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DIELECTRIC SPECTROSCOPY AS A TOOL FOR THE MEASUREMENT OF THE FORMATION OF BIOFILMS AND OF THEIR REMOVAL BY ELECTROLYTIC CLEANING PULSES AND BIOCIDES

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Dielectric spectroscopy can be used as an on-line method for the measurement of the formation of biofilms. The formation of a biofilm by the organism *Klebsiella rubiacearum* was measured by registering changes in the capacitance in the frequency range 0·1–10 MHz using a 4-terminal gold pin dielectric spectrometer. Removal and prevention of biofilms by electrolytic cleaning pulses was investigated. The effect of the addition of the biocides cetrimide, chlorine and glutaraldehyde was studied. The usefulness of the method in the on-line control of biofilms was demonstrated in an experiment in which the biofilm level was controlled by the automated addition of chlorine in response to appropriate changes in the electrical capacitance.

KEY WORDS: Biofilm, electrolytic cleaning, biocides, dielectric spectroscopy.

INTRODUCTION

Biofilms are important in many areas, and are abundant in Nature (Berkeley *et al.*, 1980; Daniels, 1980; Characklis & Cooksey, 1983; Savage & Fletcher, 1985; Costerton *et al.*, 1987; Characklis & Wilderer, 1989; Characklis & Marshall, 1990). In some cases they may be used to human advantage, e.g. in waste water treatment (Winkler & Thomas, 1984) and other industrial biotransformations (Atkinson & Mavituna, 1983; Ho, 1986; Wardell *et al.*, 1984; Vega *et al.*, 1988), and for bioaccumulation of metals (Macaskie & Dean, 1987). More commonly, however, they are a nuisance, and can play a role in corrosion (Larsen-Basse *et al.*, 1987), cause reduced heat transfer and increased frictional resistance in heat transfer equipment (Lalande *et al.*, 1989) and may be a health hazard, for instance in drinking water and air conditioning systems (Characklis, 1980, 1981, 1983) and in biomedical implants (Dankert *et al.*, 1986).

For effective control of biofilm formation one requires information on the amount of biofilm actually present in the system of interest. Due to a lack of adequate equipment for direct on-line measurement of size of a biofilm present an indirect measure of biofilm size is often used, for example the increase in the heat resistance or the increase in frictional resistance (Characklis, 1983). Because of the well-known deficiencies of indirect methods generally (e.g. Harris & Kell, 1985), it would be advantageous to have a signal that gives us direct information on the accumulation of biomass in biofilms. Dielectric spectroscopy has already been proven to be a useful method for estimating biomass concentrations in suspensions (Harris *et al.*, 1987; Kell 1987a; Davey *et al.*, 1988; Boulton *et al.*, 1989; Davey & Kell, 1990; Kell & Davey, 1990; Kell *et al.*, 1990)

and in the measurement of the rates of cell death caused in such suspensions by the addition of toxic chemicals (Stoicheva *et al.*, 1989). The present article shows that dielectric spectroscopy can also be used for measuring the accumulation of biomass in biofilms and its removal by electrolytic cleaning pulses and the addition of biocides.

THEORY

The relevant principles of dielectric spectroscopy (see Schwan, 1957; Grant *et al.*, 1978; Pethig, 1979; Pethig & Kell, 1987; Foster & Schwan, 1989; Takashima, 1989), and their use in the estimation of cellular biomass (e.g. Pethig, 1979; Kell & Harris, 1985; Harris *et al.*, 1987; Kell, 1987b; Davey & Kell, 1990; Kell & Davey, 1990) have been detailed elsewhere. It will suffice here to say that the linear, passive electrical properties of biological materials can be completely characterised by their capacitance and their conductance. Capacitance and conductance can be made independent of the geometry of the measuring system by the introduction of the cell constant:

$$\varepsilon\varepsilon_o = C(d/A)$$
$$\sigma = G(d/A)$$

in which (with the respective units in parentheses):

ε = permittivity (−)
ε_o = permittivity of free space ($8{\cdot}854{\cdot}10^{-12}$ F·m^{-1})
C = capacitance (F)
σ = conductivity (S·m^{-1})
G = conductance (S)
d/A = cell constant (m^{-1})

In broad terms, the conductivity gives a measure of the ability of a material to conduct charge, whilst the permittivity gives a measure of the ability of a material to store charge. When the material is a biological tissue or cell suspension, i.e. consists of an ensemble of essentially insulating cell membranes surrounding a conductive cytoplasm, there is a drop in the permittivity (and rise in conductivity) as the frequency of the exciting electrical field is increased. This effect, known as the beta-dispersion or "Maxwell-Wagner" effect, occurs typically in the radio-frequency portion of the electromagnetic spectrum. Such a dispersion is due predominantly to the inability of electrical charge to cross the more-or-less insulating biological membrane and its large, associated membrane capacitance. For spherical cells, the drop in the permittivity can be related to the volume fraction of biomass present, using the relation (see Schwan, 1957):

$$\varepsilon_l = \varepsilon_h + (9PrC_m/4\varepsilon_o)$$

where

ε_l = low frequency permittivity (−)
ε_h = high frequency permittivity (−)
P = volume fraction of biomass (−)
r = equivalent radius of cell (m)
C_m = membrane capacitance (F/m^2)

From an experimental standpoint, material lacking biomass may also show a frequency-dependence of the permittivity, due to the set of purely electrochemical

phenomena caused by interfacial charge transfer and known collectively as "electrode polarisation" (see e.g. Schwan, 1963; Grant *et al.*, 1978; Kell, 1987b; Kell & Davey, 1990). These phenomena are beyond our present interest, and may be minimised (Schwan & Ferris, 1968; Kell, 1987b; Kell & Todd, 1989) (i) by the choice of appropriately high measuring frequencies, and (ii) by the use of a 4-terminal system in which the voltage electrodes are not permitted to pass a significant current (and hence cannot polarise).

Some efforts have already been made (Little & Mansfeld, 1989) to use the closely-related method of impedance measurements to measure biofilms, but they were unable to detect the presence of biofilms on stainless steel electrodes, mainly due to the choice of the measuring frequency (10^{-3}–10^{5} Hz), where electrode effects tend to dominate, especially in a 2-electrode system (Kell, 1987b), and to the high conductivity of the sea-water medium studied. The method employed in the present work uses frequencies between 0·1 and 10 MHz and a patented 4-pin electrode system (Kell & Todd, 1989) which overcomes these problems.

