Dielectrophoresis of Microorganisms Treated with Antimicrobial Agents

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This thesis is dedicated to Mum, Dad, Andrew and John.

ABSTRACT

Dielectrophoresis is the translational motion of particles in a non-uniform electric field. Both the extent and polarity of the dielectrophoretic response depend upon composition of the particle and the suspending medium, and vary with AC field frequency. Since biological particle (e.g. bacterial cell) composition is diverse, characteristic spectra can be built up by measuring the number of cells collected at electrodes for each frequency applied. Biological applications of dielectrophoresis are many and varied.

The suitability of the dielectrophoretic technique for assessment of *Cryptosporidium parvum* oocyst disinfection, ultra-violet irradiation of *Escherichia coli* and antibiotic treatment of tetracycline sensitive and resistant *E. coli* were explored. A computer-controlled automated system was modified and developed to measure dielectrophoresis. This incorporated a flow-through microelectrode chamber in which a particle suspension was re-circulated, allowing consecutive spectra to be produced from each sample. Image analysis was introduced as a means of counting individual particles after each applied pulse. The optimization of parameters for dielectrophoretic experiments using the system are described. Limitations of the equipment and the microelectrodes are discussed.

Cryptosporidium parvum is a protozoan parasite which produces environmentally resistant oocysts as the infective stage of its life cycle. Ingestion of oocysts can cause mild to serious enteric disease in man, and their recalcitrance to traditional water disinfection using chlorine is a concern in water treatment. Ozone has been proposed as an alternative disinfectant. Dielectrophoretic spectra of untreated, chlorine-treated and ozone-treated oocysts were produced using varying disinfectant doses. Over the frequency range 10 kHz - 10 MHz, the polarizability of oocysts was progressively reduced with increasing chlorine concentration although *in vitro* excystation was unaffected. Using ozone, polarizability decreased at frequencies approaching 10 MHz with increasing ozone concentration. Further analysis of this dose-dependency showed a non-linear increase in the ratio of collection between 100 kHz and 10 MHz. This was correlated to *in vitro* excystation. A modified two-shell mathematical model was used to compare data. A decrease in surface permittivity and a reduction of internal conductivity were proposed to explain the dielectrophoretic effects of chlorine and ozone respectively.

Ultra-violet irradiation of *Escherichia coli* (NCIMB 11459) was performed using both lethal and sub-lethal dosage. Dielectrophoresis of untreated and UV-irradiated cells demonstrated an enhancement of collection in all UV-treated samples. This effect diminished over time and the cellular regions affected by UV were not thought important in influencing cell polarization over the frequency range investigated.

Tetracycline was used to treat *E. coli* NCIMB 11459 cells, both sensitive and those rendered resistant by the inclusion of plasmid pSC101. Although of the same type, the sensitive and resistant cells could not be directly compared as the incorporation of pSC101 caused differences including slowed growth rate, altered cell morphology and general poor polarizability. Variation in tetracycline dose made little difference to the dielectrophoretic response of sensitive cells although a dose-dependent response was apparent with the resistant cells. Viability was assessed after each spectrum using total viable counts. Alteration of lipid metabolism and the inclusion of efflux proteins were proposed to explain the different polarizabilities of sensitive and resistant cells. The chelation of essential surface divalent cations by tetracycline is proposed to explain the dose-dependency of the resistant cell collection.

It was concluded that use of the dielectrophoretic system for higher frequency investigations yielded valuable information. The system was unsuitable for analysis of low frequency (below 100 kHz) dielectrophoretic effects due to problems with microelectrodes. Considerations for further work involving dielectrophoretic viability analysis of *C. parvum* oocysts and for dielectrophoretic investigation of antibiotic-treated bacteria are discussed.

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Candidate.....Carmel Quinn.....

Supervisor.....

Date...3rd...February...1996

Chapter One

INTRODUCTION

1.1 The phenomenon of dielectrophoresis

1.1.1 Definition

The phenomenon of dielectrophoresis can be described as the translational motion of particles in a non-uniform electric field (Pohl, 1951, 1978). This results from a combination of the terms *dielectric* (referring to materials which are non-conductors of electric charge, in which the formation of a dipole results through the application of an electric field) and *phoresis* (from the Greek meaning *carrying*). By contrast to electrophoresis, wherein electrically *charged* particles migrate in uniform or non-uniform electric fields, in dielectrophoresis the formation of a dipole is essential for movement to occur.

1.1.2 A brief history

Amongst the earliest recorded evidence of non-uniform electric field effects is that of the attraction of dust particles to amber, noticed by Thales of Miletus in Turkey around 600 BC. In 1600, Gilbert observed that the shape of water droplets became distorted when brought close to electrified amber. Early useful applications of non-uniform field effects were in the petrochemical industry where Lowden (1891) used the phenomena to remove metal particles from lubricating oils. Other petroleum refining processes have also exploited dielectrophoresis (Pohl, 1978). More recently, other inorganic particles have been used, either with the purpose of investigation into the dielectrophoretic phenomenon itself (Mehrle *et al.*, 1988; Burt *et al.*, 1989) or in research aimed toward possible application in materials separation (Feeley and Pohl, 1981). However it is the biological contexts of dielectrophoresis that will be considered here. These include the possibilities for cell, organelle, cyst, spore, and virus separation and characterisation (Pohl, 1978) as well as the use of the phenomenon as a "research tool" to aid the investigation of cellular structure (Ting *et al.*, 1971; Burt *et al.*, 1990; Marszalek *et al.*, 1991).

1.1.3 A physical explanation of dielectrophoresis

A simple representation of dielectrophoresis is shown in fig 1.1 which depicts two situations, that of a uniform (1.1a) and a non-uniform (1.1b) electric field. If a charged particle is placed in a uniform electric field (parallel field lines formed between parallel plate electrodes), migration of the particle along the field lines will occur toward the electrode of opposite polarity (*electrophoresis*). A neutral particle placed in this field will remain stationary as the electric field induces dipole formation within the particle but the number and displacement of the charges are exactly equal and opposite: the particle is thus balanced electrically and is unable to move. The particle will become aligned in the field to achieve a state of minimum energy.

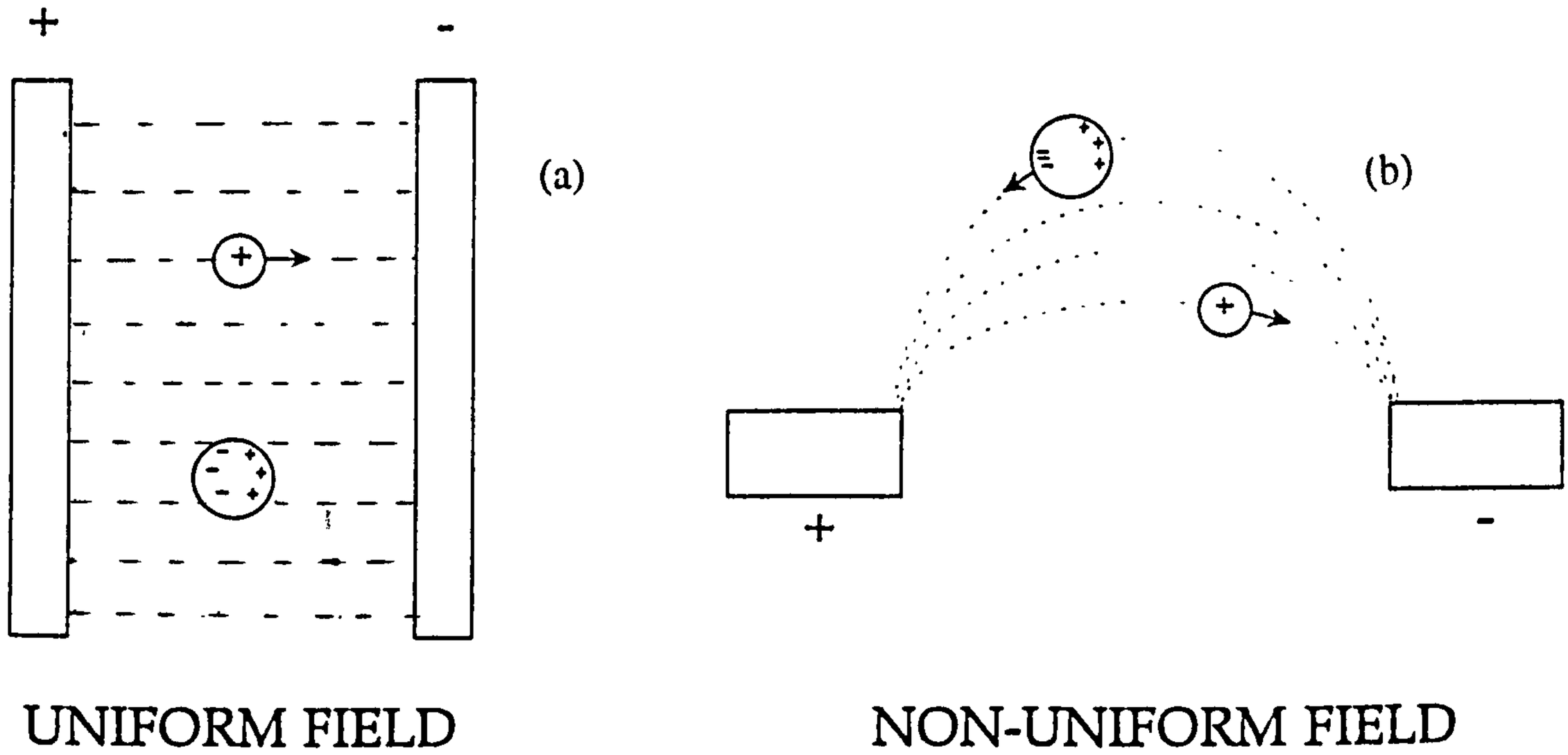

Fig 1.1 The behaviour of neutral and charged particles in electric fields.

The non-uniformity of the electric field in fig 1.1b is due to the convergence of the field lines formed between the corners of the two electrode bars. A charged particle in a non-uniform field will again move along the field lines toward the direction of opposite sign. An uncharged particle will become polarized by the field and displacement of charges within the particle occurs, forming a dipole. However, the convergence of the field lines encourages uneven charge alignment, leading to a greater charge density in one area of the particle relative to those charges of opposite sign which are aligned where the field is less intense.

The force per unit area on a particle

$$F = e.q \qquad (1.1)$$

where F = force, e = electric field strength and q = charge, becoming greater with increasing field strength. Therefore the result is a net charge on the particle enabling it to migrate toward the region of greatest field intensity (i.e. the direction in which the field lines are closest together). Dielectrophoretic movement, unlike that of electrophoresis, is independent of electrode polarity.

The geometry of the non-uniform electric field can be varied. The divergence of the field lines can be made to follow controlled patterns which are predetermined by the shape of the electrodes (Pohl, 1973). Common field geometries that have been used for biological dielectrophoresis experiments have included spherical, cylindrical or isomotive patterns.

An alternating current (AC) field can be formed by repeated reversal of electrode polarity. A charged particle in an AC electric field would react by changing direction each time the field was reversed until a maximum frequency was reached, beyond which it could not keep up. At this point the particle would remain stationary. In a neutral particle the alternation of field direction has the effect of "flipping" the induced dipole but the disparity in charge alignment remains. This allows uninterrupted movement toward the direction of increasing field intensity, irrespective of charge type.

Dielectrophoretic movement is also frequency dependent, relating to the speed of polarization (dipole-formation) for a given particle. Between each cycle of frequency, the dipole-forming charges become randomized through a process called *relaxation* (section 1.1.8.1) and subsequently re-align to re-form the dipole. When a maximum frequency is reached, at which dipole reversal cannot be completed in time with the changing field direction, a neutral particle will become static within the field.

A complication can arise at low frequencies during dielectrophoresis of cell suspensions. Due to the net negative charge on the surface of cells, electrophoretic effects may superimpose upon dielectrophoretic effects as the field reversal is slow (Burt *et al.*, 1989). This low frequency electrophoretic effect may enhance the dielectrophoretic effect by driving the particles nearer to the electrodes.

1.1.4 Negative dielectrophoresis

The previous description illustrates *positive dielectrophoresis* wherein polarization of particles results in their migration toward an electrode where field lines are converging. This can only occur when the particles are more polarizable during the application of the electric field than the medium in which they are suspended. Consequently, a medium of low ionic strength is required for positive dielectrophoresis and this can hinder some potential applications for this phenomenon. In the converse situation when the suspending medium is more polarizable than the particles it contains, repulsion of the particles *away* from the electrodes occurs. The particles congregate not at the region of greatest field strength but in *wells* within the field where the intensity is weakest. This response is also frequency dependent.

Specialist electrode geometries have been developed which can be used to display positive or negative dielectrophoresis (Pethig *et al.*, 1992). Also, it may be possible to combine the two phenomena, as has been demonstrated by Markx *et al.* (1994) in the separation and characterisation of microorganisms. In this work conditions were produced in which cells of one species collected via positive dielectrophoresis and those of a second species congregated simultaneously through negative dielectrophoresis. Section 1.1.6.5 contains a more detailed explanation of how this can occur.

1.1.5 Other AC, DC and non-uniform field effects

When a non-uniform electric field is applied to a suspension, other effects may compete with dielectrophoresis. These include heating effects (which lead to convection currents within the suspension) and electrophoresis. Another effect that may arise is *electrostriction*, the distortional response or strain resulting from electrical stress. This is not the same as dielectrophoresis as no translational movement occurs (Pohl, 1973). Other electric field effects include electrorotation, electroporation and electrofusion.

Electrorotation

Electrorotation is a non-uniform electric field phenomenon where an individual cell (or a sample of cells) is placed in a rotating AC electric field and induced to spin. The speed and direction of rotation gives information about the cells under investigation.

The first recorded evidence for electrorotation was probably accidental (Teixeira-Pinto *et al.*, 1960, in Pohl, 1978). However, the first purposeful investigations into the phenomenon were performed several years later (Arnold and Zimmerman, 1988; Mischel *et al.*, 1982). The rotating field is created using four electrodes, each driven with equal voltages and each phased apart by 90° (the equivalent is produced using n electrodes positioned 360°/n apart) (Pethig, 1991). The induced dipole moment within a cell is not formed instantaneously but takes a finite amount of time to become fully established. The dipole moment has a characteristic size and direction (section 1.1.6.3) and therefore when in a rotating field, due to the delay in its formation, the dipole moment is not precisely aligned with the field lines. This results in the formation of a rotational torque. The speed and direction of the spinning is dictated by the strength of the electric field and the angle between the dipole moment and the field. The latter is dependent on the structural composition of the cell and therefore the characterisation of cell types is feasible using this phenomenon. Further work performed by Arnold and Zimmerman (1988) demonstrated that in the kHz frequency range, *contrafield rotations* occurred wherein cells spun in the opposite direction to the applied field. Explanations offered for these observations involve the effects of surface charge and ion diffusion conductivity at the surface of the cell wall or membrane. The angular frequency of rotation within this frequency range is highly influenced by the effective conductivity of a cell whereas effective permittivity plays a relatively minor role (Fuhr and Kusmin, 1986).

Applications in cell characterisation and physiological assessment using electrorotation are being investigated, using oocysts produced by the water-borne protozoan parasite *Cryptosporidium parvum* (Smith, 1995). As electrorotation is potentially capable of assessing single particles, it has application in areas where an analysis of low numbers is required. However, it has been suggested that the field frequencies involved in electrorotation may have detrimental effects on the physiological state of cells (e.g. human erythrocytes) by increasing the efflux of ions from the cells (Georgiewa *et al.*, 1989).

Electroporation and electrofusion

These two related phenomena involve the controlled and reversible electrical breakdown of the cell membrane (Pethig, 1991). Attractive applications of these techniques can be found in genetic engineering where electrically mediated gene transfer can be performed. *Electroporation* (also called electropermeabilisation) is achieved by using a pulse which can vary in length between 40 kV cm^{-1} (for a 5 μsec pulse) down to 1 kV cm^{-1} (for millisecond pulses), according to the cell type. Genetic material can be introduced through the membrane. For *electrofusion* to occur, dielectrophoresis is first employed in order to align the cells (Mehrle *et al.*, 1988; Dimitrova and Christov, 1992). Fusion of cells including their nuclear membranes is then achieved by applying a high-strength electric pulse. In both electrofusion and electroporation a high electric field stress (approximately
1-2 x 10^6 V cm^{-1}) is formed across the cell membrane. This results in the formation of aqueous pores which enable permeation and fusion of the cells (Pethig, 1991).

1.1.6 Polarization of particles

Dielectrophoresis is dependent upon the formation of dipoles within particles (polarization) through the action of an applied electric field. A simple equation showing the relationship between variables which influence the dielectrophoretic force upon a particle is described below:

$$F = pv\,(E.del)E \qquad (1.2)$$

where p = polarizability, v = volume, E = electric field strength and del = the del vector operator (Inoue *et al.*, 1988).

Equation (1.2) emphasizes the importance of particle polarizability in determining the strength of the dielectrophoretic force. Dipole formation within a particle such as a cell is a complex and multifaceted process. The polarizability of a macromolecular particle is based upon the dipole composition of the constituent molecules, each of which is affected differentially by each AC field frequency. It is also affected by the composition of the suspending medium and by a fundamental property of every material, the *relative permittivity* which will be discussed in more detail in section (1.1.6.4) .

1.1.6.1 Dielectric properties

Study of dielectric behaviour may yield structural information at the molecular level and also aid the understanding of the dielectrophoretic polarization of biological particles. Dielectric properties of materials describe the displacement of electrons within the constituent atoms in relation to the nucleus of each atom. The resulting dipole has an electric moment in the direction of the applied field (fig 1.2). The stress exerted due to the charge displacement within the material is the *electric polarization*, P which can be defined as:

$$P = D - E\varepsilon_0 \qquad (1.3)$$

where D = charge displacement, E = electric field strength and ε_0= absolute permittivity of free space. Each dielectric material has a *dielectric strength* which is a measure of the maximum potential gradient that the material can withstand before it begins to break down.

A further description of dielectric behaviour in a simple biological system is described in section 1.1.8.2.

1.1.6.2 Modes of polarization

There are five modes of polarization known to occur in matter and these are summarized in table 1.1. The descriptions illustrate that a combination of polarization processes unite in a unique manner for a particle type at a particular frequency. When the structural complexity of cells is taken into account it is not difficult to appreciate the possibility of exploiting subtle differences using dielectrophoresis.

Table 1.1. The five modes of polarization in matter (after Pohl, 1973).

Polarization process	**Description**
Electronic polarization	The simplest molecular polarization which arises when the electronic arrangement around nuclei is distorted by an applied field.
Atomic polarization	The shift of single or grouped atoms with respect to each other, e.g. the ionic shift in NaCl when an external field is applied.
Orientational polarization	Occurs when an applied field causes reorientation of asymmetrical charge distributions between dissimilar parts of molecules e.g. in H_2O, HCl and NO which all have permanent dipoles. The orientation of water molecules contributes to polarization in aqueous solutions.
Nomadic polarization	Arises when thermally produced or excited charges (e.g. freed electrons or protons) roam over a large molecular scale.
Interfacial polarization (Maxwell-Wagner polarization)	Occurs when charged carriers migrate through mixed dielectrics. May occur in cells, which have conductive regions interleaved with barrier layers.

1.1.6.3 Permanent and induced dipoles

Typically, many of the molecules forming a dielectrophoretically susceptible particle already possess a dipole independently of electric field influence. These are *polar* molecules and their permanent dipole is a result of differing electronegativities between their constituent ions. The total number of negative and positive charges within a neutral molecule are equal but the centres of the negative and positive charge distributions are displaced with respect to one another. This asymmetrical charge grouping can be illustrated by referring to the water molecule (fig 1.2).

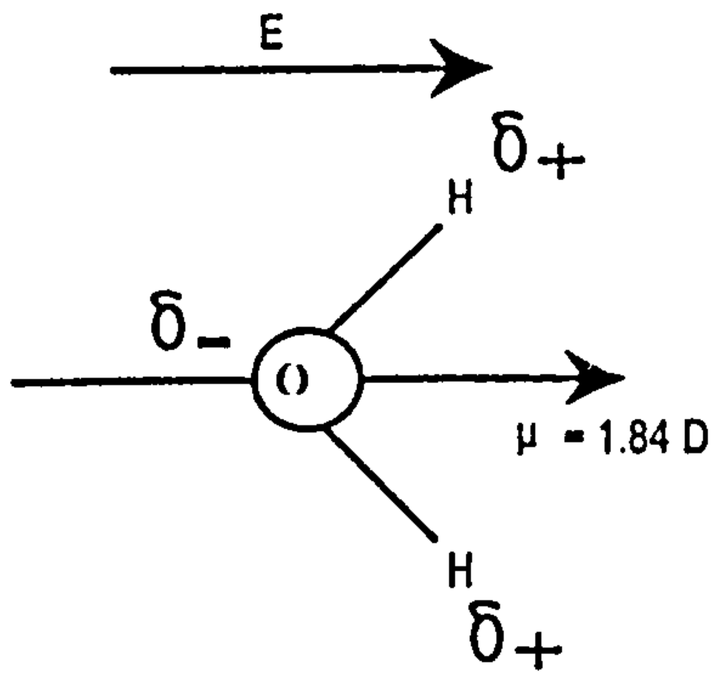

Fig 1.2 A water molecule.

A water molecule is composed of an oxygen atom which bears two extra electron pairs when compared to the two hydrogen atoms with which it is bonded. This confers a slight additional negative charge to the oxygen atom in relation to the hydrogens, therefore the centres of the positive and negative charges within the molecule do not concur and an *electric dipole* exists (Pethig, 1982). In this example, the permanent dipole is caused through the separation of *fractions* of charge but a dipole may also exist through the permanent separation of *whole* electronic charges such as in the zwitterionic forms of amino acids (Bone and Zaba, 1992).

An electric dipole possesses a size and a direction. For example, if two charges q and -q are separated by a distance l, this can be represented by a vector μ which points from the negative to the positive charge. The size of the vector is given by q.l (the total positive or negative charge multiplied by the distance between the two centres of charge) and it is called the *electric dipole moment*, μ which is commonly measured using the Debye unit, D (1D = 3.336 x 10^{-30} cm^{-1}). If the length is 1A, the dipole moment (μ) is approximately 4.8 D; for a water molecule, μ = 1.85 D.

The *polarization* of a material is determined by the average electric dipole moment per unit volume of that sample. It is logical therefore that polar materials (which contain molecules with permanent dipoles) are more polarizable in an electric field than non-polar materials such as some aromatic compounds e.g. benzene. The benzene molecule is exactly symmetrical due to its resonance hybrid state wherein all bonds are electrically equal. However, the influence of an electric field upon such a structure can induce a dipole by distorting the electronic distributions and nuclear positions. Therefore non-polar materials may also be susceptible to dielectrophoretic force. The result of electric field interaction with already polar molecules, is to further corrupt their permanent dipoles thereby increasing the overall force to which they are subjected (Pethig, 1982).

1.1.6.4 Permittivity

The permittivity of an homogeneous material is a macromolecular property which relates to the polarizability of the material. It describes the ratio of electric displacement in a medium to the intensity of the electric field producing it.

If two charges, Q_1 and Q_2 are separated by a distance r in a vacuum, the force (F) between the two charges can be described by:

$$F = \frac{Q_1 Q_2}{r^2 4\pi\varepsilon_0} \qquad (1.4)$$

Equation (1.4) is a statement of Coulombs Law where ε_0 refers to the *absolute permittivity of free space* (also called the *electric constant*) which is a constant with a value of 8.854×10^{-12} Fm^{-1}. This permittivity factor refers to the medium separating the two charges, Q_1 and Q_2 which in equation (1.4) is a vacuum. However, if the medium is anything other than a vacuum the permittivity is then a feature unique to the nature of the medium and is the *absolute permittivity of the medium*, which is always reduced compared to the situation in a vacuum. The new situation can therefore be described by:

$$F = \frac{Q_1 Q_2}{r^2 4\pi\varepsilon}$$

Every material therefore has a unique *relative permittivity*, (formerly called the *dielectric constant*) which is dimensionless but is always reduced compared to the permittivity of free space:

$$\varepsilon_r = \frac{\varepsilon}{\varepsilon_0}$$

The reason for the reduction in permittivity in a non-vacuum is the presence of particles which are polarized or become so by the application of an electric field. This causes a net reduction in the field strength known as the *polarization charge* caused through the opposing action of the polarized charges within the medium effectively creating a field in the opposite direction. The permittivity of the suspending medium is of fundamental relevance to the polarizability of any particles. The relative permittivity is larger with increasing polarity (conductivity) of the medium due to the interaction of the dipoles with the applied field. The relative permittivity of a dielectric material is measured using a capacitor. It is the name given to the factor by which that particular material is able to increase the capacitance of a capacitor. If a vacuum between the plates of a capacitor has a capacitance of C, and the capacitance with a dielectric material between the plates is C' then the relative permittivity of the dielectric is:

$$\varepsilon_r = \frac{C'}{C}$$

Conditions such as temperature which may alter molecular bonding will also alter relative permittivity.

1.1.6.5 Determination of dielectrophoretic polarity

A more specific adaptation of equation (1.2) which draws attention to the importance of effective particle conductivity, is given in equation (1.8). The influence of particle and medium conductivity on dielectrophoretic force is apparent and through the manipulation of these features it has been shown by Markx *et al.* (1994) that it is possible to predict when a positive or negative dielectrophoretic response would occur for different species of micro-organisms.

If a spherical particle (radius R) is in an electric field (E) which has an angular frequency ω, then the time-averaged value of the dielectrophoretic force ($F(\omega)$) is:

$$F(\omega) = 2\pi\, \varepsilon_0 \varepsilon_m R^3 Re \left| \frac{\sigma^*_p - \sigma^*_m}{\sigma^*_p + 2\sigma^*_m} \right| \text{del } E^2 \text{ (r.m.s.)}$$

where *Re* emphasizes that the real (as oppose to the imaginary) component of the expression inside the brackets should be considered, ε_0 is the absolute permittivity of free space, ε_m is the relative permittivity of the medium, del E defines the non-uniformity of the field and σ^*_p and σ^*_m are the complex AC conductivity values for the particle and for the medium.

If the suspending medium consists largely of water then it can be assumed that the medium conductivity and permittivity remain constant over the frequency range 10 Hz - 100 MHz because there are unlikely to be any major dielectric losses (the relaxation frequency of water is 17 GHz at 25°C). The important frequency range in the investigation of effective particle conductivity lies between 10-100 kHz and because there are no major dielectric dispersions within this range, equation (1.8) can be approximated to:

$$Re\left[\frac{\sigma^*_p - \sigma^*_m}{\sigma^*_p + 2\sigma^*_m}\right] = \frac{\sigma_p - \sigma_m}{\sigma_p + 2\sigma_m} \qquad (1.9)$$

where σ_p is the effective particle conductivity for the cells within this frequency range.

This demonstrates what has been stated in section 1.1.4, i.e. that the if the effective complex conductivity of the particle exceeds that of the medium then positive dielectrophoretic migration results. If the inverse is true, negative dielectrophoresis occurs. As a consequence of this rule, it is possible to find out conditions under which cells of one species will undergo a positive dielectrophoretic response whereas those of different species would simultaneously respond negatively. In order to predict the precise conditions of medium conductivity under which this could occur the effective particle conductivities for different species of microbes can be calculated through examination of their dielectrophoretic response within this frequency range over a variety of medium conductivity levels.

1.1.7 Importance of electric field strength and particle volume in determining dielectrophoretic force

In addition to polarizability, equation (1.2) illustrated the importance of electric field strength and particle volume in affecting the magnitude of the dielectrophoretic force. The extent of induced polarization caused through the application of an electric field is, up to a point, directly proportional to the field strength. The larger the polarizability volume of the material, the more polarizable it will be in an electric field. If a molecule has a large value for its polarizability volume, this is in part related to its atomic structure in that polarizability values reflect nuclear charge influence both on the intrinsic electron distribution and upon ability to prevent corruption of that distribution by an applied field. The nature of the constituent atoms is therefore of paramount importance to the polarizability of a molecule (i.e. as electron distance from the nucleus increases, nuclear control decreases). In addition, molecular orientation with respect to field lines is also relevant as this is directly related to the extent of distortion that the field is able to induce.

1.1.8 Dielectric dispersion

The frequency-dependent response of cells undergoing dielectrophoresis can be further explained by looking at the dielectric dispersions of some cellular constituent materials. A simple situation of a protein and water mixture is described in section 1.1.8.2.

When an electric field is applied to a particle, some of the energy of the field is absorbed by the particle. The absorbed energy acts to induce the imbalance in charge alignment causing dipole-formation, for example molecular reorientation or rotation may occur as a consequence of field energy absorption and this may aid polarization. The amount of absorbed energy is proportional to a property of the particle called the *dielectric loss* . At low enough frequencies, the AC field dipole "flipping" can be fully completed in time with the frequency and the constant re-orientation of the dipoles transmits electromagnetic energy through the medium. Each time the dipole forms, the causative charge movement produces a current within the dielectric called the *displacement current.* Because there is no lag between the time required for dipole formation and the frequency of the applied field, the displacement current is out of phase with the voltage producing the electric field, by 90°. As there is no overlap between the displacement current and the voltage, the concurrent transmission of electromagnetic energy is completed without loss. Dielectric loss begins when the frequency increases to a point where there is a lag between the reversal of the dipole and the frequency of the field. Dipole reversal still occurs in this frequency range but in this situation Joule heating of the dielectric results, i.e. this is the dielectric loss. The dielectric loss of a particle will cease to exist when a threshold frequency is reached as the process of *relaxation* is totally unable to occur: the displacement current has now fallen to zero and the dielectric is in the condition of *zero loss* once again, as it was at the lowest frequencies. The charge storage capability has now become equivalent to that of a non-polar dielectric and there is a consequent fall in permittivity (Grant *et al.*, 1978; Bone and Zaba, 1992).

1.1.8.1 Relaxation

The imbalance in charge alignment and consequent polarization of a particle is only effective whilst it is under the influence of an electric field. When the field is removed the charges revert to their original pattern of distribution by a process called *relaxation*. Relaxation must take place each time a particle dipole "flips" when the electrode polarity reverses in an AC field. For each particle type, the relaxation process takes a finite time period, the *relaxation time* which is characteristic and so can provide information regarding the types of molecules involved in the polarization of a particle. The relaxation time is the constraining factor which determines whether or not a dipole can "keep up" with the frequency as it is increased. It follows then that the relaxation time of a particle should relate to size i.e. a larger particle would be less able to reverse in time with a high frequency field than a small particle and therefore the relaxation time would be greater and the *relaxation frequency* lower. When the relaxation frequency of a particle is passed, the induced polarization is terminated and it reverts to its original conformation, remaining static within the field.

The contribution of larger molecules to the dipole formation within a macromolecular particle ceases when their threshold relaxation frequency is reached. Consequently, the particle as a whole experiences a decrease in permittivity, which falls to a lower plateau.

1.1.8.2 Dielectric dispersion of an homogenous protein solution

The situation shown in fig 1.3 may arise during the dielectric study of an aqueous solution of a globular protein, in this case haemoglobin.

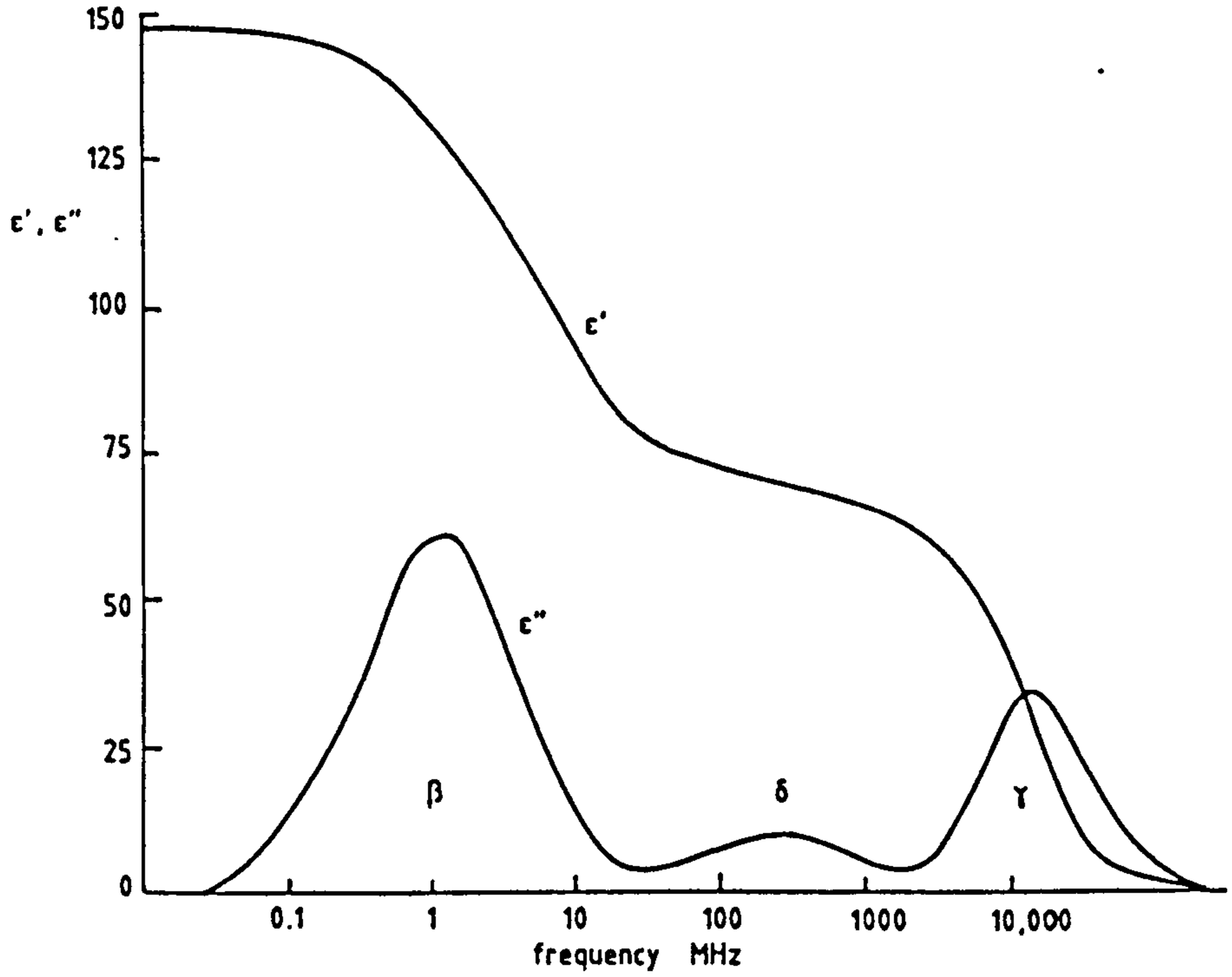

Fig 1.3 The dielectric dispersion curve of a typical globular protein (haemoglobin) in solution (taken from Bone and Zaba, 1992).

Above a frequency of 10 MHz the protein molecules cease to be polarized by the field. The portion of dielectric loss conferred to the solution by the protein is lost. The solution as a whole undergoes a decrease in permittivity (i.e this is the *dielectric increment*, ε') therefore there is a fall in the amount of charge stored by the dielectric. However at higher frequencies the solution is still polarized and it also remains subject to dielectric loss, due to smaller molecules (such as water) present in addition to the protein.

The simple situation shown in fig 1.3, that of an homogenous protein solution, has just three dielectric loss dispersions. The largest of these is for the protein molecules (the ß dispersion), the next for water associated with macromolecules, *water of hydration* (the ∂ dispersion) and the highest frequency dispersion refers to the relaxation frequency of the water molecule (the γ dispersion). The water molecules which are hydrogen-bonded to the protein molecule are hindered from field-induced free rotation therefore their relaxation times are substantially longer than water molecules in the bulk phase. Although not illustrated in fig 1.3, an earlier dispersion region, the *α dispersion* is sometimes apparent in biological suspensions. This is not well understood but is thought to relate to the relaxation of counterions which surround macromolecules and were redistributed by the electric field (Grant *at al.*, 1978; Bone and Zaba, 1992; Pethig, 1979).

When a dielectric dispersion (α, β, γ) occurs in a cell suspension, it gives information about the polarization process that was predominantly responsible for cell polarization below the dispersion frequency. A graphic demonstration of the change in dominance of electrical polarization mechanisms at different frequencies was shown by Heller (1959) working with *Euglena* cells (a unicellular algae) and also by Chen (Pohl, 1978) working with the bacterium, *Bacillus cereus*. Both of these cell types have an elongated shape. Heller in 1959 (Pohl, 1978) observed what is now known to be *mutual dielectrophoresis* by pearl chain formation in an initially uniform electric field (section 1.1.9.1). The *Euglena* cells became oriented parallel to the field lines at low frequencies but became re-oriented at right angles when the frequency of the field was increased. Similar observations were made with *B. cereus* cells which aligned themselves along the field lines at frequencies between 1 - 500 kHz, but became perpendicular to the field above 2.5 MHz. These results demonstrate that at different frequencies, different polarization mechanisms predominate. At low frequencies polarization is predominantly caused by the interaction of surface ionic counterion layers with the field as well as surface conduction, and this polarization response is at a maximum when the long axis is along the field lines and the ions can effectively "sweep" from one end to the other in time with the frequency.

As the frequency increases to a point where the ion flow cannot keep up with the reversing electrode polarity, this polarization mechanism lessens in importance. The movement of charges still able to flow at higher frequencies is hindered by the cell membrane which, acting as a capacitor allows build up of charge between layers. The capacitative effect of the membrane and therefore this mechanism of polarization, is maximized when the cell is at 90° to the field lines (Pohl, 1978)

1.1.9 Cellular dielectrophoresis

Commentary regarding biological dielectrophoresis, particularly involving different types of cells and organelles has been recorded over several years. It is helpful to review some of these results in order to relate the explanations of phenomena in previous sections to experimental observations.

1.1.9.1 Mutual dielectrophoresis

Paradoxically, studies performed using *uniform* electric fields produced the first recorded biological dielectrophoretic effects. These can be explained by *mutual dielectrophoresis*. When an initially uniform field is applied to a particle suspension the very presence of the particles acts to produce local non-uniformities within the field causing attraction of neighbouring cells. The result is the formation of a *pearl chain* of particles mutually attracted to each other, end to end (Pohl, 1978). When the field is removed, the pearl chains disassemble due to the electrostatic repulsion between the net negative charges on the cell surface (Burt *et al.*, 1989).

1.1.9.2 Polarization of cells

The reasons for the interaction of cells with non-uniform fields leading to dielectrophoresis, lie in their structural composition. Cell polarization occurs through a combination of processes many of which have already been mentioned (Table 1.1) and these are further placed into context below:

The suspending medium in cellular dielectrophoresis is water which is highly polar. The dielectrophoretic phenomenon will only occur if the field can induce an even greater polarization within the cell. Broadly speaking there are several fundamental structural features of a typical cell which enable a highly specific polarizability to be induced. These include:

- The high water content within the cell itself.
- The high concentration of polar molecules contained within the cell interior (e.g. proteins, carbohydrates and nucleic acids).
- Membranes (lipid bilayers) which act as capacitors across which potential gradients may be formed by charge distribution.
- Cell surface areas where ionic double layers are present

(Pohl, 1978).

1.1.9.3 The first cellular dielectrophoresis: yeast

The earliest published cellular dielectrophoretic experiments were performed using yeast cells (*Saccharomyces cerevisiae*). In 1966, Pohl and Hawk demonstrated dielectrophoretic separation of living and dead yeast. In their experiments and those of other workers, using very simple equipment they made observations concerning the dependence of several physical and biological variables in influencing the dielectrophoretic response. Of the physical parameters, the voltage, the cell concentration and the time period over which the field was applied, all appeared to have a linear effect on the collection yield within limits, and all three variables acted independently of the each other. Further work using live and autoclaved yeast cells (Crane and Pohl, 1968) showed the independence of these parameters by demonstrating a similar response, irrespective of the physiological state of the cells. However, the parameters of frequency and conductivity did not act independently and the effects of these were interrelated in causing cell polarization.

During this work, measurements of dielectrophoretic yield were recorded by manual counting of pearl chain length. The *Dielectrophoretic Collection Rate* (DCR) was calculated using the number of cells that accumulated per unit of time and per unit length of the electrode.

The biological parameters investigated by early workers included the age of yeast colonies, the effect of heat treatment and the effect of exposure to ultraviolet radiation. Pohl and Crane (1971) used yeast cells from colonies aged 2, 5 and 9 days. The youngest cells collected to a greater extent at higher frequencies within the range where a Maxwell-Wagner interfacial polarization would be expected to predominate. This led to the suggestion that the 2-day old cells are generally more conductive than older cells because the Maxwell-Wagner polarization mechanism depends upon the involvement of medium and particle conductivities. Subsequent work performed by Chen (in Pohl, 1978) also showed an age-dependent dielectrophoretic response using *Bacillus megaterium* and *B. cereus* bacterial cells. Additionally, Iglesias *et al.*, (1984) showed a relationship between the increasing age of a yeast culture and a decreasing dielectrophoretic yield.

Pohl and Hawk (1966) used heat treated yeast cells (60-70° for 3 min) to demonstrate the collection of living cells from a mixed suspension of living and dead. Further work using heat-killed *S. cerevisiae* cells was carried out by Mason and Townsley (1971) who also showed dielectrophoretic separation. Hawkes *et al.*, (1993) also demonstrated a differential response using live and autoclaved yeast. Mason and Townsley (1971) also investigated yeast cells grown in different media which also responded differentially to dielectrophoresis. In a more recent study, by limiting the glucose available for consumption by cultured yeast, a subsequent decrease in the magnitude of their dielectrophoretic response was observed (Iglesias *et al.*, 1984).

Irradiation of yeast cells with ultra-violet light (253.7 nm) was carried out by Pohl and Crane (1971) to induce nuclear damage sufficient to prevent cell replication but not cell metabolism. No difference in dielectrophoretic response was apparent, implying that the damage caused was not in the cellular regions most important in the polarization processes.

1.1.9.4 Dielectrophoresis of blood

The dielectrophoresis of blood was an obvious choice for study due to the diverse nature of the particular components. Work involving red blood cells (erythrocytes) (Burt *et al.*, 1990; Haigh and Betts, 1994), white blood cells (leukocytes) (Stoicheva and Dimitrov, 1986; Marszalek *et al.*, 1991) and platelets (thrombocytes) (Pohl, 1978) has been performed. Dielectrophoresis of blood has exciting applications for the differential separation and physiological analysis of individual cell types, as demonstrated recently by Becker *et al.*, (1994); Talary *et al.*, (1995) and Becker *et al.*, (1995). These workers have respectively demonstrated differential dielectrophoretic responses from leukaemic cells, bone marrow stem cells and breast cancer cells, all of human origin.

Platelet dielectrophoresis was investigated in relation to haemophilia. The deficiency of blood clotting Factor VIII leads to this hereditary condition and this substance is contained within platelets in normal blood. Samples taken from normal, haemophiliac and carrier dogs were investigated. All sample types underwent dielectrophoretic collection to yield a peak at 1 MHz. Collection diminished over time with deterioration of the samples and no collection ever occurred below 20 kHz. These experiments concluded that it was possible to show different collection levels and therefore differences in effective polarizability between normal, haemophiliac and carrier platelet samples.The frequency range where platelet dielectrophoresis occurred covered 100 kHz - 10 MHz implying that a Maxwell-Wagner interfacial polarization type was dominant, possibly modified by surface conductance. The internal medium of a platelet is very conductive and is surrounded by a membrane, separating it from the aqueous suspending medium which was kept at a low conductivity level. The resulting large disparity between the conductance of the interior and the exterior of the platelets supports the above conclusion.

Red blood cells have been the focus of several studies. These cells do not contain a nucleus and metabolize very slowly. Experiments carried out by Wiley and also by Blattler (in Pohl, 1978) investigated canine erythrocytes. Generally, dielectrophoretic collection of samples decreased with time although stabilization of levels was achieved through the incorporation of metabolic inhibitors (e.g. 1 mM iodoacetate or cyanide). The erythrocytes (irrespective of the type of inhibitor added) began to collect around 100 kHz, exhibited a plateau of collection level from 400-600 kHz, peaked at 2.5 MHz and declined until ceasing collection after 20 MHz. The Maxwell-Wagner interfacial polarization was again thought to be responsible for the peak collection frequencies, the effect of the inhibitors in stabilizing collection levels was in accordance with interfacial polarization: the prevention of ionic leakage caused by the metabolic inhibitors stopped the conductivity levels of the internal and external media of the cells, approaching equilibrium. Therefore the different polarizabilities of the two regions were not altered and decreased polarizability and collection were avoided.

Another red blood cell investigation using dielectrophoresis was performed by Burt *et al.* (1990) where information about the cell was obtained. They demonstrated that cell surface charge affected the dielectrophoretic response at low frequencies whereas cell membrane conductivity effects were more influential as the frequency increased. An increase in effective cell conductivity was demonstrated using dielectrophoresis when the differentiation of erythroleukaemia cells was induced.

Dielectrophoresis of white blood cells, particularly studies comparing normal and malignant cells is of obvious interest for applications in the diagnosis and treatment of leukaemia. Preliminary work by Stoicheva and Dimitrov (1986) has shown differences in the dielectrophoretic responses of hybridoma and B lymphocyte cells. The dielectrophoretic behaviour of myeloma cells has also been examined (Marszalek *et al.*, 1991). Recent advances have been reported by Becker *et al.* (1994) and Talary *et al.* (1995)

1.1.9.5 Dielectrophoresis of cell organelles

Chloroplasts and mitochondria are both fragile cell organelles that have received dielectrophoretic study.

Chloroplasts showed two peak collection frequencies at 300 Hz and at 1 MHz. The former was thought to be explained by polarization of the ionic double layer at the granal surface. The 1 MHz peak was again attributed to Maxwell-Wagner interfacial polarization (Ting *et al.*, 1971). Mitochondria were studied by Chen (in Pohl, 1978) who showed three peaks of dielectrophoretic collection. These were at 100 Hz, 100 kHz and 2 MHz and were explained by the expected polarization mechanisms for these frequencies. There were no regions of the frequency range over which no collection was observed indicating that there was an overlapping range of relaxation times for each type of polarization process. The dielectrophoretic response of both chloroplasts and mitochondria was seen to reflect their physiological state.

1.1.9.6 Dielectrophoresis of bacteria

Much cellular dielectrophoresis has been performed using bacteria, not least due to their ease of preparation and their resistance to deterioration over an experimental time period. The major dielectric dispersion regions responsible for whole cell polarization have been mentioned (section 1.1.8.2).

A bacterial cell may be seen as a three-component particle consisting concentrically of a conductive interior, an insulating plasma membrane and a conductive cell wall. Additional components are sometimes present outside the cell wall for example a capsule or slime layer. The α, ß and γ dielectric dispersions are all demonstrable during bacterial dielectrophoresis over a suitable frequency range.

The low frequency polarization was shown to be largely dependent on the bacterial cell wall through studies performed using intact cells and protoplasts (Carstensen 1967). More recent confirmation of this has been obtained using *Micrococcus lysodeikticus* cells (Inoue *et al.*, 1988; Burt *et al.*, 1989). The relative permittivity of the protoplast is much lower than that of a walled cell at frequencies below 100 kHz. The relaxation at frequencies of 1-10 MHz of *Micrococcus lysodeikticus* protoplasts was shown to be due to interfacial polarization (ß dispersion) through involvement of the cytoplasmic membrane (Pohl, 1978).

Early investigations involved several contrasting bacterial species including *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus megaterium*, and *B. cereus*. Much of this work was performed by Chen (in Pohl, 1978).

E. coli

E. coli is a Gram negative, rod-shaped bacterium. It has been shown to collect over a frequency range of 1 kHz - 10 MHz, typically peaking around 1 MHz although the frequency dependence of collection was strongly influenced by the conductivity of the suspending medium. An increase in suspension conductivity led to a decrease in collection at low and high frequencies. When the conductivity was altered using a different valency cation, the general effect was that the decrease in collection was greater when the valency of the cation was greater. La^{3+} ions decreased collection more than Ca^{2+} ions which lowered collection more than K^{+} ions (in Pohl, 1978).

The dielectrophoretic response of *E. coli* cells over the frequency range employed was explained using the expected polarization processes. The high polarizability at lower frequencies (below 100 kHz) was due to mobility of the ionic counterion layers at the surface. The higher valency cations were thought to effectively decrease the concentration of the mobile cations at the surface and reduce polarizability and collection as a consequence.

The dielectrophoretic collection at higher frequencies (around 1 MHz) was explained principally by two types of interfacial polarization mechanisms. The classic Maxwell-Wagner type polarization was thought to be operative alongside a modified Maxwell-Wagner polarization, incorporating the conductivity of a surface layer. This additional feature explains why the addition of high valency ions would also decrease polarizability at higher frequencies, because polarization was not simply due to the Maxwell-Wagner mechanism alone.

Other bacteria

Studies using *Pseudomonas aeruginosa* cells showed decreased polarizability at low frequencies when compared with *E. coli.* After disruption of these cells, debris rich in cell wall material was shown to collect to a greater extent at lower frequencies than at higher (around 1 MHz). This evidence supported the view that the bacterial cell wall contributed to cell polarizability through a mechanism involving mobile surface counterions rather than through the interfacial type. Further evidence for this was provided using *Bacillus cereus* cells. When subjected to a chloroform extraction to damage the cytoplasmic membrane, dielectrophoretic collection was shifted toward lower frequencies (below 100 kHz). This was explained by the leaking of cytoplasmic contents (therefore the internal and external conductivities had begun to approach equilibrium) and the removal of the capacitive membrane which together were necessary for interfacial polarization. The cell wall was relatively undamaged and therefore mobile counterion polarization generally unaffected.

These early findings have been extended and it is now possible to demonstrate dielectrophoretic characterisation and separation of a range of micro-organisms (Markx *et al.*, 1994).

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1.2 Structure and charge of the bacterial cell membrane and wall.

Antimicrobials can only be effective by firstly breaching the microbial outer layers, whether or not their mode of action lies there. It is essential to have an understanding of the composition of the bacterial cell wall in order to appreciate how surface charges result, and how they may be affected by surface-acting agents.

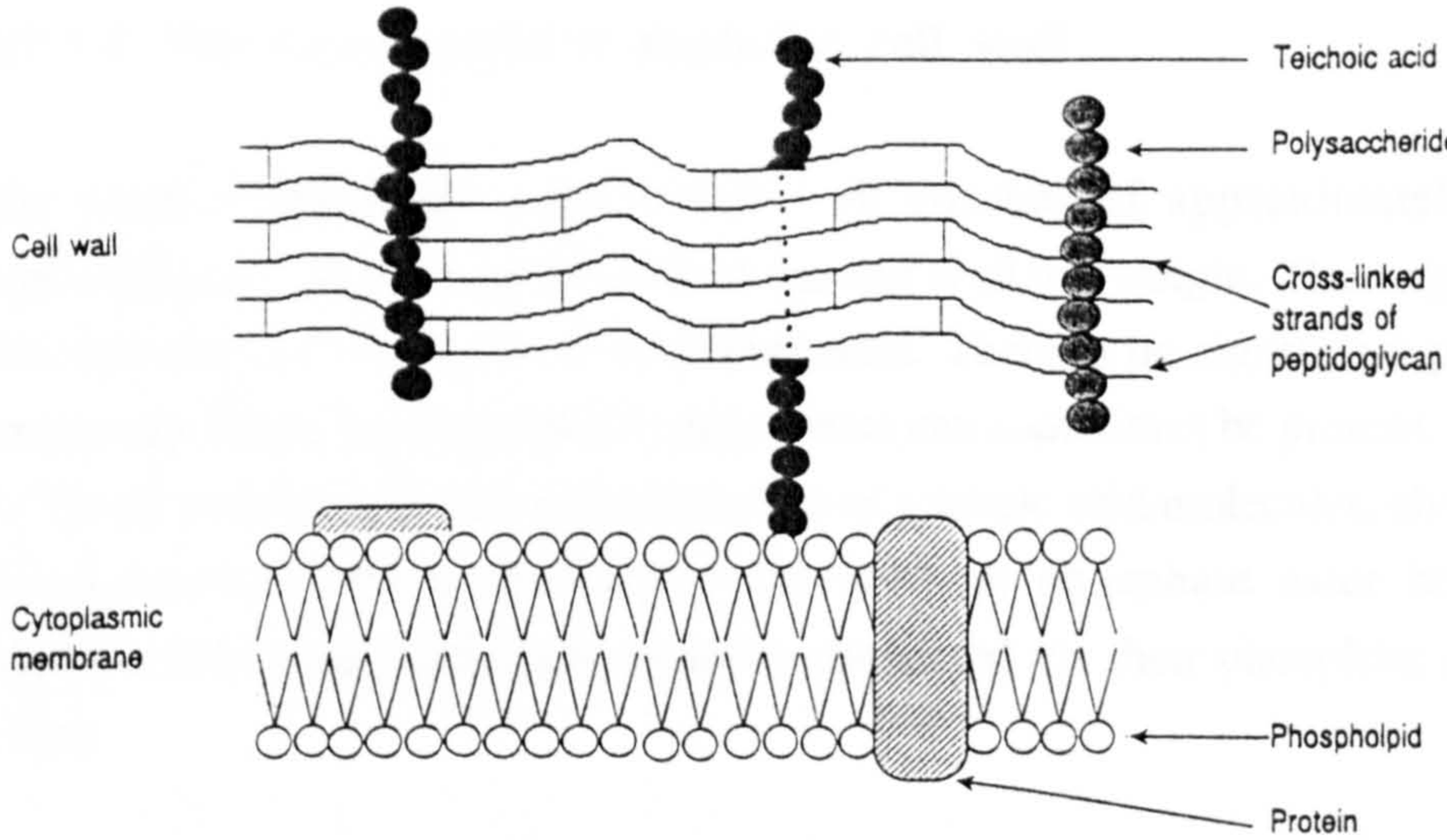
Bacterial cells typically possess a cytoplasmic membrane surrounded by a cell wall. Additional surface layers can also frequently be found. The components of each layer contribute to an overall (typically electronegative) surface charge although differences in charge distribution occur.

1.2.1 The bacterial cell membrane

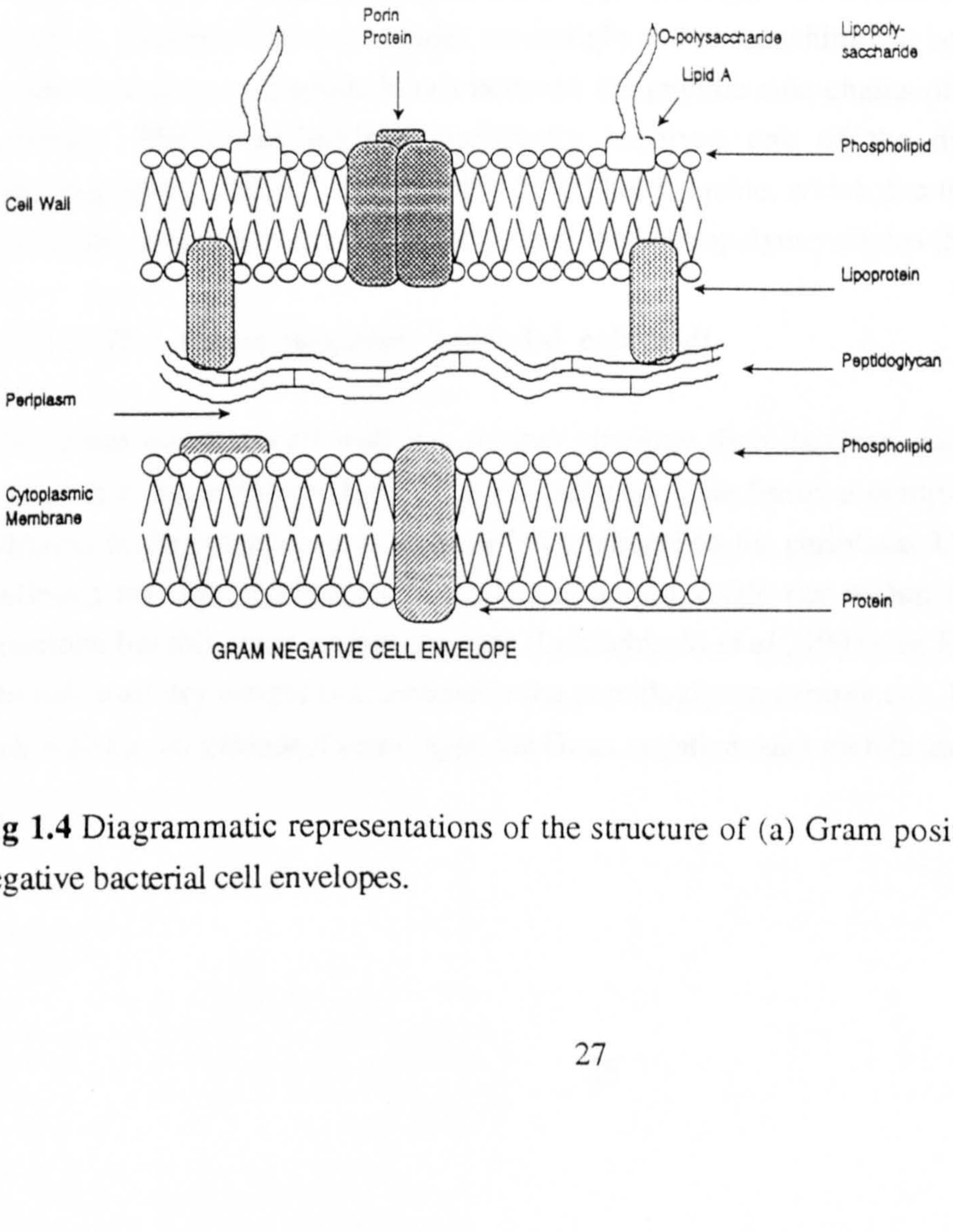
The bacterial cytoplasmic membrane has a structure similar to those found in eucaryotic cells, that of a trilaminar "unit membrane". The membrane is rich in lipids which comprise up to 80 % of total cell lipid. Integral membrane proteins can span all or part of the membrane diameter and peripheral proteins may also be attached at the surfaces. The cytoplasmic membrane forms the osmotic barrier of the cell and is the site of active transport mechanisms. The enzymes involved in electron transport and oxidative phosphorylation are also located here.

1.2.2 The bacterial cell wall

The nature of the cell wall has important taxonomical implications: it is responsible for the broad classification of bacteria into one of two divisions, based upon the ability to retain a Gram stain. Consequently, bacteria are designated as either "Gram positive" if they retain the stain or "Gram negative" if they do not (some "Gram variable" species also exist). This seemingly crude distinction relates directly to the structure of the respective cell walls, specifically the peptidoglycan (murein) content. The wall structures are illustrated in fig 1.4.



GRAM POSITIVE CELL ENVELOPE



GRAM NEGATIVE CELL ENVELOPE

Fig 1.4 Diagrammatic representations of the structure of (a) Gram positive and (b) Gram negative bacterial cell envelopes.

1.2.2.1 The Gram positive bacterial cell wall

The bulk of the Gram positive cell wall consists of approximately forty layers of peptidoglycan, comprising 30-70% of the cell wall dry weight. The large quantity imparts resistance to the extraction of the Gram stain. There is no significant protein content but covalently bound polysaccharide components can sometimes be present. A characteristic of the Gram positive cell wall is the inclusion of teichoic acid molecules, chain molecules of 8-50 glycerol or ribitol residues joined through phosphate ester bridges. These are interspersed in the peptidoglycan bulk, connected via their phosphate groups. (Schlegel, 1986).

Peptidoglycan

This macromolecule imparts mechanical strength to the bacterial cell wall. It is composed of chains of two types of alternating molecules, *N*-acetylglucosamine (NAGA) and *N*-acetyl muramic acid (NAMA) which are linked by β -(1,4) glycosidic bonds to form a regular polymer. Chains of these molecules are straight and unbranching but become cross-linked by the formation of peptide bonds between the peptide side-chains of the muramic acid residues. The cross-linking specifically involves one of the diamino acids *m*-diaminopimelic acid, LL-diaminopimelic acid or L-lysine, which due to the possession of two amino groups are able to connect two of the heteropolymer chains (Schlegel, 1986).

1.2.2.2 The Gram negative bacterial cell wall

The Gram negative cell wall is a thinner structure than its Gram positive counterpart, containing just a narrow band of peptidoglycan. This forms a complete layer which is adjacent to the cytoplasmic membrane but separated by the periplasm. Until recently it was believed that the peptidoglycan existed as only a single tier within the Gram negative envelope but this is not always the case (Labischinski *et al.*, 1991). In *E. coli*, only 10% of the cell wall dry weight is attributed to the peptidoglycan component. The Gram negative cell wall has an additional outer layer, the Gram negative outer membrane.

The Gram negative outer membrane

The Gram negative outer membrane is formed as a distinct bilayer and is composed of large quantities of phospholipids, proteins, lipoproteins and lipopolysaccharide, totalling 80% of the cell wall dry weight. The peptidoglycan layer is usually linked to the outer wall through covalent attachment of lipoprotein to diaminopimelic acids. The non-polar end of the lipoprotein molecule is anchored in the lower region of the outer membrane as this is where the majority of the phospholipid elements are located. The exterior region of the outer membrane is predominantly occupied by lipopolysaccharide (Nikaido and Varra, 1985).

Lipopolysaccharide is a unique component of the Gram negative cell wall. It has three distinct parts; the innermost segment is a complex lipid called lipid A (also known as endotoxin), attached to this is the core oligosaccharide and outermost there is the O-specific chain, a polysaccharide consisting of repeating oligosaccharide subunits. The lipopolysaccharide molecules carry a net negative charge which contributes to the strong negative surface charge of Gram negative cells (Hancock, 1984). It has been shown that lipopolysaccharide molecules possess a strong divalent cation-binding site (Coughlin *et al.*, 1981) and the stability of this component of the cell envelope is dependent upon interaction with divalent cations (in particular Mg^{2+} and Ca^{2+}) which form non-covalent cross-bridges between adjacent molecules (Hancock, 1984). Although these ions are not "true" cell wall constituents, their absence leads to significant cell wall damage (Beveridge and Graham, 1991; Hancock, 1984).

Proteins of the Gram negative outer membrane

Proteins can be peripherally or intrinsically associated with the outer membrane. They are generally individually named as the Omp proteins in relation to the genes that encode them. Some of these, particularly in cell envelopes of *E. coli*, have been studied extensively. The proteins Omp C, Omp F and Pho E (termed porins) serve as pores in the outer membrane through which small hydrophilic solutes may diffuse. This is facilitated by the positioning of charged amino acid groups at the mouth or within the porin channel which is itself filled with water (Hancock, 1984).

The *E. coli* Omp A protein spans the outer membrane and acts as an external receptor for bacteriophage whilst simultaneously its innermost portion may be cross-linked to the underlying peptidoglycan layer. There is no established function for this type of porin. Other envelope proteins which have a known involvement in nutrient requisition, can be found in differing proportions in response to varying growth conditions.

1.2.3 Capsular layers

Some bacteria secrete a polysaccharide, water-rich layer over their surface which adheres to form a capsule. In some cases the secretion may be more fluid and diffuse and may be known as a slime layer. The term glycocalyx is also sometimes used as this encompasses all types of polysaccharide layers which are adherent to the cell surface (Costerton *et al.*, 1981). The molecular composition of these capsule layers generally includes negatively charged groups amongst a combination of glucose, amino-sugars, rhamnose, 2-keto-3-deoxygalactonic acid, uronic acids of various sugars and organic acids such as pyruvate and acetate. Conversely, a few species possess capsules composed of polypeptides e.g. *Bacillus subtilis*, *Bacillus anthracis* but the major constituent of all capsules is water (Beveridge and Graham, 1991).

The possession of a capsule contributes to the overall bacterial surface charge, for example in the case of the γ-glutamyl capsule of *Bacillus licheniformis* ATCC 9945 which increased the surface charge density (McLean *et al.*, 1990). Also, the capsular polysaccharides of *Acinetobacter* increase the hydrophilicity of the bacterial surface when compared with the lone outer membrane (Beveridge and Graham, 1991).

When the electrophoretic mobility of bacteria is investigated, the presence of a capsule may influence the response. One capsular component, glucuronic acid contributes to the low dissociation value for the microbe surface and imparts a high negative charge. This is due to the predomination of carboxyl groups and this can be deduced on the basis of a characteristic pH mobility curve (James, 1991). The external pH in a suspending medium directly influences the ionization of surface groups and possibly the conformation of polymers. Variations in pH and electrolyte concentrations lead to continuous ion exchange and to fluctuations of weak bonding forces concerning surface macromolecules. Capsular polymers are particularly susceptible to alteration due to their loose arrangement (McLean *et al.*, 1990).

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1.2.4 The S-layer

Electron microscopy has revealed that some bacteria possess a regularly structured, self-assembled protein/glycoprotein covering called an S-layer (Beveridge, 1981). This is a type of sheath which has a distinctive crystalline structure due to a symmetrical subunit assembly. It has been proposed that between the subunits there are specifically sized pores which are able to selectively filter macromolecules according to their size and charge (Beveridge and Graham, 1991). The charge associated with S-layers appears to be neutral at pH 7 although this may be anionic in the case of *Thermoproteus* spp. (Messner *et al.*, 1986). There are few electronegative thiol groups in S-layers and many of the electronegative carboxyllate groups that they may contain are masked due to being enfolded within the subunit core (Koval and Murray, 1986; Sleytr and Messner, 1988).

1.2.5 Flagella, fimbriae and pili

Flagella are thread-like appendages (12-18 nm diameter and up to 20 μm long) which protrude from the cell surface and are used for bacterial motility. Not all bacteria possess flagella and the type of flagellation i.e. the number and arrangement, varies between species.

Flagella are helically wound protein comprised of a single subunit called flagellin. They are inserted into the cytoplasmic membrane and rotate in a screw-like motion from the base. They differ from eucaryotic flagella and cilia (e.g. present on some protozoa) which are composed of nine peripheral double filaments with two central single filaments, and are surrounded by the cytoplasmic membrane.

Fimbriae are long, thin, straight threads, commonly found on Gram negative organisms These may cover the whole surface of the cell or be situated in a tuft in one area, and function more for attachment than for motility. Pili ("type-1")are very similar to fimbriae and are only found on some Gram negative cells. These are distinct from the F-pili, only two of which are present per cell in certain bacteria (Schlegel, 1986).

1.2.6 Microbial surface charge composition

The surface of any cell has a unique electrical charge composition enabling it to interact optimally with its environment. Characterisation of the physical composition of the cell surface can be aided by the study of its inherent electrical charge, particularly when specific treatments are used to provoke change in this charge. The nature and extent of the surface charge may play an important role in some specific applications, including particle flocculation during water treatment (Ives, 1990) and the binding of some drugs to receptor molecules located on the cell surface; altered surface properties of bacterial cells may reflect changes in the resistance patterns toward antibiotics (Richmond and Fisher, 1973).

A well-recognized non-destructive method employed in surface charge assessment measures the electrophoretic mobility of intact cells including micro-organisms. In this way information relating to the fundamental hydrated nature of the surface can be deduced. Electrophoretic motion in a solution occurs when an electric field is applied and the extent of movement is directly related to the polarity and density of the surface charges. The surfaces of all microbes possess an electric charge which is typically negative (Richmond and Fisher, 1973).

1.2.6.1 What is the microbial cell surface ?

Defining a precise location for the microbial cell surface is a difficult process and it may be more appropriate to regard the surface not as a planar boundary, but as a narrow region with its own characteristic properties. These properties will be distinct from those of the material in either of the bulk phases i.e. the cell or the external medium. As well as the physical components which are obvious contributors to a cell surface, the surface region will also be composed of layers of electric charge. In short, the outermost area of a microbial cell surface will consist of an ionic atmosphere directly under the influence of ionogenic ("charge determining") surface groups (James, 1991).

Formation of the electrical double layer

Generally, at an interface between two phases such as a solid surface and an electrolyte solution, charges of one sign will predominantly associate with one of the phases whilst those of the opposite sign will be more prevalent in the other. When an electric field is applied, this invokes the tangential movement of one phase relative to the other. The speed of movement is dependent upon the potential at the plane of shear, the boundary between the layer of surface-associated charge that moves with the particle during field-induced movement, and the bulk of the suspending medium. The potential difference across the width of this plane of shear is called the *zeta potential* ζ (James, 1991; Dealler, 1991). The outer limit of the plane of shear (the zeta potential of any suspended particle) has no agreed definition although it can be described with reference to the electrical double layer, the *Stern layer* (fig 1.5).

