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The death and lysis of microorganisms in environmental processes

(Death; lysis; survival; starvation; maintenance; 'cryptic' growth; environment)

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1. INTRODUCTION

One of the major philosophical stumbling blocks in microbiology relates to the question of ageing. For most macrobes, the processes of birth, growth and death are tangible, observable events. The same cannot be said for the majority of microbes. Exceptions such as some filamentous microbes (e.g., *Sphaerotilus* spp.), some budding bacteria such as *Hyphomicrobium* spp. and yeasts which show effects of ageing by bud scars are well known. However, the question of the ultimate destiny of a particular microbe present at any instantaneous moment in time has as yet to be answered.

Very few papers exist that deal directly with microbial death, although a large amount of circumstantial information is available. Understanding more about the processes of ageing, death and lysis in microbes has relevance in the following areas: (1) Public health sector. Efficient testing for the presence of and mechanisms for the destruction of pathogenic organisms in food/feed and water for human and animal consumption. (2) Biological industries sector. Maximising the percentage of active strains with respect to total numbers of microbes, manipulating culture conditions to affect desired optimal system stoichiometry while maintaining activity and preventing process inhibition. (3) Medical sector. Efficacy of antimicrobial agents and qualitative disease assessment. In order to deal with such problems, an understanding of the intrinsic physiological mechanisms involved in (a) induced lysis, (b) autolysis (non-induced lysis), (c) death, (d) resistance, (e) dormancy and (f) survival is necessary.

This review will look at certain aspects of the quantification of microbial death and lysis in terms of both the methods available to investigate the phenomena and the physiological means of deferring them, and look at changes which may occur as a result of death or lysis within a population, that may enhance the survival pattern of the remaining microbes.

2. PHYSIOLOGICAL CLASSIFICATION OF A MICROBIAL CULTURE

2.1. Definitions

A culture of microbes, either in the laboratory or in their natural environment, is composed of various morphological, biochemical and physiological groups. The existence of monocultures is very rare in natural environments, even under conditions where the environment requires specialised forms such as in some thermophilic and/or

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acidogenic environments. Biochemical variance arises from the efficient interaction of different microbes in either a food or an energy network, such as in the reduction of various chemical species depending on the redox potential of the environment [1]. Microorganisms have been classified on a physiological basis as: (a) dead microbes; (b) non-viable, active microbes; (c) dormant microbes; (d) viable, active microbes.

2.1.1. Dead microbes

Strictly defined, these are organisms totally devoid of metabolic activity, but still possessing a cell wall [2]. Everything failing to meet this description must not and cannot be dead. Definitions based on the inability to reproduce [3] are false and lead to embarrassing and dangerous misinterpretations of data. Experimentally, it is almost impossible to determine quantitatively the existence of such cells. Consequently, their presence has to be inferred retrospectively from estimates of the total cell numbers and numbers of cells falling into the other categories listed above [4] for which more precise methods of quantification have been developed.

This problem has been compounded by the wide range of different methods available for quantifying microbes. This issue will be addressed in a subsequent section in this review. Dead cells have been treated as inert solids in the consideration of particulate degradation in continuous culture systems [5], although they are effectively biodegradable particulates. As such, dead cells may constitute a very large fraction of the total biomass present in trickling filters and activated sludge wastewater treatment systems, in cell recycle processes, (i.e., ethanol production) and in semi-continuous (fill and draw) fermentations where a significant portion of the biomass is retained as inoculum for each new process cycle.

2.1.2. Non-viable microbes

These are organisms which have lost the ability to reproduce. These result from genetic defects such as absence of a critical enzyme necessary for replication, or lethal breaks in the DNA of the microbe. However, such microbes can carry out substrate transformations when they possess appropriate enzymes. For example, at superoptimal growth temperatures it has been shown that substrate energy dissipation can be mediated by non-viable cells [6]. The use of non-viable microbial enzymatic conversion is the basis for immobilised whole cell biocatalysts [7].

2.1.3. Dormant microbes

These can be subdivided into two categories: (1) Spores; (2) temporarily inactive or resting microbes. These two groups differ in that spores are morphologically differentiated structures. Dormant microbes are found in a wide range of environments and may represent the largest class of naturally occurring physiological phenotypes. However, they are frequently confused with non-viable cells, due to the difficulty of promoting their growth using artificial laboratory stimuli. These two types of dormant cells also differ from one another functionally, spores serve distribution and survival functions, whilst resting microbes are only intermediates leading either to active microbes or to death or lysis [8].

2.1.4. Active microbes

These are cells which can actively assimilate substrate, increase in mass and replicate. Active microbes are most commonly found in laboratory environments, although they are also frequently the major physiological class found in industrial and technical microbial processes. More information is available on this category than on any other.

2.2. Analytical methods for physiological differentiation

The ability to grow and multiply is very often the only criterion used in differentiating between the groups mentioned above. Most working definitions for the various types of microbes are restricted by the lack of accurate methods with a sound theoretical basis, together with a fanatical adherence to historically proven inaccurate methods. Use of arcane analytical methods has been perpetuated by public health authorities, despite a wealth of evidence as to the limitations and dangers of such techniques. Some of the current

methods for assessing cell numbers and activities are discussed in the following section. For more details on methods, the reader should consult specialised reviews [9–13].

2.2.1. Cell cultivation methods

The use of agar as a solidifying agent in culture media was first proposed in 1881 [14]. Since then, its use has escalated, and today it represents the most universal microbiological technique for the cultivation of microbes [15]. Theoretically the numbers of microbes in a sample can be derived from the numbers of colonies growing on the surface of an agar solidified medium in a Petri dish [16], although it was originally intended that the use of such media should be for the isolation and growth characterisation of microbes. Despite the repeated and often vehement criticism which has appeared in the literature [15–19], agar colony counts continue to be used extensively for the enumeration of microbes for estimation of survival [20–23], in stress studies [24–26], and perhaps most surprisingly in public health [27–30], even though alternative accurate techniques are available [31].

It is now universally accepted that in most natural microbial environments only a very small fraction of the microbes present can be enumerated using the agar plate technique [18,32–34]. Estimates of this error in the literature vary depending on the environment. In soil for example, it has been claimed that only 1–10% of the true number of viable organisms are enumerated [35], whilst in seawater values below 0.1% are considered normal [33,36]. In the testing of water supplies, as many as 90% of the coliforms, the bacteria used to indicate the presence of pathogens, may not be enumerated [37].

Some of the problems associated with agar plate counting of microbes include the lack of a single universal medium which will allow growth of all organisms [38], since most organisms are sensitive to the type of medium used [17,39–43]. In the literature 'improved' media for the growth of microorganisms are frequently reported, suggesting that existing compositions are inadequate. This process of new medium formulation is never-ending, and although improvements are continually being made, an acceptable status will never be reached. The technique has to be redefined for use only under those circumstances for which it is suitable. Despite these criticisms it should also be noted that if the method is used, interpretation must embrace all of the limitations of the method, if the results obtained in routine screening work are to be valid.

Failure to grow on an agar surface has been ascribed to environmental fastidiousness, dormancy [21], inhibition by neighbouring cells [16], physicochemical differences between the laboratory and natural environment [38], clumping [9] and to the fact that stressed and injured cells may have some difficulty in reproduction [44] since sublethal damage and inactivity are not distinguishable [21,45]. Further comment on the widespread misuse of agar-based cultivation media is superfluous, and the reader may consult the reviews of Buck [17] and Fry [18] for more detailed discussion.

2.2.2. Activity measurements

Metabolic activity, as measured by the rate of oxygen consumption, has been widely used in microbial ecology for the assessment of microbial activities in different environments [46]. Electron transport system (ETS) activity measurements are usually used as a guide to metabolic activity, due to their relative simplicity. The ETS is mediated by the action of several dehydrogenase enzymes such as succinate dehydrogenase, and direct measurement of dehydrogenase enzyme activity is assumed to be a reliable indication of ETS activity in a specific environment [47,48]. A large proportion of the total metabolic activity has been shown to be linked to ETS activity [49]. The most frequently encountered method for ETS activity assessment involves the reduction of the tetrazolium salts 2,3,5-triphenyltetrazolium chloride (TTC), 2,2′-di-*p*-nitrophenyl-5,5-diphenyl-3,3′-dimethoxy-4,4′-diphenylene (NBT) or 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) to insoluble formazan compounds [50,51], a technique pioneered by Lenhard [52] for the assessment of bacteria in soil. The tetrazolium salts compete with oxygen for electrons [50,53]. Since ETS activity is common to virtually all microbes

[48,49] the technique has provoked increasing interest, and since it is applicable under both aerobic and anaerobic conditions [46,49,50] it can thus be used for samples from most environments.

Most of the methods described involve disruption of the cells after incubation with a tetrazolium salt [54–56] followed by solubilisation of the formazan using, typically, Triton X-100 [57,58] followed by measuring the absorption at 490 nm [46,47]. Direct microscopic examination has also been used to give a more precise estimation of the number of active cells based on the assumption that only living cells will contain the formazan crystal [59–61]. However, it has been suggested that not all bacteria present in natural environments are actively metabolising, and may be in a state of dormancy [60,62,63]; that not all bacteria are capable of tetrazolium salt reduction [48]; and that tetrazolium salts may suppress ETS activity [48]. Addition of substrate has also been shown to affect the results, although some disagreement still exists as to the necessity of substrate addition. Tabor and Neihof [49] found no increase in counts of microbes from water samples when succinate was included in the INT-incubation tubes. However, Trevors [48] found an increase of 100% at 4°C and 327% at 10°C in water samples when substrate was included, and Bright and Fletcher [64] obtained increased counts when the growth substrate (leucine) was included in their bacterial counts in a series of attachment studies. Work in this laboratory (unpublished results) has also clearly shown an enhancement of active cell numbers after inclusion of an ETS-activating substrate.

