Experimental Microbial Ecology

edited by Richard G. Burns

and J. Howard Slater

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Contents

List of contributors, vii

Preface, ix

Part 1
Microbial components of the biosphere

1 Procedures for the isolation, cultivation and identification of bacteria, 3
Rodney A. Herbert

2 Procedures for the isolation, cultivation and identification of fungi, 22
Denis Parkinson

3 Procedures for the isolation, cultivation and identification of algae, 31
Frank E. Round

4 Procedures for the isolation, cultivation and identification of protozoa, 44
Bland J. Finlay

5 Methods for the study of virus ecology, 66
Sandy B. Primrose, Nigel D. Seeley and Kelvin B. Logan

6 Enumeration and estimation of microbial biomass, 84
Ronald M. Atlas

Part 2
The biosphere

7 The soil environment: clay–humus–microbe interactions, 105
Guenther Stotzky and Richard G. Burns

8 Mineralisation of carbon, 134
Karl-Erik Eriksson and Susanna Christl Johnsrud

9 Nitrogen mineralisation in soils and sediments, 154
Charles M. Brown

10 Microbial succession during the decomposition of organic matter, 164
Michael J. Swift

11 Nitrification, 178
James I. Prosser and Dimity J. Cox

12 Denitrification, 194
Dennis D. Focht

13 Nitrogen fixation, 212
David W. Smith

14 Phosphorus cycle, 221
John W. B. Stewart and Robert B. McKercher

15 Primary production of the oceans, 239
Ian Morris

Part 3
Microbe–microbe interactions

16 Microbial ecology in the laboratory: experimental systems, 255
J. Howard Slater and David J. Hardman

17 Microbe–microbe interactions at surfaces, 275
J. William Costerton and Kou-Joan Cheng

18 Lichens, 291
Brian W. Ferry

19 Protozoal symbionts, 320
Dale S. Weis

20 Microbial competition in continuous culture, 342
J. Gijs Kuenen and Wim Harder

21 Bacteriocins, 368
Kimber G. Hardy

22 Bdellovibrios—intraperiplasmic growth, 379
Sydney C. Rittenberg

Part 4
Microbe–plant interactions

23 The rhizosphere, 395
James M. Lynch

24 The phylloplane and other aerial plant surfaces
Colin H. Dickinson

25 Leguminous root nodules, 431
Frank B. Dazzo

26 Mycorrhizae, 447
Conway Ll. Powell

27 Pathogenic interactions of microbes with plants, 472
Richard N. Strange

Part 5
Microbe–animal interactions

28 Sampling the skin surface, 493
William C. Noble

29 Microbe–animal interactions in the rumen, 501
Colin G. Orpin

30 Termite microbial communities, 519
Betsey Dexter-Dyer Grosovsky and Lynn Margulis

31 Nematode-destroying fungi, 533
George L. Barron

Part 6
Stressed environments

32 Extreme natural environments, 555
David W. Smith

33 Microbial ecology of domestic wastes, 575
Ken P. Flint

34 Oil degradation in hydrocarbon- and oil-stressed environments, 591
Sally Stafford, Paul Berwick, David E. Hughes and David A. Stafford

35 Effect of pesticides on Soil microorganisms, 613
Michael P. Greaves

36 Gaseous and heavy metal air pollutants, 631
Harvey Babich and Guenther Stotzky

Index, 671

Preface

The subject of microbial ecology has undergone a quiet revolution in the last decade. It has become respectable: a legitimate field of research attractive to a large number of first-rate microbiologists. Explanations for this change of attitude are not difficult to find.

First, the concern voiced by the environmental lobby in the 1950s and 1960s was gradually translated into financial support for investigations into the effects of man-made chemicals on the microbiota (Chapters 34 to 36). This research has necessitated the study of microbial ecosystems in the *absence* of pollutants (Chapters 7 to 15) in order to establish a baseline against which any perturbations can be assessed. Second, exploration of the Moon and Mars has stimulated research into extreme terrestrial environments (Chapter 32) for the purpose of establishing the parameters for microbial growth and survival and thereby designing suitable microbiological experiments to be carried to these places. Third, the demands of an increasing World population for the efficient use of agricultural resources have served to advance our understanding of microbe–plant interactions (Chapters 7 to 14, 23 to 27, 29 and 31). There is also a fourth and rather more subtle factor which has persuaded microbiologists to study the ecology of protists. This has been the gradual realisation that the term mixed-culture microbiology need not be a euphemism for contaminated cultures. Indeed, the study of mixed cultures is now a branch of microbiology in its own right (Chapters 16 and 20) and, furthermore is likely to reflect more accurately the multi-species nature of the microbial environment. A parallel development in the study of microbe–eukaryote interactions (Chapters 23 to 31) has reinforced this trend.