MATERIALS AND METHODS

Biofilm formation

The organism used was *Klebsiella rubiacearum* (NCIB 9478). It was grown on a medium of the following composition ($g \cdot l^{-1}$): glucose (BDH) 2, low salt yeast extract (lab M) 1, bacterial peptone (lab M) 1. The pH was set at 7 before autoclaving, and the growth temperature was 25°C. The conductivity of this medium was 0·50 $mS \cdot cm^{-1}$.

The organism was first grown in a cylindrical glass fermentor (34 × 5 cm). The volume of liquid in the fermentor was kept at 325 ml. Medium was pumped through continuously in order to wash out any cells in suspension and make sure that only biofilm was present. Samples from the liquid coming out of the fermentor confirmed that the overwhelming majority of the biomass was present in the biofilm and not in the fluid. The flow rate used was 8 $ml \cdot min^{-1}$ (i.e. the dilution rate was 1·48 h^{-1}). The Bugmeter probe (see later) was inserted vertically into the base of the cylinder.

All other fermentations were performed in the system shown in Figure 1A, which consists of two plates between which the medium flows. The system has the advantage of having a more reproducible flow along the surface on which the biofilm grows (Sjollema *et al.*, 1988,1989). In most experiments the material of the plates was silicone rubber, as this is autoclavable and thus allows one to work aseptically. During the experiments on the effectiveness of the electrolytic cleaning pulses, perspex was used as the plate material. These experiments were not carried out under aseptic conditions. Where indicated, medium was passed through the system at a flow rate of 7·9 $ml \cdot min^{-1}$ (dilution rate of 6·0 h^{-1}).

All pumping was carried out using a Watson-Marlow peristaltic pump. Pumping has only a very small effect on the electrical signals measured, and it can be compensated for by measuring at both low and high frequencies and taking the difference signal (see later).

Dielectric measurements

The dielectric properties of the biofilm were measured using a 4-terminal dielectric spectrometer, the Bugmeter™, produced by Aber Instruments, Aberystwyth Science Park, Cefn Llan, Aberystwyth SY23 3AH, Dyfed, UK. The Bugmeter uses an electrode

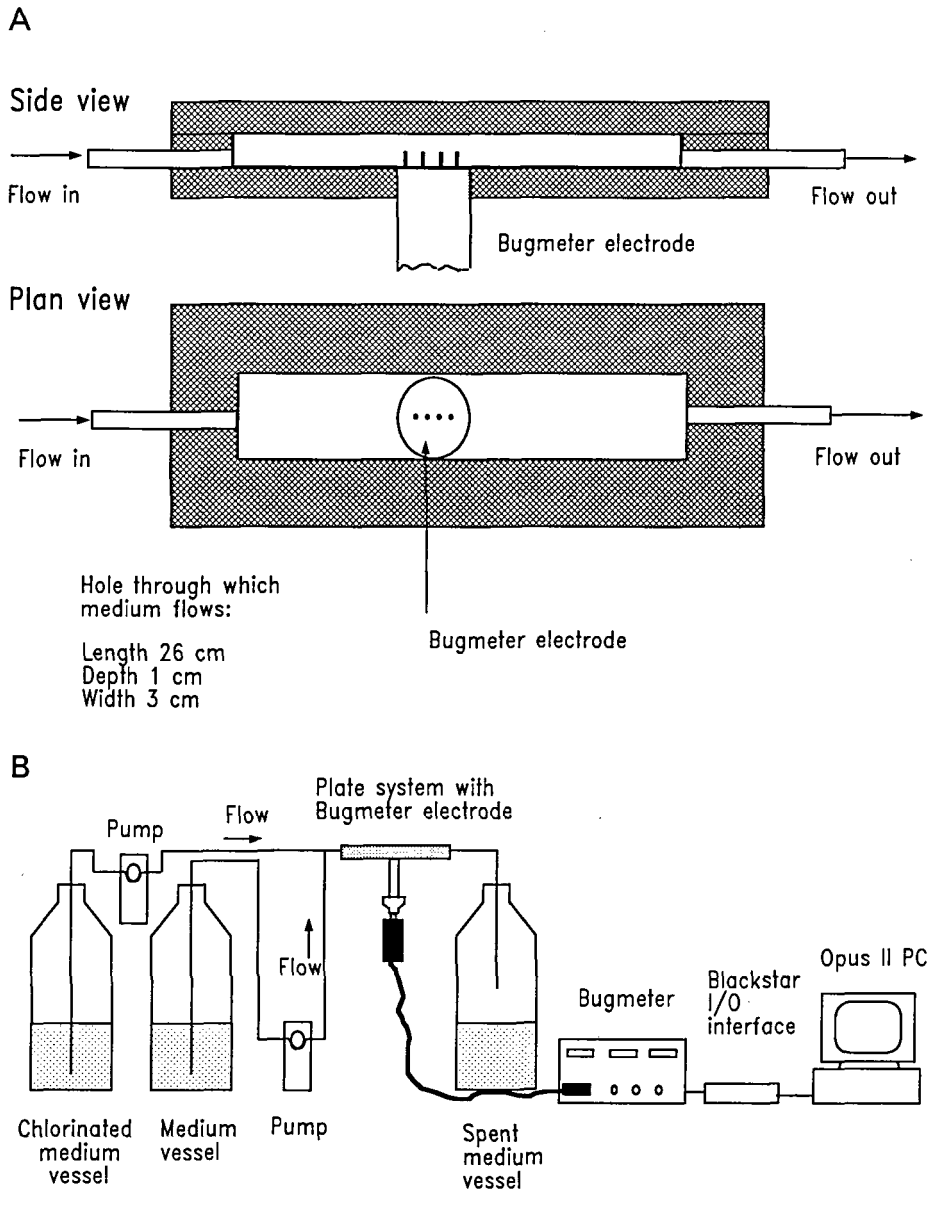


Fig. 1 Diagrams of (A) the parallel plate system used for studying biofilm formation, and (B) the arrangement used for the automated control of biofilm formation in the parallel-plate system.

probe consisting of 4 gold pins to measure the dielectric properties of systems of interest. The advantage of this approach is that one can measure samples with relatively high conductivities sensitively and with little interference from electrode polarisation phenomena (Kell, 1987b). The Bugmeter was controlled using an OPUS II IBM-XT-compatible PC and a Blackstar 2308 Interface (ADC). Initially, measurements were

