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Microbial consortia for multiple pollutant biodegradation

Geoffrey Hamer

Department of Chemical Engineering, University College Dublin, Belfield, Dublin 4, Ireland

Abstract: In spite of major emphasis that is placed on both clean(er) technology and clean(er) production routes, the time necessary for such developments will be measured in terms of decades rather than years. During the interim, end-of-pipe technologies, particularly biotreatment, will remain the primary defence against the ravages of environmental pollution. In addition, questions of effective biodegradation for the *in situ* amelioration of historical pollution are of increasing concern.

Unlike microbially mediated production processes, microbially mediated environmental protection and restoration processes involve process cultures comprizing multiple microbial consortia and it is individual consortium performance, rather than individual strain performance, that is critical as far as both process efficiency and economics are concerned.

For its first hundred years as an independent discipline, microbiology was very largely restricted to the study of pure mono-cultures growing on single carbon energy substrates. Biotreatment processes involve multiple substrates (pollutants) and highly complex mixed microbial cultures, irrespective of whether it is wastewater, waste gas or waste slurry streams that are undergoing treatment. Furthermore, biotreatment processes function in the continuous flow mode, frequently under unsteady state conditions, and involve multiple elemental (biogeochemical) cycles, whereas most physiological data have been generated under either batch or steady state continuous culture conditions. Such data have often been used to provide an erroneous basis for biotreatment process design.

INTRODUCTION

In spite of the fact that aerobic biotreatment remains a preferred technology for the elimination of biodegradable pollutants from wastewaters, waste slurries, waste gas streams and seriously polluted environmental compartments, including soils, sediments, groundwaters and surface waters, remarkably little research concerning the dynamics of multiple pollutant degradation by microbial consortia has been conducted. Biodegradation research has emphasized biochemical pathways, assumed kinetic relationships, disregarded mixed cultures, particularly quasi stable consortia, and failed to relate physical and chemical changes in process conditions with either biodegradative potential or capacity. This has resulted in distorted understanding of factors affecting process performance and, hence, retardation of the development of a sensible basis for biotreatment process optimization.

Until recently it has been common practice to classify biotreatment processes on the basis of the physical characteristics of the waste stream undergoing treatment. Essentially, wastewater streams containing soluble pollutants, polluted waste air streams and waste slurries have been examined on the basis of their different physical properties, rather than on the basis of the frequently common microbially mediated reactions responsible for their effective treatment. Biotreatment seeks to harness, control and accelerate reactions normally involved in natural self-purification, i.e., the reactions of the geobiochemical (or elemental) cycles for carbon, nitrogen, sulphur, etc. In biotreatment processes, the elemental cycles are invariably considered in isolation whereas it is frequently interactions between cycles that are of primary importance for effective comprehensive treatment.

Microorganisms can be present in biotreatment processes as discretely dispersed cells, as flocs or as biofilms. The latter two are by far the most common and both flocs and films can be considered as matrices of naturally immobilized cells. Since immobilized microbial cells were first investigated, there have been repeated claims that immobilization results in enhanced performance, claims which will most probably be explained on the basis of phenotypic responses to environmental gradients at a future date.

Koch (ref. 1) was the first to describe an effective procedure for the isolation of pure cultures of bacteria, a problem that had tested both the ingenuity and skill of all microbiologists prior to that time. Without doubt Koch's achievement revolutionized the whole gamut of microbiology and an experimental tradition involving the exclusive use of pure monocultures for some ninety years not only profoundly influenced the course of microbiology but resulted in a most unfortunate consequence, i.e. extensive study of what are essentially laboratory artifacts. The failure to recognize that the interactions within microbial systems represented real conditions pertaining to unprotected environments was a direct result of Koch's mono-culture philosophy (ref. 2).

Since Monod (ref. 3) published his pioneering work on the batch growth of bacteria, it has been widely accepted that binary mixtures of carbon energy substrates are, during heterotrophic growth, utilized sequentially in a biphasic manner, with the substrate that supported the higher maximum specific growth rate coefficient generally being utilized first. This phenomenon, known as diauxic growth, has been contradicted by examples where sequential utilization occurs without the intermediate lag that characterizes diauxic growth and by simultaneous substrate utilization in batch cultures. In the case of chemostat cultures, substrate mixtures that exhibit sequential utilization during batch growth are utilized simultaneously as has been shown (refs. 4,5), suggesting that diauxic growth is also a laboratory artifact.

A third artifact that should be mentioned involves oscillations that result from the intermittent addition of feed streams to laboratory-scale continuous flow bioreactors. This particular occurrence was extensively discussed by Harrison and Topiwala (ref. 6), when particular emphasis was placed on the intermittent addition of methanol to cultures of methylotrophs and its effect on biomass yield coefficient depression. Interestingly, the biomass yield coefficient depression also became a problem, fortunately resolvable, in scaled-up pressure cycle bioreactors used for single cell protein production from methanol. Essentially, the key assumption of growth theory that yield coefficients remain constant over a significant range of substrate concentrations could no longer be upheld, certainly as far as potentially inhibitory and/or toxic substrates are concerned.

BIOTREATMENT PROCESS CULTURES & POLLUTANT CATEGORIES

As far as the biodegradation of carbonaceous pollutants in activated sludge treatment plants is concerned, it is chemoorganotrophic and mixotrophic bacteria that function as specific organic pollutant degraders. As far as the predominant genera and species that comprize the biodegradativecapacity are concerned, remarkably few definitive studies have been undertaken. Microbiological investigations of activated sludge processes frequently emphasize the rôles of biopolymer producing microbes in floc formation, of filamentous microbes in bulking, scum and foam formation and protozoa in maintaining low suspended solids contents in the treated water discharge.

General statements concerning the composition of activated sludge treating municipal sewage (ref. 7) indicate that it comprizes *Pseudomonas*, *Flavobacterium*, *Alcaligenes*, *Acinetobacter* and *Zoogloea* spp. although clearly, these are only the predominant genera present (refs. 8,9). Other researchers who have investigated the composition of activated sludge from municipal treatment plants have found *Pseudomonas* and *Acinetobacter* spp. and *Enterobacteriaceae* (refs. 10,11) to be the dominant bacteria present, while under conditions where restriction in oxygen supply occurs, the dominant species are from the genera, *Acinetobacter*, *Pseudomonas*, *Aeromonas* and *Flavobacterium* (ref. 12). As far as activated sludge processes treating chemical industry wastewater are concerned, Blaim *et al.* (ref. 13) have reported that under conditions of good performance *Alcaligenes* and *Pseudomonas* spp. are dominant. However, it should be mentioned that the designation of many strains as *Pseudomonas* spp. is not in accord with the strict criteria proposed for this genus *Pseudomonas* (ref. 14). Unfortunately, the bacterial consortium concept is virtually undeveloped as far as activated sludge is concerned, although Hawkes (ref. 15) has suggested that individual flocs can be regarded as "mini"-communities. However, it is worthy of note that many strains belonging to both the dominant and the subordinate species present in activated sludge are common ancillary constituents of various specific substrate degrading consortia that have been characterized.

Organic pollutants are only one category of pollutants that are removed from wastewaters during biotreatment. Others include, nitrogenous pollutants, phosphates, ions and salts of various metals and metalloids and pathogenic organisms.

