MICROBIAL INVOLVEMENT IN TYPE 1½ PITTING OF COPPER

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by

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ABSTRACT

This thesis reports upon an investigation into the involvement of microorganisms in an unusual form of pitting corrosion occurring in copper water pipes, which over the last few years has come to be termed Type 1½ pitting.

Type 1½ pitting has led to the failure of copper tube carrying warm or cold water in a very small number of institutional buildings spread throughout the world. This form of pitting is very characteristic and has so far been seen mainly in areas supplied with soft waters. Failures of the pipes can be rapid with perforations being reported only a few months after replacement pipe has been installed.

This work has clearly demonstrated, for the first time, the involvement of microorganisms with Type 1½ pitting, through surveys using; the scanning electron microscope (SEM), along with field isolations from service water pipes and subsequent examination for corrosion characteristics. A novel form of static bed reactor was designed in which the pitting of copper rings was clearly associated with the presence of a consortium of bacteria.

Having demonstrated this link, an investigation of the physiology of the isolated bacteria, particularly in relation to exopolysaccharide production and biofilm formation, clearly showed a link with the conditions known to prevail in water pipes exhibiting Type 1½ pitting. From these studies it has been possible to propose a mechanism for the formation of biofilms within such pipes. Particular attention has been paid to temperature, nutrient levels and nutrient limitation. Flow conditions, including flow rates and stagnation periods were also found to be important. It has been possible to demonstrate that the conditions prevailing in pitted water pipes will favour biofilm formation.

Partial characterisation of the isolated exopolysaccharide material produced by the bacteria has been carried out and shown to be similar to those produced by other bacteria known to be involved in corrosion. This has led to the speculation that the exopolysaccharide is directly involved in the pit initiation. The potential for the bacteria and their associated exopolysaccharides to cause the dissolution of copper has also been demonstrated. The possible involvement of low molecular weight metabolic products has been considered with some preliminary studies using hydrogen peroxide yielding a form of pitting reminiscent of Type 1½ pitting.
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Chapter 1
INTRODUCTION

1.1 Types of pitting corrosion affecting copper

Copper along with brass was one of the first metals employed by man having been in use since 3000 BC. Copper is a relatively noble metal, which means that copper has a high position in the electromotive force series. Mattsson (1980) lists a number of applications for which copper has been used and for which it could be used in the future. In many countries copper has become the material of choice for the construction of water pipe systems for fresh water supplies, due to its excellent resistance to general corrosion and good workability. However, copper is not totally resistant to attack and it is well known that under certain conditions copper is an unsuitable material. In soft, aerated waters containing ions such as ammonia, corrosion is known to be severe. General attack or cuprosolvency is known to be a problem, normally in very soft, acidic waters. Also, under conditions of severely turbulent flow, localised pitting, known as impingement attack, may develop (Gilbert, 1969, Mattsson, 1980). This report, however, is concerned with a specific form of attack known as pitting corrosion. Pitting is attack in a defined area resulting in the establishment of a pit with both anodic and cathodic reactions which are self-sustaining. For a number of years the pitting corrosion of copper could be divided into two forms, which Campbell (1954) initially designated “hard” and “soft” water pitting, but later renamed Types 1 & 2 (Campbell, 1970). Both forms have been well researched over the last forty years and are well understood. A Type 3 pitting was described in the mid seventies (von Franqué, 1975), and in the last few years two further types of pitting have been identified which are thought to have a microbial association (Fischer et al., 1988; Keevil et al., 1989 and Chamberlain and Angell, 1991).
1.1.1 Classification of Types 1, 2 & 3 pitting corrosion of copper.

Type 1 pitting is dependent on the presence of an impermeable, usually carbon, film on the inner surface of the pipe. These films may be formed during the manufacturing process by the breakdown of drawing lubricants during bright annealing. Copper tubes are now blasted e.g. with chilled iron grit, to remove any carbon films formed during their manufacture. The characteristics of Type 1 pitting are as follows: well defined hemispherical pits are formed, which contain soft crystalline copper (I) oxide below a coherent but perforated membrane of copper (I) oxide which is pseudomorphous with the original copper surface. Copper (I) chloride is also present in varying amounts depending on the rate of corrosion. Immediately above the pit is a mound of basic copper carbonate. Surrounding the pits, there is typically a deposit of calcium carbonate crystals, stained green with corrosion products, overlying a very thin film of carbon, beneath which a smooth shiny layer of copper (I) oxide is formed.

The currently accepted mechanism of pitting was described by Lucey (1967). His proposals for the structure and reactions occurring within the pit are shown in Figure 1.1. In summary, an essential feature of the corrosion process appears to be the formation of an electrically conducting porous membrane of copper (I) oxide, separated from the copper surface by a pocket of electrolyte with a high copper (I) ion concentration. These two conditions will be met if chloride ions are present in sufficiently high concentrations, such that the rate of formation of copper (I) chloride exceeds the rate of loss of copper (I) ions into the bulk water, leading to the formation of a film of copper (I) chloride between the copper and the oxide layer first formed. Lucey showed that the copper (I) oxide membrane acts as a bipolar electrode. Copper (I) ions, diffusing outwards through the membrane come
into contact with aerated water and are oxidised to copper (II) ions. Some are then precipitated as insoluble basic salts, while others are reduced back to copper (I) and are available to repeat the oxidation/reduction cycle. The overall driving force of this reaction is maintained by the large differences in copper (I) ion concentration at either side of the membrane. From the above discussion it can be seen that the role of the chloride ions is of immense importance to the pitting process.

Lucey (1982) also showed that the pitting propensity of waters capable of supporting Type 1 pitting depends on a complex interaction between pH and dissolved oxygen, sulphate, sodium and nitrate contents.

Type 2 pitting does not involve carbon films and occurs in soft waters above 60°C with a bicarbonate to sulphate ratio greater than one. Type 2 pitting is characterised by the formation of deep, narrow corrosion pits which often show branching as they progress through the tube wall. The pits contain hard, crystalline...
copper (I) oxide and are usually covered by small nodules of corrosion product consisting of copper oxides and basic copper sulphate. The tube surface between the pits carries an adherent dull film consisting principally of copper (II) oxide, but with some copper (I) oxide present. A thin layer of water-deposited silt commonly overlies the whole surface, including the nodules above the pits.

Lucey (1982) thought that Type 3 pitting (von Franqué, 1975) was a modification of Type 1 pitting, however, the term Type 3 pitting was accepted by Linder & Lindman (1983) and used to designate two cases of failure of copper pipes in Sweden. The failures were in cold water systems with high pH, low hardness and low mineral content. The pitting was characterised by the formation of rather broad areas comprising numerous small hemispherical, pits under a common covering of basic copper sulphate. The surface between the pitted areas carried a thin copper (I) oxide film - in some places little more than a heavy tarnish. The pits contained copper (I) oxide with up to 1% sulphide.

1.1.2 New forms of pitting.

The two recently reported forms of pitting both occurred mainly in large institutional buildings supplied with soft water. One form resembles that described previously as Type 3 pitting in that it produces multiple pits below a basic copper sulphate crust; it has been termed “pepper pot” pitting and has mainly been reported from a few areas in south west Scotland.

The other “new” type of pitting was first reported in 1985 from a hospital in Saudi Arabia. Pitting was found to occur in both the cold and warm water circuits and had some of the characteristics of both Types 1 & 2 pitting, it has therefore been termed Type 1½ pitting. The next reported incidence was in a recently opened
German hospital in 1985 and has been well documented by Fischer et al. (1991). Earlier, Fischer et al. (1988) had noted the presence of a gelatinous film which could be lifted from the pipes with dilute acid. Corrosion chemists frequently use 25% nitric acid to lift the carbon films responsible for Type 1 pitting from copper pipes, in such cases the carbon film floats to the surface. However, Fischer noted that in this case the films lifted were of a gelatinous nature and sank. Chamberlain, Angell & Campbell (1988), using one of the techniques that they had specially adapted for this purpose, were able to show that the film contained patches of polysaccharide which stained magenta with the periodic acid-Schiff reagent. This specifically stains the vic-glycol groups found in polysaccharides. These results fuelled the speculation that there was a microbial influence on the pitting process. This thesis describes the subsequent work carried out to investigate the Type 1½ pitting occurring at the Hellersen County Hospital, West Germany and at two further hospitals subsequently identified as exhibiting Type 1½ pitting in the south west of England.

1.2 Microbially Influenced Corrosion (MIC).

MIC has been described as the venereal disease of industry; it is powerful, incapacitating and extremely expensive but usually unmentionable (White et al., 1991). At the outset of any discussion on microbially associated corrosion it needs to be noted that there are a number of mechanisms whereby microorganisms can exert an effect. Menzies (1971) lists five such methods as follows;

1. the production of corrosive metabolic products;

2. the production of differential aeration and concentration cells;

3. depolarization of cathodic processes;
4. disruption of natural and other protective films; and

5. the breakdown of corrosion inhibitors.

The bulk of the literature on the involvement of microorganisms in the corrosion of metals details anaerobic corrosion of mild steel, caused by the sulphate reducing bacteria (SRB) and characterised by the presence of black iron sulphides. These act through cathodic depolarisation (point 3 above) and/or the corrosive nature of the metabolically formed sulphide ions (point 1 above). This form of corrosion is discussed here as initially SRB were suspected of being responsible for Type 1½ pitting of copper.

SRB are obligate anaerobes that use sulphate as the terminal electron acceptor for their respiratory metabolism, thereby producing significant amounts of sulphide. SRB are generally unable to utilise carbohydrate sources or biopolymers for growth, preferentially utilising short chain organic acids or alcohols (Hamilton, 1991). Many species can additionally oxidise hydrogen as a source of metabolic energy and reducing power (Widdel, 1988).

During corrosion processes metal ions are lost from the surface by the passage into solution of positively charged metal ions at the anode of an electrochemical cell. This needs to be counter balanced by the transfer of the excess electrons to a cathodic area of the metal substratum, where a secondary reaction can absorb the electrons. Therefore the simple, non-stoichiometric, equation describing the aerobic corrosion, or rusting, of iron can be defined as follows:

\[
\begin{align*}
Fe & = Fe^{2+} + 2e^- & \text{anode} \\
H_2O + \frac{1}{2}O_2 + 2e^- & = 2OH^- & \text{cathode}
\end{align*}
\]
This leads to the formation of complexes of hydrated iron oxides and hydroxides as corrosion products.

In a situation where oxygen is absent, an anaerobic environment, it has been suggested (Hamilton, 1991) that protons or hydrogen sulphide might act as an electron acceptor at the cathode.

\[ 2H^+ + 2e^- = 2H = H_2 \]

\[ 2H_2S + 2e^- = 2HS^- + H_2 \]

N. B. HS\(^-\) will only be formed in waters with a pH above 7 otherwise H\(_2\)S will be formed. The subsequent oxidation of the molecular hydrogen by SRB, would prevent polarisation of the cathode by adsorbed hydrogen and give rise to sulphide and the potential for metal sulphide corrosion products.

\[ 4H_2 + SO_4^{2-} = 4H_2O + S^{2-} \]

\[ Fe^{2+} + S^{2-} = FeS \]

The cathodic depolarisation theory outlined above was at one stage felt not to account for the levels of corrosion seen in some experiments, it was shown that corrosion could also be brought about by the presence of iron sulphide itself. It was therefore proposed that sulphide could act as the cathode and that the role of the bacteria would therefore be a) to "regenerate" (or depolarize) iron sulphide, b) to produce more iron sulphide by their growth reaction, or even c) to bring fresh iron sulphide surfaces constantly into contact with the steel by their movement (Miller and King, 1971). This was accepted, but was felt to be part of a complex set of reactions (Hamilton, 1985). Further evidence for the cathodic depolarisation theory was given by Pankhania et al. (1986), who demonstrated that SRB can oxidise cathodic hydrogen and use it as a source of metabolic energy.
SRB have been shown to be active in the corrosion of copper and its alloys in the marine environment (Chamberlain et al., 1988). SRB are not felt to be active in Type 1½ pitting for the following reasons: no significant levels of sulphide have been detected in any of the hospitals exhibiting Type 1½ pitting (Campbell, personal communication), and where SRB have been detected in the systems they have been in very low numbers (McEvoy & Colbourne, unpublished data).

However, the formation of a pit in copper does follow a number of the basic electrochemical principles as described above and demonstrated by Lucey, (1967). It is generally accepted that the propagation of a corrosion pit is as follows. Firstly, a corrosion cell must be established between the surface of the metal and the pit. The corrosion current density is concentrated within the pit where it is very large, due to the hydrolysis of the dissolving metal ions which leads to an acidification of the local environment. Chloride ions are concentrated in the pit from the external solution since they carry the current flow, maintaining the metal surface in an active state (Fischer et al., 1991).

It is of interest that the SRB are able to promote pitting by the use of hydrogen as a terminal electron acceptor. This leads to the depolarisation of the cathode allowing the pit to propagate. Gerchakov et al., (1985) demonstrated that a Pseudomonas sp. was able to utilise copper in its electron transport chain with copper (I) being oxidised and copper (II) being reduced as shown in Figure 1.2. This would not only provide an electron sink but could also lead to the constant removal of dissolved copper ions and maintain the gradient necessary to keep a pit active. The bacteria responsible for microbially associated corrosion are normally present on the metal surface as part of a consortium forming a biofilm.
Figure 1.2 Electron transport chain for reduction/oxidation of copper ion by bacteria, from Gerchakov et al. (1985).

1.3 Biofilms

“A biofilm consists of cells immobilised at a substratum and frequently embedded in an organic polymer matrix of microbial origin” (Characklis & Marshall, 1989). The ubiquitous nature of biofilms was emphasised by Wardell, (1988) who stated that microbial populations can be found associated with all non-toxic surfaces found in natural environments. The formation of biofilms on a surface can be regarded as a universal bacterial strategy for survival and for optimum positioning with regard to available nutrients. Bacterial cells living in this protected mode of growth produce planktonic cells, with much reduced chances of survival. These detached cells may colonise new surfaces or may burgeon to form large planktonic populations in those rare environments where nutrients are plentiful and bacterial antagonists are few (Costerton et al., 1987).
Bacteria have a marked tendency to interact with surfaces (Zobell, 1943), this could be due to the fact that at the surface it is probable that nutrients, which are either absent or deficient in the bulk liquid, may be concentrated (Zobell and Anderson, 1936). This could either be due to adsorption of nutrients to the surface, or that the surface itself could be metabolised by the organisms (Sutherland, 1983).

In experiments specifically designed to investigate Zobell's thesis, Heukelakian and Heller (1940) grew *Escherichia coli* in phosphate buffer at a range of concentrations of glucose and peptone. It was shown that there was a critical or threshold concentration of nutrients above which cells grew distributed throughout the medium, and below which cells grew mainly associated with a surface. Similar results were obtained by Corpe (1970) investigating the colonization of glass slides by a *Pseudomonas* sp.. It has been suggested that, the surface represents a zone of nutritional affluence in a surrounding sea of poverty. Therefore the many microorganisms which are able to bind to a surface are capable of deriving great nutritional benefit (Sutherland, 1983).

Hamilton and Characklis, (1989) described the phases of biofilm development as follows,

1) transport of organic molecules and cells to the surface;

2) adsorption of organic molecules to give a "conditioned" surface;

3) adsorption of cells to the conditioned surface:

4) growth of adsorbed cells with associated synthesis of exopolymeric substances (EPS).

5) detachment of portions of the biofilm.

Wardell *et al.*, (1980), pointed out the need to distinguish between the
nonspecific attachment of bacteria as opposed to the development of microcolonies, the difference between points three and four above. Marshall et al. (1971) noted that the adsorption of microorganisms to a surface seems to follow two distinct phases. The process is initiated by the reversible binding of a bacterial cell to the surface, this phase being independent of time. The second phase, which is time dependent, involves the irreversible binding of bacteria to the surface which is achieved using exopolysaccharide glycocalyx polymers (Costerton et al., 1987).

Most of the microorganisms which adsorb to the surface are probably in a metabolically dormant state, therefore the attachment to a surface, conditioned with adsorbed nutrients, would lead to a rapid growth response (Marshall, 1980). A biofilm is produced as the cells start to divide on the surface producing daughter cells that are also bound within the glycocalyx matrix, leading to the initiation of microcolonies. Therefore, the eventual production of a continuous biofilm is the result of cell division producing microcolonies and new recruitment from the bulk planktonic phase. This means that within the biofilm considerable heterogeneity exists both with respect to the constituent microorganisms and the physicochemical microenvironments, established by the action of the organisms within the physical matrix created by the EPS and inorganic inclusions. This heterogeneity is particularly evident in the horizontal plane of the biofilm where it is referred to as "patchiness" and may have an effect on microbial corrosion (Hamilton and Characklis, 1989).

Once the heterogeneous biofilm is established it follows that throughout the biofilm there are gradients of oxygen, essential nutrients and dissolved organic carbon; this may well lead to an increased metabolic diversity with cross feeding from both autotrophic growth and cell lysis products entrapped within the biofilm. In deep biofilms, nutrient succession can become established with aerobic and
anaerobic species interacting in the degradation of complex polymer matrices, followed by the action of fermentative and acetogenic organisms carrying the catabolic activity through to the level of bacterial fermentative products.

As can be seen from the above discussion of biofilms their structure is complex involving many interactions, this therefore makes their study equally complex. A fundamental aspect of biofilms is that they are composed of structured consortia of bacteria. Many of these bacteria can be observed by direct methods of examination, but, are not seen in pure cultures especially when grown on rich media. (Costerton et al., 1987). This means that in any work on biofilms it is essential to note the nutrient levels present in their natural environment. However, the same authors go on to state that in their opinion data generated from the culture of bacteria in single species batch culture continues to be valuable. The data so obtained must be viewed as a single, and perhaps unrepresentative, point in a broad spectrum of bacterial characteristics expressed in response to altered environmental conditions. Planktonic cells grown in rich media at high growth rates in batch culture need to be seen as being structurally and functionally different from biofilm bacteria. This has led to a number of special methods being developed for the study of bacterial biofilms, some of which will be discussed later.

One of these methods, the RotoTorque biofilm reactor, has been extensively and successfully used by Characklis and his co-workers in biofilm research (Hamilton & Characklis, 1989). Using a monospecies biofilm developed in a RotoTorque biofilm reactor and the same organism in a chemostat, good correlation was seen between the maximum growth rates ($\mu_{\text{max}}$) and substrate affinity ($K_s$). This finding has been confirmed by Gilbert et al., (1989) using a novel technique for growth rate control of biofilms, showing that $\mu_{\text{max}}$ was the same for biofilm $sic$ grown cells and planktonic cells. In relation to the comparative growth of biofilm
cells and those in the surrounding bulk phase in a chemostat it has been noted that biofilm cells grew at twice the growth rate as the same organisms in the surrounding medium (Keevil et al., 1987), however, no suggestion was made that the rate of growth was higher than $\mu_{\text{max}}$ for the planktonic cells.

### 1.4 Possible role of bacterial polymers in Type 1½ pitting.

There are several possible ways in which bacteria could participate in Type 1½ pitting, but all of these are likely to involve extracellular products. These could be in the form of macromolecular products, or low molecular weight metabolic products including; CO$_2$ and organic compounds. It is likely however, that it is neither one nor the other but a result of a number of contributing factors. This feeling is confirmed by Hamilton (1985) who stated that corrosion in general and microbial corrosion in particular are complex phenomena and seldom, if ever, involve a single mechanism of a single species. In order to determine any possible effects of the polymer it is necessary to have an understanding of how bacteria produce exopolymers and what the composition of any polymers produced is, for this reason a review of the literature on the structure and synthesis of xanthan gum is given below as a representative detailed example. This is followed by a discussion of the known ability of many bacterial polymers to bind copper.

#### 1.4.1 Structure and synthesis of xanthan.

In a review of bacterial exopolysaccharides Sutherland (1977) divided them into two main types based on their monosaccharide composition; homopolysaccharides and heteropolysaccharides. The vast majority of research into bacterial polysaccharides has been carried out on just two of the many bacterial exopolysaccharides
known to be produced. These are xanthan gum, produced by species of *Xanthomonas* and alginate produced by *Ps. aeruginosa* and others. *Xanthomonas* spp. are plant pathogens and it is thought that in nature the exopolysaccharides are involved in their infection of host plants. Research into xanthan has followed two main interests, one is its role in plant infection. The other arising from its extremely high specific viscosity and pseudoplasticity, is its increasingly common use in foods as a thickening agent, in secondary and tertiary oil recovery for mobility control, and in petroleum drilling fluids. Alginates have proved to be of additional interest due to their involvement in the human illness, cystic fibrosis as well as their almost ubiquitous use in processed foods etc.. The bacterial alginates are similar to the alginates produced by algae, being composed of repeating units of D-mannuronic acid and L-guluronic acid. The detailed structure and synthesis of alginates is well detailed in the literature (Piggott *et al*., 1981; Anderson *et al*., 1987).

As most bacterial exopolysaccharides are synthesised from nucleoside diphosphate sugars (Sutherland, 1977), in this review the structure and synthesis of xanthan will be considered as a typical example. Xanthan gum was known to contain D-glucose, D-mannose, D-glucuronic acid in a 2:2:1 ratio, acetal linked pyruvic acid, and O-acetyl substitutents (Sloneker *et al*., 1962) and many tentative structures were proposed. The accepted structure of the xanthan repeating unit was first proposed by Jansson *et al*. (1975). It was proposed that the repeating unit had five sugar residues as shown in Figure 1.3. This structure was independently confirmed by Melton *et al*. (1976) using a different experimental procedure. Most of the recent literature now credits both papers with the structure of the xanthan repeating unit.

As mentioned earlier the mechanism for the formation of most exopolysaccharides by Gram negative bacteria involves *de novo* formation of glycosidic bonds from activated hexoses. This mechanism represents a significant drain on the carbon
source assimilated by the organism and probably on the energy made available to the organism (Sutherland 1977). As high energy \textit{sic} phosphate bonds will be utilised in the proposed transference reaction for polymerisation involving polyisoprenyl phosphates, it was assumed that one high energy \textit{sic} phosphate bond is utilised per "subunit" of xanthan. It has therefore been estimated that eleven ATP equivalents are utilised for each subunit of five hexoses polymerised (Jarman and Pace, 1984). Despite this high demand for ATP in xanthan production it has been shown that \textit{X. campestris} is an extremely efficient immobilised multi-enzyme system converting carbohydrate substrate to polymer to an extent of 70 % or more, hence the widespread use of this species in the commercial production of xanthan.

The first evidence on the mechanism of the \textit{in vitro} assembly of xanthan was described by Ielpi \textit{et al.} (1981). It was shown that UDP-glucose, UDP-mannose and
UDP-glucuronic acid sequentially donated their sugar moieties to a lipidic acceptor to form a pentasaccharide-P-P-lipid which was subsequently polymerised into xanthan gum as a two stage process. It was later demonstrated that the incorporation of the pyruvic acid acetal residues occurs at the first stage, once the pentasaccharide is assembled on the "lipid intermediate", and that the donor is phospho-enol-pyruvate (Ielpi et al., 1981b). It has been demonstrated that acetylation occurs also at the "lipid intermediate" stage and that acetyl-CoA is the donor (Ielpi et al. (1983).

The above evidence was summarised by Betlach et al. (1987) with the diagrammatic pathway shown in Figure 1.4. The pathway was also described as follows: “In this pathway, the pentameric repeating unit is assembled on an isoprenoid lipid carrier represented here as C55. The five sugars are donated by sugar nucleotide diphosphates via five specific glycosyl transferases. The repeating unit is acetylated by a specific enzyme, acetylase, utilising acetyl coenzyme A as the donor substrate. It is also pyruvylated by a specific ketalase utilising phospho(enol)pyruvate. Each step requires specific substrates and a specific enzyme to take place. If either the substrate or the enzyme is absent, the step is blocked. The activated xanthan molecule is thought to be added to the lipid-linked repeating unit by a “tail-to-head” polymerisation. The carrier lipid displaced from the xanthan molecule is recycled through a dephosphorylation step. The polymer is ultimately released into the medium.”

Betlach et al. (1987) also showed that mutational interruption of the pathway for assembly of the lipid-linked pentasaccharide repeating unit of xanthan leads to the synthesis of lipid-linked truncates of the repeating unit, for example, lipid-linked trisaccharide. These trisaccharide repeating units are then assembled on the lipid carrier and subsequently polymerised.
Figure 1.4 Diagrammatic pathway for the synthesis of xanthan. UDPG=uridine-5'-diphosphoglucose; GDPM=guanidine-5'-diphosphomannose; UDP-GA=uridine-5'-diphosphoglucuronic acid; GG=cellobiose; C55=isoprenoid lipid carrier; PEP=phospho(enol)pyruvate; Acetyl Co A= acetyl coenzyme A; I-V=glycosyltransferases I-IV; Ac=acetate; and Pyr=pyruvate. (Betlach et al. 1987)

An extensive genetic and biochemical study on the biosynthesis of xanthan gum was carried out by Vanderslice et al. (1989) which reported that the genes involved in the biosynthesis of the xanthan repeating unit, its polymerisation and extrusion make up a cluster of twelve genes.

A cosmid clone pIJ3020 containing DNA from a pathogenic strain of Xanthomonas campestris was shown to be able to complement a non-pathogenic mutant defective in the synthesis of extracellular enzymes (Daniels et al. 1984). Transposon mutagenesis of pIJ3020 Tang et al. (1991) demonstrated the presence of a cluster of, probably, seven genes, mutation in any of which resulted in the abolition of extracellular polysaccharide synthesis. They were able to sequence these genes for which the designation rpf (regulation of pathogenicity factors) was proposed.
1.4.2 Bacterial exopolymers in metal binding and corrosion.

Many bacteria are known to be able to produce copious amounts of "slime", i.e. extracellular polysaccharides (EPS) and proteinaceous material. It has been demonstrated that EPS has the ability to bind many heavy metals, including copper, as some residues at least have a net negative charge. This has lead to the suggestion of a role in corrosion. However, to date their role in pitting has not been demonstrated, although they are associated with general corrosion. Their role in anchoring cells to surfaces and holding biofilms together has been well documented, as have the environmental conditions under which many bacteria produce EPS.

The role of bacteria and their polymers in the removal of heavy metals from sewage sludge was reviewed by Brown and Lester, (1979). It was shown that EPS plus proteins and nucleic acids could all bind heavy metals including copper. Many examples of metal binding by bacterial EPS were cited.

The stability constants (K_j) are a useful measure of the metal binding capacity of bacterial polymers. The stability constant for complexes formed between Klebsiella aerogenes extracellular polymer and copper, cadmium, cobalt and nickel have been calculated using a gel chromatographic technique (Rudd et al., 1984). Copper was seen to have the highest \log K_j of 7.69 compared to 5.16, 5.48 and 5.49 respectively for the others, this shows that copper has a high affinity for forming complexes with this polymer, it also needs to be noted that the paper does not state whether natural logs are used or logs to the base 10.

