Springer Series on Biofilms

Steven L. Percival
Derek C. Knottenbelt
Christine A. Cochrane *Editors*

Biofilms and Veterinary Medicine

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Steven L. Percival • Derek C. Knottenbelt • Christine A. Cochrane
Editors

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Editors
Prof. Steven L. Percival
Medical School, Department of Pathology
West Virginia University
Morgantown, WV 26506
United States
biofilms@hotmail.co.uk

Dr. Christine A. Cochrane
Faculty of Health and Life Sciences
Institute of Ageing and Chronic Disease
University of Liverpool
Leahurst
Neston. CH64 7TE
United Kingdom
C.A.Cochrane@liverpool.ac.uk

Prof. Derek C. Knottenbelt
University of Liverpool
School of Veterinary Science
Equine Hospital, Leahurst
Chester High Road
CH64 7TE Neston
United Kingdom
knotty@liverpool.ac.uk

Series Editor
J. William Costerton
Director of Microbiology Research
Department of Orthopedic Surgery
Allegheny General Hospital and
Director of Biofilm Research
Allegheny-Singer Research Institute
320 East North Ave.
Pittsburgh PA, 15212
USA

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Steven L. Percival would like to dedicate this book to a number of people who have provided support, guidance and reassurance throughout the 20 years he has been studying and researching microbiology and biofilms. These include Carol, Alex, Tom, Mum, Dad, Nigel, Emma, Jorge, Sam, Elisha and Kara.

Christine A. Cochrane would like to dedicate this book to Paul (for your constant support and encouragement), our sons Paul and Mark and grandchildren Jennifer and Callum. Finally, to my Mother Betty who never doubted my ability to become a scientific researcher.

Preface

> Imagination should give wings to our thoughts but we always need decisive experimental proof, and when the moment comes to draw conclusions and to interpret the gathered observations, imagination must be checked and documented by the factual results of the experiment. (Pasteur L)

Louis Pasteur (1822–1985) was an amazing, persevering, perceptive and determined scientist who today is widely regarded as the father of the "Germ Theory" and bacteriology. He is revered for possessing the most important qualities of a scientist: he had an unrivalled ability to scrutinise data on almost any subject and then to develop profound and often fundamental questions from them. He had an uncanny ability to identify the solutions to problems based on analytical scrutiny of data – even without any of the sophisticated statistical tools we have today. He possessed an almost unique and certainly an enviable reputation for patience and drive to research under strictly controlled conditions regardless of the contemporary scepticism that accompanied the current dogma. These are also the characteristics of the scientists who have taken on the needs associated with biofilm research in the modern era. While Pasteur was not the first to propose that disease was the result of pathogenic microorganisms, he developed the principals and theories and conducted the experiments that clearly indicated their relevance.

Since the advent of the antibiotic age man has sought to find "chemical" strategies to overcome pathogens in particular. These have also involved the "devious" manipulation of the immune system through the development of vaccines and hyperimmune sera. The immune stimulating approach to disease is "super-efficient" in that the various cascades of the cellular and humoral immune systems are mobilised specifically at target organisms and in this process, there are a few adverse side effects. The organisms have difficulty overcoming the amazing versatility and target accuracy of the immune system. This results in prevention of disease in the case of vaccine production and/or the limitation of a disease to the extent that the pathogen causes mild or sub-clinical changes. In the event that a pathogen is introduced into a totally susceptible host, there is a race between the immune system and its attempts to both overcome and to eliminate the pathogen and the pathogen's own ability to trigger inflammatory, cytopathic, toxic or other

damaging processes. Viruses, bacteria, fungi, yeasts, protozoa and parasites are all capable of causing disease and the survival of any species is surely a testament to the "innate" and "acquired" immune systems that through evolution have developed strategies to at least limit the damage and in many cases to prevent any effect whatever. However, when an infection challenges a naive host, there is a significant delay in the mobilisation of the immune systems resources. During this time, disease can develop and so the objective of modern medicine (including the veterinary and related science and biological professions) is to try to limit the effects of the infection without harming the host animal. Having been exposed to a disease the immune system will react in a co-ordinated fashion to ensure that the disease is as short and as mild as possible and so antimicrobial drugs in these situations would become largely unnecessary.