Ammonium ion is the predominant nitrogenous pollutant present in settled sewage and in view of the fact that it is present in disproportionately high concentrations to be removed by bacterial growth and that it is toxic to fish (ref. 16), a major sewage treatment process objective where the treated water is discharged directly to surface water bodies is modification of ammonium, by the action of nitrifying bacteria, via nitrite, to nitrate. The responsible bacteria are virtually always considered to be a chemolithotrophic consortium of *Nitrosomonas* and *Nitrobacter* spp. (ref. 16), although such statements ignore any possible rôle that chemoorganotrophic nitrifying bacteria (ref. 17) might have in activated sludge processes. The denitrification of nitrate to dinitrogen will increasingly become a requirement in sewage treatment, but relatively little of presently installed plant capacity has been designed to achieve effective denitrification. However, in spite of this, some denitrification is inevitable in plants where nitrification is occurring and, in the future, denitrification will undoubtedly become a major process objective.

Traditionally, denitrification processes are thought to produce dinitrogen, an environmentally acceptable end product that can be released to the environment without adverse effects. However, there is increasing evidence that intermediates in the denitrification pathway, particularly nitrous oxide, are frequently formed and liberated during denitrification. Nitrous oxide is one of the gases that is implicated in both the tropospheric "greenhouse" effect and in the destruction of the stratospheric ozone layer, so that microbiological processes leading to incidental nitrous oxide production and release into the atmosphere need to be controlled. It has been suggested that oxygen availability and acidic pHs are primarily responsible for nitrous oxide production during denitrification (refs. 18,19). Hochstein *et al.* (ref. 20) reported that for steady state nitrate-limited chemostat cultures of *Paracoccus halodenitrificans* increasing concentrations of dissolved oxygen in the culture supernatant caused the end product of denitrification to change first from dinitrogen under anoxic conditions, to nitrous oxide and then to nitrite, prior to the dissolved oxygen concentration completely inhibiting the process. These changes were explained by inactivation of the nitrous oxide reductase by oxygen and by diversion of electrons from nitrite to oxygen. Certainly no reduction in the availability of reducing equivalents would have occurred, because these experiments were conducted under nitrate limitation.

During growth in carbon energy substrate-limited continuous cultures, where nitrate was the only nitrogen source available in the culture medium, *Pa.denitrificans* was restricted with respect to the availability of reducing equivalents so that an inadequate supply of electrons was available to the denitrification enzymes for completion of the denitrification process, so that considerable production of nitrous oxide occurred (ref. 21). This finding has important implications for biotreatment process operation, where operating conditions that tend to minimize sludge yield coefficients are generally sought, because such operating conditions will also markedly reduce the availability of reducing equivalents. In biotreatment processes, where simultaneous carbonaceous pollutant removal and nitrification occur, simultaneous denitrification will also occur. However, as denitrification will most probably be occurring under conditions where the availability of reducing equivalents is restricted, nitrous oxide production and its attendant release to the atmosphere can be predicted. While the results of Omlin (ref. 21) provide considerable insight into the specific problems that cultures of *Pa.denitrificans* experienced in adapting from an oxic to an anoxic environment, they also highlighted the enormous need for much more experimentation on defined transient state behaviour in order to allow an improved understanding and even prediction of bacterial behaviour in activated sludge biotreatment processes where transient state conditions predominate.

Phosphates are another category of pollutants that are present in settled sewage at concentrations that markedly exceed the possibilities offered for removal by normal bacterial growth processes. Although phosphate levels in sewage can be significantly reduced by the substitution of polyphosphates in laundry detergent formulations by chelating agents such as nitrilotriacetate, levels will still be about twice those that can be removed by typical bacterial growth.

Various methods have been proposed for the elimination of phosphates during sewage treatment. Among these are chemical techniques which involve the addition of iron salts either during biotreatment or in a tertiary treatment process (ref. 22), to precipitate phosphates and biological techniques where "luxury phosphate uptake" is encouraged during biotreatment. The consistency of biological techniques for phosphate elimination have been disputed for 25 years and considerable controversy exists with respect to modes of action, i.e., the process biology involved, although *Acinetobacter* spp. are generally considered to be the bacteria that are primarily responsible (ref. 23). It has been claimed (ref. 24) that biological

phosphate removal is economically superior to chemical techniques for phosphate removal and should, therefore, become the widely used technology. However, the sensitivity and fastidiousness of biological removal processes are such as to make them unreliable.

Municipal sewage, in most industrial cities and towns, is frequently seriously contaminated with toxic metal ions. The origin of much heavy metal pollution is trade effluents, particularly from the metal finishing industries, that are discharged into the sewers. The metal ion concentrations of such discharged effluents usually range between 0.1 and 10 mg l^{-1}, levels that do not permit economic and/or effective recovery by conventional recovery technologies. The heavy metals that are of the greatest concern, with respect to human health, are mercury, cadmium, chromium and lead. The uptake and/or removal of toxic metal ions by microbes in conventional activated sludge treatment processes has been an accepted process feature for many years and the importance of this in overall treatment has never been questioned. Even so, in spite of the obvious importance of the mechanisms involved in metal removal, such mechanisms have been subjected to little more than cursory investigation as far as wastewater treatment is concerned.

Microbes have developed various strategies in order to maintain low intracellular concentrations of toxic metals. The strategy adopted is very largely influenced by whether the metal in question is either essential or non-essential for growth. Essential metal ions have many important biological functions and specific regulated systems for their transport into microbes have evolved. Many essential metals are toxic to microbes at high concentrations and homeostatic mechanisms function in microbial cells to maintain the appropriate balance for effective biological function. The uptake of individual elements by microbes depends on both their chemical and physical properties. According to Wood and Wang (ref. 25), 30 of the 92 elements that comprize the Periodic Table have been found to be essential to the sum total of microbes. Carbon, nitrogen, oxygen and hydrogen are all required in bulk amounts, whilst some 26 other elements are required in intermediary to trace amounts. The apparent reason for the selection of these 26 elements seems to have been determined by their relative abundance in the earth's crust and their solubility in water under anaerobic conditions. Of the four metals that are of greatest concern with respect to adverse affects on human health, only chromium is an essential nutrient. Both lead and cadmium are non-essential for microbial growth, whilst mercury concentrations are below the threshold for inclusion in the list of crustal abundance.

It is not at all unusual to find populations of bacteria which can tolerate high concentrations of toxic metal ions. The ability to tolerate such environments rests on the ability of the microbes in question to prevent intracellular accumulation of metals to toxic levels. Five mechanisms have been identified that impart metal toxicity resistance to microbial cells; biomethylation and transport through the cell membrane of the resulting metal alkyl by diffusion controlled processes; biosynthesis of intracellular polymers which serve as traps for the removal of metal ions from solution; binding of metal ions to cell surfaces; precipitation of insoluble metal sulphides and oxides at cell surfaces; and alteration of the uptake enzyme in transport negative strains. All these natural processes have an important impact on the effectiveness of treatment processes in which effective removal of toxic metal ions is required. The enhancement of any or all of these mechanisms is dependent on the microbial community mediating the treatment process and on the environmental conditions pertaining within the process. In considering this latter aspect, it must be remembered that wastewater treatment processes never operate under steady state conditions and their process microbes inevitably function in gradients and are subject to both unpredictable transient changes in process conditions and more predictable short and longer term cyclical physical and chemical changes. In activated sludge biotreatment processes the process microbes are present as flocs which comprize a complex matrix of not only process microbes, but also extracellular, microbially produced, biopolymers. The composition of the biopolymers comprizing the non-microbial fraction of flocs depends on both environmental factors and on substrate and nutrient availability. The extracellular biopolymers in which microbes undergoing immobilized growth are embedded are of particular importance when considering metal sorption onto microbial flocs and films in biotreatment processes there has been a distinct failure to differentiate between the rôle of the microbial surface, on the one hand, and that of the extracellular associated biopolymer, on the other hand.