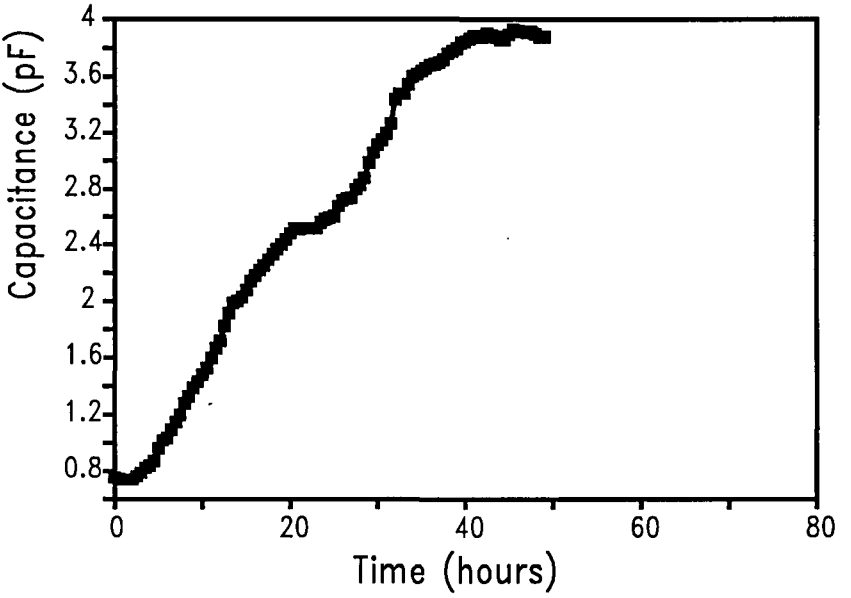


Fig. 2 Capacitance changes due to biofilm formation in the fermentor system. Measurements were carried out at 1 MHz as described in the Methods section and in the text.

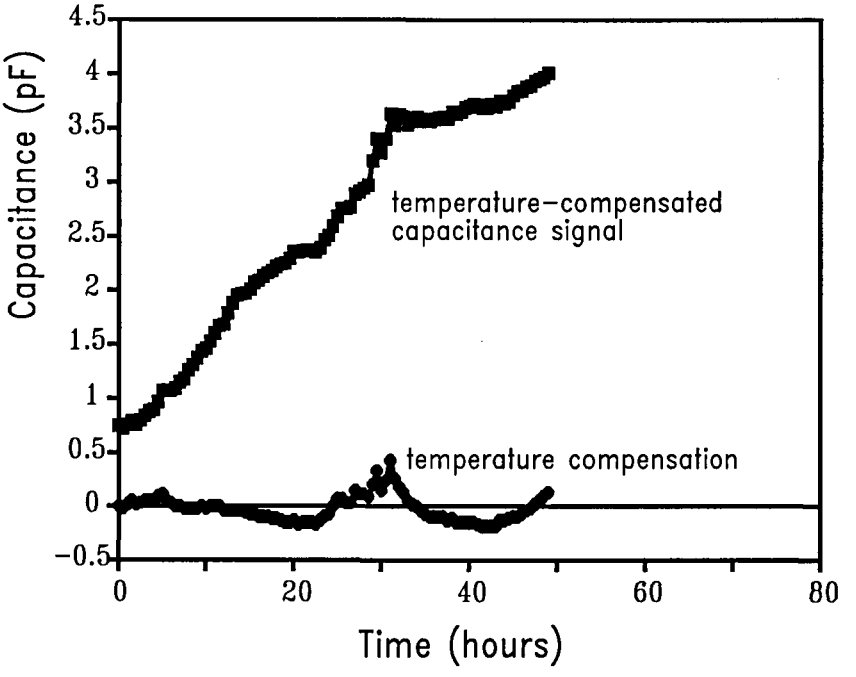


Fig. 3 Capacitance changes due to biofilm formation in the fermentor system. Measurements of capacitance and of temperature were carried out at 1 MHz as described in the Methods section and in the text. The temperature compensation was effected assuming a temperature coefficient of 0·187 $pF \cdot K^{-1}$.

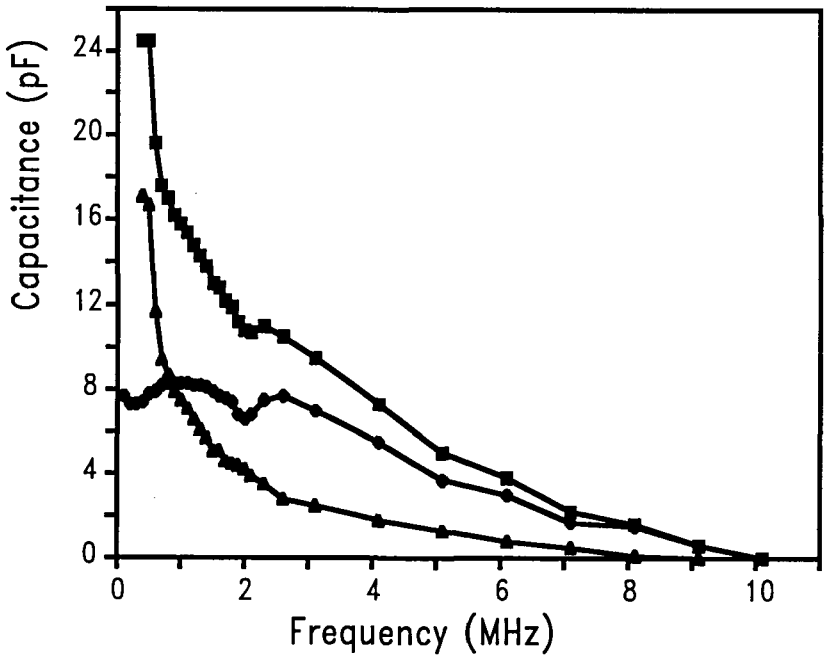


Fig. 4 Frequency-dependence of the apparent capacitance of the growth medium (▲), the biofilm-*plus*-medium (■) and their difference (◆). A biofilm was allowed to form in the fermentor system and the capacitances measured at the frequencies indicated using the Bugmeter.

made at a single frequency (1 MHz), whilst later measurements were made by comparing a low-frequency signal (1 MHz) with a high-frequency signal (9·5 MHz). Since influences such as outside temperature and flow along the pins will have essentially equal effects upon the signals at low and high frequencies, this allows one to compensate the signal for such artefacts.

The Bugmeter contains a unit which allows one to generate gas bubbles on the electrode surface by electrolysis of the medium in order to clean the electrode ("zapping"). This regime consists of a bipolar, current-limited pulse which generates a potential difference sufficient to cause electrolysis of the aqueous media employed. When using the electrolytic cleaning pulses, the interval between the pulses was 4 min, a cleaning pulse lasted 4 sec (2 sec at each polarity), and the output signal was held constant for 8 sec during and after a pulse ("hold time"), to allow gas bubbles so generated to be removed from the electrode surfaces.

