J Ind Microbiol Biotechnol (2010) 37:993–1021
DOI 10.1007/s10295-010-0816-4

REVIEW

The renaissance of continuous culture in the post-genomics age

Alan T. Bull

Received: 21 July 2010 / Accepted: 11 August 2010 / Published online: 11 September 2010

Abstract The development of continuous culture techniques 60 years ago and the subsequent formulation of theory and the diversification of experimental systems revolutionised microbiology and heralded a unique period of innovative research. Then, progressively, molecular biology and thence genomics and related high-information-density omics technologies took centre stage and microbial growth physiology in general faded from educational programmes and research funding priorities alike. However, there has been a gathering appreciation over the past decade that if the claims of systems biology are going to be realised, they will have to be based on rigorously controlled and reproducible microbial and cell growth platforms. This revival of continuous culture will be long lasting because its recognition as the growth system of choice is firmly established. The purpose of this review, therefore, is to remind microbiologists, particularly those new to continuous culture approaches, of the legacy of what I call the first age of continuous culture, and to explore a selection of researches that are using these techniques in this post-genomics age. The review looks at the impact of continuous culture across a comprehensive range of microbiological research and development. The ability to establish (quasi-) steady state conditions is a frequently stated advantage of continuous cultures thereby allowing environmental parameters to be manipulated without causing concomitant changes in the specific growth rate. However, the use of continuous cultures also enables the critical study of specified transition states and chemical, physical or biological perturbations. Such dynamic analyses enhance our understanding of microbial ecology and microbial pathology for example, and offer a wider scope for innovative drug discovery; they also can inform the optimization of batch and fed-batch operations that are characterized by sequential transitions states.

Keywords Continuous culture · Theory and applications · Chemostat · Microbial behaviour · Systems biology · Microbial physiology · Ecophysiology · Pathology · Steady state and transition state growth

A. T. Bull (✉)
School of Biosciences,
University of Kent, Canterbury, Kent CT2 7NJ, UK
e-mail: A.T.Bull@kent.ac.uk

Introduction

"The study of the growth of bacterial cultures does not constitute a specialised subject or a branch of research: it is the basic method of microbiology" (Jacques Monod [132]). Twenty-five years after this salutary message I opined [26] that the principles of microbial growth often still received only cursory attention, a practice that inevitably compromised the study of all aspects of microbial behaviour. With the gradual adoption of continuous cultures (strictly continuous-flow cultures) in the 1960s and 1970s, well-defined cultivation conditions could be imposed such that the effects of environmental parameters on growth and behaviour could be distinguished unequivocally. During this period research blossomed in such fields as phenotypic variation, metabolic control, dynamics of microbial communities and microbial evolution, while the perceived benefits of continuous cultures were explored for industrial production and processing.

Essentially there are two basic modes of culturing organisms and cells: (1) batch cultures during which no addition or removal of materials occur with time (gas exchange being the exception) and which approximate to

closed systems; (2) continuous cultures that receive a constant feed of fresh nutrients while spent medium, metabolic products and biomass are removed at the same rate, and are referred to as open systems. There are several variants of these culture types, the applications of some of which will be discussed later. The unique feature of continuous cultures that has made them so attractive as a research tool is that time-independent steady states can be established, in contrast to the time-dependent changes that characterise batch cultures. As we shall discover later, the concept of steady state in this context requires some qualification.

A very wide range of organisms (e.g. bacteria, fungi, algae, protozoa), cells and tissues (animal, plant) can be grown in one form or another of continuous culture. Strictly the biomass should be homogeneous and well mixed and for a time microbiologists considered that filamentous organisms (e.g. some fungi, actinobacteria, algae) neither grew exponentially, nor with a homogeneous morphology; reliable means for avoiding pellet or other heterogeneous growth of these organisms now are well documented in the literature.

Continuous culture became the method of choice for microbiologists exploring the interplay of environment and behaviour but the level of interest—as in growth physiology in general—faded in the latter part of the last century. Science like other human endeavours is not immune to fashion and thus the excitement heralded at that time by molecular science and thence genomics came to dominate biological research. While the achievements of such reductionist approaches have been truly revolutionary, entry into a post-genomics era is driving a return to greater holistic enquiry and the emergence of what has become known as systems biology—what van der Greef refers to as "mapping patterns of relationships" [190]. Systems biology approaches will only be as good as the technology platform on which they are based. The workflow advocated by van der Greef comprises a comprehensive technology platform (e.g. genomics, transcriptomics, proteomics, and metabolomics), from which the resulting data are integrated into a master dataset that in turn can be subject to a wide range of statistical and computational interrogations in order to generate a meaningful model of the system under study. Critically the technology platform also needs to incorporate a means of growing organisms and cells under strictly defined and controlled conditions so that data collection is reproducible and that the data obtained are referable specifically to the physico-chemical-biological environment imposed on the population. Continuous culture techniques, especially chemostat cultures (see below), provide the best means of achieving these aims and are being used increasingly in this post-genomic era although theory and practice do not always appear to be fully appreciated by contemporary users. The purpose of this article is several fold: to present briefly the historical background, the types of continuous culture and aspects of theory; to appraise major discoveries in the first age of continuous culture; to highlight recent theoretical and technical developments; and, principally, to demonstrate the appropriateness of continuous culture applications during its renaissance.

Historical background

The practical and theoretical basis of continuous culture was established simultaneously by Monod [133] at the Institut Pasteur in Paris, and by Novick and Szilard [140] at the University of Chicago (but see "Pathology" below). Novick and Szilard developed their *chemostat* in order to follow spontaneous mutations in bacteria over long periods of cultivation, what they referred to as the "manifestation of 'evolution' in the chemostat". In an understatement that was only eclipsed by Watson and Crick in their 1953 paper, Novick and Szilard suggested that studies using the chemostat promised "to yield information of some value on metabolism, regulatory processes, adaptation and mutation" [141]. The *bactogène*, on the other hand, evolved out of Monod's earlier work on the kinetics of bacterial population growth that established the relationship between its specific growth rate and the concentration of a single growth rate-limiting nutrient [132]. Notably, 30 years earlier M'Kendrick and Pai [128] had published a paper deserving of much greater recognition that anticipated the later and more precise definitions of nutrient-limited growth, growth yield and even maintenance energy requirements, from a series of simple, elegant experiments when M'Kendrick was serving in India; he later became director of the Pasteur Institute in Kasauli. Nevertheless, chemostat was the term adopted by the microbiology community to describe continuous culture of the type defined by Monod.

Significantly the subsequent intensive exploitation of continuous culture occurred in England, at the Microbiological Research Establishment, Porton Down, and in Czechoslovakia, at the Institute of Microbiology, Prague, a key feature of which was the publication of a defining paper by Denis Herbert and colleagues describing how a simple mathematical theory of continuous culture could be derived from the basic features of batch cultures established by Monod [88]. Two years later, under the guidance of Academician Ivan Málek, the first of a series of seminal international symposia on the continuous cultivation of microorganisms was convened in Prague. In total eight such symposia were organized over the next 26 years in Czechoslovakia and England, the contributions to which

Table 1 Fundamental equations of continuous culture

(1) $\ln x = \ln x_0 + \mu t$	Basic growth equation [μ, h^{-1}]
(2) $\mu = \mu_{max} \times s/K_s + s$	Growth-limiting substrate equation
(3) $dx/dt = (\mu - D)x$	Steady state equation for a simple chemostat
when zero, $\mu = D$	
(4) $D_c = \mu_{max} \times s_R/(K_s + s_R)$	Equation for the critical dilution rate; when $s_R \gg K_s$, $D_c \approx \mu_{max}$
(5) $\bar{s} = K_s(D/K_s - s)$	Steady state equation for the concentration of growth-limiting substrate in a simple chemostat
(6) $\bar{x} = Y[s_R - K_s (D/\mu_{max} - D)]$	Steady state equation for the concentration of biomass in a simple chemostat
(7) $\mu = [1 - R]\ D$	Specific growth rate equation in a recycle chemostat
(8) $\bar{x} = Y_{x/s} \times s_R/(1 - R)$	Steady state biomass concentration in a recycle chemostat
(9) $q = m + \mu/Y_G$	Equation defining maintenance coefficient
(10) $dx/dt = \mu x - ax$	Equation defining maintenance rate
(11) $q_{ATP} = \mu/Y_{ATP} = \mu/Y^{max}_{ATP} + m$	Equation for the energy of maintenance
(12) $\bar{x} = DY_{EG}(s_R - s)/(D + mY_{EG})$	Steady state equation for a simple chemostat corrected for maintenance energy requirement

$\bar{x}$ and $\bar{s}$ [g L^{-1}] define steady state values; s_R is the reservoir concentration of the growth rate-limiting substrate fed to the culture; K_s is the saturation constant for the growth-limiting substrate; Y (=$Y_{x/s}$) is the growth yield = dx/ds; R, the recycle ratio, producing a biomass concentration effect of $1/[1 - R]$; q is the specific metabolic rate; q_{ATP} is the specific rate of ATP production [mol ATP produced g^{-1} dry biomass h^{-1}]; Y_{ATP} is the growth yield per mol ATP; Y^{max}_{ATP} is the growth yield per mol ATP corrected for the maintenance energy requirement; m is the maintenance coefficient [mol ATP consumed g^{-1} dry biomass h^{-1}]; a is the specific maintenance rate [h^{-1}]; Y_{EG} is the true growth yield when s is the energy source and $m = 0$

For fuller explanations and derivations of the equations see Herbert et al. [88], Pirt [148], Bull and Young [31]

form the bedrock of a substantial part of twentieth century microbiology (see Sect. "The renaissance"). That gatherings of microbiologists from the Soviet Union, Czechoslovakia, and the USA could convene in a British defence establishment[1] at the height of the Cold War to discuss continuous culture almost defies belief, but it happened and to very great effect. The innovative applications made by Czechoslovak scientists in this field are recorded in the classic text of Málek and Fencl [124].

Members of the Porton group including S. John Pirt, John R. Postgate and David W. Tempest played a major role in expanding continuous culture research into British and other European universities and, in turn, promoting the next generation of scientists in this experimental approach to studying microbial behaviour. My own induction occurred in the 1960s as a young faculty member in the exhilarating research atmosphere of John Pirt's department in the University of London.

Types and features of continuous cultures

Although the chemostat is the most familiar and widely used form of continuous culture (cited in ca. 90% of publications in the past 5 years on continuous culture), several other types have been developed each offering particular advantages for research and industrial processes. Continuous culture cognoscenti may wish to skip this section but for those new to the subject and anticipating its use, it is hoped that the following synopsis of options will prove helpful. The operating principles and the advantages of the main types of continuous culture are as follows, while some of the defining equations are given in Table 1.

Chemostat

The flow rate (F) of fresh medium to a culture of volume V is set externally and the specific growth rate (μ) is controlled by the rate of supply of a growth-limiting substrate (s). When steady state conditions are established μ becomes equivalent to the dilution rate D (=F/V). Note that when D approaches the maximum specific growth rate of an organism the system becomes unstable and if D exceeds a critical value (D_c) washout of the culture occurs. Under ideal conditions mixing in the culture should be perfect, i.e. the time for distributing a small volume of incoming medium should be as short as possible compared with the culture replacement time (V/F), and the biomass should be homogeneous and not adhering to vessel walls or to probes. The chemostat can be operated at very low dilution rates to those approaching the maximum value of μ of the organism under the nutritional and environmental conditions set by the experimenter. Advantages comprise the ability to: (1) vary μ without changing medium composition or operating conditions such as temperature, pH; (2) fix μ in order to evaluate the specific effects of environmental parameters; (3) establish unique conditions by changing the nature of the

[1] MRE, Porton Down, Wiltshire 1967.

growth-limiting substrate, e.g. C-, N-, PO_4-, Mg-limited growth; and (4) allow analysis of steady state and defined transient state cultures, the latter providing a useful means of investigating metabolic regulation.

At this point the issue of *steady state* as applied to continuous cultures in general needs to be examined. As emphasized by Ferenci [60] nutrient-limited growth induces strong selection pressure for mutational changes that rapidly sweep microbial populations, the very objective sought by Novick and Szilard [140, 141]. Ferenci also argues that no true steady state can exist in chemostats because the residual concentration of the limiting nutrient continues to drop for hundreds of hours in a culture. Therefore, a qualified interpretation of steady state needs to be taken by continuous culturists whether in the context of "the estimations of supposed constants, like K_s, (that) are subject to complex environmental effects and changes in populations", or, adopting "chemostat studies to provide reproducible conditions for global regulation" [60]. One practical but not foolproof precaution is to return to the initial conditions of a continuous culture experiment and remeasure the values of a number of extensible variables to check against unacceptable shifts in the population. However, studies of *Trichothecium reesei* continuous-flow cultures reveal that such checks are not invariably sound measures of a good steady state [153]. Thus, perturbations to the steady state were always evident at the transcriptional level, even when they were not measurable as changes in biomass or product concentrations. Both unintentional and intentional perturbations of the steady state demonstrated that a number of genes involved in growth, protein production and secretion are sensitive markers for culture disturbances.

Turbidostat and other auxostats

The turbidostat is an example of a continuous culture whereby a growth-associated parameter (e.g. partial pressure of CO_2, bioluminescence) is held constant by automatic adjustment of the dilution rate. This general type of continuous culture is called an auxostat and, in the case of the turbidostat, the feedback parameter is culture density that is controlled by photoelectric or laser devices. The specific growth rate in turbidostats is maximized due to the excess of all substrates and, because it is not fixed, selection of faster growing mutants occurs.

The pH auxostat is one in which the pH is the feedback parameter. The power of this type of continuous culture was demonstrated recently by Groeneveld et al. [71] in selecting for increased specific growth rate of the yeast *Kluyveromyces marxianus*. Other applications include the investigation of sulphate reduction under acidic conditions favourable for metal bioleaching [17].

Multistage continuous culture

Linking chemostats together in series provides opportunities for imposing different conditions at each stage. Multistage systems can enable physiology to be investigated at specific growth rates close to or at zero, or, at μ_{max}, and also to study the effects of growth-inhibitory substrates and other inhibitory conditions, enzyme inducers, and the synthesis of secondary metabolites. The important feature in all of these cases is that a stable population can be maintained in the 2nd (to *n*th) stage that would not be possible in a single stage chemostat.

An interesting elaboration of multistage continuous culture is that of operating a bidirectional flow of materials, one such type being the gradostat [115] in which linear gradients (e.g. substrates, dissolved oxygen, pH) are established between sources and sinks. Such bidirectional systems are particularly useful in modelling ecosystem determinants (an early example being estuarine salinity gradients [41]) and resource competition among microbial communities [40].

Recycle continuous culture

Chemostats can be operated such that a fraction of the biomass is fed back to the culture vessel and can be achieved practically by external feedback via a separator in order to generate a concentrated biomass stream, or internally via a filtration device to generate a diluted biomass outflow stream. The effect of recycle is to greatly extend the range of dilution rates over which the system can be operated (notably values of $D > \mu_{max}$) [149] and thence increase the output of biomass and growth associated products (e.g. lactic acid [121]); and to study behaviour at very low specific growth rates which may be difficult or impossible to achieve in simple chemostats (see [176] for details). Biomass recycle also is particularly appropriate in circumstances where the metabolism of the growth-limiting substrate produces inhibitory compounds, where the growth-limiting substrate is very dilute (and preset) or poorly soluble, and where a product is non-growth associated (e.g. sevenfold increase in vancomycin production rate [127]).

The first age of continuous culture

My intension here is to show how the adoption of continuous culture during the first age of its history, that for convenience coincides with the period spanning the first and last continuous culture symposia (1958–1984), brought about radical new understanding across microbiology. The crux of this argument is the fact that the growth of organisms under a range of environmental constraints,

including defined nutrient limitations, induces the expression of properties not, or only fleetingly, expressed when grown in batch cultures. Chemostat applications par excellence revealed a remarkable phenotypic plasticity in microorganisms, provided insights into metabolic switches provoked by the environment, opened up new approaches to the study of microbial ecology and microbial pathogenicity, and provided tractable experimental systems for probing genetic stability and aspects of microbial evolution. Moreover, continuous culture technology was developed or refined for a variety of industrial processes. During this period continuous culture theory also progressed as exemplified by the definition of maintenance energy requirements to sustain metabolic turnover and ionic homeostasis, for example.

Landmark impacts of continuous culture in this first age are summarized in Table 2. The selection of discoveries and developments is a personal one and not exhaustive, and, where possible, reference is given to comprehensive reviews rather than to primary sources. While the outcome of most of this research is now firmly embedded in the microbiologist's experience, lessons learned from one area of continuous culture exploitation, namely large-scale industrial processing, often go unnoticed. Advantages of continuous versus batch operations were recognized early on in industry and regarding fermentation processes, increased productivity, higher processes rates and the general diseconomies of non-continuous processing were deemed to be particularly important. Moreover, continuous culture techniques proved to be very effective in process optimization, for example in defining conditions for (1) optimum product yield and quality and strain stability in xanthan gum production, and (2) removal of biological oxygen demand, phosphate and nitrate in activated sludge digesters [27]. Brief reference here to a selection of industrial-scale continuous cultures developed through the 1960s and 1980s reveals highly innovative microbiological and engineering that was required to resolve a variety of process constraints.