It is widely accepted that antimicrobial drugs (whether antiviral, antibacterial, antifungal, or antiprotozoal or antiparasitic) are inherently flawed as a long-term strategy for controlling and treating disease because of the evolutionary pressure that will inevitably result in resistance. The concept that "there is an antibiotic that will work if the dose is high enough" is definitely counter to all principles of antimicrobial therapy, and yet it is one of the commonest approaches. It is born out of frustration and lack of understanding as to why bacteria can survive against all the odds. It is surely far better to understand the reasons for failures of efficacy and to address these specifically than it ever is to simply add more and more antibacterial drugs! Whilst there is no doubt at all that antimicrobial strategies have reduced the incidence of disease and reduced the duration of illness associated with infections, the rate of new-molecule development has not kept pace with the ability of the microorganisms to resist them. In many circumstances, failure of efficacy is simply blamed on "resistance" but it is clear from biofilm research that there is much more to "resistance" than meets the eye. The spectrum of drugs used in veterinary species is relatively narrow – a few antibiotics (largely those that are not used in human's medicine!) are used widely. Veterinarians have taken their responsibility for rational use seriously and it is unfair to blame the veterinary profession for the development of antibiotic resistance. There are certainly specific circumstances when antibiotics and the hosts' own immune and reparative processes fail to control infections and one of the most interesting of these is the development of biofilms that protect and "shield" the organisms from potentially damaging environmental and host defences. Biofilms are the most common mode of bacterial growth in nature and are highly resistant to antibiotics.

Biofilms are implicated in many common medical problems including urinary tract infections, catheter infections, middle-ear infections, dental plaque, gingivitis, and some less common but more lethal processes, such as endocarditis, infections in cystic fibrosis. However, biofilms have only recently been given their true importance in the overall process of disease pathogenesis. Bacterial biofilms are one of the fundamental reasons for incipient wound healing failure in that they may impair natural cutaneous wound healing and reduce topical antimicrobial efficiency in infected skin wounds. Their existence explains many of the enigmas of microbial infection and a better grasp of the process may well serve to establish a different

approach to infection control and management. Biofilms and their associated complications have been found to be involved in up to 80% of all infections. A large number of studies have been performed targeted at the bacterial biofilms and many of these are referred to in this book, which is the first of its kind. These clinical observations emphasise the importance of biofilm formation to both superficial and systemic infections and the inability of current antimicrobial therapy to "cure" the resulting diseases even when the in vitro tests suggest that they should be fully effective.

In veterinary medicine, the concept of biofilms and their role in the pathogenesis of disease has lagged seriously behind that in human medicine. This is the more extraordinary when one considers that much of the research has been carried out using veterinary species in experimental situations. The clinical features of biofilms in human medicine are certainly mimicked in the veterinary species but there is an inherent, and highly regrettable indifference to the failure of antimicrobial therapy in many veterinary disease situations and this is probably at its most retrograde in veterinary wound management.

Leahurst, UK
March 2011

Derek C. Knottenbelt

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Contributors

Lucy A. Brunton Faculty of Biological Sciences, University of Leeds, Leeds, LS2 9JT, UK

Peter D. Clegg Department of Musculoskeletal Biology, University of Liverpool, Leahurst Campus, Neston, Cheshire, CH64 5SD, UK

Christine A. Cochrane Faculty of Health and Life Sciences, Institute of Ageing and Chronic Disease, University of Liverpool, Leahurst, Neston. CH64 7TE, United Kingdom

Helena Cruz Division of Microbiology and Risk Assessment, National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, Building H, 2860 Søborg, Denmark

Ana B. García Division of Microbiology and Risk Assessment, National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, Building H, 2860 Søborg, Denmark

Alice J. Gardner Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Ceredigion, SY23 3AL, UK

John Heritage Faculty of Biological Sciences, University of Leeds, Leeds, LS2 9JT, UK

Samuel J. Hooper Oral Microbiology Group, Tissue Engineering and Reparative Dentistry, School of Dentistry, Cardiff University, Heath Park, Cardiff, CF14 4XY, UK

Jerry S. Knapp Faculty of Biological Sciences, University of Leeds, Leeds, LS2 9JT, UK

Derek C. Knottenbelt Faculty of Health and Life Sciences, Department of musculoskeletal Biology, University of Liverpool Leahurst, Neston, South Wirral CH64 7TE, UK

Tomoari Kuriyama Department of Oral and Maxillofacial Surgery, Graduate School of Medical Science, Kanazawa University, Kanazawa 920-8640, Japan

Michael A.O. Lewis School of Dentistry, Cardiff University, Heath Park, Cardiff, CF14 4XY, UK

Thomas W. Maddox Faculty of Health and Life Sciences, School of Veterinary Science, University of Liverpool, Leahurst Campus, Neston CH64 7TE, UK

Sladjana Malic Oral Microbiology Group, Tissue Engineering and Reparative Dentistry, School of Dentistry, Cardiff University, Heath Park, Cardiff CF14 4XY, UK

Marielle B. Melchior MBM Veterinary Consultancy, 8111 RA, Heeten, The Netherlands

Steven L. Percival Department of Pathology, Medical School, West Virginia University, Morgantown, WV, USA

Marcello P. Riggio Infection and Immunity Research Group, Dental School, College of Medical Veterinary and Life Sciences, University of Glasgow, 378 Sauchiehall Street, Glasgow G2 3JZ, UK

Sónia da Silva Centre for Biological Engineering, IBB-Institute for Biotechenology and Bioengineering, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal

Samantha J. Westgate Faculty of Health and Life Sciences, Department of musculoskeletal Biology, University of Liverpool, Leahurst, Neston, South Wirral CH64 7TE, UK

David W. Williams Oral Microbiology Group, Tissue Engineering and Reparative Dentistry, School of Dentistry, Cardiff University, Heath Park, Cardiff, CF14 4XY, UK

Introduction to Microbiology, Zoonoses and Antibiotics

Steven L. Percival, Jerry S. Knapp, David W. Williams, John Heritage, and Lucy A. Brunton

Abstract Microorganisms are biological entities (organisms) which are so small they cannot be visualised without the aid of some type of microscope. There are six groups that make up the microorganisms – archaea, bacteria, protozoa, fungi, algae and viruses. Despite their small size, it is clear that microorganisms have a profound influence on human and animal life and indeed on all aspects of the biosphere. Prokaryotes come in a variety of shapes and sizes. Probably the most frequently encountered are cocci (coccus – singular) (round or oval cells), bacilli (bacillus singular) (rod-shaped) and vibrios (curved). Algae are photosynthetic eukaryotes with the cells containing chloroplasts. Algae are autotrophic primary producers and do not cause infections; they are thus of limited importance in the veterinary field. The fungi are an important and diverse group of eukaryotes; although formerly considered to be plants, they are now known to be more closely related to animal cells. Protozoa, otherwise known as protists, are also a very varied group. Protozoa are nearly all chemoheterotrophs ranging from free-living cells to obligate parasites. Viruses are infectious particles which lack a cellular structure. Since viruses do not possess the mechanisms needed to produce energy and the ribosomes required to synthesise proteins, they are incapable of independent metabolism, replication or movement. As a result, viruses are completely dependent on the host cells, which they effectively hijack, to produce new virus particles. For survival microbes require sources of energy, carbon and several other elements including nitrogen, oxygen, phosphorus, sulphur, potassium, sodium, calcium,

S.L. Percival (✉)
Department of Pathology, Medical School, West Virginia University, Morgantown, WV, USA
e-mail: Biofilms@hotmail.co.uk

J.S. Knapp • J. Heritage • L.A. Brunton
Faculty of Biological Sciences, University of Leeds, Leeds LS2 9JT, UK

D.W. Williams
Oral Microbiology Group, Tissue Engineering and Reparative Dentistry, School of Dentistry, Cardiff University, Heath Park, Cardiff CF14 4XY, UK

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magnesium and iron. Trace elements are also needed but in relatively small amounts. All these elements are required for the maintenance of life processes and the synthesis of new biomass. Animals are host to large numbers of microbes, many of which contribute to the health of their host. However, the majority of these microbes have the ability to cause disease. Many of these microbes may only be able to infect a single species, but others are able to cross the species barrier to infect other species, including humans. Diseases that can be passed between vertebrate animals and humans are known as zoonotic diseases, or zoonoses.

1 What Are Microorganisms?

Microorganisms are biological entities (organisms) which are so small; they cannot be visualised without the aid of some type of microscope. This is an unusual definition since the overall grouping of microorganisms includes some members (viruses) that cannot truly be considered to be organisms since they have no cellular structure and are the ultimate parasites, incapable of independent reproduction. In many ways, the term microbe might be preferable to microorganisms. There are six groups that make up the microorganisms – archaea, bacteria, protozoa, fungi, algae and viruses. It is notable that despite the prefix "micro", there are some members of these groups which are in fact macroscopic and visible to the naked eye. So, for example, while many fungi (e.g. yeasts) are microscopic for their entire life cycle, others have large macroscopic fruiting bodies (mushrooms and toadstools); it is paradoxical that the largest living organism (certainly in terms of its area) is probably a fungus, *Armillaria ostoyae*, which is known to grow to over 1,500 acres in size. Similarly many algae are relatively large (e.g. some of the seaweeds such as kelp), and although most protozoa are microscopic, some are visible to the naked eye (e.g. *Amoeba proteus* which can reach 0.75–1 mm in length).

The existence of microorganisms was first demonstrated by Anthonie van Leeuwenhoek in the later part of the seventeenth century. However, the systematic study of microorganisms only really started with the work of Louis Pasteur and Robert Koch from the 1860s onwards. Despite their small size, it is clear that microorganisms have a profound influence on human and animal life and indeed on all aspects of the biosphere.

Within the group of microorganisms, there exists great diversity not only of microbial size but also of structure, nutrition, ecology and genetics. The term microorganism has no real taxonomic significance as the overall grouping contains many entities with no taxonomic relationships. In many ways, what unifies the microorganisms is that they are studied by a range of similar laboratory techniques encompassed by the science of microbiology; these include microscopic observation, artificial cultivation (often in pure culture) and a range of biochemical and genetic/molecular biological techniques. As mentioned above, in many ways the term "microbe" is preferable to microorganisms as it makes fewer assumptions about taxonomy, structure and "lifestyle".

