced very brightly when tested with the pure culture. Rhodamine and fluorescein fluoresced very weakly. When microbial films were tested with the fluorochromes, only acridine orange was able to differentiate clearly between bacteria and detritus (Figs.8 and 9). Cells fluoresced a bright orange (Figs.10 and 11) which did not fade, while organic material glowed a dull brick red, which faded quickly. The optimum pH, concentration, and contact time of acridine orange agrees well with that determined by Zimmerman and Meyer-Reil (1974), but their decolourising procedure reduced the intensity and time of fluorescence.

——— 10μm

Fig. 8 Glass substrate immersed in seawater for 10 hours.
Stain:- crystal violet.

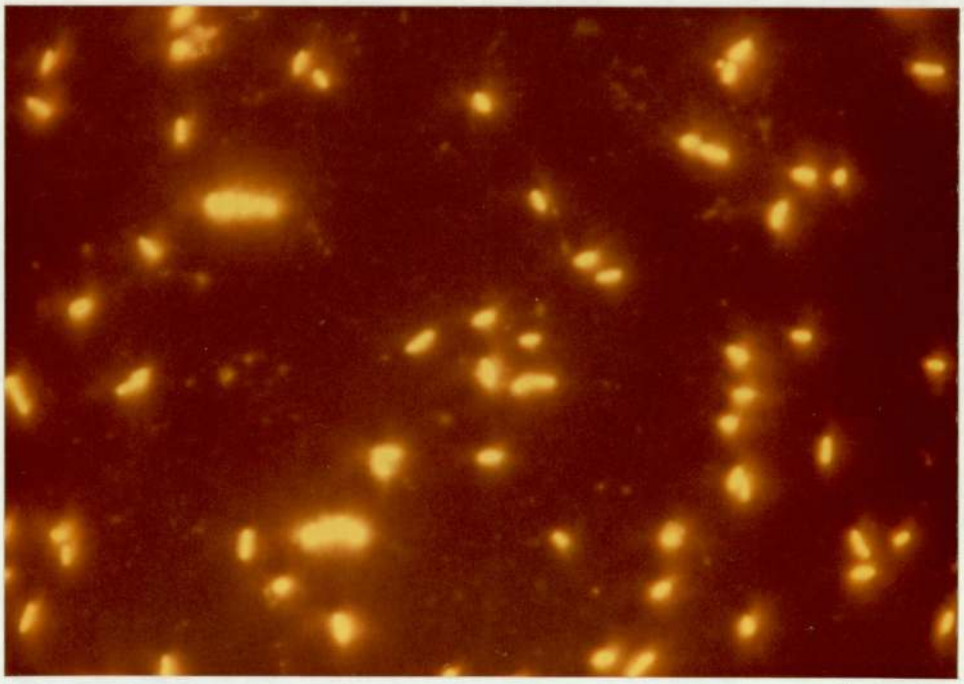
——— 10μm

Fig. 9 Glass substrate immersed in seawater for 10 hours.
Stain:- acridine orange.



——— 10µm

Fig. 10 Single cell discrimination with acridine orange staining/UV-epifluorescence microscopy (4 hours immersion, substrate-resin).



——— 10µm

Fig. 11 Multiple cell discrimination with acridine orange staining/UV-epifluorescence microscopy (156 hours immersion, substrate-glass).

### 2.5.3 Discussion

The selectiveness and sensitivity of acridine orange in differentiating bacteria from detritus is due to its affinity for DNA and RNA. Acridine orange in solution will only fluoresce when it combines with DNA (or RNA) to form a complex co-polymer (Ignatov, Shcherbakov & Krestyaninov, 1975). The configuration of acridine orange binding to a polymer (DNA or mucopolysaccharide) is affected by pH (Ikeda & Imae, 1971). It is significant that the optimum fluorescence was obtained at a specific pH value (6.5) when the AO co-polymer configuration determined by pH, gave the maximum emission intensity. The emission spectra of bound acridine orange appears to be dependent on the binding molecule or site. Dutta (1968) found that AO and DNA produced a yellow-green fluorescence, while RNA and acridine orange fluoresced orange-red; also, Ignatov *et al.* (1975) determined that the emission spectrum of acridine orange bound to DNA (505 nm) was significatly different from acridine orange bound to the cell walls (494 nm).

The colour of fluorescence may well be a reflection of the binding site, and not an indicator of metabolic activity, physiological state (Strugger, 1948), or a function of acridine orange concentration, pH, or contact time (Trolldenier, 1973). Studies using leukocytes show a red fluorescence for cell bound acridine orange, and green fluorescence for nuclear bound acridine orange (Liedman, Matveyeva, Vostricova & Prilipko, 1975). This agrees well with conclusions reached by Dutta (1969) and the observations made here while studying microbial films. To summarise, the selectiveness of acridine orange binding is the basis of the differentiation between bacteria and detritus. The intensity of emission may be influenced by pH acting on the co-polymer configuration. Only orange-red fluorescence was observed, and may be attributed to the interaction of acridine orange and binding site in or on the cell.

The efficiency of acridine orange staining as an enumeration technique has been compared with conventional light and scanning electron microscopy (Larson, Weibull & Cronberg, 1978). Overall, the efficiency was slightly less compared to SEM with the proviso that differentiation was significantly easier using epifluorescence microscopy, and that subjective interpretation was required with SEM estimations.

In conclusion :

(i) The epifluorescence technique using the concentration and pH stated was able to clearly differentiate bacteria from detritus.

(ii) Cell counts by epifluorescence microscopy are extremely efficient when compared with light and SEM (Larsson *et al.*, 1978).

(iii) The simplicity of preparation and technique with epifluorescence microscopy enables numerous samples to be examined for cell density and distribution, which would be restrictive factors if SEM (Weise & Rheinheimer, 1978) or Limulus amebocyte lysate techniques were used.

# CHAPTER 3

## SAMPLING AND ENUMERATION OF BACTERIA ON SURFACES

3.1 Introduction

### PART I

### Substrates Sampling and the Measurement of Coincidental Environmental Parameters

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- 3.2.2 Preparation of Substrates

3.3 Measurement of Surface Energies

- 3.3.1 Introduction
- 3.3.2 Materials and Methods
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- 3.4.1 The Sampling Site
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3.5 Environmental Parameters

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3.6 Enumeration Procedure

### PART II

### Analysis of the Sampling Data

3.7 Density of Bacteria on Surfaces

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# SAMPLING AND ENUMERATION OF BACTERIA ON SURFACES

## 3.1 Introduction

"The enumeration of bacteria in seawater, taken at various locations and depths at various times of the year, is a favourite occupation of the marine microbiologist." (Lewin, 1974).

This criticism can also be applied to a considerable body of repetitive work where surfaces (usually glass) are immersed in seawater and the densities of attached bacteria recorded (ZoBell & Allen, 1935; Henrici, 1936; Wood, 1950; Cviic, 1953; Skerman, 1956; O'Neil & Wilcox, 1971). It would be erroneous to suggest, however, that this type of information is valueless; historically and factually, it is a background for comparison and established the microbial film as a unique habitat in the marine ecosystem. Subsequent work defined laboratory models to determine attachment processes and sequences (Marshall *et al.*, 1971; Fletcher, 1977; Fletcher & Loeb, 1976,1979) as a mechanistic description of microbial film development.

Studies based on information obtained from direct observations and mechanistic descriptions have attempted to reconcile laboratory modelling with events occurring in the natural environment (Corpe, 1973, 1974; Sechler & Gundersen, 1973; Dexter *et al.*, 1975). The success of unification is hard to assess because sampling from temporal sampling series has not been adequate; too few types of substrate were examined, numerical analysis was not sufficiently bold, and observed data was not clearly related to laboratory models.

The objectives of this section are to :

(i) establish a sampling series over a definitive period of time which will enable analysis of significant stages during microbial film development;

(ii) determine the density, rate of attachment and distribution of bacteria on a wide range of substrates;

(iii) measure relevant environmental factors to determine their influence (if any) on attachment.

# PART I : Substrates Sampling and the Measurement of Coincidental Environmental Parameters

## 3.2 Description of Substrates

### 3.2.1 Introduction

Glass has traditionally been used as a substrate because it was suitable for direct counts by light microscopy, was inert, cheap, and familiar. It would be unwise to base conceptions of microbial attachment solely on the reaction to glass (or to any other single substrate) as the character of a surface, expressed as wettability (surface energy), can greatly influence the attachment of bacteria (Fletcher & Loeb, 1976,1979). A range of substrates (Table 3) have been studied (Sechler & Gundersen, 1973; Dexter *et al.*, 1975), but enumeration was either inaccurate or not capable of extracting the maximum amount of information.

The substrates used in the present study can be divided into two groups (Table 4): Group I, substrates used in all the sampling studies represent a range of surface energies and materials used in marine technologies. Group II is a more detailed range of substrates with respect to surface energies,and supplemented Group I in certain experiments.

### 3.2.2 Preparation of Substrates

*Glass* : (Chance Bros. Ltd.) standard glass microscope slides were cleaned in Decon 90 (BDH Ltd.) and rinsed exhaustively in distilled water. Slides were stored in ethanol (100%) and flamed prior to immersion.

*Stainless Steel* : (EN58J) 1 mm gauge sheet was cut to the dimensions of standard microscope slides. The surface was polished with 600 grit carborundum, cleaned in Decon 90, and rinsed exhaustively in distilled water. Slides were stored in ethanol (100%) and flamed prior to immersion.

TABLE 3 : List of substrates used by other workers.

| Sechler & Gundersen, 1973 | Dexter *et al.*, 1975 |
|---|---|
| Glass | Copper |
| Plexiglass | Nickel |
| Stainless Steel | Polystyrene |
| Aluminium | Polypropylene |
| Monel | Polyvinylfluoride |
| Phosphor Bronze | Polytetrafluoroethylene |
| Zinc | |

TABLE 4 : List of substrates used in this study.

| Group I | Group II |
|---|---|
| Glass | Chromium-plated Steel |
| Stainless Steel | Aluminium |
| Marine Glass Fibre Resin | Perspex (Methylmethacrylate) |
| Marine Antifouling Paint (without toxin) | Polystyrene |
| | Polypropylene |
| | Polytetrafluoroethylene |

*Marine Glass Fibre Resin* : Type A (Strand Glass Fibre Ltd.). Two sheets of 600 x 1000 x 4 mm plate glass were coated thinly with domestic silicone polish and a border of glass slides arranged on the perimeter of one sheet. Glass fibre resin was activated in accordance with manufacturer's specifications and the resultant mix spread evenly over the sheet of plate glass. After 15 minutes, to allow for the dispersion of air bubbles, the resin was sandwiched with the other sheet of glass and allowed to cure for 15 hours. The cast resin sheet was released from the 'mould' and traces of wax and surface imperfections removed with 600 grit carborundum. The resin was cut into standard microscope size slides, cleaned in Decon 90, rinsed exhaustively in distilled water, dried in a 60°C oven, and stored in a closed container.

*Antifouling Paint* : (I C I Ltd. For formulation, see Appendix III). Standard glass microscope slides were sand-blasted (Guyson Ltd. with Saftigrain E) on both surfaces. The paint was diluted 1:4 (v/v) with acetone and sprayed onto the slides so that all surfaces (including edges) were evenly coated. The slides were stored in a closed container.

*Group II Substrates* : Samples were cut to standard microscope size from 1 mm gauge sheet, cleaned in Decon 90, and rinsed exhaustively in distilled water. The chromium-plated steel and aluminium were stored in ethanol (100%) and flamed prior to use, while the plastics were oven dried at 45°C and stored in closed containers.

## 3.3 Measurement of Surface Energies

### 3.3.1 Introduction

Surface phenomena are the reactions and interactions which occur at the surface of a liquid or solid, and are influenced by various parameters, notably surface energy. Its value is a characteristic of the

solid (or liquid) and is defined as the work (ergs/cm$^2$) necessary to increase the surface area by unity, i.e. it is the work necessary to bring molecules from the interior of a liquid to the surface and increase the surface area by 1 cm$^2$ (Nachtrieb, 1971). Solids may be grouped into two categories, based on their surface energies; soft solids such as plastics have low surface energies ( < 100 ergs/cm$^2$), while hard solids such as glass and metals have high surface energies (> 500 ergs/cm$^2$) (Baier, 1970). Relative surface energies for a set of solids can be determined by calculating the critical tension of wetting ($\gamma_c$) defined as the extrapolation of the cosine contact angles for a series of liquids to 1.0, plotted against the liquid-vapour surface tension (dynes/cm$^{-1}$) of the test liquids (Zisman, 1972). Determination of $\gamma_c$ also indicates the 'wettability' of the solid — an important factor in the adhesion of cells to surfaces (Baier, Shafrin & Zisman, 1968).

The critical surface tension of wetting was determined for those substrates where no value of $\gamma_c$ has been established. The contact angle of seawater ($\theta_{sw}$) was also determined for each substrate, to ascertain if there was a significant departure of $\theta_{sw}$ from $\gamma_c$.

### 3.3.2 Materials and Method

All substrates tested were prepared and cleaned as described in Section 3.2.2. The surface tension of the test liquids was measured using a 'White' torsion balance with a platinum loop, and is given in Table 5. The contact angle of a test liquid, delivered from capillary tubing, was measured on the right and left-hand transect of the drop, using a 'Swift' goniometer. The mean contact angle for each liquid was calculated from five replicate drops.

TABLE 5 : Surface tension values of test liquids used in $\gamma_c$ measurements.

| Liquid | Surface Tension dynes/cm$^{-1}$ |
|---|---|
| Glycerol | 63.4 |
| Formamide | 58.3 |
| Ethanediol | 47.7 |
| Propyleneglycol | 40.1 |

3.3.3 Results and Discussion

The critical surface tension of wetting was calculated by :

$$m = \frac{\Sigma xy - n\cdot\bar{x}\cdot\bar{y}}{\Sigma y^2 - n(\bar{y}^2)}$$

where $m$ is the slope, $x$ is the surface tension of a test liquid, $y$ is the cosine of $\theta$ the contact angle, and $n$ is the number of test liquids used. Also, $\bar{x} = m\bar{y} + c$, $\therefore$ $c = \bar{x} - m\bar{y}$, where $c$ is a constant. The critical surface tension of wetting is given by the intercept on the $y$ axis when $\cos\theta = 1.0$ (i.e. $y = 1.0$), $\therefore$ $\gamma_c = m\bar{y} + c$ dynes/cm$^{-1}$.

The critical surface tension of wetting $\gamma_c$, and contact angle of seawater $\theta_{sw}$ for the substrates is given in Table 6. The relationship of critical surface tension of wetting ($\gamma_c$) to the wetting of a substrate by seawater was examined to determine the similarity between these two indices of surface character. From the graph in Fig.12, it can be clearly seen that the correlation is non-linear, and the necessity of estimating wetting by these two methods is justified. The value of $\gamma_c$ determined by wetting is affected by hydrogen bonding between the test

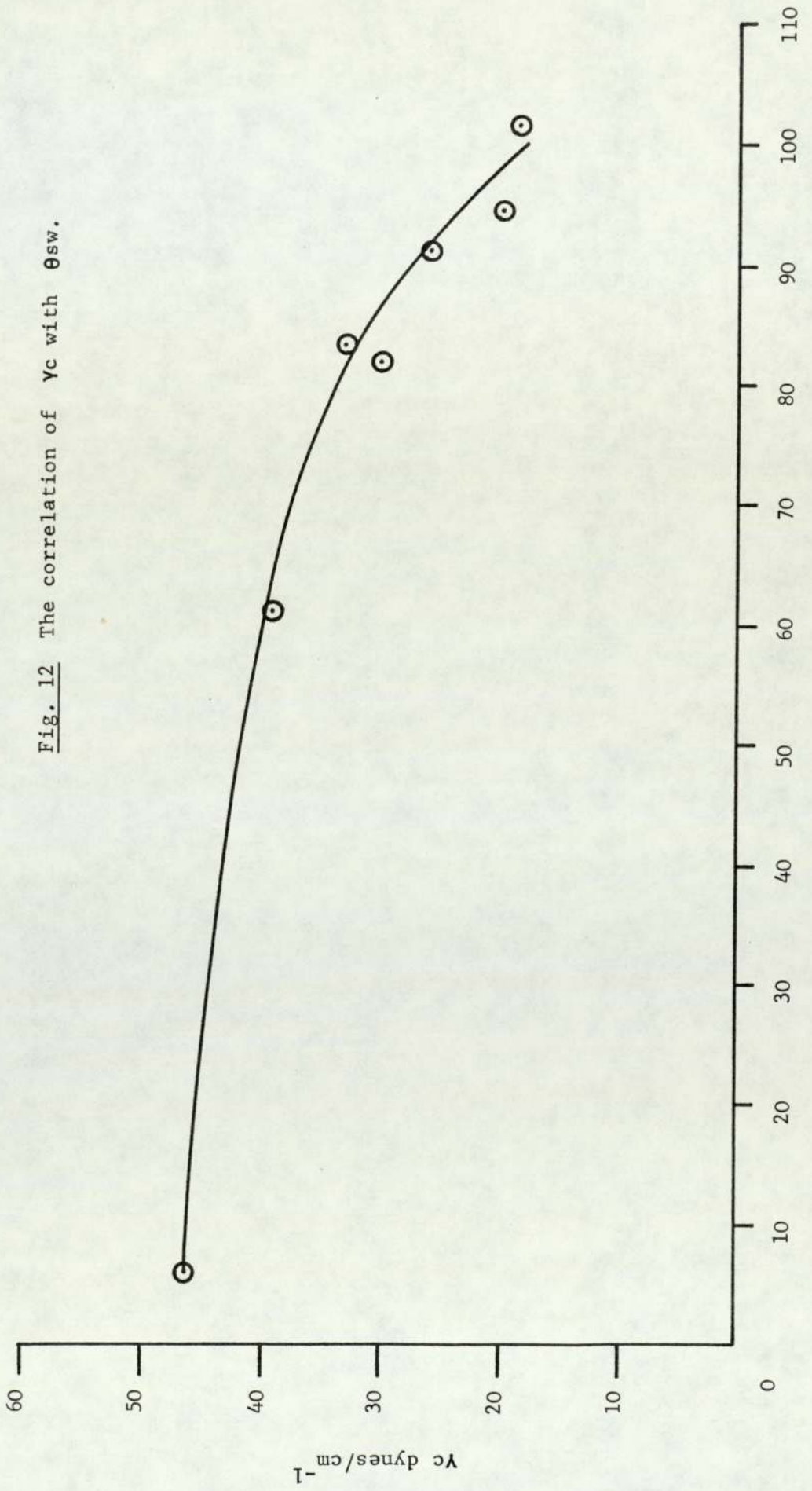



Fig. 12 The correlation of γc with θsw.

liquid and surface (Zisman, 1972) and the use of a heterologous series of liquids ensures a median value of $\gamma_c$ is obtained. It is most likely, therefore, that the heterogeneous nature of seawater and the complexity of bonding at the solid-liquid interface is the major factor for the deviation of $\theta_{sw}$ from the $\gamma_c$ series of the substrates.

TABLE 6 : $\gamma_c$ and $\theta_{sw}$ values of substrates used in this study.

| Substrate | $\gamma_c$ dynes/cm$^{-1}$ | $\theta_{sw}$ |
|---|---|---|
| Glass | 46.0 [a] | 6 |
| Chrome | ND [1] | 23.4 |
| Steel | ND | 24.4 |
| Aluminium | ND | 28.1 |
| Perspex | 39.0 [d] | 60.9 |
| Polystyrene | 33.0 [b] | 83.5 |
| Resin A | 30.0 [e] | 82.3 |
| Polypropylene | 26.0 [c] | 91.5 |
| Paint | 20.26 [e] | 94.5 |
| PTFE [2] | 18.5 [b] | 102.1 |

a - Zisman, 1972; b - Baier, *et al.*, 1968;

c - Fowkes, 1964; d - Andrade, 1973; e - calculated.

1 not determined
2 Polytetrafluoroethylene

## 3.4 The Sampling Site, Intervals, and Seasons

### 3.4.1 The Sampling Site

The sampling site for this investigation was located at Menai Bridge, Anglesey. The Isle of Anglesey is situated at the extreme north

western tip of Wales. The tract of water known as the Menai Straits was created by post-glacial flooding of the lowland valley between the high mountains of Gwynedd to the south-east and the Menai platform, which constitutes nearly half the island, to the north-west. Sampling in the Straits was from the floating pier at Menai Bridge, whose full 1000 metre national grid reference number (Ordnance Survey, 1974) is SH 559720 or latitutde 53° 10' 37" North and longitude 4° 9' 8" west. The sea-bed of the Straits is covered by a mixture of sand, mud, and silt; a maximum tide height of 20 ft. and a current of 3 to 8 knots. Situated on the western margin of a continental land mass, North Wales has a maritime climate determined by the interplay of tropical maritime and polar air streams. There is a mean annual rainfall of 1000 mm and a temperature range of 5 to 16°C.

3.4.2 Sampling Intervals

The length of immersion, and the sample intervals, were chosen so that the development of the microbial film could be followed and a detailed analysis made of the attachment dynamics.

The time scales and sampling intervals were chosen to complement each other, such that the total time of the shortest immersion period equalled the sampling intervals of the succeeding and longer time scale.

(i) Time Scale I was one hour long, and samples were taken at five minute intervals.

(ii) Time Scale II was ten hours long, and samples were taken at hourly intervals.

(iii) Time Scale III was twelve hours long, and samples were taken after one and two hours immersion, and then at two-hour intervals, up to twelve hours of immersion.

(iv) Time Scale IV was 168 hours (seven days) long, and samples were taken at twelve-hour intervals. The sampling sequences are summarised in Fig.13.

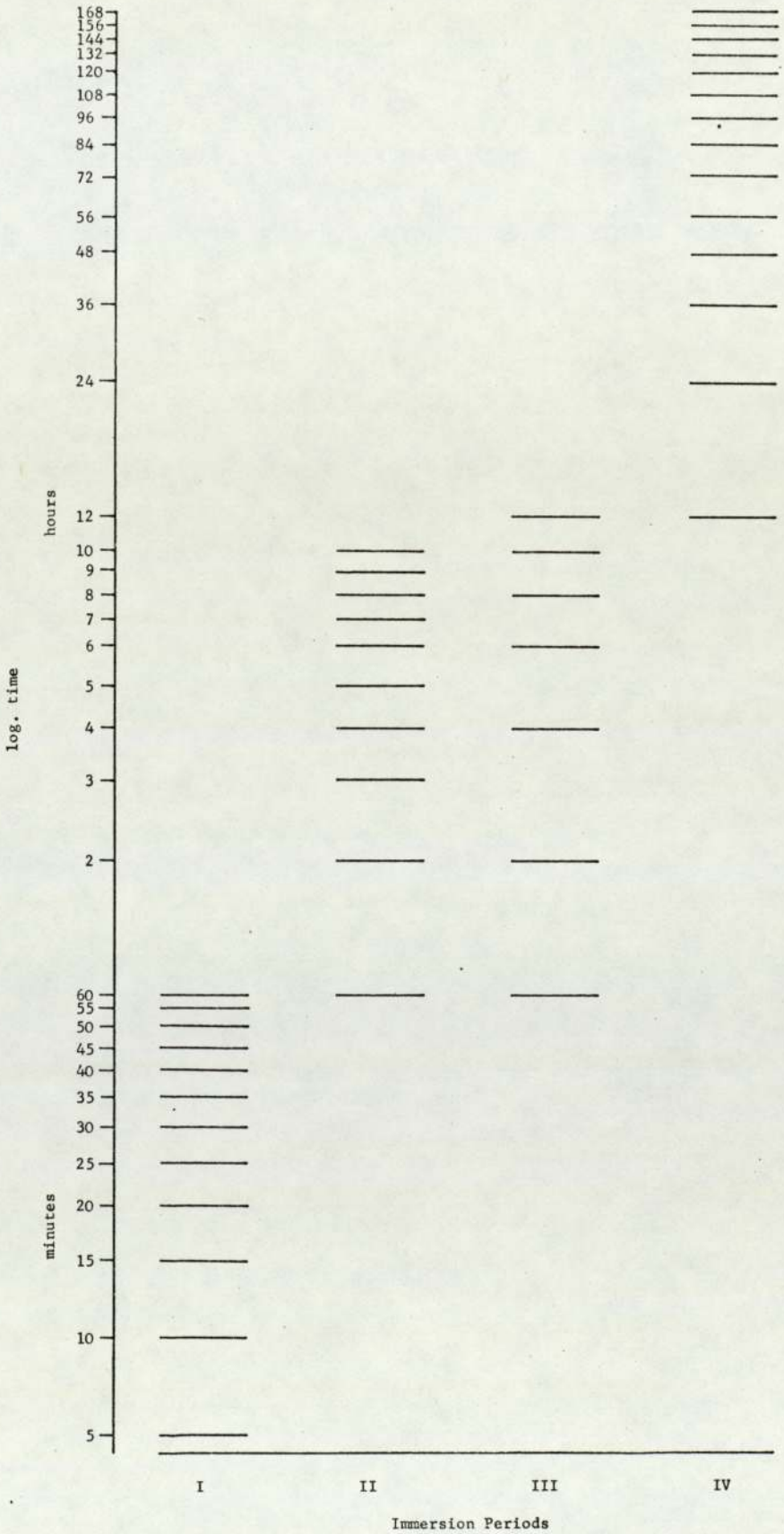



Fig. 13 Summary of sample intervals and immersion periods.

### 3.4.3 Sampling Programme

Sampling was conducted in August and December to determine the effect of environmental conditions. The sea temperature, which directly or indirectly affects microbial number and activity, shows marked seasonal variation, and there is also a significant difference in the level of dissolved organic carbon. All, or a combination of these factors might influence attachment patterns.

Four blocks of sampling were executed; the first block covered sampling during the winter on time scales III and IV, using the 'core' substrates, glass, steel, resin, and paint. The second block complemented the first, with sampling during the summer on time scales III and IV, using the 'core' substrates. The third block tested a hypothesis derived from the previous season's sampling, and required a short immersion period (Time Scale II) and substrates with a wide range of surface energy characteristics. The final block of sampling determined the density and speed of cell attachment to substrates by sampling at five minute intervals during the first hour of immersion (Time Scale I).

TABLE 7 : Summarised combinations of immersion times and substrate types.

| Season | Time Scale | Substrate |
|---|---|---|
| Winter | III 0 - 12 hrs | Glass Steel Resin Paint |
| | IV 0 - 168 hrs | Glass Steel Resin Paint |
| Summer | III 0 - 12 hrs | Glass Steel Resin Paint |
| | IV 0 - 168 hrs | Glass Steel Resin Paint |
| Summer | II 0 - 10 hrs | Glass Chrome Steel<br>Aluminium Perspex<br>Polystyrene Resin<br>Polypropylene Paint<br>Polytetrafluoroethylene |
| Winter | I 0 - 1 hr | Glass Steel Resin Paint |

## 3.5 Environmental Parameters

### 3.5.1 Introduction

A number of environmental parameters, including physical, chemical, and biological processes, may influence the dynamics of microbial attachment. A selection of relevant parameters was monitored and their significance assessed by correlation with attachment rates.

(a) Total Organic Carbon (mg $C/l^{-1}$) :

Dissolved organic carbon is adsorbed onto surfaces (Loeb & Niehof, 1977) and is considered an important factor in microbial film development (ZoBell, 1943).

(b) Microbial Number (cells/$ml^{-1}$) :

Levels of microbial biomass in seawater indicate the frequency of collision between a cell and a surface such that, with increasing cell number, there may be a proportional increase in attachment.

(c) Salinity (g/Kg, $S^{o}/oo$) :

Attachment of cells to surfaces is mediated by divalent ions such as $Ca^{++}$ and $Mg^{++}$ (Marshall *et al.*, 1971). There is a proportional relationship between $Ca^{++}$ and $Mg^{++}$ ions and salinity (Barnes, 1954); fluctuations in the concentration of these divalent ions in seawater can be monitored by measuring the index parameter, salinity.

(d) Temperature ($^{o}C$) :

Temperature influences the parameters outlined above, but most significantly it determines the rate of microbial activity, and hence, possibly attachment.

### 3.5.2 Materials and Methods

*Total Organic Carbon :*

Organic carbon was determined by ICI (Organics Division, Huddersfield), using an automated high temperature pyrolysis analyser. Replicate samples for analysis were collected from surface water in 25 ml glass, chromic acid cleaned, polypropylene capped bottles and stabilised to prevent microbial depletion of dissolved organic carbon (May, 1960). The most effective method was steam sterilisation (121$^{o}$C, for 15 minutes in sealed containers) compared with mercuric chloride (1 ppt) sterilisation and untreated samples ( $F > p = 0.001$).

*Microbial Number :*

Samples were taken from surface water and processed immediately. Biomass was estimated, using the spread plate technique. A 0.1 ml innoculum delivered from a fixed-volume disposable tip pipette (Jencons Ltd.) was distributed over replicate plates of Johnson's Marine Agar (see Section 2.4) and incubated at 15$^{o}$C for three weeks (Floodgate, 1964).

*Salinity :*

Surface samples were collected and analysed using a silver nitrate titration (Clark, 1966) to determine total chlorides. Five replicate measurements of chlorinity were made for each sample. Salinity ($S^{o}/oo$, grams/$Kg^{-1}$ of seawater) was calculated from the chlorinity using the equation $S^{o}/oo = 1.80655 \times Cl^{o}/oo$ (Johnson, 1964).

*Temperature :*

An electronic meter (Comark Ltd.) with a thermocouple probe was used to determine water temperature.

### 3.5.3 Results

*Total Organic Carbon (TOC) :*

The mean TOC during the immersion series ranged from 8.6 (July, 1978), 7.9 (December, 1976), and 3.0 mg $C/l^{-1}$ (August, 1977). This compares favourably with annual TOC cycles in northern waters which peak from April to June, decline towards August, and rise again in December (Duursma, 1965).

TOC estimates for December 1976 ranged from 8.34 to 16.8 mg $C/l^{-1}$, 14.4 to 3.0 mg $C/l^{-1}$ during August 1977, and 5.0 to 14.2 mg $C/l^{-1}$ in July 1978. These levels are considerably higher than those found in open water (Duursma, 1965; Westrum & Meyers, 1978), but are near the values expected for coastal and near-shore water (Hood, 1963). The high concentration of organic carbon is probably due to the sewage outfall at Bangor, a factor which reduces seasonal extremes. The variation in TOC levels during the sampling series was analysed. There was a significant difference between TOC levels on successive days, which was shown to be a random event (as expected), and not an environmental trend or experimental deficiency (Table 8). The accuracy of TOC sampling and measurement was gauged by the percentage standard deviation of the mean (Appendix I); none of the TOC values exceeded the 10% limit, indicating that the variation between replicates was acceptable.

*Microbial Cell Number :*

Microbial biomass expressed as cells/$ml^{-1}$ was measured during time scales II and IV. The highest counts were obtained during the summer season, and ranged from $9.97 \times 10^2$ to $1.28 \times 10^3$ cells/$ml^{-1}$ for period II (0 - 10 hrs Summer), and $5.47 \times 10^2$ to $1.2 \times 10^3$ cells/$ml^{-1}$ for period IV (0 - 168 hrs Summer), but only $1.6 \times 10^2$ to $5.24 \times 10^2$ cells/$ml^{-1}$ for period IV (0 - 168 hrs Winter), a magnitude ratio of 1:3:4 for December, August, and July sampling.

TABLE 8 : Summary of Total Organic Carbon level variance during sampling series.

| Sampling Series | Substrate | Variation | Distribution |
|---|---|---|---|
| 0 - 10 hrs Summer II | All | $F = 4.37$ $p < 0.01 < 0.05$ | Poisson $\chi^2 = 10.39$ |
| 0 - 7 days Winter IV | Glass/Steel | $F = 3.66$ $p < 0.01 < 0.05$ | Poisson $\chi^2 = 4.77$ |
| 0 - 7 days Winter IV | Resin/Paint | $F = 18.58$ $p < 0.01 < 0.05$ | Poisson $\chi^2 = 11.67$ |
| 0 - 7 days Summer IV | Core | Data not suitable for analaysis $n = 6 < 3$ mg $C/l^{-1}$ | |

The accuracy of counts was measured by the percentage standard deviation of the mean (Appendix I), which should not exceed 5% (Jennison & Wadsworth, 1939). The number of replicates used had a marked effect on accuracy; estimates made during period II and IV (0 - 7 days Summer) with ten replicates did not exceed the limit. In period IV (0 - 7 days Winter) only five replicates were used, and although in most cases the percentage standard deviation did not exceed 10%, in some instances it was as high as 20%. Overall, there was a good degree of accuracy in estimating cell number.

The variation between counts on successive days sampling for each time scale was significantly different. This may be attributed to the negative binomial type of distribution over the time scales, indicating a considerable range of cell number from one day to the next (Table 9). This type of distribution is typical of microbial populations in seawater (Ashby & Rhodes-Roberts, 1976), and is probably the result of using too small a sampling volume. Increasing the size of the sampling unit will increase the randomness of the distribution (Elliott, 1971), but the relationship between individual size of sampling unit and distribution is such that, to increase the sampling volume will make analysis impractical, if not impossible.

*Salinity :*

Salinity was measured during Time Scale II (0 - 10 hrs Summer) and part of period IV (0 - 7 days Summer). The salinity of 'Normal Water' is given as 35.04 $g/Kg^{-1}$ (Barnes, 1954) and the salinities for Time Scale IV ranged from 34.78 to 35.48 $g/Kg^{-1}$, while for period II they ranged from 33.67 to 33.97 $g/Kg^{-1}$. The percentage standard deviation of the mean was used to gauge the accuracy of measurement, and did not exceed

TABLE 9 : Summary of bacterial count in seawater variance during sampling series.

| Sampling Series | Substrate | Variation | Distribution |
|---|---|---|---|
| 0 - 10 hrs Summer II | All | K = 45.98 P < 0.01 < 0.05 | NB* $\chi^2$ = 176.44 |
| 0 - 7 days Winter IV | Glass/Steel | K = 22.13 P < 0.01 < 0.05 | NB $\chi^2$ = 73.79 |
| 0 - 7 days Winter IV | Resin/Paint | K = 23.09 P < 0.01 < 0.05 | NB $\chi^2$ = 474.71 |
| 0 - 7 days Summer IV | Core | K = 25.88 P < 0.01 < 0.05 | NB $\chi^2$ = 67.78 |

* Negative Binomial

TABLE 10 : Summary of salinity level variance during sampling series.

| Sampling Series | Substrate | Variation | Distribution |
|---|---|---|---|
| 0 -10 hrs Summer II | All | F = 2.80 P < 0.01 < 0.05 | PB[1] $\chi^2$ = 0.06 |
| 0 - 7 days Summer IV | Core | F = 4.44 P < 0.01 < 0.05 | PB $\chi^2$ = 0.05 |

[1] Positive Binomial

1.0%, well within the limit of 10%. The variations between successive samples were calculated as significantly different, but the distribution was regular, i.e. a positive binomial. This indicates that differences between samples were uniform, possibly a techniques error (see Table 10).

*Temperature :*

Sea temperatures were stable over each period and did not shift significantly. The mean temperatures for period II (0 - 10 hrs Summer) were 11.15$^{o}$C, period IV (0 -7 days Winter, Glass and Steel) 7.3$^{o}$C, period IV (0 - 7 days Winter, Resin and Paint) 7.7$^{o}$C, and period IV (0 - 7 days Summer) 16.25$^{o}$C.

The data for the measured parameters has been summarised and is shown in Figs.14 to 18.

## 3.6 Enumeration Procedure

Bacterial density on surfaces was determined by direct counting using UV epiflourescence microscopy and acridine orange staining (Section 2.5).

For each sample, the number of bacteria within the known area of a 10 x 10 occular matrix was counted in 22 consecutive fields in a horizontal and vertical scan (Hanks & James, 1940). Substrates, irrespective of the preparation and cleaning regimen, had a very low but significant background count of 'resident' bacteria. As the density was approximately equal for each type of substrate, a mean 'resident' cell density was calculated. This was estimated at 4.75 cells per 22 fields counted (2159 cells/cm$^{2}$), and was used as a correction factor when calculating true densities on samples. Bacterial density was expressed as cells/cm$^{2}$, calculated as $\bar{x}$ count - 4.75 x 10$^{4}$ cells/cm$^{2}$.