Geesey and Mittelman, (1985) reported that many of the polymers which anchored cells to surfaces had a high affinity for copper. A stability constant of 6.1 x 10^8 was reported for copper (II) ions at neutral pH and EPS from a bacterium
isolated from a metal-laden river sediment. It was also noted that pH affected the copper binding ability of the polymer; approximately six times more copper could be bound at pH 5 than at 7. However, the metal appeared to be bound more firmly at the higher pH. Geesey and Mittelman (1985) also noted that it was the free copper (II) ions which are toxic to bacteria. The binding of the copper (II) ions by such polymers was suggested as a mechanism by which bacteria overcome the toxic effect of copper. The polymer produced by *Deleya marina* has also been shown to bind strongly to copper (Ford *et al.*, 1987).

Auger electron spectroscopy and x-ray photoelectron spectroscopy have been used to show that gum arabic and a bacterial culture supernatant (BCS) were able to oxidise copper to copper (II) and that copper was also incorporated into the polymer matrix by gum arabic, BCS and *Ps. atlantica* exopolymer (Jolley *et al.*, 1989). Although this work showed the removal of copper from a surface this needs to be distinguished from actual pitting. However, the oxidation of the copper surface to copper (II) is of interest as an unusual feature of Type 1½ pitting is that black copper (II) oxide is formed in both cold and warm water pipes. Copper (II) oxide is normally formed only in pipes full of water above 60 °C. No mention is made in the paper as to what might be causing this oxidation of the copper.

Fourier transform - infrared (FT-IR) spectroscopy may be used as a rapid and non-destructive method for the analysis of bacteria and bacterial polymer matrices. Nichols *et al.*, (1985) were able to distinguish amide I bands and a strong carbonyl stretch at 1740 cm⁻¹ using FT-IR on a biofilm. The technique was thought to have great potential for the non-destructive, real-time measurement of biofilm formation and MIC studies.

Iwaoka *et al.*, (1986) took the method a stage further by developing a technique
for coating a copper film onto the surface of a cylindrical germanium internal reflectance element suitable for use in the attenuated total reflection (ATR) version of FT-IR spectrometry. The copper coat was found to be sufficiently stable to be exposed to an aqueous solution of polysaccharide for more than forty hours. These internal reflection elements were then used to sample the solid-liquid interface of metallic copper films submerged in an aqueous acidic polysaccharide solution.

The technique of attenuated total reflectance FT-IR spectroscopy (Geesey et al., 1989) was used to detect components of the exopolymer elaborated by the film forming bacteria that accumulated on the thin copper surface.

Attenuated total reflectance works on the principle that as infrared radiation is reflected internally through a germanium internal reflectance element (IRE), submerged in water, an evanescent wave of radiation penetrates the element surface into the surrounding aqueous phase, Figure 1.5. An IR spectrum can be obtained for the molecular species in the aqueous environment near the surface of the element. The depth of penetration of the evanescent wave is dependent upon the refractive indices of the IRE, the medium surrounding the element, and the wavelength of the radiation. The shallower the depth of penetration, the narrower the lens of water sampled. A penetration of approximately 350 nm is obtained with radiation of 1640 cm\(^{-1}\) through a germanium IRE into water. It has been shown (Geesey et al., 1989) that the depth of penetration can be decreased by depositing a thin copper film onto the surface of the IRE. A water absorption peak can still be detected with a copper film of up to 3.5 nm. A film between 1 & 2 nm in thickness provided a water absorption intensity that was twenty to forty percent of the bare germanium IRE. This means that differences in the thickness of the copper film can be monitored with extreme sensitivity (0.2-0.3 nm) by the differences in the intensity of the absorption bands of the water surrounding the film.
Figure 1.5 Schematic diagram of circular internal reflection element in open boat cell

Geesey and Bremer (1991) have used this technique to demonstrate that a bacterial culture designated CCI 8 could cause corrosion of the thin copper film whilst another culture CCI 11 could not. Both bacteria were isolated from a corroding copper coupon exposed to flowing municipal water. Both bacteria were described as rod shaped, Gram variable, facultative anaerobes.

1.5 Effect of growth conditions on exopolymer production

An important question, if polymers are involved in the pitting process, is under what conditions are the polymers produced and are these conditions likely to occur in the pipes where pitting is seen to take place. Many studies on the production of exopolymers by various bacteria have been reported in the literature, this research has normally been due either to the commercial value of the bacterial polymer (e.g.
xanthan) or due to the possible implication of the bacterial polymer in certain diseases.

The bulk of the early work involved studying the growth of bacteria on agar plates. This has now been superseded by studies on bacteria grown in planktonic phase using either batch or chemostat cultures. Recent work on the growth and polymer production of bacteria attached to a surface, will be dealt with in a later section.

Polymer production can be affected either by the limitation of a substance essential to growth or by environmental conditions such as pH or temperature.

1.5.1 Planktonic growth

1.5.1.1 Effects of growth limitation

Christensen et al., (1985) showed that a Pseudomonas sp. grown in batch culture produced two different exopolysaccharides during the later stages of exponential growth. The polymer production then continued well into the stationary phase. This indicated that the polymers were probably not a primary metabolite and that their synthesis occurred when growth of the culture had become limited. Growth is said to be limited if further addition of a growth factor will promote a further increment of growth. However, it should be noted that growth in a batch culture is not homogeneous, but contains bacteria at all stages of growth. The chemostat overcomes this problem by maintaining a steady state where all the cells are growing at approximately the same rate, and as described in section 1.6.1.2 the dilution rate equals the specific growth rate, within certain defined limits.

When Pseudomonas NCIMB 11264 was grown in a chemostat it was shown that polymer synthesis declined as the growth rate was increased. This showed that
the polymer synthesis was not growth linked and therefore by definition not a
primary metabolite (Williams & Wimpenny, 1978). A similar result was noted with
Ps. mendocina (Sengha et al., 1989) and glycogen production by E. coli also
decreased as the dilution rate increased (Holme, 1957).

However, in the case of Xanthomonas juglandis grown under phosphate
limitation, the amount of polymer increased as the dilution rate decreased, but the
specific rate of polymer production remained constant (Jarman, 1979). Similar
results were obtained over a range of dilution rates between 0.03 and 0.06 h⁻¹ using
Xanthomonas campestris (Tait et al., 1986). With Ps. aeruginosa a third option
was seen in that the amount of polymer increased with dilution rate (Main et al.,
1978). Similar results were obtained with Xanthomonas campestris (Silman and
Rogovin, 1972), and Ps. mendocina showed increased polymer production with
dilution rates up to 0.06 h⁻¹ above which polymer production fell sharply (Hacking
et al., 1983). From the above it can clearly be seen that the effect of growth rate
on polymer production varies from species to species.

1.5.1.2 Effect of nitrogen as growth limiting substrate.

A number of reports show that most bacteria capable of synthesising EPS do so
under conditions of nitrogen limitation (Duguid & Wilkinson, 1953; Wilkinson et
al., 1954 (Aerobacter aerogenes); Hacking et al., 1983; Sengha et al., 1989 (Ps.
mendocina); Williams & Wimpenny, 1978 (Pseudomonas NCIMB 11264)).
Williams & Wimpenny (1978) were able to show that the Pseudomonas NCIMB
11264 produced the maximum amount of polymer under nitrogen limitation and that
EPS levels were increased as nitrogen levels increased until the nitrogen was no
longer limiting.
1.5.1.3 Effect of phosphate limitation

The effect of phosphate limitation on the synthesis of EPS varies with the organism under test. Duguid & Wilkinson (1953) reported that *Aerobacter aerogenes* grown under phosphate limitation gave a ten to twenty fold increase in polymer levels compared with growth in a carbon limited medium. The polymer level with phosphate limitation was comparable to that obtained with nitrogen or sulphur limitation. Similar results were reported by Jarman *et al.* (1978), Evans *et al.* (1979) and Tait *et al.* (1986). However, Williams & Wimpenny (1977;1978) reported that phosphate limitation had no effect on polymer synthesis by *Pseudomonas* NCIMB 11264.

1.5.1.4 Effect of carbon limitation

As all the polymers in question are long chain polysaccharides it is not surprising that the literature reveals that a carbon source is necessary for polymer synthesis, and that very little polymer is produced under conditions of carbon limitation. However, it has been suggested that polymer synthesis is possible under carbon limitation when another form of "stress" is applied to the bacteria. But at the other end of the scale, Duguid & Wilkinson (1953) suggested that an upper limit for polymer levels existed even when excess carbon remained in the medium. Williams & Wimpenny (1977) noted that a high C:N ratio favoured polymer production in batch culture. However, using continuous culture (1978) they reported that carbon levels, which were non-limiting, had little effect on polymer production under nitrogen limitation.

Evans *et al.* (1979) showed that under carbon limitation polymers were produced which could be recovered by alcohol precipitation, but viscosity
measurements indicated that no polymer had been produced. From this they concluded that the polymers produced under carbon limitation differed from those produced under carbon excess, but it is possible that these polymers were proteinaceous.

It has also been shown (Dudman, 1964; Uhlinger & White, 1983), that different carbon sources affect the level of polymer produced by *Ps. atlantica*. This was not the case with NCIMB 11264, (Williams & Wimpenny, 1978).

### 1.5.1.5 Effect of oxygen levels.

Hacking *et al.* (1983) found that the optimal level of oxygen for alginate production by *Ps. mendocina* was in the range of 18-25% of saturation. No comment was made as to whether this level was limiting for growth or not. Sengha *et al.* (1989) using the same species were able to show that no alginate was produced when growth was oxygen limited, but this time no indication was given as to the level of oxygen which gave limitation. Jarman (1979) demonstrated that for *Azotobacter vinelandii* aeration rate had little effect on the specific rate of EPS production. Work on *Aerobacter aerogenes* revealed that the greatest amount of polymer was produced under conditions of aerobic growth (Duguid & Wilkinson, 1953) whereas *Rhizobium meliloti* gave the greatest yields with low aeration (Dudman, 1964). From the above results it would appear that polymers are not produced under conditions of oxygen limitation but that low, non-limiting, levels might promote production.

### 1.5.1.6 Effect of pH and temperature

Most of the work on polymer production, as cited above, has been carried out using medium at, or near, pH 7. The only paper reviewed which examined the
effect of pH was that of Williams & Wimpenny (1978) which demonstrated that polymers could be produced at pH's between 6.5-8, with an optimum at 7.

The effects of temperature vary depending on the organism in question. Wilkinson et al. (1954) reported that for *E. coli* an increase in temperature from 15-20 °C to 35 °C resulted in an increase in polymer production. This was the opposite to *Aerobacter cloacae* which gave greater yields at the lower temperatures. Duguid & Wilkinson (1953) confirmed this finding showing that *Aerobacter aerogenes* gave twice the yield of polymer at 15-20 °C than at 35 °C. Similar results were obtained for *Ps. aeruginosa* (Evans & Liniker, 1973). Norval and Sutherland (1969) grew *Klebsiella* on both agar and in liquid culture and found that a mutant produced less polymer than the parent at 20 °C but the same at 37 °C.

1.5.2 Effect of environmental conditions on bacterial attachment and physiology.

As shown above much work has been carried out into the responses of planktonic bacteria to varying growth conditions. Due to the problems mentioned previously in studying attached growth of biofilms, far less information on their physiology and polymer production is available in the literature.

Several workers have demonstrated that the maximum specific growth rate ($\mu_{max}$) for growth in a biofilm *sic* is the same as that for planktonic growth (Gilbert *et al.*, 1989; Hamilton & Characklis, 1989). It has been shown that biofilm thickness decreases as the residence time for a population of SRB's in a RotoTorque increases, that is, as the flow rate is decreased. It was reported that a residence time of 25 hours produced a film 5 µm thick. However, as the flow was increased to give a residence time of 1.3 hours the biofilm thickness increased to 1000 µm (Characklis, 1990).
Characklis (personal communication) has pointed out that biofilm reactors are often run with a dilution rate \( (D) \) greater than \( D_{crit} \), the point at which wash-out of planktonic cells occurs leading to the selection of an attached community. A further increase in the dilution rate leads to an increase in the supply of nutrients allowing faster growth, this growth will be dependent on the diffusion of nutrients through the biofilm. However, as shown above, the maximum growth rate will still be the same as that determined in planktonic culture.

Kjelleberg (1984) showed that starvation leads to a number of marine bacteria actively forming smaller cells and that increasing starvation times leads to a greater number of cells irreversibly binding to a surface.

It was observed that increased levels of glucose led to less cells attaching to a surface in a batch culture (Marshall et al., 1971). Using a chemostat Allison and Sutherland (1987) were able to demonstrate that glucose limitation lead to a greater number of cells attaching to a glass surface than under conditions where glucose was in excess. It has also been shown that biofilm thickness and cell adsorption is dependent on pH, with extensive biofilm formation at pH 7, much less at pH 9 and negligible biofilm formation at pH 5 (Sutherland, 1983).

### 1.6 Growth kinetics of microorganisms

The culturing of bacteria can be classified under two separate headings; 'open' and 'closed' systems. An open system is one in which all the components of the system may enter and leave it. In the closed system some essential part of the system is unable to enter or leave. Systems which employ a continuous flow having an input of culture medium and an output of biomass and other products are open systems. The simple batch system, which starts with an initial volume of medium to
which an inoculum is added, is a closed system. In a closed system the growth rate of the biomass must always tend towards zero as nutrients are used up or the level of some product becomes inhibitory, such systems are always, therefore, in a transient state.

1.6.1 Planktonic growth

1.6.1.1 Batch culture

In a batch culture a number of distinct phases can be distinguished. Lag phase occurs after the addition of the inoculum during which the bacteria are thought to adapt to the new environmental conditions. This is followed by a period of accelerating growth as the growth rate increases to its maximum value. Growth then continues at this maximum value (exponential growth) until the level of substrate becomes limiting, or one of the products of metabolism becomes inhibitory. At this point, stationary phase is entered where the growth rate has fallen to zero. However, this is a misnomer in terms of the physiology of the cells, as the population is still active metabolically and often produces what are termed secondary metabolites. This is followed by the death phase in which the number of cells starts to decline as the rate of cell death exceeds the rate of cell replication.

The growth rate in the exponential phase is described by the equation:

\[
\frac{dx}{dt} = \mu x
\]

Equation 1.

where \( x \) is the concentration of microbial biomass,

\( t \) is time, in hours

and \( \mu \) is the specific growth rate, in hours\(^{-1}\).
integration of the above equation gives:

\[ x_t = x_0 e^{\mu t} \]  \hspace{1cm} \text{Equation 2.}

where \( x_t \) is the biomass concentration after the time interval, \( t \) hours,
\( x_0 \) is the original biomass concentration,
e is the base of the natural logarithm.
If natural logs are taken of equation 2 the following equation is obtained:

\[ \ln x_t = \ln x_0 + \mu t \]  \hspace{1cm} \text{Equation 3.}

Therefore a plot of \( \ln x \) against time will be a straight line with slope \( \mu \).

Batch culture is useful in determining the maximum specific growth rate. Batch culture can help to determine during which phase of growth a metabolite is produced and therefore give an indication of whether it is a primary or secondary metabolite. Production only during late exponential and stationary phase tends to indicate a secondary metabolite, however, production in mid-exponential can be either primary or secondary due to the heterogeneous nature of a batch culture. Primary metabolites are growth linked and this can be best determined with the use of a chemostat where the growth rate of an homogeneous population can be controlled.

1.6.1.2 Chemostat culture

The chemostat culture is an open system in which nutrients are continuously added and biomass removed at the same rate. In an ideal chemostat it is assumed that mixing is complete and that on addition of a drop of nutrient it is immediately dispersed. The continuous addition of fresh media allows the growth to be held in
exponential phase. Provided the medium is so designed such that growth is limited by a substrate, rather than a metabolic toxin, a steady state can be reached, that is, formation of new biomass by the culture is balanced by the loss of cells from the vessel.

The dilution rate of the vessel \((D)\) is a function of the volume of the vessel \((V)\) and the rate of medium flow into the vessel \((F)\) and is defined by the equation:

\[
D = \frac{F}{V}
\]  
\text{Equation 4.}

The net change in cell concentration over a period of time may be expressed:

\[
\frac{dx}{dt} = \mu x - D x - k_p
\]  
\text{Equation 5.}

where \(k_p\) is the cell mortality rate. N.B. The death rate of the cells under most conditions is considered to be zero, it only becomes significant at very low dilution rates.

Under conditions of steady state \(\frac{dx}{dt} = 0\) so:

\[
\mu x = D x
\]  
\text{Equation 6.}

and \(\mu = D\)  
\text{Equation 7.}

thus under conditions of steady state the dilution rate controls the specific growth rate.

The chemostat can therefore be used to determine whether a product is growth
linked (a primary metabolite) or not. If product level rises with increased growth it is growth linked, if on the other hand product levels are highest at the lower dilution rates, i.e. lower growth rates, than the product is a secondary metabolite. The chemostat can also be used to study the effect of substrate limitation on product formation.

1.6.1.3 Fed batch culture

Yoshida et al., (1973) described a system in which batch cultures were fed either continuously or sequentially without the removal of culture fluid. Thus the volume of the culture will increase with time. Growth will occur until the substrate becomes limiting. By the addition of fresh medium at a dilution rate lower than \( \mu_{\text{max}} \) the substrate will be utilised as soon as it enters the vessel. Therefore the input of substrate is equalled by consumption of substrate, \( ds/dt \sim 0 \). Although the total biomass will increase with time, biomass concentration will remain constant as the volume increases, \( dx/dt \sim 0 \), thus \( \mu \sim D \). This situation is known as a 'quasi' steady state. As the time progresses further, the dilution rate decreases as the volume increases. The obvious difference between this 'steady' state and that of a chemostat is that here the dilution rate, and hence the specific growth rate, decreases, whereas in a chemostat it remains constant. A further description of the growth kinetics is given by Winkler (1991). The fed batch system will best model the process occurring in the water pipes where there are periods of stagnation followed by a high throughput of water.

1.6.1.4 Cyclic fed batch culture

A repeated or cyclic fed batch culture is achieved when part of the culture is removed at intervals, causing the culture volume, dilution rate and various metabolic
parameters to undergo cyclical variations. The fraction of the volume removed is termed the amplitude and the time between removals is termed the period. During a cyclic fed batch culture, a quasi steady state will be achieved whilst the volume is increasing. When the volume reaches a certain value a fixed fraction is removed. This sudden decrease in the volume will increase the dilution rate. If the amplitude is large enough, then the shift in $\mu$ will not occur instantly, and a transient period where $\mu$ is not equal to $D$ may occur. Fed batch and cyclic fed batch cultures provide a unique means of inducing controlled-transient conditions. As a means of achieving substrate-limited-growth they may avoid unwanted repressive or toxic effects from the culture medium.

1.6.2 Attached growth

Characklis (1990) has classified three ideal reactors for the experimental study of biofilms. These are:

a) continuous flow stirred tank reactor (CFSTR) in which no concentration gradients exist;

b) plug flow reactors, in which reactants and products move as a “plug” from inlet to outlet;

c) batch reactors with no inputs or outputs.

The CFSTR provides significant advantages for separating and evaluating the kinetics and stoichiometry of each biofilm process:

i) The bulk liquid phase is uniform, which makes sampling, chemical analysis, and mathematical modelling simple. Intensive quantities, such as reaction rates, are determined easily because the environmental conditions
are fairly uniform in the bulk liquid volume as well as across the reactive surface area.

ii) The steady state condition is convenient and reproducible.

iii) Biofilms developed in CFSTRs with constant shear stress at the walls are fairly uniform.

1.6.2.1 RotoTorque (Rotating annular reactor)

The Roto Torque is operated as a CFSTR and is claimed to be an excellent laboratory system for monitoring biofilm development, due to its sensitivity, particularly to changes in fluid friction resistance (Characklis, 1990). The RotoTorque consists of two concentric cylinders; a stationary outer cylinder and a rotating inner cylinder. Removable slides form an integral part of the inside wall of the outer cylinder, permitting sampling of the biofilm so that thickness/mass or biofilm chemical and microbial composition can be determined. The RotoTorque has been shown to have “near instantaneous mixing”. Shear forces are determined by the speed of rotation of the inner cylinder and are independent of the dilution rate. High dilution rate, selecting for attached growth can be achieved at low flow rates due the large surface to working volume ratio.

1.6.2.2 Tubular reactors

These are simple tubes which again can provide a high colonisable surface area to working volume ratio (depending on the diameter of the tube used). Tubular reactors can be operated as CFSTRs if recycling of the media is employed. At high recycling rates the reactor contents are completely mixed and no longitudinal concentration gradients exist in the liquid phase. However, a once through system
can be operated as a CFSTR if the above conditions are met by using short lengths of tube and flow rates high enough to ensure no significant concentration gradients are set up and that the flow does not become turbulent (Characklis 1990). In the case of a tubular reactor the shear forces are controlled by the flow rate.

1.6.2.3 Packed beds

By adding aggregates to a CFSTR the surface area for biofilm formation can be greatly increased. In a packed bed mixing is restricted in the axial direction and may mean that it appears to function as a plug flow reactor. However, this may be overcome by a high recycling rate/flow rate or by the use of well-spaced aggregates.

1.6.2.4 Mathematical approach

As has been said earlier bacterial accumulation at the surface is the result of several process: transport and adhesion of bacteria to the surface, growth and decay in the biofilm, and detachment of portions of the biofilm. The cell balance in the biofilm can be expressed thus:

$$
AdX_b/dt = \mu_bX_bA + R_pX_bA - R_bX_bA \quad \text{Equation 8.}
$$

Where $A$ is the surface area for biofilm accumulation in the reactor; $X_b$ is the attached cell concentration; $\mu_b$ is the attached cell growth rate; $R_p$ is the planktonic cell deposition rate; $k_b$ is the attached cell mortality rate and $R_b$ is the attached cell detachment rate. For steady state it can be considered that

$$
dX_b/dt = 0 \quad \text{Equation 9.}
$$
thus equation 8 simplifies to:

$$\mu_b = k_b + R_b - R_p$$  \hspace{1cm} \text{Equation 10.}

(Bryers and Characklis, 1982; Benbouzid-Rollet et al., 1991; Characklis, 1990 and personal communication)

1.7 Estimation of biomass

Biomass is a general term used to refer to the organisms in culture. The biomass is the parameter which allows the amount of growth to be measured and is useful in indicating when a steady state has been achieved. Pirt (1975) lists eight methods of estimating the biomass as follows: mass, volume or linear extent, mass of a biomass component, mass of substrate consumed or product formed, metabolic rates, light scattering, cell counts, staining methods. Three methods have been utilised in this work: dry weight, light scattering and cell counts.

1.8 Project aims.

As seen in the preceding literature review there is currently no satisfactory evidence demonstrating any link between the pitting of copper and aerobic bacteria in fresh water. The only evidence for any involvement of bacteria in this type of pitting was the presence of the gelatinous film reported by Fischer et al. (1988). Various work already reported in section 1.4.2 had shown that bacteria could bind copper and even that bacteria could bring about the dissolution of copper from a thin film coated on an IRE. However, it needs to be noted that this could not be defined as pitting which is very distinct from general corrosion. Due to the very nature of the thin films coated onto a germanium IRE it is impossible to create a
true pit. However, the information gained from FT-IR studies is often useful when considered in the light of other experiments.

In order to demonstrate conclusively the involvement of bacteria it is necessary to reproduce recognisable pits in copper. These pits need to be attributed to the action of specific bacteria and not some other factor, such as water chemistry. Part 1 describes the work carried out in order to determine any link between microorganisms and the pitting of copper. Chapter 3 reports on the examination of failed pipes using the scanning electron microscope (SEM). Chapter 4 reports on a site survey of hospitals, where Type 1½ pitting was suspected, in which the numbers of attached bacteria isolated from pipe samples are compared with the presence of any of the characteristics of Type 1½ pitting. Chapter 5 describes the use of a novel static bed system in order to try and reproduce the pitting in the laboratory, applying a form of Koch’s postulates.

During the course of the surveys reported in Part 1 and those by other research groups it became evident that a discernible pattern was emerging, with regards to the type of pipe installation and the flow regimes and an upper cut-off temperature above which Type 1½ pitting did not occur was observed.

Part 2 describes investigations of the physiology of bacterial growth and exopolymer production, with regard to the conditions identified in the operational systems which seemed to lead to pitting failures. Chapter 6 details the growth of bacteria isolated from a failed copper pipe, in a simple, closed, mixed batch culture. Chapter 7 contains experiments to determine the levels at which nutrients become limiting for growth and the effect of various nutrient limitations on exopolymer production. Chapter 8 examines the effect of temperature on planktonic and attached bacteria respectively. Chapter 9 considers the interplay between the planktonic and
attached populations in a cyclic fed batch reactor, while Chapter 10 examines the effect of growth rate on EPS production in a nitrogen limited chemostat. Chapter 11 details a very preliminary structural characterisation of the EPS produced by planktonic and attached bacteria.

Some electrochemical experiments involving the action of bacterial biofilms and hydrogen peroxide (a possible metabolic product) on the corrosion of copper are considered in Chapter 12. Finally, Chapter 13 provides a detailed discussion of all the results when drawn together to give an overview of the work that has been carried out in relation to the microbially influenced corrosion of copper.

In order to provide data which could be directly applied to the operational systems were failures were occurring in the copper water pipes, throughout this study a mixed population was used along with low levels of nutrients. This is contrary to the normal practise for physiology studies were a single model organism is grown in medium containing high nutrient levels.
Chapter 2.
MATERIALS AND METHODS

2.1 Bacteria

Three strains of bacteria were selected from a piece of failed copper pipe from a County Hospital in West Germany and distinguished by colony morphology and pigmentation. A white colony-forming, atypical strain close to *Ps. solanacearum*, a yellow colony-forming isolate of *Pseudomonas paucimobilis* and a brown pigmented, atypical strain of *Ps. paucimobilis*. A full characterisation of these organisms is given in Chapter 4 and Appendix I.

2.2 Media

All solutions were prepared with laboratory grade water from a Millipore reverse osmosis system (RO water).

2.2.1 Complex media

Initial bacterial isolation was carried out on ¼ strength nutrient agar (¼NA), g/l: Gibco nutrient agar 6; bacteriological agar No. 1 (Oxoid) 12.

Maintenance and enumeration of bacteria for laboratory experiments was carried out with ½ strength nutrient agar (½NA), g/l: Gibco nutrient agar 12; bacteriological agar No. 1 (Oxoid) 8.

2.2.2 Defined liquid media

Initially, a defined medium was required for the temperature block experiments and modified ARJ basal medium for pseudomonads was selected (Burnett *et al.*, 1957), g/l: NH₄H₂PO₄ 1, MgSO₄·7H₂O 0.2, KCl 0.2, ferric citrate 3.35 x 10⁻⁶. Glucose was added to give nominal final concentrations of 2 mg ml⁻¹ and 200 μg ml⁻¹.
2.2.2.1 Medium for fermentors

All fermenter work was carried out using synthetic pitting water supplemented with 200 mg l\(^{-1}\) glucose and 50 mg ml\(^{-1}\) ammonium dihydrogen orthophosphate.
A synthetic pitting water was devised based upon analysis of waters from several areas exhibiting Type 1½ pitting, (recipe from H. Campbell, Department of Material Science and Engineering, University of Surrey). g/l: CaCO₃ 0.02, NaCl 0.025, CaSO₄ 0.015, Na₂SO₄ 0.015, pH 8.