The prospect of a world shortage of protein-rich foods catalyzed a great upsurge of interest during the 1960s in single cell protein (SCP; microbial protein) and using the then considered abundance of fossil hydrocarbons as substrates. In 1965 Shell initiated a research programme that targeted methane as a substrate [82] and, against most industrial practice and perceived wisdom, opted for reconstituted mixed cultures as the source of protein. The mixed culture strategy was shown to promote higher specific growth rates, biomass yields and greater process stability than conventional monocultures, while additional benefits included greater resistance to contamination and reduced foaming [113]. On the other hand ICI developed a monoculture SCP process based on methanol (the company was a world leader in its production) as a source of high-protein animal feed. Crucial problems to be solved in the development of this massive process (eventual scale 150,000 L continuous culture) were oxygen transfer and heat removal, both of which are significantly greater than carbohydrate-based processes, and mixing. The solution was achieved by the development of a novel pressure cycle fermenter and a monoculture of an obligate methylotroph, *Methylophilus methylotrophus* [169]. Stable, uncontaminated continuous production runs of several months were operated and the product, Pruteen, proved to be an excellent feed additive for fish and livestock. The innovative technology notwithstanding, the process became non-competitive in face of soya meal and was abandoned. However, the large-scale continuous culture technology established for Pruteen subsequently was used to produce a successful high-value food rather than low-value animal feed. Rank Hovis McDougall decided in the 1960s to develop a carbohydrate-based (initially starch but finally glucose) continuous process for a protein-rich food. Partnership with ICI enabled the latter's pressure cycle reactor technology to be deployed in the production of fungal protein (ex. *Fusarium graminearum*) initially at a scale of 40,000 L and eventually at 140,000 L. The product, Quorn®, was first commercialized in the UK in 1985 as a 'healthy food' and now is available worldwide in several foods. A major problem requiring solution in this process was the gradual takeover of the culture by highly branched mycelia mutants of *F. graminearum* that adversely affected the formulation of Quorn® and resulted in termination of the run after ca. 6 weeks. Chemostat studies provided several options for controlling such colonial mutants: operation at a low D (inevitable reduction in yield), switching the growth-limiting substrate e.g. glucose → Mg^{2+}→glucose, and most attractive, the selection of morphologically stable mutants [186].

My final example of large-scale continuous culture comes from an entirely different branch of industry and involves the retrofitting of an established chemical manufacturing process. In the production of the wet strength resin Kymene® epichlorohydrin reacts with poly(aminoamides) with the formation of potentially toxic haloalcohols (typically ca. 9,000 ppm) that remain in the product. In the 1980s the producer Hercules Inc. assessed several schemes for removing or preventing the formation of these contaminating by-products and finally opted for a bioprocess to degrade the haloalcohols in situ. The constraints imposed on this R&D programme were considerable: removal of haloalcohols to zero or near zero concentrations, no adverse effects on the performance of the resin, a septic process because the production stream could not be sterilized, and retrofitting that would be compatible with a continuous-flow operation. A two-membered consortium of bacteria comprising *Agrobacterium tumefaciens* and

Table 2 Major impacts of continuous culture during the first age of application

Physiology	
Definition of the 'physiological state'	Málek [123]
Maintenance requirements	Pirt [147]
Definition of the cell quota model	Droop [54]
Stoichiometry of microbial growth	Herbert [87]
Uncoupled growth and energy-spilling mechanisms	Stouthamer [175], Neijssel and Tempest [137]
Growth of filamentous fungi	Bull and Trinci [30]
Growth of mixed cultures, utilization of mixed substrates	Bull [28]
Metabolism	
Regulation of enzyme synthesis	Clarke and Lilly [39]
Dual pathways and fluctuating environments	(e.g. Bull and Brown [29]; glycerol, ammonia)
Utilization of mixed homologous substrates	Harder and Dijkhuisen [78]
Effects of dissolved oxygen	Harrison [81]
Phenotypic variation of chemistry and morphology	
Effects of inorganic cations on bacterial wall polymers	Tempest [183]
Lipid transitions in bacterial membranes	Minnikin et al. [130]
Rod-coccus dimorphism in *Arthrobacter*	Luscombe and Gray [117]
Microbial and enzyme evolution	
Selection for bacteriophage resistance and other traits	Kubitschek [107], Sikyta [168]
Periodic selection in filamentous fungi	Wiebe et al. [197]
Plasmid and cloning vector stability	Primrose et al. [151]
Mechanisms of enzyme evolution	Hall [75]
Microbial pathogenicity	
Antibiotic resistance	Dean et al. [47], Lambert [109]
Virulence determinants	Robinson et al. [155]
Modelling disease syndromes	Hamilton and Ellwood [77]
Ecophysiology	
Mixed culture studies	Veldkamp and Jannasch [192]
Enrichment isolation	Parkes [142]
Competition studies	Kuenen and Gottschahl [108]
Simulation of natural ecosystems	Tempest et al. [184]
Degradation of xenobiotic chemicals	Senior et al. [162]
Industrial application	
Brewing	Hospodka [89], Hough et al. [90]
Strain selection	Brown and Oliver [25]
Waste treatment	Hamer [76], Nicholls et al. [138]
Microbial protein	
Exogenous C_1 compounds	Harrison et al. [83], Smith [169]
Exogenous carbohydrates	Trinci [186]
Biopolymers	Sutherland and Ellwood [177], Senior [163]
Process retrofitting (e.g. wet strength resin production)	Hardman et al. [79]

Arthrobacter histidinolovorans was selected that remained in a stable 4:1 ratio for up to 5 months in laboratory-scale chemostats operating with Kymene® and was very tolerant of perturbations in haloalcohol concentrations [79]. This pilot system was scaled and retrofitted as a septic process into a Kymene® production plant in France in the early 1990s with haloalcohol concentrations reduced to just a few parts per million. After initial operation in batch mode Kymene® was introduced at a ramped flow rate to a final value of 500 L h^{-1} (hydraulic retention time ca. 7 h); the flow rate could be modified to meet production requirements as long as such a ramping procedure was used. This continuous bioreactor (3,000 L—the largest ever operated by the writer!) was subsequently integrated into two plants

and proved to be robust, reliable, and responsive to manufacturing schedules [79].

The renaissance

The era of genomics opened up access to the sequence of genes and ultimately to whole genomes that culminated with that of the gold standard sequence for the human genome in 2003. Sequence information over the past 30 years or so has accumulated at an exponential rate and together with the multitude of high-information-density techniques (omics) has provided "unprecedented means of collecting data at the deepest molecular level of living systems" [23]. However, Brenner [23] contends that the understanding of all this information has lagged far behind that of its accumulation: for many, systems biology is seen to be the means of addressing this problem. Systems biology implies a return to holistic rather than reductionist approaches in biology, the term entering common parlance from the turn of the century. The definition presented in the special issue of *Science* devoted to systems biology is worth recalling: "problems of organization, phenomena not resolvable into local events, dynamic interactions manifest in the difference of behaviour of parts when isolated or in higher configuration, etc."; in short, "systems of various orders not understandable by investigation of their respective parts in isolation…integration and application of mathematics, engineering, physics, and computer science to understanding a range of complex biological regulatory systems" [36]. Others claim that rigorous definitions of the aims and methodology of systems biology are wanting, or, because "it is so broad and has few recognized boundaries…it is attractive to anyone who has ever thought about life and has some relevant technical expertise" [196]. Similarly, systems biology as a 'big picture viewpoint' of biology is not useful "to the ecologists, physiologists, clinicians, and evolutionary biologists who have had a big picture approach to biology for decades" [193]. In a typically trenchant and thought-provoking essay Sydney Brenner opines that the claims of systems biology will fail because the modelling of complex systems is an inverse problem[2] that cannot be solved [23].

[2] The prediction of observations is a forward problem that has a unique solution; the use of actual observations to infer properties of a system is an inverse problem that may not have a unique solution. "As an example, consider measurements of the gravity field around a planet: given the distribution of mass inside the planet, we can uniquely predict the values of the gravity field around the planet (forward problem), but there are different distributions of mass that give exactly the same gravity field in the space outside the planet. Therefore, the inverse problem—of inferring the mass distribution from observations of the gravity field—has multiple solutions (in fact, an infinite number)" [180].

Criticisms of these sorts notwithstanding, the global attraction of post-genomics systems biology is driving a renewed interest in the use of continuous cultures for probing microbial physiology in a wide range of situations. The advantages of working with microorganisms are twofold: first, because of their small genomes, modelling of their physiology is a more tractable problem [126]; second, data collection can be made under rigorously defined and regulated conditions, hence the interest in continuous culture, particularly chemostasis, that offers unique advantages with regard to reproducibility and experimental design and increasingly making them the methods of choice for systems microbiologists.

Before moving to a detailed discussion of post-genomic deployment of continuous culture I wish to single out a number of technology developments that bear on some of the fields of application.

Technology developments

The renaissance of continuous culture in part is occurring due to its combination with other enabling technologies prominent among them being devices based on microfluidics. The impact of microfabrication techniques on microbiology is covered in an excellent review by Weibel et al. [194] while the extraordinary development and exploitation of this technology is evidenced by the construction and application of a million-well culture chip [65, 92]. Microfluidic continuous cultures are finding applications in several fields including the exploration of microbial physiology at the level of single cells, high-throughput screening of drugs and toxicity testing, and high-throughput optimization programs. Most early microfluidic bioreactors were operated in batch mode and used for studying phenomena such as colony growth and bacterial persistence, and claims for some chemostatic devices are not admissible e.g. [72]; the use of terms like "batch culture chemostats" not only is confusing but unwarranted. The device described by Balagadde et al. [11] comes closer to the reality of a chemostat. It comprises a growth chamber (V, 16 nL) formulated as a fluid loop that is operated on a pseudo-continuous basis (purged periodically with lysis buffer to remove wall growth) for extended periods (>500 h) at a range of quasi dilution rates. This microchemostat has been used to determine the dynamics of *Escherichia coli* strains engineered to carry a synthetic population control circuit that regulates population density via a quorum sensing feedback mechanism. The authors claim that the ability to culture and monitor populations as small as 10^2–10^4 reduces the mutation rate thereby enabling extended analyses of genetically homogeneous populations that could be advantageous in drug discovery campaigns. The design and operation of a genuine microchemostat (V, 150 μL) that

incorporates on-line measurement of optical density, pH and dissolved oxygen concentration was reported by Zhang et al. [199]. Accumulated growth on vessel walls and probes has long been known to cause deviations in culture behaviour from chemostat theory. Adherent growth was reduced in the reactor designed by Zhang and his colleagues by coating the walls of the chamber with bio-inert co-polymers thereby enabling prolonged chemostatic operation over a wide range of dilution rates. A comparable strategy for preventing adherent growth of filamentous fungi in chemostats was recommended nearly 40 years ago [157]. Finally, because of difficulties involved in sampling and assaying at the nanolitre scale, a number of analytical devices including on-a-chip PCR and RT–PCR, have been developed for microfluidic platforms [24].

Corruptions of physiological profiles resulting from off-line sampling and analysis are driving the development of on-line analytical procedures. Metabolome profiles can be distorted during even the few seconds required for off-line sampling, a problem that can be avoided in part by innovations such as the recently described ^{1}H-NMR continuous culture system produced for in situ extracellular metabolomic studies [122]. Among the advantages posited by these authors is the ability to collect metabolomic data prior to any off-line transcriptome and proteome analyses that are less susceptible to sampling artefacts. A useful guide for optimizing procedures for off-line extraction required for intracellular metabolomics is provided by Canalas et al. [34] who used ^{13}C-labelled standards for determining yeast metabolome recoveries in chemostat experiments.

Other new developments in continuous culture technology have been directed at strain improvement. The cytostat developed by Srienc and colleagues is controlled and monitored by automated flow cytometer feedback positioned off-line in a sample loop [98]. In this bioreactor cell number concentration is defined by $F_{new} = F_{ss} - K_c(C_{x,sp} - C_{x,m})$ where F_{new} is the new medium feed rate, F_{ss} the theoretical equilibrium feed rate at a given substrate concentration, $C_{x,sp}$ the set point cell number concentration, $C_{x,m}$ the observed cell number concentration, and K_c is a control parameter (ml^2 h^{-1} cells^{-1}); thus the feed rate and thereby the washout rate of cells increase when $C_{x,m} > C_{x,sp}$. The cytostat has been used to select for mutants (M) that have only small growth advantages over wild types (WT) within a time frame that is claimed to be significantly shorter than that obtained in conventional chemostats [67]. The mutant isolation time is held to be a function of a total cell number, μ_{WT}, μ_M and beneficial mutation probability and by optimizing total cell number and μ_{WT} a minimum isolation time is achieved. Gilbert et al. [68] have used the cytostat to select for mutants of *Saccharomyces cerevisiae* that are resistant to high concentrations of acetate and show increased specific growth rates under such conditions. Mutants with these properties are important in producing ethanol from lignocellulosic hydrolysates rich in acetate. Over-expression of ENA genes encoding P-type ATPase Na^+ pumps appear to contribute to acetate tolerance via a gene dosage dependency. A continuous culture device called the Evolugator^{TM} [48] that can be operated in quasi-chemostat and turbidostat modes has been developed to circumvent problems associated with wall growth and, again, to provide a system for the experimental evolution of microorganisms. An interesting deployment of this continuous culture was reported recently by de Crécy et al. [49] who used it to select for thermotolerance in the entomopathogenic filamentous fungus *Metarhizium anisopliae*.

The final example of innovative continuous culture technology designed to meet the specific needs of field-based research is that of shipboard systems that enable the behaviour of natural plankton communities to be analysed in close to real-time conditions and circumstances. Despite the acknowledged advantages of continuous cultures they have been little used in such studies due to problems of maintaining culture homogeneity, constant flow rates, and overall robustness. Thus, the recently reported design, operation and field evaluation of a seagoing continuous culture by Pickell et al. [145] is a landmark for field-based ecophysiological research. The system consists of 14 duplicate cylindrical culture vessels mounted horizontally in rotating cradles that provide good mixing and uniform illumination. The vessels are fed freshly obtained, filtered seawater that can be amended as necessary with macro- and/or micro-elements, and are inoculated with natural phytoplankton communities taken from the same ocean locality. Pickell and her colleagues have used their seagoing platform to assess the effects of nitrate on phytoplankton communities and, especially interesting, that of iron concentration, a factor that is known to be a major determinant in these communities. Here simulated seagoing investigations promise to be important alternatives to very large scale iron fertilization projects conducted in the open ocean, and also to progress understanding on how pCO_2 and temperature affect phytoplankton trajectories over predicted long-term global changes.

The post-genomics age

The focus on quantitation is the common theme of systems biology and of continuous culture. With regard to the former, quantitation and mathematical modelling aim to provide predictive and explanatory power such that the understanding of biology is complete, or as complete as possible [50, 193]; and with regard to the latter, a timely reminder that chemostat cultivation was rendered essential

by the need for quantitative studies of microbial growth. A further imperative here is the requirement to unravel what Daran-Lapujade et al. [45] term "context-dependency" of transcriptional and related responses based on high-information-density analytical approaches for which chemostat cultivation is the sine qua non (see below). Given this emphasis on quantitative microbiology, a few points on continuous culture theory advances preface the discussions of physiology, evolution, ecophysiology, pathology, and industrial applications. The reader will quickly discern that such division of the topics is somewhat arbitrary and that considerable overlap (inter-discipline) characterises the overall field, nevertheless I hope that this arrangement will make for easier reading.

Theory

Some cautionary remarks on classic chemostat theory based on Monod kinetics (see Table 1) were noted above. Criticisms in particular of the empirical constants K_s and Y have been made by Ferenci [59] who remarked on the lack of attention given to determining the mechanistic bases for such "supposed constants". A recent attempt to address this issue was made by Snoep et al. [171] who explored the relationship between the saturation constant and the affinity of the transporter for the growth-limiting substrate in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. These authors have developed a core model and theory based on metabolic control analysis such that in the chemostat the pump rate controls μ via its effect on the growth-limiting substrate concentration (G_P^S) where the change in steady state concentration of s is given by $G_P^S = \frac{1}{\varepsilon_S^{v_{tr}} \cdot C_{v_{tr}}^{\mu}} \equiv \frac{1}{R_S^{\mu}}$, where R_S^{μ} is the response coefficient of μ with respect to s $\left(R_S^{\mu} = \frac{K_S}{K_S+S}\right)$, $\varepsilon_S^{v_{tr}}$ is the elasticity coefficient for the transporter activity $\left(\varepsilon_S^{v_{tr}} = \frac{K_m}{K_m+S}\right)$, and $C_{v_{tr}}^{\mu}$ is the control coefficient of the transporter on μ. From these equations the relationship between K_m and K_s could be established as: $C_{v_{tr}}^{\mu} = \frac{1+\frac{S}{K_m}}{1+\frac{S}{K_S}}$.

It follows from the relationship between K_S and K_m that it is possible to calculate the control of the transporter on μ if the residual substrate concentration is known. Thus, for *S. cerevisiae* it was estimated that the transporter has 78 and 49% control over specific growth rate at glucose concentrations of 0.03 and 0.29 mM respectively. The authors conclude: "The interplay between a good theoretical framework such as metabolic control analysis, modelling and experiment, as used in this study, is illustrative of the type of approaches that we think will be essential for addressing systems biology problems".

Comparable difficulties are met in the quantification of maintenance (see Table 1), the term originally used by Pirt [147] to describe energy consumed for functions other than production of new biomass. van Bodegom [187] points to four chief reasons why quantification has been compromised: a definition that includes all non-growth components without distinguishing those that are physiological in character; the treatment of maintenance as a constant rather than a variable; an overlap of concepts—maintenance coefficient and specific maintenance rate and attempts to show a constant relationship between them [e.g. $m = a/Y_{EG}$]; and, the frequent exclusion of cell death from attempts to model microbial dynamics. A conceptual model for maintenance that defines an overall maintenance coefficient, m_{tot}, incorporating relative death rate (d), relative and maximum specific growth rates (μ_r; μ_{max}), growth yield (Y_G), and a physiological maintenance requirement (m_p; related to osmoregulation, motility, proof-reading and internal turnover of nucleic acids and proteins, defence mechanisms) has been developed by van Bodegom [187] that shows m_{tot} not to be a constant but a linear function of all the variables involved $m_{tot} = d\frac{\mu_r}{\mu_{max}}\left(\frac{\frac{1}{Y_G}+m_p}{1-\frac{d}{\mu_r}}\right)$. Although relevant data are lacking for several of these variables, sensitivity analyses have allowed relative death rates and relative growth rates to be identified as dominant ones affecting variability of the overall maintenance. Two important considerations derive from this model: first the need to generate appropriate experimental data; and, second because of the variability in m_{tot} and its nonlinearity, it should caution experimental microbiologists in fields such as ecology, biochemical engineering and waste treatment on the need to question the equations being used to describe microbial dynamics.

