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The Estimation of Microbial Biomass

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ABSTRACT

Methods that have been used to estimate the content, and in some cases the nature, of the microbial biomass in a sample are reviewed. The methods may be categorised in terms of their principle (physical, chemical, biological or mathematical/computational), their speed (real-time or otherwise) and the amount of automation/expense involved. For sparse populations, where the output signal is to be enhanced by growth of the organisms, physical, chemical and biological approaches may be of equal merit, whilst in systems, such as laboratory and industrial fermentations, in which the microbial biomass content is high, physical methods (alone) can permit the real-time estimation of microbial biomass.

Key words: biomass measurement, automated microbiology, microbial biomass, microbial growth, fermentation control.

1. INTRODUCTION AND SCOPE

'The study of the growth of bacterial cultures does not constitute a specialised subject or a branch of research; it is the basic method of microbiology.' (Monod, 1949.)

In accepting at once the foregoing truism, one appreciates immediately that it is of the first importance to be able to quantify the number (and/or

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TABLE 1
Features of an Ideal Probe Suitable for Monitoring Biomass Concentration in Fermentors

1. Continuous, real-time assay, i.e. rapid response.
2. Sensitive (resolution of approximately 0·02 mg/ml:10^7cfu/ml).
3. Electrodes (probes) biologically inert.
4. Non-destructive assay; no added reagent.
5. Many electrodes (probes) using same equipment.
6. Good lifetime.
7. Low cost.
8. Can be used in optically opaque and turbid solutions.
9. Cleanable *in situ*.
10. Sterilisable (autoclavable).

nature) of viable microorganisms, or to measure the microbial biomass, in a variety of circumstances. Thus, it would be especially desirable to have a general 'ideal biomass probe' that would meet the needs both of industrial and research fermentation scientists (who generally wish to encourage microbial growth) and of others (who may not). Nevertheless, despite the plethora of possible techniques available for measuring biomass, no single method exists which is really satisfactory for either of these groups. An ideal biomass probe should possess the same characteristics as any other electrode or probe (Table 1: (Harrison, 1976; Kell, 1980; Sikyta, 1983)). However, reliable biomass measurements are notoriously difficult to obtain, in particular because of the variety of environments in which the microorganisms exist, and the diversity, both in population size and in characteristics, of the microorganisms themselves.

In 1963 Levin commented, 'It is abundantly clear that there is great room and need for improvement in classical procedures for quantitative microbiological determinations'; to a large extent this remains true some 20 years later. During this period, many attempts have been made to introduce alternative, improved ways of monitoring microbial growth (e.g. Bascomb, 1981).

Although it is usually taken that *growth* and *division* constitute the key criteria of microbial viability (e.g. Postgate, 1976), and *ergo* distinguish biomass from necromass, such an analysis (of growth and division) requires time, such that there is a lower limit to the time required to establish the content of biomass *sensu stricto*. Evidently, therefore, the goal of a 'real-time biomass probe' is in this sense unattainable. For an entrée to the literature on the rather marked, and by no means irrelevant,

philosophical difficulties involved in distinguishing life from non-life even in higher organisms, Watson's popular and thoughtful treatment (Watson, 1974) is recommended. Parenthetically, we may mention that such paradoxes, well known in physics and chemistry (Primas, 1981; Wheeler & Zurek, 1983; Garden, 1984), are more prevalent in biology than is perhaps often realised (Welch & Kell, 1985). However, leaving such difficulties aside, it is pertinent first briefly and loosely to consider the appropriate means by which one might express the microbial *biomass* content of a sample.

Microbial content may be expressed in terms of the following:

(i) *The number of viable cells present:* In many cases, especially in clinical analyses and ecological studies, the number of viable cells provides the required information concerning the population within the sample.

(ii) *The total microbial biomass:* Viable counts may provide the required information for industrial and research fermentation processes, but this may not always be so. For example, it is biomass, and in some cases also necromass, and not the number of viable cells, that is important in many fermentations, such as those which produce glycerol, poly-β-hydroxybutyrate, and single cell protein (e.g. Dawes & Senior, 1973; Smith, 1980; Anthony, 1982; Reed, 1982). Further, biomass may change independently of cell number, for instance when a change in growth rate induces a change in the morphology of *Arthrobacter*, accompanied by a reduction in the number of cells, but no reduction in the biomass (Luscombe & Gray, 1971).

Biomass may be calculated from the number of viable cells, using the cell dimensions and density of the cellular protoplasm, but the innate errors are large, possibly as high as 50% (Hobbie & Watson, 1980).

(iii) *The enclosed volume (fraction):* This quantity, the volume (fraction) enclosed within the cytoplasmic membrane of the micro-organisms, may also be used to describe the microbial biomass content (Nestaas & Wang, 1981; Harris & Kell, 1983), and has a bioenergetically significant basis (Konings & Veldkamp, 1980).

Whilst our own interests are primarily focussed on techniques suitable for measuring biomass in axenic laboratory and industrial fermentors, this review will also endeavour to cover the perhaps more numerous approaches to the monitoring of microbial growth under other conditions.

One possible categorisation of methods for determining microbial

biomass, as employed, for example, by Bascomb (1981), differentiates between those methods which depend upon growth (to enhance the output signal) and those which do not. Such a division may be relevant to clinical work, but for the applications to fermentation processes, the distinction between physical and chemical methodologies is initially more appropriate, and is used herein. Such a taxonomy distinguishes those methods which exploit physical characteristics of the microorganisms, and/or their effects upon their surroundings, and those which rely upon chemicals added to the sample, or the measurements of cellular components or metabolites produced in the sample. Chemical methods almost always require that the sample is removed from the fermentor, and so are not appropriate for real-time monitoring. Conversely, the physical methods are non-destructive, and, in principle, can allow continuous monitoring of biomass *in situ* and in real time. For completeness, microscopical and mathematical methods are also covered. A discussion of the problem of obtaining a representative sample is not given here.

The difficulties inherent to even the qualitative determination of microbial biomass in general are very well illustrated both by a controversy concerning the existence of extreme thermophiles in 'black smokers' (Baross & Deming, 1983; Trent *et al.*, 1984) and by the well-known but inconclusive experiments carried out by the Viking Lander on the planet Mars (Sagan, 1981).

That the subject of biomass determination is of great importance (e.g. Marten, 1972) is evident from the quantity of published reviews, symposia and articles on the subject (e.g. Mallette, 1969; Collins & Lyne, 1970; Strange, 1972; Mitchell, 1974; Heden & Illeni, 1975; Pirt, 1975; Curby & Gall, 1976; Isenberg & MacLowry, 1976; Johnston & Newsom, 1976; Mitruka, 1976; Jones, 1979; Dermer *et al.*, 1980; Bascomb, 1981; Bergan, 1981; Cundell, 1981; Washington, 1981; El-Shaarawi & Pipes, 1982; Geldreich & Kennedy, 1982; Tilton, 1982*a*, *b*; Anon, 1983*b*; Coonrod *et al.*, 1983; O'Toole, 1983*a*; Clarke *et al.*, 1985; Leach, 1984).

2. CHEMICAL METHODS

As well as destroying the sample, chemical methods generally require disposable reagents, which makes frequent sampling expensive. The measurements may be reasonably rapid, perhaps minutes after sampling,

but they are certainly not in real time, and cannot provide a continuous monitor of microbial biomass *in situ*. Nevertheless, they are useful for many purposes (Dermer *et al.*, 1980; Leach, 1984).

2.1. Bioluminescence and chemiluminescence

Several chemical reactions have been designed or identified such that light is produced quantitatively in response to a certain factor that may be used as an indicator of biomass. Of these reactions, bioluminescence is a method that is currently favoured by microbial ecologists, since it may be automated, is fairly rapid (output in under 2 min) and instrumentation based on this principle is commercially available (e.g. Karl, 1980). The bioluminescence assay is based upon the assumption that living cells of a given type contain a reasonably constant amount of ATP, which is lost rapidly upon cell death. Biomass may therefore be expressed in terms of ATP concentration (and also of NADH under certain conditions (Lovgren *et al.*, 1982)) by the amount of luminescence produced as a result of a reaction catalysed by firefly luciferase:

$$\text{ATP} + \text{luciferin} + O_2 = \text{oxyluciferin} + \text{AMP} + PP_i + CO_2 + \text{light} \qquad (1)$$

(e.g. Allen, 1972; Thore *et al.*, 1975; Jones, 1979; Thore, 1979; Anon, 1981*d*; O'Toole, 1983*a*; Thore, undated).

The light produced is detected photometrically, and the method is sensitive to cell numbers as low as 10^5 cells/ml (Chappelle *et al.*, 1978). Errors in the estimated biomass may arise, for instance, from one or more of the following sources:

(i) incomplete extraction of ATP;
(ii) quenching by extraction chemicals, buffers or other substances in the sample;
(iii) use of impure, though relatively inexpensive luciferase (Picciolo *et al.*, 1978);
(iv) stress on cells;
(v) activity of ATPases and other kinases;
(vi) variation of cellular ATP content with physiological conditions (Chapman *et al.*, 1971; Atkinson, 1977; Chappelle *et al.*, 1978);
(vii) the presence of free ATP of non-microbial origin (Picciolo *et al.*, 1977);
(viii) degradation of ATP by the extraction reagents.

A further method, using firefly luciferase, has been developed to overcome the problem of the variation in the cellular ATP content (or the adenylate energy charge) (Karl & Holm-Hansen, 1978; Jones, 1979). Although ATP concentration varies with physiological conditions, it has been observed (Atkinson & Walton, 1967; Chapman *et al.*, 1971) that the total concentration of adenine nucleotides (ATP, ADP, and AMP) remains essentially constant, and may be used as an indicator of metabolic activity and the potential for growth.

The concentrations of AMP and of ADP are determined by separation, enzymatic conversion to ATP, and a subsequent assay with firefly luciferase (Karl & Holm-Hansen, 1978). The adenylate energy charge is then calculated from:

$$\text{adenylate energy charge (AEC)} = \frac{[\text{ATP}] + 0{\cdot}5[\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]} \qquad (2)$$

For growing cells, the AEC is typically between 0·8 and 0·9 (Atkinson & Walton, 1967; Atkinson, 1977; Knowles, 1977). Absolute growth rates cannot be predicted from the AEC ratio, but the rate of protein synthesis and the capacity for cell growth correlate more closely to this than to absolute ATP concentrations; hence, although it has little thermodynamic basis, the AEC is a useful indicator of metabolic activity and the potential for cell growth.

The main disadvantage of the AEC method, regarding its application to the monitoring of biomass in fermentors, is the separation and subsequent conversion of ADP and AMP to ATP, which would require removal of the sample from the fermentor, and a delay of approximately 30 min before the results were available.

Bioluminescence is probably the most convenient and reliable method for measuring total microbial biomass in most environmental samples (Karl, 1980). It has been used to assay biomass in fermentor broths (Cochet *et al.*, 1984; Anon, undated, *a*), and Sharpe *et al.* (1970) suggested it to be 'a better indicator of biological activity in food than the number of colony-forming units'.

Less successful than bioluminescence is the detection of microorganisms by the chemiluminescence of luminol. This measures the light emitted by the haem-protein-catalysed oxidation of luminol in the presence of hydrogen peroxide (Neufeld *et al.*, 1965; Coates, 1976; Ewetz & Thore, 1976; Jones, 1979).

An automated luminol chemiluminescence method for measuring the

microbial content of water samples, seeded with one of six common contaminants, was developed by Oleniacz *et al.* (1968). They proposed two separate systems, both capable of detecting 10^3 to 10^5 cells/ml of water. These systems measured the light emitted from the reaction of alkaline luminol in the presence of either sodium perborate or sodium pyrophosphate peroxide. Both methods were non-specific amongst organisms; they could not differentiate quantitatively one organism in the presence of a mixed population. For each type of microorganism seeded, however, the method exhibited a linear response to varying cell numbers. It was also recognised that luminol chemiluminescence may be affected by certain transition metals, such as iron, which are known to catalyse chemiluminescence. In the same study, Oleniacz *et al.* (1968) examined the effect of ferric, ferrous, and calcium chlorides on the luminol perborate system. Iron (III) chloride provoked luminol luminescence whether *E.coli* was present or not; iron (II) chloride, below a critical concentration, similarly increased luminescence, but above this critical concentration iron (II) chloride had a quenching effect; calcium chloride exhibited a similar enhancing/quenching effect about a critical concentration. The effect of these ions and various transition metal ions, on the luminol chemiluminescence reaction could be eliminated by the appropriate use of filters, so as to permit the subtraction of the background chemiluminescence from that produced by the biomass in the sample. Miller & Vogelhut (1978) have also shown that the method is sensitive to haem moieties in the culture fluid, necessitating the isolation of microorganisms from the growth medium, and hence greatly reducing the ease of application of the luminol chemiluminescence technique. They have also indicated that the method is not as sensitive to microbial biomass as was first reported.

2.2. Other chemical methods for the assay of cellular compounds

A variety of other cellular components has also been used as indicators of biomass. Some of these methods are outlined below.

'Phospholipids constitute a part of every cellular membrane and form a relatively constant proportion of the membranes of different microbes' (White, 1983). Measurements of the phospholipid content of a sample can thus offer a convenient assessment of microbial content, though they cannot distinguish biomass from necromass. Phospholipids are quantitatively extractable, and the lipid phosphate content is readily measured,

either, following perchloric acid digestion, by colorimetric analysis (White, 1983), or by conversion to fluorescent derivatives and HPLC analysis (White, 1980). Both methods have a sensitivity equivalent to approximately 10^9 cells of the size of *E.coli*. Alternatively, glycerol from phospholipids may be assayed somewhat more sensitively, in amounts equivalent to approximately 10^6 cells of the size of *E.coli*, using GLC (Gehron & White, 1983). Martz *et al.* (1983) have used HPLC to assay one particular phospholipid, phytanylglycerol ether, which is found in methanogenic bacteria.

Watson *et al.* (1977) and Hobbie & Watson (1980) have used limulus amebocyte lysate (LAL), isolated from the blood cells of the horseshoe crab, to estimate biomass in the marine environment. LAL reacts specifically with lipopolysaccharide (LPS), which comprises a relatively constant proportion of the cell wall of Gram-negative bacteria, to form a turbid solution. The amount of turbidity measured photometrically, minus that caused by free LPS, is linearly proportional to the amount of LPS and hence to biomass. This method provides a reasonable estimate for total biomass in marine samples, since they are comprised of 80–95% Gram-negative bacteria (e.g. Watson *et al.*, 1977). The accuracy of the LAL method compares well with ATP methods and direct counts (Hobbie & Watson, 1980), but the consistency of the LAL method in different hands is poor (Watson *et al.*, 1977).

Newman & O'Brien (1975) developed a method for detecting metabolically produced ethanol in *E.coli* using gas chromatography. The time between inoculation and detection was related to the initial cell concentration.

Ribbons (1970) discussed the relationship between media constituents (e.g. carbon, energy source, nitrogen, potassium, magnesium, phosphorus, sodium and trace elements) and cell yields and composition. A relatively simple element to assay, using a commercially available organic carbon analyser, is carbon. The amount of total organic carbon represents approximately 50% of the biomass present in the (washed) sample. However, carbon alone is not a good indicator of growth and viability, since, like oxygen and the energy source, it may be incorporated or consumed after cell growth has ceased. Errors also arise owing to the presence of non-microbial carbon (e.g. eukaryotic carbon in ecosystems) and incompletely utilised but adsorbed medium constituents in fermentors.

Muramic acid, which is found only in bacterial cell walls, including

those of blue green bacteria, has been correlated to biomass (e.g. King & White, 1977; Jones, 1979). To this end, it may be hydrolysed to lactate, either chemically, using alkali (King & White, 1977; Jones, 1979), or enzymatically (e.g. Jones, 1979). The resulting lactate may then be assayed in a number of ways, with sensitivities corresponding to 10^8 bacterial cells/ml, for instance using GLC, colorimetry or pulse labelling (King & White, 1977). The most obvious disadvantage, that the relative proportion of Gram-negative to Gram-positive bacteria needs to be known for ecological studies (Moriarty, 1980), is not important in axenic fermentations, but the method is lengthy, labour-intensive, and certainly not practical for monitoring fermentors.

Obviously DNA, protein and, to a lesser extent because of the strong dependence of its content on growth rate (Herbert, 1961), RNA (Koliander *et al.*, 1984) may be regarded, in favourable cases, as reasonably biomass-specific chemicals. Methods for their assay are well known, and since they suffer the same disadvantages as other chemical assays when applied to growth in fermentors, we do not consider them further here.

At least two enzyme-based methods have been proposed, one to measure faecal coliforms by a colorimetric β-galactosidase assay (Warren *et al.*, 1978), and the other to measure glutamic acid decarboxylase activity in water samples (Trinel *et al.*, 1980). The hydrolysis of *o*-nitrophenyl-β-D-galactoside by faecal coliforms, recovered by membrane filters (LeChevallier *et al.*, 1983), can be followed colorimetrically, and the time taken to reach half the maximum possible absorbance change is proportional to the initial coliform concentration. Detection times using this method are between 8 and 20 h depending on the size of the inoculum. The assay for glutamic acid decarboxylase activity has been automated (LeChevallier *et al.*, 1983). The sample is concentrated by ultrafiltration and incubated for 12 h. Both methods rely on cell growth and so are unsuitable for real-time measurements in fermentors.

2.3. Radiometry

Radiometric procedures were pioneered by Levin and others in the 1950s. Since then radiometry has been applied to a wide range of problems, including the enumeration of coliforms, particularly in water, the search for extraterrestrial life, selection of antibodies, prospecting for oil and gas, and general microbiological research (Levin, 1963).

The principle is to supply the microorganisms with a radioisotopically labelled substrate, the most common radioisotope being ^{14}C. $^{14}CO_2$ is produced metabolically, and the amount of radioactivity which is detected, for instance with a scintillation counter, is representative of the microbial activity (e.g. Bachrach, 1976). Alternatively, heterotrophic microorganisms capable of rapid uptake of the labelled metabolite may be filtered, washed and dried, and the amount of intracellular activity counted. The amount of intracellular radioactivity may then be correlated to microbial activity (Bourgeois & Mafart, 1976). Early experiments by Levin detected 125 coliform cells in an hour, a sensitivity which was previously unobtainable, but the experimental protocol has now been modified, sacrificing the low detection times for the benefits of lower cost and lower levels of radioactivity (Levin, 1963).

Radiometry has subsequently been automated (e.g. Buddemeyer, 1976). The commercially available automated system, Bactec, has been tested, using blood and simulated blood samples, by Washington & Yu (1971), who reported, 'it is clear from our studies that this system of early detection of bacteremia lacks the sensitivity to be a suitable alternative to conventional broth culture techniques'. This report was criticised by DeBlanc *et al.* (1971), who, in testing nearly 3000 blood cultures, found a sensitivity comparable to conventional methods, and detected 70% of the positive cultures more quickly using Bactec.

Waters (1972) studied the sensitivity of the radiometric method in bacterial detection. He reported on the dependence of the detection time upon the initial size of the inoculum. Thus, 10^7 cells were detected in 1·5–2·5 h, and 1 cell in 8–16 h. Bachrach & Bachrach (1974) obtained similar results in detecting $^{14}CO_2$ produced by *E.coli* grown on ^{14}C lactose; they reported a detection time of 6 h for 1–10 cells in water samples. Rowley *et al.* (1976) found that 6–7 h was a time sufficient to differentiate suspect from non-contaminated food samples by radiometric procedures.

Radiometric techniques have obvious applications in the early and sensitive detection of microorganisms in clinical analyses, monitoring of water supplies, pharmaceutical and food industries, and ecological studies. Unfortunately, such a method, although extremely sensitive, is not performed in real time and is therefore unsuitable for monitoring fermentation processes. Its use is also limited to the detection of microorganisms which metabolise the ^{14}C-source added.

2.4. Mass spectrometry

In recent years a variety of dissolved gases and volatile substances have been assayed by membrane inlet quadrupole mass spectrometry (e.g. Lloyd & Scott, 1983; Lloyd *et al.*, 1983). Such measurements, together with a knowledge of respiratory quotients and yield coefficients in aerobic organisms, may in principle be used to assess the biomass content of a fermentor. Obviously, gaseous CO_2 may also be estimated by infra-red spectroscopy, and such measurements may be correlated in certain cases to biomass content (e.g. Park *et al.*, 1983). Whilst such methods may certainly be used to gain useful information about the state of a fermentation, it is to be assumed that any relations between the production of an extracellular chemical and the content of microbial biomass are likely to be casual rather than causal. We do not therefore pursue such approaches further.