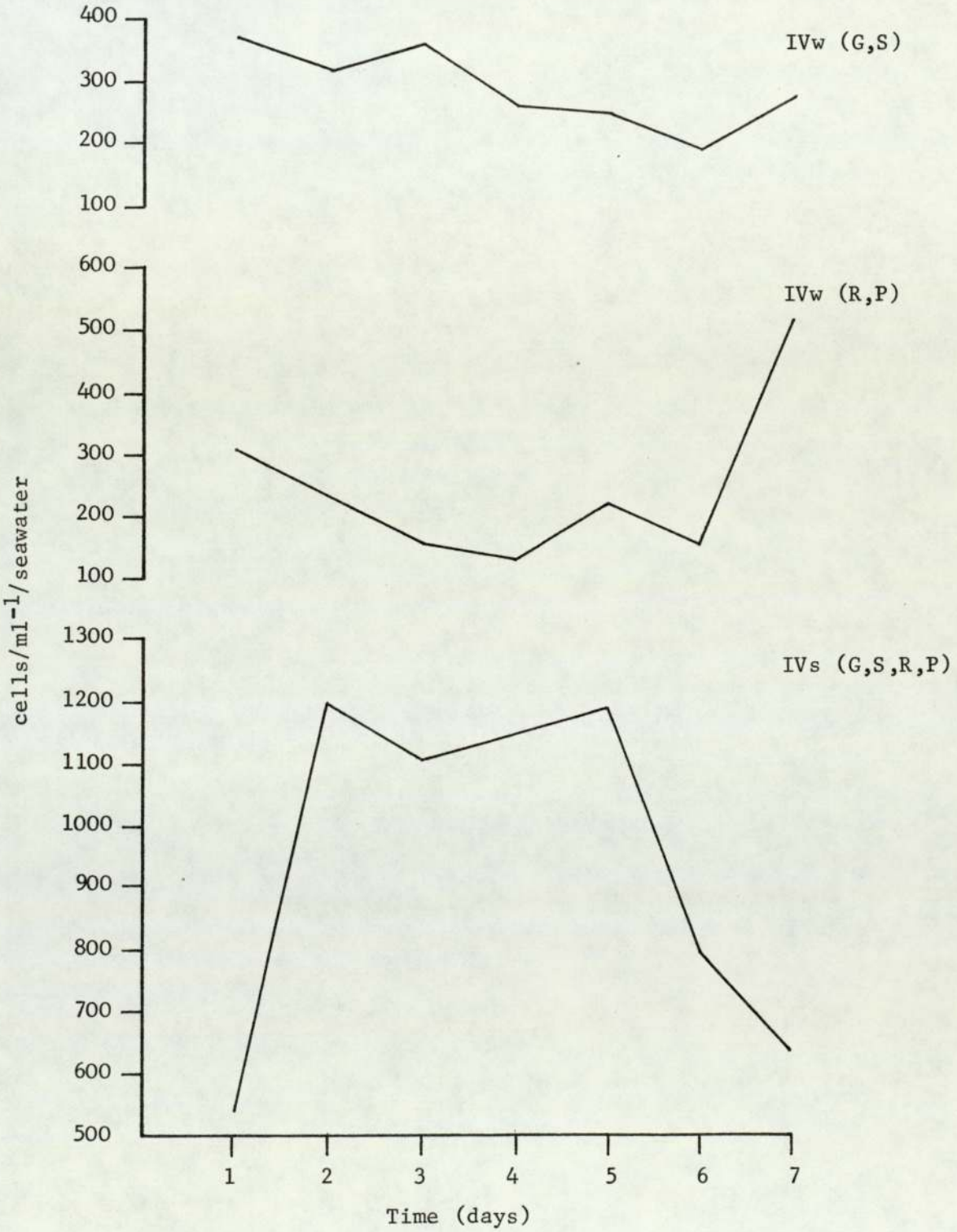



Fig. 14 Summary of environmental parameter, cells/ml$^{-1}$/seawater during summer and winter sampling period IV (0-168hrs.)

IVw (G,S) - winter;glass and steel
IVw (R,P) - winter;resin and paint
IVs (G,S,R,P) - summer;glass,steel,resin and paint

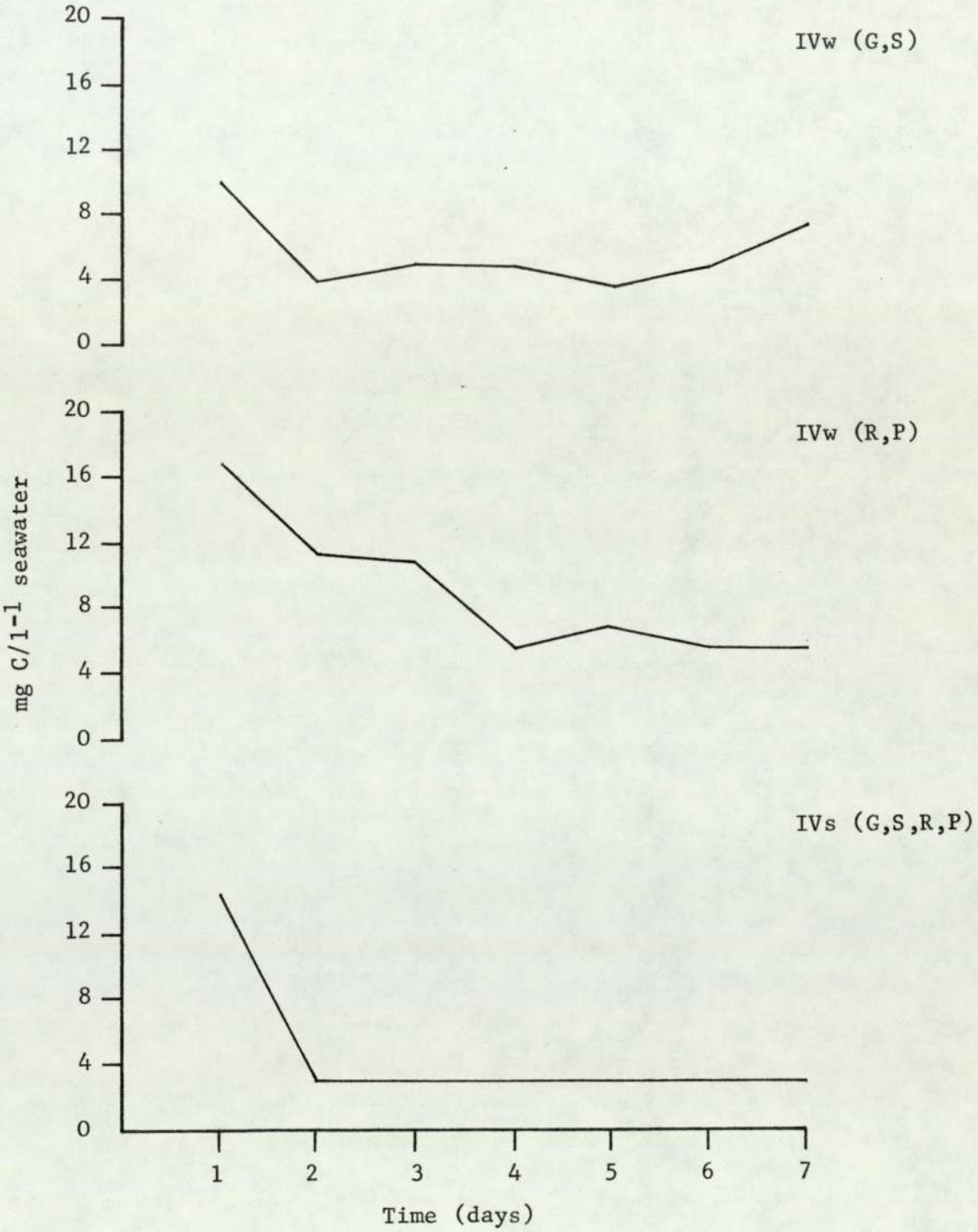



Fig. 15 Summary of environmental parameter, total organic carbon during summer and winter sampling period IV (0-168hrs.)

IVw (G,S) - winter; glass and steel
IVw (R,P) - winter; resin and paint
IVs (G,S,R,P) - summer; glass, steel, resin and paint

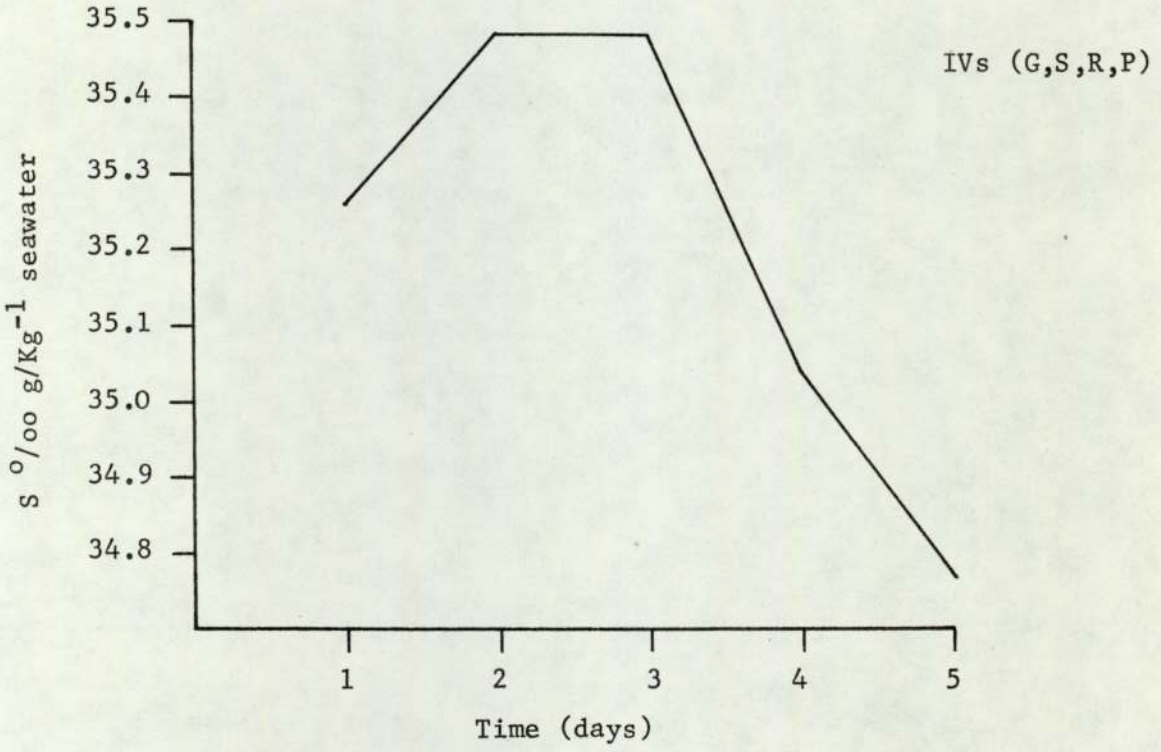



Fig. 16 Summary of environmental parameter, salinity during summer sampling period IV (0-168hrs.)

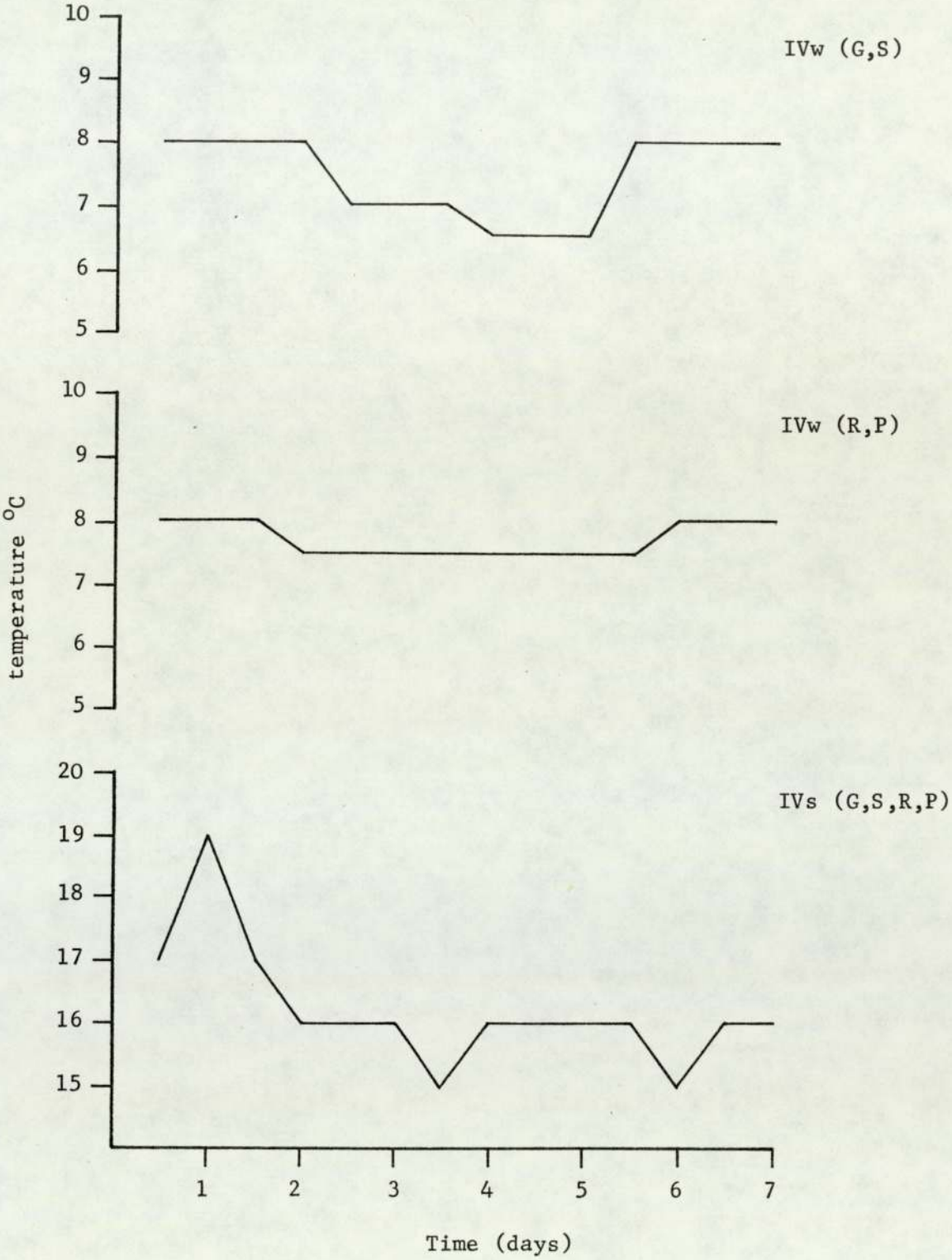



Fig. 17 Summary of environmental parameter, temperature during summer and winter sampling period IV(0-168hrs.)

IVw (G,S) - winter;glass and steel
IVw (R,P) - winter;resin and paint
IVs (G,S,R,P) - summer;glass,steel,resin and paint

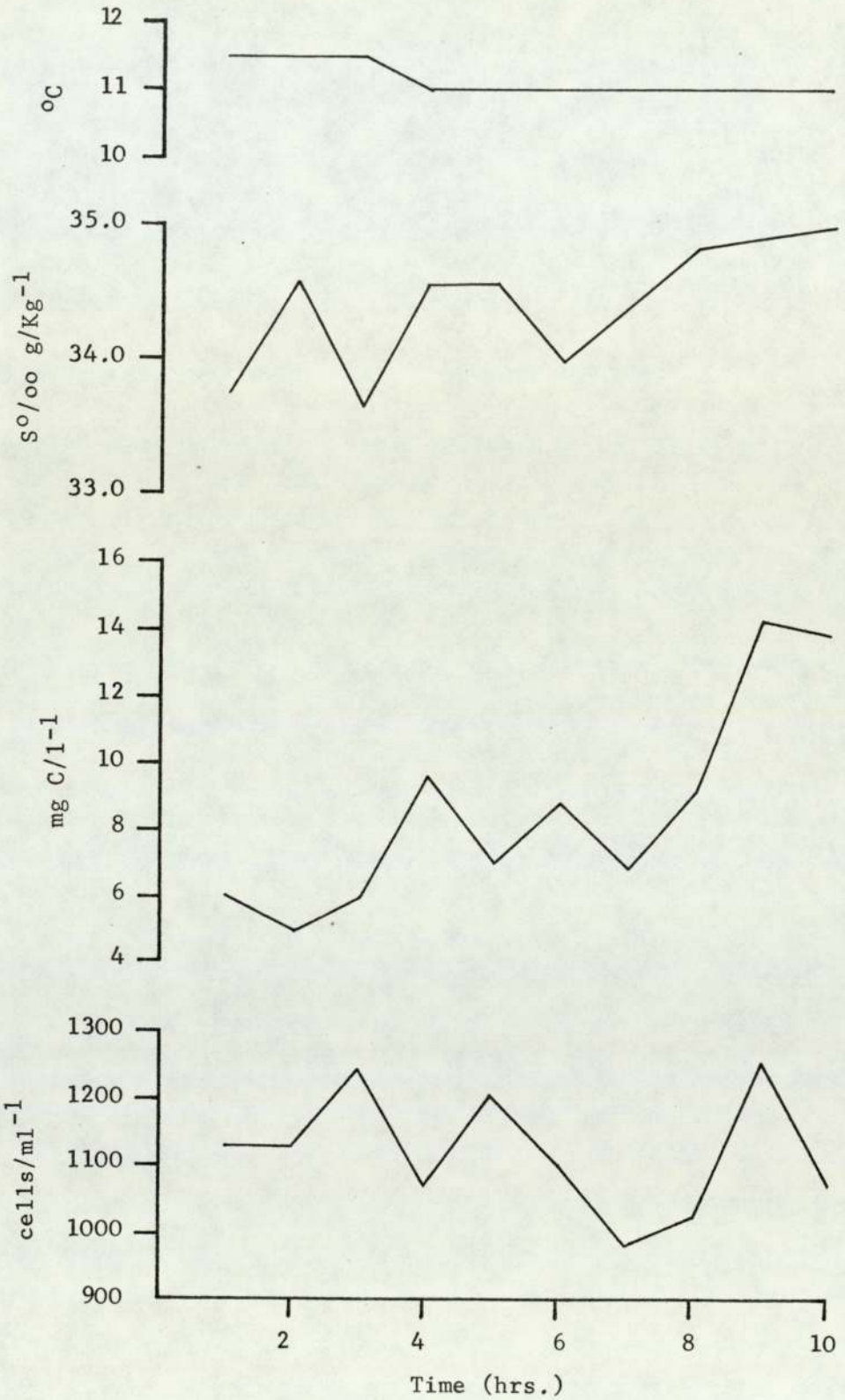



Fig. 18 Summary of environmental parameters measured during sampling period II (0-10hrs.,Summer).

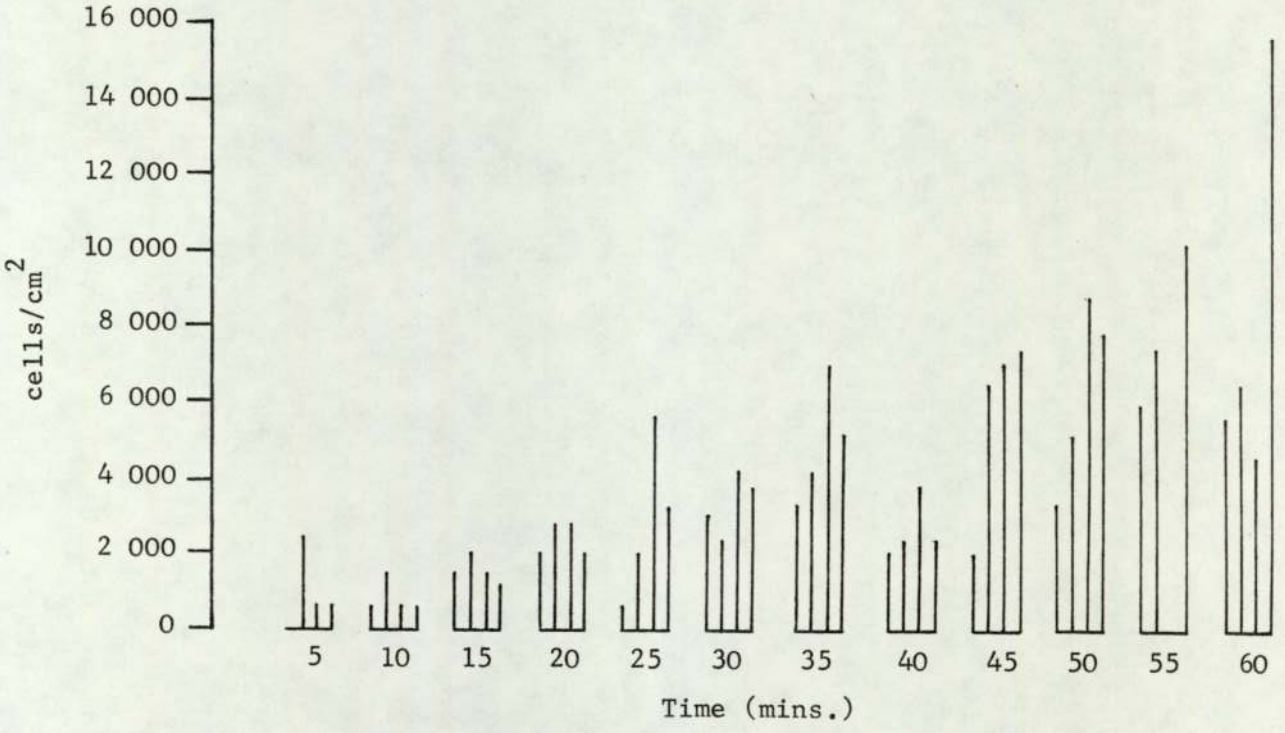
## PART II : ANALYSIS OF THE SAMPLING DATA

Part I of this chapter described the materials and methods used for the sampling programmes; here in Part II, the data are analysed quantitatively. Four aspects of attachment were examined: cell density, attachment rate, cell distribution, and the influence of specific environmental parameters.

### 3.7 Density of Bacteria on Surfaces

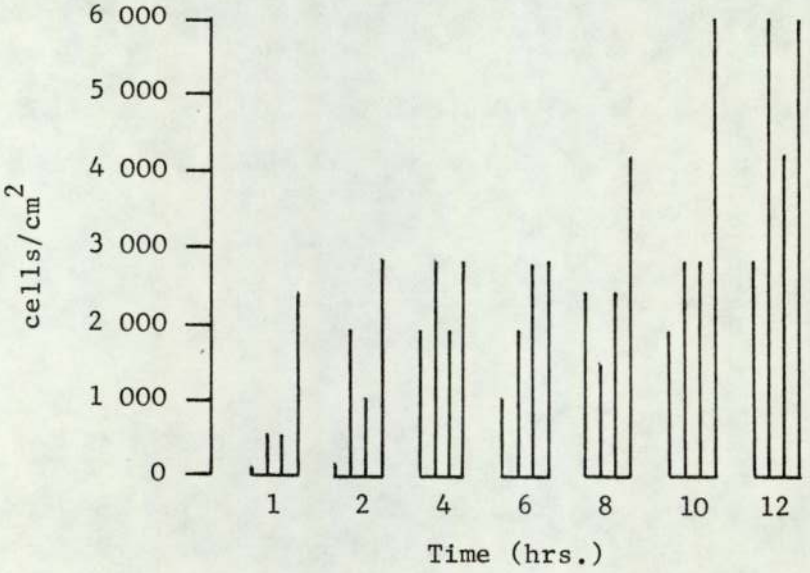
3.7.1 Six sampling programmes were run during the summer and winter, using various immersion periods, so that seasonal effects and the influence of substrate characteristics on attachment densities could be ascertained. Complementary sampling during the winter and summer used the core substrates glass, steel, resin, and paint over immersion periods III (0 - 12 hrs) and IV (0 - 168 hours), with additional summer sequences on scale II (0 - 10 hrs) using substrates of different surface energies and also on time scale I (0 - 1 hr) to test the speed of colonisation on newly immersed substrates. The cell density data have been summarised in Figs. 19 to 24.

3.7.2 Glass immersed for 12 hours during the winter had a cell density after one hour of $1.14 \times 10^2$ cells/cm$^2$, which reached $2.48 \times 10^3$ cells/cm$^2$ after 12 hours, and $9.78 \times 10^4$ cells/cm$^2$ after 7 days. In the summer season when the sea temperature and cell count of seawater was higher, cell densities on glass ranged from $2.0 \times 10^4$ cells/cm$^2$ after one hour to $5.46 \times 10^4$ cells/cm$^2$ after 12 hours, and $2.12 \times 10^5$ cells/cm$^2$ after 7 days immersion. Although comparisons of data obtained from different studies are difficult to interpret because of different experimental conditions, sampling, and counting techniques, there is nevertheless



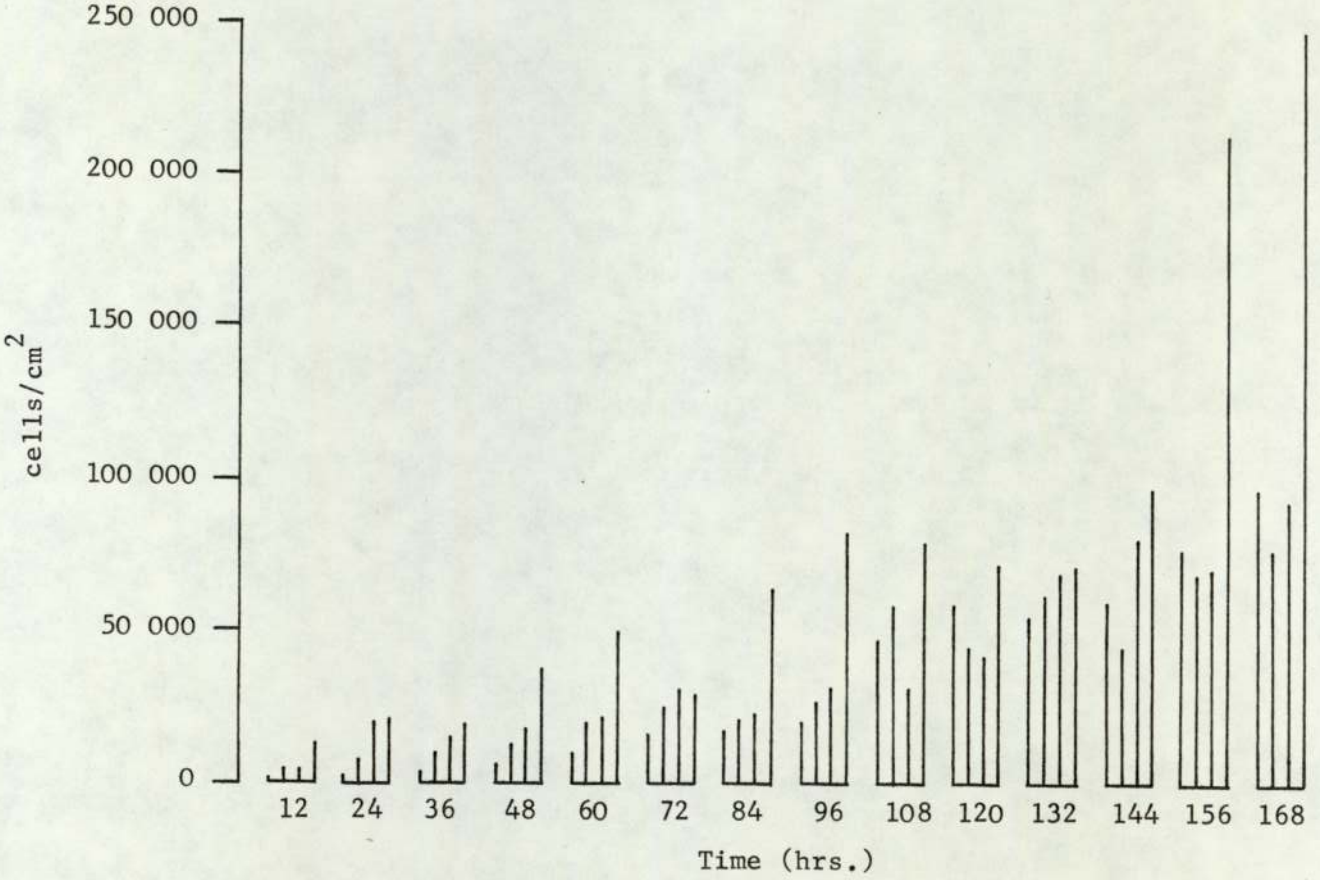

Bars in each block are in order of glass,steel,resin and paint

Fig. 19 Histogram of cell density.Sampling period I (0-1 hr.) winter



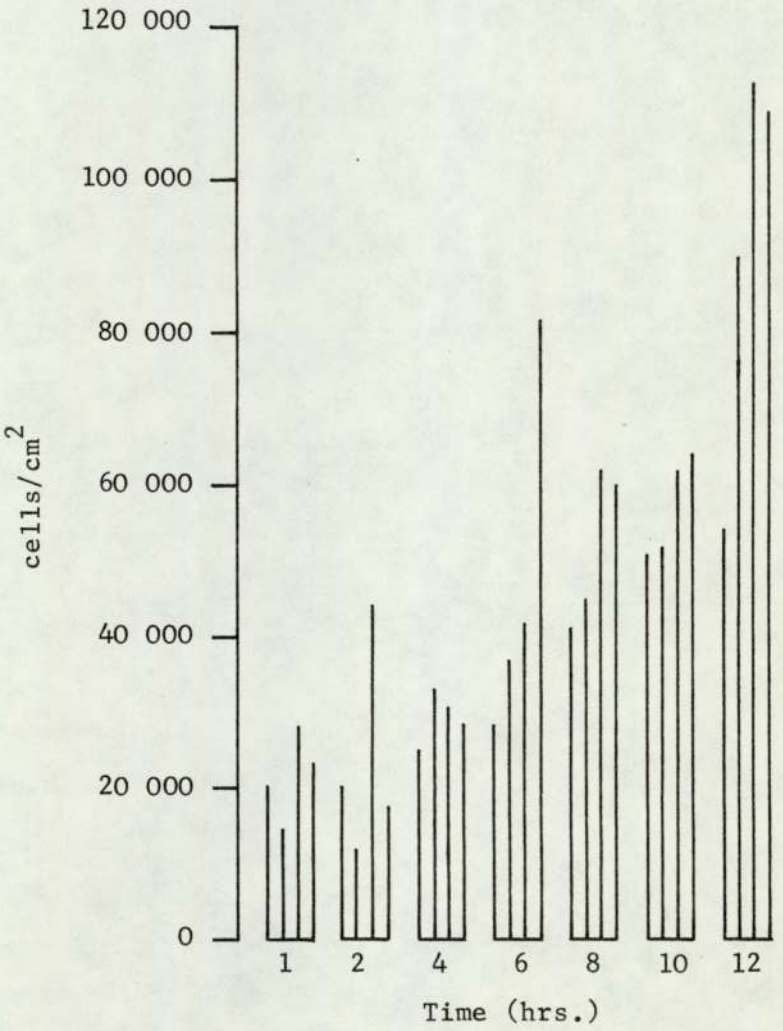

Bars in each block are in order of glass,steel,resin and paint

Fig. 20 Histogram of cell density.Sampling period III (0-12 hrs.) winter



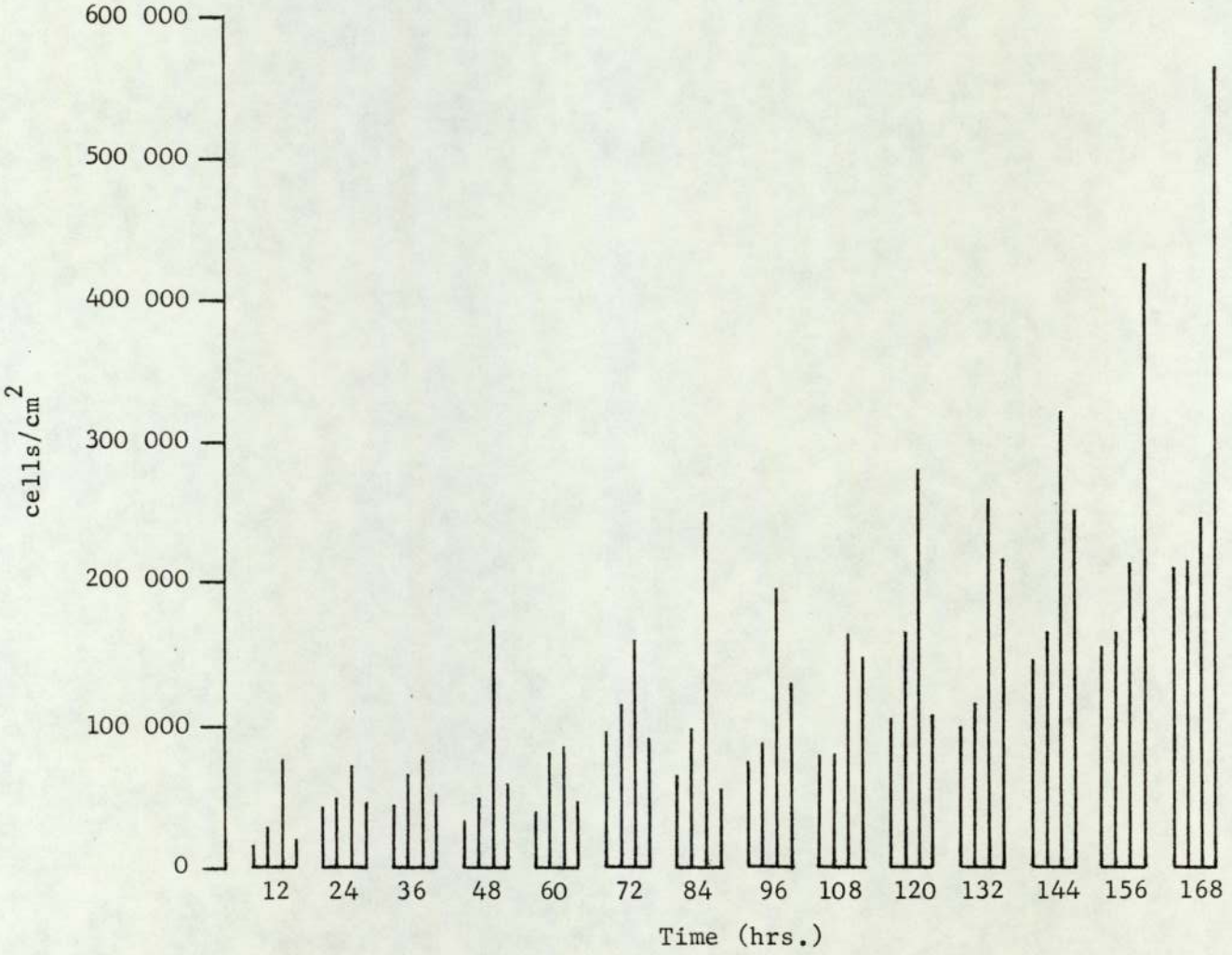

Bars in each block are in order of glass,steel,resin and paint

Fig. 21 Histogram of cell density.Sampling period IV (0-168 hrs.) winter



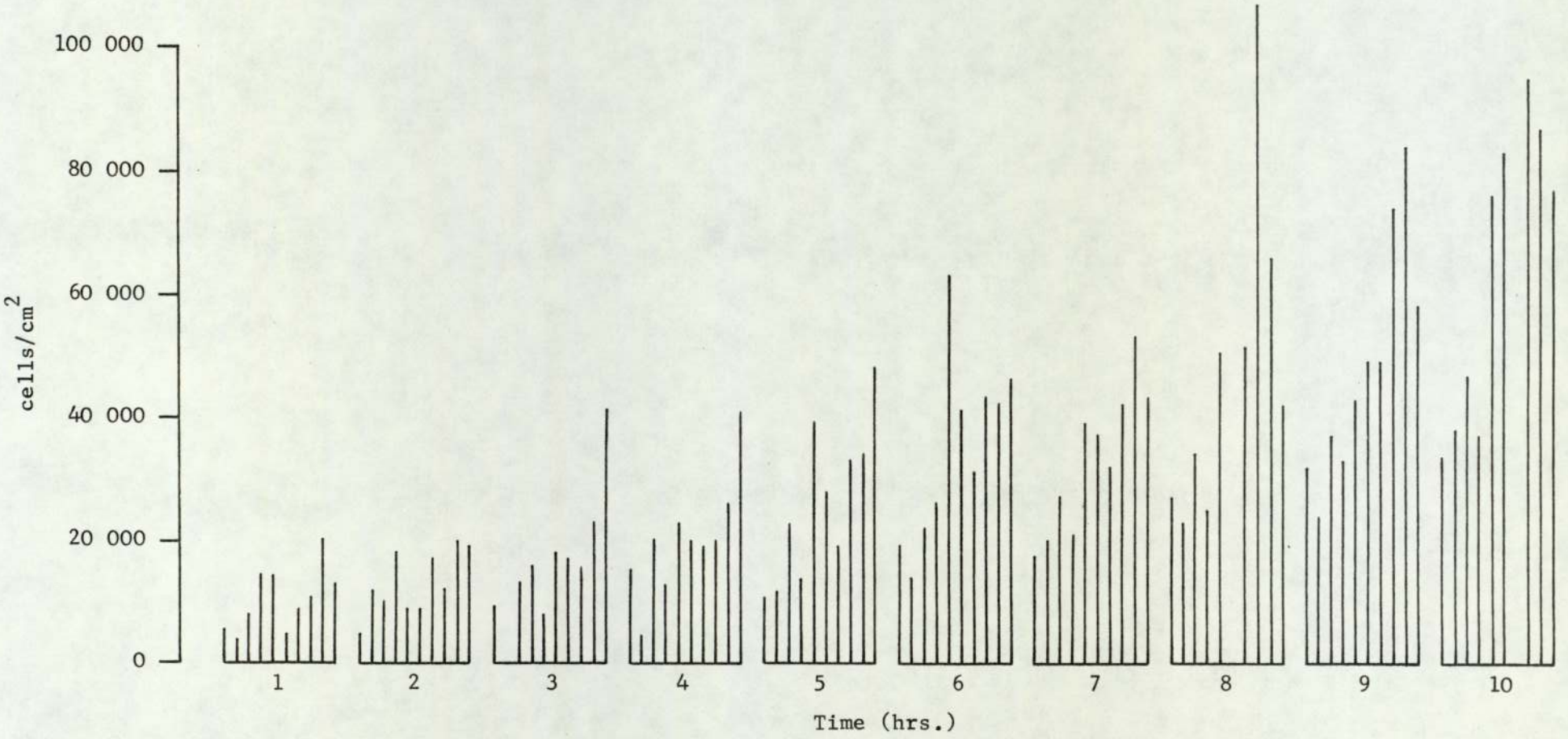

Bars in each block are in order of glass,steel,resin and paint

Fig. 22 Histogram of cell density.Sampling period III (0-12 hrs.) summer




Bars in each block are in order of glass,steel,resin and paint

Fig. 23 Histogram of cell density.Sampling period IV (0-168 hrs.) summer




Bars in each block are in order of glass,chrome,steel,aluminium,perspex,resin,polystyrene,polypropylene paint and PTFE

Fig. 24 Histogram of cell density.Sampling period II (0-10 hrs.) summer

a broad agreement with reported figures. Estimates generally range from $10^2$ - $10^3$ cells/$cm^2$ for a 12-hour immersion period, and up to $10^5$ cells/$cm^2$ for a 7-day period. Skerman (1956), using glass slides, immersed off the coast of New Zealand, found cell densities ranging from $7.1 \times 10^2$ to $4.02 \times 10^3$ cells/$cm^2$ for a 1 - 7 hour immersion period, and up to $1.19 \times 10^4$ cells/$cm^2$ after 7 days. Similarly, results for other substrates such as 'Plexiglass' (methylmethacrylate) with a density range of $8.6 \times 10^2$ to $5.3 \times 10^4$ cells/$cm^2$ after 6 hours immersion (O'Neil & Wilcox, 1971), compare favourably with densities of $1.4 \times 10^4$ cells/$cm^2$ found on 'Perspex' (methylmethacrylate) used in this study.