Various authors have commented on the problems of detection of small microbes which may be present in natural environments [65] and have suggested modifications to the basic technique [19,49,66] while other authors have chosen different compounds [67]. Much attention has also been given to the other methods for assessment of metabolic activities in natural environments, including the measurement of uptake rates of radiolabelled substrates [68] and measurement of production of metabolic products. A technique involving direct examination of cells whose nucleic acid synthesis has been repressed without apparently affecting their growth has also been successfully used [36]. The measurement of in situ metabolic activity was the subject of a recent review by Findlay and White [69].

2.2.3. Direct counts

Information concerning the physiological state of a microbe is usually expressed with respect to the total numbers of microbes present [18]. Despite the persistence of some agar based counting procedures, most total counts are presently carried out by microscopy. The use of bright-field microscopy is rare and has limited application [70,71], as has phase contrast microscopy [72]. However, epifluorescence (incident light fluorescence) microscopy has been successfully used for enumeration purposes [73]. Cells are counted on a membrane filter after filtration of a known sample volume. This technique was first developed by Strugger [74] and has since been modified and improved by the introduction of different filters [75,76] and microscope lamp/filter arrangements [77]. Various stains have been employed, including fluorescein isothiocyanate (FITC) for soils [12,78] and acridine orange for aquatic samples [2]. FITC reacts specifically with proteins and fluoresces green [79]. Acridine orange stains nucleic acids, and the technique is very good when used for enumerating total cell numbers, but does not distinguish between live and dead cells [71,80]. It has been shown, for example, that acridine orange is still taken up by autoclaved cells [81]. By controlling the pH it has been suggested that differentiation between live and dead cells is possible [82] although experimental verification has yet to be reported. A further restriction of this technique is that acridine orange does not distinguish between dormant and growing cells [2]. The accuracy of the method has been examined by comparing counts using epifluorescence and electron microscopy [83]. Using the scanning electron microscope, the counts were found to agree, but epifluorescence gives higher counts than can be obtained with transmission electron microscopy [64].

Other fluorescent compounds have also been used and some claims have been attached to their ability to accurately determine numbers of actively metabolising microbes. Rhodamine 123, for

example, relies on the existence of a proton motive force for uptake [85] although its use so far has been shown to be restricted to Gram-positive cells. Similarly, fluorescein diacetate (FDA) has been used for enumerating freshwater microbes, where this non-fluorescent compound is attacked within the cells by non-specific esterases [86] with the resultant release of fluorescent products [87]. However, FDA also has difficulty in penetrating the Gram-negative cell wall [81] and its effective use is most probably restricted to mammalian cells [88], yeasts [89] and some cyanobacteria [12]. The stain fluorescamine has also been used successfully to differentiate between microbial cells and detritus particles in marine samples, due to the high affinity of the stain for amino groups [90].

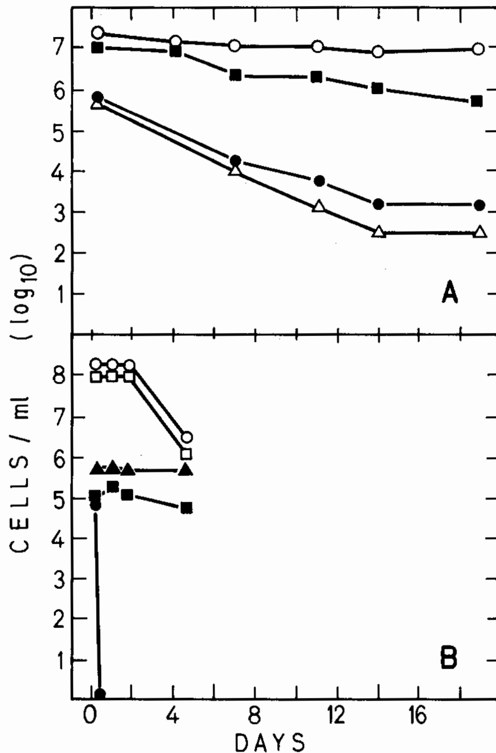

Fig. 1. Detection of *E. coli* h10407 inoculated into (A) aged estuarine water where the pH and salinity were adjusted to simulate environmental conditions; and (B) in situ experiments in semitropical water using membrane chambers. ○, Acridine orange direct count; □ acridine orange direct count control; ▲ fluorescent antibody counts; ●, standard plate count; △, eosin methylene blue agar count; ■, direct viable count (nalidixic acid). Redrawn from [34] with permission.

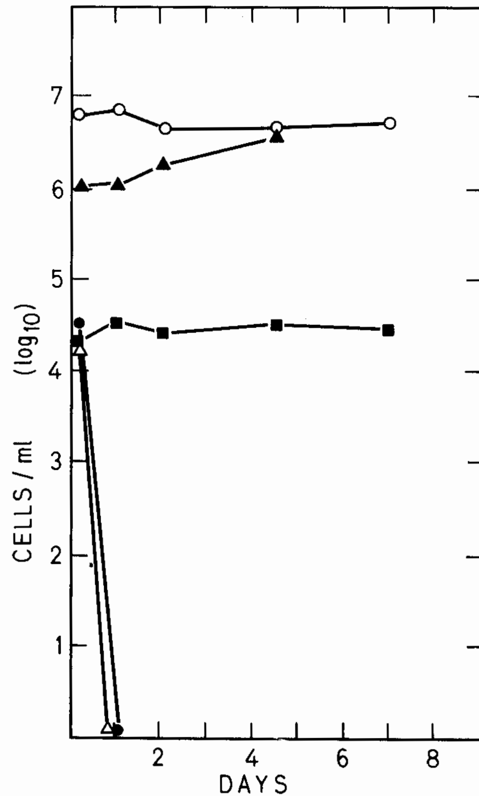

Fig. 2. Detection of *V. cholerae* CA401 exposed to Patuxent River water in microcosms. ○, Acridine orange direct count; ▲, fluorescent antibody count; ■, direct viable count (nalidixic acid); △, thiosulpate citrate bile sucrose agar count; ●, tryptic Soy agar count. Redrawn from [34] with permission.

Epifluorescence microscopy using acridine orange has also been combined with autoradiography for the detection of metabolising cells [91]. Another approach in total cell enumeration is that of fluorescent antibody labelling. The staining of cells by fluorescent antibodies allows the observation of particular strains in a mixed culture [92,93] thus allowing a direct count of individual species. However, the technique does not provide any information as to the physiological state of the cells. The use of fluorescent antibody technique for public health testing for the presence of pathogenic organisms is currently being investigated. A recent paper clearly shows the advantages of this technique over the presently used standard methods [34]. Their paper reports results from water samples from Bangladesh, tested for the presence

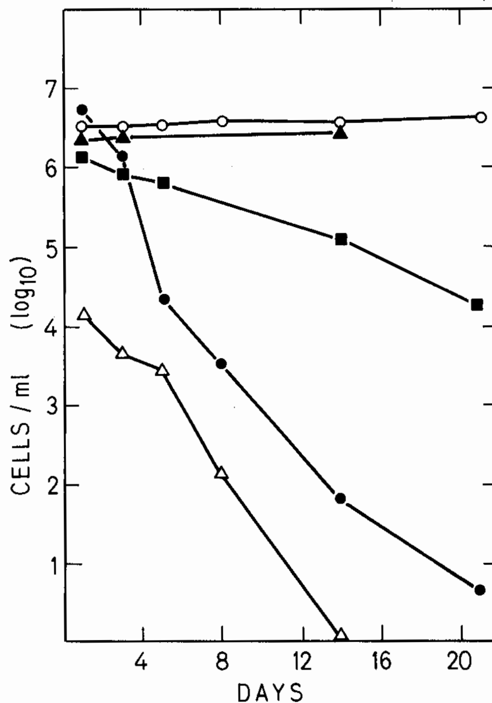

Fig. 3. Detection of *Shigella sonnei* 53G inoculated into aged estuarine water where the pH and salinity were adjusted to simulate environmental conditions. ○, Acridine orange direct count; ▲, fluorescent antibody count; ■, direct viable count; ●, tryptic soy agar count; △, MacConkey agar count. Redrawn from [34] with permission.

of *Vibrio cholerae*. Using conventional techniques, 7 of the 52 samples tested proved positive. By the fluorescent antibody technique, 51 out of the 52 were positive. Similar comparisons are made between acridine orange direct counts and plate counts (Figs. 1–3). Clearly, these direct count methods are better suited for the enumeration of organisms, especially where potential public health hazards exist.

A summary of the various conditions for and enumeration possibilities of the different methods available is given in Table 1.

3. STARVATION

Under laboratory conditions, microbes are generally grown in artificially rich environments the like of which are rarely found in nature. Unlike laboratory culture environments, organisms in natural environments are subjected to variations in substrate availability, (i.e., nitrogen, phosphorus), in temperature, oxygen, toxic chemicals as well as to spatial variations, and if the cells are attached, differences in substratum composition may exist. Despite these conditions, indigenous microbes readily survive in their natural environ-

Table 1

Evaluation of microbial enumeration methods

Act, Non-Rep, and Dorm refer to the physiologically differentiated cell types discussed in section 2.1. Parentheses imply that only a part of this population was enumerated.