The electrode used was a standard 25 mm fermentation-type probe with 4 gold-pin electrodes. The cell constant of the probe (measured using standard KCl solutions of known specific conductivity) was 0·6987 cm^{-1} (1 MHz) in the glass vessel and 1·335 cm^{-1} (1 MHz) in the plate system. (Higher changes in the capacitance may be obtained by using a lower cell constant.) Temperature was measured using a semiconductor probe (IC 590 KH, RS Components).

Biocides

The following substances were used as biocides: glutaraldehyde (BDH), cetrimide (cetyltrimethyl ammonium bromide) (Sigma) and chlorine. Chlorine was made by

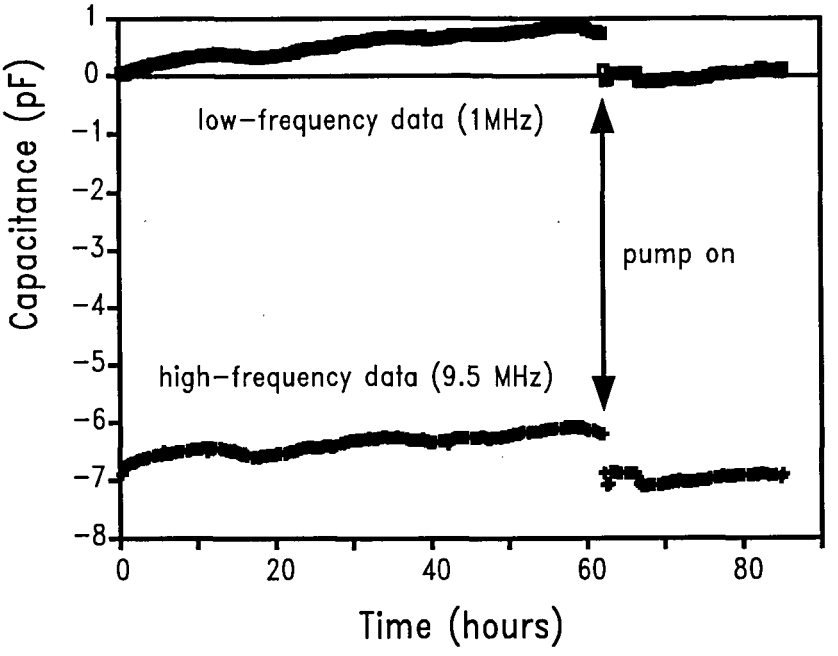


Fig. 5 Capacitance of the parallel plate system when no biofilm is present. Measurements were performed at 1 MHz and 9·5 MHz in uninoculated medium as described in the Methods section. At the point indicated, fresh sterile medium was pumped into the system.

adding HCl to household bleach and sparging the evolving chlorine through the medium.

Biofilm control

The system diagrammed in Figure 1B was used to control the biofilm level in the parallel-plate flow cell. When the signal due to the biofilm (i.e. the capacitance at 1 MHz *minus* that at 9·5 MHz) exceeded 8·1 pF, medium that had been pre-chlorinated was pumped in for a period of 5 min. Because the conductivity of the chlorinated medium (2·75 $mS \cdot cm^{-1}$) was very much higher than that of the growth medium an artefact occurred, in the sense that a large transient increase in signal occurred which was not related solely to biomass level but to a change in the baseline of the Bugmeter. This could have given rise to a continuous flow of chlorine, and therefore a time of 2 h was allowed for the medium to be refreshed, during which no chlorine could be added.

RESULTS

Biofilm formation

Typical data for the measured time-dependent changes in the capacitance at 1 MHz due to the formation of a biofilm in the fermentor system are given in Figure 2. The influence of external temperature on the capacitance recorded by the Bugmeter was

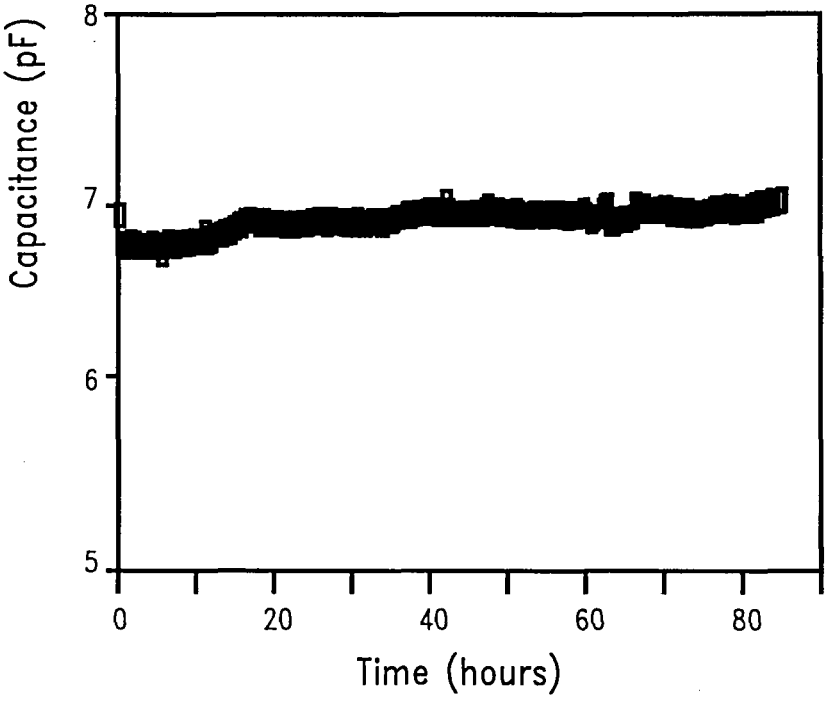


Fig. 6 Capacitance of the parallel plate system when no biofilm is present. Measurements were performed at 1 MHz and at 9·5 MHz as described in the Methods section. The data represent the difference in capacitance between the 2 frequencies for the data points in Figure 5.

measured to be 0·187 pF·(°C)$^{-1}$. The changes in the temperature, and the capacitance signal after compensation for the temperature are given in Figure 3. The formation of the biofilm proved to be generally linear with time. Some interference occurred in the later stages when the amount of gas bubbles that were generated metabolically within the biofilm started to have an influence on the measurements and also to dislodge the biofilm. The frequency scans of the medium and of the biofilm at the end of the fermentation, over the range 0·1 to 10 MHz, are given in Figure 4. It may be observed that there is a large increase in signal at the lowest frequencies, part of which is due to an increase in electrode polarisation at the lowest frequencies. However, the difference signal (biofilm-*minus*-medium) plateaus at approximately 1–2 MHz, indicating that the beta-dispersion has reached its low-frequency plateau value at these frequencies under these conditions.