3. MICROSCOPY

3.1. Direct counts

The microscope is an essential tool for the microbiologist, not only in revealing the structures of microorganisms, but also in quantifying them. With the use of appropriate staining and a counting chamber, e.g. a haemocytometer, an accurately predetermined volume of cell suspension may be counted; from this the cell concentration may be determined, and biomass estimated (e.g. Norris & Swain, 1971; Quesnel, 1971; Adams, 1980).

Suitable staining is of the first importance, to ensure that only viable cells, and not non-viable cells or background material, are counted. Many stains are pH indicators, e.g. neutral red and phenolphthalein, and emphasise the difference between living and non-living cells on the basis of the commonly observed difference, in the former, between intracellular and extracellular pH (see Padan *et al.*, 1981). Other stains are specific to nucleic acids, staining only the viable cells with intact nucleic acids. The use of nalidixic acid to differentiate viable and non-viable cells was introduced by Simidu & Kogure (1980). The basis of the method is that nalidixic acid, a DNA gyrase inhibitor (Sugino *et al.*, 1977), inhibits

the division of cells without affecting their growth. Thus, the viable cells become greatly enlarged, enabling easy identification and enumeration. However, the method would seem to be too involved to be of widespread practical use.

A further consideration prior to executing direct counts is that particulate material may be present, for instance in food samples. To eliminate the possibility of these particles being counted as colonies the sample may be filtered. However, filtration procedures themselves are liable to introduce secondary problems (e.g. Entis, 1981).

3.2. Epifluorescence microscopy

Fluorochromes, e.g. acridine orange, ethidium bromide, euchrysine, etc. (Jones, 1979), are greatly superior to the traditional stains. Of these, acridine orange seems to be the most commonly used. It has a high affinity for nucleic acids, and is used as a viable cell stain, since nucleic acids are rapidly degraded upon cell death. When viewed under ultra-violet light, stained RNA and single-stranded DNA fluoresce orange/red, whilst double-stranded DNA appears green.

Fluorochromes are used in conjunction with a black membrane background for contrast. They have proved particularly useful for environmental samples, which are filtered onto the black membrane and stained. Two types of filters are available: cellulose and polycarbonate. Cellulose filters have the disadvantage that the cells are not retained on a flat membrane surface, but on a rough surface, and since the counting is in one plane only, it may in principle result in an underestimated cell population size—Hobbie *et al.* (1977) claimed an improved accuracy in epifluorescence counts using nuclepore polycarbonate membranes dyed with Irgalan black. These membranes have a flat surface, thus allowing all the retained cells to be counted. However, this observed increase in accuracy was based on a comparison between filters that differed not only in composition but also in pore size; Jones (1979) repeated this comparison and found that the epifluorescence counts on the two types of membrane of comparable pore size did not differ significantly, and further found the cellulose filters to have the added advantage that they enhance fluorescence contrast.

Larsson *et al.* (1978) compared epifluorescence microscopy with light and electron microscopic techniques for determining the microbial numbers in lake water. The samples were stained with acridine orange

(which is five times more effective than erythrosine (Jones, 1979)), and the microorganisms collected on a nuclepore filter. Since bacteria retain their morphology when stained as described (e.g. Jones, 1979), and so are distinguishable from detritus for counting purposes, Larsson *et al.* (1978) were able to conclude that acridine orange epifluorescence microscopy is the most accurate microscopic method available for determining the total number of microorganisms in water samples.

Zimmermann (1980) applied a statistical analysis of the data obtained from epifluorescence microscopy to the estimation of bacterial biomass. Rosendal & Valdivieso-Garcia (1981) used this technique to enumerate mycoplasmas. They reported a good correlation between counts of acridine orange-stained cells and the number of colony-forming units in exponentially growing cultures, but a low correlation with organisms taken from batch cultures in their lag and death phases.

Pettipher *et al.* (1980) (see also Anon, undated, *b*), in their studies on direct enumeration of the bacterial content of raw milk, reported the direct epifluorescence filter technique, DEFT, to be rapid (of the order of 25 min) and sensitive, in the range of 5×10^3 to 5×10^8 cells/ml. Pettipher recognised the potential of DEFT as a rapid, sensitive and inexpensive method for enumerating the bacterial content of samples, but with the major problem of operator fatigue. Pettipher & Rodrigues (1982) therefore developed a semi-automated epifluorescence technique, utilising closed-circuit television and computer analyses. The closed-circuit television allowed quality control checks to be undertaken, and the computer recorded the amount of fluorescence. The semi-automated DEFT studies on raw milk have been compared with manual DEFT (94% agreement), plate counts (83% agreement), and Coulter Counter methods (81% agreement) (Pettipher & Rodrigues, 1982). Complete automation would be possible, simply by coupling the available system to a computer-controlled movement of the microscope stage (Pettipher & Rodrigues, 1982). Such a system might, with appropriate sampling procedures, be applied to the control of fermentors, albeit with a restricted response time. Pettipher's recent (1983) monograph gives an extensive discussion of the technique.

3.3. Viable counts

In contrast to direct counts using traditional stains or fluorochromes, viable counts rely on the growth of microorganisms, and hence the

resulting colonies are counted rather than the microorganisms themselves. Briefly, the method requires the dilution (or otherwise) of the sample, aliquots of which are dispensed onto a suitable nutrient agar plate, followed by incubation to allow the viable cells present to form colonies (e.g. Postgate, 1969). The obvious disadvantage of such a method is the long incubation period required before enumeration is possible, often as long as 72 h. This is a real disadvantage in clinical analyses and renders the method valueless as an on-line monitor in fermentors. Nevertheless, viable counts are commonly used in microbiology laboratories as a 'benchmark' method.

Several attempts have been made to reduce operator time in the preparation, and in the counting of colonies. Sharpe *et al.* (1972) explored the use of a foot pump dispenser, in conjunction with serial dilutions. In this arrangement, various dilutions of the sample are added to molten nutrient agar, aliquots of which are then dispensed via the foot pump into a sterile Petri dish. Don Whitley Scientific Ltd market a semi-automated spiral plating device, which, after incubating the plates, allows either manual or laser counting (see, e.g. Kramer *et al.*, 1979; Couse & King, 1982; Anon, undated, *c*). The counting time in this system has been reduced to approximately 5s, which is itself a great advance in reducing operator fatigue, but the long incubation time is still necessary. Many other commercial video-based image analysers may be used to count colonies on a Petri dish in well under 5s.

Other problems encountered with viable counts are the contamination of the sample by growth-inhibiting substances, or by particles which may be mistaken for colonies. The food industry, in particular, suffers from the effect of particulate food constituents inhibiting growth, or themselves being counted as colonies. To reduce this problem, Basel *et al.* (1983) explored a technique using density centrifugation to remove any such particles prior to incubation and counting.

Attention has also been focussed on adapting the technique of viable counts to water samples, in which the microorganisms are generally fairly sparse. Cells of *E.coli* have been collected from water samples on membrane filters, which are then placed onto a selective medium, incubated, and the resulting colonies counted (Dufour *et al.*, 1981). This method does have the advantage that it is non-lethal to bacteria. Some previous methods, which involved measurement of the conversion of tryptophan to indole, were bactericidal (e.g. Dufour *et al.*, 1981). However, any method utilising filters is likely to result in some fraction of

the microbial content being lost or damaged, yielding an underestimated population size. Perhaps a better method for concentrating the micro-organisms present in water samples is centrifugation. The pellet can then be spread onto agar on a microscope slide, covered with a cover slip and incubated. The number of colonies produced are counted as a percentage of the total number of colony-forming and single-cell units, giving the percentage and absolute number of viable cells (Fry & Zia, 1982).

Viable counts are used as a reference technique throughout the field of the enumeration of microorganisms. The method's considerable disadvantages, of long incubation time and operator fatigue, are outweighed by its high specificity (obtainable with the use of suitable nutrients), its reproducibility, and the fact that an initial high outlay is not required. It is conceivable that this procedure could be used as an occasional check on the progress of a continuous fermentor, but it is in no way suitable for an on-line biomass monitoring device.

4. PHYSICAL METHODS

In principle, the physical methods are the better methods for adapting to continuous, *in situ* monitoring of biomass in fermentors. They exploit and measure physical characteristics within the sample, leaving it unaffected, and do not require the removal of the sample from the system. Many physical techniques have already been fully automated, and are available commercially; others are still in their infancy.

4.1. Dry weights

Dry weight is often taken as the definitive measure of biomass. However, the measuring of dry weights is one exception amongst the physical methods, in that the sample is removed from the bulk and subsequently lost. As such, it is not a suitable technique for the continuous monitoring of fermentors, but may, like viable counts, provide a one-off spot check. The method entails the isolation of a known volume of sample, which is washed with distilled water, to remove any medium constituents, and dried to a constant mass. The drying process can be lengthy, and the preparation labour-intensive. Difficulties may arise when adsorbed but non-microbial substances are present, for instance media constituents or metabolites of high molecular weight. Neufeld & Zajic (1982) have

studied this problem with regard to growth on hexadecane, to which some cells adhere; they separated the adhered cells by freezing and centrifuging a known volume of sample. The free cells pelleted at the bottom of the centrifuge tube, allowing straightforward measurement of dry weight in this fraction. Lyophilisation of the frozen hexadecane and any adhered cells released the remaining cells, allowing their dry weight to be determined.

O'Toole (1983*b*) investigated the effect of the hygroscopic nature of microbial cells on dry weight measurements. Dried cells adsorb moisture during weighing, which results in an error of approximately 0·6% of the total dry weight. This error, albeit small, may easily be eliminated, by taking readings of mass over 3 min. Good laboratory practice of linear extrapolation to time zero gives the correct dry weight.

In spite of their disadvantages, dry weight methods are commonly used as a spot check for fermentors, and as a standard in microbial physiological research, where the technique has been widely applied to both general and specific problems (e.g. Stouthamer, 1979; Tempest *et al.*, 1983). The use of dry weights in clinical and ecological studies is limited, owing to the paucity of the microorganisms in the samples.

4.2. Photometric systems

'One of the strange coincidences of nature is that the size of bacterial cells and the wavelength of light are approximately the same. . . As a consequence of this closeness in size, bacterial cells will scatter visible radiation in a rather complicated manner, since they are in resonance with the incident waves.' (Wyatt, 1973.) The scatter is mainly in the forward direction (Powell, 1963). The turbidity of a microbial suspension depends not only on the geometry of the instrument, the wavelength of light, relative and absolute refractive indices, and the cuvette path length; it is also a function of microbial size, shape, which may alter with growth phase (e.g. Gates, 1983), and concentration, and may therefore be used to evaluate microbial growth.

Light scattering and transmission techniques have been made more accurate, reproducible and easier with the advent of lasers, microelectronics, and digital computers. Lasers provide an almost ideal source of light, which is monochromatic, well collimated, easily polarised, coherent and available at a variety of powers and wavelengths. Microelectronics and computers facilitate rapid manipulation of the data.

The measurement of scattered light (nephelometry) and transmitted light or optical density (turbidimetry) may be considered separately.

4.2.1. Nephelometry

Straightforward measurements of light scattered by microorganisms in a suspension give signals which are directly proportional to the cell mass (e.g. Koch, 1961; Powell & Stoward, 1962). However by measuring the scattered light under certain conditions, nephelometry has been found to give a signal proportional to cell numbers as determined by plating and counting techniques. As such it has been used, for instance, to follow the effect of bacteriophages on the cell division of *E.coli* (Starka, 1962) and to monitor the change in length of rod-shaped bacteria during growth (Powell & Stoward, 1962). However, in mitochondria, the intensity of light scattered is a function not only of the volume enclosed by the suspension but also of the energetic status of the inner mitochondrial membrane (Knight *et al.*, 1981).

For reliable, self-consistent data, both in nephelometry and turbidimetry, it is necessary to calibrate a growth curve against, say, dry weights. Subsequent measurements should be made using the same instrument, since the geometry varies between different instruments. Later checks on the stability of the instrument may be made using manufactured 'turbidity stable blocks' (e.g. Mallette, 1969). Values of light scattered (or absorbed) by the microorganisms in a suspension must be measured against a cuvette containing the suspending medium only.

At low cell concentrations, nephelometers have the advantage over turbidimeters that the scatter is measured against a background of almost zero, whilst the turbidimeter has to discriminate a small signal on a large background. The lower limits of detection of *E.coli* by the two techniques have been given as: $1{\cdot}6 \times 10^5$ cells/ml for nephelometry and $2{\cdot}4 \times 10^6$ cells/ml (OD $= 0{\cdot}002$) for turbidimetry (Mallette, 1969). At higher cell concentrations multiscatter occurs. This does not affect the linearity of the transmitted light with cell concentration until the scatter along the forward direction competes with the magnitude of the forward beam; this occurs around $1{\cdot}8 \times 10^9$ cells/ml for *E.coli* (Mallette, 1969). The upper limit for the linearity with cell number of nephelometry is of the order of 6×10^7 *E.coli* cells/ml. Nephelometers are not widely used in the laboratory since it has proved difficult to provide the necessary temperature control, but automated light scattering detectors are available. For clinical work, the Autobac system monitors light scattered in the forward

direction, during an incubation period of 5 to 6 h, and correlates the detection time with the initial size of the inoculum (Bascomb, 1981).

Keilmann *et al.* (1980) have utilised the sensitivity of nephelometers at the lower cell concentrations, and the linearity of turbidimeters at the higher cell concentrations, in a multichannel photo-nephelometer, which is able to measure either light scatter or transmission, or both simultaneously.

Parenthetically, we may mention that light scattering techniques have proved to be particularly useful in assessing the viability of bull spermatozoa, on the basis of their motility (Chen & Hallett, 1982). The quasi-elastic light scattering technique may be used as a rapid and powerful means for determining the motile properties of these and other cell populations. It measures the spectral broadening of scattered light, at a given scattering angle (Chu, 1974; Pusey & Tough, 1982; Earnshaw & Steer, 1983), caused by the motile cells (Chen & Hallett, 1982). The total light scattered by a population of viable (motile) and non-viable cells is a function of that scattered from each component. However, the deconvolution of the measured autocorrelograms is subject to a number of assumptions (Woolford & Harvey, 1982; Dahnecke, 1983).

An extension to the study of viability using quasi-elastic light scattering is the use of a twin-beam laser velocimeter, which acts on the principle of timing the transit of cells across the two laser beams in a direction perpendicular to the beam axes (Wilson & Harvey, 1983).

4.2.2. Turbidimetry

Routine photometric determinations as commonly executed in the laboratory measure, indirectly, the amount of light scattered by microorganisms in suspension. They do so by recording the amount of light that is absorbed or transmitted by the sample, rather than that which is actually scattered. This provides a relatively quick and convenient way of estimating cell concentrations in a fermentor, in general requiring only the removal and appropriate dilution of the sample. The physics of light scattering in this context is discussed, for instance, by Koch (1961), Mallette (1969), Jennings & Morris (1974), Bohren & Huffman (1983) and Dahnecke (1983).

Highly automated turbidimeters are available, being designed primarily for clinical analyses. Despite their very widespread use (e.g. Titus *et al.*, 1984), *in situ* optical measurements of fermentation processes

(e.g. Hancher *et al.*, 1974) are generally unsatisfactory for the following reasons:

(i) Commercially, the microorganisms are normally grown at concentrations outside the limits of linearity between optical density and cell concentrations (i.e. outside the range in which the Beer–Lambert law holds).
(ii) Other (non-microbial) particulate material may be present, particularly in industrial fermentors, which often utilise media containing particulate matter.
(iii) Gas bubbles, produced by aeration or metabolically, may be present.
(iv) In the absence of the above three problems, accurate measurements may still be impossible, because the microorganisms can grow preferentially on the photosensor.

Probably the most fully automated photometric (or other) system for monitoring relatively sparse microbial populations is the AutoMicrobic System (AMS) (Vitek Systems Inc). This automated, computerised system was developed by Aldridge *et al.* (1977) for the detection, enumeration and identification of bacteria and yeasts in clinical specimens. The biological basis for the system resides in lyophilised, selective media enclosed in wells of a disposable plastic cuvette. The introduction of the sample into these wells both rehydrates and inoculates the media. An automated optical system monitors the change in light transmission by solid state optics. This system does not require lenses or optical condensers, and uses an array of light-emitting diodes with a peak emission at 665 nm. The computer interprets any optical changes within the sample, and provides enumeration within 13 h. The use of selective media enhances the detection time; they are also formulated to allow specific microbes to thrive whilst suppressing others. The media thus provide a means for the identification of individual types of microorganisms and/or groups of microorganisms by the visual observation of both colour and turbidity changes. The effect of bubbles produced by the microorganisms during growth is eliminated by scanning the entire sample-containing cuvettes (Aldridge *et al.*, 1977; Heslop, 1980; Bascomb, 1981; Gibson, 1982). This system contains all the elements of a fully automated system for detecting microorganisms by their growth and division (Fig. 1).

The sensitivity of the AMS has been extensively studied using urine specimens. The AMS has detected 7×10^4 colony-forming units (cfu)/ml, with a 92% agreement with plate counts. The detection time varied between 4 and 13 h, depending on the pathogen present and its concentration (Aldridge *et al.*, 1977). Sonnenwirth (1977) used AMS to enumerate simulated and actual urine samples; he reported an agreement with the number of colony-forming units of 83·5%, and an agreement in correctly evaluating negative samples of 97·5%. Hasyn *et al.* (1981) reported the correct identification of 97% of Enterobacteriaceae samples using the Vitek AMS.

Another automated system for use in clinical work, that measures optical density using light-emitting diodes, is the Abbott MS-2. It monitors the growth in a sample at 5-min intervals, and is able to

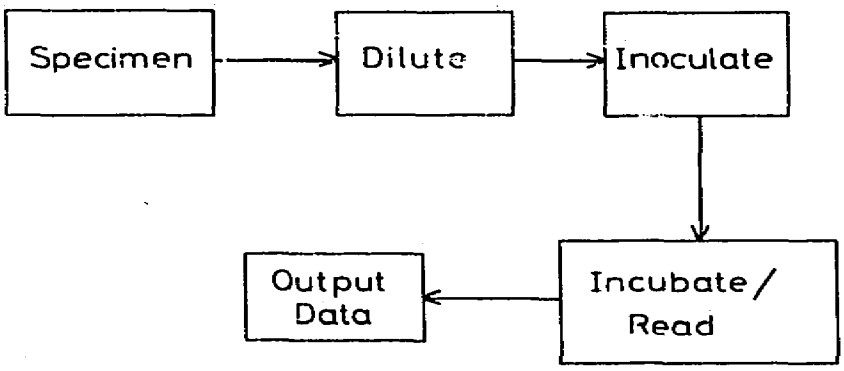

Fig. 1. Block diagram of the elements (modules) required for the full automation of microbial enumeration. Regardless of the detection principle(s) employed, all growth-associated methodologies require the steps indicated, which are embodied in the system described by Aldridge *et al.* (1977).

enumerate and identify the microorganisms present with the use of specific and selective media. Clinical studies have shown a 97·9% correct detection of 'positive' specimens containing greater than 10^5 cfu/ml, and correctly defined 99·7% of 'negative' specimens containing less than 10^3 cfu/ml (Anon, 1980).

Coleman *et al.* (1983), motivated by the high cost of commercial equipment, have devised their own automated system for measuring bacterial concentration by optical means. They have interfaced 24 sampling channels to a Commodore PET microcomputer, X–Y digital plotter, biophotometer, interface box, disc drive and printer, to study the

effect of inocula of antibiotics on the growth of bacteria. The growth curves obtained from the transmission data taken over a 24-h incubation period, have been compared (satisfactorily) with viable counts taken over the same period. The photometer was unable to detect any decrease in cell number below a concentration of 5×10^6 cfu/ml, only an increase, and, of course, antibiotics which do not affect the cell wall generally did not produce a fall in turbidity.

