



BASIC COURSE

IN

WATER MICROBIOLOGICAL ANALYSIS

Prepared By:
Microbiology Laboratory
Reference Laboratory for Drinking Water
Cairo, 2012

Contents

1. Introduction.....	5
2. Cell Structure and organization:	15
3. Microscopic Techniques.....	38
4. Bacterial Staining.....	43
5. Bacterial Growth factors and Conditions	53
6. Bacterial Metabolism.....	66
7. Types of media	79
8. Control of Microorganisms.....	87
PART 2 Water Microbiology	
1. History of Water Microbiology:	100
2. Waterborne Diseases:	101
3. Bacterial Waterborne Diseases:	102
4. Water Quality Indicators.....	133
5. WASHING AND STERILIZATION	141
6. PREPARATION OF CULTURE MEDIA	142
7. SAMPLES.....	143

- **Goal:**

- Improving level of personnel working in drinking water analysis fields that advances the HCWW commitment to protect human health and improve water quality in Egypt by providing environmental data that are credible, scientifically sound, and of sufficient and adequate quality.

- **Objective:**

- Improving level of analysts working in water microbiological analysis to provide credible, scientifically sound, and of sufficient and adequate quality data.

- **Contributed laboratory:**

- Microbiology Analysis Laboratory- Reference laboratory for Drinking Water.

- **Trainers:**

- Microbiologist: Ahmad K. Abd-Elhady
- Microbiologist: Mahmoud G. Hussein

- **Brief:**

- This course is the first part of water microbiology analysis series, Basic course is mainly targeting personnel those had freshly graduated and/or newly employed.
- As in objective, improving scientific knowledge for personnel working in microbiological analysis is the first step to know how? And understand mechanisms of bacterial growth, metabolism, nutrition..etc which help him/her to know what beyond analysis and give him/her the ability to interpret any strange situation may accidently occur during the analysis.

- **Resources Required:**

- Meeting room sufficient to train 20 persons.
- Microbiology Lab full equipped with traditional equipment and tools.

First Part

Science of Microbiology

1. Introduction

1.1. General

- Microbiology is the science of the invisible world and its effect on other forms of life.
- It is the science that deals with the study of all kinds of microorganisms: bacteria, viruses, yeasts, molds, fungi, protozoa, and algae.
- The term: microorganism refers to any of the microscopic forms of life found in nature.
- Bacteria are found everywhere; in soil, in the air, in every kind of organism, from humans to plants, living or dead.
- Single cell, free-living bacteria, is one of the simplest life forms, existing long before human life began.
- Without bacteria, our world, as we know it, could not exist for bacteria perform many varied functions.
- Decomposition of matter, for example, is a basic bacterial activity, returning to nature materials necessary to the revitalization of the earth.