3.7.3 From the data illustrated (Figs.19 to 24), it can be seen that cell density levels were greater during the summer sampling period. This variation may be explained by interpretation of the environmental parameters monitored during sampling and correlated with models of attachment processes. Dissolved organic carbon is absorbed onto surfaces in seawater (Loeb & Neihof, 1977) and, accordingly, becomes a site of nutrient concentration in a nutrient-limited environment. ZoBell (1943) suggests that bacteria which attach to surfaces are able not only to utilise the organic material but also to accelerate its accumulation, due to detrital particles collecting in cell/surface interstices and on sticky exopolymers exuded by the cell. It was observed that TOC levels during sampling programmes were much lower in the summer than the winter (Section 3.5), indicating a negative relationship between cell density and TOC levels. Undoubtedly, organic carbon plays an essential role in the attachment process, but it is obvious that other factors have a greater influence on microbial film development.

Attachment of bacteria to surfaces has been shown to occur in two stages (Marshall *et al.*, 1971), where in the first phase a cell is held at the surface but not fixed, and in the second phase is irreversibly bound by extracellular polymeric fibrils. It was also found that low levels of carbon increased polymer production and accelerated adsorption, although the cell does not have to actively metabolise to effect attachment as an existing coat of exopolymer mediates in the process (Fletcher & Floodgate, 1973; Corpe, Matsuuchi & Armbruster, 1976). During winter sampling (Time Scale I, 0 - 1 hr), under conditions precluding rapid metabolism, irreversible attachment to cleaned glass slides occurred after ten minutes immersion in seawater at $6^{o}C$ (Fig.19). Attachment, therefore, appears not to be a time-dependent metabolic response but one of compatibility between cell and surface characteristics effecting a rapid physico-chemical adhesion. It has been shown (Fletcher, 1977) that attachment dynamics can be described using the Langmuir molecular adsorption isotherms, indicative of a physico-chemical attachment process that would account for the rapid attachment of cells to surfaces. Assuming that attachment can be described in this manner, the influence of temperature and bacterial concentration in seawater on attachment densities can be assessed. Both parameters can influence adsorption but, of the two, the concentration of cells in the surrounding seawater is more significant, as it will increase the probability of cell/surface collisions.

The difference between summer and winter data can therefore be attributed to the greater bacterial concentration in seawater observed during the summer. This interpretation also explains the anomaly between the sampling series 0 - 1 hr (Time Scale I) and 0 - 12 hrs (Time Scale III) (Figs. 19 & 20), where identical cell densities were

observed during dissimilar immersion times. In both instances, TOC and temperature were approximately equal, but the bacterial concentration of seawater was considerably higher by a factor of three times during the 0 - 1 hr sampling period. These conclusions are in agreement with data obtained from mountain streams (Geesey, Mutch & Costerton, 1978) where fluctuations in cell density correspond with those of bacterial concentration in water, but not for temperature.

3.7.4 A feature of the attachment data, particularly during the first hours of immersion, was the noticeable difference in density between types of substrates. The levels of cell density for immersion period II (0 - 10 hrs) were compared with surface energy, a characteristic measured as the critical surface tension of wetting ($\gamma_c$), and wettability as the seawater contact angle of wetting ($\theta_{sw}$) (Section 3.3), and it was found that, with increasing wettability, there was a decrease in cell surface density (Table 11). The two variables could be correlated using a rectilinear regression equation ( $r = 0.8655$ , $p = 0.001$) (see Fig.25), suggesting that cell density is a function of the surface measured as the seawater contact angle of wetting, and that attachment involves some form of hydrophobic interaction between cell and surface (Fletcher & Loeb, 1979).

TABLE 11 : Comparison of cell density with substrate characteristics of $\theta_{sw}$ and $\gamma_c$.

| Substrate | $\theta_{sw}$ | $\gamma_c$ dynes/cm$^{-1}$ | cells/cm$^2$ |
|---|---|---|---|
| Glass | 6.0 | 46 | 34205 |
| Chrome | 23.4 | ND | 38295 |
| Steel | 24.4 | ND | 46477 |
| Aluminium | 28.1 | ND | 36932 |
| Perspex | 60.9 | 39 | 67932 |
| Resin | 82.3 | 30 | 82841 |
| Polystyrene | 83.5 | 33 | 49205 |
| Polypropylene | 91.5 | 26 | 95114 |
| Paint | 94.5 | 20.26 | 86932 |
| PTFE | 102.1 | 18.5 | 76932 |

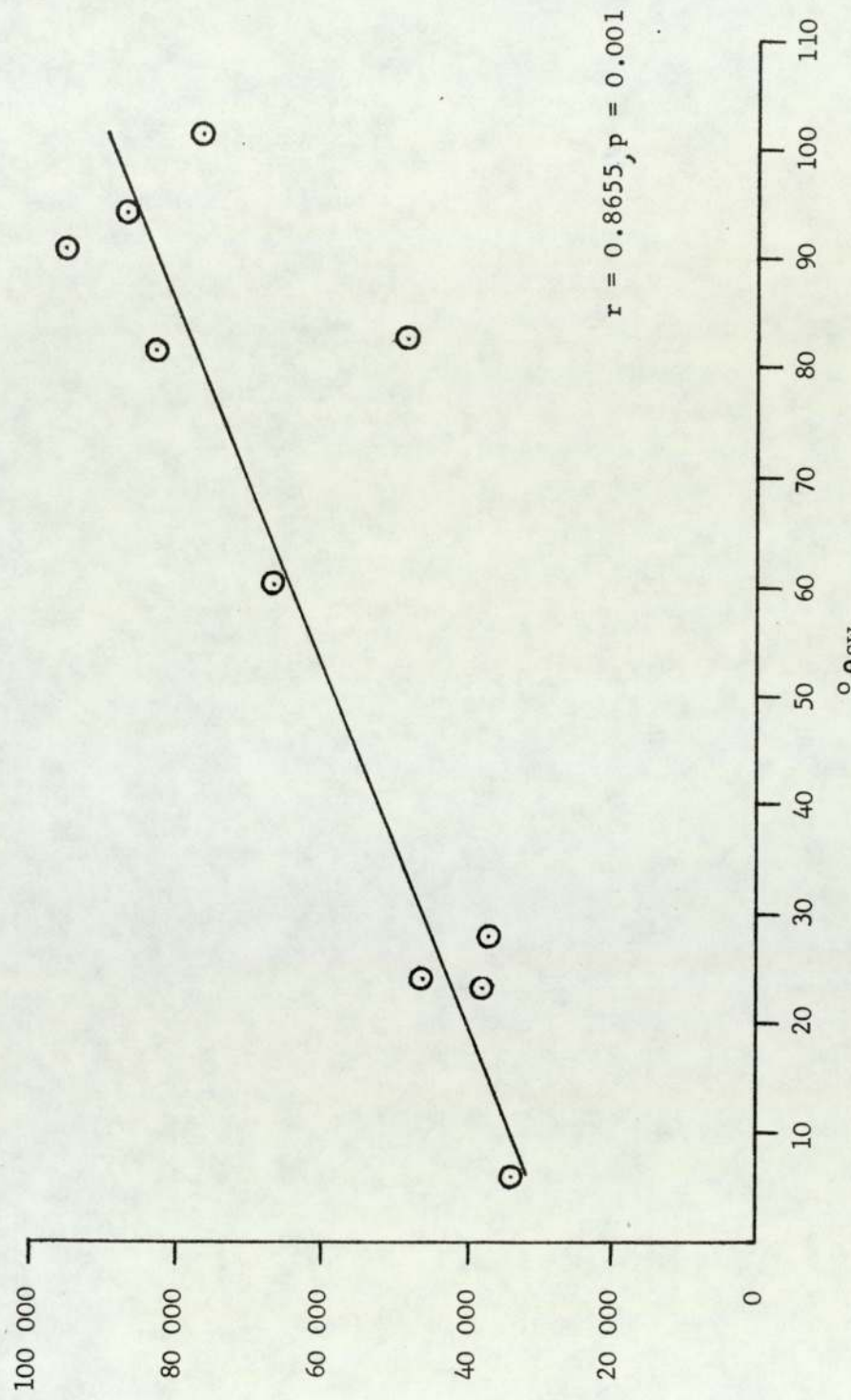



Fig. 25 Relationship between cell density and substrate hydrophobicity. Sampling period II (0-10 hrs.) summer.

A similar relationship between substrate character and density using a marine pseudomonad, has been described (Fletcher & Loeb, 1976, 1979), and it was noted that this trend was opposite to cell tissue models (Taylor, 1970). The data from this study, obtained in the natural environments, confirm the predictions of the marine model and supports the suggestion that attachment dynamics in the marine environment are based on different precepts from the cell tissue process.

The distinction between substrates becomes less clear with increasing time of immersion, and is possibly due to the surface properties of the different substrates becoming masked with adsorbed organic material (Baier *et al.*, 1968) and attached bacteria.

## 3.8 Attachment Rate of Bacteria to Surfaces

3.8.1 Attachment rate was determined from data obtained from the sampling periods given in Section 3.7.1, using the core substrates on Time Scale I (0 - hr, Winter), Time Scale III (0 - 12 hrs), and Time Scale IV (0 - 168) hrs) during the summer and winter, and all the substrates on Time Scale II (0 - 10 hrs, summer).

3.8.2 Samples from a population may, by the nature of the population itself, be unrepresentative and, combined with enumeration errors, result in inaccurate estimations of population size. This can lead to difficulties in determining the curve and hence, rate, for population data from a time series, and may require the application of rectilinear or curvilinear equations to calculate the line of best fit. Three curve equations were used to calculate the regression of y on x, as $y = a + (b.x)$ for a linear curve, $\log y = \log a + (\log b.x)$ for a logarithmic curve, and $y = x \div \left(a + (b.x)\right)$ for a hyperbolic curve, where

y = a variable on the y axis, x= a variable on the x axis, a = the intercept, and b= the slope of the curve. The degree of correlation between the two variables (x) time and cell density (y) was determined as the correlation coefficient r, where perfect agreement is indicated as +1 or -1, and is taken as significant when $r > p = 0.05$ (Table VII, Fisher & Yates, 1963). Rate,as the increase in cell density per unit time,can be determined from the slope of a straight line, and for curvilinear data, by differentiation of the curve equation. Alternatively, the data can be plotted on a log/log scale and the gradient determined from arbitrarily designated straight line segments (Dexter *et al.*, 1975).

Comparisons of rate with populations of approximately equal values of $\mu$ can be misleading when sampling and enumeration errors of data, used in the regression equation, distort the gradient of the slope. Where no specific value of rate is necessary, an order of rate can be established based on the values of the correlation coefficient r for the curve equations considered, so obviating the need to differentiate, while remaining independent of sampling and enumeration error. An order of rate was established by determining the correlation coefficient r for a linear ($r^{lin}$), logarithmic ($r^{log}$), and hyperbolic ($r^{hyp}$) equation of the data sets for comparison, assuming that population values were similar. The magnitude of r determined the curve type for each data set and indicated, by nature of the curve species, the rate. The order of rate was calculated as the relative rate coefficient $r'$ as :

$$r' = r^{x} - r^{lin}$$

where $r^{x}$ was the correlation coefficient for the curve type of greatest increase/unit time, and $r^{lin}$ the linear correlation coefficient. Large values of $r'$ indicate greater rate, because $r^{x}$ is closer to 1 and

$r^{lin}$ to 0, signifying a non-linear curve (greater increase/unit time); but if $r'$ is small, $r^{x}$ and $r^{lin}$ are closer in value, indicating linearity of $r^{x}$ (i.e. less increase/unit time).

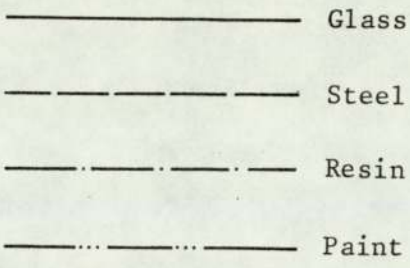
3.8.3 The curve species for each substrate immersed during the various sampling series was determined (Table 12) and the regression equations used to plot curves of attachment density against time (Figs. 26 - 31). During the winter, for the first hours of immmersion (0 - 12 hrs.), attachment rates on wettable substrates (glass and steel) were linear, while for less wettable substrates (resin and paint) they were logarithmic, indicating a faster attachment rate. During the 0-1 hr. sampling period, only glass had a linear function and the remaining substrates a logarithmic function -- an effect of the high bacterial count of seawater accelerating the attachment rate. No such difference was observed for substrates immersed for longer periods of time (0 - 168 hrs.) where the rate of attachment was considerably faster, as denoted by the hyperbolic curves. During the short summer sampling period (0 - 10 hrs.), using substrates with a range of surface energy values (wettability), a variety of attachment curve types was observed. On the wettable substrates (glass, chrome, steel, and aluminium) attachment rates were logarithmic; on only two substrates (resin and PTFE) could attachment rate be described by a hyperbolic function. The attachment rates observed during summer sampling sequences II (0 - 10 hrs.) and III (0 - 12 hrs.) are at variance with each other. It is possible that the uniform hyperbolic attachment rates of sampling period III (0 - 12 hrs.) may be a reflection of high bacterial numbers in seawater during that sampling period, and a more likely pattern of attachment rates was observed during samling period II.

Table 12 : Bacterial attachment curve types for various substrates

| | WINTER | | | | | | SUMMER | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0-1hr | r | 0-12hrs | r | 0-168hrs | r | 0-10hrs | r | 0-12hrs | r | 0-168hrs | r |
| Glass | lin | 0.8554 | lin | 0.8709 | log | 0.9542 | lin | 0.9627 | hyp | 0.9768 | hyp | 0.9316 |
| Chrome | - | - | - | - | - | - | lin | 0.8861 | - | - | - | - |
| Steel | log | 0.8572 | lin | 0.7375 | log | 0.9619 | lin | 0.9802 | hyp | 0.9850 | hyp | 0.9437 |
| Aluminium | - | - | - | - | - | - | lin | 0.8524 | - | - | - | - |
| Perspex | - | - | - | - | - | - | log | 0.8616 | - | - | - | - |
| Resin | log | 0.8126 | log | 0.8561 | log | 0.9273 | hyp | 0.9960 | hyp | 0.9546 | hyp | 0.7599 |
| Polystyrene | - | - | - | - | - | - | log | 0.9562 | - | - | - | - |
| Polyproylene | - | - | - | - | - | - | log | 0.9705 | - | - | - | - |
| Paint | log | 0.9503 | log | 0.9417 | log | 0.9441 | log | 0.9630 | hyp | 0.8511 | hyp | 0.9249 |
| PTFE | - | - | - | - | - | - | hyp | 0.9382 | - | - | - | - |

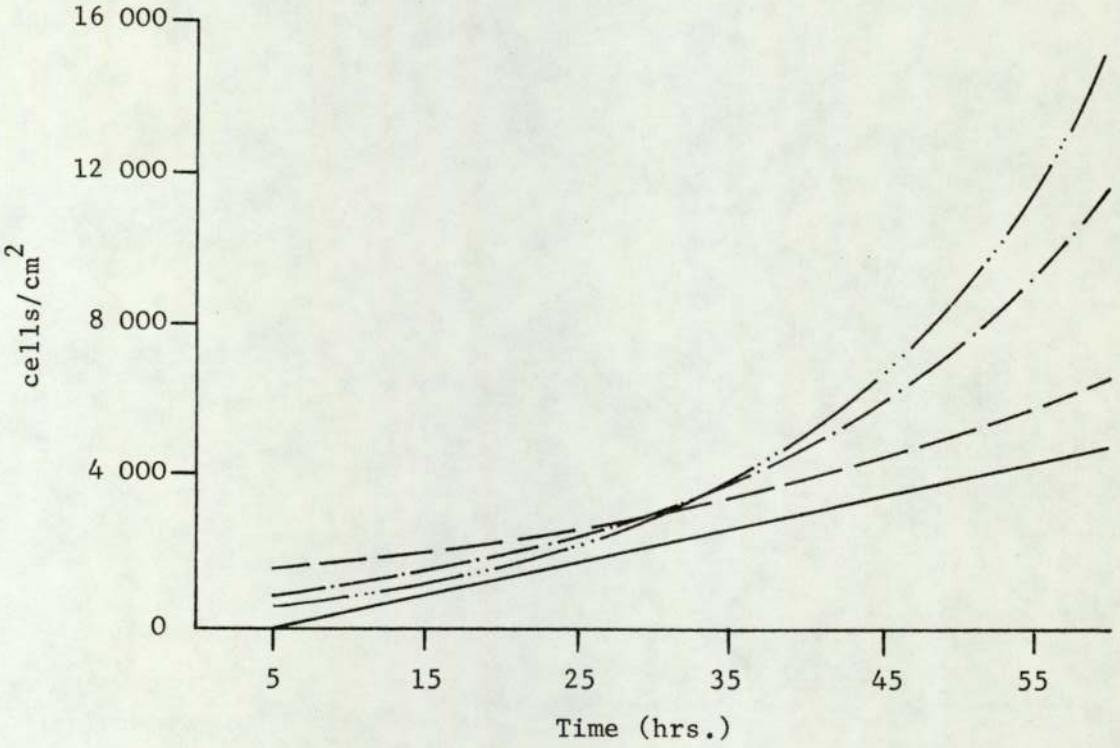
lin = linear; log = logarithmic; hyp = hyperbolic.

Substrates in order of decreasing wettability[a]




Substrates arranged in order of
decreasing wettability

Key for figs. 26 - 30




Glass $r^{lin} = 0.8554$

Steel $r^{log} = 0.8572$

Resin $r^{log} = 0.8126$

Paint $r^{log} = 0.9502$

Fig. 26 Bacterial attachment during sampling period I (0-1 hr.) winter

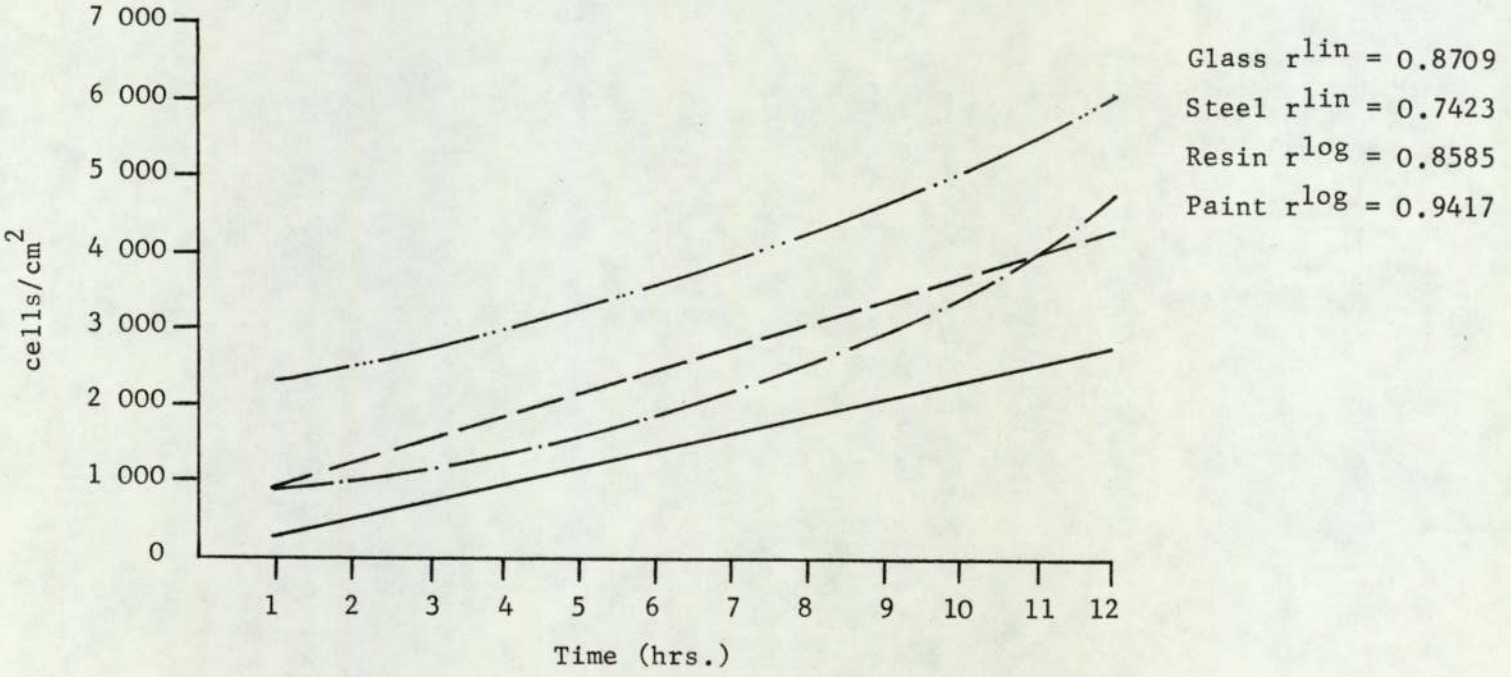



Fig. 27 Bacterial attachment during sampling period III (0-12 hrs.) winter

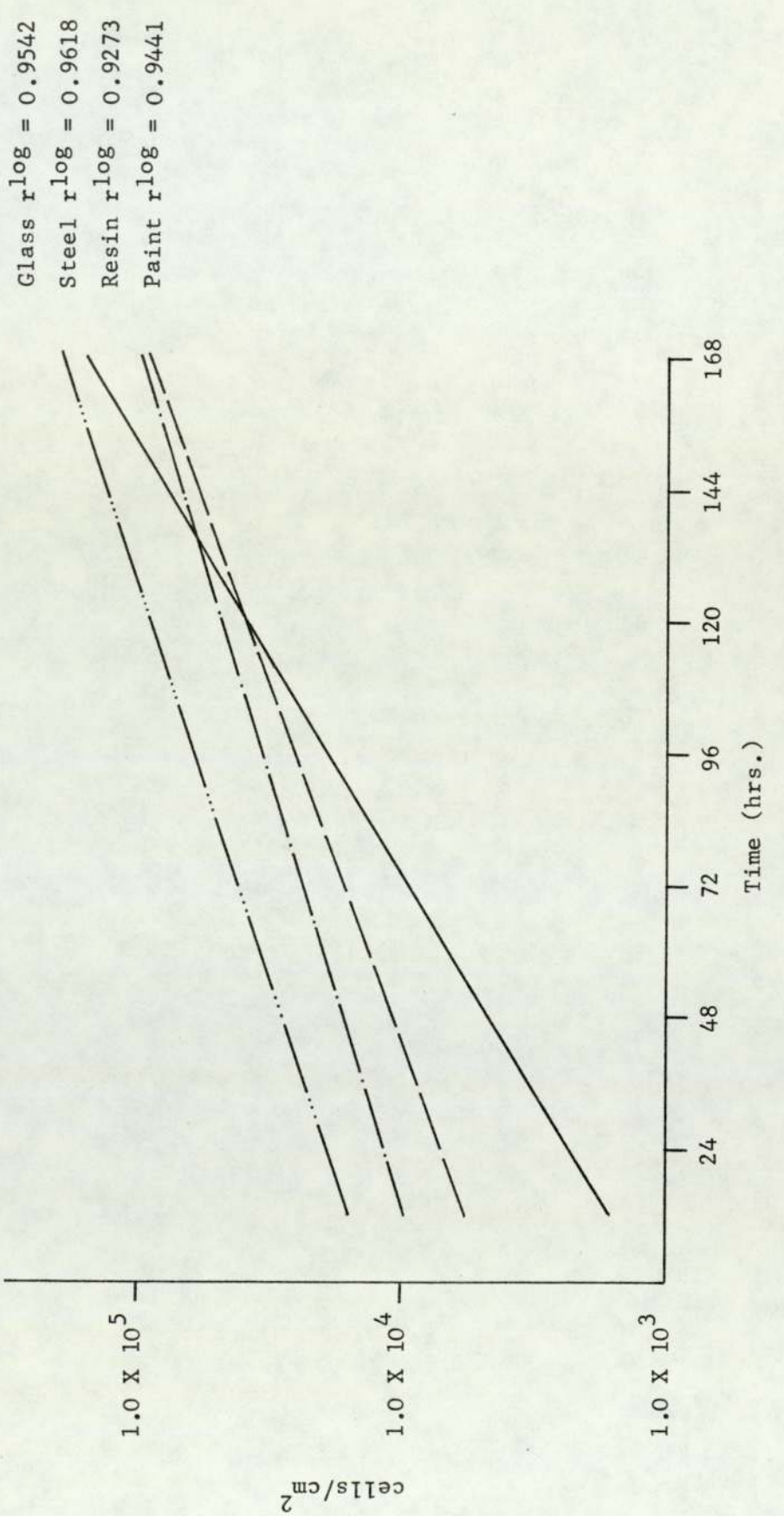



Fig. 28 Bacterial attachment during sampling period IV (0-168 hrs.) winter

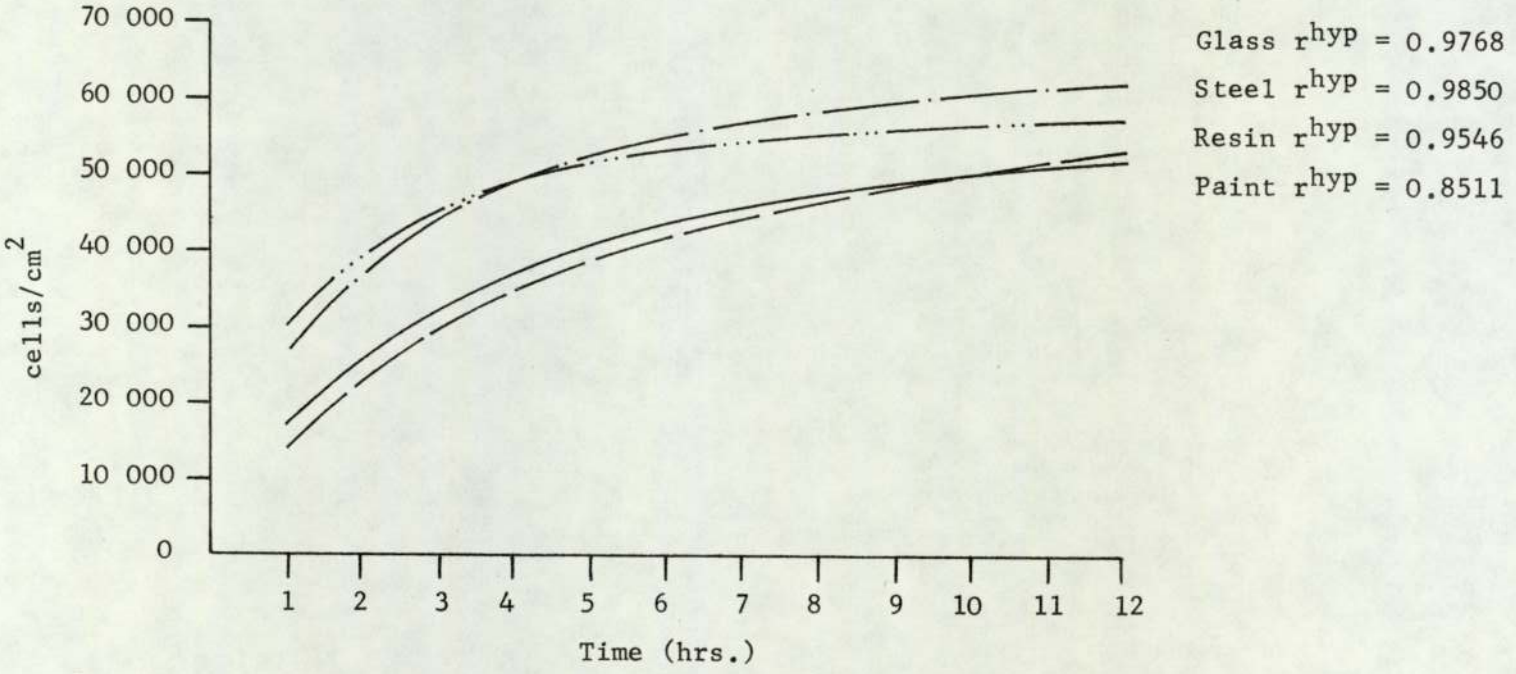



Fig. 29 Bacterial attachment during sampling period III (0-12 hrs.) summer

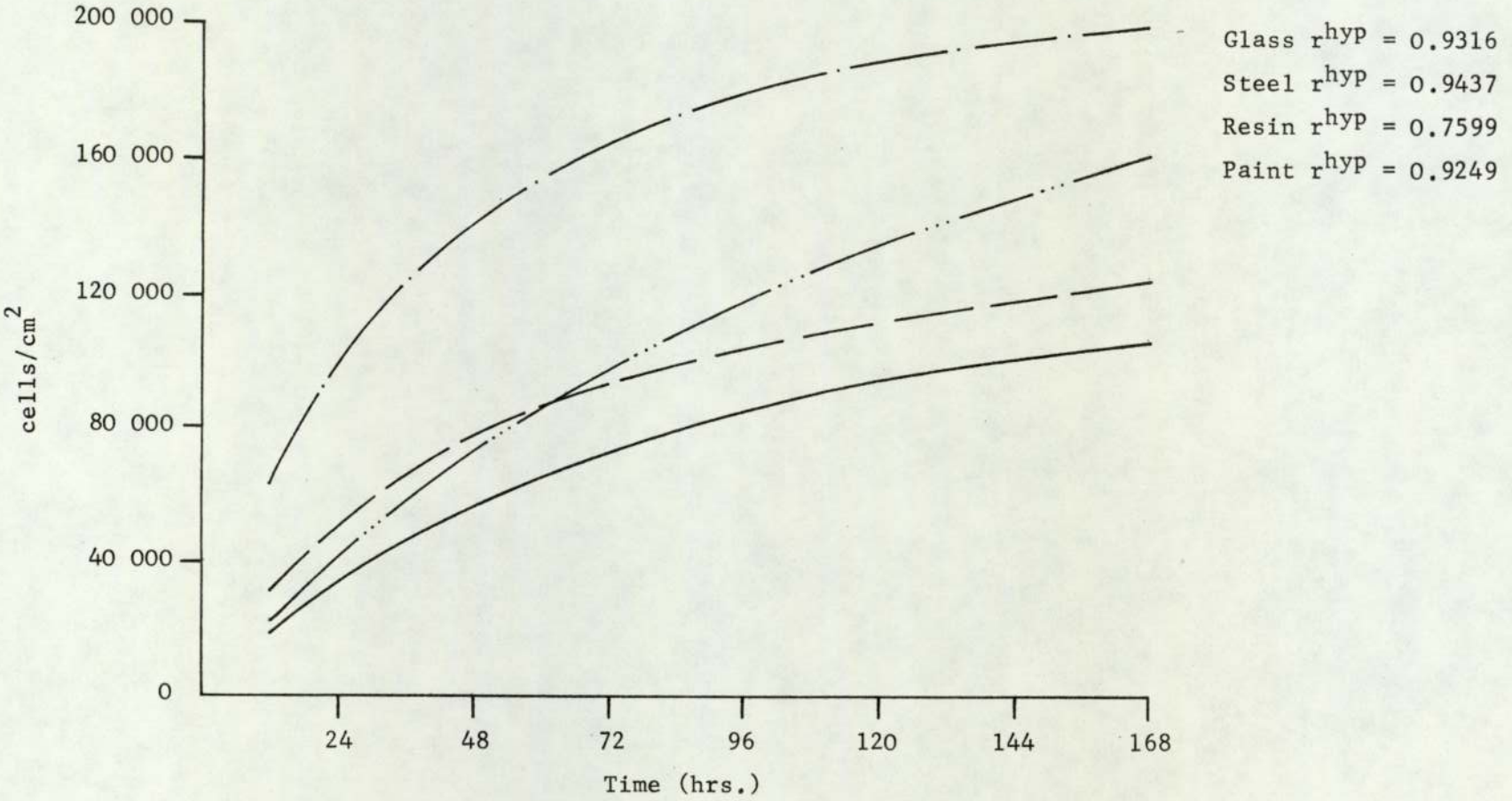
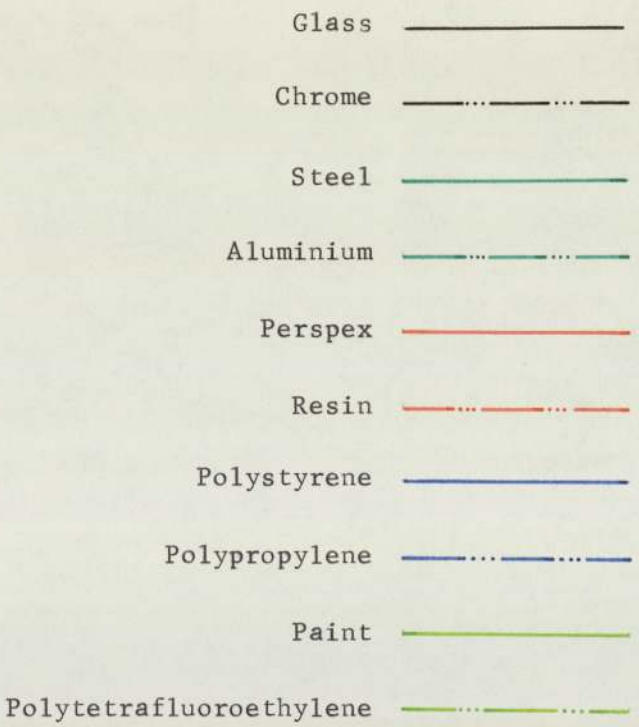



Fig. 30 Bacterial attachment during sampling period IV (0-168 hrs.) summer




Substrates arranged in order of decreasing wettability

Key for fig. 31

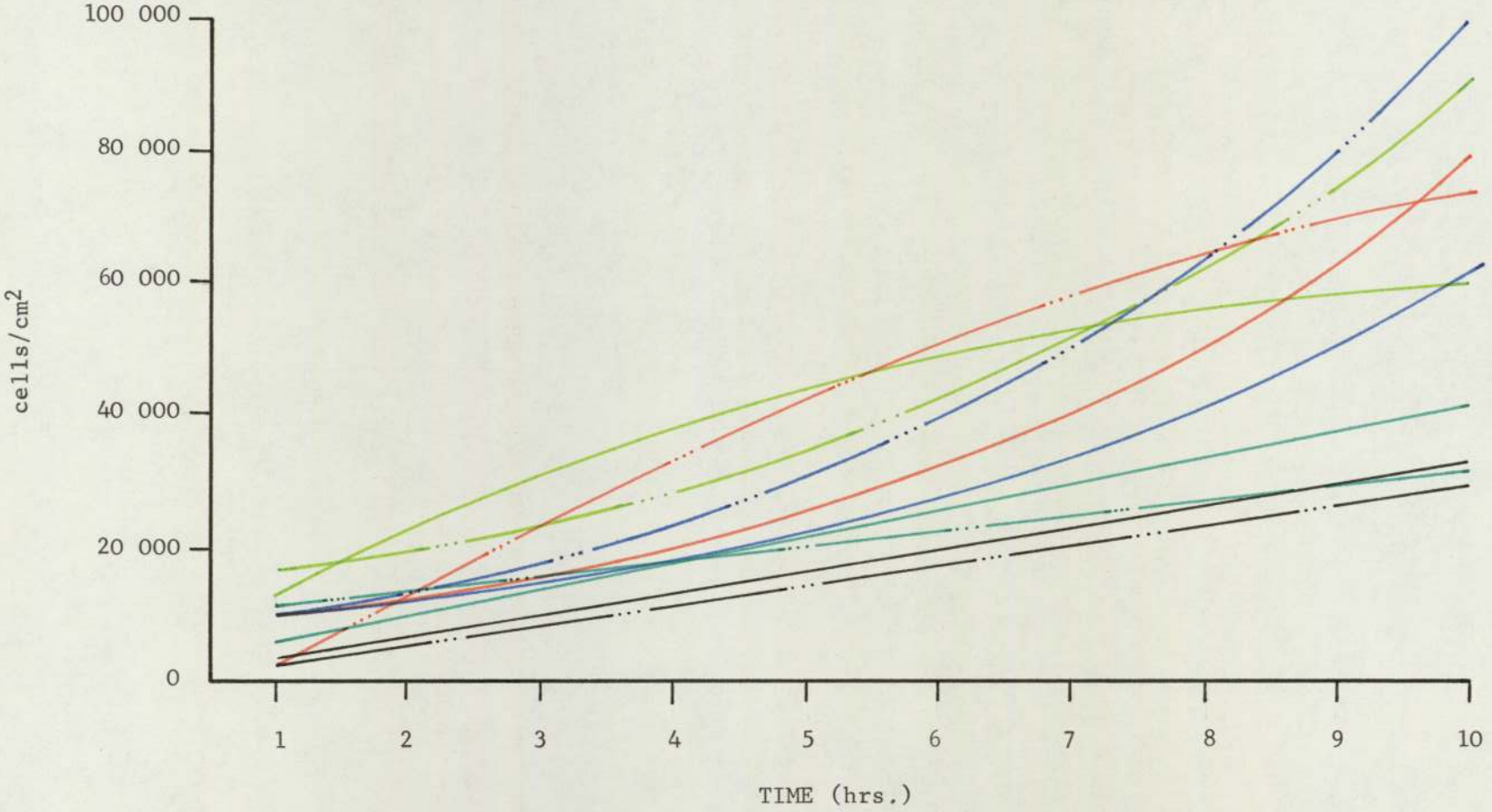



Fig. 31 Attachment of bacteria to a range of substrates during sampling period II (0-10 hrs., Summer)

The Langmuir adsorption isotherm has been used to describe attachment as a physico-chemical process (Fletcher, 1977), and it is interesting to note that a time plot of the isotherm is a hyperbolic function (Colquhoun, 1971). It is significant that curves of population data from the summer sampling series were hyperbolic, suggesting that the physico-chemical model can be used to describe attachment processes in the natural environment. Although the rate of attachment observed during the winter sampling was logarithmic rather than hyperbolic, it is likely that attachment occurred by the same physico-chemical process, but the dynamics were not apparent, due to limiting environmental conditions.