Method	Time required for test	Environment of test	Requirement for cell replication	Cell types enumerated	Differentiation between cell types	Accuracy
Cultivation						
Plate Count	24–48 h	Modified	Yes	(Act), (Dor)	Yes	Low
MPN	24–48 h	Modified	No	Act, Non-Rep, (Dorm)	(Yes)	Low
Activity						
ETS (INT)	1 h	Original/ modified	No	Act, Non-Rep, (Dorm)	No	High
ATP	2 h	(Cell extract)	No	Act, Non-Rep	No	Unknown
Direct						
AODC/FITC	30 min	Original/ modified	No	All	No	High
FDA	2 h	Original/ modified	No	Act, Non-Rep, (Dorm)	Yes/no [a]	Unknown
Slide culture	24–48 h	modified	Yes	Act, Non-Rep, (Dorm)	Yes/no	Low
Immunofluorescence	2 h	Original	No	All	Yes	Very high

[a] Only taken up by Gram-positive cells.

ments. Therefore, mechanisms obviously exist conferring the necessary properties to compete and survive. In this section those mechanisms by which microbes compete and survive under variable nutrient conditions (i.e., feast/famine or starvation) will be discussed with emphasis placed on the modes by which cells are able to defer the death process.

In order to grow, a microbial cell requires a carbon source, an energy source, and nutrients for biomass synthesis and metabolic regulation. However, it is known that bacteria are able to survive, sometimes for very long periods, in the absence of any or all of these requirements. As pointed out by Morita [65] many publications deal with short time survival (days to weeks) or with survival under specific stress conditions. Stevenson [63] suggested that in most aquatic environments a significant proportion of the bacterial community can be described as being physiologically dormant. Similarly, in soil there is evidence to suggest the dormant bacteria outnumber the active ones [94].

For a cell to survive during starvation, only a very small part of its metabolic potential needs to be expressed. These have been collectively referred to as maintenance functions, and include maintenance of osmotic potential, turnover of essential cell materials, and maintenance of the membrane potential. If energy for these processes is not provided, it is said that the cell will irreversibly cease to function. Microbial cells are biochemically sophisticated, and many of the intermediates of the chemical reactions have higher free energies than their original substrates. Energy must be supplied to counteract a natural tendency towards disorder and the energy required to maintain the basic requirements of cellular activity is termed maintenance energy [95]. Maintenance energy in the absence of an exogenous energy source has to be derived from the oxidation of either endogenous cellular constituents or storage products. This degradation is known as endogenous metabolism and can be defined as the summation of all metabolic reactions which occur when a cell is deprived of either compounds or elements which may serve specifically as exogenous substrates [96].

The theories resulting in the development of the concept of maintenance are complex. Beauchop and Elsden [97] introduced the concept $Y_{x/ATP}$ relating the mass of cells produced per mol ATP obtained from the energy source in the medium. Theoretical calculations of $Y_{x/ATP}$ can be made under anaerobic growth conditions, since the catabolic pathways for anaerobic breakdown of substrates are known [98]. Despite original theories to the contrary $Y_{x/ATP}$ is now known not to be a constant for different microorganisms [99]. Furthermore, experimental $Y_{x/ATP}$ values are nearly always much lower than those based on theoretical calculations [100]. Much of the early work was carried out in batch culture in a dynamic environment such that yield values were affected by physico-chemical changes [101]. In chemostatic culture, yield values can be obtained under conditions without such variations. One method is to use the ratio between the specific growth rate and the specific rate of substrate consumption, i.e.,

$$Y_{x/s} = \mu / q_s \tag{1}$$

Where $Y_{x/s}$ is the microbial biomass yield coefficient (mass of cells produced per mass of substrate utilized), μ is the specific growth rate constant (t^{-1}) and q_s the specific substrate consumption rate (mass substrate consumed per mass of biomass per unit time). When this is carried out over a range of different growth rates a plot of μ against q_s will give a straight line, which when extrapolated fails to go through the origin. The implied assumption is that as the growth rate decreases to zero the value for the specific substrate uptake rate tends towards a positive value. Pirt [102] explained this with his theory of maintenance energy, and deduced that the consumption of substrates was partly for growth dependent processes and partly for growth independent processes. Stouthamer and Bettenhausen [103] expressed this mathematically as:

$$q_{ATP} = \mu / Y_{x/ATP} \tag{2}$$

or

$$q_{ATP} = \mu / Y_{x/ATP}^{Max} + m_e \tag{3}$$

where q_{ATP} is the specific rate of ATP production (mol ATP $\cdot$ g^{-1} dry wt. $\cdot$ t^{-1}), $Y_{x/ATP}$ is the molar growth yield for ATP, and $Y_{x/ATP}^{Max}$ is the growth yield per mol ATP corrected for the energy of

maintenance (m_e). Values for the maintenance energy requirements have been published by Stouthamer [98]. However, Neijssel and Tempest [104] question the basic assumption made by Pirt [102], namely that the maintenance rate does not vary with growth rate. Instead, these authors propose that the maintenance rate does vary with growth rate. This led Pirt [105] to propose a modified model, whereby a growth rate-dependent maintenance term is included in Eqn. 1 for specific substrate utilisation, i.e.,

$$q_s = \mu / Y_{x/s} + m_1 + m'(1 - \mathrm{k}\mu) \qquad (4)$$

where m_1 is the constant maintenance energy coefficient m', the growth rate dependent maintenance energy coefficient when $\mu = 0$ (mass substrate per mass cells $\cdot\, t^{-1}$) and k is a constant. Thus the expression $m'(1\text{-k}\mu)$ is the growth rate-dependent maintenance energy, and m' its value when $\mu = 0$ [105]. This treatment of the maintenance energy concept was similar to an approach suggested by Neijssel and Tempest [104]. They later clearly expressed their interpretation of maintenance energy terms by stating that the maintenance energy rate and maximum growth yield value derived from linear regression analysis of either yield or metabolic rate versus growth rate are essentially mathematical constants and not biological constants. In a strict physiological sense, both may vary with growth rate [106].

One of the major problems in experimental determinations of maintenance energies is that when growing the cells at a very slow growth rate it is almost impossible to attain steady-state conditions, due to non-steady-state medium addition and spent medium removal rates, etc., such that experimental verification that the q_s/μ diagram does indeed tend naturally towards zero at lower growth rate exists and thus the conclusions derived cannot be satisfactorily resolved [107].

Microbes possess two mechanisms by which energy (ATP) can be produced from the oxidation of an energy substrate. The first of these is substrate level phosphorylation where the production of ATP is catalysed by soluble enzyme systems within the cell cytoplasm. The second mechanism is oxidative phosphorylation whereby ATP synthesis is coupled to electron transport reactions which are driven, in most cases, by the oxidation of either organic compounds or of inorganic ions of negative redox potential, with concomitant reduction of electron acceptors with higher redox potentials [108]. The mechanism by which ATP is produced in oxidative phosphorylation is almost universally agreed to be based upon the chemiosmotic theory of Mitchell [109]. Synthesis of ATP is catalysed by anF_0F_1 ATPase enzyme located in microorganisms in the cytoplasmic membrane [108], although it is generally considered that large portions of the membrane itself can be considered to be 'energy transducers' [110]. The theory in its simplest form states that these energy transducing systems act as electrogenic proton pumps and translocate protons across the cytoplasmic membrane. Since the membrane is effectively impermeable to OH^- and H^+ ions the result of their translocation is the generation of a proton gradient (ΔpH) and since they are ions, of an electrical gradient ($\Delta\psi$) between the cytoplasm of the cell and its immediate environment. Consequently the cell interior becomes alkaline and electrically negative with respect to the exterior. Since both gradients exert an inwardly directed force on the protons, this force can be expressed by the sum of these two components, namely:

$$\Delta\tilde{\mu}_{H^+} = \Delta\psi - Z\Delta \mathrm{pH} \qquad (5)$$

where $\Delta\tilde{\mu}_{H^+}$ is the proton motive force (mV) and is a measure of the combined chemical and electrical forces acting on the protons, $\Delta\psi$ is the electrical potential difference across the membrane, ΔpH is the pH difference across the membrane and $Z = 2.3\ RT/F$, where R is the gas constant, T, the absolute temperature and F, the Faraday constant. The factor Z converts the pH gradient into mV. The $\Delta\tilde{\mu}_{H^+}$ generated by electron transfer is used to drive an ATP-hyrolysing proton pump in reverse, i.e., in the direction of ATP synthesis. Thus, the energy transducing membrane contains two proton pumps, one driven by electron transfer and one driven by ATP hydrolysis.

The energetic activation of the membrane is supposedly a major regulatory mechanism in the physiology of the cell. Its possible influence in the action of autolysing enzymes will be discussed in section 4, but it has also been implicated as a

regulatory control for other physiological functions [111]. The proton motive force supplies the energy for flagella movement [112], solute transport, [113], ppGpp breakdown [114], nitrogen fixation [115], DNA transport [116], and pH homeostasis [117]. The processes driven by ATP are different from those driven by the proton motive force. Since ATP is a general intermediate in biosynthetic processes, supplying energy for the transport of some solutes, it can also be used to generate a proton motive force [118]. The interdependence between $\Delta\psi$ and ATP is one mechanism for metabolic control. In the presence of a high NADH concentration resulting from a high substrate flux, $\Delta\tilde{\mu}_{H^+}$ will be enhanced and ATP will be produced. When the ATP content is high, and a low proton motive force is operative, the latter can be regenerated by ATP hydrolysis, thus diverting ATP away from biosynthesis.