Lester and Sterritt (ref. 26) have provided data on heavy metal removal during activated sludge type secondary treatment which suggest that in general, mercury, cadmium, chromium and lead are removed with efficiencies in excess of 50 percent. However, they clearly make the point that removal is variable. The general ignorance concerning the microbiology of metals is alarming and requires remedial action just

as does the failure of many microbiologists to recognize the physical chemical properties of the systems with which they are dealing. A result that can be envisaged from such ignorance and lack of appreciation of known facts could be process operating protocols that result in the remobilization of toxic metals during biotreatment, thereby enhancing, rather than eliminating, the dangers from such pollutants when present.

Any wastewater stream that contains sanitary wastes, i.e., faeces and urine, will contain pathogenic organisms and, clearly, a major objective of treatment processes should be pathogen removal. The pathogens found in sanitary wastes include bacteria, protozoa, worm eggs and viruses. It is generally accepted that during activated sludge treatment the pathogens are concentrated in the waste sewage sludge, the major byproduct of these processes and it is assumed that pathogens are either absent or present in very low concentrations in both aerosols produced by aeration and in the treated water discharged from the treatment process. Virtually no actions are taken to eliminate aerosol formation, but in the USA disinfection of the treated water is practised and the use of chlorine in this respect has been extensively discussed both with respect to positive and negative effects. The primary problem in evaluating the quality of treated wastewater is the lack of accurate and reliable methods for determining the number of living organisms, particularly specific pathogens, present in water samples. In recent decades this problem has been exacerbated by the discovery of a non-recoverable state when certain viable pathogenic bacteria are present in aquatic environments (ref. 27).

MIXED CULTURES, COMMUNITIES AND CONSORTIA

It is inevitable that waste biotreatment processes are operated as unprotected systems allowing continuous reinoculation. The process cultures that develop are the result of continuous enrichment pressures and are inevitably mixed. The prediction of mixed culture behaviour still relies to a considerable extent on the classical ecological concepts that were extended to the discussion of binary strain/single carbon energy substrate interactions by Bungay and Bungay (ref. 28).

In the discussion of mixed culture interactions it is helpful to consider the organizational hierarchy that exists in microbial systems, namely communities, which are mixtures of interacting and non-interacting consortia, consortia, which are quasi stable interacting mixtures of diverse strains, individual strains of microbial cells and, finally, intra-cellular macromolecules. In spite of widespread acceptance of this hierarchy, the techniques employed for investigating microbial systems still emphasize fractionation, uncoupled from resynthesis, with the result that discoveries at the cellular level dominate. Biotreatment processes generally involve mixed carbon energy substrates and multiple nutrients serving each particular physiological requirement, clearly emphasizing a need for concept expansion in order to allow such processes to be evaluated. The ultimate performance of mixed process cultures employed in biotreatment processes depends on both intra-consortium and inter-consortia interactions within the overall community comprizing the process culture with respect to imposed process operating and environmental conditions.

Two distinct situations occur when the functioning of mixed cultures for specific pollutant biodegradation under essentially aerobic conditions is considered. These involve either the employment of a culture in which a complementary sequence of catabolic activities from several associated strains is harnessed in order to generate a complete degradative pathway for the pollutant under consideration or employment of a culture in which the fastidiousness of the primary pollutant degrading strain is diminished by the actions of several associated ancillary strains. Both types of mixed culture can result from enrichments but the former can also result from constitution using independently isolated strains from diverse sources. In the case of the latter, reconstitution can only follow complete fractionation of a mixed enrichment culture. In the case of the former type of mixed culture, the number of strains necessary for the constitution of the complete degradative pathway, in laboratory situations, reduce as a result of plasmid transfer (ref. 29), and such occurrences during the biodegradation of strictly xenobiotic compounds, have encouraged proposals for the use of genetically manipulated monocultures as process cultures for waste treatment processes (ref. 30). However, what has frequently been forgotten in the development of such concepts is that the transfer of a characteristic, i.e., a portion of a degradative pathway, allows neither the construction of entirely novel pathways nor the development of novel enzyme specificities. Furthermore, manipulation enhances fastidiousness and, hence, reduces strain competitiveness and performance under actual operating conditions.

The basis developed for describing mixed culture interactions (ref. 28) implies major direct interaction between primary substrate utilizing strains. Hence, one would expect that when a diverse mixed culture is enriched for strains that utilize the single carbon substrate supplied, the result would be either an essentially pure monoculture or a mixture of strains, both obligate specialists and facultative generalists, that are all capable of utilizing the specific carbon substrate under the changing conditions occurring during the enrichment procedure employed. However, what seems to be the common result for enrichments of inocula for specific carbon substrate utilization is a microbial consortium comprizing a single primary substrate utilizing strain growing in obligate association with between one and five ancillary, non-primary substrate utilizing strains that, as a result of protocooperation, optimize the microenvironment for the primary substrate utilizing strain, but collectively represent only a minor fraction of the consortium. Some of the best known examples of such consortia are the various methanotrophic consortia which were selected for single cell protein production from natural gas, but which have, more recently, been evaluated with respect to their potential utility in bioremediation processes (ref. 31). One of the most intriguing features concerning microbial consortia is the relatively restricted and frequently common identities of the ancillary strains present in diverse consortia, prompting questions concerning the effectiveness of possible exchange of ancillary strains between consortia, a feature which could well provide the key for effective bioaugmentation. The development of a dominant consortium rather than of a dominant strain during enrichment for a specific biodegradative capacity suggests that in diverse mixed process cultures of the type that mediate the spectrum of biodegradative activities that occur in aerobic biotreatment processes, primary interactions occur not between individual primary substrate utilizing strains, as has been assumed from the concept of binary strain interactions, but between the various specific primary substrate utilizing consortia present.

Some of the best examples of enrichment cultures comprizing several specific consortia involve the biooxidation of synthetic wastewaters containing simple organic compounds. In the case of the simultaneous biooxidation of methanol, phenol, acetone and *i*-propanol, Wilkinson and Hamer (ref. 32) found that three separate consortia were responsible. One consortium oxidized methanol, the second oxidized phenol, whilst the third oxidized both acetone and *i*-propanol, but perhaps most remarkable of all was the fact that the three consortia were sufficiently non-interactive so that a state of neutralism prevailed between them. Further, culture response to transient changes in operating conditions resulted in the rapid reestablishment of stability, unlike the prolonged instabilities that can be observed for primary substrate utilizing binary cultures when they are subject to transient operating conditions (ref. 33). Bitzi *et al.* (ref. 34) have reported that for the simultaneous biooxidation of methanol, acetone, *i*-propanol and dichloromethane, superior rates could be achieved when two primary substrate utilizing consortia, rather than when a binary mixed culture comprizing only the two primary substrate utilizing strains, were responsible. One of the most important questions with respect to the mediation of biooxidation in wastewater treatment plants concerns the rôle of mixotrophs, particularly *Alcaligenes* spp . Clearly, mixotrophy confers markedly enhanced metabolic versatility on microbes with such capabilities and, probably, also marked competitive advantages, particularly under unsteady state operating conditions. In general, the rôles of chemoorganotrophic bacteria in wastewater treatment processes are separated from the more restricted rôles attributed to chemolithotrophic bacteria, but whether such separation can be justified on the grounds of process functionality is questionable.