3.8.4 The relationship of attachment rate to wettability was determined by correlating relative rate coefficients ($r'$) with substrate wettability ($\theta_{sw}$) (Tables 13 and 14). The correlation of these two variables was determined as a linear relationship such that attachment rate increased with decreasing wettability, i.e. the rate of bacterial attachment is a function of the surface (Figs. 32 - 35). This relationship, however, could only be observed during the first hours of immersion (0 - 12 hrs.); with increased length of immersion the influence of the substrate on attachment rates decreased due possibly to the change in surface characteristics, the result of organic adsorption.

The influence of the substrate characteristic wetting on bacterial attachment must be regarded as a significant factor in attachment dynamics.

TABLE 13 : Comparison of relative rate $r'$ with the surface characteristic $\theta_{sw}$.

Sampling period I and III

| | | 0 - 1 hr Winter | 0 - 12 hrs Winter | 0 - 12 hrs Summer |
|---|---|---|---|---|
| Substrate | $\theta_{sw}$ | $r'$ | $r'$ | $r'$ |
| Glass | 6.0 | - 0.0176414 | - 0.035915 | - 0.160265 |
| Steel | 24.4 | 0.004422 | - 0.004813 | - 0.091877 |
| Resin | 82.3 | 0.036623 | 0.002435 | - 0.060453 |
| Paint | 94.5 | 0.050432 | 0.014294 | - 0.047833 |

TABLE 14 : Comparison of relative rate $r'$ with surface characteristic $\theta_{sw}$.

Sampling period II.

| Substrate | $\theta_{sw}$ | $r'$ |
|---|---|---|
| Glass | 6.0 | - 0.0041768 |
| Chrome | 23.4 | - 0.0072655 |
| Steel | 24.4 | - 0.0018706 |
| Aluminium | 28.1 | - 0.0100845 |
| Perspex | 60.9 | 0.0035083 |
| Resin | 82.3 | 0.0297719 |
| Polystyrene | 83.5 | 0.0245863 |
| Polypropylene | 91.5 | 0.0678949 |
| Paint | 94.5 | 0.020393 |
| P T F E | 102.1 | 0.0596654 |

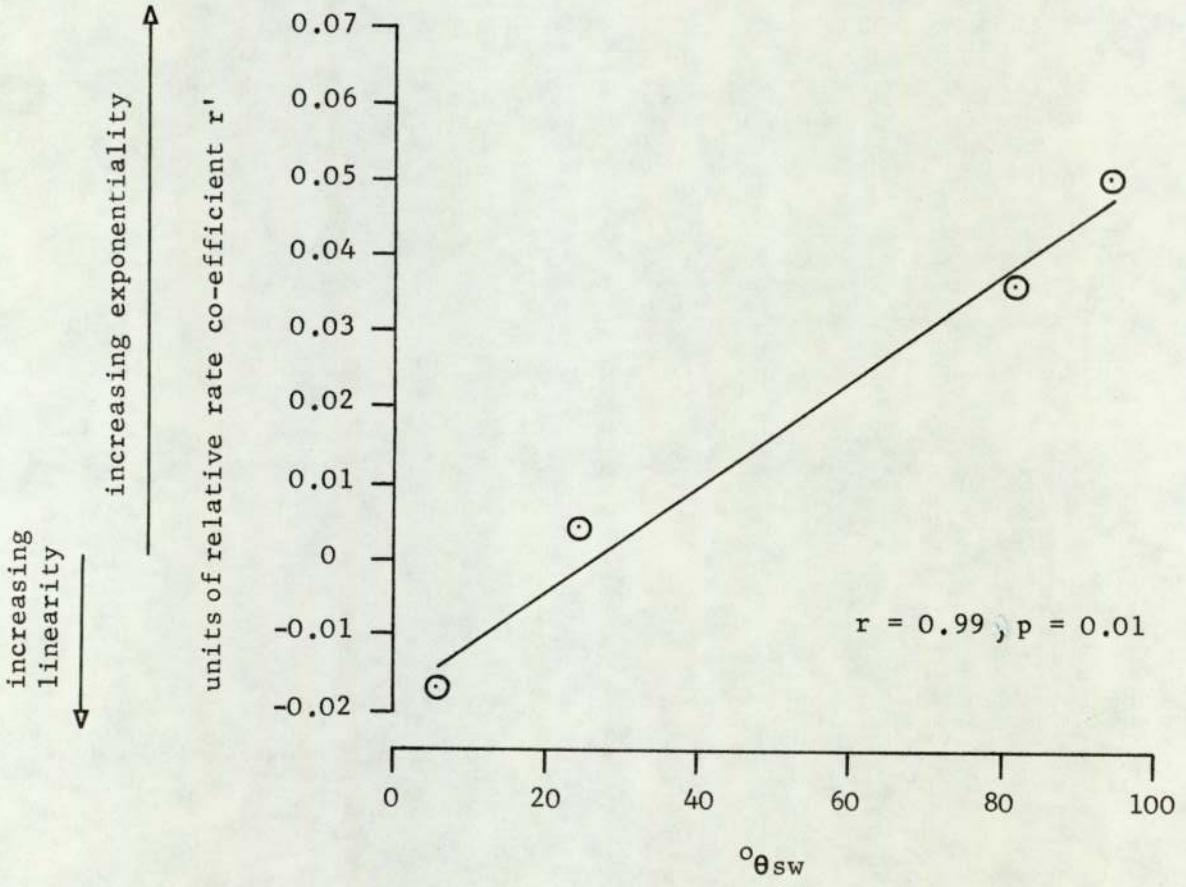



Fig. 32 Relationship between relative rate and substrate hydrophobicity. Sampling period I (0-1 hr.) winter

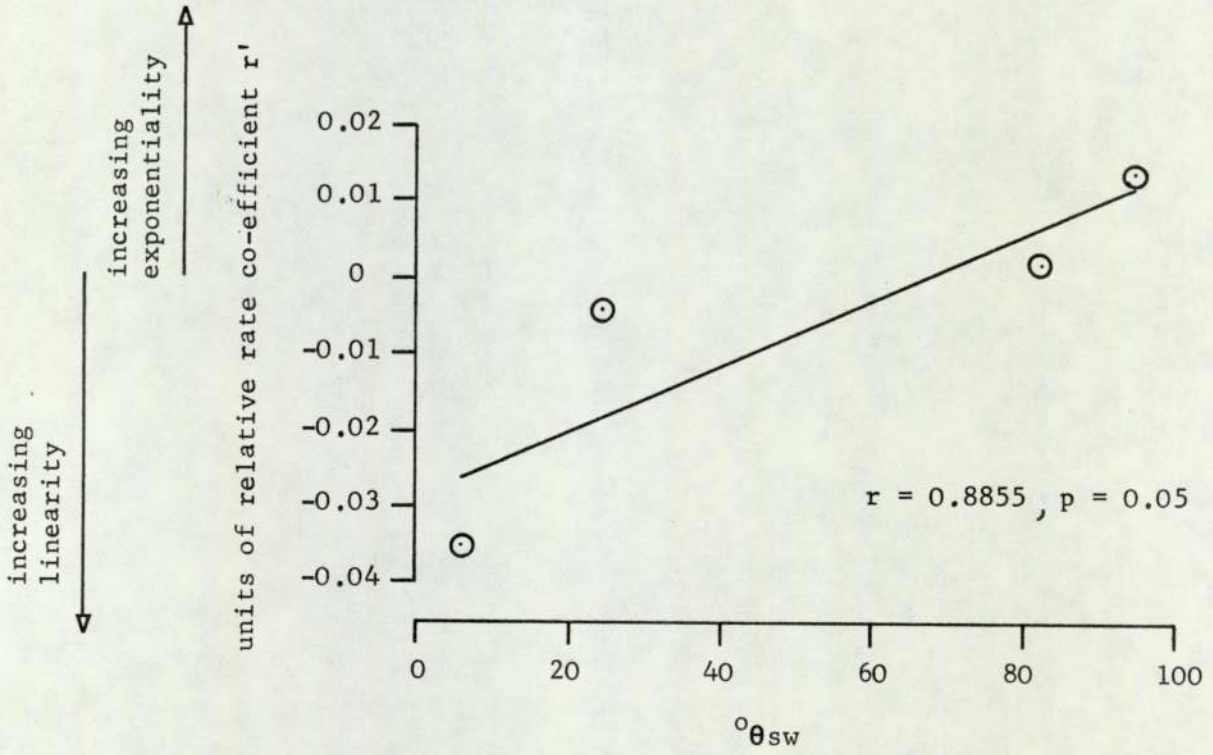



Fig 33 Relationship between relative rate and substrate hydrophobicity. Sampling period III (0-12 hrs.) winter

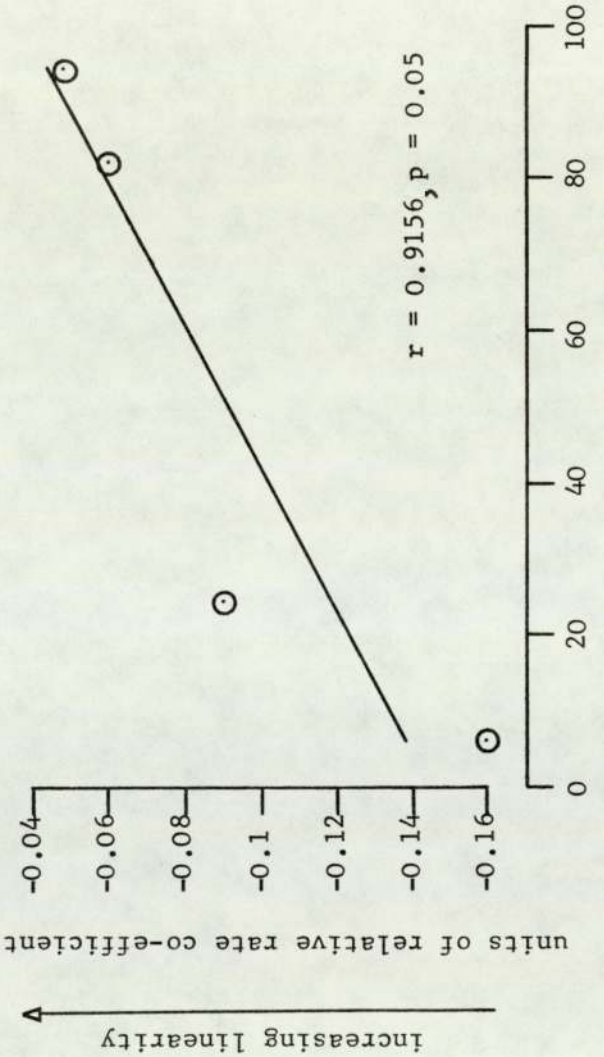



Fig. 34 Relationship between relative rate and substrate hydrophobicity. Sampling period III (0-12 hrs.) summer.

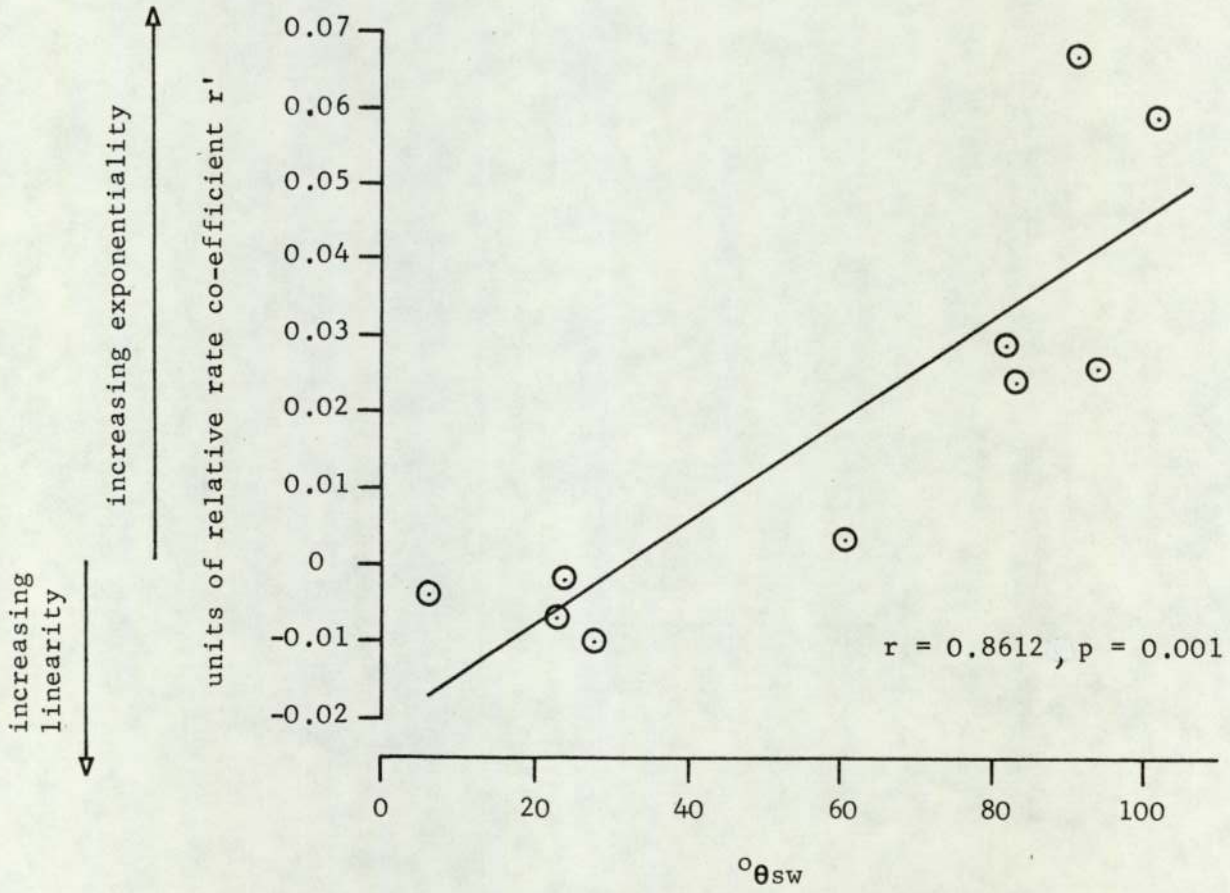



Fig. 35 Relationship between relative rate and substrate hydrophobicity. Sampling period II (0-10 hrs.) summer.

## 3.9 Distribution of Bacteria on Surfaces

3.9.1 The spatial dispersion of a population describes the attachment of its component individuals, which may conform to a specific type of distribution; it is a population characteristic. Change in the form of temporal or environmental factors will alter the dispersion of the population, which can be measured by the change in distribution of its individuals and provides an index to the reaction of the population. Two forms of distribution commonly occur when describing populations: the random distribution (Poisson) where individuals are arranged haphazardly without system, and the contagious (clumped) distribution, where groups of individuals are dispersed in specific patterns. The contagious distribution has various forms, depending on the arrangement of clumps. The most frequent types include the negative binomial (logarithmically dispersed groups), Poly-Aeppli (geometrically dispersed individuals within groups), and the Neyman type A (randomly dispersed groups) distributions.

The aim of this analysis is to describe the distributions of bacteria on substrates tested and to determine the effect of time on the spatial dispersion of the populations.

3.9.2 The distribution type was determined using the variance to mean ratio test (Elliott, 1971), and agreement with a Poisson series confirmed by $\chi^2$ test of observed and expected frequencies. Agreement with a contagious distribution and the identification of its exact form (negative binomial, Polya-Aeppli, or Neyman Type A) was determined from the observed and expected moments of the distribution, using the method of Evans (Elliott, 1971). Statistical calculations were made using a

computer programme provided by ICI Ltd. The departure of a population from randomness was determined using Taylor's Power Law (Elliott, 1971) and the parameter $b$, an index of dispersion, which varies from 0 for a regular distribution to infinity for a highly contagious distribution. It was calculated as the regression of log $s^2$ (variance) on log $\bar{x}$ (mean) for samples from an immersion series, where parameter $b$ was the slope of the rectilinear equation, and $a$ was the intercept.

3.9.3 For each substrate in the sampling series the frequency of occurrence of particular distribution types was determined (Figs.36 and 37). During short immersion periods in the Winter (0 - 1 hr, 0 - 12 hrs) the population dispersions were predominantly random, with a few instances of negative binomial distributions. During the 0 - 168 hour period the populations were characterised by random distributions with an increasing development of contagious distributions, mainly of the Neyman type A and Polya-Aeppli forms.

The short sampling period in the Summer (0 - 12 hrs) showed distributions which were predominantly random, but differed from the Winter (0 - 12 hrs) sampling period in that contagious distributions of the Neyman type A and Polya-Aeppli types occurred. In contrast to the long immersion period of the Winter sampling programme ( 0 - 168 hrs) where there was a high proportion of Poisson distributions, during the Summer, 0 - 168 hour period, Neyman type A and Polya-Aeppli contagious distributions were predominant.

The population dispersions described suggest that surface phenomena do not exert an influence on the spatial distribution of bacteria, demonstrated by the random distribution of attached cells during the first hours of an immersion period. Subsequently, there was a change in the population dispersion from a random to a contagious distribution with time.

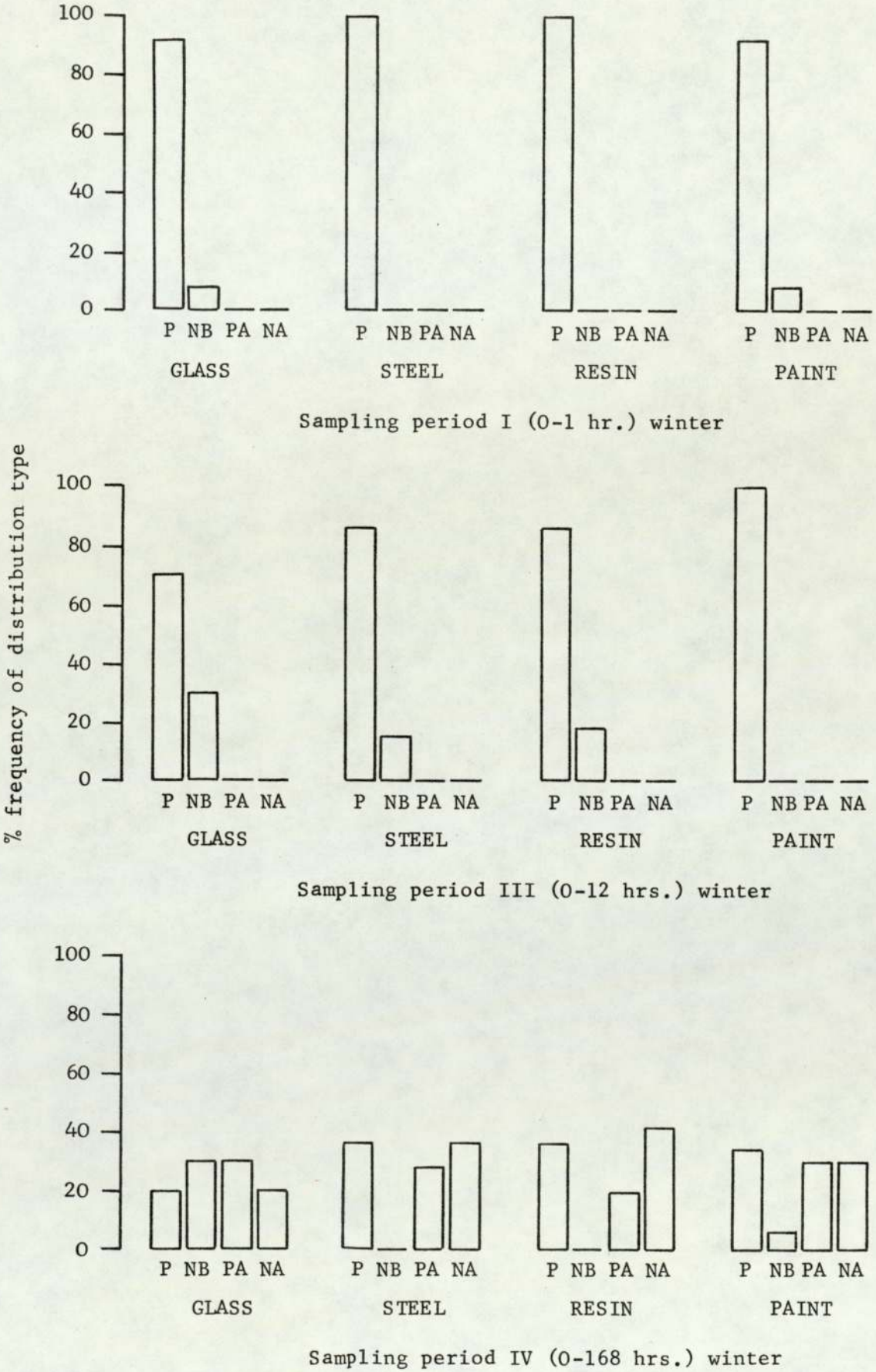



Fig. 36 Bacterial distribution types on substrates during winter sampling periods.

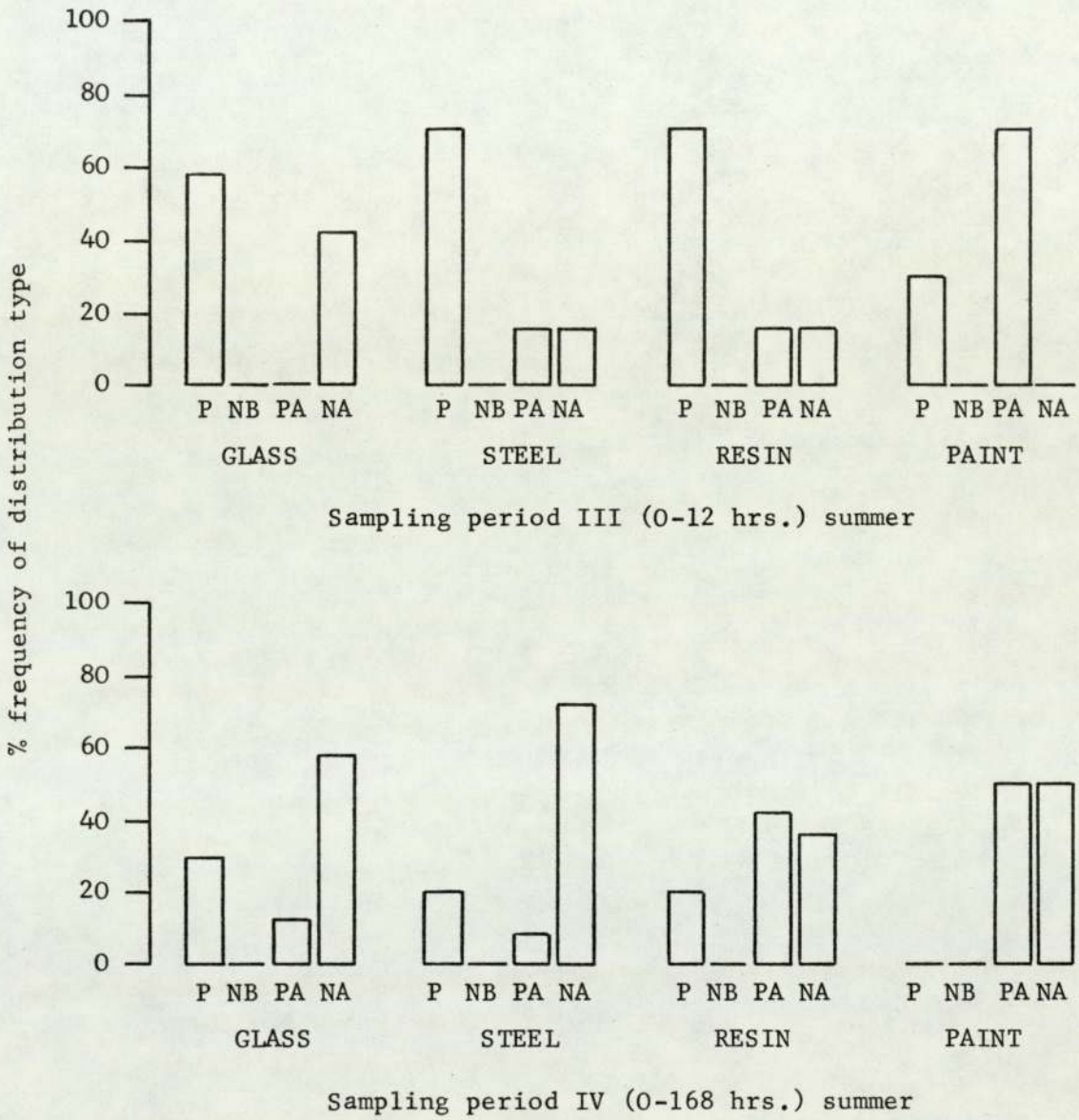


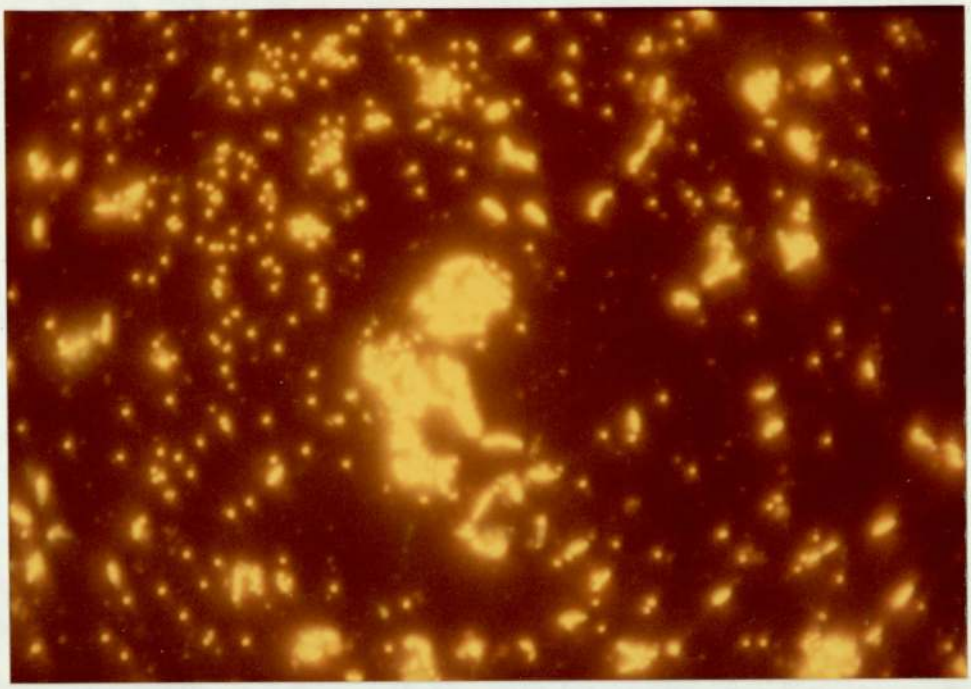

Fig. 37 Bacterial distribution types on substrates during summer sampling periods.

Key : P - poisson distribution
NB - negative binomial distribution
PA - polya-aeppli distrbution
NA - neyman type-A distribution

The occurrence of Neyman type A and Polya-Aeppli distributions indicate that the two processes may operate in microbial film development. It has been shown that most of the increase in cell density is the result of attached cells proliferating across the surface (Bott & Brock, 1970). A Polya-Aeppli distribution resulting from randomly attached cells dividing to form a nucleus of daughter cells, could describe this sequence of events (Fig.38). The Neyman type A format, however, describes randomly arranged clumps of bacteria, and it is suggested that this form of distribution may arise as a result of cells, attached during the early part of immersion, conditioning the area in their immediate vicinity by the production of expolymers, for example, so enhancing or accelerating the attachment process of subsequent cells (Fig.39).

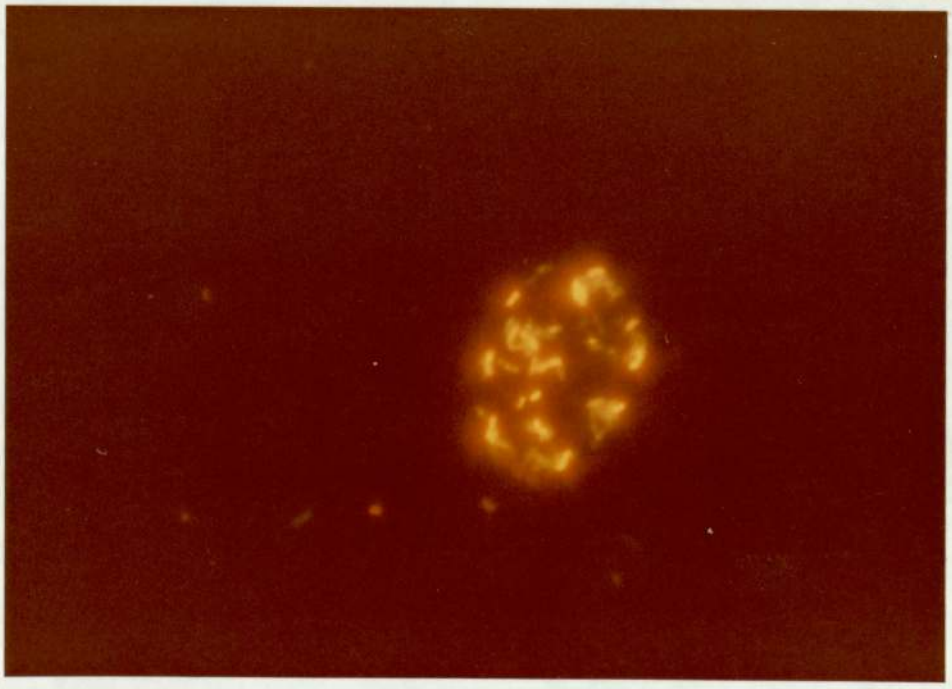
3.9.4 Changes with time in the spatial dispersion of attached microbial populations were determined using the parameter $b$ from Taylor's Power Law. For each immersion period during Summer and Winter sampling, it was found that the degree of clumping - and hence, contagiousness of the distribution - increased with proportion to time (Figs. 40 to 44). From the analysis of the distribution types, this trend is predictable, because Polya-Aeppli and Neyman type A distributions have greater frequencies of high counts (positive skewness) which results in greater variance to mean ratio ($s^2 > \bar{x}$), which increases the size of parameter $b$.

Except for the long immersion period (0 - 168 hrs) during the Summer, when there was a greater degree of clumping, no significant difference between the two seasons could be detected. This suggests that the rate of development of contagious distributions is relatively stable, and independent of environmental factors.



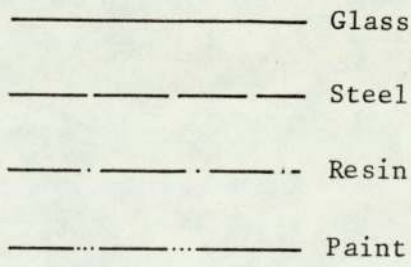
——— 10μm

Fig. 38 Micro-colony development by daughter cell division. Glass,156 hour immersion,summer.



——— 10μm

Fig. 39 Micro-colony development by localised attached cell conditioning.Glass,12 hour immersion,summer.




Substrates arranged in order of
decreasing wettability




Key for figs. 40 - 44

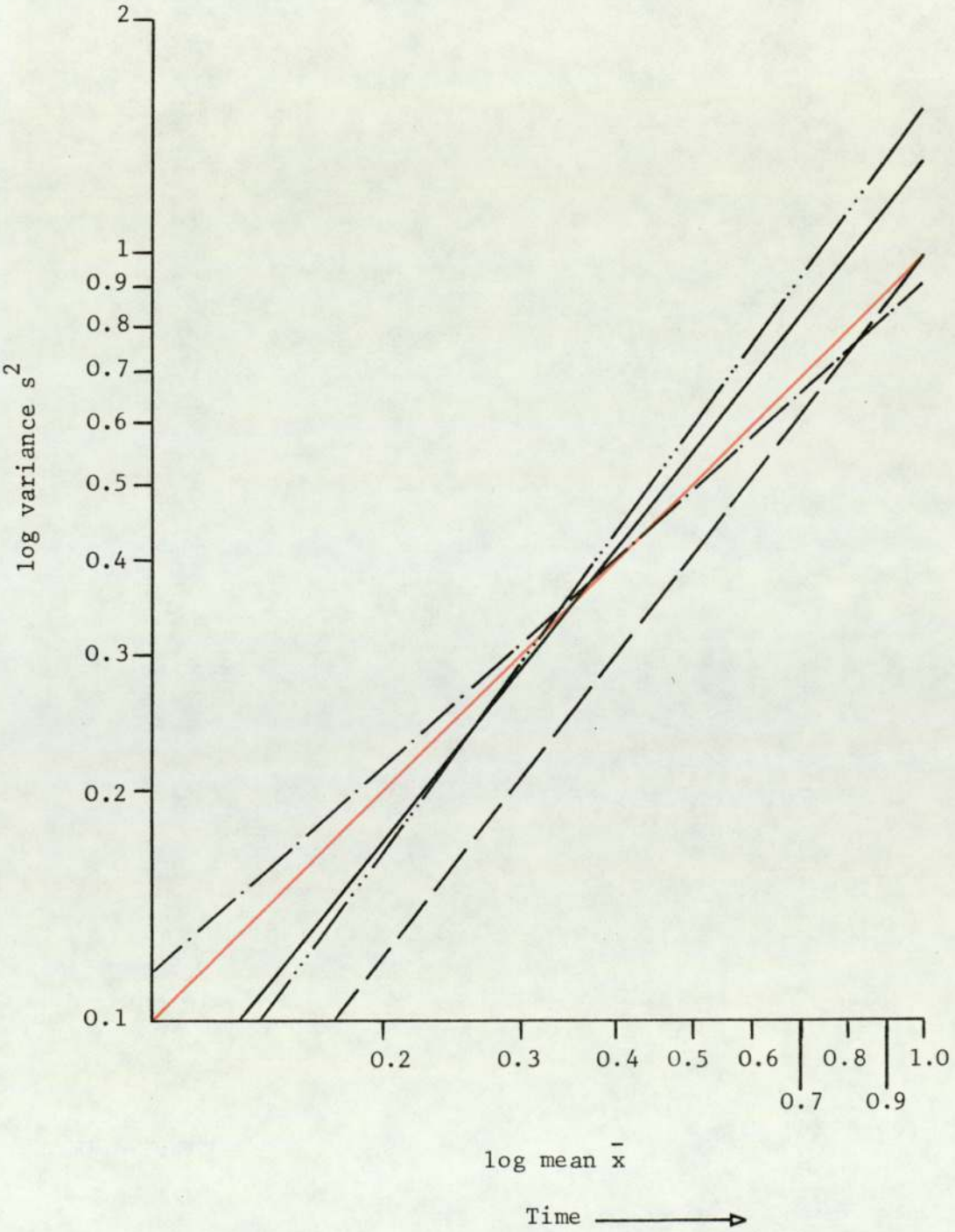



Fig. 40 Population departure from randomness during sampling period I (0-1hr.) winter.