Tempest and Neijssel [106], in a critique of maintenance energy suggest that most of the energy required is necessary for the maintenance of an ionic gradient. This suggestion is based on experiments on the K^+ ion concentration gradient in *Klebsiella aerogenes*. It was demonstrated that the transmembrane K^+ gradient increases with increasing growth rate (since the K^+ requirement of the cells also increased) which was accompanied by concommitant increases in either the specific respiration rate or the oxygen consumption rate of the culture [119]. Calculating the amount of O_2 required to maintain the ionic gradient from the specific respiration rate at each of the different growth rates, the authors demonstrated that a plot of q_{O_2} versus growth rate constant would then indeed go through the origin. Tempest and Neijssel thus conclude that more than 90% of the maintenance energy requirement for glucose-limited cultures of *Klebsiella aerogenes* is necessary for the maintenance of membrane ionic potential. Since the extracellular K^+ concentration diminishes as the growth rate and intracellular K^+ concentration increase, then the maintenance energy must vary with growth rate even in carbon substrate limited cultures with a 10-fold excess of K^+ [106].

Maintenance energy has also been associated with solute uptake. However, this function can also be related to membrane function, and therefore, to the chemiosmotic theory. Accordingly, there are several mechanisms by which solutes can be translocated across the cytoplasmic membrane. Most solutes are transported by the so called secondary transport systems which can be either passive, i.e., without the interaction of specific membrane proteins, or active, whereby such mediation occurs [111]. As such a process requires energy, a 'driving force' is derived from the proton motive force of the cells which in general can be described by the expression:

$$Z \log\left(\frac{[A]_{in}}{[A]_{out}}\right) + (n+m)\Delta\psi - nZ\Delta pH \qquad (mV) \qquad (6)$$

where m is the charge of the solute A and n is the number of protons translocated in the symport.

More recently, Michels et al. [120] have described an 'energy recycling model' which in essence is the reverse process of secondary transport of solutes, whereby the energy of an electrochemical product gradient is converted into the energy of an electrochemical proton gradient. Such systems have been detected in the homolactic fermentative *Streptococcus cremoris* and in *Escherichia coli* when the microbes are growing fermentatively and therefore have no means for proton extrusion by functional electron transport systems [121].

Another so called 'maintenance function' is microbial motility, but the motor for flagella motion is not an ATPase as has been demonstrated by the continued motility of microbes even after their ATP pool has been significantly reduced [115], the energy for both flagella motion [122] and the signal mechanism in chemotaxis [123] being derived from the proton motive force (pmf). This has been shown to be the case by using a model system of ghost cell envelopes on which flagella rotation could be initiated by imposing a pH gradient across the envelope membrane [112]. There is also the suggestion that there is a tight stoichiometric coupling between proton transfer and flagella rotation [113], although there appears to be a threshold pmf below which flagellar motion is impossible [124].

Three questions concerning survival need to be answered: (i) How can a microbe in a non-hostile, neutral environment without nutrient source survive for extended periods? (ii) Is energy lost via the non-growth associated membrane functions in microbes with a nutrient flux sufficient to meet its growth requirements? (iii) What effect do such energy losses have on the growth/survival behaviour of microbes in which they are occurring? Obviously two different points of reference are indicated. In the first question, natural environments are envisaged, whilst the second and third questions relate predominantly to microbes in technical processes and laboratory systems.

Under starvation conditions, the microbe no longer receives an adequate nutrient flux and is compelled to divert its energy towards specific survival functions. Cellular replication is not possible due to the high energy requirement for synthesis of new cell biopolymers. Energy has to be diverted to the so-called maintenance functions. In order to achieve this, the cell has at its disposal several possible mechanisms. Initially if the cell has been under a feast regime, it will contain an excess of polymers such as RNA which are now superfluous for metabolic sustenance. Oxidation of these compounds together with the mobilisation and oxidation of any reserve polymers constitutes the observed effect known as endogenous metabolism. However, this step is only the start of the process of survival under starvation conditions.

A theory has recently been proposed explaining the relationship between substrate consumption and biomass production at low growth rates [125–127]. In order to prevent the 'apparatus effect' whereby substrate addition to slow-growing cultures becomes pulsed as opposed to continuous thereby changing substrate uptake characteristics [107], a recycle reactor was used to grow cells with very long residence times. It was found that the microbe used (*E. coli*) went through various 'phases' before activating the 'stringent response', as the residence time (reciprocal dilution rate or reciprocal growth rate constant) was extended. The stringent response is a series of biochemical adjustments resulting from the limitation of amino-acyl-tRNA. As a result the cell makes a number of major readjustments of activity including (1) a reduction in RNA synthesis and accumulation; (2) increased protein turnover; (3) reduced membrane transport; (4) reduced endogenous synthesis of nucleotides; glycolytic intermediates, carbohydrates, lipids, fatty acids, polyamines and peptidoglycans; (5) increased control ('kinetic proof reading') over protein translation and (6) cAMP accumulation [128]. This reaction results from the accumulation of guanosine 5′diphosphate 3′diphosphate (ppGpp) due to an idling reaction of the ribosomes and uncharged tRNA via a protein known as stringent factor [129]. A threshold concentration of ppGpp must be exceeded for the response to manifest. Stouthamer [127] suggests that as the growth rate of the organisms slows down three phases can be distinguished, the first with sufficient tRNA, followed by a phase in which aminoacyl tRNA becomes limiting and ppGpp formation begins, and finally a phase where the stringent response is manifested, i.e., where cellular metabolism is put in check at the expense of some limited energy expenditure (from cAMP and ppGpp formation and from the proof-reading of proteins). Whether this energy expenditure is equivalent to the maintenance energy is not yet clear. This theory is one possible mechanism for survival in nutrient poor environments, whereby the accumulated cAMP may be used by the microbes to activate a range of enzymes when suitable substrates become available.

During endogenous metabolism and long-term survival experiments, it has as yet been impossible to attribute death to the loss of a specific cellular function [96]. However, several theories have been proposed associated with the concept of maintenance energy. Correlations between the proton motive force and the metabolic state of the cell have been proposed as well as theories relating the death of the microbe to energy exhaustion [130]. Konings and Veldkamp [120] have also suggested that the gradual decrease in the proton motive force found during starvation experiments could be attributed to the gradual accumulation of 'non-viable' cells in the culture.

Zilberstein et al. [117] in a study on the effects of external pH on the proton motive force ob-

served that *E. coli* shifted its membrane potential and pH in a homeostatic mechanism to maintain the internal pH at a constant value. Variations in the external pH were compensated for by variations in the membrane potential such that the $\Delta\tilde{\mu}_{H^+}$ also remained effectively constant. However, when they subjected a mutant, defective in its Na^+/H^+ antiporter activity, to a pH change they found that growth ceased when the ΔpH collapsed, although a high membrane potential was still maintained. The actual death of the microbes (as measured by lack of colony formation) did not occur for at least 12 h after the change in ΔpH.

Recently, Otto et al. [131] reported that in the homolactive fermentative *S. cremoris* the initiation of lactose starvation caused the membrane potential to collapse almost immediately, with an accompanying collapse in internal ATP concentration. However, at any time up to 24 h, both can be restored by addition of lactose, thus allowing the microbes to survive for short periods in the absence of a measurable pmf. Unfortunately, no data were provided as to what happened during extended periods of starvation.

In acidophilic bacteria, the pH of the cell cytoplasm is maintained at values close to neutrality despite the extremely low pH values of their growth environments. In a starvation study with *Thiobacillus acidophilus* growing in an environment at pH 3, some decline in ΔpH could be detected, together with some minor changes in $\Delta\psi$ during a starvation period of 530 h [130]. Of particular interest is that as soon as all the internally oxidizable substrates had been exhausted, notably poly-β hydroxybutyrate, ΔpH declined, and as soon as all the available ATP had been used up, $\Delta\psi$ collapsed (Fig. 4). The authors were able to show the dependence of colony-forming ability on the presence of a measurable $\Delta\tilde{\mu}_{H^+}$. When the $\Delta\tilde{\mu}_{H^+}$ approached zero, no 'viable cells' could be detected.

Ten Brink and Konings [132] found that in batch cultures with *S. cremoris* with no pH control, the proton motive force ($\Delta\tilde{\mu}_{H^+}$) collapsed to zero as soon as the logarithmic growth phase ceased (Fig. 5). These researchers concluded that energy was required to maintain the pH gradient, as a result of the changing pH in the external medium due to lactate production in this organism. Padan et al. [133] have also shown that in aerobically grown *E. coli*, inhibition of respiration by either anaerobiosis or KCN leads to a collapse in the ΔpH. Zychlinski and Matin [130] showed with

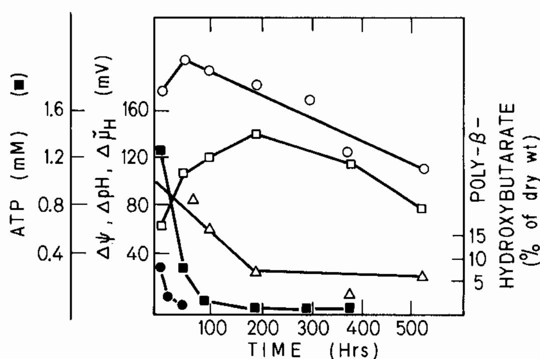

Fig. 4. Effects of starvation on various parameters in *T. acidophilus*. The organism was grown heterotrophically at pH 3.0 in a mineral salts–glucose medium. The cells were harvested by centrifugation and resuspended at time = 0 in deionized water at pH 3.0 at 29°C in an incubator shaker. ○, ΔpH; □, $\Delta\psi$; △, $\Delta\tilde{\mu}_{H^+}$; ■, cellular ATP level; ●, cellular poly-β-hydroxybutyric acid level. The scale for ΔpH and $\Delta\tilde{\mu}_{H^+}$ is in negative mV, that for $\Delta\psi$ in positive mV. Redrawn from [130] with permission.