One of the problems that is encountered when using optical density as an estimator of biomass, as mentioned above, is the interference caused by bubbles. Ohashi *et al.* (1979) overcame this by designing a submersible colorimetric probe, that permits easy and continuous measurement of the optical density whilst removing the air bubbles from the sample. Their cell mass sensor consists of a double-layer cylinder for defoaming, a measuring chamber with a light source, a photocell or phototransistor, and a discharge port. The sample is forced through the double-layer cylinder by the agitation of the fermentor broth, dispelling any air bubbles; the bubble-free suspension then enters the measuring chamber, where the photocell or phototransistor measures the transmitted light. The optical density is determined from the photoelectromotive force given by a d.c. voltmeter connected to the detector. This is, as are all optical density sensors, accurate to a maximum concentration in the order of 5×10^9 cells/ml, which is often well below the operating cell concentration for fermentors. The problem of cell growth on the surface of the probe does not yet seem to have been solved generally; for photometric systems, electrolysis using optically transparent electrodes would appear to offer the most realistic solution.

Optical density measurements combined with 'growth delay' analysis have been used for estimating the total injury of a bacterial cell population which has been subjected to potentially bactericidal conditions (Takano & Tsuchido, 1982). The resulting growth reflects a decrease in the number of viable cells, and/or an increase in the lag time which is required by the injured cells for the necessary repair processes. Automated and continuous optical density measurements have been compared with the number of colony-forming units obtained by plating techniques (Takano & Tsuchido, 1982).

4.2.3. Other photometric techniques

A major problem with measurements involving the turbidity of a microbial suspension is that they are unable to distinguish viable cells

from non-viable cells and non-microbial particulate material. Thus Kull & Cuatrecasas (1983) have estimated the number of viable cells using the absorbance of a sample after staining with neutral red, a stain specific, under many conditions, to viable cells. This method utilises commonly available photometers, but the necessary preparative procedures are lengthy and labour-intensive, thus reducing its practicability.

Zabriskie & Humphrey (1978) applied culture fluorescence to the problem of estimating biomass in fermentors. They irradiated the culture with near-ultra-violet light (366 nm), and detected the resulting fluorescence (at 460 nm) using a photometer. Over half of the fluorescence of the intracellular material was caused by NADH, the rest being caused by NADPH and unidentified fluorophores. The log of the fluorescence was generally found to be linear with the log of the biomass, but the relationship is very dependent upon the culture environment, making the technique somewhat unreliable for accurate *in situ* monitoring of biomass.

4.3. Microcalorimetry

One of the most obvious changes which occur during the growth of all microorganisms is the production of heat. The study of this thermogenesis can in principle be used to quantify microbial populations (Forrest, 1972; Mor, 1976). Further, the heat profile produced by a given microorganism is peculiar to that microorganism growing on a particular medium, allowing identification (Boling & Blanchard, 1973).

Beezer *et al.* (1976, 1978) (see also Bettleheim *et al.*, 1976) used the linear relationship of heat production with microbial population to study the survival of yeast cells that had been frozen in liquid nitrogen, and to detect the heat produced by 15 strains of bacteria which commonly cause urinary infection. They reported that 10^5 organisms/ml could be detected, after a 2-h incubation period, using flow microcalorimetry, this being the population size that is considered to cause urinary infection (Kass, 1957). Of the 299 urine specimens tested 15% were false positives and 1% false negative. The measurement of heat production was regularly and reproducibly detected for all strains of bacteria studied. This could allow fast screening of urine samples in clinical analysis, and could eliminate a subsequent proportion of urine specimens from further investigations within 2·5 h of receipt in the laboratory, on the basis of their containing fewer than 10^5 microorganisms/ml.

Ishikawa & Shoda (1983) have used calorimetric analyses to follow the growth of *E.coli* in continuous culture. Their prime purpose was to understand the growth kinetics, rather than to estimate the biomass concentration. They followed the growth of *E.coli* in a glucose-limited medium using *in situ* measurements from a twin-type heat conduction calorimeter, in which the vessel also acted as a fermentor. They also measured the optical density at 660 nm, viable counts, dry weight, and the concentrations of glucose (substrate) and acetate (metabolite). Enthalpies of the biomass were obtained on the harvested, washed and dried cells in an oxygen bomb calorimeter. It was found (Ishikawa & Shoda, 1983) that the growth yields based upon calculations of the free energy of catabolism or the total available energy were constant, but that growth yields based upon the glucose consumed decreased as the dilution rate increased; this was almost certainly due to the production of metabolites other than acetate and to the transition to O_2-limiting conditions at high dilution rates.

Nevertheless, temperature changes, or heat evolved from sources other than the metabolism of microorganisms, may be large in any fermentor. For example, in batch cultures heat exchange effects are associated with the wetting of the vessel walls above the liquid phase on stirring, the stirring action itself, and many other factors (Biltonen & Langerman, 1979). This may make it difficult microcalorimetrically to detect temperature changes due to the heat production of the microorganisms against such a large background. This, coupled to the heat evolution of respiring but non-growing cells, reduces the accuracy and probably prevents the practical application of such a technique to the successful monitoring of biomass concentration in fermentation processes.

4.4. Filtration methods

Nestaas and Wang (Nestaas & Wang, 1981, 1983*a*,*b*; Nestaas *et al.*, 1981) developed a semicontinuous automated biomass probe to monitor the growth of *Penicillium chrysogenum* during the production of penicillin, in an attempt to overcome the 'major bottleneck' due to 'the lack of reliable biomass sensors for mycelial fermentations' (Nestaas & Wang, 1981). Penicillin is not produced at a consistent rate; its production greatly depends upon the changing morphology of the mycelia throughout their life cycle (e.g. Nestaas & Wang, 1981). Thus, to achieve maximum

production of penicillin it is desirable to use a feedback loop in order to control the mycelial morphology by means of the fermentation conditions. Nestaas and Wang (Nestaas & Wang, 1981, 1983*a*,*b*; Nestaas *et al.*, 1981) proposed such a feedback control system using a filtration probe. Using mathematical models and experimental evidence, Nestaas *et al.* (1981) and Nestaas & Wang (1983*a*,*b*) showed that the filtrate volume and filter cake volume, taken as functions of time, may be related to the total biomass in accordance with the following equation:

$$X = \frac{V_c}{V_c + V_f} \frac{1000}{\bar{\nu}}$$

where: X = biomass (g/litre), V_c = filter cake volume, V_f = filtrate volume, and $\bar{\nu}$ = specific filter cake volume (ml cake/g dry biomass).

The measurements with the semicontinuous, automated filtration probe were initiated by pumping 50–100 ml of sample from the fermentor into the filtration unit, where the sample was degassed. A constant pressure was then applied across the filtration unit, and both the filter cake volume, measured either with an optical sensor or a pressure transducer, and the filtrate volume, measured with a load cell, were recorded as a function of time. Once the measurements had been completed the cake and filtrate were recycled into the fermentor, by way of solenoid valves triggered by a sequential timer, and the whole cycle was repeated after 30 min (Nestaas & Wang, 1983*a*). The resulting values for cell biomass, taken at different times during the fermentation, were in excellent agreement with those determined from dry weight measurements (Nestaas *et al.*, 1981). Furthermore, the number of cells obtained from these data, in conjunction with the average dry weight of a mycelial particle obtained microscopically, were in very good agreement with the number of spores inoculated, as determined from plate counts: $1{\cdot}3 \times 10^9$ and $1{\cdot}5 \times 10^9$ cfu/ml, respectively (Nestaas *et al.*, 1981).

The filtration probe exploits simple physical phenomena which naturally occur during filtration; their measurement, however, may involve relatively complex devices. In addition, the nature of the filtration process as described limits the response time, and prevents genuinely continuous monitoring of biomass in fermentors. However, it is not unreasonable that measurement of the pressure transfer function using this probe could lead to a real-time estimation of microbial biomass. Similar remarks may be made about the viscometric approach (Perley *et al.*, 1979).

4.5. Impedimetry

Changes in the electrical impedance (Z) of microbial cultures have been known to be associated with microbial growth since the 19th century (see e.g. Stewart, 1899; Green & Larson, 1922; Brooks, 1923; Zoond, 1927; Schanne & Ceretti, 1978; Anon, 1981*b*). They were first noticed in putrefying blood and samples containing *Clostridium* spp. and *Streptococcus lactis*. However, it is only in the last decade or so that the impedance measurements have been widely exploited. This is due to the advent of commercially available impedance bridges that are suitable for the specific application of this principle to the monitoring of microbial growth. Several groups of workers have developed devices which monitor these impedance (or conductance) changes, which are sometimes expressed as the ratio of the reference impedance to the sum of the reference and sample impedance:

$$\text{impedance change} = \frac{Z_{\text{ref}}}{Z_{\text{ref}} + Z_{\text{sample}}}$$

This ratio is then related to microbial growth.

The measured change in impedance is a function of the type and number of microorganism(s), the medium in which they are growing, the frequency of the applied signal, the surface properties and geometry of the measuring electrodes, the surface to volume ratio, the temperature and the interelectrode distance. For a given set of conditions, however, the impedance (or conductance) changes arise from two primary sources, outlined below:

(i) Microbial catabolism: the conversion of uncharged substrate molecules in the medium into charged metabolites, e.g. carbohydrates and lipids into lactic acid, acetic acid and bicarbonate. Such catabolic reactions create ion pairs and smaller, more mobile, and hence more conducting, molecules, resulting in an increase in conductivity and a decrease in impedance (e.g. Cady, 1975; Hadley & Senyk, 1975; Ur & Brown, 1975; Cady, 1978; Cady *et al.*, 1978; Anon, 1981*a*,*b*; Hause *et al.*, 1981). Conversely, the uptake of charged substances (NH_4^+, $H_2PO_4^-$, etc.) will tend to raise the impedance (decrease the conductance).

(ii) Electrode impedance: the impedance changes are measured between two electrodes in response to an applied sinusoidal electric field. The impedance of this system, which consists of the electrodes separated

by microorganisms and medium, may be represented by a capacitor, C, and a resistor, R, in series, where

$$Z^2 = R^2 + \frac{1}{(2\pi fC)^2}$$

The reactive (capacitative) component undergoes a greater change than the resistive (conductive) component (e.g. Cady, 1975), suggesting that in addition to any conceivable permittivity changes occurring in the medium, there may be changes in the surface properties of the electrodes, thereby changing the capacitance of the electrode/electrolyte interface. Cady (1975) presented evidence supporting this hypothesis. He followed the growth of *E.coli* impedimetrically. After 8 h the sample was carefully removed and the electrodes washed in sterile medium. Immediate measurements of the sterile medium gave an impedance value that closely approximated the final value attained by the *E.coli* culture, and not the expected value of the uninoculated medium. This demonstrated the presence of an altered electrode impedance. The measured impedance returns to its expected value after a few hours' immersion, or immediately upon mechanical cleaning of the electrodes or on the application of an appropriate alternating current across them.

The frequency- and potential-dependent electrode impedance is a function of the electrode metal (Schwan, 1963, 1966; Smith, 1966; Geddes & Baker, 1968; Schwan, 1968; Sluyters-Rehbach & Sluyters, 1970; Geddes, 1972; Ferris, 1974; Schanne & Ceretti, 1978; Bard & Faulkner, 1980; Bond, 1980; Gielen & Bergveld, 1982), platinum, gold and stainless steel all giving satisfactory resolution in microbial impedimetry. Stainless steel is often preferred for its cheaper price and its relative biological inertness (Kagan *et al.*, 1977; Hause *et al.*, 1981); nevertheless, extremely thin and inexpensive platinum layers may be deposited on a variety of substrates. The impedance of the electrodes is also a very strong function of the frequency of the applied voltage (Schwan, 1963, 1968; Geddes, 1972; Ferris, 1974; Kagan *et al.*, 1977; Bard & Faulkner, 1980; Bond, 1980; Hause *et al.*, 1981).

Further evidence for a contribution of the electrode impedance to the measured impedance was presented by Hause *et al.* (1981). They reported that the use of a two-electrode system is likely to include concomitant effects both on the medium and the electrodes. To this end, they tested the relative roles of the medium and the electrodes at different frequencies (100 Hz, 1 kHz and 10 kHz). The impedance changes were

calculated as a percentage of the values recorded after electrode stabilisation. They (Hause *et al.*, 1981) found an increased microorganism-dependent change in the impedance measurements at the lower frequencies, suggesting a significant role of the electrode impedance in the impedimetric system. This same effect was noted by Kagan *et al.* (1977), and others. Furthermore, Hause *et al.* (1981) calculated the real and imaginary components of the measured impedance. Electrical impedance is a complex quantity, comprising a real component (resistance) and an imaginary component (reactance):

$$Z = R + iX$$

where

$$\text{resistance}, R = \frac{G}{G^2 + (\omega C)^2}$$

$$\text{reactance}, X = \frac{\omega C}{G^2 + (\omega C)^2}$$

$$i = \sqrt{-1}$$

$$\omega = 2\pi f$$

and G and C are the equivalent parallel conductance and capacitance, respectively (e.g. Schwan, 1963).

Hause *et al.* (1981) found that at low frequencies the impedance change closely follows the change in the imaginary component, whilst at higher frequencies the impedance change closely follows the change in the real component. That is, the reactance, and hence the capacitance, predominates at low frequencies. The capacitative term, as mentioned earlier, is overwhelmingly due to the electrode impedance, and not to changes in the permittivity of the medium (e.g. Cady, 1975).

There are at least three different manufacturers of impedance bridges, who market their impedimeters for the specific monitoring of microbial growth. These bridges were developed independently from the work of the following groups: Bactometer, produced by Bactomatic (see Anon, 1982, 1983*a*), from the work of Cady (1975) in particular; Malthus Instruments Ltd based their system on the work of Richards *et al.* (1978) (see also Jason, 1983); and TEM developed Bactobridge as a result of Ur & Brown's (1975) research. Each system offers slightly different

operating conditions (see Table 2). In the latter (Bactobridge) system, measurements are made relative to a reference well containing sterile medium; this eliminates environmental effects, which are common to both wells, that do not arise from the growth of the inoculum. These include, for example, fluctuations in the temperature, electrochemical reactions, adsorption of gases and evaporation (Ur & Brown, 1975; Cady, 1978). Also with the aim of eliminating environmental influences, the impedance ratio previously given is measured in the case of the Bactometer, whilst the Malthus instrument uses the conductance *minus* the conductance at $t = 0$.

The shape of the growth curve, measured conductimetrically or impedimetrically, for a given microorganism, generally corresponds to the *shape* of the growth curve measured optically or by plate counts (Jason, 1983; Eden & Eden, 1984). However, they are not super-

TABLE 2
Operating Conditions for Commercial Impedimeters

Signal measured	*Electrode material*	*Input frequency (kHz)*	*Voltage (V)*	*Temperature control (°C)*	*Detection time for 10^5 cfu/ml (h)*	*Selected references*
Bactometer						
Impedance Conductance Capacitance	stainless steel or Au	0·4 or 2·0	0·04	0·1	2·6	Cady (1975); Cady *et al.* (1978)
Typically: $\frac{Z_{ref}}{Z + Z_{ref}}$						
Malthus Instruments Ltd						
Conductance change $G - G_0$	Pt	10	<1	0·002	4	Richards *et al.* (1978); Jason (1983)
Bactobridge						
Conductance change $G - G_{ref}$	Au	10	1·5	0·001	2	Ur & Brown (1975); Ur (1976)

imposable, owing to the effect of changes in the electrode surface properties (Cady, 1978). The time which elapses before detection of a certain impedance change is a function of the initial size of the inoculum, generation time, lag time and the impedimetrically detectable threshold characteristics of the medium–organism–electrode combination. Therefore, if the threshold for the system and the generation and lag times of the microorganism are known, the initial cell concentration may be estimated (e.g. Hardy *et al.*, 1977; Cady, 1978; Cady *et al.*, 1978; Anon, 1981*b*,*c*).

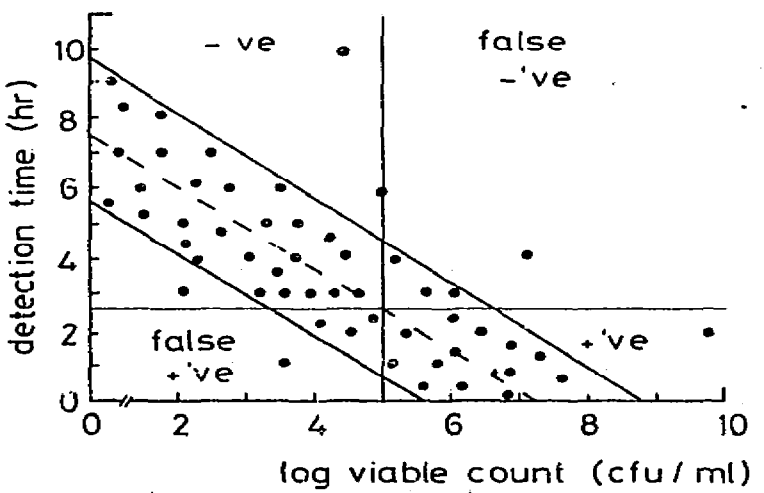

Fig. 2. Diagram of a typical scattergram depicting the decrease in detection time with viable counts for instruments such as impedimeters whose output signal is growth-associated. The broken line indicates an 'ideal' response, whilst the solid diagonals indicate the limits between which, say, 90% of all samples fall. The quadrants indicated give the false positive and negative regions using cut-off values of 2·6 h and 10^5 cfu/ml.

A characteristic scattergram (Fig. 2) is produced when the initial cell concentration, as measured for example by plate counts, is plotted against the impedimetric detection time. The large spread in the data comprising these scattergrams may be due to error associated with the plate count, or it may arise from the impedimetric measurements. Variation in the exact composition of the microorganisms, or types of microorganisms, within the sample, may give rise to different lag and generation times, whilst the impedance method assumes the same growth and lag times for all microorganisms present (Hardy *et al.*, 1977; Silverman & Munoz, 1979; Jason, 1983). Furthermore, as a result of their microscopic examinations on milk flora, Gnan & Luedecke (1982)

showed that different microorganisms grow to differing extents on the agar plates and the impedance media, which would also create a wide scattering of points in the scattergram. The scattergrams do not currently seem to possess the required accuracy for the very exact direct estimation of the number of viable cells present, but they do provide a useful quality control method, for example, in food processing and in clinical analyses, by choosing an appropriate cut-off time. The cut-off time corresponds to the detection time for a specified level of a microbial population, usually 10^5 cfu/ml (see e.g. Brown & Warner, 1982). Any microbial growth detected within this time means that the sample is positive, and if no growth is detected within this cut-off time, the sample is considered to be negative. The skill in using the cut-off time as a screening technique lies in the selection of a range of cut-off points such that the number of uncertain samples is minimised without increasing the number of false positives or false negatives (Fig. 3). When only a simple organism/medium combination is considered, the degree of scatter in such scattergrams is greatly reduced (Eden & Eden, 1984).

Impedance measurements have been used widely to assess the bacterial contamination in various samples. The majority of papers published to

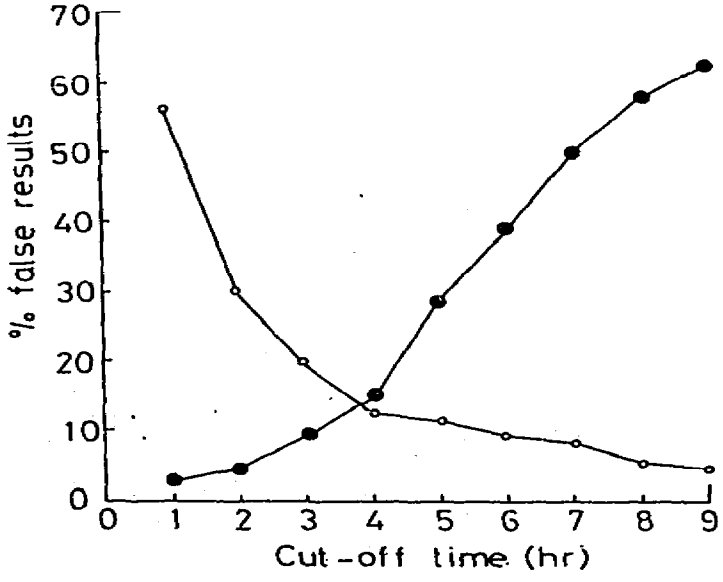

Fig. 3. The effect of cut-off time on the percentage of false positives (●) and false negatives (○) in an arbitrary experiment similar to that shown in Fig. 2. The existence of an optimum choice of cut-off time is evident. Points are the means of experimental data obtained by Brown & Warner (1982).

date on the use of impedimetric techniques seem to have involved the application of one of the Bactomatic systems. Some examples are now considered.