The chemostat-based researches of Tempest and others (Table 2) strongly postulated that avenues for energy dissipation other than for maintenance operated in at least some bacteria. It is now recognized that organisms do not always utilize energy in an efficient manner and that many bacteria and eukaryotes appear to have energy-spilling reactions, a phenomenon also referred to as metabolic uncoupling, spillage, overflow metabolism, and futile cycling (see [158] for review). In at least some cases, such energy spillage involves the active transport of protons through the cell membrane and van Bodegom [187] considers that energy-spilling reactions of this sort can be incorporated into his physiological maintenance variable (m_p). Also apposite to these discussions are aspects of overflow metabolism [135], and the concept that as an organism adapts to maximize its growth rate, the number of active metabolic reactions tend to be significantly reduced compared to non-optimal growth states, both of which have their basis in

continuous culture investigations. Nishikawa et al. [139] have argued that the reduced number appears to be constant for species of bacteria and yeast that they studied and was only slightly larger than the minimum number required for the organism to grow. The authors term this response 'massive spontaneous reaction silencing' and propose that it is triggered by the irreversibility of a large fraction of the metabolic reactions that propagates through the network as a cascade of inactivity.

Many studies of photosynthetic microorganisms and phytoplankton communities have been made in continuous cultures. Growth-limiting nutrients in standard chemostats are mixed homogeneously but in contrast light in light-limited chemostats is not so mixed but forms a steep gradient on passage through the liquid medium such that the photon flux decays exponentially with depth. Moreover, light is supplied independently of the dilution rate. Although there have been previous attempts to advance theories for light-limited growth, the work of Huisman's group [91] is especially valuable. These authors have developed a dynamic chemostat theory that relates population density to the light environment, taking account of primary production and population decline resulting from culture washout and maintenance requirements; and a quantitative description of the light gradient dependency on phytoplankton density. The dynamics of Huisman's light-limited chemostat predict steady state population densities that are related to the background turbidity of the chemostat and the mixing depth (i.e. higher in shallow, lower in deep culture systems). Full details of theory development and experimental evaluation are to be found in [91]. These authors discuss the applications of light-limited chemostats in the analysis, for example, of species interactions where competitive advantage is afforded to species having low critical light intensities, defined as the light penetration at steady state (see also "Ecophysiology" below).

Finally, in carbon-limited chemostats and in natural ecosystems simultaneous utilization of several substrates is frequently observed. Such mixed (auxiliary) substrate utilization may enable growth at enhanced specific rates compared to that on any of the component substrates. In a recent review of the auxiliary substrate concept [9] it has also been shown that it is possible to increase the yield coefficients and, based on the energetic evaluation of organic substrate, to predict improvements in carbon conversion efficiencies. Thus, yield coefficients from glucose can be increased from about 0.5 to 0.7 g g^{-1} using formate as the auxiliary substrate while yields from hexadecane are enhanced from about 0.9 to 1.3 g g^{-1} also by means of formate. These modulating effects of auxiliary substrates on μ and Y have important consequences for bioprocess design and in the understanding of competition in microbial communities.

Physiology/systems biology

The exploration of microbial physiology has continued to be the major application of continuous culture in the post-genomics period and has benefited enormously by the introduction of high-throughput-information platforms. Over the past 5 years more than 120 papers have been published that discuss physiology (ca. 80%) and systems biology (ca. 20%) approaches with the greatest focus being on *Saccharomyces cerevisiae*. As Delneri et al. [50] opined the first systems biology models have been erected for yeast both for sound technical and conceptual reasons. In this review a small selection of these studies that reflect the current interests in the field are noted while a more comprehensive range of papers is summarized in Table 3. This selection is necessarily personal and also to an extent its categorization arbitrary because of the close interrelatedness of the research.

The proponents of chemostats frequently refer to them as the culture methods of choice because single parameters can be varied while others are held constant, and hence steady state conditions maintained over long periods. The cautionary comments of Ferenci [60] on the definition of steady state mentioned above are clearly substantiated by work with *Trichoderma reesei*. Steady state growth conventionally is assessed on the basis of measuring concentrations of biomass or product, culture density, gas exchange or titrant consumption but even when biomass and heterologous protein concentrations were constant, perturbations to perceived steady states of this filamentous fungus could be detected at the transcriptional level [153]. A transcript analysis with aid of affinity capture method was used to monitor 31 genes among which a number that are involved in growth, protein synthesis and secretion showed up as sensitive markers of unintentional and intentional perturbations in a large number of chemostat cultures. Thus compelling evidence is provided for transcriptional monitoring of continuous cultures prior to embarking on costly systems-wide analyses. Standardization of culture systems and experimental procedures is recognized as de rigueur for achieving the objectives set by systems biology. Recently a collaboration between the Dutch Vertical Genomics Consortium, the European Yeast Systems Biology Network and the Standards for Reporting Enzymological Data Commission has taken this argument a stage further by reasoning that data must be representative of in vivo conditions. Focusing on pathway fluxes this group proceeded to formulate an enzyme assay medium that resembled the intracellular conditions in which the enzymes function [188]. Intracellular elemental composition was determined for *S. cerevisiae* growing in aerobic, glucose-limited chemostats at $D = 0.1$ h^{-1} from which an in vivo-like medium was designed. The V_{max} values of

Table 3 Recent applications of continuous culture for studying the physiology and systems biology of microorganisms

Systems biology	
Transcriptional monitoring of the quality of *Trichoderma* chemostat cultures prior to genome-wide analysis	Rautio et al. [153]
Standardization criteria yeast assay medium for measurements under in vivo-like conditions	van Eunen et al. [188]
Regulatory systems coordinating growth rate, cell division, and metabolism in yeast	Brauer et al. [22]
Haploproficient (increased μ) and haploinsufficient (reduced μ) high flux control genes of yeast	Delneri et al. [50]
Respiration-related functional genomics of yeast	Pir et al. [146]
Global metabolic stability of *E. coli* in response to genetic and environmental perturbations	Ishii et al. [93]
Global transcription regulation of aerobic glucose catabolism by *E. coli*	Nanchen et al. [136]
Chemostasis the ideal tool for functional genomics investigations (*Thermatoga maritima*)	Shockley et al. [167]
Genome-scale metabolic analysis of fermentative behaviour of *Lactobacillus plantarum*; model case for biotechnological/biomedical situations necessitating complex, nutrient-rich conditions	Teusink et al. [185]
Physiology: context-dependency	
Transcriptional responses of yeast to low temperature and low μ	Tai et al. [178]
Individual/combined effects of nutrient limitations and oxygen availability on transcription factor activity in yeast	Knijnenburg et al. [104]
Proteome expression in *Shewanella oneidensis*	Elias et al. [56]
Physiology: transition state behaviour	
Transcript profiling of the anaerobic → aerobic transition in *E. coli*	Partridge et al. [144]
Analysis of *E. coli* subjected to pulsed metabolites	Guebel et al. [74]
Intracellular metabolomes of glucose-pulsed *E. coli*	Schaub and Reuss [161]
Proteomics of heat-shocked *E. coli* in 2-stage chemostats	Luders et al. [116]
SFP1 and nutrient-dependent regulation of yeast ribosome biogenesis and cell size	Cipollina et al. [38]
Bioenergetics of yeast during glucose → galactose transitions	van der Brink et al. [189]
Metabolic studies	
Transcriptional up-regulation and post-transcriptional up-regulation are principal controls in yeast in response to C- and N-limited growth	Daran-Lapujade et al. [44], Kolkman et al. [106]
Definition of a Zn-specific regulon in yeast involved in storage carbohydrate metabolism	De Nicola et al. [52]
Dynamics of storage carbohydrate pools in aerobic and anaerobic yeast	Aboka et al. [1], Hazelwood et al. [84]
Yeast metabolism at near zero μ in 100% cell recycle chemostats	Boender et al. [19]
Determination of growth-limiting intracellular metabolites in yeast	Boer et al. [18]
Transcriptional response to secretion stress by *Trichoderma reesei*	Arvas et al. [5]
Polyol production by *Aspergillus niger* at low O_2 concentrations	Diano et al. [53]
Bacterial responses to nitric oxide and nitrosative stress	Pullan et al. [152]
Responses of *Methanococcus maripaludis* to nutrient limitations and μ	Hendrickson et al. [85, 86]
Thermodynamic analyses of anaerobic, acetogenic sludge mixed cultures	Bastidas-Oyanedel et al. [12]

some enzymes measured in this new medium differed appreciably from assays made under enzyme-specific optimal conditions. The authors recognize that a single assay medium devised in this way will not be universally appropriate but opine that their strategy presents a further critical stage in obtaining and integrating realistic experimental data into systems biology models.

In recent years the term 'context-dependency' has appeared in the literature to emphasize the fact that microbial behaviour (of whatever type being monitored) is contingent on the experimental conditions imposed; the old school of continuous culturists will be amused by this rediscovered maxim! Such context-dependency is demonstrated in recent cold acclimation studies of *S. cerevisiae* in [178]. The results of this study make the point very conspicuously that even in well-controlled chemostats it is impossible to change a single cultivation parameter without causing impacts on others. Thus profiling transcriptomes at 12 and 30°C, even at the same dilution rate, produces the inevitable consequence of higher residual glucose

concentration in the cultures grown at 12°C, a result at least partly due to a specific growth rate of 0.03 h^{-1} representing ca. 75% μ_{max} at 12°C, but only ca. 10% at 30°C. If the study had been restricted to a comparison of glucose-limited cultures it would have been compromised by a "contamination" of temperature-responsive gene sets with genes whose transcription is influenced by glucose. A similar caveat applies under ammonium-limited conditions but Tai and colleagues [178] deployed a combinatorial experimental design involving both glucose- and ammonium-limited cultures to reduce the impact of these secondary effects. Thus, transcriptional responses to the intrinsically linked parameters in batch cultures of low temperature and low μ can be dissected by using chemostat techniques.

This research group adopted the same strategy to reveal the combinatorial effects of oxygen status and nutrient limitation regimes on changes in transcription factor activity *S. cerevisiae* [104].

Schaub and Reuss [161] have argued the case for metabolome profiling in order to improve pathway design strategies in metabolic engineering, and for gaining greater insight into the control and optimization of industrially relevant processes. Intracellular steady state concentrations of glycolytic intermediates were measured in *E. coli* at different dilution rates (0.1, 0.2, 0.3, and 0.4 h^{-1}) and in response to glucose pulses. Steady state concentrations exhibited opposite trends for intermediates of the upper and lower parts of glycolysis; while metabolic responses to glucose pulses were shown to differ with respect to relative concentration changes. The aggregated data demonstrate the need for growth-rate dependent (dynamic) metabolomics experiments, especially for investigating industrial fed-batch processes that are characterized by changing specific growth rates.

Two recent papers illustrate the ingenious use of chemostats for investigating microbial physiology. The first explores microbial behaviour at near-zero specific growth rates, a physiological state that has direct ecological relevance (e.g. deep biospheres [143]) and may be important in optimizing growth-dissociated production processes. Boender et al. [19] have used a recycle chemostat with 100% retention of yeast to develop populations growing at $\mu < 0.001$ h^{-1} within ca. 3 weeks. It appears that maintenance energy requirements in *S. cerevisiae* do not substantially change at near-zero specific growth rates and that glucose metabolism was directed predominantly towards alcoholic fermentation to meet maintenance energy requirements. Partial loss of viability and cessation of the cell cycle were features of these populations and the authors suggest that retentostats of this type might be suitable for distinguishing between ageing and starvation responses. It is interesting to recall that physiological states of this sort were being investigated in the early days of continuous culture using simple single stage chemostats [182]; the Porton group established glycerol- and ammonium-limited cultures of *Aerobacter* (now *Enterobacter*) *aerogenes* achieving a minimum specific growth rate of 0.004 h^{-1} (NB this required 1,250 h of chemostat cultivation and, thereby, very rigorous instrument reliability). The second study is of a familiar phenomenon, heat shock response, but employing temperature up-shifts in the second of a two-stage chemostat system enabling, the authors opine, the study of temperature stress under well-defined steady state conditions [116]. Proteome analyses revealed that proteins involved in the defence against oxygen stress, functional cell envelopes, chaperones, protein and amino acid biosyntheses, and energy metabolism were differently expressed at high cultivation temperatures, and that the patterns (not surprisingly) differed from those when *E. coli* was grown under batch conditions.

In concluding this section I refer briefly to recent developments in systems microbiology that have benefited from the chemostat experimental platform. Ishii and colleagues [93] were concerned to examine the wide-range robustness of cellular systems and assembled multi-omics data (transcriptome, proteome, metabolome and fluxome) for chemostat cultures of *E. coli.* Their results suggest that *E. coli* can actively respond to changes in the concentration of the growth-limiting substrate by regulating the level of enzyme expression to maximize growth rate, whereas it does not respond significantly to the disruption of most single metabolic genes by regulating other mRNA or protein levels. In the latter case structural redundancy in the metabolic network likely provides the necessary robustness, allowing the levels of most metabolites to remain constant. Also working with chemostats at similarly low dilution rates Nanchen et al. [136] quantified the control of a number of global regulators of metabolic fluxes (O_2 sensing, catabolite repression, carbohydrate assimilation, modulation of carbon flux at growth arrest, osmotic regulation, global gene regulation, and chromosome organization) in isogenic mutants of *E. coli*. ^{13}C-based metabolic flux analysis was used to identify regulation mechanisms that determined flux distributions and the most relevant mechanism was found to be cyclic AMP-dependent catabolite repression of the newly discovered phosphoenolpyruvate (PEP)-glyoxylate cycle and thus low tricarboxylic acid cycle fluxes. These and other flux data from *E. coli* batch and glucose-limited chemostat cultures demonstrated that despite active catabolite repression there is substantial cyclic operation thus dispelling the common misconception that catabolite repression of TCA cycle *sdh* and other genes effectively splits the *E. coli* TCA cycle into a two-branched pathway during growth on readily fermentable substrates.

Oliver's group has used competition experiments in glucose-, ammonium- and phosphate-limited continuous cultures to explore haploproficiency and haploinsufficiency in yeast [50] (haploinsufficiency phenotypes show reduced growth rate when hemizygous; haploproficiency phenotypes show increased growth rate when hemizygous). This study revealed that 192 and 348 genes respectively show haploproficiency and haploinsufficiency under all three nutrient limitations and that among these, genes whose transcript levels are under growth-rate control occur no more frequently than expected by chance (13 and 14% respectively). Thus, the authors posit that a general rule emerges: genes that are major controllers of growth rate in nutrient-limited environments are not themselves subject to growth-rate control. Moreover, haploproficient genes appeared to be randomly distributed in the genome, whereas haploinsufficient genes were over-represented on chromosome III leading the authors to suggest that their accumulation on chromosome III may be the result of "intense selection pressure against the loss of one copy of this chromosome from a diploid. Such a loss would have severe consequences for the yeast population, as cells that had lost a copy of chromosome III would be diploid maters. This loss would also disadvantage the individual carrying it because it would prevent the cell from forming spores". The results of a specific growth rate–genome-wide expression study made under a large range of nutrient limitations [22] have raised several interesting aspects of yeast biology. Over 25% of all expressed genes correlated linearly with μ irrespective of the limiting nutrient, including the many genes whose expression changes regardless of the nature of the environmental stress imposed and what were previously called the environmental stress response (ESR) genes. However, not all of these putative ESR genes significantly correlated with growth rate and it is likely that many of the ESR genes as defined previously may not be responding directly to stress, but instead are responding to a reduction in growth rate secondarily to the stress. These authors also reported a new phenomenon observed in yeast that appears to be analogous to the Warburg effect in cancer cells. When growth was limited by auxotrophic requirements (uracil, leucine) the cultures wasted excess glucose but not when growth was ammonium-, sulphate- or phosphate-limited. Mutants limited in chemostat cultures by their auxotrophic requirements also failed to arrest predictably at the G_0/G_1 stage of the cell cycle, a situation that Brauer et al. [22] interpret as implying at least two mechanisms that connect metabolism and entry into the cell cycle.

Evolution

The chemostat was invented by Novick and Szilard and among their first reported experiments was the clear demonstration of mutational adaptation and the takeover of the inoculum-derived population by fitter bacterial mutants [141]. Despite such early evidence for population divergence in chemostat populations the phenomenon was largely underestimated until quite recently because as Ferenci [61] remarked "limited numbers of isolates from any population were analysed in earlier studies and few differentiating phenotypes were recognized". However, with the advent of molecular biological screening techniques such as DNA microarray probing, the ability to follow mutational sweeps in populations now has become precise and rapid. Ferenci's review [61] provides an excellent digest of adaptation events in chemostat populations, only a few of which will be related here; rather this section will focus on new information relating to adaptation, and some aspects of directed evolution using continuous cultures.

At the outset it may be helpful to restate a few key facts that are more, or less, known: (1) adaptation in this context refers to properties that increase an organism's fitness to its environment and can be acted upon by natural selection; (2) adaptation mechanisms in substrate-limited continuous cultures include spontaneous mutations in regulatory systems and structural genes, gene duplication and gene deletion; (3) chemostat populations can become highly heterogeneous in a very short time, a fact rarely appreciated in early continuous culture studies. An illustration of the last point is the rapid appearance of *rpoS* null mutants (stationary phase/stress resistance σ^{38}) in nutrient-limited *E. coli* after only 30 h continuous culture [100]. This study also revealed an interesting and unexpected trade-off between a requirement for RpoS-dependent stress resistance and that of RpoS loss leading to improved fitness under aerobic and anaerobic chemostat cultivation. Antagonstic pleiotropic trade-off has been reported to occur under other environmental circumstances one of which is considered below [200].

Work in Ferenci's group also has revealed the remarkable degree of phenotypic diversity that can evolve within chemostat populations. For full details the reader is directed to Maharjan et al. [120] but in essence regulatory, physiological and metabolic divergencies were commonly detected in chemostat cultures of *E. coli* although such divergencies were not identical in replicated chemostat cultures, this last point affirming that there can be many alternative strategies for enhancing fitness "in even a relatively constant selective environment" as pertains in chemostats.