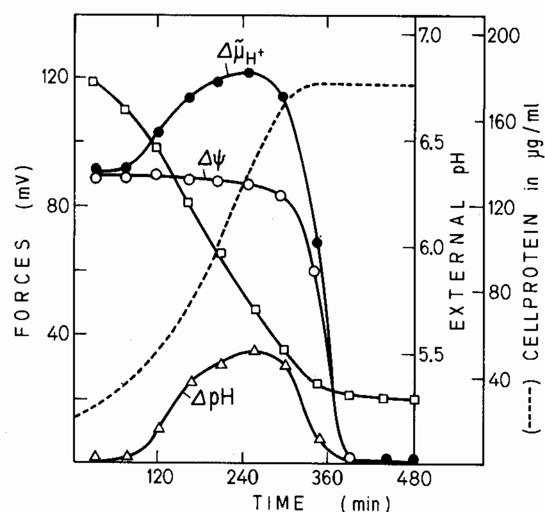

Fig. 5. The electrochemical proton gradient in *S. cremoris* during growth in batch culture on a complex medium with lactose (2 gl^{-1}) as sole energy source. △, ΔpH; ○, $\Delta\psi$; ●, $\Delta\tilde{\mu}_{H^+}$, □, pH; (- - - - - -), time course of protein synthesis. Redrawn from [132] with permission.

azide-treated cells of the acidophilic *T. acidophilus* that the pH gradient could be maintained purely passively.

A major criticism of much of the published material regarding the starvation of microbes is that the effects of death and lysis during the experiments are rarely considered. However, these factors can have serious implications in the interpretation of the biochemical changes in the cultures under study. A typical example can be seen in a recent study on the survival of the organism *Brevibacterium linens* during starvation [134]. The authors carried out a very broad range of tests during the starvation period including viability by slide culture, dry weight, oxygen consumption, total sugars, intracellular protein, intracellular amino acids, DNA, RNA, and ATP. They obtained what appear at first sight to be interesting changes in the concentration of these parameters with time (Figs. 6–8). ATP was only measured during the first 10 h of the experiment, and showed a rapid decline. However, since the viability, which was unfortunately based on a cell propagation method, decreased to 70% of its initial value, and the total cell mass also decreased together with an increase in extracellular NH_4^+, lysis processes cannot be ignored. The authors suggest that 'cryptic'

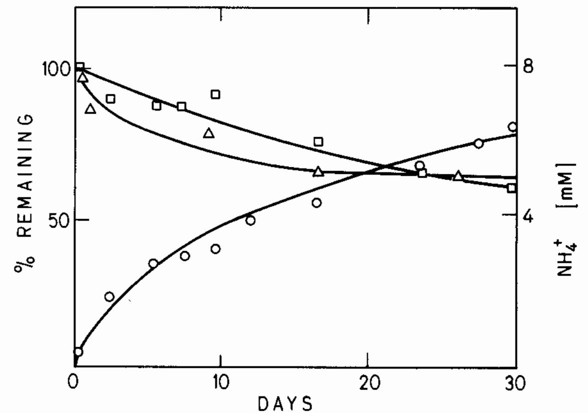

Fig. 7. Changes in the contents of □, intracellular protein; △, free amino-acids; and ○, the quantity of ammonium ions in the extracellular medium in a suspension of *B. linens* during 30 days of nutrient starvation. The cells were grown aerobically at 21°C in a 0.35% bacto-tryptone, 0.25% yeast extract and 0.125% glucose medium with mineral salts at pH 7.0. The cells were resuspended in a 0.5 M Tris–HCl buffer solution (pH 8.0) on day 0 and incubated at 21°C. Cells initially contained 310 μg protein and 110 μg of free amino acids·mg^{-1} dry weight. Redrawn from [134] with permission.

growth was not occurring, based on their failure to observe microscopically either cell wall or cell membrane fragments. The observation of such cell debris, even under conditions where lysis is un-

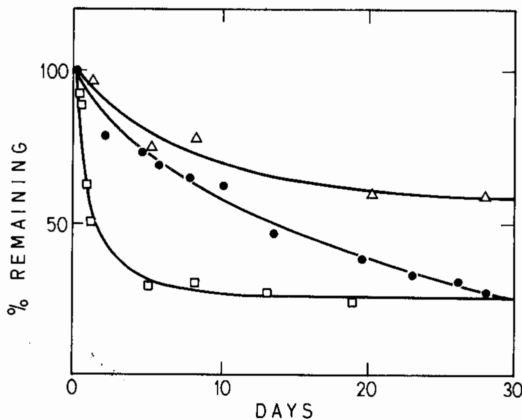

Fig. 6. Changes in ●, turbidity; □, viability; and △, dry weight in a suspension of *Brevibacterium linens* during 30 days of nutrient starvation. Viability and turbidity are expressed as % of initial values. The cells were grown aerobically at 21°C in a 0.35% bacto-tryptone, 0.25% yeast extract, 0.125% glucose medium with mineral salts at pH 7.0. The cells were resuspended in a 0.5 M Tris–HCl buffer solution (pH 8.0) on day 0 and incubated at 21°C. Redrawn from [134] with permission.

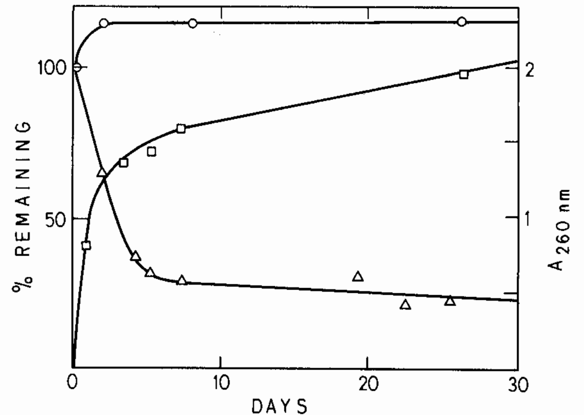

Fig. 8. Changes in the intracellular levels of ○, DNA; △, RNA; and □, extracellular material absorbing at 260 nm. The mean initial quantity of DNA was 27 μg·mg^{-1} dry weight and that of RNA was 70 μg·mg^{-1} dry weight. The bacterium *B. linens* was grown aerobically at 21°C in a 0.35% bacto-tryptone, 0.25% yeast extract and 0.125% glucose medium with mineral salts at pH 7.0. The cells were resuspended in a 0.5 M Tris–HCl buffer solution (pH 8.0) on day 0 and incubated at 21°C. Redrawn from [134] with permission.

doubtedly occurring, requires the use of either electron microscopy or of highly specialised microscopic methods such as immunofluorescence, so that this evidence for the lack of lysis is inconclusive. This aside, if one looks at the data, these are presented in terms of mass of the parameter per unit mass dry weight. However, if death and lysis occur in the culture as suggested by the NH_4^+ accumulation and the accumulation of 260 nm absorbing compounds, then the biophysical composition of what actually constitutes the reference quantity, i.e., unit mass dry weight, must be questioned. If this dry weight were composed entirely of living, active cells, then the data could be accepted as presented. However, after 30 days it is most likely to be predominantly composed of inert cell particulates and dead biodegradable biomass, so that the data have to be recalculated and expressed on a more meaningful basis such as the mass of a particular component per cell or, better still, mass per living/surviving cell.

The authors measured a reduction in protein content from 310 μg/mg dry weight to 60% of its original value, i.e., 190 μg/mg dry weight after 30 days starvation. However, if the latter solids were composed of only 60% intact cells then the amount of protein per surviving intact cell is unchanged. This result is rather surprising, since a definitive reduction certainly occurs within the population and intuitively one would expect a cell under starvation to degrade some of its proteins to supply any energy that may be necessary for the survival process. Exactly how such results are best interpreted is now a problem.

Breuil and Patel [135] have looked at the depletion of cellular contents in the anaerobic microbe *Methanospirillum hungatei* GP1 during enforced starvation. Their results were effectively similar to those for the aerobic organism *B. linens* discussed above, namely that DNA appeared to increase in concentration whilst RNA was degraded, initially rapidly and then somewhat more slowly. ATP was also found to rapidly decline in the system. However, all the results were also expressed on a per unit biomass basis and did not take into account the change in the physiological matrix of the suspended solids. A detailed analysis of cell numbers and particulate composition is required before, during and after experiments where changes in the intracellular pools of starved cells are being investigated. The foregoing description of the approach to starvation experiments is common. Most of the reported results are expressed in terms of the amount of cell component per ml cell suspension. Unfortunately the use of cell propagation methods is also extremely common in starvation experiments, thus reducing the confidence level in the interpretation of most published results.

In a subsequent section, it will be shown that microorganisms possess enzymes capable of degrading their own cell material. This will be limited to the class of enzymes known as autolysins, which function specifically on the cell walls. However, it has been recognised for some time that other enzymes are active within the cell, modifying internal structures and ensuring substrate availability during times of exogenous nutrient deprivation. However, these enzymes can also function in catabolism of exogenous nutrients resulting from cellular lysis. For example, Parquet et al. [136] reported on the possible physiological function of the enzyme *N*-acetylmuramoyl-L-alanine amidase in *E. coli* K12. This enzyme was found to be either loosely bound to peptidoglycan or entrapped in the outer membrane–peptidoglycan complex, and to specifically cleave the bond between *N*-acetylmuramic acid and alanine in the bacterial peptidoglycan. Paradoxically, such an enzymatic cleavage was found to be extremely rare in normal growing cultures. Hence, it was suggested that since the enzyme was active under autolytic conditions, it functioned as a hydrolase for growth on peptidoglycan fractions. Parquet et al. [136] were able to demonstrate growth of *E. coli* on MurNAc–Lala fractions alone as well as on a MurNAc-Lala-Xaa fraction suggesting, therefore, the presence of other hydrolases. A vast array of enzymes specific for turnover of endogenous macromolecules are present in microbial cells and under normal growth conditions they function as control mechanisms of the growth and metabolic processes. However, under conditions where exogenous substrate becomes limited, they serve to provide the cell with a continued substrate supply for energy generation in the absence of resynthesis.