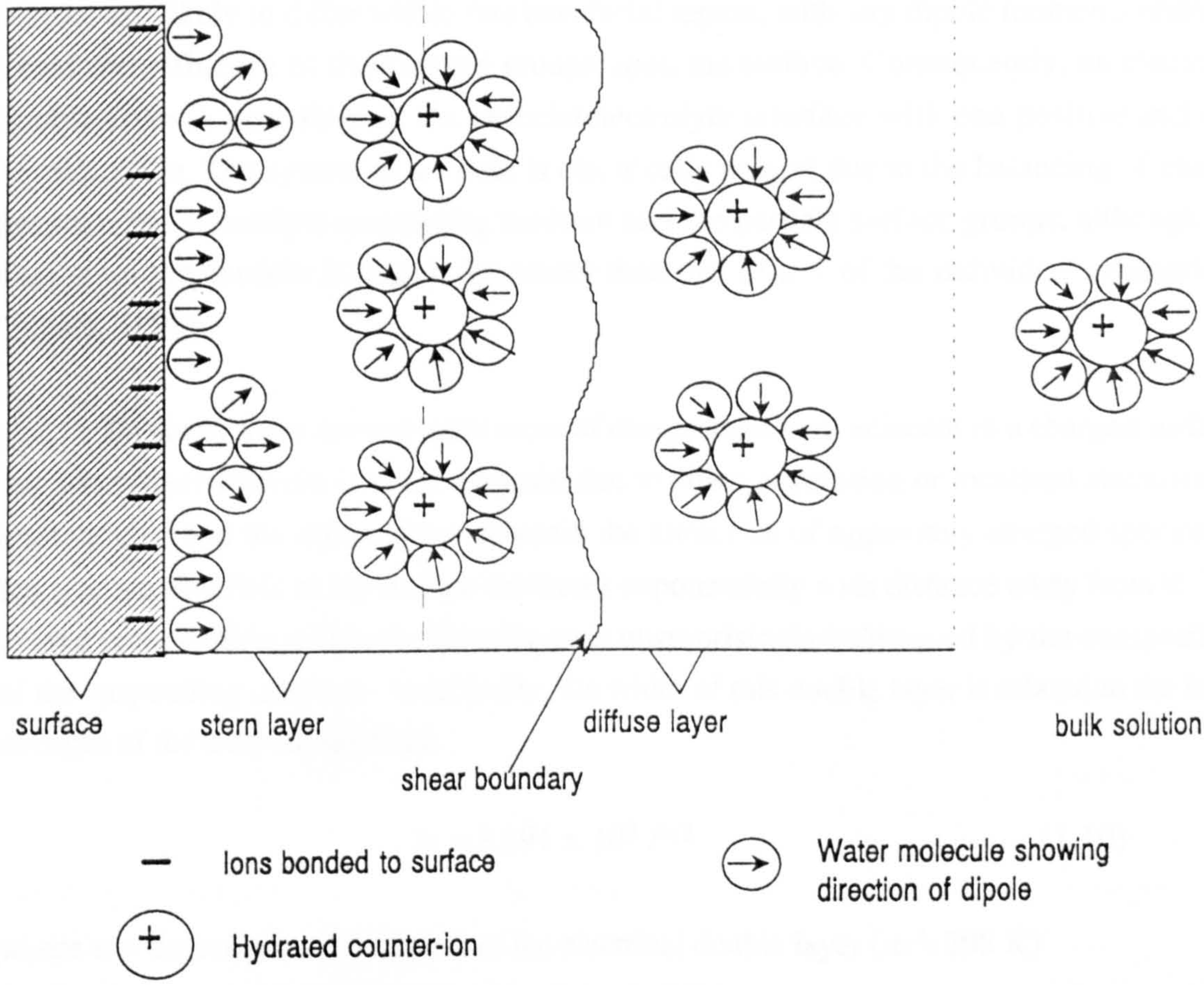


Fig 1.5. The structure of an electrical double layer. (Adapted from Oja *et al.*, 1989).

When particles of any type are suspended in aqueous media, typically they assume an electric charge as a result of ionization of various surface groups as well as adsorption of ions from the medium onto the particle surface. The relative concentrations of charged species are likely to differ within this interfacial region, with any dipole moments oriented under the influence of the charged groups upon the surface. Consequently, an electrical double layer is assembled at the particle/electrolyte interface with one positive and one negative side. The system as a whole is electrically neutral due to the balancing of charge between the electrolyte suspending medium and the particle surface groups, although the charge on the particle is massively greater than that of any of the individual counterions (James, 1990).

Basically there are two main areas of charge alignment adjacent to a charged surface: the *Stern layer* wherein ions are attracted due to direct adsorption or localised electrostatic interactions; and the *diffuse layer* wherein the attraction of oppositely charged species by the electrostatic field at the surface decreases exponentially with distance away from it. The charge composition within the Stern layer is unsurprisingly influenced by the composition of the suspending medium - specifically, the width of this double layer is related to the ionic strength of the external medium:

$$\kappa = 3.291 \times 10^9 I^{1/2} \qquad (1.10)$$

where κ = the reciprocal thickness of the electrical double layer (m^{-1}; 298 K)
I = the ionic strength of the medium (mol dm^{-3}, 298 K)

If a field is then applied, the particle has a tendency to move in one direction whilst the oppositely charged ions that are present in the mobile part of the ionic double layer (those attracted from the medium) move in an equivalent magnitude in the opposite direction. The plane of shear is thought to extend further into the suspending medium than the width of the Stern layer, inferring that the zeta potential is additionally influenced by a proportion of charges in the diffuse layer.

Overall, electrophoretic mobility depends upon the zeta potential at the plane of shear between the charged surface and the electrolyte solution. This relationship is also applicable to a microbial cell in suspension. Electrophoretic mobility can be defined by the equation:

$$u = \upsilon/X \qquad (1.11)$$

where u = the electrophoretic mobility ($m^2\ V^{-1}\ s^{-1}$), υ = the linear velocity ($m\ s^{-1}$) and X = unit field gradient ($V\ m^{-1}$).

The forces acting on a particle subject to an applied electric field can be described by:

$$u = C\,D\,\zeta/\eta \qquad (1.12)$$

where D = the dielectric constant and η = the viscosity of the suspending medium, ζ = the zeta potential and C = a constant, related to the size and shape of the particle.

In the SI format:

$$u = \upsilon/X = \varepsilon_r\,\varepsilon_0\,\zeta/\eta \qquad (1.13)$$

where ε_0 = the permittivity of free space and ε_r = the relative permittivity of the suspending medium.

Equation (1.13) can be used to calculate the measured electrophoretic mobility (10^8 $m^2\ V^{-1}\ s^{-1}$) and therefore the zeta potential ζ, in mV:

$$\zeta = 12.85\,u \quad \text{(at 298 K)} \qquad (1.14)$$

However, rather than quoting the zeta potential it is common practice to directly compare electrophoretic mobility values of different particle types, under standardised measurement conditions (James, 1991).

1.2.6.2 Factors influencing microbial cell charge

Electrophoretic mobility of bacteria is characteristic and can often be associated with "types" of cell surface structures and events. These could include ion diffusion through the cell membrane (James *et al.*, 1964) or the presence of fimbriae or a capsule associated with the cell wall (James & List, 1966). Cell surface treatments can affect the electrophoretic mobility of bacteria by modifying the charge function of the surface components - biological molecules often possess a characteristic charge structure, the response of which can be used to help elucidate surface composition. Even under controlled experimental conditions however, a specific mobility value is not enough alone to enable identification of a cell type or surface group. Nonetheless, it is often the case that comparison between the pH mobility curve of a treated micro-organism and that of an untreated counterpart can assist in identification of surface components.

Specific chemical or enzymatic treatments can be used to alter the electrophoretic properties of an organism enabling suggestions to be made as to which surface groups may have been altered and so by inference, which surface groups were present initially.

As discussed in section 1.2.6.1, the width of the cell ionic double layer is related to the ionic strength of the suspending medium. A further consideration concerns the "unmasking" of charged surface groups, which previously had associated counterions from the medium. As a decrease in ionic strength occurs, they become "free" to contribute to an overall increase in cell surface charge. Therefore, progressive change in the ionic strength of an electrolyte directly influences which charged structures are able to contribute to the net charge upon the cell, depending on the distance that they extend away from the surface (James, 1991). It is also feasible that such changes in ionic strength may effect conformational changes within macromolecular components of outer cell layers.

Effects of specific cell surface treatments upon surface charge

Treatment of cells with enzymes capable of attacking specific surface groups would be expected to alter the surface charge accordingly. Problems with the practicalities of such treatments including impurities in enzyme preparation and non-specific adsorption have hampered the widespread use of this approach to investigate relative contributions to surface charge (James, 1991).

Dyar and Ordal (1946, in James, 1991) were the first to suggest that the amount of lipid within a microbial cell surface can be assessed using surfactants. If there is lipid present, the non-polar end of the amphipathic surfactants associate with the lipid through Van der Waals forces and the polar end which has a positive or negative charge, is extended outwards into the suspending medium. The surface charge of the organism is enhanced depending upon the amount of surface lipid present and the anionic or cationic nature of the surfactant (James, 1991).

Further modifications to capsular properties affecting overall cell surface charge can be caused by specific chemical treatments. For example, exposure of *E. coli* cells to carboiimide and glycinamide resulted in the discharge of carboxyl groups and therefore the surface electrical properties were dominated by the amino groups which remained (Neihof and Echols, 1978).

In the investigation of *E. coli* and *Klebsiella aerogenes* (James and List, 1966) it was found that the electrophoretic mobility was independent of the presence of fimbriae. If cells possess neither capsular layers or fimbriae, the electrophoretic mobility is typically greater, due to the larger cell surface area to volume ratio.

(i) Antibiotic effect on electrophoretic mobility

Studies performed upon methicillin-resistant *Staphylococcus aureus* concluded that there was no relationship between the resistance to the antibiotic and the total surface lipid present. The increase in electrophoretic mobility seen with resistant *Staph. aureus* cells above their isoelectric point is of a lower magnitude to that of methicillin sensitive cells. However, upon adaptation to increasing concentrations of the antibiotic, the mobility of the sensitive cells decreased (Richmond and Fisher, 1973).

When the pH mobility curve of sporangia produced by *Pseudoperonospora humuli* was studied, the addition of 1 mg l^{-1} streptomycin was sufficient to show a considerable decrease in the mobility at pH 5.6. This was caused through binding of the antibiotic to ionic species located on the particle surface (Richmond and Fisher, 1973).

1.2.7 Surface charge of *Cryptosporidium parvum* oocysts

Work performed regarding oocyst surface charge has been necessary due to the direct relevance of this property to the ease of oocyst extraction during water treatment. Flocculation is a long established method of removing colloidal particles during drinking water purification. Any small particles in water naturally aggregate to form "clumps" unless they are prevented from doing so by forces such as those produced by electrostatic repulsion between charge layers of adjacent particles. If the zeta potential of the particles is less than -15 mV this is small enough to produce only a very weak repulsive force, as long as there are sufficient dissolved ions in the water. *Cryptosporidium* oocysts appear to have a zeta potential of below -10 mV and so consequently they can be termed "destabilized" meaning they offer very little resistance to aggregation. However in practical terms, oocysts present in water are not in close enough contact with each other to form aggregates but this situation can be improved by the addition of chemical flocculant aids (Ives, 1990).

1.3 Coccidian protozoa

The coccidia are a group of obligate intracellular protozoa with predominantly vertebrate hosts. Coccidia of medical and veterinary importance are classified into 3 families: Eimeriidae, Sarcocystidae and Cryptosporidiidae. The Eimeriidae incorporate the genera, *Eimeria*, *Isospora* and *Caryospora*. Amongst others, the Sarcocystidae family includes the six genera: *Sarcocystis*, *Frenkelia*, *Toxoplasma*, *Besnoitia*, *Hammondia* and *Neospora*. Cryptosporidiidae is comprised of only a single genus, *Cryptosporidium*. *Cryptosporidium* means "hidden sporocysts". When *Cryptosporidium* was first described, unlike the oocysts of the known coccidia of the time, this organism did not have sporocysts surrounding its sporozoites (Current, 1988).

The medical importance of another coccidian, *Cyclospora* has recently been recognized.

1.3.1 The Eimeriidae

Infections caused by these genera are transmitted by release of oocysts in the host faeces. Intestinal coccidiosis in poultry and livestock is widespread. Anticoccidial drugs are essential to the poultry industry as almost all commercially raised chickens must be given these to survive infection. *Isospora* causes diarrhoeal disease in a variety of mammalian hosts and one species, *I. belli* may cause gastroenteritis in humans travelling through endemic areas. It may also cause serious, unremitting infection in AIDS patients. *Caryospora* species may have intestinal stages in reptiles and tissue-dwelling stages in mammals (Upton *et al.*, 1986).

1.3.2 The Sarcocystidae

The genera *Sarcocystis* and *Frenkelia* are in a subfamily (Sarcocystinae) because they both have sexual stages (including oocyst formation) only in the intestine of their definitive host. Also, their asexual stages only occur in the tissues of their intermediate hosts. The route of transmission from the intermediate to the definitive host is via ingestion of tissue cysts. *Sarcocystis* species have been reported to cause intestinal and non-intestinal infection in humans.

The four genera *Toxoplasma*, *Besnoitia*, *Hammondia* and *Neospora* belong to the subfamily Toxoplasmatinae because these species are able to transmit from one intermediate host to another via asexual life cycle stages. *Toxoplasma gondii* is known to cause human disease. In immunocompetent individuals infection is usually mild although tissue cysts are formed (generally in the central nervous system) which remain for life. In immunodeficient humans e.g. AIDS patients or unborn foetuses, life threatening infection often results. Abortion in livestock is also caused by this organism. Both the definitive and the intermediate hosts can become infected by ingestion of infected animal tissues or milk, by transplacental transmission, by blood transfusion or by organ transplantation (Kogut, 1990). *Besnoitia* is closely related to *T. gondii* and one species (*B. besnoiti*) can cause significant cattle morbidity in certain areas of the world. *Hammondia* species produce tissue cysts in mammals and ingestion leads to transmission to their definitive hosts, dogs and cats. *Neospora caninum* has been shown to produce an infection similar to toxoplasmosis in dogs (Dubey *et al.*, 1988).

1.3.3 The Cryptosporidiidae

The number of species belonging to the single genus *Cryptosporidium*, has been a subject for debate. However it is generally agreed that *C. parvum* is the species causing diarrhoeal disease in man, as well as in a number of other mammalian hosts (Angus, 1988). A respiratory illness may also be caused by this parasite in immunocompromised hosts. Severe respiratory disease is caused in birds (usually chickens) infected with *C. baileyi* (Current *et al.*, 1986; Blagburn *et al.*, 1991). Another species *C. meleagridis* can cause enteritis and diarrhoea in commercially raised poultry (Blagburn, 1988). Cryptosporidiosis of zoo and pet birds has also been recorded although the causative species is not clear (Lindsay *et al.*, 1991).

C. muris was the first species of the *Cryptosporidium* genus to be described, by E.E. Tyzzer in 1907. It is the type species for the genus and is infective to mammals including cattle (Upton and Current, 1985; Anderson, 1988; Casey, 1991), though not to humans.

A further species of *Cryptosporidium* has been proposed, *C. serpentis* which infects reptiles. Undoubtedly *Cryptosporidium* spp. can cause disease in reptiles and one study has shown that oocysts produced show significant differences to those of *C. parvum* (Tilley *et al.*, 1990a).

1.3.4 Cyclospora

Cyclospora is thought to have been recognized as a protozoan by Ashford *et al.* in 1978. *Cyclospora cayetanensis* has been reported to cause protracted diarrhoea in humans from locations all round the world (Bendall and Chiodini, 1995; Sterling, 1995). This coccidian produces environmentally resistant oocysts of similar dimensions to *C. muris* (8-10 μm), which contain 4 sporozoites. *Cyclospora* oocysts are not sporulated when excreted in the faeces of the host and sporulation can take 4-6 months to occur when stored at 4°C. It is thought likely that transmission of *Cyclospora* could be waterborne although this has not been proven. The sensitivity of the oocysts to disinfection is not known (Sterling, 1995).

1.3.5 Coccidian oocysts

Eimeria, *Isospora* and *Cryptosporidium* are amongst the coccidians which produce infective oocysts as the transmissible stage of their life cycle. Ultrastructural investigation of oocysts has proved difficult because conventional tissue preparation methods used for transmission electron microscopy are ineffective. The robust wall of the oocyst hinders fixation. Scanning electron microscopy has been used to look at oocysts but problems of distortion and collapse are still common (Ball and Pittilo, 1990).

Coccidian oocysts are formed from 2 types of wall-forming bodies. Type 1 bodies give rise to the outer oocyst wall and type 2 form the inner layer. The wall-forming bodies start to disappear from the cytoplasm as the layers begin to form (Ball and Pittilo, 1990). When *C. muris* oocyst walls are developing they consist of 3 layers (Uni *et al.*, 1987). The excreted *C. parvum* oocyst wall has 2 layers. The outer layer has an irregular shape. It is separated from the inner layer by an electron-lucent space (Reduker *et al.*, 1985a). The inner layer itself has an inner and an outer zone. An atypical type of *C. parvum* oocyst has also been reported which has 3 layers in the wall (Baxby and Blundell, 1988).

1.3.5.1 Resistance to disinfection

Oocysts produced by coccidian protozoa are robust structures evolved to survive in the environment until they are ingested and can initiate infection. Their survival is based upon the sturdy nature of the oocyst wall. However as a consequence, *Cryptosporidium* oocysts in particular are very resistant to traditional disinfectants (Campbell *et al.*, 1982; Blewett, 1988, Peeters *et al.*, 1989; Korich *et al.*, 1990). This is a particular problem in water treatment as the levels of chlorine used cannot inactivate *Cryptosporidium* oocysts (Anon, 1990; Smith and Rose, 1990). Avian *Cryptosporidium* spp. also exhibit resistance to disinfection which has important implications for the poultry industry (Sundermann *et al.*, 1987). It is thought that human cryptosporidiosis may be caused by ingestion of just ten oocysts (Miller *et al.*, 1989) or even just one oocyst (Blewett *et al.*, 1993). *Cryptosporidium* may also pose a threat in the contamination of foods (Hoskin and Wright, 1991) and the possible application of dielectrophoresis in this area has been discussed (Betts, 1995).

1.4 Giardia

Giardia is a genus of flagellated parasitic protozoa which cause diarrhoeal illness in man. Although these organisms are not coccidians, they are transmitted by the ingestion of contaminated food or water and are therefore commonly associated with the occurrence of *Cryptosporidium* oocysts. The parasite has a two-stage life cycle in which it exists as a trophozoite (the free-living stage) or a cyst. The cysts are oval (8-12 μm by 7-10 μm) and are enclosed within a cyst wall approximately 0.3-0.5 μm thick (Feeley *et al.*, 1984). Ingestion of 10-100 cysts can result in human infection (Simmons, 1991) although theoretically one cyst could be sufficient (Jarroll, 1988). While these cysts do not share the same high level of disinfectant resistance as *Cryptosporidium* oocysts they are several times more resistant to inactivation with free chlorine than *E.coli* (van Roodselaar, 1988). Chlorine C*t* values of 44.8-25.5 mg.min l^{-1} for *Giardia* and 0.014 mg.min l^{-1} for *E. coli* have been recorded (Jarroll, 1988). This in combination with the low number needed to cause infection makes them a cause for concern in water treatment.

There have been numerous recorded outbreaks of giardiasis caused by contamination of the water supply. These are primarily responsible for the large number of studies and the high degree of research regarding both the occurrence of *Giardia* in water and the efficacy of disinfectant inactivation (Craun, 1984; Jakubowski, 1984; Jarroll *et al.*, 1984; Neuwirth *et al.*, 1988; Wallis *et al.*, 1989; Hibler, 1988; Nebel *et al*, 1988; Rose *et al.*, 1988; LeChevallier *et al*, 1991; Gilmour *et al.*, 1991; Mahbubani *et al.*, 1991).

1.5 Antimicrobial agents

Many microorganisms with roles in spoilage and disease can be targeted with antimicrobial agents. This term includes all externally added compounds which result in microbial inactivation. Antimicrobial agents can be split into two groups, distinguishing antimicrobial drugs (antibiotics and antimetabolites) from biocides (preservatives, antiseptics and disinfectants).

1.5.1 How do antibiotics, antimetabolites and biocides differ ?

Antibiotics are commonly regarded as chemotheraputic agents. They are substances produced by microorganisms which in small amounts are able to inhibit other microorganisms (Tortora *et al.*, 1995). Antimetabolites are synthetic chemical compounds which act against microbes (Dawes and Sutherland, 1992). Antibiotics are primarily "natural" organic compounds but nowadays the term is often used to include semi or wholly synthetic compounds. Some are sufficiently specific in their antibacterial activity to be used in treating local and systemic infection.

Chemicals may be grouped together as biocides on the basis that they are non-therapeutic compounds but are capable of killing microorganisms (Russell and Chopra, 1990b). Disinfectants are traditionally regarded as suitable for use on inanimate objects (e.g. surfaces and equipment) but are too toxic to be applied to body tissue. Antiseptics are generally non-toxic to animal tissue and are used in topical applications but remain too non-specific to be taken internally as treatment for infection. Preservatives are chemicals which can prevent microbial contamination and proliferation in the products in which they are incorporated. They are widely used in pharmaceuticals, cosmetics and foods to protect against contamination and spoilage.

Some compounds are suitable for use as either disinfectants, antiseptics or preservatives by varying their concentration (Russell and Chopra, 1990b).

1.5.2 Antibiotics

Since the discovery of penicillin in 1928, a large number of different antibiotics have been investigated. A diverse range of compounds can be classed as antibiotics but only a limited number can be used to treat infection (Tortora *et al.*, 1995). Many antibiotics in clinical use are modified versions of those produced by microorganisms and potency and efficacy are often improved as a result.

Most clinical antibiotics in use today originate from either *Streptomyces* species (filamentous soil bacteria), *Bacillus* species and the fungal genera *Penicillium* and *Cephalosporium* (Bowman and Rand, 1980). Table 1.2 lists some commonly used antibiotics and their general site of action.

Table 1.2 Some antibiotics in common use.

Antibiotic family	Examples in clinical use	Primary mode of action
Penicillins	Natural penicillin; penicillin G	Inhibition of cell wall synthesis.
Semi-synthetic penicillins	Ampicillin; methicillin; oxacillin	Inhibition of cell wall synthesis.
Cephalosporins	Cefotaxime, cephalexin	Inhibition of cell wall synthesis.
Carbapenems	Imipenem	Inhibition of cell wall synthesis.
Aminoglycosides	Streptomycin; gentamicin; neomycin	Inhibition of protein synthesis.
Tetracyclines	Tetracycline; chlortetracycline; oxytetracycline	Inhibition of protein synthesis.
Rifamycins	Rifampicin	Inhibition of RNA synthesis.
Macrolides	Erythromycin	Inhibition of protein synthesis.
Glycopeptides	Vancomycin	Inhibition of cell wall synthesis.
Quinolones	Norfloxacin, ciprofloxacin	Inhibition of DNA replication.
Polypeptides	Bacitracin	Inhibition of cell wall synthesis.
	Polymyxin B	Injury of plasma membranes
Antifolates	Sulphanilamide	Inhibit folate synthesis
	Trimethoprim	

1.5.2.1 Common modes of action for antibiotics and antimetabolites

Antibiotics and antimetabolites either cause either direct bacterial death (bactericidal action) or prevent further bacterial growth (bacteriostatic action). There are several major ways in which antibiotics can act against pathogens, summarized in fig 1.6.

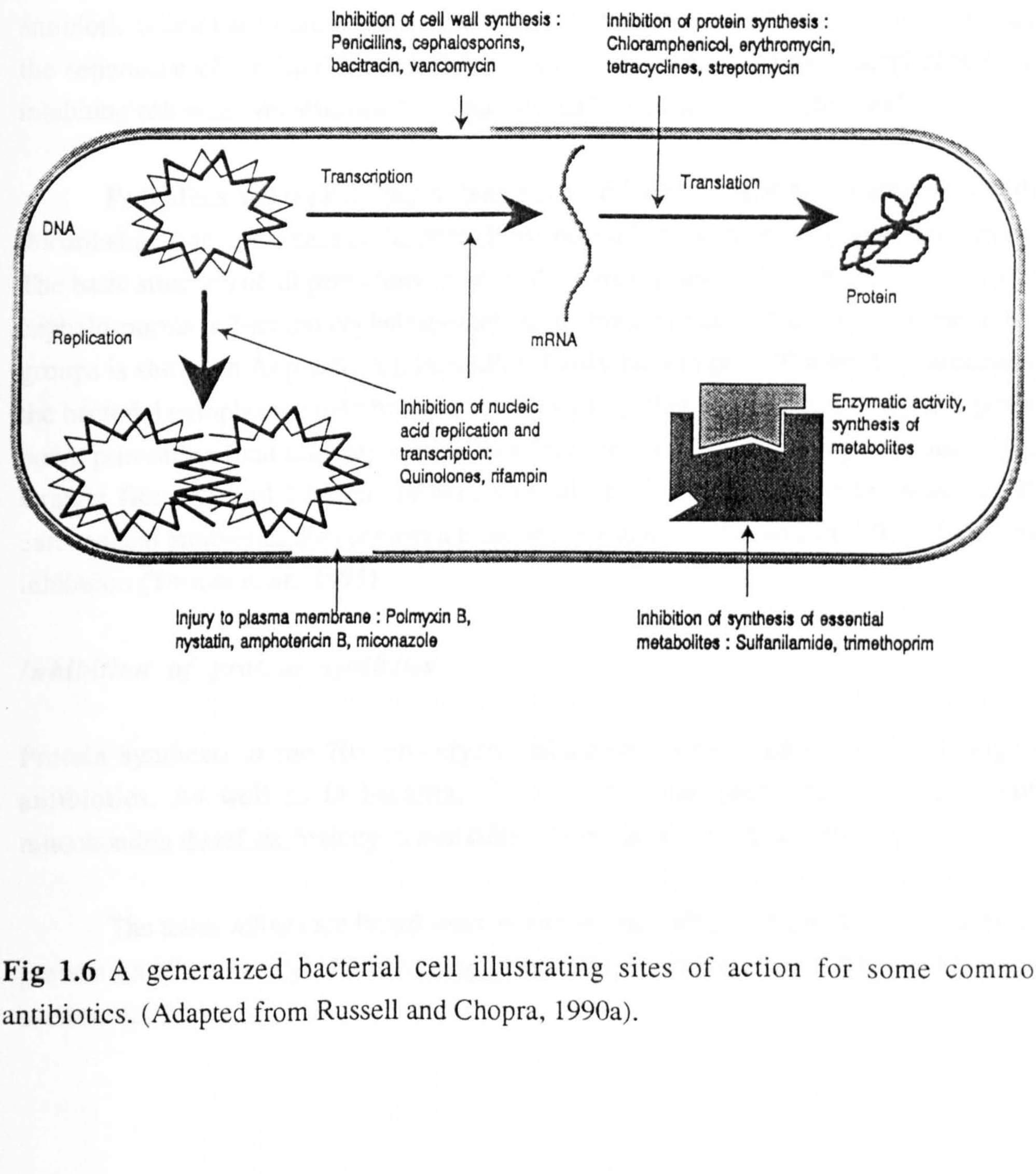


Fig 1.6 A generalized bacterial cell illustrating sites of action for some common antibiotics. (Adapted from Russell and Chopra, 1990a).

Inhibition of cell wall synthesis

Interference during peptidoglycan assembly can occur during the formation of the linear strands or during the final cross-linking between the strands.

Bacitracin, a polypeptide antibiotic containing four D-amino acids (Appendix VI), impairs cell wall synthesis by inhibiting the dephosphorylation of lipid pyrophosphate to lipid phosphate. This step is essential to regenerate the lipid carrier therefore strand formation cannot continue (Bowman and Rand, 1980). Vancomycin is a glycopeptide antibiotic which has a core heptapeptide. This also prevents strand formation, by inhibiting the separation of the lipid intermediates from the membrane bound phospholipid, thus inhibiting cell wall synthesis one stage earlier than bacitracin (Nagarajan, 1991).

Penicillins and cephalosporins both have a ß-lactam ring at their core, fused with a thiozoladine ring in the case of the penicillins and a dihydrothiazine ring in cephalosporins. The basic structure of all penicillins consists of 6-amino penicillinic acid whereas that of the cephalosporins is 7-amino cephalosporanic acid (Jawetz *et al.*, 1982). An example of both groups is shown in Appendix VI. Penicillins firstly bind to penicillin binding proteins on the bacterial cytoplasmic membrane. They act by targeting the D-alanyl-D-alanine peptide bond, preventing final transpeptidation therefore inhibiting cross linking of peptidoglycan strands (Russell and Chopra, 1990a). Cephalosporins act in a similar manner. The carbapenem antibiotics also possess a ß-lactam ring and are effective in cell wall synthesis inhibition (Tortora *et al.*, 1995).

Inhibition of protein synthesis

Protein synthesis at the 70S procaryotic ribosomes is the target site for a range of antibiotics. As well as in bacteria, 70S ribosomes are also present in eucaryotic mitochondria therefore, toxicity to host cells may occur with some antibiotics.

The tetracyclines are broad spectrum antibiotics which inhibit protein synthesis by prevention of amino acyl tRNA binding at the 30S ribosomal subunit (discussed further in section 4.1.2.2).

The aminoglycosides include amongst others, streptomycin, gentamicin (Appendix VI), neomycin, kanamycin, amikacin and tobramycin. They all attach to and inhibit the function of the 30S ribosomal subunit. Streptomycin interferes with the binding of formylmethionyl-tRNA (preventing correct protein synthesis initiation) and also leads to a misreading of mRNA. The streptomycin site of action is the S12 protein in the 30S ribosomal subunit (Gale *et al.*, 1981; Jawetz *et al.*, 1982).

Macrolides have a large lactone ring of 12-16 atoms substituted with sugar residues (Appendix VI) which are very important to their activity. Erythromycin inhibits protein synthesis by attachment to a 23S rRNA receptor on the 50S bacterial ribosomal subunit, therefore preventing translocation of the growing polypeptide chain from the amino acid binding site to the peptidyl binding site. Peptide chain elongation is disrupted (Bowman and Rand, 1980; Jawetz *et al.*, 1982).

Injury to the plasma membrane

Increased permeability of the bacterial plasma membrane results in loss of metabolites from the cell. Polymyxins, polypeptide antibiotics (Appendix VI) act as cationic detergents: they coat the bacterial cell membrane and destroy its active transport and function as a permeability barrier, causing rapid loss of small molecules including nucleotides from bacteria (Tortora *et al.*, 1995; Kimura *et al.*, 1992).

Inhibition of nucleic acid replication and transcription

Interference with bacterial DNA replication and transcription is dependent on the selective toxicity of antibiotics. The level of specificity for procaryotic nucleic acids over eucaryotic can vary dramatically depending on the antibiotic and the target pathogen.

Rifampicin specifically inhibits the initiation of RNA synthesis. Its structure is shown in Appendix VI. Rifampicin does not prevent the initial binding of RNA polymerase to the DNA template but interferes with the formation of the first phosphodiester bond in the RNA chain. Elongation of chains which have already been synthesized are not affected. The rifampicin site of action is the ß subunit of RNA polymerase (Stryer, 1988).

Nalidixic acid is a synthetic antimicrobial which inhibits bacterial DNA replication. It does not bind directly to the DNA but targets a subunit of the enzyme DNA gyrase which is necessary for the replication of circular chromosomes. Nalidixic acid was the first quinolone antimicrobial and lead to the development of the fluoroquinolones (including norfloxacin and ciprofloxacin) which also target DNA gyrase (Gale *et al.*, 1981; Tortora *et al.*, 1995).

Inhibition of essential metabolite synthesis

Antimetabolites included in this category often prevent metabolite synthesis through competitive inhibition, where the structure of the drug is sufficiently similar to that of the metabolite to enable irreversible binding of the drug to the enzyme involved. The drug affinity for the enzyme is typically greater than that of the normal substrate. This therefore effectively halts the metabolic pathway. Trimethoprim and sulfanilamide both act in this manner in the bacterial folic acid synthesis pathway, where they can be used synergistically (Gale *et al.*, 1981; Tortora *et al.*, 1995).

1.5.2.2 Some clinical applications of antibiotics

Penicillins

Natural penicillins have a narrow spectrum of activity although they are one of the most frequently used clinical antibiotic groups. Penicillin G is the drug of choice against many organisms including streptococci, pneumococci, meningococci, clostridia, aerobic rods, non-penicillinase producing staphylococci and gonococci, and some spirochaetes. Semi-synthetic penicillins can be used against a wider range of Gram negative as well as Gram positive bacteria. Ampicillin can be used in treatment of some urinary tract infections. Carbenicillin is similar in efficacy to ampicillin but is more active against *Pseudomonas* and *Proteus*. A disadvantage of natural and semi-synthetic penicillins is their susceptibility to penicillinases (see section 1.5.2.5) (Jawetz *et al.*, 1980; Wright and Wilkowske, 1991).

Cephalosporins

Though similar to penicillins, they are more effective against a broader spectrum of Gram negative organisms and are resistant to the penicillinases (although they are subject to attack from other ß-lactamases). Cephalosporins are effective against Gram positives including penicillinase-producing staphylococci and against several strains of the Gram negatives *Klebsiella*, coliforms and *Proteus*. Cefotaxime and Cefoxitin are active against several anaerobes including *Bacteroides* (Jawetz *et al.*, 1980; Bowman and Rand, 1980). The range of cephalosporins becoming available is increasing (Bryan, 1991a).

Carbapenems

The carbapenems have a very broad spectrum of activity. The first carbapenem antibiotic, Primaxin is made from a combination of imipenem (a ß-lactam antibiotic) and cilastatin sodium (Tortora *et al.*, 1995). A newer carbapenem, meropenem is stable against dehydropeptidase therefore, the co-administration with cilastatin is not necessary. Meropenem is more effective against Gram negative bacilli than imipenem but less so against Gram positive bacteria (Bryan, 1991a).

Aminoglycosides

The different members of the family cover a broad range of antibacterial activity. Clinically they are used against Gram negative enteric bacteria or where there is evidence of severe sepsis. The low cost of aminoglycosides ensures their continued use despite introduction of newer antimicrobials (Edson and Terrell, 1991). In the treatment of bacteraemia or endocarditis, aminoglycosides are sometimes given in conjunction with penicillin which enhances cell permeability allowing increased entry of the aminoglycoside (Jawetz *et al.*, 1980; Bowman and Rand, 1980). The suppression of aminoglycoside activity under conditions of low pH and oxygen tension (as may occur in infected tissue), can be usurped by co-administration with a ß-lactam (Bryant *et al.*, 1992).

Tetracyclines

Tetracyclines are very broad spectrum antibiotics and their clinical uses are discussed in section 4.1.4.

Rifamycins

The only widely used rifamycin is rifampicin. In general rifampicin is prescribed for Gram positive bacterial infections. These include the mycobacteria which cause tuberculosis and leprosy. Rifampicin is particularly effective due to an ability to penetrate tissues and to reach the cerebrospinal fluid (Jawetz, *et al.*, 1980; Nau *et al.*, 1992).

Macrolides

They are fairly narrow spectrum, effective against Gram positive organisms but only a few, albeit important Gram negative organisms (*Legionella*; some *Neisseria* species). Erythromycin is active against Gram positive organisms including pneumococci, streptococci and corynebacteria. Also susceptible are *Mycoplasma, Chlamydia trachomatis, Legionella pneumophila* and *Campylobacter jejuni* (Jawetz, *et al.*, 1982). Newer macrolides include clarithromycin and azithromycin which have an extended antibacterial spectrum, increased absorption and improved intracellular penetration. They are hailed as an improvement for respiratory tract infection chemotherapy (Bryan, 1991b; Ball, 1991).

Polypeptides

The polypeptide antibiotics are not widely used due to their relative toxicity. Their modes of action differ although essentially they affect the bacterial cell envelope. Bacitracin is useful for topical application against Gram positive bacteria. Polymyxin B is effective against Gram negatives and can be injected intravenously for serious Gram negative infections including *Pseudomonas* sepsis. Its most common use today is in topical treatment of superficial infections (Jawetz, *et al.*, 1982).

Quinolones

Nalidixic acid, the first quinolone antimicrobial has limited clinical use due to toxicity but can be used in treatment of urinary tract infections. The recently developed fluoroquinolones, norfloxacin and ciprofloxacin are broad spectrum antimicrobials and are widely used to treat adult urinary tract infections and enteric infection (Tortora *et al.*, 1995).

Glycopeptides

For many years the only glycopeptide in clinical use was vancomycin though recently teicoplanin has been launched in some European countries including the UK. Vancomycin is toxic and cannot be absorbed from the gastrointestinal tract but is used to treat severe Gram positive infections including those of methicillin-resistant *Staphylococcus aureus.* (Nagarajan, 1991).

1.5.2.3 Entry of antibiotics into bacterial cells

As discussed above, the primary target of most antibiotics is the inhibition of some part of the bacterial cell metabolism, rather than to physically break up the cell. However interaction with and transport across the cell surface layers is necessary before a compound can reach the target site.

The different structure of Gram positive and Gram negative cell envelopes is the basis of the differential specificity of some antibiotics. The entry of antibiotics across the outer membrane is achieved through passive diffusion or self-promoted uptake (Russell and Chopra, 1990a). The former is facilitated by the porins and the speed of entry determined by the molecular size, charge and lipophilic properties of the antibiotic i.e. hydrophilic molecules of less than 600 daltons should pass through with ease. Self-promoted uptake involves the destabilization and disorganization of the outer membrane caused by polycationic antibiotics such as aminoglycosides as well as some quinolones (Nikaido, 1989; Chopra, 1990). These displace divalent cations so causing outer membrane disruption, allowing access to antibiotics, though this mode entry for the aminoglycosides is not widespread among Gram negative bacteria (Hancock, 1984). Recent work has suggested that quinolones may not utilize the self-promoted uptake pathway (Marshall and Piddock, 1994).

Antibiotics usually cross the cytoplasmic membrane of bacterial cells by passive diffusion or by active transport mechanisms which derive energy from ATP or the proton motive force (pmf). Active transport usually can only occur when the drug is similar in structure to a naturally occurring molecule and is able to interact with the normal transport carrier (Russell & Chopra, 1990a).

1.5.2.4 Resistance

The phenomenon of resistance to antimicrobial agents is a huge problem and limits the extent to which certain drugs can be prescribed. Random mutation occurs at a low order of frequency in any microbial population. A selective advantage is conferred to a single cell if its mutation results in the development of resistance during exposure to the relevant antimicrobial.

Resistance may be either intrinsic or acquired. A common intrinsic means of resistance is the Gram negative outer membrane which is a permeability barrier to some large or hydrophobic antimicrobial molecules (e.g. macrolides and hydrophobic ß-lactams). These molecules are unable to diffuse through the porins (Zimmermann and Rosselet, 1977; Nikaido, 1989). Acquired resistance is based on either mutations in chromosomal genes or from the possession of extrachromosomal DNA (plasmids or transposons) which encode products conferring resistance (Russell and Chopra, 1990b).

A resistant cell population may spread rapidly throughout a community, diminishing the efficacy of the relevant antimicrobial drug. Careful administration of drugs is essential to keep the development of resistant strains to a minimum. Steps that can be taken include controlling the dosage, aiming at the optimum strength to minimize the survival chances for resistant cells and prescribing two or more drugs in combination with different action mechanisms. A population resistant to one of the drugs is less likely to become established if a second drug can kill the cells using an alternative mode of attack (Russell and Chopra, 1990b).

1.5.2.5 Common modes of resistance

There are several ways in which resistance is achieved by microbial cells, including:

Drug inactivation

This commonly occurs when ß-lactam antibiotics are used. ß-lactamase enzymes (penicillinases and cephalosporinases) synthesized by resistant microorganisms cleave a covalent bond in the ß-lactam ring (Tortora *et al.*, 1995; Gale *et al.*, 1981).

Chloramphenicol can be inactivated when resistant cells produce chloramphenicol transacetylase, which donates an acetyl group from acetyl-CoA to the antibiotic molecule (Gale *et al.*, 1981).

Prevention of access to the target site

This can include permeability differences and active efflux mechanisms, both of which prevent the build up of an adequate inhibitor concentration within the cell. As described in section 4.1.3, tetracycline efflux is a well recognized resistance mechanism (Chopra, 1985; Chopra, 1992; Chopra *et al.*, 1992).

The Gram negative outer membrane confers intrinsic resistance against antibiotics which are unable to penetrate it effectively (Hancock, 1984).

Alteration of the target site

Small changes in a specific target site may modify it so that the drug is no longer able to act there. In the bacterial folic acid synthetic pathway, sulphonamides usually have a much higher affinity for their target enzyme, tetrahydropteric synthetase, than the normal substrate, *p*-amino benzoic acid (PABA). However, a single enzyme mutation may decrease the sulphonamide affinity and increase that of PABA therefore conferring resistance. Macrolide resistance may occur due to the methylation of the rRNA receptor, therefore translocation can proceed. Also, resistance to rifampicin can occur when the ß chain of the RNA polymerase enzyme is altered (Gale *et al.*, 1981).

Bypassing the target metabolic step

Gram negative bacteria, particularly *Pseudomonas* spp. are resistant to ß-lactam antibiotics, as they possess very little peptidoglycan. Gram positive cells rely primarily upon peptidoglycan for cell structural integrity but Gram negative cells have other macromolecules to contribute to this, therefore damage to peptidoglycan does not inevitably cause cell lysis (Gale *et al.*, 1981; Hancock, 1984). Some microbes e.g. mycoplasmas do not synthesize peptidoglycan therefore, are resistant to ß-lactam antibiotics (Jawetz *et al.*, 1982).

Overproduction of an inhibited enzyme

In the case of trimethoprim resistance, a modified, resistant version of the target enzyme is synthesized. Trimethoprim attacks the dihydrofolate reductase enzyme in the folic acid synthetic pathway. Sensitive cells produce this enzyme which is targeted, but resistant cells produce this enzyme plus a modified version with a lower affinity for trimethoprim, which therefore allows sufficient folic acid production. (Gale *et al.*, 1981).

Most of the above mechanisms are caused through the inclusion of an extrachromosomal plasmid, containing genes coding for cellular changes which allow a resistance mechanism to develop. Plasmids known as R factors can be transferred between cells in a population (by conjugation) and may often contain genes coding for resistance against several antibiotics simultaneously.

1.5.3 Biocides

The term biocide covers a very diverse range of compounds used for their disinfectant, antiseptic or preservative properties. It is often difficult to ascribe a primary target site to biocides. They may be active at several sites within a cell. Alternatively a primary target may trigger additional effects and the inhibitory action may depend upon the culmination of events (Russell and Chopra, 1990c).

Biocides can be classified by their application as either disinfectants, antiseptics or preservatives (although some overlap exists). Table 1.3 lists some common biocide genera and some of their uses.

Table 1.3 Some common biocide genera and some of their uses (information taken from Hugo and Russell, 1992).

Type of biocide		Uses	Examples
Phenols		preservative	phenol
QACs		antiseptic, disinfectant	cetrimide
Biguanides		antiseptic	chlorhexidine
Aldehydes		disinfectant	glutaraldehyde
Acridines		wound treatment	aminacrine
Quinones		agricultural fungicides	chlorancil
Iodine compounds:	iodine	wound treatment (old)	
	iodophors	disinfectant, antiseptic	
Chlorine compounds			
	hypochlorites	disinfectant (e.g. water)	sodium hypochlorite
	chloroform	pharmaceutical preservative	
Alcohols:	ethanol	disinfectant	
	isopropanol	preservative (cosmetics)	
Hydrogen peroxide		disinfectant; antiseptic	
EDTA		chelator which potentiates disinfection	
Permeabilisers		potentiates disinfection	poly-L-lysine
Heavy metal compounds			
	copper salts	disinfectant (fungicides); preservative	
	silver salts	antiseptic (burn treatment)	
Radiation		disinfectant (e.g. water; surfaces)	ultra-violet

1.5.3.1 Adsorption of biocides to cells

In order for a chemical biocide to target a cell it must first interact with the surface. Biocide adsorption can be assessed by adding a known concentration of cells to a biocide solution (also of known concentration), for a predetermined time interval. By removing the cells using centrifugation, the amount of biocide remaining in the supernatant can be calculated - the deficit has been removed by the cells. Absorption isotherms can be plotted for different cell/biocide combinations. There are five common forms of absorption isotherms (fig 1.7), termed S, L, H, C and Z (Hugo, 1992).

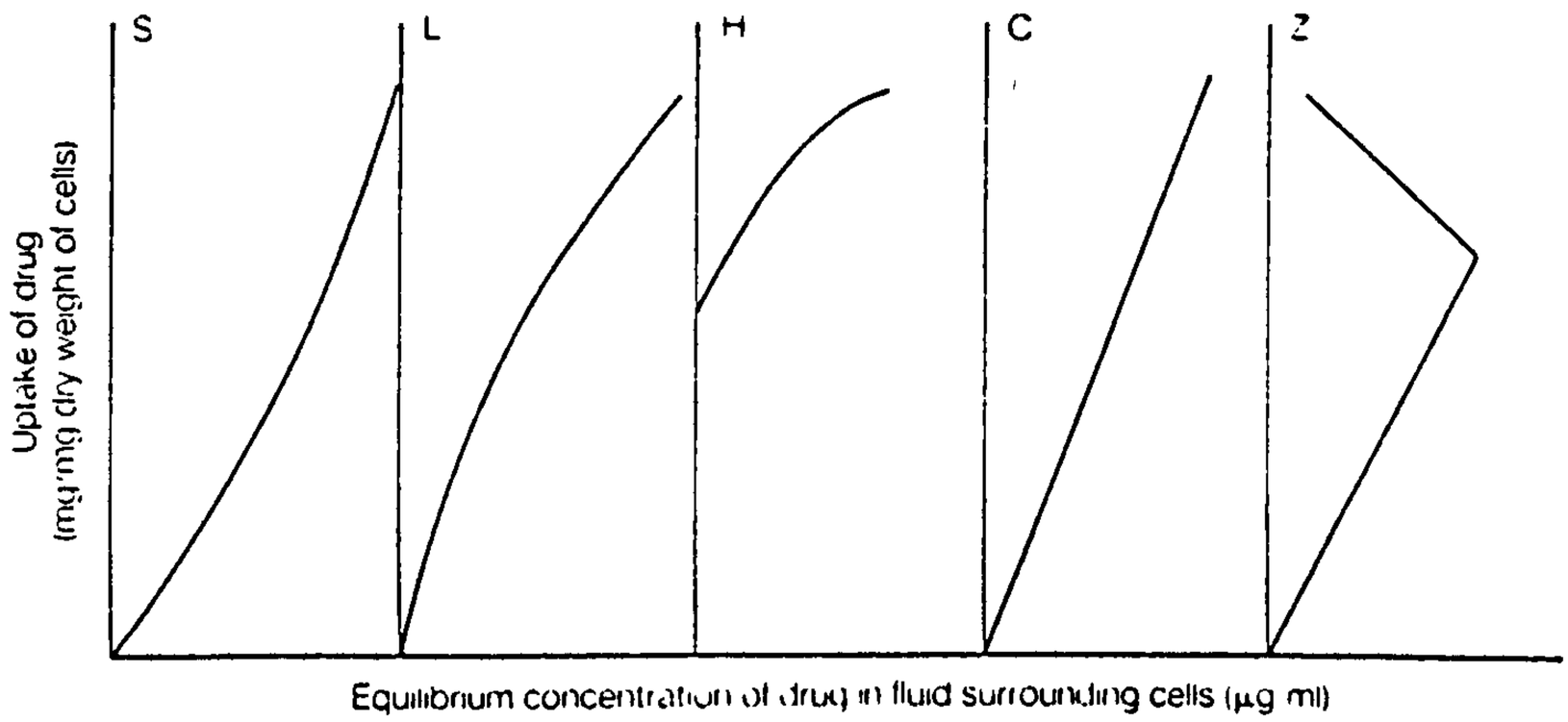


Fig 1.7 Patterns of absorption isotherms. (Taken from Hugo, 1992)

- The S pattern. This occurs with singular solute (biocide) molecules which have strong competition for adsorption sites on the cell from the solvent molecules.
- The L pattern. As more cell adsorption sites are filled, it becomes increasingly difficult for remaining solute molecule to find a vacant site.
- The H (high affinity) pattern. This occurs when the solute is almost completely absorbed
- The C (constant partition) pattern. This results when the solute molecules are able to adsorb to the cell more easily than the solvent molecules.
- The Z pattern. The isotherm shows a peak after which uptake of the solute increases. This occurs when solute adsorption causes a breakdown of cell surface absorption sites, unveiling new absorption sites.

1.5.3.2 Factors influencing biocide activity

Some of the most important factors influencing biocide activity include temperature, concentration, exposure, pH and presence of organic matter (Russell and Chopra, 1990c).

- Temperature. Antimicrobial activity of biocides generally increases with increasing temperature. Some phenols can become sporicidal at elevated temperatures whereas they are ineffective at room temperature.
- Concentration. When biocide concentration rises, the rate of microbial inactivation generally rises. This effect varies between biocides.
- Exposure time. All biocides need time to act as death is never instantaneous. Generally higher concentrations produce higher inactivation rates.
- pH. Variations in pH can significantly alter the efficacy of some biocides. This is due to effects upon the biocide molecules (e.g. at acid pH levels, hypochlorous acid predominates in hypochlorites which is beneficial for disinfection efficiency), and to effects upon the microbial surface (e.g. bacterial surface charge becomes more negative at high pH therefore cationic biocides including chlorhexidine and QACs are more effective).
- Organic matter. Some biocides can lose chemical activity by binding to organic matter e.g. chlorine-releasing compounds.

1.5.3.3 Uptake of biocides into bacterial cells

Unlike antibiotics, there are no specific permeases or receptor molecules to aid bacterial biocide uptake. Entry into Gram positive cells is usually achieved easily, via passive diffusion or partitioning. Gram negative cells are more resistant and modes of biocide uptake are not well understood. However, quaternary ammonium compounds (QACs) and chlorhexidine are thought to operate self-promoted uptake by first displacing the divalent cations in the Gram negative outer membrane and consequently rendering the cells more permeable for their entry.

1.5.4.4 Modes of action

Biocides may be broadly split into 3 groups based upon the way in which they work: chemicals which interact with outer cellular components; chemicals which act at the cytoplasmic membrane; chemicals which interact with the constituents of the cytoplasm (Russell and Chopra, 1990c; Hugo, 1992). These are summarized in fig 1.8.

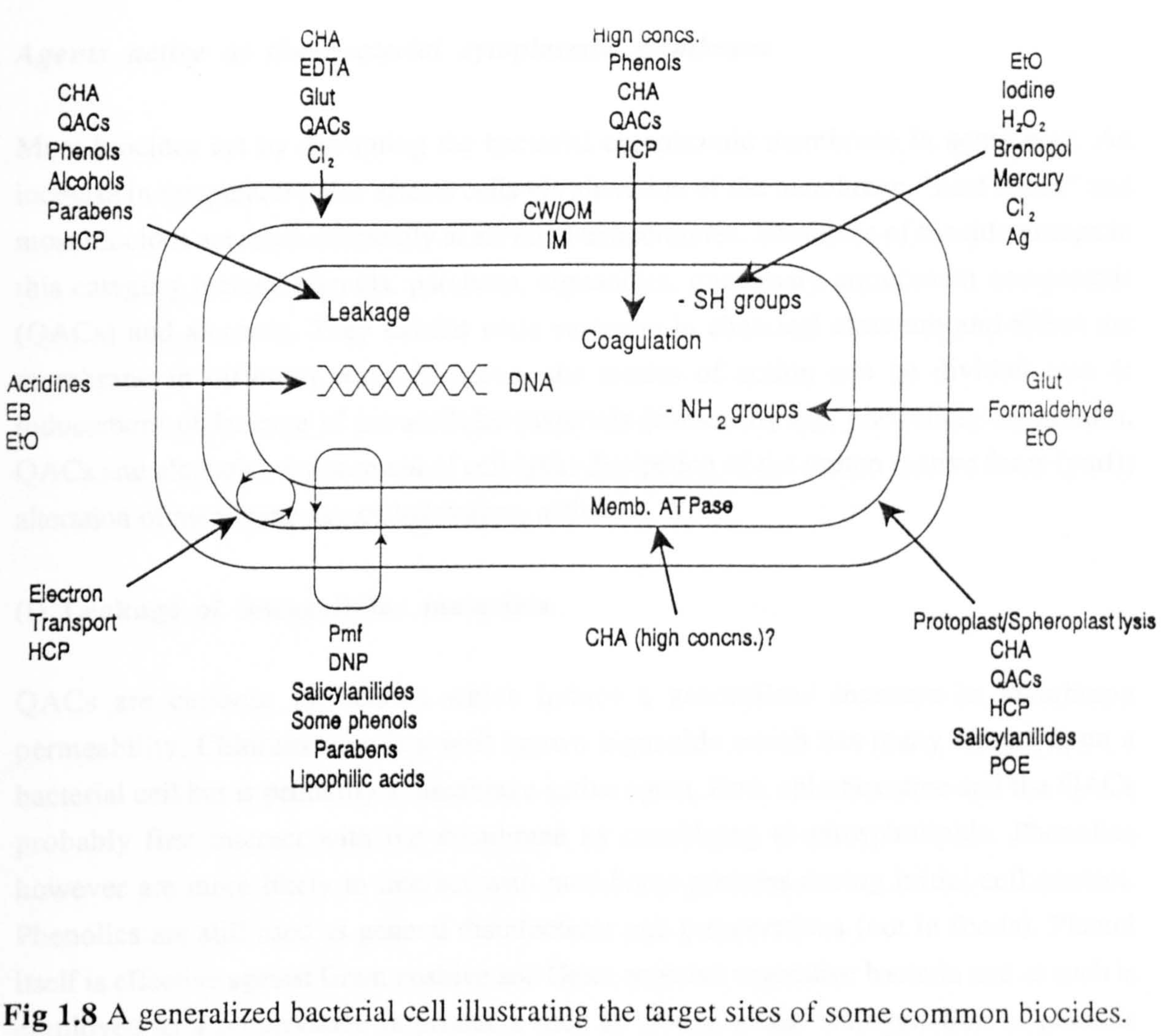


Fig 1.8 A generalized bacterial cell illustrating the target sites of some common biocides. (Adapted from Russell and Chopra, 1990c)

Agents active against outer cellular components

Glutaraldehyde interacts with amino groups and therefore binds to many outer cell constituents. Other agents act by increasing the permeability of the outer cell membrane in Gram negative bacteria e.g. ethylenediamine tetraacetic acid (EDTA), polycations, lactoferrin and transferrin. Lysozyme is also included in this group as it can digest peptidoglycan by acting upon the ß (1-4) linkages.

Agents active at the bacterial cytoplasmic membrane

Most biocides act by disrupting the bacterial cytoplasmic membrane in some way. An increase in temperature also affects cells via alteration of the membrane, "heat shock" and most biocides act more efficiently at elevated temperatures. Examples of biocide groups in this category include phenols, parabens, biguanides, quaternary ammonium compounds (QACs) and alcohols. They exhibit wide variation in chemical structure and affect the membrane in different ways. However the modes of action can be divided into 4: inducement of leakage of intracellular materials (caused by e.g. phenolics, biguanides, QACs and alcohols); inducement of cell lysis; dissipation of the proton motive force (pmf); alteration of membrane-associated enzyme activity.

(i) Leakage of intracellular materials

QACs are cationic surfactants which induce a generalized increase in membrane permeability. Chlorhexidine is a well known biguanide which has many effects upon a bacterial cell but is primarily a membrane active agent. Both chlorhexidine and the QACs probably first interact with the membrane by combining to phospholipids. Phenolics however are more likely to interact with membrane proteins during initial cell contact. Phenolics are still used as general disinfectants and preservatives (not in foods). Phenol itself is effective against Gram positive and Gram negative vegetative bacteria and as such is employed as a preservative in products such as animal glues. Halogenation of phenols generally increases antimicrobial activity and successful examples of disinfectants produced by this process include 2,4,6-trichlorophenol (preservative and antiseptic applications) and pentachlorophenol (used as a preservative for adhesives, textiles and wood).

Alcohols also induce membrane leakage to varying extents although ethanol and isopropanol are specifically membrane disrupters.

(ii) Cell lysis

Total bacterial cell lysis through biocide action is rare and usually involves protoplasts (cells without a cell wall) or spheroplasts (which have a modified cell wall).

(iii) Dissipation of the pmf

The proton motive force (pmf) is generally responsible for powering active transport, oxidative phosphorylation and ATP synthesis within chloroplasts, mitochondria and bacterial cells. The pmf is produced via ATP hydrolysis or by metabolic oxido-reductions. It forms a gradient across the bacterial cytoplasmic membrane making the cell interior relatively negatively charged. Compounds responsible for the dispersion of the pmf include parabens which are esters of the organic acid para(4)-hydroxybenzoic acid. The parabens are widely used as preservatives in the pharmaceutical, cosmetic and food industry. Other food preservatives include benzoic and proprionic acids and vinegar (acetic acid). Their preserving ability is due to their action as uncoupling agents.

(iv) Alteration of membrane-associated enzyme activity

Although many biocides have an effect upon enzymes associated with the membrane there are few which target these as their primary site of action. More usually, inactivation of membrane-associated enzymes is one of a number of biocide effects. However, hexachlorophane, a phenolic compound, is an example of a biocide which produces a specific effect upon the electron transport chain. Many other biocides react with thiol groups (-SH) within enzymes.

Agents which interact with cytoplasmic constituents

Biocides which interact with components of the cytoplasm must first cross the bacterial cell envelope. In many cases this process itself is damaging to the cell. At high concentrations chlorhexidine can cause coagulation of cytoplasmic proteins and nucleic acids which is responsible in part, for its inhibitory action. Some biocides act by alkylating intracellular proteins, e.g. ethylene oxide. Others cause cross-linking of intracellular proteins and/or nucleic acids, e.g. formaldehyde. Intercalating agents, e.g. acridines, also act primarily within the cell interior, their most important binding site is DNA.

Oxidising agents are a powerful antimicrobial group which include the cell interior amongst their sites of action. Important examples include hypochlorites, peroxygens (hydrogen peroxide and peracetic acid) and ozone.

1.6 Research objectives

The fundamental aims of the research in this thesis were to develop further a dielectrophoretic system incorporating image analysis and to use the equipment to assess dielectrophoretic responses of microorganism treatment with antimicrobial agents.

Questions proposed for investigation included:

- Was dielectrophoresis suitable for use in the analysis of disinfectant efficacy (using ozone and chlorine) against *Cryptosporidium parvum* oocysts ?

- Could internal bacterial cell damage caused by ultra-violet irradiation be detected using dielectrophoresis, in "real time" or after an extended time period ?

- Was there potential for the use of dielectrophoresis in bacterial antibiotic sensitivity analysis ? This investigation involved analysis of the dielectrophoretic response upon addition of tetracycline to *E. coli*. The dose-dependency of the response and variation when using tetracycline resistant bacteria were of interest.

Chapter Two

OPTIMISATION OF A DIELECTROPHORETIC SYSTEM INCORPORATING IMAGE ANALYSIS

2.1 INTRODUCTION

The equipment used for all dielectrophoresis experiments described in this thesis was developed at the University of York over the period 1990-1995. The current system bears similarities with original ideas reported in the 1960's and over recent years the measurement system has evolved to utilize image analysis.

2.1.1 Early measurement of biological dielectrophoresis

The first dielectrophoretic experiments using living cells were performed by Pohl and Hawke in 1966 using yeast (*Saccharomyces cerevisiae*). Their simple equipment consisted of a set of electrodes, a microscope and a power supply, with manual measurement of dielectrophoretic collection. The electrodes were bare wires with rounded tips, laid upon a microscope slide in a pin and plate configuration. A chamber was created by placing a hollowed-out cover slip over the top and the whole arrangement viewed using a phase contrast microscope fitted with a graticule. When a field was applied to yeast cells suspended in deionized water, they formed pearl chains parallel to the field lines. Measurement was made by counting the average length of the chains collected over a specified time period and was termed "dielectrophoretic collection rate". To produce a frequency spectrum could take hours, over which time it was likely that cell physiological status was altered, increasing suspension conductivity.

2.1.2 Development of the dielectrophoresis equipment prior to the incorporation of image analysis

Flow-through electrode array

The electrodes used in experiments described here were microelectrode bars laid down onto a glass microscope slide using photolithographic techniques. The method used is described in Appendix I. The non-uniformity of the field arises from the convergence of field lines at the edges of the bars (fig 1.1). A chamber was constructed over the electrodes so that a particle suspension could flow over them continuously. The flow-through system allowed repeated measurements to be taken from a cell sample and spectra could be produced more rapidly (Betts and Hawkes, 1994).

Measurement of dielectrophoretic collection using absorbance

The first measurement system using the flow through electrode array involved the use of a spectrophotometer (Hawkes *et al.* 1993). The cuvette chamber was removed and electrodes were positioned so that the light beam passed though the gap downstream of the electrode bars. A cell suspension was re-circulated through the electrode chamber using a peristaltic pump. Both this and a signal generator were controlled by an IBM 286 computer, using software written "in-house". The measured absorbance of the cell suspension decreased when an electric pulse was applied and increased when it was removed as the package of cells passed through the spectrophotometer light beam. Spectra of yeast cells and a range of different bacteria were produced using this system. One problem concerned the difficulty in aligning the electrodes in the light beam. Also it was only possible to use very high cell concentrations as turbidity only became measurable at concentrations above 10^7 cells ml^{-1}.

Measurement of dielectrophoretic collection using a video capture method

The spectrophotometer was replaced with a video capture method to facilitate measurement of dielectrophoresis of lower concentration particle samples. The method was used to perform the first ever dielectrophoretic experiments using *Cryptosporidium parvum* oocysts (Archer *et al.*, 1993).

The flow-through electrodes were positioned on a microscope stage. A charge coupled device (CCD) video camera was attached to the top of the microscope and the image of the electrode bars was viewed on an adjacent monitor. A suspension of *C. parvum* oocysts was re-circulated through the electrode chamber and a series of electric pulses of varying frequency were applied. The pump and signal generator were computer controlled as before. The whole experiment was video-taped and on completion, the tape was analysed and the number of collected oocysts at each frequency were manually counted using the digital freeze-frame facility, to produce a frequency dependent spectrum. The most obvious limitation of this measurement approach was lack of speed in the analysis of results. Although significantly quicker than early dielectrophoretic measurements (section 2.1.1) the delay in the procedure caused by the manual counting method needed improvement.

Advantages of image analysis counting for dielectrophoretic measurement

The main advantage from incorporation of an image analysis package to replace manual counting was that measurement became possible in "real-time". This increased the speed with which experiments could be performed. As with the video capture method, dielectrophoretic collection could be observed directly. Image analysis allowed rapid production of results immediately at the end of an experimental sequence, in a time period over which physiological change was minimal. The programme was able to take several particle counts per second therefore maximising accuracy.

2.2 MATERIALS AND METHODS

2.2.1 The dielectrophoretic experimental system

2.2.1.1 The equipment

Aluminium electrodes (fig 2.1) were constructed using a photolithographic technique (Appendix I). The electrodes (50 μm wide separated by a 50 μm or 5 μm space) were deposited onto a glass microscope slide and a chamber was constructed over them to allow suspensions of particles to pass through (Betts and Hawkes, 1994; Hawkes *et al.*, 1993). The walls of the chamber were produced from a layer of photoresist, built up using double-sided sticky tape. A perspex or glass lid with two holes for the inward and outward flow of the sample was then placed on top, and its edges glued on top of the inlet and outlet holes to secure the tubes. Connector wires were attached to tabs on either side of electrodes in order to link them to a signal generator (Hewlett Packard 8116A, Germany).

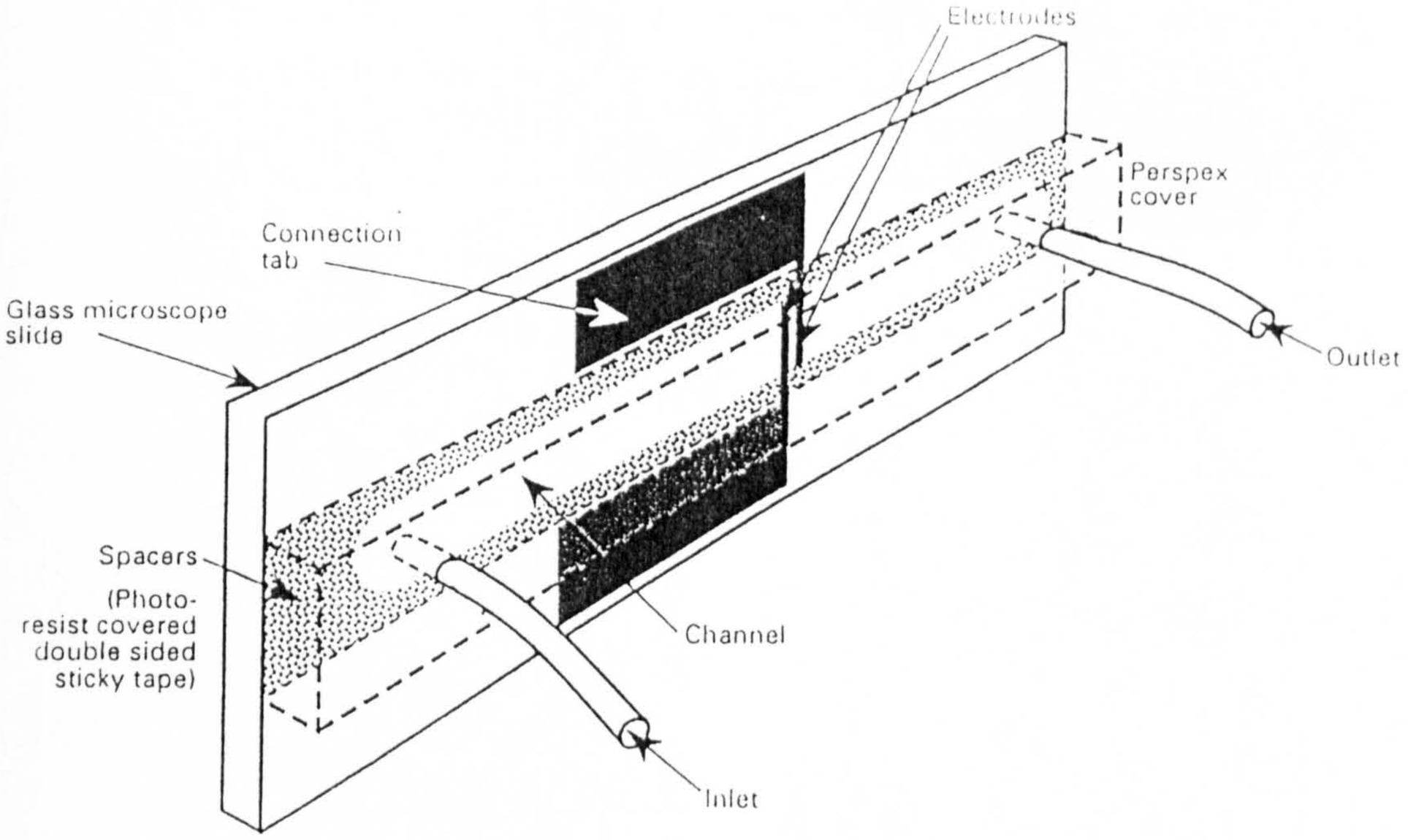


Fig 2.1 A flow through electrode array.

The experimental equipment was arranged as shown in figs 2.2 and 2.3. The particle suspension was re-circulated through the electrode chamber, through an on-line conductivity probe (RE 387 Tx, EDT Instruments, Dover, U.K.) and back to the reservoir by a peristaltic pump (Gilson Minipuls 3, France). The electrodes were mounted on a microscope (Nikon Labophot 2, Japan) to which a high resolution charge coupled device (CCD) camera (Ikegami ICD-42E, Japan) was attached. The electrode bars were observed in bright field using a 10 x objective or in phase contrast using a 40 x objective, depending on the size of the particles under investigation. A signal generator was connected through leads attached to tabs on the electrodes. The whole system was managed by a computer (AST 486 Bravo) which housed software controlling all parameters including frequency, pulse voltage and period (signal generator control via a Hewlett Packard Interface Bus HP 82335A), and timing and speed of the pump. A second monitor was used to set and control the experimental parameters and to monitor the experiment in progress. Each experiment was "traced" on the second monitor as it proceeded. The trace was colour-coded so that each part of the sequence was evident. A typical trace is shown in fig 2.4.