Martins *et al.* (1982) evaluated the use of the Bactometer 32 against standard plate counts, psychrotrophic plate counts and coliform counts, for determining the keeping quality of milk. They found that pasteurised milk samples with a detection time of 13·5 h or more had a shelf life of 12 days or longer, and a sample with a detection time of under 13·5 h spoiled within 11 days. The cut-off time of 13·5 h was a great improvement on the 48 h previously required for quality checks, undertaken by plating methods. Gnan & Luedecke (1982), also using the Bactometer 32 to determine the microbial population of untreated milk, found the mean detection time for 10^5 cfu/ml could be reduced to 8·9 h by enhancing the growth rate of the microorganisms by the addition of yeast extract.

The Bactometer 32 was also used by Silverman & Munoz (1979), to detect the concentration of coliforms in the effluent from sewage plants. They measured the impedance changes in an inoculated sample relative to those in an uninoculated control. They noted a large variation in the detection times, caused by the different growth phases of the inoculum and the dependence of the lag time on environmental conditions at the plant, such as temperature and rainfall. However, an extension of this work overcame the problem of this large variation. Munoz & Silverman (1979) incubated the inoculated media for 24 h, recording any increase in the impedance ratio. A recorded increase in the impedance ratio greater than or equal to 0·0039 in 1 h, and continuing for at least 1·5 h, was considered a positive indication of growth, if it occurred inside the 24-h incubation period. All the positive samples tested gave indication of growth within 18 h, thus satisfactorily reducing the detection time as compared with conventional methods. However, they reported (Munoz & Silverman, 1979) that the detectable impedance change given above was associated with between 10^6 and 10^7 coliforms/ml, which is the level that produces visible turbidity. So, providing there are no particles present in the medium, a simple visual check might determine whether the sample was positive or not.

Hardy *et al.* (1977) achieved a 96% agreement between standard plate counts and the Bactometer in quantifying the microbial contamination of 367 frozen-vegetable samples. They reported that the impedance measurements themselves did not appear to interfere with microbial growth, and found that the threshold concentration of 10^6–10^7 cfu/ml

necessary for detection was attained in 5 h for a sample initially containing 10^5 cfu/ml.

A large proportion of the clinical microbiologist's work is concerned with the detection of microorganisms in urine samples. Conventional methods require up to 48 h before the results are available, and also occupy a great deal of time in counting procedures. Impedance methods aim, as do the other novel methods reviewed herein, to decrease the test time and to free personnel for other laboratory tasks. Urine samples have been monitored for microbial contamination using the Bactomatic system, with a reduction in test time, for the 10^5 coliform cells/ml which are considered to produce bacteriuria (Kass, 1957), to approximately 3 h (e.g. Specter *et al.*, 1976; Zafari & Martin, 1977; Cady *et al.*, 1978; Anon, 1981*b*). On assaying 200 urine samples with the Bactometer 32, Specter *et al.* (1976) found that the impedance method provided a rapid screen for clinical urine specimens whilst permitting relatively accurate detection of bacterial growth. They reported 4% false positives and 2% false negatives in an average detection time of 2·5 h. Zafari & Martin (1977) also used the Bactometer to examine urine specimens. Of the 156 samples tested, they found a 96·8% correlation with standard plate counts in the classification of positive (those containing greater than 10^5 cfu/ml) and negative samples. However, they pointed out (Zafari & Martin, 1977) that bacteria with slow generation times may fail to give an impedimetrically detectable response before the cut-off time, and thereby increase the number of false negatives. Cady *et al.* (1978) monitored the impedance changes of 1133 urine samples. Using a cut-off time of 2·6 h to be an indicator of the presence of greater than 10^5 cfu/ml, they found 95·8% of the samples tested were correctly classified in accordance with the standard plate counts. They noted that the correct choice of cut-off time was important. A cut-off time longer than the 2·6 h chosen does not necessarily improve the correlation between impedimetry and conventional procedures, since, whilst it may produce a decrease in the number of false negatives, it also gives an increase in the number of false positives.

Clinical analyses also involve determining microbial populations in blood specimens, another area which has lent itself to impedimetric studies. For example, Hadley (1976) examined 1271 blood samples using the Bactometer 32. He found that shaking the cultures and providing the optimal medium markedly improved the detection time of those microorganisms which were otherwise detected very slowly or not at all. Hadley (1976) considered the blood samples to be positive when the microbial

population was greater than 10^6 cells/ml, which corresponded to a detection time of between 6 and 8 h. Of the total number of samples tested (1271), 161 showed microbial growth by conventional methods, whilst a further 9 were detected as being positive by the impedance method alone. Sixteen of the samples that were considered positive by the conventional methods were classed as negative by the Bactometer. Half of these false negatives were thought to be caused by colonial growth of the micro-organisms away from the electrodes, and consequently the number of false negatives was reduced (to 8) by shaking the culture to obtain a homogeneous suspension.

Kahn *et al.* (1976) also explored the use of the Bactometer 32 to study blood samples, together with cerebrospinal fluid of children. In testing 500 blood samples they found that positive samples could be detected with an average detection time of 8·5 h. Some 15% of the samples were considered positive by both impedance and conventional methods, and 14·5% were positive by the impedance method only. On investigating cerebrospinal fluid, Kahn and co-workers found a common cause of bacterial meningitis to be the presence of *Haemophilus influenzae* at concentrations above 10^4 cells/ml. Using the Bactometer 32 they were able to detect 10^4 cells/ml and 10^7 cells/ml in 5·8 and 2·4 h, respectively, thus greatly reducing the test time normally required by conventional methods (see above).

Throm *et al.* (1976) used the presence of bacterial activity in blood as a means of comparing radiometric methods, using the Bactec system, with the impedance technique embodied in the Bactometer 32. At that time the radiometric system was well established; conversely automated impedance systems were new to the market. Throm *et al.* (1976) appreciated the potential of the Bactometer 32, in that it offered more rapid detection times (up to 10 times faster than the standard plating methods, compared with Bactec which was only three times faster). Other advantages were said to be:

(i) greater consistency between the detection times;
(ii) lower cost per sample;
(iii) easy adaptation to the monitoring of samples other than blood; and
(iv) that impedimetry is non-hazardous both to sample and operator.

However, in 1976 the Bactometer instrumentation did not offer standardised electrodes, and was capable of monitoring only 32 samples simul-

taneously. Hence, Throm *et al.* (1976) selected the radiometric method for use in their routine laboratory work. Bactomatic have now improved the performance of their system, in that their latest model is capable of monitoring the microbial growth in over 500 sample wells simultaneously, and the gold and stainless steel electrode arrangements used have now been standardised. The Malthus instruments use platinum electrodes, which from a heuristic standpoint are probably more satisfactory, owing to their lower impedance. According to Richards *et al.* (1978), the use, as in the Malthus instrument, of conductance rather than impedance, is also conceptually to be preferred.

In summary, the impedance methods offer advantages in their ease of use, reduced test time, ability to function in opaque media, and their ability to handle large numbers of samples while retaining a high level of agreement with conventional counting techniques. More specialised applications of this methodology have included the selective detection of bacteria by the use of selective media (Martins & Selby, 1980). Currently, however, the prime objective, by way of the cut-off times, is that it be used as a quality control procedure, and not for providing very accurate estimations of the total number of viable cells in a sample. Large errors may arise in the accuracy and consistency of measured values of the detection time for a given number of colony-forming units per unit volume, owing to different lag- and generation-times of the micro-organisms present in the sample. These large errors, coupled with the detection time (which, although a great improvement in conventional quality control procedures, is by no means in real time), make impedimetric methods of the type discussed above unsatisfactory for monitoring biomass in fermentors.

Wheeler and Goldschmidt (Goldschmidt & Wheeler, 1975; Wheeler & Goldschmidt, 1975) investigated the potential of an approach in some respects similar to that of the impedance method, for the rapid screening of urine cultures from patients suspected of suffering bacteriuria. The purpose of this approach was to eliminate the time involved in plate counts or the accumulation of a measurable amount of metabolite. Using relatively simple instrumentation, Wheeler & Goldschmidt (1975) applied a 10 V (*sic*) peak-to-peak square-wave signal across microbial suspensions, via the outer pair of a tetra-polar stainless steel electrode probe, and measured the change in electrical output as a function of the frequency and the input voltage. They found the maximum signal for the different types and numbers of bacteria studied to occur at an input

frequency of 10 Hz. Further, they found a linear relationship between voltage change recorded on an oscilloscope at this 'frequency of maximum resolution' and the logarithm of the microbial cell concentration. This method allows, in principle, the total electrical properties of the system to be measured instantaneously, without the need for the accumulation of metabolites. Hence, the advantage of this adaptation of the impedance method is the claimed 10-min detection time, compared with the hours necessary for the impedance detection time. Wheeler and Goldschmidt tested 30 fresh urine samples against a calibration curve of output voltage for different concentrations of *E.coli*. They reported a 90% correlation with plate counts for samples containing greater than 10^5 cells/ml, and detected several samples containing 10^3 and 10^4 cells/ml which were considered to be negative by the conventional methods.

However, it has been shown that the capacitative and resistive properties of microorganisms and similar charged objects at low frequencies are a function not only of the applied field, but also of the particle radius and cell wall (i.e. surface) properties (see, e.g. Schwan, 1957; Schwarz, 1962; Einolf & Carstensen, 1973; Dukhin & Shilov, 1974; Carstensen & Marquis, 1975; Schanne & Ceretti, 1978; Pethig, 1979; Harris & Kell, 1983; Schwan, 1983; Harris *et al.*, 1984; Kell, 1983). Wheeler & Goldschmidt (1975) tested their system for microorganisms of different size and shape, and found no effect on the output signal, thus demonstrating that their measurements of the passive electrical properties of the microbial suspension are in fact dominated by the high impedance of the electrode/electrolyte interface at this frequency. Also, the electrical properties of microorganisms in the audio frequency range are mainly thought to be a result of the counterion relaxation tangential to their charged surfaces, and as such are, in principle (Harris *et al.*, 1984; Kell, 1984), a strong function of the ions present in the suspending phase as well as of the biomass. They also scale with the microbial concentration and not its logarithm. Therefore, although the resolution obtained with this technique was apparently high, the signal was not wholly (or even mainly) due to the microbial population. Despite the reasonable results presented by Wheeler and Goldschmidt, further investigations would seem to us to be necessary before either the value or the operating principle of this method may be accurately assessed. Finally, the fact that the cells had to be dispersed in distilled water (Wheeler & Goldschmidt, 1975) seriously diminishes the utility of their method for measurements in fermentors.

4.6. Electrical counting and sizing

The optical assessment of cell size and numbers, and hence the estimation of biomass, has been superseded in many cases by the advent of electronic counting and sizing devices, such as the Coulter Counter and Channelyser. Electronic counting techniques monitor the effect of microorganisms on an electric field as the microorganisms traverse the field. The microorganisms are suspended in an electrically conducting fluid, normally the growth medium, and are forced to flow through a small aperture across which an electric field is applied via a constant current source (Fig. 4). As the relatively non-conducting microorganism passes through the field, the electrical resistance within the aperture is increased, giving rise to a transiently increased voltage drop across the aperture. It was assumed that under certain conditions the magnitude of this pulse is proportional to the size of the microorganism (e.g. Grover *et al.*, 1969*b*, 1982; Dow, 1976), such that particles giving pulses of a given magnitude may be differentiated and counted. However, the principle does not permit significant discernment of particle shape, and coccoid or rod-shaped bacteria are counted as spheres of equivalent size (e.g. Kubitschek, 1969; Hobson & Mann, 1970; Curds *et al.*, 1978; Anon, undated, *d*, but cf. Bator *et al.*, 1984).

Coulter Electronics Inc. indicated that their original electronic device could count and size particles in the range of 0·4 to 800 μm diameter, from the pulse heights produced. Empirical evidence presented by Kubitschek

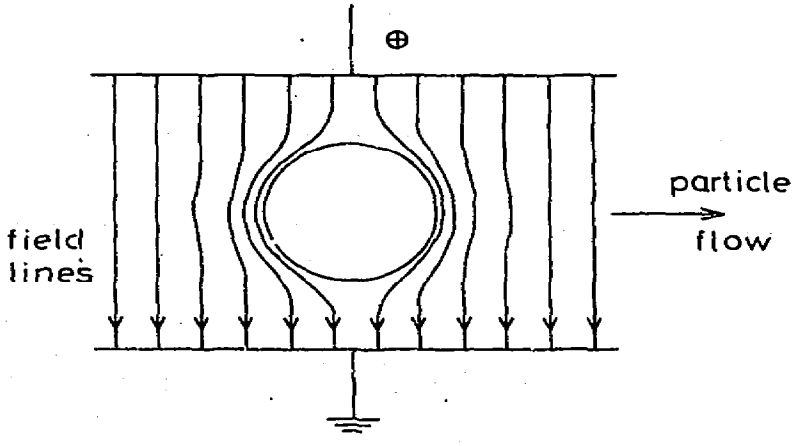

Fig. 4. The principle of the Coulter Counter. See text for further explanation.

(1969) supported this claim by demonstrating a fair degree of proportionality between measurements of optical density and the electronically measured cell volume for bacterial cells of differing mean cell volumes.

Potentially successful applications of the Coulter Counter require that the following conditions be met:

(i) The pulse height should be much greater than the background signal. Noise may be generated by other electrical equipment in the vicinity of the counter, e.g. fluorescent lights, and so the instrument must be suitably shielded to increase the reliability of the results (Hobson & Mann, 1970). Modern versions incorporate a Faraday cage.

(ii) Unusually large pulses must be minimised since they momentarily perturb the pulse-height analyser and prevent immediate, subsequent counting. Large signals may be produced by contaminating particles, or by two or more cells traversing the field simultaneously. An upper limit to the size of the pulse counted and analysed may be enforced, but there may still be momentary disturbance of the system (Kubitschek, 1969; Curds *et al.*, 1978).

(iii) The concentration of the suspension must be optimised; large concentrations increase the occurrence of coincident counts and low concentrations decrease the signal to noise ratio (e.g. Kubitschek, 1969; Hobson & Mann, 1970; Zimmermann *et al.*, 1973).

(iv) Samples of low concentration, or those containing a high proportion of large non-microbial particles, should be filtered to remove any material that may otherwise be counted (Kubitschek, 1969; Hobson & Mann, 1970).

(v) Aperture length, flow rate and amplifier response must be matched to produce output voltages of suitable magnitude (Kubitschek, 1969).

(vi) For sizing, the suspension should be hydrodynamically focussed to counteract the non-uniformity of the current density at different locations in the orifice, such that the path of the microorganisms through the field is uniform (Jeltsch & Zimmermann, 1979; Zimmermann, 1982), since the magnitude of the output voltage is also a function of rotation and deflection of the individual microbe as it traverses the field (Grover *et al.*, 1969*a*; Zimmermann *et al.*, 1973; Roberts, 1980).

(vii) Very important, and yet often overlooked, since it has only been noted rather recently, is that the magnitude of the applied field must be below a certain critical value, above which the measured size of the cells decreases with increasing field strength (Zimmermann *et al.*, 1973; Jeltsch & Zimmermann, 1979). This critical value, causing dielectric breakdown, is of the order of 1 to 10 kV/cm for most microbial systems (Hamilton & Sale, 1967; Grover *et al.*, 1972; Zimmermann *et al.*, 1973; Zimmermann *et al.*, 1980; Hulsheger & Niemann, 1981). This point will be discussed in some detail.

This non-proportionality between peak height and field strength may be due to one or a combination of two factors in particular: either a change in the electrical properties of the suspending medium caused by electrolysis at the higher voltages, or the dielectric breakdown of the microbial membranes at the higher field strengths. In theory, non-conducting particles should produce a square-wave voltage pulse that is proportional to the size of the particle when this particle crosses a d.c. (or low frequency) electric field. However, this assumes that the conductivity of the particle is constant under different experimental conditions. For solid particles, e.g. pollen and latex spheres, this is indeed the case, but the electrical properties of microorganisms change with the magnitude of the applied field. If the electric field is small or absent, microorganisms consist of a relatively non-conducting membrane enclosing a relatively highly conducting cytoplasm. An electric field applied across a microbial cell induces a potential gradient across the cell membrane. This potential gradient causes, *inter alia*, an electrical alignment of the dipolar molecules comprising the membrane, to a degree dependent upon the field strength and the size of the microorganism. Such an alignment of the membrane dipoles (electrostatic versus elastic restoring forces—see e.g. Zimmermann *et al.*, 1973, 1980, 1981) can result in the dielectric breakdown of the membrane as seen by a decrease in the capacitance and an increase in the conductance of the suspension of microorganisms. The extent to which dielectric breakdown occurs is a function of the applied field strength per unit length (e.g. Hamilton & Sale, 1967; Zimmermann *et al.*, 1973, 1980; Hulsheger & Niemann, 1980, 1981).

Microorganisms being counted and sized in the Coulter Counter are often subjected to an electric field which is capable of producing dielectric

breakdown. Under such conditions, the Coulter Counter will record a smaller change in conductance than if the breakdown had not occurred. Hence, on the assumption that pulse height is proportional to cell size, a smaller cell size than is actually the case is estimated. Providing that dielectric breakdown is not complete, and the conductance of the micro-organism at this field strength is not equal to that of the suspending medium, a change in conductance will still be recorded, and although this change may not give correct *sizing*, it may be used for *counting* purposes. Grover *et al.* (1972) noted this effect, for human red blood cells, about a critical field strength of 0·8 kV/cm, but gave no explanation. Other workers (e.g. Morrison & Tomkins, 1973; Ricketts & Rappitt, 1974; Curds *et al.*, 1978; Roberts, 1980) have observed a lower cell size measured with the Coulter Counter than with other techniques.

Morrison & Tomkins (1973) used the Coulter Counter to measure the mean cell size of *Tetrahymena* under conditions of growth and starvation. They compared the values of cell size obtained from the electronic sizing with those obtained by tracing, cutting out and weighing the silhouettes of a series of cells from photomicrographs of the suspension. This comparison revealed that the Coulter Counter underestimated the cell volume by a factor of 4·9 for normally growing cells, and this underestimation increased during starvation of the protozoa. Morrison & Tomkins (1973) explained this discrepancy in terms of the error involved in the optical method because of the shape of the protozoa, whereas the error was more likely to be in the Coulter Counter method owing to the dielectric breakdown of the *Tetrahymena* membranes. The increased underestimation of the cell volume of the starved protozoa may be ascribed to the conditions of starvation giving rise to an electrically weaker membrane. A weaker membrane would undergo dielectric breakdown at a lower field strength, or the breakdown would be further advanced at the given field strength.

Ricketts & Rappitt (1974) compared values of the cell size of *Tetrahymena* obtained using the Coulter Counter with cell dimensions measured optically. They, too, found that the Coulter Counter underestimated the cell size, the electronically determined size being 1·2 to 2·6 times lower than that determined from optical measurements. Again the full error was assigned to the optical method and the asymmetry of the protozoa, and no part of it to the Coulter Counter.

Curds *et al.* (1978) used the Coulter Counter to size ciliated protozoa, in conjunction with optical sizing methods. They found that the mean cell

volume of an axenic population of *Tetrahymena*, measured over a wide range of sizes, was on average 3·3 times greater by optical methods than by the electronic device. No reason was proposed for this discrepancy. However, for a series of six separate electronic sizings they found the values of the mean cell volume to be reproducible to within 1%, and further found that although the absolute values of the cell volumes measured by the two different methods did not agree, the ratio between them was consistent, and also that the electronically determined mean cell volume was linear with cell dry weight.

Roberts (1980) similarly reported an underestimated cell size (in comparison with microscopic sizing techniques) when sizing *Tetrahymena* with the Coulter Counter and Channelyser. In an attempt to overcome this problem he calibrated the electronic device with pollen grains of a known size, osmotically stabilised the cells in mannitol, and hydrodynamically focussed the flow of cells through the field; nevertheless, he consistently found a four-fold underestimation in cell size (Roberts, 1980). Roberts concluded that the cell size was not proportional to the peak height under these conditions, and proposed that the underestimated cell size was due to an increase in the conductivity of the protozoa in the electric field. However, he offered no explanation as to why an increase in conductivity should occur.