1.2. Tree of life

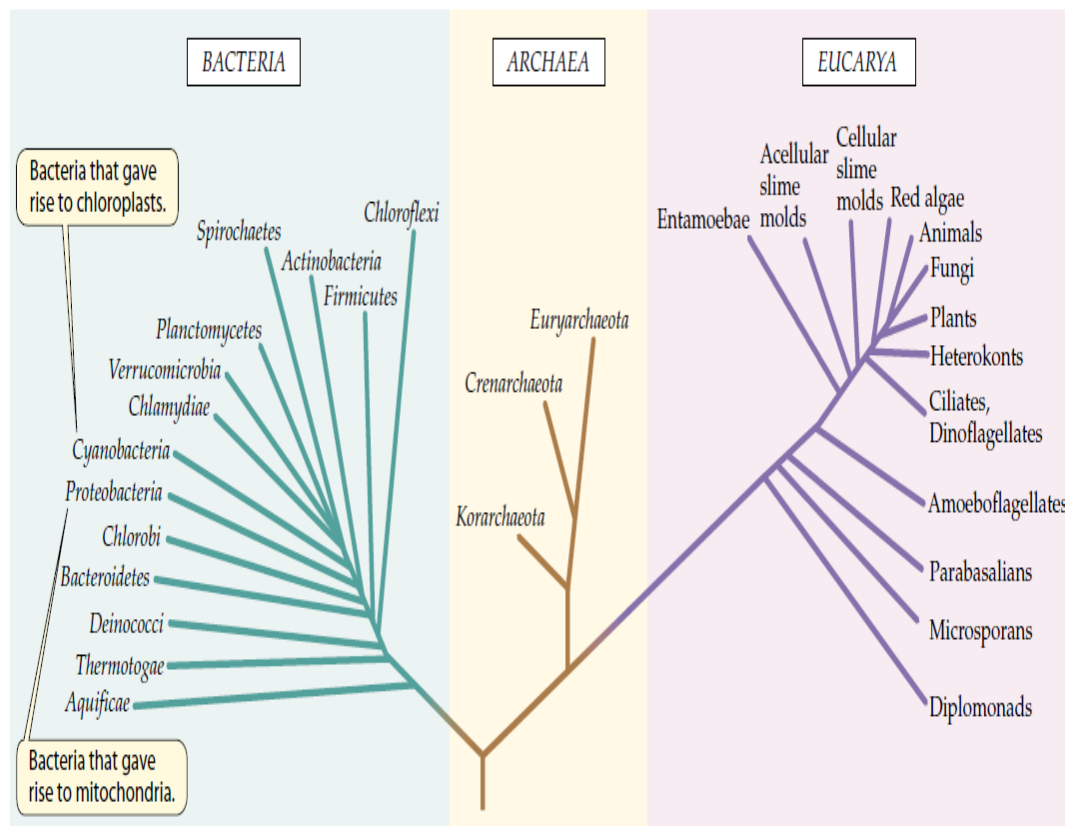


Figure 1.6 Tree of Life

This diagram shows the evolutionary tree of various groups of organisms based on 16S and 18S rRNA sequence analysis. The two prokaryotic domains are the *Bacteria* and *Archaea*. All eukaryotic microorganisms are placed in a separate domain, the *Eucarya*, along with the plant and animal "king-

doms." The *Eucarya* contains many "kingdoms" of microorganisms, including the fungi and various protists. The *Bacteria* and *Archaea* also contain many "kingdoms," which in this book we call phyla. The *Bacteria* contains at least 30 phyla, many of which have never been studied in the laboratory.

1.3. Prokaryotic Versus Eukaryotic Microorganisms:

- When examined under the light microscope, bacteria appear different from eukaryotic microorganism.
- Bacterial cells are usually very small and have no apparent nucleus. In contrast, cells of algae, protozoa, fungi, plants, and animals are typically much larger and have a distinct nucleus.
- Bacteria have a much simpler cell structure and are referred to as **Prokaryotic** (from the Greek meaning “before nucleus”) organisms. In contrast, algae, fungi, and protozoa are called **Eukaryotic** (“good nucleus” or “true nucleus”).

1

Table 1.2 Characteristics of Prokaryotic (Eubacteria) and Eukaryotic (Fungi, Protozoans) Microorganisms

Characteristic	Prokaryotes (bacteria)	Eukaryotes (fungi, protozoans)
Nuclear structure	Circular DNA molecule not covered with proteins	Complex of DNA and basic proteins
Localization of nuclear structure	Dense tangle of DNA in cytoplasm; no nuclear membrane; nucleoid or nuclear equivalent	In nucleus surrounded by nuclear membrane
DNA	Nucleoid and plasmids	In nucleus and in mitochondria
Cytoplasm	No mitochondria and no endoplasmic reticulum, 70S ribosomes	Mitochondria and endoplasmic reticulum, 80S ribosomes
Cell wall	Usually rigid wall with murein layer; exception: mycoplasmas	Present only in fungi: glucans, mannans, chitin, chitosan, cellulose
Reproduction	Asexual, by binary transverse fission	In most cases sexual, possibly asexual

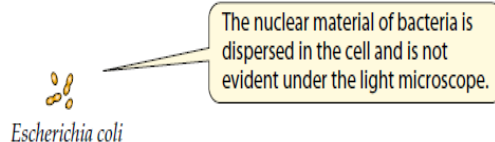
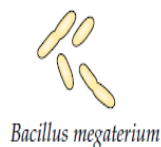
Table 1.2 Major differentiating characteristics of the three domains of life

	<i>Bacteria</i>	<i>Archaea</i>	<i>Eucarya</i>
Nuclear membrane	No	No	Yes
Plastids	No	No	Yes
Peptidoglycan cell walls	Yes ^a	No	No
Membrane lipids	Ester-linked	Ether-linked	Ester-linked
Ribosome size	70S	70S	80S