The following media were formulated to study the effects of nutrient limitation on the growth and extracellular polymer production of a mixed community of bacteria. Observations made on the level of glucose, ammonia and phosphate utilisation by the bacteria when grown in ARJ medium, allowed levels to be predicted above which these nutrients would not be limiting. By setting two nutrients at these higher levels and adjusting the third to various lower levels the point at which nutrient limitation occurs is deemed to be the point at which addition of that variable nutrient causes no further increase in growth, providing the other nutrients are present in excess. All media were adjusted to pH 8. A detailed description of the use of these media is given in Chapter 7.

AL series media g/l: Glucose 0.2; NaH₂PO₄ 0.063; (NH₄)₂SO₄ 0.00366, 0.01831 & 0.03661 for AL1, AL5 & AL10 respectively.

PL series media g/l: Glucose 0.2; (NH₄)₂SO₄ 0.09154; NaH₂PO₄ 0.00126, 0.01263 & 0.03158 for PL1, PL10 & PL25 respectively.

GL series media g/l: NaH₂PO₄ 0.063; (NH₄)₂SO₄ 0.09154; Glucose 0.010, 0.025 & 0.050 for GL10, GL25 & GL50 respectively.

NL series media g/l: Glucose 0.2; NaH₂PO₄ 0.063; NaNO₃ 0.00649, 0.02844, 0.06499 for NL1, NL5 & NL10 respectively.

2.3 Methods

2.3.1 Maintenance and cultivation of bacteria

Bacteria were routinely maintained by streaking onto ½NA and incubating at 30 °C. Stock cultures were then maintained at 4 °C.
2.3.2 Batch culture in a fermenter

The fermenter used for this study was an LH 1 litre fermenter. Agitation was provided by a magnetically driven impeller at 500 rpm. Temperature was maintained at 30 °C by a “cold finger” connected to a Churchill thermocirculator and monitored by a thermistor connected to a temperature controller (LH Module 503). The pH was monitored but not controlled.

The completed fermenter assembly was sterilised by autoclaving.

2.3.3 Continuous culture

Continuous culture employed many of the techniques and instrumentation used for batch culture with an LH 1 litre fermenter with temperature control, agitation and sterilisation as described above. In addition, pH was controlled using a pH controller (LH Module 505) connected to reservoirs of 1N HCl and 1N NaOH. Sterile air was pumped through the system at a flow rate of 3 litres per minute. A peristaltic pump was used to add fresh medium and to remove excess medium through a descending tube set to maintain a working volume of one litre.

Medium was made up in twenty litre volumes and sterilised by filtration through a Sartobran two stage capsular filter (pore sizes 0.45 μm and 0.22 μm, Sartorius, Goettingen, Germany). Culture samples were taken regularly and enumerated for colony forming units (cfus) (section 2.4.1). Steady state was deemed to have been reached when constant cell counts were obtained, normally after four fermenter volumes had been replaced.

2.3.4 Static beds

These were set up as shown in Figure 2.1. The glass reactor vessel had a working volume of 60 ml and was filled, to a level leaving a 15 mm headspace, with 10 mm copper rings cut from 15 mm copper pipe (IMI Yorkshire Copper Tube Ltd. Table X). Medium was pumped through at a flow rate of 1 ml per minute for
ten hours a day using a Watson Marlow 202U/AA peristaltic pump controlled via a BBC model B computer. The system was devised such that the medium covered the rings at all times. Silicone rubber was used for the bung and tubing to allow the system to be autoclaved, and to minimise the risk of the material being utilised as a carbon source by the microorganisms. Autoclaving was carried out with the vessel full of water to prevent excessive oxidation of the copper. Due to the size and design of the vessel it was necessary to monitor the pH and dissolved oxygen on the outflow as close as possible to the vessel (N.B. no evidence was seen for interference caused by the close proximity of the probes to each other). These data were logged via the computer. A listing of the control program is given in Appendix III. These systems were either run sterile or after inoculation as described in detail in Chapter 5.

Figure 2.1 Schematic diagram of static bed reactors, (not to scale).
2.3.5 Tubular reactors.

These were constructed from three 100 mm lengths of 22 mm copper tube (IMI Yorkshire Copper Tube Ltd. Table X), surface area 6908 mm$^2$, connected by silicone tube. Total working volume was approximately 120 ml. Temperatures were maintained ± 1 °C by immersing the tubular reactors in water baths. The copper was artificially aged by autoclaving full of water. Medium was pumped through continuously to give a dilution rate of 0.5 h$^{-1}$. Sterilisation was carried out by a second autoclaving of the integral system full of water, for the reasons detailed in section 2.3.4. The system was inoculated by passing culture from a chemostat at steady state through each tube in turn for ten minutes at a flow rate of 186 ml h$^{-1}$. The tubes were then left overnight to allow attachment of the bacteria. A slow flow (2 ml h$^{-1}$) was then applied for a further eight hours to consolidate surface colonisation before the full dilution rate of 0.5 h$^{-1}$ was set.

2.3.6 Temperature block

For investigations into the effect of temperature on planktonic growth a custom made temperature block was utilised. The temperature block was made of solid aluminium, drilled to accept thirty six rows of eight glass 15 ml tubes. A gradient was established by heating one end of the block with water from a thermo-circulator whilst cooling the other end with cold ethylene glycol from a chiller circulator. The entire apparatus was heavily insulated with expanded polystyrene (5 cm thickness), including a lid through which thermometers protruded. This maintained individual tube temperatures at ±0.5 °C and also ensured linearity of the gradient which was found to be excellent allowing five degree temperature intervals to be obtained. Temperatures were measured using thermometers placed in a test tube, containing 10 ml of oil, placed in the holes drilled to accommodate the experimental tubes.
2.3.7 Bacterial sampling from in vivo pipe work

Sections of pipe from operational systems in hospitals were rinsed immediately after removal with sterile RO water to clear any loosely associated bacteria. The end of the pipe interior was thoroughly swabbed with a cotton swab (approximately 1.5 cm sampled), which was then broken off into 10 ml of sterile ¼ Ringer’s solution and shaken well to resuspend the bacteria. The ¼ Ringer’s solutions were stored in a cool box for a maximum of eight hours. As soon as possible after taking the samples a tenfold dilution series was made in ¼ Ringer’s and 0.1 ml of each dilution was spread on a ¼NA plate and incubated at 30 °C.

SRB isolations were carried out at a later stage on the same tubes which, after the initial isolations, were refilled with system water to prevent excessive air contact and/or drying. A further tube removed and maintained full of system water was used solely for SRB isolation. Tubes were placed in an anaerobic cabinet and cut in the middle to allow sampling well away from the areas previously sampled. The tubes were again rinsed in sterile, deaerated RO water, before sampling was carried out by removing materials from both the surface and from deep within the biofilm, these were resuspended in deaerated ¼ Ringer’s and used to inoculate Postgate C medium, which was incubated anaerobically at 30 °C for up to three weeks.

2.3.8 Sampling of tubular reactors.

Each individual 100 mm pipe section was rinsed gently with 100 ml of sterile RO water prior to removal of any attached material with a sterile, tight-fitting, test tube brush which was “pistoned” within the tube for one minute (avoiding aerosol formation). The brush was then agitated in 10 ml of sterile ¼ Ringer’s for a further minute, this was then used for the assays required.

2.3.9 Isolation of bacterial EPS

The extracellular polymers produced by bacteria may either remain adherent to
the organism or pass into solution in the medium. The adherent material can be collected as detailed below, extracellular polymers from the culture medium can be isolated by first spinning down the cells as detailed below and then following the procedure as detailed, starting with the propan-2-ol precipitation.

Adherent EPS was collected by centrifuging the bacteria from a culture at 2,400 x g for forty minutes. The pellet of bacteria was resuspended in 40 ml of sterile RO water and treated with a mixture of 0.8 ml 0.5M Na₂EDTA and 0.8 ml of 5M NaCl for ten minutes at room temperature. The cells were then removed by centrifugation as above and the supernatant treated with three volumes of cold propan-2-ol. The precipitate formed was collected and thoroughly dialysed against running tap water overnight, then against two changes of RO water (8 hours each). Cellular debris was removed by ultracentrifugation for four hours at 20,000 x g. The supernatant was then lyophilised and stored in a desiccator.

2.3.10 Chromatography of EPS

2.3.10.1 Acid hydrolysis of carbohydrates

Approximately 20 mg of polymer were added to 1 ml of 0.5N H₂SO₄ (0.25M), flame-sealed in a freeze-drying ampoule and maintained at 100 °C overnight. 0.5 ml of Amberlite MBY resin was added to neutralise the hydrolysate and allowed to stand for two hours. The treated hydrolysate was then carefully removed from the beads and freeze dried.

2.3.10.2 Paper chromatography

The freeze dried material was redissolved in 100 μl of water and 5 μl spotted onto Whatman No. 1 chromatography paper along with sugar standards including glucose, mannose, galactose and fructose. (1 μl of 0.1M solution). The chromatogram was run overnight in descending mode with 6:4:3 mixture of
butan-1-ol: pyridine: water, removed and air dried after 16-20 hours. It was developed with silver nitrate solution (1ml saturated aqueous silver nitrate with 200ml of ‘Analar’ acetone; store in dark bottle), air dried for 30 sec, and treated with 2% w/v ethanoic sodium hydroxide. Finally, the spots were fixed with 5% w/v sodium thiosulphate solution and air dried.

2.3.11 Preparation of samples for scanning electron microscopy.

Samples were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4 overnight. The samples were then given three one hour washes in cacodylate buffer followed by three washes in 30 % ethanol, then cooled to -70 °C before being freeze dried and stored in a desiccator until required.

2.3.12 PAS reaction (Chamberlain et al., 1988).

Sections of film lifted from copper pipes with 25% nitric acid were treated with freshly prepared 0.1 M sodium chlorite in 1% acetic acid for 10 minutes to preblock any aldehyde groups present. The film was then rinsed for 1 minute with RO water before being oxidised in 1% aqueous periodic acid for an absolute maximum of 10 minutes. Sections were then washed gently for 5 minutes in RO water prior to staining with Schiff’s reagent for between 5 and 10 minutes. Material was then rinsed extensively with RO water until the effluent was colourless and examined for the magenta colouration indicative of vic-glycol groups typical of polysaccharides.

2.4 Assays

All spectrophotometer absorbance readings were carried out using either a Pye-Unicam SP8-400 uv/vis spectrophotometer or an SP6-500 uv spectrophotometer. For each of the assays detailed below standard curves were prepared for every run, samples of which are give in Appendix II.
2.4.1 Cell counts

Bacteria were enumerated by the plate count technique of Miles and Misra (1938). A tenfold dilution series of cultures was made, down to $10^{-6}$. Six times 20μl drops, of each dilution, were made on a ½NA plate and incubated at 30 °C. Dilutions with between 3 and 30 cfus per drop are counted, divided by 6 and multiplied by $50 \times 10^n$ to give cfus ml$^{-1}$, where $n=$ dilution factor at which the drops were counted, for a dilution of $10^{-5}$ $n=5$.

2.4.2 Glucose assay

2.4.2.1 [Trinder] system (Trinder, 1969)

Materials:
Glucose oxidase (Trinder system, Sigma)
Dissolve one vial of glucose oxidase in 100 ml of RO water (as described by Sigma)
Standard glucose solution 1000 μg ml$^{-1}$ diluted as necessary to give: 200, 150, 100, 50 and 10 μg ml$^{-1}$ standards.

Procedure:
To 0.1 ml of sample 3 ml of glucose oxidase were added and mixed. After 20 minutes at room temperature spectrophotometer absorbance readings were taken at 505 nm.

2.4.2.2 Hexokinase system (Bondar & Mead, 1974)

Materials:
Glucose [HK] reagent (Sigma)
Dissolve one vial of glucose [HK] reagent in 20 ml RO water (as described by Sigma)
Standard glucose solution as in 2.4.2.1

Procedure:
To 0.1 ml of sample 3 ml of glucose [HK] reagent were added and absorbance readings taken immediately at 340 nm using a spectrophotometer.
2.4.3 Phosphate assay (extracellular) (McDermott, 1991)

Materials:

'Analar' acetone
Sulphuric acid (2.5 M)
Ammonium molybdate (10 mM)

Immediately prior to use mix 2:1:1 ratio of above solutions respectively to give the A.A.M. solution.

Citric acid (1 M)

Standard phosphate solution 1 mg PO₄³⁻ ml⁻¹ (1.43 g of KH₂PO₄ per litre of RO water. Diluted where necessary to give 100, 80, 60, 40, 20 and 0 µg ml⁻¹ PO₄³⁻ standards.

Procedure:

To 0.5 ml of sample 4 ml of A.A.M. were added, the solutions were mixed, 0.4 ml of citric acid added and absorbance readings taken immediately at 335 nm using a spectrophotometer.

2.4.4 Ammonium assay (McDermott, 1991)

Materials:

Salicylate reagent: sodium salicylate 85 g and sodium nitroprusside 0.6 g per litre of RO water.

Fichlor's reagent: Dichloroisocyanurate 2.5 g l⁻¹ of sodium hydroxide (1 M)

Standard ammonium solution: 25 µg ml⁻¹ NH₄-N (117.97 mg (NH₄)₂SO₄ l⁻¹), diluted as necessary to give 25, 20, 15, 10, 5 and 0 µg of NH₄-N ml⁻¹.

Procedure:

Samples were diluted to ensure the level NH₄-N fell within the range of the standards.

To 2 ml of salicylate reagent 0.25 ml of sample were added, followed by 2 ml of Fichlor's reagent. The solutions were then mixed and incubated at 30 °C for 30 minutes. To each tube 5 ml of distilled water were added, absorbance readings were then taken at 660 nm using a spectrophotometer.
2.4.5 Protein assay (Smith et al. 1985)

Materials:
Solution A: bicinechonic acid solution (B-9643, Sigma)
Copper sulphate solution (CuSO₄·5H₂O), (4% (w/v) in H₂O)
Mix above in ratio 50:1 respectively to give BCA reagent
Standard Protein solution: 2 mg ml⁻¹ of bovine serum albumin (BSA) diluted as necessary to give 0, 0.4, 0.8, 1.2, 1.6, and 2 µg ml⁻¹ standards.

Procedure:
Samples (150 µl) were added to 3 ml aliquots of the BCA reagent, incubated at 37 °C for 30 minutes, allowed to cool to room temperature and absorbance readings taken at 526 nm using a spectrophotometer.

2.4.6 Total carbohydrate assay (Dubois et al., 1956)

Materials:
Concentrated sulphuric acid
Phenol solution: phenol 5 g 100 ml⁻¹ of RO water
Glucose standard solution 1000 µg ml⁻¹ diluted as necessary to give 100, 75, 50, 25, 10 and 0 µg ml⁻¹ standards.

Procedure:
Samples were diluted where necessary to give readings within the range of the standards.

0.5 ml of sample were added to 0.5 ml of phenol solution and mixed, then 2.5 ml of concentrated sulphuric acid were added rapidly and mixed to obtain maximum heating (suitable safety precautions need to be taken in line with COSHH regulations). After 20 minutes at room temperature absorbance readings were taken at 488 nm using a spectrophotometer.
2.4.7 Uronic acid assay. (Blumenkrantz & Asboe-Hansen, 1973)

Materials:

- m-hydroxy biphenyl 0.15 g 100 ml⁻¹ of sodium hydroxide solution (0.5% (w/v))
- Sodium tetraborate 0.0125 M solution in concentrated sulphuric acid.
- Standard alginate solution 1000 μg ml⁻¹ diluted as necessary to give 100, 75, 50, 25 and 0 μg ml⁻¹ standards.

Procedure:

To 0.2 ml of sample 1.2 ml of acidified tetraborate were added at 0 °C and mixed, heated at 100 °C for 5 minutes and cooled before 20 μl of m-hydroxy diphenyl reagent were added. Absorbance readings were taken immediately at 520 nm using a spectrophotometer.

2.4.8 Hydrogen peroxide (Schumb et al., 1955)

Materials:

- Titanium (IV) sulphate (11% v/v) in 28% v/v sulphuric acid
- Concentrated sulphuric acid
- Hydrogen peroxide standards as follows 3, 7.5, 15 & 30 ppm

Procedure:

To 4 ml of sample 0.1 ml of concentrated sulphuric acid was added followed by 0.02 ml of titanium (IV) sulphate solution absorbance readings were taken immediately at 400 nm using a spectrophotometer.
2.4.9 Nitrate (Beutler & Wurst, 1986)

Materials:
Boehringer, Nitrate UV-method test combination (cat. No. 905 658) comprising:
- Bottle 1, imidazole buffer, pH 7 plus stabilizers
- Bottle 2, tablets of 0.5 mg NADPH plus stabilizers
- Bottle 3, with approx. 3 units nitrate reductase (as lyophilisate)

1 tablet from bottle 2 was dissolved in 1 ml of solution from bottle 1 in a cuvette (reagent 2).

The contents of bottle 3 were dissolved in 0.6 ml RO water (reagent 3)

Procedure:
To a cuvette containing reagent 2, 0.1 ml of sample (containing 3-30 µg nitrate) was added, followed by 1.9 ml of RO water. A reaction blank was also prepared using a cuvette with reagent 2 and 2 ml of RO water. The absorbance was then read at 340 nm ($A_1$). The reaction was started by the addition of 0.05 ml of reagent 3 and the absorbance read at 340 nm after 40 minutes ($A_2$). After a further 20 minutes the absorbance at 340 nm was again read ($A_3$).

Calculation:
\[
\Delta A = [(A_1 - A_2)_{\text{blank}} - 2(A_2 - A_3)_{\text{blank}}] - [(A_1 - A_2)_{\text{sample}} - 2(A_2 - A_3)_{\text{sample}}]
\]
content $\text{nitrate} = 0.3001587 \times \Delta A$
content $\text{nitrate} [\text{g l}^{-1}]$. 
RESULTS: PART 1

Investigations into a link between microorganisms and Type 1½ pitting of copper.
Chapter 3

SCANNING ELECTRON MICROSCOPE EXAMINATION OF TYPE 1½ PITTING.

One of the original investigations into the first reported cases of Type 1½ pitting, which occurred in Saudia Arabia, was carried out by Campbell, Garner and Chamberlain (unpublished). The general characteristics of the pitting and corrosion products were identified using light microscopy, x-ray diffraction and standard microchemical tests, however, their detailed morphology was not ascertained.

In order to answer a number of basic questions regarding the pitting phenomenon a detailed study was undertaken using the SEM and associated technique of energy dispersive x-ray analysis (EDXA). As a polysaccharide film had been identified on the pipes it was necessary to locate this film in situ, along with associated bacteria, and to determine whether the biofilm distribution was correlated with any of the corrosion products. At this stage it was not known whether the biofilm was specifically associated with the actual pits, either overlaying or within them, or if it was covering the entire tube surface. The reason for the presence of the unusual, but characteristic, black copper (II) oxide was also unknown. It seemed likely, however, that the biofilm was in some way involved with its formation, as these were the two characteristic features of this form of pitting.

Sample tubes were received, full of water, courtesy of Prof. Dr. W. Fischer from the Hellersen County Hospital in West Germany. One cm² samples were cut, fixed and processed as detailed in Methods (section 2.3.11). These were examined using the scanning electron microscope (SEM) and EDXA. This was carried out on a Jeol 35CF scanning electron probe microanalyser equipped with a Link Systems detector and spectrometer, which was operated in a windowless mode allowing the detection of the lighter elements such as carbon, nitrogen and oxygen.
Results:

(In the following discussion the author acknowledges the help of Mr. H. S. Campbell in providing the results of his unpublished observations and microchemical tests).

Visual examination of the samples (Figure 3.1) revealed the following characteristics of Type 1½ pitting. A black “sooty” deposit, which often formed small “walls” running along the pipe parallel to the direction of flow and adjacent to the mounds of blue corrosion products, which covered the pits. These corrosion products were separated from the red crystalline material within the pit by a perforated membrane. Between the mounds of corrosion product was a more or less continuous, black, adherent layer. When the corrosion products were removed to reveal the pits, it was noted that the pitting occurred primarily in the lower half of the tube and comprised several large, almost equidistant, pits surrounded by numerous much smaller pits.

Figure 3.2, a scanning electron micrograph, shows the adherent black layer which covered most of the area between the pits. It was composed of masses of small nodules with a covering of organic material which charged under the electron beam. Campbell, using microchemical spot tests and powder x-ray diffraction analysis, confirmed this as copper (II) oxide. Figure 3.3 shows the area above a pit from which the raised corrosion products have been removed. A number of pores are visible passing through the thickened ‘membrane’ which covers the pit. EDXA of this ‘membrane’ indicated the presence of copper and oxygen in roughly a two to one ratio suggesting the presence of copper (I) oxide. A similar area is shown in Figure 3.4 at a higher magnification. Two pores in the perforated copper (I) oxide membrane are seen in the top left corner. The area around the pit still retained some of the plate like crystals which once covered the whole area of the pit. These crystals were the blue/green material seen by eye. The EDXA of this material, as shown in Figure 3.5, indicated the presence of copper, oxygen, sulphur and a trace of carbon. This was consistent with Campbell’s earlier investigation using x-ray
Figure 3.1a View of inside of a failed copper pipe showing corrosion products.

Figure 3.1b Line drawing of Figure 3.1a detailing corrosion products.
Figure 3.2 Low power SEM showing mounds of copper (II) oxide with coating of organic material

Figure 3.3 SEM showing pores (P) passing through the thickened oxide membrane which covers the pits.
Figure 3.4 SEM showing perforated copper (I) oxide membrane (OM) and plate-like basic copper sulphate corrosion products (CP).

Figure 3.6 SEM of cubic copper (I) oxide crystals, showing unusual morphology, and needle-shaped copper (I) chloride crystals (Cu Cl) found in the pits.
diffraction which showed such crystals to be basic copper sulphate. As shown in Figure 3.6 the red crystals in the pits, beneath the membrane had a basic cubic structure. EDXA of these crystals (Figure 3.7) showed them to contain mainly copper and oxygen, again in a two to one ratio, with traces of phosphorus and chlorine. Normally, copper (I) oxide crystals are cubic, occasionally with one corner removed. The crystals seen in Type 1½ pits were atypical, having all the corners removed. It is suggested that the presence of the small amounts of chlorine and phosphorus seen by EDXA could account for the unusual morphology of these crystals. Also present in a number of the pits were white, needle-shaped crystals, which contained copper, chlorine and oxygen; these were copper (I) chloride which had been partially hydrolysed and oxidised to copper (II) by exposure to air.

The "sooty" black material is seen by SEM to be composed of spherical masses (Figure 3.8), which appear to be coated with a layer of amorphous organic material. In the late eighties, when investigations into Type 1½ pitting started, a number of workers mistakenly identified these spheres as bacteria. EDXA (Figure 3.9) shows the presence of copper and oxygen in roughly equal amounts, consistent with copper (II) oxide being present. Transmission electron microscopy (TEM) of sections of this material (Angell and Chamberlain, unpublished) showed none of the structural detail of microorganisms. Again EDXA showed the whole structure to be composed of copper (II) oxide. Numerous rod shaped bacteria were visible, normally associated with the spherical copper (II) oxide. Figures 3.10 and 3.11 show the bacteria and their associated exopolymeric material coating the copper (II) oxide.
Figure 3.5 EDXA analysis of plate-like crystals overlaying pits.

Figure 3.7 EDXA analysis of cubic crystals found filling the pits.
Figure 3.8 SEM of "sooty" black material, identified as spherical copper (II) oxide, with associated organic material.

Figure 3.10 SEM showing bacteria and exopolymeric material.
Figure 3.9 EDXA analysis of spherical black deposits overlaying the copper (I) oxide membrane.
Figure 3.11 SEM showing bacteria and exopolymeric material.
Discussion

The results of the SEM survey served to confirm the observations originally made on the morphology and chemical properties of Type 1½ pitting. It was found that the general morphology of the pitting was similar to that described by Lucey (1967) and shown in Figure 1.1, with a pit containing copper (I) chloride and copper (I) oxide. The pit is overlain by a perforated membrane of copper (I) oxide above which is a mound of basic copper sulphate corrosion products. In Type 1 pitting Lucey noted the presence of basic copper carbonate as the mounds of corrosion products, this difference is due mainly to the fact that Type 1 pitting occurs in hard waters which have a high level of carbonate. By far the most common form of Type 1½ pitting occurs in soft waters where the sulphate will be formed in preference to the carbonate. The other important observation was the presence of the bacteria and their association with the unusual and characteristic black copper (II) oxide. As noted in the Introduction, copper (II) oxide is commonly found associated with Type 2 pitting, which occurs at temperatures above 60 °C, but would not be expected in pipes full of water below this temperature. However, copper (II) oxide can form below 60 °C when water-saturated air is present in the tube. In order to obtain copper (II) oxide a powerful oxidising agent needs to be present, the association of the bacteria with this material suggests that the bacteria could be involved in this process. The mere presence of bacteria on the failed pipes obviously does not prove their involvement, but does provide further circumstantial evidence.
Chapter 4

BACTERIAL ISOLATIONS FROM HOSPITALS EXHIBITING TYPE 1½ PITTING.

The original bacterial isolations were carried out on the sections of failed pipe received from the German hospital and used for the SEM examination reported in the preceding Chapter. Bacteria were isolated by swabbing the inner surface of the pipes as detailed in Methods (section 2.3.7).

Following information from the National Corrosion Service three hospitals in the south west of England were identified as possibly exhibiting Type 1½ pitting. Having received sections of pipe from these hospitals and made preliminary visits it was confirmed that Type 1½ pitting was occurring at two of the hospitals, but not at the third, which was subsequently used as a control. Visits were arranged to remove sections of pipe allowing immediate sampling for attached bacteria.

A number of surveys carried out at other sites, by various research groups, were restricted mainly to sampling the planktonic communities of bacteria within the pipes, and then simply characterising the attached bacteria without carrying out any enumeration. This was felt to be unsatisfactory as it was the biofilm bacteria which were more likely to be responsible for the corrosion process as they were in contact with the copper. In line with this aim a sampling regime was devised which allowed counts to be made on the biofilm bacteria as well as those in the planktonic phase, this is detailed in Methods (section 2.3.7). The pipes were then examined independently for the characteristics of Type 1½ pitting.

In order to remove the pipes the site engineers drained the relevant section of pipe work and quickly cut out samples. These sections were immediately rinsed through with sterile distilled water to remove any loose bacteria and sampled for attached bacteria as described in Methods (section 2.3.7). The tubes were then quickly refilled with system water, capped and transported back to the laboratory.
where anaerobic facilities were available for SRB isolation as described in Methods (section 2.3.7). Detailed examination of the pitting characteristics were carried out courtesy of Mr. H. S. Campbell.

The location of the pipes and likely conditions prevailing within them were also noted. Discussions were also held with the site engineers to discover if any particular type of pipe run (e.g. vertical/horizontal, continuous/intermittent flow, stagnation periods etc.) was more prone to Type 1½ pitting failure.

Results

Bacteria forming three distinct colony types were isolated from the failed pipe from the German hospital. They were distinguished initially by their colony morphology and pigmentation. A yellow colony forming isolate was identified by the API 20NE test system as having a 99% fit for *Pseudomonas paucimobilis*. The other two were also *Pseudomonas* sp. according to the API data base but could not be classified further and were sent to the National Collection of Industrial and Marine Bacteria (NCIMB) for species identification. These were determined as a white colony-forming, atypical strain close to *Ps. solanacearum* and a brown pigmented, atypical strain of *Ps. paucimobilis* (Appendix I).