Zimmermann *et al.* (1973), Jeltsch & Zimmermann (1979) and Zimmermann *et al.* (1980) noted that the voltage applied across the orifice of the Coulter Counter was of the same order of magnitude as that which is required for the dielectric breakdown of microbial membranes. If membrane breakdown should occur, the high conductance of the cytoplasm contributes to the total conductance of the system, giving a smaller change in conductance with respect to the suspending medium, and hence an underestimation of the cell volume. Zimmermann *et al.* (1973) found that dielectric breakdown did not occur in solid particles, such as latex spheres and pollen, nor in *E.coli* when the membranes had been fixed with glutaraldehyde.

The implication of the phenomenon of dielectric breakdown of microbial membranes in electronic counting and sizing systems has not been widely appreciated, and it may be assumed that results obtained from such systems have often been misinterpreted. However, the technique may still be used for counting cells, and indeed for sizing if the applied field is below the critical level, and has had many successful applications (e.g. Zimmermann *et al.*, 1973; Dow, 1976; Dow *et al.*, 1979;

Zimmermann *et al.*, 1980). Dow (1976) used the Coulter Counter linked to a Channelyser to study population dynamics and morphology in microbial cultures. Further work of Dow *et al.* (1979) utilised this same system for particle distribution analysis of the microbial populations of 600 urine samples. The microorganisms present in the samples were sized, and those with a diameter greater than 0·4 μm were counted. The result was purely qualitative, in that urine was classed as being either positive or negative, but an agreement of 98·8% was obtained between classification based on this technique and that based on microscopy and culture methods.

A major disadvantage of the electrical counting technique (it should not be affected by the magnitude of the applied field so long as a change in conductance is recorded) is its inability to distinguish viable cells from non-viable cells and non-microbial particles. Several attempts have been made to overcome this problem (e.g. Seydel & Wempe, 1980; Matsushita *et al.*, 1982). Seydel & Wempe (1980) devised a method using the Coulter Counter to differentiate viable and total cell counts in *E.coli* cultures inhibited by chemotherapeutic agents and compared these results with the usual viable counts obtained through plating procedures. The principle was to administer the drugs in such concentrations that they would inhibit the growth of some cells whilst killing others, producing a suspension of viable non-multiplying cells and killed cells, with a fairly constant cell count. The inoculated culture may then be diluted with fresh broth such that the concentration of the inhibitor is negligible, allowing the viable but inhibited cells to regrow. Coulter Counter measurements were taken at hourly intervals between three and six hours after dilution, thus minimising the error due to the killed cells and allowing the re-growing cells to reach a concentration suitable for counting. From a simple kinetic approach the logarithm of the cell count was seen to be linear with the incubation time, and extrapolation to the time of dilution gave the number of viable cells after the addition of the drugs. This principle was applied to cultures of *E.coli* treated with three different drugs: HN-32 (a nitrofuran derivative), TMP (trimethoprim) and tetracycline. The death rates obtained with the Coulter Counter were identical with those obtained from plating techniques for both HN-32 and TMP, but the Coulter Counter underestimated the death rate, that is, over-estimated the cell population, for tetracycline. Seydel & Wempe (1980) suggested that the measured low death rate was probably due to the mode of action of tetracycline, and not to an error in the technique, since it is

known that cells recovering from the action of tetracycline grow more slowly on agar than they do in liquid cultures. Such a method has obvious advantages over plate counts in the saving of time; this Coulter Counter method would require a 6 h incubation period compared with the 24 h needed for plate counts, but further experiments are necessary.

Matsushita *et al.* (1982) attempted to distinguish viable and non-viable mouse myeloma cells, using a Coulter Counter linked to a Channelyser, by suspending the cells in phosphate-buffered saline (PBS) solution. The resulting electrical size distribution profile was bimodal and showed an increase in the fraction of smaller cells in the presence of PBS. Trypan blue staining, and microscopic examination of the PBS-treated culture, showed that PBS was lethal and that the killed cells were indeed smaller than the viable population, hence providing a means to differentiate live and dead cells by electrical sizing and counting. However, the membranes of the killed cells exhibited an increased permeability to ions, and the cells would therefore in any event generate pulses of decreased size.

Electrical counting techniques by themselves are not capable of providing viable counts. The attempts to extend the technique have not been wholly satisfactory in the specific systems which they were designed to study, and could certainly not be applied universally to the estimation of viable counts in fermentors. Electrical sizing techniques appear to possess the potential for providing an insight into cell population dynamics, but have been shown to suffer from certain artifacts, in particular those caused by the dielectric breakdown of microbial membranes, under the conditions of field strength commonly used (e.g. see Zimmermann *et al.*, 1973; Jeltsch & Zimmermann, 1979; Zimmermann *et al.*, 1981). Electronic counts do have the advantage that they monitor passive electrical properties of the microorganism and do not rely on growth, and so the counts are available in minutes rather than hours, although they are not quite in real time. Time-dependent changes in the medium conductivity would also tend to confound their applicability to on-line measurements in fermentors.

4.7. Electrochemically based detection systems

Many electrochemical detection systems have been proposed for the estimation of microbial populations. Their aim, as with other physical techniques discussed above, is to reduce the time necessary for the detection of microorganisms, with the view ultimately to provide a quick, effective, and simple-to-use biomass probe.

Wilkins (Wilkins, 1974, 1979; Wilkins *et al.*, 1974, 1978, 1980) developed an electrical detection system for the evaluation of microbial populations, based on the detection of gases produced by actively metabolising microorganisms. Initially the detection of these gases was effected by means of a pressure transducer linked to a chart recorder (Wilkins, 1974). The delay period, for given experimental conditions, between the inoculation of the sample and the detection of the response was found to be linear with the logarithm of the inoculum size. As expected, the detection time increased for decreasing inoculum size; for example, the detection time for 10^7 cells/ml was 3 h, and for 1 cell/ml was 12 h. Despite the sensitivity of the pressure transducer, Wilkins (Wilkins *et al.*, 1974) abandoned this method in favour of a yet more sensitive electrochemical method.

Wilkins (Wilkins *et al.*, 1974) reported the use of an electrochemical method, consisting of a platinum electrode and a reference (standard calomel) electrode, which monitored the production of hydrogen by the metabolising microorganisms. The principle behind this detector is that the evolution of hydrogen decreases the apparent E_h (redox potential) (see Morris, 1974) owing to the redox equilibrium established between (principally) the H_2/H^+ couple and the platinum electrode:

$$H_2 \rightleftharpoons 2H^+ + 2e^-$$

Subsequently, Wilkins *et al.* (1978) reported that responses to this electrode system were also obtained with non-hydrogen-producing micro-organisms. It thus seems more likely that this electrode configuration was monitoring changes in redox potentials resulting from the microbial production of electroactive substances including, but not solely consisting of, molecular hydrogen (see Kjaergaard, 1977). Using the platinum and calomel electrode arrangement, Wilkins *et al.* (1974) recorded response curves containing the characteristic lag period before 'detection' was possible. The lag period was found to be linear with the logarithm of the initial inoculum size as determined by viable counts. Typical detection times for *E.coli* using this method were 1 h for 10^6 cells/ml, and 7 h for 1 cell/ml, which is indeed quicker than for the pressure transducer. In view of this improved sensitivity, Wilkins *et al.* (1978) developed a multi-channel electrochemical detection unit capable of detecting and indicating the detection times for each of the eight channels simultaneously and automatically.

Wilkins (Wilkins *et al.*, 1978) further found that the response curves obtained with two platinum electrodes of differing surface area were

similar to those obtained with the platinum/calomel electrode system. Here the experimental arrangement consisted of a test tube containing two platinum electrodes of differing length immersed into the sample medium. The response curve arising from the potential difference between the two electrodes again exhibited a lag period which was found to be linear with the logarithm of the size of the inoculum. The platinum–platinum electrode system was (not surprisingly) less sensitive than the combination electrode system, but was attractive by virtue of its lower cost and ease of handling. It also lent itself to adaptation for a combined membrane filtration–electrochemical method for microbial enumeration (Wilkins *et al.*, 1980). The objective of this adaptation was to decrease the detection time by increasing the initial concentration of the sample through filtration, thereby enabling the monitoring of microorganisms in sparsely populated environments. In practice, cells were collected on a 0·45 μm filter, on to which two platinum electrodes were clamped, and the potential difference between them was then measured. Wilkins *et al.* (1980) used this system to test nearly 100 aquatic samples. They found a certain degree of linearity, with a correlation coefficient of 0·825, between the logarithm of the inoculum size and the detection time. However, filtration techniques are known to induce the death of a proportion of cells, and should be avoided for consistent results (Jones, 1979). The *modus operandi* of this system (Wilkins *et al.*, 1980) is not at all evident; it may involve fuel cell-like reactions.

Holland *et al.* (1980) used a similar system to that of Wilkins (1979), but with two stainless steel (rather than platinum) electrodes of differing size. They inoculated 100-ml samples with 300 or 50 cfu of one of several microbial species commonly found in the blood of patients suffering from bacteraemia, and recorded changes in the apparent potential difference at 15-min intervals. The sample was considered to be positive if the voltage change was greater than or equal to 0·1 mV/min and increased for the following 45 min. Of the 163 test samples seeded with the larger inoculum, 90·8% were considered to be positive; 88·7% of the 53 samples seeded with 50 cfu were deemed positive using the electrochemical technique. This principle was then applied to the detection of microbial growth in blood specimens from 156 patients (Holland *et al.*, 1980). This electrical detection system correctly identified the 13 positive samples that had also been identified by conventional turbidimetric methods, with an average detection time 18 h less than for the conventional method.

Junter and co-workers (Junter *et al.*, 1980, 1984; Junter & Selegny,

1982*a*,*b*) followed bacterial growth using electrochemical measurements of the decrease in redox potential associated with (i) the oxygen consumption during aerobic glycolysis and (ii) the subsequent reduction of exogenous lipoic acid, and from this estimated the number of viable cells present in an axenic population of *E.coli*. During the oxidation–reduction reactions of glucose catabolism, lipoic acid (LA) enters the bacterial cells where it is reduced to dihydrolipoic acid (LAH_2). The reduced form may then be transported from the cells (e.g. Junter & Selegny, 1982*a*). The output voltage, measured using a gold electrode with respect to a standard calomel electrode, therefore follows the modification of redox potential due to the reduction of dissolved oxygen, and of the lipoate/dihydrolipoate couple, resulting from bacterial activity. The gold electrodes were found to be highly sensitive to the dihydrolipoate produced, down to concentrations of the order of 1 μM, owing to adsorption of the dihydrolipoic acid onto the gold (Junter & Selegny, 1982*a*).

In order to maximise the consistency in the measured redox potentials of samples containing inocula of the same size, the number of possible metabolic pathways was restricted by growing the bacteria in a relatively defined medium containing only salts and glucose (but sometimes a small amount of yeast extract). The time preceding the characteristic drop in potential of the output voltage was found to represent the number of viable cells initially present in the inoculum, enabling as few as 10 cells/litre to be detected in 11 h. The experimentally observed linear relationship between the logarithm of the initial inoculum size and the time for a 100 mV variation in potential to occur was supported by a mathematical model (Junter *et al.*, 1980). The kinetic expression obtained was related to the rate and mechanism of lipoic acid uptake, the excretion and reoxidation of the dihydrolipoic acid and the concentration and generation time of the microorganisms. Junter & Selegny (1982*a*) proposed a more complex mathematical model by describing the system at three distinct levels, and simulating potential–time curves from the resulting series of equations. The three levels considered were:

(i) the electrode: this expressed the potential of the gold electrode as a function of the concentration of the electroactive species present;
(ii) the cell or metabolic level: involving the time evolution of the concentrations of the relevant electroactive species;

(iii) the culture broth: involving the physical and chemical conditions of the culture, such as the dissolution of molecular oxygen from the air into the broth.

The relationships of the cell population determined by colony counts with the theoretical minimum detection time, and with the detection time found experimentally, were of the same form for inocula sizes in the range 10–10^{10} cells/litre. Thus the theory agreed with the experimental observations.

Using this electrochemical technique, 10^5 *E.coli* cells/ml, grown in a medium enriched with yeast extract, may be detected in under 7 h (Junter *et al.*, 1980), and compliance with the International Standards for drinking water, which require that disinfected drinking water contains only 1 bacterium/100 ml, may be gauged within 12 h (Junter *et al.*, 1980). Although this method offers features which are desirable for a probe to monitor biomass in fermentors, such as the comparative simplicity of an electrochemical approach, and the sensitivity obtainable, it does, however, have its disadvantages. First, detection relies on microbial growth, and therefore cannot be in real time; secondly, the addition of lipoic acid might conceivably have an adverse effect on the fermentation process under study; and thirdly, the detection time for 10^5 cells/ml is longer than by other growth methods (e.g. impedimetry, radiometry, etc., see above). Nevertheless, the elegant simplicity of this system seems to indicate that it would provide a convenient assay for microbial activity in clinical, ecological and food samples.

The detection systems of Wilkins *et al.* (Wilkins *et al.*, 1974, 1978, 1980; Wilkins, 1979), Holland *et al.* (1980), Junter *et al.* (1980) and of Junter & Selegny (1982*a*,*b*) rely on the changes in potential caused by the growth of the microorganisms and the production of electroactive metabolites. As such, these methods have the potential to detect very low cell numbers, but the detection time may still be relatively long, in the order of 10 to 12 h for less than 10 cfu/ml, and 3 h for 10^7 cfu/ml. Karube, Suzuki and coworkers (Matsunaga *et al.*, 1979, 1980; Ishimori *et al.*, 1981; Nishikawa *et al.*, 1982), and Turner *et al.* (Turner *et al.*, 1983; Aston & Turner, 1984) have developed a fuel cell-type electrode system (see also Wingard *et al.*, 1982), in which the detection time, some 15 to 20 min, is independent of microbial growth. This 15-min response time, although not in real time, could in principle enable the continuous monitoring of biomass in fermentors.

Both pairs of reference and measuring electrodes of the system proposed by Matsunaga *et al.* (1979) consisted of a platinum anode and silver peroxide cathode. One pair of electrodes was covered by a cellulose dialysis membrane, which allowed the free passage of the medium into this electrochemical cell, whilst preventing the access of the microorganisms. Thus, the total electrode system measured the difference in current produced in the two circuits. In this, the changes in electroactive substances in the medium caused by the metabolic activities of the microorganisms (e.g. the production of molecular hydrogen, formic acid and

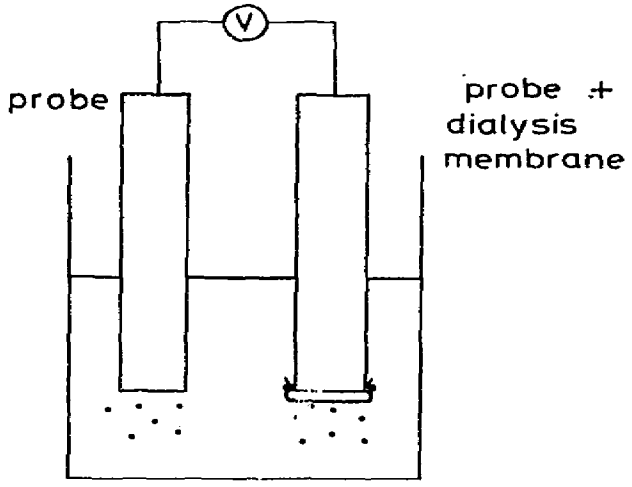

Fig. 5. The principle of using dual probes, one covered with a dialysis membrane, a nuclepore filter or other microorganism-impermeable membrane, to nullify the effect of environmental changes on purportedly biomass-specific signals. The main disadvantages of this method are its requirement for the calibration of both probes and a decreased response time.

other metabolites) may be cancelled, and the measured difference in current is due to the microorganisms alone. This principle (Fig. 5) is discussed in some detail by Clarke *et al.* (1982) and by Clarke *et al.* (1985). Further, the system is apparently measuring the current output generated by viable cells, and not that generated by non-viable cells or other particulate material (prevented from entering the reference electrode by the dialysis membrane); heating, ultrasonic irradiation, and the addition of nystatin (an antibiotic that destroys cell membrane integrity) produced a decrease in the output signal (Matsunaga *et al.*, 1979).

Matsunaga *et al.* (1979) found the measured current difference to be proportional to the number of cells in the sample, estimated by colony counts, to within an average error of 6%, and evaluated the effect of pH, temperature, and phosphate buffer and oxygen concentrations on this relationship. They reported that the current output was independent of the oxygen and phosphate buffer concentrations. However, an increasing signal was obtained with increasing pH, and also with increasing temperature up to a maximum at 50°C, thus necessitating temperature and pH control of the suspension. The linearity of the electrode response was tested using suspensions of known concentrations of *Lactobacillus fermentum* and *Saccharomyces cerevisiae*, and the device was then successfully applied to the continuous monitoring of cell populations of *S. cerevisiae* in a fermentor.

This proposed electrochemical system (Matsunaga *et al.*, 1979) has the distinct advantage over those of Wilkins (Wilkins, 1974, 1979; Wilkins *et al.*, 1974, 1978, 1980), Holland *et al.* (1980) and Junter *et al.* (Junter *et al.*, 1980; Junter & Selegny, 1982*a*,*b*) in that the detection time is a function of the response time of the dialysis-membrane-covered electrode (some 15 to 20 min) and does not depend on the growth of the microorganisms *per se*. This faster response time, which would be improved by the substitution of a nucleopore filter for the dialysis membrane, when coupled with the simplicity of an electrode system, enables continuous, on-line monitoring of biomass in fermentors. However, the platinum–silver peroxide system has two disadvantages: (i) the silver peroxide cathode decomposes on autoclaving, and (ii) the system is not capable of providing the sensitivity required over the range of cell populations that are initially found in fermentation processes (the minimum number of detectable cells being 10^7 yeast cells/ml, and 10^8 *L. fermentum* cells/ml), nor those that are typically found in clinical or ecological specimens.

To overcome the first disadvantage, Matsunaga *et al.* (1980) tested another electrode system, similar to the platinum–silver peroxide system. This new system consisted of two cells, measuring and reference, each comprising a platinum working electrode, platinum counter electrode, and saturated calomel electrode (SCE). Both of these electrode cells were immersed into a culture of *B. subtilis*, the reference cell again being covered with a cellulose dialysis membrane to maintain a microorganism-free 'reference solution'. The platinum working electrodes of each electrode system were held at a constant potential with respect to the SCE, and the difference in current flowing in the 'measuring' and

'reference' circuits was recorded. Matsunaga *et al.* (1980) found that the maximum current difference was obtained at a working potential of 0·2 V (versus SCE), and that the output signal increased with increasing temperature to a maximum at 50°C, owing to the increased reactivity of the electroactive substances and the subsequent inactivation of the bacteria at 50°C. They found that it was also necessary to neutralise the organic acids produced by actively metabolising *B.subtilis*, and hence to control the pH of the culture.