Table 1.3 Major differentiating characteristics of prokaryotes and eukaryotes

Characteristic	Prokaryote	Eukaryote
<i>Nuclear structure and function</i>		
Nucleus with membrane	No	Yes
Chromosomes	One	Two or more
Mitosis	No	Yes
Sexual reproduction	Rare; only part of genome involved	Common; all chromosomes involved
Meiosis	No	Yes
<i>Cytoplasmic structures</i>		
Mitochondria	No	Yes ^a
Chloroplasts	No	Yes (if photosynthetic)
Ribosomes	70S	80S ^b
Typical cell volume	<5 μm^3	>5 μm^3

(A) Prokaryotes



(B) Eukaryotes

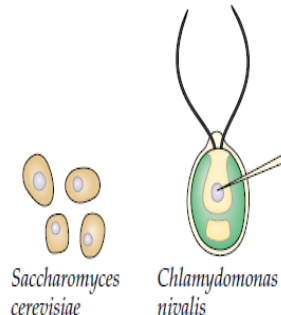
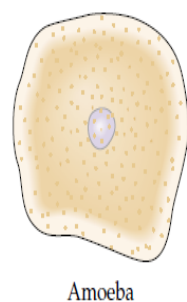


Figure 1.7 Drawings of representative microorganisms, as they appear by light microscopy

The two examples of bacteria are a large rod, *Bacillus megaterium*, and a small rod, *Escherichia coli*. The eukaryotic organisms are an amoeba (a protozoan), a yeast (*Saccharomyces cerevisiae*), and an alga (*Chlamydomonas nivalis*). Note the cup-shaped chloroplast and the two flagella of *C. nivalis*.

Bacteria

- **Classic bacteria.** These organisms reproduce asexually by binary transverse fission. They do not possess the nucleus typical of eucarya. The cell walls of these organisms are rigid (with some exceptions, e.g., the mycoplasma).
- **Chlamydiae.** These organisms are obligate intracellular parasites that are able to reproduce in certain human cells only and are found in two stages: the infectious, nonreproductive particles called elementary bodies (0.3 μm) and the noninfectious, intracytoplasmic, reproductive forms known as initial (or reticulate) bodies (1 μm).
- **Rickettsiae.** These organisms are obligate intracellular parasites, rod-shaped to coccoid, that reproduce by binary transverse fission. The diameter of the individual cell is from 0.3–1 μm .
- **Mycoplasmas.** Mycoplasmas are bacteria without rigid cell walls. They are found in a wide variety of forms, the most common being the coccoid cell (0.3–0.8 μm). Threadlike forms also occur in various lengths.

Fungi and Protozoa

- **Fungi.** Fungi (*Mycophyta*) are nonmotile eukaryotes with rigid cell walls and a classic cell nucleus. They contain no photosynthetic pigments and are carbon heterotrophic, that is, they utilize various organic nutrient substrates (in contrast to carbon autotrophic plants). Of more than 50 000 fungal species, only about 300 are known to be human pathogens. Most fungal infections occur as a result of weakened host immune defenses.
- **Protozoa.** Protozoa are microorganisms in various sizes and forms that may be free-living or parasitic. They possess a nucleus containing chromosomes and organelles such as mitochondria (lacking in some cases), an en-

Subcellular Infectious Entities

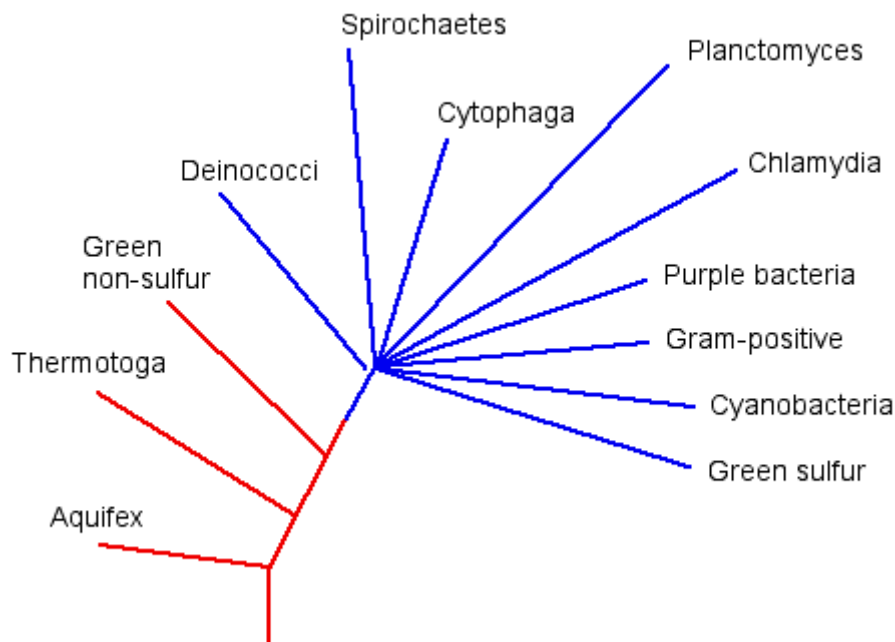
- **Prions (proteinaceous infectious particles).** The evidence indicates that prions are protein molecules that cause degenerative central nervous system (CNS) diseases such as Creutzfeldt-Jakob disease, kuru, scrapie in sheep, and bovine spongiform encephalopathy (BSE) (general term: transmissible spongiform encephalopathies [TSE]).