A similar approach was adopted by Gaudy et al. [149] who used sonicated sludge as a nutrient source for 'cryptic' growth experiments. By following the COD reduction after reinoculating with sludge organisms, it was found that approx. 50% of the COD was removed in the first 15 min, and 90% by the end of the experiment (Fig. 12), and is indicative of the greater potential for 'cryptic' growth to occur under mixed culture conditions than in pure culture. Obviously a tremendous amount of work still needs to be carried out to assess correctly the impact of 'cryptic' growth in processes such as wastewater and waste sludge treatment and in other industrial processes.

4. AUTOLYSIS

The growth of bacteria requires the interaction of biosynthesis and degradation of various structural polymers, notably cell wall polymers such as peptidoglycan, to allow volumetric expansion and cell division. The class of enzymes responsible for cell wall polymer hydrolysis have been termed autolysins and their action can be broadly divided into (a) constructive or voluntary and (b) destructive or involuntary. Constructive properties are exhibited while the control mechanisms of the autolysins are still effective and include daughter cell separation [150,151], turnover and expansion of cell wall biopolymers [152–156], and morphological differentiation [151,157]. Should this tight control of autolysin activity break down, then the destructive roles of the autolysins are manifested by the rupture of cell walls, leading to loss of cell activity and eventually to total dissolution of cellular integrity. While several reviews have been published on the constructive (voluntary) roles of autolysins [158–160], less attention has been focused on the destructive potential of this class of enzymes and the quantitative realisation of this potential. Some suggestion has been made that the destructive aspect of autolysins may be continuously manifested at low levels in populations grown under what are assumed to be ideal conditions [146,161,162].

The dual potential of the autolysins demands a highly sensitive and efficient control mechanism, possibly one of the most demanding in the whole cell, since a single error can result in irreversible damage. A close relationship has been demonstrated between protein synthesis and autolysis [153,163–165] and inhibitors of autolysin activity [164] or activating substances [166–169] have been implicated in the control of autolytic enzymes. Evidence suggesting that cell wall turnover and autolysin activity are controlled by different genes was presented by Vitcović [170] who found no correlation between the two processes.

The location of the autolysins is also unknown. Pooley [171,172] and Glaser and Lindsay [154] have suggested that wall turnover occurs on the outer cell surface, the autolytic enzymes being transported through the cell wall and activated in the region of the older peptidoglycan. New peptidoglycan is laid down in the inner wall area and protected from degradation by its structural and spatial configuration [156]. The matrix in which peptidoglycan is embedded is a highly organised three-dimensional structure and as such should precisely tune the activity of the autolysins. The presence of specific teichoic acids in this matrix have been shown to be necessary for the activity of some of these enzymes in Gram-positive microbes [150,173–175]. An alternative explanation was proposed by Joliffe et al. [166] who suggested that the surface chemistry of the cell membrane as determined by the magnitude of the proton motive force, has an inhibitory effect on the activity of the autolytic enzymes. As these move further away from the sphere of influence of the membrane their activity increases. They derived this hypothesis from the fact that the addition of agents which were capable of dissipating either electrical or pH gradients resulted in both inhibition of growth and the rapid lysis of exponentially growing cells. They also found that bacteria subjected to starvation conditions, such as those suspended in buffer solutions in the absence of an oxidisable carbon substrate, rapidly lyse, but lysis was immediately inhibited by re-energising the membrane by the addition of electron-donating agents.

Membrane de-energisation was also implicated as a possible cause of cell lysis following addition of medium chain fatty acids to cultures of *Bacillus subtilis* [176]. Anomalies are also known where the

polymeric particulates up into easily assimilable monomers. However, the heat killing process does not necessarily lead to cell lysis so that most of the soluble material which was potentially available for incorporation into new cells was unavailable, being entrapped in heat-stabilised 'dead cells'. Once again, the enumeration method used was the bacterial plate count technique, and therefore, little reliance can be placed on the numbers obtained. Nioh and Furusaka [145] also found that addition of ammonium chloride led to a considerable increase in the numbers of cells growing in the heat killed suspensions, suggesting that their suspensions were nitrogen limited, and therefore, the full potential of the 'cryptic' growth process could not be realised.

The growth of bacteria in their heat-killed suspensions results in lower biomass yield coefficients than are to be expected from theoretical calculations. Hamer and Bryers [146] used the relationship between maximum biomass yield coefficient and the heat of combustion proposed by Linton and Stevenson [147] to predict a theoretical maximum yield value of 0.66 g bacterial biomass per g substrate bacterial biomass, assuming complete conversion of the substrate carbon to either new cells or CO_2, although these authors suggest deoptimization to a yield coefficient of about 0.33 would probably be encountered in real 'cryptic' growth situations. Mason et al. [148] have shown this estimate to be valid with reported yield coefficients around 0.4 (g cell carbon/g substrate carbon) in pure cultures of *K. pneumoniae.*

In this same study, Mason et al. [148] grew *K. pneumoniae* in continuous culture in a defined medium with glucose as growth limiting substrate. A volume of the culture was removed and used as the growth medium for the same cells after sonication, centrifugation and sterile filtration. The resulting solution, comprised of soluble organics, was used as growth medium for the same cells. The cells for inoculation were derived from the same culture after steady state conditions were reestablished. With this method there was no apparent lag phase before exponential growth occurred, suggesting that the microbes were already fully equipped with the necessary battery of enzymes for growth on the complex nutrient source

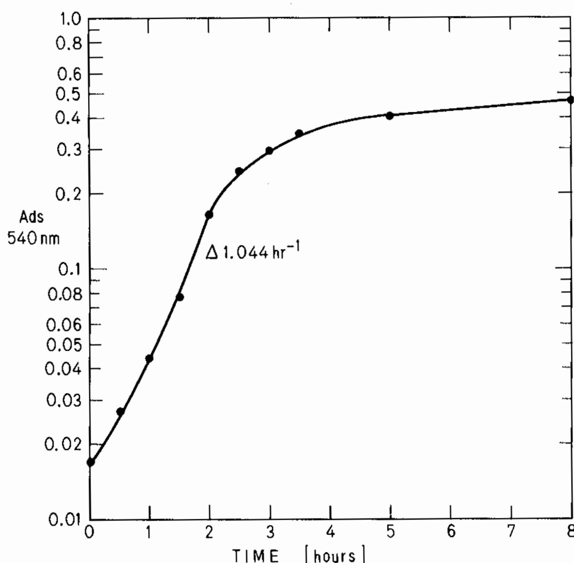

Fig. 11. 'Cryptic' growth curve of *K. pneumoniae* NCIB418 growing on a mixture of carbon sources derived from cell sonicate preparation. *K. pneumoniae* was grown in a chemostat at $D = 0.5\ h^{-1}$. The 'cryptic' growth medium was prepared by sonication followed by sterile filtration of the chemostat culture. The inoculum (10 μl in 50 ml) was taken from the original culture after steady state conditions had been reestablished. (From [148].)

(Fig. 11). Deoptimisation of the maximum theoretical yield coefficient to values of approx. 0.4 nevertheless represents highly efficient conversion, despite the very large range of potential carbon sources of varying complexity in the sonicated suspension.

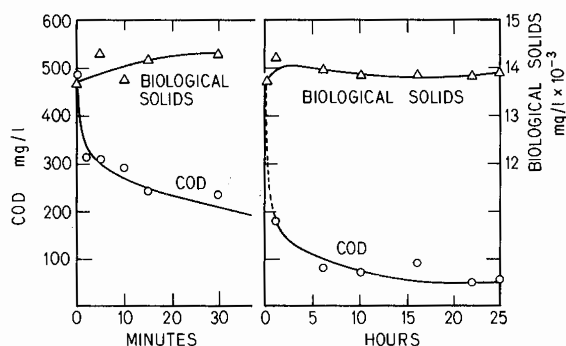

Fig. 12. Metabolism of soluble cell components (prepared by subjecting sludge to sonication) by extended aeration activated sludge. The left-hand graph shows the trend during the first 30 min, whilst the extended scale on the right shows the long-term results. Redrawn from [149] with permission.

active, mobile, semipermeable structure which mediates transport between the cell and its environment. Since the elucidation of the chemiosmotic theory of energy generation, the importance of the membrane and membrane potential have become apparent. Many of the enzyme-mediated reactions in a cell occur attached to the membrane, and the electron transport chain is an integrated part of the membrane structure. Either rupture or damage to the cell membrane will, therefore, result in injury (if the cell can recover) or death by eventual cessation of metabolic functions or by lysis.

Potential sites for damage within the cytoplasm also exist. The DNA, for instance, which encodes the information necessary for the correct functioning of a cell and the genetic information for replication is particularly vulnerable. The presence of lesions in the DNA will lead to the production of nonsense proteins and enzymes and, thereby, to the loss of cellular function or to the inability to reproduce. The ribonucleic acids are also potential sites of damage. They are found as structural components in ribosomes, as well as functioning as messengers between the DNA and the ribosomes and for transporting amino acids during protein synthesis. Therefore, damage to this component will also result in non-faithful reproduction of enzymes and other proteins and lead to loss of cellular function.

In the following section a cursory glance will be directed towards the question of methods of inducing death in microbes.