A fundamental problem in assessing the total microbial resource and the total metabolic potential in biotreatment processes is the inability to either isolate or characterize most of the microbes present in such systems. Traditional techniques for analysing the composition of complex mixed microbial populations require that any microbe present must grow and multiply under laboratory culture conditions before it can be isolated and, subsequently, characterized. Further, a prerequisite of traditional characterization procedures is that similar strains must previously have been characterized. Estimates of the undiscovered microbial resource in natural ecosystems suggest that this probably represents more than 80 percent of the total microbes present in such ecosystems (ref. 35). Recently, molecular genetic techniques for exploring the extent of the undiscovered microbial resource have become available. Using one such technique, involving the determination of 16 S ribosomal RNA sequences, Ward *et al.* (ref. 36) have shown that in a microbial community that had previously been thought to be well characterized using conventional methodology, in excess of 80 percent of the microbes present still remained to be isolated and characterized.

PHYSIOLOGICAL STATES AND PERTURBATIONS

It is only relatively recently that the dynamic behaviour of microbial cells during growth and respiration has become widely accepted. Previously, many studies considered microorganisms as if they were abiotic particles of constant composition, size and properties, irrespective of the environmental conditions to which they were exposed. In fact, the important pioneering work of Herbert (ref. 37) concerning the phenotypic variability of bacterial cells was largely ignored by those responsible for the research and development of microbially mediated processes. Fortunately, it is now accepted that bacteria have both mechanisms that allow rapid growth under balanced (favourable) environmental conditions and different mechanisms that permit survival under a diverse range of conditions that are adverse to growth. The intracellular proteins necessary for rapid growth are present in the cell in requisite quantities, but those required for protection, growth under adverse conditions and/or damage repair are usually presently only in low concentrations in a bacterium undergoing rapid growth, but are synthesized when needed. This suggests that when a bacterium that is undergoing rapid growth under essentially optimum conditions is subjected to a change in conditions, either as a result of an externally imposed change or as a result of an environmental change mediated by the growth process itself, it initiates a sequence of protective measures, involving both gene expression and control of enzyme activity, which enhance its survival potential. However, what actually constitutes adverse conditions remains to be defined, but clearly, it seems to be intimately associated with the stress protein response. The problem that now arises is reconciling the observation that in chemostat culture of *Escherichia coli* , increasing levels of heat stress protein synthesis occurs throughout the optimum temperature range for growth with increasing temperature (ref. 38), but without significantly affecting overall culture performance measured in terms of specific growth rate. Even so, in the case of chemostat cultures, the apparent steady state conditions that occur represent, at best, a pseudo-steady state, even under conditions where complete mixing is said to exist. By using two-dimensional gel electrophoresis, Harendeen *et al.* (ref. 39) investigated the levels of 133 individual intracellular proteins, including heat shock proteins, present in *E. coli* B/r during exponential growth throughout most of the temperature permissible range for growth. The most important conclusion from this work was that within the Arrhenius temperature range, metabolic coordination was achieved primarily by modulation of the amount of enzyme present, but within the optimum and superoptimum temperature ranges for growth, conditions where the effects of heat stress were either starting to or had become evident, the levels of many proteins either decreased or increased, indicating temperature dependent regulation of their synthesis at both the transcriptional and translational levels.

The heat shock response is the best known example of regulation at the level of transcription. However, in addition to the heat shock (or heat stress) response, a wide range of other physical and chemical stresses, particularly starvation, have been shown to invoke stress responses in which enhanced intra-cellular levels of sub-sets of heat shock proteins are produced (refs. 40,41). The stress proteins have now been grouped by Völker *et al.* (ref. 42) into general stress proteins and heat-specific stress proteins.

Anaerobiosis has, in the case of *Salmonella typhimurium*, been cited by Spector *et al.* (ref. 43) as a promoter of stress protein formation, but in their study it was not entirely clear whether it was oxygen limitation or anaerobiosis that was under consideration, as the two terms were used interchangeably. In spite of extensive discussions of nutrient and substrate starvation, it is not possible to ascertain the point where starvation and limitation coincide, particularly when oxygen is the nutrient under consideration.

In the case of obligate aerobes, oxygen limitation must be regarded as a stress, in a similar context to heat shock, but in the case of facultative anaerobes, it might also be regarded as a promoter of alternative metabolism, under which entirely different conditions for optimum (balanced) growth apply. The proteins induced by both anaerobiosis and aerobiosis in *E. coli* have been examined by Smith and Neidhardt (refs. 44,45) and, in addition, examination of shifts between aerobic and anaerobic conditions were also investigated. Because of the uncontrolled nature of the culture systems employed, measurements of the dynamics involved were not possible and the data reported refer to the two end-point situations that occurred during balanced aerobic and balanced anaerobic growth.

Incomplete mixing on both the micro and macro scales in aerobic waste treatment processes will allow the intermittent occurrence of anaerobic regions within the process environment, a feature that is of undoubted significance when facultative anaerobic bacteria are concerned with process mediation. In the particular case of facultative anaerobes, the dynamics of the transition from aerobic, through oxygen limited, to anaerobic growth and *vice versa* is little understood even as far as macro effects are concerned.

One of the first attempts to evaluate the macro effects of aerobic-anaerobic and anaerobic-aerobic switches on continuous cultures of the facultative anaerobes, *Klebsiella aerogenes* and *E. coli*, was reported by Harrison and Loveless (refs. 46,47). In these studies, it was shown that reduced cell yield coefficients and increased oxygen uptake rates occurred at low dissolved oxygen concentrations, but without any increase in the formation of dissolved extra-cellular carbonaceous products, in spite of the ability of *E. coli* to mediate a mixed acid fermentation from glucose. In addition, a loss of tight coupling between growth and energy conservation was identified during transitions between anaerobic and aerobic growth. Clearly, the aerobic and the anaerobic utilization of glucose by *E. coli* involves different enzymic activities and it was Thomas *et al.* (ref. 48) who found that the change from respiration to fermentation was initiated well before the dissolved oxygen concentration reached zero, on the basis of an increase in the concentrations of those enzymes that mediate the latter process and a concomitant decrease in those mediating the former process. In addition, changes in glucose regulation were also reported to be involved. Attempts to exploit the effects of oxygen availability switches for biomass yield coefficient minimization in activated sludge processes have been reported(refs. 49,50). Essentially, the mechanism that appeared to be involved was the uncoupling of oxidative phosphorylation.