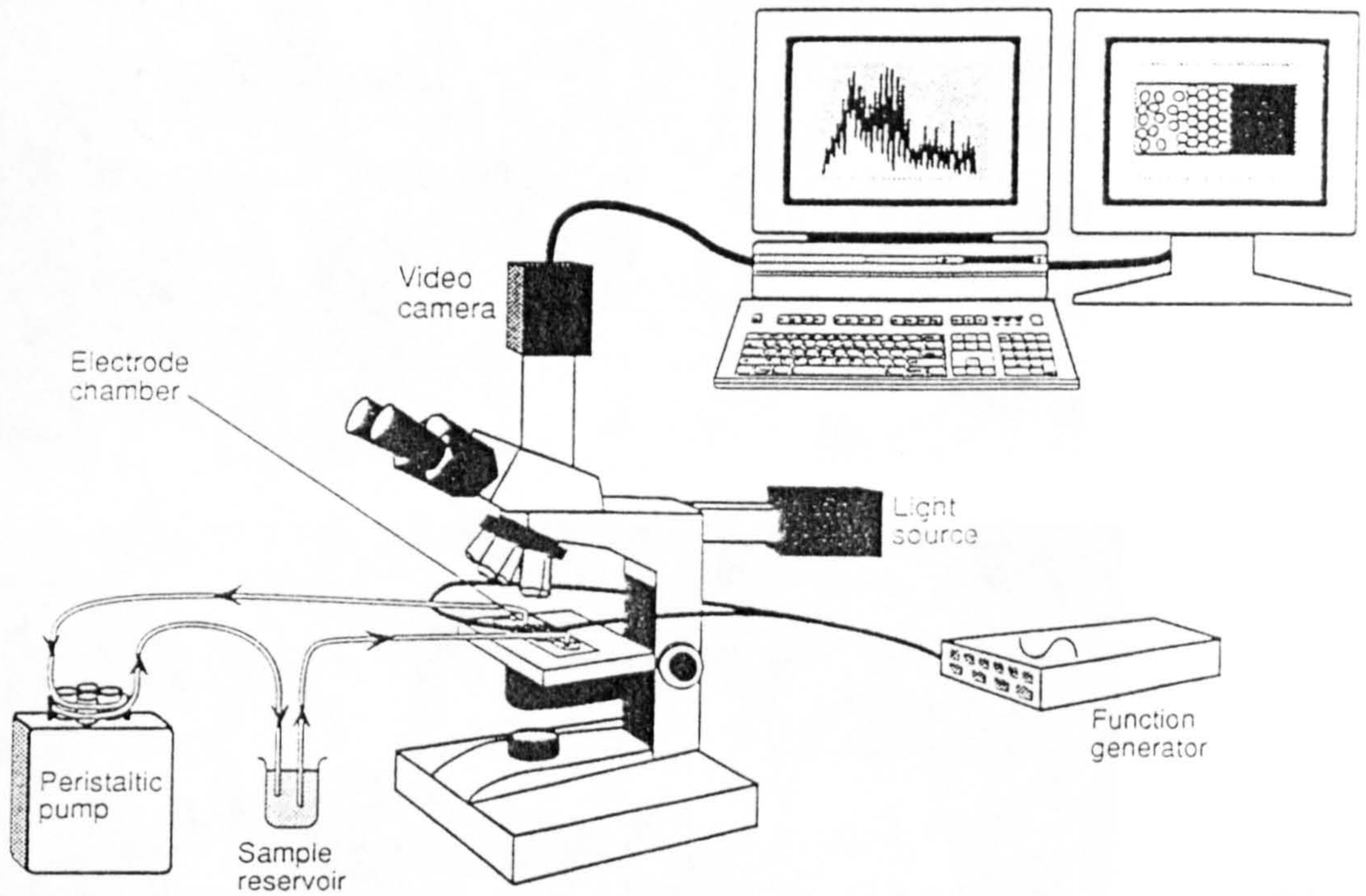
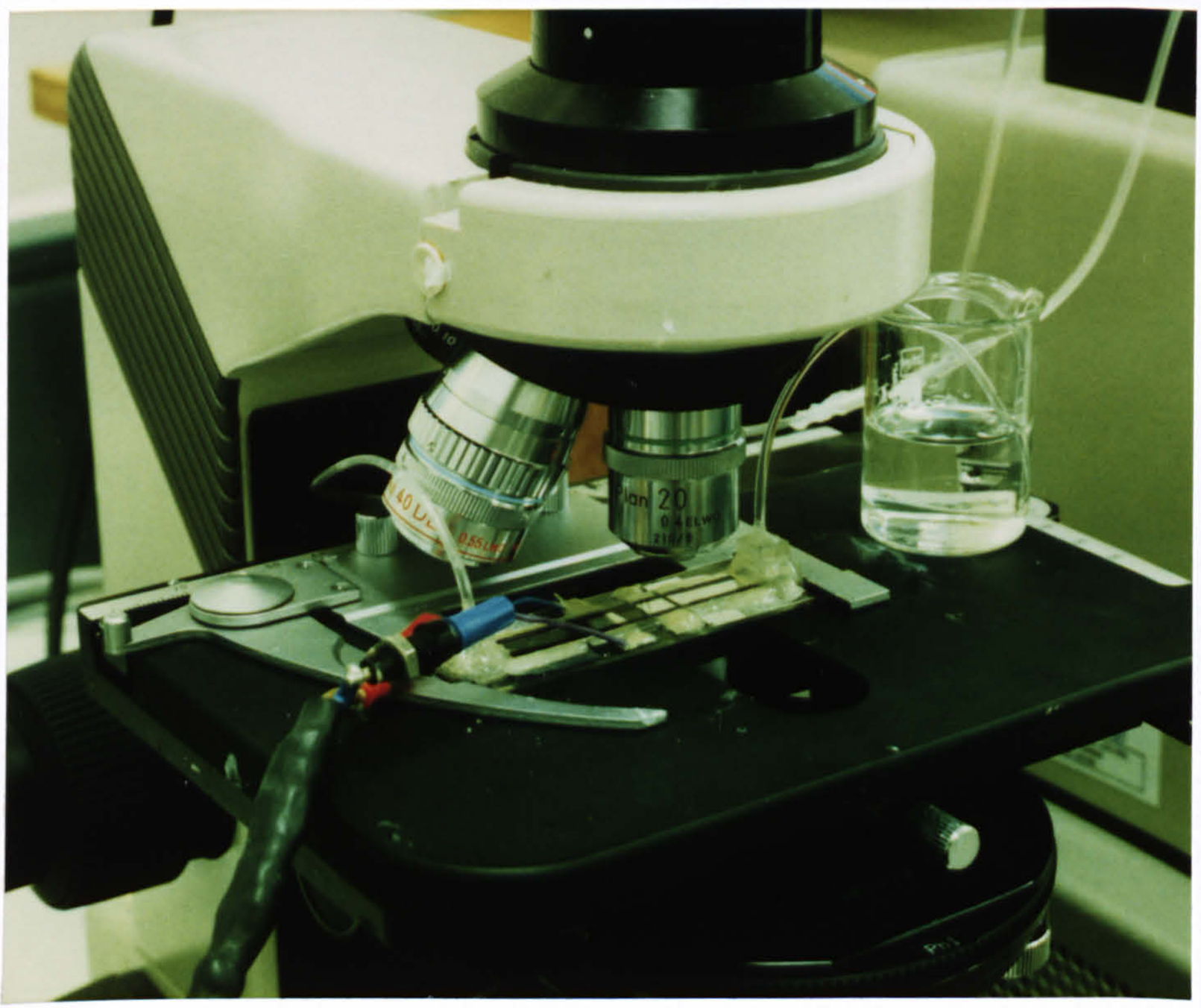


Fig 2.2 The dielectrophoresis equipment incorporating image analysis

(a)



(b)

Fig 2.3 The dielectrophoretic equipment (a) including the microelectrode chamber (b).

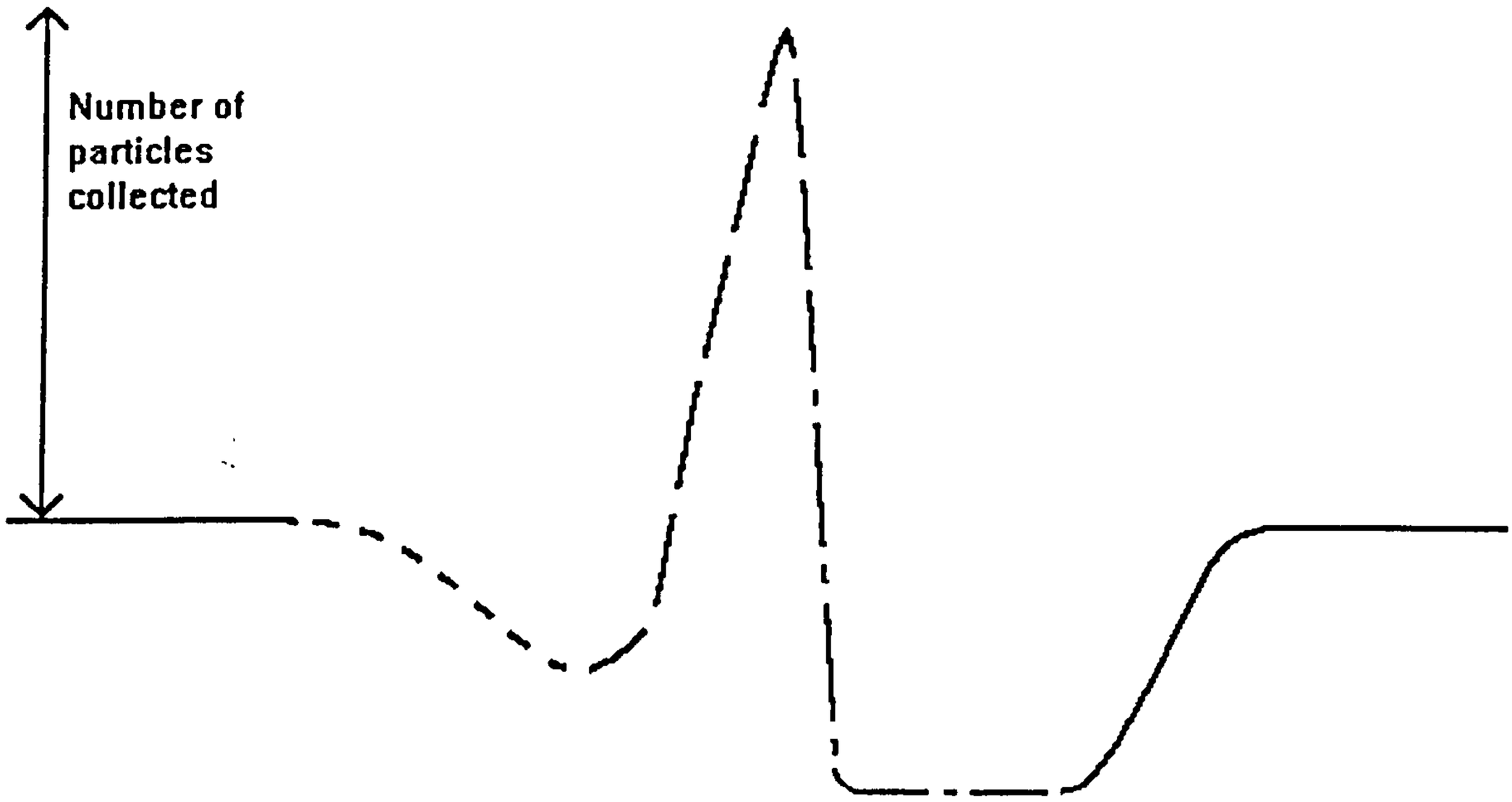


Fig 2.4 A typical "trace" illustrating base line (———), pulse applied (– – –), pulse removed (— —) and pump flushing through (—– –—). The baseline count was subtracted from the total peak height to calculate the number of particles collected.

By observing the trace it was possible to determine whether the parameters had been set appropriately, e.g. if the peak time was too short and the maximum particle count was not being recorded this would be apparent. The number of particles collected was automatically calculated by the subtraction of the baseline count from the total peak count. The software used in this part of the system was developed "in house" and in addition contained subroutines allowing variation of voltage, pulse length, and pump speed, wherein the frequency was kept constant.

2.2.1.2 The image analysis package

The image analysis package selected was "Domino" (Perceptive Instruments, U.K.) originally designed as an automatic colony counting system (Pover, 1990). This was specifically modified for use with the dielectrophoretic system. The hardware, an image analysis board, was installed in the computer in order to provide accessibility to software suitable for image analysis and storage.

Video signals from the CCD camera were fed to the image analysis board which contained circuitry to condition the signal before digitising it into a binary image memory. This was then accessed by special digital processing logic, allowing measurements to be obtained in real-time from each successive frame of the picture. The video signal was displayed on the monitor used to view the electrodes, with colour coded overlays showing the objects detected by the system and allowing the operator to define regions in which they were to be measured, i.e. the frame size. A dedicated control and data processing software programme communicated with the board via the computer expansion bus and converted measurements into calibrated data which could then be displayed, printed or transferred to proprietary spreadsheet or statistical analysis packages. The results of dielectrophoresis experiments were automatically transferred to the FigP graphics package (Biosoft, Cambridge, U.K.).

The programme was operated using a menu screen, called up on the control monitor, which invited the user to select the various parameters desired. The size of the frame on the monitor within which cells were counted could be varied, as could the level of detection (contrast) required. Particles were detected by virtue of contrast differences with the suspending medium. The "Domino" software operated in conjunction with the "in house" software designed to control the timing of an experimental sequence.

The "settings" option on the Domino menu bar allowed some counting conditions to be set, which generally remained unchanged for all dielectrophoretic experiments. Because Domino was primarily a colony counting system, some of the parameters in the "settings" option were obsolete in dielectrophoresis measurement. The parameters used in the "settings" option for dielectrophoresis experiments are listed in table 2.1.

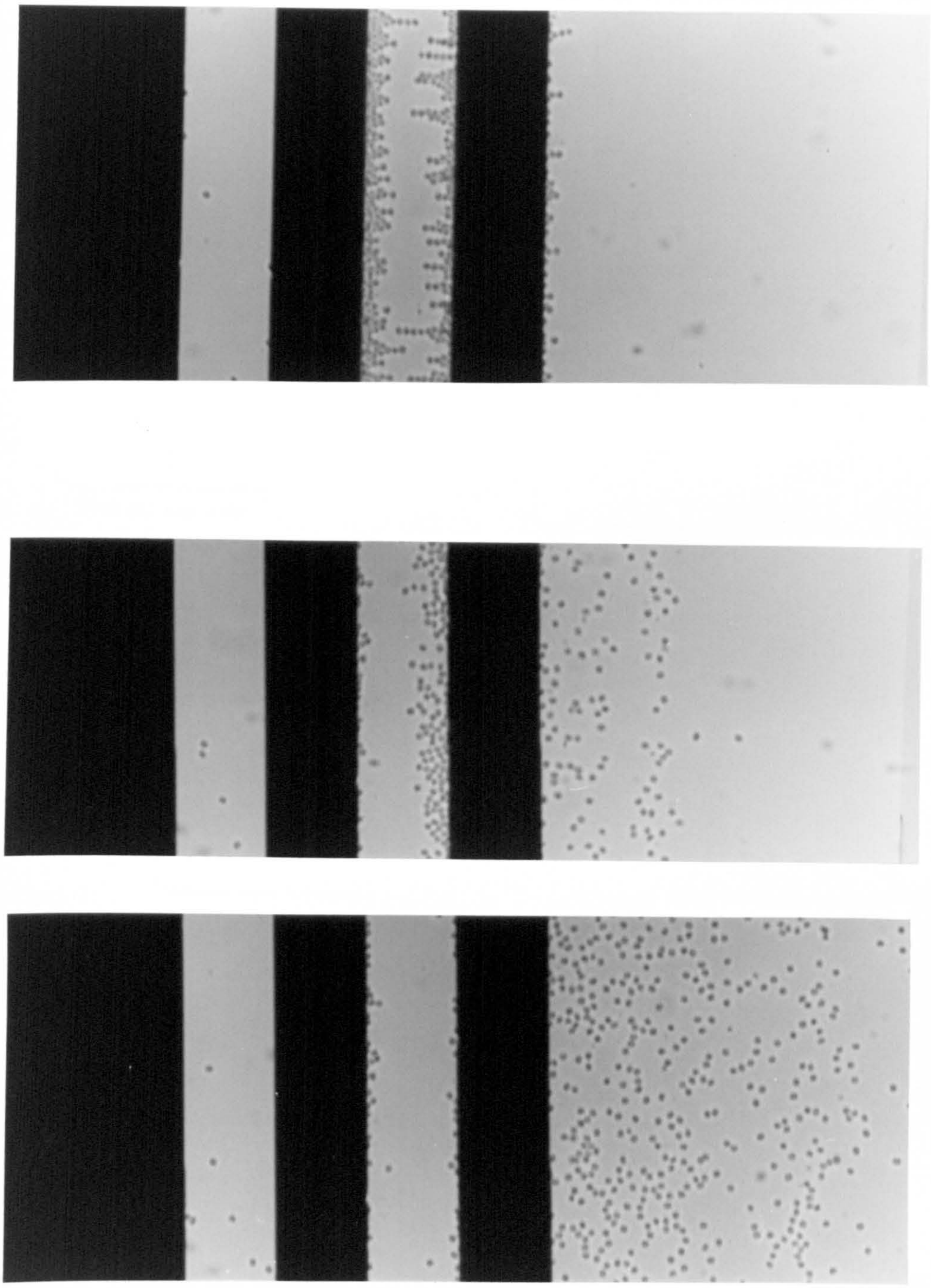
Table 2.1 The parameters used in the Domino image analysis package "settings" option.

Setting	Parameter chosen	Comments
Video mode	Normal	Can be normal or inverted.
Shade compensation	On	Corrects for shading in the background of the counting frame.
Type of frame	Square	Can be circular.
Sizing option	Off	For use in colony counting.
Sizing limit	2	Value irrelevant as only operates when sizing option "on".
Fill holes	Off	For use in measuring zones of inhibition in agar diffusion assays.
Full count	Off	Makes provision for objects which are touching to be counted as individual particles.
Auto detect	Off	An alternative to the manual setting of detection.
Multiple initiate	On	An additional feature added for the purpose of dielectrophoretic measurement. Takes several counts per second, each count separated by a pre-determined time interval.

Twelve counts per second were taken by the image analysis system. For each applied frequency, an average of the highest three measurements was taken and this information then transferred to the graphics package to create a spectrum.

2.2.1.3 Dielectrophoretic collection measurements

The parameters used were chosen so as to yield optimum particle collection. The frequencies of the applied pulse ranged from 1 kHz to 10 or 50 MHz, with measurements taken ten times per decade of log frequency. Other typically used parameters were: 10 s applied electric pulse; 10-14 v; 6-8 s collection counting period; 5 s flush through period; 5 s baseline counting period; 2 rpm pump speed (increasing to 20 rpm during the application of the electric pulse). A count of particles collected at each frequency was taken upon their subsequent release from the electrodes at the end of each applied pulse. It was found that counting the package of particles released rather than counting particles as they collected yielded a more accurate measurement by the image analysis (see sections 2.3.1 and 2.4.1). Upon removal of the electric field the particles were released from the electrodes and passed by the counting frame of the image analysis system as individual objects. A sequence of photographs illustrating collection, pearl chain formation and release are shown in fig 2.5.



(a)

(b)

(c)

Fig 2.5 Photographs illustrating (a) *C. parvum* oocyst pearl chain formation during collection at microelectrodes, (b) release of oocysts as pulse is removed and (c) dispersion of oocysts as they pass through the image analysis counting frame to be detected as individual objects

A typical experimental sequence is presented in fig 2.6. Each replicate experiment took approximately 13 min to complete.

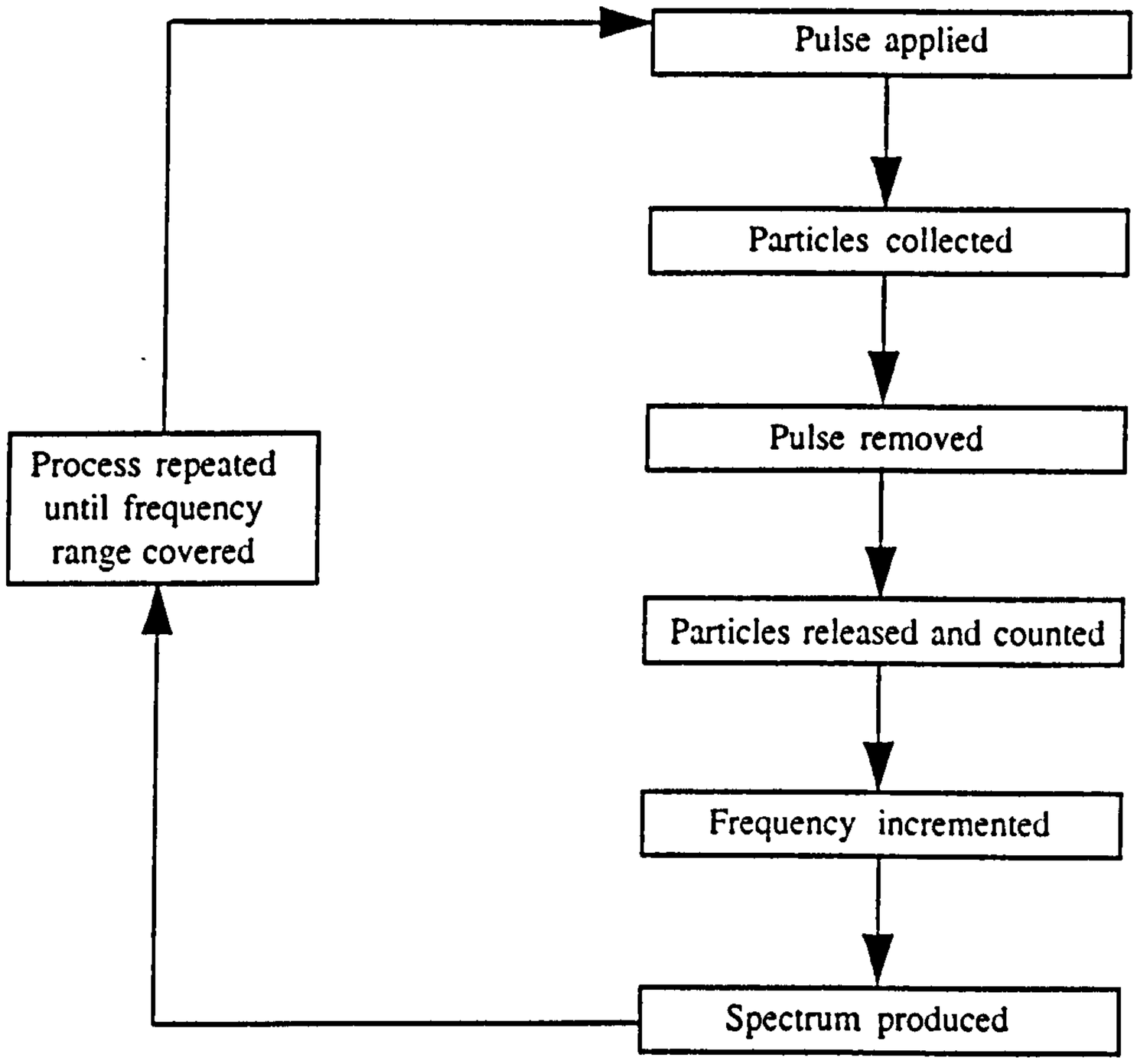


Fig 2.6 A typical experimental sequence to produce a frequency-dependent dielectrophoretic spectrum.

2.2.2 Optimisation of experimental parameters

In order to achieve the most accurate measurement of dielectrophoretic response over a frequency range, it was necessary to optimize the experimental parameters. Some parameters respond independently (e.g. pulse length, pump speed) and most behave in a similar manner for whatever particle type is involved. A range of different particle types have been used to demonstrate which parameter settings are optimal.

2.2.2.1 Comparison of measuring dielectrophoresis during collection and during release

When the image analysis system was first incorporated into the dielectrophoretic system it was necessary to establish whether particles should be counted as they were collecting or upon their release.

Particle type

Reconstituted dried yeast, *Saccharomyces cerevisiae* (Allinson Dried Active Baking Yeast, Maidenhead, Berks) was used to demonstrate this effect. Approximately 1 g of dried yeast was placed into a 10 ml syringe. A sterile 0.2 μm pore-size syringe filter (Minisart, Sartorius) was placed on the end and sterile deionized water was drawn through. The water was expelled through the filter and the process repeated 3-4 times to ensure the yeast was properly washed. The final suspension in a few ml of deionized water was re-circulated through the electrodes.

Experimental procedure

The procedure used in this experiment was primitive compared to all other dielectrophoresis described here. This experiment was performed before the image analysis package had been configured with the "in house" programme and the FigP graphics package. Also the pump speed did not increase during the application of the pulse (see section 2.2.2.4).

The electrodes were positioned so that the 50 μm gap between the 50 μm electrodes was viewed on the monitor and the image analysis counting frame was altered to cover this area. A frequency range of 1 kHz - 50 MHz was applied to the electrodes (14 V, 3 s pulse, 3 s base time, 2 s pump time, 3 s peak time, 3 measurements per decade of log frequency) whilst the counts taken by Domino were scrolled on the screen. The point at which the pulse was applied and removed was recorded manually. The process was repeated 4 times and at the end of each run, the maximum count registered during the collection period of each frequency was recorded. An average of the 4 counts per frequency was used to form a spectrum.

The electrodes were repositioned so that the electrodes were adjacent to the edge of the counting frame. The process was repeated taking measurements when the pulse was released.

2.2.2.2 The effects upon measurement of dielectrophoresis by varying pulse length

Particle type

C. parvum oocysts suspended in deionized water (prepared as described in section 3.2.1.1) were used to demonstrate the effects of varying pulse length.

Experimental procedure

Oocysts were re-circulated through the electrode chamber and a programme was used to vary the length of applied pulse. A range of pulses (1-30 s) were applied within the limitations of the programme. This process was repeated 13 times. All other parameters remained unchanged throughout (400 kHz, 14 V, 6 s peak time, 5 s base time, 5 s pump time, conductivity 10 μS cm^{-1}, pump speed 2 rpm, concentration
6×10^6 ml^{-1}).

2.2.2.3 The effects upon measurement of dielectrophoresis by varying voltage

Particle type

C. parvum oocysts (preparation described in section 3.2.1.1) were used to demonstrate the effects of varying voltage.

Experimental procedure

A range of voltage was applied (3-16 V) limited by the capacity of the signal generator and the process was repeated 16 times. All other parameters remained unchanged throughout (400 kHz, 6 s peak time, 5 s base time, 5 s pulse time, 5 s pump time, conductivity 9 μS cm^{-1}; pump speed 2 rpm; concentration $6x10^6$ ml^{-1}).

2.2.2.4 The effects upon measurement of dielectrophoresis by varying pump speed

In order to improve dielectrophoretic collection when using low particle concentrations, the pump speed was increased. The speed entered at the beginning of an experiment would typically increase ten-fold whilst the field was applied and would return to normal when the pulse was removed. Therefore, more particles were brought into the vicinity of the electrodes during the collection period and higher counts were achieved.

Particle type

A suspension of *E. coli* NCIMB 11459 cells in deionized water (preparation described in section 4.2.7), was used to demonstrate the effects of increasing pump speed during application of the pulse.

Experimental procedure

The cell suspension was re-circulated through the electrode chamber and dielectrophoresis was performed. A series of different pump speeds (1-30 rpm) were operated whilst the pulse was applied but the speed remained at 1.5 rpm for the rest of the sequence. The other parameters remained unchanged throughout (frequency 1 MHz, 10 s pulse length, 10 s peak time, 5 s pump time, 5 s base time, conductivity
38 $\mu S\ cm^{-1}$, concentration $9 \times 10^7\ ml^{-1}$).

2.2.2.5 The effect of increasing medium conductivity upon dielectrophoretic collection

The effect of increasing medium conductivity upon dielectrophoresis was assessed by monitoring the frequency at which positive dielectrophoretic collection began for each conductivity level.

Particle type

A suspension of *C. parvum* oocysts in deionized water (prepared as described in section 3.2.1.1) was used to demonstrate the effects of increasing medium conductivity.

Experimental procedure

The *C. parvum* suspension was re-circulated through the electrode chamber. The image analysis package was not employed for this experiment which was intended to determine whether collection was apparent at each frequency level, not the extent of collection. The 5 μm electrodes were viewed on the monitor and a series of experiments were performed using 10 kHz - 10 MHz, 12 V, 10 s pulse length, 10 s peak time, 3 s pump time, 3 s base time, pump speed 2 rpm, concentration 5 x 10^6 ml^{-1}. Between each experiment, the medium conductivity was increased by addition of small amounts of dilute KCl (Pohl, 1978).

2.2.2.6 The effect of particle concentration upon measurement of dielectrophoresis

A series of spectra were produced using progressively lower particle concentrations, to demonstrate the limitations of the system in this respect.

Particle type

A suspension of *E. coli* NCTC 10418 cells in deionized water was used to demonstrate the effect of decreasing concentration. Cells were taken from the same stationary phase stock culture and after preparation (described in section 3.2.4.1) were diluted appropriately to achieve the lower concentrations.

Experimental procedure

The first sample gave an OD_{510} measurement of 0.053, approximately half the concentration used in routine bacterial dielectrophoresis experiments. The actual concentration was estimated to be approximately 6 x 10^7 cells ml^{-1}, using a calibration graph (absorbance versus total viable counts). A series of three dielectrophoretic experiments were performed (frequency range 1 kHz - 50 MHz, 10 s pulse length, 10 V, 8 s peak time, 5 s pump time, 5 s base time, pump speed 1.5 rpm, conductivity 9 μS cm^{-1}, 5 measurements per decade of log frequency).

The second sample was prepared from the stock culture. The concentration was too low to give an optical density measurement but was calculated to be approximately 6 x 10^6 cells ml^{-1}. The parameters remained unchanged for the first experimental sequence apart from an increase in pulse length to 20 s. The following two spectra were obtained using 14 V. The medium conductivity was 7 $\mu S\ cm^{-1}$.

The third sample was also prepared from the stock culture. The concentration was approximated to be 6 x 10^5 ml^{-1}. Parameters were basically unchanged but the first spectrum was produced using a 20 s pulse and 16 V whereas the pulse was increased to 30 s for the remaining two spectra. The medium conductivity was 10 $\mu S\ cm^{-1}$.

The final sample was prepared to give an approximate concentration of 10^5 cells ml^{-1}. Three consecutive spectra were produced using 16 V, 30 s pulse length and an increased pump speed of 1.2 rpm. The medium conductivity was 11 $\mu S\ cm^{-1}$.

2.3 RESULTS

2.3.1 Comparison of measuring dielectrophoresis during collection and during release

The results are shown in fig 2.7. It is apparent that using image analysis to measure particles upon release gives much higher counts than measurement during collection. The spectra plotted for the cells measured on release peaks at approximately 1 MHz. This is in accordance with many dielectrophoretic experiments that were performed subsequently involving live cells and always measurement upon release.

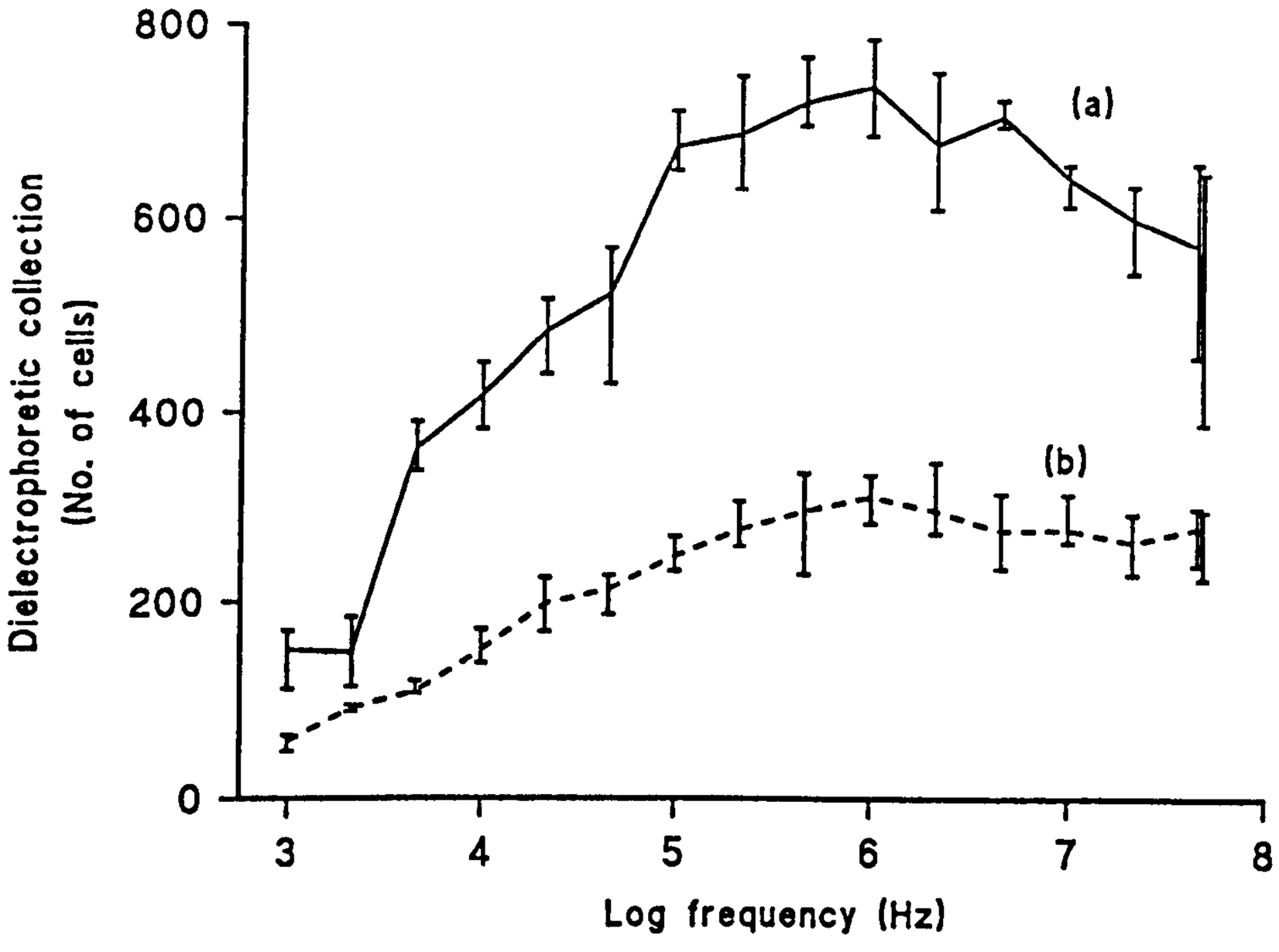


Fig 2.7 Dielectrophoretic counts of yeast cells measured (a) upon release from the electrodes and (b) during collection.

2.3.2 The effects upon measurement of dielectrophoresis by varying pulse length

The results are shown in fig 2.8. It is clear that collection of *Cryptosporidium parvum* oocysts at this concentration increases linearly with increasing pulse length up to 30 s. The small size of the error bars (SE) demonstrates the reproducibility of this relationship. Typically a pulse length of 10 s was used subsequently in dielectrophoretic experiments, which can be seen to produce a substantial collection level in fig 2.8.

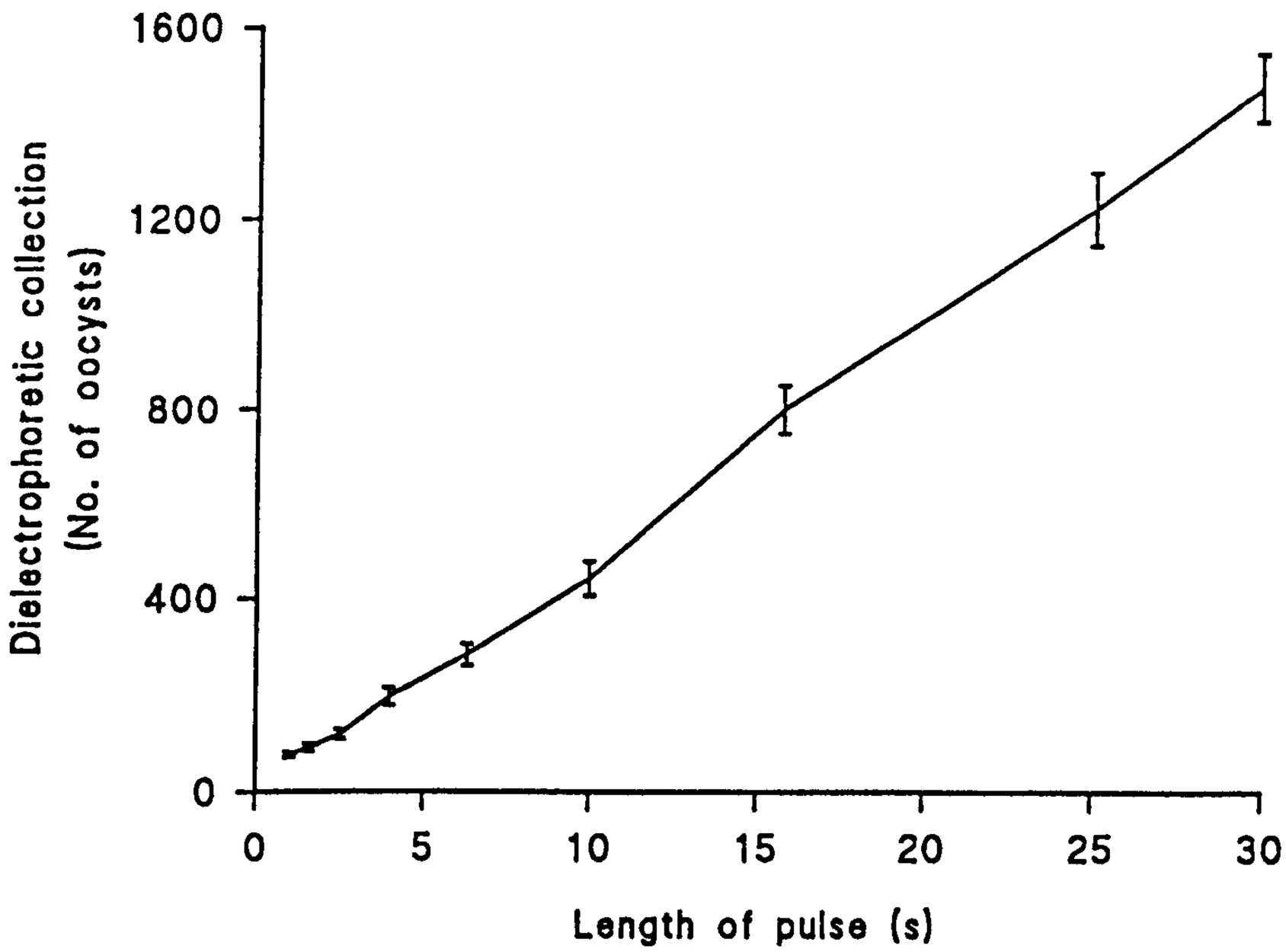


Fig 2.8 Relationship between dielectrophoretic measurement and increasing pulse length.

2.3.3 The effects upon measurement of dielectrophoresis by varying voltage

The results are shown in fig 2.9. After an initial lag phase the extent of dielectrophoretic collection increased with increasing voltage. The signal generator was unable to produce a voltage higher than 16. Typically a voltage of 10-14 V was used subsequently for dielectrophoresis, 10 V was commonly used in bacterial dielectrophoresis and 12-14 V in *C. parvum* dielectrophoresis.

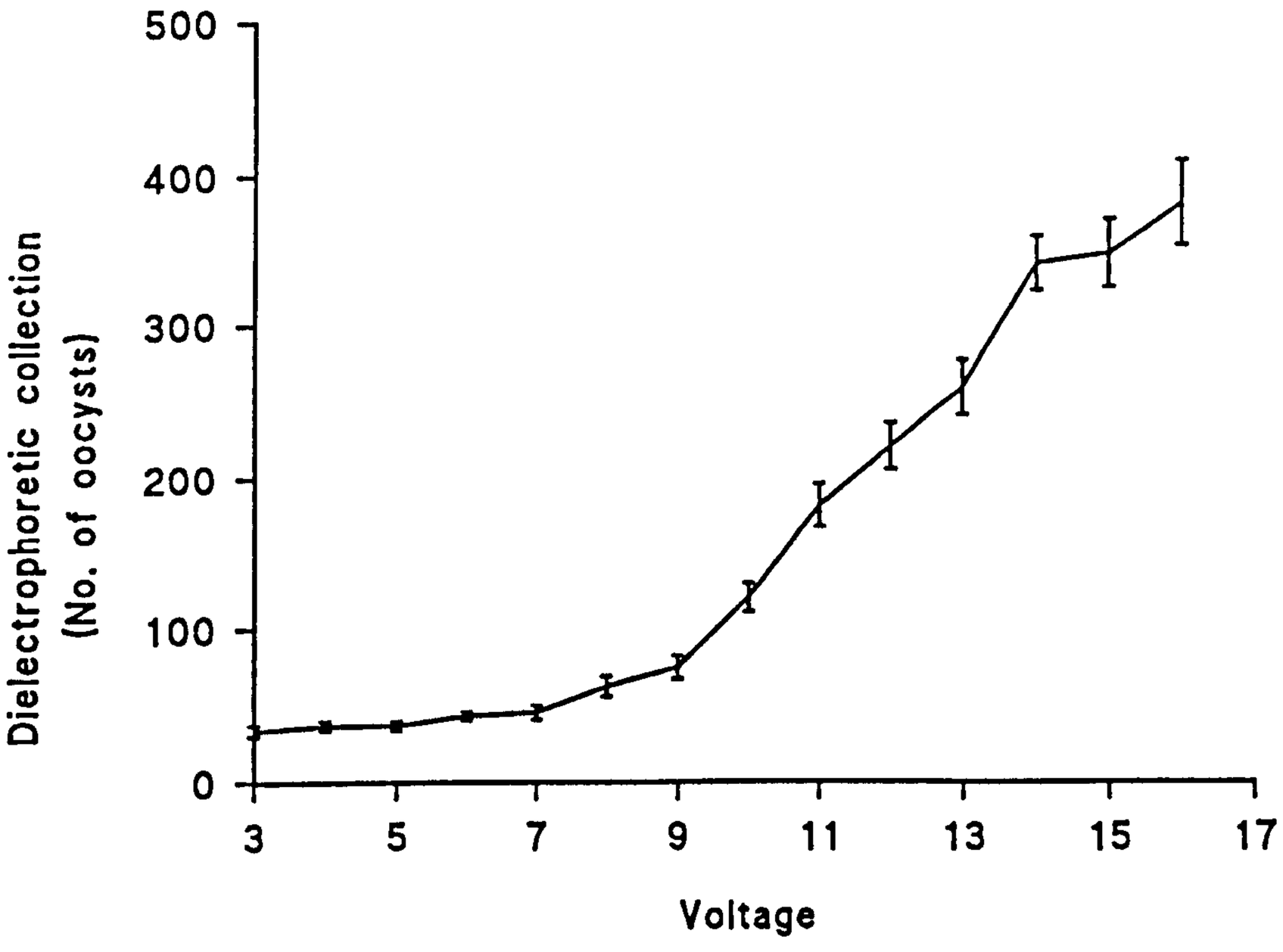


Fig 2.9 The Relationship between dielectrophoretic measurement and increasing voltage.

2.3.4 The effects upon measurement of dielectrophoresis by varying pump speed

The results are shown in fig 2.10. By increasing the speed of the pump to approximately 15 rpm whilst the pulse was applied, an increase in counts was produced. The level did not increase much further, probably due to electrode saturation and the counteraction of the dielectrophoretic force by high flow rate. Electrode saturation was likely as the cell concentration used in the experiment was high and was not limiting.

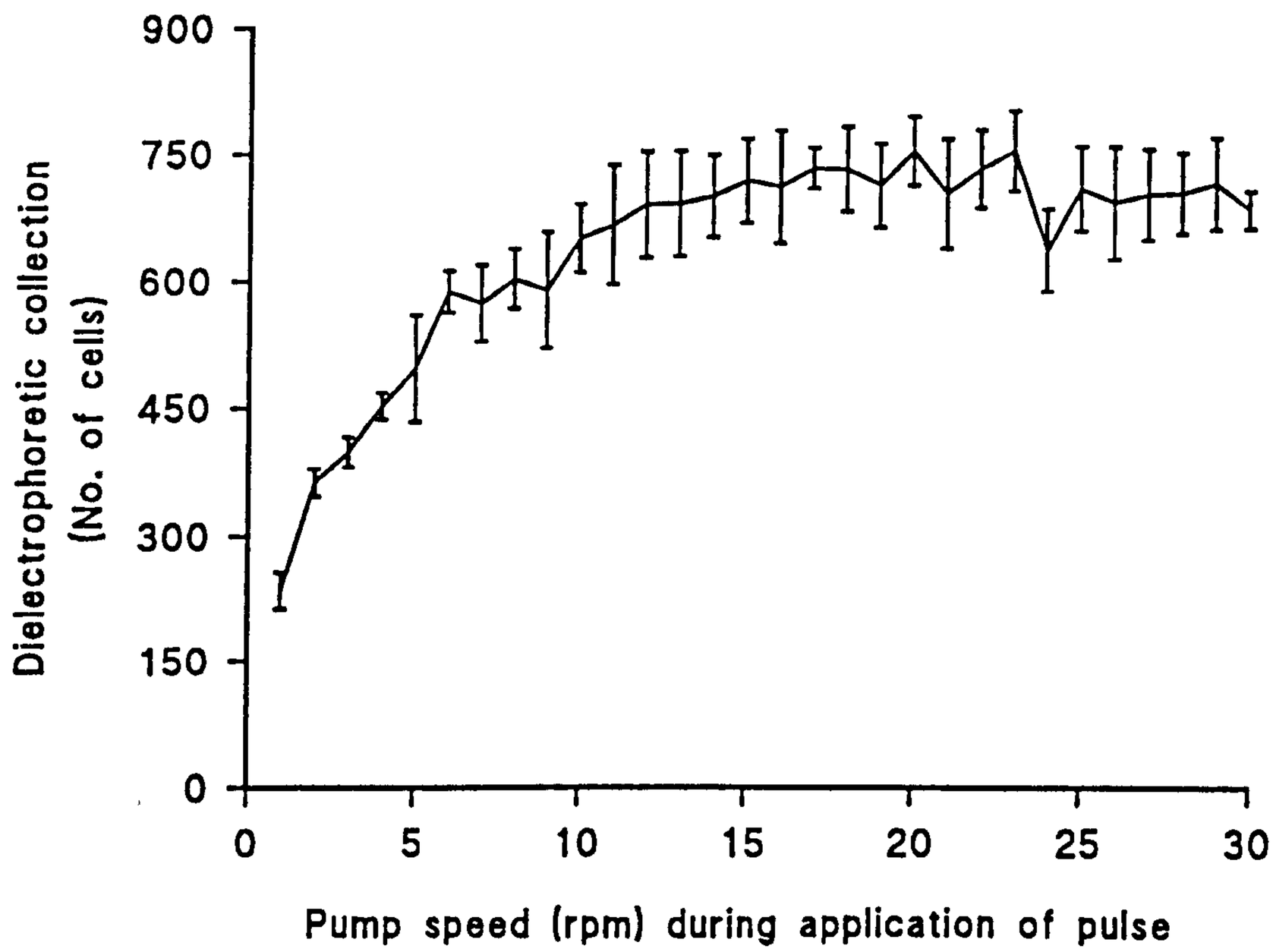


Fig 2.10 Relationship between dielectrophoretic measurement and increased pump speed during application of the pulse.

2.3.5 The effect of increasing medium conductivity upon dielectrophoretic collection

The results shown in fig 2.11 demonstrate that as medium conductivity was increased, the onset of positive dielectrophoretic collection occurred at increasingly higher frequencies. Sensitivity of positive dielectrophoresis onset to increasing medium conductivity, was greatest at lower conductivities. This effect began to diminish when the medium conductivity increased above 100 $\mu S\ cm^{-1}$.

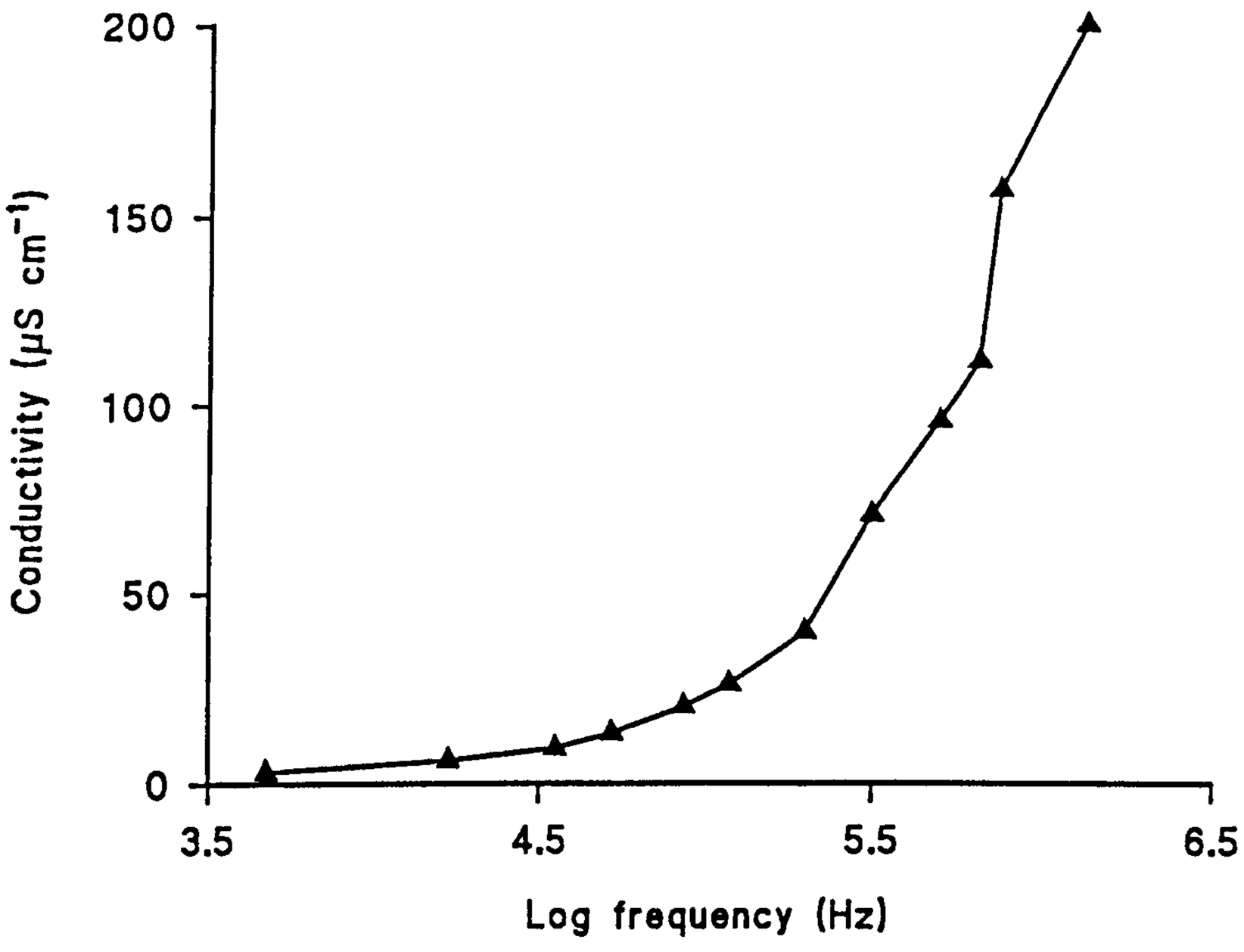


Fig 2.11 Effect of increasing medium conductivity upon the onset of positive dielectrophoresis.

2.3.6 The effect of particle concentration on dielectrophoretic measurement

The effects of altering cell concentration are shown in fig 2.12 (a)-(d). Fig 2.12 (a) using an *E. coli* cell sample approximately half of that routinely used, shows high counts under normal experimental conditions.

Fig 2.12 (b), where the cell sample used was intended to represent a concentration 5% of that normally used, shows erratic spectra. Collection levels were improved by increasing the pulse voltage, in accordance with the relationship shown in fig 2.9.

Fig 2.12 (c) shows spectra produced from a cell sample approximately 0.5% of normal sample concentrations. The results had become increasingly erratic and spectra were no longer a characteristic shape. The increased pulse length used for the second and third spectra increased total collection levels appreciably, in accordance with the results shown in fig 2.8.

Fig 2.12 (d) shows a mean of four consecutive spectra taken using a cell sample concentration 1000-fold lower than routinely used samples. The spectra were very erratic and the total numbers very low, despite the use of maximum voltage and pulse length, and an increased pump speed.

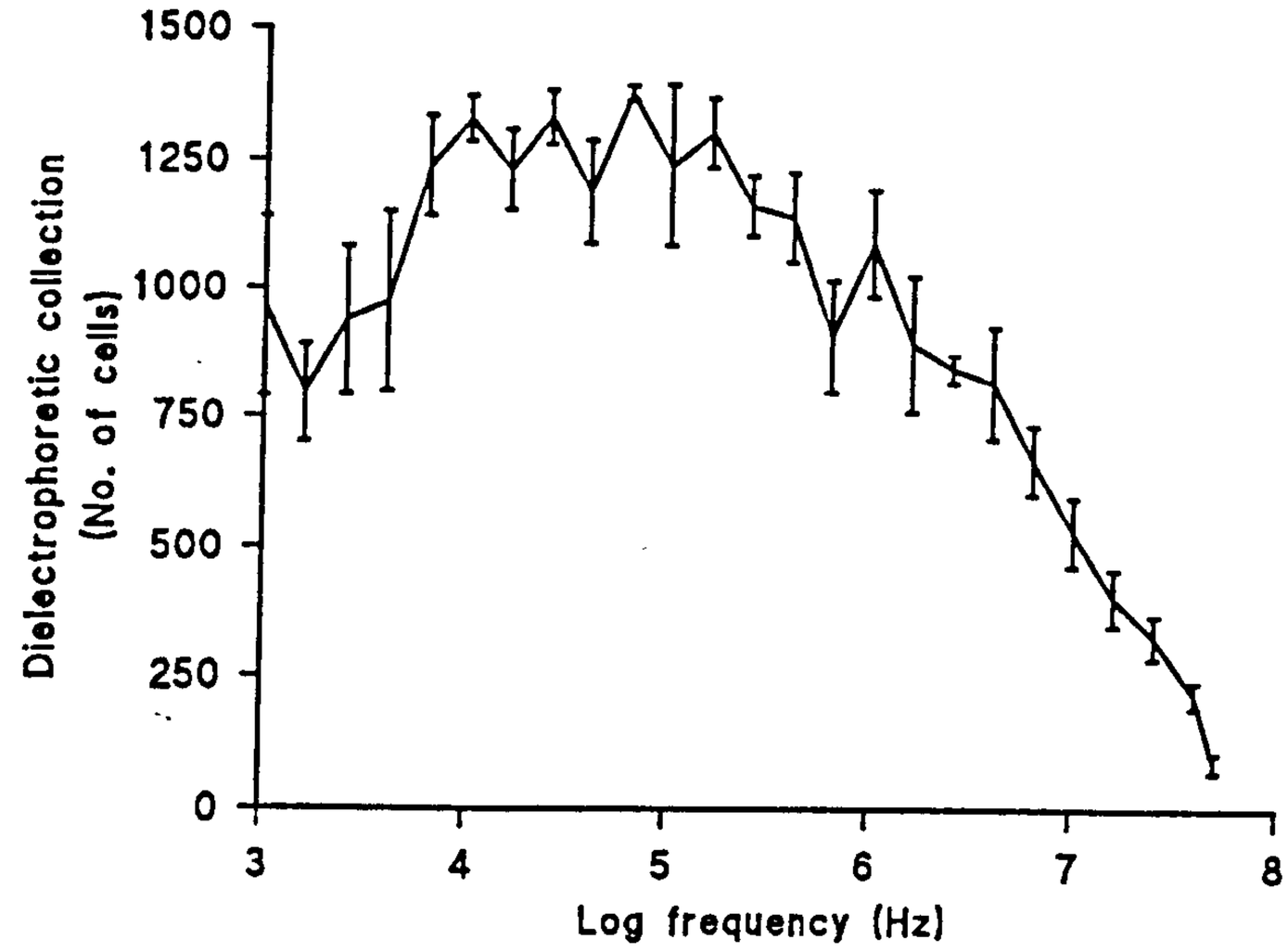


Fig 2.12 (a) Mean spectrum (n=3) of *E. coli* cells, approximate concentration 6×10^7 cells ml^{-1}.

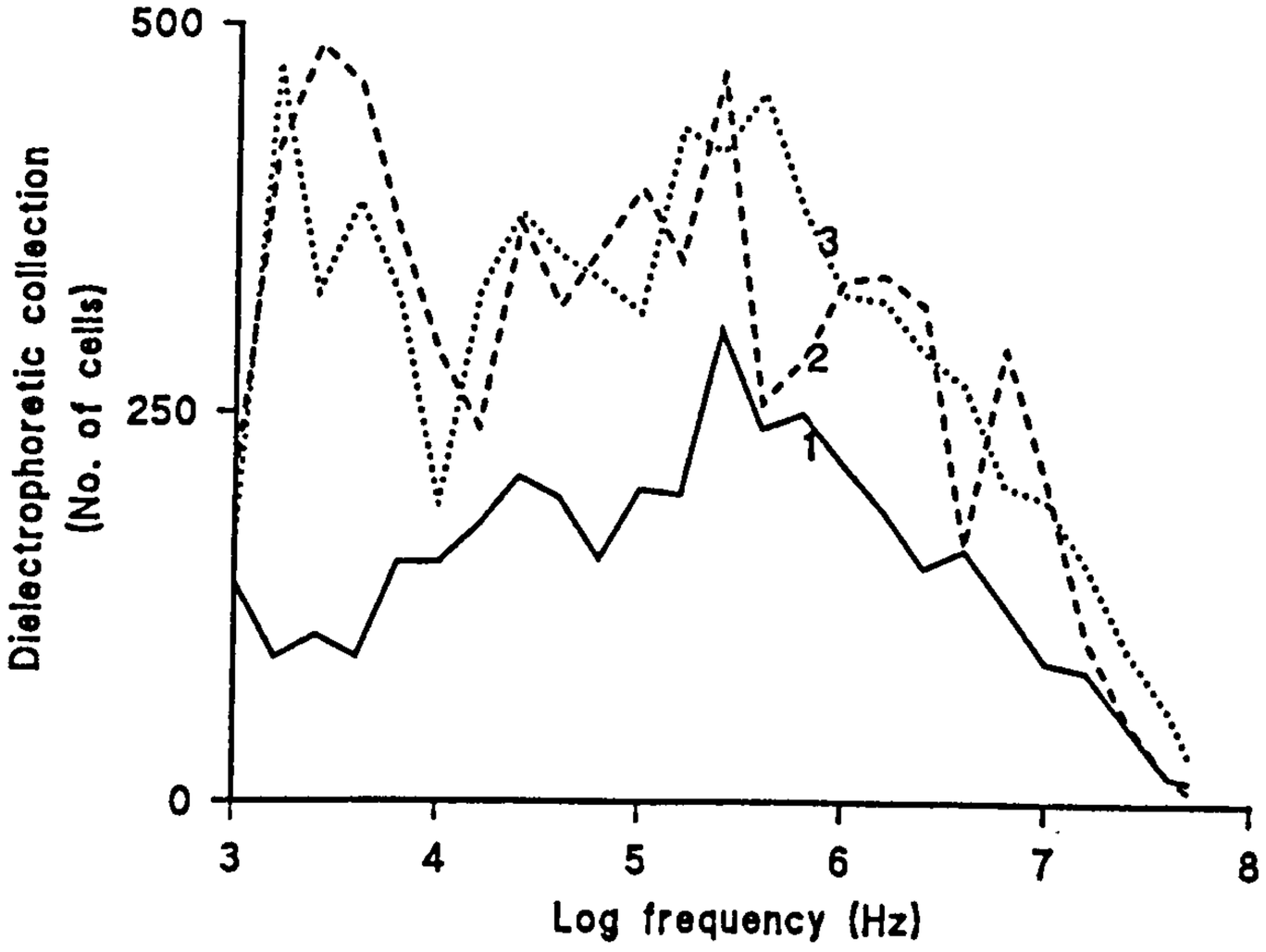


Fig 2.12 (b) Three spectra of *E. coli* cells, approximate concentration 6×10^6 cells ml^{-1}. Run 1 = 10 V; runs 2 & 3 = 14 V.

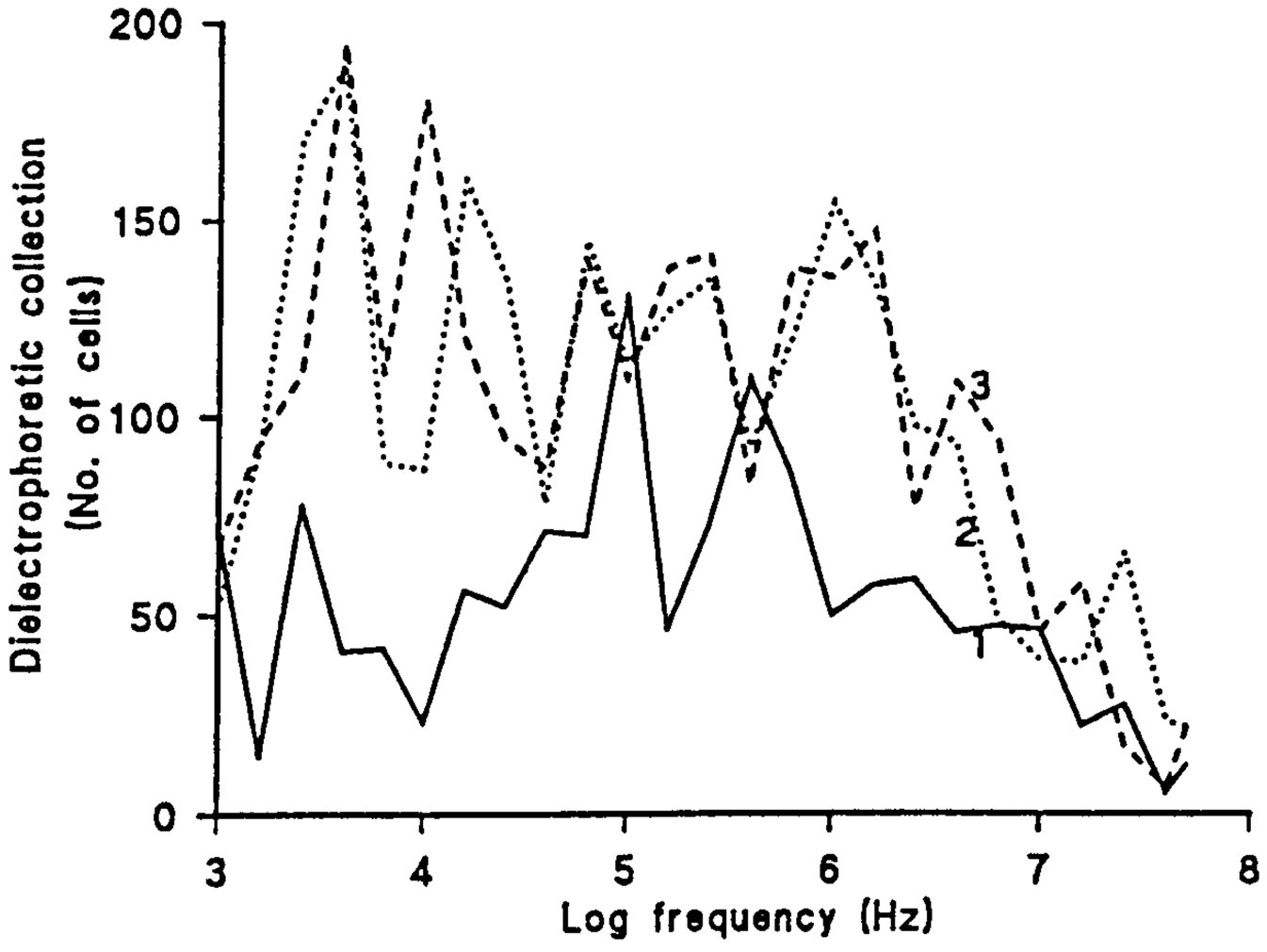


Fig 2.12 (c) Three spectra of *E. coli* cells, approximate concentration 6 x 10^5 cells ml^{-1}. Run 1 = 20 s pulse; runs 2 & 3 = 30 s pulse.

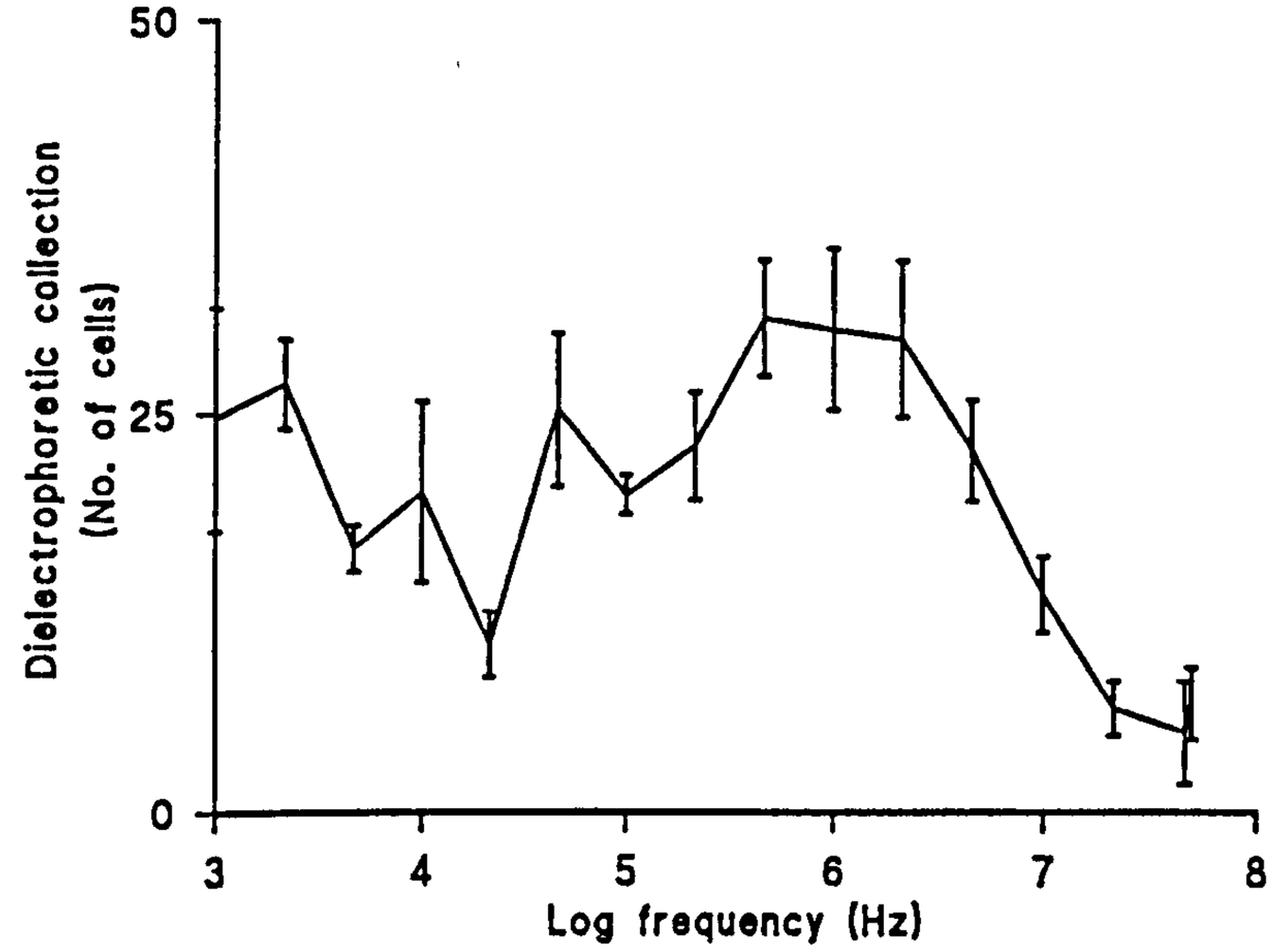


Fig 2.12 (d) Mean spectrum (n=4) of *E. coli* cells, approximate concentration 10^5 cells ml^{-1}.

2.4 DISCUSSION

2.4.1 Comparison of measurement of dielectrophoresis during collection and during release

The higher counts shown in fig 2.7 when comparing measurement of yeast cells upon release with that during collection, was to be expected due to the nature of image analysis. When particles are collecting at an electrode and forming pearl chains, they cannot easily be distinguished either from the electrode surface or other particles. Consequently an underestimation of collection levels was made. This situation could have been improved upon by the use of image analysis to detect total particle area rather than individual particles. However, this would not have circumvented the problem of particles lying on top of one another as well as those attached to the electrodes. An important factor in the measurement of released cells is that this allows all collected cells to be counted, including those which had congregated in chains or groups, which would have been completely omitted from count estimates made during collection.

When measuring released particles, it was important to position the image analysis counting frame adjacent to the electrodes, though not so close as to allow pearl chain formation to be detected and therefore increase the noise level of the trace. This was not a serious consideration when using *Cryptosporidium parvum* oocysts. These did not produce long pearl chains as they were used at relatively low concentrations, but the problem was encountered with other cells.

2.4.2 The effects upon measurement of dielectrophoresis by varying pulse length

The relationship in fig 2.8 shows a proportionate increase in collection of *C. parvum* oocysts with increasing pulse length. This is true for all particles up to the saturation level of the electrodes when it is not possible for any further increase in collection to occur. If a higher concentration was used, or the range of pulse length extended beyond 30 s, a plateau of collection level would have been expected. In addition, before complete electrode saturation was achieved, it was possible that the counting frame could have become saturated.

2.4.3 The effects upon measurement of dielectrophoresis by varying voltage

At low voltage, collection levels are very poor (fig 2.9) as the low field strength causes only weak polarization and therefore particles held at the electrodes are easily detached by the force of the flow. When a "threshold" voltage is reached, the particles are retained with enough strength to counteract the flow, and higher voltages produce higher collection. *C. parvum* oocysts were used to demonstrate this effect and their comparatively large size resulted in a relatively high "threshold" voltage to overcome the force of the flow and retain them on the electrodes. Typically 12 or 14 V was used in dielectrophoresis experiments using oocysts but 10 V was sufficient when using bacteria (which were much smaller and more easily retained).

2.4.4 The effects upon measurement of dielectrophoresis by varying pump speed

The purpose of increasing the speed of the pump whilst the pulse was applied was to maximise collection of low particle concentrations. The aim was to bring more particles into the electrode vicinity during the period of an applied pulse but not to use a flow rate so high as to counteract the dielectrophoretic force (which varied with frequency). The results in fig 2.10 show that collection increased with pulse length up to a plateau level denoting electrode saturation. An *E. coli* cell sample of relatively high concentration was used to demonstrate this and the electrodes became saturated at a relatively slow pump speed. A lower particle concentration would not have reached a plateau until a higher pump speed was used. Fig 2.10 shows a possible drop off in collection beginning to occur after 25 rpm. A decreased count at very high pump speeds would be expected (at lower pump speeds for larger particles) because eventually the flow of the suspension would be too fast to allow collection and would actively disrupt and remove collected particles.

Another, *pseudo*-decrease in measured collection at high flow rate may have resulted from rapid particle fall-off from the electrodes. When the pulse is released, although the pump speed reduces, the particle package may be swept past the image analysis counting frame by the inertia from the high flow rate. Measurement of individual particles is not possible and collection underestimated. This can be ameliorated by positioning the counting frame a short distance away from the electrode edge, so that when the package passes through it will have slowed and diffused.

2.4.5 The effect of increasing medium conductivity upon dielectrophoretic collection

An increase in medium conductivity delayed the onset of positive dielectrophoretic collection until higher frequencies. This occurred because the increased ionic strength produced an enhanced polarizability in the medium. Therefore in order for suspended particles to display a yet higher specific polarizability, progressively higher frequencies were necessary.

As frequency increased, different polarization mechanisms were employed to contribute to total particle polarizability. At low medium conductivity, positive dielectrophoresis began at low frequencies, polarization being partly due to surface charge effects. However, when the ionic strength of the medium was increased, its polarization counteracted the particles' surface charge polarization; therefore positive dielectrophoresis could not occur at the same frequency. Collection could begin at higher frequencies, when other surface groups were able to contribute to net particle polarization.