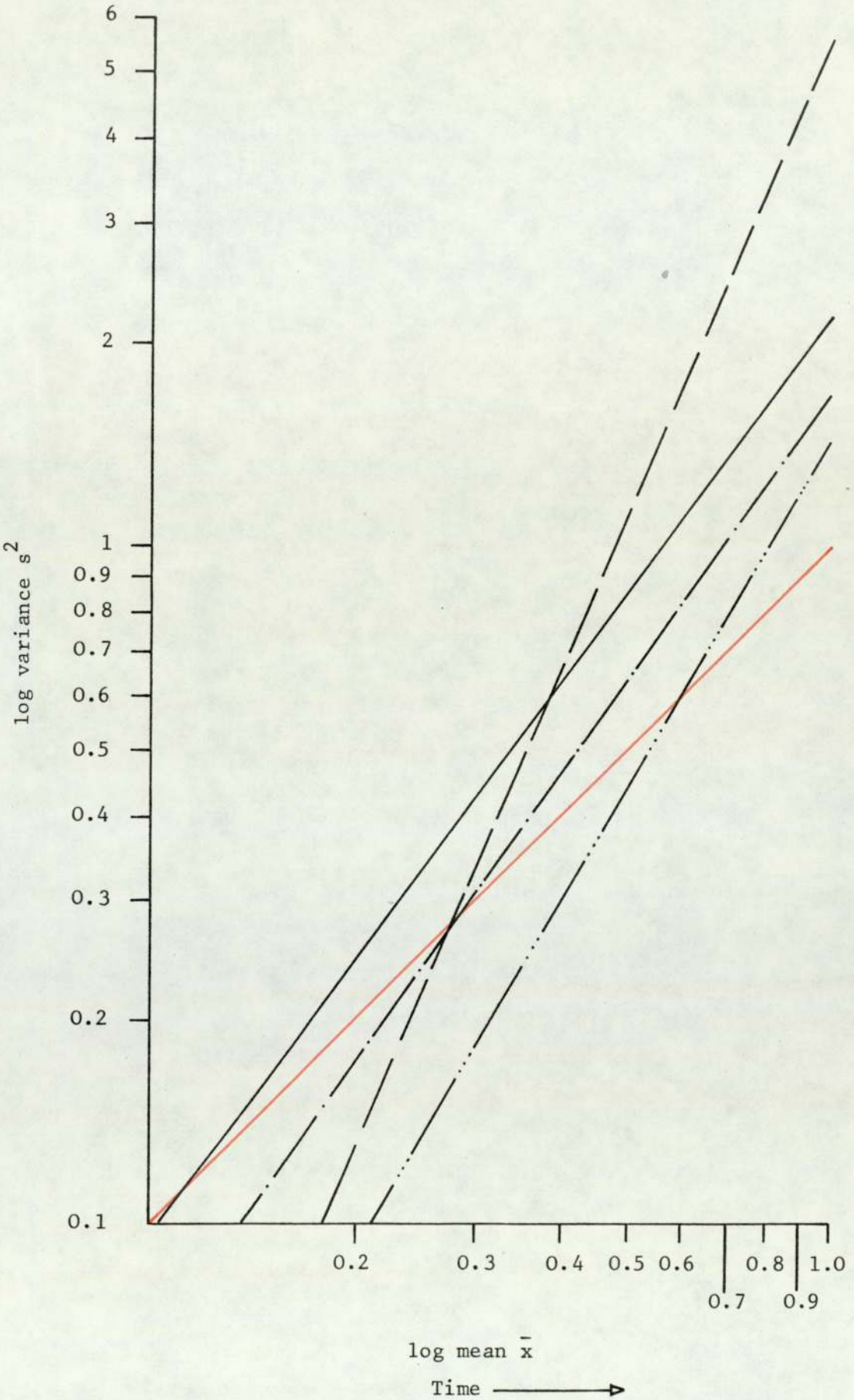



Fig. 41 Population departure from randomness during sampling period III (0-12 hrs.) winter.

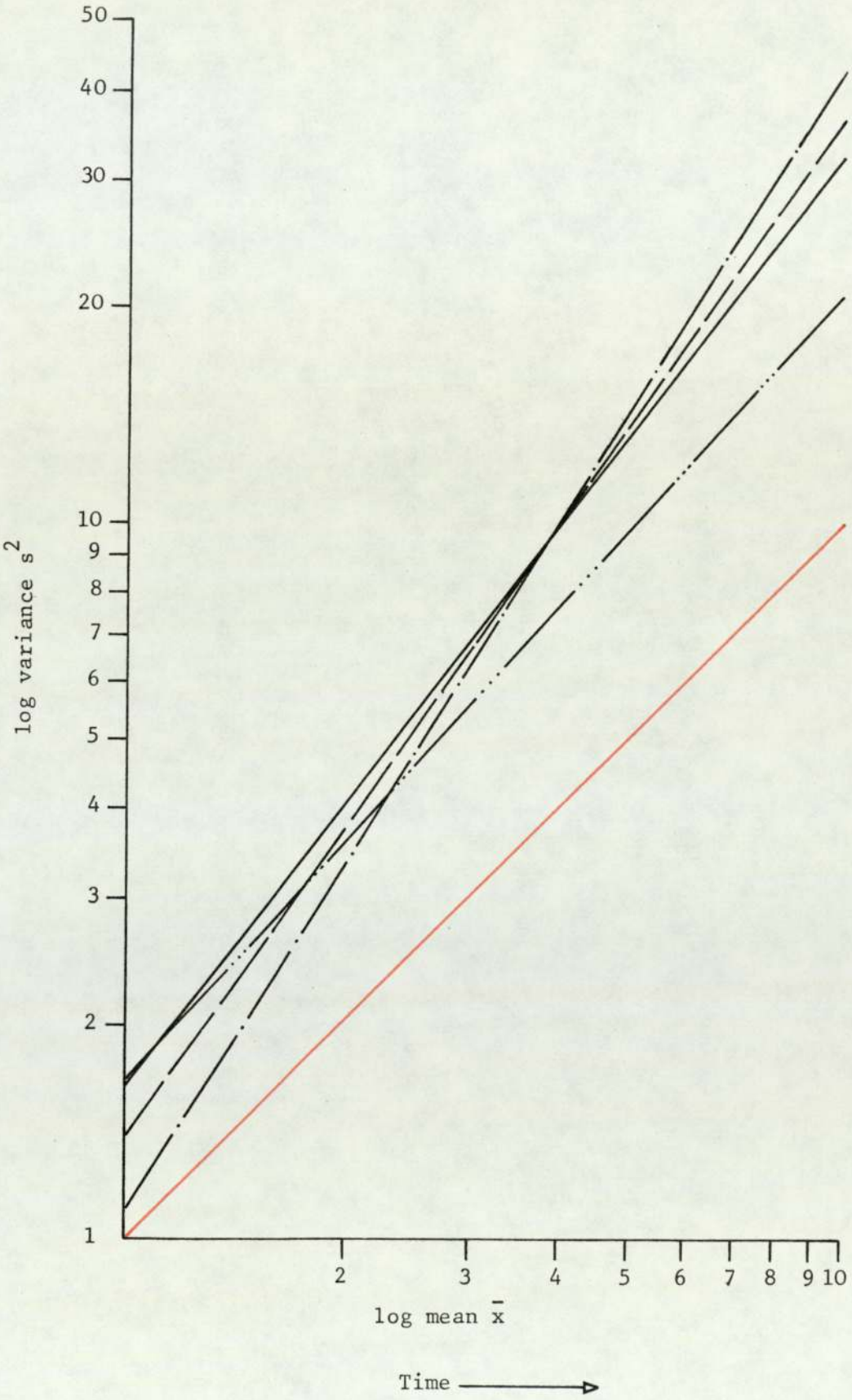



Fig. 42 Population departure from randomness during sampling period IV (0-168 hrs.) winter.

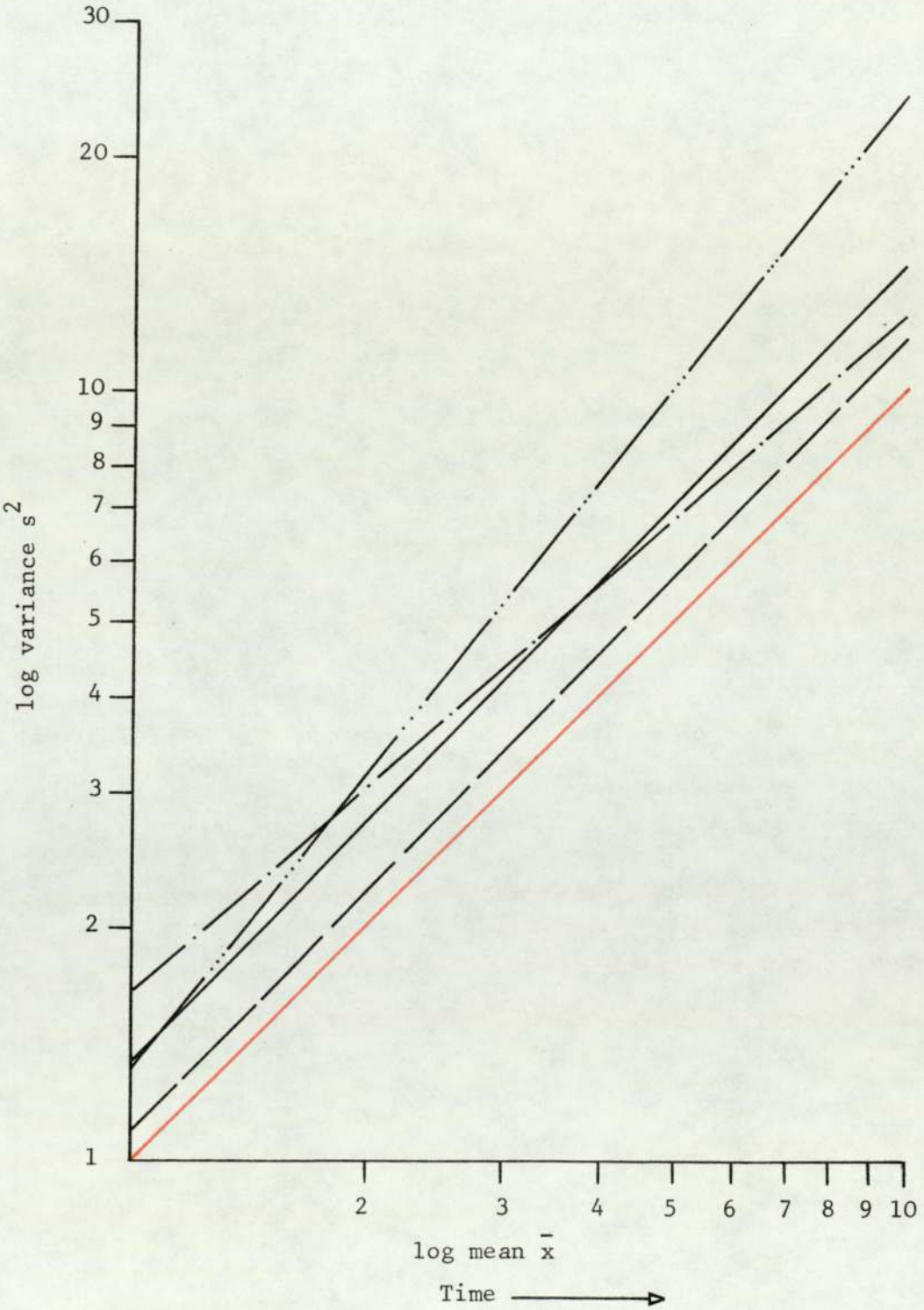



Fig. 43 Population departure from randomness during sampling period III (0-12 hrs.) summer.

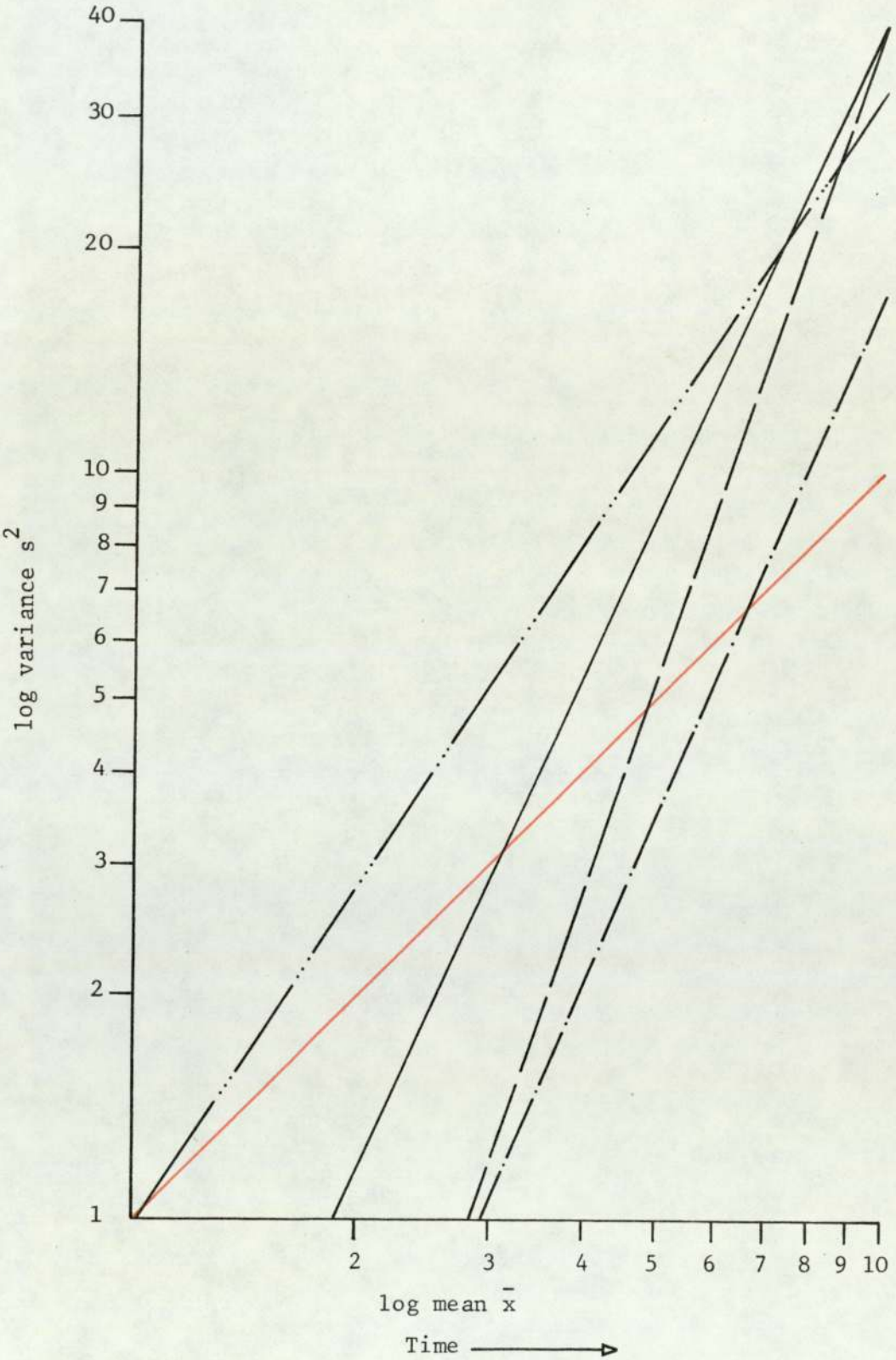



Fig. 44 Population departure from randomness during sampling period IV (0-168 hrs.) summer.

The relationship of distribution type to surface characteristics was examined by considering the variables of wetting ($\theta_{sw}$) and the index of clumping (contagiousness), parameter $b$. No correlation could be calculated ($r < p = 0.05$)[a], and it is likely that surface characteristics do not influence the spatial dispersion of attached populations.

a - Table VII, Fisher & Yates, 1963.

# CHAPTER 4

## THE ROLE OF FLAGELLA IN MICROBIAL ATTACHMENT

# THE ROLE OF FLAGELLA IN MICROBIAL ATTACHMENT

## 4.1 Introduction

4.1.1 Two major types of flagella arrangement are recognised: the single polar flagellum, and the peritrichous arrangement where numerous flagella are distributed over the soma. Two less common forms are the sub-polar monotrichous and lophotrichous positions. In the former case, a single flagellum is inserted at a sub-terminal locus, while the latter type has a number of flagella (between 2 - 6) inserted as a tuft at the polar position (Liefson, 1960). A different flagella arrangement was first reported by Houwink and van Iterson (1950), where both polar and peritrichous flagella were apparent on the one soma. It was Liefson (1963), however, who observed that this type of flagellation was a function of surfaces, i.e. some polarly flagellated bacteria (as determined by liquid culture) when grown on surfaces produced both polar and peritrichous flagellation on the one cell, and termed it "mixed flagellation". In keeping with the -trichous definitive for flagella arrangements, it is suggested that the term "poikilotrichous" (Gr. *pokilos* - varied) is used to describe the term 'mixed flagellation'. This formation is used as a taxonomic character for the genus *Beneckea* (Baumann, Baumann & Mandel, 1971). The two types of flagella are distinctly different. The polar flagellum is approximately 24 to 30 nm in diameter, has a discernible sheath (Allen & Baumann, 1971), and a long wavelength. The peritrichous flagella are narrower, only 14 to 15 nm in diameter, and have a shorter wavelength, giving them a "curly appearance".(Houwink & van Iterson, 1950). In addition, the protein units of flagellin for both types of flagella have for *Beneckea parahaemolytica* been shown to

be of two different types (Baumann & Baumann, 1977), indicating that their initiation may be regulated by different external stimuli.

4.1.2 The significance of bacteria switching flagellation patterns induced by surfaces is not clear, but the most likely function is to mediate in an attachment process (de Boer, Golten & Scheffers, 1975). The sequence of events in the attachment model (Marshall *et al.*, 1971) begins with the random event of a bacterium entering the immediate vicinity of a surface. The cell is attracted to the surface by van der Waal's forces, but fails to make contact as it is repulsed by a double-ion layer. It assumes a position very close to the surface, held at a point where the force of the cell/surface interaction is in equilibrium by the repulsion of the double-ion layer. Extra cellular polymeric fibrils are then synthesized by the loosely captive cells, and these bind it very firmly to the surface. It was suggested by de Boer *et al.* (1975) that a polarly flagellated cell, held at the double-ion interface but unable to synthesize extra cellular polymers, switches its flagellation pattern and becomes anchored to the surface by numerous peritrichous flagella. The advantages of adhering to a surface have not been fully resolved, but the most promising argument is that the surface is a site of nutrient accumulation in an environment of extremely low nutrient concentration (ZoBell, 1943).

4.1.3 The aim of this Section is to determine the frequency of mixed flagellation in relation to the hypothesis of de Boer *et al.* (1975) using bacteria previously isolated from substrates during the temporal sampling series (Section 5.2.1). In addition, the frequency would be related to the succession and development of the microbial film on the

substrates, glass, steel, marine glass-fibre resin, and antifouling base paint. These substrates also represent a range of surface energies (an expression of wettability) which has been shown to influence the dynamics of bacterial attachment (Carson & Allsopp, 1978). The influence of surface energy on mixed flagellation frequency would also be examined.

## 4.2 Materials and Method

### 4.2.1 Source of Bacteria

Bacteria were obtained from the 'core' substrates, glass, steel, resin, and paint during a Winter sampling programme. Two immersion periods were used; the first was 12 hours long, and samples were removed at 4 and 8 hour intervals, while during the second period, 168 hours long, samples were taken at 12 hour intervals.

The substrates were rinsed (Section 2.3) and placed in Johnson's marine broth (see Appendix II), as described in Section 2.4, and incubated for 14 days at 15$^{o}$C (Floodgate, 1964). After incubation, the cultures were plated out onto Johnson's marine agar (JMA) to give discrete colonies. Isolates were chosen using a numbered grid and random tables, checked for purity, and stored on slopes of JMA at 4$^{o}$C.

From a total of 380 isolates, 93 were chosen (see Appendix IV) on the criteria of colony size, morphology, and texture, so that a cross-section embracing the extremes of population variance was obtained for each substrate during the immersion periods. The isolates were taken to represent the succession of bacteria on the substrates. Sixty-nine of the isolates were motile, and were used in this study.

The isolates were coded so that the time of immersion and substrate could be readily identified, and a typical code, such as 212SB2, indicates

that the sample was taken on the second day of December (212), at the second sampling period of the day (B), and it was a steel substrate (S); the remaining digit identifies the number of the isolate (1-6) for that particular sampling time, 212SB.

### 4.2.2 Culture Methods

A marine peptone yeast agar (MPYA) and broth (MPYB) (see Appendix II) were used for maintaining and growing the cultures. When the medium was used to prepare cultures for electron microscopy, the complex ionic nature of seawater rendered specimens for examination useless. As the bacteria were halophiles, the seawater could not be removed without replacing it with a source of sodium ions. A satisfactory solution was achieved by substituting sodium chloride (1.5% w/v) for seawater in the peptone-yeast extract medium. For studying flagellation in the liquid phase, cultures were grown in MPYB, and for the solid phase MPYA slopes were used. Both types of culture were incubated at 15$^{o}$C for five days.

### 4.2.3 Preparation of Electron Microscopy Grids

"Maxtaform" (Emscope Ltd.) 300 mesh copper grids with 40% transmission were used to support specimens. The grids were plastic - coated with polyvinyl formvar by a modified method of Drummond (1950). The modification was to prepare grids for nitrocellulose coating, but use a 2% w/v solution of formvar in 50/50 (% v/v) chloroform/ethylene dichloride.

### 4.2.4 Preparation of Specimens

Solid phase cultures were suspended by pipetting MPYB onto agar slants (Houwink & van Iterson, 1950). The suspension and liquid phase cultures were then checked for motility by phase contrast microscopy.

Grid specimens were prepared by adding a drop of liquid culture or suspension to a grid, removing the excess, and allowing to air dry. The bacteria were negatively stained using phosphotungstic acid prepared as a 1% w/v solution of phosphotungstic acid in distilled water, neutrailized with 1 M KOH to pH 7.1 to form potassium dodeca phosphotungstic acid (KPTA). The grid was dipped in KPTA, the excess removed, and allowed to air dry. Using this procedure, crystals of sodium chloride were dissolved, leaving a clean specimen. The grid was examined by phase contrast microscopy to ensure a low density and even distribution of bacteria on the grid (approx. 15 cells/grid square).

### 4.2.5 Electron Microscopy

Two transmission electron microscopes were used, an AEI E6MB, and a JEOL 100B. The AEI was used predominantly and operated at an accelerating voltage of 50 KV. The JEOL was used for high magnification examinations and at an accelerating voltage of 80 KV. The photographs were taken using Kodak Electron Microscope film 4489. Dimensions were calculated using latex beads of two known sizes, 0.109 µm and 0.312 µm (E.Fulham Inc., U.S.A.).

## 4.3 Results

4.3.1 The bacteria were grown in the liquid and on the solid phase, and the flagella arrangement examined by electron microscopy. In Table 15 and Fig.45, the frequency of each type of flagellation (expressed as a percentage of the total) for the liquid and solid phase and for each type of substrate is shown.

TABLE 15 : Percent flagellation types on solid and in liquid phases of bacteria isolated from glass, steel, resin, and paint surfaces.

| Flagellation (%) | Glass | | Steel | | Resin | | Paint | |
|---|---|---|---|---|---|---|---|---|
| | Liquid | Solid | Liquid | Solid | Liquid | Solid | Liquid | Solid |
| Monotrichous | 90.48 | 57.14 | 88.24 | 76.47 | 93.33 | 53.33 | 81.25 | 75.0 |
| Sub-polar monotrichous | 4,76 | 4.76 | 11.76 | 5.88 | - | - | 12.50 | 12.5 |
| Lophotrichous | - | 4.76 | - | - | - | - | - | - |
| Peritrichous | 4.76 | 9.52 | - | 5.88 | 6.67 | 26.67 | 6.25 | 12.5 |
| Poikilotrichous | - | 23.81 | - | 11.77 | - | 20.00 | - | - |

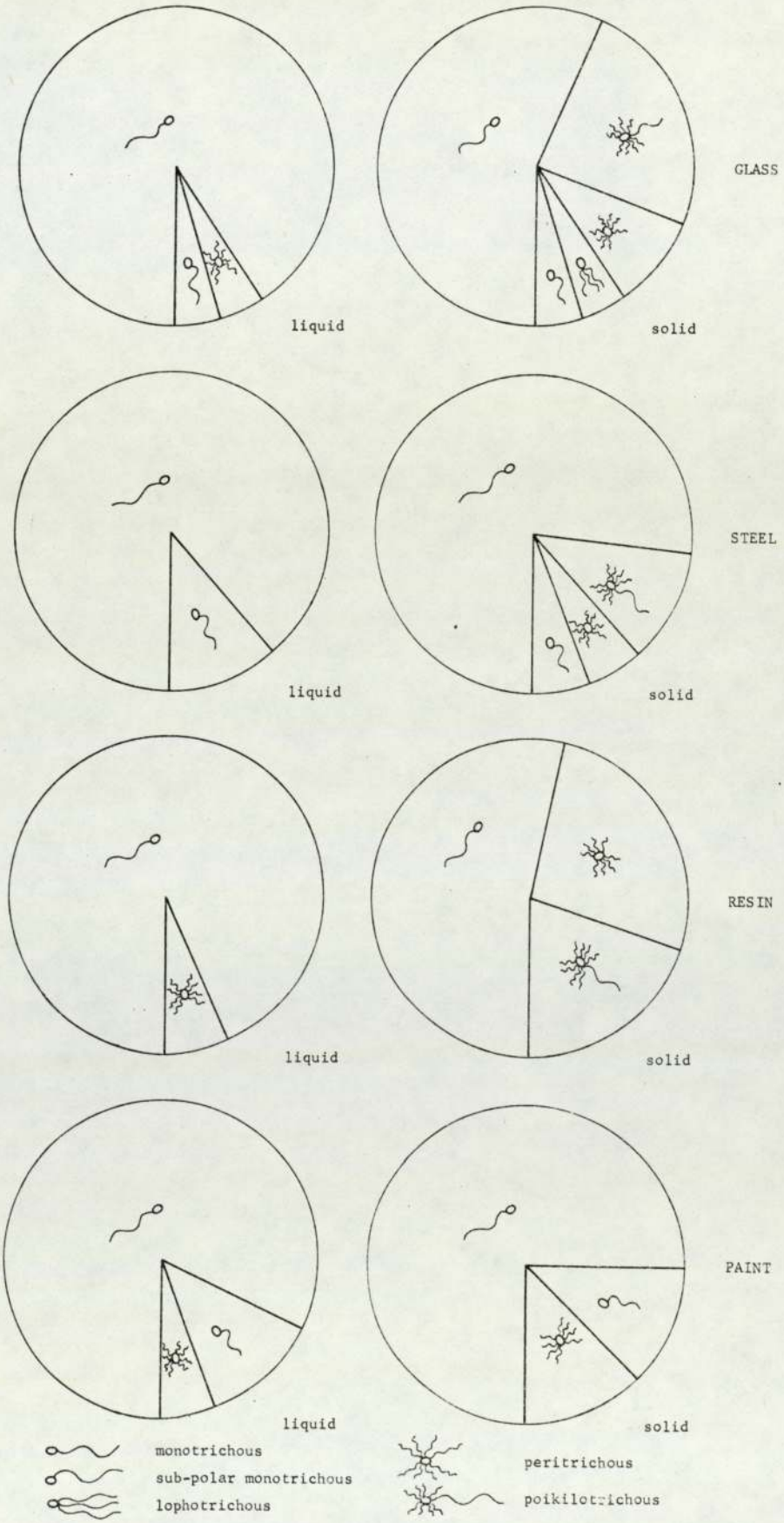
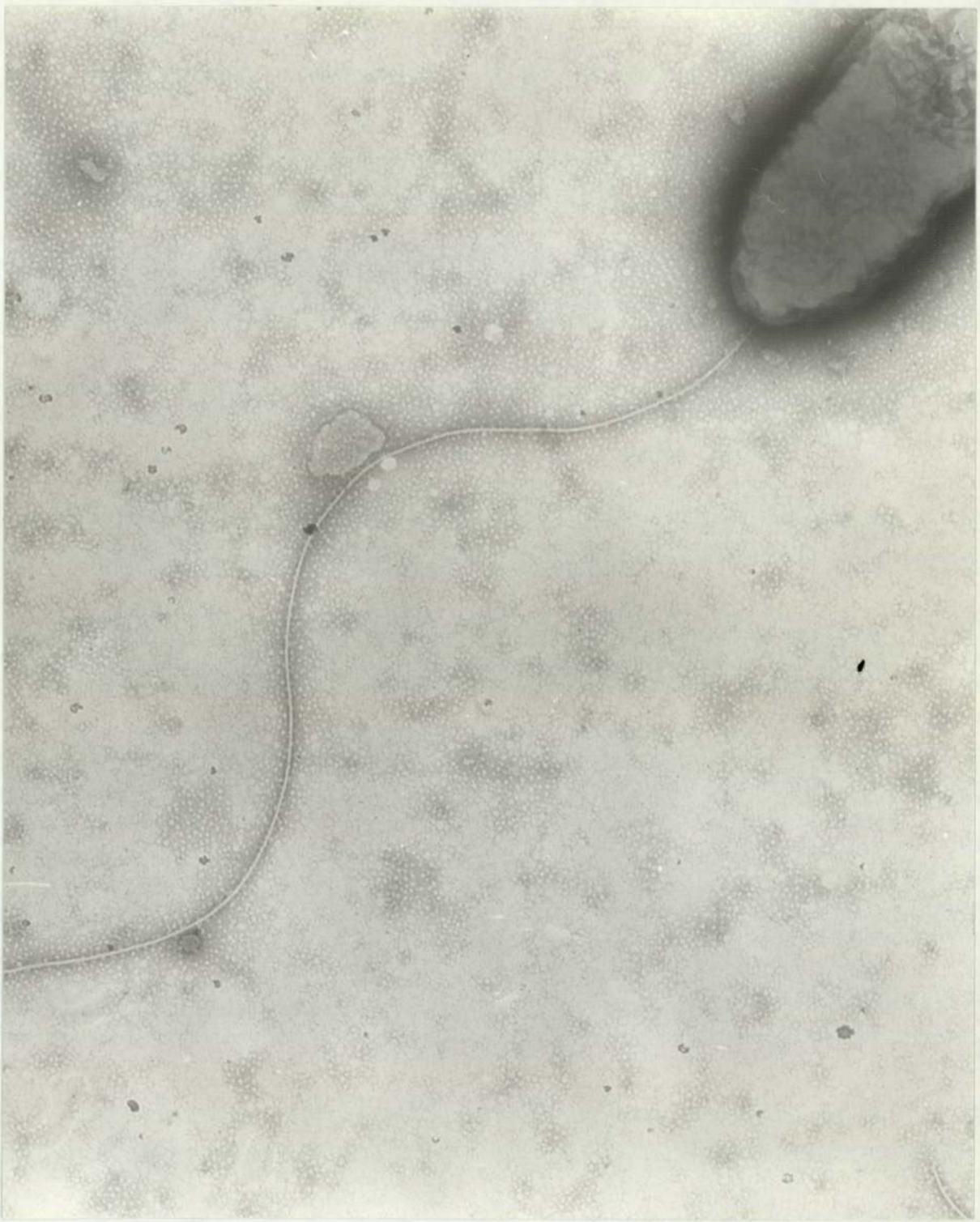



Fig. 45 Frequency of flagellation types in the liquid and on the solid phase for isolates from the substrates glass, steel, resin and paint.

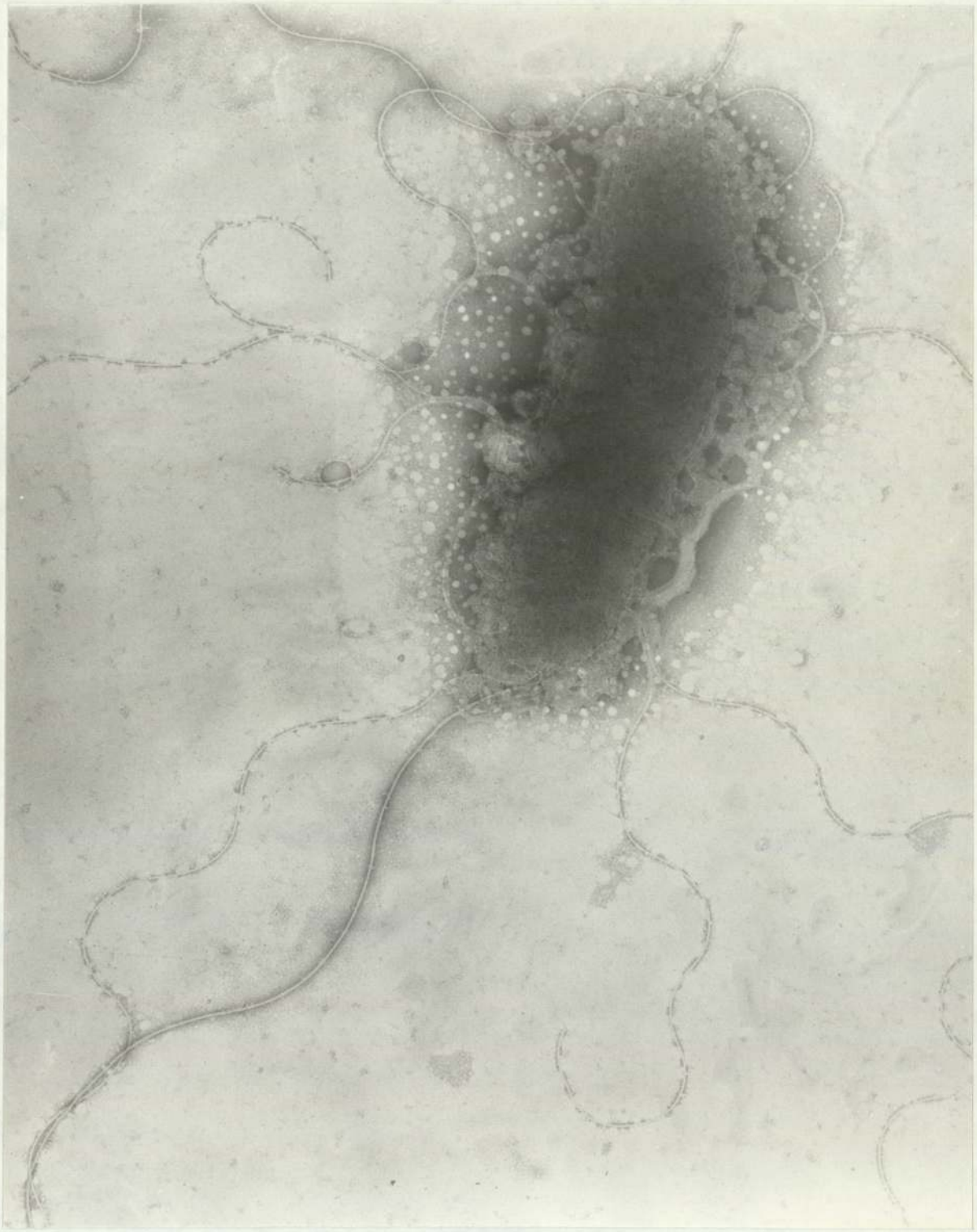
A degree of uniformity in flagellation was observed when the isolates were grown in the liquid phase; namely, a high proportion were of the polar variety (Fig.46) and, of this type, 88.57% were monotrichous, and 7.14% sub-polar monotrichous. The remaining 4.29% were peritrichous. The solid phase had a marked effect on flagellation and there was a change in types of 24.29%. Most of these were from polar to poikilotrichous flagellation (14.29%) and were identified by the differential wavelengths of the polar and lateral flagella (de Boer *et al.*, 1975). Organism 1512RA5 for example had a polar wavelength of 1.49 μm and a lateral wavelength of 0.69 μm (Fig.47). No measurable difference in diameter or structure could be detected between the polar and lateral flagella. The measured diameter of 15.6 nm agrees with that found by de Boer *et al.* (1975) and Allen & Baumann (1971) for an unsheathed flagellum, although they and Houwink and van Iterson (1950) all report that the polar flagellum is normally sheathed and has a diameter of 25 nm. Some switched flagellation was to the peritrichous arrangement (12.86%) where no distinction could be made between diameter or wavelength of any of the flagella (Fig.48). In one instance, a lophotrichous arrangement (Fig.49) was found when the isolate 3011G4 was grown on the solid phase. This arrangement was not consistent for all the cells examined, and a small proportion had a single polar flagellum.

4.3.2 The surface energies (a measure of surface wettability) of the four substrates were measured by previous experimentation. The substrates, ordered in decreasing wettability as determined by the droplet surface contact angle with respect to seawater, were found to be: glass ($6^{o}$), steel ($24.4^{o}$), resin ($83.3^{o}$), and paint ($94.5^{o}$). The change in flagellation with respect to each substrate was: glass,



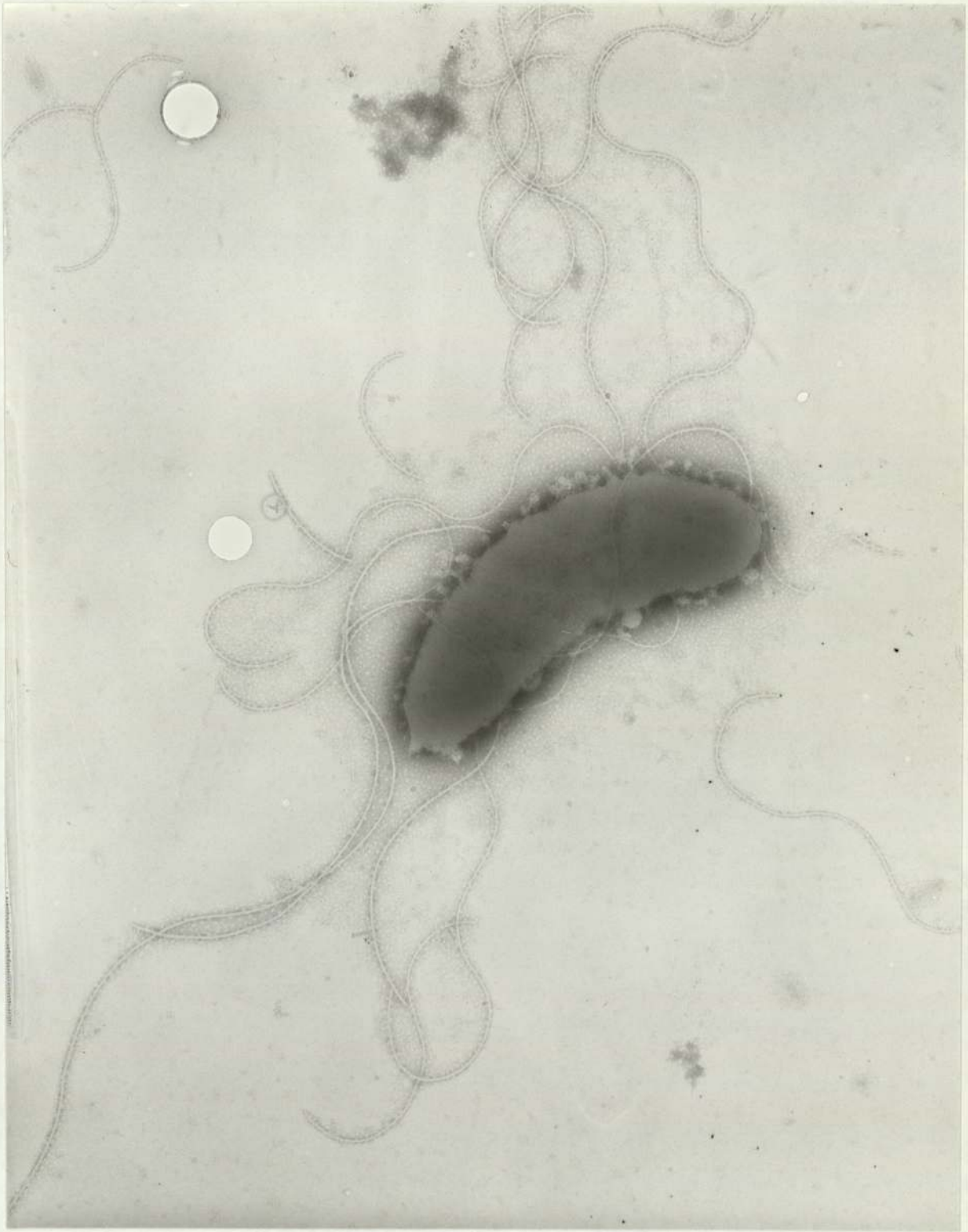
1μm

Fig. 46 Organism 1512RA5 grown in the liquid phase. Monotrichous flagellation.