A control experiment in the parallel plate system, in which the electrodes were kept in medium without cells, is shown in Figure 5. For the first 2·5 days no fresh medium was passed through the system. Then medium was flowed through the system for 1 day. The measurements at the high frequency paralleled the measurements at the low frequency. This shows clearly that measurements at high frequencies can be used to compensate measurements at low frequencies for external influences. In Figure 6 the data at high frequency have been subtracted from the data at low frequency, where it may be seen that the drift over 5 days is less than approximately 0·25 pF, averaging some +0·05 pF·day^{-1}. It may be mentioned (see Methods) that the background capacitance

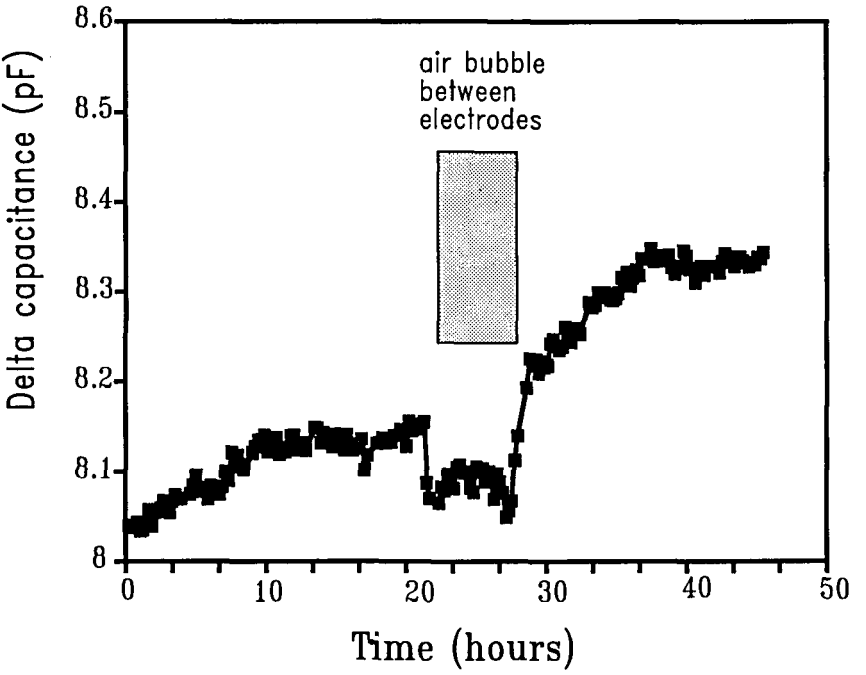


Fig. 7 Changes in capacitance due to the growth of a biofilm in the parallel-plate system. Measurements were performed exactly as described in the Methods section and represent the differential capacitance between 1 MHz and 9·5 MHz.

depends upon the cell constant, which reflects the volume (via the factor d/A) which the sensor samples. This is the reason why the background capacitance is significantly greater in the plate system than in the fermentor system. Obviously one may choose to subtract the background signal (as is possible using the Bugmeter), but for clarity we have not done so herein.

The next experiment was performed in the plate system to determine the magnitude of the changes in capacitance that one might expect due to biofilm formation under these conditions. The changes in the difference in the capacitances at 1 and 9·5 MHz due to biofilm formation in the plate system are given in Figure 7. Again there is mainly a linear increase in permittivity, indicating that the growth (accretion of biomass) of the biofilm is linear, rather than, say, exponential. The trapping of a gas bubble between the electrodes over the period 22 to 28 h may also be observed. Following a similar experiment, the biofilm was scraped off and carefully dried and weighed. From this it could be determined that for the present organism under the stated conditions the differential capacitance of the biofilm was some 0·58 pF for a coverage of 1 mg dry wt·cm^{-2}.

Electrolytic cleaning pulses

We next wished to study the potential ability (Dhar, 1986) of bipolar (Kell & Todd, 1989) electrolytic cleaning pulses to inhibit the formation of biofilm and to remove biofilm that had previously formed. To this end, a similar experiment to that described in Figure 7 was performed (Fig. 8), except that the cleaning pulse regime described in

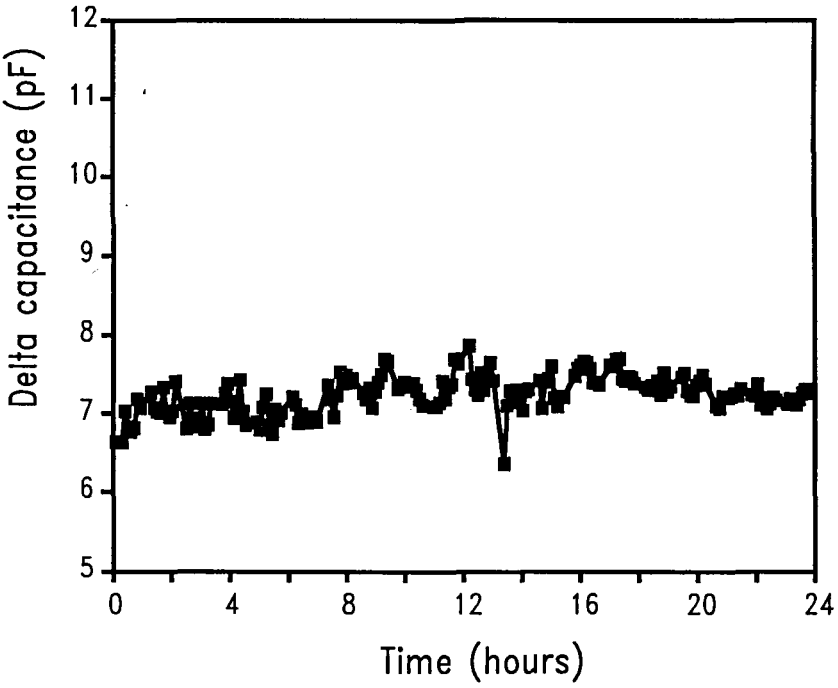


Fig. 8 Effect of electrolytic cleaning pulses on the formation of biofilm in the parallel-plate system. Measurements were performed exactly as described in the legend to Figure 7, except that electrolytic cleaning pulses were applied according to the regime described in the Methods section.

the Methods section was applied. It may be observed (Fig. 8) that no significant biofilm was formed under these conditions. It was therefore of interest to discover whether such electrolytic cleaning pulses could remove a *pre-formed* biofilm. In this experiment (Fig. 9), a biofilm was allowed to form in the absence of electrolytic cleaning pulses, which were initiated at 16 h. The heavier biofilm that had formed by this stage inhibited the removal of electrolytically generated gas bubbles (Fig. 9), giving rise to a more noisy signal. However, after several hours the electrolytic cleaning regime had succeeded in removing the overwhelming majority of the biofilm.