The samples taken from the three hospitals in the south west of England were identified as follows:

**Samples from Hospital T (Removed on 26.7.89)**

**Sample T9.** 250 mm length of 28 mm horizontal pipe feed to fire hose. Part of original installation.

**Sample T10.** 750 mm length of 28 mm horizontal pipe, adjoining T9 and known to have been replaced within the previous six months.

**Sample T11.** 195 mm length of 35 mm vertical pipe taken from the main riser for the fire hydrant at the first floor landing.
Sample T12a. 70 mm length of 28mm horizontal recent replacement pipe from second floor fire hose supply.

Sample T12b. 290 mm length of 28 mm horizontal pipe forming part of original feed to second floor fire house. Connected to T12a by brass compression coupling and upstream of it.

Sample T13. 250 mm length of 28 mm horizontal pipe from feed to first floor landing, installed 4.4.86.

Sample T14. 100 mm length of 15 mm horizontal pipe, in ceiling space, from cold water feed (from storage tank) to bath (Ward 4).

Sample T15. 100mm length of 15 mm horizontal pipe from cold water feed (from storage tank) to basin in Pathology department.

Samples from Hospital B (removed 27.7.89)

Sample B1. 140 mm length of 28 mm vertical cold water pipe.

Sample B2. 170 mm length of 28 mm horizontal cold water pipe from the same run as B1.

Sample B3. 200 mm length of 28 mm horizontal cold water pipe (recent replacement).

Sample B4. 360 mm length of 15mm horizontal cold water pipe.

Sample B5. 220 mm length of 35 mm horizontal hot water pipe, carrying outward flow in circulating system.

Sample B6. 180 mm length of 35 mm horizontal pipe from return leg of hot water flow line.
Figure 4.1 Log plot of bacterial counts from failed copper pipes, bacteria grouped by colony pigmentation.

Figure 4.2 Linear plot of bacterial counts, grouped by colony pigmentation, from failed copper pipes.
Sample B8. 300 mm length of 15 mm vertical pipe from cold water feed (from storage tank) connecting B10 to B9.

Sample B9. Capillary soldered brass stopcock with, downstream, a 190 mm vertical length of 15 mm tube connected by a flared and soft soldered joint to a 220 mm length and, upstream, a 300 mm length of 15 mm tube bent to a swan neck. The sample provided a cold water supply to a ward wash basin and was fed by sample B8.

Sample B10. 600 mm long 15 mm horizontal cold (storage tank) water pipe with right-angle bend 200 mm from one end which was joined by capillary soldered coupling to a 100 mm long 15 mm right-angle bend. In service this led directly to Sample B8.

Sample from hospital C (removed 27.7.89)

Sample C1. 150 mm length of 15 mm horizontal mains water pipe.

All the samples, except B5 & 6 which were from hot water circuits (>55 °C), were removed from sections of pipe which experienced periods of stagnation in which the temperature of the water would rise to the ambient temperature of around 20-30 °C, causing the water to be warm, not cold (<15 °C). These periods of stagnation were followed by brief periods of high throughput of water.

The results of the bacterial counts are shown in Figure 4.1 indicating that bacteria could be isolated from all the pipes sampled. In each case the highest numbers of bacteria isolated were from the heterogeneous group of bacteria forming white colonies. The pink colony forming organism was one of the most dominant species in the biofilm. It was identified tentatively as a *Methylobacterium* sp. however, they did not grow well as pure cultures with no viable cells recoverable after only 3 subculturings. The yellow colony forming isolate was identified using the API20NE test system which indicated a 99% fit for *Ps. paucimobilis*. The
brown colony forming organism was identified by the API 20NE test system and the biochemical tests used by the NCIMB (Appendix I), as the atypical strain of *Ps. paucimobilis*. Again using the API 20NE and the biochemical tests used by the NCIMB (Appendix I) the major white colony forming bacterium was found to be the atypical strain of *Ps. solanacearum*, the other white forming bacteria was tentatively identified as an *Aeromonas*.

Figure 4.2 shows the same data, this time plotted on a linear scale, which has the effect of removing the samples which gave very low counts i.e. below 1000 cfus cm\(^{-2}\). From this data it can be seen that the highest bacterial counts were obtained from samples T9, T10, T15, B2, B3, B4, B9 and B10. No SRB were isolated from any of the pipes sampled, even those where special precautions had been taken to maintain any anaerobiosis.

Table 4.1 Correlation of pitting indicators and polysaccharide biofilm.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Black film</th>
<th>Cu(_2)O beneath</th>
<th>Pitting</th>
<th>Perforation</th>
<th>Film</th>
<th>PAS reaction</th>
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<td>Hospital T</td>
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</table>
Table 4.1 shows the results of the examination for pitting characteristics together with results for the presence of a biofilm and the PAS reaction (sections 1.1.2 & 2.3.12). Perforation had occurred in pipe samples T9, B4 and B9, whilst pipe samples T13, T15, B2 and B10 showed pitting but no perforation. Samples B5 and B6, which had carried hot water, were identified as showing classic Type 2 pitting. A general trend could be discerned which showed that pitting and perforation occurred mainly in those samples with the highest bacterial numbers; T10, T13 and B3 appeared to be exceptions to this rule. T13 showed pitting but had low bacterial counts, whilst T10 and B3 showed very high numbers but no pitting indicators. However, the latter two samples were both replacement sections of pipe which had been installed within the previous year to replace failed pipe. Both sections were adjacent, in service, to pipe samples which showed high bacterial numbers and had perforated, T9 and B4 respectively. The two samples T10 and B3 indicate that pipes replaced in a “piece meal” fashion are quickly colonised by high numbers of bacteria, and although pits had not been formed at the time of sampling it is known that pits may take several years to develop.

Discussion

With the exception of the three cases noted above, two of which are explicable, it was shown that a good positive correlation exists between high bacterial numbers and pitting. As mentioned above a control section was removed from the third hospital visited along with some sections of pipe from the other two hospitals (T11 & B1), which were not themselves showing signs of failure but were upstream of failed sections. However, it needs to be borne in mind from the outset of the discussion that more controls would have been preferable, but for obvious reasons the site engineers were reticent to remove “good” sections of pipe. A number of the sections removed had perforated in the week prior to removal. In anticipation of our visit these sections of pipe had been patched only, which allowed sampling to be carried out on freshly removed material. These results show a substantial link between bacterial numbers and Type 1½ pitting. Although additional samples would have been helpful these results are sufficiently clear to add further
circumstantial evidence to that obtained from the SEM study. This is believed to be still the only survey to show a link between the numbers of attached bacteria and the presence of various pitting characteristics.

It is important to note that the three organisms isolated from the German hospital and used as the test organisms (Section 2.1) were also isolated in this survey. These same organisms along with the *Methylobacterium* were also isolated from the Scottish hospitals where "pepper-pot" pitting was occurring (Walker *et al.*, 1991b).

Another very important observation that was made for the first time was the interrelationship between the presence of the bacterial biofilm and the occurrence of the characteristic black copper (II) oxide. There was no incidence of the black copper (II) oxide being present without there also being a bacterial biofilm. However, the biofilm was occasionally seen without the copper (II) oxide on section of pipe which were adjacent to sections with both the biofilm and the copper (II) oxide, showing that the pipe system was susceptible to the formation of copper (II) oxide. This suggests that the biofilm precedes the copper (II) oxide.
Chapter 5

Modelling of pitting in vitro.

In order to conclusively demonstrate that bacteria are able to initiate pitting in copper, pits need to be reproduced in the laboratory in such a way that they can be attributed to the action of the bacteria and not some other factor such as the water chemistry alone. Historically in medical microbiology, the proof of a link between a microorganism and disease has entailed the application of Koch's Postulates as follows:

1) The organism must be present in every case of the disease:

2) the microorganism must be isolated from the diseased host and grown in pure culture:

3) the specific disease must be reproduced when a pure culture of the microorganism is inoculated into a healthy, susceptible host:

4) the microorganism must be recoverable once again from the experimentally infected host.

In the case of Type 1½ pitting of copper it is likely that no one single bacterial species would be responsible, rather a consortium of bacterial species is more likely to be the cause. Due to this Koch's Postulates cannot be applied literally, but the general principles would allow any possible link between bacteria and Type 1½ pitting to become evident. A matched sterile control was also included to ensure any pitting was due to bacteria and not just the chemistry of the water or flow conditions.

A number of systems were considered in order to model the system and hopefully reproduce the pits. The chemostat model in which coupons could be suspended (Keevil et al., 1987) was considered to be useful for many studies but
would not be suitable for modelling this system. This was due to the large bulk or planktonic phase compared to the relatively small copper surface available for colonisation (roughly a 6 : 1 ratio), it was felt that this ratio should be as close to that found in the operational systems as possible (2.7 : 1 for 15 mm pipe and 1.4 : 1 for 28 mm pipe). As shown in the site survey (Chapter 4) it was also known that the flow conditions occurring in the failed systems were important, with periods of stagnation followed by periods of high flow causing the wash out of the planktonic population. These flow regimes could not be reproduced in a simple chemostat model, which by its very nature must maintain a steady state.

The static bed system chosen to model the operational system was similar to that used to grow attached cells, such as animal cells. In these static bed systems a support matrix is used to provide a large surface area in relation to the surrounding volume of the bulk planktonic phase. The vessels detailed in Section 2.3.4 were designed to take account of these requirements. Fifteen copper rings were randomly packed to provide the support matrix, giving a ratio of 2.5 : 1 for the planktonic phase to surface area. The system was operated as described giving both periods of stagnation over night and a high dilution rate during the day leading to enhancement of attached growth. If the dilution rate exceeds the maximum growth rate of the bacteria wash out will occur removing all the planktonic bacteria. This models the actual system when water is drawn from the system via a tap.

Two such static beds were set up and operated as described above. One system was maintained sterile while the other, in view of the first of Koch's Postulates, was inoculated with the three *Pseudomonas* spp. isolated from the German Hospital as described in Methods (section 2.1). The inoculum was prepared from forty eight hour pure cultures grown on $\frac{1}{2}$NA which were picked-off and resuspened in $\frac{1}{4}$ Ringer's, giving roughly equal numbers of each strain. As has been previously noted these same species of bacteria were isolated from both the hospitals in south west England during the survey described in Chapter 4 and from other sites surveyed by us and other workers. Initially, the static beds were supplied with the synthetic pitting water as detailed in Section 2.2.2 with the addition of nutrients as
follows, mg l⁻¹: glucose, 16; sodium nitrate, 9.5; di-sodium orthophosphate, 0.3.
Routine sterility checks were carried out by plating out the effluent from the sterile system. After six months of operation, during which time occasional problems were encountered with maintaining the control system sterile, the added nutrients were omitted from the medium. This alleviated the problems in maintaining sterility. After a year of operation water from one of the hospitals, where Type 1½ pitting had been identified, became available on a regular basis and was adopted as the medium for the two systems following filter sterilisation as detailed in Methods (section 2.3.3).

Results and Discussion:

Figure 5.1 shows a thirty six hour plot for the pH and dissolved oxygen contents of the effluent from each vessel and the medium reservoir. A drop was seen in dissolved oxygen content in the inoculated system overnight when the flow was halted. The sterile system showed a lower dissolved oxygen content than the fresh medium but the level showed no significant variation between conditions overnight when the flow was stopped and during the day when the flow was restored, when D > μmax. This drop in the dissolved oxygen in the output from the sterile system compared to the medium input is not due simply to the "natural" loss of oxygen as no such drop was seen in the medium reservoir. There are several possible reasons for this drop, the most likely are, either the added nutrients were reacting with the copper and causing a drop in the level of dissolved oxygen or there was a miscalibration of the system, checks revealed the latter not to be the case.

The pH showed consistently lower values in the inoculated system than in either the other, sterile, system or the medium reservoir 6.7 versus 8.8 and 9.0 respectively. This could be due either to the production of organic acids, the release of carbon dioxide as a result of bacterial respiration or both. High pressure liquid chromatography (HPLC) analysis was carried out using an Aminex 87-H organic acid column (Mroček-Delclós, 1991). A number of peaks were detected which were
Figure 5.1 Thirty six hour plot of pH (---) and DO₂ (__) for inoculated, sterile systems and medium reservoir after four months.

Figure 5.2 Thirty six hour plot of pH (---) and DO₂ (__) for inoculated, sterile systems and medium reservoir after one year (less nutrients).
identified as constituents of the original medium. In addition, a few very small peaks were also obtained, for which the retention time did not match any of the ten organic acid standards run (lactic, acetic, propionic, butyric, formic, oxalic, tartaric, citric, malic and pyruvic acids). The production of carbon dioxide would seem the most likely explanation for the pH shift as any organic acid produced in sufficient quantity to change the pH would clearly be seen by HPLC. However, this does not mean that no organic acids are produced as evidenced by the weak positive for the production of acid from glucose obtained by the NCIMB for the brown *Ps. paucimobilis* (Appendix I).

Figure 5.2 again shows a thirty six hour plot, this time after the omission of the previously added nutrients. Similar patterns were seen, only this time the variations were smaller. In the sterile system both the dissolved oxygen and pH showed very little variation from those of the medium reservoir. This suggests that the drop originally seen in the dissolved oxygen was the result of a reaction between the oxygen, nutrients and copper. The inoculated system showed a consistently lower pH and the drop in the dissolved oxygen overnight was still evident but smaller. These results confirm the suggestion that the changes are a result of bacterial metabolism, with the uptake of oxygen and a release of carbon dioxide, and possibly small amounts of organic acids (Appendix I). A decrease in the level of nutrients would result in a decrease in growth with less oxygen uptake and carbon dioxide release.

After eighteen months of operation, when the inoculated system was showing signs of pitting, two rings were removed from each system and examined. The rings from the inoculated system were seen to be darker with a greater amount of a light blue corrosion product; a few possible areas of pitting were identified. However, the corrosion products were not disturbed to allow a detailed examination to be carried out by Mr. H. S. Campbell for pitting characteristics, his report is reproduced below.

"The samples from the sterile system showed broad areas, inside and outside,
where slight superficial corrosion had produced lacy blue/white corrosion product which was shown by chemical tests to contain copper (II) with a small quantity of carbonate and a very small quantity of chloride; sulphate was not detected. These results are interpreted as indicating that the material is principally basic copper carbonate. Most of the surface was covered with dull, light brown copper (I) oxide which was slightly thicker beneath the basic carbonate deposits. At one point, on the inside surface of one of the rings, close to the cut edge, the basic copper carbonate was in nodular form with powdery copper (I) oxide beneath but with no localised attack that could be regarded as pitting. On the extreme outer edge of the other ring from the sterile system a definite corrosion pit had developed. This was about 0.1 mm diameter, 0.05 mm deep, and contained crystalline copper (I) oxide with nodular basic copper carbonate surrounding it. This pit was situated at a point where the residual stress level from cutting (and consequently the potential for dissolution) was particularly high and where the ring was probably in point contact with another.

The two rings from the inoculated system were darker than those from the sterile system and carried a general coating of gelatinous material which was shown by staining tests to consist principally of polysaccharides. There was a broad area (15-20 mm wide and practically the full length of the sample) on the outside of one of the rings where corrosion had taken place producing a thin powdery orange layer of copper (I) oxide, partially covered by a thin, more coherent copper (I) oxide film and surrounded by a heavy deposit of corrosion products consisting principally of basic copper carbonate but with lamellar basic sulphate crystals also present. The area of attack is believed to have occurred where two rings were in close contact. On the opposite side of the same ring there was a ridge of corrosion product, about 1 mm wide and 5 mm long, consisting principally of basic copper carbonate but with small amounts of basic sulphate present, beneath which were a number of small but clearly defined corrosion pits, about 0.1 mm diameter, containing loose copper (I) oxide. The surface surrounding these pits carried an oxide film which was nearly black and probably consisted principally of copper (II) oxide. Since however the oxide film was very thin and its surface was contaminated with basic copper (II) carbonate it was not possible to carry out analysis to confirm this.
The second ring from the inoculated system showed two areas, one inside and one outside, where small pits had developed beneath a crust of basic copper carbonate. These pits were larger than those on the outside of the ring, being up to 0.3 mm diameter, and contained more crystalline copper (I) oxide. They were again surrounded by nearly black oxide films and small patches of similar dark film were observed on parts of the surface remote from pitting. It is probable that the dark film was largely copper (II) oxide but contamination with other deposits again made it impossible to carry out confirmatory analysis.

**Further analysis of static bed rings:**

After two years and three months the remaining rings were removed from each system and examined under a binocular light microscope for evidence of pitting. Sections of the rings were then prepared for SEM examination as detailed in Methods (section 2.3.11). Finally, rings were treated with dilute 25% nitric acid to lift any films. Where present, these were stained following the procedure of Chamberlain *et al.* (1988), (Method section 2.3.12) for the demonstration of polysaccharides.

In general all the rings removed from the inoculated system were darker than the rings from the sterile system. The majority of the rings removed from the inoculated system showed pitting, similar to that detailed above. Figure 5.3 shows one such pit. The pits were covered by a blue crystalline material, which under the SEM was seen to be lamellate (Figure 5.4). From its colour and morphology it was concluded that this material was basic copper sulphate. Surrounding the pits, and over the majority of the surface, was a crust of pale, greeny/blue material (Figure 5.5) which showed no definite crystal morphology under the SEM. This material produced effervescence with the addition of dilute nitric acid and was therefore identified as the basic copper carbonate, as shown by Campbell on the rings examined previously. The pits were seen to contain a dull red material, identified as copper (I) oxide. The pits were small, being up to 0.5 mm in diameter. Again the pits were surrounded by nearly black material which was believed to be copper (II) oxide, but again there was not enough material present for firm identification to be carried out.
Photographic details:

All colour photographs were taken using an OM-2 camera mounted on a Nachet binocular microscope, with additional light source (Nachet EF 50-s) using Kodak EPY 15-36 Ektachrome 64asa tungsten professional film.
Figure 5.3 View of pit in a copper ring exposed to an intermittent flow and a consortium of bacteria for two years (mag x40).

Figure 5.5 View of copper ring exposed to an intermittent flow and a consortium of bacteria for two years, showing a covering of basic copper carbonate and polysaccharide (mag x20)
Figure 5.4 SEM showing plate-like crystals of basic copper sulphate on pitted area seen in figure 5.3.

Figure 5.6 SEM of numerous bacteria on the surface of copper ring from static bed inoculated with a consortium of bacteria.
Using the SEM numerous bacteria were visible colonising the surface of the ring from the inoculated system (Figure 5.6). In contrast, the rings from the sterile system, showed no bacteria under the SEM and were a lighter colour than those described above, with no visible signs of pitting. In a few areas a thin layer of pale greeny/blue material was observed (Figure 5.7), this was identified as being basic copper carbonate. Most of the surface of the rings was as seen in Figure 5.8, having a few darker areas on an otherwise "normal" looking copper surface. The use of the SEM revealed the presence of a superficial layer of an amorphous material rather like that noted in the inoculated system but no bacteria were visible on the surface of any of the pipes, confirming the negative results of the regular plate counts carried out to check for sterility.

The use of dilute 25% nitric acid to lift any films present revealed that polysaccharide biofilms were only present in the inoculated system. A film was lifted from one of the sterile rings but this was optically denser and did not stain with the periodic acid-Schiff reagent nor the sequential alcian blue/yellow procedure as described by Chamberlain et al. (1988). It was therefore concluded that this film was not of microbial origin and was probably an inorganic film, the precise nature of this film was not determined but it was possibly a silica gel which is known to occur in water pipes.

Figures 5.9 & 5.10 show one of the rings from the inoculated system after treatment with 25% nitric acid to remove the corrosion products. It can be seen that the corroded region is composed of a number of small pits and is therefore perhaps more akin to the Type 3 or "pepper pot" pitting seen in the south west of Scotland. In line with Koch’s postulates the bacteria were recovered from the pitted rings and confirmed as only the three species originally inoculated. These were then used to reinoculate a further system.

**Conclusion:**

Although the *in vitro* pitting does not show all the characteristics of Type 1½ pitting, as the black copper (II) oxide was not present in the quantities seen *in vivo*, the involvement of bacteria, isolated from a failed pipe, in the pitting corrosion of
Figure 5.7 Copper ring exposed to a sterile intermittent flow for two years showing lacey white basic copper carbonate and darker areas of copper (I) oxide (mag x25).

Figure 5.8 Copper ring exposed to a sterile intermittent flow for two years showing lacey white basic copper carbonate and darker areas of copper (I) oxide (mag x25).
Figure 5.9 Light microscope picture of pitted copper ring after pickling with dilute 25% nitric acid to remove corrosion products (mag x50).

Figure 5.10 Light microscope picture of pitted copper ring after pickling with dilute 25% nitric acid to remove corrosion products (mag x50).
copper has clearly been demonstrated. However, as pitting occurred in both systems it appears that under suitable conditions, e.g. defects in the crystal structure caused by cutting stress or where point contact between two rings occurs, the water chemistry alone can support pitting. In the case of the sterile system the pits were only seen where two rings had been in contact initiating the pitting. In the case of the inoculated system several pits were seen on the inner surface of the rings, where no contact could have occurred. It can therefore be concluded that in this water the initiation of pitting can be brought about by contact, equating to a crevice or deposit, or the presence of organisms and/or their products.

The presence of another copper ring making contact with the copper surface will lead to a higher copper (I) ion concentration within the crevice formed between the two rings. This increase in copper (I) ion concentration will result in that part of the metal surface being cathodic to the region immediately outside of the crevice where copper ions can diffuse away. Consequently crevice corrosion of copper normally results in a trench of deep attack just outside the crevice while cathodic deposition of metallic copper often occurs within the crevice. It is frequently found that the high rate of attack in the region just outside of the crevice will result in the development of a membrane covered pit. Consequently as the deposit (in this case the contacting copper ring) has the characteristics of a water-impermeable film, which greatly restricts the diffusion of copper ions and oxygen, pit formation will be facilitated.

These results along with those from the site survey (Chapter 4) and the SEM examination of failed pipes (Chapter 3) provide for the first time the necessary evidence to prove the occurrence of Type 1½ pitting is linked with the action of the bacteria. The bacteria are probably involved in the initiation of the pit which then proceeds by the mechanisms outlined for pit propagation in the Introduction. This is supported by the similarity between the morphology and corrosion product chemistry of the Type 1½ pits and the Type 1 pits described by Lucey (1967).
RESULTS: PART 2

Physiology of the bacteria from pitted copper pipes.
Chapter 6

GROWTH OF BACTERIA IN BATCH CULTURE.

Batch culture was employed at an early stage of the work to determine the maximum specific growth rates (necessary for chemostat studies), and to indicate the growth phases at which polymer was produced in batch culture. Although these results in themselves are not of great direct significance, they are vital to underpin much of the work contained in the following chapters. The three bacterial species under examination (Methods section 2.1) were grown in a mixed batch culture (Introduction 1.6.1.1 and Methods 2.3.2) for thirty six hours at 30 °C. The inoculum was prepared by picking the bacteria from two day old pure cultures, grown on ½NA, into sterile ¼ Ringer’s solution. Samples (3 ml) were taken every two hours over a thirty six hour period. Aliquots of 100 µl were immediately used for cfu counts, (Methods 2.4.1) and the remainder frozen until further assays for residual amounts of glucose, ammoniacal nitrogen and phosphate together with total carbohydrates could be carried out as described previously in Methods (section 2.2.2.1).

Results:

The bacterial counts are shown in Figure 6.1 and the levels of residual glucose and total carbohydrate are shown in Figure 6.2. The Ps. solanacearum showed a four hour lag phase, then exponential growth proceeded for a further eight hours. The bacteria then remained in stationary phase from twelve to thirty six hours. The yellow strain of Ps. paucimobilis showed a longer lag phase of ten hours, over
Figure 6.1 Plot of bacterial counts against time for a mixed batch culture, (+) *Ps. solanacearum*, (△) yellow *Ps. paucimobilis*, (O) brown *Ps. paucimobilis*.

Figure 6.2 Plot of residual glucose (+) and total carbohydrate (△).
which time its numbers declined, and then steady exponential growth occurred for the remainder of the experiment. The brown strain of *Ps. paucimobilis* survived in the vessel for ten hours over which time it showed a steady drop in numbers until it finally disappeared. From the graphs for the two bacteria that survived their maximum growth rates could be calculated by two methods; either by dividing the natural log of two by the doubling time, or by the gradient of the line when the natural log of bacterial numbers is plotted against time. Using the latter method $\mu_{\text{max}}$ was calculated to be $0.39 \text{ h}^{-1}$ and $0.225 \text{ h}^{-1}$ for the *Ps solanacearum* and the yellow *Ps. paucimobilis* respectively.

The level of residual glucose can be seen to drop steadily over the thirty six hour period at an average rate of $1.125 \text{ g h}^{-1}$. The level of total carbohydrate was more erratic but a downward trend was observed for the first twenty hours followed by an increase until the end of the experiment. If a polynomial curve is fitted to the points as shown in Figure 6.2 the rate of decline in total carbohydrate levels is seen to be similar to that for the drop in residual glucose. This indicates that over the first twenty hours no significant level of exopolysaccharide is produced. However, after twenty hours the exopolysaccharide production is initiated at the same time as the *Ps. solanacearum* is entering its stationary phase of growth and the yellow *Ps. paucimobilis* is in mid-exponential growth. During the time course of this experiment the level of ammoniacal nitrogen decreased from $83 \text{ g ml}^{-1}$ to $57 \text{ g ml}^{-1}$ over the first eighteen hours, but never fell below the limit of detection ($1 \text{ g ml}^{-1}$). There was no decrease in the level of residual phosphate during the first twenty hours of the experiment but after this time a slight drop in the amount of residual phosphate could be detected, with a drop from $42.8 \text{ g ml}^{-1}$ to $40.7 \text{ g ml}^{-1}$.
Discussion:

These results indicate that the bulk of the polymer is produced in the later stages of exponential growth and continue into the stationary phase. This would tend to indicate that the polymer is produced as a secondary metabolite, therefore polymer is most likely to be produced in the operational systems during extended periods of stagnation, when the bacteria will follow batch culture conditions. However, it needs to be noted that this was a mixed batch culture with a heterogeneous population (information on polymer production by pure axenic cultures is given in Chapter 8). Chapter 11 details further studies carried out in a chemostat into polymer production at various growth rates, below $\mu_{\text{max}}$, under nitrogen limitation.

Figure 6.2 clearly shows some of the main problems that were encountered throughout the work concerning the detection of extracellular polysaccharides. The data appears to indicate that more carbohydrate is present at the end of the experiment than at the beginning. These results were confirmed by repeating the assays twice. It is not certain why this is happening, but it is possible that the bacteria are utilising carbon sources other than carbohydrates. This would be particularly likely as production occurred later in the experiment when a number of the bacteria were in stationary phase and growth was limited. It has been suggested that in such circumstances the bacteria produce polymers to keep the metabolic pathways active.