The medium conductivity in fig 2.11 ranged from 3 to 200 $\mu S\ cm^{-1}$, covering all levels used in subsequent dielectrophoresis experiments. It was still possible to achieve particle polarization at 200 $\mu S\ cm^{-1}$ with collection beginning at above 1 MHz.

Interfacial polarization was involved in oocyst polarization in this frequency range and contributed towards positive dielectrophoresis. This would be expected to continue until medium conductivity had reached a high enough level to over-ride this oocyst polarization mechanism. Eventually when particles could no longer be polarized the dielectrophoretic force would tend to zero. If medium conductivity was to continue rising beyond this point, negative dielectrophoresis would result. Price *et al.* (1988) reported similar observations using *Micrococcus lysodeikticus*. A medium conductivity of above 2000 $\mu S\ cm^{-1}$ was necessary to demonstrate negative dielectrophoresis.

2.4.6 The effect of particle concentration upon measurement of dielectrophoresis

The results shown in fig 2.12 (a)-(d) demonstrate the limitations of the dielectrophoretic system in its present form for the analysis of low concentration, low volume samples. However, if whole frequency spectra were not required and for example just several repeated pulses at one or two specific frequencies would suffice for a particular analysis, it may be feasible to use low concentrations and achieve enough information for statistical confidence in the results. The concentrations quoted in fig 2.12 (a)-(d) are probably underestimates of the true concentrations. They were estimated from absorbance readings which had been calibrated to total viable counts rather than to total cell counts. Minimum concentrations (approximate ideal 10^7 particles ml^{-1}) were necessary when using the current set-up due to several limiting components of the system. These included:

- **The depth of the electrode chamber.** The use of a thinner spacer, whilst difficult in practice, could reduce the chamber depth and therefore volume.
- **The inclusion of an on-line conductivity probe.** This has a chamber of approximately 2 ml therefore immediately limiting the volume size that could be used.
- **Tubing necessary to link all equipment components.** Lengths of different sized tubing influenced the sample volume that could be used.
- **Sample reservoir.** This needed to be of sufficient size to prevent air being drawn into the system.

- **The electrode area.** The total area of the electrode bars over which dielectrophoretic collection occurs was relatively large when compared with the area monitored and measured by image analysis.
- **The inclusion of a water bath.** This was frequently used for the incubation of the sample reservoir when maintenance of physiological temperature was attempted. Volume size was further limited by this.

2.4.7 Commonly encountered problems in experiments using the current dielectrophoretic equipment

When performing the dielectrophoretic experiments described here, some inherent problems recurred throughout which caused difficulty in the interpretation of results.

2.4.7.1 Lowering of dielectrophoretic collection levels with time

Perhaps the most confusing of the recurrent problems was the decrease in overall collection levels with time. This meant that in some cases consecutive spectra were represented by overlaying sets of data (e.g. in sections 3.3.2 and 4.3.1) rather than taking mean values and plotting a single spectrum with error bars. In other cases, when variation in consecutive spectra was not due to progressively decreasing collection, mean spectra were presented. Conditions during experiments were continually subject to slight changes (some of which are discussed below) therefore it may not have been strictly appropriate to present data in terms of mean values, as consecutive spectra were not true replicates. However, the extent of variation within such experiments appeared to be random and the least confusing way in which to display the results involved using mean values and error bars. The clarity in presentation of the data was thought to justify this.

The effect of lowered collection levels over time was two-fold. Peak height could decrease gradually during the course of a series of spectra, and bulk decrease in collection levels could occur between separate experiments performed at different times. Both the particle type and the electrodes contributed to the effects.

Decrease of particle collection levels within an experiment was particularly common during bacterial dielectrophoresis. When treated and untreated samples were being compared, it was often unclear whether decreased collection of the treated sample was attributable to treatment. Collection level of a control sample typically decreased less than that of a treated sample, but the extent of this effect varied even between similar samples as well as over time, therefore a differential level of decrease was not always informative.

It was likely that bacterial cells were themselves responsible for some of the decreased collection observed during an experiment. A similar observation was made by Inoue *et al.* (1988). Using *Micrococcus lysodeikticus*, they showed a decrease in collection level with time over the whole frequency range (10 Hz to >1 MHz), which was particularly prominent at the lowest frequencies. The original collection level was restored upon washing and re-suspension of the cells. The decrease was attributed to a gradual diffusion of ions from inside the microorganism into the bulk solution, therefore effectively lowering particle polarizability.

The contribution of the electrodes to decreasing collection levels during an experiment is variable. In some instances conditions deteriorate sufficiently within the time period of an experiment so that the electrode quality is affected and influences subsequent results. However, most problems caused due to electrodes are responsible for altering dielectrophoresis between separate experiments performed at different times. Broadly, problems with electrodes can be grouped into three areas:

- **Electrode deterioration.** Sets of electrodes were made in batches of 6-10 using photolithographic techniques (see Appendix I). A single mask was used in their manufacture but slight variations were inevitable between both sets and batches. One cause of this was the creation of pin point holes in the metal film during the vacuum process. Future deterioration could centre upon these defects. Oxidation of the aluminium occurred over time, further increasing deterioration during storage and therefore electrode batches larger than 10 may have led to wastage. Deteriorated electrodes produced an altered electric field and worked inefficiently.

- **Adhesion of particles to the electrode surface.** Even after a single spectrum had been produced, typically particles became stuck to the electrode surfaces. The extent to which this occurred varied according to particle type but was particularly prevalent in bacterial dielectrophoresis. Bacterial cell surface characteristics vary within and between species - capsular or slime layers would be particularly adhesive. However, in these experiments bacteria with tetracycline resistance were the most problematic in this respect. The coating upon the electrode surface effectively insulated it therefore decreasing the field strength.

- **Adhesion of particles to the background area.** As with the electrodes themselves, adhesion of particles to the background area commonly occurred. Although the electrodes themselves were not directly affected by this, dielectrophoretic *detection* was impaired. In addition to adherence of particles from the experimental suspension, in some cases organisms were observed to grow within the electrode chamber. Cells resembling yeasts were common, i.e. they were oval-spherical shaped, approximately 7-8 μm diameter and spread by budding. Growth occurred particularly during bacterial dielectrophoretic experiments incorporating a 37°C water bath for incubation of the sample reservoir but could also occur over a longer period of time if electrodes were not stored at 4°C in the dark. This effect was minimal if a cell suspension containing an antimicrobial at high concentration was used. By sticking to the microslide, particles were detected by image analysis as background (baseline) counts. When this occurred only to a small extent this did not produce a problem as the baseline count was subtracted from the peak for every applied frequency pulse to produce the value for collected particles. However, as the effect increased, particles falling off the electrodes and passing through the counting frame were masked when passing over areas of the background already covered with adhered particles. Consequently dielectrophoretic collection was underestimated and the electrode set was rendered effectively useless.

2.4.7.2 Cleaning of electrodes

Due to the time and expense involved in electrode manufacture, they were generally in demand. Often the need for a new set was due to the contamination of the chamber (described above) rather than breakage of the electrodes. Since the electrode chamber was first designed, many methods have been tried for the removal of particles adhering to the electrode bars and the counting frame area in an attempt to prolong electrode life.

A variety of detergents and disinfectants have been tried including sodium dodecyl sulphate (SDS), Tween, Lipsol, Gigasept, ethanol and hydrogen peroxide, all at varying concentrations and some at elevated temperature. Most were ineffective and/or corrosive to the aluminium. Various antibiotics and enzymes have also been tried. The agent which produced most success was an "Electrode cleaning solution for biological materials" (Whatman, cat. no. 6200 0004) which was a trypsin-hydrochloric acid combination. However, this was still unable to detach many particles and itself was very easily contaminated leading to further contamination of the electrodes themselves. When this was discovered the electrode cleaner was always filtered before use.

Electrode chambers had a finite life span and efforts to extend this were generally unsuccessful. Thorough washing and agitation using deionized water containing lots of air bubbles was routinely performed at the end of an experiment and storage at 4°C in the dark was vital to minimise further contamination and deterioration.

2.4.7.3 Effects which occur at low frequencies

Negative dielectrophoresis

The image analysis of the dielectrophoretic experiments described here was performed using a counting frame which covered the total screen area of the monitor. It was not possible to directly observe whether dielectrophoresis was positive or negative during the course of an experiment. However, it is likely that in some experiments where high collection levels were apparent at low frequencies, this may have been a negative response. The photographs in fig 2.13 illustrate how particles could be held above the electrodes during negative dielectrophoresis.

Negative dielectrophoresis was apparent over low frequencies (typically below 5 kHz) particularly in experiments involving bacteria. However this effect tended to lessen as consecutive spectra were produced.

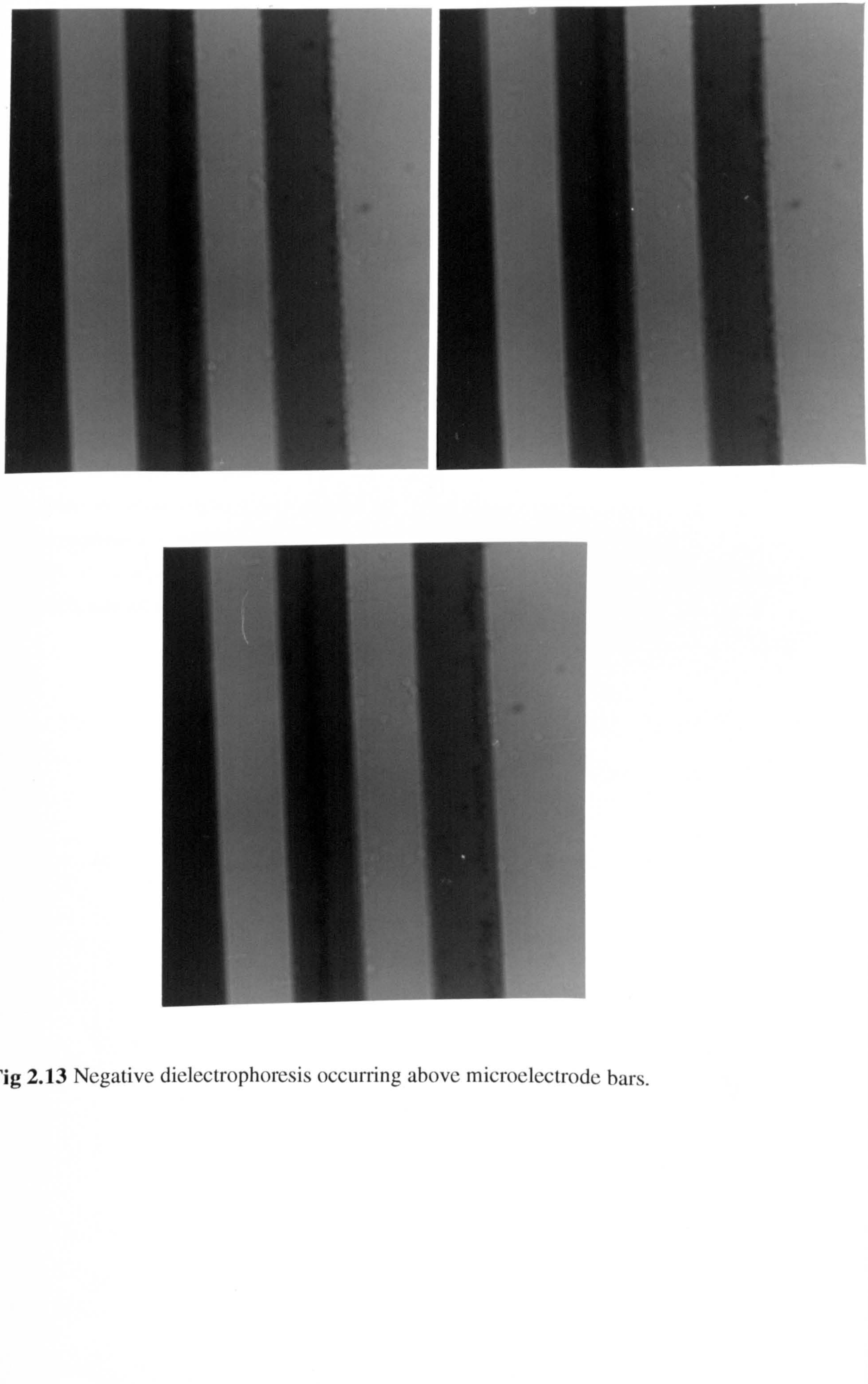


Fig 2.13 Negative dielectrophoresis occurring above microelectrode bars.

When cells were freshly washed and suspended in deionized water, it was likely that the surface ion concentration needed time to equilibrate. Inoue *et al.* (1988) attributed a decrease in positive dielectrophoretic collection levels over time to the gradual diffusion of ions from the cells out into the bulk phase. In a similar manner, at low frequencies where polarization is dominated by the conductivities of the particle surface and the medium, until diffusion of ions *into* the surface region has begun, cell polarizability would be low allowing negative dielectrophoresis.

Electrophoresis

If a DC. current was applied to a cell suspension, cells would be expected to migrate toward the cathode due to their negative surface charge. In a very low frequency AC. field, because the current is reversing very slowly, some electrophoretic mobility may occur in suspensions of low conductivity. This could have the effect of driving the cells nearer to the electrode, therefore leading to an apparent enhancement of cell polarizability as assessed by dielectrophoretic collection (Burt *et al.*, 1989). However this was not likely to affect the results of any experiments described here as the frequencies employed by the dielectrophoretic system (minimum 1 kHz) are not very low.

Chapter Three

DIELECTROPHORESIS OF *CRYPTOSPORIDIUM PARVUM* OOCYSTS AND *E. COLI* TREATED WITH DISINFECTANTS

3.1 INTRODUCTION

3.1.1 Microorganisms in water

Water provides an environment in which many specialized types of microorganisms may thrive. Natural, "raw" (untreated) water may contain approximately 10^5 microorganisms ml^{-1} although most are removed through water treatment and are non-pathogenic. (Jawetz *et al.*, 1982). If enteric pathogens are present in raw water it is a sign of contamination. In public health terms, the most important waterborne pathogens originate from human or animal faeces. Some waterborne pathogens proven to have caused enteric disease are listed in table 3.1. Other intestinal pathogens present in human and animal faeces also have the potential to cause waterborne disease e.g. *Bacillus cereus*, some clostridia, *Isospora* spp. and some enteric viruses (Anon, 1994a).

Table 3.1 Known waterborne enteric pathogens (Jawetz, 1982; Anon, 1994a)

Bacteria	**Protozoa**	**Viruses**
Campylobacter spp.	*Cryptosporidium spp.*	Hepatitis A virus
E. coli (certain serotypes)	*Balantidum coli*	Hepatitis E virus
Salmonella spp. (including *S. typhi*)	*Entamoeba hystolytica*	Small round structured viruses (e.g. Norwalk virus)
Shigella spp.	*Giardia lamblia*	Rotaviruses
Streptobacillus moniliformis		
Vibrio spp. (including *V. cholerae*)		
Yersinia enterocolitica		

In treated water, levels of *indicator organisms* are monitored to warn of faecal contamination. An indicator organism must be one that is readily detectable and consistently present in human faeces, therefore indicating contamination from human waste. If the number of indicator organisms in water is high, this implies a more serious contamination problem from faecal pathogens. The most commonly used indicator organisms are coliform bacteria. Total coliform organisms are oxidase negative and produce acid from lactose after incubation for 4 h at 30°C, followed by 14 h at 37°C. If acid is produced when the final 14 h incubation is at 44°C, the organisms are presumptive faecal coliform bacteria (Anon, 1994a). Some coliform bacteria may be associated with soil or plants but it is specifically *faecal* coliforms, typically *E. coli* that are monitored.

There are several standardized, simple tests for monitoring faecal coliform presence in water after treatment. If any faecal coliform presence is detected this demands immediate attention as a breach in treatment processes is implied.

3.1.2 Water treatment

The treatment of water to make it fit for consumption involves several separate processes which collectively should be capable of eradicating all potential pathogens. However deciding upon a treatment strategy able to cope with them all is difficult due to the variation in species number and susceptibility.

3.1.2.1 Physical treatment (sedimentation and filtration)

The type and extent of treatment necessary for raw water depends upon its original source. Ground water e.g. from springs or deep wells often only requires minimal treatment as it can be of high purity, due to filtering as the water originally percolated through the ground. Normally, precautionary chlorine disinfection is performed on ground water to be used for consumption (Anon, 1990).

Water supplies for large areas such as cities commonly originate from a polluted source, e.g. a river, into which sewage and industrial waste is deposited. Intensive treatment is necessary although the specific combination of processes can vary.

Water may be pumped into a holding reservoir containing enough water for several weeks supplies. During this time many suspended particulates (including microorganisms) may settle out and turbidity is reduced. In the UK, one of two principal forms of treatment may be employed: chemical coagulation with rapid filtration; or pre-filtration followed by slow sand filtration.

Chemical coagulation (or flocculation) involves the addition of compounds e.g. aluminium or ferric sulphate to the water. Under controlled chemical conditions, these aggregate to form precipitates (flocs) which enmesh suspended particulates. The flocs may be removed in sedimentation tanks. Rapid filtration follows, through filter beds of for example sand or granular activated carbon (Anon, 1990).

Pre-filtration before slow sand filtration is necessary to prevent blockage of the slow sand filters. No chemicals are used but raw water is passed through either course rapid sand filters or stainless steel micro-strainers. The slow sand filters themselves are very efficient at removing coliforms. The top 10-20 mm of the filter are covered in a bacterial and algal slime layer (a *schmutzdecke*, German for "dirt layer") and this contributes largely to the filtering efficiency (Anon, 1990). Sand filtration is thought to be efficient at removing *Cryptosporidium parvum* oocysts from water (Chapman and Rush, 1990).

3.1.2.2 Chlorine disinfection

Disinfection of water in the UK is routinely performed using chlorine. Coliform disinfection is achieved by exposure to 5 mg l^{-1} chlorine for 30 min (C*t* 150) (Anon, 1990). The presence of organic matter in water can inactivate disinfectants but through monitoring, chlorine levels can be adjusted accordingly. Chlorine is a powerful oxidant and the possibility that oxidation of organic material may lead to the formation of carcinogens (such as chlorinated aromatics) has been reported (Fawell *et al.*, 1987; Glaze, 1987). Aquatic life may also suffer toxic effects from chlorine residuals and by-products (Ward and DeGrave, 1978).

Chlorine is usually added in the form of chlorine gas or sodium/calcium hypochlorite (Anon, 1991) and depending on the pH of the water and if ammonia is present, it may form HOCl, ClO^-, Cl_2 or chloramines in solution. Ammonia may be added to chlorine to purposely form chloramines (e.g. monochloramine NH_2Cl, dichloramine $NHCl_2$ and nitrogen trichloride NCl_3) which are less powerful disinfectants but which have a longer residual period in the water distribution system. Generally, a lower pH results in a greater killing effect of chlorine. At pH 5, 99.7% of the free chlorine is provided by HOCl, but only 78.7% at pH 7, and 3.6% at pH 9. The hypochlorite ion ClO^- provides more than 99% of the free chlorine at pH 10 but this is 150-300 fold less cystidal to *Giardia* cysts than HOCl (Jarroll, 1988). Total disinfection of water cannot be guaranteed using chlorine alone. For example, the protozoan parasites *Giardia* and *Cryptosporidium* produce cysts and oocysts respectively, which are resistant to chlorination. *Giardia* may be controlled by increasing disinfectant levels but *Cryptosporidium* oocysts which have passed through the filtration processes are unlikely to be killed by chlorination: doses of between 8,000 and 16,000 mg l^{-1} chlorine have been reported necessary for inactivation *Cryptosporidium* oocysts (Smith *et al.*, 1990). However, agitation of oocysts with sand prior to chlorination appears to increase their susceptibility (Parker and Smith, 1993).

3.1.2.3 Ozone disinfection

Ozone (O_3) is a reactive gas and powerful disinfectant (Anon, 1990; Botzenhart *et al.*, 1993). It can be used solely for its disinfectant properties and may be particularly effective when used in combination with other agents e.g. chloramines (Nieminski and Bradford, 1991) and hydrogen peroxide (Hall and Sobsey, 1993). Ozone may also be used as an oxidant to control the flora, colour and odour in drinking water. Ozonation of water prior to treatment can enhance flocculation of suspended particles. Ozone may be preferred to chlorine for water treatment disinfection due to the lower level of mutagens produced by ozonation. However, disadvantages to the use of ozone include its instability in water and its generation of low molecular weight organic by-products. Ozone decomposition in water depends upon water pH, e.g. at pH 8 ozone has a half life of less than one hour therefore limiting the residual disinfection capacity. Low-molecular weight compounds generated when ozone and natural organic substances react are more easily degraded and thus promote microbial growth within the distribution system (Glaze, 1987; Glaze *et al.*, 1989).

Ozone is more effective than chlorine at disinfecting *Giardia* cysts (Glaze, 1987; Wallis *et al.*, 1989). Also it could be used to inactivate *Cryptosporidium* oocysts at concentrations practical for use in water treatment and it has received particular attention for this reason (Anon, 1990). The method of ozonation is important for the inactivation of oocysts i.e. direct ozonation of water is more efficient than use of residual ozone levels alone. Elevated temperatures also improve oocyst inactivation (Parker *et al.*, 1993). Water companies that have used ozone as a disinfectant maintained a minimum residual concentration of 0.4 mg l^{-1} for 6 min by injecting 1.5-4 mg l^{-1} ozone into the water (Peeters *et al.*, 1989). Studies so far suggest that this would be insufficient for complete disinfection of *C. parvum* oocysts.

3.1.3 Ultra-violet radiation

The ultra-violet (UV) wavelength range forms the 100-400 nm portion of the electromagnetic spectrum, dividing x-rays and visible light. The UV spectrum may be split into 3 or 4 regions depending on terminology (table 3.2):

Table 3.2. Regions within the ultra-violet light spectrum.

Wavelength	Type of UV radiation
100-190 nm	extreme- or vacuum-UV (absorbed by air and water).
190-300 nm	far-UV (shielded from the earth's surface by atmospheric ozone; absorbed by nucleic acids and proteins).
300-380 nm	near-UV (penetrates to the earth's surface).
100-200 nm	vacuum-UV
200-280 nm	UV-A
280-315 nm	UV-B
315-400 nm	UV-C

Microorganisms are good absorbers of far-UV radiation due to their high protein and nucleic acid content. In proteins both aromatic amino acids and peptide bonds are important UV absorbers. Proteins absorb strongly below wavelengths of 230 nm. The bases of nucleic acids are important far-UV absorbers and between approximately 240 and 290 nm, nucleic acids absorb 10-20 times as much UV radiation as equal weights of protein (Jagger, 1967).

Absorption of UV radiation by nucleic acids results in the formation of dimers, covalent bonds between adjacent bases (predominantly the pyrimidines: thymine, cytosine and uracil). Hydration and dimer formation in pyrimidines are produced by low dosage of far-UV but they are often reversible. In the presence of light, pyrimidine dimers (predominantly thymine dimers) may be split by the process of photoreactivation, mediated by a light-dependent enzyme. Pyrimidine dimers and other structural distortions in DNA caused by UV damage may also be repaired by light-independent means. Among the well characterized mechanisms in bacteria (particularly *E. coli*) are the excision-repair systems. Each system can remove mis-paired or damaged bases from DNA and synthesize an appropriate replacement DNA strand. The process begins after recognition by an endonuclease, with the incision (cleavage of the damaged DNA on both sides). An exonuclease excises the damaged DNA and the remaining single-stranded DNA section is used as a template for DNA polymerase synthesis of a new DNA segment. DNA ligase finally links the 3' end of the newly synthesized strand to the original DNA.

If the genes responsible for processes such as the excision-repair systems are damaged by UV radiation, this leads to increased UV sensitivity and increased mutation frequency in cells. The *uvr* genes of *E. coli* (*uvr A, B* and *C*) all code for different components of a repair endonuclease enzyme (Lewin, 1990).

The apparent inactivation of microorganisms through UV disinfection may be false if repair occurs subsequently. However, it has been suggested that photoreactivation at least, may not be important in the disinfection of waste water (Whitby and Palmateer, 1993).

UV radiation has several disinfection applications e.g. in surface sterilization of hospital rooms and in the treatment of waste water and drinking water. Penetration of most liquids by UV radiation is poor and it is rarely used to sterilize solutions.

3.1.3.1 Disinfection using UV radiation

In North America, UV has been accepted as an alternative to chlorination in the treatment of waste water (Venosa, 1983). While UV does not produce the undesirable by-products of chlorine, disadvantages include the difficulty in estimating an *in situ* UV dose and in monitoring disinfection efficiency (Qualls and Johnson, 1983; Sommer and Cabaj, 1993). The UV dose range necessary to kill a variety of indicator organisms may be much narrower than the equivalent range of chlorine levels (Yip and Konasewich, 1972). Generally, UV disinfection resistance of microorganisms follows the series:

Protozoan cysts > bacterial spores > viruses > vegetative bacteria (Chang *et al.*, 1985; Dolman and Dobrogowski, 1989).

Research performed on the UV susceptibility of some waterborne pathogens has been limited, e.g. hepatitis A virus (Battigelli *et al.*, 1993; Wiedenmann *et al.*, 1993) and *Cryptosporidium* oocysts (Anon, 1990) even though they are known to be more resistant to chlorine than traditional indicator organisms. *Giardia* cysts have been reported as being several times more resistant to UV than *E. coli* (Rice and Hoff, 1981; Jarroll, 1988) although the difference between the two may be as great as 1000-2000 times when chlorine disinfection is used (Rice *et al.*, 1982). UV activity can be enhanced when used with other disinfectants e.g. chlorine (Sobotka, 1993) or hydrogen peroxide (Bayliss and Waites, 1980). Little research has been performed regarding the UV susceptibility of *C. parvum* oocysts (Anon, 1990). Lorenzo-Lorenzo *et al.* (1993) indicated a high level of resistance but more recently, Campbell *et al.* (1995) have shown a significant reduction in oocyst viability after UV treatment in water, suggesting it may have an application in post-treatment water disinfection.

3.1.4 *Cryptosporidium parvum*

The robust oocysts produced by this protozoan parasite are resistant to environmental pressures (Robertson *et al.*, 1992) including traditional water treatment disinfection i.e. using chlorine (Smith *et al.*, 1990) as described earlier. Its occurrence is world-wide and ingestion of very low oocyst numbers (Miller *et al.*, 1989; Blewett *et al.*, 1993; Dupont *et al.*, 1995) may cause human infection. Cryptosporidiosis can be fatal in the immunocompromised and increasing incidence of this condition further emphasizes the need for control of this organism, e.g. approximately 10% of AIDS patients in the USA and an estimated 30-50% in the developing world, become infected with *C. parvum* (Petersen, 1993).

3.1.4.1 Life cycle of *C.parvum*

C. parvum is a monoxenous organism, i.e. completes its entire life cycle in a single host (fig 3.1). Upon ingestion of the oocysts, four motile sporozoites excyst into the small intestine. The necessary conditions including elevated temperature and presence of bile salts can be simulated *in vitro* to produce excystation. Each sporozoite invades a gut epithelial cell (enterocyte), becomes surrounded by a host cell-derived membrane and develops into a Type I meront. These are intracellular but extracytoplasmic as they remain outside the host cell cytoplasm. Asexual development follows and 8 merozoites are produced in each meront. Upon cell rupture these may either invade other epithelial cells to form further Type I meronts or develop into Type II meronts. Four Type II merozoites develop asexually within the latter and each invades an enterocyte to develop into either a microgamont (which produces "male" microgametes) or a macrogamont (containing a single "female" macrogamete). The released microgametes penetrate the macrogamete leading to fertilization to form a zygote. Approximately 20% of these proceed to form thin-walled oocysts which are "autoinfective" as the sporozoites are released and repeat the life cycle within the same host. Most zygotes develop into resistant oocysts which are excreted in the host faeces (Petersen, 1993; Current, 1986; Janoff and Reller, 1987).

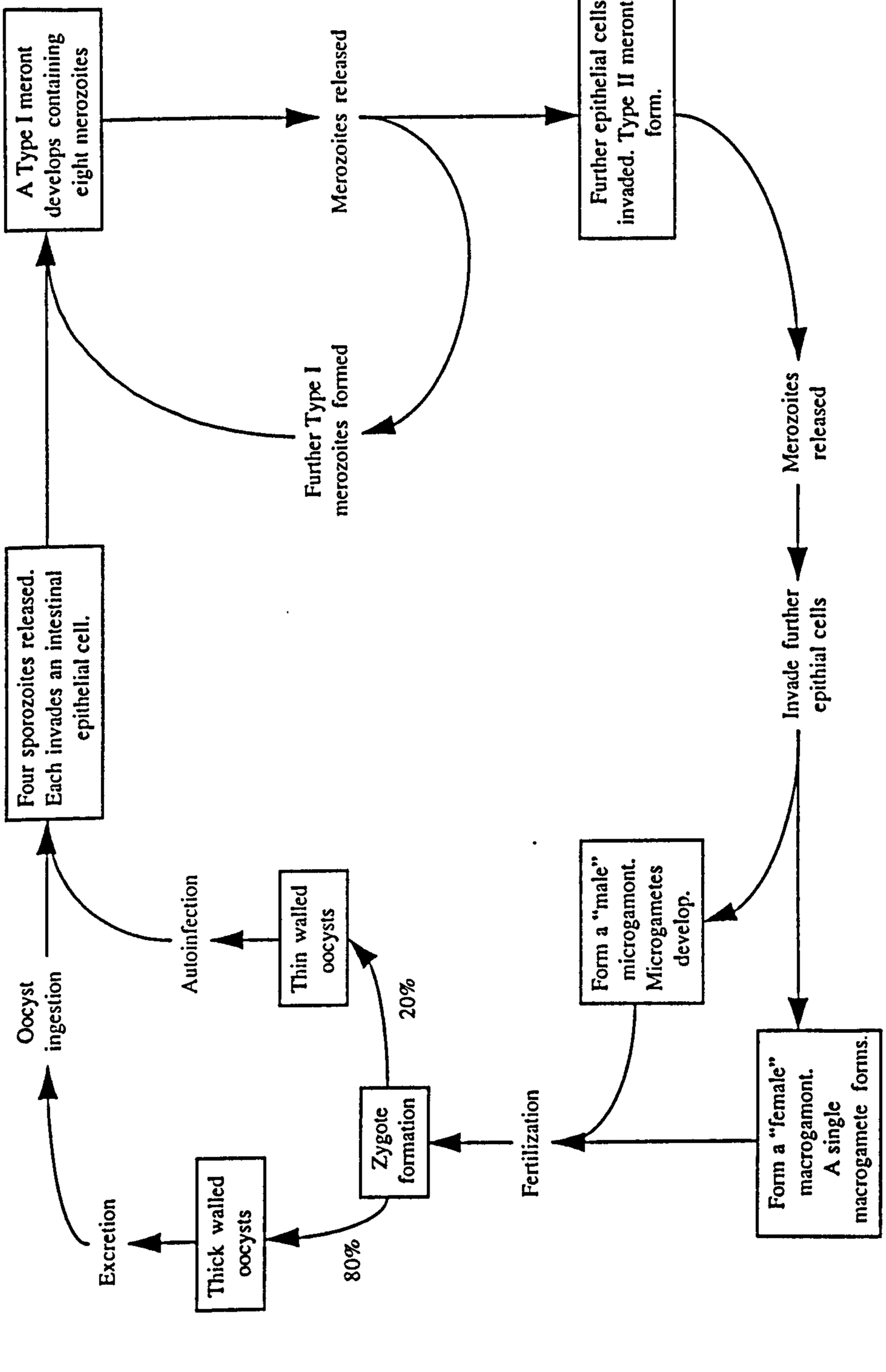
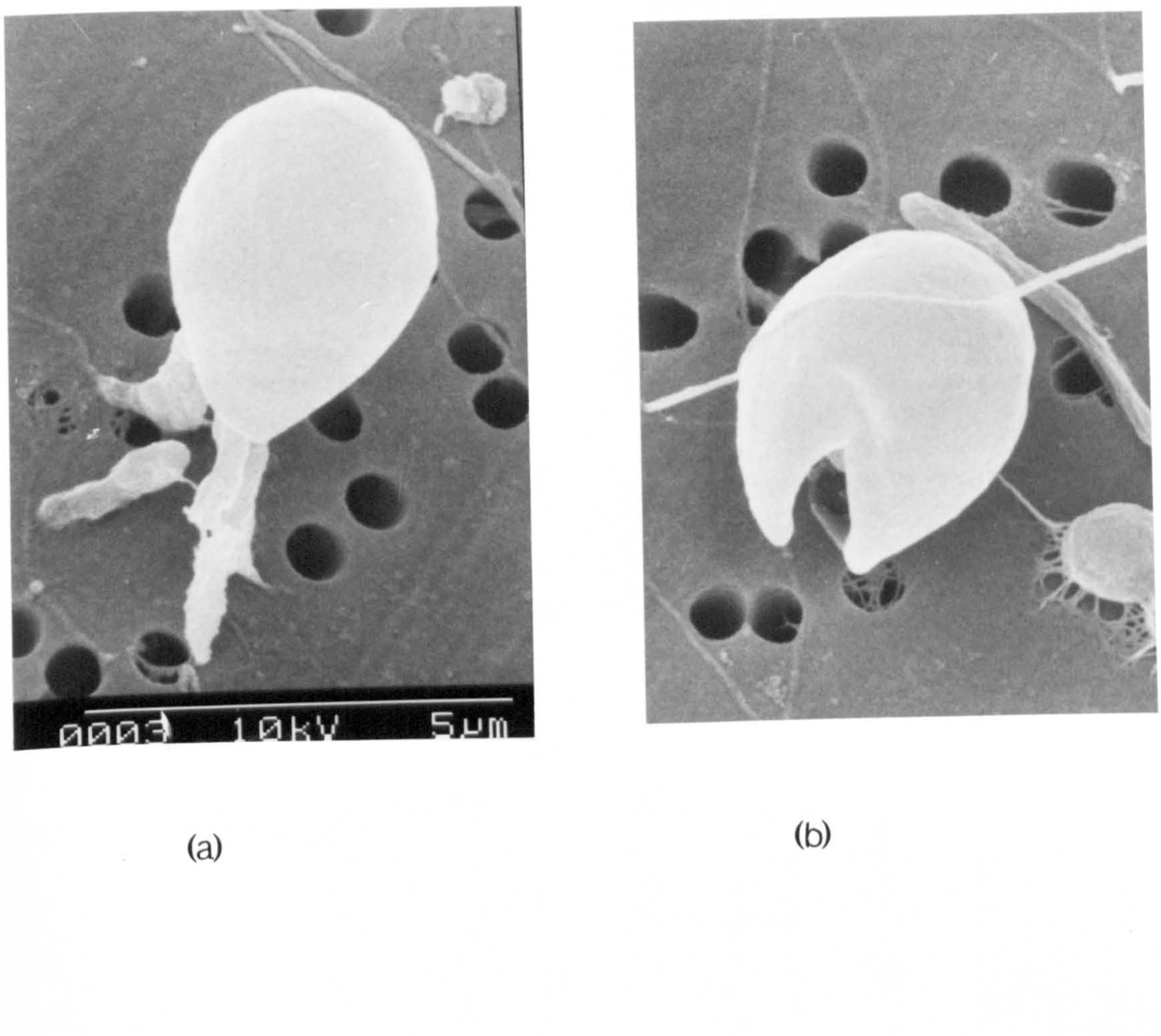


Fig 3.1 The life cycle of *Cryptosporidium parvum.*

3.1.4.2 The *Cryptosporidium* oocyst wall

C. parvum oocysts are spherical or ovoid structures, 4-6 μm in diameter (fig 3.2). Sometimes they may contain a fold or suture extending around one third of the oocyst circumference but this may not always be present and therefore is not a reliable diagnostic feature (Robertson *et al.*, 1993a).



(a) (b)

Fig 3.2 Scanning electron micrographs of *Cryptosporidium parvum* oocysts: (a) with emerging sporozoites and (b) an empty oocyst showing an open suture.

Coccidian oocyst walls typically have two layers, sometimes called the ectocyst and endocyst (Ryley, 1973). *Cryptosporidium* oocyst walls are also two-layered, with an outer layer of irregular thickness (10 nm average) and an inner layer (average 37.4 nm thick) which can be further divided into two layers (Reduker *et al*, 1985a). The coccidian oocyst wall layers can be separated by treating with high concentrations of sodium hypochlorite or hydrochloric acid. Ryley (1973) suggested that the ectocyst was likely to be composed largely of a quinone-tanned type protein because it both dissolved in sodium hypochlorite and could reduce ammoniacal silver chloride. It was thought that its inability to dissolve in sodium sulphide or sodium thioglycollate indicated that the ectocyst was unlikely to be formed from a keratin-type protein. The protein component of the *Eimeria tenella* ectocyst was characterised by its high proline content and lack of basic amino acids - carbohydrate was also present. The inner layer consisted of approximately 70% protein, 30% lipid and 1.5% carbohydrate (Ryley, 1973). A *C. parvum* oocyst wall protein amino acid sequence was also found to have a high proportion of proline as well as cysteine and histadine (Ranucci *et al.*, 1993).

The structure and composition of the *Eimeria tenella* oocyst wall was also examined by Stotish *et al.* (1978). They concluded that the relative proportions of peptide, lipid and carbohydrate were 67%, 14% and 19% respectively. The peptide was a repeating glycoprotein subunit of 10 kD within which all of the oocyst wall carbohydrate was located. It was proposed that the oocyst wall structure had a 10 nm lipid outer layer covering a 90 nm thick layer of glycoprotein linked by disulphide bridges. The outer layer of the *C. parvum* oocyst wall is thought to have a very low lipid content and the small amount present appears to be virtually immobile (H.V. Smith, pers. comm.).

Interest in *C. parvum* oocyst wall proteins, in particular those located at the oocyst surface is important with regard to both the development of monoclonal antibodies for use in diagnosis and environmental detection, and for research into the immune response to *Cryptosporidium.* It is thought that carbohydrates form a large proportion of the epitopes present on the *C. parvum* oocyst (Luft *et al.*, 1987). Analysis of the cyst wall of another protozoan intestinal parasite *Giardia*, has indicated that the filamentous layer is primarily comprised of a carbohydrate peptide complex (Manning *et al.*, 1992). Further evidence for the presence of *C. parvum* surface glycoprotein has been suggested (Bonnin *et al.*, 1991). The agglutination of oocysts through the use of lectins Tomentine and UEA-II, has identified the presence of GlcNAc (n-acetyl glucosamine) upon the *C. parvum* oocyst surface (Llovo *et al.*, 1993).

Oocyst wall protein analysis has shown different levels of antigenic variation between different *Cryptosporidium* species (Tilley *et al.*, 1990b; Nina *et al.*, 1992a), between host type, and between isolates (Nina *et al.*, 1992b). Some common wall protein bands were also found for different species (Tilley *et al.*, 1990b). Despite the antigenic variation between isolates, a common protein of 32,000 MW, located on the oocyst wall was suggested as a suitable candidate for the preparation of an antibody probe (Lumb *et al.*, 1988).

3.1.4.3 Cryptosporidiosis

Cryptosporidiosis is the disease resulting from infection with *Cryptosporidium*, which can range from being asymptomatic to life-threatening. The most common clinical feature is characteristic profuse, watery ("cholera-like") diarrhoea. This is often associated with weight loss and other less common clinical features include abdominal pain, nausea and vomiting, and low-grade fever. The severity and longevity of infection usually depends upon the immune status of the host. Generally, immunocompetent individuals experience a short-term, self-limiting illness whereas immunocompromised, e.g. AIDS patients suffer from a prolonged and life-threatening condition. A respiratory cryptosporidial infection can also occur in immunocompromised patients, with or without simultaneous intestinal disease (Navin and Juranek, 1984; Janoff and Reller, 1987; Current, 1988; Current, 1986; Current and Garcia, 1991). There is presently no effective chemotherapy for cryptosporidiosis.

The diarrhoea-causing mechanisms of *Cryptosporidium* infection are poorly defined although malabsorption and impaired digestion (via parasite induced villus damage) may occur within the gastrointestinal tract. This may lead to overgrowth of intestinal microflora, an osmotic pressure change across the wall of the gut and consequent fluid influx to the intestinal lumen (Current and Garcia, 1991).

3.1.4.4 Waterborne outbreaks of cryptosporidiosis

Cryptosporidiosis is transmitted by a faecal-oral route. The infection can be spread in many different ways, e.g. by zoonosis and person-to-person contact (Casemore, 1991; Robertson and Smith, 1992). Waterborne transmission has been the reported cause of several cryptosporidiosis outbreaks, although it cannot always be proven to be the result of inefficient treatment processes. Documented waterborne outbreaks include: Braun Station, Texas, 1984; Sheffield, 1986; Carrollton, Georgia, 1987; Ayrshire, 1988; Swindon and Oxfordshire, 1989; North Humberside, 1989; Milwaukee, Wisconsin, 1993 (Anon, 1990; Smith, 1992; Smith *et al.*, 1989; Rush *et al.*, 1990; Poulton *et al.*, 1991; Addiss, *et al.*, 1995). At the time of writing of this thesis, an outbreak was reported in Devon, UK (Anon, 1995) The EU directive relating to drinking water states that all drinking water should be pathogen free (Robertson and Smith, 1992).

3.1.4.5 Detection methods for *C. parvum* oocysts

Techniques have been developed either to detect oocysts in faeces for diagnostic purposes (Sterling and Arrowood, 1986; Garcia *et al.*, 1987; Chapman *et al.*, 1990) or for monitoring levels of oocysts in water.

The standard method for the recovery and detection of *Cryptosporidium* oocysts from water is inefficient, sometimes less than 10% (Anon, 1990; Vesey and Slade, 1991; Vesey *et al.*, 1993a). One hundred litres of water are passed through a 1 µm pore-size polypropylene cartridge filter. Oocysts and any similar sized particles are collected. The oocysts are eluted using detergent and are separated from similar sized debris by density gradient centrifugation. A fluorescently labelled monoclonal antibody is added to identify oocyst presence using microscopy (Musial *et al.*, 1987; Rose *et al.*, 1988).

Recent improvements upon this methodology are being adopted by parts of the water industry. For example, calcium carbonate flocculation may be used to concentrate oocysts in water with recovery efficiencies of 69-80% (Vesey *et al.*, 1993b). Fluorescent monoclonal antibodies are added to the concentrate and flow cytometry, which is capable of concentrating particles of defined size and fluorescent intensity, is used to sort the oocysts onto slides. Confirmation of *Cryptosporidium* oocyst identity is made using epifluorescence microscopy (Vesey *et al.*, 1993b). An increase in the number of observable features within the oocysts could be achieved by the combination of DAPI (4',6-diamidino-2-phenylindole) viability staining with epifluorescence microscopy (Grimason *et al.*, 1994).

A recently proposed method for oocyst detection in environmental samples involves the use of cooled charge coupled devices (CCDs) which detect photon emission. They are able to detect fluorescence of labelled oocysts at low magnification. The associated software can use this information to calculate the dimensions of the particle and thus identify it (Campbell *et al.*, 1992a; 1993a).

Chemiluminescence, the conjugation of a luminescent compound to an enzyme-labelled antibody has also been investigated with regard to *C. parvum* oocyst detection (Campbell *et al.*, 1993b). Presently the sensitivity of this technique is limited by the specificity of the monoclonal antibody.

The use of the polymerase chain reaction (PCR) is under investigation for the identification of *Cryptosporidium* oocysts. This has enormous potential for the detection of *Cryptosporidium* oocyst species and perhaps even strain virulence. Sporozoite nucleic acid extracted from oocysts can be detected using a labelled oligonucleotide probe but the current limits of its detection make it unsuitable for use in water samples (Smith *et al.*, 1993; Johnson *et al.*, 1993).

Detection techniques in current use are unable to determine oocyst viability. Use of flow cytometry has increased speed of analysis but gives no information regarding whether oocysts are alive or dead (Vesey *et al.*, 1995). *In vitro* excystation assay or animal infectivity are the conventional methods for viability assessment. They both involve the need for large numbers of oocysts and results are affected by the presence of contaminating debris (Smith *et al.*, 1993).

The application of electrorotation assay (ERA, see section 1.1.5) for *Cryptosporidium* oocyst viability is under investigation (Smith *et al.*, 1994). Preliminary studies indicated that at a particular frequency, viable oocysts could be induced to rotate clockwise in a non-uniform AC electric field whilst non-viable oocysts rotated anti-clockwise.

The use of vital stains has been studied (Smith *et al.*, 1991), particularly the inclusion/exclusion of fluorogenic dyes, DAPI and PI (propidium iodide) (Campbell *et al.*, 1992b). It correlates well with *in vitro* excystation and highlights the morphology of the sporozoites inside the oocyst, providing further evidence for oocyst recognition.

3.2 MATERIALS AND METHODS

3.2.1 Dielectrophoresis of *Cryptosporidium parvum* oocysts

3.2.1.1 Microorganism

Cryptosporidium parvum oocysts were originally obtained from the Moredun Research Institute, Edinburgh, UK and supplied through Yorkshire Water Service plc, York, UK as a concentrated suspension (typically 10^9 oocysts ml^{-1}) in phosphate buffered saline. They were obtained from faecal samples taken from new-born lambs and purified using sedimentation and filtration methods (Hill *et al.*, 1990). A 5% aqueous dilution of faeces was acidified (pH 5 to 6) in order to flocculate contaminating debris. Oocysts were repeatedly washed in tap water and re-suspended in 1% sodium dodecyl sulphate (SDS) for 60 min at room temperature. They were then washed a further 3 times in tap water and stored at 4°C in Hanks balanced salt solution (HBSS) containing antibiotics, penicillin and streptomycin.

3.2.1.2 Preparation of untreated oocysts

A stock suspension of oocysts was vortexed and a 50 µl sample (containing approximately 10^7 oocysts) was extracted. This was diluted to 3-4 ml with deionized water, filtered using a sterile syringe filter (0.2 µm pore size; Minisart, Sartorius) and re-suspended in 3-4 ml of deionized water. A routine count of untreated oocyst samples was performed in order to assess the number of empty oocyst shells present in the stock suspension.

3.2.1.3 Preparation of chlorine-treated oocysts

A 50 µl sample of untreated oocysts was prepared as described above but this time the oocysts were re-suspended in 10 ml of sodium hypochlorite solution (Fisons) at pH 7.0. This was previously prepared by the addition of concentrated sodium hypochlorite to 0.01 M phosphate buffer, pH 7.0 and was adjusted back to pH 7.0 by the addition of small amounts of the stock buffer solutions. Chlorine demand-free water and glassware were used both in the preparation of the buffers and throughout all other sample manipulations (Korich *et al.*, 1990). The oocysts suspension was vortexed and left for 40 min to provide a C*t* (concentration x time [in minutes]) value of 20. The required chlorine concentration of 0.5 mg l^{-1} had previously been quantified using a comparator (Lovibond 2000 Mk. II, Tintometer Ltd., Salisbury, UK). The reaction was subsequently quenched by the addition of a 2 ml volume of 10% (w/v) sodium thiosulphate solution (Fisons). A similar sample had been used previously to establish that a residual level of chlorine remained after the contact time had expired. The procedure was repeated using a further oocyst sample which had been treated with 5 mg l^{-1} chlorine providing a C*t* value of 200. A further control experiment was performed using an untreated oocyst sample which was exposed to 2 ml 10% (w/v) sodium thiosulphate solution alone. This was to demonstrate that this treatment appeared to have no additional effect on the dielectrophoretic response of chlorine-treated or ozone-treated oocysts.

The oocyst suspensions were washed twice by filtering using deionized water in order to lower their conductivity levels, before being re-suspended in 3-4 ml of deionized water ready for use.

3.2.1.4 Preparation of ozone-treated oocysts

Calibration of the laboratory ozonator

Ozonation was performed using a laboratory ozonator (Model DA 023, Wallace and Tiernan, UK). An air flow rate of 150 l h^{-1} into the ozonator was used to treat the oocyst suspension. The ozone concentration was determined by the indigo method of Bader and Hoigné (1982).

Ozone de-colourises indigo trisulfonate in acidic solution, stoichiometrically. A stock solution of indigo trisulfonate was made. To a 1 l flask, 1 ml concentrated AR grade phosphoric acid (Fisons) and 500 ml distilled water were added. The solution was stirred while 770 mg potassium indigo trisulfonate (Sigma) was added, and the flask was finally made up to 1000 ml using distilled water. A 100-fold dilution of this stock solution measured OD_{600} 0.2 ± 0.1 cm^{-1} and was useful for up to 4 months when stored in the dark.

The indigo reagent used for the ozonator calibration ("indigo reagent I") was made using the stock solution. To a 1 l flask, 20 ml of the stock solution, 10 g of AR grade sodium dihydrogen phosphate (Fisons) and 7 ml concentrated AR grade phosphoric acid were added. This was made up to 1000 ml with distilled water and had a shelf life of one week.

For the calibration procedure, two 100 ml flasks (A and B) were used. To each of the flasks, 10 ml of indigo reagent I was added. Flask A was filled up to 100 ml with distilled water. Flask B was filled up to 100 ml using the ozonated water sample. If expected ozone concentrations were greater than 0.3 mg l^{-1}, a reduced amount of ozonated water was added to flask B and the total volume made up to 100 ml with distilled water. In both flasks, the respective solutions were stirred constantly during the addition of the water samples. A 1 ml sample was removed from each flask into 1 ml quartz cuvettes (Spectrosil-UV-VIS, Phillip Harris, UK) and the absorbance at 600 nm read for both using a Model 4050 Ultrospec II spectrophotometer (LKB Biochrom, Cambridge, UK). The difference in absorbance values between the control and the ozone treated flasks was recorded and the ozone concentration worked out using an equation:

$$[O_3]\ \text{mg}\ l^{-1} = \frac{\Delta A \,.\, 100}{f.b.v}$$

where ΔA is the difference in absorbance between the sample (flask B) and the control (flask A), b is the path length of the cuvette (cm), v is the volume of ozonated sample added (ml) and f is 0.42.

Deionized water samples were ozonated for between 1 and 15 min at 1 min intervals. Ozone demand-free water and glassware were used throughout (Korich *et al.*, 1990). A replicate was taken for each of the 15 ozonations. The ozonator was run to waste for 30 min before the start of the ozonation procedure and was left running until all sample ozonation had been completed. A curve relating ozonation time (min) to residual ozone concentration (mg l^{-1}) was plotted (fig 3.3)

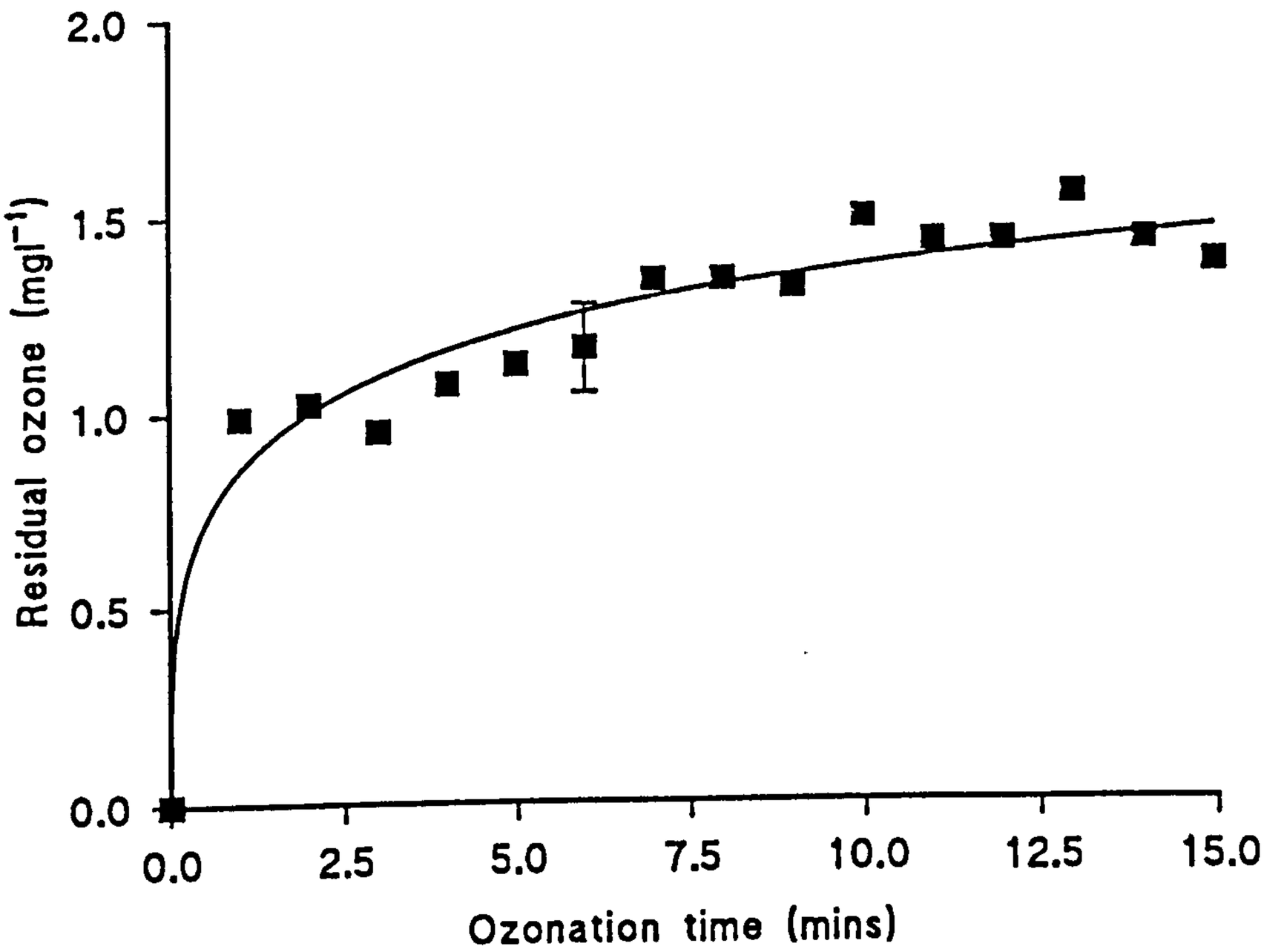


Fig 3.3 Calibration curve for the Model DA O23 laboratory ozonator (Wallace and Tiernan, UK).

Ozonation procedure

A 30 ml volume of ozone demand-free water in a 50 ml ozone demand-free flask (Korich *et al.*, 1990) was spiked with a 2 ml suspension containing approximately 2 x 10^7 *Cryptosporidium* oocysts. This amount had been previously determined to provide a residual concentration of approximately 10^7 oocysts after accounting for losses in the ozonation procedure. These were incurred due to thorough washing, performed using filtration, which was necessary to reduce the conductivity level of the suspension, as well as the inevitable formation of aerosols during ozonation. Ozone was bubbled through the oocyst suspension which was maintained at 25°C with constant stirring. The ozonation apparatus was housed in a fume cabinet. Immediately upon completion of the ozone treatment the oocysts suspension was transferred to a centrifuge tube containing 2 ml 10% (w/v) sodium thiosulphate in order to quench the reaction. The ozonated oocyst suspension was subsequently washed twice by filtration and re-suspended in 3-4 ml deionized water.

3.2.1.5 *In vitro* excystation assay

Previous studies which employed both *in vitro* excystation assays and mouse infectivity experiments to assess oocyst viability, examined the disinfection effect of 1 mg l^{-1} ozone (Korich *et al.*, 1989, 1990). These workers showed that a C*t* value of between 5 and 10 rendered 99 to 99.9% of *Cryptosporidium parvum* oocysts inactive. They also demonstrated that treatment with free chlorine using C*t* values greater than 200 did not alter oocyst viability. As a means of estimating the effects of ozone treatment performed in the current study upon oocyst viability, the *in vitro* excystation method of Robertson *et al.* (1993b) was employed. Suspensions of oocysts were centrifuged and re-suspended in 100 µl Hanks balanced salt solution (HBSS) (Gibco) and incubated at 37°C for 1 h. To each of the oocyst suspensions, 200 µl of 1% bovine bile (Sigma) in Hanks minimal essential medium (HMEM) (Gibco) and 50 µl 0.44% sodium hydrogen carbonate in deionized water were added. These reagents had been freshly prepared less than 30 min before use and incubated for a short time at 37°C.

The excystation mixture was incubated at 37°C with shaking for a minimum of 4 h and the extent of excystation subsequently examined using Hoffman Modulation Contrast microscopy (Hoffman, 1977) at a total magnification of x 1000. The percentage excystation was calculated from:

(Number of excysted/Number of excysted + intact oocysts) x 100.

Sporozoite ratios were not calculated as many were lysed by the end of 4 h incubation period.

3.2.1.6 Dielectrophoresis apparatus

The apparatus used is described in section 2.2.1. The electrodes were observed using a 10 x objective giving an image on the monitor with a total magnification of x 1360. This magnification was sufficient to allow for oocyst detection although morphological detail including excystation status could not be distinguished (this was determined subsequent to dielectrophoretic experimentation).

3.2.1.7 Dielectrophoretic collection measurements

The parameters used were chosen so as to yield optimum oocyst collection (as described in chapter two). The frequencies of the applied pulse ranged from 1 kHz to 10 MHz, with measurements taken ten times per decade of log frequency. Other typically used parameters were: 10 s applied electric pulse; 12-14 v; 6-8 s collection counting period; 5 s flush through period; 5 s baseline counting period; 2 rpm pump speed (increasing to 20 rpm during the application of the electric pulse).

3.2.1.8 Analysing the ratio of collection at two key frequencies

To establish the statistical significance in the spectra obtained from untreated and ozone-treated oocyst samples, two further experiments were performed. A range of oocyst samples were treated with different doses of ozone as described above.

In the first experiment, six oocyst samples were ozonated for 0.5, 1, 3, 4, 5 and 10 min. These corresponded to residual ozone doses of 0.42, 0.99, 2.86, 4.28, 5.56 and 14.88 mg.min l^{-1} ozone respectively, according to the ozonator calibration curve. Twenty repeated pairs of dielectrophoretic measurements were taken at 100 kHz and 10 MHz respectively for the 30 s ozonated sample and the untreated control sample. Forty pairs of measurements were taken for the other ozonated samples. All samples were subjected to an *in vitro* excystation assay subsequent to dielectrophoresis. Student's t-tests were performed between the mean ratios of each individual ozonated sample and that of the untreated control.

In the second experiment, the same procedure was followed but this time, eight oocyst samples were ozonated for 0.5, 1, 2, 3, 4, 5, 7 and 10 min. These corresponded to residual ozone doses of 0.42, 0.99, 2.05, 2.86, 4.28, 5.60, 9.44 and 14.88 mg.minl^{-1} ozone respectively. Sixty repeated pairs of measurements at 100 kHz and 10 MHz were taken for all ozonated samples and the untreated control.

3.2.2 Ultraviolet irradiation

Ultraviolet (UV) radiation was used to treat stationary phase *E. coli* cells and samples were subsequently used to investigate UV treatment effects upon the dielectrophoretic response over time. Stationary phase cells were chosen for investigation as these were thought to be representative of indicator bacteria targeted in water disinfection using UV irradiation.

3.2.2.1 Preparation of microorganism for experiments

For survival curve using stationary phase cells

E. coli was purchased from NCIMB, Aberdeen, UK (NCIMB 11459). This was grown up into stationary phase (20 h at 37°C with shaking) in nutrient broth (Oxoid). A growth curve had been established previously (section 4.2.4). Aliquots of 2 ml were removed sequentially, washed 3 times in sterile deionized water using a 0.2 µm syringe filter (Minisart, Sartorius) and re-suspended in sterile deionized water to an OD_{510} of approximately 0.13 (± 0.01). A 25 ml volume was aseptically transferred to a sterile 9 cm diameter Petri dish (Phillip Harris Scientific, UK) for UV treatment.

For dielectrophoretic experiments

Stationary phase cells were grown as described above. A sample was irradiated for a time period determined by the death curve. Upon completion, two 1 ml samples were taken for viability assessment and a 10 ml sample was used for dielectrophoresis. This was washed twice in sterile deionized water using a 0.2 μm sterile syringe filter (Minisart, Sartorius) and finally re-suspended in sterile deionized water to OD_{510} approximately 0.13. The pH measurement of each sample was recorded at the beginning and end of each experiment and the conductivity levels were monitored throughout.

3.2.2.2 UV irradiation apparatus

The UV apparatus is shown in fig 3.4 The UV source was provided by a Phillips TUV6 bulb (Starna Ltd., Essex, UK) installed into a mains lamp. Both the wavelength range and the intensity of the radiation produced from the UV source were measured using a radiometer (Macam Photometrics Ltd., Livingstone, Scotland).

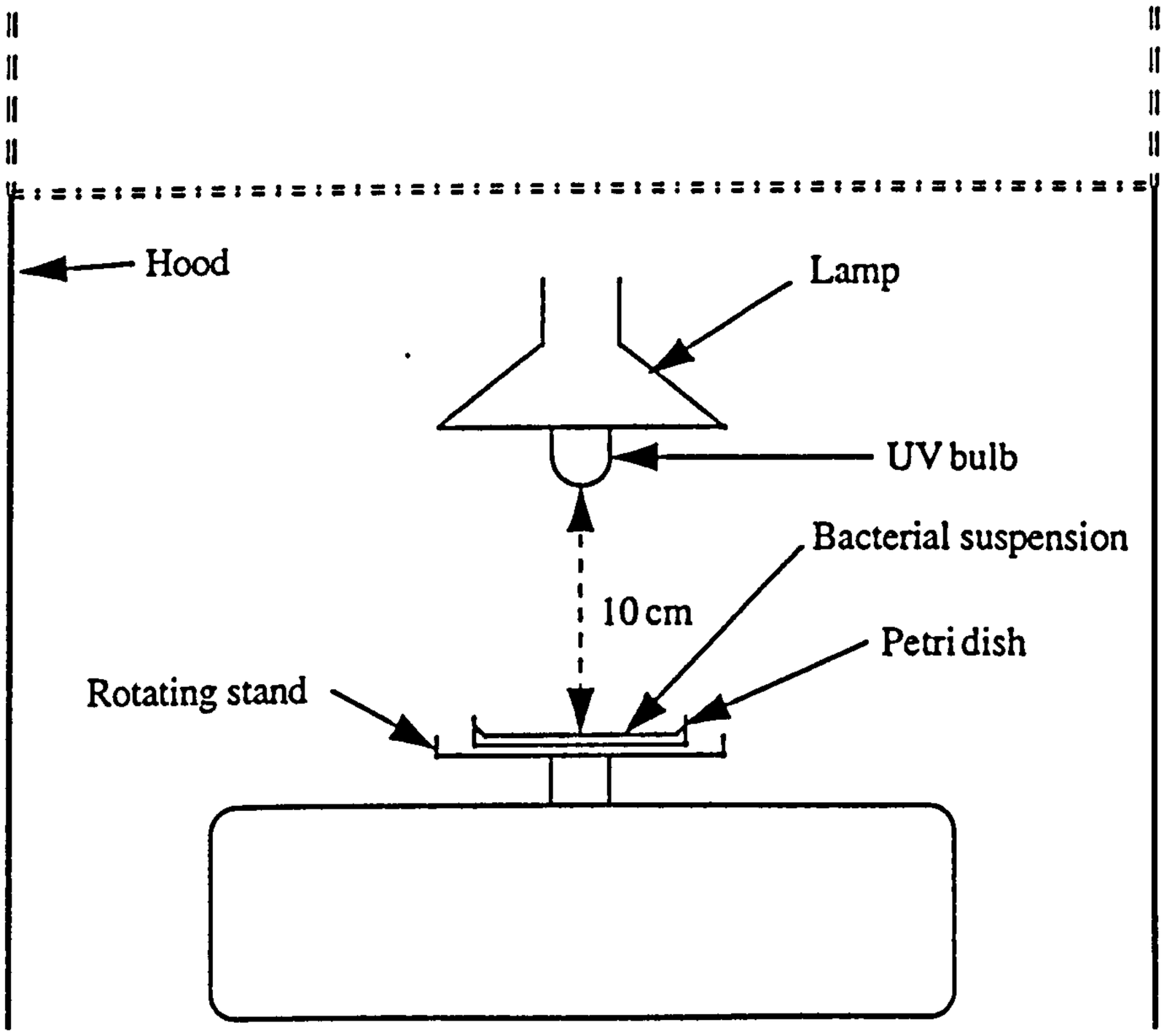


Fig 3.4 Apparatus used for UV irradiation of *E. coli* cell suspensions

A rotating plater suitable to hold a 9 cm diameter plastic Petri dish was constructed. This rotated at a speed of 34 rpm. The stand was positioned so that the centre of the Petri dish was perpendicular to the UV bulb at a distance of 10 cm below. A hood was placed over the entire apparatus to shield the surrounding area during UV irradiation. The temperature beneath the hood during prolonged irradiation was found to increase to over 30°C therefore the hood was modified by increasing the height and omitting the top panel so that the temperature remained relatively unchanged.

Calibration of UV lamp

(i) Wavelength measurement

The wavelengths of UV light emitted from the Phillips TUV 6 bulb were measured using the radiometer. The detector of the radiometer was placed perpendicularly below the UV source. A 10 cm distance below was selected (Dr G. Shama, pers. comm.). The dose emitted from the UV source was measured ($\mu W.m^{-2}$) for selected wavelengths in the range 200 to 400 nm. The maximum dose from this UV source was expected at a wavelength of approximately 254 nm.

(ii) Intensity measurement

The intensity of the UV radiation covering an area equivalent to that occupied by a 9 cm diameter Petri dish, 10 cm below the UV source, was measured. The purpose of this was to optimize positioning of the lamp to achieve maximum UV light intensity. It was necessary to modify the set-up of the radiometer for this application The intensity detector of the radiometer was placed into position and the dose (for this application measured in $w.m^{-2}$) was recorded at a variety of positions over a Petri dish sized area. An optimal position was identified over which the maximum intensity was produced.

3.2.2.3 Determination of lethal UV dose

Survival curve for stationary phase cells

A microbial survival curve was produced by irradiation of cell suspensions for varying time intervals (1-60 min). Cell suspensions were prepared as described in section 3.2.2.1. The first samples (1, 2, 5, 7, 10, 12, 15, 20 and 25 min irradiated) were plated out immediately after UV treatment. Two 1 ml aliquots were removed from each sample and 10-fold serial dilutions were performed down to 10^{-6} before 6 replicate 0.1 ml volumes were plated onto nutrient agar. Later samples (30, 40, 45, 50 and 60 min irradiated) were incubated for 3 h at 37°C in daylight before aliquots were removed for plating out. This was to allow for the inclusion of UV repaired cells in the viable counts. Due to the reduction in viability through longer UV exposure it was not necessary to dilute these latter samples down to 10^{-6}. The number of dilutions performed was reduced as the UV exposure period was lengthened. A survival curve including two sets of data (for cell viability immediately after UV treatment and for 3 h post-treatment incubation) was produced. The total UV dose applied for lethal and sub-lethal injury of cells was calculated.

3.2.2.4 Dielectrophoresis of stationary phase *E. coli* NCIMB 11459

Dielectrophoresis equipment

The dielectrophoretic equipment described in section 2.2.1 was used with certain modifications. Phase contrast microscopy of x400 magnification was needed to provide a sufficient resolution level for cell detection due to the small size of the bacteria (typical length 1-2 μm). A long working distance 40x objective was installed (MTN,LWD, DLC 40x, Nikon, Japan) to give increased magnification and still allow for use of the electrode chamber. The electrode chamber was modified by using a top cover-slip made out of glass. This was necessary for the improved resolution required at the higher magnification. A 37°C water bath was also installed alongside the dielectrophoretic equipment.

Dielectrophoretic procedure

The cell suspension was re-circulated through the electrode chamber and the detection level of the "Domino" image analysing system was set. The parameters used during dielectrophoresis were:

Frequency range	1 kHz-50 MHz
Measurements per decade of log frequency	10
Voltage	10 V
Length of pulse	10 s
Length of peak time	8 s
Length of base time	5 s
Length of flush-through	5 s

The pump speed was set at 1.5 rpm but this was increased to 15 rpm during the 10 s time period that the electric field was applied to the electrodes. Maximized collection levels were achieved by increasing the number of cells brought in closer to the electrode area. Both the sample and the conductivity probe were contained within a 37°C water bath.

3.3 RESULTS

3.3.1 Dielectrophoresis of chlorine-treated *C. parvum* oocysts

The mean of two spectra using untreated oocysts, the mean of two spectra using 0.5 mg l^{-1} chlorine-treated oocysts (Ct = 20) and the mean of two spectra using 5.0 mg l^{-1} chlorine-treated oocysts (Ct = 200) are shown in fig 3.5.