The output signal was attributed to the presence of viable microorganisms, since an autoclaved sample containing $1{\cdot}4 \times 10^9$ *B.subtilis*/ml produced a greatly reduced signal in comparison with a viable cell population of the same size. The difference in current between the measuring and reference (electrochemical) cells was found to be linear with cell numbers (measured by colony counts) in the range 10^8 to 4×10^9 *B.subtilis*/ml, with a mean error of 4%. This potentiostatic device, as well as being stable to autoclaving, has the added advantage over the platinum–silver peroxide system in that the response time has been reduced from 20 min to only 5 min. However, the operating range of bacterial concentration for this system, and for the platinum–silver peroxide electrodes, is restricted, and although their sensitivities will suffice in some instances, many cultures are grown at concentrations outside the limits of linearity of these devices. The second disadvantage of the platinum–silver peroxide system is therefore shared by the platinum–platinum–calomel arrangement, and in order to overcome this lack of sensitivity, Karube, Suzuki and colleagues (Nishikawa *et al.*, 1982) adapted the fuel cell-type electrode system to include the filtration of dilute populations: they also added redox mediators ('signal amplifiers'). Initially, they evaluated the efficiency of various redox dyes as signal amplifiers, using suspensions of *E.coli*. Of the redox dyes tested, 2, 4-dichlorophenolindophenol (DCIP) was found to be the most effective signal amplifier, and like the other redox dyes it enhances the signal by acting as an electron carrier between the electron donors in the microbial membrane and the platinum anode (Turner *et al.*, 1983). The signal was maximal under the following conditions of pH, temperature, and DCIP concentrations (Nishikawa *et al.*, 1982): pH 7–8, 35°C, and 40 μM DCIP, respectively. Subsequently, suspensions of *E.coli* and three other types of bacteria, *Pseudomonas aeruginosa*, *Frankia arbrescens*, and *Bacillus subtilis*, were concentrated onto acetylcellulose membranes. These membranes were attached to the anode of the measuring electrode and

immersed in phosphate buffer containing DCIP. A linear relationship was obtained between the logarithm of the output current and the logarithm of the cell population determined from colony counts, within the range of 10^4 to 10^6 cells/ml (Nishikawa *et al.*, 1982). Samples of industrial water, from a paper mill and a petrochemical plant, were similarly treated and their respective microbial populations estimated from a calibration curve for *E.coli* (Nishikawa *et al.*, 1982). The lower limit of detection of the platinum–silver peroxide electrode system was decreased from 10^7 to 10^4 cells/ml by the use of DCIP (Nishikawa *et al.*, 1982). Different mediators would presumably be needed for use in anaerobic fermentation processes.

Ishimori, Karube and Suzuki (Ishimori *et al.*, 1981) presented a novel electrochemical technique for the continuous monitoring of yeast populations in fermentors, exploiting the properties of piezoelectric membranes. Essentially, the probe consisted of two parallel piezoelectric membranes 2·5 mm apart which were immersed into the yeast culture. When the input piezoelectric membrane was charged to a certain voltage by an oscillator, the resulting ultrasonic waves were transmitted through the culture separating the two membranes, and induced a voltage on the output membrane. Ishimori *et al.* (1981) recorded the individual responses obtained from a yeast culture, the supernatant of the yeast culture, and water, and found that approximately 40% of the output signal could be assigned to the effect of the microorganisms on the transmission of the ultrasonic waves between the two piezoelectric membranes. The transmission of ultrasonic waves through fluids is also a function of (i) the frequency of the applied voltage, (ii) the pH and buffering capacity of the sample, (iii) the temperature, (iv) the density of the medium, and (v) the adiabatic compressibility of the sample. All these variables were duly tested, and those which were found significantly to affect the signal were suitably controlled.

The piezoelectric membrane probe was used to estimate microbial populations in suspensions of *B.subtilis* and *Klebsiella* spp., and to monitor the growth of yeast in a fermentor. The calibration curve for yeast showed a linear response between the output voltage and the number of cells/ml (counted with a haemocytometer) in the range 10^6 to 10^8 cells/ml, and a different linear response between 10^8 and 10^{10} cells/ml, with a mean error of 6%. The level of sensitivity is below that at which yeast is grown in a typical commercial fermentation (Matsunaga *et al.*,

1979), but it may not be suitable for monitoring other fermentation processes. Ishimori *et al.* (1981) also found that although the piezoelectric membrane seemed promising as a biomass probe for yeast, small cracks appeared in the epoxy resin during autoclaving which resulted in the loss of the output signal. This is hardly an insuperable problem, and further investigation of devices based on this principle seems warranted.

The electrochemical detection systems probably offer the best techniques for on-line and continuous monitoring of biomass in fermentors. They are inexpensive (particularly when compared with the currently available impedimetric, light scattering and radiometric devices), easy to use, the electrodes may be effectively sterilised by autoclaving, they function in the normal growth media, and they may be easily automated. However, further work is necessary in order to provide a system which has the desired sensitivity and a very fast, preferably real-time, response.

4.8. Neutron scattering

In certain circumstances, particularly in sewage treatment, microbial growth is carried out in a film on an inert support matrix such as rock or plastic. In the former case, one method which has been proposed for the determining of microbial biomass content involves the measurement of the scattering of thermal (slow) neutrons (Harvey *et al.*, 1963). As is well known, the efficiency of hydrogen as a moderator of fast neutrons is so much higher than that of any other element that the flux of slow neutrons surrounding a source of fast neutrons at equilibrium is determined almost entirely by the hydrogen content of the medium. Measurement of the flux of slow neutrons then gives a measure mainly of the water content of a typical sample of interest (Marais & Smit, 1960), and can thus, in favourable cases, be used to assess the microbial biomass content of percolating filters. The method does not seem to have enjoyed widespread use to date.

5. MATHEMATICAL METHODS AND COMPUTATION

Problems with the direct determination of microbial biomass have led to the use of mass balancing, by which all components other than biomass are monitored, the biomass content being assessed by difference. This has

obvious difficulties connected with the summation of errors. However, mathematical models (e.g. Robinson & Tiedge, 1983) have the potential for quantitative explanation of the phenomena observed during fermentation processes, coupled to the estimation of cell biomass. Nevertheless, their limitations must be appreciated, and apparently successful models should not be considered final proof of any of the assumptions incorporated into the model, since two or more alternative models may lead to similar conclusions (Topiwala, 1973; Roels, 1983).

The two main obstacles preventing on-line, real-time monitoring of fermentations are the lack of real-time sensors, and insufficient understanding of the interaction of process variables and the dynamic response of a fermentation to changes in environmental conditions (Zabriskie *et al.*, 1976; Kell, 1980; Clarke *et al.*, 1982; Clarke *et al.*, 1985). Despite these problems, mathematical models have been proposed to describe some simple fermentation processes (e.g. Sinclair & Topiwala, 1970; Curds, 1971; Zabriskie *et al.*, 1976; Cooney *et al.*, 1977; Wang *et al.*, 1979; Junter & Selegny, 1982*a*,*b*; Park *et al.*, 1983; Roels, 1983; Solomon *et al.*, 1983). However, further discussion of the use of the mass balancing technique lies outside the scope of the present considerations.

6. SUMMARY AND PROSPECTS

(i) Many methods for the estimation of biomass have been proposed; in cases where the microbial population is sparse, growth leading to an increase in the output signal of interest is required; given this delay, both chemical and physical methods are suitable.

(ii) No wholly satisfactory biomass sensor suitable for use in typical fermentation processes presently exists. If a real-time probe is desired, its operating principle must rely on a physical characteristic which distinguishes microorganisms from aqueous solutions, gas bubbles and particulate material.

(iii) It is to be expected that the advent of sophisticated signal processing techniques may contribute significantly to the realisation of a 'microbial biomass probe' for use in laboratory and industrial fermentors. It is our belief that the attainment of such a goal, based upon one or more of the methods alluded to herein, will be possible within the next quinquennium.

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REFERENCES

Adams, R. P. L. (1980). Cell counting procedures. In: *Laboratory Techniques in Biochemistry and Molecular Biology* (Ed. T. S. Work and R. H. Burdon), Vol. 8, Elsevier Scientific Publishers. Amsterdam, pp. 103–8.

Aldridge, C., Jones, P. W., Gibson, S., Lanham, J., Meyer, M., Vannest, R. & Charles, R. (1977). Automated microbial detection/identification system, *J. Clin. Microbiol.*, **6**, 406–13.

Allen, P. D. (1972). Development of the luminescence biometer for microbial detection, *Dev. Ind. Microbiol.*, **14**, 67–73.

Anon. (1980). *Automated Microbiology*, Abbott Diagnostics Division.

Anon. (1981*a*). *Bactometer Models 32 and 120.*

Anon. (1981*b*). Impedance microbiology, a current perspective, *Detection Times* (Bactomatic Inc.), **1**(1).

Anon. (1981*c*). The impedance screen: An effective tool for the food microbiologist, *Detection Times* (Bactomatic Inc.), **1**(2).

Anon. (1981*d*). Turner Designs, Luminescence Review, Bulletin 202.

Anon. (1982). Impedance screening of raw meat samples using the model 120B, *Detection Times* (Bactomatic Inc.), **2**(1).

Anon. (1983*a*). Rapid Automated Microbiology. Bactomatic Model 123.

Anon. (1983*b*). Bacterial colonies by numbers, *Lab. Equip. Digest*, **21**, 87–9.

Anon. (undated, *a*). The bioluminescence of yeast biomass in fermentor broths, *Luminescence Today*, LKB Wallac, Bioluminescence Application Note 508.

Anon. (undated, *b*). Direct Epifluorescent Filter Technique. DIFCO Technical Information.

Anon. (undated, *c*). The Spiral Plating Method. Don Whitley Scientific Ltd.

Anon. (undated, *d*). Microbial Growth Analysers, Malthus Instruments Ltd.

Anthony, C. (1982). *The Biochemistry of Methylotrophs*, Academic Press, London.

Aston, W. J. & Turner, A. P. F. (1984). Biosensors and biofuel cells, *Biotechnol. Genetic Eng. Rev.*, **1**, 89–120.

Atkinson, D. E. (1977). *Cellular Energy Metabolism and its Regulation*, Academic Press, New York.

Atkinson, D. E. & Walton, G. M. (1967). Adenosine triphosphate conversion in metabolic regulation, *J. Biol. Chem.*, **242**, 3239–41.

Bachrach, U. (1976). Radiometric methods for the detection of bacteria in biological fluids and water. In: *Proc. 2nd Int. Symp. on Rapid Methods and*

Automation in Microbiology (Ed. H. H. Johnston and S. W. B. Newsom), Learned Information (Europe) Ltd, Oxford, p. 3.

Bachrach, U. & Bachrach, Z. (1974). Radiometric method for the detection of coliform organisms in water, *Appl. Microbiol.*, **28**, 169–71.

Bard, A. J. & Faulkner, L. R. (1980). *Electrochemical Methods*, Wiley, Chichester.

Baross, J. & Deming, J. W. (1983). Growth of 'Black Smoker' bacteria at temperatures of at least 250°C, *Nature*, **303**, 423–6.

Bascomb, S. (1981). Application of automation to the general and specific detection of bacteria, *Lab. Pract.*, **30**, 461–4.

Basel, R., Richter, E. & Banwart, G. (1983). Monitoring microbial numbers in food by density centrifugation, *Appl. Env. Microbiol.*, **45**, 1156–9.

Bator, J. M., Groves, M. R., Price, B. J. & Eckstein, E. C. (1984). Erythrocyte deformability and size measured in a multiparameter system that includes impedance sizing, *Cytometry*, **5**, 34–41.

Beezer, A. E., Newell, R. D. & Tyrrell, H. J. V. (1976). Application of flow microcalorimetry to analytical problems: The preparation, storage and assay of frozen inocula of *Saccharomyces cerevisiae*, *J. Appl. Bacteriol.*, **41**, 197–207.

Beezer, A. E., Bettleheim, K. A., Al-Salihi, S. & Shaw, E. J. (1978). The enumeration of bacteria in culture media and clinical specimens of urine by microcalorimetry, *Sci. Tools*, **25**, 6–8.

Bergan, T. (1981). Rapid methods for the detection of aerobic bacteria in blood and spinal fluid, *Proc. 3rd Int. Symp. Rapid Methods and Automation in Microbiology* (Ed. R. C. Tilton), American Society for Microbiology, Washington, pp. 36–40.

Bettleheim, K. A., O'Farrell, S. M., Al-Salihi, S., Shaw, E. J. & Beezer, A. E. (1976). Clinical investigation into the potential use of microcalorimetry for the diagnosis of bacteriuria. In: *Proc. 2nd Int. Symp. on Rapid Methods and Automation in Microbiology* (Ed. H. H. Johnston and S. W. B. Newsom), Learned Information (Europe) Ltd, Oxford, p. 8.

Biltonen, R. L. & Langerman, N. (1979). Microcalorimetry for biological chemistry: Experimental design, data analysis and interpretation, *Meth. Enzymol.*, **61**, 287–318.

Bohren, C. F. & Huffman, D. R. (1983). *Absorption and Scattering of Light by Small Particles*, Wiley, Chichester.

Boling, E. A. & Blanchard, G. C. (1973). Bacterial identification by microcalorimetry, *Nature*, **241**, 472–3.

Bond, A. M. (1980). *Modern Polarographic Methods in Analytical Chemistry*, Marcel Dekker, New York.

Bourgeois, C. & Mafart, P. (1976). The uptake of a labelled metabolite as a technique for microorganism detection. In: *Proc. 2nd Int. Symp. on Rapid Methods and Automation in Microbiology* (Ed. H. H. Johnston and S. W. B. Newsom), Learned Information (Europe) Ltd, Oxford, p. 4.

Brooks, S. C. (1923). Conductivity as a measure of vitality and death, *J. Physiol.*, **5**, 365–81.

Brown, D. F. J. & Warner, M. (1982). Impedance and conductivity methods for detecting bacteriuria. In: *Proc. 3rd Int. Symp. Rapid Methods and Automation in Microbiology* (Ed. R. C. Tilton), American Society for Microbiology, Washington, pp. 171–5.

Buddemeyer, E. U. (1976). Automated quantitative radiometric measurement of bacterial growth. In: *Proc. 2nd Int. Symp. Rapid Methods and Automation in Microbiology* (Ed. H. H. Johnston and S. W. B. Newsom), Learned Information (Europe) Ltd, Oxford, p. 4.

Cady, P. (1975). Rapid automated bacterial identification by impedance measurements. In: *New Approaches to the Identification of Microorganisms* (Ed. C.-G. Heden and T. Illeni), Wiley Biomedical, New York, pp. 73–99.

Cady, P. (1978). Progress in impedance measurements in microbiology. In: *Mechanising Microbiology* (Ed. A. Sharpe and D. Clarke), Charles C. Thomas, Springfield, pp. 199–239.

Cady, P., Dufour, S. W., Lawless, P., Nunke, B. & Kraeger, S. J. (1978). Impedimetric screening for bacteriuria, *J. Clin. Microbiol.*, **7**, 273–8.

Carstensen, E. L. & Marquis, R. E. (1975). Dielectric and electrochemical properties of bacterial cells. In: *Spores VI* (Ed. P. Gerhardt, R. N. Costilow and H. L. Sadoff), American Society for Microbiology, Washington, pp. 563–71.

Chapman, A. G., Fall, L. & Atkinson, D. E. (1971). Adenylate energy charge in *E.coli* during growth and starvation, *J. Bacteriol.*, **108**, 1072–86.

Chappelle, E. W., Piccioli, G. L. & Deming, J. W. (1978). Determination of bacterial content in fluids, *Meth. Enzymol.*, **57**, 656–72.

Chen, S.-H. & Hallett, F. R. (1982). Determination of motile behaviour of prokaryotic and eukaryotic cells by quasi-elastic light scattering, *Quart. Rev. Biophys.*, **15**, 131–222.

Chu, B. (1974). *Laser Light Scattering*, Academic Press, New York.

Clarke, D. J., Kell, D. B., Morris, J. G. & Burns, A. (1982). The role of ion-selective electrodes in microbial process control, *Ion Selec. Elec. Rev.*, **4**, 75–131.

Clarke, D. J., Calder, M. R., Carr, R. J. G., Blake-Coleman, B. C. & Moody, S. C. (1985). The development and application of biosensing devices for bioreactor monitoring and control, *Biosensors* (in press).

Coates, D. A. (1976). The use of the limulus pyrogen assay for the rapid detection of contamination by Gram negative bacteria. In: *Proc. 2nd Int. Symp. Rapid Methods and Automation in Microbiology* (Ed. H. H. Johnston and S. W. B. Newsom), Learned Information (Europe) Ltd, Oxford, pp. 19–20.

Cochet, N., Tyagi, R. D., Ghose, T. K. & Lebeault, J. M. (1984). ATP measurement for cellulose production control, *Biotechnol. Lett.*, **6**, 155–60.

Coleman, K., Hunter, P. A. & Ridgeway Watt, P. (1983). *Antibiotics: Assessment of Microbiological Activity and Resistance*, Soc. Appl. Bacteriol. Technical Series no. 18 (Ed. A. D. Russell and L. B. Quesnel), Academic Press, London, pp. 317–30.

Collins, C. H. & Lyne, P. M. (1970). *Microbiological Methods*, Butterworths, London.

Cooney, C. L., Wang, H. & Wang, D. I. C. (1977). Computer aided material balancing for prediction of fermentation parameters, *Biotechnol. Bioeng.*, **19**, 55–67.

Coonrod, J. D., Kunz, L. J. & Ferraro, M. J. (Eds) (1983). *The Direct Detection of Microorganisms in Clinical Samples*, Academic Press, New York.

Couse, N. L. & King, J. W. (1982). Quantitation of the spiral plating technique for use with *Salmonella*/mammalian microsome assay, *Environ. Mutagen*, **4**, 445–55.

Cundell, A. M. (1981). Rapid counting methods for coliform bacteria, *Adv. Appl. Microbiol.*, **27**, 169–83.

Curby, W. A. & Gall, L. S. (1976). Instrumental approach to microbiological analysis of body fluids, *Pub. Health Lab.*, **35**, 118–39.

Curds, C. R. (1971). A computer simulated study of predator–prey relationships in a single-stage continuous-culture system, *Water Res.*, **5**, 793–812.

Curds, C. R., Roberts, D. McL. & Wu, C.-H. (1978). The use of continuous cultures and electronic sizing devices to study the growth of two species of ciliated protozoa. In: *Techniques for the Study of Mixed Populations* (Ed. D. W. Lovelock and R. Davies), Academic Press, London, pp. 165–77.

Dahnecke, B. E. (Ed.) (1983). *Measurement of Suspended Particles by Quasi-Elastic Light Scattering*, Wiley, Chichester.

Dawes, E. A. & Senior, P. J. (1973). The role and regulation of energy reserve polymers in microorganisms. In: *Advances in Microbial Physiology* (Ed. A. H. Rose and D. W. Tempest), Vol. 10, Academic Press, London, pp. 135–266.

DeBlanc, H. J., DeLand, F. & Wagner, H. D. (1971). Automated radiometric detection of bacteria in 2967 blood cultures, *Appl. Microbiol.*, **22**, 846–9.

Dermer, O. C., Curtis, V. S. & Leach, F. R. (1980). *Biochemical Indicators of Subsurface Pollution*, Ann Arbor Science, Ann Arbor.

Dow, C. S. (1976). Cell volume analysis. In: *Proc. 2nd Int. Symp. Rapid Methods and Automation in Microbiology* (Ed. H. H. Johnston and S. W. B. Newsom), Learned Information (Europe) Ltd, Oxford, pp. 97–8.

Dow, C. S., France, A. D., Khan, M. S. & Johnson, T. (1979). Particle size distribution analysis for the rapid detection of microbial infection of urine, *J. Clin. Pathol.*, **32**, 386–90.

Dufour, A. P., Strickland, E. R. & Cabelli, V. J. (1981). Membrane filter method for enumerating *E.coli*, *Appl. Env. Microbiol.*, **41**, 1152–8.

Dukhin, S. S. & Shilov, V. N. (1974). *Dielectric Phenomena and the Double Layer in Disperse Systems and Polyelectrolytes*, Wiley, Chichester.

Earnshaw, J. C. & Steer, M. W. (Eds) (1983). *The Application of Laser Light Scattering to the Study of Biological Motion*, Plenum Press, New York.

Eden, G. & Eden, R. (1984). Enumeration of microorganisms by their dynamic ac conductance patterns, *IEEE Trans. Biomed. Eng.*, **31**, 193–8.

Einolf, C. W. & Carstensen, E. L. (1973). Passive electrical properties of microorganisms. V. Low frequency dielectric dispersion of bacteria, *Biophys. J.*, **13**, 8–13.

El-Shaarawi, A. H. & Pipes, W. O. (1982). Enumeration and statistical inferences. In: *Bacterial Indicators of Pollution* (Ed. W. O. Pipes), CRC Press, Boca Raton, Florida, pp. 43–66.

Entis, P. (1981). Practical considerations in the filtration of foods. In: *Proc. 3rd Int. Symp. Rapid Methods and Automation in Microbiology* (Ed. R. C. Tilton), American Society for Microbiology, Washington, pp. 58–61.

Ewetz, L. & Thore, A. (1976). Factors affecting the specificity of luminol reaction with hematin compounds, *Anal. Biochem.*, **71**, 564–70.

Ferris, C. D. (1974). *Introduction to Bioelectrodes*, Plenum Press, New York.