YIELD COEFFICIENT MINIMIZATION

Biomass yield coefficient minimization is a major requirement in processes for the biotreatment of sewage and industrial wastewater, waste slurries and sludges and waste gases. In the case of the first two types of waste stream, high biomass from substrate yield coefficients result in excessive production of a major byproduct, i.e., waste biomass (sludge). In such cases, the waste biomass is unstable and potentially putrefactive, is frequently difficult to dewater by simple cost effective means and is the sink for accumulated sorbed noxious pollutants and potentially pathogenic agents. In the case of waste gas biotreatment, excessive biomass production results, in system fouling, in the case of biofilters, and in waste sludge disposal problems, in the case of bioscrubbers.

As has already been mentioned, biomass yield coefficients for particular strains and carbon energy substrates are system operating parameter dependent and, accordingly, are frequently subject to marked variations. In the case of a pure mono-culture growing on a single carbon energy substrate under steady state conditions, the biomass yield coefficient will approach a maximum value, usually some 80 percent of the theoretical maximum. Under virtually all other operating conditions, biomass yield coefficients are depressed. However, from a biotreatment process performance point of view, depression must not detract from biodegradation capacity.

In the laboratory, major emphasis with respect to biomass yield coefficient reduction is placed on the effects of what is termed either endogenous metabolism or maintenance requirement. However, in practical biotreatment processes, where one is dealing with mixed cultures and multiple substrates and nutrients satisfying particular physiological requirements, the major factors contributing to biomass yield coefficient reduction are cometabolism (cooxidation), the uncoupling of growth and respiration, and death by lysis and subsequent "cryptic" growth (exogenous metabolism).

Cometabolism incorporates oxidations, dehalogenations, condensations and rearrangements, but as far as yield coefficient minimization is concerned, it is cooxidation that offers the greatest impact. The strict definition of cometabolism is the microbial transformation of a compound which is unable to support cell replication in the requisite presence of a transformable co-substrate that supports cell replication. Essentially, oxidation is a zero biomass from a transformable compound yield coefficient process. The strict definition excludes situations, described as fortuitous oxidation, where the non-specific nature of certain monooxygenases results in the transformation of non-growth substrates in the absence of a co-substrate, in spite of the fact that the effects of such processes can only be deemed positive as far as biotreatment processes are concerned.

In the case of multi-consortia that oxidize mixtures of methanol, phenol, acetone and *i*-propanol present as primary carbon energy substrates (ref. 32), essentially neutralistic (non-interactive) behaviour was observed between the three distinct consortia that developed. These utilized methanol, phenol and acetone, respectively, with *i*-propanol being cooxidized by the acetone utilizing consortium. Clearly, it could have been expected that four distinct consortia would have been involved in the oxidation of four primary substrates, particularly because consortia utilizing *i*-propanol can be readily enriched in the absence of

acetone. Certainly the identification of a cooxidizing consortium was a surprize, but perhaps more important was the observation that energy resulting from the cooxidation of *i*-propanol enhanced the biomass from acetone yield coefficient (ref. 51), and that to achieve cooxidation, an acetone: *i*-propanol ratio in excess of 1.18 was necessary. Cooxidation has yet to be systematically exploited in biotreatment processes. Prior to achieving systematic exploitation, it will be necessary to determine the ratios of co-substrate:cooxidized substrate required.

In a recent study dealing with the simultaneous bioxidation of 2-butanone and 4-methyl-2-pentanone (ref. 52) by an enrichment culture in continuous flow culture, dramatic reduction of the yield coefficient for growth on 2-butanone occurred as a result of both major perturbations in the feed concentrations to the bioreactor and by pulse additions of the ketones to the bioreactor. During unsteady-state operation, accumulation of 2-butanone was also a common feature of operation.

Very few studies concerning the growth of microorganisms take any account of microbial death. So much so, that Mason *et al.* (ref. 53) commented that microorganisms are not immortal. The fundamental problem that is encountered in differentiating between living and dead microorganisms is that for a microbe to be identified as live in conventional test procedures, it is also necessary for the microbe to be viable. Microbes can, on a physiological basis, be classified as viable and active, non-viable and active, dormant or dead (ref. 54). However, in investigations where efforts have been made to identify dead microbes as a component state in growing cultures, numbers have been shown to be vanishingly small (ref. 54), prompting the hypothesis that death occurs as a result of lysis, such that under typical growth conditions death and lysis are coincident events.

"Cryptic" growth is the process whereby microbes of either the same or of different strains grow on the solubilized lysis products of a particular strain. The process is widely known as an important step in both anaerobic and aerobic sludge digestion processes, but under most other circumstances, it is ignored, in spite of the fact that it has been shown to be of much more significance in the growth of pure cultures of *K. pneumoniae*, as a mechanism for yield coefficient reduction, than is endogenous metabolism. Very clearly, "cryptic" growth is a major feature during the growth of consortia. Essentially, the process involves repeated reuse of substrate carbon, such that the repeated passage of carbon through the death/lysis - "cryptic" growth cycle results in progressively reduced biomass yield coefficients (ref. 55).

BIODEGRADATIVE CAPACITY

Modern biodegradation research has emphasized the elucidation of metabolic pathways, but failed to recognize most of the important aspects of microbial physiology that are also involved. This has resulted in a surfeit of kinetically barren metabolic maps. However, as Kacser (ref. 56) has pointed out, the individual chemical transformations which constitute activity in microbial cells are primarily organized in a kinetic manner. Therefore, when considering the biodegradation of an individual pollutant it is necessary to examine the system as a whole rather than examine individual enzymes and the specific reactions they mediate. In fact, all the enzymes comprizing a specific pathway are clearly coupled to one another by the metabolites they share and further, the rates and fluxes through particular enzymes are significantly affected by adjacent enzymes, so that the concept of a single rate-determining enzyme in any pathway is invalid.

During the last 20 years, a methodology known as metabolic control analysis, has emerged for characterizing metabolic systems by using response, control and elasticity coefficients. The methodology seeks to characterize the sensitivity of metabolic responses with respect to either changes in enzyme activities or parameters without the use of comprehensive structured mathematical models. Metabolic control analysis can be applied to metabolic systems in general, but with the restriction that they possess an asymptotically stable steady state or quasi steady state. In addition to enzymic reactions, both non-enzymic reactions and transport processes can be included as additional steps in the analysis. Spatial variations with systems can be lumped into compartments and transport processes between them are modelled as reactions.

Metabolic control analysis has primarily been concerned with description of metabolic regulation at the steady state, essentially quantifying how changes in parameters modify steady state responses, but it has been extended to the control of transient states. Metabolic control analysis has been applied mostly to the flux substrates and products during the growth of pure monocultures, but recently has been extended to consider the binary interaction known as commensalism in chemostat cultures (ref. 57).