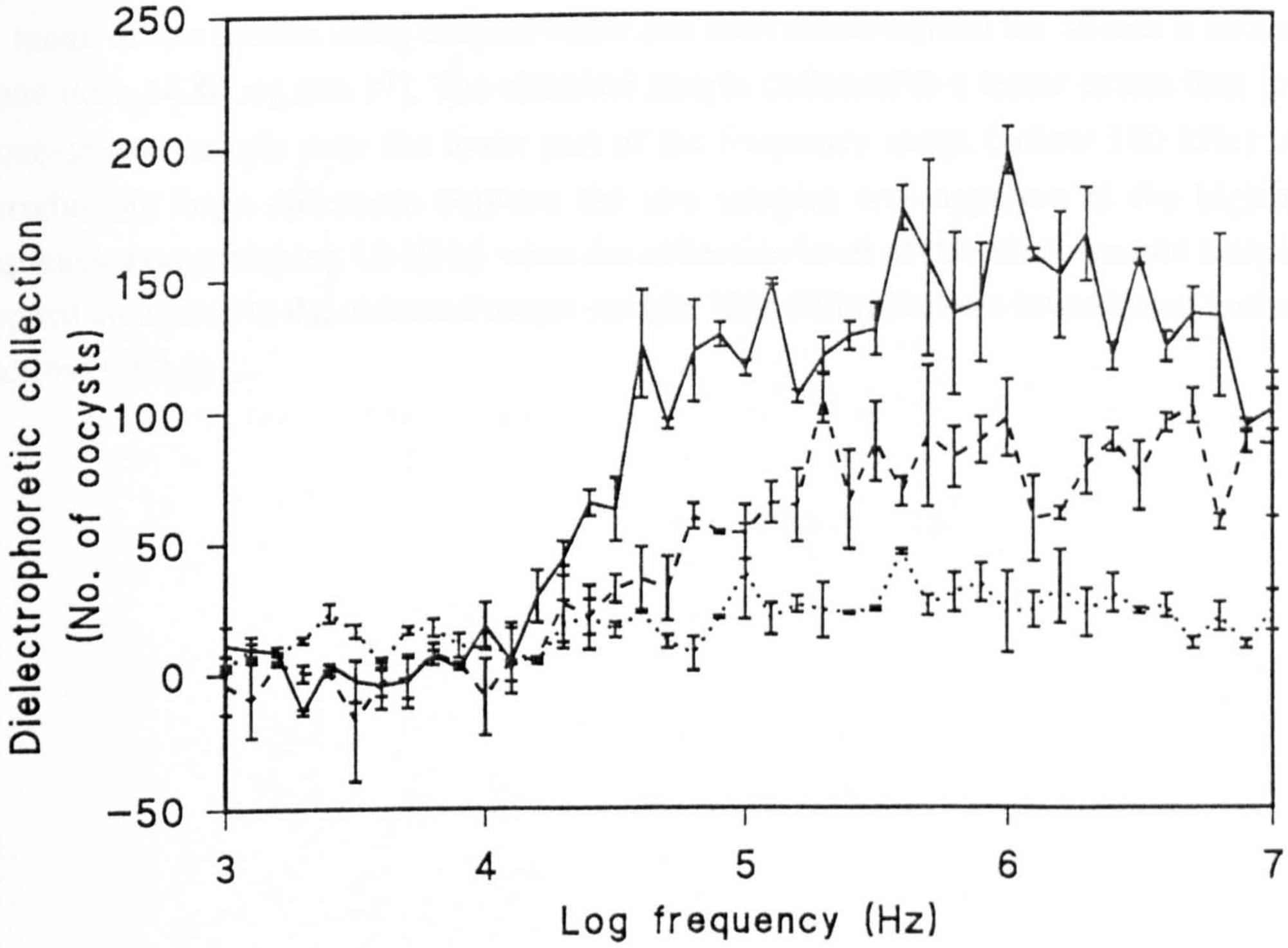


Fig 3.5 Mean spectra (n=2) of untreated (——), 0.5 mg l^{-1} chlorine-treated (- - - -) and 5.0 mg l^{-1} chlorine-treated (• • • • •)*C. parvum* oocysts.

The untreated oocyst spectrum is quite typical in that collection begins to rise between 20-30 kHz for a sample in low conductivity medium. The treatment with chlorine caused a progressive decrease in collection over the whole frequency range with increasing chlorine concentration. The *in vitro* excystation of the samples were 80.27% (SE = 2.52), 79.13% (SE = 2.68) and 71.48% (SE = 2.25) for the untreated, 0.5 mg l^{-1} chlorine-treated sample and the 5.0 mg l^{-1} chlorine-treated sample respectively. A minimum of three fields of view were counted in each case.

3.3.2 Dielectrophoresis of ozone-treated *C. parvum* oocysts

Fig 3.6 shows the mean of 2 spectra using untreated *Cryptosporidium parvum* oocysts and the mean of two spectra using oocysts which had been ozone-treated for 10 min (residual ozone dose 14.88 mg.min l^{-1}). The untreated sample collected to a lesser extent than the ozone-treated sample over the lower part of the frequency range (below 100 kHz). A reproducible large difference between the two samples was apparent at the highest frequencies (approaching 10 MHz) when the collection level of the ozone-treated sample dropped compared to the untreated oocyst sample. This difference was investigated further (section 3.3.2.1).

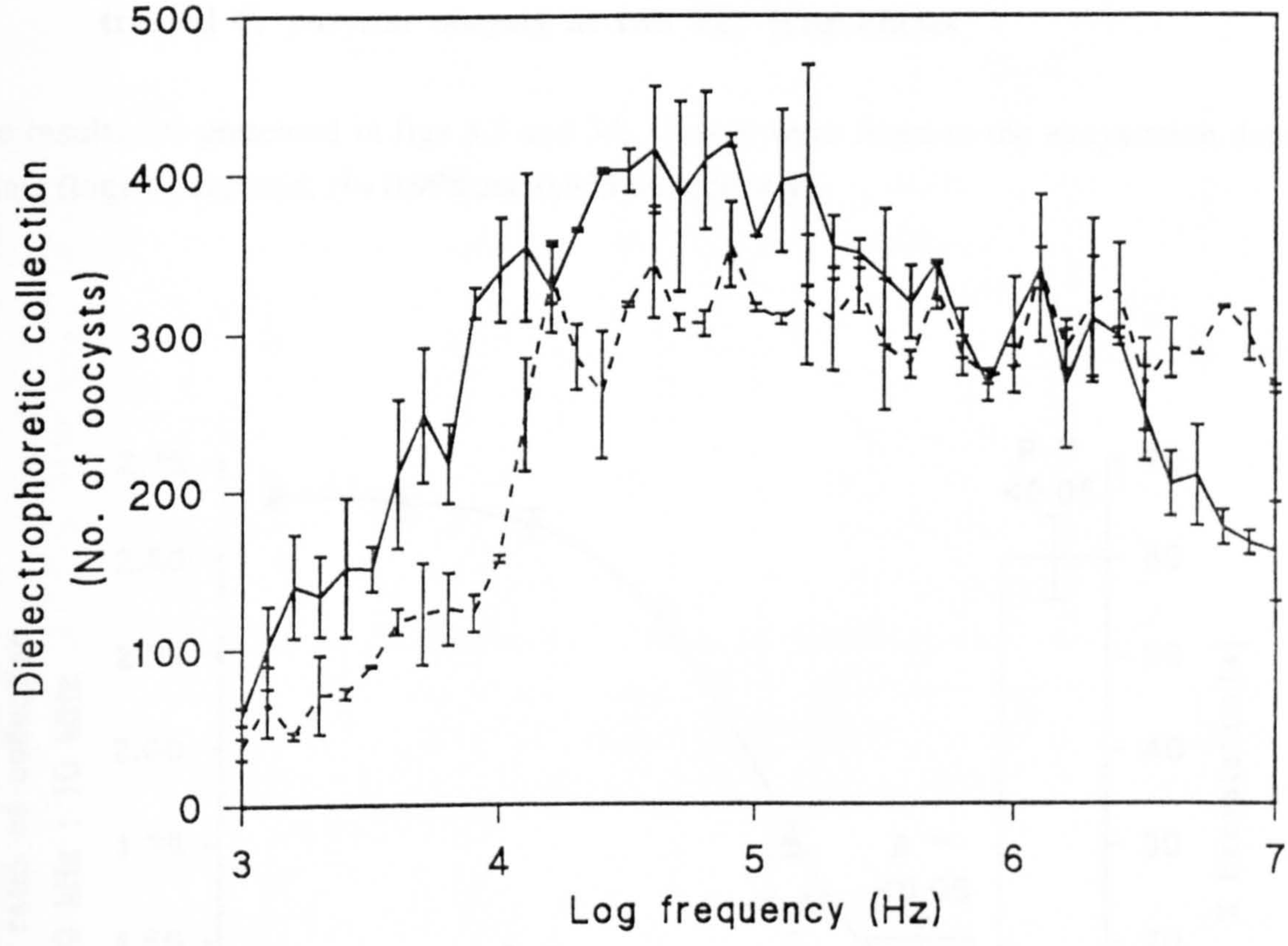


Fig 3.6 Mean spectra (n=2) of untreated (———) and ozone-treated [residual 14.88 mg.minl^{-1}] (- - - - - -) *C. parvum* oocysts.

The percentage excystation for the untreated sample was 82.12%, (SE 0.83) and for the ozone-treated sample was 5.62% (SE 0.84) with 16 fields of view counted in each case.

3.3.2.1 Analysis of dose-dependent dielectrophoretic collection of ozone-treated *C. parvum* oocysts at two key frequencies

The results are presented in figs 3.7 and 3.8. Curves were fitted to the excystation data points (logistic sigmoid, r^2= 0.998 and 0.955 respectively).

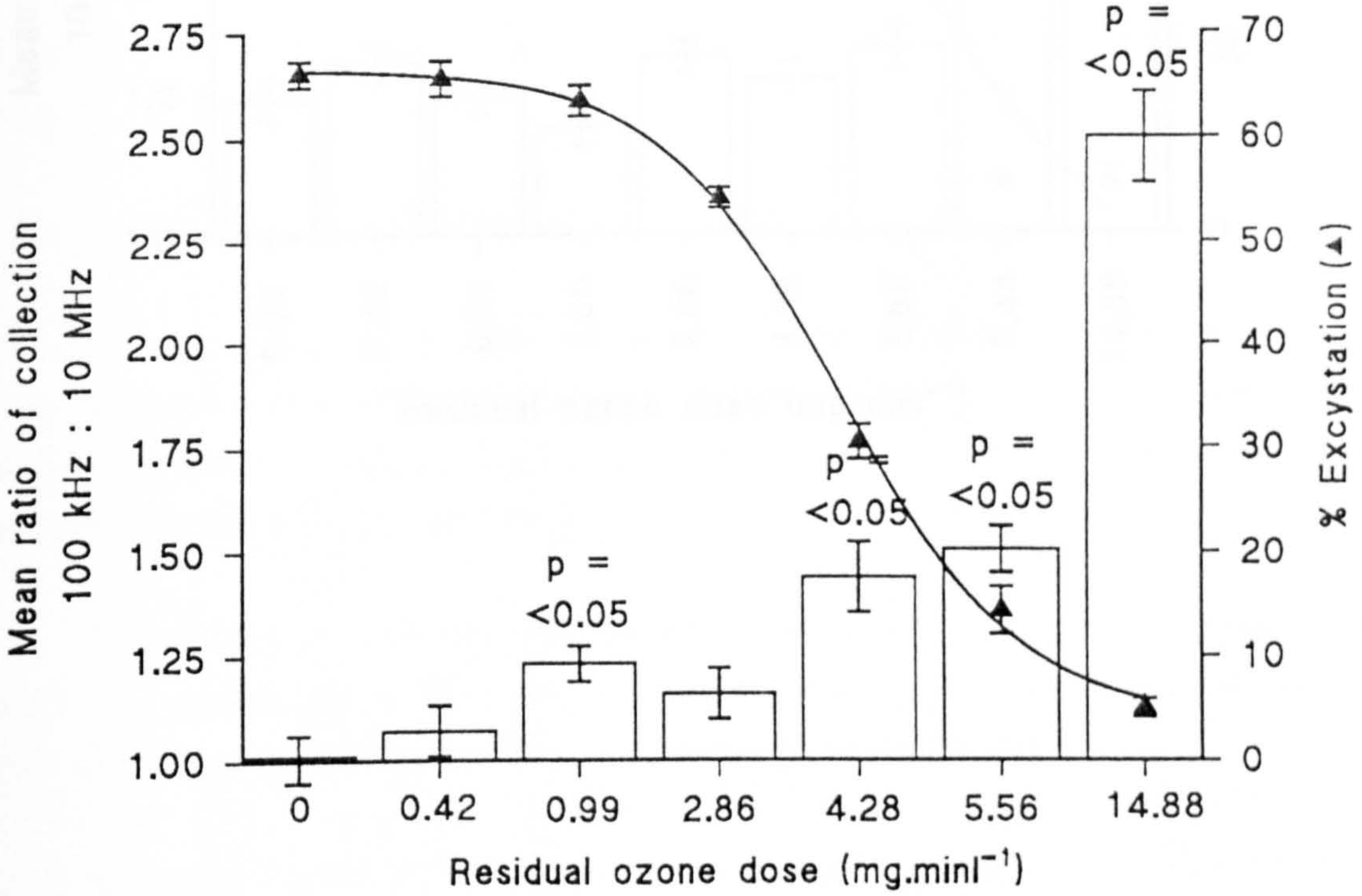


Fig 3.7 Mean ratios of untreated and ozone-treated C. *parvum* oocyst dielectrophoretic collection, between 100 kHz and 10 MHz. Six oocyst samples with varying ozone doses were used. The *in vitro* excystation level of each oocyst sample (▲) is included.

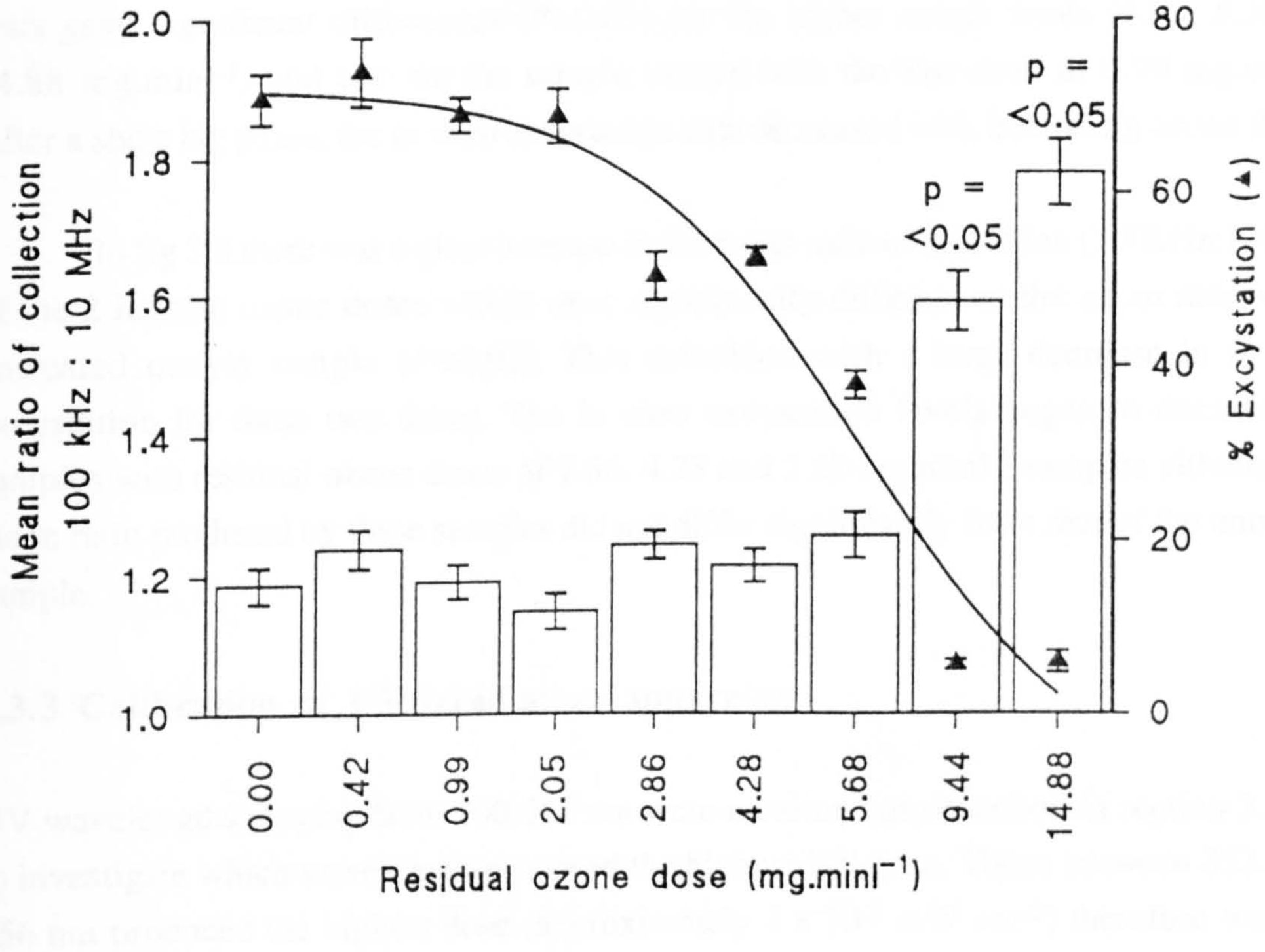


Fig 3.8 Mean ratios of untreated and ozone-treated *C. parvum* oocyst dielectrophoretic collection, between 100 kHz and 10 MHz. Eight oocyst samples with varying ozone doses were used. The *in vitro* excystation level of each oocyst sample (▲) is included.

In fig 3.7 the mean ratio of collection between 100 kHz and 10 MHz for all of the ozone-treated samples differed from that of the untreated sample, with the greatest difference occurring with the most heavily dosed oocysts (14.88 mg.minl^{-1}). Students t-tests gave significant differences (P<0.05) for the higher ozone doses (4.28, 5.56 and 14.88 mg.minl^{-1}) and also for the sample treated with the low dose of 0.99 mg.minl^{-1}. After a short lag phase, the *in vitro* excystation data decreased with increasing ozone dose.

In fig 3.8 there was a clear increase in the mean ratio of collection (100kHz:10MHz) of the 2 highest ozone doses which were significantly different to the mean ratio of the untreated oocyst sample (P<0.05). This coincided with a large decrease in *in vitro* excystation for these two doses. The *in vitro* excystation levels began to decrease for samples with residual ozone doses of 2.86, 4.28 and 5.60 mg.minl^{-1} samples although the mean ratio produced by these samples did not differ significantly from that of the untreated sample.

3.3.3 Calibration of UV irradiation apparatus

UV wavelengths ranging from 200-380 nm were measured as described in section 3.2.2.2, to investigate which wavelength produced the highest UV dose. Those between 253.7 and 256 nm produced the highest dose (approximately 4 x 10^{-3} mW cm^{-2}) therefore were the dominant wavelengths produced by the UV source.

The maximum intensity (the dose described above) was simultaneously found to occur (at a wavelength of 253.7 nm) at a position where the centre of the 9 cm diameter Petri dish was located 10 cm below and 3 cm "north" of the UV source.

3.3.4 Survival curve of UV-treated stationary phase *E. coli* NCIMB 11459

Fig 3.9 shows the number of survivors (cfu) over time. When samples were plated out immediately after irradiation the number of survivors able to grow to form colonies was reduced to zero at 25 min UV exposure. The inactivation rate was low for samples irradiated for time periods 0-12 min as shown by the shoulder on the survival curve. An exponential inactivation rate occurred for samples irradiated for time periods from 15-25 min.

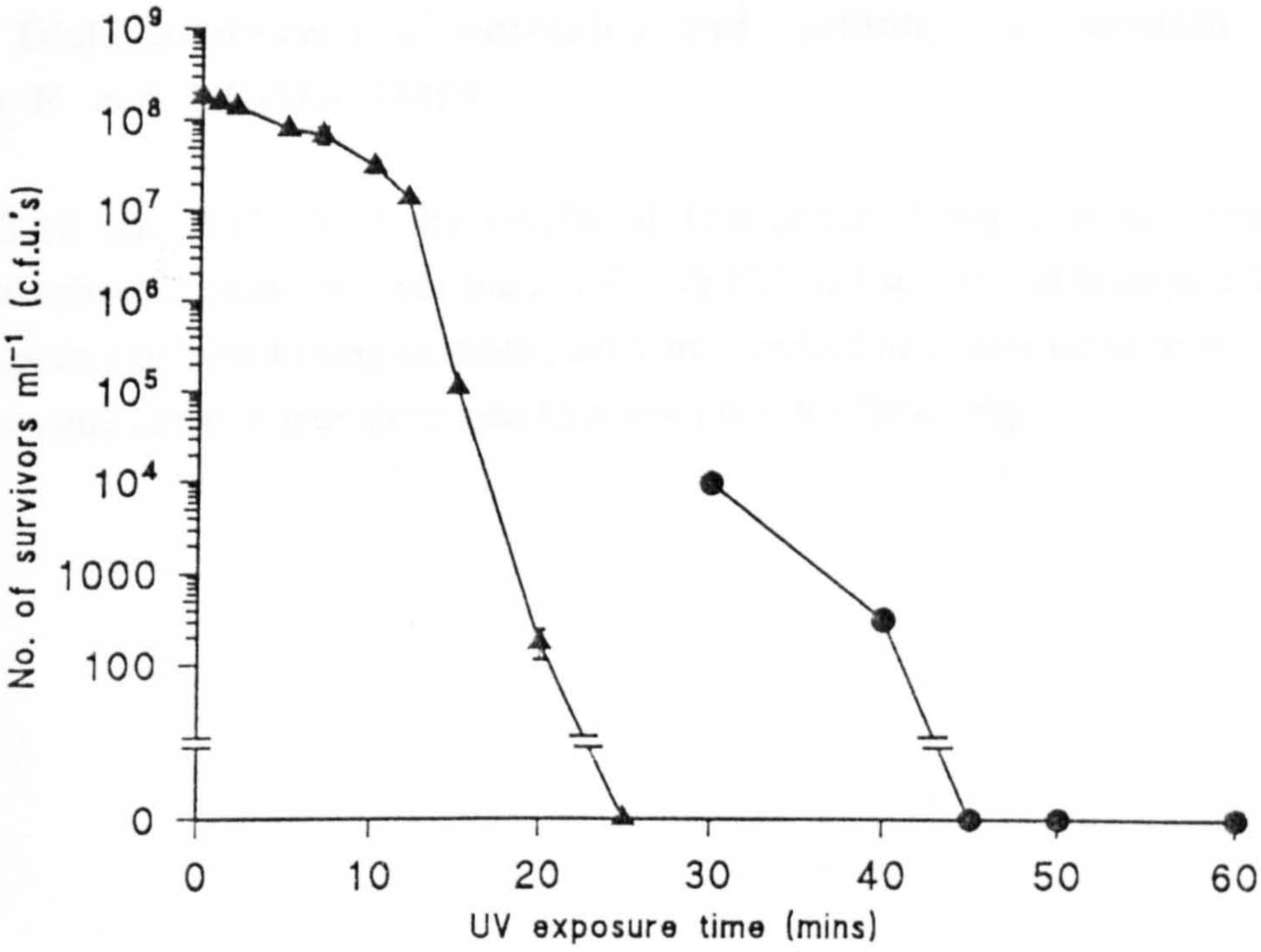


Fig 3.9 Survival curve of *E. coli* (NCIMB 11459) irradiated with 257 nm UV light: (▲) samples plated immediately after exposure (n=6), (●) sample plated after 3 h incubation, 37°C in daylight (n=4).

By plating out samples 3 h after UV treatment it was clear that a greater exposure time (45 min) was needed to ensure that no survivors remained. The total dose (intensity x exposure time) was calculated to be 7.2 mW sec^{-1} cm^{-2} for 30 min exposure and 14.4 mW sec^{-1} cm^{-1} for 60 min exposure.

3.3.5 Dielectrophoresis of untreated and "lethally" UV-treated stationary phase *E. coli* NCIMB 11459

Figs 3.10 and 3.11 show the results of two separate experiments measuring the dielectrophoretic response over time of *E. coli* NCIMB which had been irradiated for 60 min. Spectra obtained using untreated cells are overlaid in colour for comparison. In both figures some of the intermediate runs have been omitted for clarity.

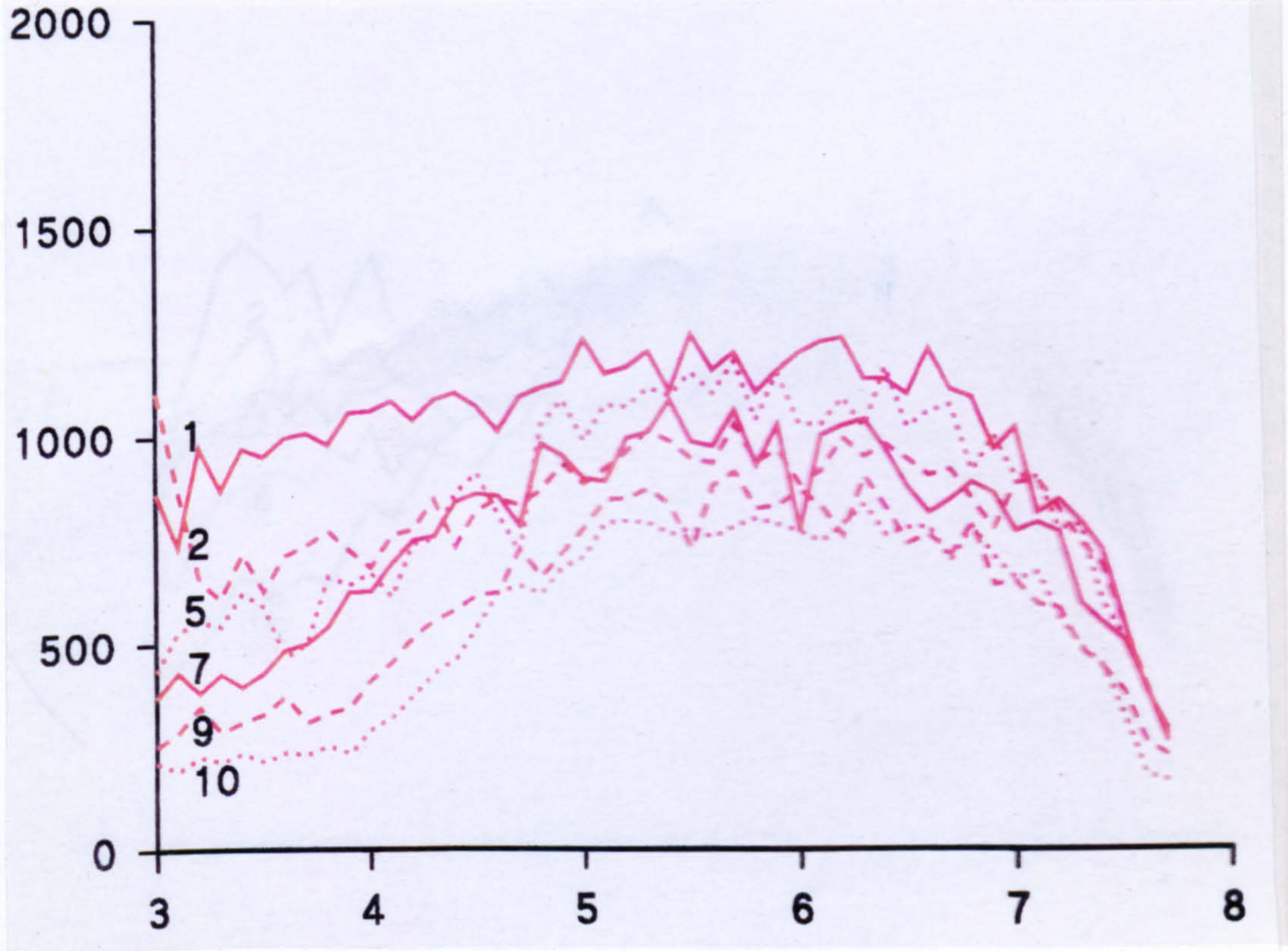


Fig 3.10 Dielectrophoretic spectra of untreated and 60 min UV-treated *E. coli* NCIMB 11459. Runs 1, 2, 3, 4, and 12 from 12 consecutive spectra using UV irradiated cells are plotted. Runs 1, 2, 5, 7, 9 and 10 from 10 consecutive spectra using untreated cells are overlaid in colour.

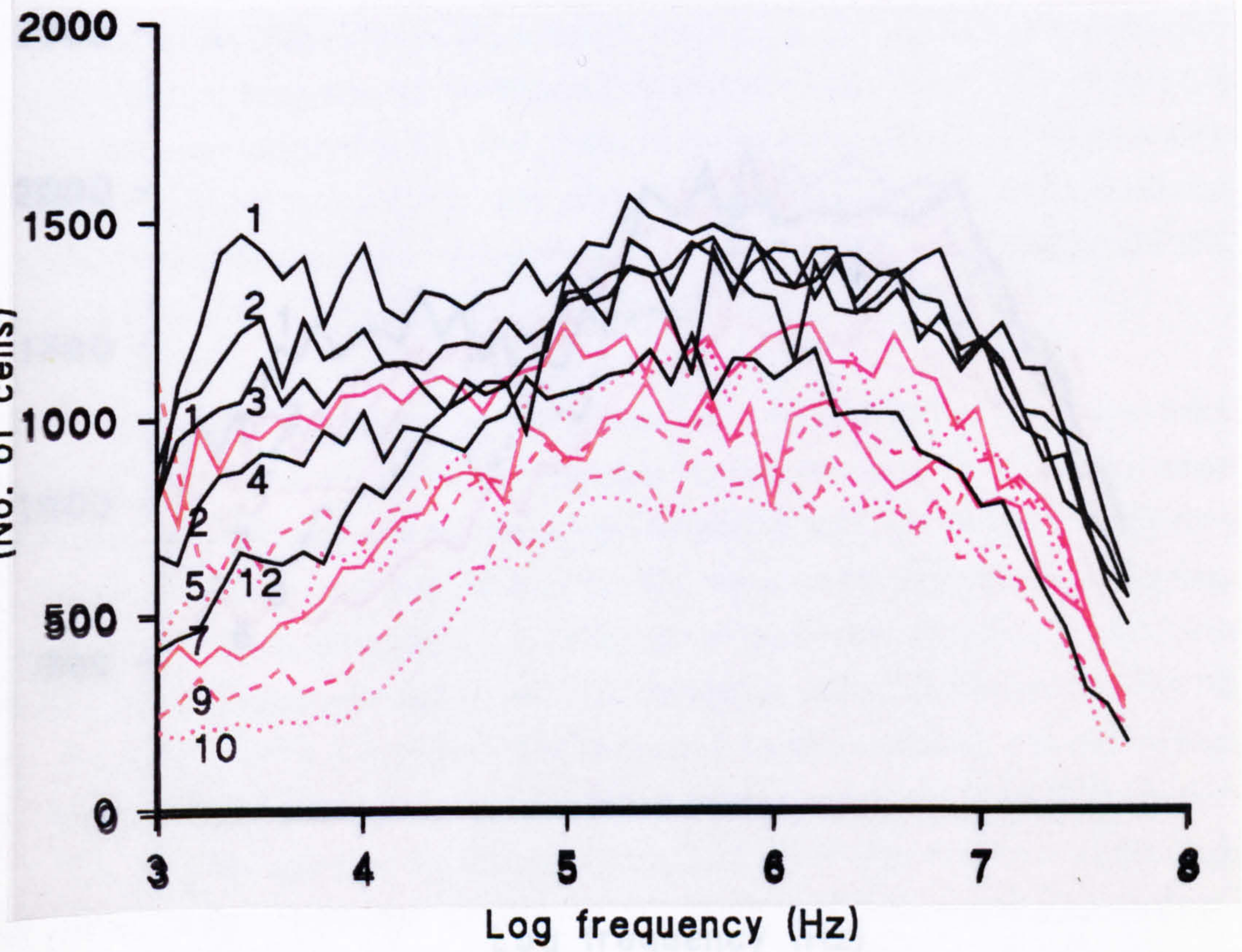


Fig 3.10 Dielectrophoretic spectra of untreated and 60 min UV-treated *E. coli* NCIMB 11459. Runs 1, 2, 3, 4, and 12 from 12 consecutive spectra using UV irradiated cells are plotted. Runs 1, 2, 5, 7, 9 and 10 from 10 consecutive spectra using untreated cells are overlaid in colour.

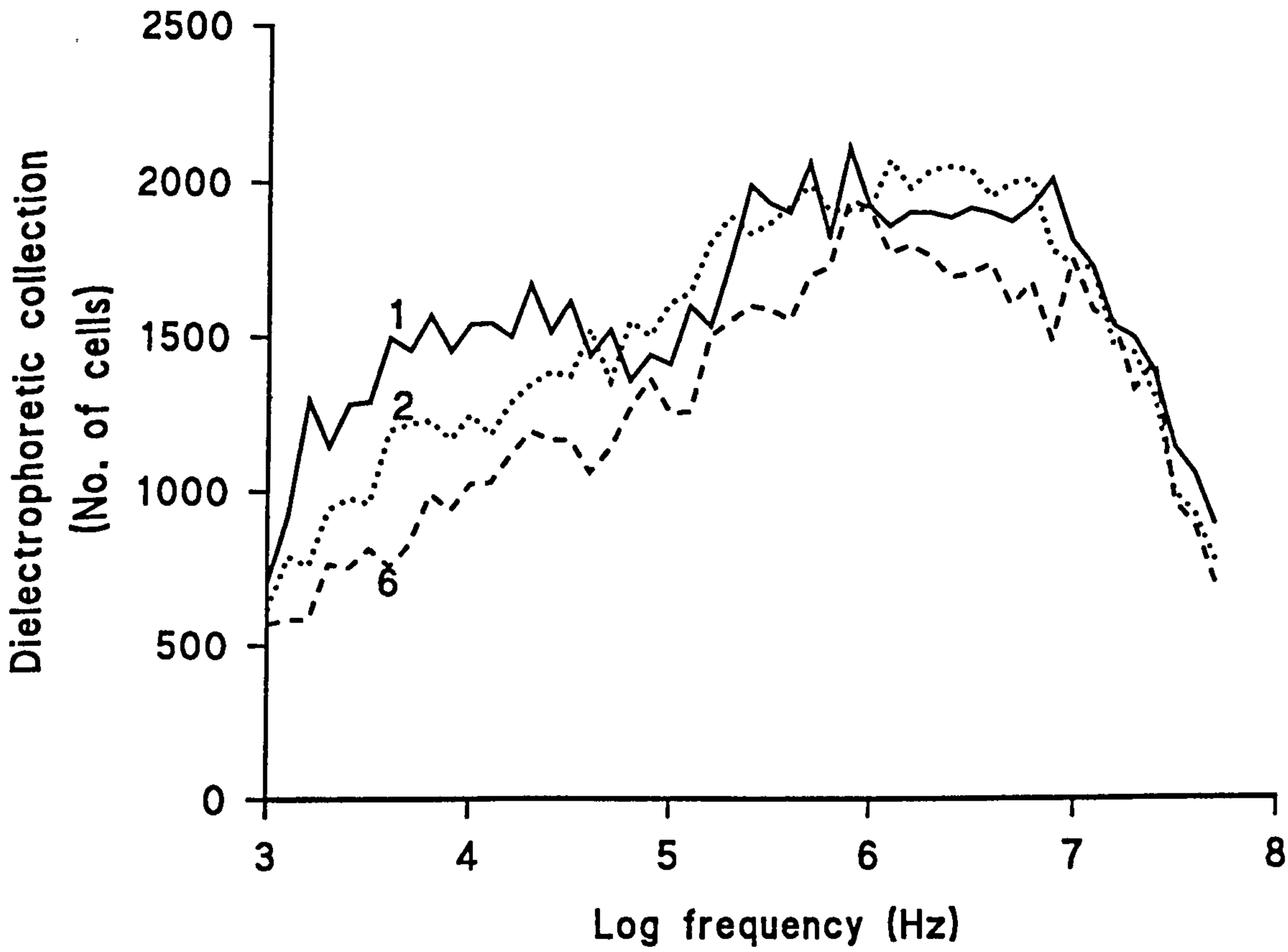


Fig 3.11 Dielectrophoretic spectra of untreated and 60 min UV-treated *E. coli* NCIMB 11459. Runs 1, 2 and 6 from 6 consecutive spectra using UV irradiated cells are plotted. Runs 1, 3 and 6 from 6 consecutive spectra using untreated cells are overlaid in colour.

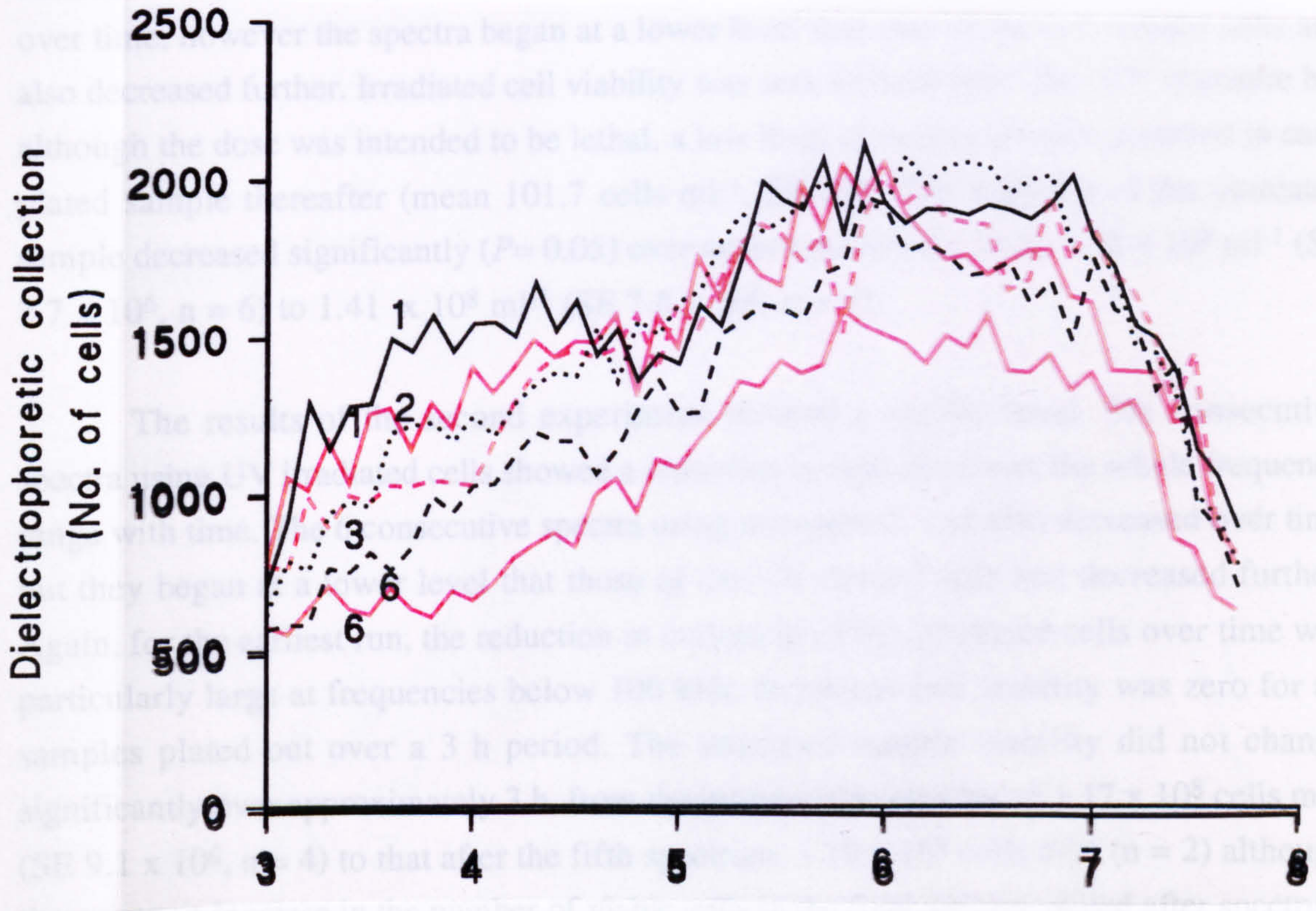


Fig 3.11 Dielectrophoretic spectra of untreated and 60 min UV-treated *E. coli* NCIMB 11459. Runs 1, 2 and 6 from 6 consecutive spectra using UV irradiated cells are plotted. Runs 1, 3 and 6 from 6 consecutive spectra using untreated cells are overlaid in colour.

In the first experiment, the level of collection for 12 consecutive spectra using UV irradiated cells decreased over time. This occurred over the whole frequency range but was particularly dramatic at the lower frequencies (below 100 kHz) for the earliest spectra. Ten consecutive spectra using untreated cells also showed an overall decrease in collection level over time, however the spectra began at a lower level than that of the UV-treated cells and also decreased further. Irradiated cell viability was zero immediately after UV exposure but although the dose was intended to be lethal, a low level of colony growth occurred in each plated sample thereafter (mean 101.7 cells ml^{-1}, SE 8.9). The viability of the untreated sample decreased significantly (P= 0.05) over approximately 4h, from 1.78 x 10^8 ml^{-1} (SE 7.7 x 10^6, n = 6) to 1.41 x 10^8 ml^{-1} (SE 7.6 x 10^6, n = 6).

The results of the second experiment showed a similar trend. Six consecutive spectra using UV irradiated cells showed a reduction in collection over the whole frequency range with time. The 6 consecutive spectra using untreated *E. coli* also decreased over time but they began at a lower level that those of the UV-treated cells and decreased further. Again, for the earliest run, the reduction in collection of the irradiated cells over time was particularly large at frequencies below 100 kHz. Irradiated cell viability was zero for all samples plated out over a 3 h period. The untreated sample viability did not change significantly over approximately 3 h, from the initial viable number of 1.17 x 10^8 cells ml^{-1} (SE 9.1 x 10^6, n = 4) to that after the fifth spectrum, 1.19 x 10^8 cells ml^{-1} (n = 2) although there was an increase in the number of viable cells in the final aliquot, plated after spectrum number 6 (1.97 x 10^8 cells ml^{-1}, SE 2.5 x 10^6, n = 2). This was likely to be due to errors within the dilution or plating of the sample.

3.3.6 Dielectrophoresis of untreated and sub-lethally UV-treated stationary phase *E. coli* 11459

Fig 3.12 shows the results of an experiment showing the dielectrophoretic response over time of *E. coli* irradiated for 30 min. Spectra obtained using an untreated sample are overlaid for comparison. Again, some intermediate runs have been omitted for clarity. Fig 3.12 shows a similar pattern to those described for the 60 min irradiated samples. Collection is reduced over the whole frequency range. The collection level of the irradiated cells tended toward that of the mean of 6 consecutive spectra using untreated cells.

The viability of the irradiated cells was zero immediately after UV exposure but had risen to 7.3 x 10^3 cells ml^{-1} (SE 7.5 x 10^2, n = 4) after the first spectrum approximately 30 min after treatment. The viability did not change significantly over the remaining time period. The untreated cell sample viability did not vary significantly over time (mean 1.10 x 10^8 cells ml^{-1}, SE 6.8 x 10^6, n = 24).

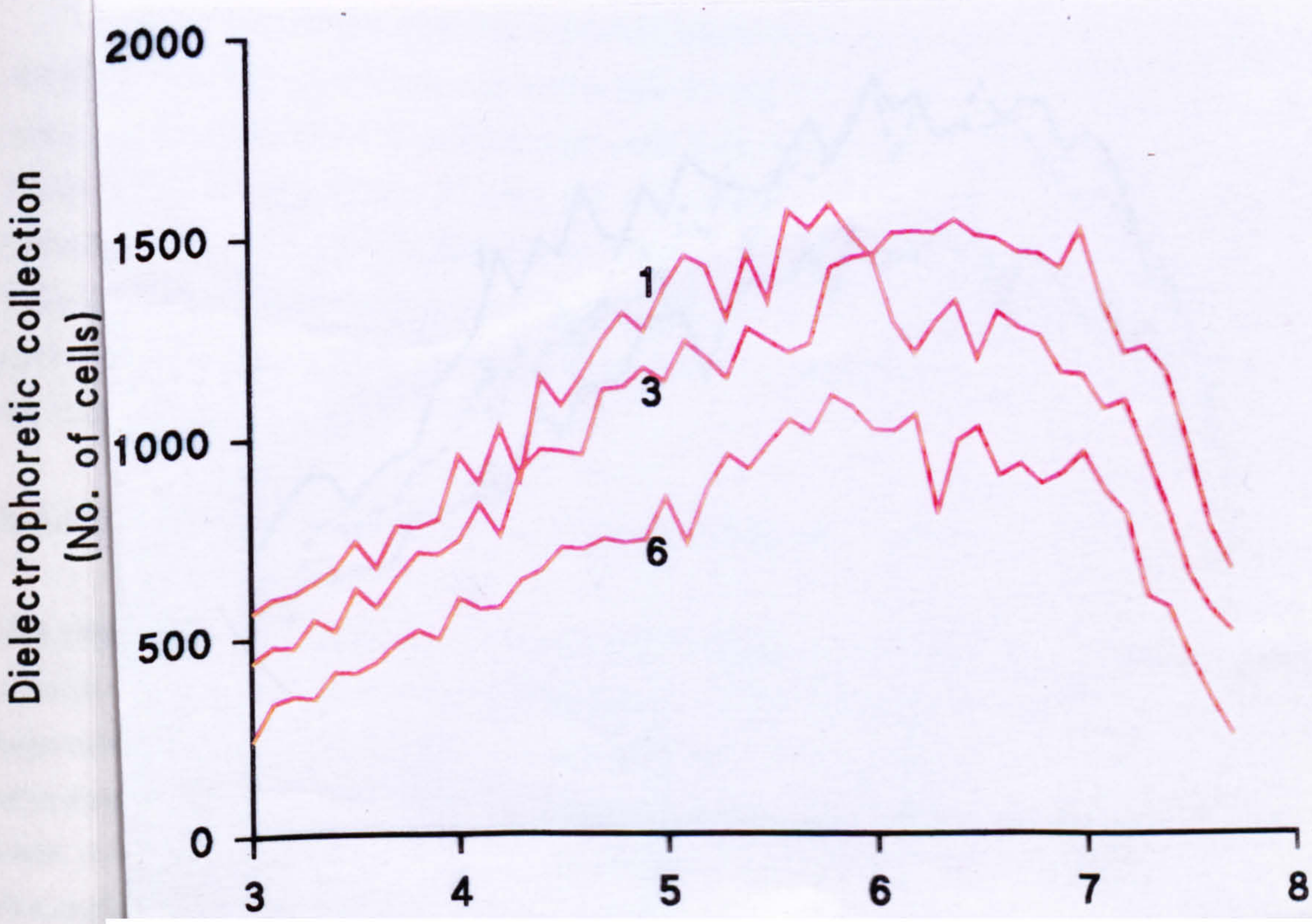


Fig 3.12 Dielectrophoretic spectra of untreated and 30 min UV-treated *E. coli* NCIMB 11459. Runs 1, 3 and 6 from 6 consecutive spectra using UV irradiated cells are plotted. Runs 1, 3 and 6 from 6 consecutive spectra using untreated cells are overlaid in colour.

The viability of the irradiated cells was zero immediately after UV exposure but had risen to 7.3 x 10^3 cells ml^{-1} (SE 7.5 x 10^2, n = 4) after the first spectrum approximately 30 min after treatment. The viability did not change significantly over the remaining time period. The untreated cell sample viability did not vary significantly over time (mean 1.10 x 10^8 cells ml^{-1}, SE 6.8 x 10^6, n = 24).

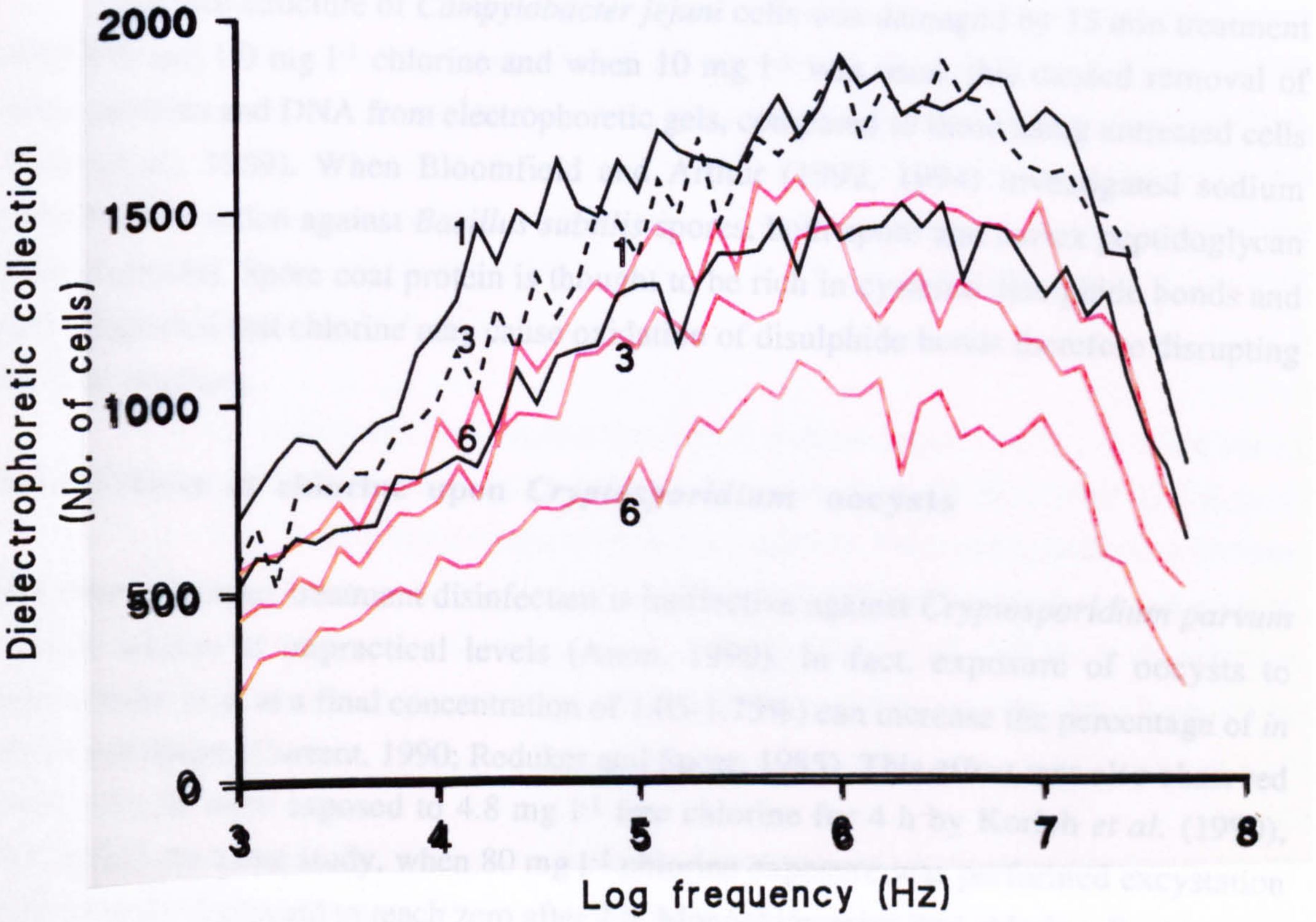


Fig 3.12 Dielectrophoretic spectra of untreated and 30 min UV-treated *E. coli* NCIMB 11459. Runs 1, 3 and 6 from 6 consecutive spectra using UV irradiated cells are plotted. Runs 1, 3 and 6 from 6 consecutive spectra using untreated cells are overlaid in colour.

3.4 DISCUSSION

3.4.1 Effects of chorine upon microorganisms

Chorine is routinely used for the disinfection of potable water. It attacks chemical constituents of viruses and bacterial cells. The exact mechanisms are unknown but it is thought that chlorine may affect the enzyme triose-phosphate-dehydrogenase, specifically the sulphydryl groups (Gray, 1989).

Surface structure of *Campylobacter jejuni* cells was damaged by 15 min treatment with 0.5 and 1.0 mg l^{-1} chlorine and when 10 mg l^{-1} was used, this caused removal of some proteins and DNA from electrophoretic gels, compared to those using untreated cells (Yun *et al.*, 1989). When Bloomfield and Arthur (1992, 1994) investigated sodium hypochlorite action against *Bacillus subtilis* spores, both spore and cortex peptidoglycan were degraded. Spore coat protein is thought to be rich in cysteine disulphide bonds and they suggested that chlorine may cause oxidation of disulphide bonds therefore disrupting the coat structure.

3.4.2 Effects of chlorine upon *Cryptosporidium* oocysts

Chlorine as a water treatment disinfectant is ineffective against *Cryptosporidium parvum* oocysts except at impractical levels (Anon, 1990). In fact, exposure of oocysts to hypochlorite (e.g. at a final concentration of 1.05-1.75%) can increase the percentage of *in vitro* excystation (Current, 1990; Reduker and Speer, 1985). This effect was also observed when oocysts were exposed to 4.8 mg l^{-1} free chlorine for 4 h by Korich *et al.* (1990), although in the same study, when 80 mg l^{-1} chlorine exposure was performed excystation progressively decreased to reach zero after 2 h. Monochloramine and chlorine dioxide were also investigated. Monochloramine was slightly less effective at oocyst inactivation than free chlorine (2% excystation remaining after 2 hour exposure to 80 mg l^{-1}) but chlorine dioxide was several times more effective (1.3 mg l^{-1} exposure for 1 h decreased excystation from an initial 87% down to 5%). Mice infectivity trials were also carried out and the results correlated well with *in vitro* excystation.

Peeters *et al.* (1989) found that chlorine dioxide (0.4 mg l^{-1}) significantly reduced the level of oocyst *in vitro* excystation and mouse infectivity after 15 min contact time. However some viable oocysts still remained after 30 min. Blewett (1988) found that *Cryptosporidium* oocysts failed to be completely inactivated by 1% sodium hypochlorite solution when incubated for 30 min at either 22°C or 37°C. Robertson *et al.* (1993b) found no difference in viability as assessed by staining (though a decrease in excystation) when oocysts were incubated with 100 mg l^{-1} free chlorine but a significant viability reduction occurred when 1000 mg l^{-1} was used. In this study a higher incubation temperature (1h at 37°C) and longer period may have affected the result. Woodmansee (1987) found enhanced excystation after suspending oocysts in full strength commercial bleach (5.25%) for 5 min on ice. However this observation may have been oocyst degradation caused by the high chlorine concentration (Robertson *et al.*, 1993b). Fayer (1995) looked specifically for the effects upon infectivity of oocysts treated with sodium hypochlorite. He found that oocysts suspended in 5.25%, 2.63% or 1.31% bleach for 10, 30, 60 or 120 min at 21°C were all infective to neonatal BALB/c mice.

Reduker and Speer (1985) found that 5.25% sodium hypochlorite treatment for 12 min on ice caused oocysts to partially collapse. Reduker *et al.*, (1985a) used 1.75% (v/v) sodium hypochlorite for 12 min on ice to clean their oocysts before using scanning electron microscopy to reveal that compared to untreated oocysts they had a smoother outer surface. When transmission electron microscopy was used (Reduker *et al.*, 1985b) it showed that the treatment perforated or removed the outer layer and outer zone of the inner layer of the oocyst wall.

The action of chlorine treatment upon *Giardia* cysts showed varying degrees of morphological changes (including cyst wall deterioration, ruffling of the plasma membrane and progressive granularity of the cytoplasm) with different doses and exposure times (Neuwirth *et al.*, 1988; Wallis *et al.*, 1989).

3.4.3 Dielectrophoresis of untreated and chlorine-treated *C. parvum* oocysts

The levels of chlorine used in this experiment were intended to be of the order used in drinking water disinfection and were not intended to inactivate the oocysts. As expected, the excystation levels suggested little difference between any of the samples.

The reason for the decrease in collection and, by implication the reduced relative polarization of chlorine-treated oocysts over the whole frequency range is not known. However the results suggested that the effect is dose-dependent. The stripping of the outer oocyst wall caused by e.g. 1.75% v/v sodium hypochlorite for 12 min on ice (Reduker *et al.*, 1985b) was unlikely with the much lower concentrations used in this experiment, although it was possible that outer oocyst wall stripping may have occurred at a low level.

A smeared out two-shell model modified from that of Huang *et al.* (1992) was used to model the experimental data. Values for the parameters were obtained from the literature where available e.g. oocyst diameter and wall thickness (Reduker *et al.*, 1985b). Others were estimated from dielectric studies of other cells (Marszalek *et* al, 1991, Huang *et al.*, 1992, Fuhr *et al.*, 1990). The medium conductivity had been measured directly during experimentation.

Due to its distinctive characteristics, the parameter values for oocyst wall conductivity and relative permittivity were estimated to lie between those reported in the literature for cell membranes (Marszalek *et al.*, 1991; Huang *et al.*, 1992) and for a yeast wall (Huang *et al.*, 1992). The *C. parvum* oocyst wall is not a cell membrane although it provides more permeability barrier properties and has a greater protein component than a yeast wall, which is largely carbohydrate. Therefore lower conductivity and permittivity values than those for a yeast wall were used.

The estimated dielectric parameters were adjusted to produce predicted polarization spectra over an external conductivity range, which were consistent with dielectrophoretic spectra obtained experimentally. Using the modified Huang model it was found that a decrease in oocyst wall permittivity caused a progressive reduction in polarization matching that obtained in the experiment (fig 3.13).

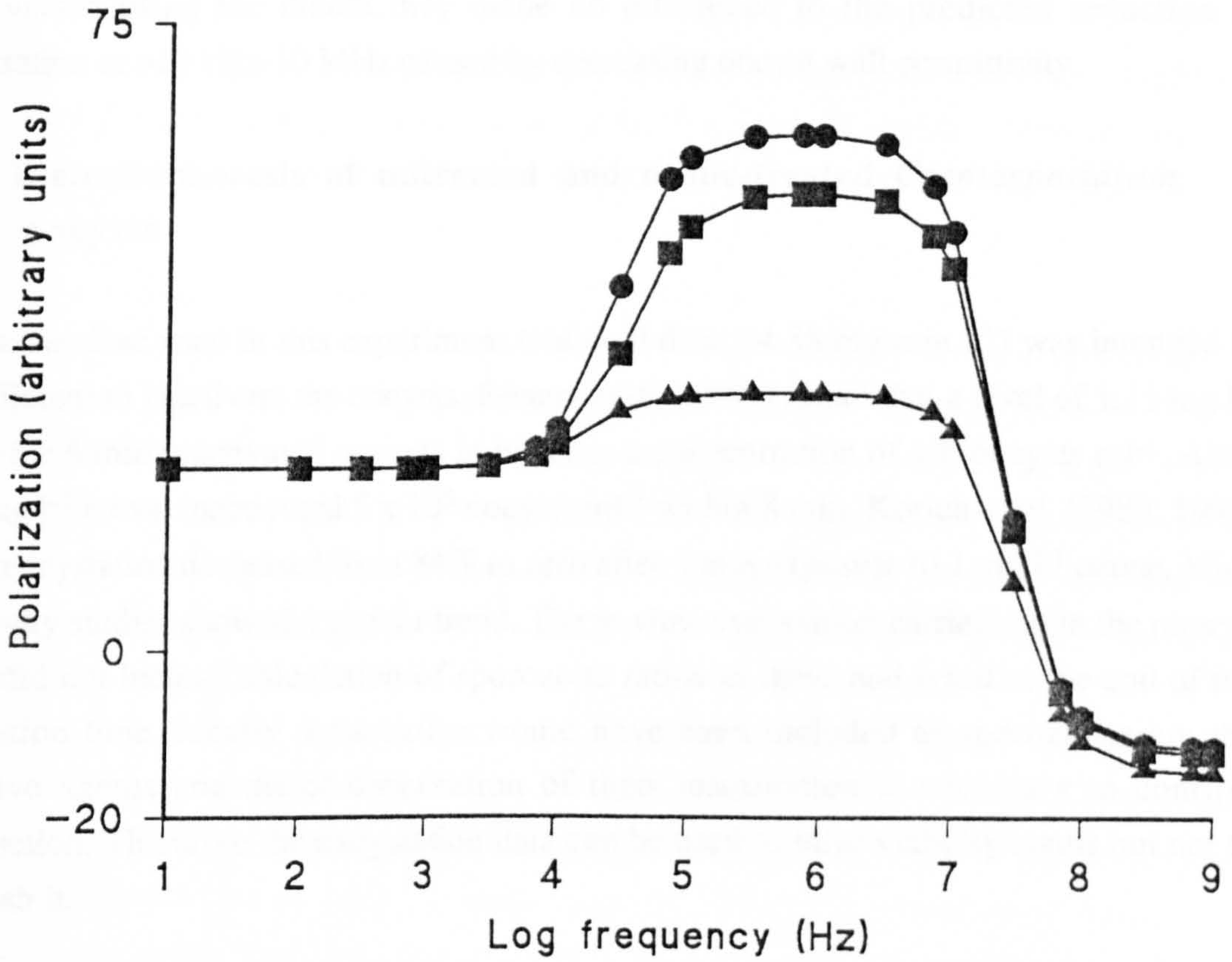


Fig 3.13 Predicted polarization spectra for three *C. parvum* oocyst samples: (●) an untreated sample, and (■) (▲) two samples with progressively reduced wall permittivity values

A decrease in permittivity may have been caused by a small reduction in the oocyst wall thickness resulting from chlorine action. If chlorine had acted in this way, other parameters, including the width of the counterion layer and the conductivity of the oocyst wall, would also have been expected to change. However, when these parameter values were varied using the model they made no difference to the predicted reduction of polarization at >10 kHz-10 MHz caused by decreasing oocyst wall permittivity.

3.4.4 Dielectrophoresis of untreated and ozone-treated *Cryptosporidium* oocysts

The ozone dose used in this experiment (residual dose 14.88 mg min l^{-1}) was intended to be sufficient to inactivate the oocysts. Peeters *et al.* (1989) found that a level of 1.11 mg l^{-1} ozone for 6 min inactivated oocysts in water at a concentration of 10^4 oocysts ml^{-1}. Also, 2.27 mg l^{-1} ozone inactivated 5 x 10^5 oocysts ml^{-1} within 8 min. Korich *et al.* (1989; 1990) found excystation decreased from 84% to zero after 5 min exposure to 1 mg l^{-1} ozone. Mice infectivity studies showed a similar trend. The *in vitro* excystation carried out in the present study did not include calculation of sporozoite ratios as these had lysed at the end of the incubation time. Ideally these ratios would have been included as sporozoites are the infective agents and direct observation of their inactivation is necessary to confirm disinfection. Therefore the excystation data can be used to infer viability status but not to establish it.

Differences in the frequency dependence of the untreated oocyst sample mean spectrum and that of the ozone-treated oocysts occurred over the lower frequency range (1-100 kHz) and the highest frequencies (2-10 MHz). The extent of the lower frequency differences varied between experiments. If oocyst damage was causing leakage of intra-oocyst medium, extra ions could have been associating with the oocyst surface and enhancing polarizability at the lower frequencies. The difference at the highest frequencies was reproducible between experiments so 10 MHz was chosen for further investigation. It was thought that the difference in collection between frequencies around 100 kHz and that at 10 MHz might be due to a decrease in oocyst internal conductivity and consequent decreased polarizability. This is further discussed in section 3.4.5.

3.4.5 Dielectrophoresis of untreated and ozone-treated *C. parvum* oocysts: ratios of collection at 100 kHz and 10 MHz

The levels of ozone used in this study included those shown to be sufficient to inactivate *Cryptosporidium parvum* oocysts (Peeters *et al.*, 1989; Korich *et al.*, 1989, 1990). The relationship between the low level of excystation and the high mean ratio of collection is clear for the 2 highest dose samples, indicating that the ratio of collection between two key frequencies under defined conditions might be used to assess viability after ozone treatment.

The level of excystation began to decrease with increasing ozone dose before the mean ratio of collection showed any significant change. This suggests that the dielectrophoretic response is related to oocyst viability, but it occurs in a dose-dependent manner only after a threshold of serious ozone damage has been breached. Ozone damage to oocysts might be in two phases. Initial damage to the oocyst wall could be responsible for a proportion of total damage but serious damage to the sporozoites may lead to the large decrease in excystation.

The data described in figs 3.7 and 3.8 show different values for the mean ratios. Both however show a large difference in the ratio of the most heavily ozone-treated samples compared to the low dosed and the untreated samples. In the first experiment, four of the mean ratios showed a significant difference to that of the untreated sample whereas only two were significantly different in experiment 2. This can be partly explained by examining the ratio values. In experiment 1 the ratio for the untreated sample was 1.01 whereas the corresponding ratio in the second experiment was 1.188. Therefore, it is more likely that the ratios of the ozone-treated samples could be significantly different to the untreated sample ratio if the latter is a smaller value, particularly as less data points were used to calculate the mean in experiment 1 than in experiment 2 (20 and 60 data points respectively). This is the likely explanation for the significant difference of the 0.99 mg min l^{-1} ozone-treated sample ratio in experiment 1 to the untreated sample ratio. It may also in part explain the significant differences of the 4.28 and 5.56 mg.minl^{-1} ozone-treated sample ratios (the corresponding doses were not significantly different in experiment 2).

A likely contributor to the differences in the untreated sample mean ratios between the two experiments is the age of the *Cryptosporidium* oocysts. The oocysts used were taken from the same stock and were three months old at the time of experiment 1 and four months old when experiment 2 was performed. Oocysts are known to deteriorate during storage over a few months (Reduker *et al.*, 1985b; Sterling, 1990). If a decrease in polarization at 10 MHz is related to oocyst physiological status then a slight decrease in collection of untreated oocysts at this frequency might be expected with the older oocyst sample. This could lead to a greater value for an untreated ratio as occurred in experiment 2. However, the excystation percentages were similar for the untreated oocyst samples in both experiments (67% and 71%) and a decrease in initial excystation might have been expected if oocyst stock deterioration was occurring over time. It is possible that when oocysts first begin to deteriorate they may still be able to excyst when in ideal conditions but be in depleted physiological condition compared to those in a fresh stock. Recent interest in the relationship between *Cryptosporidium* oocyst viability and infectivity suggests that damaged/aged oocysts may still be able to excyst. However, the sporozoites may not be able to infect host cells possibly due to depletion of endogenous reserves.

Even accepting this, if the physiological status of the oocysts differed in such a way that the ratio of the untreated samples were affected, this might be expected to carry on proportionately i.e. if a better physiological status in experiment 1 led to a lower mean ratio value for the untreated sample compared to that in experiment 2, then the ozone-treated samples would also have proportionately lower ratios. However, if a certain level of physiological deterioration has been caused over time, the addition of comparable amounts of ozone to different oocyst samples, may not cause an equivalent percentage lowering of the excystation level. The same may apply to their decrease in polarization at 10 MHz.

There is an apparently clear relationship between an increase in the mean ratios of the ozone-treated samples and a decrease in *in vitro* excystation levels in experiment 2. This is less apparent in the first experiment. However, when comparing the ratios and ozone doses between the two experiments, it can be seen that the excystation at 4.28 mg.minl^{-1} in experiment 1 is down to 30.4% and the ratio is "high" (significantly different to the untreated sample mean ratio) whereas the corresponding excystation value in experiment 2 is much higher (52.4%) and the ratio is low (and not significantly different to the untreated sample mean ratio).

The same relationship between the two experiments exists for the 5.56 mg min l^{-1} ozone-treated samples - the first experiment has a low excystation level (14.3%) and a higher, significantly different ratio whereas in the second experiment, excystation is higher (at 37.7%) and the ratio lower. Therefore although the ratios and excystation levels produced differed between experiments despite the use of equivalent ozone doses, the suggestion of a relationship between increasing ratio and decreasing excystation still applies when each experiment is considered individually. In both experiments the 14.88 mg min l^{-1} ozone-treated sample resulted in high ratio values and very low excystation values.

3.4.6 Evidence for ozone damage in biological systems

It is not known for certain why this change in dielectrophoretic collection occurs at 10 MHz when ozone treatment has been performed but some suggestions can be made. Ozone in aqueous solution decomposes to give hydrogen peroxide, superoxide and hydroxy radicals. The mode of action of ozone against *Cryptosporidium* oocysts is unknown although the free radical nature of ozone reaction and decomposition products are likely to be involved in primary and secondary reactions.

There are two main mechanisms responsible for the action of ozone in the destruction of biomolecules: (i) the oxidation of polyunsaturated fatty acids to acid peroxides and (ii) the oxidation of sulphydryl groups and amino acids of enzymes, proteins and peptides. This means that all membranes are sensitive targets to ozone (Victorin, 1992).

The action of ozone on the environmentally resistant cysts produced by *Giardia* spp., has been documented (Wallis *et al.*, 1989). Ozone appeared to degrade components within the cytoplasm without disrupting the plasma membrane or cyst wall. It was suggested that ozone either penetrated the cyst wall and plasma membrane to react with cell organelles, or that ozone reacted with the cyst wall, changing its physiological properties and causing cyst damage by disrupting equilibrium. The action of ozone could create small holes in the *Cryptosporidium* oocyst wall and the sporozoite membranes. Observations have since been made where long periods of ozonation (greater than those used in this study) have caused oocyst walls to rupture (personal observation). This has also been reported for prolonged ozonation of *E. coli* where initial leakage of cell contents culminated in lysis of cells (Hamelin *et al.*, 1978).

Several studies have been undertaken to observe the effects of ozone upon whole cells and individual components. Ozone damage studies in plant tissue implicate the plasma membrane as the site of initial injury, possibly due to membrane protein damage, in particular amino acid sulphydryl groups, therefore affecting membrane permeability (Dominy and Heath, 1985). Chevrier *et al.*, (1990) showed a two-phased damage of *Euglena gracilis* cells caused by ozone. Within 15 min ozone exposure there was a decrease in uptake of both vitamin B_{12} and acetate, indicating the ozone sensitivity of both of these transport systems. However, a second step in the oxidative effects was seen after 30 min exposure when K^+ ion leakage began. The effects of ozone upon cell membranes and essential enzymes were thought to contribute to bacterial inactivation (Heath and Frederick, 1979; Johnson, 1980).

There have been other reports of the effects of ozone upon cell enzyme systems (Dominy and Heath, 1985) and cellular proteins (Pryor and Uppu, 1993). Ozone is known to cause alterations in membrane transport of Ca^{2+} ions in plant cells by increasing the passive permeability of Ca^{2+} influx (Castillo and Heath, 1990). Chevrier and Sarhan (1992) found that energy metabolism in *Euglena gracilis* cells was reversibly decreased by ozonation. They found a decrease in total adenine nucleotide concentration due to a decrease in ATP and ADP although AMP levels remained virtually unchanged. They suggested that in the initial minutes of ozonation the size of the individual adenine nucleotide pools are regulated to maintain an appropriate energy balance within the cell under ozone stress.