Forrest, W. W. (1972). Microcalorimetry. In: *Methods in Microbiology* (Ed. J. R. Norris and D. W. Ribbons), Vol. 6B, Academic Press, London, pp. 285–318.

Fry, J. C. & Zia, T. (1982). A method for estimating the viability of aquatic bacteria by slide culture, *J. Appl. Bacteriol.*, **53**, 189–98.

Garden, R. W. (1984). *Modern Logic and Quantum Mechanics*, Adam Hilger, Bristol.

Gates, M. A. (1983). Trajectories of cell volume distributions during the growth cycle of *Tetrahymena*, *J. Gen. Microbiol.*, **129**, 895–900.

Geddes, L. A. (1972). *Electrodes and the Measurement of Bioelectric Events*, Wiley Interscience, New York.

Geddes, L. A. & Baker, L. E. (1968). *Principles of Applied Biomedical Instrumentation*, Wiley Interscience, New York.

Gehron, M. J. & White, D. C. (1983). Sensitive assay of phospholipid glycerol in environmental samples, *J. Microbiol. Meth.*, **1**, 23–32.

Geldreich, E. E. & Kennedy, H. (1982). The cost of microbial monitoring. In: *Bacterial Indicators of Pollution* (Ed. W. O. Pipes), CRC Press, Boca Raton, Florida, pp. 131–47.

Gibson, S. (1982). An automated approach to clinical microbiology—The automicrobic system. In: *Proc. 3rd Int. Symp. Rapid Methods and Automation in Microbiology* (Ed. R. C. Tilton), American Society for Microbiology, Washington, pp. 80–2.

Gielen, F. L. H. & Bergveld, P. (1982). Comparison of electrode impedances of Pt, Pt–Ir (10%) and Ir/AIROF electrodes used in electrophysiological experiments, *Med. Biol. Eng. Comput.*, **20**, 77–83.

Gnan, S. & Luedecke, L. O. (1982). Impedance methods in raw milk as an alternative to the standard plate count, *J. Food Prot.*, **45**, 4–7.

Goldschmidt, M. & Wheeler, T. C. (1975). Determination of bacterial cell concentration in urine specimens by electrical measurements. In: *Microbiology 1975* (Ed. D. Schlessinger), American Society for Microbiology, Washington, pp. 6–11.

Green, R. G. & Larson, W. P. (1922). Conductivity of bacterial cells, *J. Infect. Dis.*, **30**, 550–8.

Grover, N. B., Naaman, J., Ben-Sasson, S. & Doljaniski, F. (1969*a*). Electrical sizing of particles in suspension, I. Theory, *Biophys. J.*, **9**, 1398–414.

Grover, N. B., Naaman, J., Ben-Sasson, S. & Doljaniski, F. (1969*b*). Electrical sizing of particles in suspension, II. Experiments with rigid spheres, *Biophys. J.*, **9**, 1415–25.

Grover, N. B., Naaman, J., Ben-Sasson, S. & Doljaniski, F. (1972). Electrical sizing of particles in suspension, III. Rigid spheroids and red blood cells, *Biophys. J.*, **12**, 1099–117.

Grover, N. B., Ben-Sasson, S. A. & Naaman, J. (1982). Electrical sizing of cells in suspension. In: *Cell Analysis*, Vol. 1 (Ed. N. Catsimpoolas), Plenum Press, New York, pp. 93–109.

Hadley, W. K. (1976). The use of electrical impedance measurements for the detection of microorganisms in blood cultures. In: *Proc. 2nd Int. Symp. Rapid Methods and Automation in Microbiology* (Ed. H. H. Johnston and S. W. B. Newsom), Learned Information (Europe) Ltd, Oxford, p. 14.

Hadley, W. K. & Senyk, G. (1975). Early detection of microbial metabolism and growth by measurements of electrical impedance. In: *Microbiology 1975* (Ed. D. Schlessinger), American Society for Microbiology, Washington, pp. 12–21.

Hamilton, W. A. & Sale, A. J. H. (1967). Effects of high frequency electric fields on microorganisms, *Biochim. Biophys. Acta*, **148**, 781–8.

Hancher, C. W., Thacker, L. H. & Phares, E. F. (1974). A fibre-optic retro-reflective turbidimeter for continuously monitoring cell concentration during fermentation, *Biotechnol. Bioeng.*, **16**, 475–84.

Hardy, D., Kraeger, S., Dufour, S. & Cady, P. (1977). Rapid detection of microbial contamination in frozen vegetables by automated impedance measurements, *Appl. Env. Microbiol.*, **34**, 14–17.

Harris, C. M. & Kell, D. B. (1983). The radio-frequency dielectric properties of yeast cells measured with a rapid, automated, frequency-domain dielectric spectrometer, *Bioelectrochem. Bioenerg.*, **11**, 15–28.

Harris, C. M., Hitchens, G. D. & Kell, D. B. (1984). Dielectric spectroscopy of microbial membrane systems. In: *Charge and Field Effects in Biosystems* (Ed. M. J. Allen and P. N. R. Usherwood), Abacus Press, Tunbridge Wells, pp. 179–85.

Harrison, J. (1976). Rapid detection of microorganisms. In: *Proc. 2nd Int. Symp. Rapid Methods and Automation in Microbiology* (Ed. H. H. Johnston and S. W. B. Newsom), Learned Information (Europe) Ltd, Oxford, pp. 101–2.

Harvey, B. R., Eden, G. E. & Mitchell, N. T. (1963). Neutron scattering: A technique for the direct determination of the amount of biological film in a percolating filter, *J. Inst. Sew. Purif.*, 495–506.

Hasyn, J. J., Cundy, K. R., Dietz, C. C. & Wong, W. (1981). Clinical laboratory evaluation of the automicrobic system for rapid identification of Enterobacteriaceae, *J. Clin. Microbiol.*, **13**, 491–7.

Hause, L. L., Komorowski, R. A. & Gayon, F. (1981). Electrode and electrolyte impedance in the detection of bacterial growth, *IEEE Trans. Biomed. Eng.*, **28**, 403–10.

Heden, C.-G. & Illeni, T. (Eds) (1975). *New Approaches to the Identification of Microorganisms*, Wiley, Chichester.

Herbert, D. (1961). The chemical composition of microorganisms as a function of their environment, Symp. Society for General Microbiology, **11**, 391–416.

Heslop, J. (1980). Automation in microbiology, *Clin. Lab. Products*, November issue, 1–10.

Hobbie, J. E. & Watson, S. W. (1980). Comparison of direct count and LPS estimations of bacterial biomass, Abstr. 2nd Int. Symp. Microbial Ecol., Warwick, pp. 175–6.

Hobbie, J. E., Daley, R. J. & Jasper, S. (1977). Use of nuclepore filters for counting bacteria by fluorescence microscopy, *Appl. Env. Microbiol.*, **33**, 1225–8.

Hobson, P. N. & Mann, S. O. (1970). *Applications of the Coulter Counter in Microbiology. Automation, Mechanisation and Data Handling in Microbiology*, Soc. Appl. Bacteriol. Technical Series no. 4 (Ed. A. Baillie and R. J. Gilbert), Academic Press, London, pp. 91–105.

Holland, R. L., Cooper, B. H., Helgeson, N. G. P. & McCracken, A. W. (1980). Automated detection of microbial growth in blood cultures by using stainless-steel electrodes, *J. Clin. Microbiol.*, **12**, 180–4.

Hulsheger, H. & Niemann, E. G. (1980). Lethal effects of high voltage pulses on *E. coli* K12. *Radiat. Env. Biophys.*, **18**, 281–8.

Hulsheger, H. & Niemann, E. G. (1981). Killing of bacteria with electrical pulses of high field strength. *Radiat. Env. Biophys.*, **20**, 53–65.

Isenberg, H. D. & MacLowry, J. D. (1976). Automated methods and data handling in bacteriology, *Ann. Rev. Microbiol.*, **30**, 483–505.

Ishikawa, Y. & Shoda, M. (1983). Calorimetric analysis of *E.coli* in continuous culture, *Biotechnol. Bioeng.*, **25**, 1817–27.

Ishimori, Y., Karube, I. & Suzuki, S. (1981). Determination of microbial populations with piezoelectric membranes, *Appl. Env. Microbiol.*, **42**, 632–7.

Jason, A. C. (1983). A deterministic model for monophasic growth of batch cultures of bacteria, *Antonie van Leeuwenhoek*, **49**, 513–36.

Jeltsch, A. & Zimmermann, U. (1979). Particles in a homogeneous electric field: A model for the dielectric breakdown of living cells in a Coulter Counter, *Bioelectrochem. Bioenerg.*, **6**, 349–84.

Jennings, B. R. & Morris, V. J. (1974). Light scattering by bacteria. II. Size and electrical properties of *E.coli*, *J. Coll. Interf. Sci.*, **49**, 89–97.

Johnston, H. H. & Newsom, S. W. (Eds) (1976). *Proc. 2nd Int. Symp. Rapid Methods and Automation in Microbiology*, Learned Information (Europe) Ltd, Oxford.

Jones, J. G. (1979). *A Guide to Methods for Estimating Microbial Numbers and Biomass in Fresh Water*, Freshwater Biol. Assoc., Scientific Publication 39, Kendal.

Junter, G.-A. & Selegny, E. (1982*a*). Evolution with time of the zero-current potential of a gold electrode in *E.coli* cultures supplied with lipoic acid, Part I: Mathematical modeling and computer simulation, *Bioelectrochem. Bioenerg.*, **141**, 679–97.

Junter, G.-A. & Selegny, E. (1982*b*). Evolution with time of the zero-current potential of a gold electrode in *E.coli* cultures supplied with lipoic acid, Part II: Properties of the model and their application to investigations on drug effects on bacterial activity, *Bioelectrochem. Bioenerg.*, **141**, 699–709.

Junter, G.-A., Lemeland, J.-F. & Selegny, E. (1980). Electrochemical detection and counting of *E.coli* in the presence of a reducible coenzyme, lipoic acid, *Appl. Env. Microbiol.*, **39**, 307–16.

Junter, G.-A., Bogaczyk, M. C., Lemeland, J.-F. & Selegny, E. (1984). Potentiometric characterisation and monitoring of antibiotic effects on bacterial activity. In: *Charge and Field Effects in Biosystems* (Ed. M. J. Allen and P. N. R. Usherwood), Abacus, Tunbridge Wells, pp. 195–200.

Kagan, R. L., Schuette, W. & Zierdt, C. H. (1977). Rapid and automated diagnosis of bacteremia by impedance detection, *J. Clin. Microbiol.*, **5**, 51–7.

Kahn, W., Friedman, G., Rodriguez, W., Coutroni, C. & Ross, S. (1976). Rapid detection and isolation of bacteria in blood and cerebrospinal fluids in children by the electrical impedance methods. In: *Proc. 2nd Int. Symp. on Rapid Methods and Automation in Microbiology* (Ed. H. H. Johnston and S. W. B. Newsom), Learned Information (Europe) Ltd, Oxford, pp. 14–15.

Karl, D. M. (1980). Cellular nucleotide measurements and applications in microbial ecology, *Microbiol. Rev.*, **44**, 739–96.

Karl, D. M. & Holm-Hansen, O. (1978). Methodology and measurement of adenylate energy charge ratios in environmental samples, *Marine Biol.*, **48**, 185–97.

Kass, E. H. (1957). Bacteriuria and the diagnosis of infections of the urinary tract, *Arch. Int. Med.*, **100**, 709–14.

Keilmann, F., Bohme, D. & Santo, L. (1980). Multichannel photometer–nephelometer, *Appl. Env. Microbiol.*, **40**, 458–61.

Kell, D. B. (1980). The role of ion-selective electrodes in improving fermentation yields, *Process. Biochem.*, **15**, 18–23, 29.

Kell, D. B. (1983). Dielectric properties of bacterial chromatophores, *Bioelectrochem. Bioenerg.*, **11**, 405–15.

Kell, D. B. (1984). Dielectric Spectroscopy of the Rotational and Translational Motions of Membrane Proteins: Theory and Experiment, *EBEC Reports*, **3**, 645–6.

King, J. D. & White, D. C. (1977). Muramic acid as a measure of microbial biomass in estuarine and marine samples, *Appl. Env. Microbiol.*, **33**, 777–83.

Kjaergaard, L. (1977). The redox potential: its use and control in biotechnology, *Adv. Biochem. Eng.*, **7**, 131–50.

Knight, V. A., Wiggins, P. M., Harvey, J. D. & O'Brien, J. A. (1981). The relationship between the size of mitochondria and the intensity of light that they scatter in different energetic states, *Biochim. Biophys. Acta*, **637**, 146–51.

Knowles, C. J. (1977). Microbial metabolic regulation by adenine nucleotide pools, *Symp. Soc. Gen. Microbiol.*, **27**, 241–83.

Koch, A. L. (1961). Some calculations on the turbidity of mitochondria and bacteria, *Biochim. Biophys. Acta*, **51**, 429–41.

Koliander, B., Hampel, W. & Roehr, M. (1984). Indirect estimation of biomass by rapid ribonucleic acid determination, *Appl. Microbiol. Biotechnol.*, **19**, 272–6.

Konings, W. N. & Veldkamp, H. (1980). Phenotypic responses to environmental change. In: *Contemporary Microbial Ecology* (Ed. D. C. Ellwood, J. N. Hedger, M. J. Latham, J. M. Lynch and J. H. Slater), Academic Press, London, pp. 161–91.

Kramer, J. M., Kendall, M. & Gilbert, R. J. (1979). Evaluation of the spiral plate and laser colony counting techniques for the enumeration of bacteria in food, *Eur. J. Appl. Microbiol. Biotechnol.*, **6**, 289–99.

Kubitschek, H. E. (1969). Counting and sizing microorganisms with the Coulter Counter. In: *Methods in Microbiology*, Vol. 1 (Ed. J. R. Norris and D. W. Ribbons), Academic Press, London, pp. 593–610.

Kull, F. C. & Cuatrecasas, P. (1983). Estimation of cell number by neutral red content. Applications for proliferative and survival assays, *Appl. Biochem. Biotechnol.*, **8**, 97–103.

Larsson, K., Weibull, C. & Cronberg, G. (1978). Comparison of light and electron microscope determinations of the number of bacteria and algae in lake water, *Appl. Env. Microbiol.*, **35**, 397–404.

Leach, F. R. (1984). Biochemical indicators of groundwater pollution. In: *Groundwater Pollution Microbiology* (Ed. G. Bitton and C. P. Gerba), Wiley, London, pp. 303–51.

LeChevallier, M., Cameron, S. & McFeters, G. (1983). Comparison of verification procedures for the membrane filter total coliform technique, *Appl. Env. Microbiol.*, **45**, 1126–8.

Levin, G. V. (1963). Rapid microbiological determinations with radioisotopes, *Adv. Appl. Microbiol.*, **5**, 95–133.

Lloyd, D. & Scott, R. I. (1983). Direct measurement of dissolved gases in microbiological systems using membrane inlet mass spectrometry, *J. Microbiol. Methods*, **1**, 313–28.

Lloyd, D., Scott, R. I. & Williams, T. N. (1983). Membrane inlet mass spectroscopy-measurements of dissolved gas in fermentation liquids, *Trends Biotechnol.*, **1**, 60–3.

Lovgren, T., Peacock, R., Lavi, J., Karp, M. & Raunio, R. (1982). The bioluminescent assay of NADH and NADPH, *Int. Lab.*, **12**, 58–67.

Luscombe, B. M. & Gray, T. R. (1971). Effect of varying growth rate on the morphology of *Arthrobacter*, *J. Gen. Microbiol.*, **69**, 433–4.

Mallette, M. F. (1969). Evaluation of growth by physical and chemical means. In: *Methods in Microbiology*, Vol. 1 (Ed. J. R. Norris and D. W. Ribbons), Academic Press, London, pp. 521–66.

Marais, P. G. & Smit, W. B. De V. (1960). Laboratory calibration of the neutron moisture meter, *South Afr. J. Agr. Sci.*, **3**, 581–601.

Marten, J. (1972). Automatic and continuous assessment of fermentation parameters. In: *Methods in Microbiology*, Vol. 6B (Ed. J. R. Norris and D. W. Ribbons), Academic Press, London, pp. 319–30.

Martins, B. B., Hodapp, S., Dufour, S. W. & Kraeger, S. J. (1982). Evaluation of a rapid impedimetric method for determining the keeping quality of milk, *J. Food Prot.*, **45**, 1221–6.

Martins, S. B. & Selby, S. J. (1980). Evaluation of a rapid method for the quantitative estimation of coliforms in meat by impedimetric procedures, *Appl. Env. Microbiol.*, **39**, 518–24.

Martz, R. F., Sebacher, D. I. & White, D. C. (1983). Biomass measurements of methane forming bacteria in environmental samples, *J. Microbiol. Meth.*, **1**, 53–61.

Matsunaga, T., Karube, I. & Suzuki, S. (1979). Electrode system for the determination of microbial populations, *Appl. Env. Microbiol.*, **37**, 117–21.
Matsunaga, T., Karube, I. & Suzuki, S. (1980). Electrochemical determinations of cell populations, *Eur. J. Appl. Microbiol. Biotechnol.*, **10**, 125–32.
Matsushita, T., Brendzel, A., Shotola, M. & Groh, K. (1982). Electrical determination of viability in saline treated mouse myeloma cells, *Biophys. J.*, **39**, 41–7.
Miller, C. A. & Vogelhut, P. O. (1978). Chemiluminescent detection of bacteria: experimental and theoretical limits, *Appl. Env. Microbiol.*, **35**, 813–16.
Mitchell, R. (1974). *Introduction to Environmental Biology*, Prentice-Hall Series in Environmental Sciences, Englewood Cliffs, NJ, p. 84.
Mitruka, B. (1976). *Methods of Detection and Identification of Bacteria*, CRC Press Inc., Boca Raton, Florida.
Monod, J. (1949). The growth of bacterial cultures, *Ann. Rev. Microbiol.*, **3**, 371–94.
Mor, J. R. (1976). Flow microcalorimetry in microbiology. In: *Proc. 2nd Int. Symp. Rapid Methods and Automation in Microbiology* (Ed. H. H. Johnston and S. W. B. Newsom), Learned Information (Europe) Ltd, Oxford, p. 7.
Moriarty, D. J. W. (1980). Measurement of bacterial biomass using muramic acid, Abstr. 2nd Int. Symp. Microbial Ecology, Warwick, p. 177.
Morris, J. G. (1974). *A Biologist's Physical Chemistry*, 2nd Ed., Edward Arnold, London.
Morrison, G. A. & Tomkins, A. L. (1973). Determination of mean cell size of *Tetrahymena* in growing cultures, *J. Gen. Microbiol.*, **77**, 383–92.
Munoz, E. & Silverman, M. (1979). Rapid, single step most-probable-number method for enumerating fecal coliforms in effluents from sewage treatment, *Appl. Env. Microbiol.*, **37**, 527–30.
Nestaas, E. & Wang, D. I. C. (1981). A new sensor, the 'filtration probe' for quantitative characterisation of the penicillin fermentation. I: Mycelial morphology and culture activity, *Biotechnol. Bioeng.*, **23**, 2803–13.
Nestaas, E. & Wang, D. I. C. (1983*a*). A new sensor, the 'filtration probe' for quantitative characterisation of the penicillin fermentation. III: An automatically operating probe, *Biotechnol. Bioeng.*, **25**, 1981–7.
Nestaas, E. & Wang, D. I. C. (1983*b*). Computer control of the penicillin fermentation using the filtration probe in conjunction with a structured process model, *Biotechnol. Bioeng.*, **223**, 781–96.
Nestaas, E., Wang, D. I. C., Suzuki, H. & Evans, L. (1981). A new sensor, the 'filtration probe' for quantitative characterisation of the penicillin fermentation. II: The monitoring of mycelial growth, *Biotechnol. Bioeng.*, **23**, 2815–24.
Neufeld, H. A., Conklin, J. & Towner, R. D. (1965). Chemiluminescence of luminol in the presence of hematim compounds, *Anal. Biochem.*, **12**, 303–9.
Neufeld, R. J. & Zajic, J. E. (1982). A technique for monitoring biomass levels and cell adhesion in hexadecane fermentations, *Biotechnol. Lett.*, **4**, 701–4.
Newman, J. & O'Brien, R. T. (1975). Gas chromatographic presumptive test for