Despite these stimuli the progress towards an understanding of microbes in their environment has been less than rapid. One basic problem is the difficulty in carrying out controlled, reproducible and informative experiments in the field. On the other hand, extrapolating observations made in the laboratory to the natural environment is a dangerous and controversial exercise. This apparent paradox, in the collection and interpretation of data in microbial ecology, is a continuing theme in this volume and is returned to again and again by the various authors. One way of resolving the problem is to design laboratory experiments which show a gradual increase in complexity, a step-by-step approach to a realistic microbial ecosystem: for example, a progression from monoaxenic cultures through co-cultures to mixed cultures containing three or more species; the use of simple defined media at optimum concentrations as well as complex media at realistic (often sub-optimal) concentrations; the choice of 'climatic' factors (e.g. pH, temperature, oxygen levels); that are likely to reflect conditions *in situ*; and encouraging interfacial phenomena characteristic of microbial activity in the environment (Chapters 7 and 17).

This volume is not intended as a recipe book for experiments in microbial ecology but rather an attempt to put research workers in touch with the advantages and shortcomings of the experimental techniques which are currently available. The experimental tools used at present to probe the complex microbial environment are barely adequate. However, given the recent surge of interest we feel that a collection of essays concerned with the plethora of techniques with which microbial ecologists are armed currently will be a spur to the development of novel techniques aimed at unravelling the mysteries of microbial ecology. Already some techniques are quite specific to microbial ecology because they have evolved from the study of highly integrated relationships (e.g. Chapters 18, 19, 22, 26, 30 and 31). Others have been borrowed from a more established microbial methodology such as that discussed in Chapters 1 to 6. Inevitably, therefore, a few authors have biased their chapters towards a review of their subject whilst others have concentrated upon a critical discussion of methodology.

The burgeoning interest in microbial ecology is

illustrated by the convening of three international meetings in the past ten years (Uppsala, Sweden 1972; Dunedin, New Zealand 1977; Warwick, England 1980) with a fourth planned for East Lansing, Michigan, USA in 1983. In addition, the major American and British Microbiology Societies (ASM and SGM) have thriving microbial ecology groups and publish a large number of ecology papers in their house journals (*Applied and Environmental Microbiology, Journal of General Microbiology*). Finally the research journal *Microbial Ecology* (Springer-Verlag, New York) is now in its 9th year and *Advances in Microbial Ecology* (Plenum Press, New York), established in 1977, has reached volume 5.

In conclusion, the editors would like to thank Anne Brown and Bob Campbell of Blackwell Scientific Publications for their encouragement, assistance and patience throughout the long gestation period of this volume.

Richard G. Burns, Canterbury
J. Howard Slater, Coventry

Part 1
Microbial Components of the Biosphere
Chapter 1 · Procedures for the Isolation, Cultivation and Identification of Bacteria

Rodney A. Herbert

1.1 Introduction

Experimental

1.2 Types of enrichment systems

1.3 Selective agents used in enrichment cultures

- 1.3.1 Temperature
- 1.3.2 pH as a selective agent
- 1.3.3 Light as a selective agent
- 1.3.4 Aerobic or anaerobic conditions as selective agents
- 1.3.5 Trace element and cofactor requirements
- 1.3.6 Inhibitors as selective agents

1.4 Isolation methods

- 1.4.1 Winogradsky column
- 1.4.2 Soil perfusion methods
- 1.4.3 Chemostat enrichment systems
- 1.4.4 Other procedures
- 1.4.5 Membrane filtration

1.5 Media for the isolation and cultivation of bacteria

1.6 Identification of bacteria

Recommended reading

References

1.1 Introduction

Natural environments are extremely diverse and the majority contain a wide range of microorganisms which reflect the nature of the habitat and the ability of individual members to compete successfully and coexist within that given ecosystem. In general terms the greater the heterogeneity of the environment, the more diverse and complex will be the microflora. For example, in an environment such as garden soil where numerous microenvironments exist, the microbial flora is extremely complex whereas in thermophilic or hypersaline environments where one physical or chemical characteristic dominates over all others only a few specialised species can grow under such extreme ecological conditions.

The microbial ecologist, seeking to determine what types of microorganisms are to be found in a particular environment, is, therefore, faced with the dilemma of trying to isolate them from a complex microbial community. In many instances the organism of interest is present in relatively low numbers and some form of enrichment is necessary before isolation can be attempted. The deliberate encouragement (selection) of one microorganism at the expense of others dates back to the work of Schloesing and Müntz (1877) who showed that the oxidation of ammoniacal liquors to nitrate was a biological process. This technique led Winogradsky and Beijerinck, early in this century, to lay the foundations of microbial ecology and physiology. Van Niel (1955) emphasised that, with the alteration of only a few words, Koch's postulates concerning the conditions which must be fulfilled before a microorganism may be considered responsible for causing a disease, can also be applied to enrichment cultures. The postulates can be stated as follows:

(1) the microorganism must always be present when the relevant chemical process is occurring;

(2) it must be possible to isolate and grow the microorganism in pure culture in the laboratory;

(3) the chemical process should occur when a suitable growth medium is inoculated with a pure culture of the microorganism and it should be possible to obtain the isolate from the growth medium at the end of the experiment.