Tests carried out using glucose as the only added carbon source in uninoculated medium showed that the levels detected by the two methods were occasionally different, even though the same standards were used. Simple checks ruled out the possibility that this variation was due to the presence of L-glucose which would not be detected by the glucose assays. One reason for the variation was possibly due to
the fact that the assays were often carried out at the lower extreme of their sensitivity. This was often particularly true of the glucose assays. It is known that the deviation in the absorbance readings of the spectrophotometer was ±0.003 in the case of the phenol sulphuric acid assay and that 10 µg ml\(^{-1}\) glucose gave an absorbance of about 0.100 therefore the error is negligible. In the case of glucose Trinder assay 10 µg ml\(^{-1}\) of glucose gave an absorbance of about 0.010, therefore the error is far more significant.

A number of possible alternatives were sought for determining the level of EPS produced, but were found to be unsuitable. A common method employed by other workers is the collection of the polymer by propan-2-ol precipitation. This was impracticable due to the large number of samples often employed and the low amounts of polymer generated due to the low levels of glucose provided in the media. These were a compromise in order to maintain the nutrient levels close to those in natural potable waters, whilst still being able to assay them. HPLC was considered as a technique which could have been both quantitative and specific enough, however on checking in the literature and with the major suppliers it was found there were no methods available for the identification of long chain bacterial polysaccharides. It was found to be possible to detect polymers by gel filtration, but this method was neither quantitative nor specific enough. A possible solution could have been found in an Elisa type assay but no such assay has yet been developed.
Chapter 7

TIME STUDIES IN SHAKE FLASKS.

Due to the low levels of nutrients present within potable waters (dissolved organic carbon $1 \mu g \text{ ml}^{-1}$, ammoniacal nitrogen $0.03 \mu g \text{ ml}^{-1}$ and phosphate $0.76 \mu g \text{ ml}^{-1}$, values obtained from S.W. Water for hospital T) it is likely that nutrient limitation will rapidly occur. It is therefore necessary to know the effects of nutrient limitation on planktonic bacteria, in relation to their polymer production and also whether nutrient limitation will enhance the attachment of bacteria to a surface. The level at which nutrient limitation occurs can be determined by growing the bacteria in a series of media in which all the nutrient levels are kept high except the nutrient under test. This test nutrient is added to give a series of increasing concentration; the point of incipient limitation is then said to occur when further addition of the test nutrient results in no increment of growth, provided all other growth requirements are satisfied.

Time studies were performed using shake flasks to determine the level at which various nutrients became limiting for the mixed population of bacteria isolated from failed pipes (Methods section 2.1). The effects on polymer production due to various nutrient limitations were also determined. These nutrient levels were then related to the values commonly found in water supplied to the buildings exhibiting pitting failure, and subsequently used to select which nutrient should be limiting in the chemostat studies reported in Chapter 11.

Flasks (250 ml) containing $50 \text{ ml}$ of the following media, PL1, PL10, PL25, GL10, GL25, GL50, AL1, AL5 and AL10 as detailed in Methods (section 2.2.2),
(PL-phosphate limitation, GL-glucose limitation and AL-ammonia limitation number - μg ml⁻¹ of limiting substrate), were incubated at 30 °C and shaken at 220 rpm. It has been previously calculated that these conditions will prevent oxygen limitation occurring (Mc Dermott, 1991). Due to nitrate normally being the nitrogen source present in waters at the highest levels a further set of flasks was used to compare the levels of limitation and exopolysaccharide production by the bacteria using ammoniacal nitrogen and nitrate. Assays were carried out as described in Methods (section 2.4) for cfus, the residual levels of glucose, ammonium and phosphate along with total carbohydrate, also the absorbance at 600 nm was read for the 10 day assay set, to give a value of total biomass (viable and non-viable). Triplicate flasks were used for each medium. The inocula were two day pure cultures grown on ½NA picked off into 9 ml of sterile ¼ Ringers, initially giving the following bacterial counts: Ps. solanacearum 2.56 x 10⁶ cfu ml⁻¹; brown Ps. paucimobilis 7.5 x 10⁶ cfu ml⁻¹ and yellow Ps. paucimobilis 3.3 x 10⁶ cfu ml⁻¹ (t=0 values).

**Results:**

**7.1 Growth limitation levels.**

Figure 7.1 shows that over the entire experiment in each flask the phosphate level increased by 3-10 μg ml⁻¹. The greatest rise was seen in the AL and GL series of media, the PL series showed smaller increases. Table 7.1 shows the total number of bacteria (cfus) for the PL series of media at five, seven and ten day intervals.
Figure 7.1 Plot of residual phosphate for PL1 (+), PL10 (●), PL25 (●) and average of AL & GL series media (+).

Figure 7.2 Plot of residual glucose levels for GL10 (+), GL25 (△) & GL50 (○).
Table 7.1 Determination of level at which phosphate becomes limiting for a mixed population of bacteria isolated from a failed copper pipe.

<table>
<thead>
<tr>
<th>Phosphate µg ml⁻¹</th>
<th>Total cfus ml⁻¹ 5 days</th>
<th>Total cfus ml⁻¹ 7 days</th>
<th>Total cfus ml⁻¹ 10 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>80x10⁶</td>
<td>19x10⁶</td>
<td>11x10⁶</td>
</tr>
<tr>
<td>10</td>
<td>57x10⁶</td>
<td>22x10⁶</td>
<td>25x10⁶</td>
</tr>
<tr>
<td>25</td>
<td>69x10⁶</td>
<td>93x10⁶</td>
<td>83x10⁶</td>
</tr>
</tbody>
</table>

Table 7.1 indicates that perhaps phosphate was limiting, in that, with 25µg ml⁻¹ higher bacterial counts were obtained for the seven and ten day samples compared to the lower levels in PL1 and PL10. The five day sample however shows higher bacterial counts for PL1 than the other two. This clearly shows that phosphate was not limiting at this stage in any of the cultures. The residual glucose assay for the five and seven days samples, where limitation appeared to be occurring, showed that the level of glucose had fallen below 10 µg ml⁻¹, the level at which glucose limitation was seen to occur, therefore no phosphate limitation occurred. When the levels of residual phosphate are compared it would appear that no phosphate is used in any of the flasks as all the residual phosphate levels rose. However, as zero phosphate utilisation cannot be the cause, this could be due either to stored phosphate being released by the cells or some unknown metabolic product interfering with the assay and giving falsely high results. It is known that many commercial detergents contain high levels of phosphate which could leave residual phosphate on the glassware used for the experiments and assays.

In order to ensure this was not the source of phosphate contamination all glassware was cleaned in '7x' phosphate-free detergent (Flow Laboratories), acid washed in 2% 'Analar' sulphuric acid over night, followed by extensive rinsing in
RO water (six complete changes). A further set of flasks was set up using PL1, PL0 (as the other PL series medium but with no added phosphate), AL1 and AL5, with an inoculum grown in PL1 for three days. Both PL media were unable to sustain bacterial growth whilst growth occurred in both the AL series media. This would indicate that either one of the other nutrients was present at an inhibitory level, or the phosphate is present at a limiting level. The former seems unlikely as good growth was observed in the AL series media which had the same level of glucose as the PL media and good growth was seen in the earlier experiments using GL series media which has the same level of ammonia as the PL series media. However, there is a possibility that there is a combined inhibitory effect due to high glucose and ammonia. The residual phosphate assay showed levels of phosphate starting at 8 µg ml⁻¹ and rising to 10 µg ml⁻¹ after ten days, the level of phosphate initially added was 0 and 1 µg ml⁻¹ respectively for the PL0 and PL1 medium. The conclusion drawn from the latter experiment is that phosphate levels are limiting at some level below 10µg ml⁻¹ and that due to interference in the phosphate assay this level was not determined. The former experiment showed that the bacteria used in the experiment are able to store phosphate and recycle it thus preventing phosphate limitation. In this case the drop in bacterial numbers coincided with the glucose levels dropping to 10 µg ml⁻¹ or below, suggesting glucose limitation. The ability of the bacteria to store phosphate was confirmed using Albert’s stain for volutin granules (Laybourn, 1924). The increase seen in the phosphate level cannot be totally accounted for by storage. A major factor is thought to arise from the assay which was used close to its limit of detection. Checks have shown that accuracy declines at levels below 10 µg ml⁻¹. It is felt that limitation should not be occurring with such high levels of phosphate as the bacteria have been seen to grow in tap water which is known to have low levels of phosphate.
Glucose limitation was clearly achieved in the GL series of media by reducing the glucose concentration to 10 µg ml\(^{-1}\) (Table 7.2).

Table 7.2 Investigation into levels at which carbon limitation occurs for a mixed population for bacteria isolated from a failed copper pipe.

<table>
<thead>
<tr>
<th>Glucose µg ml(^{-1})</th>
<th>Total cfus ml(^{-1}) 5 days</th>
<th>7 days</th>
<th>10 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>53x10(^6)</td>
<td>42x10(^6)</td>
<td>23x10(^6)</td>
</tr>
<tr>
<td>25</td>
<td>179x10(^6)</td>
<td>108x10(^6)</td>
<td>47x10(^6)</td>
</tr>
<tr>
<td>50</td>
<td>80x10(^6)</td>
<td>57x10(^6)</td>
<td>32x10(^6)</td>
</tr>
</tbody>
</table>

In each case the drop in bacterial numbers coincided with the residual glucose level falling below 10 µg ml\(^{-1}\) (Figure 7.2). This accounts for the low bacterial count for GL50, initially, the assays showed approximately 50 µg ml\(^{-1}\) of glucose but by the time the first sample was taken, at five days, this had dropped to below 10 µg ml\(^{-1}\), suggesting that the higher glucose level had allowed growth to occur at a higher rate than at the lower levels and that the cells have entered the decline phase of the batch growth cycle.

Nitrogen limitation was clearly evident when the level of ammonia-N was reduced from 5 µg ml\(^{-1}\) to 1 µg ml\(^{-1}\) (Table 7.3)

Table 7.3 Investigation into the levels at which nitrogen limitation occurs for a mixed population of bacteria isolated from a failed copper pipe.

<table>
<thead>
<tr>
<th>Ammonium µg ml(^{-1})</th>
<th>Total cfus ml(^{-1}) 5 days</th>
<th>7 days</th>
<th>10 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.7x10(^6)</td>
<td>13.6x10(^6)</td>
<td>8.58x10(^6)</td>
</tr>
<tr>
<td>5</td>
<td>49.7x10(^6)</td>
<td>66.2x10(^6)</td>
<td>41.5x10(^6)</td>
</tr>
<tr>
<td>10</td>
<td>14.6x10(^6)</td>
<td>13.0x10(^6)</td>
<td>31.3x10(^6)</td>
</tr>
</tbody>
</table>
7.2 Product formation

During the course of the time studies in shake flasks the level of total carbohydrate increased as the biomass (bacterial counts) decreased where phosphate and nitrogen limitation had occurred. Table 7.4 shows the specific polymer yield for each medium determined by the subtraction of residual glucose from the total carbohydrate level, expressed per viable cfu at five and seven days and per absorbance unit at 600 nm after ten days, giving an estimation of both viable and non-viable bacteria.

Table 7.4 Time course of polymer yield by a mixed population of bacteria isolated from a failed pipe grown under various nutrient limitations.

<table>
<thead>
<tr>
<th>Media</th>
<th>Yield of polymer after 5 days (µg ml⁻¹)/cfu⁵#</th>
<th>Yield of polymer after 7 days (µg ml⁻¹)/cfu⁵#</th>
<th>Yield of polymer after 10 days (µg ml⁻¹)/OD₆₀₀*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PL1</td>
<td>0.049</td>
<td>0.088</td>
<td>0.069</td>
</tr>
<tr>
<td>PL10</td>
<td>0.057</td>
<td>0.071</td>
<td>0.087</td>
</tr>
<tr>
<td>PL2'</td>
<td>0.006</td>
<td>0.006</td>
<td>0.065</td>
</tr>
<tr>
<td>GL10</td>
<td>0.004</td>
<td>0.006</td>
<td>0.050</td>
</tr>
<tr>
<td>GL25</td>
<td>0.003</td>
<td>0.005</td>
<td>0.044</td>
</tr>
<tr>
<td>GL50</td>
<td>0.005</td>
<td>0.005</td>
<td>0.079</td>
</tr>
<tr>
<td>AL1</td>
<td>0.095</td>
<td>0.298</td>
<td>0.180</td>
</tr>
<tr>
<td>AL5</td>
<td>0.042</td>
<td>0.105</td>
<td>0.139</td>
</tr>
<tr>
<td>AL10</td>
<td>0.007</td>
<td>0.175</td>
<td>0.113</td>
</tr>
</tbody>
</table>

#cfu⁵ - colony forming units (Viable bacteria) x 10⁵
*OD₆₀₀ - Optical Density at 600nm (viable & nonviable bacteria)

The above results clearly show that the greatest yield of polymer is produced under conditions of nitrogen limitation. The yield values for PL series of media
suggest that polymer is produced at the two lower levels of phosphate after five and
seven days. However, this effect becomes obscured for the yield at ten days when
absorbance at 600 nm was used. It is possible that some polymer is produced for
GL50 after ten days as this yield is similar to those for the PL series of media.

7.3 Comparison of nitrate and ammonia as nitrogen sources

In the initial study above, ammonium sulphate was used as the nitrogen source
due to the ease with which ammoniacal nitrogen could be measured in the
laboratory. However, when the chemistry of the waters known to sustain Type 1½
pitting is examined it is evident that the major nitrogen source present is nitrate (1
μg ml⁻¹). Therefore a further set of shake flasks was set up containing the following
media AL1, AL5, AL10, NL1, NL5 & NL10. The bacterial inoculum was similar to
that used in the previous experiments, giving roughly the same numbers and ratio of
bacteria, and the incubation was for three, five, seven and ten days at 30 °C. After
each time period a sample was removed and bacterial counts carried out as
described in Methods section 2.4.1. Residual glucose, ammonia and nitrate were
assayed along with total carbohydrate as detailed in Section 2.4.

Results

When the bacterial counts were examined (Table 7.5) it was noted that due to
the high inoculum used the results were not clear cut, however, a number of trends
were discernible. The *Ps. solanacearum* grew better in the ammonia limited media
than it did in the nitrate media. The counts for both the media showed that
increasing the levels of nitrogen had no effect on the numbers of *Ps. solanacearum,*
suggesting that limitation was not occurring after five days growth. Again the bacterial counts for the yellow *Ps. paucimobilis* were higher with ammonia as the nitrogen source, compared to nitrate. With both nitrogen sources the levels had no discernible effect on the bacterial counts. The counts for the brown *Ps. paucimobilis* dropped in both media as the nitrogen levels were increased; this result was greater in the case of the ammoniacal nitrogen, which constantly had lower counts than the nitrate. In order to determine the overall effect on growth of the mixed culture either the level of glucose utilisation or the optical density at 600 nm can be compared, both of which will give a reliable indicator of growth. These results are shown in Table 7.6.

Table 7.5 Bacterial counts for a mixed culture when grown in media containing differing levels of nitrate or ammonium salts as the nitrogen source after 5 days incubation at 30 °C.

<table>
<thead>
<tr>
<th>Media</th>
<th><em>Ps. solanacearum</em></th>
<th>Yellow <em>Ps. paucimobilis</em></th>
<th>Brown <em>Ps. paucimobilis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>NL1</td>
<td>3.8 x 10⁶</td>
<td>5.8 x 10⁴</td>
<td>7.8 x 10⁶</td>
</tr>
<tr>
<td>NL5</td>
<td>4.3 x 10⁶</td>
<td>6.8 x 10⁴</td>
<td>1.2 x 10⁷</td>
</tr>
<tr>
<td>NL10</td>
<td>4.5 x 10⁶</td>
<td>2.8 x 10⁴</td>
<td>1.0 x 10⁶</td>
</tr>
<tr>
<td>AL1</td>
<td>1.1 x 10⁷</td>
<td>9.8 x 10⁴</td>
<td>2.7 x 10⁶</td>
</tr>
<tr>
<td>AL5</td>
<td>6.5 x 10⁶</td>
<td>8.0 x 10⁴</td>
<td>3.9 x 10²</td>
</tr>
<tr>
<td>AL10</td>
<td>9.2 x 10⁶</td>
<td>1.2 x 10⁵</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 7.6 Glucose utilisation and absorbance at 600nm used as indicators of growth for a mixed culture when grown in media containing differing levels of nitrate or ammonium salts as the nitrogen source (after ten days incubation).

<table>
<thead>
<tr>
<th>Media</th>
<th>OD.600nm</th>
<th>Glucose utilised µg ml⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>NL1</td>
<td>36</td>
<td>29</td>
</tr>
<tr>
<td>NL5</td>
<td>28</td>
<td>54</td>
</tr>
<tr>
<td>NL10</td>
<td>30</td>
<td>43</td>
</tr>
<tr>
<td>AL1</td>
<td>54</td>
<td>31</td>
</tr>
<tr>
<td>AL5</td>
<td>87</td>
<td>45</td>
</tr>
<tr>
<td>AL10</td>
<td>107</td>
<td>90</td>
</tr>
</tbody>
</table>

From Table 7.6 it is clearly evident that growth is greater in the medium with ammonia as the nitrogen source and that, as the level of ammoniacal nitrogen is increased, so does the growth indicating that limitation is occurring. In the case of nitrate it is less clear cut and would appear to indicate that the level of nitrate has little positive effect on growth. This was confirmed by the results of the assay for residual nitrate which showed that no nitrate was utilised over the period of the experiment and the levels in fact rose.

The polysaccharide yields (Table 7.7) for the ammonia series of media showed the same trend as described above, namely that as the amount of ammonia is increased the yield of polymer decreases. In the case of the yield for the nitrate series of media the reverse is the case, with polymer yield being higher with increased levels of nitrate. The yield of polymer is higher for each of the nitrate media than for any of the ammonia media. This result would be expected if indeed nitrate is not utilised as a nitrogen source for growth, further it would suggest that
beyond a certain level nitrate is inhibitory to growth, but promoting polymer production instead.

Table 7.7 Polymer yield by a mixed population of bacteria isolated from a failed pipe grown under various nutrient limitations (after ten days incubation).

<table>
<thead>
<tr>
<th>Media</th>
<th>Yield of polymer (µg ml⁻¹)/OD₆₀₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>NL1</td>
<td>0.107</td>
</tr>
<tr>
<td>NL5</td>
<td>0.303</td>
</tr>
<tr>
<td>NL10</td>
<td>0.303</td>
</tr>
<tr>
<td>AL1</td>
<td>0.114</td>
</tr>
<tr>
<td>AL5</td>
<td>0.058</td>
</tr>
<tr>
<td>AL10</td>
<td>0.043</td>
</tr>
</tbody>
</table>

From these results it can be seen that in the waters where Type 1½ pitting is evident and the levels of nutrients are low, the production of exopolysaccharides will be enhanced. These exopolysaccharides will then have a beneficial role in the attachment of the bacteria as seen in the later chapters. The inhibitory effect of nitrates and the apparent preference for organic nitrogen sources would be of great benefit in biofilm growth where lysis products will be retained within the biofilm matrix. The fact that ammonium ion limitation gave the highest yield of polymer was instrumental in the choice of ammoniacal nitrogen as the limiting nutrient in the chemostat studies reported in Chapter 11.
Chapter 8

8.1 EFFECTS OF TEMPERATURE ON PLANKTONIC GROWTH AND POLYMER PRODUCTION

From the observations made in Chapter 4, regarding the temperatures of the waters in which pitting occurred, it appears that this is an important factor in the corrosion process. This was confirmed by a survey carried out into the effect of temperature on pitting in a number of hospitals in the south west of Scotland, where no occurrences of non-Type 2 pitting were found in systems maintained throughout above 55 °C (Walker et al., 1991).

8.1.1 Growth

In order to determine the interrelationships between temperature, growth, extracellular polymer production and copper tolerance of pure axenic cultures of the bacteria and a mixed community of all three bacterial species, the temperature block described in Methods (section 2.3.6) was used. The bacteria were grown in modified ARJ medium with 2000 µg ml\(^{-1}\) of glucose (Methods section 2.2.2). Assays were carried out for cfus, residual glucose, total carbohydrate and total protein as detailed in Methods (section 2.4). The tubes were incubated at 5 °C intervals over the 10 - 50 °C range.

Results:

When grown in pure cultures (Figure 8.1a) the \textit{Ps. solanacearum} showed good growth over the 10 - 50 °C temperature range reported. A preliminary run had
Figure 8.1a Variation of growth with temperature for three bacterial strains cultured individually. (+) *Ps. solanacearum*, (o) yellow *Ps. paucimobilis* & (Δ) brown *Ps. paucimobilis*. (N.B. no growth at 15 °C for the brown *Ps. paucimobilis* for three replicate tubes in three separate experiments.

Figure 8.1b Variation of growth (as cfus ml⁻¹) with temperature for *Ps. solanacearum* grown as a pure culture.
shown no growth at 55 °C (Figure 8.1b). The yellow *Ps. paucimobilis* showed high bacterial numbers when grown at 10, 15 and 20 °C. At the higher temperatures the numbers declined until no bacteria were detected at 40 °C. The brown *Ps. paucimobilis* showed growth at 10 °C, none at 15 °C, then growth was recorded again at ALL intervals up to 45 °C. This result of no growth at 15 °C is inexplicable but was seen in each of nine individual tubes, but was not seen in the later experiment with the lower glucose level and no added copper. The link between the upper cut-off temperatures for growth and pitting, serves as extra evidence for the microbial involvement in Type 1½ pitting.

When grown as a mixed culture (Figure 8.2) a number of interactions were seen between the various strains. In mixed culture the yellow *Ps. paucimobilis* appeared to derive some temperature resistance at the higher temperatures with higher growth between 25 and 40 °C. However, at the lower temperatures, 15 and 20 °C it appeared to have a slightly inhibitory effect with decreased bacterial numbers for the *Ps. solanacearum*. This effect could be caused by the yellow *Ps. paucimobilis*

![Figure 8.2 Variation of growth with temperature for three bacterial strains grown as a mixed culture, (+) *Ps. solanacearum*, (A) brown & (o) yellow *Ps. paucimobilis*.](image)
out competing the *Ps. solanacearum* for some growth limiting substrate. The assay for residual glucose showed that this was not at a limiting level. Ammoniacal nitrogen was not assayed but this was felt to be present in excess at an initial concentration of 273 μg ml⁻¹. It is most likely that oxygen was the limiting factor as the cultures were grown in narrow test tubes which would allow little oxygen diffusion into the medium. Each of the three species of bacteria also showed a peak in numbers at 35 °C.

The results for protein and carbohydrate production for the pure and mixed cultures are shown in Figures 8.3-8.6. Tests on the initial medium alone showed an apparent protein level of around 100 μg ml⁻¹, possibly due to the reducing effect of glucose which has been shown to cause interference (Smith *et al.*, 1985). Figure 8.3 clearly shows carbohydrate polymer production, in mixed culture, at the higher temperatures between 25 and 45 °C, with a peak occurring at 40 °C. Extracellular protein in the medium is elevated at temperatures below 30 °C. A small amount was detected at 45 °C, if the yield of extracellular protein per cfu is plotted a peak is still seen at 20 °C but the peak at 45 °C becomes very large.

In pure culture carbohydrate polymer is produced between 15 and 45 °C by the *Ps. solanacearum* (Figure 8.4). Figure 8.5 shows that no detectable polymer was produced by the yellow *Ps. paucimobilis* and Figure 8.6 shows that the brown strain of the same species shows a barely detectable trace above 30 °C.

Protein release in the pure cultures was much lower than in the mixed culture. The *Ps. solanacearum* showed no detectable levels of extracellular protein (Figure 8.4). The yellow strain of *Ps. paucimobilis* showed signs of protein release between 15-40 °C (Figure 8.5) while the brown strain of the *Ps. paucimobilis* showed steadily increasing levels from 20 °C up to 50 °C (Figure 8.6).
Figure 8.3 Temperature profiles of residual glucose (+), total carbohydrate (▲) and Protein levels (○) in a mixed culture (note tests showed the media gave a reading of approximately 100 µg ml⁻¹).

Figure 8.4 Temperature profiles of residual glucose (+), total carbohydrate (▲) and Protein levels (○) for a pure culture of *Ps. solanacearum* (note tests showed the media gave a reading of approximately 100 µg ml⁻¹).
Figure 8.5 Temperature profiles of residual glucose (+), total carbohydrate (Δ) and Protein levels (o) for a pure culture of the yellow strain of *Ps. paucimobilis* (note tests showed the media gave a reading of approximately 100 μg ml⁻¹).

Figure 8.6 Temperature profiles of residual glucose (+), total carbohydrate (Δ) and Protein levels (o) for a pure culture of the brown strain of *Ps. paucimobilis* (note tests showed the media gave a reading of approximately 100 μg ml⁻¹).
8.1.2 Copper Tolerance

The above experiment was repeated with a lower level of glucose (200 μg ml⁻¹) in modified ARJ media (Methods section 2.2.2), with added copper (II) ions to give final copper concentrations of 50, 100 and 150ppm; a control with no added copper was also used. The reasons for the reduction in the level of glucose were twofold; firstly it was noted that in the previous experiments only a small amount of the glucose was utilised, secondly it was known that glucose could bind small amounts of copper (Chapter 10). The amount of bound copper needed to be minimised. Assays were carried out for bacterial counts, residual glucose and total carbohydrate as described in Methods (section 2.4)

Results:

Although the assays were all carried out as above it proved impossible to interpret the results of the total carbohydrate assay. The levels of total carbohydrate showed no sensible relation to the residual glucose levels. Tests carried out on the total carbohydrate assay using standard glucose solutions loaded with increasing levels of copper showed that copper ions interfered with the assay. For this reason the bacterial counts alone are reported.

Figures 8.7-8.12 show the effect of increasing copper concentrations on bacterial counts for the three strains of bacteria tested when grown both as pure axenic cultures and as a mixed culture.

The most noticeable effect of increasing copper concentration is seen at temperatures below 25 °C, with a marked decline in growth as the copper concentration is increased. In all the cultures, at all copper concentrations, the
bacterial counts are very similar at temperatures between 25 and 40 °C. Above this temperature the effect of increasing copper concentrations was less marked but trends were seen which are summarised in Table 8.1.

Table 8.1 Summary of effects of increasing copper concentrations on maximum temperature at which growth occurred.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Single spp. culture</th>
<th>Mixed spp. culture</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ps. solanacearum</em></td>
<td>Decrease in max. temp. of growth.</td>
<td>Variable</td>
</tr>
<tr>
<td><em>Ps. paucimobilis</em></td>
<td>Decrease in max. temp. of growth.</td>
<td>Increase in max. temp. of growth.</td>
</tr>
<tr>
<td>(Yellow)</td>
<td>Variable.</td>
<td>Increase in max. temp. of growth.</td>
</tr>
<tr>
<td>(Brown)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The link that is clearly demonstrated between copper tolerance and temperature is particularly striking, with only the pure culture of the brown *Ps. paucimobilis* recovered at 20 °C when 150 ppm of copper were added. However, at 35 °C no significant difference could be detected between the bacterial counts at any of the copper concentrations. The earlier work using the temperature block to look at polymer production (Section 8.1.1) showed that carbohydrate polymers were produced between 25 and 45 °C in the mixed culture. Where carbohydrate polymer was detected for pure axenic cultures it was again mainly in this temperature range, where copper levels up to 150 ppm appear to have no effect on bacterial growth. This is complementary to the findings of Geesey and Mittelman (1985) that carbohydrate polymers produced by *Pseudomonas* spp. have the ability to bind free copper ions, which has been suggested as a mechanism for copper tolerance by several authors (Brown & Lester, 1979; Rudd *et al.*, 1984 & Geesey & Mittelman, 1985). These results would appear to confirm this suggestion.
Figure 8.7 Effects of temperature and copper (II) ion concentration, (+) 0 ppm, (△) 50 ppm, (○) 100 ppm & (+) 150 ppm, on the growth of a pure culture of *Ps. solanacearum*.