coliform bacteria in water, *Appl. Env. Microbiol.*, **30**, 584–8.
Nishikawa, S., Sakai, S., Karube, I., Matsunaga, T. & Suzuki, S. (1982). Dye-coupled electrode system for the rapid determination of cell populations in polluted water, *Appl. Env. Microbiol.*, **43**, 814–18.
Norris, J. R. & Swain, H. (1971). Staining bacteria. In: *Methods in Microbiology*, Vol. 5A (Ed. J. R. Norris and D. W. Ribbons), Academic Press, London, pp. 105–34.
Ohashi, M., Watabe, T., Ishikawa, T. & Watanabe, Y. (1979). Sensors and instrumentation: Steam-sterilisable dissolved oxygen sensor and cell mass sensor for on-line fermentation system control, *Biotechnol. Bioeng. Symp.*, **9**, 103–16.
Oleniacz, W. S., Pisano, M. A., Rosenfeld, M. H. & Elgart, R. L. (1968). Chemiluminescent method for detecting microorganisms in water, *Env. Sci. Technol.*, **2**, 1030–3.
O'Toole, D. K. (1983*a*). Methods for the direct and indirect assessment of the bacterial content of milk, *J. Appl. Bact.*, **55**, 187–201.
O'Toole, D. K. (1983*b*). Weighing technique for determining bacterial dry weight based on rate of moisture uptake, *Appl. Env. Microbiol.*, **46**, 506–8.
Padan, E., Zilberstein, D. & Schuldiner, S. (1981). pH homeostasis in bacteria, *Biochim. Biophys. Acta*, **650**, 151–66.
Park, S. H., Hong, K. T., Lee, J. H. & Bac, J. C. (1983). On-line estimation of cell growth for glutamic acid fermentation systems, *Eur. J. Appl. Microbiol. Biotechnol.*, **17**, 168–72.
Parley, C. R., Swartz, J. R. & Cooney, C. L. (1979). Measurement of cell mass concentration with a continuous flow viscometer, *Biotechnol. Bioeng.*, **21**, 519–23.
Pethig, R. (1979). *Dielectric and Electronic Properties of Biological Materials*, Wiley, Chichester.
Pettipher, G. L. (1983). *The Direct Epifluorescent Filter Technique for the Rapid Enumeration of Microorganisms*, Research Studies Press, Letchworth.
Pettipher, G. L. & Rodrigues, U. M. (1982). Semi-automated counting of bacteria and somatic cells in milk using epifluorescence microscopy and television image analysis, *J. Appl. Bacteriol.*, **53**, 323–9.
Pettipher, G. L., Mansell, R., McKinnon, C. H. & Cousins, C. M. (1980). Rapid membrane filtration–epifluorescence microscopy for direct enumeration of bacteria in raw milk, *Appl. Env. Microbiol.*, **39**, 423–9.
Picciolo, G. L., Chappelle, E. W., Thomas, R. R. & McGarry, M. A. (1977). Performance characteristics of a new photometer with a moving filter tape for luminescence assay, *Appl. Env. Microbiol.*, **34**, 720–4.
Picciolo, G. L., Deming, J. W., Nibley, D. A. & Chappelle, E. W. (1978). Characteristics of commercial instruments and reagents for luminescent assays, *Meth. Enzymol.*, **57**, 550–9.
Pirt, S. J. (1975). *Principles of Microbe and Cell Cultivation*, Blackwell, Oxford.
Postgate, J. R. (1969). Viable counts and viability. In: *Methods in Microbiology*, Vol. 1 (Ed. J. R. Norris and D. W. Ribbons), Academic Press, London, pp. 611–28.

Postgate, J. R. (1976). Death in macrobes and microbes, *Sym. Soc. Gen. Microbiol.*, **26**, 1–18.

Powell, E. O. (1963). Photometric methods in bacteriology, *J. Sci. Agri.*, **14**, 1–8.

Powell, E. O. & Stoward, P. J. (1962). A photometric method for following changes in length of bacteria, *J. Gen. Microbiol.*, **27**, 489–500.

Primas, H. (1981). *Chemistry, Quantum Mechanics and Reductionism*, Springer-Verlag, Heidelberg.

Pusey, R. N. & Tough, R. J. A. (1982). Dynamic light scattering, a probe of Brownian particle dynamics, *Adv. Coll. Interf. Sci.*, **16**, 143–59.

Quesnel, L. B. (1971). Microscopy and micrometry. In: *Methods in Microbiology*, Vol. 5A (Ed. J. R. Norris and D. W. Ribbons), Academic Press, London, pp. 2–103.

Reed, G. (1982). Microbial biomass. Single cell protein and other microbial products. In: *Prescott and Dunn's Industrial Microbiology*, 4th Edn (Ed. G. Reed), Macmillan, London, pp. 541–92.

Ribbons, D. W. (1970). Growth relationships between growth media constituents and cellular yield and composition. In: *Methods in Microbiology*, Vol. 3A (Ed. J. R. Norris and D. W. Ribbons), Academic Press, London, pp. 297–304.

Richards, J. C. S., Jason, A. C., Hobbs, G., Gibson, D. M. & Christie, R. H. (1978). Electronic measurements of bacterial growth, *J. Phys. E: Sci. Instrum.*, **11**, 560–8.

Ricketts, T. R. & Rappitt, A. F. (1974). Determination of the volume and surface area of *Tetrahymena pyriformis* and their relationship to endocytosis, *J. Protozool.*, **21**, 549–51.

Roberts, D. McL. (1980). Aspects of cell size measurements in *Tetrahymena ellioti*, *J. Gen. Microbiol.*, **120**, 211–18.

Robinson, J. & Tiedge, J. (1983). Non-linear estimation of Monod growth kinetic parameters from a single substrate depletion curve, *Appl. Env. Microbiol.*, **45**, 1453–8.

Roels, J. A. (1983). *Energetics and Kinetics in Biotechnology*, Elsevier, Amsterdam.

Rosendal, S. and Valdivieso-Garcia, A. (1981). Enumeration of mycoplasmas after acridine orange staining, *Appl. Env. Microbiol.*, **41**, 1000–2.

Rowley, D. B., Brevite, J. J. & Srinivasa, H. (1976). A radiometric screening method for estimating the level of aerobic mesophilic bacteria in foods. In: *Proc. 2nd Int. Symp. on Rapid Methods and Automation in Microbiology* (Ed. H. H. Johnston and S. W. B. Newsom), Learned Information (Europe) Ltd, Oxford, p. 3.

Sagan, C. (1981). *Cosmos*, Macdonald, London, p. 122ff.

Schanne, O. F. & Ceretti, E. R. P. (1978). *Impedance Measurements in Biological Cells*, Wiley Interscience, New York.

Schwan, H. P. (1957). Electrical properties of tissue and cell suspensions, *Adv. Biol. Med. Phys.*, **5**, 147–208.

Schwan, H. P. (1963). Determination of biological impedances. In: *Physical*

Techniques in Biological Research, Vol. VIB (Ed. W. L. Nastuk), Academic Press, New York, pp. 323–407.

Schwan, H. P. (1966). Alternating current electrode polarisation, *Biophysik*, **3**, 181–201.

Schwan, H. P. (1968). Electrode polarisation impedance and measurements in biological materials, *Ann. New York Acad. Sci.*, **148**, 191–209.

Schwan, H. P. (1983). Dielectric properties of biological tissues and cells at ELF-frequencies. In: *Biological Effects and Dosimetry of Nonionizing Radiation* (Ed. M. Grandolfo, S. M. Michaelson and A. Rindi), Plenum, New York, pp. 549–59.

Schwarz, G. (1962). A theory of the low-frequency dielectric dispersion of colloidal particles in an electrolyte solution, *J. Phys. Chem.*, **66**, 2636–42.

Seydel, J. K. & Wempe, E. (1980). A simple, fast, and inexpensive kinetic method to differentiate between total and viable bacteria using the Coulter Counter technique, *Drug Research*, **30**, 298–301.

Sharpe, A. N., Woodrow, M. N. & Jackson, A. K. (1970). ATP levels in food contaminated by bacteria, *J. Appl. Bacteriol.*, **33**, 758–67.

Sharpe, A. N., Dyett, E. J., Jackson, A. K. & Kilsby, D. C. (1972). Technique and apparatus for rapid and inexpensive enumeration of bacteria, *Appl. Env. Microbiol.*, **24**, 4–7.

Sikyta, B. (1983). *Methods in Industrial Microbiology*, Ellis Horwood, Chichester, p. 300ff.

Silverman, M. & Munoz, E. (1979). Automated electrical impedance technique for rapid enumeration of fecal coliforms in effluents from sewage treatment plants, *Appl. Env. Microbiol.*, **37**, 521–6.

Simidu, U. & Kogure, K. (1980). Measurements of living and non-living marine bacteria using nalidixic acid, Abstr. 2nd Int. Symp. Microbial Ecology, Warwick, p. 176.

Sinclair, C. G. & Topiwala, H. H. (1970). Model for continuous culture which considers the viability concept, *Biotechnol. Bioeng.*, **12**, 1069–96.

Sluyters-Rehbach, M. & Sluyters, J. H. (1970). Sine wave methods for the study of electrode processes. In: *Electroanalytical Chemistry* (Ed. A. J. Bard), Marcel Dekker, New York, pp. 1–128.

Smith, D. E. (1966). A.C. polarography and related techniques; theory and practice. In: *Electroanalytical Chemistry, A Series of Advances*, Vol. I (Ed. A. J. Bard), Edward Arnold, London, pp. 1–155.

Smith, S. R. L. (1980). Single cell protein, *Phil. Trans. R. Soc. B.*, **290**, 341–54.

Solomon, B. O., Erickson, L. E. & Yang, S. S. (1983). Estimation of biomass concentration in the presence of solids for the purpose of parameter estimation, *Biotechnol. Bioeng.*, **25**, 2469–77.

Sonnenwirth, A. C. (1977). Preprototype of an automated microbial detection and identification system: A developmental investigation, *J. Clin. Microbiol.*, **6**, 400–5.

Specter, S., Throm, R. & Friedman, H. (1976). Screening for bacteriuria in the clinical microbiology laboratory by electrical impedance monitoring. In: *Proc. 2nd Int. Symp. Rapid Methods and Automation in Microbiology* (Ed.

H. H. Johnston and S. W. B. Newsom), Learned Information (Europe) Ltd, Oxford, pp. 13–14.

Starka, J. (1962). Cellular division and reproduction of bacteriophage in synchronised cultures of *E.coli*, *J. Gen. Microbiol.*, **29**, 83–90.

Stewart, G. N. (1899). The changes produced by the growth of bacteria in the molecular concentration and electrical conductivity of culture media, *J. Exp. Med.*, **4**, 235–43.

Stouthamer, A. H. (1979). The search for correlation between theoretical and experimental growth yields. In: *International Reviews of Biochemistry, Series 2, Microbial Biochemistry* (Ed. J. R. Quayle), University Park Press, Baltimore, pp. 1–47.

Strange, R. E. (1972). Rapid detection and assessment of sparse microbial populations, *Adv. Micr. Physiol.*, **8**, 105–41.

Sugino, A., Peebler, C. L., Kreuzer, K. N. & Cozzarelli, N. R. (1977). Mechanism of action of nalidixic acid: Purification of *Escherichia coli nalA* gene product and its relationship to DNA gyrase and a novel nicking–closing enzyme, *Proc. Natl. Acad. Sci.*, **74**, 4767–71.

Takano, M. & Tsuchido, T. (1982). Availability of growth delay analysis for the evaluation of total injury of stressed bacterial populations, *J. Ferment. Technol.*, **60**, 189–98.

Tempest, D. W., Neijssel, O. H. & Zevenboom, W. (1983). Effect of supply and limitation of a cell substrate on the size of bacteria and their growth rate. In: *Microbes in Their Natural Environments*, Symp. 34, Society for General Microbiology (Ed. J. H. Slater, R. Whittenbury and J. W. T. Wimpenny), Cambridge University Press, Cambridge, pp. 119–52.

Thore, A. (1979). Technical aspects of the bioluminescent firefly luciferase assay of ATP, *Sci. Tools*, **26**, 30–4.

Thore, A. (undated). Bioluminescence Assay, Extraction of ATP from Biological Samples. LKB Wallac.

Thore, A., Ansehn, A., Lundin, A. & Bergman, S. (1975). Detection of bacteriuria by luciferase assay of adenosine triphosphate, *J. Clin. Microbiol.*, **1**, 1–8.

Throm, R., Strauss, R., Specter, S. & Friedman, H. (1976). Automated blood culture testing using radio-isotope and electrical impedance monitoring equipment. In: *Proc. 2nd Int. Symp. Rapid Methods and Automation in Microbiology* (Ed. H. H. Johnston and S. W. B. Newsom), Learned Information (Europe) Ltd, Oxford, pp. 21–3.

Tilton, R. C. (Ed.) (1982*a*): *Rapid Methods and Automation in Microbiology*, American Society for Microbiology, Washington.

Tilton, R. C. (1982*b*). The laboratory approach to detection of bacteremia, *Ann. Rev. Microbiol.*, **36**, 475–93.

Titus, J. A., Luli, G. W., Dekleva, M. L. & Strohl, W. R. (1984). Applications of a microcomputer-based system to control and monitor bacterial growth, *Appl. Env. Microbiol.*, **47**, 239–44.

Topiwala, H. H. (1973). Mathematical modeling in microbiology. In: *Methods in Microbiology*, Vol. 8 (Ed. J. R. Norris and D. W. Ribbons), Academic Press, London, pp. 35–59.

Trent, J. A., Chastain, R. A. & Yayanos, A. A. (1984). Possible artefactual basis for apparent bacterial growth at 250°C, *Nature*, **307**, 737–40.

Trinel, P. A., Hanoune, N. & Leclerc, H. (1980). Automation of water bacteriological analysis: Running test of an experimental prototype, *Appl. Env., Microbiol.*, **39**(5), 976–82.

Turner, A. P. F., Ramsay, G. & Higgins, I. J. (1983). Applications of electron transfer between biological systems and electrodes, *Biochem. Soc. Trans.*, **11**, 445–8.

Ur, A. (1976). Prospects and limitations of the impedance technique. In: *Proc. 2nd Int. Symp. Rapid Methods and Automation in Microbiology* (Ed. H. H. Johnston and S. W. B. Newsom), Learned Information (Europe) Ltd, Oxford, pp. 245–7.

Ur, A. & Brown, D. F. J. (1975). Monitoring of bacterial activity by impedance measurements. In: *New Approaches to the Identification of Bacteria* (Ed. C.-G. Heden and T. Illeni), Wiley, Chichester, pp. 61–71.

Wang, H. Y., Cooney, C. L. & Daniel, I. (1979). Computer control of bakers' yeast production, *Biotechnol. Bioeng.*, **21**, 975–95.

Warren, L. S., Benoit, R. E. & Jessee, J. A. (1978). Rapid enumeration of fecal coliforms in water by a colorimetric β-galactosidase assay, *Appl. Env. Microbiol.*, **35**, 136–41.

Washington, J. A. (1981). Rapid methods for detection of microorganisms in septicemia and meningitis. In: *Proc. 3rd Int. Syp. Rapid Methods and Automation in Microbiology* (Ed. R. C. Tilton), American Society for Microbiology, Washington, pp. 33–5.

Washington, J. A. (II) & Yu, P. K. W. (1971). Radiometric method for the detection of bacteremia, *Appl. Env. Microbiol.*, **22**, 100–1.

Waters, J. (1972). Sensitivity of the $^{14}CO_2$ radiometric method for bacterial detection, *Appl. Microbiol.*, **23**, 198–9.

Watson, L. (1974). *The Romeo Error*, Hodder & Stoughton, London.

Watson, S. W., Novitsky, T. J., Quinby, H. L. & Valois, F. W. (1977). Determination of bacterial numbers and biomass in marine environments, *Appl. Env. Microbiol.*, **33**, 940–6.

Welch, G. R. & Kell, D. B. (1985). Not just catalysts. The bioenergetics of molecular machines. In: *The Fluctuating Enzyme* (Ed. G. R. Welch), Wiley, Chichester, in press.

Wheeler, J. A. & Zurek, W. H. (1983). *Quantum Theory and Measurement*, Princeton University Press, Princeton.

Wheeler, T. G. & Goldschmidt, M. (1975). Determination of bacterial cell concentrations by electrical measurements, *J. Clin. Microbiol.*, **1**, 25–9.

White, D. C. (1980). Lipid extracts as a measurement of biomass and community structure of detrital, benthic and biofouling assemblies, Abstr. 2nd Int. Symp. Microbial Ecology, Warwick, p. 77.

White, D. C. (1983). Analysis of microorganisms in terms of quantity and activity in natural environments. In: *Microbes in Their Natural Environments*, Society for General Microbiology, Symp. 34. (Ed. J. H. Slater, R. Whittenbury and J. W. T. Wimpenny), Cambridge University Press, Cambridge, pp. 37–66.

Wilkins, J. R. (1974). Pressure transducer method for measuring gas production by microorganisms, *Appl. Microbiol.*, **27**, 135–40.
Wilkins, J. R. (1979). Use of Pt electrodes for the electrochemical detection of bacteria, *Appl. Env. Microbiol.*, **36**, 683–7.
Wilkins, J. R., Stoner, G. & Boykin, E. (1974). Microbial detection method based on sensing molecular hydrogen, *Appl. Env. Microbiol.*, **27**, 949–52.
Wilkins, J. R., Young, R. & Boykin, E. (1978). Multichannel electrochemical microbial detection unit, *Appl. Env. Microbiol.*, **35**, 214–15.
Wilkins, J. R., Grana, D. & Fox, S. (1980). Combined membrane filtration–electrochemical microbial detection method, *Appl. Env. Microbiol.*, **40**, 852–3.
Wilson, M. C. & Harvey, J. D. (1983). Twin-beam laser velocimeter for the investigation of spermatozoa mobility, *Biophys. J.*, **41**, 13–21.
Wingard, L. B., Shaw, C. H. & Castner, J. F. (1982). Bioelectrochemical fuel cells, *Enz. Microb. Technol.*, **4**, 137–42.
Woolford, M. W. and Harvey, J. D. (1982). Light scattering studies of bull spermatozoa. II Interaction and concentration effects, *Biophys. J.*, **40**, 7–16.
Wyatt, P. (1973). Differential light scattering techniques for microbiology. In: *Methods in Microbiology*, Vol. 8 (Ed. J. R. Norris and D. W. Ribbons), Academic Press, London, pp. 183–263.
Zabriskie, D. W. & Humphrey, A. E. (1978). Estimation of fermentation biomass concentration by measuring culture fluorescence, *Appl. Env. Microbiol.*, **35**, 337–43.
Zabriskie, D. W., Armiger, W. B. & Humphrey, A. E. (1976). *Computer Applications in Fermentation Technology*, GBF Monograph Series, No. 3, Verlag Chemie, Weinheim, pp. 1–15.
Zafari, Y. & Martin, W. (1977). Comparison of bactometer microbial monitoring system with conventional methods for detection of microorganisms in urine specimens, *J. Clin. Microbiol.*, **5**, 545–7.
Zimmermann, R. (1980). Microscopical determination of microbial biomass, Abstr. 2nd Int. Symp. Microbial Ecology, Warwick, p. 175.
Zimmermann, U. (1982). Electric field-mediated fusion and related electrical phenomena, *Biochim. Biophys. Acta*, **694**, 227–77.
Zimmermann, U., Schulz, J. & Pilwat, G. (1973). Transcellular ion flow in *E.coli* and electrical sizing in bacteria, *Biophys. J.*, **13**, 1005–12.
Zimmermann, U., Groves, M., Schnabl, H. & Pilwat, G. (1980). Development of a new Coulter Counter system: Measurement of the volume, internal conductivity and dielectric breakdown of a single guard cell protoplast of *Vicia faba*, *J. Membrane Biol.*, **52**, 37–50.
Zimmermann, U., Scheurich, R., Pilwat, G. & Benz, R. (1981). Cells with manipulated functions: New perspectives for cell biology, medicine and technology, *Angew. Chem. Int. Ed.*, **20**, 325–44.
Zoond, A. (1927). The interpretation of changes in the electrical resistance accompanying the death of bacterial cells, *J. Bacteriol.*, **14**, 279–86.