Experimental

1.2 Types of enrichment systems

Two main types of enrichment cultures are used by microbiologists. The most commonly used enrichment procedure is the closed system in which the inoculum is added to a liquid or solid growth medium whose physico-chemical conditions are such that the desired microorganism is enriched

relative to other components of the microflora. The desired bacterium forming the dominant population under these circumstances may be isolated by conventional techniques. A major attraction of the closed system is that it only requires simple equipment: bottles or flasks plus a few inexpensive chemicals. The particular microorganism which develops in enrichment culture clearly depends upon the chemical composition of the medium used, together with other factors such as temperature, Eh, pH, presence of selective inhibitors, light, gas phase and others. It is, therefore, apparent that to enrich successfully for a particular bacterium some knowledge of its physiology is a prerequisite yet, paradoxically, this can only be obtained from pure-culture studies. Many microbiologists have observed under the microscope microorganisms which never develop in any type of enrichment culture and our current knowledge does not yet allow us to overcome this hiatus. Traditionally in closed enrichment systems the initial nutrient levels are high in order to promote a large population of microorganisms. However, as growth proceeds there is a continuous change in the medium as the original nutrient levels are depleted and metabolic end-products accumulate. Since the chemical changes are due to the metabolic activities of the inoculum the chemical composition of the medium cannot be controlled and as a consequence it is important not to miss the stage when the desired organism becomes dominant.

The complexity of changes occurring during growth in closed enrichment systems are such that it is often difficult to predict with any certainty which species will become dominant. Another major disadvantage is that the nutrient levels in closed systems are often unnecessarily high particularly in comparison with many natural environments which are usually nutritionally poor, e.g. ocean waters. In an attempt to overcome some of the problems of the closed enrichment system Jannasch (1965, 1967) pioneered the use of continuous culture methods to enrich for microorganisms (see Chapters 16 and 20). In open enrichment systems, such as the chemostat, the exhaustion of nutrients and accumulation of end-products, which are inherent defects in the closed system, present no problems since fresh medium is continuously added and waste products are removed. Jannasch (1965, 1967) has argued that continuous culture enrichment methods have three particular advantages:

(1) no succession of species occurs and if there is no wall growth or interaction, the predominance of one species increases with time;

(2) the growth advantages of the successful competitor are not dependent upon substrate specificity but on the particular growth parameters of the organism and the cultural conditions provided: if these parameters are known and stable, then the enrichment is reproducible;

(3) enrichments may be carried out in the presence of extremely low concentrations of one particular growth-limiting nutrient and, therefore, at low population densities.

The theory and practice of the chemostat has been well reviewed (Herbert *et al.* 1956; Powell 1958; Kubitschek 1970; Tempest 1970; Veldkamp 1976; Slater 1979; see also Chapter 20) and therefore only those aspects relevant to competition will be discussed here.

The key to the chemostat lies in the way in which the specific growth rate (μ) of a microbial population depends upon the concentration of a growth-limiting substrate (s) in the culture medium. In its simplest form the relationship between μ and s can be described by the Monod equation (Monod 1942):

$$\mu = \mu_{max} \cdot \frac{s}{K_s + s}$$

where μ is the specific growth rate; s the concentration of the growth-limiting nutrient; μ_{max} the maximum specific growth rate at saturating values of s; and K_s the saturation constant which is equal to that concentration of s producing a growth rate which is half that of μ_{max}.

In a chemostat one nutrient in the incoming medium is usually maintained at a relatively low concentration and s in the growth vessel is fixed by the dilution rate. This, in turn, controls μ at some point on the μ/s curve. As a consequence μ is maintained at values below μ_{max} and is fixed by the culture dilution rate.

When two bacteria are competing for the same growth-limiting substrate in a chemostat, assuming no interaction between the two populations, then the outcome will be determined by the μ/s relationships of the isolates involved (Veldkamp 1970). Fig. 1 shows the possible relationships between the two isolates. If bacterium A has higher μ_{max} and lower K_s values than isolate B then it will outgrow B at all μ values imposed by the dilution