Plasmid DNA can be broken through the action of ozone (Hamelin, 1985). Further evidence using *E. coli* has suggested that ozone can penetrate into cells to cause nucleic acid degradation, the guanine and thymine residues being targeted (Ishizaki *et al.*, 1987). Ozone degradation of tobacco mosaic virus coat proteins by particular damage to tryptophan and tyrosine residues caused protein aggregation and subsequent cross linking to virus RNA (Shinriki *et al.*, 1988).

3.4.6.1 Suggested explanation for decreased polarization of ozone-treated *C. parvum* oocysts at 10 MHz

Puncturing of *Cryptosporidium* sporozoite membranes through ozone damage would be likely to kill the delicate sporozoites, which are incapable of survival outside of an oocyst or host, and begin to lyse within 30 min incubation in excystation medium (Robertson *et al.*, 1993b). Ozone damage to the oocyst wall could lead to leakage of intra-oocyst medium. Sporozoite death inside intact oocysts may lead to membrane leakage, and with further leakage outside of the oocyst wall consistent with its permeation or puncturing by ozone, the internal conductivity level would decrease and subsequently begin to approach that of the external medium. When the difference between the conductivity levels was decreased the capacitive effect was lowered. Consequently, polarization of oocysts above 1 MHz where the interfacial polarization mechanism dominates (Pohl, 1973), was reduced whereas collection at lower frequencies where different polarization mechanisms dominate, remained unchanged.

In order to demonstrate this, a smeared-out two shell sphere model (as used in section 3.4.3), was used to model oocysts. Whilst leaving the other parameter values unchanged from those used to model chlorine treatment of oocysts, internal conductivity was found to be the only one which could be varied sensibly to reduce polarization at 10 MHz in a sphere. For example, lowering this from a value of 2.5 mS cm^{-1} to 1.4 mS cm^{-1} was sufficient to increase the ratio between 100 kHz and 10 MHz from 1.175 to 1.772 (fig 3.14). This was achieved by a reduction in polarization at 10 MHz whilst the 100 kHz level remained unchanged. This corresponded with the changes in dielectrophoretic collection ratio obtained from the highest dosed ozone-treated oocysts. An earlier study investigating dielectrophoresis of autoclaved *C. parvum* oocysts found a decrease in polarization after 1 MHz when compared to untreated oocysts (Archer *et al.*, 1993). It is probable that the harsh nature of autoclaving caused more severe oocyst wall damage than caused through ozonation, and that more of the oocyst contents leaked out. Consequently the internal conductivity level would have been similar to that of the external medium, therefore reducing oocyst polarization at an earlier frequency as the interfacial polarization mechanism was unable to function.

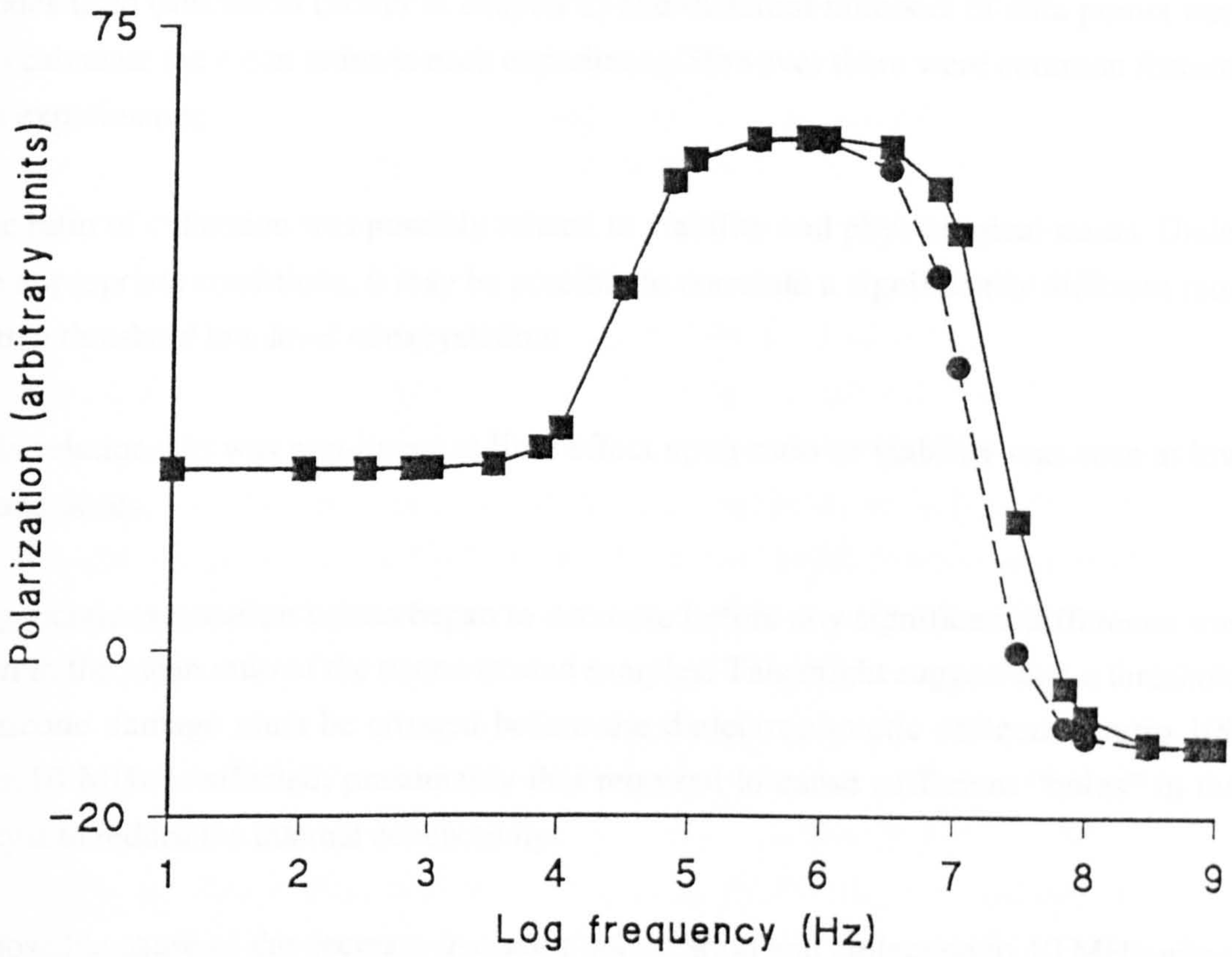


Fig 3.14 Predicted polarization spectra for two *C. parvum* oocyst samples: (■) an untreated sample and (●) a sample with a reduced internal conductivity.

Analysis of ratio of collection between two key frequencies: In conclusion

The two experiments undertaken to determine ratios were not directly comparable because of changes in the status of oocysts such as age. There were also differences in the electrodes used (discussed further in chapter 2) and different numbers of data points were used to calculate the mean ratios in each experiment. However there were common features to both experiments:

- The ratio of collection was possibly related to viability and physiological status. Under the appropriate conditions, it may be possible to correlate a significantly different ratio with a threshold low level of excystation.

- This relationship was non-linear as little effect upon ratio or viability was seen at low ozone doses.

- In general, excystation values began to decrease before any significant difference was seen in the mean ratio of the ozone-treated samples. This might suggest that a threshold of ozone damage must be crossed before the dielectrophoretic collection ratio 100 kHz:10 MHz is affected, presumably that required to cause sufficient "holes" in the oocyst to reduce the internal conductivity.

- A possible cause of the decrease in oocyst polarization and collection at 10 MHz was a reduction in the internal conductivity, as demonstrated using a two-shell model. This may have been due to leakage of material from both damaged sporozoites and intra-oocyst medium, out from the oocyst wall.

3.4.6 Survival curve of UV-irradiated, stationary phase *E. coli* NCIMB 11459

Measurement of microbial death caused by disinfectants may have practical problems, as the number of individuals surviving often depends on the way the cells are treated after exposure. Sometimes more cells can be resuscitated by use of an optimised medium and a longer incubation period (Dawes and Sutherland, 1992). Nutrient agar and overnight incubation at 37°C were used for the survival curve and results are based upon bacterial recovery under these conditions.

Stationary phase *E. coli 11459* cells exhibited disinfection death kinetics characteristic for a bacterial population, following an exponential function. Such exponential decrease in viability is a sign that the cause of death relates to a common "target" within the cells therefore the greater the dosage of radiation, the greater the probability that the target will be "hit". A shoulder at the lowest doses relates to the ability of the bacteria to repair DNA damage after treatment and to the need for multiple "hits" to cause death (Dawes and Sutherland, 1992).

Chang *et al.* (1985) investigated the UV inactivation of pathogenic and indicator microorganisms. All samples were assayed for viability immediately after UV treatment and inactivation of stationary phase *E. coli* by 3 log units (99.9%) required a total UV dose of 7.5 mW sec^{-1} cm^{-2}. An irradiation period of 30 min in the survival curve (fig 3.9) produced an apparently total kill when cells were plated out immediately after exposure and a reduction of greater than 99.99% occurred when samples were incubated for 3 h before plating. The initial cell concentration was omitted from the study performed by Chang *et al.* and this may have been partly responsible for the slight difference in the results seen in fig 3.9.

3.4.7 Dielectrophoresis of untreated, "lethally" and sub-lethally UV-treated stationary phase *E. coli* 11459

Treatment of cells using UV radiation of predominantly 253.7 nm wavelength causes damage primarily to nucleic acids. Therefore cell death resulting from this may not be expected to cause significant cell surface damage immediately, rather this would become apparent over time. However, UV photons need to pass through the cell envelope to cause nucleic acid damage and therefore some concurrent effects upon the cell surface are inevitable.

Results from the experiments using both 60 min UV exposed *E. coli* and those exposed for 30 min exhibited a decrease in collection level of the UV-treated sample over time, tending toward the lower collection levels for the untreated cell samples. Due to the nature of the dielectrophoretic equipment, there was inevitably some decrease in collection level over time and the extent to which this occurs was variable. As discussed in chapter 2, during the course of an experiment bacterial cells adhered to the surface of the electrodes and this to some extent decreased effective field strength. In addition, as some cells adhered in the area of the electrode that comprised the counting frame, this effectively decreased the available frame size for cells to be detected and counted.

However despite this, an initial enhancement of collection level after UV treatment was clear, suggesting increased polarization. Because both the lethally and sub-lethally dosed samples demonstrated a similar response, direct UV action upon the peripheral regions of the cells were more likely to have influenced polarizability than effects caused as a consequence of nucleic acid damage. Enhanced collection did not appear to be directly dependent upon viability levels that were identified subsequently to dielectrophoresis.

AC fields of high frequencies are needed to enlist the involvement of internal structures in the cellular dielectrophoretic response. Although the induced polarization of DNA molecules (due to asymmetrical distribution of counterions) has been documented, studies have used naked DNA which had been extracted from cells (Bone and Zaba, 1992). If DNA damage within intact cells could directly influence the dielectrophoretic response, the frequencies available (maximum 50 MHz) may have been too low to allow this effect to be seen.

In both of the experiments using bacteria irradiated for 60 min, enhancement of polarization in the early spectra appeared greatest over the lower frequency range. However the presence and extent of this effect was variable and probably related in part to the nature of the equipment as mentioned above. A low-frequency decrease in dielectrophoretic collection was seen in experiments using *Micrococcus lysodeikticus* (Inoue *et al.*, 1988), albeit at lower frequencies than in the experiments described here. The results were consistent with a gradual diffusion of ions from inside the cells, which were entering the *bulk phase* around the cell surface. This disrupted the particle conductivity/membrane conductivity balance and reduced polarization. The effect could be stopped by washing and re-suspending cells in fresh media, although it occurred again over time. A decrease in collection over the whole frequency range (20 Hz - 4 MHz) also occurred with time (within 4 h) although to a lesser degree than that at low frequencies.

Early research into dielectrophoretic changes caused through UV treatment of yeast cells (Pohl and Crane, 1971), suggested that any damage caused did not affect the cellular regions important for polarization at the frequencies investigated (see section 1.1.9.3). Eventually lethal UV treatment causing nucleic acid damage would lead to serious cell envelope deterioration. In one of the experiments using 60 min irradiated cells, 12 consecutive runs over approximately 6 h were performed to try and detect this change. However, other factors also affected the dielectrophoretic response over this time period and so any subtle changes may have been masked. Membrane damage, as assessed by leakage of cell contents, would have been expected to significantly alter overall polarization by affecting the contributions made by the cell surface and interior. However, when using 253.7 nm UV radiation with *E. coli*, potassium leakage is only induced at doses much greater than those needed for killing (Jagger, 1967).

3.4.7.1 Suggested explanation for enhanced polarization of UV-treated *E. coli*

UV radiation is utilised in both "ultraviolet photoelectron spectroscopy" (UPS) and "photoelectron microscopy" as the ultraviolet photons are sufficiently energetic to eject electrons from atomic outer shells. UPS is used to analyse molecules or to investigate the nature of chemical bonds and electronic properties. Photoelectron microscopy uses a UV beam to produce photoelectrons near the surface of a specimen which are used to form an image of the sample (Rouxhet and Genet, 1991).

Also, in photoelectrospectrometry, UV irradiation is used to generate an electrochemical gradient across a pigment-containing lipid bilayer (Lopez and Tien, 1980). These techniques illustrate that UV radiation must be able to affect bacterial surfaces and membranes, perhaps by altering surface charge or other structural components.

The extent of surface group ionization may be increased through the production of photoelectrons and additionally, UV energy may be able to alter bond strength. Absorption of UV energy might also distort orientation of surface or membrane groups and consequently their polarizability in an applied electric field. Enhancement of overall cell polarizability by UV irradiation seems probable and may "fall off" over time as UV energy is dissipated.

3.4.7.2 UV effects upon biomolecules

Although the dominant wavelength emitted from the UV source primarily affects nucleic acids, UV radiation at this wavelength can to a lesser extent alter proteins and other biological molecules. Also, UV wavelengths above and below the principal 253.7 nm will inevitably be generated to a small extent (Dr G. Shama, pers. comm.; Jagger, 1967). Consequently, relatively minor effects upon the structural features on and within the surface region of bacterial cells may be caused by UV absorption although these may have no effect upon viability.

Molecules involving conjugated rings are important far-UV absorbers. These include phenolic structures, rings containing nitrogen (pyridine, pyrimidine, imidazole), double ring structures (purine, indole), triple ring structures (riboflavin) and quadruple rings (steroids, porphyrins). The protein components which most effectively absorb UV are the aromatic amino acids and the peptide bond. Aromatic amino acid bases show high absorption of far-UV radiation. Tryptophan and tyrosine are the major absorbers of far-UV but if a protein has low proportions of these, phenylalanine, cysteine and cystine may be the dominant UV absorbers, partially due to the presence of disulphide bonds which increase the UV lability of proteins. Peptide bonds are relatively weak UV absorbers but as one is present for each amino acid within a protein, their contribution to total UV absorption (particularly below 240 nm) is significant (Jagger, 1967).

Chapter Four

DIELECTROPHORESIS OF TETRACYCLINE SENSITIVE AND RESISTANT *ESCHERICHIA COLI*

4.1 INTRODUCTION

The effect of antibiotics upon bacterial dielectrophoresis was first reported by Fomchenkov *et al.* in 1979. Although differences were observed, no correlation between cell physiological status and dielectrophoretic response was investigated. The use of an automated dielectrophoretic system as described in this thesis introduced the possibility of investigating antibiotic effects on bacterial cells over time. Additionally, the possibility of investigating differences between antibiotic sensitive and resistant strains, in the presence and absence of an antibiotic could be considered.

4.1.1 The importance of antibiotic sensitivity testing

Diagnosis of infection is primarily based upon the symptoms presented, and a suitable antibiotic may be prescribed. Simultaneously a sample of e.g. blood or urine may be obtained from the patient for testing to confirm the nature of the infection and the suitability of the treatment. However, test results take time, sometimes several days in the case of some blood infections and confirmation of an infective agent does not reveal its antibiotic sensitivity, for which further testing is necessary. Meanwhile, if a resistant isolate is involved the therapy prescribed may have been ineffective, causing both prolonged discomfort and expense. The ability to perform real time evaluations upon antibiotic sensitivity would be of great benefit.

In U.K. hospital laboratories, agar diffusion assays are commonly performed to assess antibiotic sensitivity of clinical isolates. The isolated organism is plated onto a specialist agar medium (e.g. Iso-sensitest, Oxoid) and a series of paper discs are placed on top, each pre-impregnated with a specific antibiotic at a defined dose. After overnight growth, the level of bacterial inhibition for each antibiotic is assessed and the isolate classed as resistant, sensitive or intermediate, compared to the response of a known sensitive control organism. Some typical MIC values for a range of clinical isolates and antibiotics are listed in table 4.1.

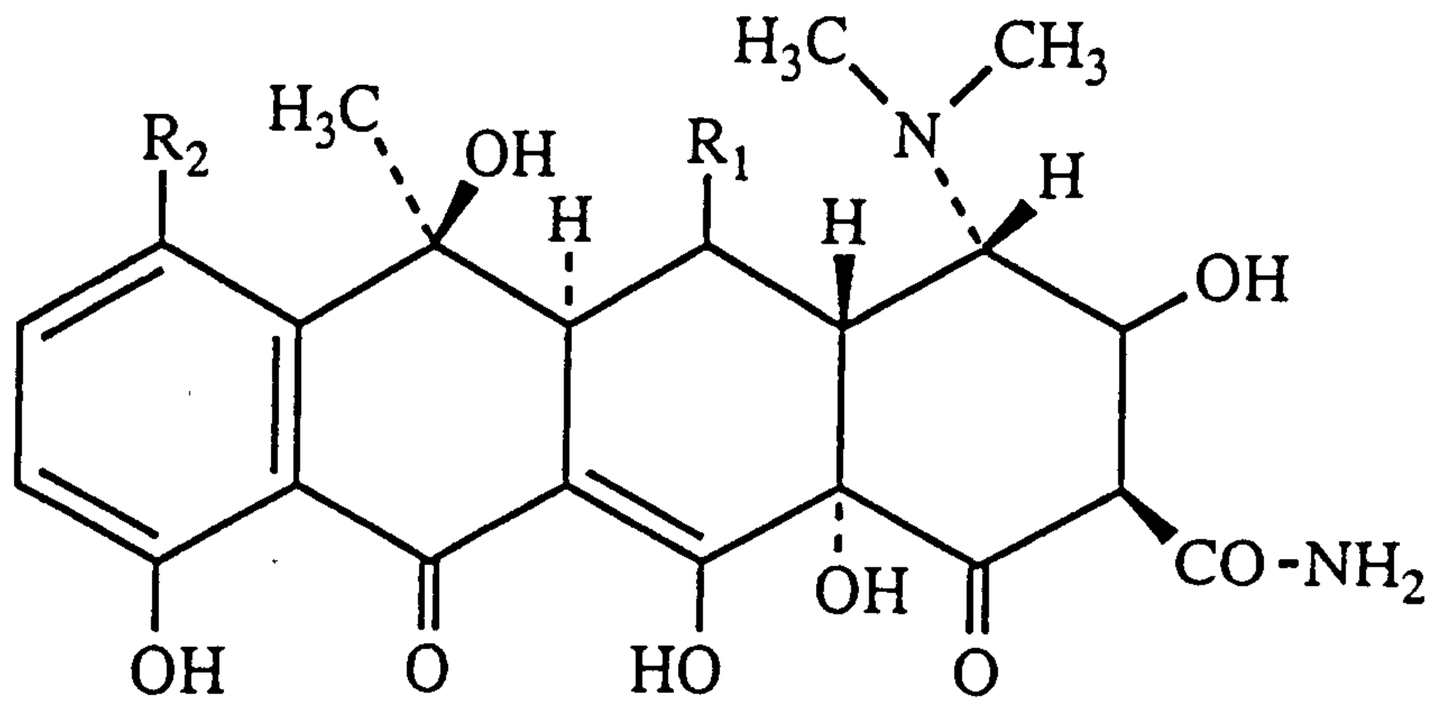
Table 4.1 Some "typical" MIC's ($\mu g\ ml^{-1}$) for a range of clinically important organisms and antibiotics (Dr A.W. Anderson, pers. comm.).

	Tetracycline	**Streptomycin**	**Rifampicin**	**Cefotaxime**
Proteus	32	8	4	0.1
Salmonella	1	2	8	0.5
Shigella	2	4	4	-
Serratia	16+	4	8	0.5
Pseudomonas aeruginosa	32+	16	20	16+
Staphylococcus aureus	<0.01	2	0.02	2
Streptococcus sanguis	<0.01	32	0.1	2
Enterococcus faecalis	0.5	32	4	2
Listeria	0.25	2	0.3	-

4.1.2 Tetracycline

The first tetracycline, aureomycin was discovered in 1948, isolated from *Streptomyces aureofaciens*. Terramycin followed in 1950, obtained from *S. rimosus*. When their structures were elucidated in 1952, they were re-named chlortetracycline and oxytetracycline respectively as they differed only in that one had a chlorine group and the other an hydroxy group. Tetracycline itself (also known as achromycin) was discovered in 1953. The tetracyclines are a closely related family of antibiotics all with a four-ring structure (fig 4.1). Although over 1000 tetracycline analogues are known, only seven are in routine clinical or veterinary use (Chopra *et al.*, 1992). They are broad spectrum, inhibiting Gram negative and Gram positive bacteria, as well as rickettsial organisms and mycoplasmas.

Fig 4.1 Tetracycline structure.



	R1	R2	R3
Tetracycline	H	CH_3	H
Chlortetracycline	Cl	CH_3	H
Oxytetracycline	H	CH_3	OH
Demethylchlor-tetracycline	Cl	H	H

The hydrochloride form of tetracycline is more soluble than the compound alone (approximately 10% compared to 0.05%) and as such is used in therapeutic formulations (Garrod *et al.*, 1973).

4.1.2.1 Tetracycline selectivity

The basis of tetracycline selectivity is not immediately obvious. Tetracycline is able to enter eucaryotic cells, with the ability to penetrate into most organs of the body (Chopra, 1985). Also tetracycline can be used to treat chlamydial infection (Cunha, 1985) therefore must be able to cross the host eucaryotic cell membrane to achieve this. In addition, tetracycline is able to inhibit eucaryotic protein synthesis although doses used in antibiotic chemotherapy do not profoundly affect 80S ribosomes. However, mitochondrial 70S ribosomes are readily inhibited by tetracycline, although up to 50% inhibition of the terminal enzyme of the respiratory chain does not cause any apparent deleterious effects (Chopra, 1985). Tetracycline selectivity therefore relies to an extent upon the abundant production of mitochondrial protein synthesis. In addition, the active accumulation of tetracycline in bacterial cells enhances the selective action (Chopra *et al.*, 1992).

4.1.2.2 Tetracycline mode of action

Tetracycline binds to most nucleoproteins including both of the ribosomal subunits. Most binding is readily dissociable although a small proportion may bind irreversibly. When bacteriostatic concentrations of tetracycline are used, bacterial cells are able to resume growth if transferred to a drug-free medium. (Franklin and Snow, 1971).

Tetracycline acts by disrupting the codon-anticodon interactions between tRNA and mRNA, therefore inhibiting protein synthesis by preventing binding of amino-acyl tRNA to ribosomal receptor site A. It is not known precisely how tetracycline prevents attachment of amino-acyl tRNA to site A, but it is thought to interact with the 30S subunit as many tetracyclines bind strongly to a single site there. Interaction at the 30S subunit is reversible which explains the bacteriostatic action of tetracycline.

Within the tetracycline binding domain of the 30S subunit, proteins S3, S7, S14 and S19 are present. Tetracycline probably binds directly to S7 (Chopra *et al.*, 1992). In addition to the proteins, 16S rRNA may also contribute to the tetracycline binding domain (Buck and Cooperman, 1990). The bacterial ribosome is not the primary site of action of all tetracycline analogues, although all clinically used tetracyclines act in this way. Others e.g. chelocardin, promote bacterial membrane damage and are bactericidal (Oliva *et al.*, 1992; Chopra *et al.*, 1992).

4.1.2.3 Divalent metal ion chelation

The ability of tetracycline molecules to chelate divalent metal cations (Gale *et al.*, 1981) has important implications for its entry into the cell, efflux from the cell and possible effects upon the Gram negative cell surface. Tetracycline molecules form a complex with the magnesium ions present in the bacterial cell wall (Hammond and Lambert, 1978).

At physiological pH (generally between pH 5 and 8) chemical groups present on a bacterial cell surface are ionized making the surface hydrophilic. Surface groups are commonly carboxylates, phosphates and amines whereas sulphydryl groups are rarely exposed here. At low pH, H^+ and H_3O^+ cations can predominate in the electronegative surface sites (Beveridge, 1989).

The outer membrane of the Gram negative cell wall is a lipid-protein bilayer with an asymmetric lipid distribution. The outermost lipid is lipopolysaccharide (LPS) whereas phospholipid predominates at the inner face. The LPS has more electronegative sites per molecule and it points toward the surrounding medium (Beveridge, 1989).

It has been shown that the *E. coli* cell wall can bind metal ions from solution (Hoyle and Beveridge, 1983) and that metal chelators can have a destabilizing effect upon the outer membrane (Leive, 1965). Metal ion chelators (e.g. EDTA) can extract small vesicles from the outer membrane. LPS predominates on the outer membrane surface where it contains 75% of total outer membrane phosphate. At physiological pH, the electronegative phosphoryl groups point toward the external medium and form strong interactions with Ca^{2+} and Mg^{2+} ions. Once the divalent ions have become complexed with the outer membrane their presence is essential to maintain stability.

Cation chelation disturbs the lipid motion and packing order, so much so that they require a more curved surface than the shape of the bacterium. Therefore they form small vesicles which leave the outer membrane. LPS is most dramatically affected by chelation but the less electronegative phospholipid and some protein are also contained within the vesicles (Beveridge, 1989).

4.1.2.4 Entry of tetracycline into bacterial cells

Tetracycline is thought to cross the outer membrane of Gram negative bacteria by passive diffusion. Due to the hydrophilic nature of tetracycline, entry is believed to be facilitated by the water-filled porins, OmpC and preferentially, OmpF (Chopra, 1985, Nikaido, 1989). The octanol/water partition coefficient of tetracycline is 0.036, below the level of 0.07 designated as being hydrophoebic (Chopra, 1985). OmpF is known to be cation selective (Hancock, 1984; Nikaido, 1989) and the chelation of tetracycline with magnesium cations in the cell wall would be consistent with this route of crossing (Russell and Chopra, 1990a; Chopra *et al.*, 1992). OmpF also favours diffusion of zwitterionic antibiotics (Russell and Chopra, 1990a).

Tetracycline can be ionized in aqueous solution and at neutral pH, the forms "TH_2" and "TH^-" predominate. A Donnan equilibrium exists across the outer membrane therefore a negative charge exists inside (Stock *et al.*, 1977). This will not hinder diffusion of the uncharged "TH_2" molecules but would be expected to prevent diffusion of the anionic "TH^-" species. However the formation of cationic chelates through the binding of divalent cations enables the "TH^-" molecules to diffuse through the outer membrane without hindrance from the Donnan equilibrium (Chopra, 1985).

One study investigating the accumulation of several antibiotics in porin-deficient mutants of *E. coli* suggests an equal role for both OmpF and OmpC in tetracycline accumulation (Mortimer and Piddock, 1993). The loss of OmpF alone caused a decrease of accumulation to 82% compared with the wild type strain whereas loss of OmpC alone caused virtually no decrease. Loss of both OmpF and OmpC resulted in only 52% accumulated tetracycline compared to the wild type. These results were used to suggest that tetracycline may use either porin to cross the cell outer membrane and when one is lost, the other can act as efficiently as the pair.

Tetracycline uptake across the bacterial cytoplasmic membrane involves both energy independent and energy dependent processes. Passive diffusion, an energy independent process, is known to occur across the membrane (Chopra, 1985; Russell and Chopra, 1990a; Chopra *et al.*, 1992). However, the nature of the energy dependent processes are controversial. Much research has been directed toward finding a carrier-mediated transport system for tetracycline uptake (reviewed by Chopra, 1985). It was suggested that two active transport systems were involved in tetracycline accumulation, an ATP-dependent system and a proton motive force (pmf) dependent system (Smith and Chopra, 1984; Russell and Chopra, 1990a). However, it is now thought that such systems are unlikely to be involved, due to evidence including uptake studies using radioactive tetracycline which did not show saturation kinetics, expected in the presence of a carrier.

Tetracycline is a relatively lipid soluble molecule and the more likely explanation for energy dependent uptake is its distribution across the cytoplasmic membrane in response to a pH gradient (Chopra *et al*, 1992). The conformation adopted by different tetracycline analogues probably influences their effectiveness at interacting with the bacterial cytoplasmic membrane. At physiological pH, many tetracyclines can exist as an equilibrium between a mixture of two free base forms, a low energy, lipophilic, non-ionized form and a high energy, hydrophilic, zwitterionic structure. The former conformation is thought to be important in uptake across the cytoplasmic membrane whereas the latter is believed important for binding to the ribosome (Hughes *et al.*, 1979). Energy dependent uptake can lead to an intracellular accumulation of tetracycline in *E. coli* up to 100-fold greater than the external concentration (Chopra *et al.*, 1992).

4.1.3 Mechanisms of resistance to tetracycline

Bacterial resistance to tetracyclines in clinical use is usually "acquired" i.e. the resistance mechanism is encoded by genes upon a plasmid or transposon (Chopra *et al.*, 1992). Cross-resistance between the members of the tetracycline family is common.

There are 3 known biochemical mechanisms for bacterial resistance to tetracyclines: an energy dependent efflux mechanism facilitated by the insertion of efflux proteins into the cytoplasmic membrane; a mechanism imparting protection to ribosomes whereby tetracyclines can no longer bind effectively; a little understood, chemical alteration of the tetracycline molecule in an oxygen-requiring reaction rendering the drug unable to inhibit protein synthesis (Chopra, 1992).

Active efflux systems which are responsible for the resistance of a number of structurally unrelated antibiotics are constantly being recognized (Levy, 1992; Chopra, 1992). Tetracycline efflux proteins fall into 3 families of determinants according to their lack of cross hybridisation. The first two families both mediate drug efflux although the second family has been detected only in Gram positive aerobic organisms. Determinants of the third family specify ribosomal protection (Levy, 1989). It has been suggested that tetracycline efflux proteins have evolved on 2 or 3 separate occasions, probably from other transport proteins (Sheridan and Chopra, 1991). The related classes Tet A to Tet E (from within the first family) all contain a structural gene (to encode for the efflux protein) and a repressor gene, the latter demonstrating that resistance to tetracycline is inducible. These classes are all found in several species including some *Enterobacteriaceae*.

Efflux of tetracyclines from resistant cells maintains a low intracellular concentration of the antibiotic. Efflux is dependent on the proton motive force (pmf) (Rothstein *et al.*, 1993) and is driven mainly by the pH gradient (Kaneko *et al.*, 1985; Russell and Chopra, 1990b). Efflux occurs when the electrically neutral proton/tetracycline antiport system exchanges a monocationic tetracycline-magnesium complex with a proton. Tetracycline chelation of divalent cations as well as the high intracellular magnesium concentration (1-4 mM) both accommodate the formation of monocationic complexes within the cell (Levy, 1992; Chopra *et al.*, 1992).

The secondary structure of the efflux proteins themselves consists of 12 hydrophoebic regions (separated by shorter hydrophilic sequences) which span the cytoplasmic membrane. Most of the protein is contained within the lipid bilayer with small hydrophilic amino acid loops extending into the periplasm or cytoplasm (Yamaguchi *et al.*, 1990; Levy, 1992).

4.1.3.1 Tetracycline resistance encoded by plasmid pSC101

The pSC101 plasmid contains enough genetic information to allow for its replication and for the generation of tetracycline resistance. The resistance determinant belongs to class Tet C. The mechanism for control of tetracycline resistance by the pSC101 plasmid was investigated in detail by Tait and Boyer (1978). At this time it was known that this mode of resistance was caused through a decreased intracellular accumulation of the antibiotic rather than the possession of resistant ribosomes. It was not known that active efflux of tetracycline occurred until the discovery that energy inhibitors increased tetracycline uptake in resistant cells (Levy and McMurray, 1978).

Tait and Boyer found that pSC101 plasmid mediated resistance was inducible as cells containing the plasmid grew more efficiently when high tetracycline concentrations were present. Using ^{14}C-amino acid incorporation, it was found that plasmid containing cells were evidently more resistant to protein synthesis inhibition upon addition of tetracycline, than cells without pSC101. When pSC101 cells were pre-incubated with tetracycline, the level of resistance was further enhanced. Introduction of tetracycline to cells containing pSC101 caused an initial reduction in the rate of protein synthesis although this returned to normal after approximately 45 min. Pre-incubation of cells with tetracycline prevented this temporary rate reduction. These results suggested that enhanced resistance to tetracycline involved a mechanism that was itself stimulated by tetracycline.

The presence of tetracycline was found to increase specifically the synthesis of 14, 26 and 34 kD polypeptides, indicating their involvement in the resistance mechanism. The 34 kD protein, along with another plasmid-encoded 18 kD polypeptide were thought to be sufficient alone to impart resistance. Both of these proteins were encoded by a derivative of pSC101, pMB9, which also generated tetracycline resistance mediated by decreased accumulation (Tait and Boyer, 1978). However, it was found that the 18 kD protein was not after all a product of the tetracycline resistance region and therefore its role in tetracycline resistance has been discounted (Chopra, 1985). The 14 kD protein which was inserted into the cell wall (Chopra, 1985) was thought to reduce the initial adsorption of tetracycline to cells, and the role of the 26 kD protein was unknown (Tait and Boyer, 1978; Chopra, 1985).

Tait and Boyer also performed experiments using ^{3}H-tetracycline and an ATP uncoupler, arsenite, to investigate the energy dependence of uptake in cells with and without the pSC101 plasmid. The plasmid-free cells exhibited a very rapid initial binding of tetracycline, then a rapid loss of bound antibiotics, followed by a slow accumulation of bound tetracycline. When arsenite was added at the same time as the ^{3}H-tetracycline, the initial binding was prevented. When arsenite was added to cells after they had been allowed to bind tetracycline, the rate of bound antibiotic release was greatly reduced. The slow accumulation of tetracycline was unaffected in both cases. These results were used to suggest that in pSC101-free cells, the initial binding and release were ATP dependent whereas the slow accumulation was ATP independent.

When the same experiment was repeated using pSC101 cells, the rate and total amount of initial tetracycline binding was reduced compared to the plasmid-free cells, and the slow tetracycline accumulation was completely prevented. When arsenite was included it transpired that the initial binding and loss were also ATP dependent but the mechanism preventing the accumulation was ATP independent. These findings are consistent with the now established pmf-driven, cell entry diffusion that is utilized by tetracycline.

4.1.4 Clinical usage of tetracyclines

The tetracyclines have been widely used clinically since their discovery in the 1950's. The introduction of the second generation tetracyclines, so-called "long acting" (including minocycline and doxycycline) increased the scope for tetracycline chemotherapy (Cunha, 1985). The spread of resistance and concurrent availability of new chemotheraputic agents has reduced their applications in recent years although their low cost and relative safety ensures continued usage (Chopra *et al.*, 1992).

Tetracyclines are the first choice drugs against a variety of organisms and can be a good alternative when other drugs cannot be used, for example due to allergic reaction. In addition, tetracyclines are used in the treatment of clinical symptoms of disease syndromes.

For a long time, treatment of respiratory disease utilized tetracyclines. Pneumonia caused by *Streptococcus pnemoniae* and *Haemophilus influenzae* are now usually treated with alternative drugs although *Mycoplasma pneumoniae* infections are best treated with tetracyclines (Cunha, 1985, Chopra *et al.*, 1992). In particular, minocycline and doxycycline are effective as they can better penetrate into oropharyngeal secretions (Cunha, 1985).

Tetracyclines have been extensively used in treatment of sexually transmitted disease and are still prescribed today. *Neisseria* spp. are treatable with all tetracyclines and concomitant infection with *Chlamydia trachomatis* is also susceptible (Chopra, 1985). Doxycycline can be useful against *N. gonorrhoea* but minocycline is the most effective tetracycline. In general, Gram positive organisms are more susceptible to doxycycline than to other members of the tetracycline family (Cunha, 1985).

All tetracyclines are potentially effective in chemotherapy of *Shigella* dysentery but resistance is widespread. Treatment of cholera (*Vibrio cholerae*) is effective but only in the early stages as the disease is primarily toxin-mediated. All tetracyclines are ineffective against *Proteus mirabilis* and *Pseudomonas aeruginosa* (Cunha, 1985; Chopra *et al.*, 1992).

Tetracyclines have been the drug of choice against infections of *Pasteurella* spp. For example, tetracycline can be used alone or in conjunction with streptomycin in the treatment of *Pasteurella pestis* infection (bubonic plague). Meliodosis (caused by *Pseudomonas pseudomallei*) can be treatable using tetracyclines, as can brucellosis and infection by *Borrelia recurrentis* (Cunha, 1985). Tetracyclines are also useful therapeutic agents for rickettsial infections including typhus and spot fevers (Chopra *et al.*, 1985).

A large single area of use for tetracyclines is in the treatment of "non-specific ureteritis" caused by infection with *Chlamydia* or *Ureaplasmas*. Also tetracyclines have been used for the prophylaxis of travellers diarrhoea. Doxycycline is effective in this situation against toxigenic strains of *E. coli*. The same antibiotic is concurrently active against *Vibrio cholerae*, *Shigella* dysentery, *Campylobacter*, *Yersinia*, *Salmonella* and to a lesser extent, *Entamoeba hystolytica* (Cunha, 1985).

Tetracyclines are used as an alternative therapy for infections of *Listeria* (first choice drug, ampicillin), *Yersinia enterocolitica* (first choice, an aminoglycoside) and *Haemophilus influenzae* (Cunha, 1985).

Tetracyclines are useful in treatment of the clinical symptoms manifested in chronic bronchitis syndrome, the primary cause of which is usually viral but secondary bacterial infection by e.g. *H. influenzae* is responsible for the inflammatory response and the bronchial edema. The amelioration of clinical symptoms of acne may also be performed by tetracyclines for which they are still widely prescribed (Chopra *et al.*, 1992). They can penetrate the dermis layers and reduce the inflammation response.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of tetracycline stock solution

Stock solutions of 1 mg ml^{-1} were prepared by dissolving 100 mg tetracycline (Sigma) in 0.1 ml 1M HCl and making up to 100 ml with sterile distilled water. Aliquots of 1 ml were stored in sterile eppendorf tubes at -70°C.

4.2.2 Microorganisms

Tetracycline resistant and sensitive *E. coli* isolates were obtained from NCIMB, Aberdeen, U.K. for use in dielectrophoretic experiments. The isolates were both strain NCIMB 11459 but one was resistant to tetracycline due to the inclusion of the plasmid pSC101. The isolates were stored on slopes at 4°C and sub-cultured regularly. The resistant isolate was maintained on a medium containing:

12.5 mg l^{-1}	tetracycline (Sigma)
10 g l^{-1}	tryptone (Oxoid)
5 g l^{-1}	yeast extract (Oxoid)
5 g l^{-1}	sodium chloride (Oxoid)
1 g l^{-1}	glucose (BDH)
12 g l^{-1}	technical agar (Oxoid) (Anon, 1994b).

4.2.3 Growth curve of tetracycline sensitive *E. coli*

During dielectrophoresis experimentation with antibiotic-exposed bacteria it was important to use organisms taken from an exponential growth phase (i.e. when growth was unlimited by factors such as nutrient competition or toxic waste accumulation). These cells were more susceptible to antibiotics than those at other growth phases. The growth curve is detailed in appendix II. Growth of cultures was monitored over time by taking absorbance measurements. Samples were taken simultaneously for serial dilution and plating to obtain total viable counts (TVCs).

4.2.4 Growth curve of tetracycline resistant *E. coli*

The plasmid pSC101 confers bacterial resistance to tetracycline by encoding for membrane and wall proteins which prevent tetracycline binding and intracellular accumulation (Chopra, 1985). Commonly, the inclusion of a plasmid in a bacterial cell lengthens bacterial replication time. Therefore it was necessary to produce a second growth curve as detailed in appendix III.

4.2.5 Agar diffusion assays

Agar diffusion assays modelled on those carried out in clinical laboratories were performed to assess the tetracycline sensitivity of bacteria. Tetracycline sensitive and resistant *E. coli* NCIMB 11459 were tested against a control organism known to be sensitive, *E. coli* NCTC 10418. Details of the procedure and the sensitivities of the organisms are in appendix IV.

4.2.6 Determining the MIC of tetracycline against sensitive and resistant *E. coli*

This method was used to give a more quantitative sensitivity assessment of tetracycline sensitive and resistant *E. coli* NCIMB 11459 than the agar diffusion assay.

The MIC is the lowest concentration of an antibiotic which inhibits *visible* growth of an isolate after a suitable period of incubation. MIC tests can be undertaken using either the *agar dilution* method (where a range of antibiotic concentrations are added to agar medium before plates are poured - the test organism is applied as a series of spots to the agar surface), or the *broth dilution* methods (where a test organism is used to inoculate broth media containing a series of antibiotic concentrations).

The broth dilution method was used to obtain a concentration range including the MIC for tetracycline sensitive and resistant *E. coli*. A known sensitive organism, *E. coli* NCTC 10418 was also tested as a control. The procedure and results are detailed in appendix V.

4.2.7 Preparation of *E. coli* for use in dielectrophoresis experiments

Exponential phase cells of tetracycline sensitive and resistant *E. coli* NCIMB 11459 were grown up by inoculating 100 ml sterile nutrient broth with 1 ml overnight broth culture. The flasks were incubated at 37°C with shaking for a period of time predetermined by a growth curve (appendix II) to produce an exponential growth phase. Approximately 5 ml of the broth culture was removed into a sterile 10 ml syringe (Terumo, Belgium), washed three times by filtering through a 0.2 μm pore-sized sterile syringe filter (Minisart, Sartorius, Germany) and back-washing with sterile deionized water. The final bacterial suspension in sterile deionized water was diluted to achieve an OD_{510} of approximately 0.12.

4.2.7.1 Addition of tetracycline

A suspension of exponential phase cells in sterile deionized water was prepared as described above. An appropriate volume of a thawed 1000 μg ml^{-1} tetracycline stock solution was made up to a 10 ml total volume with the bacterial suspension to produce the required antibiotic concentration (e.g. to produce a 40 μg ml^{-1} tetracycline concentration, 0.4 ml of a 1000 μg ml^{-1} stock concentration was added to 9.6 ml bacterial suspension).

4.2.8 Dielectrophoresis of tetracycline sensitive *E. coli* with and without tetracycline

4.2.8.1 Microorganisms

E. coli (NCIMB 11459) was prepared as described in section 4.2.7. Six flasks were inoculated and kept at 4°C until they were incubated at 37°C. Each flask was incubated in sequence so that it reached an exponential growth phase when needed. Exponential phase *E. coli* samples containing 80 μg ml^{-1}, 40 μg ml^{-1}, 20 μg ml^{-1} and 10 μg ml^{-1} tetracycline, respectively, were prepared and used in sequence for dielectrophoresis.

Two control exponential phase samples were prepared subsequently to the tetracycline-containing samples, one containing 8 µl of 0.1M HCl in a 10 ml sample and one with nothing added. Small volumes (less than 50 µl) of 0.1M KCl were added to all samples immediately prior to dielectrophoresis to ensure that the conductivity levels of samples lay within a narrow range. Each sample was used for dielectrophoresis over approximately a 2 h period. A 200 µl sample was removed after each spectrum had been completed and serially diluted before plating out for viability assessment (section 3.2.2.3).

4.2.8.2 Dielectrophoresis equipment and procedure

The equipment and procedure described in section 3.2.2.4 were used. A minimum of 4 repeated spectra were produced using each of the *E. coli* tetracycline containing samples and the controls.

4.2.9 Dielectrophoresis of tetracycline resistant *E. coli* with and without tetracycline

4.2.9.1 Microorganisms

E. coli (NCIMB 11459) with the inclusion of the tetracycline resistance pSC101 plasmid was prepared as described in section 4.2.7. Samples containing 80 µg ml^{-1} tetracycline, no tetracycline and 20 µg ml^{-1} tetracycline were made up and used in sequence for dielectrophoresis. Two further samples were then used, containing 10 µg ml^{-1} tetracycline and another containing no tetracycline. The same preparation procedure was followed, as in the experiment using the tetracycline sensitive *E. coli*. Viability assessment was also performed in the same way.

4.2.9.2 Dielectrophoresis equipment and procedure

The equipment and procedure described in section 4.2.7 were used.

4.2.10 Use of a pin and plate electrode configuration to observe dielectrophoretic effects.

The experiments described in sections 4.2.8 and 4.2.9 involved the use of the microelectrodes described in chapter 2. The nature of the dielectrophoretic responses of the cells could not be seen as the electrode bars were not in view. Another microelectrode design, that of pin and plate electrodes (Archer *et al.*, 1995) were used to directly observe dielectrophoresis of the cells.

4.2.10.1 The pin and plate electrodes

The pin and plate electrodes were laid down onto a glass microscope slide using photolithographic techniques, similar to those described in chapter 2. Connector wires were attached to tabs on the electrodes in the same way as with the microelectrodes. The plate electrode was 400 μm x 10 μm and the pin electrode was 200 μm x 10 μm. They were separated by a 50 μm gap.

4.2.10.2 The experimental equipment

The pin and plate electrodes were placed on a microscope stage and viewed at 200x magnification. They were connected to a signal generator (Hewlett Packard 8116A, Germany). The electrode image was displayed on an adjacent monitor (Sanyo VMC7213) via a video camera (Hitachi KP-C500) and a video recorder (S-VHS DS6000K) with a digital freeze-frame facility was set up to record the experiment.

4.2.10.3 Microorganism preparation

A sequence of exponential phase cultures of tetracycline sensitive and resistant *E. coli* NCIMB 11459 were prepared as described in section 4.2.7. The samples chosen for observation were tetracycline sensitive *E. coli* (untreated, 80 μg ml^{-1} and 20 μg ml^{-1}) and tetracycline resistant *E. coli* (untreated and 80 μg ml^{-1}). Conductivity measurements of each sample were made prior to use and 0.1M KCl was added when necessary so that all samples had a similar conductivity level (approximately 60 μS cm^{-1}). Cell concentrations of all samples were similar to those used in the dielectric experiments described in sections 4.2.8 and 4.2.9.

4.2.10.4 Experimental procedure

A 10 µl drop of cell suspension was placed on top of the pin and plate electrodes using a 20 µl Gilson pipette. A coverslip was placed on top to create a thin film of cells and the electrodes were re-focused on the monitor. A series of 10 V pulses were manually applied over a frequency range and the dielectrophoretic responses were observed and video recorded. The frequencies varied between 1 kHz and 50 MHz depending on the sample under investigation. Each drop upon the electrodes was used for just a few min before being replaced by a fresh sample to avoid deleterious heating effects from distorting the dielectrophoretic response. The electrodes and coverslip were washed with deionized water between each drop of sample. A 1 ml aliquot was removed from each stock of cell suspension for serial dilution and plating at the beginning and the end of its experimental use.

4.3 RESULTS

4.3.1 Dielectrophoresis of tetracycline sensitive *E. coli* NCIMB 11459 with and without tetracycline

4.3.1.1 Dielectrophoresis

The spectra obtained using the tetracycline-containing *E. coli* samples and the control samples are shown in fig 4.2. A spectrum has been plotted from the mean value of each sample. The peak of collection for all samples occurred in the 1 MHz range. The level of collection of 80 μg ml^{-1} tetracycline sample appeared to be higher over the lower frequency range (below 100 kHz) compared to the samples containing no tetracycline. There was little variation in the dielectrophoretic response of the other tetracycline containing samples (40 μg ml^{-1}, 20 μg ml^{-1} and 10 μg ml^{-1}) although all showed a lower level of collection than that of the 80 μg ml^{-1} sample below 1 MHz.

4.3.1.2 Cell viability

The viability counts were erratic for all of the tetracycline-containing and the control samples. For some samples the counts appeared to increase after the first aliquot was plated out but this did not continue. As the cells were taken from exponential phase it was possible that some dividing cells were counted as one colony forming unit when plated out immediately after preparation but had completed their division after a few min i.e. by the time the second aliquot was plated out. The untreated sample showed a slight though significant increase over the experimental time period (1.19×10^7 cells ml^{-1} [SE 1.5×10^6] to 2.2×10^7 cells ml^{-1} [SE 5×10^5]). The 80 μg ml^{-1} tetracycline sample showed a significant decrease over time (3.36×10^7 cells ml^{-1} [SE 1.33×10^5] to 1.03×10^7 cells ml^{-1} [SE 1.2×10^5]).

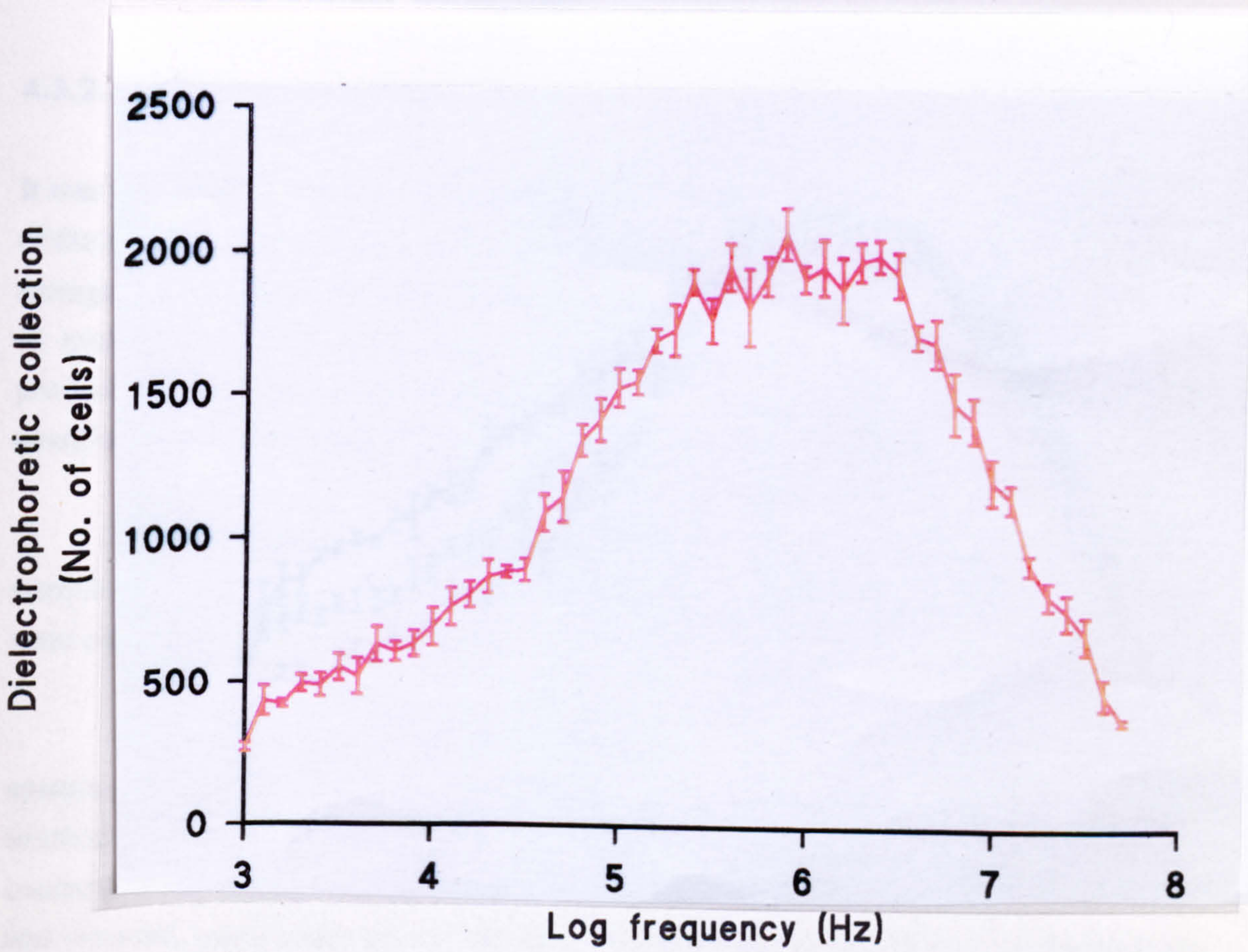


Fig 4.2 Mean dielectrophoretic spectra (n=4) of tetracycline sensitive *E. coli* NCIMB 11459 (80 μg ml^{-1} = black; 40 μg ml^{-1} = yellow; 20 μg ml^{-1} = green; 10 μg ml^{-1} = blue). A mean spectrum using an untreated cell sample is overlaid in colour.

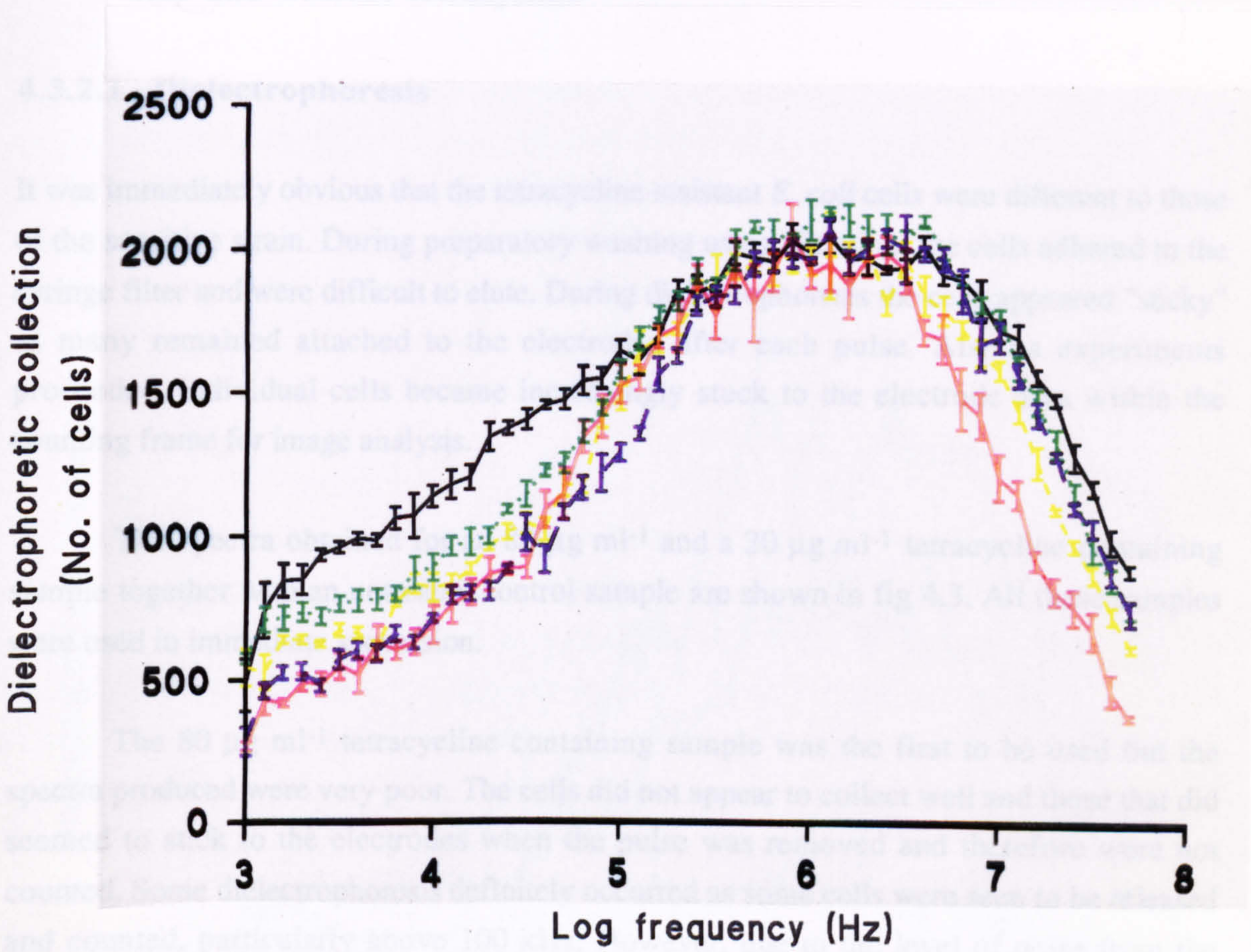


Fig 4.2 Mean dielectrophoretic spectra (n=4) of tetracycline sensitive *E. coli* NCIMB 11459 (80 μg ml^{-1} = black; 40 μg ml^{-1} = yellow; 20 μg ml^{-1} = green; 10 μg ml^{-1} = blue). A mean spectrum using an untreated cell sample is overlaid in colour.

4.3.2 Dielectrophoresis of tetracycline resistant *E. coli* NCIMB 11459 with and without tetracycline

4.3.2.1 Dielectrophoresis

It was immediately obvious that the tetracycline resistant *E. coli* cells were different to those of the sensitive strain. During preparatory washing using filtration, the cells adhered to the syringe filter and were difficult to elute. During dielectrophoresis the cells appeared "sticky" as many remained attached to the electrodes after each pulse. Also as experiments proceeded, individual cells became increasingly stuck to the electrode area within the counting frame for image analysis.

The spectra obtained for an 80 μg ml^{-1} and a 20 μg ml^{-1} tetracycline containing sample together with an untreated control sample are shown in fig 4.3. All these samples were used in immediate succession.

The 80 μg ml^{-1} tetracycline containing sample was the first to be used but the spectra produced were very poor. The cells did not appear to collect well and those that did seemed to stick to the electrodes when the pulse was removed and therefore were not counted. Some dielectrophoresis definitely occurred as some cells were seen to be released and counted, particularly above 100 kHz. However, due to the level of noise from the deteriorating electrodes and the sticking of the cells (discussed further in chapter 2) the spectra produced were erratic. The untreated sample was next prepared for use and although dielectrophoretic counts were low compared to those of the sensitive strain, repeatable spectra were produced. This infers that the addition of the tetracycline caused some difference to the cells which decreased their polarizability and the effect could not be simply due to deteriorating electrodes alone. When the 20 μg ml^{-1} tetracycline sample was used, repeatable spectra were produced. Collection levels lay between those of the untreated sample and the 80 μg ml^{-1} tetracycline containing sample.

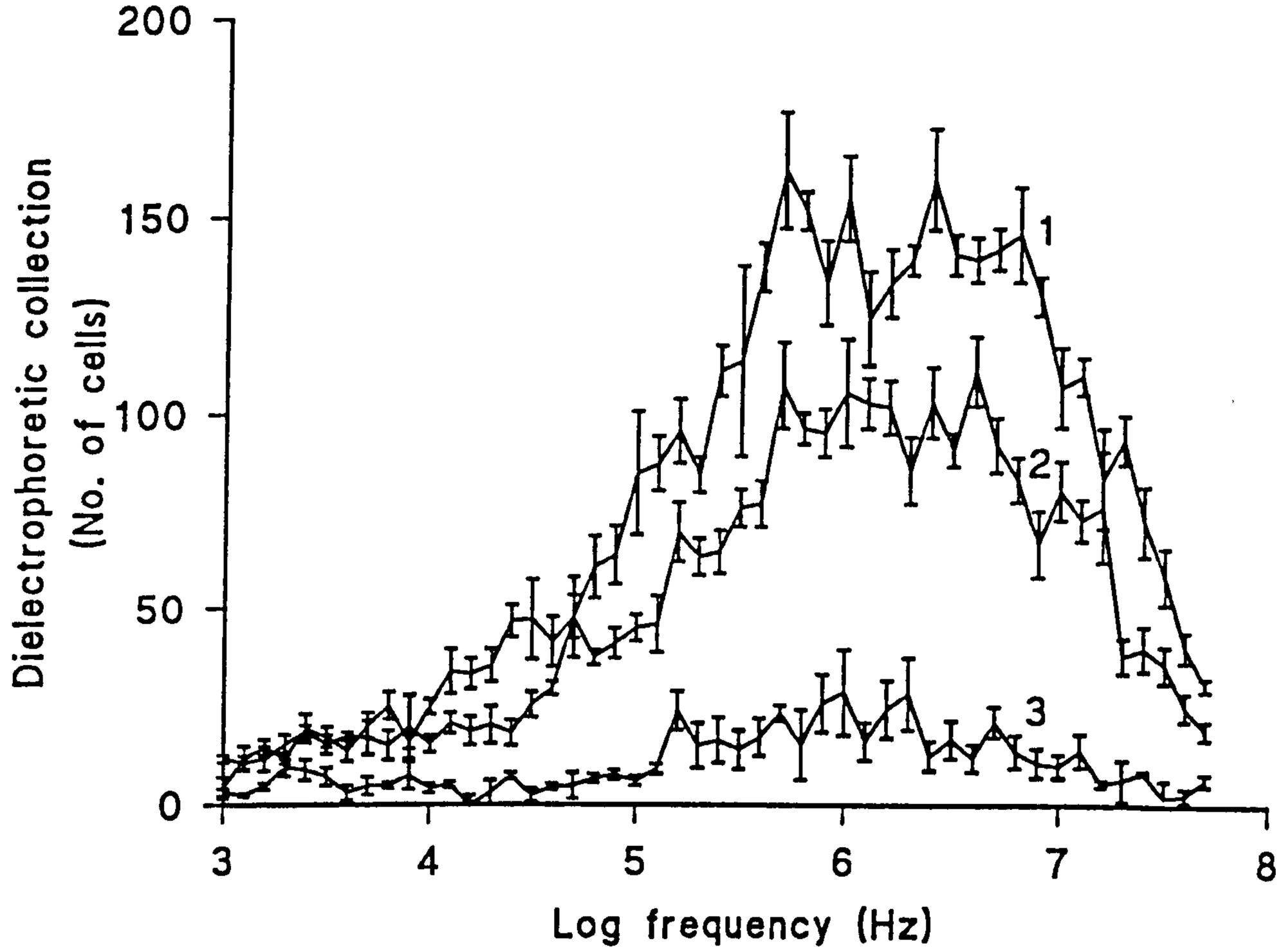


Fig 4.3 Mean dielectrophoretic spectra (n=4) of tetracycline resistant *E. coli* NCIMB 11459: (1) untreated, (2) with 20 μg ml^{-1} tetracycline and (3) with 80 μg ml^{-1} tetracycline.

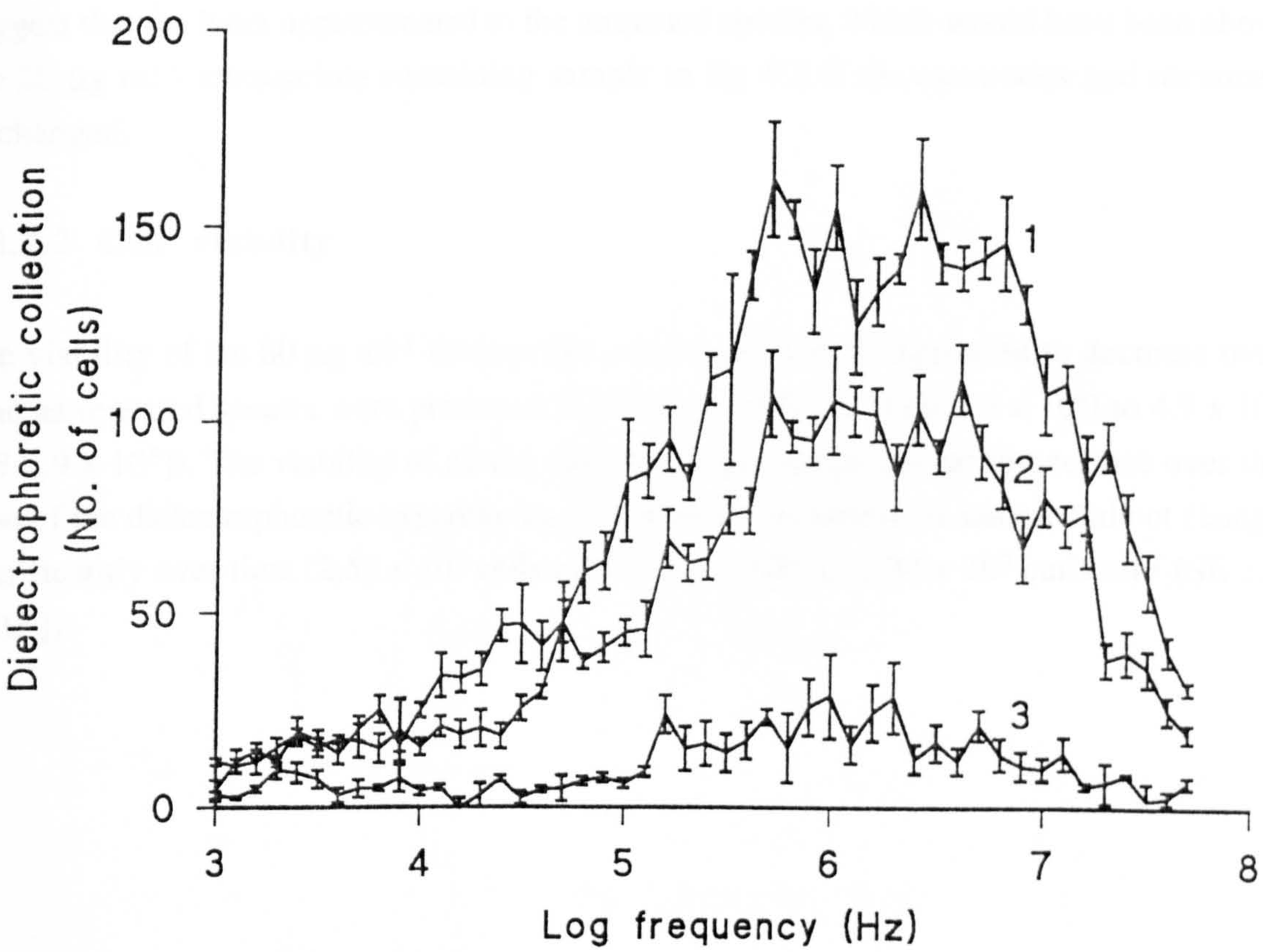


Fig 4.3 Mean dielectrophoretic spectra (n=4) of tetracycline resistant *E. coli* NCIMB 11459: (1) untreated, (2) with 20 μg ml^{-1} tetracycline and (3) with 80 μg ml^{-1} tetracycline.

The next two samples used were a 10 μg ml^{-1} tetracycline containing sample and a further untreated sample. The spectra are shown in fig 4.4. Both samples produced erratic spectra but appeared to collect to a similar degree. By the time these samples were used the electrodes had deteriorated badly and therefore they cannot be directly compared with the samples used in fig 4.3. However, the observation that an untreated and a 10 μg ml^{-1} tetracycline containing sample collected to a similar extent over a frequency range, may suggest that the latter approximated to the untreated spectra, which would have been above the 20 μg ml^{-1} tetracycline containing sample in fig 4.3 if the electrodes had remained unchanged.

4.3.2.2 Cell viability

The viability of the 80 μg ml^{-1} tetracycline containing sample appeared to decrease over time as repeated spectra were produced (2.74×10^7 cells ml^{-1} [SE 2.5×10^6] to 4.9×10^6 [SE 2.9×10^5]). The viability of all the other samples did not appear to decrease over the time of the dielectrophoretic experiments, for example the untreated sample did not change significantly over time (2.53×10^7 cells ml^{-1} [SE 3×10^6] to 2.34×10^7 cells ml^{-1} [SE 1.5×10^6]).

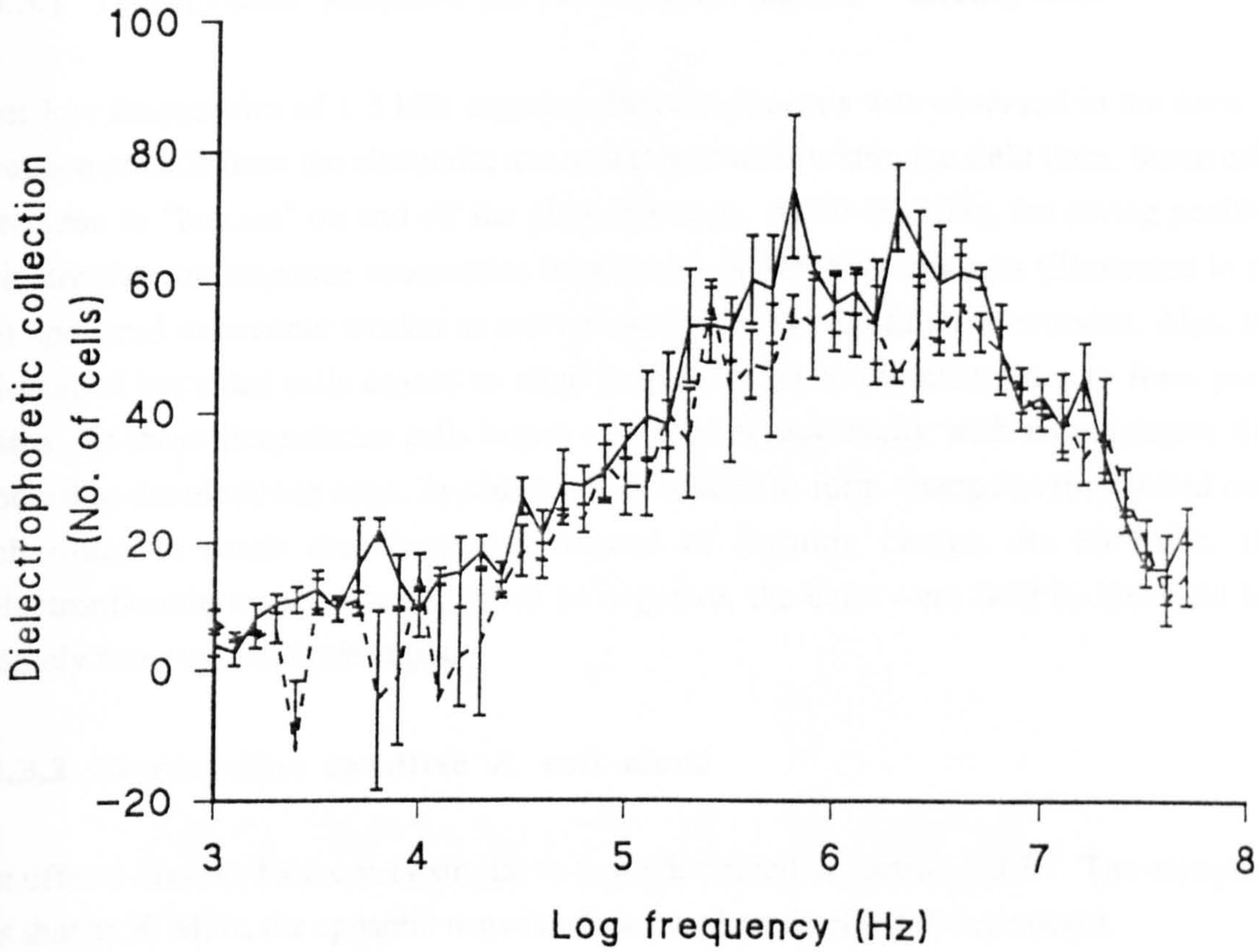


Fig 4.4 Mean dielectrophoretic spectra (n=4) of tetracycline resistant *E. coli* NCIMB 11459: (——) untreated and (– – –) with 10 μg ml^{-1} tetracycline.