It is now thought that living organisms can be divided into three distinct domains based on cell structure. These domains are the archaea, the bacteria and the eukarya, the latter group including all plants and animals as well as the eukaryotic microorganisms. The relationship between the domains can be confirmed by a detailed analysis of the structure and sequences of the RNA molecules in the small subunit of the ribosomes – 16S for the prokaryotes and 18S for eukaryotes. The genes for these RNA molecules are present in all organisms, always have the same function, are large enough to be able to show divergence in sequence and appear to have changed slowly over time.

Microorganisms can also be divided into three sections.

1. Non-cellular obligate parasites – the viruses. These entities are infectious particles containing a nucleic acid genome and some proteins and occasionally lipids, but they lack the complete cellular "machinery" to generate energy and to synthesise proteins and nucleic acids. Viruses are completely reliant on the cells of the host to reproduce new particles and so cannot on their own be considered living organisms. There appear to be viruses which parasitize all types of living cellular organisms.
2. Prokaryotic microbes – the archaea and bacteria. Prokaryotes are true cellular organisms, but have a relatively simple basic cell structure lacking internal membrane-bound nuclei and organelles. Prokaryotes have 70S ribosomes consisting of 30S and 50S subunits. Most species are unicellular and in general they do not exhibit differentiated cell types – although there are some exceptions such as production of resting stage spores by *Bacillus* and *Clostridium* species and the production of heterocysts (cells specialised for di-nitrogen fixation) by some cyanobacteria such as *Nostoc*. Archaea and bacteria are similar in many respects and until recently archaea were all classed as bacteria. However, it is now clear that there are significant differences between the groups in relation to the presence and structure of cell walls, the composition of cell membranes, and also in relation to the molecular biology of their genomes and the processes used for protein synthesis. Many archaea are extremophiles (able to live in very hot, saline or acidic conditions) or have unusual metabolic pathways, such as the ability to generate energy during the production of methane.
3. Eukaryotic microorganisms – this group includes the algae, the protozoa and the fungi, along with other lesser known groups. Eukaryotes have more complex cells with a true membrane-bound nucleus and membrane-bound organelles (normally including mitochondria). The classification of eukaryotic microorganisms has changed greatly in recent years and taxonomy based on gene and protein sequences has revealed previously unsuspected evolutionary relationships. Eukaryotes are a diverse group; all possess the more complex eukaryotic cell structure, and while some are simple unicellular organisms, others are more complex and multicellular and display considerable differentiation and complexity of life cycles. Eukaryotic cells are typically more complex than prokaryotes. They have a membrane-bound nucleus, which contains more than one linear chromosome, and generally have membrane-bound structures or organelles, such as mitochondria, hydrogenosomes and chloroplasts.

The flagella (singular *flagellum*) (or cilia) of eukaryotic cells are more complex in structure than those of prokaryotes. Eukaryote cytoplasm also has larger 80S ribosomes than prokaryotes. It is now accepted that eukaryotes evolved partly due to the acquisition of symbiotic bacterial cells, which were incorporated into ancestral cell types and over time became permanent. This is highlighted by the fact that mitochondria and chloroplasts have their own genomes and prokaryote-type ribosomes.

2 Bacteria and Archaea

2.1 Cell Shape and Arrangement of Bacteria and Archaea

Prokaryotes come in a variety of shapes and sizes. Probably the most frequently encountered are cocci (coccus – singular) (round or oval cells), bacilli (bacillus – singular) (rod-shaped) and vibrios (curved). Very short rod-shaped bacilli are referred to as coccobacilli. A spirillum is a helical or spiral-shaped cell, while long slender spiral cells with flagella within the turns of the helix are called spirochaetes. A significant number of bacteria and archaea exist as long filaments. In recent years, more bacteria have been discovered with unusual shapes, some are rather irregular and lobed; indeed, there is even a flat square archaea. Certain types of prokaryotes are pleiomorphic with cells varying in shape and size at different stages of growth.

While many bacteria and archaea grow as single cells, others form distinctive arrangements and are often found as chains (filaments), pairs or fours, or cubical packets of eight (these are called sarcinae). Other bacteria, such as staphylococci, form irregular clumps giving the classical appearance of a "bunch of grapes". These characteristic arrangements are caused by the failure of daughter cells to fully separate after cell division and reflect the symmetry, or otherwise, of successive rounds of cell division.

2.2 Cell Size

Prokaryotes are always small, but do show wide variation in size. Typical dimensions are in the order of 0.75 to 2 μm in diameter for cocci, and for bacilli 0.75 to 2 μm in width to about 3 to 8 μm in length. There are, however, many exceptions; some species of *Mycoplasma* have cells of about 0.2 to 0.3 μm. These *Mycoplasma* cells have very small genomes and are often parasitic, with some being able to be grown in complex artificial media. At the other extreme, the largest bacterium is the recently discovered *Thiomargarita namibiensis* which is a spherical marine chemolithotrophic bacterium that is theoretically visible to the naked eye with a diameter of 750 μm. In the case of this species, it has to be said that a large part of the cell's volume is occupied by a vacuole and it is not all cytoplasm.