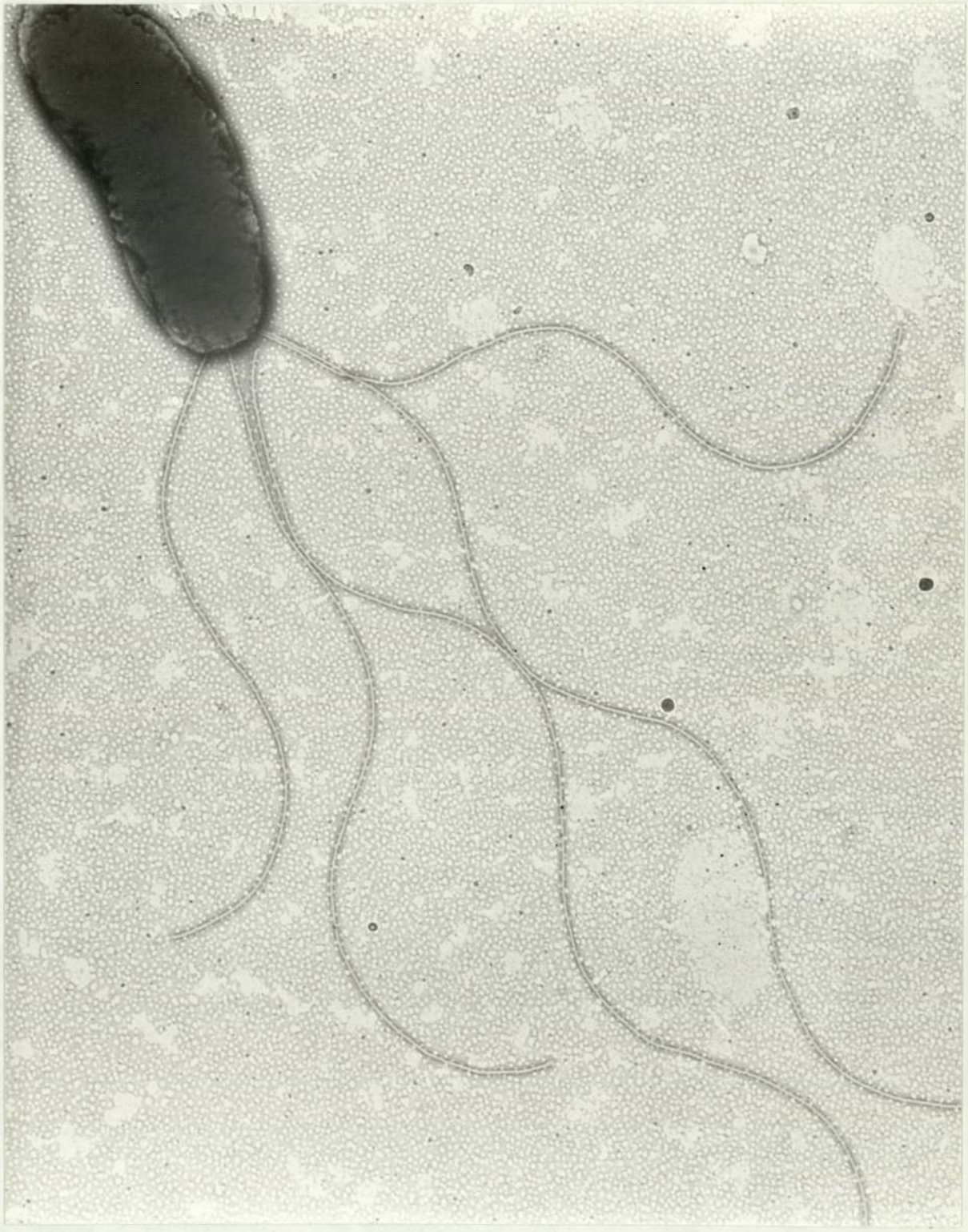
1μm

Fig. 47 Organism 1512RA5 grown on the solid phase.
Poikilotrichous flagellation.



1μm

Fig. 48 Organism 912PA1 grown on the solid phase. Peritrichous flagellation.



1μm

Fig. 49 Organism 3011S1 grown on the solid phase. Lophotrichous flagellation.

33.33%; steel, 17.6%; resin, 40%; paint, 6.25%. No correlation between frequency of flagella switching and substrate could be seen. However, it is possible that organisms isolated from the resin may have been unrepresentative and the results from the remaining substrates, which show a strong relationship between flagella switching and surface energy, go some way to supporting this view. It will require the use of a wider range of substrates before any firm conclusions can be drawn.

4.3.3 The isolates for this study were obtained from a temporal sampling series and represent the development and succession of bacteria in the microbial film. For each substrate, the percentage change of flagellation between liquid and solid phase was determined at each sample interval up to 156 hours of immersion. The results are summarised in Table 16. No significant co-relation ($p > 0.05$) was found for the data from steel, resin, and paint substrates, but a good correlation was found for the glass data where $r = -0.9449$ and is the correlation coefficient for a recti-linear regression, where $p < 0.001$. This data is plotted in Fig.50.

TABLE 16 : Percentage change of flagellation type on solid and in liquid phases of bacteria isolated at successive time intervals.

| Hours | Change (%) | | | |
|---|---|---|---|---|
| | Glass | Steel | Resin | Paint |
| 12 | 9.52 | 5.88 | 6,67 | 6.25 |
| 36 | 9.52 | 0 | 6.67 | 0 |
| 60 | 4.76 | 5.88 | 0 | 0 |
| 84 | 4.76 | 0 | 13.33 | 0 |
| 108 | 4.76 | 5.88 | 6.67 | 0 |
| 132 | 0 | 0 | 0 | 0 |
| 156 | 0 | 0 | 0 | 0 |

A degree of consistency was observed for the last 48 hours of immersion where no switching of flagellation was detected.

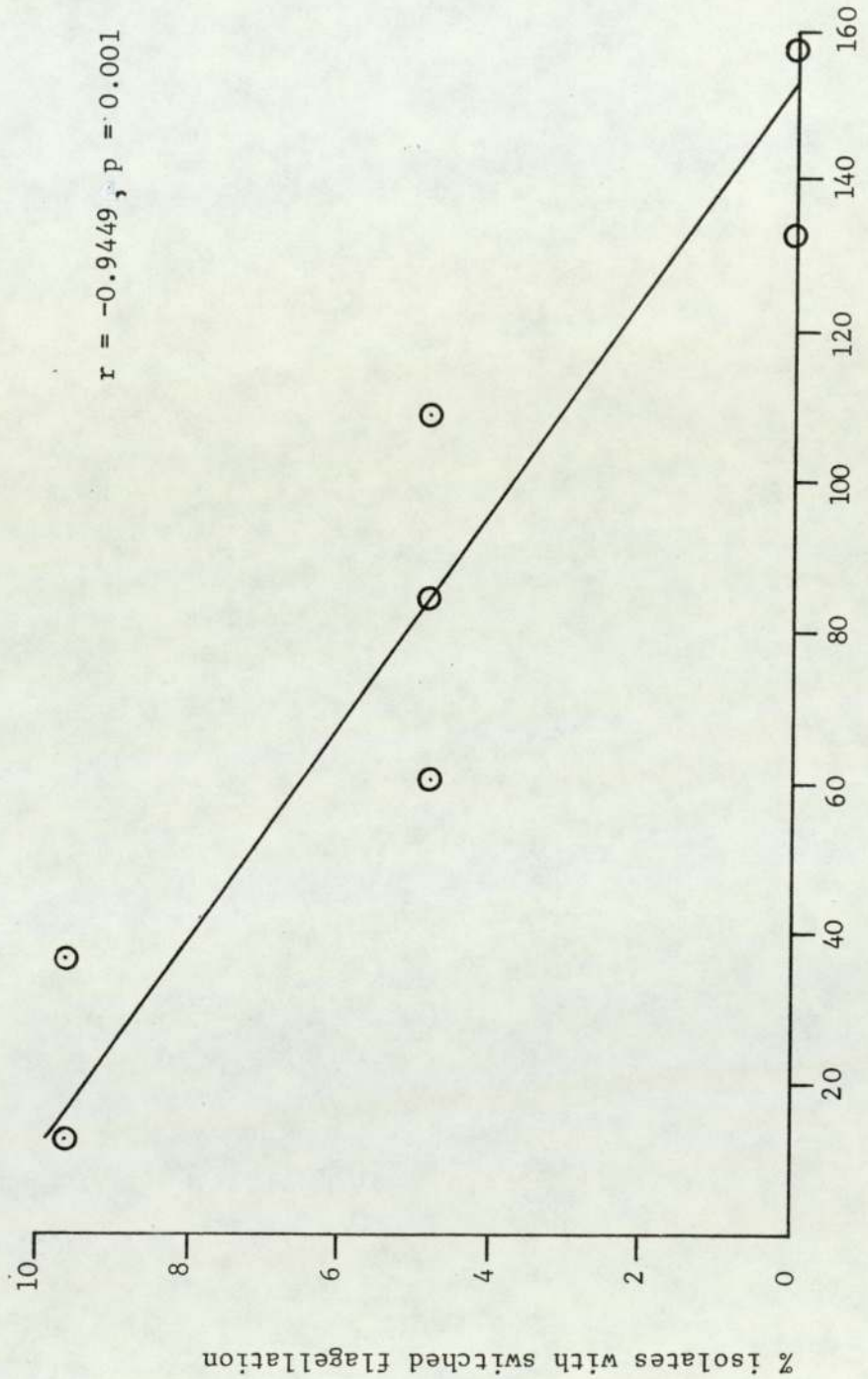



Fig. 50 Graph of decrease in switched flagellation frequency with time.
Substrate : glass.

## 4.4 Conclusions

4.4.1 A high proportion of bacteria (nearly 25%) had a form of switched flagellation, the predominant form being the single polar flagellum with a wavelength of 1.49 μm and a contrasting lateral flagellum with a shorter wavelength of 0.69 μm.

4.4.2 The frequency of flagella switching appears to be influenced by substrate surface energy such that, with decreasing wettability, there is a reduction in frequency.

4.4.3 The occurrence of flagella switching reduced with increasing time of immersion for at least a high surface energy (wettable) substrate. No relationship could be detected for the other substrates. The occurrence of switched flagellation bacteria appears to be limited to the early period of immersion.

## 4.5 Discussion

4.5.1 The switching frequency from polar to mixed flagellation of 24.29% found for isolates from the microbial film, indicates the significance of this process when compared with the frequency of 17.5% for isolates from the intestines of various marine animals, and only 1.71% for isolates from the open sea (Liefson, 1963). The majority of switched flagellation was to the poikilotrichous type, identified by the longer wavelength of the polar flagellum but, in contrast, some peritrichous and monotrichous forms were also found. All the reported forms of switched flagellation have, however, been of the one type, namely poikilotrichous, based on the one group of organisms from the

overlapping genera *Vibrio* and *Beneckea*. Also, Houwink and van Iterson (1950) and Liefson, Cosenza, Murchelano and Cleverdon (1964) report that the switched forms of unidentified isolates were to the poikilotrichous type. The dichotomy which exists is not unreasonable in presuming that other multiflagellate forms may exist, as it is the abundance of flagella to anchor the cell which is the important factor. The significance of the sheath on the polar flagellum is not apparent, and was not detected on any of the isolates studied. It is possible that the sheath is a feature of the *Vibrio/Beneckea* group, and not one of poikilotrichous flagellation. As it is not a consistent feature (Allen & Baumann, 1971) and would not be detected using flagella stains and light microscopy, the important distinction between peritrichous and poikilotrichous flagellation should be made on the differential wavelengths of the polar and lateral flagella.

4.5.2 In assessing the importance of flagella switching in microbial film development, some consideration was given to the effect of surface energy (wettability) on selecting organisms able to switch flagellation patterns. If the results of the substrates: glass, steel, and paint are considered, a good correlation was found between switching frequency and wettability, such that, with decreasing wettability (glass → paint), there was a decrease in frequency. However, the results obtained from the resin substrate show an extremely high switching frequency which negates the relationship, but it is likely that the bacteria from this substrate were unrepresentative. The effect of surface energy has also been shown to affect attachment rates and cell density, so that, with decreasing wettability, there is an increase

in rate and density (Carson & Allsopp, 1978). The factor or factors which surface energy influences is not clear, but it may be the rate or nature of organic adsorption to the substrate. Nevertheless, there is a good indication to suppose that with increasing substrate wettability a greater proportion of bacteria, able to switch flagellation type, will be selected.

4.5.3 It is during the initial stages of immersion of a substrate that the greatest changes take place, both physically and biologically. In the first twelve hours, there is a rapid increase in cell density and rate of attachment (Dexter *et al.*, 1975), and there is a change in the physical properties of the surface due to organic adsorption (Loeb & Niehof, 1977). The distribution of switched flagellation bacteria was examined for the 156-hour immersion period, and a strong correlation between length of immersion and switching frequency was found for the glass substrate. The relationship describes the reduction of switching frequency with time, indicating that during the initial stage of immersion, bacteria with switched flagellation were most numerous. For none of the other substrates could such a relationship be found, but it was observed that no bacteria with switched flagellation were found once the film had become established. The significance of this result is hard to determine, but it is possible that bacteria with switched flagellation can successfully colonise high surface energy (wettable) surfaces during the initial stages of immersion, while other potential groups of bacteria which adhere by polymer bridging cannot do so.

4.5.4 Although the data establishes the frequency of switched flagellation of isolates from microbial films and assesses its relevance

in microbial film development, it does not establish whether bacteria with poikilotrichous flagellation are able to anchor themselves to surfaces in the manner suggested by de Boer *et al.*, (1975). Further experimentation is required which will, first, enable direct observation to be made on the attachment of cells using flagella, and second, provide information on the frequency of flagella switching using a wider range of substrates.

# CHAPTER 5

## IDENTIFICATION OF ATTACHED BACTERIA

# IDENTIFICATION OF ATTACHED BACTERIA

## 5.1 Introduction

It has been argued (Park, 1977) that the identification of individuals is an irrelevant contribution to an ecological study and that a more apposite description of microbial processes in the ecosystem should be based on the functionalism of the population. Undoubtedly this latter form of analysis will define in precise terms the role of the microbial population, but identification of individuals provides not only recognizable points of reference to an ecological survey, but also a broad and rapid assessment of population function, indicated by genus descriptions based on morphological, biochemical, and physiological similarities. Identification and functionalism should not be regarded as mutually exclusive terms.

The identification of bacteria attached to surfaces has been limited to isolates obtained from glass (Floodgate, 1971; O'Neil & Wilcox, 1971; Corpe, 1973). The range of genera (Table 17) is typical of a seawater flora, and may be a reflection of the techniques used in obtaining isolates from surfaces (see Section 2.3).

The aim of this section was to identify, using determinative keys for marine bacteria, representatives of populations from developing microbial films on various types of substrates. The range of organisms on each substrate and the effect of time on microbial film composition was also assessed.

TABLE 17 : List of genera isolated from surfaces by other workers.

| Floodgate, 1971 | O'Neil & Wilcox, 1971 | Corpe, 1973 |
|---|---|---|
| *Pseudomonas* | *Pseudomonas* | *Pseudomonas* |
| *Vibrio* | *Vibrio* | *Achromobacter* |
| *Spirillum* | *Achromobacter* | *Flavobacterium* |
| *Arthrobacter* | *Flavobacterium* | *Caulobacter* |
| *Bacillus* | *Bacillus* | *Hyphobacterium* |
| *Sarcina* | *Micrococcus* | |
| *Corynebacterium* | | |
| *Caulobacter* | | |

## 5.2 Materials and Method

### 5.2.1 Source of bacteria

Bacteria were obtained from the 'core' substrates: glass, steel, resin, and paint, during a winter sampling programme. Two immersion periods were used; the first was 12 hours long, and samples were removed at 4 and 8 hour intervals, while during the second period, 168 hours long, samples were taken at 12 hour intervals.

The substrates were rinsed (Section 2.3) and placed in Johnson's marine broth (Appendix II), as described in Section 2.4, and incubated for 14 days at 15°C (Floodgate, 1964). After incubation, the cultures were plated out onto Johnson's marine agar (JMA) to give discrete colonies. Isolates were chosen using a numbered grid and random tables, checked for purity, and stored on slopes of JMA at 4°C.

From a total of 380 isolates, 93 were chosen (see Appendix IV) on the criteria of colony size, morphology, and texture, so that a cross-section embracing the extremes of population variance was obtained for each substrate during the immersion periods.

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

### 5.2.2 Identification keys

The following keys were used in the identification of the isolates:

(i) *Pseudomonas* and *Altermonas*, Gibson Hendrie, Houston and Hobbs, 1977; Lee, Gibson, and Shewan, 1977; Baumann, Baumann, Mandel and Allen, 1972.

(ii) *Vibrio*, Gibson *et al.*, 1977.

(iii) *Flavobacterium*, McMeekin and Shewan, 1978; Hayes, 1977.

(iv) *Beneckea*, Baumann, Baumann, Mandel, 1971.

(v) *Moraxella*-like group, Gibson *et al.*, 1977.

(vi) *Micrococcus*, Anderson, 1962; Bergey's Manual of Determinative Bacteriology, (Buchanan & Gibbons, 1974).

(vii) Coryneform group, Austin, Allen, Mills and Colwell, 1977; Shewan, 1973.

### 5.2.3 Identification Tests

Where diagnostic tests (Cowan, 1974) gave incubation times of 24 hours, this was extended to 72 hours, the growth period of the isolates incubated at 15°C. The maintenance and growth medium, unless stated otherwise, was Johnson's marine agar (Appendix II) formulated with artificial seawater (MacLeod *et al.*, 1954).

(i) *Gram Reaction :*

Gram stained films were prepared from 72-hour cultures using Jensen's modification (Cruickshank, Duguid, Marmon and Swain, 1975). The KOH method (Gregersen, 1978), a rapid test for gram reaction, was also used, and the close agreement between both procedures suggests that the KOH method can be used with confidence.

(ii) *Morphology :*

Cell shape was determined by examination of a 72-hour liquid culture with phase contrast microscopy.

(iii) *Motility :*

72-hour liquid cultures were examined by phase contrast microscopy for motility. Non-motile isolates, or those with uncertain activity, were checked using a filter-paper bridge method. A JMA plate was divided (5 mm ditch) and bridged with a piece of sterile filter-paper (5 x15 mm) which was inoculated at one end. If, after incubation, growth was observed at the opposite end of the strip, motility due to active migration across the bridge was indicated.

(iv) *Flagella position :*

Flagella arrangements were determined by electron microscopy. The methods are described in Section 4.2.

(v) *Oxidase Test :*

72-hour cultures were tested for oxidase activity using Kovac's method,modified by the addition of ascorbic acid to prevent auto-oxidation (Cowan, 1974).

(vi) *Oxidation - Fermentation Test :*

The type of glucose dissimilation was determined by Hugh and Liefson's method, modified by an increase of sodium chloride from 5 $g/l^{-1}$ to 15 $g/l^{-1}$ w/v (Chaina, 1968). None of the organisms, tested by streaking onto plates of oxidation-fermentation medium, were inhibited by bromo-thymol blue, the pH indicator (Liefson, 1963 A). The fermentation test tubes were sealed with petroleum jelly (Andrews, 1976), but shrinkage and hardening at the incubation temperature of 15°C resulted in oxygen penetration into the medium, rendering the test invalid. Anaerobiosis was achieved by placing tubes in a 'Gaspak' (Beckton Dickinson Ltd.) anaerobic jar and removing oxygen with alkaline pyragallol (Willis, 1969).

(v) *DNA'se Test :*

DNA agar was prepared by the method of Jeffries, Holtman and Guse (Cowan, 1974) as JMA modified by the addition of 2 mg/ml w/v of DNA. Cultures were incubated for 96 hours at 15°C.

(vi) *Nitrate Reduction :*

Method 1 given by Cowan (1974) was used to determine nitrate reduction. JMA was modified by adding 0.1% $KNO_3$. After incubation for seven days, sulphanilic acid and α-naphthylamine were added to the culture and the presence of nitrite indicated by a red colouration.

(vii) *Arginine Hydrolysis :*

The method of Niven *et al.*, (Cowan, 1974) was used to determine arginine hydrolysis. The formulation was modified by the addition of

sodium chloride at 1.5% w/v; the arginine was an L-isomer. After incubation for 72 hours, Nessler's reagent was added, and hydrolysis was indicated by a brown colouration.

(viii) *Antibiotic sensitivities :*

JMA plates were surface-dried in a heated sterile airflow cabinet (Hepaire Ltd.). Antibiotic discs of Penicillin (1.5 i.u.), Chloramphenicol (10 µg), and Novobiocin (30 µg) (Oxoid Ltd.) were placed on seeded plates and incubated for 72 hours.

## 5.3 Results and Discussion

5.3.1 The bacteria isolated from core substrates sampled over a period of time were subjected to a battery of tests and identified using the keys indicated. The genera and groups to which the isolates could be assigned include *Pseudomonas*, *Alteromonas*, *Pseudomonas* and *Alteromonas* group, *Beneckea*, *Vibrio*, *Moraxella*-like group, *Flavobacterium*, Coryneform group, and *Micrococcus* (see Appendix IV). A proportion of the isolates (6.45%) could not be identified (see Table 18 for their primary characteristics), but resembled the *Pseudomonas/Alteromonas* group in that they were gram negative rods, motile with a polar flagellum, did not ferment glucose, but were oxidase negative. It is possible that they were atypical forms, verifiable by DNA base composition determination. Isolates assigned to the *Pseudomonas/Alteromonas* group (19.35%) were those which were gram negative rods, motile by a polar flagellum, and oxidase positive, but could not be identified to genus level because of atypical responses to the DNA's activity test, arginine dihydrolase activity test, and sensitivity tests to penicillin and chloramphenicol (see Table 19). Identification could be made if the oxidation/fermentation (glucose) test was weighted (Cowan & Liston, 1974), but the need for other definitive tests for the group is indicated.

TABLE 18 : Primary characteristics of unidentified isolates.

| Organism No. | Gram Reaction | Motility | Flagella | Oxidase | O/F Glucose | Amylase | Gelatinase | $NO_3^-$ Reduction | DNA'se | Argn. Dihydrolase |
|---|---|---|---|---|---|---|---|---|---|---|
| 3011G1 | - | + | Polar | - | O/- | + | + | + | + | - |
| 112SB2 | - | + | Polar | - | -/- | - | + | - | - | - |
| 312SB4 | - | + | Polar | - | -/- | - | + | - | + | + |
| 412GB2 | - | + | Polar | - | -/- | + | + | + | + | + |
| 912PA2 | - | - | NA | + | -/F | ND | ND | ND | - | - |
| 212SB4 | - | + | Polar | - | -/- | - | + | - | + | + |

ND - Not determined; NA - Not applicable.

Three isolates, 412GA3, 512GB4, and 141GA2 were identified as *Alteromonas vaga* an oxidase negative form of *Alteromonas* (Baumann *et al.*, 1972) and isolate 612GB3 was identified as *Pseudomonas perfectomarinus*, a non-motile pseudomonad (Baumann *et al.*, 1972).

The assignation of gram positive asporogenous rods to specific genera is not satisfactory as only a few discrete genera are considered homogeneous. Bergey's manual (Buchanan & Gibbons, 1974), has not defined the genus *Corynebacterium* but has created the coryneform group, divided into three sections: animal pathogens, plant pathogens, and non-pathogens.

TABLE 19 : Characteristics of atypical isolates assigned to the *Pseudomonas/Alteromonas* group.

| Organism Number | O/F Glucose | DNA'se | Arginine Dihydrolase | Penicillin Sensitivity | Chloramphenicol Sensitivity |
|---|---|---|---|---|---|
| 3011S1 | O/- | - | - | + | + |
| 612SB2 | -/- | + | + | - | - |
| 612GB1 | -/- | + | + | - | - |
| 612GB6 | -/- | + | + | - | - |
| 712R2 | -/- | + | + | - | - |
| 712P2 | O/- | + | + | - | - |
| 712P3 | O/- | + | ND | ND | ND |
| 812PB2 | -/- | + | + | - | - |
| 812PB3 | -/- | + | + | - | - |
| 1012PB3 | -/- | + | + | - | - |
| 1112RB3 | O/- | + | - | ND | ND |
| 1212PB1 | O/- | + | + | - | + |
| 1312PB3 | O/- | + | + | - | - |
| 1312PB5 | -/- | + | + | - | - |
| 1412SA3 | -/- | + | + | - | - |
| 1512RA4 | O/- | + | - | + | - |

ND - Not determined.

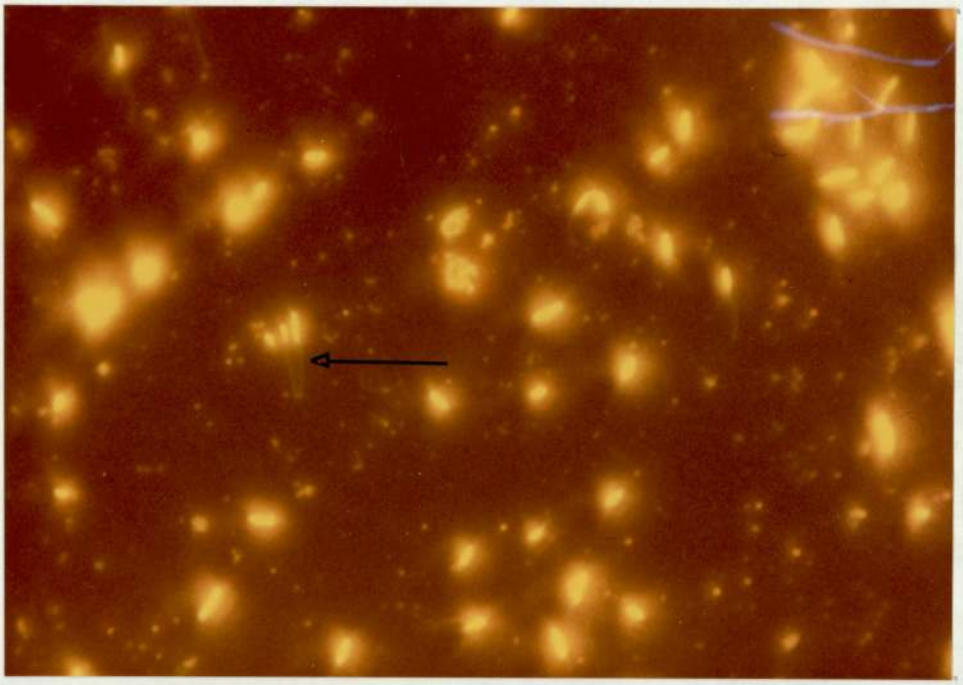
Candidates for election to the group must be gram-positive asporogenous rods which are catalase positive. The organisms isolated from the substrates and identified as coryneform have diverse properties (see Table 20), indicating the heterogeneity of the classification.

5.3.2 The range of organisms on the four substrates was greater than that found by O"Neil and Wilcox (1971) and Corpe (1973), a reflection, in part, of the isolation procedures, but more significantly due to the advances made in identification procedures for marine bacteria. This is particularly noticeable in the *Pseudomonadaceae* with the creation of a new genus *Alteromonas*, which describes the non-oxidative (glucose) pseudomonads with a mol % GC ratio of 43.2 - 48.0% (Baumann *et al.*, 1972). Similarly, no isolates were assigned to the genus *Achromobacter*, used as a depository for unreactive, unpigmented rods which have subsequently been assigned to the genera *Alcaligenes*, *Acinetobacter* and the *Moraxella*-like group. No sporogenous bacteria were detected as found by O'Neil and Wilcox (1971), and Floodgate (1971). Corpe (1973) identified prosthecate bacteria of the genera *Caulobacter* and *Hyphomicrobium*. No stalked bacteria were observed during the 0 - 168 hour immersion periods used in this study, but in one unique exploratory sampling programme of six weeks duration, using glass as a substrate, stalked bacteria were detected (see Fig.51).

5.3.3 Over half (55.91%) of the organisms isolated from the substrates came from the family *Pseudomonadaceae* of which the genus *Alteromonas* comprised 25.81% of the total isolates, *Pseudomonas* 10.75%, and the *Pseudomonas/Alteromonas* group 19.35% (see Fig.52).

TABLE 20 : Characteristics of coryneform isolates.

| Organism Number | Gram Reaction | Motility | Flagella Position | Oxidase | Oxidation/ Fermentation (Glucose) | Arginine dihydrolase | Catalase |
|---|---|---|---|---|---|---|---|
| 512GB2 | + | + | Peritrichous | + | O/- | - | + |
| 512SB3 | + | - | - | + | O/- | + | + |
| 712P4 | + | - | - | + | -/F | + | + |
| 812RB4 | + | - | - | + | -/F | + | + |
| 812RB2 | + | + | Polar | - | -/F | + | + |
| 212GB4 | + | + | Polar | - | O/- | - | + |
| 512SB6 | + | + | Polar | - | O/- | - | + |
| 912RB1 | + | - | - | + | -/F | + | + |
| 912PA1 | + | + | Peritrichous | - | -/F | - | + |
| 1012RB1 | + | + | Peritrichous | + | -/F | + | + |
| 1112PA2 | + | - | - | - | -/- | + | + |
| 1412GA1 | + | - | - | - | -/- | + | + |
| 1412SA1 | + | + | Polar | + | O/- | - | + |
| 1512RA6 | + | - | - | + | O/- | - | + |
| 1512PA6 | + | - | - | - | -/F | - | + |
| 1512RB5 | + | - | - | - | O/- | + | + |



——— 10 µm

Fig. 51 Stalked bacteria (arrowed) on glass, immersed for 6 weeks.

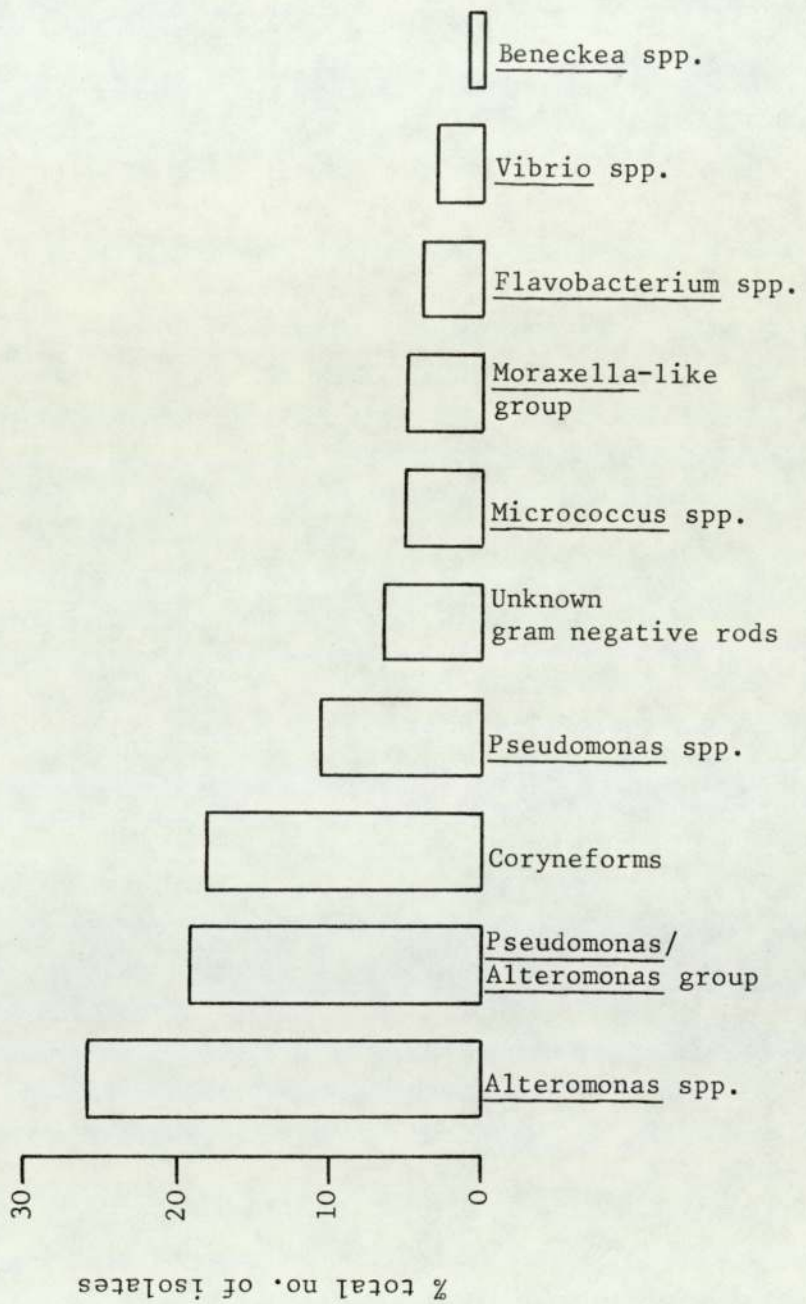
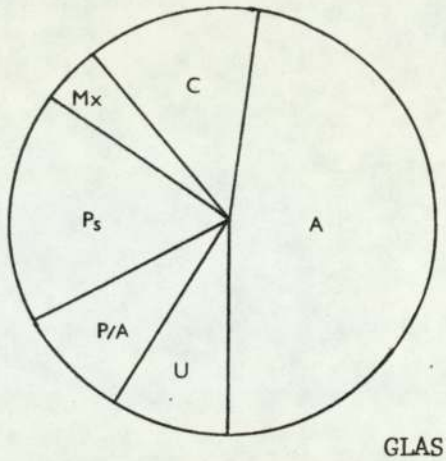


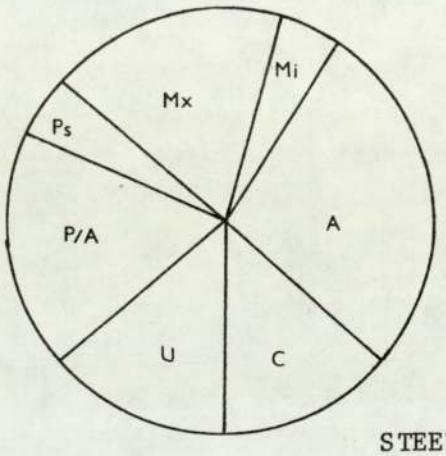

Fig. 52 Genus frequency of microbial film isolates.

This suggests that the major component of the attached flora are gram negative rods, characterized by their versatile nutrition and ability to utilise low levels of nutrient. The substrates, when considered more specifically and evaluated in terms of the genera recognised and their implied group descriptions, as distinct from specific characteristics, the high surface energy, wettable substrates glass and steel, had a small range of genera (Fig.53). In contrast, the low surface energy, less wettable substrates, resin and paint, had a greater range of genera. This suggests that the physico-chemical properties of hydrophobic substrates enable a wide variety of bacteria to enter the attachment sequence (Marshall *et al.*, 1971), while hydrophilic substrates have limiting conditions restricting the range of organisms able to irreversibly adsorb. The selectiveness of the surface for particular groups has been shown (Marshall, Stout & Mitchell, 1971 A), eg. glass will preferentially select only small rods for irreversible sorption.