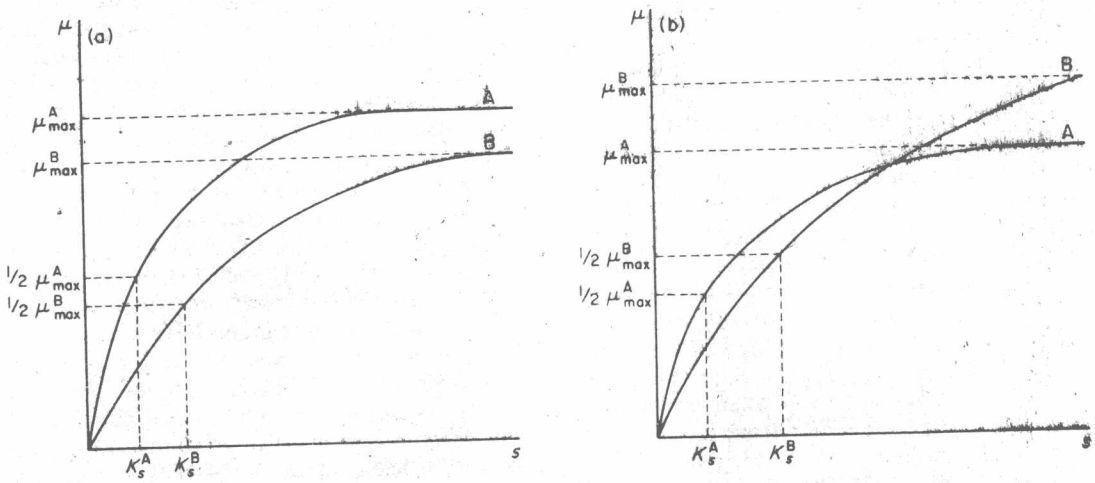

Figure 1. The μ/s relationship of two bacteria A and B (after Veldkamp 1970).

rate and will predominate in both batch and chemostat culture enrichments (Fig. 1(a)). If, however, the μ/s curves of isolates A and B intersect (Fig. 1(b)), then at high growth rates isolate B will outgrow isolate A whereas A will outgrow B at low growth rates. In a closed enrichment system with saturating values of s, selection is based only on μ_{max} and isolate B would always predominate. In an open flow system, such as a chemostat, the outcome depends upon the dilution rate and by using low flow rates, low K_s, low μ_{max} bacteria of type A would predominate, a situation which would never occur in closed-system enrichments. Several examples of μ/s curves which intersect have now been reported and these result in different bacteria predominating at different growth rates (Jannasch 1967; Meers 1971; Kuenen *et al.* 1976). Jannasch (1965, 1967) has shown that when samples of ocean water were inoculated into chemostats run at different dilution rates different bacteria became predominant. If these bacteria were isolated in pure culture, mixed and reinoculated into chemostats the enrichments were reproducible. The reason for the reproducibility was due to the fact that the growth conditions were closely defined. In the chemostat it is possible to select conditions favouring a particular bacterium of low substrate specificity. No other experimental system provides such well-defined conditions for the study of microbial processes in natural environments.

1.3 Selective agents used in enrichment cultures

The literature on enrichment methods is extensive. In this section the general principles underlying the use of particular selective agents will be discussed.

1.3.1 Temperature

Temperature is clearly an important selective agent in enrichment cultures as it is in natural environments. Most aquatic environments are at relatively low temperatures and it has been shown that microorganisms which commonly occur in these ecosystems are rapidly killed when exposed to temperatures above 20°C (Morita & Haight 1964; Stanley & Rose 1967). Thus, if these cold-loving (psychrophilic) microorganisms are to be obtained in enrichment cultures it is imperative that the inoculum be processed and incubated at low temperatures. Taking these precautions Sieburth (1967) was able to follow the seasonal variation in bacterial populations in Narragansett Bay, Rhode Island. In winter when the water temperature was low (minimum −2°C) psychrophiles predominated, whereas in summer (maximum temperature +23°C) mesophilic bacteria with temperature optima between 20 to 33°C were predominant. If samples taken during the winter had been enriched and incubated at 30°C, the dominant population of psychrophiles would have been missed.

Conversely thermophiles can be readily isolated by incubation at elevated temperatures (55 to 65°C). If *Bacillus stearothermophilus* is present in a sample, it can be isolated easily from other components of the microflora by incubation at 65°C (Wolfe & Barker 1968). Similarly, thermoactinomycetes, such as *Thermoactinomyces vulgaris*, can be readily isolated from mouldy hay on yeast extract agar by incubating at 55°C (Cross 1968).

Temperatures may also exert more subtle effects which need to be considered when isolating microorganisms in enrichment culture. At higher incubation temperatures the dissolved oxygen tensions are lower and this greatly affects the cell density of the enriched bacterial population (Sinclair & Stokes 1963). *Lactobacillus arabinosus* when grown in air at 39°C had a specific requirement for aspartate, whilst at 35°C there was no such requirement (Borek & Waelsch 1951). The effect of the increased temperature was to reduce the CO_2 concentration and the dependence on aspartate at 39°C was relieved by increasing the CO_2 concentration.