Subjecting microbes to sub-lethal concentrations of chemical inhibitors can result in the loss of their ability to either reproduce or grow under certain environmental conditions. Specific agar-based enumeration media used routinely to determine the extent of bactericidal activity are thus inappropriate, and may result in unacceptable health risks when used for testing for food- and water-borne pathogens. Chemical agents for killing microbes are used in water treatment, pharmaceutical therapy, disinfection and food industries. Microbes are susceptible to attack by chemical agents. In contrast to most physical stresses, chemical agents often interact directly with either the metabolism or the physical structure of the microbe, and as such, chemical damage occurs at the molecular level. The effects of many chemical agents have still to be elucidated.

The cell wall is the target of many antibiotics, but compounds like EDTA, lysozyme, phenol, and chlorine are also known to act at the outer boundary of the cell [187] together with various surfactants such as Triton X-100 [188]. Similarly, the cell membrane succumbs easily to attack by agents capable of disorganizing the structure, or changing specific activities towards ions, or by inhibiting the membrane-bound proteins involved in transport processes such as by the action of some antibiotics [189], or at superoptimal temperatures or with strong oxidising agents. The membrane is also susceptable to attack by agents capable of uncoupling oxidative phosphorylation reactions. Organic solvents such as butanol [190], ethanol [191], alkali metal ionophores [192] and detergents [193], have their site of action at the cell membrane.

Cytoplasmic targets for chemicals include cytoplasmic enzymes, nucleic acids and the ribosomes. Coagulation of the cytoplasm can occur at high drug concentrations [194]. A range of different antibiotics affect the biosynthesis and functioning of the nucleic acids. The ribosomes tend to be susceptible to agents which cause instability through removal of the Mg^{2+} necessary for the association of the 30S and 50S subunits [195,196].

Often ignored under the heading of chemical injury is the effect of aerobiosis and anaerobiosis in obligate aerobes and obligate anaerobes. The sensitivity of anaerobes exposed to oxygen varies with different strains [197]. The time required to deactivate a cell appears to be relatively long. Shoesmith and Warsly [198] in a review on the exposure of anaerobes to oxygen, summarised the oxygen tolerance data derived from other authors using various strains and found that despite little agreement in assessment methods, most of the strains were still alive 1 h after exposure.

During refrigeration death occurs as a result of a number of different effects not least of which is the rate of cooling and subsequent thawing processes. Thus, experiments designed to investigate the effects of refrigeration on microbes must be very carefully controlled [199]. Two types of

control mechanism over autolysin activity breaks down. Cultures of *Myxococcus coralloides* D exhibit such behaviour whereby all the cells in a batch culture spontaneously lyse at the end of the exponential growth phase [177]. This behaviour appears to be due to the synthesis of an autolysin activating substance which builds up in the medium and on reaching a certain concentration activates the autolytic enzymes and triggers lysis [167].

Autolytic enzyme activity is not just limited to the bacteria. In fungi, autolysis of mycelia has also been investigated and the same precise mechanisms of control are apparent. In *Botrytis cinerea*, the activity of several different autolytic enzymes varied independently during the autolytic phase in the growth of the mycelium [178]. Autolytic activity has also been demonstrated in archaebacteria [179] and in yeasts [180]. Microbial autolysis was the subject of a recent symposium [181]. It should not be forgotten that anomalies also exist especially in the case of the Mycoplasmas. Since these organisms completely lack a cell wall, they do not succumb to autolysis in the same sense as cell-wall-bound microbes. However, lysis by cell membrane rupture in such organisms will lead to loss of function.

5. MICROBIAL DEATH

Microbes can be killed by various means, although in order for a microbe to die it must either lose cellular integrity or incur irreversible damage to its genome. Agents by which one or both of these conditions arise include; chemical damage (including antibiotics), refrigeration, freezing, heating, and irradiation. Very often these results in only temporary damage to the microbe, if cellular integrity is not lost or if irreversible damage to the genome does not occur. However, if sub-lethal damage has occurred, it does not necessarily follow that the microbe will recover. It has been clearly shown that a damaged microbe is more susceptible to further injury than a healthy microbe [182,183].

Unfortunately, in some instances, damage or injury have become synonymous with failure to illicit growth on an agar surface [45,184]. The accuracy and meaning of this interpretation is open to doubt. In some instances the test may indeed be indicative of some form of damage. However, the type and extent of damage varies considerably. Some forms of injury tend to make the microbes more susceptible to the stresses during either surface or submerged cultivation than others. An example of this was reported by Hurst [184] citing results from Curran and Evans [185] where one medium used for enumerating the microbes as a test for injury resulted in one set of results, but changing the medium composition resulted in a totally different picture. Similarly, injury has been described as being the inability of microorganisms to form colonies on a defined minimal medium, while retaining the colony forming capability when complex nutrients are present in the medium or vice versa [45]. In experiments designed to investigate the effects of either physical or chemical maltreatment of microbes, very often no differentiation is made between injured and dead microbes.

There are various possibilities for inflicting injury on a microbial cell. The outer layer of a microbe ignoring the capsular material and cell appendages, is the cell wall/membrane complex. Gram-positive microbes possess a simpler wall structure than Gram-negative microbes. In the latter, a three-layered structure exists consisting of an innermost layer of peptidoglycan covalently linked to lipoprotein molecules. The outermost layer is the outer membrane and is covalently linked to the peptidoglycan middle layer and consists of lipopolysaccharides (LPS), phospholipids, and proteins [186]. The Gram-positive wall, in contrast, is much simpler and consists of peptidoglycan chains embedded in a teichoic acid matrix. The cell wall serves as a barrier between the internal and external cell environments and confers stability on the cell. Due to the higher osmotic pressure inside the cell, any damage to the wall/membrane structure can lead to rupture of this structure and loss of cellular integrity. Strictly speaking the cell does not die, but loss of microbes from a population results from either the disintegration or lysis of living individuals.

The cytoplasmic membrane is a metabolically

refrigeration are common, namely chilling and freezing. These practices are commonly used in the preservation of microbial cultures in technical processes, but are equally likely to occur under the influence of seasonal changes in natural environments. The effects of refrigeration are also important in the food industry where this practice constitutes a major form of preservation, whereby the destruction and inhibition of microbial growth and activity are desired.

Death often results as a consequence of the loss of control of cell permeability—a phenomenon known as cold shock [199]. One theory for the ensuing death after chilling is the loss of activity of the enzyme DNA ligase as a result of the depletion of magnesium ions from the cell [200]. At temperatures below 0°C cell damage can occur as a result of either extracellular or intracellular ice crystal formation, depending on the cooling rate [201] and from changes in the ice-crystal structure under different physical (i.e., pressure or temperature) conditions [203]. Death during extended frozen storage occurs at a fast initial rate but slows down with time until a stage is reached where constant numbers remain 'viable' [204]. Death is thought to occur as a result of exposure to the raised solute concentration as a consequence of ice crystal formation, as well as from physical damage.

Without knowing the physiological reasons for its effectiveness, heat treatment has been used for over 100 years as a means of either destroying or limiting the growth of microbes. Temperature affects different microbes in different ways. The growth of a particular microbe normally takes place within a narrow range of temperatures. It has been suggested that heat principally affects the DNA by inducing the action of exo- and endonucleases resulting in multi-strand breaks in the DNA [204,205]. In *E. coli,* the effect of heat has been suggested to result in the denaturation of the cell wall leaving the peptidoglycan layer weakened at one or two points after which the plasma membrane ruptures [206]. Some association of proteins with the nucleoids also occurs as a result of mild heat treatment [207].

Radiation results in a range of alterations in the DNA, including phosphodiester strand breaks, nucleic acid-protein cross links, pyrimidine dimer formation, etc. [208].

For detailed information on the mechanisms of destruction of microbes, the reader is recommended to consult specialised texts [209–211].

6. MATHEMATICAL MODELLING OF DEATH AND LYSIS

Models in general can be physical structures, verbal descriptions, or sets of mathematical equations that aid the user in comprehending complex phenomena. In some instances, a model serves as a hypothesis, other models attempt to predict behaviour and still others aid in understanding. No model will reflect all aspects of 'reality'. Nevertheless, all models should force the investigator to think in a concise manner and to consolidate what information is known plus what new information is required.

Mathematical modelling of microbial systems has been used to help understand the dynamics of microbial behaviour in laboratory, natural, industrial and man-made environments. Application of such models to scale-up laboratory operations into industrial processes and for extrapolation of laboratory behaviour to natural environments has resulted from the construction of such system modelling. Mathematical models also serve to direct and to optimise the research experimentation and to allow prediction of microbial behaviour. Biological systems are extremely complex and the actual system has frequently to be replaced by an imaginary model system which is mathematically tractable [212]. As a result, a very large number of models can be proposed for any single system each depending on the assumptions made for simplifying the real biological case. Many theoretical dynamic studies have been based on models formulated from steady state or equilibrium kinetics and thus inadequately describe dynamic behaviour [213].

The modelling of death and lysis processes in living cultures requires a broad understanding of the physiology of the organisms concerned. The traditional black box approach, whilst describing the behaviour of a population under a given set of

constant conditions, might be totally inadequate when those conditions change. Calibration of the mathematical model with careful experimentation is also necessary.

One of the earliest mathematical considerations of bacterial growth was presented by Buchanan [214], who presented equations describing the lag, logarithmic, stationary, accelerated death and logarithmic death phases during the batch cycle. Buchanan suggested that cell death begins during the late exponential phase and increases to a level in the stationary phase where it exactly balances the rate of growth of the bacteria, and finally exceeds the rate of growth such that a net decrease in the number of live bacteria present results. He described each of the individual phases using mathematical equations.