2.3 Genetic Material

Prokaryotes, unlike eukaryotes, do not have a true membrane-bound nucleus, but have a single circular chromosome. In addition, prokaryotes may carry a range of plasmids. Plasmids are small DNA elements, usually circular, which generally encode a range of non-essential, though often useful, characteristics such as antibiotic resistance or pathogenicity determinants.

2.4 Cell Membranes

Bacterial and archaeal cytoplasm is enclosed within a cytoplasmic membrane which controls the integrity of the cell and is situated within the cell wall, if the cell has one. The cell membrane is a phospholipid bilayer containing a range of embedded functional proteins. It controls the entry and exit of materials and has a crucial role in energy generation though the establishment of transmembrane gradients. Though similar, there are a range of differences between bacterial and archaeal cell membranes.

2.5 Cell Walls

Prokaryote cells are generally contained within an outer rigid cell wall which protects the cell from osmotic stress. However, there are some exceptions, with some cells having cell walls that lack rigidity. In a few genera, such as *Mycoplasma* and *Chlamydia* in the bacteria and *Thermoplasma* in the archaea, there is even a complete absence of a cell wall. While there are various types of bacterial cell walls, all contain a layer of the polymer peptidoglycan, otherwise known as murein, this provides the rigidity and strength. Peptidoglycan consists of many polysaccharide chains cross linked by short peptide bridges and it is uniquely found in bacteria.

Within the bacteria there are two major groups distinguished by their cell wall structure and differentiated by the classical Gram stain. Gram-positive bacteria retain a crystal violet-iodine complex and appear purple, whereas Gram-negative cells lose this complex and display the pink counter stain. Gram-positive bacteria contain thick, multiple layers of peptidoglycan along with other polymers including teichoic and teichuronic acids. Gram-negative cell walls have a single layer of peptidoglycan, but outside this they have an outer membrane layer, composed of lipopolysaccharide and protein. The archaeal cell walls do not contain peptidoglycan, but have a variety of different substances including pseudomurein, which is structurally similar to peptidoglycan. Other materials found in archaeal cell walls include polysaccharides, glycoproteins and proteins.

The cell wall and cytoplasmic membrane together are often referred to as the cell envelope.

2.6 External Structures

Outside the cell wall, some bacteria and archaea have an additional layer called a capsule or a slime layer. Capsules and slime layers are normally made of polysaccharide, although some are made of protein. Capsules are much more defined and tightly attached, whereas slime layers are more diffuse, covering a greater area. Capsulated bacteria tend to resist engulfment by phagocytic cells and therefore help pathogens evade the immune response. Even when the bacterium is engulfed, a capsule can protect the microorganism from intracellular killing. These layers also assist attachment of microbes to surfaces and are important in formation of biofilms.

Other external structures found in some bacteria are fimbriae and pili. These filamentous protein structures have a range of functions. Fimbriae are involved in attachment to cells and other surfaces and may have a role in pathogenic processes. They also aid attachment of bacteria to each other and are involved in formation of pellicles and biofilms. Pili have a range of functions including attachment, genetic exchange and movement.

Some prokaryotes are capable of movement and motile archaea and most motile bacteria move by means of flagella. These proteinaceous appendages protrude through the cell wall and move with a rotary motion. They may be attached at the poles of the cell or all around it (polar and peritrichous flagellation, respectively). A range of motile bacteria do not possess flagella and can only move in contact with surfaces, some of these move with the aid of retractile pili (twitching motility), but others with "gliding motility" have no obvious locomotor organelles and mechanisms for their movement are still a matter of debate.

2.7 Algae

Algae are photosynthetic eukaryotes with the cells containing chloroplasts. Algae are autotrophic primary producers and do not cause infections; they are thus of limited importance in the veterinary field.

2.8 Fungi

The fungi are an important and diverse group of eukaryotes; although formerly considered to be plants, they are now known to be more closely related to animal cells. Fungi are chemoheterotrophs and classically comprise a mass of protoplasm contained within a filamentous structure called a hypha. Fungal cell walls, in some ways, resemble plant cell walls but are chemically different, generally consisting largely of chitin. Most fungi grow as a network of branching hyphae

known as a mycelium which grows centrifugally and can spread out to cover a very large area. Fungi generally reproduce by production of spores, which may be a sexual or asexual process. However, some fungi, known as yeasts, grow as single cells rather than hyphae and generally reproduce by budding rather than binary fission. The yeasts are not a taxonomic grouping, with the term "yeast" describing a type of morphology. Other fungi include moulds, mushrooms and toadstools.