Chromosomal evolution in chemostat populations of *E. coli* has been investigated by Zhong et al. [200] who followed adaptations to lactulose or methyl-galactoside, or, to a mixture of these substrates. First, the evolutionary events observed in single substrate chemostats were found

to be specific adaptations to growth limitation by specific sugars, namely: *lac* duplications were adaptations to prolonged growth on lactulose not methyl-galactoside, whereas mutations at *mgl* were adaptations to methyl-galactoside but not to lactulose. Second, although adaptation in the presence of sugar mixtures might be expected to favour the evolution of generalists, such outcomes were extremely rare with only one isolate being recovered from 13 chemostat cultures. This strain carried *lac* duplication and was *galS*(−) (methyl-galactoside specialist trait) and showed increased transcription and translation of both operons [201] Paradoxically the generalist was prevented from sweeping to fixation because its competitor [*lac*(+), *galS*(+)] expressed an adhesin gene product that enabled it to attach to the culture vessel and thereby avoid washout. More usually the adaptation to mixed sugars led to the evolution of specialists, a result that argues for an antagonistic pleiotropic trade-off that curtails the evolution of generalists. In addition selected mutations were not restricted to resource use and included deletions affecting genes controlling flagella and capsule synthesis, resulting in overall energy and material sparing.

Yeast continues to be a favoured model organism for investigating adaptation trajectories in continuous cultures, a few examples of which we considered here. Following prolonged glucose-limited growth of *Saccharomyces cerevisiae* a progressive decrease of the residual glucose concentration (20 to 8 mg L^{-1} after 200 generations) was accompanied by a major reduction in fermentative capacity. These observations led Jansen et al. [95] to make transcriptomic and physiological studies of an evolved strain that remained stable when used to establish new chemostat cultures. The increased affinity for glucose was correlated with a nearly threefold decrease in the K_m for glucose transport while the lower fermentative capacity related to significant decreases in the specific activities of most glycolytic enzymes, a finding largely corroborated by transcriptome analyses. On the other hand, mechanistic bases for explaining the reduced K_m were not provided by measuring the transcript levels of known glucose transporters. However, a genome-wide transcriptome survey revealed that 186 genes had a higher transcript level in the evolved strain, among them being those involved in the cell cycle and DNA processing, and in determining cell morphology. The authors opine that up-regulation of these genes may contribute to a decreased μ_{max} of the evolved strain observed in batch cultures. This study epitomizes the importance of integrated, multidisciplinary approaches for gaining an understanding of the evolutionary dynamics and the physiology of microorganisms in continuous cultures.

Most investigations in this field to date have followed adaptation events under conditions of a constant dilution rate (usually well below that of μ_{max}) and most frequently of carbon limitation. As pointed out early in this review continuous culture offers a wealth of cultivation options that include variation of the growth-limiting substrate, exposure to defined transition states and shock conditions, and establishment of mixed microbial communities. These options remain to be exploited in the study of evolutionary adaptation that is why the report of Gresham et al. [70] warrants attention. Prolonged growth (ca. 200 generations) of *Saccharomyces cerevisiae* was made in replicate chemostats limited either by glucose, phosphate or sulphate. First, the authors inferred that the mutations observed and the subsequent dynamics of adaptation were determined by the batch phase of growth prior to initiation of continuous cultivation, i.e. the large diversity of genetic variation generated during batch growth directed subsequent evolution within the chemostat population. Unfortunately the use of non-standard terms such as 'culture saturation' to describe batch growth (presumably stationary phase population) is confusing but given that prior batch phase growth is so decisive in determining the course of mutational adaptation it would be interesting to compare its progression when initiated with batch populations of different physiological states. While some adaptive mutations related to known physiology were identified, others for the moment remain obscure and offer tantalizing prospects for further research. Second, the monitoring of genetic changes under conditions of different nutrient limitation goes some way to simulating selective pressures imposed in natural environments, "since competition for nutrients is a driving evolutionary pressure that is thought to have shaped the long-term evolution of biological networks". Thus, the phenotypes of strains adapted to sulphate limitation (determined on the basis of DNA microarrays) were found to be much less variable than those adapted to either glucose or phosphate limitation. Variations of this sort should not surprise us because as the authors state "the diversity of adaptive outcomes will vary as a function of the distribution of fitness effects of beneficial mutations, which differs dramatically depending on the selective pressure". Nevertheless, adaptation studies of this nature are infrequent and should encourage more innovative uses of continuous cultures to this end.

Further studies on *S. cerevisiae* have focused attention on the adaptive evolution of protein material costs, whereby highly expressed proteins may have reduced contents of growth-limiting elements than other proteins in the proteome. Bragg and Wagner [20] analysed previously published genome-scale transcripts obtained from chemostat populations [22, 58, 95] with the object of erecting a working hypothesis for 'element sparing' in proteins under circumstances where the element is growth limiting. Thus, under C-limitation adapted strains might up-regulate genes encoding C-poor proteins and down-regulate those that are

C-rich; alternatively, an opposite scenario might be plausible if, for example, other adaptive changes increased the affinity for the C-limiting substrate as demonstrated by Jansen et al. [95]. The analyses provided no support for the first hypothesis as up-regulated genes in adapted strains were not depleted in carbon, indeed proteins that were expressed in the ancestral strains at higher levels than in adapted strains had lower C contents than those of the rest of the proteome. The conclusion is that other adaptations appear to decouple nutrient limitation from protein composition. Moreover, genes that had at least one duplicate (ca. 25% of those examined) encoded proteins having significantly lower C and N contents than those of singleton genes. Bragg and Wagner suggest that gene duplication may confer selective advantage if one paralogue encoded protein with a reduced requirement for an element can be up-regulated when that element become growth limiting. Subsequently these authors have developed a model that predicts how natural selection can operate on the material costs of gene expression, by showing that mutations increasing the quantities of C, N or S costs of expression may cause sufficiently large impacts to be opposed by selection [21].

Finally in this section I refer to how continuous cultures have been used for directed evolution of microorganisms, or what has also been termed evolutionary engineering, that can be an adjunct to empirical strain improvement. Here the principle is to challenge microorganisms with a selective environment of choice such that natural selection delivers mutants with desired phenotypes. During the first age of continuous culture research such directed evolution was concentrated on 'improvements' of enzyme affinities and substrate specificities pioneered among others by Clarke and Hartley and their groups, see [35] for review, while its use in selecting for complex phenotypes was rather neglected (but see above the successful selection of morphological mutants for the production of Quorn®). More recently purpose designed continuous culture systems have been constructed for the selection of complex phenotypes that have relevance for industrial processes and products [49, 67, 68]. The entomopathogenic fungus *Metarhizium anisopliae* is currently being investigated for deployment as a bioinsectide but wild type strains have limited use because of their relatively low tolerance to abiotic stresses such as heat (upper growth limit of 32°C). de Crécy et al. [49] have successfully adapted this fungus to grow at 37°C using their Evolugator™ continuous culture technology (see "Technology developments" above). The most promising evolved strain obtained to date showed pleomorphic changes including reduced conidiation and lowered infectivity and a faster kill time, some features of which could be improved by passage through the insect host. The authors hypothesize that insect target specificity and/or virulence might be increased via continuous culture adaptation on specific insect cuticular extracts as the growth substrate; and that the technology could be applied to adapt strains for greater resistance to other abiotic stresses such as UV irradiation.

Two other studies illustrate the power of continuous culture selection of improved strains for deployment as 'cell factory' organisms, the first in increasing μ_{max} (and hence potential process productivity), and the second in producing enhanced resistance to bacteriophages. Groeneveld et al. [71] made use of pH auxostats to select for adapted strains of *Kluyveromyces marxianis* that had increased specific growth rates. While this yeast is notable among eukaryotic microorganisms in displaying a high μ_{max} (0.6 h^{-1}) this is substantially lower than that of the most rapidly growing prokaryote (ca. 4 h^{-1}), consequently any improvements would benefit industrial production of single cell protein or heterologous products. Adaptive evolution experiments produced strains whose μ_{max} was increased to 0.8 h^{-1}; this result was reproducible and the evolved strains were stable when tested in batch culture. Accompanying this growth rate change was a 40% increase in the cell surface while the surface to volume ratio increased to almost the same extent as the μ_{max}, suggesting that the cell membrane is the site of major limitation of the growth rate. The susceptibility of *Escherichia coli* to phage infections has concerned a group at the Tokyo Institute of Technology who have attempted to improve the robustness of the heterologous protein host [179]. Chemostat cultures maintained at a dilution rate of 1.0 h^{-1} were challenged with a cocktail of phages obtained from a sewage influent. Increasingly phage resistant strains were subject to a succession of mixed phage challenges under the same chemostat conditions and after four of which one highly resistant strain was obtained that had a 209-kbp genome deletion. The deletion was shown to include the whole *OmpC* and other genes necessary for lipopolysaccharide synthesis. Although this strain retained low susceptibility to some phages and OmpC complementation did not fully restore phage susceptibility, no deleterious effects on μ or on the expression of recombinant plasmids were observed.

Ecophysiology

Ecophysiology explores the interaction between an organism's physiology and the environment in which it exists. The advantages of continuous culture systems for studying microbial ecophysiology have long been appreciated (see Table 2) and more recently the case for using them has been stated cogently by Huisman et al. [91]: "Chemostats have contributed considerably to the development of general ecological theories, because they offer several convenient advantages for the pragmatic ecologist". Microorganisms

typically have short generation times enabling many generations to be investigated within a short time span; chemostats can simulate a wide variety of environmental conditions under well-controlled laboratory (and field) conditions; their spatial features provide an ideal test bed for many ecological models; and, the accuracy of chemostat experiments allows a precision of replication almost unprecedented in ecological research. Interest has continued to be focused on population dynamics—modelling and the mechanisms of inter-species reactions, and on ecosystem simulations, with omic techniques being used to gain understanding of responses to changing environmental conditions.

The advantages of using continuous cultures for enrichment isolation were demonstrated during its first age of application (Table 2), and the practice continues to provide new insights into ecosystem diversity. Recent studies of marine hydrothermal vent populations [3, 150] prove the point emphatically. Postec and her colleagues [150] inoculated continuous and batch culture systems with a sample obtained from a black chimney located in the Mid-Atlantic Ridge and monitored the diversity of thermophilic bacteria and archaea that were enriched. Whereas sequences representing several heterotrophic organisms were detected in both types of culture, those of the autotrophic bacteria *Deferribacter* and *Thermodesulfitator* were specific to the continuous enrichment. The authors suggest that a community of autotrophic organisms was enriched over time as a consequence of being able to utilize CO_2 and H_2 produced by the heterotrophs in the community. A further example of continuous culture enrichment revealing the wider diversity of organisms in ecosystems was reported recently by Alain et al. [3] who isolated a new species of the strictly anaerobic, thermophilic chemolithotroph *Thermodesulfatator atlanticus* from the same site (Rainbow field) at the Mid-Atlantic Ridge, using a gas-lift bioreactor operated with a mean residence time of 40 h.

Simulations of aquatic ecosystems frequently have made use of chemostats and recent illustrations of their relevance in this context include the development of riverine antibiotic resistance, the effect of viruses on aquatic food webs, and carbon turnover throughout an estuarine–marine transect. Thus challenging river water bacteria with high (800 μg L^{-1}) concentrations of chlortetracycline (CTC) had the anticipated effect of selecting for antibiotic resistant strains; the higher the concentration, the higher the number of CTC-resistant bacteria and the greater diversity of *tet* resistant genes [134]. Low concentrations of CTC (8 μg L^{-1}) did not select for increased resistance in these populations. Viruses are considered to exert both strong top-down and bottom-up effects on ecosystems, a situation that Lennon and Martiny [110] have explored in replicate, model chemostats limited by phosphate and comprising the marine cyanobacterium *Synechococcus*, an unspecified myovirus, and a community of non-target heterotrophic bacteria. Initially virus infection caused a major crash of *Synechococcus* numbers with an immediate fivefold increase in available phosphate and, although this effect decreased with time, chemostats did not return to their initial phosphate conditions even after 120 days operation. The presence of this particular virus exerted only a weak effect on the heterotrophic population despite the increased resource availability. After 50 days the *Synechococcus* population started to increase, showing oscillatory behaviour but not returning to pre-virus challenge levels. This attenuation of viral parasitism coincided with the appearance of resistant phenotypes that, the authors suggest, buffered the effect of the virus on nutrient cycling. Furthermore they opine that the results of this simulation experiment support current ideas on the importance of evolutionary changes on ecologically relevant timescales and for the understanding of community dynamics such as the top-down role of viruses in phytoplankton control.

The approach to ecosystem simulation taken by Kisand et al. [102] was to use chemostats in series to follow the degradation of humic-rich dissolved organic carbon (DOC) by bacterial populations obtained from a sequence of estuarine (oligohaline, salinity 5; mesohaline, salinity 15) and coastal (polyhaline, salinity 30) waters in the Weser Estuary (NW Germany). Each of three-stage chemostats were inoculated with one of the of the water samples and fed freshwater rich in humic substances (HS), adjusted to the salinity of the inoculant water, and supplemented with N, P and vitamins. Only in the polyhaline system was DOC removed (ca. 60%) and analysis of HS showed major reductions in the fulvic, humic and hydrophilic acid fractions; small reductions in some HS fractions were observed in the less saline chemostats. The authors conclude that these results are compatible with current knowledge of humic-rich DOC transformations during estuarine transport to the sea; thus degradation in extended shallow coastal ecosystems, typified by the Wadden Sea into which the Weser empties, is predicted to be higher than in those where riverine discharge is directly into deep coastal ecosytems. The residence times selected for the multistage chemostat simulations were 6–7 days (polyhaline) and 10 days (oligo- and mesohaline) and it would be interesting to see how the transformation dynamics change with significantly shorter residence times.

It is important that caution is taken when extrapolating the results from continuous culture experiments to real ecosystems and most authors are careful to indicate limitations in their laboratory simulations. Muñoz-Aguayo et al. [134] for example, point out that the 'non-effect' of low CTC levels may be misleading because concentration effects in sediment may enable selection for resistance;

likewise, anaerobic populations are known to act as reservoirs of antibiotic resistance whereas their study only considered aerobic conditions. Anomalies also are likely to result from observing too simplified models such as the *Synechococcus* parasitism described above. Finally, problems of scale are often invoked in this context. Huisman et al. [91] rehearse the limitations of scale when discussing their light-limited chemostat studies, one being the higher densities of phytoplankton in chemostats than usually seen in field populations that might cause more intense allelopathic (see below) interactions. The need for effective scaling rules has been addressed in part by Smith et al. [170] who analysed the scale dependency of phytoplankton diversity over very large (oceanic) to very small (chemostat) systems, i.e. $a > 15$ orders of magnitude spatial extent. They used the power law $S = cA^z$ where S is species richness, A the area of the system, c a taxon/environment-dependent constant, while the exponent z quantifies the scaling of richness with area. Manifest similarity was found for the power relationship in the natural and experimental ecosystems ($S = 66.5A^{0.114}$ and $S = 67.6A^{0.139}$ respectively). As Smith et al. [170] conclude "Phytoplankton species richness thus scales smoothly and consistently from laboratory microcosm to the world's oceans, with 74% of the observed variance in S being attributable to variations in ecosystem surface area alone". Thus, although challenges remain in fully understanding scale effect, this work shows not only that model (simulated) aquatic ecosystems can successfully inform about potential determinants of biodiversity in natural ecosystems, but should stimulate further research on what regulates such diversity, and encourage laboratory ecophysiologists in their endeavours.

The use of chemostats for investigating phenomena such as ecological stoichiometry, phenotypic plasticity, and competition in, and adaptation to, particular environments has been reported by several groups. The studies I have selected here illustrate the range of problems being addressed by microbial ecologists and the appropriateness of chemostat-based experiments. The concept of ecological stoichiometry, advanced only recently [173], is becoming an important component in the understanding of trophic dynamics in aquatic ecosystems. Of the few data relating to this issue those generated by Chrzanowski and Grover [37] are clearly pertinent. *Pseudomonas fluorescens* was chosen as a model prey organism to investigate the relationship between growth rate, an environmental variable (temperature) and element stoichiometry in phosphate-limited chemostats. In summary, the element composition of the bacteria varied as an interactive function of μ and T, most evidently in C to N and C to P ratios. The stoichiometry theory predicts two possible outcomes if there is an imbalance between the ratios of elements in the prey and its predator: (1) if the element ratio of the prey is within the range of that of the predator, the latter will excrete that element consumed in excess, i.e. the outcome is nutrient recycling; or (2) if the ratio of the prey is not within that of the predator, the latter is displaced in the trophic structure of the ecosystem by one that takes greater advantage of the resource pool. These postulates imply that the efficiency by which elements are transferred from a dissolved state through microorganisms by predators will vary according to prevailing environmental conditions. As the authors state, a common prey "may be a high quality food at some times and a poor-quality food at others, even though the resource environment does not change". Subsequent experiments by Grover and Chrzanowski [73] in which the *Pseudomonas* prey was grown in C-, N-, or P-limited chemostats showed that P-limited prey sustained the lowest growth rate of the protozoan predator *Ochromonas danica*, a result consistent with the growth rate hypothesis of ecological stoichiometry (Jones and Ellner [97]), namely high μ is predicated by large ribosome pools, ribosomes have a high P content, ipso facto P-limitation reduces growth relative to other limitations. Experimental support for some of these ideas was provided by Shannon et al. [165] who found that *O. danica* preferentially ingested *Pseudomonas fluorescens* cells of high food value (low C to P or C to N ratios) than those of low food value, though digestion rates were not correlated with food value. Critical experiments are now needed to assess the outcome of predation when the prey is provided as a mixture of high- and low-value food, and when it is live rather than dead, as used by Shannon et al. and to further test the predictions referred to above.

The phenotypic plasticity of microorganisms, or phenotypic variability as it was then known, was one of the dramatic discoveries during the first age of continuous culture (see Table 2) and now is seen to confer competitive advantage to organisms subject to fluctuating environments. The elegant complementary chromatic adaptation (CCA) work by Huisman's group [174] once again reinforces the power of chemostat studies for dissecting complex ecological situations. Using light-limited chemostats (see "Theory"), competition experiments between marine red and green picocyanobacteria (*Synechococcus* strains) and a strain of *Pseudanabaena* that changes colour by CCA, were made under conditions of red/green light fluctuations at different periodicities ranging from 0.5 days (6 h red/6 h green) to 28 days (14 days red/14 days green). Under all conditions the flexible *Pseudanabaena* excluded both the red and green picos, results that were in good agreement with model predictions based on monoculture data. On the basis of these and other findings Stomp and her colleagues [174] questioned in which environments CCA might be advantageous. The under water light

spectrum in the Baltic Sea, from which these three bacteria were isolated, changes from white (surface) to green (deep water). Accordingly at the surface *Pseudanabaena* would be expected to adjust its pigments to complement those of competing phytoplankton species, while under prevailing green light it would be expected to turn red; such predictions are consistent with previous competition experiments. The authors also comment on the significance of the timescales for chromatic adaptation. Positively buoyant cyanobacteria may be able to escape from turbulent ocean mixing caused by storms but those like *Pseudanabaena* could be transported by intense mixing to deeper water under which conditions CCA would be highly advantageous. Again it is important to note that experiments of this sort do not give complete answers and situations can be conceived where trade-off in competitive abilities occur, e.g. flexible phenotypes may be weaker competitors for a single spectrum light than specialists on that light.