Figure 8.8 Effects of temperature and copper (II) ion concentration, (+) 0 ppm, (△) 50 ppm, (○) 100 ppm & (+) 150 ppm, on the growth of a pure culture of the yellow strain of *Ps. paucimobilis*. 
Figure 8.9 Effects of temperature and copper (II) ion concentration, (+) 0 ppm, (Δ) 50 ppm, (o) 100 ppm & (+) 150 ppm, on the growth of a pure culture of the brown strain of *Ps. paucimobilis*.

Figure 8.10 Effects of temperature and copper (II) ion concentration, (+) 0 ppm, (Δ) 50 ppm, (o) 100 ppm & (+) 150 ppm, on the growth of *Ps. solanacearum* when grown as a mixed culture of the three test organisms.
Figure 8.11 Effects of temperature and copper (II) ion concentration, (+) 0ppm, (△) 50ppm, (o) 100ppm & (+) 150ppm, on the growth of the yellow strain of *Ps. paucimobilis* when grown as a mixed culture of the three test organisms.

Figure 8.12 Effects of temperature and copper (II) ion concentration, (+) 0ppm, (△) 50ppm, (o) 100ppm & (+) 150ppm, on the growth of the brown strain of *Ps. paucimobilis* when grown as a mixed culture of the three test organisms.
8.2 EFFECTS OF TEMPERATURE ON ATTACHED GROWTH AND POLYMER PRODUCTION

It has been shown that pipes maintained above 55 °C do not suffer Type 1½ pitting failure (Chapter 4 & Walker et al., 1991). In the previous section it was demonstrated that this could be due to the inability of planktonic bacteria to grow at this temperature and/or produce polymer. The following experiment was carried out to determine the effect of temperature on growth and exopolysaccharide production for attached bacteria. These are likely to differ in their physiology from planktonic populations (see section 1.3). A series of tubular reactors were set up as described in Methods (section 2.3.5). These were operated for four weeks, supplied with filter sterilised (Methods section 2.3.3) water collected from a hospital in southwest England exhibiting Type 1½ pitting. Material collected from the each of the three pipes at the same temperature (Methods section 2.3.8) was assayed in triplicate for bacterial counts and total carbohydrate as described previously (Methods section 2.4.1 & 2.4.6). Initially tubular reactors were operated at 15, 25, 30, 35, 40 and 45 °C in two separate experiments. In the first run, temperatures of 25, 35 and 45 °C were used, the other temperatures were used for the second run.

Results and Discussion:

Figure 8.13 shows the exopolysaccharide produced at these temperatures, while Table 8.2 shows the bacterial counts for each temperature.
Table 8.2 Attached bacterial counts from copper tubular reactors

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>Ps. solanacearum</th>
<th>Yellow Ps. paucimobilis</th>
<th>Brown Ps. paucimobilis</th>
<th>Total cfus cm⁻²</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>1.3x10⁵</td>
<td>2.8x10²</td>
<td>-</td>
<td>1.3x10⁵</td>
</tr>
<tr>
<td>25</td>
<td>7.1x10⁴</td>
<td>1.6x10³</td>
<td>1.5x10⁴</td>
<td>8.8x10⁴</td>
</tr>
<tr>
<td>30</td>
<td>2.1x10⁴</td>
<td>-</td>
<td>6.6x10³</td>
<td>2.7x10⁴</td>
</tr>
<tr>
<td>35</td>
<td>1.7x10⁵</td>
<td>3.9x10³</td>
<td>1.8x10⁴</td>
<td>1.9x10⁶</td>
</tr>
<tr>
<td>40</td>
<td>7.4x10⁴</td>
<td>-</td>
<td>64</td>
<td>7.4x10⁴</td>
</tr>
<tr>
<td>45</td>
<td>8.1x10³</td>
<td>40</td>
<td>-</td>
<td>8.1x10³</td>
</tr>
</tbody>
</table>

The results of bacterial counts confirm those obtained for planktonic growth of *Ps. solanacearum* and the brown *Ps. paucimobilis* (section 8.1); in which the *Ps. solanacearum* grew well over the whole temperature range and the brown *Ps. paucimobilis* showed higher bacterial numbers at the mid-range temperatures. The yellow *Ps. paucimobilis* showed higher counts at the lower temperatures. The results for exopolysaccharide production (Figure 8.13) showed conflicting results. When all the results from both runs were considered it would appear that good production occurred at the lower temperatures with a peak at 30 °C and little production at 45 °C. Considering the two runs separately a different picture is obtained from the first run which shows a higher level of production at 15 °C with a gradual decline as the temperature was increased. As the two runs were carried out at separate times the inoculum might have varied although this should have been minimised by the use of the chemostat. A more likely explanation can be found in the water used as the medium. This was collected from the hospital on two separate occasions and it is known that variations do occur in the level of nutrients in the water.

In view of these conflicting results the experiment was repeated, with an extra
Figure 8.13 Level of polymer isolated from tubular reactors operated at various temperatures in two separate experiments (25, 35 & 45 and 15, 30 & 40 °C)

Figure 8.14 Level of polymer isolated from tubular reactors operated at 20, 25, 30 and 35 °C in one experiment.
set of tubes allowing 20, 25, 30 and 35 °C to be examined in the same run. Figure 8.14 shows the results for exopolysaccharide production, which confirm the results seen in the first run. The levels of polymer produced decreased with increasing temperatures, however, planktonic bacteria (section 8.1.1) showed increased polymer production with increasing temperature, with no detectable carbohydrate polymer produced below 25 °C. The production of polymer by attached bacteria at these lower temperatures is of significance as Type 1½ pitting occurs in "cold" water pipes which in hospitals run at about 25 °C (Chapter 4).

From these results it is evident that the bacteria are able to colonise copper at temperatures between 15 and 45 °C. This is relevant to the studies on Type 1½ pitting as this is the temperature range over which pitting is seen to occur. It is of interest that the greatest amount of polymer is produced at the lower temperatures for attached growth compared to the higher temperatures seen for planktonic seen in section 8.1 (N.B. units of polymer production are not comparable, data needs to be treated qualitatively). This clearly supports the observation made by Costerton et al. (1987) reported in Section 1.3 that planktonic cells are often structurally and functionally different from biofilm bacteria. It can be seen that some of the data obtained on planktonic populations (Figure 8.2) are relevant to biofilm bacteria (Table 8.2), for instance their temperature growth profiles. Alternatively, it can be seen that the temperature profiles for exopolysaccharide production differ (Figure 8.3 & Figure 8.14), this latter point is perhaps not surprising as the exopolysaccharide is an integral part of the ability to form a biofilm.
Chapter 9

9.1 A STUDY OF THE INTERACTION BETWEEN PLANKTONIC AND ATTACHED GROWTH IN A FED BATCH CULTURE.

As already mentioned in Chapter 5 the operational system does not comply with a simple chemostat model in relation to flow regimes, with high flow followed by extended periods of stagnation. These two extremes of flow will favour different forms of growth (Characklis, 1990). A high, non-turbulent, flow rate giving a dilution rate in excess of $\mu_{\text{max}}$, will enhance biofilm growth and the stagnation will initially enhance planktonic growth until the nutrients are depleted. The results of the survey reported in Chapter 4 revealed the likely importance of stagnation in the pitting process. This was confirmed by an extensive survey carried out in a German Hospital, which showed that pitting occurred primarily in horizontal pipes which experienced extended periods of stagnation (Fischer et al., 1991). In order to study the effect of extended stagnation in a closed system on the interplay between planktonic and attached growth, a fed batch culture was established. After two hundred and sixteen hours, the bulk phase was removed and replaced with fresh medium to model effects of stagnation periods followed by a water flow delivering fresh nutrients to the biofilm.

The basic one litre batch culture fermenter was used as described in Methods (section 2.3.2) for culturing the mixed community isolated from the failed systems as described in Methods (section 2.1). The culture was stirred slowly at 20 rpm to aid mixing. The fermenter top plate was specially modified to provide seven ports through which copper coupons could be removed. The coupons were 1 cm$^2$ with
two 1.5 mm holes drilled in diagonally opposite corners through which tinned copper wire, 1 mm diameter, was passed to connect six coupons together. The end of the wire from the top coupon was then secured into a silicone bung which plugged the port. The coupon assembly is shown in Figure 9.1. Sampling was carried out by first sterilising the top plate with 70 % IMS before a bung was removed and the lower coupon separated using sterile tin snips. The coupon was collected in 9 ml of sterile ¼ Ringer’s to remove any loosely attached bacteria, and fixed, ready for SEM examination as described in Methods (section 2.3.11). At the same time as each coupon was removed the pH, dissolved oxygen level and temperature were noted. A sample (10 ml) of the medium was removed and bacterial counts measured using a 0.1 ml aliquot (Methods section 2.4.1). The rest

![Diagram of coupon array in fed batch reactor.](image)

Figure 9.1 Diagram of coupon array in fed batch reactor.
of the sample was frozen for subsequent assays of residual glucose (Methods section 2.4.2.1), residual ammonia (Methods section 2.4.4) and total carbohydrate (Methods section 2.4.6). Subsequently, it was decided to assay for residual glucose using the hexokinase assay (Methods section 2.4.2.2) as explained in the following results section. Medium as detailed in Methods (section 2.2.2.1).

After two hundred and sixteen hours of operation the entire medium volume was removed and one litre of fresh medium added and stirred for twenty minutes at 200 rpm, this in turn was replaced by a further litre of fresh medium and assays continued as before.

**Results and Discussion:**

Figure 9.2 shows the results for the bacterial counts over the four hundred hour run. The *Ps. solanacearum* quickly went into exponential growth which lasted for approximately twenty hours. The number of bacteria then remained fairly constant as they maintained a stationary phase for a further fifty hours, after which the counts started to drop slowly. At two hundred and sixteen hours, when the fresh sterile medium was added, the *Ps. solanacearum* counts showed a rise, due to detachment of attached cells from the walls of the vessel and the copper coupons. It has been reported that a pulse of nutrients led to the detachment of *Legionella pneumophila* from the walls of a chemostat (Keevil, personal communication). Over the course of the next two hundred hours, the counts for *Ps. solanacearum* followed the same pattern as seen in the first two hundred hours, reaching similar levels of growth, $3 \times 10^7$ cfus ml$^{-1}$.

During the first twenty hours, while the *Ps. solanacearum* was in exponential
Figure 9.2 Bacterial growth (as cfus ml\(^{-1}\)) for a fed batch culture with fresh medium added after 216 hours, (___) \textit{Ps. solanacearum}, (....) yellow strain of \textit{Ps. paucimobilis} and (----) brown strain of \textit{Ps. paucimobilis}.

Figure 9.3 Variation in dissolved oxygen and residual ammonia with time during a fed batch culture with the addition of fresh nutrients after 216 hours, (___) \% DO\(_2\) and (----) ammonical nitrogen.
growth, both strains of *Ps. paucimobilis* showed a rapid decline in numbers to $10^2$ cfu ml$^{-1}$. The levels then remained fairly constant at this level for a further eighty hours, the time during which the *Ps. solanacearum* was in stationary phase. The yellow strain of *Ps. paucimobilis* then dissapeared and no further planktonic colonies were counted. The brown strain of *Ps. paucimobilis* showed a slow but steady rise in numbers.

With the addition of fresh medium, after two hundred and sixteen hours, the brown strain showed a large rise in counts to a level slightly higher than the *Ps. solanacearum*, due to the size of the increase ($10^4$ cfu ml$^{-1}$) and the time frame (30 minutes, this again could only be due to sloughing from the vessel walls and copper coupons, brought about by the addition of fresh nutrients. The bacterial counts then dropped over the next twelve hours and remained at a low level for a further fifty hours, before exponential growth occurred over the remainder of the experiment taking the counts from $8 \times 10^1$ cfus ml$^{-1}$ to $1 \times 10^5$ cfus ml$^{-1}$, correlating with the decline in numbers of the *Ps. solanacearum*, the growth rate calculated over this period for the *Ps. paucimobilis* was found to be $0.106 \ h^{-1}$ (using the equation $\mu = \frac{d(\ln x)}{dt}$)

Figure 9.3 shows that during the time that the *Ps. solanacearum* was in exponential growth the level of dissolved oxygen rapidly fell to zero percent where it remained for twelve hours. This fall in dissolved oxygen shows that the uptake of oxygen from the medium by the *Ps. solanacearum*, during exponential growth, exceeds the rate of diffusion of oxygen into the medium, causing the level to drop to zero as any oxygen diffusing into the medium is immediately utilised by the bacteria. This drop in dissolved oxygen could account for the decrease in numbers for both strains of *Ps. paucimobilis*, as no nitrate was added to the system it is
unlikely that their anaerobic respiration could have taken place. Soon after the *Ps. solanacearum* reached its stationary phase, at about twenty four hours, the level of dissolved oxygen rose back to a level of eighty percent of saturation. It then remained fairly steady at this level until the fresh medium was added at two hundred and sixteen hours. The fresh medium added showed a decrease in dissolved oxygen as it had been autoclaved. However, it quickly rose to eighty percent of saturation before falling sharply to twenty percent of saturation as the *Ps. solanacearum* again underwent exponential growth. The level rose again as the *Ps. solanacearum* declined in numbers. From these results it can be seen that the *Ps. solanacearum* has the greatest effect on the level of dissolved oxygen causing it to become the dominant species in the planktonic community while ammoniacal nitrogen levels were detectable, however, the brown strain of *Ps. paucimobilis* is not limited by ammoniacal nitrogen and is probably capable of using other lysis products (Chapter 7) and therefore grows later when the ammonia level is limiting for the *Ps. solanacearum*.

The level of residual ammonia, also shown in Figure 9.3, fell to 0 μg ml⁻¹ within the first twenty four hours of the experiment. Again it would appear to be the *Ps. solanacearum* which is responsible for the utilisation of the ammonia. The growth of the brown strain of *Ps. paucimobilis* when there appeared to be no ammonia available would confirm it has either a very low requirement (low Kₘ) for ammonia or that it is capable of using other nitrogen sources, such as lysis products.

With the addition of fresh medium, at two hundred and sixteen hours, the level of residual ammonia became erratic for about twenty hours before it rapidly fell to 3 μg ml⁻¹ and then slowly fell to 1 μg ml⁻¹ at the end of the experiment. The erratic nature of the residual ammoniacal nitrogen, which initially increased with time,
would indicate that ammoniacal nitrogen was being released, possibly as a protein lysis product.

The levels of residual glucose and total carbohydrate are shown in Figure 9.4. The initial glucose assay was carried out using the [Trinder] assay (Methods section 2.4.2.1). A rapid decline in the level of residual glucose was observed over the first seventy hours, which correlated with the exponential growth and stationary phase of the *Ps. solanacearum*. The residual glucose level then remained at approximately 40 µg ml⁻¹ until the fresh medium was added. Over the same time period the level of total carbohydrate showed a rapid rise in the first twelve hours after which it fell rapidly to mimic the residual glucose from thirty six hours until the fresh medium was added at two hundred and sixteen hours. The initial peak in total carbohydrate is due to the lysis of the *Ps. paucimobilis* strains and the release of storage material accumulated during their growth on the “rich” ½ NA plates prior to seeding the batch culture. These results would appear to indicate that no exopolysaccharide

![Figure 9.4 Levels of residual glucose (---) [Hexokinase], (...) [Trinder] and total carbohydrate (----) in a fed batch culture with the addition of fresh nutrients after 216 hours, (due to the large number of sample points these have been omitted).](image)
was produced during this phase of growth, but from Chapter 6 it is known that this
is unlikely to be the case, the results of the SEM observations reported below would
suggest that any soluble polymer could be bound to the coupons. This finding is
supported by the work reported in section 1.4.2 which showed that some bacterial
polymers at least have a strong affinity for copper.

With the addition of fresh medium the residual glucose level rose to
approximately 150 µg ml⁻¹ as expected. However, it then remained at this level
throughout the rest of the experiment suggesting that no glucose was utilised. In
comparison the total carbohydrate assay started at 150 µg ml⁻¹ and showed a steady
decline for four data points after the first twenty four hours to 111 µg ml⁻¹, N. B.
sampling was every three hours, over a twelve hour period each day. After falling to
this level it then steadily rose until the experiment was ended. As the bacterial
counts in the second phase, after the addition of fresh medium, were identical to
those for the first phase, it seemed inconceivable that a similar level of glucose was
not utilised, especially as in the first phase the inoculum was from a nutrient rich
environment and the second phase was from a nutrient deficient environment. The
level of total carbohydrate shows the expected pattern with carbohydrate utilisation
followed by the production of a carbohydrate exopolymer. It is possible that the rise
in bacterial numbers is a result of utilisation of surface bound polymers. A rise in
bacterial numbers in sea water was observed during conditions of starvation
(Kjelleberg, 1984). In this case the rise in bacterial numbers was accounted for by
the cell actively dividing and producing a number of physically smaller daughter
cells. However, in the case of the second phase of the experiment above, active
division as a response to starvation is not thought to be the cause for the rise in
bacterial numbers as there is no evidence that any of the major nutrients were
limiting.
In view of these unexpected results all the samples were reassayed using both the [Trinder] and the Hexokinase methods (Methods section 2.4.2). Both methods gave similar results, to each other and the original data, throughout the whole experiment (Figure 9.4). (N.B. Assay blanks were RO water).

It would appear that both assays for glucose are giving rather unexpected results for the second phase of the experiment. Despite similar levels of growth (bacterial numbers) in the two phases, one utilised approximately 80 μg ml⁻¹ of glucose but the other apparently utilised none. The total carbohydrate is a direct measure of the level of carbohydrates present, whilst both the glucose assays are indirect measurements of glucose. For this reason the total carbohydrate is felt to be a more reliable indication of the levels present.

The [Trinder] assay relies on the reaction of glucose oxidase with glucose producing gluconate and hydrogen peroxide. The hydrogen peroxide is then assayed as it is formed at a level which is directly proportional to the amount of glucose originally present. Similarly the hexokinase assay relies on the production of NADH in the following reaction:

\[
\text{Glucose} + \text{ATP} \rightarrow \text{HK} \rightarrow \text{Glucose-6-Phosphate} + \text{ADP}
\]

\[
\text{Glucose-6-Phosphate} + \text{NAD} \rightarrow \text{G-6-PDH} \rightarrow \text{6-Phosphogluconate} + \text{NADH}
\]

Therefore the presence of endogenous hydrogen peroxide or NADH respectively in the samples would lead to the falsely high results. Growth of all three species of bacteria on "glucose oxidase" media (Quay et al., 1972) has shown that
they all contain the enzyme glucose oxidase, which has been described as being membrane associated (Quay et al., 1972). Other workers have claimed it to be extracellular (Keevil, personal communication). Therefore hydrogen peroxide could be generated extracellularly by any of the three bacteria. However, as all three bacteria are pseudomonads they all have the enzyme catalase which breaks down hydrogen peroxide. Catalase being membrane bound will only act on any hydrogen peroxide within the cell which could allow low levels of peroxide to accumulate in the media leading to the falsely high values for the glucose [Trinder] assay. It needs to be noted that the bacterial inoculum in the second phase of the experiment comprised attached bacteria from the nutrient-deficient primary phase whereas the initial inoculum was of bacteria grown on nutrient-rich agar plates. As reported in the Introduction (section 1.3), it is known that bacteria from nutrient-rich environments may be structurally and functionally different from biofilm bacteria (Costerton et al., 1987).

SEM examination of the coupons revealed the following general trends. Figure 9.5 shows the coupon removed after six hours, with a small amount of material attached to the surface, but very few bacteria were present. Over the next thirty hours the amount of material on the surface increased as did the bacterial numbers. Figure 9.6 shows the increase of material and the readily detectable bacteria on the thirty six hour sample. It appeared that the number of bacteria on the surface increased due to adsorption, as no microcolonies were visible, even after a further seventy eight hours, at which point a sloughing event appears to have taken place (Figure 9.7), Figure 9.2 also confirmed this with an instantaneous rise in the numbers of \textit{Ps. paucimobilis} in the planktonic phase.

Figure 9.8 shows the surface of the coupon removed six hours after fresh media
Figure 9.5 Copper coupon removed after six hours from a fed batch culture.

Figure 9.6 Copper coupon removed from a fed batch culture after thirty six hours, note the attachment of bacteria to the surface.
Figure 9.7 Copper coupon removed from a fed batch culture after one hundred and eight hours, showing a sloughing event has taken place.

Figure 9.8 Copper coupon removed from a fed batch culture six hours after the addition of fresh nutrients showing a build up of material on the copper surface.
Figure 9.9 and 9.10 Copper coupons removed from a fed batch culture after the addition of fresh nutrients showing the formation of bacterial microcolonies.
had been added, two hundred and twenty two hours total time. The surface of the coupon appears to have a good general covering of material, with a number of large aggregates of material. After two hundred and twenty eight hours microcolonies were clearly evident. Over the remaining time course of the experiment the numbers, and size, of the microcolonies continued to increase (Figures 9.9 & 9.10).

The SEM observations on the coupons show that a conditioning layer accumulated on the surface of the copper. With the initial high nutrient levels, very few bacteria attached to the surface, whilst exponential growth occurred in the bulk liquid phase. As both the dissolved oxygen and residual ammonia levels fell, at thirty hours, more bacteria appeared to attach to the surface, but no microcolonies appeared to be formed while the residual nutrient levels remained low. The sloughing event at approximately one hundred and eight hours appeared to coincide with the dissolved oxygen levels returning to nearly ninety percent of saturation (Figure 9.3) and an increase in the numbers of planktonic *Ps. paucimobilis*. With the addition of fresh nutrients, after two hundred and sixteen hours, rapid growth appears to have occurred on both the copper surface and in the bulk liquid phase. However, in order to obtain the high numbers of bacteria present in the bulk phase, there must have been another sloughing event as the majority of the planktonic community would have been removed during the emptying and rinsing carried out prior to the addition of fresh medium. Sloughing would be likely to occur from the glass wall of the vessel as well as from the copper.

From the above results it is clear that there are differences between the physiology of planktonic cells from a rich nutrient environment and cells which have been detached from a surface, as shown by the differences in nutrient utilisation. It has also been demonstrated that the bacteria attach to a surface when nutrient levels become limiting, it has been further demonstrated that the bacteria
can overcome the toxicity of copper (Chapter 7) and attach to a copper surface. Another point of relevance to the formation of biofilms in copper pipes is the detachment of some cells when fresh nutrients are added, this will play an important part in the formation of a "patchy" biofilm which is believed to be important in the pitting process. The relevance of this work is discussed in greater detail in the final discussion (Chapter 13).

9.2 Further studies into hydrogen peroxide production in a fed batch culture.

Following the unexpected results for the glucose assays in the previous experiment (Section 9.1) it was decided to repeat the experiment, but to include an assay for hydrogen peroxide, along with a control culture with no added copper coupons. The possible presence of hydrogen peroxide could be important to the pitting process. Hydrogen peroxide is a strong oxidising agent, likely to be capable of oxidising copper (I) to copper (II) under the prevailing conditions in the operating system and therefore could account for the formation of the black copper (II) oxide which is characteristic of this form of pitting. The coupons in the system were not removed this time for analysis. Both fermenters were operated as described above and in Methods (section 2.3.2). No coupons were removed at each sampling time but the pH and dissolved oxygen level were noted and 10 ml of culture removed for bacterial counts (Methods section 2.4.1), residual glucose [Trinder] (Methods section 2.4.2.1), residual ammonia (Methods section 2.4.4), total carbohydrate (Methods section 2.4.6) and hydrogen peroxide (Methods section 2.4.8).
Results:

Figures 9.11 and 9.12 show the results of the bacterial counts in both systems over the first one hundred and ninety four hours before fresh medium was added. In both systems the numbers of the *Ps. solanacearum* showed normal batch growth but the lag phase was longer in the culture with the copper coupons. The yellow strain of *Ps. paucimobilis* quickly dropped below the level of detection in the culture with the copper coupons. However, it was still present at the end of the first phase as seen by the occasional count later on in the time course. In the system without any copper coupons the same strain showed a similar decrease, although it remained at a higher level than the system just described. The counts for the brown strain of the *Ps. paucimobilis* again showed a drop but remained at a countable level, (but not statistically valid [Pirt, 1975]) in both systems, again higher in the system without the copper coupons. From these results for the bacterial counts it can be seen that copper has an initial inhibitory effect on all three bacteria under observation. The inhibition being greatest for the yellow, followed by the brown, strain of *Ps. paucimobilis* and least on the *Ps. solanacearum*, suggesting the latter has a greater copper tolerance and is therefore possibly the primary coloniser allowing the other species to colonise the surface.

The results for the residual glucose and total carbohydrate assays during the first phase of culture are shown in Figures 9.13 and 9.14. The levels of residual glucose in the culture with the copper coupons were similar to those noted above, in that, they declined as the *Ps. solanacearum* grew, to a level of approximately 40 µg ml\(^{-1}\) and remained at this level. In the other, copper free, system the levels fell below the level of detection (< 1 µg ml\(^{-1}\)). The initial level of total carbohydrate in both cases was approximately 40 µg ml\(^{-1}\) higher than the residual glucose level but quickly fell to similar levels. This would suggest either cell lysis with the release of a stored polymer or the carry over of carbohydrate from the inoculum. The results
Figure 9.11 Growth of bacteria in a fed batch culture in the presence of copper coupons, *Ps. solanacearum* (+), yellow *Ps. paucimobilis* (Δ) and brown *Ps. paucimobilis* (o).

Figure 9.12 Growth of bacteria in a fed batch culture in the absence of copper coupons, *Ps. solanacearum* (+), yellow *Ps. paucimobilis* (Δ) and brown *Ps. paucimobilis* (o).
Figure 9.13 Residual glucose (+), total carbohydrate (Δ) and residual ammoniacal nitrogen levels (o) in a fed batch culture in the presence of copper coupons.

Figure 9.14 Residual glucose (+), total carbohydrate (Δ) and residual ammoniacal nitrogen levels (o) in a fed batch culture in the absence of copper coupons.
for the residual glucose would indicate that up to 40 µg ml\(^{-1}\) glucose was unavailable in the system with the copper coupons. If this is the case it is most likely that this glucose has bound copper which could prevent the glucose from being taken up by the bacteria. The ability of glucose to bind copper was demonstrated by mixing a solution containing copper (II) nitrate and glucose which was allowed to react for thirty minutes at room temperature, and then passed through a column containing Amberlite IRC 718. This specifically binds free copper ions. Assays were then carried out on the glucose and copper levels in the eluate from which the levels of bound copper could be calculated. A control containing an equal amount of copper (II) nitrate without the added glucose was passed through a similar column. In this case all the copper was bound by the column, demonstrating that all the the copper passing through in the presence of glucose was indeed bound to the sugar and not simply a result of saturating the column.