Viruses. Ultramicroscopic, obligate intracellular parasites that:

- contain only one type of nucleic acid, either DNA or RNA,
- possess no enzymatic energy-producing system and no protein-synthesizing apparatus, and
- force infected host cells to synthesize virus particles.

1.4. Classification of bacteria:

- **Bacteria consist of approximately 12 distinct groups** most of these groups appear to have radiated from the same point. These are called the "main radiation" groups. A few branches are deeper and earlier, and appear to represent more primitive bacterial groups.



1.4.1. Aquifex-Hydrogenobacter group.

- Thermophilic bacteria. *A. pyrophilus* is the most thermophilic bacterium known, 85-95° C. optimal growth temp.

1.4.2. Thermotoga and relatives.

- All are thermophilic, anaerobic, fermentative rods. Cells enclosed in a sheath.

1.4.3. Green non-sulfur bacteria.

- Also thermophilic. Includes Chloroflexus, Thermomicrobium, Thermoleophilum, Herpetosiphon. These organisms are filamentous and move by gliding. Chloroflexus grows as anaerobic phototrophs or aerobically by fermentation; other groups are typically heterotrophic.

Note: these 3 groups are all **thermophilic**, suggesting that the ancestors of the bacterial domain were thermophiles.

1.4.4. *Deinococcus*, *Thermus* and relatives:

- *Deinococci* are very resistant to radiation, including gamma rays, X-rays, and UV. More than 20 x as resistant as *E. coli*. Cells have very efficient DNA repair systems, multiple copies of DNA.
- Often show up in the spoilage of radiation-pasteurized food. Dose of gamma-rays used in food sterilization is very high precisely because of the need to kill *Deinococcus*. Compare with use of very high temperatures to preserve food, because of need to kill heat-resistant spores.
- *Thermus* is another thermophilic genus. *Thermus aquaticus* is the source of Taq polymerase used in PCR, an enzyme with great commercial success. View [phase micrographs of thermus bacteria](#).

1.4.5. *Spirochaetes* and relatives.

- Only 9 genera, including *Borrelia* (cause of Lyme disease) and *Treponema* (cause of syphilis). All have an axial fiber around which the cell is "wound", producing spiral shape.

1.4.6. *Cytophaga*

- Group includes genera *Cytophaga*, *Flavobacterium*, *Bacteroides*.
- All heterotrophic, rod-shaped. Common in soils and waters, not pathogenic and relatively poorly studied. *Cytophaga* move by interesting gliding motility.

1.4.7. *Planctomyces*

- Have never been cultivated, but common in pond water.
- Distant cousins of Chlamydia, also lack peptidoglycan
- Free-living aquatic oligotrophs; divide by budding, not binary fission.
- All have fimbriae & flagella and some have nuclear envelopes, like eukaryotes.

1.4.8. *Chlamydia*.

- Obligate intracellular parasites, unable to grow outside host cells because they cannot synthesize many basic biomolecules (e.g., amino acids, ATP) and require these from their host cell.
- Can exist in two states: metabolically inert elementary body (EB) and as metabolically active reticulate body (RB) found only inside host cells.
- EB is analogous to virion stage of virus, a transmissible form that can travel to different body regions, must be ingested by phagocytosis to enter a cell.