The ecophysiological question posed by Lin et al. [112] was what determines the dominance of members of the *Geobacteraceae* (*δ-Protobacteria*) in certain sub-surface, iron-reducing environments undergoing petroleum remediation or subject to metal contamination? *Geobacter metallireducens* was grown in a chemostat operated with 100% biomass feedback and mimicking the environmentally relevant very low supply of metabolic energy so enabling extremely low growth rates (down to 0.0008 h^{-1}; i.e. doubling times $>$ 850 h!). Maintenance energy requirements under these conditions (growth limited by acetate or a humic acid analogue) are exceptionally low or insignificant (e.g. -0.37 ± 0.60 mmol acetate g biomass^{-1} h^{-1}). Finally *G. metallireducens* has the ability for the simultaneous utilization of alternative electron acceptors, without the necessity of gene induction, that may aid its adaptation to varying redox conditions and the rapid scavenging of available electron acceptors. This combination of physiological attributes makes *Geobacter* eminently suited for dominating under certain iron-reducing, sub-surface conditions. The authors conclude that such chemostat experiments combined with omic analyses will provide additional understanding of microbial behaviour at low growth rates characteristic of many ecosystems.

One such transcriptomic study has been reported recently on *Rhodopirellula baltica*, a marine member of the budding order of bacteria *Planctomycetales*, and the first one for which a complete genome sequence is available. The stress responses of *R. baltica* to changes in salinity and temperature were analysed by whole genome microarray transcriptomics [195]. In total more than 40% of this bacterium's genes were affected by such changes: high salinity resulting in the modulation of genes coding for compatible solutes, and ion transporters, cold shock resulting in altered expression of lipid metabolism and stress genes, and heat shock causing the induction of known chaperone genes. All of these stresses triggered down-regulation of the ribosomal machinery and up-regulation of transposases and certain extracytoplasmic functioning sigma factors, all of which enables Wecker and her colleagues to posit that *R. baltica* regulates its gene activity on a global rather than operon scale; however, given that more than 50% of the regulated genes remain to be ascribed a function, further insights into the responsive behaviour of this model planctomycete can be anticipated as data analysis is intensified.

Results of a large number of chemostat researches that attempt to model various population dynamics have appeared in the past few years, the brief descriptions of a few of which illustrate the range of problems being addressed and how the data produced might inform real ecosystem predictions. (1) *Resource competition*. Competition experiments between the N_2-fixing cyanobacterium *Cyanothece* and either the non-N_2-fixing *Synechococcus bacillaris* or *Chlorella* in low nitrate-limited chemostats revealed that *Cyanothece* outcompeted *Chlorella* but established a stable coexistence with *S. bacillaris* as fixed nitrogen released by *Cyanothece* enabled much denser populations of the picobacterium than occurred in monoculture. In contrast in light-limited chemostats at high nitrate concentrations *Synechococcus* displaced *Cyanothece*, while the latter could displace *Chlorella*. These results were in agreement with model predictions of lowest critical nitrogen requirements and critical light intensities of the three competing species [2]. (2) *Allelopathic interference*. Allelochemicals are produced by organisms as a competitive response, a recent described example being that of chlorellin production by mixed populations of micro green algae [51, 62]. Co-cultures of *Chlorella vulgaris* and *Pseudokirchneriella subcapitata* in P-limited chemostats showed mutual growth stimulation by low concentrations of chlorellin but at higher levels *P. subcapitata* was displaced. The effects of allelochemicals of this type in more complex ('natural') simulations of phytoplankton now need to be explored. (3) *Predator–prey dynamics*. Chemostat studies in this field have retained their popularity and importance and with recent focus, for example, on the effects of rapid prey evolution on predator–prey cycles [97] and on inducible and constitutive prey defences [191]. (4) *Strain diversification*. Virus interactions with marine cyanobacteria were referred to earlier in this section; further consequences of virus–host contact have been described by Middelboe et al. [129]. The effects of virulent bacteriophages on the marine *Cellulophaga baltica* were examined in chemostats and shown to drive strain diversification both in terms of viral resistance and a reduced capacity to assimilate carbon substrates. During a 3-week chemostat run the initial strain (sensitive to 24 *Cytophaga* phages)

diversified into to a multi-strain, multi-resistant population. No direct correlation could be made between resistance and loss of metabolic capacity although such trade-off may be found to exist if higher differentiating assays are employed.

Mathematical modelling has been a mainstay of ecophysiology research but will not be discussed in detail here. Although considerable output continues in this field the inadequacy of dialogue between biologists and modellers has caused Flynn [63] to argue, in the context of plankton research, that their "ultimate communication failings and associated waste of effort are all too obvious". Thus greater effort is needed from microbiologists to ensure that the data collected are the most commensurate for modelling, a need that is imperative as systems biology approaches come to dominate much of biology. Flynn's recent reappraisal of the Droop quota model [64], for example, reflects many of the dilemmas at the theory–experiment interface. The quota model [54] developed specifically for phytoplankton, states that growth is a function of internal nutrient availability not as stated by the Monod model on external nutrient availability, an irony as Flynn points out because both were based on steady state chemostat studies. In dynamic situations, therefore, an organism may continue to grow in the absence of sufficient external resource; attempts to simulate ecological and disease situations almost invariably neglect this concept which in turn questions the reliance on steady state chemostats rather than exploiting defined non-steady-state continuous cultures. Recent modelling that approaches some of these issues include non-equilibrium coexistence of many species on few resources [154], coexistence of microorganisms having different nutritional lifestyles (e.g. hetero-, auto- and mixotrophy [43]), and synchronization within unstructured populations resulting from individuals interacting with one another via a common nutrient pool [125].

Pathology

Continuous culture was used almost from its inception as a means of investigating the behaviour of pathogenic microorganisms. These early studies were driven by a need to determine nutritional requirements for growing pathogenic bacteria in the laboratory, their antigenic properties and virulence factors, and the rapidly expanding development of vaccines. Indeed Phil Gerhardt should at least share the credit for inventing the first continuous cultures as he described the operation of both "cyclic" and completely continuous systems for the cultivation of *Brucella suis*, the causal agent of swine and human brucellosis [66]. Post-1950 research saw a wide range of pathogenic bacteria brought into chemostat culture including *Bordetella pertusis, Clostridium* spp., *Corynebacterium diphtheriae, Neisseria gonorhoeae, Pasteurella* (*Yersinia*) *pestis, Salmonella typhimurium* and *S. typhosa* (*typhi*). These early investigations were reviewed by Stejskal [172]. During this period continuous cultures were used to mimic disease conditions and for studying interactions between pathogenic bacteria (e.g. dental caries [57]). The impact of omic technologies is very evident in this field and, as part of the continuous culture platform, is a powerful means for the further understanding of pathogenesis. Progress in this area can be illustrated by case studies of tuberculosis and peridontal disease.

In an appraisal of the present fight against human tuberculosis (TB) Russell and his colleagues conclude that "Despite recent increases in research activity, we remain hampered by large gaps in our knowledge of the biology of this pathogen" (*Mycobacterium tuberculosis*, *Mtb*), a situation that is exacerbated by a failure of the BCG vaccine to protect against adult pulmonary TB, a failure of chemotherapy to cure many patients, and a failure of target-based drug discovery programmes [159]. Currently there are less than 10 new drugs in clinical trials for TB [55] and urgent needs are for a broader range of drugs for containing resistant strains, and ones capable of being effective over shorter regimens (<6 months) in order to facilitate completion of treatment protocols.

The systems biology approach that a multidisciplinary team at the University of Surrey is developing aims to gain a better understanding of TB and ultimately the development of new drugs. Working with the BCG vaccine strain of *Mycobacterium bovis*, this group's first objective was to make a detailed study of how macromolecular composition was influenced by the growth environment. The murine model has proved to be a valuable one for deciphering some of the shifts in metabolism that define growth of *M. bovis* in vitro as opposed to that in the infection state. Thus, as an initial stage of developing a systems biology investigation of *Mtb*, specific growth rates for *M. bovis* in chemostat culture were chosen to simulate persistence (low μ) and the acute phase of infection (high μ) [13]. The physiological state known as persistence remains poorly understood but it enables the pathogen to remain quiescent in the host for extended periods before re-emerging. This study revealed that lipids were the major cell components, the proportions of which were very sensitive to changes in specific growth rate. It was concluded that under the imposed conditions of carbon-limited chemostat growth, the lipid values represented the minimum necessary to maintain metabolic and structural (cell wall, storage) requirements. Subsequent transcriptome analyses of *M. bovis* growing at low and high specific growth rates revealed that several genes of the dormancy survival regulon were expressed in slowly growing mycobacteria [14]. Moreover, the transcriptome profile from the latter physiological state showed good, though not complete,

congruence with that of *M. tuberculosis* growing inside the macrophage, thereby encouraging the use of chemostat models for deconstructing aspects of the in vivo status of pathogens. The ability to grow mycobacteria in chemostat culture provides opportunity to apply steady state modelling techniques such as flux balance analysis and in this context the group's macromolecular composition data have been incorporated into a genome-scale metabolic model of the TB pathogen [15]; the model was based on 849 unique reactions, 739 metabolites, and 726 genes and calibrated against steady state chemostat behaviour of *M. bovis*. Substrate consumption rates were calculated from flux balance analysis, while essential genes were predicted with nearly 80% success by flux balance analysis simulations and compared with published mutagenesis data for *M. tuberculosis*. An interactive, web-based version of the model is available (http://sysbio.sbs.surrey.ac.uk/tb/) and is continually updated to take account of gene annotations and experimental testing. As Russell et al. [159] opine an understanding of the adaptations of the pathogen and its points of vulnerability under the microenvironments it experiences in the host represents a logical route to develop drugs that will be potent in the disease situation. Most recently Beste et al. [16] have screened a transposon mutant library in order to define genetic requirements for slow and fast growth rates of *M. bovis* in chemostat cultures and the requirements for switching specific growth rate. A total of 84 genes were identified that are required exclusively for slow growth (simulating persistence physiological state) and 256 genes that are required for switching from slow to fast (simulating infection physiological state). In order to validate these results the group analysed knock-out mutants of *M. bovis* and *Mtb* and among the findings showed that the *mce1* gene locus appears to be a component of the fast to slow growth rate switch, a result consistent with its proposed involvement in modulating the host inflammatory response so that *Mtb* can enter a persistent state without being eliminated or causing disease in the host [166].

Another study of mycobacteria that further vindicates the use of chemostat cultivation relates to exploring the host preferences of *Mtb* and *M. bovis*. Because the genome sequences of these organisms are close to identical (99.95% at nucleotide level), Golby et al. [69] opted to focus on gene expression as a means of probing the molecular basis of this host predilection. These authors stress the point that in defining core gene expression differences it is essential that identical growth conditions are used for the two bacteria, hence the use of continuous culture "since it allows environmental conditions and bacterial growth rates to be tightly and reproducibly controlled, reducing variation between biological replicates", and gives confidence in identifying key gene expression differences between the strains, rather than them being growth rate or medium effects. Although the 'minimal' set of differentially expressed genes between *Mtb* and *M. bovis* remains to be defined, this group nevertheless has identified differentially expressed candidate genes encoding a range of functions (including cell wall and secreted proteins, transcriptional regulators, PE/PPE proteins, lipid metabolism and toxin–antitoxin pairs) that could have pivotal roles in the pathogenic process.

Periodontitis is the advanced type of periodontal disease in which subgingival dental plaque develops in the tissues that support the teeth. The major causal agent of chronic periodontitis is believed to be *Porphyromonas gingivalis*. The group of Reynolds in the University of Melbourne has made extensive use of chemostats to study the adaptation of virulent invasive strains of *P. gingivalis* to biofilm growth. Initial studies focused on comparative proteomics of planktonic and biofilm populations growing together in chemostats. Among the proteins that increased in abundance in biofilm organisms were those of the cell-surface-located C-terminal domain [4]. The latter included RgpA, HagA, thiorhodoxin, cag70 carboxypeptidase, API extracellular protease and the Pg99 immunoreactive protein. These C-terminal domain proteins are likely to have significant impacts on virulence because they are surface exposed and probably participate in secretion and attachment. Reynolds' group have followed up this work with a comparative transcriptomics analysis of the two growth forms [114] and found that ca. 18% of the genome is differentially expressed when *P. gingivalis* develops as a biofilm. Particularly interesting is the down-regulation of genes involved in bacterial envelope biogenesis and energy production, suggesting an adaptation to the biofilm niche where low growth rate and a contracted metabolic status could have significant survival value. The authors are careful to acknowledge that their data were obtained from mono-species chemostat cultures, consequently results from the analysis of gene expression when *P.gingivalis* is part of the multi-species disease community will be especially important in understanding the pathology. *Porphyromonas gingivalis* has a requirement for Fe and protoporphyrin IX that is met by heme, and most recently this group has reported on its response when switched from heme-excess to heme-limited continuous culture conditions [46]. Transcriptome analysis revealed a shift from an energy-efficient physiological state to one less efficient when growth is heme-limited. Moreover, the response to heme limitation is far reaching, involving as it does up-regulation of genes encoding proteins involved in the invasion of host cells, oxidative stress response, Fe and heme transport, and overall virulence.

Fusobacterium nucleatum also has a defining role in the establishment of periodontal disease, having been

described as an "initiator organism" that affects changes in the gingivae that enable acid-sensitive pathogens to co-colonize. The expression of soluble proteins in chemostat populations of *F. nucleatum* grown under either acid or alkaline conditions was determined by Zilm et al. [202]. At sub-optimal acid pH the bacterium responded by up-regulating proteins involved in glutamate catabolism and thereby facilitating the alkalinization of the gingival milieu. Furthermore, growth in chemostats under conditions (μ, alkaline pH) mimicking those of the pathology caused *F. nucleatum* bacteria to co-adhere and form biofilms, behaviour that was attended by increased cell-surface hydrophobicity [203]. The most recent report from Reynolds' laboratory concerns another organism implicated in periodontal disease, *Treponema denticola* [131]. Transcriptome analysis of biofilm and planktonic organisms grown in continuous cultures produced the major revelation of up-regulation of putative prophage genes in biofilms, following which bacteriophage and circular DNA were isolated from the spirochaete. Also up-regulated in this population were genes encoding virulence factors, toxin-antitoxin systems, and transposases. The group opine that these data bespeak of an increased potential for genetic mobility in biofilms of *Treponema denticola* that in turn is significant for biofilm persistence and hence virulence.

Dissolved oxygen tension (DOT) has been known to influence the ability of group B streptococci (GBS) to invade human cells and recently transcriptome and proteome profiles of chemostat cultures have been obtained and used to extend our understanding of this pathology. GBS type V organisms grown at a high specific growth rate (0.385 h^{-1}), that is considered to mimic in vivo growth, and a DOT of 12% were shown to invade cervical epithelial cells in large numbers compared to those grown at the same rate but without oxygen, or at a low specific growth rate (0.063 h^{-1}) [96]. Invasive organisms of this opportunistic pathogen had up-regulated genes encoding several adherence proteins that supports their role in the early physiopathological events. Chemostat studies aimed at gaining greater insight into the pathology of another streptococcus have been reported recently by Cornejo et al. [42] who found that population densities of clinical and laboratory strains of *Streptococcus pneumoniae* oscillate dramatically with a constant period in chemostat cultures. Population death was attributable to a protein toxin that was effective against different strains of *S. pneumoniae* and closely related species leading to the conclusion that it acts as an allelopathic agent. The authors postulate that such toxins have evolved as a means of preventing niche colonization of the established populations by different, closely related competitors.

Scientists at the Netherlands Vaccine Institute have adopted a systems biology approach incorporating a continuous culture platform for optimizing the efficacy and production of the outer membrane vesicle vaccine against the causal agent of human meningitis. A genome-scale metabolic model was derived that enabled a chemically defined, minimal growth medium to be formulated, the validity of which was confirmed using flux balance analysis obtained from chemostat cultures [7] (cf. Beste et al. [15] above), and subsequent trials in batch and chemostat cultures. Although *N. meningitides* maintained a constant macromolecular composition over a wide range of specific growth rates (0.041–0.161 h^{-1}) (cf. Beste et al. [15]), the PorA antigen content of the outer membrane vesicles was significantly reduced at high specific growth rate [8] a finding that has major bearing on the vaccine production operation.

Fluoroquinolones have been acclaimed as one of the more recent successes in antimicrobial chemotherapy and, for example, their administration was considered low risk for the development of *Clostridium difficile* infection (CDI). This proposition has been tested by using a validated three-stage chemostat model of the human gut [118] in which epidemic strains of *C. difficile* were challenged with several widely prescribed fluoroquinolone antibiotics (ciprofloxacin, levofloxacin and moxifloxacin) [160]. The chemostat model comprised three fermentation vessels (proximal, medial and distal) having an overall retention time of 66.7 h (vessel 1, 16.7 h; vessels 2 and 3, 25 h) and a dilution rate of 0.015 h^{-1}. Vessel 1 was operated at low pH (5.5) and high substrate availability, whereas vessels 2 and 3 were operated at more neutral pH (6.2 and 6.8 respectively) and lower substrate availability to reflect the increasing alkalinity of the colon from proximal to distal. The model was inoculated with faecal emulsion from pooled *C. difficile* negative faeces of healthy elderly volunteers having no history of antimicrobial therapy in the preceding 3 months, and with *C. difficile* endospores (ca. 10^7 cfu) on days 14 and 21. All of the fluoroquinolones caused germination of the *C. difficile* spores, growth, and a high level of cytotoxin production that causes mucosal inflammation and diarrhoea in hospitalized elderly patients. These common effects of fluoroquinolones were observed despite differences in the degree of inhibition of the gut microbiota but it remains to be confirmed whether they directly induce germination, or provide an environment conducive for germination. One of these compounds, moxifloxacin, also appeared to trigger increased resistance to this fluoroquinolone. In the clinical context it is pertinent to note that the elderly have larger gut populations of enterobacteria and clostridia than young adults [119] and the implications that antibiotic use and hospitalization may have for this cohort of patients. As in our discussions of ecophysiology it must be emphasized that in vitro chemostat models are but a mirror of reality; in this case the gut model does not mimic immunological or

secretory events within the colon, but it is a reliable indicator of whether a drug may have a propensity to induce CDI in vivo. The glycylcycline antibiotic tigecycline on the evidence of a small case series appears promising as a treatment of refractory CDI and offers a potential new alternative for intravenous therapy [164]. The report by Baines et al. [10] that tigecycline does not induce growth or cytotoxin production by epidemic strains of *C. difficile* in the chemostat gut model should encourage further trials of this antibiotic.