4.3.3 Dielectrophoretic observations using tetracycline sensitive and resistant *E. coli* with pin and plate electrodes

The results of the experiments are described in the order in which they were performed.

4.3.3.1 Tetracycline sensitive *E. coli* with 80 μg ml^{-1} tetracycline

Over low frequencies of 1-5 kHz negative dielectrophoresis was observed in the form of repulsion of cells from the electrodes and swirling of cells within the field lines. Some cells were seen to "bounce" on and off the plate electrode. At 10-50 MHz, the strong positive dielectrophoretic response observed at frequencies of 100 kHz upwards (illustrated in fig 4.5) appeared to become weaker as cells moved more slowly to the electrodes. Also, the rod-shaped bacterial cells ceased to align perpendicular to the electrodes to form pearl chains. At these frequencies cells began to collect transversally with their longest side exposed to the electrode edge. In addition cells tended to form clumps as they rolled over each other to reach the electrodes instead of forming chains. At 50 MHz, the dielectrophoretic response appeared to be negative, the cells were held by the field but remotely from the electrode edges.

4.3.3.2 Tetracycline sensitive *E. coli* alone

The effects observed were very similar to those described in section 4.3.3.1. The exception was that at 50 MHz, the apparent negative dielectrophoretic effect did not occur.

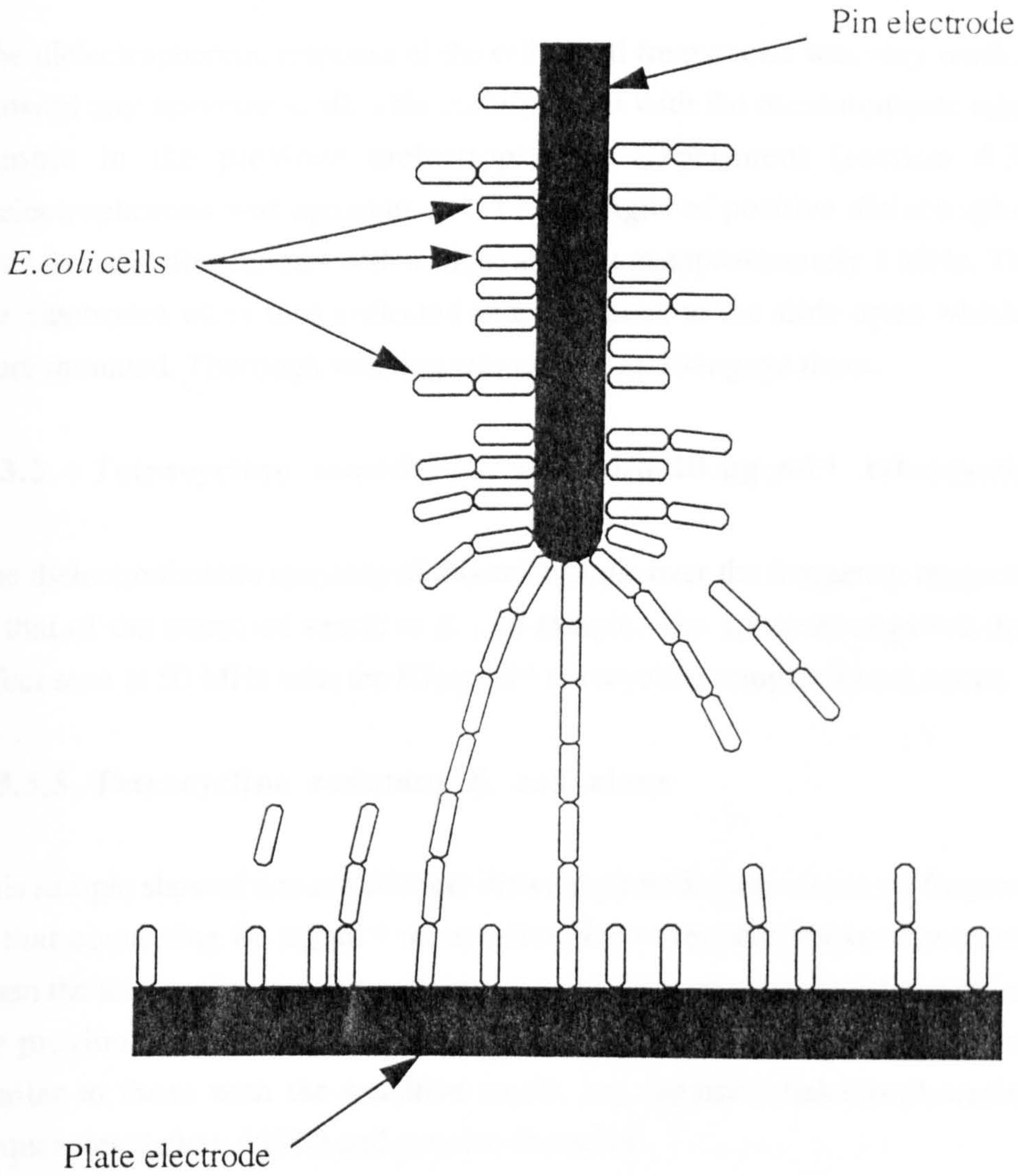


Fig 4.5 A diagrammatic representation of *E. coli* cells undergoing positive dielectrophoresis, collecting along the field lines between the pin and plate electrodes.

4.3.3.3 Tetracycline resistant *E. coli* with 80 μg ml^{-1} tetracycline

The dielectrophoretic response of the cells at all frequencies was very weak. Very few cells showed any response at all. This corresponded with the measurements taken for a similar sample in the previous dielectrophoretic experiment (section 4.3.2). Negative dielectrophoresis was apparent at 1-3 kHz. Signs of positive dielectrophoresis occurred over the other frequencies with a slight increase at approximately 1 MHz. The cells stuck to the electrodes when they collected and also stuck to the slide upon which the electrodes were mounted. Thorough washing was needed to disengage them.

4.3.3.4 Tetracycline sensitive *E. coli* with 20 μg ml^{-1} tetracycline

The dielectrophoretic response of this cell sample over the frequency range was very similar to that of the untreated sensitive *E. coli* sample. The apparent negative dielectrophoretic effect seen at 50 MHz with the 80 μg ml^{-1} tetracycline sample did not occur.

4.3.3.5 Tetracycline resistant *E. coli* alone

This sample showed a much stronger dielectrophoretic response at all frequencies compared to that containing 80 μg ml^{-1} tetracycline. However, the response was not as strong as when the tetracycline sensitive strain was used. This correlated with the effects measured in the previous dielectrophoretic experiment (section 4.3.2). The series of responses were similar to those with the sensitive strain, i.e. negative dielectrophoresis over the low frequencies (below 6 kHz) and positive thereafter.

4.3.3.6 Cell viability

The viability of the tetracycline sensitive *E. coli* with 80 μg ml^{-1} tetracycline was decreased over the period of use. That of the untreated sensitive sample also decreased though slightly. The tetracycline resistant *E. coli* with 80 μg ml^{-1} tetracycline decreased slightly over the experimental time period whereas that of the untreated resistant sample showed a slight increase in the total viable counts.

4.4 DISCUSSION

4.4.1 Experimental design

Dielectrophoresis of bacteria to assess changes due to the addition of an antibiotic could have been investigated using two approaches:

- Direct addition of the antibiotic to cells in suspension at the time of dielectrophoretic analysis.
- Pre-incubation of cells with the antibiotic before preparation of the cell suspension.

In experiments the antibiotic tetracycline was added to a bacterial suspension immediately prior to dielectrophoresis to determine changes in response over a period of time. The addition of antibiotic to cells already suspended in water implied that conditions were not ideal for active uptake, as the cells were in a slowed metabolic state. Therefore it was possible that the biological mode of action of tetracycline i.e. inhibition of protein synthesis might not occur and any dielectrophoretic changes upon addition of the antibiotic may not have been due to the usual mode of action.

Although cells suspended in water are likely to experience shock, they are unlikely to be rendered immediately dormant as they possess endogenous reserves (e.g. volutin, polysaccharide granules, polyhydroxybutyrate and sulphur). In fact when motile bacterial strains have been used in dielectrophoresis the cells have been observed to "swim" on the screen and therefore could not have been dormant. Some uptake of tetracycline may thus be possible, and effects of physico-chemical binding and of internal biological action may both have played a role in the dielectrophoretic response.

An alternative route for investigation would have involved the incorporation of the antibiotic into the growth medium for a predetermined time period, allowing the antibiotic to be taken up by the cells. In this situation an alteration in bacterial cell morphology may be observed when particular classes of antibiotics were used (e.g. lengthening of cells, as they continue to grow but not to divide when a cell wall inhibitor is added). This was observed in a preliminary experiment using cefotaxime (a cephalosporin) and clinical isolates of *E. coli*.

This effect prevented the use of the current image analysing system to measure dielectrophoresis as it did not take into account cell size, just the number of objects. If total cell area were measured instead of total cell number this may have been one way of accounting for cells of increased size. In the case of tetracycline, its bacteriostatic action may have prevented further cell growth, but the polarizability of the cells which had incorporated the antibiotic may not have been expected to change significantly over the frequency range and time period investigated.

Use of the former route was more likely to yield information regarding the binding of the tetracycline molecule to the bacterial cell surface. This could alter the dielectrophoretic response in itself, and if it did so in a way which correlated with the effect of the tetracycline on cell viability, this information could be valuable. In a clinical situation, if sensitivity could be assessed by the direct addition of an antibiotic to a bacterial suspension without the need for a pre-incubation step, obviously the reduction in analysis time could be advantageous.

4.4.2 Dielectrophoresis of tetracycline sensitive *E. coli* with and without tetracycline

The enhanced collection of the 80 μg ml^{-1} tetracycline *E. coli* sample over the lower frequency range (below approximately 70 kHz) may have been partly attributable to a negative dielectrophoretic response. However, the enhanced polarization was more likely to have been due to the electrodes, which were un-used prior to the experiment and began to deteriorate after the 80 μg ml^{-1} tetracycline sample had been used (discussed further in section 2.4.7). Electrode polarization effects upon collection of cell samples subsequent to the 80 μg ml-1 sample were probably involved. This effect can occur up to frequencies as high as 100 kHz (Burt *et al.*, 1989).

During positive dielectrophoresis, cells were held on or at the edges of the electrodes. However if the cells became immobilized remotely from the electrode bars but still remained close enough to the electrodes so that they were not visible on the screen, a negative dielectrophoretic response might have occurred. Photographs illustrating this effect have been included in chapter 2. This could not be observed during the course of an experiment as the electrode bars were out of view. When the electric field was removed and the cells were released into the counting frame, it was not possible to distinguish between the two types of dielectrophoretic response. When a pulse of a low frequency was applied during the experiment, it appeared as though more cells left the package associated with the electrodes and visibly streamed across the screen than when higher frequencies were applied. This was known to be indicative of negative dielectrophoresis, where the cells appeared to be held more weakly than during positive dielectrophoresis and therefore could be dragged away readily by the force of the flow.

However, it was highly unlikely that negative dielectrophoresis would have occurred up to 70 kHz at the low conductivity level used in this experiment. Also, although the involvement of negative dielectrophoresis has been proposed at low frequencies with other bacteria, this has usually been supported by a different spectral shape: counts have decreased when negative dielectrophoresis lessened as the dielectrophoretic force tended to zero, before positive dielectrophoresis began and counts increased.

To further investigate the dielectrophoretic response (whether negative or positive) of the 80 $\mu g\ ml^{-1}$ tetracycline *E. coli* sample, the response was observed directly using a static pin and plate electrode design. If negative dielectrophoresis had occurred, the addition of 80 $\mu g\ ml^{-1}$ tetracycline to *E. coli* must have decreased their polarizability over the low frequencies. However, negative dielectrophoresis was only observed at frequencies up to approximately 5 kHz, no greater than with the other samples used with this electrode design.

Polarizability of cells at lower frequencies (e.g. below 100 kHz) are thought to involve mechanisms which utilise the peripheral areas, e.g. the counterion layer and charged surface groups. Addition of tetracycline which may have bound to the outer surface could have affected the polarizability of the cells at these frequencies by cancelling or restricting some of the surface charge. Thus the electric field would be unable to interact or distort the charges to enhance cell polarization.

4.4.2.1 Antibiotic effects upon bacterial surface charge

In studies performed by Morris and Jennings (1975, 1977), electric polarization of *E. coli* at low frequencies was found to be reduced upon addition of antibiotics. The method they used to measure the induced dipole involved monitoring the orientation of cells in an applied field by measuring the intensity of scattered light. In the first study, the polycationic antibiotics streptomycin and neomycin were used. Both had the effect of decreasing polarizability, which they attributed to a negative surface charge neutralization effect. It was suggested that rather than a change in the number of available counterions, a reduction in the number of available cell surface charged sites was caused through antibiotic adsorption. Polarization at the frequency used (780 Hz) was thought to arise from the distortion of counterions in the particle-medium interface, caused by the applied electric pulse.

The same explanation was proposed by Fomchenkov *et al.* (1979), for the decrease in positive dielectrophoretic response of *E. coli* caused by addition of the same antibiotics. However a decrease over a large frequency range (100 Hz - 10 MHz) was observed so the same polarization mechanism cannot be solely responsible for effects seen at higher frequencies. The possible action of the antibiotics in causing cell envelope destabilization through displacement of surface divalent cations (Hancock, 1984) may have contributed to the observed effects. No decrease in polarizability was recorded by Fomchenkov *et al.* when penicillin, a negatively charged molecule, was used.

The surface charge neutralization explanation of Morris and Jennings (1975) and Fomchenkov *et al.* (1979) is contradicted in the second study performed by Morris and Jennings (1977). In addition to the original antibiotics, bacitracin, penicillin and polymyxin-B were also used. All antibiotics had a similar effect, causing a sharp decrease in polarizability. However, neutralization of surface charge could not be primarily responsible, as positively charged, negatively charged and neutral (bacitracin) antibiotics had all produced a similar effect.

The alternative explanation involved a decrease in cell surface permittivity caused by antibiotic adsorption which increased the strength of counterion binding and effectively made them more difficult to polarize. Electrophoretic mobility can be reduced either by a reduction in cell surface charge or by a change in surface permittivity, therefore this correlated with a decrease in electric polarizability.

Conversely, Dealler (1991) suggested that adsorption of antibiotics is not responsible for the change in bacterial cell zeta potential (important in electrophoretic mobility) caused through addition of antibiotics. A study using ampicillin, colistin and gentamicin showed the formation of a separate population of cells with altered zeta potential in each cell sample. The population size increased over time (approximately 1 h), then began to decrease. Evidently antibiotics had altered zeta potential although if adsorption had been responsible, this would have occurred more rapidly than the zeta potential change and would have remained over time. Instead, it was suggested that the effects may be due to altered transmembrane potential caused through organism stress.

However, in general the effects of altered zeta potential upon cell polarization are manifested at frequencies too low for measurement using the dielectrophoretic set-up described in this thesis. If lower frequency effects had been investigated, larger differences between the dielectrophoretic effects of the antibiotic doses may have been apparent, although practical problems including electrode polarization and oxidation of the aluminium electrodes would have made this difficult.

The peak collection at 1 MHz for all samples (those with and without tetracycline) suggested that addition of the antibiotic could not have affected the predominant polarization mechanisms (interfacial polarization) responsible for dielectrophoresis of cells at this frequency.

4.4.2.2 The negative dielectrophoretic response at 50 MHz of tetracycline sensitive *E. coli* with 80 μg ml^{-1} tetracycline

Tetracycline sensitive cells with 80 μg ml^{-1} were repelled from the electrodes at 50 MHz. A large decrease in cell polarizability was probable, as cells needed to be less polarizable than the suspending medium for negative dielectrophoresis to result. The high level of tetracycline used was above the MIC of the bacteria and the reduction in viability inferred the damaging effect of this antibiotic dose. Cell damage caused through the action of tetracycline may not be primarily membrane targeted but any cell damage may affect the transmembrane potential and therefore allow for some degree of leakage to occur. Membrane leakage would serve to reduce the internal conductivity with respect to the medium conductivity and the decrease in capacitance leads to decreased polarizability and collection.

However, another consideration is the small volume size used in these dielectrophoretic investigations. Contamination through ineffective cleaning of the electrodes or through impurities on the coverslip might have resulted in a high local conductivity upon the electrodes (therefore an effective increase in medium polarizability rather than a decrease in cell polarizability), although that of the stock suspension may have remained unchanged.

In fig 4.2 no reduction in cell polarization of the 80 μg ml^{-1} sample at 1 MHz was observed which would have been expected if leakage had occurred. If conductivity of the medium was so high that even at 50 MHz the cells remained less polarizable, negative dielectrophoresis would result.

Energy dependent uptake of tetracycline is likely to be limited with cells suspended in deionized water. Tetracycline can cross both the outer membrane and to an extent, the cytoplasmic membrane of *E. coli* by passive diffusion (Chopra, 1985; Chopra *et al.*, 1992).

Incubation of cells with a high tetracycline level of 80 μg ml^{-1} may have lead to a significant amount of antibiotic entering the cell. Cells may have been unable to achieve tetracycline accumulation by energy dependent means due to suspension in deionized water, but if diffusion resulted in equilibrium between the intracellular and extracellular concentrations, a significant amount of tetracycline may be available to inhibit protein synthesis. If this effect was dependent on diffusion occurring over time, this would be consistent with the reduction in total viable counts over the course of the experiment.

4.4.3 Dielectrophoresis of tetracycline resistant *E. coli* with and without tetracycline

The electrodes used to perform these experiments had deteriorated since their use with tetracycline sensitive cells and all spectra produced were erratic to some degree due to noise (discussed in chapter 2).

According to the results of this experiment, the 80 μg ml^{-1} tetracycline sample either collected to a much lesser extent over the whole frequency range, and/or collection caused the cells to stick to the electrodes from where they could not be released. The subsequent experiment using the pin and plate electrodes showed that both of these effects occurred although the cells did appear to be very unresponsive to dielectrophoresis in general. The addition of tetracycline to these cells reduced the polarizability of this already poorly polarizable strain.

The viability of the 80 μg ml^{-1} tetracycline containing sample showed a decrease over time whereas none of the other samples did. Although the MIC broth dilution assay designated this organism to be resistant to tetracycline at a dose of 80 μg ml^{-1}, the MIC probably lay not far from this dose (certainly between 80 and 160 μg ml^{-1}) and the additional stress of the preparation and re-suspension of the cells in deionized water may have reduced their resistance to this high level of antibiotic. An outer membrane damage effect caused through the chelating action of this high dose may have made a contribution to the decrease in viability.

The untreated resistant cells showed higher collection levels over almost the entire frequency range than the 80 μg ml^{-1}, whilst the 20 μg ml^{-1} produced an intermediate response. This suggests that the tetracycline caused more of a dose-dependent response with the resistant than with the sensitive organism, certainly over the 100 kHz-50 MHz frequency range.

4.4.3.1 A proposed explanation for the dielectrophoretic response of tetracycline resistant *E. coli*

The low level collection of the 80 μg ml^{-1} tetracycline sample at 100 kHz - 50 MHz, indicates that tetracycline must have affected cells to decrease polarization in this region. At around 1 MHz the interfacial polarization mechanism operates. Cell leakage could lead to decreased membrane capacitance, therefore decreased polarizability and collection. It is not clear whether tetracycline either directly or indirectly led to this effect.

As the cells are suspended in deionized water, it is uncertain whether they are sufficiently metabolically active to allow uptake and subsequently, efflux of tetracycline. However, the phenotypic surface differences of the resistant strain compared to the sensitive strain may be at least partly caused by the induction of the 34kD, 26kD and 14kD resistance proteins in the presence of tetracycline.

Upon their induction, proteins involved in efflux-mediated tetracycline resistance must have become integrated into the bacterial cytoplasmic membrane and wall, and assumed the correct conformation for their function. The resistance proteins 34 and 26 kD are present in the cell membrane and the 14 kD protein in the cell wall. All are induced in the presence of tetracycline. Some studies have suggested that the inclusion of tetracycline resistance encoding plasmids lead to altered phospholipid and fatty acid metabolism within the cell (Chopra, 1985). This seemed logical with the creation of new membrane domains to accommodate resistance proteins. However, the changes in lipid content of such cells are thought to be non-specific. Nevertheless, inclusion of plasmids invariably results in an altered growth rate which may indirectly lead to altered lipid metabolism (Chopra, 1985). If this were the case in the *E. coli* cells incorporating pSC101 it may explain the poor polarizability of the strain.

It has been reported that insertion of other biological molecules into lipid membranes has resulted in altered dielectric properties. The insertion of cholesterol into membranes composed of the lipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) altered the rotational mobility of the lipid charged head groups. Cholesterol causes an increase in the head group separation, therefore decreasing head group-head group electrostatic interaction. In addition it sterically hinders acyl chain motion (Bone and Zaba, 1992). Insertion of gangliosides of a particular structural shape into a phospholipid bilayer, has also been shown to alter electrical behaviour (Cametti *et al.*, 1993). It seems probable that altered lipid arrangement or metabolism, as well as the introduction of membrane proteins would affect electrical properties of bacterial membranes, and consequently their polarizability.

4.4.3.2 A proposed explanation for the dose-dependent differential collection levels of tetracycline resistant *E. coli*

It was discovered that tetracycline resistant cells carrying the transposon *Tn*10 or the plasmid pBR322, were preferentially inhibited by a variety of lipophilic compounds (Bochner *et al.*, 1980; Maloy and Nunn, 1981). Although the molecular basis for this is unclear, it is possible that chelation of essential membrane cations may be involved. The level of cations may already have been lowered due to the insertion of resistance proteins into the membrane and wall (Bochner *et al.*, 1980).

An effect similar to this may have been influential in the further decrease in polarizability of the pSC101-containing cells upon the addition of 80 $\mu g\ ml^{-1}$ tetracycline. Tetracycline is also known to be a relatively lipid soluble compound (Chopra *et al.*, 1992). The direct effects of other antibiotics upon Gram negative cell surfaces have been documented, including the displacement of divalent cations by aminoglycosides (Hancock, 1984) and chelation, causing membrane destabilization, by quinolones (Nikaido, 1989).

The plasmid pBR322 is a derivative of pSC101, containing part of the tetracycline resistance encoding region, including the genes coding for the 34 kD efflux protein. The high concentration of tetracycline (80 $\mu g\ ml^{-1}$) may have acted to disrupt the membrane by chelating divalent cations, a function that tetracycline is known to possess (Gale *et al.*, 1981). This may have lead to the differential level of dielectrophoretic response observed with different tetracycline doses.

In some classes of tetracycline efflux determinants (e.g. Tet A and Tet B),the resistance determinant is flanked by direct repeats which provide sequence homology for gene amplification. Generally, increased gene copy numbers of plasmid-located antibiotic resistance determinants are relatively common and frequently lead to increased levels of resistance, due to e.g. the production of higher enzyme levels. However, an increase in tetracycline efflux does not occur when these resistance determinants are amplified (Chopra, 1985).

Between 1-36 copies of the class Tet A resistance determinant were used per *E. coli* cell in an experiment to study the effects of increased copy number (Wiebauer *et al.*, 1981). No increase in efflux was detected implying that there was a limited number of sites within the membrane, capable of incorporating efflux proteins. However, increased osmotic lability also occurred indicating that the increase in efflux protein insertion into the cell envelope lead to instability. It was concluded that the number of sites available for efflux protein insertion may not itself have been the limiting factor, but the functional sites from which efflux could be promoted probably was (Wiebauer *et al.*, 1981).

In the case of class Tet B determinants, increased gene copy number lead to decreased tetracycline efflux. It was proposed that lowered resistance was due to membrane destabilization, caused through the accumulation of multiple copies of the 36 kD protein which decreased efflux efficiency (Moyed *et al.*, 1983).

CHAPTER FIVE
GENERAL DISCUSSION

5.1 The dielectrophoretic measurement system

Although several biological applications for dielectrophoresis have been investigated, there is still potential for exploitation of this non-invasive phenomenon within many other areas of biology. However, while dielectrophoresis can be employed to good use, measurement of the phenomenon does have its limitations. Some of the strengths and weaknesses of the dielectrophoretic investigation of biological particles using the image analysis enhanced equipment are described in this thesis.

Dielectrophoresis was used to investigate *Cryptosporidium parvum* oocysts and *E. coli* before and after treatment with certain antimicrobial agents. The current dielectrophoretic measurement system was able to assess responses over time which is not easily achieved using a non-automated system.

Analysis of dielectrophoretic response for particle suspensions before and after antimicrobial treatment generally showed either a change in the frequency dependence of collection and/or an alteration in collection level over a frequency range. The former occurred in the treatment of *C. parvum* oocysts with ozone (section 3.3.2) and was quantified further by calculating the ratio of collection at two key frequencies with different applied ozone doses (section 3.3.2.1). However, reduction of collection levels as described for oocysts treated with 0.5 and 5 mg l^{-1} chlorine, and tetracycline resistant *E. coli* with varying tetracycline doses, were more difficult to quantify. In each case the levels of collection were altered when compared to other samples and to an untreated control. However, quantifying this reduction was complicated by variability within and between experiments due largely to problems with microelectrodes (section 2.4.7).

The microelectrodes used in experiments were not ideal for use at low frequencies. Below 1 kHz, application of a pulse could cause electrolysis of water; in this situation H_3O^+ and OH^- ions are produced and the OH^- ions damage the aluminium. This can be prevented by use of a less reactive metal in electrode manufacture, e.g. gold plated chromium has been tried. Even at frequencies above 1 kHz, the problem of electrode polarization was thought to lead to the underestimation of the dielectrophoretic force. Burt *et al.* (1989) and Bone and Zaba (1992) have reported that this can occur up to frequencies as high as 100 kHz. In addition, any occurrence of negative dielectrophoresis at low frequency can confuse results although direct observation of collection would clarify this.

Methods for elimination of and correction for electrode polarization have been described:

- Use of a set of electrodes where the distances separating the electrodes can be varied, allows for correction of electrode polarization. Larger inter-electrode distances provide a relatively larger contribution of sample polarization rather than electrode polarization.
- A substitution method involving dielectric measurements of an electrolyte solution of equivalent conductivity to the sample can be employed. The dielectric measurement of the sample can then be corrected for the electrode polarization.
- Treatment of electrodes to increase the surface area available for ions can be performed by roughening the surface or using platinum electrodes coated in platinum black.
- A "four electrode technique" can be used wherein two electrodes produce the AC electric field whilst the remaining two probe the potential across the sample. This monitors for the presence of electrode polarization - it is not present if the potential electrodes do not draw any current. (Bone and Zaba, 1992).

The surface of cells including surface groups and ions associated with the cell wall and membrane, predominantly contribute to cell polarizability in an applied AC field typically below 100 kHz (Inoue *et al.*, 1988; Burt *et al.*, 1989; Markx *et al.*, 1994). However as most of the problems associated with the microelectrodes occur below this frequency, effects of antimicrobial agents upon these cellular features may not be fully characterized using the system described in this thesis. Some of the treatments may have been expected to alter surface charge/particle conductivity and consequently dielectrophoresis. The detection of such changes using the current system may not have been fully realized. However, treatment which altered capacitive properties of the cell membrane, or which affected internal features were possibly more suitable for investigation as these were measured using higher frequencies.

5.2 Future considerations for the dielectrophoresis of *C. parvum* oocysts

Using the modified Huang method to model the electrical properties of the outer shell of particles (Huang *et al.*, 1992), explanations were proposed for the action of ozone and chlorine upon oocysts which lead to their altered dielectrophoretic responses (sections 3.4.3 and 3.4.6.1). However, for these results to be of use when examining oocysts found in water treatment systems several other factors need to be considered and investigated.

Oocysts extracted from water will have been exposed to a number of environmental stresses which could affect their response to treatment with ozone and/or chlorine. A consideration of how individual and combined endured pressures would affect dielectrophoretic response is essential. Important factors include:

- oocyst age / length of time spent in the environment
- fluctuating temperature
- desiccation
- shear forces and abrasion (e.g. during filtration)
- chemical flocculation
- pH alterations (e.g. as may be experienced during water treatment)
- grazing effects
- exposure to chemicals e.g. pesticides and fertilisers
- the combined action of ozone and chlorine.

At present ozone is not routinely employed in UK water treatment for disinfection purposes. The potential for exploitation of the dielectrophoretic response described in this thesis (sections 3.4.4 and 3.4.5) would depend on widespread adoption of this disinfection method, although it is likely that other oocystidal agents could produce damage resulting in a similar dielectrophoretic response.

Little is known about the physiology of *C. parvum* oocysts, including whether they are able to utilize nutrients in the environment. This may have some bearing on how oocysts would be expected to respond dielectrophoretically. The question of oocyst viability equating to infectivity also needs to be resolved, so that dielectrophoresis and other technologies with the potential to assess viability can be focused more accurately.

If oocysts are exposed to partial excystation conditions, it is conceivable that stimulation of sporozoite activity could be induced, though not to a sufficient extent for excystation to occur. If this situation results in depletion of sporozoite endogenous reserves, sporozoites excysting subsequently may be no longer able to infect host cells. If nutrient uptake by intact oocysts is feasible, replenishment of reserves may be possible and this situation may not arise. The ability of oocysts to take up and to utilize nutrients is uncertain but may affect oocyst survival time in different environments. Also, it is feasible that oocysts may be able to take up nutrients when exposed to certain excystation triggers such as elevated temperature, when first entering the host gastrointestinal tract. This may be relevant to their ability to infect host cells. Experiments to investigate this could involve direct measurement of uptake of radio-labelled compounds, e.g. glucose, or to look for utilization of glucose by monitoring CO_2 evolution.

5.3 Future considerations for the dielectrophoresis of antibiotic-treated bacteria

The aims of the dielectrophoretic investigations involving tetracycline described in this thesis were two-fold:

- to determine the effects of antibiotic treatment upon bacterial dielectrophoretic response
- to measure dielectrophoresis of antibiotic sensitive and resistant cells, with and without antibiotic.

Antibiotics include a variety of chemical compounds with different polar properties. Use of compounds with stronger positive (e.g. streptomycin) or negative (e.g. penicillin) charges may have been expected to have a more dramatic effect upon dielectrophoresis below 100 kHz. However the effects of antibiotics in reducing surface permittivity must also be considered (section 4.4.2.1). The difficulties associated with dielectrophoretic measurement below 100 kHz using the current system made investigations difficult to design and interpret. The addition of any agent which may alter surface polarizability cannot be easily explained.

When investigating the dielectrophoretic response of treated cells which may be expected to have an altered surface charge, measures could be taken to isolate lower frequency effects (below 100 kHz) from "noise" caused by features of the microelectrodes. The problem of negative dielectrophoretic immobilization at electrodes causing confusion with positive dielectrophoretic collection could be overcome by direct observation of experiments before using the image analysis system to quantify the response. In general, performing experiments solely in order to observe dielectrophoretic effects may be useful for the analysis of spectra before the experiments to generate repeated data sets and assess the reproducibility of an effect are performed. The problem of electrode polarization could be corrected for by using one of the methods described in section 5.1. A relatively straightforward method is that of Burt *et al.* (1989) where the second of the four methods in section 5.1 was used. This involved measuring the frequency dependence of the suspending medium conductivity, to determine the frequency at which electrode polarization ceased. Below this frequency, ion layers upon the electrodes reduced the availability of the electric field for particle polarization. Therefore, correction of dielectrophoretic responses below this frequency to give their "true" polarizability is possible.

Initially the use of tetracycline with *E. coli* seemed to indicate marked differences between sensitive and resistant strains, where collection of resistant cells reduced with increasing tetracycline concentration but collection of the sensitive cells remained virtually unchanged (sections 4.3.1 and 4.3.2). However, the inclusion of the plasmid pSC101 (to confer tetracycline resistance) altered the morphology of the cells in such a way that comparison between the resistant and sensitive cells was not straightforward. This is likely to be the case in many situations of sensitive and resistant bacterial strains, particularly as resistance is commonly plasmid mediated.

Any future dielectrophoretic investigation of antibiotic sensitive and resistant organisms would involve a wide range of considerations. If a clinical application was intended, these may include:

- the dielectrophoretic characterization of a wide range of microorganisms
- the variation in dielectrophoretic response between isolates of the same species due to, for example, different growth conditions
- the alteration of dielectrophoretic response upon addition of a range of antibiotics
- the direct and indirect effects of resistance (likely to vary depending on genetic origin and mode of action)
- the effect of varying antibiotic doses, including sub-MIC which may be important in a post-antibiotic effect
- the effects of microorganism age
- the effects of culture conditions and suspending medium
- the optimisation of electrodes designed for dielectrophoretic investigation of particle conductivity or correction for electrode polarization effects (discussed in section 5.1 and above)
- the effects of pre-incubation and subsequent incorporation of the antibiotic (section 4.4.1) and consequent modifications necessary for measurement.

The dielectrophoresis experiments using antibiotics were not well supported by the traditional total viable count method of viability assessment. This involved assessing the ability of cells to form colonies when a suspension was serially diluted and aliquots plated onto nutrient agar. Hence, the results were not available until the following day and yielded little information about the cells at the time of dielectrophoresis. Also, if antibiotic uptake did affect cell viability, this would have meant that cells unable to grow on nutrient agar during dielectrophoresis may have remained metabolically active though unable to reproduce.

A method of assessing physiological damage, not necessarily a presence/absence of viability, would be more suitable for dielectrophoresis experiments using bacteria. However the reliability of many physiological assessment methods are controversial, e.g. some fluorescent staining techniques are plagued by false positives due to inadequate specificity. The increasing use of flow cytometry in microbiology may be one suitable method which could become more available in the future.

A further future consideration for dielectrophoresis is the possibility of incorporation with existing technologies. For example, the dielectrophoretic viability analysis of an environmental *C. parvum* oocyst sample initially identified using flow cytometry. This could eliminate the need for fluorescent staining and microscopy, currently performed manually after flow cytometric sorting. Also, the possibility exists for the combination of dielectrophoretic analysis with blood culture enrichment technology. It has been shown that organisms can be extracted directly from blood cultures grown in instruments such as the BacT/Alert (Organon Teknika) and used to produce dielectrophoretic spectra (Betts, 1994). However research is insufficiently advanced to assess the suitability of such a combination.

The phenomenon of dielectrophoresis has been investigated with respect to applications in many areas including some within biology. In practice, measurement of the phenomenon can be problematic with respect to both the particles and frequency range under investigation. Consequently, the development of equipment to assess the dielectrophoretic response of biological particles must take such considerations into account. The equipment used for dielectrophoretic analyses described in this thesis, incorporated image analysis for rapid, automated measurement of the response. This adaptation introduced the possibility of rapid production of consecutive spectra and generation of results in "real time". Although the nature of the system used was not ideal for the measurement of low frequency effects steps could be taken to improve this in the future. However the work in this thesis demonstrates both the potential for dielectrophoresis as a tool to investigate biological particles and the value of an automated, image analysis enhanced system to generate rapid results important in many applications.

APPENDIX I

Protocol for aluminium microelectrode manufacture

Manufacture was carried out in the clean room of the University of York Department of Electronics.

1. ***Cleaning of slides***

Glass microscope slides (BDH Super Premium microslides, cat. no. 406/0180/02) were each cleaned with mechanical abrasive, followed by storage in deionized water. The slides were subsequently immersed in boiling acetone and dried using a pressurised nitrogen gun.

2. ***Metal-coating of slides***

A vacuum evaporator was used to coat one side of each slide with aluminium.

3. ***Application of the first photoresist layer***

A layer of syringe-filtered photoresist (Shipley, cat. no. 1400-27) was used to cover the metal-coated surface of each slide. They were placed on a slide spinner at 1500 rpm for 30 s to produce a uniform thin coating. The slides were transferred to a hot plate for a pre-exposure bake at 95°C for 1 min.

4. ***Exposing slides to the electrode template***

Each photoresist-coated slide was positioned together with the mask defining the electrode configuration using a mask aligner (Micro Controle Sulzer Electro technique, model no. MA750). The slide was exposed to ultra-violet light for 7 s.

5. ***Development of the photoresist***

The electrode design was revealed upon the microslide after photoresist exposure. The development of the design meant that the exposed photoresist and the aluminium layer underneath were resistant to acid-etching. Therefore this was used to remove excess metal, leaving the electrode design on the slide. The slide was immersed in developer (MF 319) and gently agitated, therefore any unexposed photoresist was removed until the electrode image could be seen (approximately 40 s). The slide was then baked for 15 min, 95°C.

6. ***Etching***

A solution of <1 part nitric acid : 1 part acetic acid : 1 part orthophosphoric acid, in turn diluted to 6 parts etch solution : 1 part water, was used for acid etching. The aluminium which was not protected by the exposed photoresist was removed by immersion in the solution until the glass slide could be seen.

7. ***Removing the excess photoresist***

Boiling acetone was used to remove the excess photoresist, taking care not to rub the metal.

8. ***Adding the adhesion promoter***

Adhesion promoter was freshly prepared, consisting of 1 part deionized water : 1 part Selectiplast HTR AP3 (Ciba Geigy) : 98 parts propan-2-ol. The slides were covered with promoter and left for 15 s before being spun at 1500 rpm for 15 s. The slides were then baked at 95°C for 1 min.

9. ***Applying the second coat of photoresist to delineate chamber boundaries***

The aluminium surface of the slides was covered with a second photoresist (Selectilux HTR3-200, Ciba Geigy) and spun at 550 rpm for 15 s, then the speed increased to 1500 rpm for an additional 3 s. The slides were baked prior to exposure at 95°C for 35 min. The combination of this photoresist and conditions resulted in a durable layer approximately 70 μm thick.

10. ***Exposing the slide to the channel template***

The slide and channel template were positioned using the mask aligner as before. The photoresist was exposed using ultra-violet illumination for 1 min 45 s.

11. ***Developing the photoresist***

Excess photoresist was removed by gentle rubbing of the surface with a cotton bud moistened with Selectiplast HTR D2 (Ciba Geigy) until the channel motif was revealed between (60-90 s). The slide was then rinsed in a 50/50 solution of HTR D2 and IPA for 15 s, then in IPA for 15 s before being dried with a pressurized nitrogen gun. The slide was then inspected to evaluate the success of the developmental process which could be repeated if necessary. When satisfactory the slides were baked at 150°C for 80 min and were then ready for use.

APPENDIX II

Growth curve of tetracycline sensitive *E. coli* NCIMB 11459

Procedure

An overnight culture of *E. coli* NCIMB 11459 was made by inoculating into 100 ml sterile nutrient broth in a 250 ml conical flask which was then incubated at 37°C with shaking. Six similar flasks each containing 100 ml of sterile nutrient broth were each inoculated with 1 ml overnight broth culture. Three flasks were incubated at 37°C with shaking and three were refrigerated on ice. The number of flasks was limited to three by the amount of available incubator space.

At regular time intervals, two 1 ml samples were aseptically removed from each inoculated flask and the control flask, one into a 1.5 ml cuvette and the second into a 9 ml volume of sterile quarter strength Ringer solution. After 12 h the three refrigerated flasks were put into the 37°C incubator. After a further 12 h these had reached a similar growth phase to that of the first 3 flasks after 12 h so the second set of flasks were used to continue the growth study.

Absorbance measurements

Growth was monitored by measuring the OD_{510} of each sample against a sterile nutrient broth blank. The OD measurement wavelength was selected by varying the wavelength when a sample of nutrient broth alone was placed in the spectrophotometer until the maximum absorbance value was established. This wavelength was subsequently used for OD. measurements. When the OD_{510} of a sample was >0.2, the sample and the blank were diluted and re-read.

Viable counts

Ten-fold serial dilutions of each sample were made down to 10^{-6}, 10^{-7} or 10^{-8} depending on how long the cultures had been growing. Aliquots of 0.1 ml were plated out in triplicate from each of the last 3 dilutions of each sample onto nutrient agar plates. These were incubated at 37°C overnight. Over the first 180 min, samples were taken every 30 min and hourly thereafter for a total of 22 h.

Results

The absorbance values were plotted on a log scale over time to give a growth curve (fig A1). The total viable counts were also plotted on a log scale over time (fig A2).

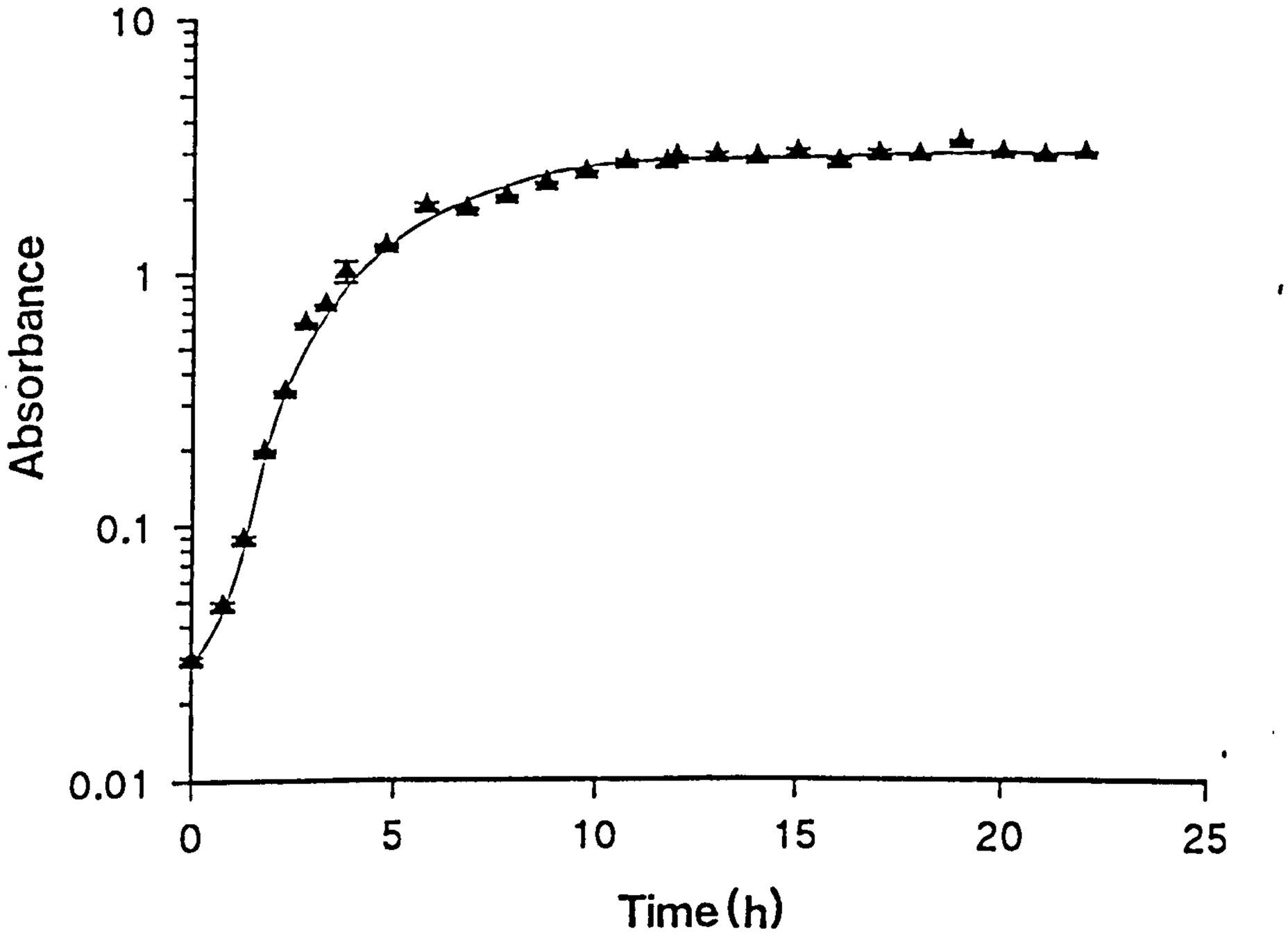


Fig A1 Growth curve of *E. coli* NCIMB 11459: Absorbance (at 510 nm) over time.

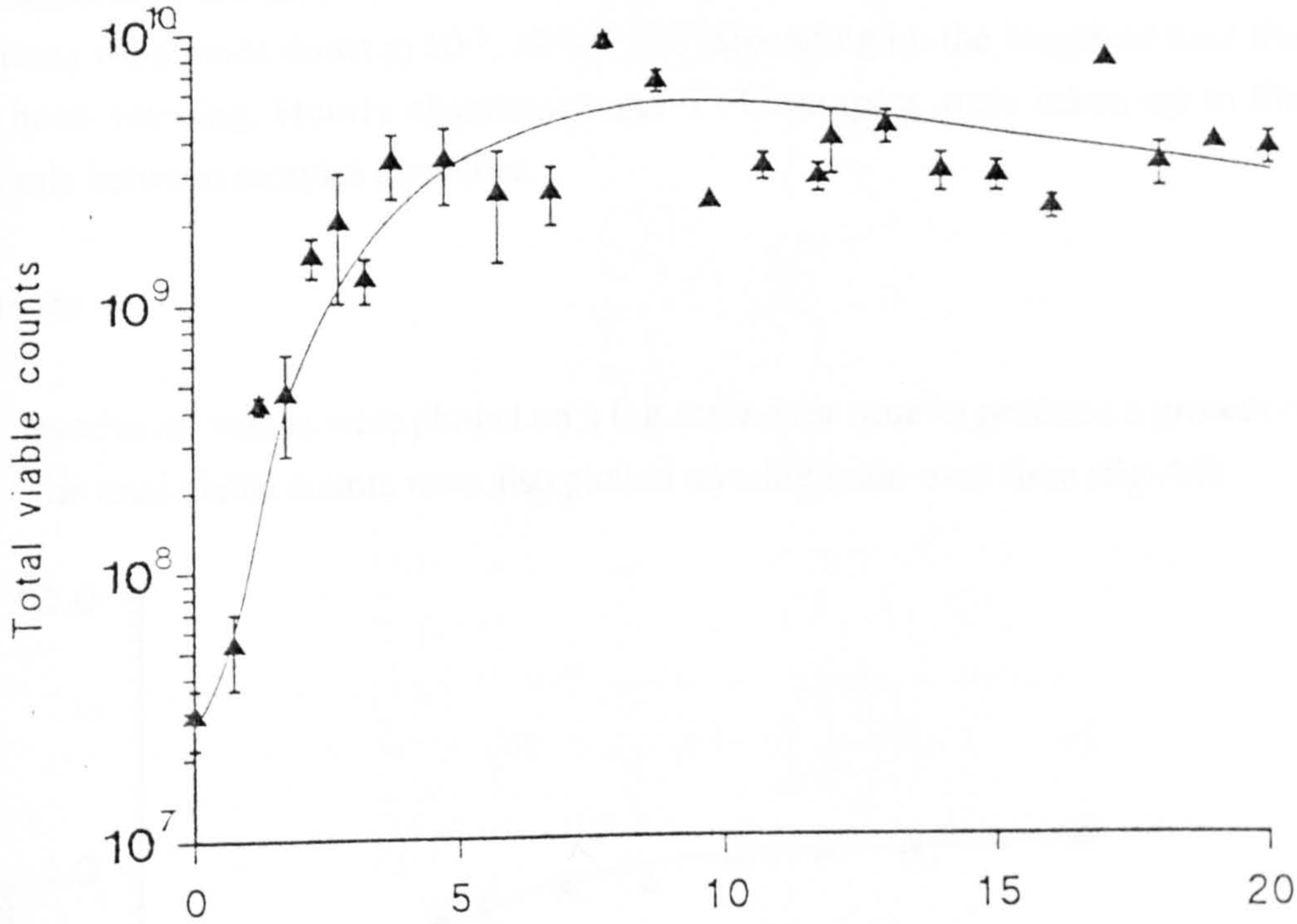


Fig A2 Growth curve of *E. coli* NCIMB 11459: Log total viable counts over time.

APPENDIX III

Growth curve of tetracycline resistant *E. coli* NCIMB 11459

The procedure was similar to that for sensitive *E. coli* NCIMB 11459. Two sets of five replicate flasks were used for absorbance measurements (one refrigerated on ice for 12h) and samples were also taken from two flasks from each set to estimate TVCs. Serial dilutions were made down to 10^{-5}, 10^{-6} or 10^{-7} depending on the length of time the cultures had been growing. Hourly absorbance and TVC samples were taken up to 8h with 2h intervals between samples thereafter.

Results

The absorbance values were plotted on a log scale over time to produce a growth curve (fig A3). The total viable counts were also plotted on a log scale over time (fig A4).

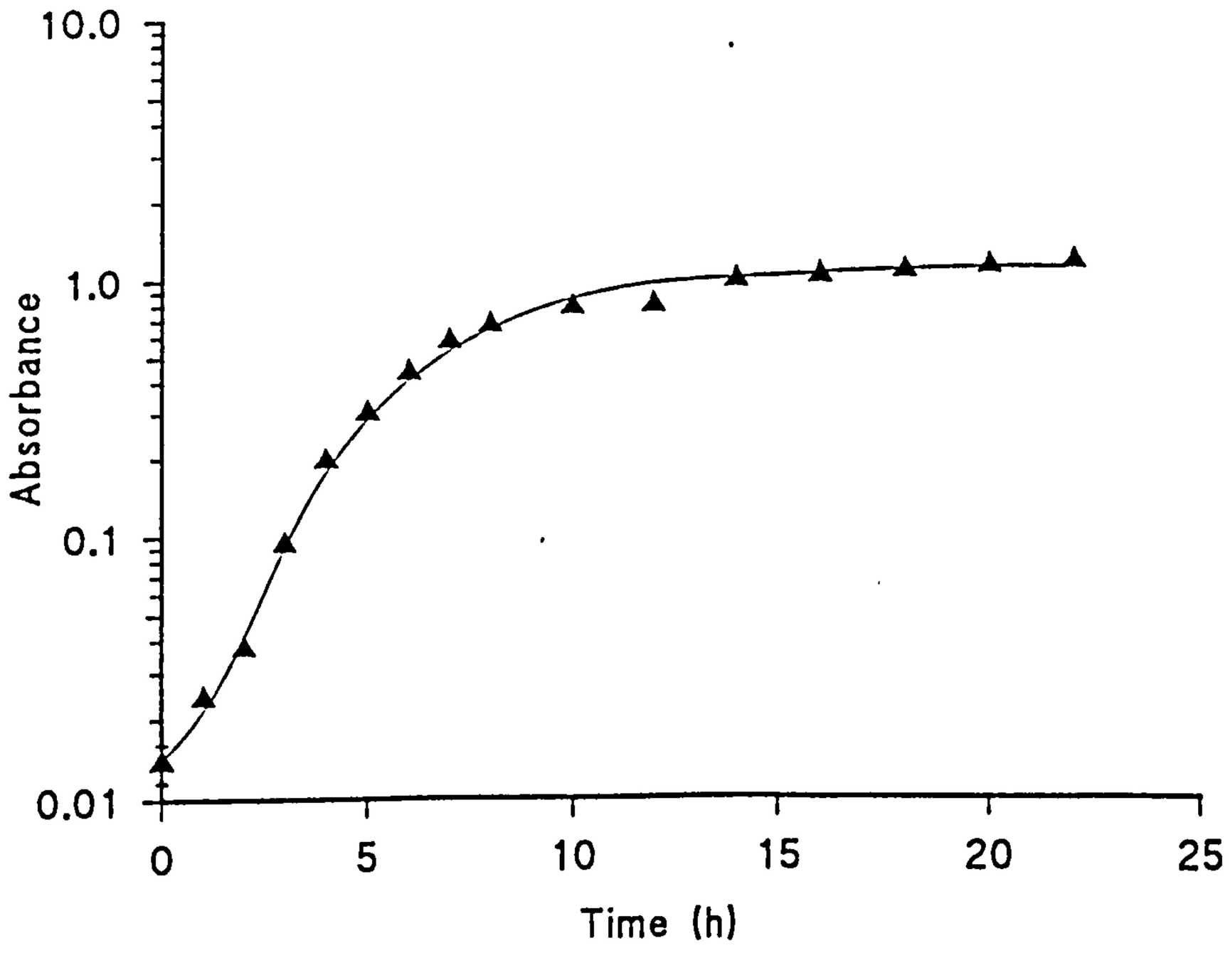


Fig A3 Growth curve of tetracycline resistant *E. coli* (*E. coli* NCIMB 11459 with plasmid pSC101): Absorbance (at 510 nm) over time.

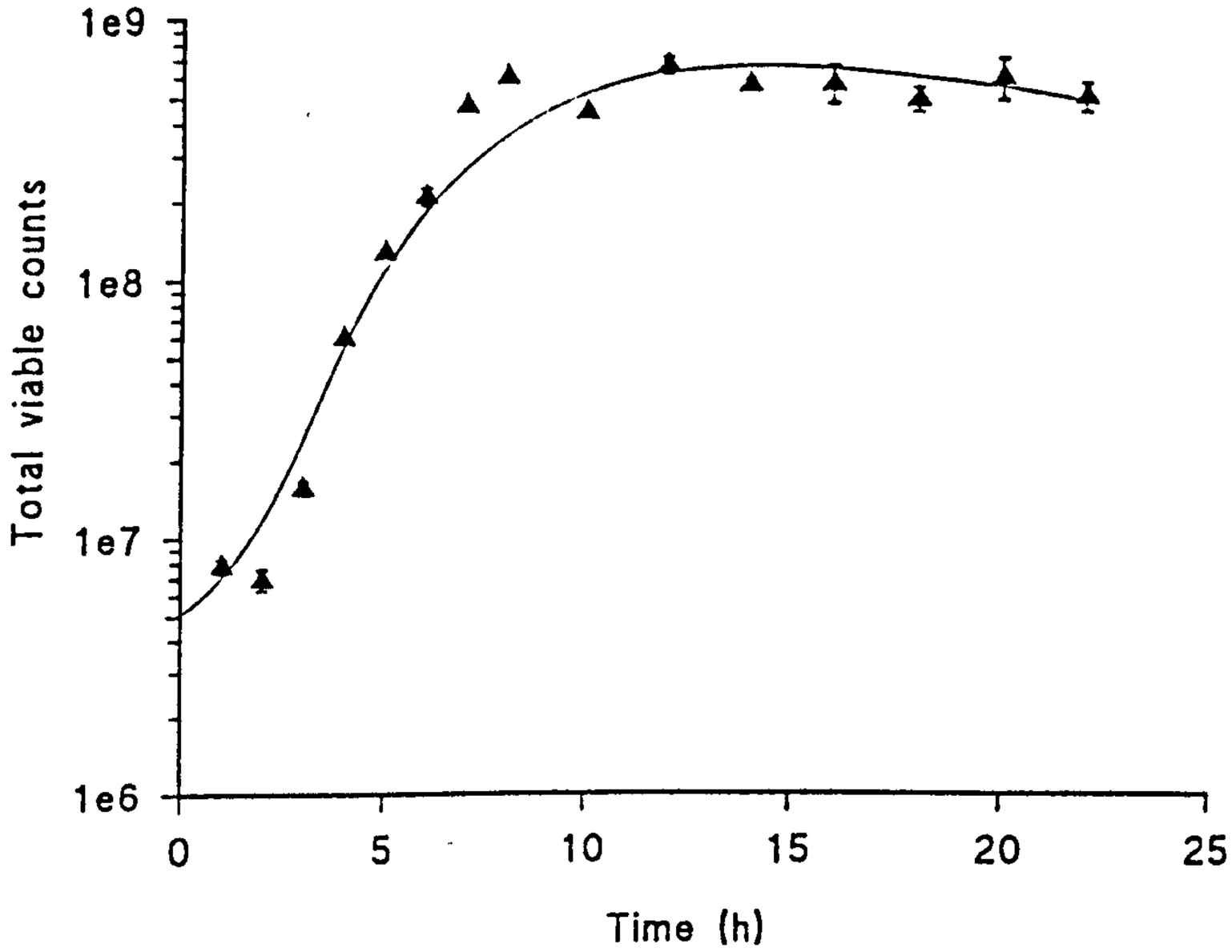


Fig A4 Growth curve of tetracycline resistant *E. coli* (*E. coli* NCIMB 11459 with plasmid pSC101): Log total viable counts over time.

APPENDIX IV

Agar diffusion assays

Introduction

Agar diffusion assays are routinely performed in clinical laboratories to assess the sensitivity of a bacterial isolate to an antibiotic. The methodology used varies around the world, for example methods followed in Europe and the United States are outlined by the ECCLS (European Committee for Clinical Laboratory Standards) and the NCCLS (National CCLS) respectively and use numerical values to assess antibiotic sensitivity. In the U.K. *comparative methods* are used. Test and control organisms are plated out at the same time under identical conditions and paper discs impregnated with standardised amounts of antibiotics are placed on top of the inoculated media. The size of the zones of inhibition around the discs are compared between the test and the control organisms. The control organisms used in the U.K. are listed in table A1. The control may be plated onto a separate identical plate or onto the same plate as the test organism. The latter is known as the Stokes' method where using rotary plating the control organism is plated onto the inner part of the plate and the test organism is plated around the outside. Antibiotic containing discs are then positioned so that half of each disc is covering the test organism and the other half is covering the control.

Table A1. Control organisms for the comparative methods.

Control organism	Organism for comparison with
E. coli NCTC 10418	Coliforms
Ps. aeruginosa NCTC 10662	*Pseudomonas* spp.
Haemophilus influenzae NCTC 11931	*Haemophilus* spp.
Neisseria gonorrhoea 'sensitive strain'	*N. gonorrhoea*
Staph. aureus NCTC 6571	Other organisms that grow aerobically
Clostridium perfringens NCTC 11229	*Clostridium* spp.
Bacteroides fragilis NCTC 9343	Other anaerobic organisms

The results obtained classify the organism as either sensitive, resistant or intermediate (compared to the control organism). Sensitive isolates are inactivated if a manufacturer's recommended dose of an antibiotic is prescribed to a patient. Resistant isolates are unlikely to respond to treatment with the manufacturer's recommended dose. Intermediate isolates may respond to doses higher than routinely used (Holt and Brown, 1991).

E. coli (NCIMB 11459) both with and without the inclusion of the pSC101 plasmid (resistant and sensitive to tetracycline respectively) were tested to assess their susceptibility to tetracycline using the agar diffusion assay comparative method.

Media

Iso-Sensitest agar (Oxoid) which is made commercially for antibiotic sensitivity testing was used. The depth of the medium was 3-4 mm. These were stored at 4°C and used within 1 week.

Antibiotic

Tetracycline stocks were prepared as described in section 4.2.1. A 0.4 ml volume was removed from the thawed stock solution and diluted with 9.6 ml sterile distilled water to give a final concentration of 40 $\mu g\ ml^{-1}$. Two-fold serial dilutions were made to give further concentrations of 20, 10, 5, 2.5 and 1.25 $\mu g\ ml^{-1}$ tetracycline.

Choice of control organism

E. coli NCTC 10418 was chosen as the control organism because the test organisms are coliforms.

Growth of cultures

Flasks containing 100 ml of sterile Iso-sensitest broth (Oxoid) were each inoculated with a single colony taken from a plate. The plates were re-streaked fortnightly. All cultures were grown up overnight at 37°C with shaking.

Preparation and application of inocula

The inoculum was sufficient to produce semi-confluent colony growth. If growth was too heavy the effects of the antibiotics were masked and if colonies were spread too far apart it was difficult to define zones of inhibition. A wet swab inoculation method was used. A 1 ml aliquot of overnight broth culture was diluted in 99 ml sterile distilled water. A sterile swab was dipped into the suspension and the excess removed by turning the swab against the side of the tube. The inoculum was spread over the surface of the plate in 3 directions, 60° apart and the plate was left to dry. Triplicate plates of the control and the two test organisms were inoculated.

Application of discs

A series of concentrations of tetracycline were used. A template was made so that the antibiotic sensitivity discs could be arranged in the same order on each plate. Paper antibiotic sensitivity discs (Whatman) were sterilized. Fine forceps were flamed in alcohol and used to pick up one disc and dip it into an antibiotic solution and then onto blotting paper to remove the excess. The disc was placed in the appropriate position on the inoculated plate and pressed down gently. The process was repeated using all antibiotic concentrations and a control of sterile distilled water alone. Triplicate plates were produced for the control and the two test organisms. The plates were incubated overnight at 37°C.

Measuring zone size

Zone diameters, including the diameter of the disc (6 mm) were measured using a pair of Verniers callipers and are shown in fig A5. In a UK clinical situation formal measurement of zone size is not routinely performed.

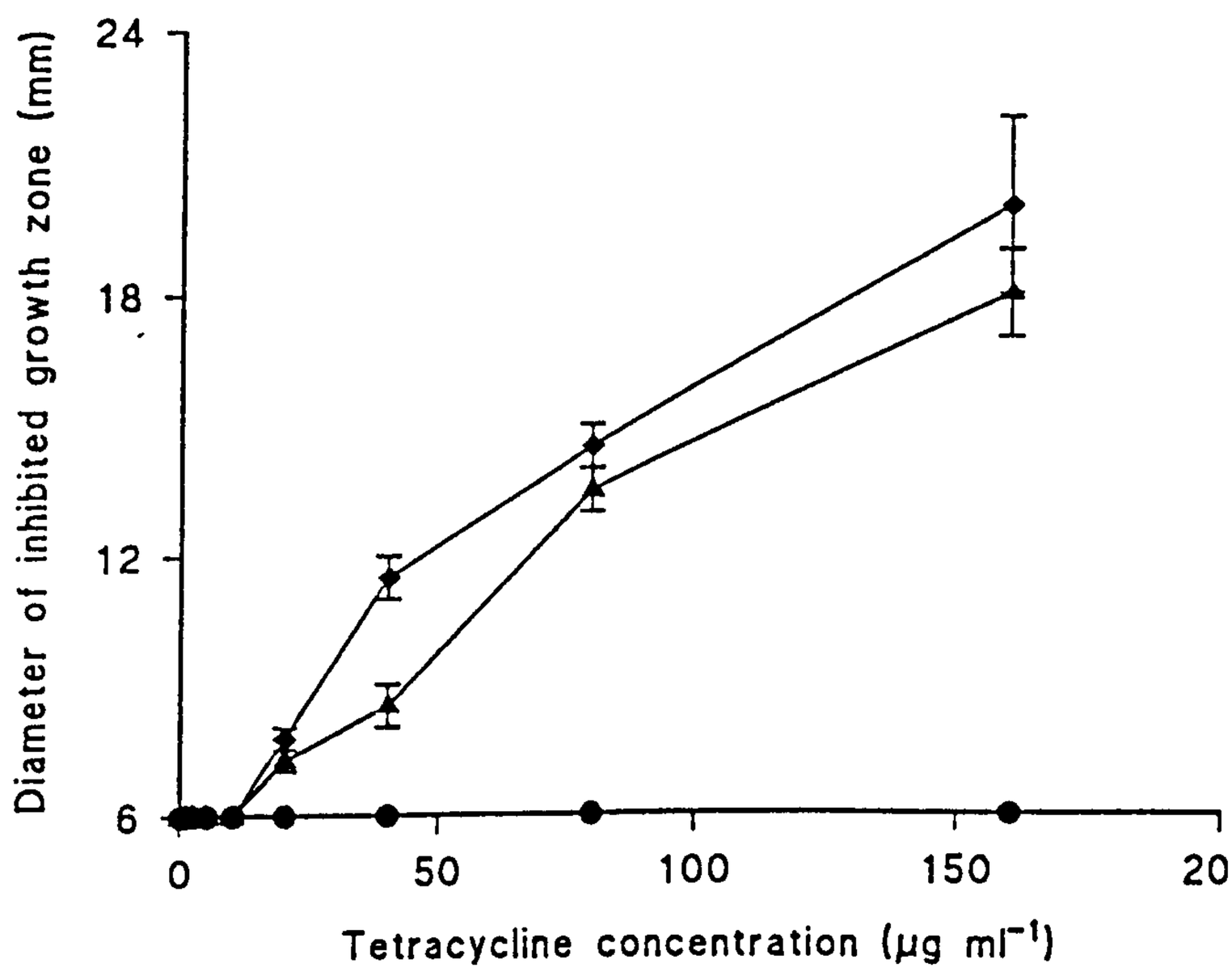


Fig A5 Agar diffusion assay of tetracycline with: *E. coli* NCTC 10418 (◆), *E. coli* NCIMB 11459 (▲) and *E. coli* NCIMB 11459 with plasmid pSC101 (●). Diameter of assay disc = 6 mm.

APPENDIX V

Determining the MIC of tetracycline against sensitive and resistant *E. coli* NCIMB 11459

Introduction

This method was used to give a more quantitative sensitivity assessment of tetracycline sensitive and resistant *E. coli* NCIMB 11459 than the agar diffusion assay. The MIC is the lowest concentration of an antibiotic which inhibits visible growth of an isolate after a suitable period of incubation.

Preparation of media

A 1.6 ml volume from a thawed tetracycline stock solution (preparation described in section 4.2.1) was added to 8.4 ml sterile Iso-sensitest broth (Oxoid) to give a concentration of 160 $\mu g\ ml^{-1}$. Doubling dilutions were to give further concentrations of 80, 40, 20, 10, 5, 2.5, 1.25 $\mu g\ ml^{-1}$. A 5 ml volume was removed from the final dilution and discarded giving a 5 ml total volume for each dilution. A control of 5 ml sterile Iso-sensitiest broth with no added tetracycline was included and another control of a serially diluted set of tetracycline broths which remained un-inoculated. A replicate of the whole series was produced for each of the organisms tested.

Inoculation of broths

A 50 µl volume from an overnight broth culture was added to each of the tubes in the appropriate sets of broth and shaken gently. The tubes are incubated for 18-24 h at 37°C.

Measuring the MIC

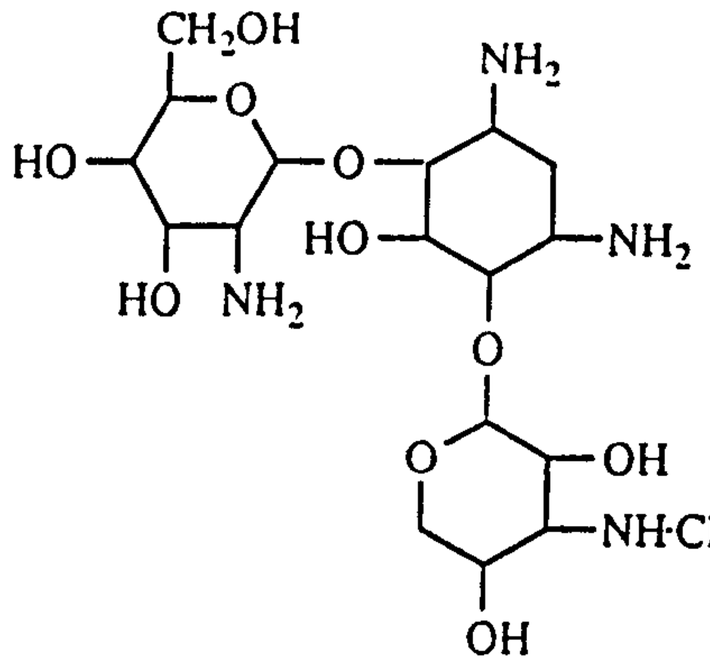
For each set of broth dilution tubes, the presence or absence of growth was assessed by eye in comparison to the two controls i.e. one with no tetracycline therefore maximum growth and one with no bacteria which was sterile. For a more quantitative assessment of growth, absorbance measurements were taken by removing a 1 ml aliquot from each tube and reading on a spectrophotometer (Model 4050 Ultrospec II, LKB Biochrom, Cambridge, UK) against a sterile Iso-sensitest broth blank. Dilution of the broth culture samples was performed where absorbance was >0.2. A relationship between the tetracycline concentration and the bacterial growth for each organism tested was produced and a dose range was calculated within which lay the MIC for each organism.

Results

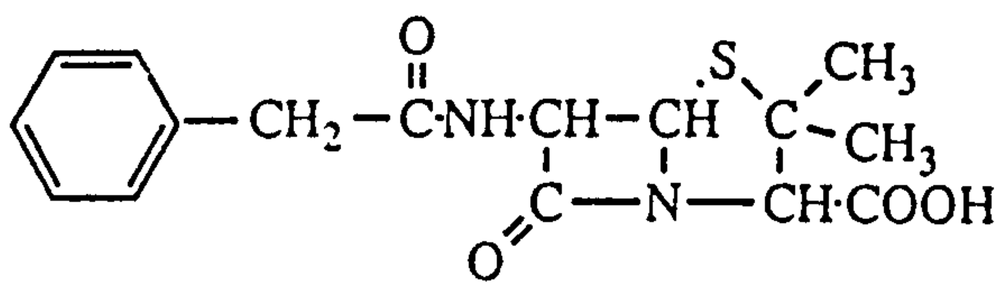
The MIC of *E. coli* NCTC 10418 lay between 10-20 μg ml^{-1} tetracycline. The MIC of tetracycline sensitive *E. coli* NCIMB 11459 lay between 40-80 μg ml^{-1} tetracycline. The MIC of tetracycline resistant *E. coli* NCIMB 11459 lay between 80 and 160 μg ml^{-1} tetracycline.