Most fungi are aerobes, although there are some strict anaerobes. Indeed, a number of anaerobic fungi play an important role in digestion of herbage in the rumen and caecum of herbivores. Most fungi are saprophytic absorbing dissolved nutrients, or secreting enzymes which decay, and solubilise macromolecules which can then be used as nutrients. In this regard, fungi play a major role in the decomposition of dead tissues particularly plant matter. In fact, fungi are the agents *par excellence* of decomposition of woody materials. However, a number of fungi are pathogenic, particularly towards plants, but there are also a wide range of human and animal pathogens. Some cause superficial infections of the skin (e.g. *Trichophyton* spp.) or in the mouth and vagina (e.g. *Candida* spp.). Other species cause systemic infections (e.g. of the lungs, *Cryptococcus*, *Pneumocystis* and *Coccidioides*). *Pneumocystis* is an interesting organism as it was once thought to be a protozoan, but genetic analysis now shows it to be a fungus, although it has little resemblance to one. In recent years, it has been demonstrated that some chytrid fungi are severe pathogens of frogs and are causing widespread death of frogs worldwide.

Fungi also have a range of industrial uses that make them economically very important. Best known of these is the use of yeast in the production of alcoholic beverages and bread. Fungi are also a source of some of the major antibiotics such as penicillin (*Penicillium chrysogenum*) and cephalosporins (*Cephalosporium acremonium*) and a range of valuable fine chemicals.

2.9 Protozoa

Protozoa, otherwise known as protists, are also a very varied group. Protozoa are nearly all chemoheterotrophs ranging from free-living cells to obligate parasites. *Euglena* are exceptions in that they have chloroplasts and thus are able to photosynthesize, although some can also ingest and digest bacteria. Of the heterotrophs, some use dissolved nutrients, while others are predatory. Some protozoa are free-living, and some are involved in symbioses (e.g. some anaerobic ciliates are symbionts in the rumen, while cellulose-degrading flagellates are important symbionts in the guts of wood-eating termites). Examples of parasitic protozoa and the diseases they cause are given in Table 1, and some of these diseases are zoonotic.

Table 1 Examples of some parasitic protozoa and the diseases

Organism	Disease caused
Entamoeba histolytica	Amoeboid dysentery
Giardia lamblia	Gasteroenteritis
Cryptosporidium parvum	Gasteroenteritis
Trypanosoma brucei	African sleeping sickness
Plasmodium spp.	Malaria
Toxoplasma sp.	Toxoplasmosis
Eimeria	Coccidiosis
Trichomonas vaginalis	Vaginitis

2.10 Viruses

Viruses are infectious particles which lack a cellular structure. Since viruses do not possess the mechanisms needed to produce energy and the ribosomes required to synthesise proteins, they are incapable of independent metabolism, replication or movement. As a result, viruses are completely dependent on the host cells, which they effectively hijack, to produce new virus particles.

Outside the host cell, viruses exist as tiny particles called virions, but when they invade host cells their components are separated and become interspersed within the host cytoplasm. The infecting virus remains devoid of a defined structure until the point when new virus components are constructed and assembled by the host cell. These new viruses can then be released by the host cell and will serve to infect other host cells.

Virions contain a nucleic acid genome and some proteins which may have structural roles or enzymic activity (which can be crucial to the viral life cycle – e.g. reverse transcriptase in retroviruses). The viral genome may be composed of DNA or RNA, which may be either single- or double-stranded; the former may be either positively or negatively stranded. Some viruses may use both DNA and RNA at different stages of their life cycle, but the virion will only contain one of these at a given point in time.

Viral nucleic acid is contained within a protein coat called a capsid; these structures vary greatly in shape and size but are always very small – generally below the resolution of light microscopy (ca. 20–300 nm), although giant mimiviruses exist and are ~750 nm in diameter. In addition to the capsid, some virions are enveloped, surrounded by a lipid bilayer membrane containing proteins or glycoproteins. Not surprisingly given their small size and very limited capabilities, the genomes of viruses are very small ranging from 3.5 to 150×10^3 base pairs, although some are a little smaller and the largest genome is about 1.2×10^6 base pairs (mimivirus). Viral genomes are sufficient enough to code for necessary proteins to instigate viral replication or persistence in a host cell.

Classification of viruses is principally according to the type of host organism (animal, plant, bacterium, etc.) they infect, the type of nucleic acid in their genome (RNA/DNA, single or double strand, retrovirus) and whether they are enveloped or not.

By following the old adage "big fleas have little fleas upon their backs to bite them, little fleas have lesser fleas and so ad infinitum", viruses are known to parasitize all forms of life including animals, plants, fungi, protozoa and bacteria (such viruses are known as bacteriophages) and even other viruses. In the case of the latter, these are called virophages and an example of this is the Sputnik virus that exploits the replication processes of a mimivirus.