Industrial applications

Wastewater treatment remains the largest continuous industrial bioprocess but whereas biofuel (principally bioethanol) continuous production has been stimulated by the need for greater economic sustainability, continuous beer fermentation still has limited commercial application. In each of these cases technological advances have been incremental rather than radical. Thus, despite several decades of research the once anticipated adoption of continuous brewing has not occurred due largely to the difficulty of achieving acceptable organoleptic quality. Methods for on-line monitoring of aroma compounds in continuous beer fermentations are available [181], while genome-scale transcriptome analysis of *Saccharomyces cerevisiae* during industrial bioethanol production has recently been reported [111]. Such developments can provide important insights into yeast performance in the course of these fermentations and, thereby, lead to improved continuous processes.

A constant and universal challenge for industrial microbiology is to maximize productivity and here chemostat theory predicts higher productivities of biomass (Dx) and growth-related metabolites compared with batch processes operating with similar yields, and final biomass and inoculum concentrations. Moreover, the advantage of continuous cultures in this regard increases as a function of D, and particularly with recycle systems because processes can be operated at $D > \mu_{max}$ [27, 121, 127]. Of course productivity can also be enhanced via strain improvement programs and although these mostly rely on a random approach continuous culture selection systems have been designed that deliver desired, complex phenotypes that have relevance for industrial processes and products [49, 68]. Alternative options discussed in a comprehensive review by Rokem et al. [156] in the context of antibiotic production include metabolic engineering, and obtaining detailed physiological insight into the natural producer organism, something that is now feasible relatively quickly and at comparative low cost through a systems biology approach. Genome-scale metabolic models aim to reconstruct complete metabolic networks and these coupled with flux analyses enable the simulation of growth and product formation. A number of recent applications of these tools to *Streptomyces* and *Penicillium* antibiotic syntheses demonstrate the value of the approach.

In a series of papers Bushell and his colleagues [6, 94, 101] reported the application of flux and cluster analysis to chemostat cultures of *Streptomyces clavuligerus* growing under different nutrient limitations. Clavulanic acid titres were affected by the nature of the growth-limiting substrate and it appeared that a high anaplerotic carbon flux might be responsible for the stoichiometric limitation of a C_3 precursor. *S. clavuligerus* has limited capacity to assimilate glucose thereby prompting the use of glycerol as a carbon source; however, competition from other C_3-utilising pathways can influence its availability. This group made predictive use of metabolic flux analysis (MFA) of comparatively small networks to design amino acid feed formulations to enhance clavulanic productivity [32]. Phosphate-limited chemostats fed mixtures of amino acids whose biosynthesis require oxaloacetate as a precursor (i.e. aspartate, threonine, asparagine and arginine) improved clavulanic acid yields by more than tenfold compared with that found in non-supplemented cultures. Crucially the amino acid feeds causing yield increases are not clavulanic acid precursors per se, consequently they are not obvious and are only identified using a predictive model. Subsequently Bushell et al. [33] extended their work to genome-scale metabolic flux variability analysis (MFVA) in order to determine rational feed design for actinorhodin production by *S. coelicolor*. The *S. coelicolor* genome contains two genes encoding glucose-6-phosphate dehydrogenase and deletion of either gene caused both an increase in the in vitro activity in the remaining gene product, and in actinorhodin synthesis. The authors rightly assert that this effect could not have been predicted by simple MFA; only when the MFVA was made on both strains was "a pivotal role for intracellular glutamate as an actinorhodin precursor identified", a prediction again borne out by elevated antibiotic production in glutamate-fed phosphate-limited chemostats. Thus, MFVA has the significant ability to reveal areas of metabolism from relatively disparate pathways that might be connected in the biosynthesis of complex secondary metabolites. The authors provide further discussion of this topic in Khannapho et al. [99].

Approaches to the improvement of β-lactam production have been guided by a similar logic. As Kleijn et al. [103] have argued classical strain improvement strategies based on increasing the levels of enzymes in secondary metabolic pathways "will, at some point, lead to shift of the metabolic bottleneck from secondary metabolism towards primary metabolism". These authors examined the synthesis of penicillin G by *Penicillium chrysogenum* in carbon-limited chemostat cultures in order to test the hypothesis that the supply of NADPH (critical for the biosynthesis of

β-lactam amino acid precursors such as cysteine) is principally through the oxidative branch of the pentose phosphate pathway (PPP). Analysis of growth under penicillin G producing and non-producing conditions revealed significantly higher oxidative PPP fluxes in antibiotic producing chemostats, suggesting that penicillin production exerts a major burden on the supply of cytosolic NADPH. Subsequent MFA showed that the oxidative branch of the PPP indeed produced the majority of the cytosolic NADPH needed for penicillin synthesis. The authors conclude that limiting the cytosolic NADPH demand for penicillin synthesis (for example by introducing a cytosolic direct sulfhydrylation pathway for cysteine synthesis) could provide an interesting target for future metabolic engineering of *P. chrysogenum.*

The production of penicillin G and its side-chain precursor phenylacetic acid (PAA) has been investigated in a high-producing strain of *P. chrysogenum* and a construct lacking the functional penicillin-biosynthesis gene cluster [80]. The use of transcriptome analyses on glucose-limited chemostat cultures of these strains, grown in the presence and absence of PAA, showed that the homogentisate pathway for PAA catabolism was strongly transcriptionally up-regulated in PAA-fed cultures; and that several genes involved in nitrogen and sulfur metabolism were transcriptionally up-regulated only under penicillin G producing conditions suggesting a drain of amino acid precursor pools. Most recently this group has again used chemostat-based transcriptome analysis to identify the gene encoding the acyl-CoA ligase involved in the activation of adipate prior to its incorporation into the β-lactam backbone [105]. The results from both of these studies indicate clear-cut targets for metabolic engineering, in the former case genes beyond those of the β-lactam pathway per se, and in the latter case for exploring the production of cephalosporin analogs.

Reflections and outlook

There can be little argument with the view expressed by Pronk's group [45] that the renewed interest in continuous culture is linked strongly with the advent of high-information-density (HID) technology and the consequent need for rigorously controlled and reproducible conditions for microbial growth. Moreover, the days are long gone when equipment had to be constructed and assembled by the enthusiast; commercially available units are widely accessible and in my experience manufacturers are very adept at custom building to meet specific research objectives. Detailed practical advice on continuous culture systems is published regularly, the latest appearing in the new edition of the *Manual of Industrial Microbiology and Biotechnology* [198]. So my main conclusion is that the revival of continuous culture will be long lasting because its recognition as the growth system of choice is increasingly appreciated. A converse view that might regard batch and fed-batch systems as unregulated and unacceptable for basic research is, of course, absurd but further thought on this point later.

Given this overall conclusion at least two caveats need to be emphasized. First, the renaissance of continuous culture and the widespread adoption of HID technologies brings with it the danger of data overload and confusion particularly when different (even slightly different) culture platforms are used by different groups. The approach taken by van Eunen et al. [188] in one specific context might provide an exemplar for resolving problems of this type. We would do well to keep Brenner's counsel in mind in this context: the conversion of data into knowledge constitutes a great challenge for future biological research [23]. Massive data accumulation that increasingly is part and parcel of systems biology needs to the tempered by careful experimental design and conduct, and intelligent evaluation of outputs in order to achieve greater biological insight. Second, an understanding of basic chemostat theory (and that of related continuous cultures) is mandatory if the full benefits of the methodology are to be realized, and to prevent unwarranted statements appearing in the literature—and with respect this admonition is made particularly to first-time users. This is not to imply that theoretical matters are being ignored during this post-genomics revival as the re-examination of maintenance requirements [187] and the extended theory of light-limited growth [91] testify. The early researches on continuous culture merit serious revisiting for their wealth of insightful exploration of microbial behaviour; this is a major purpose of this review.

This review has attempted to make an assessment of the impact of continuous culture across a comprehensive range of microbiological research and development. Some discussions will appear immediately relevant to industrial microbiology, others perhaps less so. Focus on ecophysiology could, at first sight, seem inappropriate but if we fail to understand the factors affecting microbial interactions, population dynamics or adaptation mechanisms, for example, our capacity to design and operate improved waste treatment and environmental management systems, or to predict and prepare for local and global environmental perturbations assuredly will be compromised. Consideration of this latter point prompts another significant advantage associated with the use of continuous culture, namely the ability to analyse and thence exploit data derived from the study of specified transition states or any one of an infinite number of chemical, physical or biological perturbations. The analysis of dynamic situations of this sort can enhance our understanding of microbial

ecology, and of microbial pathology offering a wider scope for innovative drug discovery; they also can inform the optimization of batch and fed-batch operations that, by definition, are characterized by sequential transition states.

The direct influence of continuous culture on industrial processes has been less that at one time envisaged. It is possible that just as the adjudged shortage of food and feed protein stimulated industrial-scale SCP production, the escalating concerns over energy and fuel availability might encourage comparable continuous culture options beyond those of bio-alcohol or biogas. To date, however, the contributions of continuous culture to industrial processes and products have been incremental in the sense that that they have impacted on process development and optimization.

The outlook for continuous culture undoubtedly is bright—just as growth of (bacterial) cultures is the basic method of microbiology [132], continuous culture is reasserting itself as *the* method of choice for revealing many of its intricacies. In my experience this facet of microbiology—growth physiology—has been severely diluted (practically and theoretically) in many university degree programs and now would be an opportune time to redress ill-considered curriculum decisions. To borrow some words of Bill Shankly, the iconic manager of Liverpool FC in the 1960s and 1970s: some people think continuous culture is a matter of life and death, it's much more serious than that!

Acknowledgments It is a pleasure to acknowledge the inspirational inputs of numerous students and post-docs in my group over so many years, and of the wider community of continuous culturalists the names of most of whom can be found in Table 2 and from whose friendship my thinking and research has greatly benefited. My thanks to those colleagues who sent me papers prior to publication. Michael Bushell read the review at draft stage and provided valuable comments. This review is dedicated to the next generation of scientists who will carry the torch for continuous culture.

References

1. Aboka FO, Heijnen JJ, van Winden WA (2009) Dynamic ^{13}C-tracer study of storage carbohydrate pools in aerobic glucose-limited *Saccharomyces cerevisiae* confirms a rapid steady-state turnover and fast mobilization during a modest stepup in the glucose uptake rate. FEMS Yeast Res 9:191–201
2. Agawin NSR, Rabouille S, Veldhuis MJW, Servatius L, Hol S, van Overzee MJ, Huisman J (2007) Competition and facilitation between unicellular nitrogen-fixing cyanobacteria and non-nitrogen-fixing phytoplankton species. Limnol Oceanogr 52:2233–2248
3. Alain K, Postec A, Grinsard E, Lesongeur F, Prieur D, Godfroy A (2010) *Thermodesulfatator atlanticus sp. nov.,* a thermophilic, chemolithoautotrophic, sulfate-reducing bacterium isolated from a Mid-Atlantic Ridge hydrothermal vent. Int J Syst Evol Microbiol 60:33–38
4. Ang CS, Vieth PD, Dashper SG, Reynolds EC (2008) Application of ^{16}O/^{18}O reverse proteolytic labeling to determine the effect of biofilm culture on the cell envelope proteome of *Porphyromonas gingivalis* W50. Proteomics 8:1645–1660
5. Arvas M, Pakula T, Lanthaler K, Saloheimo M, Valkonen M, Suortti T, Robson G, Pentilla M (2006) Common features and interesting differences in transcriptional responses to secretion stress in the fungi *Trichoderma reesei* and *Saccharomyces cerevisiae*. BMC Genomics 7:32
6. Avignone-Rossa C, White J, Kuiper A, Postma PW, Bibb MJ, Texeira de Mattos MJ (2002) Carbon flux distribution in chemostat cultures of *Streptomyces lividans*. Metab Eng 4:138–150
7. Baart GJE, Zomer B, de Haan A, van der Pol LA, Beuvery EC, Tramper J, Martens DE (2007) Modeling *Neisseria meningitidis* metabolism: from genome to metabolic fluxes. Genome Biol 8:r136
8. Baart GJE, Willemsen M, Khatami E, de Haan A, Zomer B, Beuvery EC, Tramper J, Martens DE (2008) Modeling *Neisseria meningitides* B metabolism at different specific growth rates. Biotechnol Bioeng 101:1022–1035
9. Babel W (2009) The auxiliary substrate concept: from simple considerations to heuristically valuable knowledge. Eng Life Sci 9:285–290
10. Baines SD, Saxton K, Freeman J, Wilcox MH (2006) Tigecycline does not induce proliferation or cytotoxin production by epidemic *Clostridium difficile* strains in human gut model. J Antimicrob Chemother 58:1062–1065
11. Balagadde FK, You LK, Hansen CL, Arnold FH, Quake SR (2005) Long-term monitoring of bacteria undergoing programmed population control in a microchemostat. Science 309:137–140
12. Bastidas-Oyanedel JR, Aceves-Lara CA, Ruiz-Filippi G, Steyer JP (2008) Thermodynamic analysis of energy transfer in acidogenic cultures. Eng Life Sci 8:487–498
13. Beste DJV, Peters J, Hooper C, Avignone–Rossa C, Bushell ME, McFadden JJ (2005) Compiling a molecular inventory for *Mycobacterium bovis* BCG at two growth rates: evidence for growth rate-mediated regulation of ribosome biosynthesis and lipid metabolism. J Bacteriol 187:1677–1684
14. Beste DJV, Laing E, Bonde C, Avignone-Rossa C, Bushell ME, McFaddern JJ (2007) Transcriptomic analysis identifies growth rate modulation as a component of the adaptation of Mycobacteria to survival inside the macrophage. J Bacteriol 189:3969–3976
15. Beste DJV, Hooper T, Stewart G, Bonde B, Avignone-Rossa C, Bushell M, Wheeler P, Klamt S, Kierzek AM, McFadden JJ (2007) GSMN-TB: a web-based genome scale network model of Mycobacterium tuberculosis metabolism. Genome Biol 8:r89
16. Beste DJV, Espasa M, Bonde B, Kierzek AM, Stewart GR, McFadden JJ (2009) The genetic requirements for fast and slow growth in mycobacteria. PLoS One 4:e5349
17. Bijmans MFM, Dopson M, Peeters TWT, Lens PNL, Bujisman CJN (2009) Sulfate reduction at pH 5 in a high-rate membrane bioreactor: reactor performance and microbial community analyses. J Microbiol Biotechnol 19:698–708
18. Boer VM, Crutchfield CA, Bradley PH, Botstein D, Rabinowitz JD (2010) Growth-limiting intracellular metabolites in yeast growing under diverse nutrient limitations. Mol Biol Cell 21:198–211
19. Boender LGM, de Huslster EAF, van Maris AJA, Daran-Lapujade PAS, Pronk JT (2009) Quantitative physiology of *Saccharomyces cerevisiae* at near-zero specific growth rates. Appl Environ Microbiol 75:5607–5614
20. Bragg JG, Wagner A (2007) Protein carbon content evolves in response to carbon availability and may influence the fate of duplicated genes. Proc R Soc B 274:1063–1070