1.3.2 pH as a Selective Agent

The growth and reproduction of microorganisms is greatly influenced by the pH of the growth medium. Most bacteria can only grow within the range pH 4.0 to 9.0 (Thimann 1964) with optimal growth occurring between pH 6.5 and 8.5. Only a very few bacteria can grow at pH 3.0 or below, e.g. the acidophilic thiobacilli and lactobacilli. By using selective media which have a low buffering capacity, acid production allows the enrichment of acidophilic bacteria at the expense of the majority of the microflora of the original inoculum. In an analogous manner alkalophilic bacteria can be enriched by incubating at high pH (pH 10.0 to 11.0). Wiley and Stokes (1962) demonstrated that *Bacillus pasteurii* could be enriched and isolated from soil by inoculating onto a simple ammonium salts-yeast extract agar at pH 9.0. Under more extreme alkaline conditions (pH 10.0 to 11.0) *Bacillus* spp. still predominate (Gee *et al.* 1980) but alkalophilic strains of *Micrococcus* (Akiba & Horikoshi 1976), *Pseudomonas* (Hale 1977) and *Ectothiorhodospira* (Grant *et al.* 1979) have also been reported. Caldwell and Hirsch (1973) demonstrated the use of a two-dimensional steady-state diffusion system in which pH gradients can be developed to enrich for fastidious bacteria in a natural community.

When enriching for bacteria which are sensitive to pH changes, buffers are often incorporated into the medium. Usually they are of limited value in preventing pH changes since the buffering capacity is too low. Phosphates, whilst useful at low molarities, suffer from the disadvantages of giving rise to precipitates of insoluble phosphates. Tris buffers, since they are organic, provide a potential source of both carbon and nitrogen which may be undesirable in the enrichment medium. Calcium carbonate is frequently included in enrichment media when excessive acid production is anticipated but in stationary cultures it is not particularly effective and it makes microscopical examination of the enrichment difficult. To minimise pH changes occurring in closed system enrichments the concentration of the compound(s) responsible for the pH change should be reduced to a minimum. Alternatively frequent transfers of the developing enrichment into fresh media ensures that excessive pH changes do not occur.

1.3.3 Light as a Selective Agent

The selective enrichment of phototrophic microorganisms is dependent not only on the light wavelengths supplied but the light irradiance levels used. Since the photosynthetic purple and green sulphur bacteria are obligately phototrophic they can be selectively enriched in mineral media in the presence of H_2S, anaerobic conditions and light. Purple non-sulphur bacteria can be similarly enriched except that the appropriate organic electron donor is substituted for H_2S. By using filters to exclude particular wavelengths of light, e.g. infrared filters that will only transmit wavelengths greater than 800 nm, purple sulphur bacteria can be selectively enriched (Pfennig 1967). Using infrared light with a wavelength greater than 900 nm as the selective agent, Eimhjellen *et al.* (1967) were able to isolate a hitherto unknown purple sulphur bacterium which contained a chlorophyll species with an in-vivo absorption maxima of 1017 to 1020 nm.

The light irradiance applied to enrichments for phototrophs may also significantly affect the enrichment and subsequent isolation of phototrophic bacteria. At high irradiance levels (500 to 2000 lux) and high sulphide levels (0.2% w/v) *Chlorobium* spp. usually predominate. In sharp contrast the enrichment of the green sulphur bacterium *Pelodictyon* sp. requires low light irradiance (50 to 100

Table 1. Optimal light irradiance levels for the enrichment of purple sulphur bacteria.

Light irradiance (lux)	Bacteria likely to predominate	Other conditions
100–300	*Chromatium okenii* *Chromatium weissii* *Thiospirillum jenense*	Alternating 16 h light and 8 h dark periods. Light from tungsten lamps
300–700	*Chromatium warmingii*	Continuous illumination from tungsten lamps
700–2000	Small *Chromatium* spp. *Thiocystis* sp. *Thiocapsa* sp. *Amoebobacter* sp.	Continuous illumination from tungsten lamps

lux). Pfennig (1965) has determined the optimum light irradiance for the enrichment of purple sulphur bacteria from fresh waters and the data are summarised in Table 1.

In order to exclude green sulphur bacteria the enrichment cultures were first exposed to infra-red light with a wavelength greater than 800 nm.

1.3.4 Aerobic or Anaerobic Conditions as Selective Agents

In closed enrichment systems aerobic conditions are usually achieved by dispensing the media as a shallow layer either in conical flasks or Petri dishes. However, even under these conditions the oxygen tension is high only at the surface of the medium and once pellicle growth develops the concentration declines even at the surface. Nevertheless the successful enrichment of species of *Azotobacter, Thiobacillus, Acetobacter, Nitrosomonas* and *Nitrobacter* occurs under these stationary conditions when the appropriate medium is used. If high oxygen tensions are required, these can be readily provided either by shaking the culture or by sparging with sterile air. However, even with strict aerobes the effect of oxygen tension on growth is subtle and in many instances enrichments are more successful in stationary culture, where oxygen is limiting, than when oxygen is provided in excess.