Biocides

Whilst the electrolytic cleaning regime proved a convenient and efficient means for removing biofilms from the electrode surfaces, chemical agents are by far the most common means of removing biofilms (e.g. Ruseska *et al.*, 1982; Bryers, 1988; LeChevalier *et al.*, 1988a,b). We therefore wished to use the present approach to study their efficacy. A biofilm was allowed to grow in the plate system (Fig. 10A), and at the point indicated the medium was changed to one of the same composition but containing in addition 1% (w/v) of the biocide cetrimide. The results in Figure 10A show that the biofilm is neither killed nor removed by the stated dose of cetrimide, but that its growth is immediately halted. When the medium was changed for medium without biocide the biofilm broke off (Fig. 10A), leaving clean electrode pins behind.

The effect of glutaraldehyde on a similar biofilm is shown in Figure 10B. In this experiment, the biocide was added to the inlet of the plate system as a pulse of 5 ml of a

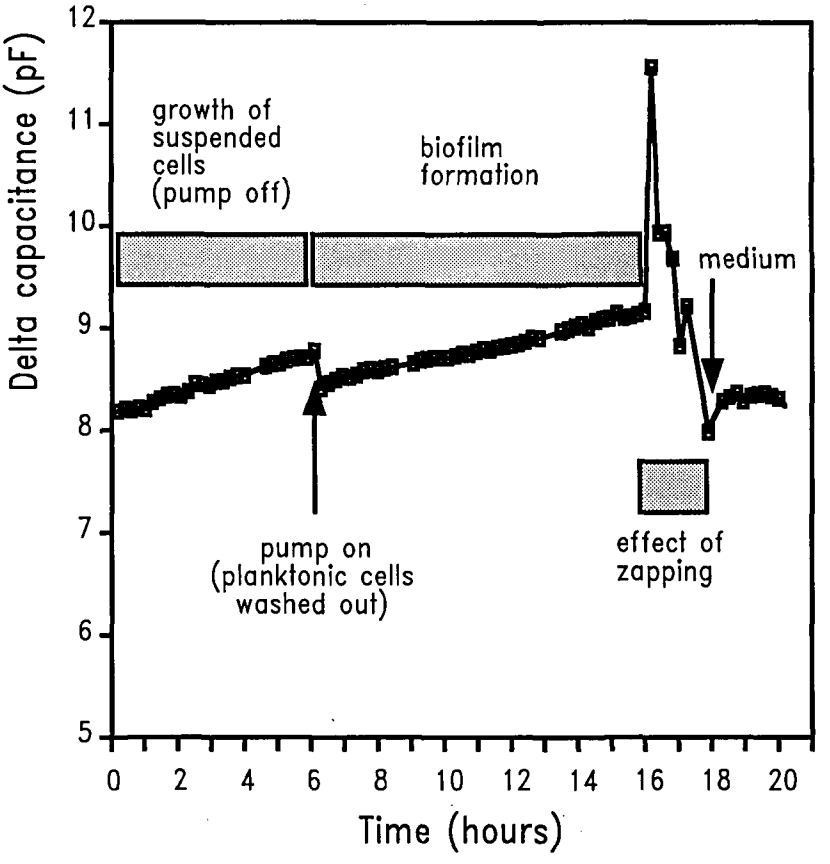


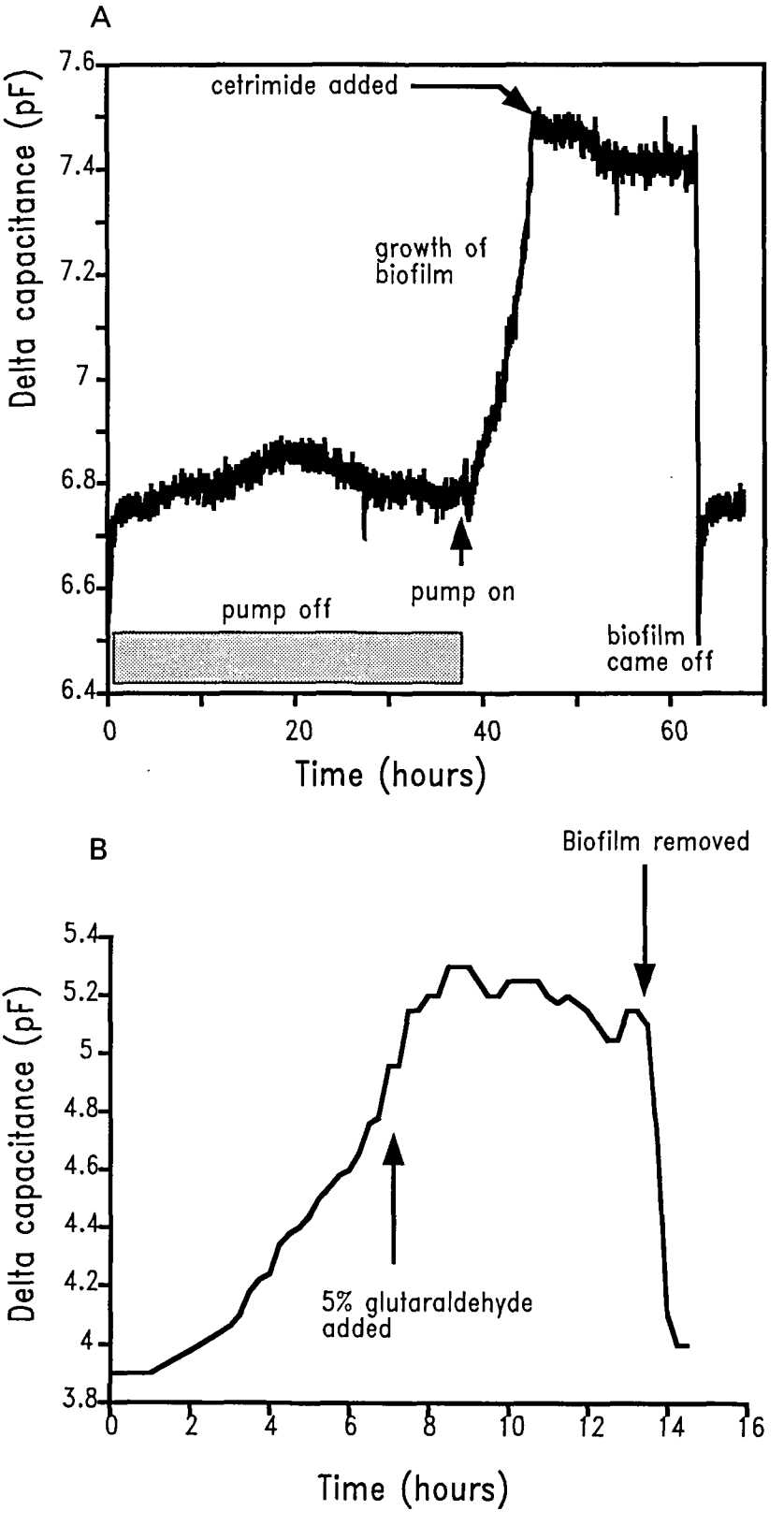
Fig. 9 The ability of electrolytic cleaning pulses to remove a preformed biofilm. At time zero, cells were inoculated into the parallel-plate system. Measurements were performed as described in the legend to Figure 7. At the time indicated, the medium pump was turned on and the growth of the biofilm monitored. At 16 h the electrolytic cleaning pulses were begun. At the point marked "medium", the system was flushed with tap water to remove flocs of biofilm and the medium supply continued. The return to the initial capacitance indicates that all biofilm had been removed.