As parasites, viruses damage the host cells generally causing diseases, although some are well tolerated by their normal hosts within which they coexist, causing little apparent damage. Some such viruses can, on occasion, spread to new hosts which have not evolved tolerance often with disastrous results; this ability to "jump the species barrier" can give viruses zoonotic potential.

Although generally adverse in their effects, viruses can have a positive role in causing genetic variation and this has been exploited through the use of viruses as biological control agents for insect pests. Similarly, bacteriophage use in a limited way has been proposed, especially in the former USSR, to treat human bacterial infections, although to date their value is currently limited.

2.11 Microbial Nutrition and "Lifestyle"

For survival microbes require sources of energy, carbon and several other elements including nitrogen, oxygen, phosphorus, sulphur, potassium, sodium, calcium, magnesium and iron. Trace elements are also needed but in relatively small amounts. All these elements are required for the maintenance of life processes and the synthesis of new biomass. The type of energy source utilised varies with phototrophs using the energy of light, and chemotrophs the energy generated from the oxidation of reduced compounds or elements. The types of energy sources and nutrition used are described by a range of terms defined in Table 2.

Table 2 Types of energy source and nutrition used

Autotroph	Obtains all of its carbon from carbon dioxide
Heterotroph	Obtaining its carbon from organic compounds
Phototroph	Able to use the energy of light via photosynthesis
Chemotroph	Obtains energy from the oxidation of reduced organic compounds or elements
Lithotroph	Obtains energy from oxidation of inorganic compounds or elements
Chemoheterotroph	Obtains both energy and carbon from the organic compounds
Photoautotroph	Obtains energy from light and carbon from CO_2
Photoheterotroph	Obtains energy from light and carbon from organic compounds
Chemolithoautotroph	Obtains energy from oxidation of inorganic compounds (e.g. hydrogen, ammonia or sulphide) and carbon from CO_2
Mixotroph	Uses inorganic energy sources but organic carbon sources
Parasite	Obtain some of their nutrients from a host organism in a relationship which damages the host. Some organisms are obligate parasites while others may be capable of independent life

Many microorganisms employ only one type of nutrition, but some are able to use different modes of metabolism according to the environment they find themselves in and the relative availability of sources of carbon and energy. So, for example, a cyanobacterium may be a photoautotroph when light is available or chemoheterotrophic in the dark and when suitable organic chemicals are present.

Between them, microorganisms cover all the different nutritional types and lifestyles available in the biosphere. Microorganisms vary from the extreme autotrophs capable of growth on inorganic chemicals alone, to parasites like viruses, which obtain all their requirements from their host. Some heterotrophic microorganisms only obtain their full nutrient and/or environmental requirements by living within another organism. These organisms are either parasites or obligate symbionts. In the case of symbionts, both the host and the microbe derive mutual benefit, and with parasites the host organism is damaged or even killed by the relationship. Some parasitic bacteria e.g. rickettsias or chlamydias are obligate parasites and cannot be grown outside their hosts, but still have certain, if incomplete, metabolic capabilities. Others such as some mycobacteria and mycoplasmas may be parasitic but can be cultured on artificial media if suitable nutrients and conditions are provided. These microorganisms are described as facultative parasites.

The majority of bacteria are chemoheterotrophs, although there are also many phototrophs and chemolithotrophs too. Chemoheterotrophs require carbon sources for both energy generation and synthesis of biomass. In some cases, the same compounds are used for energy generation and biosynthesis, but this is not always the case and organic substrates which serve as precursors for biosynthesis of macromolecules cannot be degraded to generate energy.

Many bacteria can use a wide range of individual organic carbon and energy sources (in excess of a hundred) and such bacteria are referred to as being nutritionally versatile. These bacteria, typified by species of *Pseudomonas*, *Rhodococcu*s, *Acinetobacter* and many *Mycobacterium* species, have very important roles in the degradation of chemical pollutants. Other bacteria may be very limited in the types of carbon sources they use and this is restricted to one or a very few energy substrates.

The nutritional groupings of microorganisms depend mainly on the carbon and energy sources used, although obviously other elements are important. Some elements are available in many forms and those utilised by particular microbes depend on the organism's ability to synthesise particular metabolites and allow cellular uptake. Some organisms can synthesise all their required nutrition from just inorganic ions, whereas others need preformed organic molecules. For example, bacteria of the genus Pseudomonas can grow in the presence of a single organic carbon source together with inorganic sources of nitrogen, phosphorus, sulphur, magnesium potassium and iron salts. From these nutrients, *Pseudomonas* can synthesise all amino acids, nucleotides, vitamins, and carbohydrates required to produce new biomass.