The incubation of enrichments in the absence of oxygen allows the development of facultative and obligate chemo-organotrophs and, if light is present, phototrophs. Terms such as micro-aerophiles, non-exacting anaerobes and strict anaerobes should be avoided since they are imprecise. A more reliable index is to express anaerobiosis in terms of the oxidation-reduction potential (Eh) which can be precisely measured. Numerous methods are available to grow anaerobic bacteria. These include the use of anaerobic jars (Willis 1969), roll-tube techniques (Hungate 1969), agar shakes (van Niel 1931) and Pankhurst tubes (Pankhurst 1966). The simplest form of anaerobic enrichment is to use a screw-topped bottle and it is preferable to use a high ratio of liquid to gas volume thereby minimising the ingress of oxygen. In primary enrichments the presence of slight traces of oxygen is not critical since it is rapidly removed by aerobes present in the inoculum and strict anaerobes, such as methanogens, which will only grow at low Eh values (-350 mV), will develop in the appropriate medium. However, where it is essential to maintain anoxic conditions in sample preparation prior to inoculation, an inflatable anaerobic glove bag of the type described by Leftley and Vance (1979) is eminently suitable. When working with anaerobes the media should always be freshly prepared since even when stored under allegedly anoxic conditions some ingress of oxygen often occurs.

Whilst absolutely anoxic conditions are not essential in primary enrichments, for reasons mentioned above, it is vital that in subsequent isolation procedures the medium be poised at the correct redox potential for growth. For example, Postgate (1966) showed that sulphate-reducing bacteria will not initiate growth unless the Eh is less than -100 mV and methanogens need an Eh in the range of -350 mV. To develop the desired Eh values reducing agents are frequently included in the growth medium: cysteine, mercapto-ethanol, mercapto-acetate, sulphide, dithionite, ascorbic acid and iron wire or nails are frequently used. To ensure that the medium has become sufficiently reduced, redox indicators are usually incorporated into the media. The most commonly used are resazurin (E'_0 at pH 7.0, -30 to -40 mV), indigocarmine (E'_0 at pH 7.0, -123 mV) and benzyl viologen (E'_0 at pH 7.0, -359 mV) at concentrations ranging from 0.0001 to 0.0005% (w/v). Reducing agents are often toxic and thus their concentrations in the media are critical. Cysteine is very useful at concentrations not exceeding 0.05% (w/v) but at higher concentrations it is toxic.

Sulphide is also extremely useful but the toxicity level is pH dependent.

Agar shakes (van Niel 1931) provide a convenient method for isolating anaerobes but for the more exacting forms the Hungate roll-tube method (Hungate 1969) is probably a more reliable technique. When working with strict anaerobes the inocula used should be about 10% (w/v) of the fresh medium used since too small an inoculum often fails to grow.

1.3.5 Trace Element and Co-factor Requirements

Under normal circumstances there are sufficient trace elements present in the reagents used to prepare the enrichment media. However, to avoid any trace element deficiency occurring they are often included in enrichment media.

One of the most useful trace element solutions is that of Hoagland as modified by Pfennig (1965). It comprises the following components in distilled water ($g\ l^{-1}$): $AlCl_3$, 1; KI, 0.5; KBr, 0.5; LiCl, 0.5; $MnCl_2 \cdot 4H_2O$, 7; H_3BO_3, 11; $ZnCl_2$, 1; $CuCl_2$, 1; $NiCl_2$, 1; $CoCl_2$, 5; $SnCl_2 \cdot 2H_2O$, 0.05; $BaCl_2$, 0.5; Na_2MoO_4, 0.5; $NaVO_3 \cdot H_2O$, 0.1; and selenium salt, 0.5. All the salts are dissolved separately in distilled water and mixed to give a final volume of 3.6 l at pH 3.0 to 4.0. Normally 5.0 to 6.0 ml of the trace element solution is added per litre of medium.

An important feature regarding trace elements is that not only should they be present but also available. A frequent problem when incorporating trace elements into enrichment media is that they co-precipitate out during autoclaving. To overcome this trace element solutions should be filter sterilised and added aseptically when the medium is cool.

In addition to trace element requirements some bacteria also need growth factors. For example, many strains of purple and green sulphur bacteria have a requirement for vitamin B_{12} whilst some purple non-sulphur bacteria, such as *Rhodopseudomonas sphaeroides*, require thiamine, biotin and nicotinic acid for growth (Pfennig & Trüper 1974). Many lactobacilli require a large number of growth factors, i.e. amino acids, purines, pyrimidines and vitamins. Growth factor requirements can often be satisfied by adding small quantities of yeast extract which contains a wide range of amino acids and vitamins of the B group. Vitamin B_{12}, however, is not present in yeast extract and must be added separately.