5% glutaraldehyde solution. It may be observed (Fig. 10B) that the signal does not drop immediately upon addition of the glutaraldehyde, indicating that at least the majority of the cell membranes stay intact and that these biofilms are highly resistant to the action of glutaraldehyde.

Finally, the effect of chlorine was studied, using the system diagrammed in Figure 1B. As shown in Figure 10C, following a conductivity-dependent artefact (see Methods), the addition of chlorine-saturated medium at high concentrations kills the biofilm and subsequently causes its removal.

Biofilm control

Since it was possible to obtain an accurate, on-line measurement of biofilm formation, it was in principle possible to arrange for a capacitance-dependent signal to act as the feedback element in a control loop for the addition of biocide. The results of such an


A
Delta capacitance (pF)
7.6
7.4
7.2
7
6.8
6.6
6.4
cetrimide added
growth of biofilm
pump on
pump off
biofilm came off
0
20
40
60
Time (hours)
B
Biofilm removed
Delta capacitance (pF)
5.4
5.2
5
4.8
4.6
4.4
4.2
4
3.8
5% glutaraldehyde added
0
2
4
6
8
10
12
14
16
Time (hours)

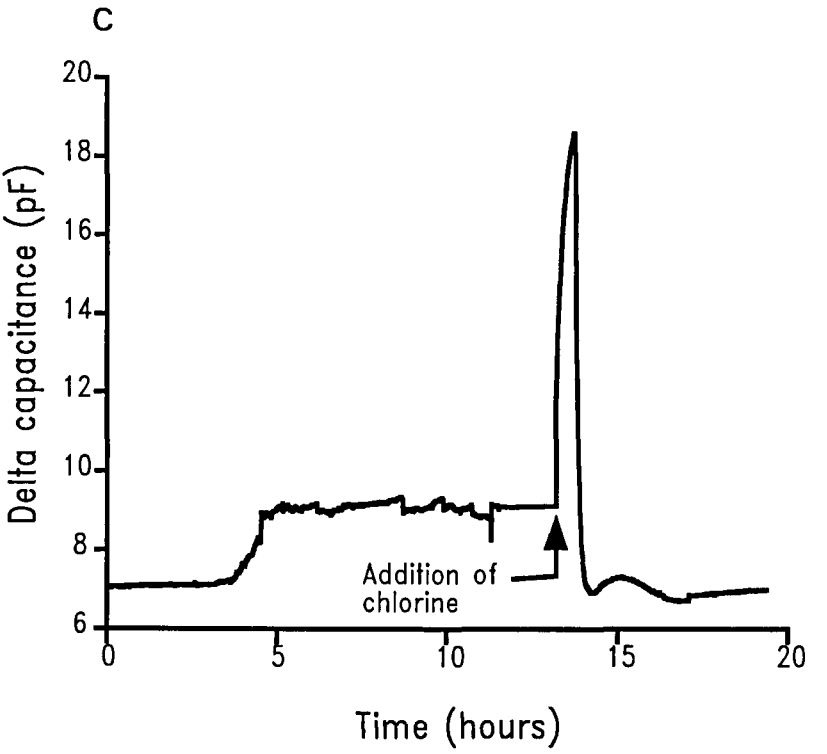


Fig. 10 Effect of biocides on the growth of biofilm. **A.** Cetrimide. Measurements were performed as described in the legend to Figure 7. At time zero, cells were inoculated into the parallel-plate system. At the time indicated, the medium pump was turned on and the growth of the biofilm monitored. At the point indicated, the medium flow was changed to one containing in addition 1% (w/v) cetrimide. At the point indicated, the biofilm was dislodged. **B.** Glutaraldehyde. A biofilm was grown up as described in **A.** At the time indicated, 5 ml of 5% glutaraldehyde was added as a single pulse, and at the time further indicated the biofilm was dislodged. **C.** Chlorine. A biofilm was grown up as described in **A.** At the time indicated, the medium was changed, for a period of 30 min, to one that was saturated with chlorine.

experiment, in which it was attempted to control the biofilm level in the parallel plate system, are shown in Figure 11. A less concentrated chlorine solution than in the former experiment with chlorine was used. Because of the conductivity-dependent artefact shown in Figure 10, the algorithm used to control the biofilm formation was designed so as to initiate the addition of chlorinated medium for 5 min after the capacitance reached a "trigger level" of 8·1 pF and then to ignore any increases above the trigger level for a period of 2 h. It may be observed (Fig. 11) that this regime effected a consistent, but only partial, removal of the biofilm. By leaving the chlorinated medium pump on for longer periods, complete biofilm removal could be effected (Fig. 11).