21. Bragg JG, Wagner A (2009) Protein material costs: single atoms can make an evolutionary difference. Trends Genet 25:5–8
22. Brauer MJ, Huttenhower C, Airoldi EM, Rosenstein R, Matese JC, Gresham D, Boer VM, Troyanskaya OG, Botstein D (2008) Coordination of growth rate, cell cycle, stress response, and metabolic activity in yeast. Mol Biol Cell 19:352–367
23. Brenner S (2010) Sequences and consequences. Phil Trans R Soc B 365:207–212
24. Breslauer DN, Lee PJ, Lee LP (2006) Microfluidics-based systems biology. Mol Bio Syst 2:97–112
25. Brown SW, Oliver SG (1982) Isolation of ethanol-tolerant mutants of yeast by continuous selection. Eur J Appl Microbiol Biotechnol 16:119–122
26. Bull AT (1974) Microbial growth. In: Bull AT, Lagnado JR, Thomas JO, Tipton KF (eds) Companion to biochemistry–selected topics for further study. Longman, London, pp 415–442
27. Bull AT (1983) Continuous culture for production. In: Hollaender A, Laskin AI, Rogers P (eds) Basic biology of new developments in biotechnology. Plenum, New York, pp 405–437
28. Bull AT (1985) Mixed culture and mixed substrate systems. In: Bull AT, Dalton H (eds) Comprehensive biotechnology, vol 1. The principles of biotechnology: scientific fundamentals. Pergamon, Oxford, pp 281–299
29. Bull AT, Brown CM (1979) Continuous culture applications to microbial biochemistry. In: Quayle JR (ed) International review of biochemistry, vol 21: microbial biochemistry. University Park Press, Baltimore, pp 177–226
30. Bull AT, Trinci APJ (1977) The physiology and metabolic control of fungal growth. Adv Microbial Physiol 15:1–84
31. Bull DN, Young MD (1982) Enhanced product formation in continuous fermentations with microbial cell recycle. Biotechnol Bioeng 23:373–389
32. Bushell ME, Kirk S, Zhao HJ, Avignone-Rossa C (2006) Manipulation of the physiology of clavulanic acid biosynthesis with the aid of metabolic flux analysis. Enzyme Microb Technol 39:149–157
33. Bushell ME, Sequeira SIP, Khannapho C, Zhao HJ, Chater KF, Butler MJ, Kierzek AJM, Avignone-Rossa C (2006) The use of genome scale metabolic flux variability analysis for process feed formulation based on an investigation of the effects of the *zwf* mutation on antibiotic production in *Streptomyces coelicolor*. Enzyme Microb Technol 39:1347–1353
34. Canelas AB, ten Pierick A, Ras C, Seifar RM, van Dam JC, van Gulik WM, Heijnen JJ (2009) Quantitative evaluation of intracellular metabolite extraction techniques for yeast metabolomics. Anal Chem 81:7379–7389
35. Carlile MJ, Skehel JJ (eds) (1974) Evolution in the microbial world, pp i–x, 1–430, symposium 24 Soc Gen Microbiol, Cambridge University Press, Cambridge
36. Chong L, Ray LB (2002) Whole-istic biology. Science 295:1661
37. Chrzanowski TH, Grover JP (2008) Element content of *Pseudomonas fluorescens* varies with growth rate and temperature: a replicated chemostat study addressing ecological stoichiometry. Limnol Oceanogr 53:1242–1251
38. Cipollina C, van den Brink J, Daran-Lapujade P, Pronk JT, Porro D, de Winde JH (2008) *Saccharomyces cerevisiae* SFP1: at the crossroads of central metabolism and ribosome biogenesis. Microbiology 154:1686–1699
39. Clarke PH, Lilly MD (1969) The regulation of enzyme synthesis during growth. In: Meadows PM, Pirt SJ (eds) Microbial growth, symposium 19 Soc Gen Microbiol. Cambridge University Press, Cambridge, pp 113–159
40. Codeco CT, Grover JP (2001) Competition along a spatial gradient of resource supply: a microbial experimental model. Am Nat 157:300–315
41. Cooper DC, Copeland BJ (1973) Responses of continuous-series estuarine microecosytems to point source input variations. Ecol Monogr 43:213–236
42. Cornejo OE, Rozen DE, May RM, Levin BR (2008) Oscillations in continuous cultures populations of *Streptococcus pneumoniae*: population dynamics and evolution of clonal suicide. Proc R Soc B 276:999–1008
43. Crane KW, Grover JP (2010) Coexistence of mixotrophs, autotrophs, and heterotrophs in planktonic microbial communities. J Theor Biol 262:517–527
44. Daran-Lapujade P, Jansen MLA, Daran JM, van Gulik W, de Winde JH, Pronk JT (2004) Role transcriptional regulation in controlling fluxes in central carbon metabolism of *Saccharomyces cerevisiae*–a chemostat study. J Biol Chem 279:9125–9138
45. Daran-Lapujade P, Daran JM, van Maris AJA, de Winde JH, Pronk JT (2009) Chemostat-based micro-array analysis in baker's yeast. Adv Microbial Physiol 54:257–311
46. Dashper SG, Ang CS, Veith PD, Mitchell HL, Lo AWH, Seers CA, Walsh KA, Slakeski N, Chen D, Lissel JP, Butler CA, O'Brien-Simpson NM, Barr IG, Reynolds EC (2009) Response of *Porphyromonas gingivalis* to heme limitation in continuous culture. J Bacteriol 191:1044–1055
47. Dean ACR, Ellwood DC, Melling J, Robinson A (1976) The action of antibacterial agents on bacteria grown in continuous culture. In: Dean ACR, Ellwood DC, Evans CGT, Melling J (eds) Continuous culture 6: applications and new fields. Ellis Horwood, Chichester, pp 251–261
48. de Crécy E, Metzgar D, Allen C, Pénicaud M, Lyons B, Hansen CJ, Crécy-Lagard V (2007) Development of a novel continuous culture devise for experimental evolution of bacterial populations. Appl Microbiol Biotechnol 77:489–496
49. de Crécy E, Jaronski S, Lyons B, Lyons TJ, Keyhani NO (2009) Directed evolution of a filamentous fungus for thermotolerance. BMC Biotechnol 9:74
50. Delneri D, Hoyle DC, Gkargkas K, Cross EJM, Rash B, Zeef L, Leong HS, Davey HM, Hayes A, Kell DB, Griffith GW, Oliver SG (2008) Identification and characterization of high-flux-control genes of yeast through competition analysis. Na Genet 40:113–117
51. DellaGreca M, Zarelli A, Fergola P, Cerasuola M, Pollioi A, Pinto G (2010) Fatty acids released by *Chlorella vulgaris* and their role in interference with *Pseudokirchneriella subcapita*: experiments and modeling. J Chem Ecol 36:339–349
52. De Nicola R, Hazelwood LA, De Hulster EAF, Walsh MC, Knijnenburg TA, Reinders MJT, Walker GM, Pronk JT, Daran JM, Daran-Lapujade (2007) Physiological and transcriptional responses of *Saccharomyces cerevisiae* to zinc-limitation in chemostat cultures. Appl Environ Microbiol 73:7680–7692
53. Diano A, Peeters J, Dynesen J, Nielsen J (2009) Physiology of *Aspergillus niger* in oxygen-limited continuous cultures: influence of aeration, carbon source concentration and dilution rate. Biotechnol Bioeng 103:956–965
54. Droop MR (1968) Vitamin B12 and marine ecology. IV. The kinetics of uptake, growth and inhibition in Monochrysis butleri. J Protozool 48:689–733
55. Dye C, Williams BG (2010) The population dynamics and control of tuberculosis. Science 328:856861
56. Elias DA, Tollaksen SL, Kennedy DW, Mottaz HM, Giometti CS, McLean JS, Hill EA, Punchuk GE, Lipton MS, Fredrickson JK, Gorby YA (2008) The influence of cultivation methods on *Shewanella oneidensis* physiology and proteome expression. Arch Microbiol 189:313–324
57. Ellwood DC, Hunter JR (1976) The mouth as a chemostat. In: Dean ACR, Ellwood DC, Evans CGT, Melling J (eds) Continuous culture 6: applications and new fields. Ellis Horwood, Chichester, pp 270–282

58. Ferea TL, Botstein D, Brown PO, Rosenzweig RF (1999) Systematic changes in gene expression patterns following adaptive evolution in yeast. Proc Nat Acad Sci U S A 96:9721–9726
59. Ferenci T (1999) Growth of bacterial cultures 50 years on: towards an uncertainty principle instead of constants in bacterial growth kinetics. Res Microbiol 150:431–438
60. Ferenci T (2006) A cultural divide on the use of chemostats. Microbiology 152:1247–1248
61. Ferenci T (2008) Bacterial physiology, regulation and mutational adaptation in a chemostat environment. Adv Microbial Phyisol 53:22–169
62. Fergola P, Cerasuolo M, Pollio A, Pinto G, DellaGreca M (2007) Allelopathy and competition between *Chlorella vulgaris* and *Pseudokirchneriella subcapitata*: experiments and mathematical model. Ecol Model 208:205–214
63. Flynn KJ (2005) Castles built on sand: dysfunctionality in plankton models and the inadequacy of dialogue between biologists and modelers. J Plankton Res 27:1205–1210
64. Flynn KJ (2008) Use, abuse, misconceptions and insights from quota models–the droop cell quota model 40 years on. Oceanogr Mar Biol An Rev 46:1–23
65. Gefen O, Balaban NQ (2008) The Moore's Law of microbiology–towards bacterial culture miniaturization with the micro-Petri chip. Trends Biotechnol 26:345–347
66. Gerhardt P (1946) *Brucella suis* in aerated broth culture, III. Continuous culture studies. J Bacteriol 52:283–292
67. Gilbert A, Srienc F (2009) Optimized evolution in the cytostat: a Monte Carlo simulation. Biotechnol Bioeng 102:221–231
68. Gilbert A, Sangurdekar DP, Srienc F (2009) Rapid strain improvement through optimized evolution in the cytostat. Biotechnol Bioeng 103:500–512
69. Golby P, Hatch KA, Bacon J, Cooney R, Riley P, Allnutt J, Hinds J, Nunez J, Marsh PD, Hewinson RG, Gordon SV (2007) Comparative transcriptomics reveals key gene expression differences between the human and bovine pathogens of the *Mycobacterium tuberculosis* complex. Microbiology 153:3323–3336
70. Gresham D, Desai MM, Tucker CM, Jenq HT, Pai DA, Ward A, DeSevo CG, Botstein D, Dunham MJ (2008) The repertoire and dynamics of evolutionary adaptations to controlled nutrient-limited environments in yeast. PLOS Genet 4:e1000303
71. Groeneveld P, Stouthamer AH, Westerhoff HV (2009) Super life–how and why 'cell selection' leads to the fastest-growing eukaryote. FEBS J 276:254–270
72. Groisman A, Lobo C, Campbell JK, Dufour YS, Stevens AM, Levchenko A (2005) A microfluidic chemostat for experiments with bacterial and yeast cells. Nat Methods 2:685–689
73. Grover JP, Chrzanowski TH (2009) Dynamics and nutritional ecology of a nanoflagellate preying upon bacteria. Microb Ecol 58:231–243
74. Guebel DV, Canovas M, Torres NV (2009) Analysis of the *Escherichia coli* response to glycerol pulse in continuous, high-cell density culture using a multivariate approach. Biotechnol Bioeng 102:910–922
75. Hall BG (1985) Enzyme evolution. In: Bull AT, Dalton H (eds) Comprehensive biotechnology vol 1, the principles of biotechnology: scientific fundamentals. Pergamon, Oxford, pp 553–566
76. Hamer G (1984) Continuous culture kinetics and activated sludge processes. In: Dean ACR, Ellwood DC, Evans CGT (eds) Continuous culture 8: biotechnology, medicine and the environment. Ellis Horwood, Chichester, pp 169–184
77. Hamilton IR, Ellwood DC (1978) Effects of fluoride on carbohydrate metabolism by washed cells of *Streptococcus mutans* grown at various pH values in a chemostat. Infect Immun 19:434–442
78. Harder W, Dijkhuisen L (1976) Mixed substrate utilization. In: Dean ACR, Ellwood DC, Evans CGT, Melling J (eds) Continuous culture 6: applications and new fields. Ellis Horwood, Chichester, pp 297–314
79. Hardman DJ, Huxley M, Bull AT, Slater JH, Bates R (1997) Generation of environmentally enhanced products: clean technology for paper chemicals. J Chem Technol Biotechnol 70:60–66
80. Harris DM, van der Krogt ZA, Klaasen P, Raamsdonk LM, Hage S, van den Berg MA, Bovenberg RAL, Pronk JT, Daran JM (2009) Exploring and dissecting genome-wide gene expression responses of *Penicillium chrysogenum* to phenylacetic acid and penicillin G. BMC Genomics 10:75
81. Harrison DEF (1972) Physiological effects of dissolved oxygen tension and redox potential on growing populations of microorganisms. In: Dean ACR, Pirt SJ, Tempest DW (eds) Continuous culture 5: environmental control of cell synthesis and function. Academic, London, pp 417–440
82. Harrison DEF, Topiwala HH, Hamer G (1972) Yield and productivity in single-cell protein production from methane and methanol. In: Terui G (ed) Proc IV Int Ferment Symp Fermentation Technology Today, pp 491–495
83. Harrison DEF, Wikinson TG, Wren SJ, Harwood JH (1976) Mixed bacterial cultures as a basis for continuous production of SCP from C_1 compounds. In: Dean ACR, Ellwood DC, Evans CGT, Melling J (eds) Continuous culture 6: applications and new fields. Ellis Horwood, Chichester, pp 122–134
84. Hazelwood LA, Walsh MC, Luttik MAH, Daran-Lapujade P, Pronk JT, Daran JM (2009) Identity of the growth-limiting nutrient strongly affects storage carbohydrate accumulation in anaerobic chemostat cultures of *Saccharomyces cerevisiae*. Appl Environ Microbiol 75:6876–6885
85. Hendrickson EL, Haydock AK, Moore BC, Whitman WB, Leigh JA (2007) Functionally distinct genes regulated by hydrogen limitation and growth rate in methanogenic Archaea. Proc Nat Acad Sci U S A 104:8930–8934
86. Hendrickson EL, Liu Y, Rosas-Sandoval G, Porat I, Soll D, Whitman WB, Leigh JA (2008) Global responses of *Methanococcus maripaludis* to specific nutrient limitations and growth rate. J Bacteriol 190:2198–2205
87. Herbert D (1976) Stoichiometric aspects of microbial growth. In: Dean ACR, Ellwood DC, Evans CGT, Melling J (eds) Continuous culture 6: applications and new fields. Ellis Horwood, Chichester, pp 1–30
88. Herbert D, Elsworth R, Telling RC (1956) The continuous culture of bacteria: a theoretical and experimental study. J Gen Microbiol 14:601–622
89. Hospodka J (1966) Industrial application of continuous fermentation. In: Malék I, Fencl Z (eds) Theoretical and methodological basis of continuous culture of microorganisms. Czech Acad Sci, Prague, pp 493–645
90. Hough JS, Keevil CW, Maric C, Philliskirk G, Young TW (1976) Continuous culture brewing. In: Dean ACR, Ellwood DC, Evans CGT, Melling J (eds) Continuous culture 6: applications and new fields. Ellis Horwood, Chichester, pp 226–237
91. Huisman J, Matthijs HCP, Visser PM, Balke H, Sigon CAM, Passarge J, Weissing FJ, Mur LR (2002) Principles of the light-limited chemostat: theory and ecological applications. Antonie van Leeuwen 81:117–133
92. Ingham CJ, Sprenkels A, Bomer J, Molenaar D, van den Berg A, Vlieg JETV, de Vos WM (2007) The micro-Petri dish, a million-well growth chip for the culture and high-throughput screening of microorganisms. Proc Nat Acad Sci U S A 104:18217–18222
93. Ishii N et al (2007) Multiple high-throughput analyses monitor the response of *E. coli* to perturbations. Science 316: 593–597

94. Ives PR, Bushell ME (1997) Manipulation of the physiology of clavulanic acid production in *Streptomyces clavuligerus*. Microbiol 143:3573–3579
95. Jansen MLA, Diderich JA, Mashego M, Hassane A, de Winde JH, Daran-Lapujade P, Pronk JT (2005) Prolonged selection in aerobic, glucose-limited chemostat cultures of *Saccharomyces cerevisiae* causes a partial loss of glycolytic capacity. Microbiology 151:1657–1669
96. Johri AK, Margarit I, Broenstrup M, Buttoni C, Hua L, Gygi SP, Telford JL, Grandi G, Paoletii LC (2007) Transcriptional and proteomic profiles of group B *Streptococcus* type V reveal potential adherence proteins associated with highlevel invasion. Infect Immun 75:1473–1483
97. Jones LE, Ellner SP (2008) Effects of rapid prey evolution on predator-prey cycles. J Math Biol 55:541–573
98. Kacmar J, Gilbert A, Cockrell J, Srienc (2006) The cytostat: a new way to study cell physiology in a precisely defined environment. J Biotechnol 125:163–172
99. Khannapho C, Zhao H, Bonde BK, Kierzek AM, Avignone-Rossa CA, Bushell ME (2008) Selection of objective function in genome scale flux balance analysis for process feed development in antibiotic production. Metab Eng 10:227–233
100. King T, Seeto S, Ferenci T (2006) Genotype-by-environment interactions influencing the emergence of *rpoS* mutations in *Escherichia coli* populations. Genetics 172:2071–2079
101. Kirk S, Avignone-Rossa CA, Bushell ME (2000) Growth limiting substrate affects antibiotic production and associated metabolic fluxes in *Streptomyces clavuligerus*. Biotechnol Lett 22:1803–1809
102. Kisand V, Rocker D, Simon M (2008) Significant decomposition of riverine humic-rich DOC by marine but not estuarine bacteria assessed in sequential chemostat experiments. Aquatic Microbial Ecol 53:151–160
103. Kleijn RJ, Liu F, van Winden WA, van Gulik WA, Ras C, Heijnen JJ (2007) Cytosolic NADPH metabolism in penicillin-G producing and non-producing chemostat cultures of *Penicillium chrysogenum*. Metab Eng 9:12–123
104. Knijnenburg TA, de Winde JH, Daran JM, Daran-Lapujade, Pronk JT, Reinders MJT, Wessels LFA (2007) Exploiting combinatorial cultivation conditions to infer regulation. BMC Genomics 8:25
105. Koetsier MJ and 10 other authors (2010) The *Penicillium chrysogenum* aclA gene encodes a broad-substrate-specificity acyl-coenzyme A ligase involved in activation of adipic acid, a side-chain precursor for cephem antibiotics. Fungal Genet Biol 47: 33-42
106. Kolkman A, Daran-Lapujade P, Fullaondo A, Oisthoorn MMA, Pronk JT, Slijper M, Heck AJR (2006) Proteome analysis of yeast response to various nutrient limitations. Mol Syst Biol 2:026
107. Kubitschek HE (1970) Introduction to research with continuous cultures. Prentice-Hall, Englewood Cliffs, pp iix, 1–195
108. Kuenen JG, Gottschahl JC (1982) Competition among chemolithotrophs and methylotrophs and their interactions with heterotrophic bacteria. In: Bull AT, Slater JH (eds) Microbial interactions and communities. Academic, London, pp 153–187
109. Lambert PA (1984) The role of the bacterial envelop in antibiotic resistance. In: Dean ACR, Ellwood DC, Evans CGT (eds) Continuous culture 8: biotechnology, medicine and the environment. Ellis Horwood, Chichester, pp 38–54
110. Lennon JT, Martiny JBH (2008) Rapid evolution buffers ecosystem impacts of viruses in a microbial food web. Ecol Lett 11:1178–1188
111. Li BZ, Cheng JS, Qiao B, Yuan YJ (2010) Genome-wide transcriptional analysis of *Saccharomyces cereviseae* during industrial bioethanol fermentation. J Ind Microbiol Biotechnol 37:43–55
112. Lin B, Westerhoff, Roling WFM (2009) How *Geobacteriaceae* may dominate subsurface biodegradation: physiology of *Geobacter metallireducens* in slow growth habitat-simulating retentostats. Environ Microbiol 11:2425–2433
113. Linton JD, Drozd JW (1982) Microbial interactions and communities in biotechnology. In: Bull AT, Slater JH (eds) Microbial interactions and communities. Academic, London, pp 357–406
114. Lo AW, Seers CA, Boyce JD, Dashper SG, Slakeski N, Lissel JP, Reynolds EC (2009) Comparative transcriptomic analysis of *Porphyromonas gingivalis* biofilm and planktonic cells. BMC Microbiol 9:18
115. Lovitt RW, Wimpenny JWT (1981) The gradostat: a bidirectional compound chemostat, and its application in microbiological research. J Gen Microbiol 127:261–268
116. Luders S, Fallet C, Franco-Lara E (2009) Proteome analysis of the *Escherichia coli* heat shock response under steady-state conditions. Proteome Sci 7:36
117. Luscombe BM, Gray TRG (1974) Characteristics of *Arthrobacter* grown in continuous culture. J Gen Microbiol 82:213–222
118. Macfarlane GT, Macfarlane S, Gibson G (1998) Validation of a three-stage compound continuous culture system for investigating the effect of retention time on the ecology and metabolism of bacteria in the human gut. Microbial Ecol 35:180–187
119. Macfarlane GT, Macfarlane LE (2009) Acquisition, evolution and maintenance of the normal gut microbiota. Digestive Dis 27:90–98
120. Maharjan R, Seeto S, Notley-McRobb L, Ferenci T (2006) Clonal adaptive radiation in a constant environment. Science 313:514–517
121. Major NC, Bull AT (1989) The physiology of lactate production by *Lactobacillus delbreuckii* in a chemostat with cell recycle. Biotechnol Bioeng 34:592–599
122. Majors PD, McLean JS, Scholten JCM (2008) NMR bioreactor development for live in situ microbial functional analysis. J Magn Reson 192:159–166
123. Málek I (1976) Physiological state of continuously grown microbial cultures. In: Dean ACR, Ellwood DC, Evans CGT, Melling J (eds) Continuous Culture 6: Applications and New Fields. Ellis Horwood, Chichester, pp 31–39
124. Málek I, Fencl Z (eds) (1966) Theoretical and methodological basis of continuous culture of microorganisms. Czech Acad Sci, Prague, pp 1–655
125. Massie TM, Blasius B, Weithoff G, Gaedke U, Fussmann GF (2010) Cycles, phase synchronization, and entrainment in single-species phytoplankton populations. Proc Nat Acad Sci U S A 107:4236–4241
126. McFadden J (2010) Systems biology and the TB bacillus. Microbiology Today 17:16–19
127. McIntyre JJ, Bunch AW, Bull AT (1999) Vancomycin production is enhanced in chemostat culture with biomass-recycle. Biotechnol Bioeng 62:576–582
128. M'Kendrick AG, Pai MK (1911) The rate of multiplication of microorganisms, a mathematical study. Proc R Soc Edinb 31:649–655
129. Middelboe M, Holmfeldt K, Riemann L, Nybroe O, Haaber J (2009) Ba24 bacteriophages drive strain diversification in a marine *Flavobacterium*: implications for phage resistance. Environ Microbiol 11:1971–1982
130. Minnikin DE, Abdolrahimzadeh H, Baddiley J (1972) Variation of polar lipid composition of *Bacillus subtilis* (Marburg) with different growth conditions. FEBS Lett 27:16–18