The MIC values agree with the tetracycline sensitivity results of the agar diffusion assays (appendix IV) i.e. the control organism, *E. coli* NCTC 10418 was the most sensitive, the resistant *E. coli* containing plasmid pSC101 the least sensitive, and the *E. coli* NCIMB 11459 lay intermediate between the two.

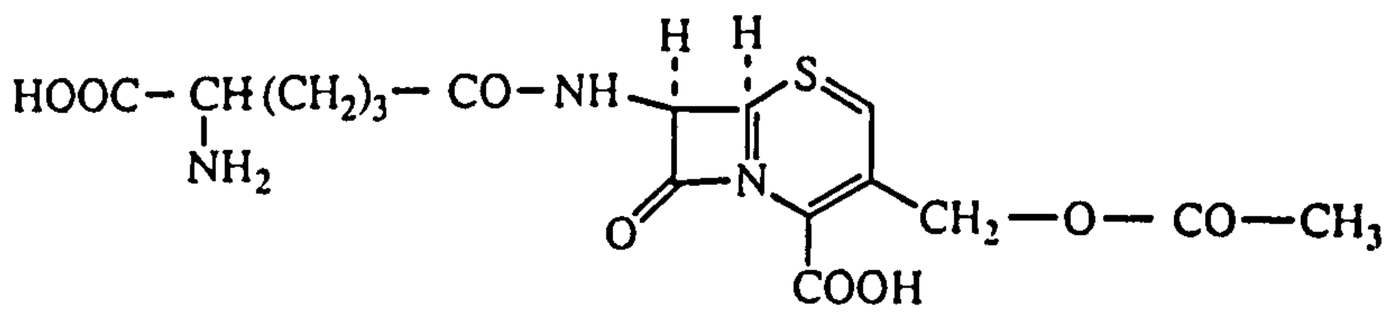
APPENDIX VI



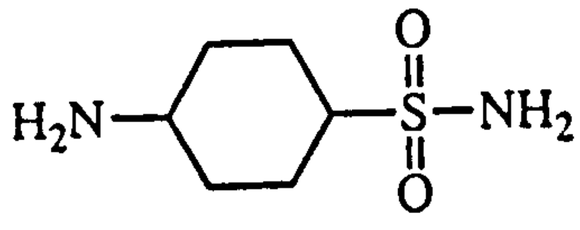
Gentamicin



Penicillin

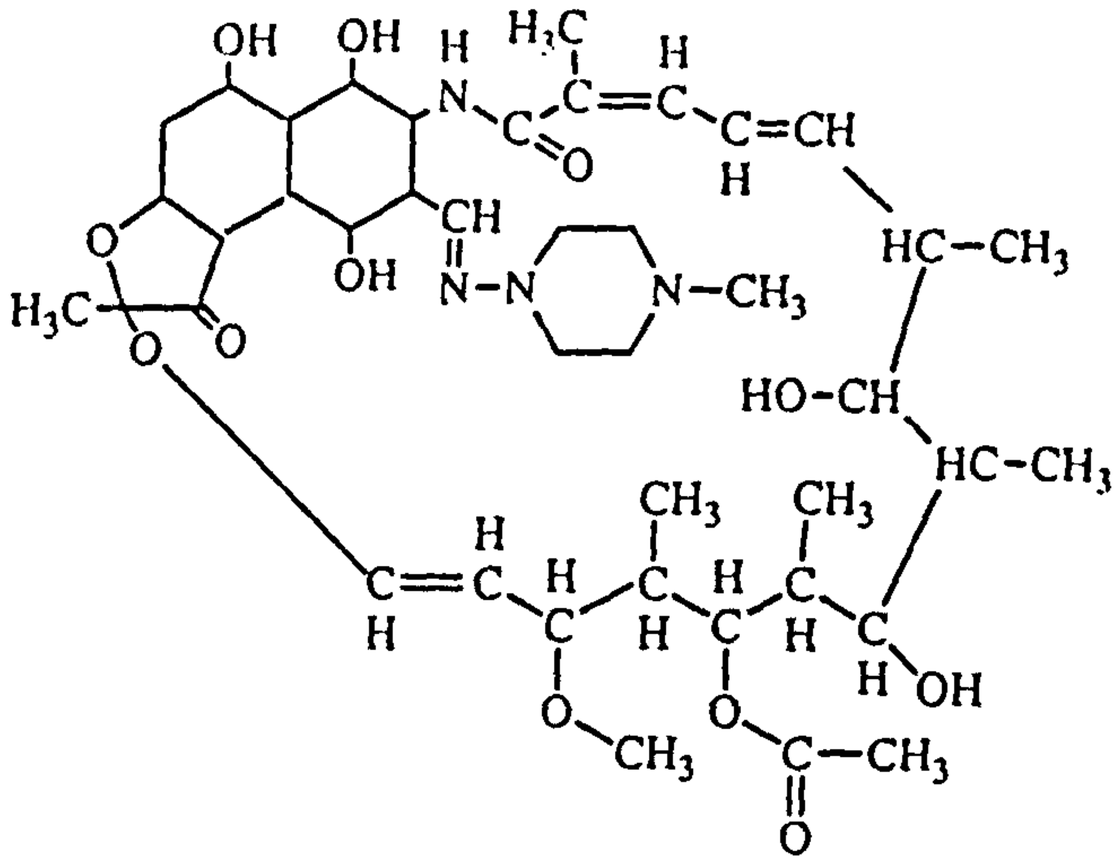


Cephalosporin C

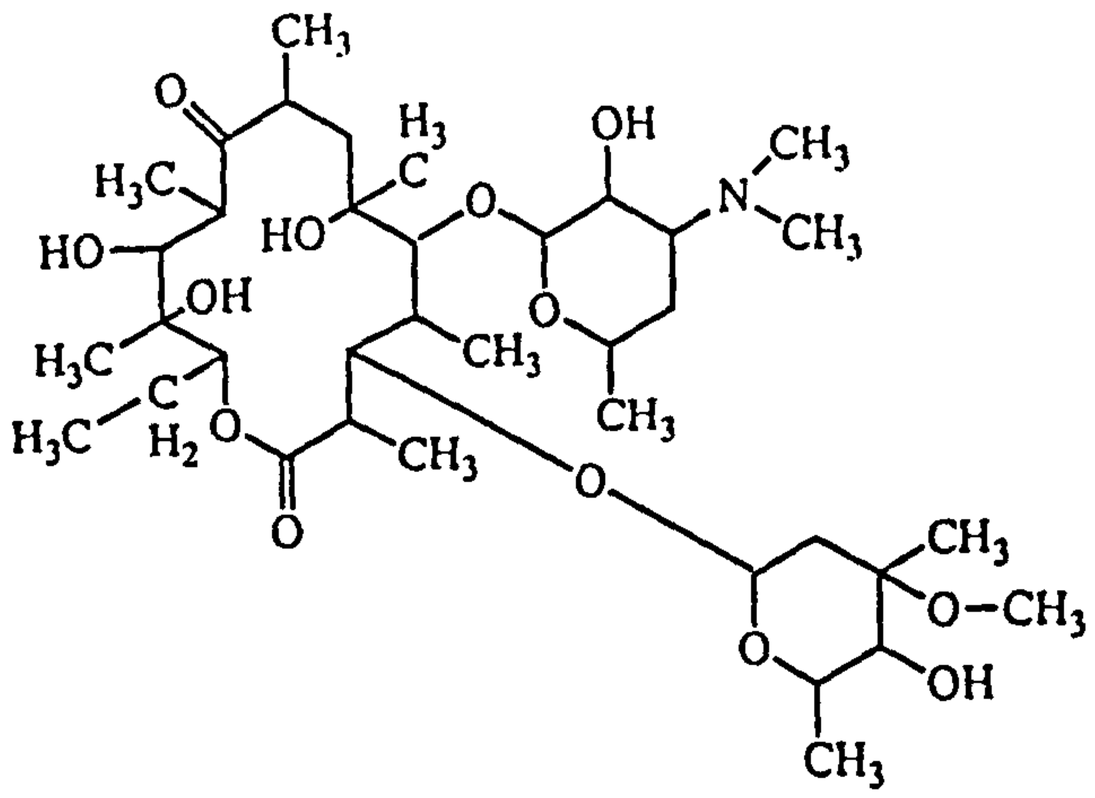


Sulphanilamide

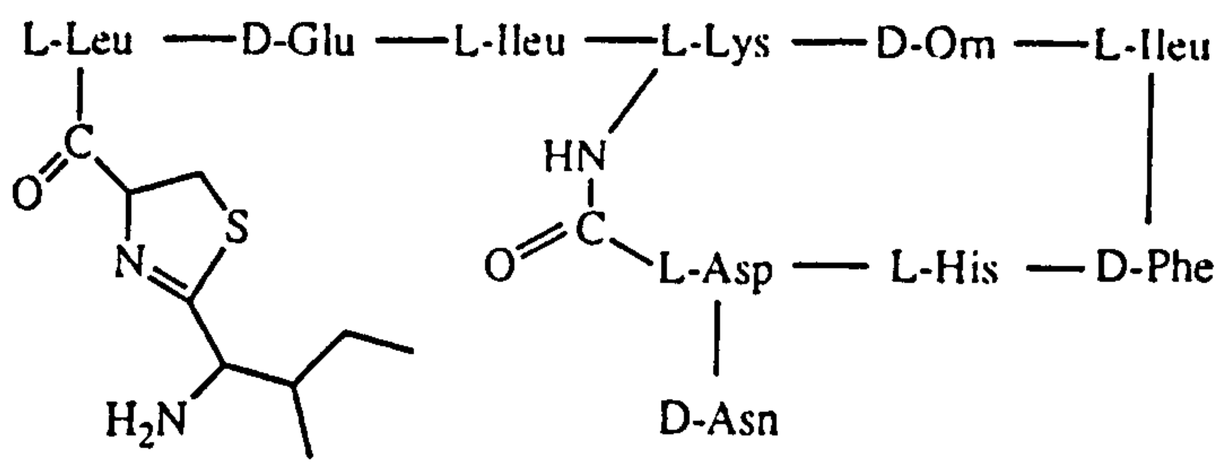
Structures of some common antibiotics.



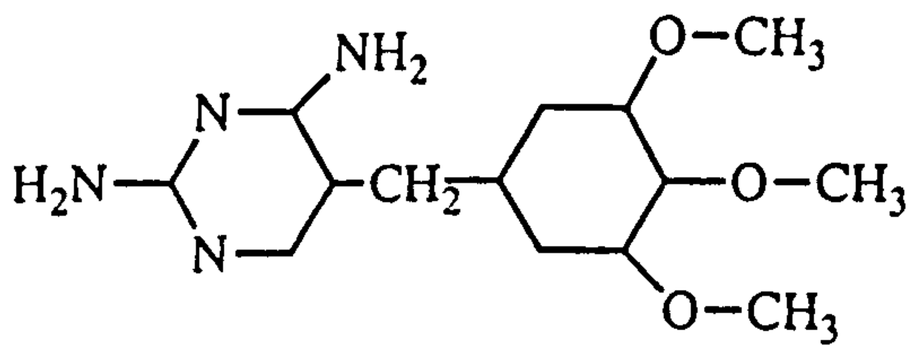
Rifampicin



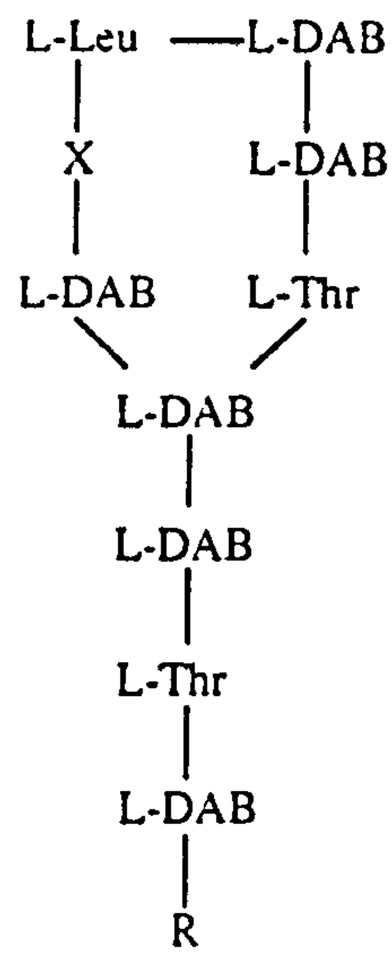
Erythromycin



Bacitracin



Trimethoprim



Polymyxin B

REFERENCE LIST

Addiss, D.G., MacKenzie, W.R., Hoxie, N.J., Gradus, M.S., Blair, K.A., Procter, M.E., Kazmierczak, J.J., Schell, W.L., Osewe, P., Frisby, H., Cicerello, H., Cordell, R.L., Rose, J.B.and Davis, J.P. (1995) Epidemiologic features and implications of the Milwaukee cryptosporidiosis outbreak. In *Protozoan Parasites and Water* eds. Betts, W.B., Casemore, D., Fricker, C., Smith, H. and Watkins, J. Cambridge: The Royal Society of Chemistry.

Anderson, A.W. (1994) Personal communication.

Anderson, B.C. (1988) *Cryptosporidium* spp. in cattle. In *Cryptosporidiosis - Proceedings of the First International Workshop* eds. Angus, K.W. and Blewett, pp. 55-63. Edinburgh.

Angus, K.W. (1988) Mammalian cryptosporidiosis: a veterinary perspective. In *Cryptosporidiosis - Proceedings of the First International Workshop* eds. Angus, K.W. and Blewett, D.A. pp. 43-53.Edinburgh.

Anon., (1990) *Cryptosporidium* in water supplies. London: HMSO.

Anon., (1991) Water Treatment Handbook. Paris, France: Lavoisier Publishing.

Anon, (1994a) The Microbiology of Water 1994 Part 1 - Drinking Water. London: HMSO.

Anon., (1994b) National Collection of Industrial and Marine Bacteria, Aberdeen: Catalogue of Strains.

Anon, (1995) Water Bulletin. No. 667, p.3.

Archer, G.P., Quinn, C.M., Betts, W.B., Allsop, D.W.E. and O'Neill, J.G. (1995) Physical separation of untreated and ozone treated *Cryptosporidium parvum* oocysts using non-uniform electric fields. In *Protozoan Parasites and Water* eds. Betts, W.B., Casemore, D., Fricker, C., Smith, H. and Watkins, J. pp. 143-145. Cambridge: The Royal Society of Chemistry.

Archer, G.P., Render, M.C., Betts, W.B. and Sancho, M. (1993) Dielectrophoretic concentration of microorganisms using grid electrodes. *Microbios* **76**, 237-244.

Archer, G.P., Betts, W.B. and Haigh, T. (1993) Rapid differentiation of untreated, autoclaved and ozone treated *Cryptosporidium parvum* oocysts using dielectrophoresis. *Microbios* **73**, 165-172.

Arnold, W.M. and Zimmerman, U. (1982) Rotating-field-induced rotation and measurement of the membrane capacitance of single mesophyll cells fo *Avena sativa*. *Z. Naturforsch* **37c**, 908-915.

Arnold, W.M. and Zimmerman, U. (1988) Electrorotation: Development of a technique for dielectric measurements on individual cells and particles. *Journal of Electrostatics* **21**, 151-191.

Ashford, R.W., Vince, J.D., Gratten, M.J. and Miles, W.E. (1978) *Strongyloides* infection associated with acute infantile disease in Papua New Guinea. *Transactions of the Royal Society of Hygiene and Tropical Medicine* **72 (5)**, 554.

Bader, H. and Hoigné, J. (1982) Determination of ozone in water by the indigo method. *Ozone Science and Engineering* **4**, 169-176.

Ball, P. (1991) The future role and importance of macrolides. *Journal of Hospital Infection* **(Suppl A)**, 47-59.

Ball S.J. and Pittilo R.M.(1990) Structure and ultrastructure. In *Coccidiosis of Man and Domestic Animals* ed. Long, P. pp. 17-41. Boca raton: CRC Press.

Battigelli, D.A., Sobsey, M.D. and Lobe, D.C. (1993) The inactivation of hepatitis A virus and other model viruses by UV irradiation. *Water Science and Technology* **27 (3-4)**, 339-342.

Baxby, D. and Blundell, N. (1988) Recognition and laboratory characteristics of an atypical oocyst of *Cryptosporidium*. *Journal of Infectious Diseases* **158**, 1038.

Bayliss, C.E. and Waites, W.M. (1980) The effect of hydrogen peroxide and ultraviolet irradiation on non-sporing bacteria. *Journal of Applied Bacteriology* **48**, 417-422.

Becker, F.F., Wang, X-B., Huang, Y., Pethig, R., Vykoukal, J.and Gascoyne, P.R.C. (1994) The removal of human leukaemia cells from blood using interdigitated microelectrodes. *Journal of Physics D - Applied Physics* **27 (12)**, 2659-2662.

Becker, F.F., Wang, X-B, Huang, Y., Pethig, R., Vykoukal, J. and Gascoyne, P.R.C. (1995) Separation of human breast cancer cells from blood by differential dielectric affinity. *Proceedings of the National Academy of Sciences of the United States of America* **92 (3)**, 860-864.

Bendall, R.P. and Chiodini, P.L. (1995) The epidemiology of human *Cytclospora* infection in the UK. In *Protozoan Parasites and Water* eds. Betts, W.B., Casemore, D., Fricker, C., Smith, H. and Watkins, J. pp. 26-29. Cambridge: The Royal Society of Chemistry.

Betts, W.B. (1994) Technical report to BTG: Dielectrophoretic assessment of antibiotic sensitivity.

Betts, W.B. (1995) The potential of dielectrophoresis for the real-time detection of microorganisms in foods. *Trends in Food Science and Technology* **6**, 51-58.

Betts, W.B. and Hawkes, J.J. (1994) Dielectrophoretic characterisation of microorganisms and other particles. *UK patent* GB2238619B.

Beveridge, T.J. (1981) Ultrastructure, chemistry and function of the bacterial wall.*International Reviews in Cytology* **72**, 229-317.

Beveridge, T.J. (1988) The bacterial surface: general considerations towards design and function. *Canadian Journal of Microbiology* **34**, 363-372.

Beveridge, T.J. (1989) Interaction of metal ions with components of bacterial cell walls and their biomineralization. In *Metal-Microbe Interactions*. eds. Poole, R.K. and Gadd, G.M. pp. 65-84 Oxford: IRL Press.

Beveridge, T.J. and Graham, L.L. (1991) Surface layers of bacteria. *Microbiological Reviews* **55 (4)**, 684-705.

Blagburn, B.L. (1988) Avian cryptosporidiosis. In *Cryptosporidiosis - Proceedings of the First International Workshop* eds. Angus, K.W. and Blewett, D.A. pp. 27-41. Edinburgh.

Blagburn, B.L., Lindsay, D.S., Hoerr, F.J., Davis, J.F. and Giambrone, J.J. (1991) Pathology of cryptosporidiosis (*C. baileyi*) in broiler chickens. *Journal of Protozoology* **38 (6), 25S-27S.**

Blewett, D.A. (1988) Disinfection and oocysts. In *Cryptosporidiosis, Proceedings of the First International Workshop*. eds. Angus, K.W. and Blewett, D.A. pp.107-115. Edinburgh.

Blewett, D.A., Wright, S.E., Casemore, D.P., Booth, N.H.E. and Jones, C.E. (1993) Infective dose size studies on *Cryptosporidium parvum* using gnotobiotic lambs. *Water Science Technology* **27 (3-4)**, 61-64.

Bloomfield, S.F. and Arthur, M. (1992) Interaction of *Bacillus subtilis* spores with sodium hypochlorite, sodium dichloroisocyanurate and chloramine-T. *Journal of Applied Bacteriology* **72**, 166-172.

Bloomfield, S.F. and Arthur, M. (1994) Mechanisms of inactivation and resistance of spores to chemical biocides. *Journal of Applied Bacteriology Symposium Supplement* **76, 91S-104S.**

Bochner, B.R., Huang, H-C., Schieven, G.L. and Ames, B.N. (1980) Positive selection for loss of tetracycline resistance. *Journal of Bacteriology* **143**, 926-933.

Bone, S. and Zaba, B. (1992) *Bioelectronics*. Chichester: John Wiley and Sons.

Bonnin, A., Dubremetz, J.F. and Camerlynck, P. (1991) Characterisation and immunolocalisation of an oocyst wall antigen of *Cryptosporidium parvum* (Protozoa: Apicomplexa). *Parasitology* **103**, 171-177.

Botzenhart, K., Tarcson, G.M. and Ostruscka, M. (1993) Inactivation of bacteria and coliphages by ozone and chlorine dioxide in a continuous flow reactor. *Water Science and Technology* **27 (3-4)**, 363-370.

Bowman, W.C. and Rand, M.J. (1980) Textbook of Pharmacology. 2nd edition. Oxford: Blackwell Scientific Publications.

Bryan, J.P. (1991a) Cephalosporins and carbapenems. *Current Opinion in Infectious Disease* **4** (6), 727-741.

Bryan, J.P. (1991b) New macrolides. *Current Opinion in Infectious Disease* **4** (6), 722-726.

Bryant, R.E., Fox, K., Oh, G. and Morthland, V.H. (1992) Beta-lactam enhancement of aminoglycoside activity under conditions of reduced pH and oxygen tension that may exist in infected tissues. *Journal of Infectious Diseases* **165** (4), 676-682.

Buck, M.A. and Cooperman, B.S. (1990) Single protein omission reconstitution studies of tetracycline binding to the 30S subunit of *Escherichia coli* ribosomes. *Biochemistry* **29**, 5374-5379.

Burt, J.P.H., Al-Ameen, T.A.K. and Pethig, R. (1989) An optical dielectrophoresis spectrometer for low-frequency measurements on colloidal suspensions. *Journal of Physics E: Scientific Instrumentation* **22**, 952-957.

Burt, J.P.H., Pethig, R., Gascoyne, P.R.C. and Becker, F.F. (1990) Dielectrophoretic characterisation of Friend murine erythroleukaemic cells as a measure of induced differentiation. *Biochimica et Biophysica Acta* **1034**, 93-101.

Cametti, C., Deluca, F., Macri, M.A.,Maraviglia, B., Misasi, R. and Sorice, M. (1993) Radiofrequency dielectric spectroscopy of synthetic bilayers containing glycolipids. *Colloids and Surfaces A - Physicochemical and Engineering Aspects* **72**, 173-176.

Campbell, I., Tzipori, S., Hutchison, G. and Angus, K.W. (1982) Effect of disinfectants on survival of *Cryptosporidium* oocysts. *The Veterinary Record* **111**, 414-415.

Campbell, A.T., Haggart, R., Robertson, L.J. and Smith, H.V. (1992a) Fluorescent imaging of *Cryptosporidium* using a cooled charge couple device (CCD). *Journal of Microbiological Methods* **16**, 169-174.

Campbell, A.T., Robertson, L.J. and Smith, H.V. (1992b) Viability of *Cryptosporidium parvum* oocysts: correlation of *in vitro* excystation with inclusion or exclusion of fluorogenic vital dyes. *Applied and Environmental Microbiology* **58 (11)**, 3488-3493.

Campbell, A.T., Robertson, L.J. and Smith, H.V. (1993a) Novel methodology for the detection of *Cryptosporidium parvum*: a comparison of cooled charge couple devices (CCD) and flow cytometry. *Water Science and Technology* **27 (3-4)**, 89-92.

Campbell, A.T., Robertson, L.J. and Smith, H.V. (1993b) Detection of oocysts of *Cryptosporidium* by enhanced chemiluminescence. *Journal of Microbiological Methods* **17**, 297-303.

Campbell, A.T., Robertson, L.J., Snowball, M.R. and Smith, H.V. (1995) Inactivation of oocysts of *Cryptosporidium parvum* by ultraviolet irradiation. *Water Research* **29 (11)**, 2583-2586.

Carstensen, E.L. (1967) Passive electrical properties of microorganisms. II. Resistance of the bacterial membrane. *Biophysics Journal* **7**, 493-503.

Casemore, D.P. (1991) The epidemiology of human cryptosporidiosis and the water route of infection. *Water Science and Technology* **24 (2)**, 157-164.

Casey, M.J. (1991) *Cryptosporidium* and bovine cryptosporidiosis: a review. *Irish Veterinary Journal* **44**, 2-7.

Castillo, F.J. and Heath, R.L. (1990) Ca^{2+} transport in membrane vesicles from pinto bean leaves and its alteration after ozone exposure. *Plant Physiology* **94 (2)**, 788-795.

Chang, J.C.H., Ossoff, S.F., Lobe, D.C., Dorfman, M.H., Dumais, C.M., Qualls, R.G. and Johnson, J.D. (1985) UV inactivation of pathogenic and indicator microorganisms. *Applied and Environmental Microbiology* **49 (6)**, 1361-1365.

Chapman, P.A. and Rush, B.A. (1990) Efficiency of sand filtration for removing *Cryptosporidium* oocysts from water. *Journal of Medical Microbiology* **32**, 243-245.

Chapman, P.A., Rush, B.A. and McLauchlin, J. (1990) An enzyme immunoassay for detecting *Cryptosporidium* in faecal and environmental samples. *Journal of Medical Microbiology* **32**, 233-237.

Chevrier, N. and Sarhan, F. (1992) Effect of ozone on energy metabolism and its relation to carbon dioxide fixation in *Euglena gracilis*. *Journal of Plant Physiology* **140 (5)**, 521-526.

Chevrier, N., Chung, Y.S. and Sarhan, F. (1990) Oxidative damages and repair in *Euglena gracilis* exposed to ozone. II. Membrane permeability and uptake of antimetabolites. *Plant Cell Physiology* **31 (7)**, 987-992.

Chopra, I. (1985) Mode of action of the tetracyclines and the nature of bacterial resistance to them. In *The Tetracyclines* ed. Hlavka, J.J. and Boothe, J.H. pp. 317-392. Berlin: Springer-Verlag.

Chopra, I. (1990) Penetration of antibiotics to their target sites. *Journal of Antimicrobial Chemotherapy* **26**, 607-611.

Chopra, I. (1992) Efflux-based antibiotic resitance mechanisms: the evidence for increasing prevalence. *Journal of Antimicrobial Chemotherapy* **30**, 737-744.

Chopra, I., Hawkey, P.M. and Hinton, M. (1992) Tetracyclines, molecular and clinical aspects. *Journal of Antimicrobial Chemotherapy* **29**, 245-277.

Costerton, J.W., Irvin, R.T. and Cheng, K. -J. (1981) The bacterial glycocalyx in nature and disease. *Annual Reviews in Microbiology* **35**, 299-324.

Coughlin, R.T., Caldwell, C.R., Haug, A. and McGroarty, E.J. (1981) A cationic electron spin resonance probe used to analyze cation interactions with lipopolysaccharide. *Biochemical and Biophysical Research Communications* **100 (3)**, 1137-1142.

Crane, J.S. and Pohl, H.A. (1968) A study of living and dead yeast cells using dielectrophoresis.*Journal of the Electrochemical Society* **115**, 584-586.

Craun, G.F. (1984) Waterborne outbreaks of giardiasis. In *Giardia and Giardiasis* ed. Erlandsen, S.L. and Meyer, E.A. pp. 243-261. New York: Plenum Press.

Cunha, B.A. (1985) Clinical uses of the tetracyclines. In *The Tetracyclines* ed. Hlavka, J.J. and Boothe, J.H. pp. 393-404.

Current, W.L. (1986) *Cryptosporidium*: its biology and potential for environmental transmission. *CRC Critical Reviews in Environmental Control* **17 (1)**, 21-51.

Current, W.L. (1988) The biology of *Cryptosporidium*. *American Society for Microbiology News* **54**, 605-611.

Current, W.L. (1990) Techniques and laboratory maintenance of *Cryptosporidium*. In *Cryptosporidiosis of Man and Animals* ed.Fayer, R., Speer, C.A. and Dubey, J.P. pp.31-49. Boca Raton: CRC Press.

Current, W.L. and Garcia, L.S. (1991) Cryptosporidiosis. *Clinical Microbiology Reviews*, **4 (3)**, 325-358.

Current, W.L., Upton, S.J. and Haynes, T.B. (1986) The lifecycle of *Cryptosporidium baileyi* n. sp. (Apicomplexa, Cryptosporidiidae) infecting chickens. *Journal of Protozoology* **33 (2)**, 289-296.

Dawes, I.W. and Sutherland, I.W (1992) *Microbial Physiology* second edition. Oxford: Blackwell Scientific Publications.

Dealler, S.F. (1991) Antibiotics induce rapid bacterial zeta potential changes. *Journal of Antimicrobial Chemotherapy* **28 (3)**, 470-473.

Dimitrova, A.P. and Christov, A.M. (1992) Electrically induced protoplast fusion using pulse electric fields for dielectrophoresis. *Plant Physiology* **100**, 2008-2012.

Dolman, P.J. and Dobrogowski, M.J. (1989) Contact lens disinfection by ultraviolet light. *American Journal of Ophthalmology* **108**, 665-669.

Dominy, P.J. and Heath, R.L. (1985) Inhibition of the K^+-stimulated ATPase of the plasmalemma of pinto bean leaves by ozone. *Plant Physiology* **77**, 43-45.

Dubey, J.P., Carpenter, J.L., Speer, C.A., Topper, M.J. and Uggla, A. (1988) Newly recognized fatal protozoan disease of dogs. *Journal of the American Veterinary Medical Association* **192**, 1269-1283.

Dupont, H.L., Chappell, C.L., Sterling, C.R., Okhuysen, P.C., Rose, J.B. and Jakubowski, W. (1995) The infectivity of *Cryptosporidium parvum* in healthy volunteers. *New England Journal of Medicine*, **332 (13)**, 855-859.

Edson, R.S. and Terrell, C.L. (1991) The aminoglycosides. *Mayo Clinic Proceedings* **66 (11)**, 1158-1164.

Fawell, J.K., Fielding, M. and Ridgeway, J.W. (1987) Health risks of chlorination - is there a problem ? *Water and Environmental Management* **1**, 61-66.

Fayer, R. (1995) Effect of sodium hypochlorite exposure on infectivity of *Cryptosporidium parvum* oocysts for neonatal BALB/c mice. *Applied and Environmental Microbiology* **61 (2)**, 844-846.

Feeley, D.E., Erlandsen, S.L. and Chase, D.G. (1984) Structure of the trophozoite and cyst. In *Giardia and Giardiasis* ed. Erlandsen S.L. and Meyer, E.A. pp. 3-31. New York: Plenum Press.

Feeley, C.M. and Pohl, H.A. (1981) The influence of resistivity and permittivity on the motion of uncharged solid particles in non-uniform electric fields (the 'dielectrophoretic' effect). *Journal of Physics D: Applied Physics* **14**, 2129-38.

Fomchenkov, V.M., Brezgunov, V.N., Gavrilyuk, B.K., Smolyaninov, V.V. and Bunina, Z.F. (1979) A study of the effects of antibiotics on orientational and dielectrophoretic spectra of bacterial cells. *Journal of Biological Physics* **7**, 45-56.

Franklin, T.J. and Snow, G.A. (1971) Interference with the translation of the genetic message: Inhibitors of protein synthesis. In *Biochemistry of Antimicrobial Action* pp. 87-111. London: Chapman and Hall.

Fuhr, G., Rösch, P., Müller, T., Dressler, V. and Göring, H. (1990) Dielectric spectroscopy of chloroplasts isolated from higher plants - characterization of the double membrane system. *Plant Cell Physiology* **31 (7)**, 975-985.

Gale, E.F., Cundliffe, E., Reynolds, P.E., Richmond, M.H. and Waring, M.J. (1981) Tetracyclines. In *The Molecular Basis of Antibiotic Action*. pp. 448-454. London: John Wiley and Sons.

Garcia, L.S., Brewer, T.C. and Bruckner, D.A. (1987) Fluorescence detection of *Cryptosporidium* oocysts in human faecal specimens by using monoclonal antibodies. *Journal of Clinical Microbiology* **25 (1)**, 119-121.

Garrod, L.P., Lambert, H.P. and O'Grady, F. (1973) Tetracyclines. In *Antibiotic and Chemotherapy* pp. 149-166. London: Churchill Livingstone.

Georgiewa, R., Donath, E., Gimsa, J., Löwe, U. and Glaser, R. (1989) Ac-field-induced leakage from human red cells at low ionic strengths - implications for electrorotation measurements. *Bioelectrochemistry and Bioenergetics* **22**, 255-270.

Gilmour, R.A., Smith, H.V., Smith, P.G., Morris, G.P. and Girdwood, R.W.A. (1991) The occurrence and viability of *Giardia* spp. cysts in UK waters. *Water Science and Technology* **24 (2)**, 179-182.

Glaze, W.H. (1987) Drinking water treatment with ozone. *Environmental Science and Technology* **21 (3)**, 224-230.

Glaze, W.H., Koga, M. and Cancilla, D. (1989) Ozonation byproducts. 2. Improvement of an aqueous phase derivitization method for the detection of formaldehyde and other carbonyl compunds formed by the ozonation of drinking water. *Environmental Science and Technology* **23 (7)**, 838-847.

Grant, E.H., Sheppard, R.J. and South, G.P. (1978) *Dielectric Behaviour of Biological Molecules in Solution* Oxford: Clarendon Press.

Gray, N.F. (1989) *Biology of Wastewater Treatment* Oxford: Oxford University Press.

Grimason, A.M., Smith, H.V., Parker, J.F.W., Bukhari, Z., Campbell, A.T. and Robertson, L.J. (1994) Application of DAPI and immunofluorescence for enhanced identification of *Cryptosporidium* spp. oocysts in water samples. *Water Research* 28 (3), 733-736.

Haigh, T. and Betts, W.B. (1994) Rapid dielectrophoretic analysis of erythrocytes. *Biomedical Letters* 50, 141-146.

Hall, R.M. and Sobsey, M.D. (1993) Inactivation of hepatitis A virus and MS2 by ozone and ozone-hydrogen peroxide in buffered water. *Water Science and Technology* 27 (3-4), 371-378.

Hamelin, C. (1985) Production of single strand and double strand breaks in plasmid DNA by ozone. *International Journal of Radiation Oncology Biology and Physics* 11 (2), 253-257.

Hamelin, C., Sarhan, F. and Chung, Y.S. (1978) Induction of deoxyribonucleic acid degradation in *Escherichia coli* by ozone. *Experimentia* 34, 1578-1579.

Hammond, S.M. and Lambert, P.M. (1978) Inhibitors of ribosome function. In *Antibiotics and Antimicrobial Action* pp. 45-50. London: Edward Arnold.

Hancock, R.E.W. (1984) Alterations in outer membrane permeability. *Annual Reviews in Microbiology* 38, 237-264.

Hawkes, J.J., Archer, G.P. and Betts, W.B. (1993) A dielectrophoretic spectrometer for characterising microorganisms and other particles. *Microbios*, **73**, 81-86.

Heath, R.L. and Frederick, P.E. (1979) Ozone alteration of membrane permeability in chlorella-I. Permeability of potassium ion as measured by a rubidium-86 traces. *Plant Physiology* **64**, 445-449.

Hibler, C.P. (1988) Analysis of municipal water samples for cysts of *Giardia*. In *Advances in Giardia Research* eds. Wallis, P.M. and Hammond, B.R. pp. 237-235. Calgary: University of Calgary Press.

Hill, B.D., Blewett, D.A., Dawson, A.M. and Wright, S. (1990) Analysis of the kinetics, isotype and specificity of serum and coproantibody in lambs infected with *Cryptosporidium parvum*. *Research in Veterinary Science* **48**, 76-81.

Hoffman, R. (1977) The modulation contrast microscope: principles and performance. *Journal of Microscopy* **110 (3)**, 205-222.

Hoskin, J.C. and Wright, R.E. (1991) *Cryptosporidium*: an emerging concern for the food industry. *Journal of Food Protection* **54 (1)**, 53-57.

Hoyle, B. and Beveridge, T.J. (1983) Binding of metallic ions to the outer membrane of *Escherichia coli*. *Applied and Environmental Microbiology* **46**, 749-752.

Huang, Y., Hölzel, R., Pethig, R. and Wang, X-B. (1992) Differences in the AC electrodynamics of viable and non-viable yeast cells determined through combined dielectrophoresis and electrorotation studies. *Physics in Medicine and Biology* **37 (7)**, 1499-1517.

Hughes, L.J., Stezowski, J.J. and Hughes, R.E. (1979) Chemical structural properties of tetracycline derivatives. 7. Evidence for the coexistence of the zwitterionic and nonionized forms of the free base in solution. *Journal of the American Chemical Society* **101**, 7655-6.

Hugo, W.B. (1992) Disinfection Mechanisms. In *Principles and Practice of Disinfection, Preservation and Sterilization* 2nd edition, eds. Russell, A.D., Hugo, W.B. and Ayliffe, G.A.J. pp. 187-209. Oxford: Blackwell Scientific Publications.

Hugo, W.B. and Russell, A.D. (1992) Types of antimicrobial agents. In *Principles and Practice of Disinfection, Preservation and Sterilization* 2nd edition, eds Russell, A.D., Hugo, W.B. and Ayliffe, G.A.J. pp. 9-88. Oxford: Blackwell Scientific Publications.

Iglesias, F.J., Lopez, M.C., Santamaria, C. and Dominguez, A. (1984) Dielectrophoretic properties of yeast cells dividing by budding and by transversal fission. *Biochimica et Biophysica Acta* **804**, 221-229.

Inoue, T., Pethig, R., Al-Ameen, T.A.K., Burt, J.P.H. and Price, J.A.R. (1988) Dielectrophoretic behaviour of *Micrococcus lysodeikticus* and its protoplast. *Journal of Electrostatics* **21**, 215-223.

Ishizaki, K., Sawadaishi, K., Miura, K. and Shinriki, N. (1987) Effect of ozone on plasmid DNA of *Escherichia coli in situ*. *Water Research* **21**, 823-827.

Ives K.(1990) *Cryptosporidium* and water supplies: treatment processes and oocyst removal. In *Cryptosporidium in water supplies*, Anon. pp. 154-184. London: HMSO.

Jagger, J. (1967) *Introduction to Research in Ultra-violet Photobiology*. New Jersey: Prentice-Hall Inc.

Jakubowski, W. (1984) Detection of *Giardia* cysts in drinking water. In *Giardia and Giardiasis* ed. Erlandsen, S.L. and Meyer, E.A. pp. 263-286. New York: Plenum Press.

James, A.M. (1991) Charge properties of microbial cell surfaces. In *Microbial Cell Surface Analysis* eds Mozes, N., Handley, P.S., Busscher, H.J. and Rouxhet, P.G. pp. 221-262. Cambridge: VCH.

James, A.M., Loveday, D.E.E. and Plummer, D.T. (1964) Some physical investigations on the behaviour of bacterial surfaces: XI. The effect of phenol and subsituted phenols on the electrophoretic mobility of *Aerobacter aerogenes*. *Biochimica et Biophysica Acta* **79**, 351-363.

James, A.M. and List, C.F. (1966) Some physical investigations on the behaviour of bacterial surfaces: XII. The effect of fimbriae on the electrophoretic mobility of some capsular and non-capsular bacteria of the *coli-aerogenes* group. *Biochimica et Biophysica Acta* **112**, 307-317.

Janoff, E.N. and Reller, B.L. (1987) *Cryptosporidium* species, a protean protozoan. *Journal of Clinical Microbiology* **25 (6)**, 967-975.

Jarroll, E.L., Hoff, J.C. and Meyer, E.A. (1984) Resistance of cysts to disinfection agents. In *Giardia and Giardiasis* ed. Erlandsen, S.L. and Meyer, E.A. pp. 311-328. New York: Plenum Press.

Jarroll, E.L. (1988) Effect of disinfectants on *Giardia* cysts. *CRC Critical Reviews in Environmental Control* **18**, 1-28.

Jawetz, E., Melnick, J.L. and Adelberg, E.A. (1982) Review of Medical Microbiology. 15th edition. California: Lange Medical Publications.

Johnson, D.A. (1980) Ozone inactivation of human alpha$_1$-proteinase inhibitor. *American Review of Respiratory Disease.* **85**, 567-576.

Johnson, D.W., Pieniazek, N.J. and Rose, J.B. (1993) DNA probe hybridization and PCR detection of *Cryptosporidium* compared to immunofluorescence assay. *Water Science and Technology*, **27 (3-4)**, 77-84.

Kaneko, M., Yamaguchi, A. and Sawai, T. (1985) Energetics of tetracycline efflux system encoded by Tn*10* in *Escherichia coli. FEBS Letters* **193**, 194-198.

Kimura, Y., Matsunaga, H. and Vaara, M. (1992) Polymyxin B octapeptide and polymyxin B heptapeptide are potent outer membrane permeability increasing agents. *Journal of Antibiotics (Tokyo)* **45 (5)**, 742-749.

Kogut, M.H. (1990) Host specificity of the coccidia. In *Coccidiosis of Man and Domestic Animals* ed. Long, P. pp. 44-62. Boca Raton: CRC Press.

Korich, R.G., Mead, J.R., Madore, M.S., Sinclair, N.A. and Sterling, C.R. (1989) Chlorine and ozone inactivation of *Cryptosporidium* oocysts. *Proceedings of the American Waterworks Association Water Quality Technology Conference*, Philadelphia pp. 681-693.

Korich, D.G., Mead, J.R., Madore, M.S., Sinclair, N.A. and Sterling, C.R. (1990) Effects of ozone, chlorine dioxide, chlorine and monochloramine on *Cryptosporidium parvum* oocyst viability. *Applied and Environmental Microbiology* **56 (5)**, 1423-1428.

Koval, S.F. and Murray, R.G.E. (1986) The superficial protein arrays on bacteria. *Microbiological Science* **3**, 357-361.

Labischinski, H., Goodell, E.W., Goodell, A. and Hochberg, M.L. (1991) Direct proof of a "more than single layered" peptidoglycan architecture of *Esherichia coli* W7. A neutron small-angle scattering study. *Journal of Bacteriology* **173**, 751-756.

LeChevallier, M.W., Norton, W.D. and Lee, R.G. (1991) Occurrence of *Giardia* and *Cryptosporidium* spp. in surface water supplies. *Applied and Environmental Microbiology* **57 (9)**, 2610-2616.

Leive, L. (1965) Release of lipopolysaccharide by EDTA treatment of *E. coli*. *Biochemical and Biophysical Research Communications* **21**, 290-296.

Levy, S.B. (1989) Evolution and spread of tetracycline resistance determinants. *Journal of Antimicrobial Chemotherapy* **24**, 1-7.

Levy, S.B. (1992) Active efflux mechanisms for antimicrobial resistance. *Antimicrobial Agents and Chemotherapy* **36 (4)**, 695-703.

Levy, S.B. and McMurray, L. (1978) Plasmid mediated tetracycline resistance involves alternative transport systems for tetracycline. *Nature* **276**, 90-92.

Lewin, B (1990) Genes IV. Oxford: Oxford University Press.

Lindsay, D.S., Blagburn, B.L., Hoerr, F.J. and Smith, P.C. (1991) Cryptosporidiosis in zoo and pet birds. *Journal of Protozoology* **38 (6)**, 180S-181S.

Llovo, J., Lopez, A., Fabregas, J. and Munoz, A. (1993) Interaction of lectins with *Cryptosporidium parvum. Journal of Infectious Diseases* **167** 1477-1480.

Lopez, J.R. and Tien, H.T. (1980) Photoelectrospectrometry of bilayer lipid membranes. *Biochimica et Biophysica Acta* **597**, 433-444.

Lorenzo-Lorenzo, M.J., Ares Mazas, M.E., Villacorta martinez de Maturana, I. and Durn-Oreiro, D. (1993) Effect of ultra-violet disinfection of drinking water on the viability of *Cryptosporidium parvum* oocysts. *Journal of Parasitology* **79 (1)**, 647-70.

Luft, B.J., Payne, D., Woodmansee, D. and Kim, C.W. (1987) Characterisation of the *Cryptosporidium* antigens from sporulated oocysts of *Cryptosporidium parvum. Infection and Immunity* **55 (10)**, 2436-2441.

Lumb, R., Lanser, J.A. and O'Donaghue, P.J. (1988) Electrophoretic and immunoblot analysis of *Cryptosporidium* oocysts. *Immunology and Cell Biology* **66**, 369-376.

Mahbubani, M.H., Bej, A.K., Perlin, M., Schaefer III, F.W., Jakubowski, W. and Atlas, R.M. (1991) Detection of *Giardia* cysts using the polymerase chain reaction and distinguishing live from dead cysts. *Applied and Environmental Microbiology* **57 (12)**, 3456-3461.

Maloy, S.R. and Nunn, W.D. (1981) Selection for loss of tetracycline resistance by *Escherichia coli. Journal of Bacteriology* **145**, 1110-1112.

Manning, P., Erlandsen, S.L. and Jarroll, E.L. (1992) Carbohydrate and amino acid analysis of *Giardia muris* cysts. *Journal of Protozoology* **39 (2)**, 290-296.

Markx, G.H., Huang, Y., Zhou, X-F. and Pethig, R. (1994) Dielectrophoretic characterisation and separation of microorganisms. *Microbiology* **140**, 585-591.

Marshall, A.J.H. and Piddock, L.V.J. (1994) Interaction of divalent cations, quinolones and bacteria. *Journal of Antimicrobial Chemotherapy* **34 (4)**, 465-483.

Marszalek, P., Zielinsky, J.J., Fikus, M. and Tsong, T.Y. (1991) Determination of electic parameters of cell membranes by a dielectrophoresis method. *Biophysical Journal* **59**, 982-987.

Mason, B.D. and Townsley, P.M. (1971) Dielectrophoretic separation of living cells. *Canadian Journal of Microbiology* **17**, 879-888.

Mehrle, W., Hampp, R., Zimmermann, U. and Schwan, H.P. (1988) Mapping of the field distribution around dielectrophoretically aligned cells by means of small particles as field probes. *Biochimica et Biophysica Acta* **939**, 561-568.

Messner, P., Pum, D., Sára, M., Stetter, K.O. and Sleytr, U.B. (1986) Ultrastructure of the cell envelope of the archaebacteria *Thermoproteus tenax* and *Thermoproteus neutrophilus*. *Journal of Bacteriology* **166**, 1046-1054.

Miller, R.A., Bronsdon, M.A. and Morton, W.R. (1989) Experimental cryptosporidiosis in a primate model. *Journal of Infectious Disease* **161**, 312-315.

Mischel, M., Voss, A. and Pohl, H.A. (1982) Cellular spin resonance in rotating electric fields. *Journal of Biological Physics* **10**, 223-226.

McLean, R.J.C., Beauchemin, D., Chapman, L. and Beveridge, T.J. (1990) Metal binding character of the γ-glutamyl capsular polymer of *Bacillus licheniformis* ATCC 9945. *Applied and Environmental Mirobiology* **56**, 3671-3677.

Morris, V.J. and Jennings, B.R. (1975) The effect of neomycin and streptomycin on the electrical polarizability of aqueous suspensions of *Escherichia coli*. *Biochimica et Biophysica Acta* **392**, 328-334.

Morris, V.J. and Jennings, B.R. (1977) The effect of antibiotics on the electrical polarizability of aqueous suspensions of bacteria. *Biochimica et Biophysica Acta* **497**, 253-259.

Mortimer, P.G.S. and Piddock, L.J. (1993) The accumulation of five antibacterial agents in porin-deficient mutants of *E. coli*. *Journal of Antimicrobial Chemotherapy* **32**, 195-213.

Moyed, H.S., Nguyen, T.T. and Bertrand, K.P. (1983) Multicopy *Tn*10 *tet* plasmids confer sensitivity to induction of *tet* gene expression. *Journal of Bacteriology* **155**, 549-556.

Musial, C.E., Arrowood, M.J., Sterling, C.R. and Gerba, C.P. (1987) Detection of *Cryptosporidium* in water by using polypropylene cartridge filters. *Applied and Environmental Microbiology* **53 (4)**, 687-692.

Nagarajan, R. (1991) Antibacterial activities and modes of action of vancomycin and related glycopeptides. *Antimicrobial Agents and Chemotherapy* **35 (4)**, 605-609.

Nau, R., Prange, H.W., Menck, S., Kolenda, H., Visser, K. and Seydel, J.K. (1992) Penetration of rifampicin into the cerebrospinal fluid of adults with uninflamed meninges. *Journal of Antimicrobial Chemotherapy* **29 (6)**, 719-724.

Navin, T.R. and Juranek, D.D. (1984) Cryptosporidiosis: clinical, epidemiologic and parasitologic review. *Reviews of Infectious Diseases* **6 (3)**, 313-327.

Nebel, C., Lally, A., Bosher, T., Hmurciak, J.W., Hmurciak, L. and Breen, D.A. (1988) Inactivation of *Giardia lamblia* cysts from a surface water by oxidation with ozone. In *Advances in Giardia Research* ed. Wallis, P.M. and Hammond, B.R. pp. 125-128. Calgary: University of Calgary Press.

Neihof, R.A. and Echols, W.H. (1978) Chemical modification of the surfaces of bacterial cell walls.*Physiology, Chemistry and Physics* **10**, 329-343.

Neuwirth, M., Roach, P.D., Buchanan-Mappin, J.M. and Wallis, P.M. (1988) Effects of chlorine on the ultrastructure of the *Giardia* cyst. In *Advances in Giardia Research* eds. Wallis, P.M. and Hammond, B.R. pp.133-135. Calgary: University of Calgary Press.

Nieminski, E.C. and Bradford, S.M. (1991) Impact of ozone treatment on selected microbioloogical parameters. *Ozone Science and Engineering* **13 (2)**, 127-145.

Nikaido, H. (1989) Outer membrane barrier as a mechanism of antimicrobial resistance. *Antimicrobial Agents and Chemotherapy* **33 (11)**, 1831-1836.

Nikaido, H. and Varra, M. (1985) Molecular basis of bacterial outer membrane permeability. *Microbiological Reviews* **49**, 1-32.

Nina, J.M.S., McDonald, V., Dyson, D.A., Catchpole, J., Uni, S., Iseki, M., Chiodini, P.L. and McAdam, K.P.W.J. (1992a) Analysis of oocyst wall and sporozoite antigens from three *Cryptosporidium* species. *Infection and Immunity* **60 (4)**, 1509-1513.

Nina, J.M.S., McDonald, V., Deer, R.M.A., Wright, S.E., Dyson, D.A., Chiodini, P.L. and McAdam, K.P.W.J. (1992b) Comparative study of the antigenic composition of oocyst isolates of *Cryptosporidium parvum* from different hosts. *Parasite Immunology* **14**, 227-232.

Oja, T., Bott, S. and Sugrue, S. (1989) Doppler electrophoretic light scattering analysis using the Coulter DELSA 440. pp. 1-11. Luton: Coulter Electronics.

Oliva, B., McNicholas, G.G., Ellestad, G. and Chopra, I. (1992) Evidence that tetracycline analogues whose primary target is not the bacterial ribosome cause lysis of *Escherichia coli. Antimicrobial Agents and Chemotherapy* **36 (5)**, 913-919.

Parker, J.F.W. and Smith, H.V. (1993) Destruction of oocysts of *Cryptosporidium parvum* by sand and chlorine. *Water Research* **27 (4)**, 729-731.

Parker, J.F.W., Greaves, G.F. and Smith, H.V. (1993) The effect of ozone on the viability of *Cryptosporidium parvum* oocysts and a comparison of experimental methods. *Water Science and Technology* **27 (3-4)**, 93-96.

Peeters, J.E., Ares Mazas, E., Masschelein, W.J., Villacorta Martinex, I. and Debacker, E. (1989) Effect of disinfection of drinking water with ozone or chlorine dioxide on survival of *Cryptosporidium parvum* oocysts. *Applied and Environmental Microbiology* **55 (6)**, 1519-1522.

Petersen, C. (1993) Cellular biology of *Cryptosporidium parvum. Parasitology* Today **9 (3)**, 87-91.

Pethig, R. (1979) Dielectric and Electronic Properties of Biological Materials, Chichester: John Wiley and Sons.

Pethig, R. (1982) A review of some present molecular and submolecular physical concepts in biology. *Journal of Biological Physics* **10**, 201-218.

Pethig, R. (1991) Application of AC electrical fields to the manipulation and characterisation of cells. In *Automation in Biotechnology* ed. Karube, I. pp. 159-185. Amsterdam: Elsevier Science Publishers.

Pethig, R., Huang, Y., Wang, X-B. and Burt, J.P.H. (1992) Positive and negative dielectrophoretic collection of colloidal particles using interdigitated castellated microelectrodes. *Journal of Applied Physics* **24**, 881-888.

Pohl, H.A. (1951) The motion and precipitation of suspendoids in divergent electric fields *Journal of Applied Physics* **22 (7)**, 869-871.

Pohl, H.A. (1973) Biophysical aspects of dielectrophoresis. *Journal of Biological Physics* **1**, 1-15.

Pohl, H.A. (1978) *Dielectrophoresis: The Behaviour of Neutral Matter in a Non-Uniform Electric Field.* Cambridge: Cambridge University Press.

Pohl, H.A. and Hawk, I. (1966) Separation of living and dead cells by dielectrophoresis. *Science* **152**, 647-648.

Pohl, H.A. and Crane, J.S. (1971) Dielectrophoresis of cells.*Biophysics Journal* **11**, 711-727.

Poulton, M., Colbourne, J. and Dennis, P.J. (1991) Thames Waters experiences with *Cryptosporidium Water Science and Technology* **24 (2)**, 21-26.

Pover, P.S. (1990) Colony counting and other petri dish applications of image analysis. *Binary* **2**, 77-79.

Price, J.A.R., Burt, J.P.H. and Pethig, R. (1988) Applications of a new optical technique for measuring the dielectrophoretic behaviour of micro-organisms. *Biochimica et Biophysica Acta* **964**, 221-230.

Pryor, W.A. and Uppu, R.M. (1993) A kinetic model for the competitive reactions of ozone with amino acid residues in preoteins in reverse micelles. *Journal of Biological Chemistry* **268 (5)**, 3120-3126.

Qualls, R.G. and Johnson, J.D. (1983) Bioassay and dose measurement in UV disinfection. *Applied and Environmental Microbology* **45 (3)**, 872-877.

Ranucci, L., Müller, H.M., La Rosa, G., Reckmann, I., Gomez Morales, M.A., Spano, F., Pozio, E. and Crisanti, A. (1993) Characterisation and immunolocalisation of a *Cryptosporidium* protein containing repeated amino acid motifs *Infection and Immunity* **61 (6)** 2347-2356.

Reduker D.W. and Speer, C.A. (1985) Factors influencing excystation in *Cryptosporidium* oocysts from cattle. *Journal of Parasitology* **71 (1)** 112-115.

Reduker, D.W., Speer, C.A. and Blixt, J.A. (1985a) Ultrastructure of *Cryptosporidium parvum* oocysts and existing sporozoites as revealed by high resolution scanning electron microscopy. *Journal of Protozoology* **32 (4)** 708-711.

Reduker, D.W., Speer, C.A. and Blixt, J.A. (1985b) Ultrastructural changes in the oocyst wall during excystation of *Cryptosporidium parvum* (Apicomplexa; Eucoccidiorida). Canadian Journal of Zoology, **63**, 1892-1896.

Rice, E.W. and Hoff, J.C. (1981) Inactivation of *Giardia lamblia* cysts by ultraviolet radiation. *Applied and Environmental Microbiology* **42**, 546-547.

Rice, E.W., Hoff, J.C. and Schaeffer, F.W.III (1982) Inactivation of *Giardia* cysts by chlorine. *Applied and Environmental Microbiology* **43**, 250-251.

Richmond, D.V. and Fisher, D.J. (1973) The electrophoretic mobility of microorganisms. *Advances in Microbial Physiology* **9**, 1-29.

Robertson, L.J. and Smith, H.V. (1992) *Cryptosporidium* and cryptosporidiosis. Part I: Current perspective and present technologies. *European Microbiology* **Nov-Dec**, 20-29.

Robertson, L.J., Campbell, A.T. and Smith, H.V. (1992) Survival of *Cryptosporidium parvum* oocysts under various environmental pressures. *Applied and Environmental Microbiology*, **58 (11)**, 3494-3500.

Robertson, L.J., Campbell, A.T. and Smith, H.V. (1993a) Induction of folds or sutures on the walls of *Cryptosporidium parvum* oocysts and their importance as a diagnostic feature. *Applied and Environmental Microbiology* **59 (8)** 2683-2641.

Robertson, L.J., Campbell, A.T. and Smith, H.V. (1993b) *In vitro* excystation of *Cryptosporidium parvum. Parasitology* **106**, 13-19.

Rose, J.B., Kayed, D., Madore, M.S., Gerba, C.P., Arrowood, M.J., Sterling, C.R. and Riggs, J.L. (1988) Methods for the recovery of *Giardia* and *Cryptosporidium* from environmental waters and their comparative occurrence. In *Advances in Giardia Research* eds. Wallis, P.M. and Hammond, B.R. pp. 205-209. Calgary: University of Calgary Press.

Rothstein, D.M., McGlynn, M., Bernan, V., McGahren, J., Zaccardi, J., Cekleniak, N. and Bertrand, K.P. (1993) Detection of tetracyclines and efflux pump inhibitors. *Antimicrobial Agents and Chemotherapy* **37 (8)**, 1624-1629.

Rouxhet, P.G.and Genet, M.J.(1991) Chemical composition of the microbial cell surface by X-ray photoelectron spectroscopy. In *Microbial Cell Surface Analysis* eds. Mozes, N., Handley, P.S., Busscher, H.J. and Rouxhet, P.G. pp. 173-220. Cambridge: VCH.

Rush, B.A., Chapman, P.A. and Ineson, R.W. (1990) A probable waterborne outbreak of cryptosporidiosis in the Sheffield area. *Journal of Medical Microbiology* **32**, 239-242.

Russell, A.D. and Chopra, I. (1990a) Mode of action of antibiotics and their uptake into bacteria. In *Understanding Antibacterial Action and Resistance* pp. 39-94. Chichister: Ellis Horwood.

Russell, A.D. and Chopra, I. (1990b) Genetic and biochemical basis of resistance to chemotheraputic antibiotics. In *Understanding Antibacterial Action and Resistance* pp. 146-181. Chichester: Ellis Horwood.

Russell, A.D. and Chopra, I. (1990c) Antiseptics, disinfectants and preservatives: their properties, mechanisms of action and uptake into bacteria. In *Understanding Antibacterial Action and Resistance* pp. 95-131. Chichester: Ellis Horwood.

Ryley, J.F. (1973) Cytochemistry, physiology and biochemistry. In *The Coccidia: Eimeria, Isospora, Toxoplasma and Related Genera* eds. Hammond, D.M. and Long, P.L. Baltimore: University Park Press.

Schlegel, H.G. (1986) General Microbiology. 6th edition. Cambridge: Cambridge University Press.

Shama, G. (1994) Personal communication.

Sheridan, R.P. and Chopra, I. (1991) Origin of tetracycline efflux proteins: conclusions from nucleotide sequence analysis. *Molecular Microbiology* **5**, 895-900.

Shinriki, N., Ishizaki, K., Yoshizaki, T., Miura, K. and Ueda, T. (1988) Mechanism of inactivation of tobacco mosaic virus with ozone. *Water Research* **22**, 933-938.

Simmons, N.A. (1991) *Cryptosporidium* and *Giardia lamblia*. *Biologist* **38 (4)**, 147-148.

Sleytr, U.B. and Messner, P. (1988) Crystalline surface layers in procaryotes. *Journal of Bacteriology* **170**, 2891-2897.

Sobotka, J. (1993) The efficiency of water treatment and disinfection by means of ultraviolet radiation. *Water Science and Technology* **27 (3-4)**, 343-346.

Sommer, R. and Cabaj, A. (1993) Evaluation of the efficiency of a UV plant for drinking water disinfection. *Water Science and Technology* **27 (3-4)**, 357-362.

Smith, H.V. (1992) Personal communication.

Smith, H.V. (1992) *Cryptosporidium* and water: a review. *Journal of the Institute of Water and Environmental Management* **6**, 443-451.

Smith, H.V. (1995) Emerging technologies for the detection of protozoan parasites in water. In *Protozoan Parasites and Water* eds. Betts, W.B., Casemore, D., Fricker, C., Smith, H. and Watkins, J. pp. 108-114. Cambridge: The Royal Society of Chemistry.

Smith, H.V. and Rose, J.B. (1990) Waterborne cryptosporidiosis. In *Cryptosporidium in Water Supplies*. Anon. pp. 146-153. London: HMSO.

Smith, H.V., Patterson, W.J., Hardie, R., Greene, L.A., Benton, C., Tulloch, W., Gilmour, R.A., Girdwood, R.W.A., Sharp, J.M.C., Forbes, G.I. (1989) An outbreak of waterborne cryptosporidiosis caused by post-treatment contamination. *Epidemiology and Infection* **103 (3)**, 703-715.

Smith, H.V., Smith, A.L., Girdwood, R.W.A. and Carrington, E.G. (1990) The effect of free chlorine on the viability of *Cryptosporidium* sp. oocysts isolated from human faeces. In *Cryptosporidium in Water Supplies* pp.185-204. London: HMSO.

Smith, H.V., Grimason, A.M., Benton, C. and Parker, J.F.W. (1991) The occurrence of *Cryptosporidium* spp. oocysts in Scottish waters and the development of a fluorogenic viability assay for individual *Cryptosporidium* spp. oocysts. *Water Science and Technology* **24 (2)**, 169-172.

Smith, H.V., Robertson, L.J. and Campbell, A.T. (1993) *Cryptosporidium* and cryptosporidiosis. Part II: Future technologies and state of the art research in laboratory detection. *European Microbiology*, **Jan-Feb**, 22-29.

Smith, H.V., Burt, J.P.H., Bukhari, Z., Pethig, R. and Parton, A. (1994) Non-uniform alternating current electrical fields and *Cryptosporidium parvum* oocyst viability. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **88**, 619.

Smith, M.C. and Chopra, I. (1984) Energetics of tetracycline transport into *Escherichia coli*. *Antimicrobial Agents and Chemotherapy* **25 (4)**, 446-449.

Sterling, C.R. (1990) Waterborne cryptosporidiosis. In *Cryptosporidiosis of Man and Domestic Animals* ed Fayer, R., Dubey, J.P. and Speer, C.A. pp. 51-58. Boca Raton: CRC Press.

Sterling, C.R. (1995) Emerging pathogens:*Cyclospora*, the Microspora, and how many more ? In *Protozoan Parasites and Water* eds. Betts, W.B., Casemore, D., Fricker, C., Smith, H. and Watkins, J. pp. 243-245. Cambridge: The Royal Society of Chemistry.

Sterling, C.R. and Arrowood, M.J. (1986) Detection of *Cryptosporidium* sp. infections using a direct immunofluorescent assay. *Pediatric Infectious Disease* **5 (1)**, S139-S142.

Stock, J.B., Rauch, B. and Roseman, S. (1977) Periplasmic space in *Salmonella typhimurium* and *Escherichia coli*. *Journal of Biological Chemistry* **252**, 7850-7861.

Stoicheva, N.G. and Dimitrov, D.S. (1986) Dielectrophoresis of myeloma cells, hybridomas and B lymphocytes. *Comptes Rendues de l'Academie Bulgare des Sciences* **39 (2)**, 105-107.

Stotish, R.L., Wang, C.C. and Meyenhofer, M. (1978) Structure and composition of the oocyst wall of *Eimeria tenella*. *Journal of Parasitology* **64**, 1074-1081.

Stryer, L. (1988) Biochemistry, 3rd edition. New York: W.H. Freeman and Company.

Sundermann, C.A., Lindsay, D.S. and Blagburn, B.L. (1987) Evaluation of disinfectants for ability to kill avian *Cryptosporidium* oocysts. *Companion in Animal Practice* **6**, 36-39.

Tait, R.C. and Boyer, H.W. (1978) On the nature of tetracycline resistance controlled by the plasmid pSC101. *Cell* **13**, 73-81.

Talary, M.S., Mills, K.I., Hoy, T., Burnett, A.K. and Pethig, R. (1995) Dielectrophoretic separation and enrichment of CD34+ cell subpopulation from bone marrow and peripheral blood stem cells. *Medical and Biological Engineering and Computing* **33 (2)**, 235-237.

Tilley, M., Upton, S.J. and Freed, P.S. (1990a) A comparative study on the biology of *Cryptosporidium serpentis* and *Cryptosporidium parvum* (Apicomplexa: Cryptosporidiidae). *Journal of Zoo and Wildlife Medicine* **21 (4)**, 463-467.

Tilley, M., Upton, S.J., Blagburn, B.L. and Anderson, B.C. (1990b) Identification of outer oocyst wall proteins of three *Cryptosporidium* (Apicomplexa: Cryptosporidiidae) species by ^{125}-I surface labelling. *Infection and Immunity* **58 (1)**, 252-253.

Ting, I.P., Jolley, K., Beasley, C.A. and Pohl, H.A. (1971) Dielectrophoresis of chloroplasts. *Biochimica et Biophysica Acta* **234**, 324-329.

Tortora, G.J., Funke, B.R. and Case, C.L.(1995) Microbiology - An Introduction. 5th edition. California: Benjamin Cummings.

Upton, S.J. and Current, W.L. (1985) The species of *Cryptosporidium* (Apicomplexa: Cryptosporidiidae) infecting mammals. *Journal of Parasitology* **71 (5)**, 625-629.

Upton, S.J., Current, W.L. and Barnard, S.M. (1986) A review of the genus *Caryospora* Leger, 1904 (Apicomplexa: Eimeriidae). *Systematic Parasitology* **8**, 3-21.

Uni, S., Iseki, M., Maekawa, T., Moriya, K. and Takada, S. (1987) Ultrastructure of *Cryptosporidium muris* (strain RN66) parasitizing the murine stomach. *Parasitology Research* **74**, 123-132.

van Rooselaar, A. (1988) Water treatment and the *Giardia* cyst. In *Advances in Giardia Research* eds. Wallis, P.M. and Hammond, B.R. pp. 85-86. Calgary: University of Calgary Press.

Venosa, A.D. (1983) Current state-of-the-art of wastewater disinfection. *Journal of Pollution Control Federation* **55**, 457-466.

Vesey, G. and Slade, J.S. (1991) Isolation and identification of *Cryptosporidium* from water. *Water Science and Technology* **24 (2)**, 165-167.

Vesey, G., Slade, J.S., Byrne, M., Shephard, K. and Fricker, C.R. (1993a) New techniques for the detection of protozoan parasites in water. In *New techniques in food and beverage microbiology* eds. Kroll, R.G., Gilmour, A. and Sussman, M. pp. 101-114. Oxford: Blackwell Scientific Publications.

Vesey, G., Slade, J.S., Byrne, M., Shepard, K. and Fricker, C.R. (1993b) Routine monitoring of *Cryptosporidium* oocysts in water using flow cytometery. *Journal of Applied Bacteriology* **75**, 87-90.

Vesey, G., Ashbolt, N., Wallner, G., Dorsch, M., Williams, K. and Veal, D. (1995) Assessing *Cryptosporidium parvum* oocyst viability with fluorescent *in situ* hybridisation using ribosomal RNA probes and flow cytometry. In *Protozoan Parasites and Water* eds. Betts, W.B., Casemore, D., Fricker, C., Smith, H. and Watkins, J. pp. 133-138. Cambridge: The Royal Society of Chemistry.

Victorin, K. (1992) Review of the genotoxicity of ozone. *Mutation Research* **277**, 221-238.

Wallis, P.M., van Roodselaar, A., Neuwirth, M., Roach, P.D., Buchanan-Mapin, J.M. and Mack, H. (1989) Inactivation of *Giardia* cysts in a pilot plant using chlorine dioxide and ozone. *American Water Works Association Water Quality Technology Conference, Philadelphia.*

Ward, R.W. and DeGrave, G.M. (1978) Residual toxicity of several disinfectants in domestic wastewater. *Journal of Pollution control Federation* **50**, 46-60.

Whitby, G.E. and Palmateer, G. (1993) The effect of UV transmission, suspended solids and photoreactivation on microorganisms in wastewater treated with UV light. *Water Science and Technology* **27 (3-4)**, 379-386.

Wiebauer, K., Schraml, S., Shales, S.W. and Schmitt, R. (1981) The tetracycline resistance transposon *Tn*1721: RecA-dependent gene amplification and expression of tetracycline resistance. *Journal of Bacteriology* **147**, 851-859.

Wiedenmann, A., Fischer, B., Straub, U., Wang, C-H., Flehmig, B. and Schoenen, D. (1993) Disinfection of hepatitis A virus and MS-2 coliphage in water by ultraviolet irradiation. *Water Science and Technology* **27 (3-4)**, 335-338.

Woodmansee, D.B. (1987) Studies of *in vitro* excystation of *Cryptosporidium parvum* from calves. *Journal of Protozoology* **34 (4)**, 398-402.

Wright, A.J. and Wilkowske, C.J. (1991) The penicillins. *Mayo Clinic Proceedings* **66 (10)**, 1047-1063.

Yamaguchi, A., Udagawa, T. and Sawai, T. (1990) Transport of divalent cations with tetracycline as mediated by the transposon *Tn*10-encoded tetracycline resistance protein. *Journal of Biological Chemistry* **265 (9)**, 4809-4813.

Yip, R.W. and Konasewich, D.E. (1972) Ultraviolet sterilization of water - its potentials and limitations. *Water Pollution Control (Canada)* **June**, 14-18.

Yun, M.S., Oh, H.S. and Kim, C.K. (1989) Disinfection effects of chlorine and monochloramine on *Campylobacter jejuni. Korean Journal of Applied Microbiology and Bioengineering* **17 (6)**, 539-544.

Zimmermann, W. and Rosselet, A. (1977) Function of the outer membrane of *Escherichia coli* as a permeability barrier to ß lactam antibiotics. *Antimicrobial Agents and Chemotherapy* **12**, 368-372.