1.3.6 Inhibitors as Selective Agents

The addition of inhibitory compounds to enrichment media suppresses the development of the majority of the microflora enabling the development of the desired species to occur. A good example of this is the use of 0.5% (w/v) bile salts to inhibit non-intestinal bacteria allowing the growth of *Escherichia coli*. In an analogous manner 0.03% (w/v) cetrimide is a useful agent for the selective enrichment of *Pseudomonas aeruginosa* (Brown & Lowbury 1965). Enrichment media containing antibiotics have been used extensively. For example actidione agar (Difco Manual 1966; Oxoid Manual 1979) is invaluable for the isolation and enumeration of bacteria in samples containing large numbers of yeasts and fungi, e.g. bacterial contamination of pitching yeast. At a concentration of 10 $\mu g\ ml^{-1}$, actidione permits the growth of bacteria but inhibits the growth of most yeasts and fungi. Similarly, Cross (1968) used a combination of novobiocin (25 $\mu g\ ml^{-1}$) and actidione (50 $\mu g\ ml^{-1}$) to isolate selectively *Thermoactinomyces vulgaris* from thermophilic *Bacillus* spp. and fungi in mouldy hay. It is clearly impracticable to review the extensive range of chemical compounds that have been added to growth media for the selective isolation of microorganisms from natural environments, foodstuffs and clinical specimens and thus the reader is referred to the Difco (1966) and Oxoid manuals (1979) for further details.

1.4 Isolation methods

1.4.1 Winogradsky Column

The Winogradsky column (Winogradsky 1888) provides an extremely simple but effective way to simulate natural sediment environments in the laboratory and by providing the necessary selective pressures the enrichment of specific microorganisms from an extremely diverse initial microflora can be achieved. Aaronson (1970) gives a clear and concise account of the setting up and sampling of a Winogradsky column and this will not be discussed here. Within the glass or plastic column (Fig. 2) a gradient of nutrients and metabolic end-products develops as a result of fermentative

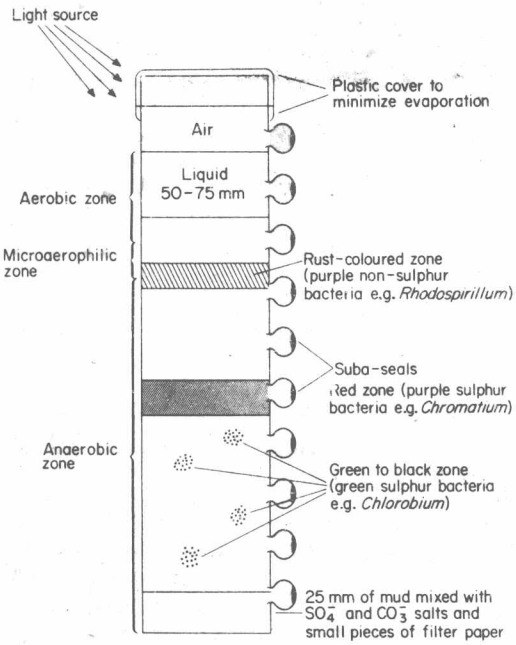

Figure 2. The Winogradsky column (modified after Aaronson 1970).

metabolism from the sediment upwards whilst oxygen diffuses in from the surface. These interacting gradients enable individual bacterial types, if present in the inoculum, to develop at specific points along the column where growth conditions are optimal. For example, in the upper regions of the column both oxygen and sulphide occur which allows the development of *Thiobacillus* spp. whilst in the depth of the sediment, where conditions are anoxic and sulphate levels are high, *Desulfovibrio* spp. predominate. Where light (artificial or natural), anaerobic conditions and hydrogen sulphide are present phototrophic bacteria develop. Green sulphur bacteria tolerate much higher sulphide levels than the purple sulphur bacteria (Pfennig 1967) and thus develop below them in the column. To facilitate the growth of purple non-sulphur bacteria which do not tolerate high sulphide levels the activities of sulphate-reducing bacteria must be suppressed. This may be achieved either by omitting exogenous sulphate from the column, that is, by replacing $CaSO_4$ with $CaCl_2$, or by using temperatures above 33°C whereupon the sulphate reduction rate is reduced (Schlegel & Pfennig 1961). As a consequence fermentation products accumulate and in the presence of light purple non-sulphur bacteria may predominate. From these few examples it can be seen that the Winogradsky column can be exploited to achieve the enrichment of a diverse range of metabolic types. In order to isolate the bacteria from the column a number of devices can be employed, for example, a spatula, wire-loop or hypodermic syringe. A more useful approach is to drill holes in the column (Fig. 2) and seal them with Suba seals or their equivalents. Samples can then be withdrawn with ease from the required sampling sites along the column.