The first application of continuous culture growth expressions appeared in 1950 [215] and these were latter assessed by using quantitative experimentation [216]. By 1958 the phenomenon of a decreasing yield coefficient at low growth rates was recognised and this biomass-reducing effect was termed 'endogenous metabolism' [217]. Herbert [217] described the constant loss of biomass at all growth rates using Eqn. 7.

$$dx/dt = (\mu - k_e)x \tag{7}$$

where k_e is the endogenous metabolism constant.

Powell [218] derived an expression for the steady state biomass concentration using this notion of endogenous decay

$$x = Y_{x/s}^{max}\left[\frac{s_0 D}{D + k_e} - \frac{K_s D}{\mu_{max} - D}\right] \tag{8}$$

A large amount of work during the 1950s and 1960s was concerned with the destruction of microbes, particularly in hostile environments as encountered in sterilisation and pasteurisation processes. This work arose primarily due to stringent hygiene requirements for food for public consumption. A distinct paucity of information exists concerning death and lysis of microorganisms under conditions where microbes are not exposed suddenly to hostile environments. Without any apparent stress, death must occur by processes such as those of involuntary autolysin activity, as described earlier. In continuous culture systems at low growth rates it has long been recognised that a large proportion of the microbes is 'non-viable' or 'dead' [143,129]. Tempest et al. [219] found that the viability of *A. aerogenes* (*K. pneumoniae*) was as low as 40% at a dilution rate of 0.004 h^{-1}, but increased to 90% above 0.5 h^{-1}. Although this data represented evidence of death within a continuous culture, note that the cell enumeration method used was the slide cultivation technique of Postgate et al. [220]. The possibility that such slow-growing organisms (at $D = 0.004$ h^{-1}, the doubling time = 173 h or 7.2 days) fail to adapt to the agar environment and form colonies does exist and limits extrapolation of these results.

Sinclair and Topiwala [221] presented one of the first models for growth in continuous culture which considers the viability concept and uses the results of Tempest et al. [219] and Postgate and Hunter [143] to verify their model. Two mechanisms were assumed to act on the living biomass, x: (1) cell death, which was assumed proportional to the living biomass; and (2) endogenous metabolism, also considered proportional to living biomass. Thus, Sinclair and Topiwala present the mass balance equation for biomass as:

$$dx/dt = \mu(s)x - k_e - \gamma x - Dx \tag{9}$$

Where γ is the specific cell death rate constant (t^{-1}) and D is the dilution rate (t^{-1}). Similarly the mass balance for s appears as:

$$ds/dt = D(s_0 - s) - \frac{\mu(s)x}{Y_{x/s}^{max}} \tag{10}$$

where s_0 is the influent substrate concentration, s the residual substrate concentration and $Y_{x/s}^{max}$ the yield that would be attained in the absence of decay and cell death.

Thus, the steady-state concentration of dead cells ($\bar{x}_d$) can be written as

$$\bar{x}_d = (\gamma/D)\bar{x} \tag{11}$$

and for steady-state soluble substrate, ($\bar{s}$),

$$\bar{s} = \frac{K_s(D + K + \gamma)}{\mu_m - (D + K + \gamma)} \tag{12}$$

The viability can be calculated using Eqn. 13

$$\text{viability } (\nu) = \frac{\bar{x}}{(\bar{x} + \bar{x}_d)} = \frac{\bar{x}}{\bar{x}_t} = \frac{D}{D + \gamma} \tag{13}$$

The specific death rate constant can be determined, rearranging Eqn. 13,

$$1/\nu = 1 + \gamma/D \tag{14}$$

and plotting the inverse of viability versus inverse dilution rate, the slope would equal γ.

Using these equations, Sinclair and Topiwala [221] satisfactorily predicted a decrease in biomass at low dilution rates with concomitant low viability.

Weddle and Jenkins [222] analysed pilot- and full-scale activated sludge systems for cell viability and activity using various techniques including ATP, dissolved oxygen uptake, cell counts (using enrichment on agar) and electron transport system activity using the compound 2,3,5-triphenyltetrazolium chloride. They suggested the existence of non-viable organisms and of non-degradable inert biomass fractions. Grady and Roper [223] derived a model for the activated sludge process which predicted cell viability as a function of the mean residence time by incorporating the processes of death, maintenance (endogenous decay) and loss of viability in the absence of death. These authors recognised the existence of those cells which while not dead, were incapable of replication. They further suggested that these cells could have functional enzyme systems and were therefore susceptable to death and decay by endogenous metabolism and lysis. They also commented on the influence of 'cryptic' growth on the system but chose not to model it due a lack of experimental capabilities required for verification. The contribution of non-viable cells to the metabolic activity of the system was discussed by Jones [224], who divided biochemical activity in a heterogenous plug flow industrial process into the following categories, each group having its own appropriate growth kinetics: (i) Bacteria which grow within the system (Monod kinetics); (ii) bacteria which do not divide within the system but are viable (maintenance kinetics); (iii) Non-viable cells which still retain some metabolic (biochemical) activity (Michaelis–Menten kinetics). The growth of group (i) microbes maintains suitable concentrations of groups (ii) and (iii) in the system, such that substrate removal approaches first order kinetics [223].

The effect of non-viable, metabolically active

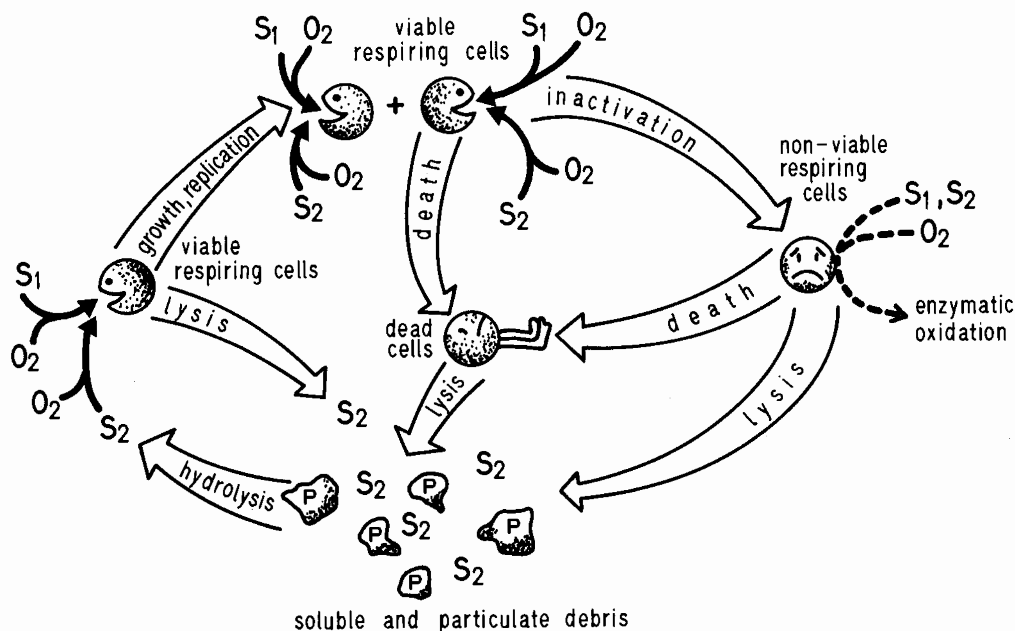

Fig. 13. Physiologically differentiated forms of biomass present in a continuous culture system and the fundamental processes occurring (From [162].)

cells on the yield coefficient was also shown by van Uden and Madeira-Lopes [6] in their model of yeast growth in chemostats at superoptimal temperatures.

Within the last 10 years, more accurate methods have evolved to determine the various types of microbe that can exist in a culture. Mason et al. [162] depicted the processes of microbial growth and death in continuous culture, as shown in Fig. 13. They recognised the possibility for the presence of each of the various categories of cells and their interrelationship and attemptted to experimentally differentiate between them. Differentiation between metabolically active and non-active (dead) cells was possible based on ETS activity. Their results suggested that a very low fraction, if any, of the cells present were indeed dead which appears to disagree with much of the earlier work suggesting the contrary. However, it should be noted, that much of that earlier data were derived using plate count methods which were totally inadequate for quantitative assessment. The model proposed by Mason et al. [162] suggests that the reduction in biomass yield results from a low level of lysis in the culture and in the model this lysis process and the subsequent growth of the intact organisms on their own lysis products ('cryptic' growth) are represented. Consequently this model may be suited to describe more accurately the behaviour of organisms in natural environments where cell decline may result from cell lysis and actual death occurs rarely, if at all. Hamer and Bryers [146] and Hamer [225] using the same principles, incorporated lysis and 'cryptic' growth in models which satisfactorily describe the effects of these processes in wastewater and waste sludge treatment processes.

Future prospects in the modelling of net microbial growth will have to focus on the utilization of particulate substrates present in the system feed and produced by particulate formation after cell lysis [95]. The possible presence of a non-biodegradable inert biomass fraction accumulating during microbial growth may also affect the calculation of the yield coefficient [226] and the assessment of the efficiency of microbial biomass production, product formation and product utilisation.

7. CONCLUDING REMARKS

Despite more than a century of microbiological research, emphasis still tends to be placed on the growth of microbes, and little, if any, attention is focused on their demise. In natural and man-made (including laboratory) environments, microbial 'death' or decline is a process which occurs hand-in-hand with microbial growth. Microbes are neither immortal, nor are they any less accident-prone than other creatures. Since they are not the highly efficient machines they are frequently made out to be, it is time that the effect of their frailness be properly considered in process design and evaluation, in public health investigations and in eco-physiological research. In order to satisfactorily achieve this, it requires a better understanding of the methods available, their limitations and the extent to which deductions can be made from the results they generate.

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