Figure 9.13 shows that in both cultures the residual ammonia quickly dropped below the limit of detection (1 µg ml\(^{-1}\)). Apart from the residual glucose level dropping to zero in the culture without the coupons all the results for the first phase are similar to those seen in the original run.

The hydrogen peroxide assay appeared to show levels quickly rising to above 10 ppm, at a similar rate to the absorbance at 600 nm (used in the assay as a measure of background absorbance due to bacteria). This suggested production was growth linked, however, no yellow colouration was visible to the eye. It was therefore concluded that a large part of the absorbance at 400 nm was due to the pigment of the \textit{Ps. paucimobilis} species and not the presence of hydrogen peroxide, hence the increase at a rate similar to growth as measured by the absorbance at 600
nm. In order to remove this obvious error the absorbance at 400 nm was also read at the start of the assay and this value was used as the background level of absorbance. This reduced the supposed levels of hydrogen peroxide to 3 ppm, but again the expected yellow colouration was not visible by eye. Cells were removed by centrifugation at 4,000 x g for twenty minutes before the assay was carried out on the supernatant. This time the level of hydrogen peroxide for the culture with the copper coupons showed negative values, whilst the other showed values of zero. When an extra reading of the absorbance at 400 nm was carried out, after the addition of the sulphuric acid, the reading dropped in the culture with copper and rose in the other. Despite the problems encountered with the assay it did, however, show that no appreciable levels of peroxide were produced certainly not at levels which could have accounted for the unexpected results seen earlier (tests showed that 3ppm of hydrogen peroxide gave an equivalent glucose level of 17 µg ml⁻¹ in the Trinder assay).

Table 9.1 shows the bacterial counts for the second phase of the experiment, with the addition of fresh medium, the counts for the *Ps. solanacearum* increased in both cultures. In both cases the highest counts recorded were 10⁷ cfus ml⁻¹, which was lower than in the first phase. The brown and yellow *Ps. paucimobilis* were not recovered from the culture with the copper coupons after twelve hours. Their numbers again showed a decline in the culture without the copper coupons. In the case of the latter culture contamination occurred initially at a level of 10⁴ cfus ml⁻¹, this had dropped to 10² cfus ml⁻¹ by the end of the run, this is therefore not felt to have a major bearing on the results discussed.
Figure 9.15 Levels of residual glucose (○), total carbohydrate (—) during the second phase of a fed batch culture in the presence of copper coupons.

Figure 9.16 Levels of residual glucose (○), total carbohydrate (—) during the second phase of a fed batch culture in the absence of copper coupons.
Table 9.1 Bacterial numbers for second phase of a fed batch cultures after the addition of fresh medium at 194 hours.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>cfu ml(^{-1}) with copper coupons</th>
<th>cfu ml(^{-1}) without copper coupons</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ps. solanacearum</td>
<td>brown Ps. solanacearum</td>
</tr>
<tr>
<td>195</td>
<td>3.3 x 10(^4)</td>
<td>3.3 x 10(^2)</td>
</tr>
<tr>
<td>198</td>
<td>2.7 x 10(^4)</td>
<td>2.5 x 10(^2)</td>
</tr>
<tr>
<td>203</td>
<td>3.5 x 10(^4)</td>
<td>8 x 10(^1)</td>
</tr>
<tr>
<td>218</td>
<td>1.2 x 10(^5)</td>
<td></td>
</tr>
<tr>
<td>222</td>
<td>1.6 x 10(^5)</td>
<td></td>
</tr>
<tr>
<td>227</td>
<td>2.9 x 10(^5)</td>
<td></td>
</tr>
<tr>
<td>242</td>
<td>1.6 x 10(^5)</td>
<td></td>
</tr>
<tr>
<td>246</td>
<td>2.0 x 10(^6)</td>
<td></td>
</tr>
</tbody>
</table>

The levels of residual glucose and total carbohydrate for both systems are shown in Figures 9.15 and 9.16. The system without copper again shows a decline in both the levels of residual glucose and total carbohydrate, which this time start at similar levels. In the case of the culture with the copper coupons there is no significant change in the level of residual glucose and the level of total carbohydrate appears to be erratic. Again a possible trend of decline followed by a rise is evident but due to the smaller numbers of samples taken this is not confirmed. Yet again in the presence of copper we see the numbers of bacteria, from a nutrient deficient environment, increasing without the expected associated drop in glucose, the supplied carbon source.

**Discussion**

From the above results no satisfactory evidence can be found for the production of hydrogen peroxide in sufficient quantities to account for the unexpected glucose levels repeatedly seen in the second phase of the fed batch cultures grown in the
presence of copper. The possibility of hydrogen peroxide being produced in very small amounts, undetectable by the assay used, and playing a part in the initiation of pitting in the copper is however not ruled out by these experiments. The effect of low levels of hydrogen peroxide on the pitting of copper were studied as a result of this work and are reported in Chapter 12. Perhaps the best explanation for the unexpected results seen in the glucose assays is again that of Costerton et al., (1987) who felt that planktonic cells grown in a rich medium at high growth rates need to be seen as being structurally and functionally different from biofilm bacteria. These results confirm this finding as the cells in the first phase had been transferred from a nutrient rich environment, growing on agar plates, whilst the inoculum to the second phase was from a nutrient deficient environment.
Chapter 10

EXOPOLYSACCHARIDE PRODUCTION IN CHEMOSTAT CULTURE

Having determined the interplay between the planktonic bacteria and the biofilm bacteria during periods of stagnation followed by high flow rates causing the wash out of the planktonic bacteria, it was of interest to discover the effect of growth rate on polymer production over a range of growth rates below $\mu_{\text{max}}$. It has already been seen how batch culture effects polymer production, but as already discussed this does not provide accurate information on the effects of growth rate.

In order to study the effect of growth rate on EPS production a chemostat culture (Introduction 1.6.1.2 and Methods 2.3.3) was used. A nitrogen limited chemostat was chosen as the time studies in shake flasks (Chapter 7.2) had demonstrated a higher yield of polymer under such conditions. Samples were taken at a range of steady states with dilution rates ranging from 0.01 to 0.1 h$^{-1}$ all known to be less than the maximum growth rates for the two species as determined in Chapter 6 & 9. The various dilution rates were set randomly and not sequentially so to prevent selection or "training" of the bacteria. The assays described previously for bacterial counts, residual glucose, residual ammonia and total carbohydrate were performed on each sample (Methods section 2.4). Medium as detailed in Methods (section 2.2.2.1).

Results and Discussion.

The data (Figures 10.1 & 10.2) showed an obvious trend; as the dilution rate increased the number of colony forming units decreased but the level of EPS
Figure 10.1 Plot of specific polymer yield with dilution rate for a chemostat culture of a mixed community of three bacteria isolated from a failed copper pipe.

Figure 10.2 Plot of total bacterial numbers against dilution rate for a chemostat culture of a mixed community of three bacteria isolated from a failed copper pipe.
increased. The increase of polymer with growth rate \((D=\mu)\) indicates a growth-linked product. This appears to be contradictory to the observation made in batch culture (Chapter 6) that the polymer is produced during the stationary phase and therefore not growth linked. Further evidence for polymer production being growth linked is provided by the plot of biomass (cfus) against polymer level yielding a straight line (Figure 10.3). What is of note is that there appears to be an inverse relationship between product formation and biomass, with a low growth rate supporting biomass production and a high growth rate supporting a high polymer production and being unable to support biomass. It is possible that there is competition for a growth limiting nutrient, with a shunt occurring between high and low growth rates. The limiting nutrient in the chemostat was nitrogen which is necessary for growth, however, the results in Chapter 7 suggest that nitrogen is not required for polymer production. Therefore the competition is unlikely to be for nitrogen, but some other unidentified factor.

Figure 10.3 Variation of polymer level produced by a mixed community of bacteria, in a chemostat, with total bacterial numbers (line fitted by linear regression).
Chapter 11

Characterisation of EPS.

Bacteria are known to produce many different polysaccharides (Sutherland 1983), two of which, xanthan and alginate, have received the most research attention; for reasons detailed in section 1.4. The EPS produced by bacteria associated with corrosion processes have received little attention. The major sugar components of biofilms formed by Desulfovibrio are glucose, mannose and galactose with their respective ratios of 1:0.7:0.6 with small amounts of rhamnose, xylose and allose detected (Beech et al., 1991). Before this the most recent report on the characterisation of polymers produced by bacteria shown to be involved in corrosion was also for SRB (Ochynski & Postgate, 1965). Following the demonstration in Part 1 that bacteria and their biofilms were involved in Type 1½ pitting of copper, the composition of any polymers produced was of interest as structural differences might provide clues to any possible role of polymers in the initiation of the pitting process.

During the course of the previous experiments it was possible to isolate polymers grown under two sets of conditions:

1) planktonic growth in synthetic medium with glucose as the carbon source (Chapter 10).

2) attached growth from a copper surface with tap water from a hospital experiencing pitting supplying the carbon source, (Chapter 8).

Exopolymeric material was isolated as detailed in Methods section 2.3.9. EPS
collected from the planktonic bacteria was then subjected to acid hydrolysis and paper chromatography as detailed in Methods section 2.3.10. Analysis of these polymers was carried out by Prof. I. W. Sutherland at the University of Edinburgh, using the above methods along with HPLC analysis using a Gilson system, SCX Brownlea cartridge in lead form held at 80 °C, with water (0.2 ml min^{-1}) as eluent (Sutherland and Kennedy, 1986; Kennedy and Sutherland, 1987).

**Results:**

**12.1 Planktonic EPS**

Several of the polymers isolated from various planktonic populations were run and all gave a strong glucose spot along with other weaker spots which were possibly galactose and mannose. Uronic acid analysis (Methods section 2.4.8) showed that uronic acids composed 1% of this material. Later analysis by Prof. Sutherland showed that carbohydrates accounted for only 14.8% of the material, the remainder was shown to be mainly composed of protein and nucleic acid by UV absorption. Both paper chromatography and HPLC showed glucose to be the main component along with a large unidentified component. The glucose reacted with glucose oxidase confirming it as D-glucose. The unidentified component did not match any of the available standards in HPLC, it was therefore judged to be possibly a degradation product from the nucleic acid known to be present.

**12.2 Biofilm EPS**

Due to the problems encountered by Prof. Sutherland with the large amount of proteinaceous material in the planktonic material the biofilm EPS was cleaned up
using DNAase and RNAase to breakdown the nucleic acids followed by phenol extraction to remove the proteins following the method of Maniatis et al. (1982). The aqueous phase was then thoroughly dialysed to give a purer carbohydrate material. The results of the paper chromatograms showed a major spot which correlated with galactose, which was confirmed enzymically to be D-galactose. A possible trace of glucose running ahead of the galactose was also seen on some of the chromatograms.

HPLC analysis revealed a major peak eluting at 16.732 minutes which correlated with galactose. Again a trace of glucose was detected at 14.032 minutes. The uncorrected ratio based on peak heights were glucose:galactose, 1:11.93. This ratio can be corrected for the differing refractive indices of the two sugars to give a ratio of approximately 1:26.85. The HPLC analysis therefore was seen to confirm the results of paper chromatography. Another trace component was seen eluting at 18.75 minutes, but it was not possible to ascertain whether this was real or an artifact caused by air bubbles which entered the system. If this peak was real it would have indicated the presence of mannose.

Discussion:

It is evident that the two polymers examined are different, it is not, however, possible to say whether this difference is attributable to the different mode of growth or the different media used. From the literature it would appear that either is possible as it has been reported (Uhlinger and White, 1983) that the carbon source affects the polymer produced by *Ps. atlantica*. Beech et al. (1991) using *Desulfovibrio* have also shown that there is a difference between the polymers produced by planktonic and attached growth, however, this difference was in the
ratio of the component sugars. It is of interest that the sugar components of the 
polymer produced on copper with the natural water were similar to those found by 
Beech et al. (1991) using *Desulfabrio* on steel coupons, but the ratios are different. 
The characterisation of the exopolymer was also similar to that described by Corpe 
(1970) for *Ps. atlantica* which contained glucose, galactose, mannose, pyruvate and 
galacturonic acid. The three basic sugar components are similar to those found in 
the attached polymer, however, due to the small size of the sample, pyruvate and 
uronic acids were not assayed. It has been demonstrated that the exopolymer 
produced by *Ps. atlantica* can cause the biocorrosion of copper (Jolley et al., 1989). 
The effect of both of the polymers, isolated in this study, on pitting of copper is as 
yet unknown, although Prof. Geesey has shown that the removal of copper on an 
IRE correlates to the formation of a biofilm by the brown strain of *Ps. paucimobilis* 
used in this work (Appendix IV).
Chapter 12

ELECTROCHEMICAL STUDIES

As detailed in the Introduction (section 1.2) the corrosion of metals depends on an electrochemical cell being established. Aqueous corrosion always involves both an anodic and a cathodic process which have to proceed at an equal rate. The possible anodic processes, which involve oxidation (electron loss) are:

Dissolution of copper to form copper (I) ions in solution.
Oxidation of copper (I) to copper (II) ions.

The possible cathodic processes which involve reduction are:
Deposition of copper from copper (I) or copper (II) ions in solution.
Reduction of copper (II) ions to copper (I).
Reduction of dissolved oxygen forming hydroxyl ions (alkali).

These anodic and cathodic processes may occur adjacent to one another or at a short distance apart, however, there must always be an electronically conducting path, formed by metal or a conducting oxide, and an electrolytically conducting path, formed by water containing dissolved salts, to complete a circuit between the anodic and cathodic sites. For corrosion to occur there needs to be an electrical (corrosion) current that flows between the anodic and the cathodic areas. A current can only flow if the electrochemical potential at the anodic site (measured against a standard reference electrode) is lower than the electrochemical potential at the cathodic site. This current will lead to the loss of metal ions at the anodic site, which is corrosion.

Valuable information on the anodic and cathodic processes can be determined
independently by plotting the current/potential, or polarisation, of a corroding metal electrode as the potential is varied linearly either side of its rest potential. The ability to separate the anodic and cathodic processes is particularly important in the case of copper pitting as the situation is complicated by the anodic and cathodic processes taking place on either side of the oxide membrane over the pit, neither of which are easily accessible to a reference electrode. As most of any impressed cathodic current will be carried by the tube surface surrounding a pit, any cathodic polarisation measurements will be of little use as these two areas will have quite different polarisation characteristics.

Anodic polarisation measurements are then of more use as the existence of pitting should be revealed by very low values, yielding high currents, due to the presence of solid copper (I) chloride which will maintain a constant copper (I) ion concentration within the pit even at high anodic dissolution rates.

Electrochemical experiments were carried out in conjunction with Mr. H. S. Campbell using specially designed electrochemical cells through which various media and water could be passed or circulated. These cells, Figure 12.1, essentially comprise the test copper coupon (the working electrode), a silver/silver chloride reference electrode and a conductive Bakelite (Buehler, Coventry) counter electrode. The potential was applied across the counter electrode and the working electrode and measured across the reference and working electrodes. The current was measured across the working and counter electrodes. The potential was swept 70 mV either side of the rest potential, at 14 mV min⁻¹, using a Chemical Electronics sweep generator and a Princeton Applied Research Model 363 potentiostat. The potential and current were recorded on a Pharmacia 2-channel chart recorder.
Two sets of experiments were carried out using this equipment. The first examined the electrochemical effect of hydrogen peroxide on a copper surface. The second studied the electrochemical effect of a biofilm on a copper surface.

The first experiment was carried out, at room temperature, pumping 1 ppm hydrogen peroxide in the synthetic pitting water (SPW) (methods 2.2.2) through a pair of cells in series at a rate of 1 ml min⁻¹, for ten hours per day. A similar pair of cells was set up as a control with SPW alone being pumped through. One cell of each pair was polarised by an applied current of approximately 4 µA (8 µA cm⁻²) with the copper electrode being anodic.

The second set of experiments was conducted by installing two cells downstream of both the sterile and inoculated static bed systems described in Chapter 5.
Results:

12.1 Effect of hydrogen peroxide on copper coupons.

A sample trace for the applied potential and resultant current for the cell exposed to 1ppm hydrogen peroxide for 5 days is shown in Figure 12.2A. The comparable trace for the control cell is shown in Figure 12.2B (in both cases the scans were carried out using just the synthetic pitting water as the electrolyte). From the traces it can be seen clearly that both the anodic and cathodic currents were larger for the coupon that had been exposed to the hydrogen peroxide; this would indicate that the hydrogen peroxide had interacted with the copper surface. Copper can catalyse the breakdown of hydrogen peroxide leading to the generation of oxygen which could enhance either the anodic or cathodic processes leading to a greater loss of metal ions. Visual examination of the copper electrodes after four weeks exposure revealed that the unpolarised electrode exposed to SPW alone showed little more than a heavy tarnish. Small amounts of basic copper salt corrosion products were seen on the unpolarised electrode exposed to hydrogen peroxide. The level of these corrosion products was greater on the polarised cell exposed only to SPW while the polarised cell exposed to hydrogen peroxide was covered with a thick crust of corrosion products (basic sulphate and basic carbonate). Removal of the corrosion products by gently pickling with 10 % citric acid revealed a patchy copper (II) oxide layer of variable thickness on the polarised electrode exposed to the synthetic pitting water. The polarised electrode exposed to hydrogen peroxide was seen to have a complete covering of copper (I) oxide of uniform thickness, but with numerous well spaced holes in it, which were presumed to correspond with the pits discovered below the oxide membrane.
Removal of the oxide films showed that the unpolarised electrode exposed to the SPW had only a light uniform etch. The unpolarised electrode, exposed to hydrogen peroxide, showed a similar etch but with what appeared to be very small pits surrounded by areas covered with a black deposit (possibly copper (II) oxide). The polarised electrode, exposed to just the SPW, showed severe corrosion over the entire surface. This general attack was fairly uniform with a few deeper areas. The polarised electrode, exposed to hydrogen peroxide, showed attack over the whole surface in the form of numerous, separate pits. As has been discussed in Chapter 9 it is theoretically possible for the bacteria involved in the biofilms to produce hydrogen peroxide but as yet it has not been directly observed, although FAB-mass spectrometry of biofilms involved in Type 1½ pitting has revealed the presence of peroxides (Fischer et al., 1988). These results suggest that in the presence of hydrogen peroxide the formation of both the characteristic copper (II) oxide and the perforated copper (I) oxide membranes may occur, but not in this case on the same electrodes.

However, this work has clearly shown that hydrogen peroxide affects the corrosion of copper and, under conditions of polarisation, pits similar to those seen in operational systems can be rapidly formed.

12.2 Effect of a bacterial biofilm on copper coupons.

In the case of the cells down-stream of the static bed system these were regularly monitored on a weekly basis over a seventeen week period. Over this time course the following sequence of events was seen to occur. Initially, both the anodic and cathodic currents of the inoculated electrodes were lower than those of the sterile ones as shown in Figures 12.3 & 12.4. This continued for approximately two
Figure 12.2 Resultant current for a potentiodynamic sweeps of copper electrode exposed to A) 1 ppm H₂O₂ in SPW for five days. B) SPW alone.

Figure 12.3 Resultant current for a potentiodynamic sweep of two copper electrodes exposed for five days to effluent from inoculated static bed.

Figure 12.4 Resultant current for a potentiodynamic sweep of two copper electrode exposed for five days to effluent from sterile static bed.
weeks after which both the anodic and cathodic currents began to increase in the inoculated cells. However, the difference was not large as the higher current of the two sterile cells was roughly equal to the lower current of the two inoculated cells. The other cell in each case being respectively lower (sterile) and higher (inoculated) as detailed in Figures 12.5 & 12.6. Initially, it can be seen that the formation of a biofilm on a copper coupon had an inhibitory effect on the corrosion of the copper, as evidenced by the drop in the anodic current. This could be due simply to the presence of a film on the copper acting as a diffusion barrier, preventing the metal ions from moving away from the copper surface. This would lead to an increase in metal ions at the copper surface and have an inhibitory effect on the production of further ions. The biofilm would therefore be exhibiting a greater resistance to the passage of copper ions and therefore causing a drop in the current.

However, this effect was short lived and at the end of the second week it was evident that the presence of the biofilm now enhanced the dissolution of copper ions, as seen by the increase in the anodic current in the inoculated cells. This result could be due to the uptake of copper ions by the negatively charged polymers produced by the bacteria, or alternatively the presence of other metabolic products (such as CO₂, organic acids or even ammonia) which will enhance the anodic loss of metal ions generating a larger anodic current. It could also be due to the presence of hydrogen peroxide, which as seen above causes an increased anodic current. Whether this effect is due to some property of the bacterial polymer or the metabolic action of the bacteria is as yet uncertain. This question can possibly be answered in part by looking at the effect of the isolated bacterial polymers coated onto the copper coupons. Whatever the cause it is clearly evident that the presence of the bacterial biofilm, formed by the bacteria isolated from a failed pipe, enhances both the anodic and cathodic currents of a copper test electrode and will therefore lead to a higher corrosion rate.
Figure 12.5 Resultant current for a potentiodynamic sweep of two copper electrode exposed for seventeen weeks to effluent from inoculated static bed.

Figure 12.6 Resultant current for a potentiodynamic sweep of two copper electrode exposed for seventeen weeks to effluent from sterile static bed.
DISCUSSION

One of the important aspects of this work has been the demonstration of the link between bacteria and Type 1½ pitting corrosion in Part 1. This was carried out as stated by several carefully selected methods, which were designed to show the interaction between biofilm bacteria and the occurrence of Type 1½ pitting. Several other workers have tried to show this link but have not been successful due in part to experimental design, however, they have provided much useful data with regards to the pitting of copper. The choice of the novel form of static bed system proved most beneficial and has clearly demonstrated that, given suitable conditions, bacteria can initiate the pitting process in copper.

The morphology of the pitting as revealed in the SEM survey (Chapter 3) showed this to be very similar to that described by Lucey (1967) for Type 1 pitting. It is now commonly accepted that Type 1 pitting is initiated by the presence of an impermeable carbon film and then proceeds by the mechanism outlined in section 1.1. Due to the similarity of the morphology of the pits and the chemistry of the corrosion products, it is highly likely that once the bacteria have initiated Type 1½, pitting it proceeds by a similar mechanism to that described for Type 1 pitting.

During the course of the site survey, detailed in Chapter 4, it was evident that the majority of failures were occurring in horizontal pipe runs which had experienced extended periods of stagnation followed by a brief, high level of water draw-off, causing fresh water to replace completely that which had become stagnant. There were no Type 1½ pitting failures recorded from the hot system, which in both hospitals was circulated and maintained above 55 °C. The cold water was assessed to be in excess of 15 °C due to heat uptake from the high ambient
temperatures of the surrounding areas.

A study carried out in a number of hospitals, in the western region of Scotland (Walker et al. 1991) where "pepper pot" pitting had been reported, showed that in those hospitals where the hot water was recycled, maintaining temperatures above 55 °C throughout the length of the run, no cases of pitting failure had occurred. However, in those hospitals which had pitting failures it was found that the hot water circuits were not maintained above 55 °C. It was also reported that in those hospitals with a pitting problem the dissolved oxygen content within the pipes fell to 0% overnight, *i.e.* when no water was being drawn from the system. This drop in dissolved oxygen would suggest that an active biofilm was present in the pipes, or that there was a very high level of corrosion, or both.

The latter results regarding temperature and dissolved oxygen are both indicative of a microbial process. It is unfortunate that no results are available concerning the effect of low temperatures on the pitting of copper water pipes. As already mentioned it is known that the cold water in many hospitals runs at relatively warm temperatures, often as high as 25 °C. This heating of the water occurs as the pipes pass through service ducts and above suspended ceilings where the temperatures are often elevated above the ambient temperature of the hospital. To date no information has been received on instances of Type 1½ pitting occurring in waters routinely operating below 15 °C.

An extensive survey was carried out in a county hospital in West Germany (Fischer et al. 1991), which correlated the number of cases of failures, from a large number of pipe sections removed from throughout the hospital, with the known flow conditions, orientations and installation of the pipes. Figure 14.1 shows the results
for the type of pipe runs located in the hospital, overlaid by the areas where the highest number of failures were recorded. It can be clearly seen that the highest number of failures occurred in the community and medical care areas, where the pipes ran horizontally, were intensively branched and experienced extended periods of stagnation. This confirmed the findings of our small scale survey in the hospitals in the south west of England. However, one important difference was noted between the two site surveys, Fischer had no failures in the vertical pipes, whereas, in one of the U.K. hospitals, numerous failures were found in the vertical pipe runs to patients sinks in a geriatric unit built in the mid-seventies. This finding seems to be unique for soft waters.

It was found that the study of the physiology of the bacteria gave further
evidence of the microbial involvement in the pitting of copper, the temperature block experiments (Chapter 8) confirmed that the bacteria, isolated from the German hospital, were unable to grow above 55 °C. Walker et al. (1991b) also showed that in a two stage chemostat with suspended copper coupons the bacteria isolated from a Scottish hospital (known to contain the three species used in the above studies) were not able to grow above 60 °C, confirming the results reported in Chapter 8. The slightly raised maximum temperature at which growth occurred in the chemostat study could have been due either to a slightly different population being present or selection caused by the temperature being sequentially increased, compared with the study in Chapter 8 where all the tubes were incubated at only the specified temperature.

The studies on bacterial physiology (Part 2) have enabled the proposal of a mechanism for the formation of biofilms in copper water pipes, incorporating the importance of temperature and stagnation on the process. The mature biofilm then leads to subsequent failure by Type 1½ pitting, if a number of other conditions prevail.

The proposal is summarised in Figure 14.2. At the onset of the process an inoculum of bacteria and nutrients are present in the pipe (Chapter 4; Walker et al., 1991b). It is known from analysis of the water supplied to hospitals exhibiting Type 1½ pitting, that the levels of ammonia are low (<0.3 μg ml⁻¹). The studies in Chapter 7, have shown that these are limiting and promote exopolymeric carbohydrate production. These results are similar to the findings for X. campestris, where polymer production was also greatest under conditions of nitrogen limitation (Tait et al., 1986). When the flow of water ceases the temperature rises (Angell, Campbell and Chamberlain unpublished data), stimulating the bacteria to grow and
Figure 13.2 Summary of processes leading to biofilm formation and Type 1½ pitting of copper water pipes.
metabolise the relatively low levels of nutrients available in the limited volume of water. As nitrogen becomes limiting polymer synthesis will occur and some of this polymer will adsorb to the wall of the pipe adding to the inorganic conditioning layer (Chapter 9). It has been shown previously that polymers produced by *Pseudomonas* sp. promote bacterial adhesion (Corpe, 1970; Fletcher and Floodgate, 1974). The exopolymer present on the pipe and in the water also binds many of the toxic copper (II) ions (Chapter 8), in line with the findings of Bitton and Freihofer (1978) and Geesey and Mittelman, (1985). The decrease in nutrient levels due to metabolism will cause the bacteria to attach to the copper walls of the pipe (Chapter 10) (Marshall *et al.*, 1971; Kjelleberg, 1984), gaining much of their resistance to the copper from the polymer that they have synthesised as demonstrated in Chapter 8.