1.4.2 Soil Perfusion Methods

Lees and Quastel (1946) developed the use of a soil reperfusion apparatus for enriching bacteria from soil and the percolation system shown in Fig. 3 is a typical design. After placing the soil sample or sediment within the main chamber of the apparatus, the enrichment medium is circulated through the soil column by applying a low vacuum pressure at the outlet. By incorporating the desired substrate, with or without inhibitors, into the percolation medium the required bacterium can often be successfully enriched by this technique. To minimise waterlogging of the soil column Jeffreys and Smith (1951) modified the soil perfusion apparatus of Lees and Quastel (1946) and incorporated a simple valve system to regulate the percolation rate. This method has an added advantage in that the percolation medium can be circulated using an inert gas, e.g. high-purity nitrogen or argon, and so anaerobes can be isolated.

1.4.3 Chemostat Enrichment Systems

The advantages of chemostat enrichments over conventional batch systems have already been discussed. A considerable number of chemostat designs are available. However, as a general principle, the simpler the design the more reliable the chemostat. The technical design and construction of chemostats has been well reviewed by Evans *et al.* (1970). In our own experience the simple one-stage all-glass chemostat of Baker (1968) has proved extremely useful in chemostat enrichment experiments (Dunn *et al.* 1978; 1980; see also Chapter 16). Jannasch (1967), in contrast, favoured a more complex arrangement involving three chemostat units and varied the dilution rate by using culture vessels of different volumes.

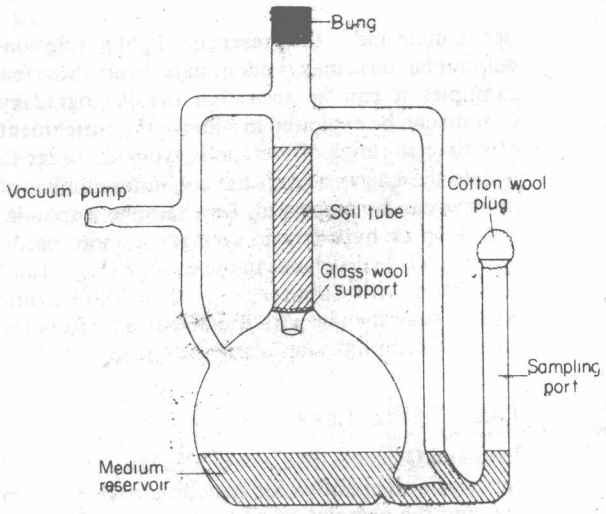

Figure 3. A typical soil perfusion apparatus.

After inoculation of the culture vessel(s) with either a water or sediment sample, small volumes of the culture are removed at daily intervals and streaked onto the corresponding agar medium. The dominant bacterial species can be classified to the genus level using conventional morphological and biochemical tests. In this manner chemostat enrichments for heterotrophic bacteria from aquatic environments have been made (Jannasch 1967; Veldkamp & Kuenen 1973; Brown *et al.* 1978). Dunn *et al.* (1980) extended this technique to isolate nitrate-dissimilating bacteria from estuarine sediments and R. A. Herbert (unpublished observations) has also successfully isolated *Desulfovibrio* spp. and *Clostridium* spp. from estuarine sediments by chemostat enrichment.

1.4.4 Other Procedures

Enrichment cultures do not provide estimates of the abundance of a particular bacterium in a given habitat or whether or not it is physiologically active in that environment. They result in the isolation of those bacteria which are best adapted to the selective conditions and thus outgrow their competitors. Interpretation of the role played by particular bacteria in natural environments, when isolated by enrichment methods, should be treated with great caution.

Direct methods for the isolation of bacteria from natural environments involve the preparation of dilution series followed by either plating out onto solid media or distribution into tubes of media. To ensure maximum viability the choice of diluent is important and buffered diluents such as phosphate buffered saline or one-quarter-strength Ringer's solution (Cruickshank 1965) are preferable to 0.9% (w/v) saline. Indicators, such as 0.0001% (w/v) resazurin, should be incorporated into the diluent when handling anaerobes to ensure that the conditions are sufficiently reduced. Agar is usually used to gel the media but in certain circumstances, particularly with autotrophs, organic materials present in the agar are toxic and so silica gel should be used (Skerman 1967). To avoid spreading of the colonies over the agar surface the plates should be dried at 37°C for 2 d before use. Finally, spread-plates are preferable to pour-plates since many bacteria are killed by the thermal shock of being exposed to molten agar.

The isolation of anaerobic bacteria can be carried out either by using agar shake tubes (van Niel 1931) or by the roll-tube technique (Hungate 1969). The agar shake method has the great advantage of requiring simple equipment and yet it is extremely effective for the isolation of anaerobes. A series of tubes, each containing 9.0 ml volumes of the required agar medium, are kept molten at 42 to 44°C in a waterbath. The first tube in the series is inoculated with a sample of soil or sediment and, after mixing by inversion, a dilution series is prepared by pouring about 1.0 ml from one tube into the next, again mixing between each transfer. After mixing, the tubes are rapidly cooled