131. Mitchell HL, Dashper SG, Catmull DV, Paolini RA, Cleal SM, Slakeski N, Tan KH, Reynolds EC (2010) *Treponema denticola* biofilm-induced expression of a bacteriophage, toxin-antitoxin systems and transposases. Microbiology 156:774–788
132. Monod J (1949) The growth of bacterial cultures. Ann Rev Microbiol 3:371–394
133. Monod J (1950) La technique de culture continuée. Theorie et application. Ann Inst Pasteur (Paris) 79:390–410
134. Muñoz-Aguayo J, Lang JK, LaPara TM, González G, Singer RS (2007) Evaluating the effects of chlortetracycline on the proliferation of antibiotic- resistant bacteria in a simulated river water ecosystem. Appl Environ Microbiol 73:5421–5425
135. Nakhu R, Valgepea K, Lahtvee PJ, Erm S, Abner K, Adamberg K, Vilu R (2010) Specific growth rate dependent transcriptome profiling of *Escherichia coli* K12 MG1655 in accelerostat cultures. J Biotechnol 145:60–65
136. Nanchen A, Schicker A, Revelles O, Sauer U (2008) Cyclic AMP-dependent catabolite repression is the dominant control mechanism of metabolic fluxes under glucose limitation in *Escherichia coli*. J Bacteriol 190:2323–2330
137. Neijssel OM, Tempest DW (1976) Role of energy-spilling reactions in growth of *Klebsiella aerogenes* NCTC-418 in aerobic chemostat culture. Arch Microbiol 110:305–311
138. Nicholls HA, Osborn DW, Buchan L, Melmed LN, Pitman AR (1984) Biological removal of phosphorus and nitrogen from waste water. In: Dean ACR, Ellwood DC, Evans CGT (eds) Continuous culture 8: biotechnology, medicine and the environment. Ellis Horwood, Chichester, pp 185–204
139. Nishikawa T, Guilbahce N, Motter AE (2008) Spontaneous reaction silencing in metabolic optimization. PLoS Comput Biol 4:e1000236
140. Novick A, Szilard L (1950) Description of the chemostat. Science 112:715–716
141. Novick A, Szilard L (1950) Experiments with the chemostat on spontaneous mutations of bacteria. Proc Nat Acad Sci U S A 36:708–719
142. Parkes RJ (1982) Methods for enriching, isolating, and analysing microbial communities in laboratory systems. In: Bull AT, Slater JH (eds) Microbial interactions and communities. Academic, London, pp 45–102
143. Parkes RJ, Wellsbury P (2004) Deep biospheres. In: Bull AT (ed) Microbial diversity and bioprospecting. ASM, Washington, DC, pp 120–129
144. Partridge JD, Scott C, Tang Y, Poole RK, Green J (2006) *Escherichia coli* transcriptome dynamics during the transition from anaerobic to aerobic conditions. J Biol Chem 281:27806–27815
145. Pickell LD, Wells ML, Trick CG, Cochlan WP (2009) A seagoing continuous culture system for investigating phytoplankton community response to macro- and micro-nutrient manipulations. Limnol Oceanogr Methods 7:21–32
146. Pir P, Kirdar B, Hayes A, Onsan ZI, Ulgen KO, Oliver SG (2008) Exometabolic and transcriptional response in relation to phenotype and gene copy number in respiration-related deletion mutants of *S. cerevisiae*. Yeast 25:661–672
147. Pirt SJ (1965) The maintenance energy of bacteria in growing cultures. Proc R Soc B 163:224–231
148. Pirt SJ (1975) Principles of microbe and cell culture. Blackwell, Oxford, pp i–x, 1–274
149. Pirt SJ, Kurowski WM (1970) Extension of theory of chemostat with feedback of organisms - its experimental realization with a yeast culture. J Gen Microbiol 63:357–366
150. Postec A, Lesongeur F, Oignet P, Ollivier B, Querellou J, Godfroy A (2007) Continuous enrichment cultures: insights into prokaryotic diversity and metabolic interactions in deep-sea vent chimneys. Extremophiles 11:747–757
151. Primrose SB, Derbyshire P, Jones IM, Robinson A, Ellwood DC (1984) The application of continuous culture to the study of plasmid stability. In: Dean ACR, Ellwood DC, Evans CGT (eds) Continuous culture 8: biotechnology, medicine and the environment. Ellis Horwood, Chichester, pp 213–238
152. Pullan ST, Monk CE, Lee L, Poole RK (2008) Microbial responses to nitric oxide and nitrosative stress: growth, "omic", and physiological methods. Methods Enzymol 437:499–519
153. Rautio JJ, Smit BA, Wiebe M, Pentilla M, Saloheimo M (2006) Transcriptional monitoring of steady state and effects of anaerobic phases in chemostat cultures of the fungus *Trichoderma reesei*. BMC Genomics 7:247
154. Revilla T, Weissing FJ (2008) Nonequilibrium coexistence in a competition model with nutrient storage. Ecology 89:865–877
155. Robinson A, Gorringe AR, Keevil CW (1984) Expression of virulence determinants in *Bordetella pertusis* and *Neisseria gonorrhoeae*. In: Dean ACR, Ellwood DC, Evans CGT (eds) Continuous culture 8: biotechnology, medicine and the environment. Ellis Horwood, Chichester, pp 22–37
156. Rokem JS, Laantz AE, Nielsen J (2007) Systems biology of antibiotic production by microorganisms. Nat Prod Rep 24:1262–1287
157. Rowley BI, Bull AT (1973) Chemostat for the cultivation of moulds. Lab Prac, April: 286–289
158. Russell JB (2007) The energy spilling reactions of bacteria and other organisms. J Mol Microbiol Biotechnol 13:1–11
159. Russell DG, Barry CE, Flynn JL (2010) Tuberculosis: what we don't know can, and does, hurt us. Science 328:852–856
160. Saxton K, Baines SD, Freeman J, O'Connor R, Wilcox MH (2008) Effects of exposure of *Clostridium difficile* PCRF ribotypes 027 and 001 to fluoroquinolones in a human gut model. Antimicrob Agents Chemother 53: 412-420
161. Schaub J, Reuss M (2008) In vivo dynamics of glycolysis in *Escherichia coli* shows need for growth-rate dependent metabolome analysis. Biotechnol Prog 24:1401–1407
162. Senior E, Bull AT, Slater JH (1976) Enzyme evolution in a microbial community growing on the herbicide Dalapon. Nature 263:476–479
163. Senior PJ (1984) Polyhydroxybutyrate, a speciality polymer of microbial origin. In: Dean ACR, Ellwood DC, Evans CGT (eds) Continuous culture 8: biotechnology, medicine and the environment. Ellis Horwood, Chichester, pp 266–271
164. Shah D, Dang MD, Hasbun R, Koo HL, Jiang ZD, DuPont HL, Garey KW (2010) *Clostridium difficile* infection: update on emerging antibiotic treatment options and antibiotic resistance. Expert Rev Anti Infect Ther 8:555–564
165. Shannon SP, Chrzanowski TH, Grover JP (2007) Prey food quality affects flagellate ingestion rates. Microbial Ecol 53:66–73
166. Shimono N, Morici L, Casal N, Cantrell S, Sidders B, Ehrt S, Riley LW (2003) Hypervirulent mutant of *Mycobacterium tuberculosis* resulting from disruption of the *mce1* operon. Proc Natl Acad Sci U S A 100:15918–15923
167. Shockley KR, Scott KL, Conners SB, Johnson MR, Montero CI, Wolfinger RD, Kelly RM (2005) Genome-wide transcriptional variation within and between steady states for continuous growth of the hyperthermophile *Thermatoga maritima*. Appl Environ Microbiol 71:5572–5576
168. Sikyta B (1991) Directed selection of microorganisms in continuous culture. Academia, Prague, pp 1–134
169. Smith SRL (1980) Single cell protein. In: Brenner S, Hartley BS, Rodgers PJ (eds) New horizons in industrial microbiology. The Royal Society, London, pp 63–76
170. Smith VH, Foster BL, Grover JP, Holt RD, Leibold MA, deNoyelles F (2005) Phytoplankton species richness scales consistently from laboratory microcosms to the world's oceans. Proc Nat Acad Sci USA 102:4393–4396

171. Snoep JL, Mrwebi M, Schuurmans JM, Rohwer JM, de Mattos MJT (2009) Control of specific growth rate in *Saccharomyces cerevisiae*. Microbiology 155:1699–1707
172. Stejskal A (1966) Fundamental advantages of continuous cultivation of pathogenic microorganisms. In: Malék I, Fencl Z (eds) (1966) Theoretical and methodological basis of continuous culture of microorganisms. Czech Acad Sci, Prague, pp 418–442
173. Sterner RW, Elser JJ (2002) Ecological stoichiometry. Princeton University Press, Princeton
174. Stomp M, van Dijk MA, van Overzee HMJ, Wortel MT, Sigon CAM, Egas M, Hoogveld H, Gons HJ, Huisman J (2008) The timescale of phenotypic plasticity and its impact on competetion in fluctuating environments. American Naturalist 172:E169–E185
175. Stouthamer AH (1979) The search for correlation between theoretical and experimental growth yields. In: Quayle JR (ed) International review of biochemistry vol 21, microbial biochemistry. University Park Press, Baltimore, pp 1–47
176. Stouthamer AH, Bulthius BA, van Verseveld HW (1990) Energetics of growth at low growth rates and its relevance for the maintenance concept. In: Poole RK, Bazin MJ, Keevil CW (eds) Microbial growth dynamics. IRL, Oxford, pp 85–102
177. Sutherland IW, Ellwood DC (1979) Microbial exopolysaccharides–industrial polymers of current and future potential. In: Bull AT, Ellwood DC, Ratledge C (eds) Microbial technology: current state, future prospects, symposium 29 Soc Gen Microbiol. Cambridge University Press, Cambridge, pp 107–150
178. Tai SL, Daran-Lapujade P, Walsh MC, Pronk JT (2007) Acclimation of *Saccharomyces cerevisiae* to low temperature: a chemostat-based transcriptome analysis. Mol Biol Cell 18:5100–5112
179. Tanji Y, Hattori K, Suzuki K, Miyanaga K (2008) Spontaneous deletion of a 209-kilobase-pair fragment from the *Escherichia coli* genome occurs with acquisition of resistance to an assortment of infectious phages. Appl Environ Microbiol 74:4256–4263
180. Tarantola A (2006) Popper, Bayes and the inverse problem. Nat Phys 2:492–494
181. Tarkiainen V, Kotiaho T, Mattila I, Virkajarvi L, Aristidou A, Ketola RA (2005) On-line monitoring of continuous beer fermentation process using automatic membrane inlet mass spectrometric system. Talanta 65:1254–1263
182. Tempest DW, Herbert D, Phipps PJ (1967) Studies on the growth of *Aerobacter aerogenes* at low dilution rates in a chemostat. In: Powell EO, Evans CGT, Strange RE, Tempest DW (eds) Continuous culture 3: microbial physiology and continuous culture. Her Majesty's Stationery Office, London, pp 240–254
183. Tempest DW (1969) Quantitative relationships between inorganic cations and anionic polymers in growing bacteria. In: Meadows PM, Pirt SJ (eds) Microbial growth, symposium 19 Soc Gen Microbiol. Cambridge University Press, Cambridge, pp 87–111
184. Tempest DW, Neijssel OM, Zevenboom W (1983) Properties and performance of microorganisms in laboratory culture: their relevance to growth in natural ecosystems. In: Slater JH, Whittenbury R, Wimpenny JWT (eds) Microbes in their natural environments, symposium 34 Soc Gen Microbiol. Cambridge University Press, Cambridge, pp 119–152
185. Teusink B, Wiersma A, Molenaar D, Francke C, de Vos WM, Siezen RJ, Smid EJ (2006) Analysis of growth of *Lactobacillus plantarum* WCFS1 on a complex medium using a genome-scale metabolic model. J Biol Chem 281:40041–40048
186. Trinci AJP (1994) Evolution of the Quorn® myco-protein fungus, *Fusarium graminearum* A3/5. Microbiology 140:2181–2188
187. van Bodegom P (2007) Microbial maintenance. A critical review on its quantification. Microbial Ecol 53:513–523
188. van Eunen K, Bouwman J, Daran-Lapujade P, Postmus J, Canelas AB, Mensonides FIC, Orij R, Tuzun I, van den Brink J, Smits JG, van Gulik WM, Brul S, Heijnen JJ, de Winde JH, Teixeira de Mattos MJ, Kettner C, Nielsen J, Westerhoff HV, Bakker BM (2010) Measuring enzyme activities under standardized in vivo-like conditions for systems biology. FEBS J 277:749–760
189. van der Brink J, Akeroyd M, van der Hoeven R, Pronk JT, de Winde JH, Daran-Lapujade P (2009) Energetic limits to metabolic flexibility: responses of *Saccharomyces cerevisiae* to glucose-galactose transitions. Microbiology 155:1340–1350
190. van der Greef J, Martin S, Juhsz P (2007) The art and practice of systems biology in medicine: Mapping patterns of relationships. J Proteome Res 6:1540–1559
191. van der Stap I, Vos M, Kooi BW, Mulling BTM, van Donk E, Mooij WM (2009) Algal defenses, population stability, and the risk of herbivore extinctions: a chemostat model and experiment. Ecol Res 24:1145–1153
192. Veldkamp H, Jannasch HW (1972) Mixed culture studies with the chemostat. J appl Chem Biotechnol 22:105–123
193. Way JC, Silver PA (2007) Why we need systems biology. Complexity 13:22–29
194. Weibel DB, DiLuzio WR, Whitesides GM (2007) Microfabrication meets microbiology. Nat Rev Microbiol 5:209–218
195. Wecker P, Klockow C, Ellrott A, Quast C, Langhammer P, Harder J, Glöckner FO (2009) Transcriptional response of the model planctomycete *Rhodopirellula baltica* SHI^T to changing environmental conditions. BMC Genomics 10:410–426
196. Werner E (2007) All systems go. Nature 446:493–494
197. Wiebe MG, Robson GD, B, SG, Trinci AJP (1993) Periodic selection in long-term continuous-flow cultures of the filamentous fungus *Fusarium graminearum* J Gen Microbiol 139: 2811–2817
198. Zeng AP, Sun J (2010) Continuous culture. In: Baltz RH, Demain AL, Davies JE (eds) Manual of industrial microbiology and biotechnology, 3rd edn. ASM, Washington DC, pp 685–699
199. Zhang Z, Boccazzi P, Choi HG, Perozziello G, Sinskey AJ, Jensen KF (2006) Microchemostat–microbial continuous culture in a polmer-based, instrumented microbioreactor. Lab Chip 6:906–913
200. Zhong S, Khodursky A, Dykhuizen DE, Dean AM (2004) Evolutionary genomics of ecological specialization. Proc Nat Acad Sci U S A 101:11719–11724
201. Zhong S, Miller SP, Dykhuisen DE, Dean AM (2009) Transcription, translation, and the evolution of specialists and generalists. Molecular Biol Evol 26:2661–2678
202. Zilm PS, Bagley CJ, Rogers AH, Milne IR (2007) The proteomic profile of *Fusobacterium nucleatum* is regulated by growth pH. Microbiology 153:148–159
203. Zilm PS, Rogers AH (2007) Co-adhesion and biofilm formation by *Fusobacterium nucleatum* in response to growth pH. Anaerobe 13:146–152