The longer the period of stagnation the greater will be the number of bacteria which irreversibly bind to the copper (Kjelleberg, 1984). When water is eventually drawn off from the tap this will introduce a fresh supply of water to the pipe, containing more nutrients and new bacteria, possibly different species. The fresh medium, with a different ionic concentration and higher nutrient levels, will cause some of the attached bacteria to detach and join the planktonic population (Chapter 9). Bacterial detachment was reported by Corpe, (1970b) who noted that marine bacteria attached to a glass microscope slide could be washed a number of times with sea water without any of the cells detaching, however when the slides were rinsed with tap water a number of the cells detached. It has been demonstrated that certain cations in the water are responsible for the structural arrangement of some polymers. Changing these cations leads to an alteration of the structural arrangement of the polymer and subsequently to cell desorption (Fletcher and Floodgate, 1976; Sutherland, 1983). Alternatively this desorption of bacteria could simply be due to a chemotaxic response to the higher nutrient levels in the fresh
medium (Young and Mitchell, 1974). Those bacteria which remain attached will be in a position to grow rapidly (Keevil et al. 1987) and form microcolonies (Chapter 9) and generate further exopolymer (Chapter 8), which serves to bind the bacteria to the copper surface.

As the nutrient levels drop further once again more bacteria will attach to the surface, this time possibly including species which previously found the copper toxic (Chapter 9). The copper toxicity being overcome due to the conditioning of the pipe surface with polymers (Chapter 8). Section 9.2 clearly showed that, in the mixed community studied, the *Ps. solanacearum* had a greater resistance to copper than the two strains of *Ps. paucimobilis*, and the temperature block experiment (Chapter 8) showed that in pure axenic culture it was this species that produced the most exoploysaccharide. Therefore, it is likely that the role of the *Ps. solanacearum* in the community of bacteria isolated is that of primary coloniser, allowing the less resistant bacteria to subsequently attach. This suggests that one role of the polymer is to neutralise the toxicity of the copper ions and that the corrosion is brought about by some other metabolic product such as organic acids or hydrogen peroxide. The results of the FTIR studies carried out by Prof. Geesey on the bacteria used in this study (Appendix IV) would appear to confirm this as the *Ps. solanacearum* had little effect on the dissolution of copper, whereas the brown strain of the *Ps. paucimobilis* was very effective at removing copper from the copper coated IRE. Thus, the main role of the polymer from the *Ps. solanacearum* is to neutralise the toxicity of the copper ions, but in view of the results obtained by Prof. Geesey it is unlikely that it is directly involved in the corrosion reactions. The corrosion is more likely brought about by some other metabolic product such as organic acids or hydrogen peroxide, most probably produced by the *Ps. paucimobilis*.

The above process will be repeated each time water is drawn from the pipe, via
a tap, causing fresh nutrients and bacteria to be delivered to the system, leading to the formation of a mature biofilm. This repeated, but occasional, turbulent flow will lead to sloughing and redeposition events, creating a patchy biofilm. Once this mature biofilm is established it will remain self-sustaining with the cycling of nutrients as bacteria die and lysis products are liberated within the biofilm.

In section 1.3 five possible mechanisms, as listed by Menzies (1971), for the involvement of bacteria in the corrosion of metals were reported:

1. the production of corrosive metabolic products;

2. the production of differential aeration cells and concentration cells;

3. depolarization of cathodic processes;

4. disruption of natural and other protective films; and

5. the breakdown of corrosion inhibitors.

From the following discussion of suggested mechanisms for Type 1½ pitting it can be seen that a number of the above points could be involved. As with SRB corrosion it is likely that Type 1½ pitting is not the result of one single bacterial species operating by just one single mechanism.

Work carried out in association with Mr. H. S. Campbell (Chapter 12) has shown that the bacteria used in the present study are capable of increasing both the anodic and cathodic currents on test coupons downstream of the inoculated static bed system, reported in Chapter 5, compared to similar coupons downstream of the sterile system. The increase in the anodic current indicates that metal ions are able
to leave the metal more easily and therefore suggests that not only are the bacteria involved in the pit initiation (Chapter 5) but that in their presence corrosion occurs at a greater rate.

A possible role of the mature biofilm in bringing about the greater anodic current and initiation of pitting was demonstrated using a scanning vibrating electrode technique (SVET) (Franklin et al., 1991). For carbon steel at least, highly localised areas of anodic current densities developed, which subsequently became inactivated as new areas developed. It was further shown that when a biofilm was present on the surface of the metal similar trends of pit initiation and repassivation were observed for several hours. The corrosion then propagated and spread, until a large area of the sample was anodic. It was concluded that the growth of bacteria altered surface conditions and prevented the initiated anodic sites from undergoing the usual repassivating. The repassivation, seen in the presence of the medium without the bacteria can be explained as follows. A rise in the anodic current will result in a drop in the open current potential (OCP), this will cause the capacitance of the passive surface to discharge, producing a cathodic current equal to the anodic "pitting" current. This mechanism would account for the equidistantly-spaced pits reported in Chapter 3, which were surrounded by smaller pits which had probably been passivated by the larger more active adjacent pit.

The further sets of cells exposed in Chapter 12 to a flow of synthetic tap water containing 1 ppm hydrogen peroxide and a control without the hydrogen peroxide showed that after just five days the anodic and cathodic currents were greater in the system containing hydrogen peroxide indicating that corrosion was occurring at a greater rate. This was confirmed when the cells were removed after a further four weeks of operation and the cell with hydrogen peroxide and induced polarisation
(N.B. the cells were anodically polarised to enhance any anodic reaction) clearly showed numerous pits. In comparison, the cells with hydrogen peroxide and no polarisation and the cell without hydrogen peroxide but with the induced polarisation showed far less pitting. No pitting at all was evident in the cell with neither hydrogen peroxide nor polarisation. As mentioned in Chapter 9 it is known that the bacteria produce hydrogen peroxide and that they possibly generate extracellular hydrogen peroxide, which has been clearly shown to enhance the pitting process by increasing the anodic current densities. It has also been shown using a SVET (Franklin et al., 1991), that bacterial culture supernatants did not produce the same effect on carbon steel coupons as an active bacterial culture in preventing the repassivation of the naturally occurring anodic sites. If this prevention of repassivation, seen with the active biofilm, was due to some metabolic product such as hydrogen peroxide, which if released at all appears to be present in only very small amounts and is naturally unstable, then used medium would not be expected to reproduce the effect. If the hydrogen peroxide reacted with the copper a "continual" supply would be needed to be produced close to the copper surface, this could only be achieved in an active biofilm. Therefore, it has been possible to demonstrate a mechanism by which the bacteria can initiate the pitting process. However, it needs to be noted that this is possibly one of many. A separate project now in progress will look at the effects of the polysaccharides on their own and the effects of other metabolic products such as organic acids.

A further mechanism of pitting initiation has been proposed by Geesey and Mittelman (1985) who suggested that different species of bacteria produce different exopolymers with different affinities for copper ions. When these occur in adjacent sites within the biofilm, the areas with a high affinity for copper will likely contain lower concentrations of dissolved free copper (II) ions sic, than adjacent areas.
bearing microcolonies of bacteria that produce less reactive exopolymers. This will lead to the establishment of copper concentration cells on the underlying copper surface. The area below the polymer with the highest copper affinity will be anodic to the surrounding areas. This condition will persist until all the free copper binding sites on the polymer are saturated. Biofilms saturated with copper ions may even promote anodic polarization by serving as a simple diffusion barrier to copper ions migrating from the surface to the bulk solution, the latter is however, felt to be unlikely due to the spatial arrangement of biofilms which are extremely hydrated and reported to contain up to 99% water (Sutherland, 1983). Costerton et al. (1987), however, suggests that the resistance of biofilm bacteria to antibiotics is a result of the antibiotics being unable to diffuse through the biofilm. This was refuted by the demonstration that, although tobramycin (an antibiotic) binds to the exopolysaccharide produced by \textit{Ps. aeruginosa}, the resulting reduction in diffusion coefficient of tobramycin within the colony or biofilm would not be enough to allow the definition of the glycocalyx as a significant penetration barrier (Nichols \textit{et al.}, 1988).

Having proposed a mechanism for biofilm formation and reviewing the possible role of biofilms in the initiation of pitting it is possible to suggest a number of remedial measures which can be taken to prevent Type 1½ pitting from occurring. Firstly, it is logical to try and prevent the formation of a biofilm on the pipe. It is impractical, as well as impossible, to attempt to prevent bacteria from entering the system. It is, however, possible to decrease their numbers and remove much of the particulate matter which serves as a source of nutrients from the supply water by simple filtration and/or chlorination. A decrease in nutrients will obviously lead to a lower level of growth unless a mature biofilm has already become established. The research detailed above has shown a link between temperature and biofilm...
formation. It can clearly be seen that maintaining the temperatures of the hot water circuit above 55 °C will alleviate the problem. The effect of lower temperatures in the cold system is less clear cut but it is felt that maintaining the cold water below 15 °C would be beneficial. This could be achieved by lagging the systems to cut down on the heat pick-up from the service ducts etc. also recirculating the cold water would help to maintain it at a low temperature (< 10 °C). The recirculation of the water would also eliminate the problems described that are caused by stagnation. A high flow rate would also enhance the erosion rate of the biofilm from the pipe wall (Characklis 1990b). It has been demonstrated that the rate of biofilm erosion is dependant both on the biofilm thickness and the flow rate of the water (Characklis et al, 1990) and that a fluid velocity of only 0.24 m s⁻¹ resulted in a biofilm erosion rate of 19 g m⁻² h⁻¹. However, it needs to be noted that if a biofilm was present and the water was circulated at too low a flow rate this would lead to a more rapid biofilm accumulation as nutrients would be continually supplied to an actively growing biofilm, which could serve only to aggravate the problem. Alternatively if the flow rate is too high (2 & 1.5 m s⁻¹ at 10 & 50 °C respectively) this could lead to erosion corrosion (Mattsson, 1980).

In conclusion this thesis has proved a link between certain bacteria and Type 1½ pitting of copper and allowed a mechanism to be proposed for the formation of a biofilm in copper pipes.
Future work

Having established the link between the bacteria and Type 1½ pitting of copper and made a start on the study of the physiology in this study the following ideas are suggested for possible future study.

Having demonstrated the ability of the EPS to anchor and maintain the biofilm and as a mechanism for the bacterial tolerance to the toxicity of the copper ions, coating the purified bacterial polymers onto the electrodes used in Chapter 12, would answer the question as to whether or not they are directly involved in the corrosion process as suggested by Geesey and Mittelman (1985).

It has been shown that the proteinaceous component of the extracellular material also has the ability to bind the toxic copper ions (Geesey and Mittelman, unpublished data). The production of extracellular proteins was studied in the work using the temperature block, however, a full study should perhaps be carried out on the physiology of extracellular protein production.

Analysis of an active biofilm for waste products and proteinaceous material by a sensitive technique such as mass spectrometry might allow the detection of a number of products such as hydrogen peroxide and organic acids which could have a role in the corrosion process. Possible "corrosive" metabolites identified could then be tested on the electrochemical cells described here or using the SVET of Franklin et al. (1991).
REFERENCES


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Lucey, V. F. (1982) Assessment of Type 1 pitting corrosion characteristics of potable waters, Symposium: Corrosion of Copper and Copper Alloys in Building, Japan Copper Development Association, Tokyo, 1982, 86.


APPENDIX I

RESULTS OF NCIMB SPECIES DETERMINATION OF TWO BACTERIAL ISOLATES FROM FAILED COPPER WATERPIPES.
Morphological descriptions are from growth on Lab M nutrient agar except as stated

<table>
<thead>
<tr>
<th>Isolate Code</th>
<th>Brown</th>
<th>White</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C Incubation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30°C</td>
<td></td>
<td>30°C</td>
</tr>
</tbody>
</table>

**Cell morphology**

**X630**

- Cells a) 3 days ambient, by phase contrast.
- Cells b) 4 hours, 30°C, nutrient broth + 0.75% agar, by phase contrast.

<table>
<thead>
<tr>
<th>Gram stain</th>
<th>Spores</th>
<th>Motility</th>
<th>Colony morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3 days.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Deep orange, round, regular, entire convex, smooth, shiny, opaque, &lt;1mm diameter.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>48 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>White, round, regular, entire low convex, Smooth, Shiny, translucent, 1mm diameter.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>°C Growth</th>
<th>Catalase</th>
<th>Oxidase</th>
<th>Fermentative in Glucose OF</th>
<th>Preliminary Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C +</td>
<td></td>
<td>+</td>
<td></td>
<td>←Pseudomonas species?</td>
</tr>
<tr>
<td>41°C -</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>45°C -</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37°C +</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>41°C -</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>45°C -</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Rapid Test (API)

Brown White

48 hours, 30°C

+ + NO₃ reduction
- - Indole production
- - Acid from glucose
- - Arginine dehydrolase
- - Urease
+ - Aesculin hydrolysis
- - Gelatin hydrolysis
+ - B galactosidase
+ + glucose assimilation
- + arabinose assimilation
- - mannose assimilation
- + mannitol assimilation
- - N-acetylglucosamine assimilation
+ - maltose assimilation
- + gluconate assimilation
- - caprate assimilation
+ + adipate assimilation
- + malate assimilation
- - citrate assimilation
- - phenylacetate assimilation
+ + cytochrome oxidase
Further Tests on "Brown" Isolate (ID20250)

Acid from glucose (LP) (+)
Phosphatase –
Gelatin (+)
Casein –
Starch +
DNase +
Levan +
Tween 80 +
Urease –
Acid from maltose (OF) (+)
Lysine decarboxlase –
Indole –
Acid from ethanol –
Flexirubin pigment –

Sensitivity to:
Penicillin G –
Streptomycin –
Chloramphenicol +
Tetracycline +
Novobiocin +
Polymyxin B +

OF: Hugh and Leifson
LP: Low peptone medium
(+) Weak positive
Further Tests on "White" Isolate

<table>
<thead>
<tr>
<th>Compound</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO$_3$ - NO$_2$</td>
<td>-</td>
</tr>
<tr>
<td>NO$_3$ - N$_2$</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin</td>
<td>(+) v. weak</td>
</tr>
<tr>
<td>Starch</td>
<td>-</td>
</tr>
<tr>
<td>Tween 80</td>
<td>+</td>
</tr>
<tr>
<td>Levan</td>
<td>+</td>
</tr>
<tr>
<td>Phenylalanine deaminase</td>
<td>-</td>
</tr>
</tbody>
</table>

**Carbon source utilization:**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>+</td>
</tr>
<tr>
<td>Betaine</td>
<td>-</td>
</tr>
<tr>
<td>Histidine</td>
<td>+</td>
</tr>
<tr>
<td>Adipate</td>
<td>+</td>
</tr>
<tr>
<td>Xylose</td>
<td>-</td>
</tr>
</tbody>
</table>
Conclusion

We consider these isolates to be strains as follows:

Brown a Pseudomonas species, most closely resembling Ps. paucimobilis, but atypical in utilising adipate. This species formerly known as "Flavobacterium devorans" will sometimes form long filaments, and has variable motility.

This strain does not appear to fit with the following species, atypical reactions are given in brackets:

Ps. vesicularis (DNase, mobility, acid from ethand)

Flavobacterium group IIB (indole, phosphatase, casein)

Ps. hydrogenothermophila (maltose CSU, glucose CSU, Starch)

Ps. glathei (starch, aesculin, urea)

Chloroflexus aurantiacus (growth on NA, morphology)

Flexibacter spp (gliding, cell morphology)

Ps. elodea Starch, urea, NO$_3$,DNA

Ps. fulva fluorescent pigment, NO$_3$, starch

Ps. elongata (NaCL requirement, NO$_3$, mannose csu, malate CSU)

Prosethecomicrobium enhydrum (cell morphology)

White a Pseudomonas species, possibly from rRNA group 2. The strain most closely resembles Ps. solanacearum but would be atypical for arginine CSU (+) and levan (+).

The reactions do not appear to fit the following species, atypical results in brackets.

Ps. pickettii (Levan, xylose CSU, mannitol CSU, arginine CSU, 41°C NCIB result)

Ps. Pseudoalcaligenes (mannitol CSU, Betaine CSU, and 41°C NCIB result)

Ps. stutzeri (Starch, maltose CSU, arginine CSU, Histidine CSU, 41°C, NCIB result).
References


APPENDIX II

SAMPLE STANDARD CURVES FOR ASSAYS

Figure 1. Glucose [Trinder]

Figure 2. Glucose [Hexokinase]

Figure 3. Phosphate

Figure 4. Ammoniacal nitrogen

Figure 5. Protein

Figure 6. Total carbohydrate

Figure 7. Hydrogen peroxide
Figure 1

Figure 2.
Figure 3.

Figure 4.
Figure 5

Figure 6.
Figure 7
APPENDIX III

STATIC BED CONTROL PROGRAM

The following program was written to control the static beds and to act as a data logger.

```
10 REM ***STATIC BED CONTROL PROG***
20 REM WRITTEN BY P. ANGELL AND P. BURROWS
25 *DRIVE 1
30 ?&FE62=255:?&FE60=0
40 MODE1: Day=1:X=100:dump=4320600:dumpl=8610000
50 PROCsetclock: PROCsinadc: PRINT nextadc%: INPUT "SPEED ", speed: PROCsetumpum
: PROCsetalevel: PROCcalprobes
60 INPUT "TITLE ", filenames: ST=OPENOUT filenames
70 CLS
80 PROCscreen
90 REPEAT
100 VDU20,3
110 PROCshowtime
120 IF TIME>8640000 THEN PROCdaycheck
130 IF TIME> nextadc% PROCadc
140 PRINT TAB(17,0) Day; ;;HR;; MIN;; SEC;;
150 PRINT TIME
160 PRINT nextadc%
170 IF TIME> nextmedla% PROCmedla
180 IF TIME> dump PROCdump
190 IF TIME> dumpl PROCdumpl
200 IF INKEY(-102) PROCmenu
210 IF INKEY(-82) THEN INPUT "NEW SPEED ", speed: ?&FE60=speed
213@%=&20209
215 IF INKEY(-56) PRINT corvall; ;;corval2; ;;corval3; ;;corval4; ;;corval5; ;;corval6; ;;corval7; ;;corval8
217@ %=10
220 UNTIL FALSE
225 IF INKEY(-56) THEN PRINT corvall; corval2; corval3; corval4; corval5; corval6; corval7; corval8
230 PRINT "TEST,200
240 CLOSER ST
250 *GIMAGE KAG
260 END
270 END
280 DEF PROCsetclock
290 PRINT "PLEASE INPUT THE TIME ": INPUT "HOURS ",H:INPUT "MINUTES ",M: INPUT "SECONDS ",S
300 TIME = H*360000 + M*6000 + S*100
310 ENDPROC
320 DEF PROCsinadc
330 INPUT "READING INTERVAL 1/100 S ", adcint%
340 nextadc%= TIME DIV adcint%
350 nextadc%= adcint% *(nextadc% + 1): ENDPROC
360 DEF PROCsetalevel
370 INPUT "LOWER AIR LEVEL ",all
380 INPUT "UPPER AIR LEVEL ",ahl
390 ENDPROC
400 DEF PROCcalprobes
420 readl=ADVAL(1): read2=ADVAL(2): read3=ADVAL(3): read4=ADVAL(4)
430 read5=ADVAL(5): read6=ADVAL(6): read7=ADVAL(7): read8=ADVAL(8)
450 C6=read6/corval6: C7=read7/corval7: C8=read8/corval8
460 ENDPROC
470 DEF PROCSHOWTIME
480 SEC= (TIME DIV 100) MOD 60
490 MIN= (TIME DIV 6000) MOD 60
500 H= (TIME DIV 360000) MOD 24
510 ENDPROC
520 DEF PROCdump
530 dump=dump+8640000
540 *GIMAGE KAG
```
DEF PROC dumpl
  dumpl = dumpl + 8640000
  *GIMAGE KAG
ENDPROC

DEF PROC daycheck
  TIME = TIME - 8640000: Day = Day + 1: nextmedia% = nextmedia% - 8640000: CLS: PROC scr
  nextadc% = nextadc% - 8640000: dump = dump - 8640000: dump1 = dump1 - 8640000
ENDPROC

DEF PROC media
  IF mpumponstate = FALSE THEN
    PRINT TAB(20, 1) "MPUMP OFF": ? & FE60 = 0
    mpumponstate = FALSE
    nextmedia% = nextmedia% + mofftimeint%
  GOTO 720
  ENDPROC

DEF PROC setmpump
  PRINT "MEDIA PUMP"
  INPUT "OFF TIME INT 1/100s", mofftimeint%
  INPUT "ON TIME INT 1/100s", montimeint%
  INPUT "INITIAL MPUMP OFF", nextmedia%
  mpumponstate = TRUE: ? & FE60 = speed
ENDPROC

DEF PROC screen
  GCOL0, 2: VDU5
  MOVE 100, 820: DRAW 100, 100: DRAW 1228, 100: DRAW 1228, 820
  FOR I% = 0 TO 24
    X = 100 + 47 * I%: MOVE X, 100: DRAW X, 90
  NEXT I%
  FOR I1% = 1 TO 10
    Y = 100 + 72 * I1%: MOVE 100, Y: DRAW 90, Y
    MOVE 0, Y + 10: PRINT I1% * 10
  NEXT I1%
  FOR I% = 1 TO 14
    Z = 100 + 51 * I%: MOVE 1228, Z: DRAW 1214, Z
    MOVE 1150, Z + 10: PRINT I%
  NEXT I%
  FOR I% = 1 TO 12
    X = 100 + 47 * (2 * I%): MOVE X - 20, 70: PRINT 2 * I%
  NEXT I%
  VDU 19, 3, 6, 0, 0, 0
  MOVE 70, 30: GCOL0, 2: PRINT "TIME(hours)"; MOV E 0, 870: GCOL0, 1: PRINT "%D02": MOVE 1
  30, 870: GCOL0, 3: PRINT " pH"
  990 MOVE110, (corval1*51)+100: PRINT "1": MOVE110, (corval2*7.2)+100: PRINT "2": MOVE1
  0, (corval3*7.2)+100: PRINT "3": MOVE110, (corval4*7.2)+100: PRINT "4": MOVE110, (corval5*7.2)+100
  1000 PRINT "5": MOVE110, (corval6*51)+100: PRINT "6": MOVE110, (corval7*51)+100: PRINT "7".
  "MOVE110, (corval8*7.2)+100: PRINT "8"
  1010 VDU 19, 3, 2, 0, 0, 0
  1020 X = 100
  1030 ENDPROC

DEF PROC adc
  REM***D02=7.2 pH=51*****
  MI = adcint%/6000: FC = 60/MI: int = 47/FC
  1080 MOVE0, (corval1*51)+100
  1090 R1 = ADVAL(1)
  1100 corval1 = R1/CI: DRAWX, int, (corval1*51)+100
  1110 MOVE0, (corval1*51)+100: R5 = ? & FCD1: corval5 = R5/C5: DRAWX, int, (corval5*51)+100
  1120 MOVEO, (corval2*7.2)+100: R2 = ADVAL(2)
  1130 corval2 = R2/2: DRAWX, int, (corval2*7.2)+100
  1140 MOVE0, (corval3*7.2)+100: R3 = ADVAL(3)
  1150 corval3 = R3/C3: DRAWX, int, (corval3*7.2)+100
  1160 MOVE0, (corval4*7.2)+100: R4 = ADVAL(4)
  1170 MOVE0, (corval5*1)+100: R6 = ? & FCD0: corval6 = R6/C6: DRAWX, int, (corval6*51)+100
  1180 MOVEO, (corval7*51)+100: R7 = ? & FCO0: corval7 = R7/C7: DRAWX, int, (corval7*51)+100
1180 MOVEX, (corval8*7.2)+100: R8=?&FCE1: corval8=R8/C8: DRAWX+int, (corval8*7.2)+100
1190 X=X+int
1200 MOVEO,0
1210 VDU2: *FX6,10
1220 VDU5
1230 @%=&20209
1240 PRINTTEST, corval1, corval2, corval3, corval4, corval5
1250 @%=10
1260 PRINTTEST, Day, HR, MIN
1270 VDU3: VDU4
1280 IF corval4>all THEN PRINT TAB(20,2) "APUMP ON": ?F62=254
1290 IF corval4>ahl THEN PRINT TAB(20,2) "APUMP OFF": ?F62=255
1300 ENDPROC
1310 DEF PROC menu
1320 INPUT "DO YOU WISH TO ABORT Y/N ", Y$
1330 IF Y$="Y" OR Y$="y" THEN 230
1340 INPUT "DO YOU WISH TO CHANGE A SETTING Y/N ", Y$
1350 IF Y$="N" OR Y$="n" THEN 90
1370 COLOUR 129: COLOUR 2
1380 PRINT: PRINT
1390 PRINT "MENU ": PRINT: PRINT "TO CHANGE TIME 1": PRINT "TO CHANGE READING INTERVAL 2": PRINT "TO CHANGE MEDIA PUMP 3": PRINT "TO CHANGE AIR PUMP 4": PRINT "TO CHANGE PROBE CALIBRATION 5"
1410 INPUT "PLEASE ENTER CHOICE ", Y$
1420 IF Y=1 THEN PROC setclock
1421 IF Y=2 THEN PROC setinadc
1422 IF Y=3 THEN PROC setmpump
1423 IF Y=4 THEN PROC setapump
1424 IF Y=5 THEN PROC calprobes
1425 CLG: COLOUR 128: COLOUR 3: PROC screen
1430 ENDPROC
Appendix IV

FT-IR STUDIES ON TWO OF THE ISOLATES BY Prof. GEESEY.

As reported in section 1.4.2 attenuated total reflectance Fourier transform infrared spectroscopy (FT-IR) has been shown to be a useful technique for the study of microbial corrosion. As part of the wider study of Type 1½ pitting it was requested by the grant body (International Copper Association) that the bacteria isolated from the failed pipes be sent to Prof. Geesey at Montana State University for FT-IR analysis. His findings are reported in this appendix as they have a direct bearing on the work contained in this thesis.

Cultures of *Ps. solanacearum* and the brown strain of *Ps. paucimobilis* were sent as freeze dried gelatine discs. Both isolates were found to grow well in defined culture medium (DCM Geesey & Bremer 1991). This medium is used as it has been shown to have no corrosive effect on the copper thin films deposited onto the internal reflection elements (IRE). Under conditions of batch culture conditions, both isolates were found to colonise copper coupons in DCM. When *Ps. paucimobilis* was then grown in batch culture in the presence of a copper coated IRE a dramatic decrease in the stability of the copper film was observed. This was seen as an increase in infrared water absorbance at 1640 cm\(^{-1}\). This deterioration of the copper was seen to coincide with with the accumulation of a biofilm over a four hundred hour period. A significant but much less dramatic deterioration of the copper was seen when the culture was grown under conditions of medium flow (dilution rate 3 h\(^{-1}\)). Visual examination of the surface of the copper film after exposure revealed a discoloured copper surface under the biofilm.

In conclusion Prof. Geesey feels that his results confirm the findings reported in Part 1, in that the bacteria *Ps. paucimobilis* is involved in the corrosion of copper.
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