What is clearly evident is that a better understanding of biodegradation kinetics will undoubtedly enhance possibilities for more effective and efficient biotreatment. In this respect, an alternative formalism to metabolic control analysis that is receiving increased attention is the mosaic non-equilibrium thermodynamic model for microbial growth (ref. 58), in which an ensemble of independent metabolic compartments, into which relevant biochemical information can be inserted, and which obey both the laws of thermodynamics and kinetic principles, has been developed. Perhaps the most important feature of the model is that it can be expanded to any desired level of complexity by the addition of specific information. In the simplest configuration of the model, microbial metabolism is considered to comprize three separate, but mutually dependent, fluxes. These are, a catabolic flux in which catabolic substrates are converted into endproducts, coupled to production of energy-rich molecules, an anabolic flux, in which energy-rich compounds are used together with anabolic substrates for the formation of new cells, and a "leakage" flux that consumes energy-rich compounds, but is not coupled to the formation of new biomass. However, the anabolic flux can be further subdivided into those for protein, DNA, RNA, lipid and polysaccharide, as has been done by Mulder *et al.* (ref. 59). Such subdivision is particularly important when one considers the, all too frequently ignored, facts that metabolic activity is a function of macromolecular, rather than the elemental, composition of microbial cells, that growth rate markedly affects macromolecular composition, and that macromolecular composition is affected by both substrate and nutrient limitations. Furthermore, it is such phenotypic variability and the intrinsic flexibility of microbes which determine culture dynamics. In many respects the mosaic non-equilibrium thermodynamic model for microbial growth seems to offer the best prospects for extension to both the markedly heterogeneous physiological environment encountered in microbial consortia and the continuously perturbed state that exists in real biotreatment processes. However, it must be borne in mind that any formalism will be invalid unless pollutant levels can be expressed in terms of individual pollutant concentrations, rather than in terms of "lumped" parameters.

METHANOTROPHIC CONSORTIA

Relatively few microbial consortia have been studied at the level of their component strains. Probably the best examples of such studies are those by Wilkinson *et al.* (ref. 60) and Linton and Buckee (ref. 61) concerning methanotrophic bacterial consortia growing on methane, the major component of natural gas. However, it was Higgins *et al.* (ref. 62) who were the first to recognize the potential, with respect to diverse pollutant biodegradation, that exists because of in vivo expression of broad-specificity methane monooxygenases in methanotrophs. The ability of methanotrophic bacteria to cometabolize various liquid *n*-alkanes had already been reported by Leadbetter and Foster (ref. 63), but identification of the ability of methanotrophs to mediate not only oxidations, but also oxidations coupled with dechlorination, condensation or rearrangement, sometimes using additional enzyme systems, producing relatively easily biodegradable intermediates from apparently recalcitrant pollutants demonstrated hitherto unsuspected biodegradative capacity. More recently, Ensley (ref. 64) has also indicated similar cometabolic capacities for the well-known chemoautotrophic nitrifying consortium comprizing *Nitrosomonas* and *Nitrobacter* spp.

Of the two definitive studies that have been performed concerning the growth of methanotrophic consortia, it is that of Wilkinson *et al.* (ref. 60) that is the most illuminating as far as intra-consortium interactions are concerned. The consortium studied comprized a type II methanotroph, a facultative anaerobic methylotrophic *Hyphomicrobium* sp., capable of denitrification at dissolved oxygen concentrations below that corresponding to saturation at an oxygen partial pressure of 0.05 bar, and two heterotrophic bacteria, an *Acinetobacter* sp. and a *Flavobacterium* sp. The explanation proposed for such a culture composition was that the obligate type II methanotroph converted a small fraction of the methane utilized into methanol, which is potentially self inhibitory, rather than producing only bacterial biomass and carbon dioxide, as a stable end product, and that the byproduct methanol produced served as a carbon energy substrate for the *Hyphomicrobium* sp., which, at low dissolved oxygen concentrations, did not compete with the methanotroph for oxygen because of its ability to use nitrate as an alternative electron acceptor. Furthermore, it was postulated that the rôle of the two heterotrophic strains was to use inhibitory lysis products for "cryptic" growth.

Equally important to its versatility as far as the carbon cycle is concerned, is the rôle the methanotrophic consortia can play with respect to the nitrogen cycle, a feature that has been frequently disregarded because of the failure of methanotrophs to assert themselves under the typical conditions used for the isolation of

aquatic and soil microorganisms. Hutton and ZoBell (ref. 65) were the first to demonstrate that some methanotrophic isolates could oxidize ammonium to nitrate, i.e., the first step in nitrification. However, it was left to Drozd *et al.* (ref. 66) to confirm that strains of both type I and type II methanotrophs were able to nitrify ammonium, via nitrite, to nitrate, i.e., complete nitrification. Dinitrogen fixation by methanotrophic bacteria was confirmed by Davis *et al.* (ref. 67) on the basis of their growth in a nitrogen-free medium. However, this important finding was not generally accepted because of the erratic behaviour of methanotrophs in the acetylene reduction assay. It was not until de Bont and Mulder (ref. 68) showed the assay to be invalid, because of cometabolism and the ethylene produced, that dinitrogen fixation by both type I and type II methanotrophs became accepted. To date, no evidence exists suggesting that methanotrophs are able to denitrify. However, as far as methanotrophic consortia are concerned, those that have a methylotrophic *Hyphomicrobium* sp. as a component have been shown to denitrify both in the presence and in the absence of dissolved oxygen (ref. 69). In the presence of nitrate, denitrifying *Hyphomicrobium* spp. do not compete with methanotrophs for oxygen as a terminal electron acceptor at reduced dissolved oxygen concentrations. However, whether they can also nitrify under the same conditions, like certain other aerobic denitrifying bacteria (ref. 17), remains to be investigated.

With respect to the complex nitrogenous matter released by the lysis of methanotrophic bacteria, the heterotrophic strains present in the consortium will hydrolyze and deaminate such matter to ammonium, which can then be assimilated by the component strains present in the consortium.

Very often, microbial growth is described as either carbon- or nitrogen-limited. However, because of the ability of microbes to change both their nitrogen content and the nature of their various intracellular nitrogen compounds, particularly the enzymes present, simultaneous carbon- and nitrogen-limitation frequently occurs (ref. 70). Under such growth conditions, enzymic activities become impaired, and hence, simultaneous carbon- and nitrogen-limitation is growth-rate dependent (ref. 71), a fact that is totally ignored when considering microbial growth in biotreatment processes.

CONCLUDING REMARKS

The performance of biotreatment processes depends on the stimulation of the activities of various microbial consortia under conditions where unsteady state operating conditions predominate. The most critical problem to be faced in the continuing development of biotreatment processes is the remarkable paucity of data concerning the physiology of the microorganisms that comprize biotreatment process cultures. However, for these to be of value, they must relate to the process environments that occur in real biotreatment processes. Only then will prediction of process performance be possible, so that guarantees of process performance can be provided.

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REFERENCES

1 Koch, R. (1881). Zur Untersuchung von pathogenen organismen. *Mitt. Kaiserl. Gesundht., Berl.*, **1**, 1–48.

2 Bull, A.T. & Quayle, J.R. (1982). New dimensions in microbiology: an introduction. *Phil. Trans. Roy.Soc.*,Lond. B, **297**, 447–457.

3 Monod, J. (1942). Rescherches sur la croissance des cultures bactériennes. pp. 1–210, Hermann, Paris. F.

4 Standing, C.N., Frederickson, A.G. & Tsuchiya, H.M. (1972). Batch and continuous culture transients for two substrate systems. *Appl. Microbiol.*, **23**, 254–259.

5 Egli,T., Bosshard,C. & Hamer, G. (1986). Simultaneous utilization of methanol-glucose mixtures by *Hansenula polymorpha* in chemostat: influence of dilution rate and mixture composition on utilization pattern. *Biotechnol. Bioengng.*, **28**, 1735–1741.

6 Harrison, D.E.F. & Topiwala, H.H. (1974). Transient and oscillatory states in continuous culture. *Adv. Biochem. Engng.*, **5**, 167–219.