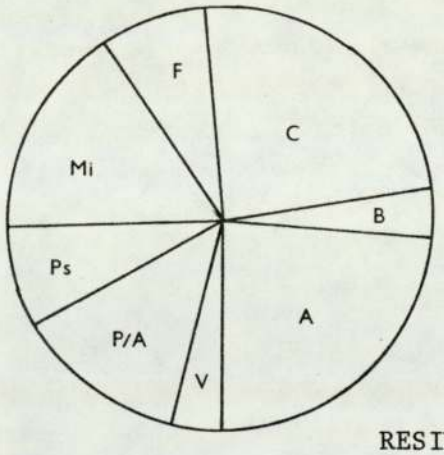
5.3.4 O'Neil and Wilcox (1971) observed that there was an increase in the range of bacteria attached to substrates with the length of immersion. During the first four hours, only gram negative rods were isolated; after eight hours, cocci; and twenty-four hours, gram positive rods. During this investigation, no clear-cut relationship between time and genera isolated was observed, but some general trends could be distinguished (Fig.54). Coryneforms were predominant during the first eight hours of immersion for low and high surface energy substrates, but for wettable substrates there was no apparent relationship between time and genera isolated after this initial period. In contrast, a range of genera were isolated from less wettable



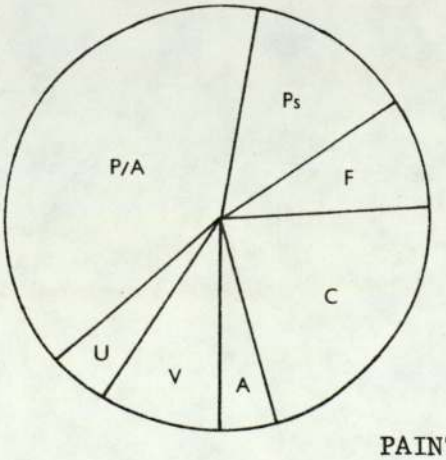

GLASS




STEEL




RESIN




PAINT

A - Alteromonas
B - Beneckea
C - Coryneform
F - Flavobacterium
Mi - Micrococcus
Mx - Moraxella-like group
Ps - Pseudomonas
P/A - Pseudomonas/Alteromonas group
U - Unknown
V - Vibrio

Fig. 53 Genus frequency of isolates from individual substrates

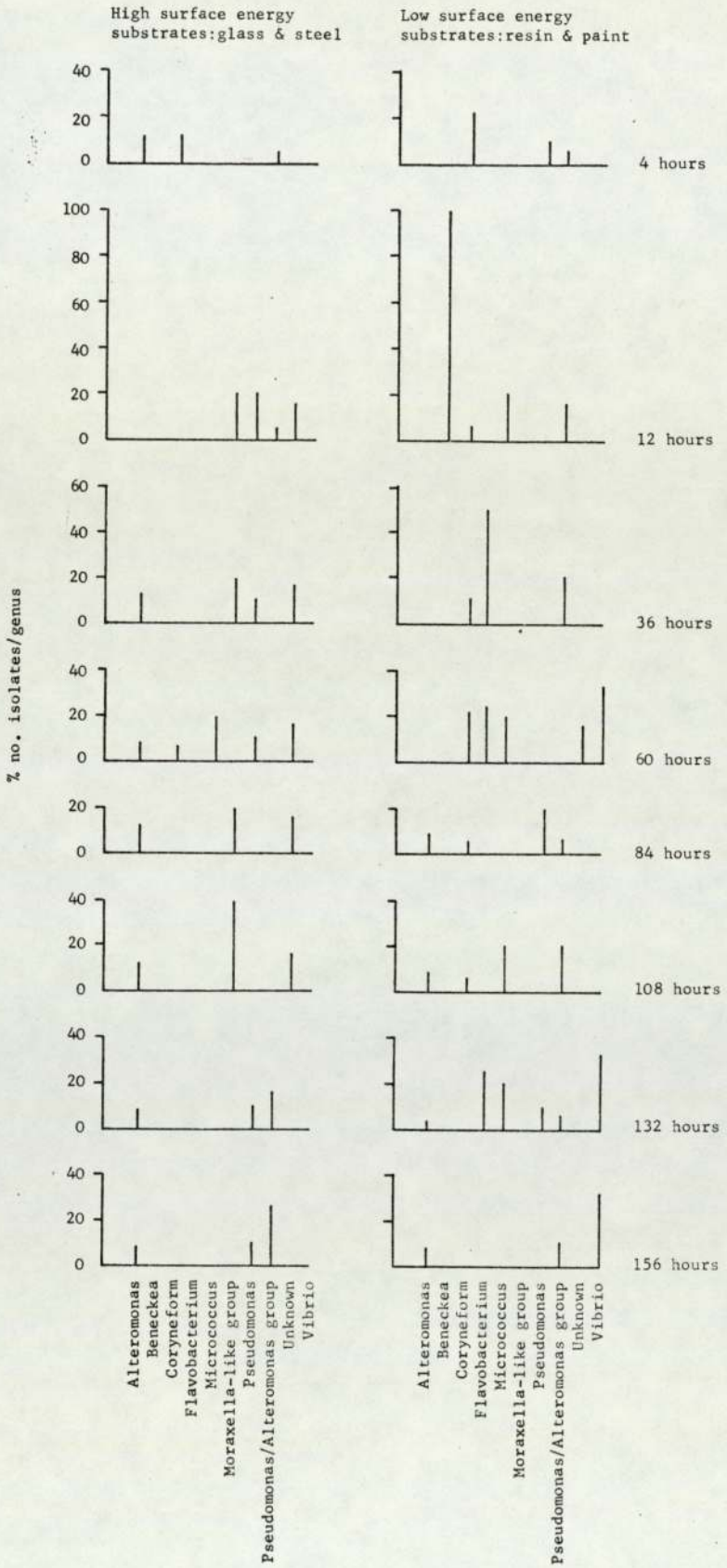



Fig. 54 Relationship of genera from high and low surface energy substrates with length of substrate immersion

substrates (resin and paint) during the early period of immersion, which became less pronounced with time when the predominance of isolates were from the *Pseudomonadaceae*. This indicates that a wide variety of organisms attach to less wettable substrates soon after immersion, and that conditioning of the surface either by organic adsorption or attached microbial processes accelerates the attachment of other organisms,and a shift in predominance is observed from primary to secondary colonisers.

# CHAPTER 6

## THE IDENTIFICATION OF ATTACHED MICROBIAL POPULATIONS ON SUBSTRATES OF DIFFERENT SURFACE CHARACTERISTICS

# THE IDENTIFICATION OF ATTACHED MICROBIAL POPULATIONS ON SUBSTRATES OF DIFFERENT SURFACE CHARACTERISTICS

## 6.1 Introduction

6.1.1 Microbial film development is subject to a number of influences, the most significant of which are substrate surface characteristics. It has been shown in this study that the relationship between attachment dynamics and the substrate is a function of surface hydrophobicity and determines the rate and density of microbial attachment. It also appears to influence the type of bacteria which become firmly attached, as it has been demonstrated that natural populations on glass can consist exclusively of short rods (Marshall, Stout & Mitchell, 1971 A).

Surfaces can be modified by adsorbed organic material (Baier *et al.*, 1968) which shifts the emphasis of surface character from the substrate to the nature of the adsorbed material. The concentration of organic adsorbate is dependent on a number of factors, and includes the surface free energy of the substrate (Eirich, 1977). Indexed as critical surface tension of wetting ($\gamma_c$) there is an increase in adsorbate concentration with decreasing values of $\gamma_c$ (hydrophobicity) (MacRitchie, 1972); in addition to the concentration factor, substrates may also show selective adsorption specificities for organic constituents (Niehof & Loeb, 1974). The organic film has a complex composition which reflects the characteristics of the underlying substrate and has an identity that is unique to the substrate.

It is suggested, therefore, that since microbial attachment is a function of the surface, substrates of different hydrophobicity will have microbial populations of distinctly separate identities due to differential heterologous organic substrate adsorption.

6.1.2 Microbial populations of unknown dimensions and activities from natural ecosystem may be described using phenotypically descriptive

characteristics and analysed by principal component and factor analysis (Rosswall & Kvillner, 1978). The population, translated into numerical terms, can then be qualified and quantified; these methods have been used successfully to characterise microbial populations in soil (Sundman, 1968,1969). To identify separate or overlapping populations in a habitat, the aim of the analysis is not quantitative characterisation of the population *per se*, but one of determining the number of occupied niches within the habitat by individuals of similar activities. This approach has been used to identify bacterial populations associated with sponges, as distinct from free living populations, by grouping organisms of similar functions, using taxometric techniques (Wilkinson, 1978).

6.1.3 The proposed hypothesis suggesting that attached microbial populations have separate and distinct identities on different substrates, was tested using representative strains of bacteria isolated from the 'core' substrates, glass, steel, resin, and paint. The strains were examined using morphological and biochemical tests and the data analysed by numerical taxonomic techniques. Groups containing organisms of similar identity were related to substrate character.

## 6.2 Materials and Method

### 6.2.1 Source of Bacteria

The organisms used in this study were isolated, selected, and identified as described in Section 5.2, and represent a cross-section of the attached microbial flora on the 'core' substrates: glass, steel, resin, and paint, during a winter sampling programme. Eighty-five organisms were used in this population study. The isolates, previously identified to genus level (Section 5), coded by their isolation number (incorporating

substrate and time of sampling details), were additionally numbered 1 - 85 for this analysis. The isolate coding, identification, and numbering are summarised in the results section (6.3) - Table 22. The isolates were grown on Johnson's marine agar (JMA) at 15°C and stored on JMA slopes at 4°C.

6.2.2 Collection of Data

Each isolate was characterised using 33 tests of a two-state type with mutually exclusive terms of positive or negative. Tests which could be done in liquid culture, including carbohydrate dissimilation, indole production, acemethylcarbinol production, methyl red test, nitrate reduction, and arginine dihydrolase activity, were performed in "Replidishes" (Sterilin Ltd.) containing 25 compartments. Test media dispensed into Replidishes were inoculated from master plates, prepared from tube-grown liquid cultures, using a simple multi-point inoculator of 5 x 5 loops (Sneath & Stevens, 1967).

The efficacy of transfer was checked by determining absence (or presence) of growth in control dishes of JMB. Tests involving zones of inhibition or hydrolysis were performed in petri dishes. All tests were incubated at 15°C, the optimum growth temperature of the isolates.

6.2.3 Tests

(i) *Gram reaction :* Gram stained films were prepared from 72-hour cultures using Jensen's modification (Cruickshank *et al.*, 1975) and Gregersen's KOH method (1978).

(ii) *Morphology :* Cell shape was determined by examination of a 72-hour culture with phase contrast microscopy.

(iii) *Pigmentation :* The presence (+) or absence (-) of a yellow pigmentation on 7-day cultures grown on JMA at 15°C was recorded.

(iv) *Motility :* 72-hour liquid cultures were examined by phase contrast microscopy and the filter-paper bridge method (see Section 5.2.3).

(v) *Oxidase Test :* 72-hour cultures were tested for oxidase activity by Kovac's method, modified by the addition of ascorbic acid to prevent auto-oxidation (Cowan, 1974).

(vi) *Catalase Test:* Drops of 3% 10 volume hydrogen peroxide were run down 72-hour slope cultures and a positive result recorded within five minutes.

(vii) *Oxidation / Fermentation :* (Glucose) Test: see Section 5.2.3.

(viii) *DNA'se Test :* DNA agar was prepared by the method of Jeffries, Holtman and Guse (Cowan, 1974) as JMA modified by the addition of 2 $mg/ml^{-1}$ (w/v) of DNA. Cultures were incubated for 96 hours at 15°C.

(ix) *Nitrate Reduction :* Method 1 given in Cowan (1974) was used to determine nitrate reduction. JMB was modified by the addition of $KNO_3$ to give a final concentration of 0.1% (w/v); cultures were incubated for seven days.

(x) *Arginine Hydrolysis :* The method of Niven *et al.* (Cowan, 1974) was used to determine arginine dihydrolase activity. The formulation was modified by the addition of NaCl at 1.5% (w/v); the L-isomer of arginine was used.

(xi) *Antibiotic Sensitivities* : Antibiotic discs of Penicillin (1.5 i.u.), chloramphenicol (10 μg), and Novobiocin (30 μg) were placed on dried JMA plates seeded with a 0.2 ml liquid culture surface inoculum.

(xii) *Starch Hydrolysis* : Soluble starch was added to JMB to give a final concentration of 1.0% (w/v). Plates were inoculated with a single streak, incubated for three weeks, and flooded with Gram's Iodine to determine zones of hydrolysis.

(xiii) *Gelatin Hydrolysis* : Gelatin agar was prepared by dissolving gelatin in JMA to give a concentration of 0.4% (w/v). Plates were inoculated with a single streak, incubated for seven days, and zones of hydrolysis detected by Frazier's method (Cowan, 1974), using acid mercuric chloride.

(xiv) *Indole Production* : Indole was detected using Ehrlich's reagent (Cowan, 1974) in 72-hour JMB cultures.

(xv) *Methyl Red and Voges - Proskauer Test* : Glucose phosphate seawater broth was prepared by the method of Chaina (1968) and cultures were incubated for seven days. Acetylmethylcarbinol production was tested by the method of Barritt (Cowan, 1974).

(xvi) *Oxidative Carbohydrate Dissimilation* : Chaina's modification (1968) of Hugh and Liefson's oxidation/fermentation medium was used as the basal indicator medium for carbohydrate dissimilation, and was prepared without agar. Carbohydrates sterilised by momentary autoclaving (Cowan, 1974) were added aseptically to the sterile basal medium to give a final concentration of 1.0% (w/v). The carbohydrates used in this study are listed in Table 22. The medium was dispensed as 4 ml aliquots into Replidishes, multi-point inoculated, and incubated for fourteen days. Dishes were inspected after seven and fourteen days.

TABLE 21 : List of carbohydrates used in oxidative carbohydrate dissimilation tests.

| Carbohydrate | Class |
|---|---|
| Arabinose<br>Xylose | Pentoses |
| Fructose<br>Galactose<br>Mannose | Hexoses |
| Cellobiose<br>Lactose<br>Sucrose | Disaccharides |
| Raffinose | Trisaccharides |
| Inulin | Polysaccharides |
| Salicin | Glycosides |
| Mannitol<br>Glycerol | Alcohols |
| Inositol | Hexahydroxycyclohexane |

6.2.4 Computation

Similarities between isolates were calculated as the Simple Matching Coefficient $S_m\%$ (Sneath, 1978). The data were organised as an unsorted similarity matrix, and significant groups obtained by single linkage and complete linkage cluster analysis (Sneath & Sokal, 1973). Levels of clustering were determined from a dendrogram, constructed from cluster analysis, which enabled a sorted similarity matrix to be drawn, illustrating the size and position of each group in relation to each other.

## 6.3 Results

6.3.1 The clustering procedures organised the 85 representatives of the attached microbial flora on the four substrates: glass, steel, resin, and paint, into nine major groups at the 60% similarity level. The choice of cluster analysis had a profound effect on the grouping of isolates; single linkage clustering, which admits candidates to groups at the highest similarity level, resulted in an overall similarity between isolates at the 76% level (Fig.55) and significant group identification could not be made. Complete linkage clustering, although producing high intra-group similarity with low inter-group similarity, did enable discrete, well defined groups to be identified (Fig.56).

The allocation of organisms, together with their generic and substrate identities, to the sorted clusters, is given in Table 22. Within Group II, IV, and VI, divisions could be recognised at the 70% similarity level and are indicated as sub-groups II A,B; IV A,B,C; and VI A,B, respectively.

6.3.2 The relationship between manifestly different organisms within a recognisable population can be determined by the clustering of individuals and ordination of groups (Fig.57).

The intra-group generic heterogeneity which may be observed is not significant; the analysis is not intended to show the level of generic relatedness, but rather the degree of population homogeneity (or heterogeneity) indicated by the number of, and distance between intra-population groups. This latter aim was achieved by evaluating the groups from the attached microbial population, individually, and in relation to each other, by interpreting clusters as a function of the substrates: glass, steel, resin, and paint.

TABLE 22 : List of sorted clusters with details of cluster composition.

GROUP I

| Analysis Number | Substrate | Isolation Time (hrs.) | Genus |
|---|---|---|---|
| 19 | S | 84 | Unknown |
| 13 | S | 60 | Unknown |
| 33 | G | 132 | *Alteromonas vaga* |
| 31 | G | 132 | *Pseudomonas /Alteromonas* |
| 56 | P | 84 | ND * |
| 70 | R | 156 | *Alteromonas* sp. |
| 39 | G | 156 | *Pseudomonas /Alteromonas* |
| 28 | S | 132 | ND |
| 62 | P | 108 | *Pseudomonas /Alteromonas* |
| 15 | G | 60 | *Alteromonas* sp. |
| 21 | G | 84 | *Alteromonas* sp. |
| 20 | G | 84 | *Alteromonas* sp. |
| 43 | P | 12 | *Pseudomonas /Alteromonas* |
| 71 | R | 156 | *Vibrio* sp. |
| 74 | P | 156 | *Pseudomonas /Alteromonas* |
| 73 | P | 156 | *Pseudomonas /Alteromonas* |
| 48 | P | 36 | *Pseudomonas /Alteromonas* |
| 47 | P | 36 | *Pseudomonas /Alteromonas* |
| 35 | S | 156 | *Pseudomonas /Alteromonas* |
| 41 | R | 12 | *Pseudomonas /Alteromonas* |
| 37 | G | 156 | *Pseudomonas /Alteromonas* |
| 34 | S | 156 | *Alteromonas* sp. |
| 65 | R | 132 | *Alteromonas* sp. |
| 18 | S | 84 | *Alteromonas* sp. |
| 79 | G | 4 | *Alteromonas* sp. |
| 55 | R | 84 | *Alteromonas* sp. |
| 24 | G | 96 | *Alteromonas vaga* |
| 23 | S | 108 | *Alteromonas* sp. |
| 63 | P | 108 | *Alteromonas* sp. |
| 26 | G | 108 | Unknown |
| 27 | G | 108 | *Alteromonas* sp. |
| 67 | P | 132 | *Pseudomonas /Alteromonas* |
| 6 | S | 36 | Unknown |

ND - Not Determined

TABLE 22 (contd.)

GROUP II (A)

| Analysis Number | Substrate | Isolation Time (hrs.) | Genus |
|---|---|---|---|
| 5 | G | 12 | *Pseudomonas* sp. |
| 4 | G | 12 | *Pseudomonas* sp. |
| 68 | P | 132 | *Vibrio* sp. |
| II (B) | | | |
| 51 | P | 60 | Coryneform |
| 16 | G | 60 | Coryneform |
| 53 | P | 60 | *Vibrio* sp. |

GROUP III

| | | | |
|---|---|---|---|
| 7 | S | 36 | *Pseudomonas* sp. |
| 2 | S | 12 | *Moraxella*-like group |
| 8 | S | 36 | *Moraxella*-like group |

GROUP IV (A)

| | | | |
|---|---|---|---|
| 10 | G | 36 | *Alteromonas* sp. |
| 9 | G | 36 | *Alteromonas* sp. |
| 11 | G | 36 | *Alteromonas* sp. |
| 59 | R | 108 | *Alteromonas* sp. |
| 42 | R | 12 | *Beneckea* sp. |
| 78 | G | 4 | *Alteromonas vaga* |
| IV (B) | | | |
| 66 | R | 132 | *Pseudomonas* sp. |
| 57 | P | 84 | *Pseudomonas* sp. |
| 72 | P | 156 | *Pseudomonas* sp. |
| IV (C) | | | |
| 84 | R | 4 | *Pseudomonas* sp. |
| 76 | S | 4 | *Pseudomonas /Alteromonas* |

TABLE 22 (contd.)

GROUP V

| Analysis Number | Substrate | Isolation Time (hrs.) | Genus Genus |
|---|---|---|---|
| 75 | S | 4 | Coryneform |
| 60 | R | 108 | *Pseudomonas /Alteromonas* |
| 54 | R | 84 | Coryneform |

GROUP VI (A)

| | | | |
|---|---|---|---|
| 25 | G | 108 | *Moraxella*-like group |
| 17 | S | 84 | *Moraxella*-like group |
| 14 | G | 60 | *Pseudomonas* sp. |
| 81 | P | 4 | Coryneform |
| 46 | R | 36 | Coryneform |
| 44 | P | 12 | Coryneform |
| 52 | P | 60 | Unkown |
| 38 | G | 156 | *Ps. perfectomarinus* |
| 22 | S | 108 | *Moraxella*-like group |
| 36 | S | 156 | *Alteromonas* sp. |
| VI (B) | | | |
| 29 | S | 132 | Coryneform |
| 12 | S | 60 | *Micrococcus* sp. |
| 64 | R | 132 | *Micrococcus* sp. |
| 50 | R | 60 | *Micrococcus* sp. |
| 77 | G | 4 | Coryneform |
| 40 | R | 12 | *Micrococcus* sp. |

TABLE 22 (contd.)

GROUP VII

| Analysis Number | Substrate | Isolation Time (hrs.) | Genus |
|---|---|---|---|
| 58 | R | 108 | *Micrococcus* sp. |
| 48 | R | 36 | Coryneform |
| 32 | G | 132 | Coryneform |
| 1 | S | 12 | *Psuedomonas /Alteromonas* |
| 61 | P | 108 | Coryneform |
| 49 | R | 60 | Coryneform |

GROUP VIII

| | | | |
|---|---|---|---|
| 69 | R | 156 | *Alteromonas* sp |
| 3 | G | 12 | Unknown |
| 83 | R | 4 | *Pseudomonas /Alteromonas* |
| 30 | S | 132 | *Pseudomonas /Alteromonas* |

GROUP IX

| | | | |
|---|---|---|---|
| 82 | P | 4 | Coryneform |
| 80 | R | 4 | Coryneform |
| 85 | R | 4 | Coryneform |

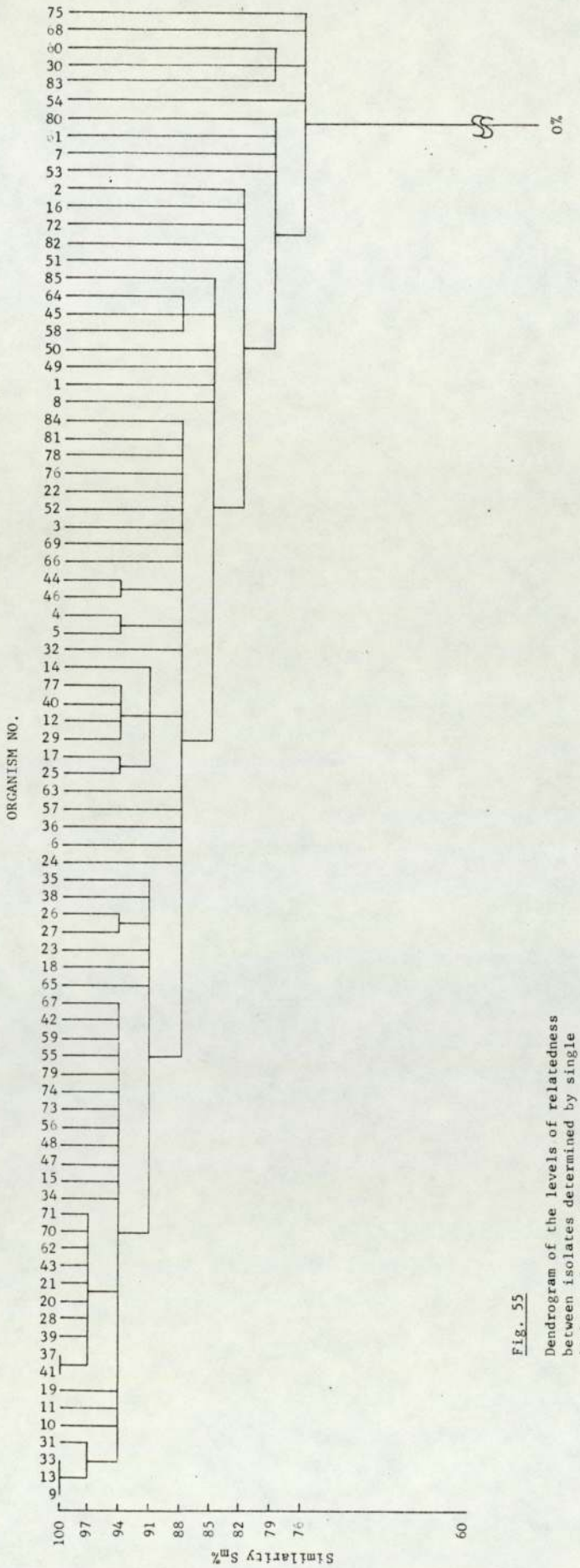



Fig. 55

Dendrogram of the levels of relatedness between isolates determined by single linkage cluster analysis.

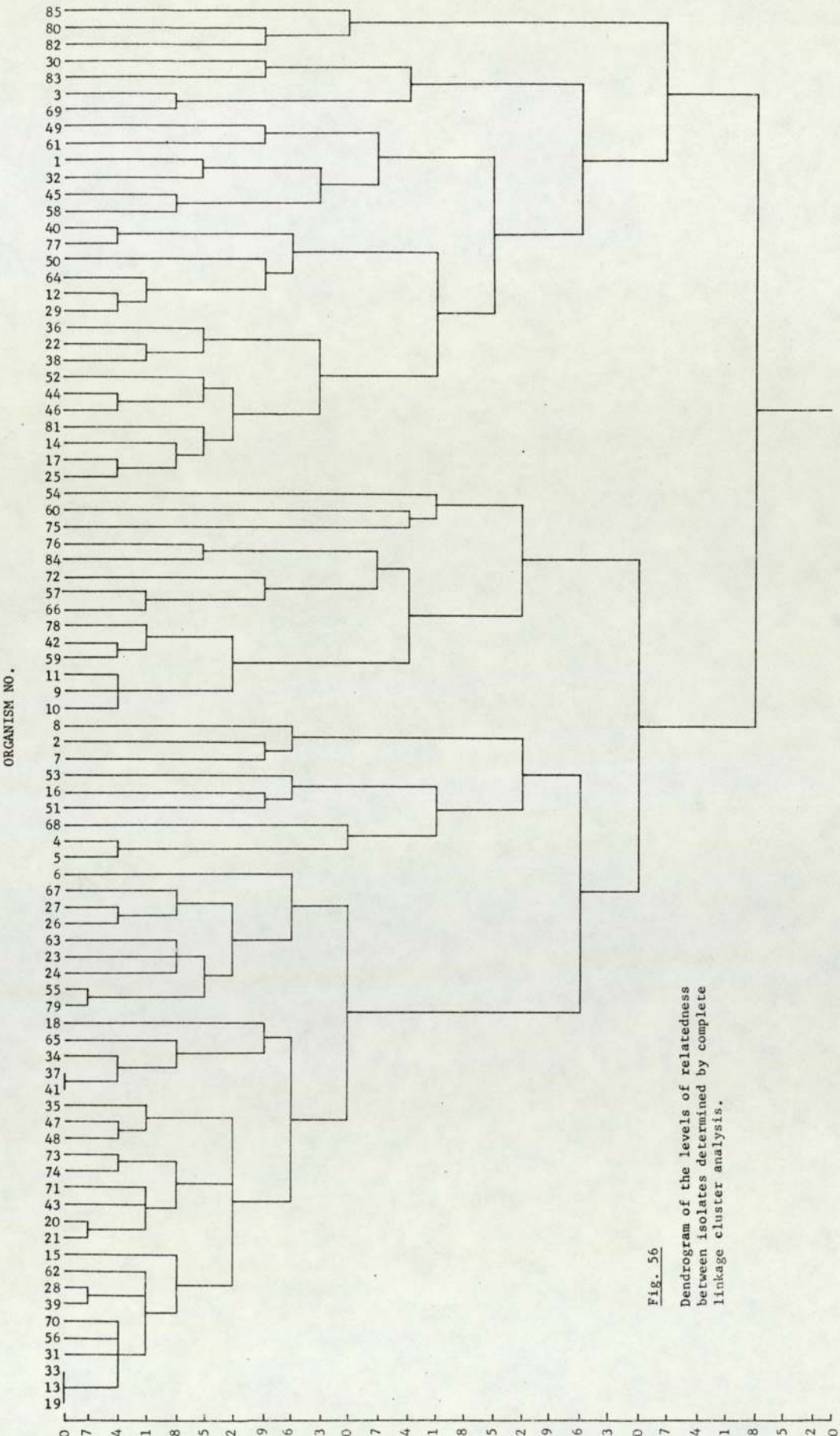



Fig. 56

Dendrogram of the levels of relatedness between isolates determined by complete linkage cluster analysis.

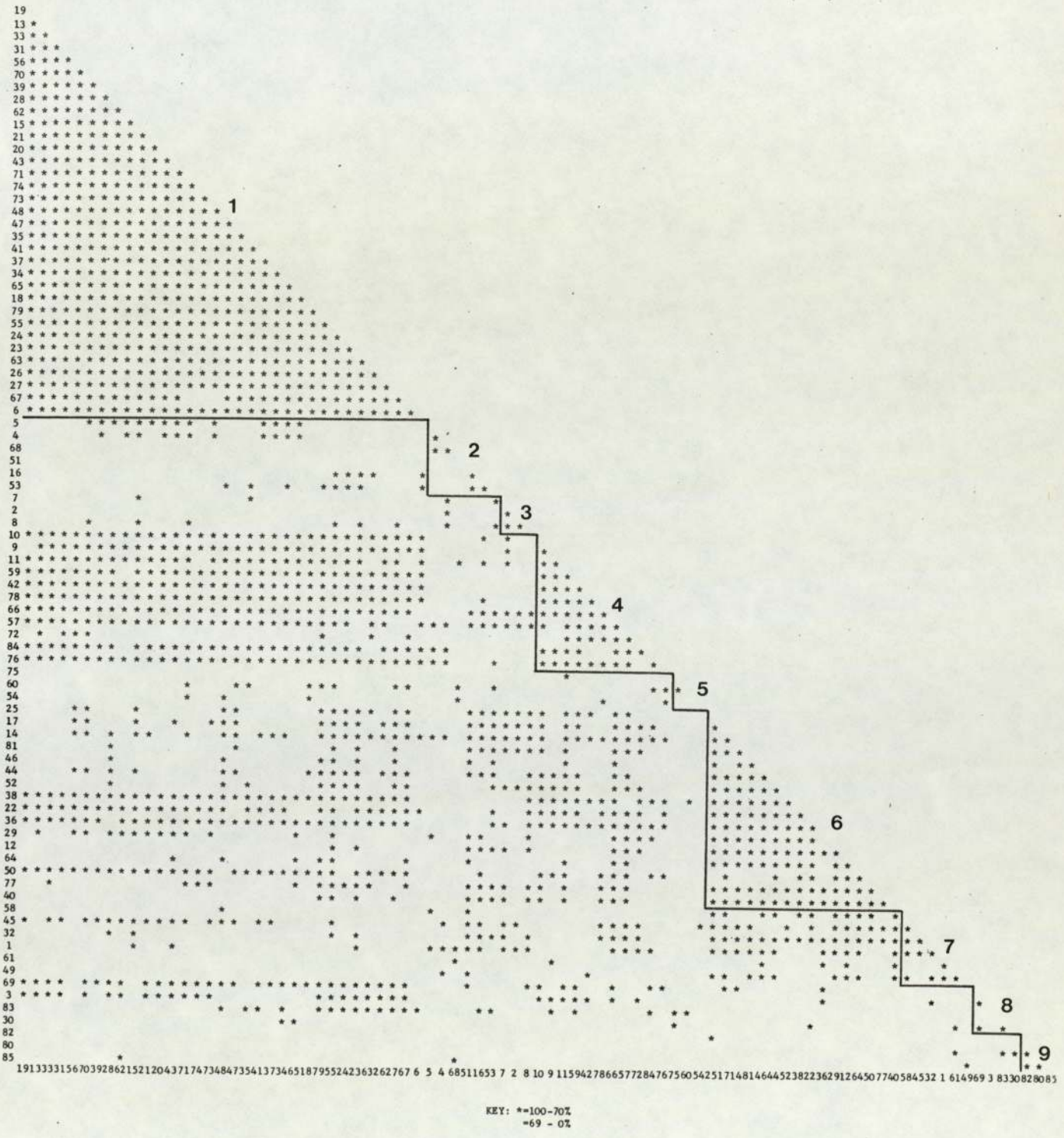



Fig. 57 Sorted similarity matrix. Clusters determined at the 60% similarity level.

For each cluster, the number of individuals from the four substrates was determined and expressed as a percentage of the total number of isolates from their respective substrates. A more significant relationship could be observed if the substrates were regarded as two broad types : glass and steel as high surface energy substrates (HSES) ; and resin and paint as low surface energy substrates (LSES). The distribution of substrate isolates between the nine clusters can be seen in Fig.58. A feature of the data presented as a histogram is the difference in level observable between low and high surface energy substrates and clusters. The significance of this aspect is masked by the different sizes of cluster, but is apparent when the difference in frequency of occurrence (as number percent) between HSES and LSES isolates for each cluster is calculated.

The difference, expressed as units of percent, was determined by arbitrarily subtracting the number percent of LSES isolates from HSES isolates. This index of level may have a positive (greater frequency of HSES isolates) or negative (greater frequency of LSES isolates) sign; it is only a relative scale. The index of level between HSES and LSES populations was plotted against the distance between clusters as increasing dissimilarity of isolates from clusters I - IX (Fig.59). The relationship between decreasing frequency of HSES isolates and increasing dissimilarity of isolates from cluster I was determined as a linear function ($r = -0.6727$, $p = 0.05$). Alternatively, a relationship is described of increasing frequency of LSES isolates with decreasing similarity of isolates from cluster I ($r = 0.6727$, $p = 0.05$).

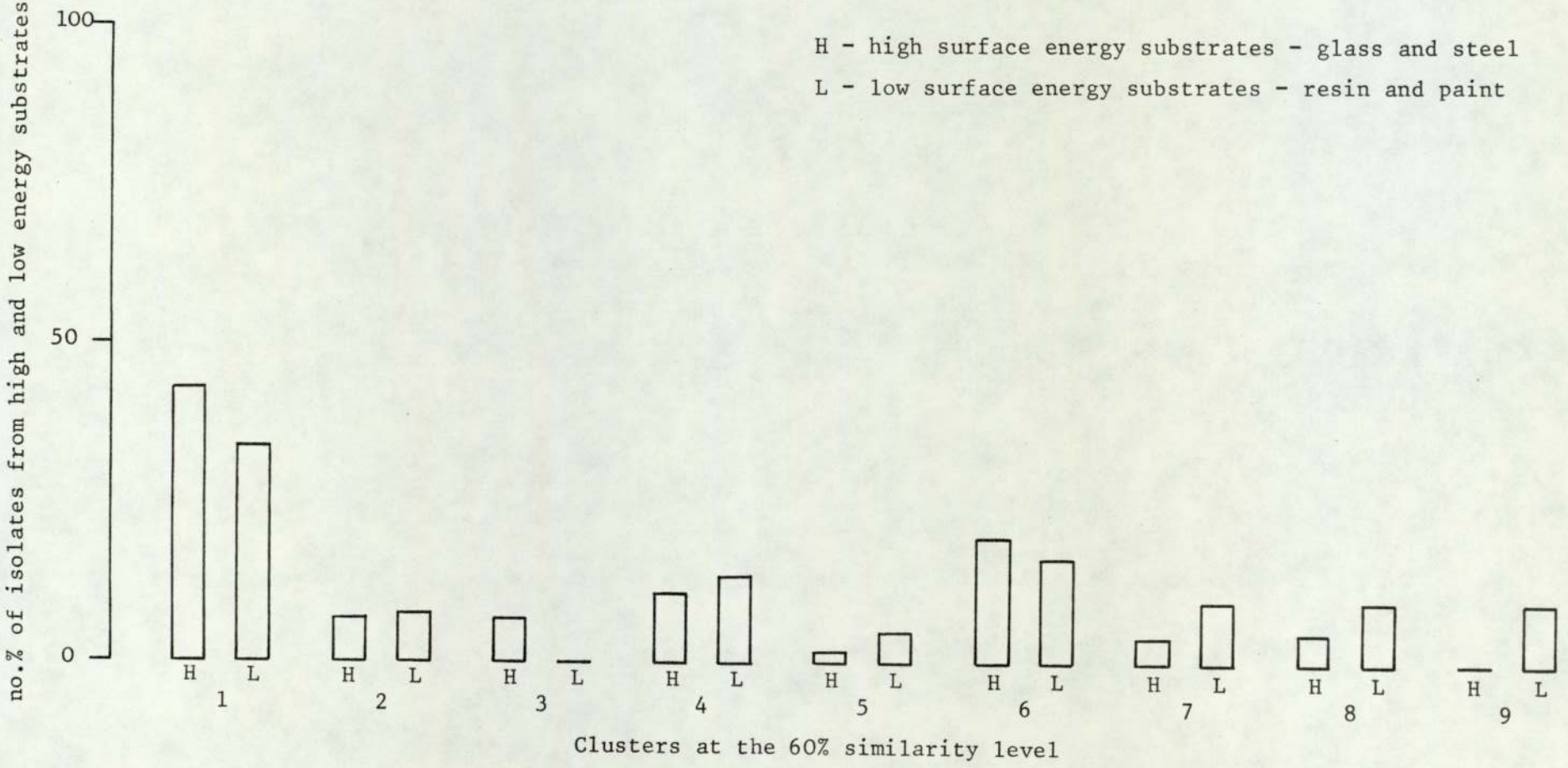



Fig. 58 Distribution of substrate isolates between clusters at the 60% similarity level

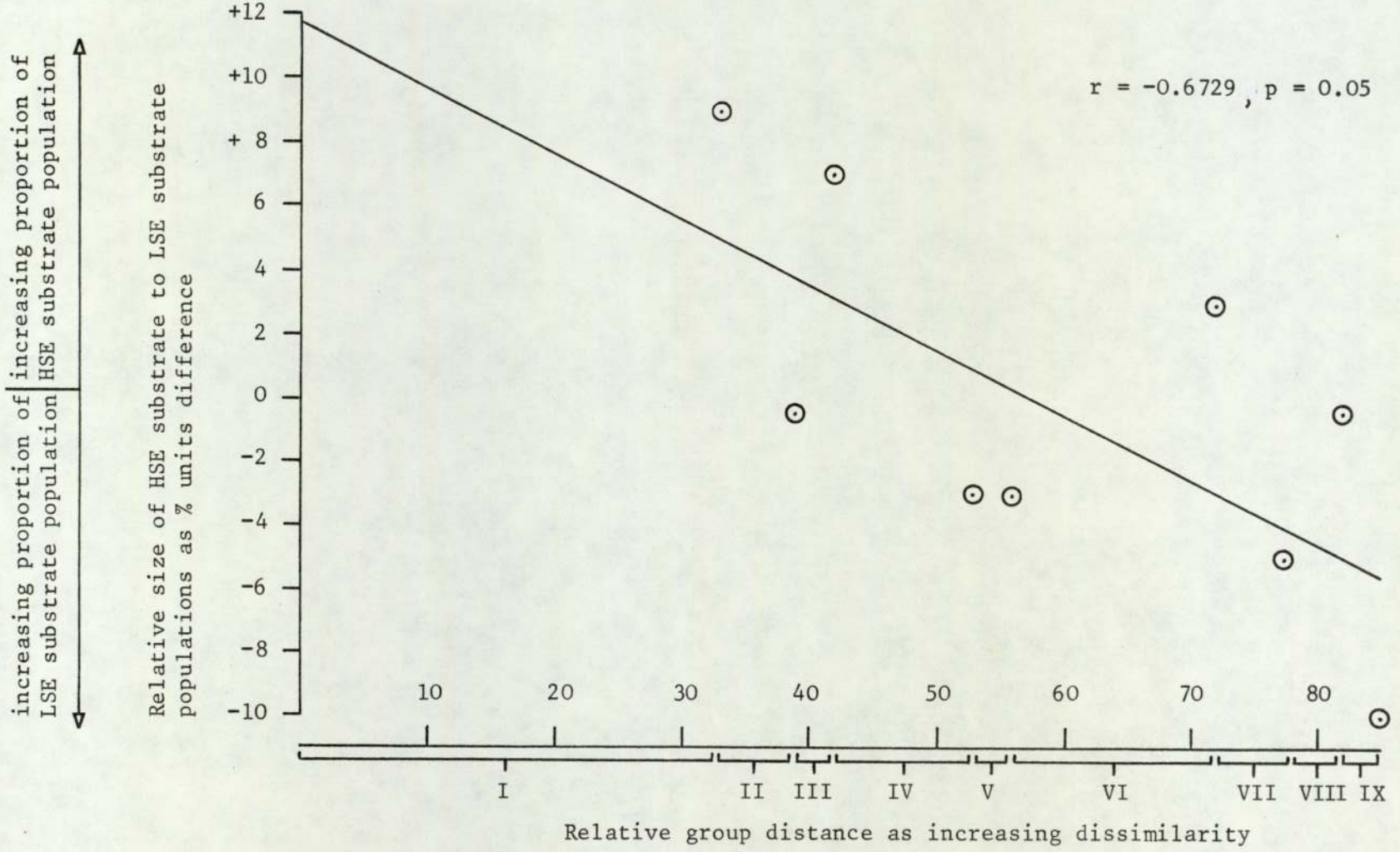



Fig. 59 Relationship between cluster composition and substrate population type.