Other bacteria are unable to synthesise the carbon skeletons needed to generate many amino acids, vitamins, etc., and so a wide range of preformed organic molecules are required to support their growth in addition to the organism's main carbon and energy sources. These compounds are referred to as "growth factors"

and like most minerals tend to be required in small amounts compared to the main carbon source. The lactic acid bacteria (including *Lactobacillus*, *Streptococcus*, and *Leuconostoc*) are well known for their complex growth factor requirements and some members of this group have greater nutritional requirements than humans! Growth factors may be obtained by some free-living microbes from their environments; for example, lactic acid bacteria may grow in nutrient-rich materials such as milk or fermenting plant products. Alternatively these bacteria may grow as harmless commensals within the bodies of animals (for example in the mouth, gasterointestinal tract or vagina). However, some microbes may infect plants or animals and so obtain their nutritional requirements by parasitizing their host organism.

Organisms requiring many growth factors typically lack the genes coding the required enzymes for their synthesis and generally these organisms have small genomes. There is a metabolic cost to organisms in making growth factors themselves (in terms of the energy required for synthesis and the carriage of a large genetic component) so if their typical environment can provide these growth factors it may be advantageous to use preformed molecules rather than to make their own. It is thought that this is because they have lost metabolic capabilities during their evolution.

Most microorganisms (including all archaea, bacteria, algae, fungi and some protozoa) are osmotrophs, which take in their nutrients by diffusion across the cytoplasmic membrane. Some (though not all) protozoans are, however, phagotrophs which engulf particles of food into their cells by phagocytosis. The food particles may include live prey (bacteria and other microbes) as well as decaying organic matter. Many ciliate and amoeboid protozoa capture live prey and can be regarded predatory; there are even a few species of bacteria which predate other bacteria (e.g. *Bdellovibrio* sp.).

2.12 Microbial Growth

Many microorganisms can be grown in the laboratory in artificial growth media. Such media may be simple or complicated to prepare, depending on the nutritional requirements of the organism. We generally refer to growth media as either being defined or complex. Defined media contain only specific pure chemical components in appropriate quantities, although they may contain many components if a lot of growth factors and trace element are needed. Complex media may contain some defined pure chemicals, but always contain some components of indeterminate composition which provide most of the growth factors and trace elements needed. Examples of such indeterminate components include meat extracts, protein hydrolysates and yeast extracts. Complex media are very simple to prepare and may contain few components, but their inexact composition makes it difficult to standardise microbial growth and so variable results may be obtained using different sources of the material.

Many microorganisms are currently non-culturable in the laboratory. One of the best known examples of this is *Treponema pallidum*, the causative organism of syphilis. There are, however, many other examples of non-culturable microbes from a wide range of environments. The reasons for an inability to culture microorganisms include the absence of a required nutrient from the medium or the provision of incorrect environmental conditions (e.g. concentration of dissolved oxygen, carbon dioxide or hydrogen, as well as temperature and pressures levels). In addition, some microbes are labile and easily damaged during transfer from their natural habitat into the laboratory. Although the existence of non-culturable bacteria has been known for many years, a large number of new non-culturable microbes are discovered every year as the results of exploration of new and often extreme environments (Hegarty et al. 2001). It is now thought that there are many more microbes which we are unable to culture in the laboratory than ones we can. For some of these microbes, the growth conditions may well eventually be defined to effect their in vitro growth.

Until recently, these non-cultivable organisms were largely studied by observation, but with the development of modern molecular biological methods, including PCR, genome sequencing and transcriptomics, it is now possible to learn a lot about the biochemistry and physiology of such organisms.

Viruses do not grow on their own in artificial media in the laboratory, but some can be propagated using tissue culture where cells of suitable host organisms (mammals, insect, plants, etc.) are grown in artificial media under well-defined environmental conditions. Similarly, bacteriophages can be grown in bacterial cell cultures. It has also proved possible to culture a number of obligately parasitic bacteria (like *Chlamydia trachomatis*) in tissue cultures of mammalian cells.

2.13 Oxygen

Microbes vary in their response to oxygen. Strict or obligate aerobes cannot grow in the absence of oxygen, which is required for energy generation via respiration. Anaerobes grow without oxygen, usually employing fermentation processes. Some microbes, however, are inhibited or even killed by exposure to oxygen (due to the inability to cope with toxic oxygen radicals) and are therefore strict/obligate anaerobes, an example being *Clostridium tetani*, the cause of tetanus. Other microbes can grow either with or without oxygen and these are called facultative anaerobes. Generally, facultative anaerobes grow better with oxygen, as respiration is more efficient for energy generation than fermentation and this is the case with *Escherichia coli*. Lactic acid bacteria, however, cannot respire aerobically and so growth is no better and sometimes a little worse in the presence of oxygen; these are referred to as aerotolerant anaerobes. Another group, the microaerophiles, require oxygen but at low concentrations (often 2–10% saturation) and *Campylobacter* species are typical examples. For some organisms which normally respire aerobically, nitrate can substitute for oxygen as an electron acceptor allowing anaerobic growth.