7 Adamse, A.D., Deinema, M.H. & Zehnder, A.J.B. (1984). Studies on bacterial activities in aerobic and anaerobic waste water purification. Antoine van Leeuwenhoek J. Microbiol. Serol., **50**, 665–682.

8 Benedict, R.G. & Carlson, D.A. (1971). Aerobic heterotrophic bacteria in activated sludge. *Water Res.*, **5**, 1023–1030.

9 Witthauer, D.P. (1980). Biocoenosis and degradation in model wastewater treatment plants. *Europ. J. Appl. Microbiol. Biotechnol.*, **9**, 151–163.

10 Seiler, H. & Busse, M. (1978). ie Floraanalyse in biolozischen Kl ranlagen und deren Aussage. *Z. Wasser-Abwasser-Forsch.*, **11**, 111–117.

11 Kappesser, S., Rude, E. & Kutzner, H.J. (1989). Microbiological studies of selected bacterial cultures for aerobic treatment of waste water. *Proc. Dechema Biotechnol. Conf.*, **3B**, 855–858.

12 Autheunisse, J. & Koene, J.I.A. (1987). Alteration of the aerobic- and facultative anaerobic bacterial flora of the A/B purification process caused by limited oxygen supply. *Water Res.*, **21**, 129–131.

13 Blaim, H., Seiler, H. & Baumgarten, J. (1984). Microbiol population in an activated sludge treatment plant of a chemical combine. *Z. Wasser-Abwasser-Forsch.*, **17**, 37–41.

14 DeVos, P. & De Ley, J. (1983). Intra-and intergeneric similarities of *Pseudomonas* and *Xanthomonas* ribosomal ribonucleic acid cistrons. *Internatl. J. System. Bacteriol.*, **33**, 487–509.

15 Hawkes, H.A. (1978). The ecology of activated sludge. In: The Oil Industry and Microbial Ecosystems (Chater, K.W.A. & Somerville, H.J., Eds.) pp.217–232, Heyden, London, G.B.

16 Hughes, D.E. & Stafford, D.A. (1976). The microbiology of the activated-sludge process. *CRC Crit. Revs. Environ. Contr.*, Sept., 233–257.

17 Robertson, L.A., Cornelisse, R., De Vos, P., Hadioetomo, R. & Kuenen, J. G. (1989). Aerobic denitrification in various heterotrophic nitrifiers. *Antonie van Leeuwenhoek J. Microbiol. Serol.*, **56**, 289–299.

18 Focht, D.D. (1974). The effects of temperature, pH and aeration on the production of nitrous oxide and gaseous nitrogen - a zero order kinetic model. *Soil Sci.*, **118**, 173–179.

19 Knowles, R. (1982). Denitrification. *Microbiol. Revs.*, **46**, 43–70.

20 Hochstein, L.I., Betlach, M. & Kritikos, G. (1984). The effect of oxygen on denitrification during steady state growth of *Paracoccus halodenitrificans*. *Arch. Microbiol.*, **137**, 74–78.

21 Omlin, D.B. (1990). Denitrification und Assimilation von Nitrat bei *Paracoccus denitrificans*: Untersuchungen zum Wechsel zwischen Oxischen und Anoxischen Bedingungen in Kontinuierlicher Kultur. *Doctoral Diss.* ETH Zurich, No. 9348, pp. 1–137.

22 Boller, M. (1984). Chemical optimization of tertiary contact filters. *Am. Soc. Civ. Engrs. J. Environ. Engng.*, **110**, 263–276.

23 Toerien, D.F., Gerber, A., Lötter, L.H. & Cloete, T.E. (1990). Enhanced biological phosphorus removal in activated sludge systems. *Adv. Microb. Ecol.*, **11**, 173–304.

24 Nicholls, H.A., Osborn, D.W., Buchan, L. Melmed, L N. & Pitman, A.R. (1984). Biological removal of phosphorus and nitrogen from waste water. In: Continuous Culture 8 (Dean, A.C.R., Ellwood, D.C. & Evans, C.G.T., Eds.) pp. 185–204, Ellis Horwood, Chichester, GB.

25 Wood, J.M. & Wang, H.K. (1985). Strategies for microbial resistance to heavy metals. In: Chemical Processes in Lakes (Stumm, W., Ed.) pp. 81–98, Wiley, New York, USA.

26 Lester, J.N. & Sterritt, R.M. (1985). Microbial accumulation of heavy metals in wastewater treatment processes. *J. Appl. Bacteriol. Symp. Suppl.* **59**, 141s-153s.

27 Xu, H.-S., Roberts, N., Singleton, F.L., Attwell, R.W., Grimes, D.J. & Colwell, R.R. (1982). Survival and viability of nonculturable *Escherichia coli* and *Vibrio cholerae* in the esturine and marine environment. *Microb. Ecol.*, **8**, 313–324.

28 Bungay, H.R., & Bungay, M.L. (1968). Microbial interactions in continuous culture. *Adv. Appl. Microbiol.*, **10**, 269–290.

29 Hartmann, J., Reineke, W. & Knackmuss, H.-J. (1979). Metabolism of 3-chloro-, 4-chloro- and 3,5-dichlorobenzoate by a pseudomonad. *Appl. Environ. Microbiol.*, **37**, 421–428.

30 Fujita, M. & Ike, M. (1994). Waste Treatment Using Genetically Engineered Organisms. pp. 1–176, Technormic, Basel, CH.

31 Hamer, G. (1992). The functioning of a microbial consortium for bioremediation: methanotrophy revisited. *Ann. N.Y. Acad. Sci.*, **665**, 435–448.

32 Wilkinson, T.G. & Hamer, G. (1979). The microbiol oxidation of mixtures of methanol, phenol, acetone and isopropanol with reference to effluent purification. *J. Chem. Technol. Biotechnol.*, **29**, 56–67.

33 Al-Awadhi, N., Hamer G. & Egli, T. (1990). The biooxidation of methanol, ethanol, and isopropanol by a defined co-culture at elevated temperatures. *Bioproc. Engng.*, **5**, 39–45.

34 Bitzi,U., Egli,T. & Hamer, G. (1991). The biodegradation of mixtures of organic solvents by mixed and monocultures of bacteria. *Biotechnol. Bioengng.*, **37**, 1037–1042.

35 Wayne, J.G., Brenner, J., Colwell, R.R., Grimont, P.A.D., Kandler, O., Krichevsky, M.I., Moore, L.H., Moore, W.E.C., Murray, R.G.E., Stackebrandt, E., Starr, M.P. & Trüper, H.G. (1987). Report of the ad hoc committee on reconciliation of approaches in bacterial systematics. *Internatl. J. System. Bacteriol.*, **37**, 463–464.

36 Ward, D.M., Weller, R. & Bateson, M.M. (1990). 16s rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature*, **345**, 63–65.

37 Herbert, D. (1958). Some principles of continuous culture. In: Recent Progress in Microbiology. (Tunevall, G., Ed.) pp. 381–396, Almqvist & Wiksell, Stockholm, S.

38 Heitzer, A., Mason, C.A. & Hamer, G. (1992). Heat shock gene expression in continuous cultures of *Escherichia coli*. *J. Biotechnol.*, 22 , 153–170.