DISCUSSION

As discussed *in extenso* by Harris and Kell (1985), there is no suitable, general, independent method for the estimation of microbial biomass. Even "benchmark"

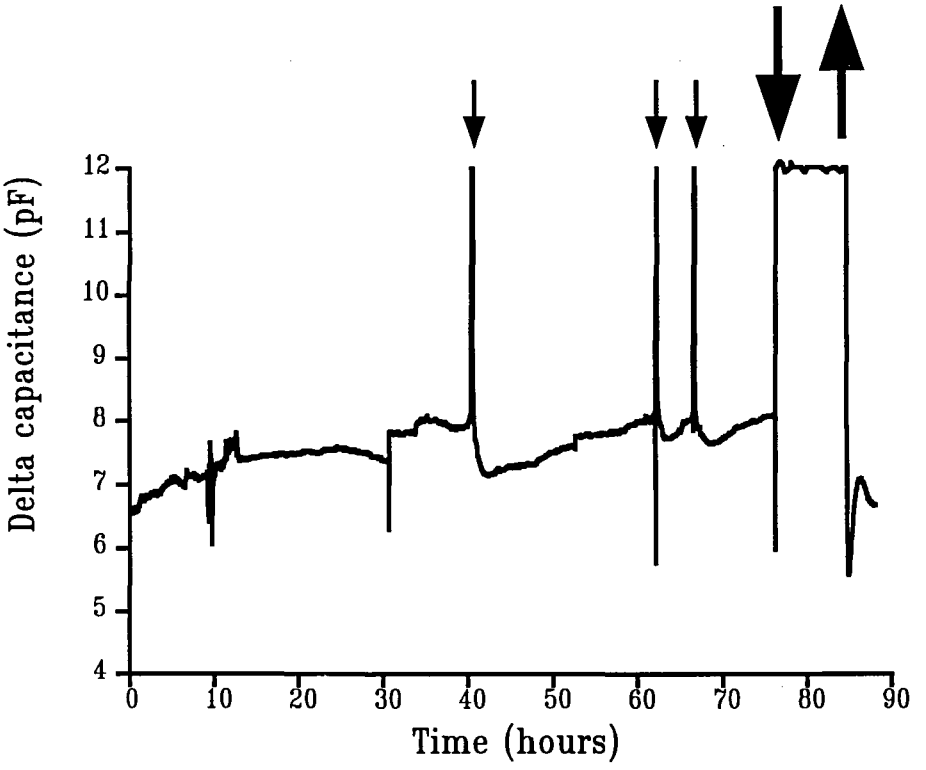


Fig. 11 The control of biofilm formation using capacitance as a feedback signal for the addition of biocide. Measurements were made in the parallel-plate system as described in the legend to Figure 10, using the system illustrated in Figure 1B. When the differential capacitance reached 8·1 pF, the medium was changed to a chlorinated medium for 5 min. No further chlorine additions were permitted for 2 h after such events (which are indicated by the smaller downward-pointing arrows). At the larger downward-pointing arrow, the medium was changed to that containing chlorine, whilst fresh medium was pumped in at the point indicated by the large upward-pointing arrow.

methods such as viable counts may be criticised on the basis that the nutrient status of the agar plates used will differ from those of the sample. Indirect methods such as dry weight, protein content, ATP, particle counting, light-scattering and so on do *not* measure biomass, but also necromass. All of these, and other difficulties described therein (Harris & Kell, 1985), are compounded when the sample of interest is in a difficult matrix such as a biofilm. For these and other reasons, an operational definition of biomass is required. Since living cells, but not other material likely to be co-existent, possess by definition an intact plasma membrane, a convenient operational definition is the biovolume, i.e. the (relative) volume of the region of interest which is surrounded by a cytoplasmic membrane. Further, to achieve a non-invasive, on-line, real-time measurement, only physical methods are appropriate (Harris & Kell, 1985; Harris *et al.*, 1987; Kell *et al.*, 1990).

Of the possible physical approaches (optical, acoustic and electrical), dielectric spectroscopy at radio frequencies is a method which permits the non-invasive, on-line registration of the accretion of cellular biovolume, due to the fact that these dielectric

properties of cell suspensions are dominated by the electrical behaviour of the (microbial) plasma membrane. The general approach has a very long history and an exact theoretical basis (see e.g. Grant *et al.*, 1978; Pethig, 1979; Pethig & Kell, 1987; Foster & Schwan, 1989), and we and others have shown that the magnitude of the so-called beta-dielectric dispersion is linear with the concentration of *suspended* biomass over an extended range (Harris *et al.*, 1987; Kell, 1987a; Davey *et al.*, 1988; Boulton *et al.*, 1989; Stoicheva *et al.*, 1989; Davey & Kell, 1990; Kell & Davey, 1990; Kell *et al.*, 1990). Thus it was of interest to enquire as to whether the method might be suitable for the registration of the accumulation of biomass (biovolume) in biofilms.

We have shown in the present work that it is possible to obtain a direct and continuous measurement of biofilm formation using dielectric spectroscopy at radio frequencies, although the capacitances determined are generally quite low. Measurement of the electrical capacitance at low *versus* high frequencies provides an excellent compensation for potentially interfering external factors such as temperature changes. Using this approach, growth of the biofilm between the lag and stationary phases was found to be linear rather than exponential (as is often observed (e.g. Bryers, 1988)), until the biofilm was dislodged by gas formation within the biofilm (Wimpenny, 1981) or by other factors.

Experiments with electrolytic cleaning pulses suggested that such pulses were able both to inhibit the formation of biofilms on metal surfaces and to assist the removal of pre-existing biofilms. Of the biocides studied cetrimide was reasonably successful, in that it caused an immediate cessation of growth and, in combination with modest hydrodynamic/mechanical forces, ultimate removal of the biofilm. Biofilms of *K. rubiacearum* were remarkably resistant to the action of glutaraldehyde, even high concentrations failing to be wholly bactericidal. The large differences in the effectivity of these biocides are not surprising, and have also been found by others (e.g. Ruseska *et al.*, 1982; LeChevalier *et al.*, 1988a, b). Indeed, it is generally found that cells present in biofilms are much more resistant to biocides than are planktonic cells (e.g. Costerton *et al.*, 1987; Exner *et al.*, 1987; Anwar *et al.*, 1989; Nichols, 1989). High concentrations of chlorine were able to remove the biofilm.

Because the biofilm could be effectively removed by chlorine, we studied the ability of a feedback regime to control chlorine addition and thereby to inhibit and remove biofilms. The lower concentration of chlorine used in this experiment did not cause the biofilm to die completely when the biocide was added for short periods. However the toxicity of the reagent was great enough to give a demonstrable decrease in biofilm size sufficient to keep it below the set point, and, when added continuously, to cause the complete removal of the biofilm. It is evident that "fine tuning" of the algorithm to particular cases would allow one fully to control the formation of a biofilm using the present approach.

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