## 6.4 Conclusions

(i) Microbial film components are of two types: substrate non-specific, and substrate specific.

(ii) A microbial film is composed predominantly of substrate non-specific types and, to a lesser extent, substrate specific types.

(iii) The low, overall similarity (27% $S_m$) between isolates indicates a wide disparity of bacterial types in microbial films.

(iv) Substrate influence on microbial film composition is a significant factor in attached microbial community structure.

## 6.5 Discussion

Substrates of different surface energies acquire organic layers of dissimilar composition and concentration by selective adsorption (MacRitchie, 1972; Niehof & Loeb, 1974; Eirich, 1977). Attachment is believed to involve the organic layer, and it was suggested (Section 6.1) that attached microbial populations have separate and distinct identities on different substrates. Using taxometric techniques, representatives from the substrates: glass, steel, resin, and paint, were clustered, and the groups ordinated to determine levels of identify within the population. This form of analysis is holarchich (Koestler, 1978) and seeks to define structure and organisation by considering the interaction of holons (individuals in the holarchy), rather than by empirical analysis of isolated individuals.

Cluster composition and ordination were interpreted in terms of substrate type; the size of cluster I and its association with high surface energy substrates such as glass and steel, and the smallness but greater

dissimilarity of clusters derived from low surface energy substrate populations, indicate that the majority of the attached microbial populations were substrate non-specific, and could be found on either glass, steel, resin, or paint. It was apparent by the shift in emphasis from HSES isolates to LSES isolates, that a proportion of the isolates was substrate specific and associated exclusively with either high or low surface energy substrates.

Microbial films have been shown to develop as integrated self-limiting communities (Hargrave & Phillips, 1977), achieved by a concert interaction of component individuals. It has been shown in this study that substrate surface characteristics influence the composition of attached microbial populations. The initial community contains a proportion of substrate specific components which may structure and specify the form of the final (climax) community by their interaction with non-specific components. Cluster analysis, using the complete linkage method, which forms discrete clusters of monolithic similarity, determined the overall relatedness between isolates from the four substrates at the 27% $S_m$ level. This analysis indicates a wide variation between bacterial types in attached microbial communities. The disparity between types indicates that the structure and activity of established populations on different substrates may be very dissimilar.

Microbial succession within the attached population was also considered as a factor determining cluster composition and ordination. Succession, measured as the sampling time during the immersion sequence, was correlated with increasing isolate dissimilarity from cluster I to IX. No relationship was established between clusters and sequential attachment patterns during the immersion period.

The form of analysis used in this study is qualitative, and may only identify populations within a particular habitat. To characterise or quantify specific activities and indicate the range of the population other forms of statistical treatment such as principal component or factor analysis are required.

# CHAPTER 7

## DISCUSSION

# DISCUSSION

7.1 The significance of microbial attachment has been realised in a number of fields, including dental pathology (Gibbons & van Houte, 1975), fermentation systems (Atkinson & Fowler, 1974), and microbial fouling (Young & Mitchell, 1973; Norrman, Characklis & Bryers, 1977). Attachment phenomena have been studied extensively, determining mechanisms and factors influencing bacterial adhesion and adsorption to surfaces; to a lesser degree, investigations of attached microbial communities in the natural environment, particularly marine situations, have been made to determine microbial film development on artificial substrates. Significantly, there has been little work to relate mechanistic descriptions of microbial attachment with observations of microbial film development in the natural environment.

The scope of this work is to redefine the concept of the microbial film as an ecological entity within the marine ecosystem. Aspects of attached microbial community ecology considered were the dynamics of microbial attachment and the factors which influence density, rate, and distribution of attached bacteria. In addition, microbial film development was examined, together with a study of microbial film components. A rationale of microbial film functionalism was derived by correlating these observational studies of the microbial film in the natural environment, with mechanistic descriptions and predictions of microbial attachment, based on laboratory models.

7.2 The liquid/solid interface in the aquatic environment is a habitat defined by its physicochemical parameters. Occupation of

niches within the habitat (attachment sites) is determined largely by the interaction of bacteria - potential residents - with surface phenomena; bacteria sequestered (passive adsorption) or attached (active adhesion) may either remain quiescent or exploit and realise the dimensions of the niche. Growth and proliferation on the substrate is believed to be due to the utilisation of adsorbed organic materials on substrate surfaces (ZoBell, 1943). In an environment of nutrient limitation, concentration of nutrients at surfaces represent sites of advantage. An inherent characteristic of cell/surface conformation is the interface interstice, which enables the cell to exploit the adsorbed organics efficiently. This region reduces exoenzyme diffusion and traps detrital material, an energy input to the system, accelerates cell proliferation, and enhances the site for subsequent microbial attachment. The increased metabolic activity of attached microbial communities compared with free-living groups (Hendricks, 1978) indicates the advantages of substrate attachment in a nutrient limited environment.

Microbial attachment has been defined as a two-stage sequence of events (Marshall *et al.*, 1971). In the first stage, a cell encounters a surface by a random event and is held, but not in direct contact with the surface; this is reversible sorption. Firm adhesion, or irreversible sorption is achieved when extracellular polymeric fibrils bridge the double-ion repulsion layer and bind the cell securely to the substrate. This latter process is time-dependent (Marshall *et al.*, 1971) the result of metabolic activity / synthesis (Marshall, 1973). During the short immersion programme (time scale I, 0 - 1 hr., winter), irreversible sorption was detected on substrates immersed for only ten minutes in seawater at 6$^{o}$C. Under these extreme conditions, attachment involving a synthesis would be unlikely and some other attachment mechanism is indicated.

Attachment dynamics have been described by the Langmuir Adsorption Isotherm, suggesting that rapid attachment could be effected by a chemisorption phenomenon (Fletcher, 1977) involving an existing but discrete expolymer around the cell (Fletcher & Floodgate, 1973). Attachment would be 'instantaneous' where bacteria are passive elements in the physico-chemical interaction at the surface. Factors influencing the dynamics of microbial attachment effected by rapid chemisorption, are predicted by the Langmuir Adsorption Isotherm. Fletcher (1977) determined that the concentration of cells in the liquid phase was the major influential factor on attachment, due to the increased chance of collision between bacteria and surface; only to a lesser extent did temperature affect the dynamics.

Attachment patterns obtained from data collected during summer and winter sampling series (time scale III, 0 - 12 hrs; time scale IV, 0 - 168 hrs.) can be interpreted with reference to the monitored environmental parameters of cells/ml$^{-1}$ in seawater, temperature, and total organic carbon — factors influencing attachment dynamics. It was found that the concentration of cells in seawater was the most influential factor in determining cell density on substrates; temperature was significant only to a lesser degree. No relationship could be established between total organic carbon and cell density.

The correlation of environmental parameters with attachment patterns indicates that the predominant and most significant attachment mechanism of microbial adhesion is by 'instantaneous' attachment, and that time-dependent attachment is a specialised mechanism of adhesion.

An alternative attachment mechanism to time-dependent polymeric fibril synthesis and 'instantaneous' adhesion has been described by

flagella binding of the cell to the surface (de Boer, Golten, Scheffers, 1975). It was projected that monotrichous flagellated cells, on entering the reversible stage of the Marshall *et al.* model (1971), switch their flagella pattern to a peritrichous arrangement in response to nutrient accumulation at the surface. The flagella then anchor the cell firmly to the substrate. It has been shown in this study, using bacteria isolated from surfaces, that a significant proportion were capable of switching flagella patterns, indicating that this mechanism of attachment may be used by some bacteria.

Attachment mechanisms can be divided into two categories: the first involves non-specific 'instantaneous' chemisorption attachment, and secondly, a selective, adaptive mechanism in which extracellular polymeric fibrils or flagella are synthesised to effect a time-dependent attachment. Chemisorption attachment of bacteria does not imply that irreversibly sorbed bacteria are capable of exploiting the niche potential; it only indicates a cell/surface interaction. Conversely, time-dependent attachment does suggest that attached bacteria have responded positively to the nutrient conditions of the surface, and may colonise the substrate.

From data collected in the natural environment during this study, it is suggested that all three mechanisms are involved in microbial attachment, but the most significant process, particularly under limiting conditions, is the chemisorption mechanism.

7.3 Microbial attachment involves an interaction between cell and substrate surface, with irreversible adsorption effected by the adhesion of bacterial extracellular polysaccharides to the substrate. Cohesiveness is affected by the surface characteristic of wetting

(Baier *et al.*, 1968) which can be measured as the critical surface tension of wetting, $\gamma_c$ (Zisman, 1972), or hydrophobicity as the contact angle of wetting by seawater, $\theta_{sw}$ (Section 3.3). The attachment of cells to surfaces can be influenced by substrate wettability; tissue cells preferentially attach to wettable surfaces (Baier, 1970), while a marine pseudomonad has been shown to attach selectively to less wettable substrates (Fletcher & Loeb, 1976). In this study, using substrates with a range of wetting values, attachment of microbial populations from the natural environment was described as a linear increase of cell density with increasing hydrophobicity. The correlation of these two variables suggests that the surface characteristic, wettability, is a significant factor influencing attachment patterns. The preferential attachment of marine bacteria to less wettable substrates suggests a hydrophobic interaction (Weiss, 1970; Fletcher & Loeb, 1979) between hydrophobic sites on bacteria and surfaces in a hydrophilic system (Marshall, 1976). It has also been suggested that cell attachment can only proceed once the substrate has adsorbed a glycoprotein layer (Baier, 1973), but for marine bacteria, adsorbed proteins may inhibit their attachment (Fletcher, 1967). Clearly, attachment processes in the marine environment are influenced by factors other than those determining attachment dynamics described for blood and cell tissue systems.

The influence of the substrate surface characteristic, wetting, on attachment density predicts that this factor may influence attachment rate. Using a range of substrates immersed over different lengths of time and during different environmental conditions, relative attachment rates were compared with substrate surface hydrophobicity. The relationship between these two factors was described as a linear

increase in relative attachment rate with decreasing wettability, indicating the significance of wettability on attachment rate.

Flagella mediated adhesion of bacteria to surfaces, denoted by the ability of isolates to switch flagella patterns from polar to peritrichous arrangements, may be influenced by substrate surface characteristics. During this study, a significant proportion of attached bacteria on the high energy wettable substrate, glass, were of types able to switch flagella patterns. Reversible sorption of flagella has been demonstrated by Meadows (1972), and it is suggested that the relationship between flagella mediated adhesion and hydrophilic substrates is due to an undefined specific interaction between flagella protein sub-units, flagellin, and the surface. The specificity of protein/surface adhesion is due to polymer conformation, functional side group size, polarity, and charge (Eirich, 1977) interacting with surface wettability and charge characteristics (MacRitchie, 1972).

The influence of substrate surface wettability on attachment dynamics was only discernible during the first twelve hours of substrate immersion. In blood and cell tissue systems, the influence of surface energy is limited to determining the nature of the adsorbed organic layer, the principal factor in that type of attachment system. In the marine environment, however, because substrate organic adsorption is slow due to the dilute nutrient status of seawater (Baier, 1973), surface energy is initially a major factor influencing attachment dynamics. The observed decline of substrate wettability influence with time is due to organic adsorption and microbial attachment masking the substrate and modifying surface features (Loeb & Niehof, 1977).

In conclusion, it is apparent from this study that substrate surface wettability is a major factor influencing microbial attachment dynamics on newly immersed substrates in the marine environment. Surface influence, however, appears to be limited to approximately the first twelve hours of substrate immersion, and once the adsorbed organic layer has become established, no significant differences between substrates could be observed.

7.4 The development of attached microbial communities on substrates immersed in seawater is dependent on a number of factors; the most significant were found to be microbial cell number in seawater and substrate surface characteristics.

Attachment to substrates during the first twelve hours of immersion in seawater during the winter months was typified by low cell densities and attachment rates — a direct effect of reduced bacterial numbers in seawater. During this period of immersion, density and attachment rate were also influenced by substrate characteristics. Wettable substrates such as glass, had attachment densities of $2.83 \times 10^3$ cells/$cm^2$, while less wettable substrates in the region of $6.02 \times 10^3$ cells/$cm^2$ after twelve hours immersion. Rates of attachment, hence microbial film development, were correspondingly lower; density increase on glass was linear, while on paint it was faster — denoted by the logarithmic increase.

In contrast, microbial film development for a similar immersion time during the summer period was characterised by greater cell densities and attachment rates. The greater densities observed for this period were due to the increased bacterial cell number of seawater

and, to a lesser extent, sea temperature. It has been suggested (Section 7.2) that most attachment is by some form of chemisorption, but it is likely that a significant proportion of the immersed cell density for this period was the result of time-dependent microbial synthesis attachment, facilitated by higher sea temperatures. Densities were also influenced by substrate wettability, with levels on glass at $5.47 \times 10^4$ cells/cm$^2$, and paint $1.09 \times 10^5$ cells/cm$^2$ after twelve hours immersion. Attachment rates during the summer were considerably faster than the winter period, denoted by the hyperbolic curve of cell density increase.

The dynamics of microbial film development viewed over longer immersion periods (0 - 168 hrs.) largely reflect the prevailing environmental status. Attachment during the early immersion period, together with organic adsorption, sufficiently modify the substrates so that no significant differences could be observed in density or rate of attachment between substrate types. Densities were considerably higher during the summer ($2.12 \times 10^5$ - $6.02 \times 10^5$ cells/cm$^2$) than the winter ($9.78 \times 10^4$ - $2.5 \times 10^5$ cells/cm$^2$), due to low winter temperatures and bacterial numbers in seawater. Similarly, attachment rates were slower during the winter, a logarithmic increase, while a hyperbolic increase in cell density indicates the rapid development of the microbial film during summer conditions.

In conclusion, attachment is influenced significantly by environmental factors, particularly bacterial number in seawater. During the early period of immersion, attachment rate and density is influenced by substrate wettability. Attachment rate and density is considerably greater during the summer than in the winter.

7.5 The structure and development of attached microbial populations was found to be a characteristic of microbial films, independent of substrate and environmental factors. Typically, populations developed from random attachment events on the substrate which, with increasing length of immersion, changed to a clumped (contagious) distribution where definitive aggregates of bacteria would be recognised. The degree of clumping (population structure) was found to be a consistent factor between populations on different substrates, and suggests that microbial film development, by and large, conforms to a defined pattern. The development of population structure, indicated by the types of bacterial clumping, suggests that two mechanisms of nucleation may operate. The Newman, type A, distribution indicates that random clumps of cells develop by favourable conditioning of the immediate vicinity (Marshall *et al.*, 1971 A) around the initial randomly attached cell. The other mechanism, derived from the Polya-Aeppli distribution, suggests that clumps develop by daughter cell division of the initial attached cell.

It was apparent from this study that, although population structure was a constant factor, independent of substrate type, the diverse variety of bacteria comprising attached microbial communities was influenced by substrate surface characteristics. High surface energy (wettable) substrates had a limited range of genera, the majority either *Pseudomonas* or *Alteromonas* spp., noted for their nutritional versatility; the low surface energy (less wettable) substrates had a greater range of organisms, including isolates from the genera *Pseudomonas, Alteromonas, Vibrio, Beneckea, Flavobacterium,* and the coryneform group, suggesting a more complex population on this group of substrates.

The complexity and organisation of substrate populations were considered in this study, and it was suggested (Section 6.1) that attached microbial communities were substrate-specific. Attached populations were found to be heterogeneous with respect to genera composition and activity, but had a distinct order which could be correlated with the type of substrate. Populations appeared to be composed largely of substrate non-specific types, and only a few substrates specific types, but it is considered that the interaction of these two components results in an attached microbial community, typical of the substrate. The large proportion of non-specific substrate types in attached communities may be bacterial types which attach by non-selective chemisorption, while the substrate specific types may be bacteria which attach by time-dependent polymer synthesis mechanisms in response to surface conditions.

In conclusion, the structure of substrate populations is a factor independent of substrate type or environmental factors. The composition of the attached population is influenced by the substrate and there is evidence to suppose that populations are substrate specific.

7.6 The microbial film does not exist as an isolated uninvolved community in the marine environment; it is thought (Mitchell & Young, 1972) that attached bacteria precondition the substrate and so enable secondary colonisers such as algae and barnacles to adhere and develop. The attachment of oyster larvae, *Crassostrea virginica* is influenced by the nature of the microbial film (Young & Mitchell, 1973), and clearly demonstrates the significance not only of the microbial film, but also its composition and character on the attachment of secondary

colonisers. Microbial films have been shown to develop as integrated communities (Hargrave & Phillips, 1977), achieved by a concert interaction of component individuals. The established community may consist of a diverse variety of bacterial types, which may confer specific characteristics on the microbial film either by their interaction with other components, or as individual entities. The development of specific attached microbial film characteristics, particularly by bacteria able to synthesise vitamin $B_{12}$ (Starr, Jones & Martinez, 1957), an algal growth factor, or bacteria which precipitate calcium carbonate (Greenfield, 1963; Billy, Blanc & Rouvillois, 1976), a component of algal holdfasts, may have a significant influence on algal settlement and development.

7.7 In this study, the mechanism of microbial attachment, the dynamics of microbial film development, and attached microbial community structure, were examined for populations in the natural environment. It is felt that expansion of this work could be made in specific areas, namely the development and structure of attached communities and the interaction of microbial films with secondary colonisers, particularly algae. Work in these two areas would be complementary in that an understanding of community structure, function, and activity would be of direct relevance to an understanding of the role of the microbial film in algal zoospore settlement.

7.7.1 Particular areas of research in microbial film structure are required to determine :

(i) the nature and influences of microbial interactions during the development of the microbial film;

(ii) ascertain the activities of individuals and quantify the predominance of the activity in the community, e.g. vitamin $B_{12}$ production, by principle component and factor analysis.

7.7.2 The influence of the microbial film on algal zoospore settlement has been considered by Tosteson and Corpe (1975) and, more especially, by Dempsey and Fletcher (1976). Suggested work in this area could include a detailed study of zoospore settlement on microbial films of mixed communities of known composition and activity, in order that zoospore / bacterial interaction can be accurately quantified and settlement influencing factors identified.

# APPENDIX I

## STATISTICAL ANALYSIS OF VIABLE COUNTS

# APPENDIX I

## STATISTICAL ANALYSIS OF VIABLE COUNTS

### I.1 Statistical Analysis of Viable Counts on Spread Plates

Plate counts are a discontinuous variable, and are not normally distributed. In order that confidence limits can be calculated, the distribution species were determined using the chi-squared variance to mean ratio test (Elliott, 1971). The precision and hence confidence, which can be placed in the cell counts were determined by calculating the percentage standard deviation ($\%\delta_n$) (Jennison & Wadsworth, 1939) where an index of reliability is accepted if the percent standard deviation does not exceed 5% of the mean. For other biological data, an index of reliability is demonstrated when the $\%\delta_n$ value does not exceed 10% (Southwood, 1971).

#### I.1.1 Chi-squared variance to mean ratio test

Small samples ($n < 31$)

The chi-squared value was calculated by

$$\chi^2 = \frac{\Sigma(x^2) - \bar{x}\,\Sigma x}{\bar{x}}$$

where $x$ was the bacterial count on a replicate plate. Agreement with a Poisson distribution was accepted ($p > 0.05$) if the value of $\chi^2$ lay between the 5% significance levels for $n-1$ degrees freedom ($n$ = the number of replicate plates) in Table 8 (Pearson & Hartley, 1966). If the $\chi^2$ value exceeded the value for $Q = 0.025$, $v = n-1$, then a contagious distribution was indicated.

I.1.2 Calculation of standard deviation of sample means

(Elliott, 1971)

Sample means ($n < 30$)

*Poisson distribution* : The standard deviation of sample means ($\sigma n$) was calculated as :

$$\bar{x} \pm \sqrt{\frac{\bar{x}}{n}}$$

*Contagious distribution* : When the variance exceeded the mean ($s^2 > \bar{x}$), the counts (x) were normalised, and for small samples this was done by a logarithmic transformation. The values of x were transformed (log. $x = y$), and the mean of the transformed counts ($\bar{y}$) calculated. The variance of the transformed counts was given by :

$$s^{2'} = \frac{\Sigma(\log. x - \bar{y})^2}{n-1}$$

where n = the number of replicates. The standard deviation of sample means was calculated as :

$$\bar{y} \pm \sqrt{\frac{s^{2'}}{n}}$$

The product was expressed as a derived mean (the antilog. of $\bar{y}$) and derived standard deviation of sample means as :

$$\bar{x} \quad \stackrel{x}{\div} \quad \delta n$$

I.1.3 Calculation of percentage standard deviation (% $\sigma n$)

The standard deviation of sample means was expressed as a percentage of the mean :

$$\% \, \delta n = \frac{\delta n}{\bar{x}} \times 100$$

and should not exceed 5% for plate counts, or 10% for general biological data.

## 1.2 Kruskal-Wallis Test

The Kruskal-Wallis test is a non-parametric one-way analysis of variance, where the null hypothesis (*Ho*) states that there is no difference in mean levels between samples. The efficiency of this method compared to the parametric analysis of variance method is 96% (Elliott, 1971); the marginal inefficiency of the method outweighs the disadvantages of tedious transformations and complex calculations required before parametric tests could be applied.

### I.2.1 Ranking

Samples (media tested) were ordered as a vertical column, and the counts (replicates) arranged horizontally opposite their respective sample. Each count was ranked, the lowest designated 1 up to the highest x. Equal counts were given tied ranks. The ranks for each sample were then totalled to give $R_1$, $R_2$, to $R_i$. The number of counts per sample was totalled to give $N$.

### I.2.2 Calculation of K

K was calculated as :

$$K = \frac{12}{N(N+1)} \sum \frac{(R_i^2)}{n_i} - 3(N+1)$$

K was referred to in Table 8 (Pearson & Hartley, 1966) in column Q = 0.05, where $v = i - 1$ degrees of freedom, and $i$ = the number of samples. If K exceeded the tabulated value, the *Ho* was rejected at the 5% level ($P < 0.05$).

# APPENDIX II

## MEDIA

# APPENDIX II

## MEDIA

All quantities shown are given on a w/v basis. Peptone, unless indicated, is Bacto-peptone - Difco Ltd; yeast extract and agar, Oxoid Ltd; all reagents are standard laboratory grade - B DH Ltd. Seawater was collected from the Menai Straits and stored in a closed circuit, aerated 450 litre seawater system . All media were steam sterilised at 121$^{o}$C for 15 minutes.

(i) Anderson's Marine Agar (Anderson, 1962)

| | |
|---|---|
| Bacto-peptone | 2.5 g |
| Yeast Extract | 2.5 g |
| $FePo_4$ | 0.1 g |
| Agar | 12.0 g |
| Seawater | 900 ml |
| Distilled Water | 100 ml |
| pH | 7.6 |

(ii) Johnson's Marine Agar (Johnson, 1968)

| | |
|---|---|
| Bacto-peptone | 5.0 g |
| Yeast Extract | 1.0 g |
| $FeSo_4.7\,H_2O$ | 0.2 g |
| $Na_2\,S_2\,O_3$ | 0.3 g |
| Agar | 12.0 g |
| Seawater | 900 ml |
| Distilled Water | 100 ml |
| pH | 7.5 - 7.6 |

(iii) Johnson's Marine Broth

Johnson's Marine Agar formulated without agar.

(iv) Marine Peptone Yeast Agar

| | |
|---|---|
| Bacto-peptone | 5.0 g |
| Yeast Extract | 2.0 g |
| NaCl | 15.0 g |
| Distilled Water | 1000 ml |
| pH | 7.6 |

(v) Marine Peptone Yeast Broth

Marine Peptone Yeast Agar formulated without agar.

(vi) Nutrient Agar (Oxoid Ltd.)

| | |
|---|---|
| "Lab-Lemco" Beef Extract | 1.0 g |
| Peptone (L 37) | 5.0 g |
| Yeast Extract | 2.0 g |
| Agar | 12.0 g |
| Seawater | 900 ml |
| Distilled Water | 100 ml |
| pH | 7.6 |

(vii) Peptone Yeast Agar with Synthetic Seawater

| | |
|---|---|
| Bacto-peptone | 5.0 g |
| Yeast Extract | 2.0 g |
| Agar | 12.0 g |
| Synthetic Seawater | 900 ml |
| Distilled Water | 100 ml |
| pH | 7.6 |

(viii) Peptone Yeast Agar with Seawater

Peptone Yeast Agar formulated with natural seawater.

(ix) ZoBell's Marine Agar 2216 E (Oppenheimer & ZoBell, 1952)

| | |
|---|---|
| Bacto-peptone | 5.0 g |
| Yeast Extract | 1.0 g |
| $FePO_4$ | 0.1 g |
| Agar | 12.0 g |
| Seawater | 900 ml |
| Distilled Water | 100 ml |
| pH | 7.5 - 7.6 |

(x) Synthetic Seawater (MacLeod, Onofrey, Norris, 1954)

| | |
|---|---|
| NaCl | 23.476 g |
| $Na_2SO_4$ | 3.917 g |
| $NaHCO_3$ | 0.192 g |
| K Cl | 0.664 g |
| K Br | 0.096 g |
| $Mg\ Cl_2$ | 4.981 g |
| $Ca\ Cl_2$ | 1.102 g |
| $Sr\ Cl_2$ | 0.024 g |
| $H_3BO_3$ | 0.026 g |
| Distilled water | 1000 ml |

# APPENDIX III

## FORMULATION OF ANTIFOULING BASE PAINT

# APPENDIX III

## FORMULATION OF ANTIFOULING BASE PAINT

The paint sample was provided by I.C.I. Ltd., Mond Division - Paint Code 3/19 T.

| | wt(%) |
|---|---|
| Alloprene R 10 | 16.4 |
| Titanium Dioxide | 46.0 |
| Toxin | 0 |
| Plasticiser (TCP) | 7.2 |
| Resin | 3.4 |
| Solvent | 27.0 |

TCP - Tricresyl phosphate

Solvent - 4:1 w/w Xylene : Aromosol H

# APPENDIX IV

## ORGANISM ISOLATION CODES AND IDENTIFICATION DETAILS

| | ORGANISM NUMBER | LENGTH OF IMMERSION HOURS | SUBSTRATE | | ORGANISM NUMBER | LENGTH OF IMMERSION HOURS | SUBSTRATE |
|---|---|---|---|---|---|---|---|
| | 1412GA1 | 4 | Glass | * | 912PA5 | 60 | Paint |
| * | 1412GA2 | 4 | Glass | * | 312GB1 | 84 | Glass |
| * | 1412GA3 | 4 | Glass | * | 312GB3 | 84 | Glass |
| * | 1412SA1 | 4 | Steel | | 312SB1 | 84 | Steel |
| * | 1412SA2 | 4 | Steel | * | 312SB3 | 84 | Steel |
| * | 1412SA3 | 4 | Steel | * | 312SB4 | 84 | Steel |
| * | 1512RA4 | 4 | Resin | * | 1012RB1 | 84 | Resin |
| * | 1512RA5 | 4 | Resin | * | 1012RB3 | 84 | Resin |
| | 1512RA6 | 4 | Resin | * | 1012RB6 | 84 | Resin |
| | 1512RB5 | 8 | Resin | * | 1012PB1 | 84 | Paint |
| | 1512PA2 | 4 | Paint | * | 1012PB3 | 84 | Paint |
| | 1512PA6 | 4 | Paint | * | 1012PB4 | 84 | Paint |
| * | 3011G1 | 12 | Glass | * | 412GA3 | 96 | Glass |
| * | 3011G2 | 12 | Glass | | 412GB1 | 108 | Glass |
| * | 3011G4 | 12 | Glass | * | 412GB2 | 108 | Glass |
| * | 3011S1 | 12 | Steel | * | 412GB3 | 108 | Glass |
| | 3011S5 | 12 | Steel | | 412SB1 | 108 | Steel |
| | 712R1 | 12 | Resin | * | 412SB4 | 108 | Steel |
| * | 712R2 | 12 | Resin | | 1112RB1 | 108 | Resin |
| * | 712R3 | 12 | Resin | * | 1112RB2 | 108 | Resin |
| * | 712P2 | 12 | Paint | * | 1112RB3 | 108 | Resin |
| * | 712P3 | 12 | Paint | | 1112PA2 | 108 | Paint |
| | 712P4 | 12 | Paint | * | 1112PA4 | 108 | Paint |
| * | 112GB3 | 36 | Glass | * | 1112PA5 | 108 | Paint |
| * | 112GB4 | 36 | Glass | * | 512GB2 | 132 | Glass |
| * | 112GB5 | 36 | Glass | * | 512GB4 | 132 | Glass |
| * | 112SB2 | 36 | Steel | | 512SB3 | 132 | Steel |
| * | 112SB3 | 36 | Steel | * | 512SB5 | 132 | Steel |
| | 112SB4 | 36 | Steel | * | 512SB6 | 132 | Steel |
| * | 812RB2 | 36 | Resin | | 1212RB1 | 132 | Resin |
| | 812RB4 | 36 | Resin | * | 1212RB5 | 132 | Resin |
| | 812RB5 | 36 | Resin | * | 1212RB6 | 132 | Resin |
| * | 812PB2 | 36 | Paint | * | 1212PB1 | 132 | Paint |
| * | 812PB3 | 36 | Paint | | 1212PB4 | 132 | Paint |
| | 812PB4 | 36 | Paint | * | 1212PB6 | 132 | Paint |
| * | 212GB1 | 60 | Glass | * | 612GB1 | 156 | Glass |
| * | 212GB2 | 60 | Glass | | 612GB3 | 156 | Glass |
| * | 212GB4 | 60 | Glass | * | 612GB6 | 156 | Glass |
| * | 212SB2 | 60 | Steel | * | 612SB2 | 156 | Steel |
| | 212SB3 | 60 | Steel | * | 612SB4 | 156 | Steel |
| * | 212SB4 | 60 | Steel | * | 612SB6 | 156 | Steel |
| | 912RB1 | 60 | Resin | * | 1312RB1 | 156 | Resin |
| | 912RB4 | 60 | Resin | * | 1312RB2 | 156 | Resin |
| | 912RB5 | 60 | Resin | * | 1312RB5 | 156 | Resin |
| * | 912PA1 | 60 | Paint | * | 1312PA1 | 156 | Paint |
| | 912PA2 | 60 | Paint | * | 1312PB3 | 156 | Paint |
| | | | | * | 1312PB5 | 156 | Paint |

* Motile organisms

| ORGANISM NUMBER | GENUS |
|---|---|
| 1412GA1 | Coryneform |
| 1412GA2 | Alteromonas vaga |
| 1412GA3 | Alteromonas sp. |
| 1412SA1 | Coryneform |
| 1412SA2 | Alteromnas sp. |
| 1412SA3 | Pseudomonas/Alteromonas grp. |
| 1512RA4 | Pseudomonas/Alteromonas grp. |
| 1512RA5 | Pseudomonas sp. |
| 1512RA6 | Coryneform |
| 1512RB5 | Coryneform |
| 1512PA2 | Coryneform |
| 1512PA6 | Coryneform |
| 3011G1 | Unknown |
| 3011G2 | Pseudomonas sp. |
| 3011G4 | Pseudomonas sp. |
| 3011S1 | Pseudomonas/Alteromonas grp. |
| 3011S5 | Moraxella-like group |
| 712R1 | Micrococcus sp. |
| 712R2 | Pseudomonas/Alteromonas grp. |
| 712R3 | Beneckea sp. |
| 712P2 | Pseudomonas/Alteromonas grp. |
| 712P3 | Pseudomonas/Alteromonas grp. |
| 712P4 | Coryneform |
| 112GB3 | Alteromonas sp. |
| 112GB4 | Alteromonas sp. |
| 112GB5 | Alteromonas sp. |
| 112SB2 | Unknown |
| 112SB3 | Pseudomonas sp. |
| 112SB4 | Moraxella-like group |
| 812RB2 | Coryneform |
| 812RB4 | Coryneform |
| 812RB5 | Flavobacterium sp. |
| 812PB2 | Pseudomonas/Alteromonas grp. |
| 812PB3 | Pseudomonas/Alteromonas sp. |
| 812PB4 | Flavobacterium sp. |
| 212GB1 | Pseudomonas sp. |
| 212GB2 | Alteromonas sp. |
| 212GB4 | Coryneform |
| 212SB2 | Alteromonas sp. |
| 212SB3 | Micrococcus sp. |
| 212SB4 | Unknown |
| 912RB1 | Coryneform |
| 912RB4 | Flavobacterium sp. |
| 912RB5 | Micrococcus sp. |
| 912PA1 | Coryneform |
| 912PA2 | Unknown |
| 912PA5 | Vibrio sp. |

| ORGANISM NUMBER | GENUS |
|---|---|
| 312GB1 | Alteromonas sp. |
| 312GB3 | Alteromonas sp. |
| 312SB1 | Moraxella-like group |
| 312SB3 | Alteromonas sp. |
| 312SB4 | Unknown |
| 1012RB1 | Coryneform |
| 1012RB3 | Alteromonas sp. |
| 1012RB6 | Alteromonas sp. |
| 1012PB1 | Pseudomonas sp. |
| 1012PB3 | Pseudomonas/Alteromonas grp. |
| 1012PB4 | Pseudomonas sp. |
| 412GA3 | Alteromonas sp. |
| 412GB1 | Moraxella-like group |
| 412GB2 | Unknown |
| 412GB3 | Alteromonas sp. |
| 412SB1 | Moraxella-like group |
| 412SB4 | Alteromonas sp. |
| 1112RB1 | Micrococcus sp. |
| 1112RB2 | Alteromonas sp. |
| 1112RB3 | Pseudomonas/Alteromonas grp. |
| 1112PA2 | Coryneform |
| 1112PA4 | Pseudomonas/Alteromonas grp. |
| 1112PA5 | Alteromonas sp. |
| 512GB2 | Coryneform |
| 512GB4 | Alteromonas vaga |
| 512SB3 | Coryneform |
| 512SB5 | Pseudomonas/Alteromonas grp. |
| 512SB6 | Coryneform |
| 1212RB1 | Micrococcus sp. |
| 1212RB5 | Alteromonas sp. |
| 1212RB6 | Pseudomonas sp. |
| 1212PB1 | Pseudomonas/Alteromonas grp. |
| 1212PB4 | Flavobacterium sp. |
| 1212PB6 | Vibrio sp. |
| 612GB1 | Pseudomonas/Alteromonas grp. |
| 612GB3 | Pseudomonas sp. |
| 612GB6 | Pseudomonas/Alteromonas grp. |
| 612SB2 | Pseudomonas/Alteromonas grp. |
| 612SB4 | Alteromonas sp. |
| 612SB6 | Alteromonas sp. |
| 1312RB1 | Alteromonas sp. |
| 1312RB2 | Alteromonas sp. |
| 1312RB5 | Vibrio sp. |
| 1312PB1 | Pseudomonas sp. |
| 1312PB3 | Pseudomonas/Alteromonas grp. |
| 1312PB5 | Pseudomonas/Alteromonas grp. |

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