39 Harendeen, S.L., van Bogelan, R.A. & Neidhardt, F.C. (1979). Levels of major proteins of *Escherichia coli* during growth at different temperatures. *J. Bacteriol.*, 139 , 185–194.

40 Martin, A. (1992). Genetics of bacterial stress response and its applications. *Ann. N.Y. Acad. Sci.*, **665**, 1–15.

41 Martin, A. (1992). Physiology, molecular biology and applications of the bacterial starvation response. *J.Appl. Bacteriol. Symp. Suppl.*, **73**, 49s-57s.

42 Völker, U., Engelmann, S., Maul, B., Riethdorf, S., Völker, A., Schmid, R., Mach, H. & Hecker, M. (1994). Analysis of the induction of general stress proteins of *Bacillus subtilis*. *Microbiol.*, **140**, 741–752.

43 Spector, M.P., Aliabadi, Z., Gonzalez, T. & Foster, J.W. (1986). Global conrol in *Salmonella typhimurium* : two dimensional electrophoretic analysis of starvation -, anaerobiosis-, and heat shock-inducible proteins. *J. Bacteriol.*, **168**, 420–424.

44 Smith, M.W. & Neidhardt, F.C. (1983). Proteins induced by anaerobiosis in *Escherichia coli*. *J. Bacteriol.*, **154**, 336–343.

45 Smith, M.W. & Neidhardt, F.C. (1983). Proteins induced by aerobiosis in *Escherichia coli*. *J. Bacteriol.*, **154**, 344–350.

46 Harrison, D.E.F. & Loveless, J.E. (1971). Transient responses of facultatively anaerobic bacteria growing in chemostat culture to a change from anaerobic to aerobic conditions. *J. Gen. Microbiol.*, **68**, 45–52.

47 Harrison, D.E.F. & Loveless, J.E. (1971). The effects of growth conditions on respiratory activity and growth efficiency in facultative anaerobes grown in chemostat culture. *J. Gen. Microbiol.*, **68**, 35–43.

48 Thomas, A.D., Doelle, H.W., Westwood, A.W. & Gordon, G.L. (1972). Effects of oxygen on several enzymes involved in the aerobic and anaerobic utilization of glucose in *Escherichia coli*. *J. Bacteriol.*, **112**, 1099–1105.

49 Millis, N.F. & Ip, S.-Y. (1986). Effect of uncoupling oxidative phosphorylation on the economics of the activated sludge process. *Chem. Engng. Commun.*, **45**, 135–144.

50 Ip, S.-Y., Bridger, J.S. & Millis, N.F. (1987). Effects of alternating aerobic and anaerobic conditions on the economics of the activated sludge system. *Water Sci. Technol.*, **19** , 911–918.

51 Hamer, G. (1983). A biotechnological approach to the treatment of wastewater from petrochemicals manufacture. *Instn. Chem. Engrs. Symp. Ser.*, **77**, 87–101.

52 Geoghegan, D.P., Hamer G. & Deshusses, M.A. (1996). Effects of unsteady state conditions on the biooxidation of methyl ethyl and methyl isobutyl ketone in continuous flow liquid phase cultures. *Bioproc. Engng.*, in press.

53 Mason, C.A., Hamer, G. & Bryers, J.D. (1986). The death and lysis of microorganisms in environmental processes. *FEMS Microbiol. Revs.*, **39**, 373–401.

54 Mason, C.A., Hamer, G. & Bryers, J.D. (1986). Activity, death and lysis during microbial growth in a chemostat. *Chem. Engng. Commun.*, **45**, 163–176.

55 Hamer G. & Bryers, J.D. (1985). Aerobic thermophilic sludge treatment and utilization. *Conserv. Recycl.*, **8**, 267–285.

56 Kacser, H. (1988). Regulation and control of metabolic pathways. In: Physiological Models in Microbiology 1. (Bazin, M. J. & Prosser, J. I., Eds.) pp. 1–23, CRC Press, Boca Raton, Fl., USA.

57 Allison, S.M., Small, J.R., Kacser, H. & Prosser, J.I. (1993). Control analysis of microbial interactions in continuous culture: a simulation study. *J. Gen. Microbiol.*, **139**, 2309–2317.

58 Westerhoff, H.V., Lolkema, J.G., Otto, R. & Hellingwerf, K.J. (1982). Thermodynamics of growth, non-equilibrium thermodynamics of bacterial growth, the phenomenological and the mosaic approach. *Biochim. Biophys. Acta.* **683**, 181–220.

59 Mulder, M.M., van der Gulden, H.M.L., Postma, P.W. & van Dam, K. (1988). Effect of macromolecular composition of microorganisms on the thermodynamic description of their growth. *Biochim. Biophys. Acta*, **936**, 406–412.

60 Wilkinson, T.G., Topiwala, H.H. & Hamer, G. (1974). Interactions in a mixed bacterial population growing on methane in continuous culture. *Biotechnol. Bioengng.*, **16**, 41–59.

61 Linton, J.D. & Buckee, J.C. (1977). Interactions in a methane-utilizing mixed bacterial culture in a chemostat. *J. Gen. Microbiol.*, **101**, 219–225.

62 Higgins, I.J., Hammond, R.C., Soriaslani, F.S., Best, D., Davies, M.M., Tryhorn, S.E. & Taylor, F. (1979). Biotransformation of hydrocarbons and related compounds by whole organism suspensions of methane-grown Methylosinus trichosporium OB 3b. *Biochem. Biophys. Res. Commun.*, **89**, 671–677.

63 Leadbetter, E.R. & Foster, J.W. (1960). Bacterial oxidation of gaseous alkanes. *Arch. Mikrobiol.*, **35**, 92–104.

64 Ensley, B.D. (1991). Biochemical diversity of trichloroethylene metabolism. *Annu. Revs. Microbiol.*, **45**, 283–299.

65 Hutton, W.E. & ZoBell, C.E. (1953). Production of nitrite from ammonia by methane-oxidizing bacteria. *J. Bacteriol.*, **65**, 216–219.

66 Drozd, J.W., Godley, A. & Bailey, M.L. (1978). Ammonia oxidation by methane-utilizing bacteria. *Proc. Soc. Gen. Microbiol.*, **5**, 66–67.

67 Davis, J.B., Coty, V.F. & Stanley, J.P. (1964). Atmospheric nitrogen fixation by methane-oxidizing bacteria. *J. Bacteriol.*, **88**, 468–472.

68 de Bont, J.A.M. & Mulder, E.G. (1974). Nitrogen fixation and co-oxidation of ethylene by a methane-utilizing bacterium. *J. Gen Microbiol.*, **83**, 113–121.

69 Wilkinson, T.G. & Hamer, G. (1972). Some growth characteristics of a *Hyphomicrobium* sp. in batch culture. *J. Appl. Bacteriol.*, **35**, 577–588.

70 Gräzer-Lampert, S.D., Egli, T. & Hamer, G. (1986). Growth of *Hyphomicrobium* ZV620 in the chemostat: regulation of NH_4^+ assimilating enzymes and cellular composition. *J. Gen Microbiol.*, **132**, 3337–3347.

71 Egli, T. (1991). On multiple-nutrient-limited growth of microorganisms, with special reference to dual limitation by carbon and nitrogen substrates. Antonie van Leeuwenhoek *J. Microbiol. Serol.*, **60**, 225–234.