- Once ingested, organism grows and divides inside host cell in RB form. When cell is completely wasted, EB forms accumulate, cell lyses, and EBs are released for possible infection of other cells.
- Includes Chlamydia trachomatis, the most common STD.
- Chlamydia does not have peptidoglycan, but are sensitive to beta-lactam antibiotics (mechanism not understood).

1.4.9. Purple bacteria and relatives (aka Proteobacteria):

- Includes Purple photosynthetic + non-photosynthetic Gram-negative bacteria.
- Most common gram-negative heterotrophs are in this group: Escherichia, Salmonella, Pseudomonas, etc.
- Also called "Proteobacteria" because of broad range of phenotypes.
- Thousands of species, many diverse forms.
- Includes most "common" Gram-negative bacteria.
- Gram-positive (including Mycoplasmas)

1.4.10. Cyanobacteria:

- Oxygenic phototrophs carry out photosynthesis much like plants, split water and produce oxygen as waste product.
- Electron transport and pigments are located on thylakoid membranes. Membranes are lined with particles called phycobilisomes Cells vary greatly in shape.

1.4.11. Green sulfur bacteria:

- Ex: Chlorobium a few related genera. Photosynthetic, use sulfide as electron donor, not water like cyanobacteria.

1.4.12. Archaea consist of 3 distinct groups:

- Archaea means "ancient" because use ancient energy mechanisms
- Many found in harsh, early earth-like environments.
- Introduction to the Archaea
- Thermal vents at bottom of ocean.
- Extreme salt conditions (Great Salt Lake, Dead Sea).
- High acid conditions.

1.4.13. Major Archaeal groups

- Halophiles

- Example: Halobacterium, Found only in very concentrated brines, evaporating salt basins, Dead Sea, etc.
- Brightly colored due to purple pigments (Bacteriorhodopsin) Use light energy to pump protons across cell membrane, generate proton gradient; make ATP from this

1.4.14. Methanogens

- Example: Methanococcus jannaschii , the first archaeal organism to have its genome sequenced.

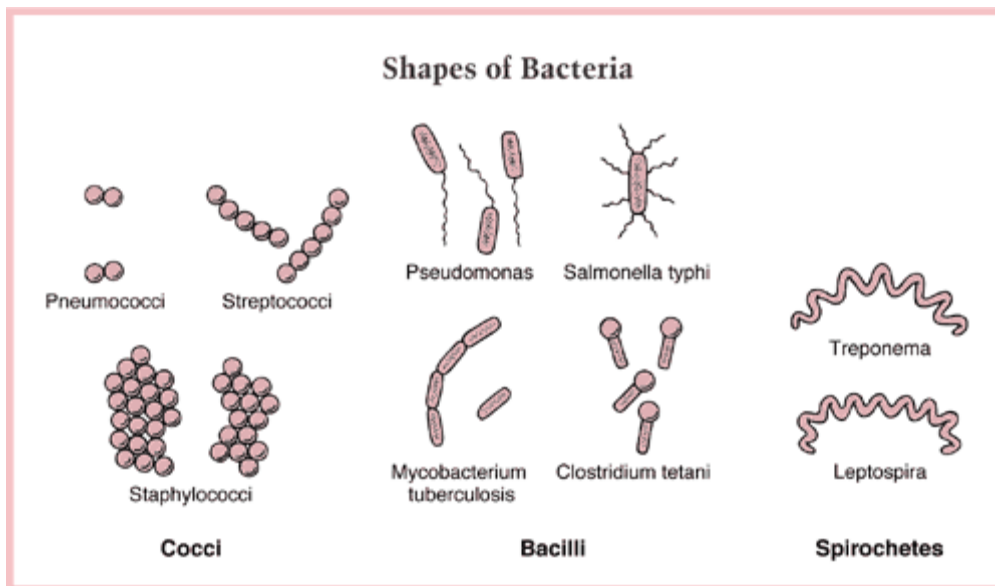
1.4.15. Extreme Thermophiles.

- Example: Pyrococcus furiosus; grows well at temperatures above boiling!

2. Cell Structure and organization:

2.1. Forms of Bacteria

- **Cocci** (kock'-si): Spherical bacteria are called cocci, which means —berries in Greek. Among the cocci group of bacteria are the **Streptococci**. **Staphylococcus aureus** is also a coccus and is a common pathogen that causes pneumonia, meningitis and food poisoning, among others.
- **Bacilli** (ba-sil'-i): The rod-shaped cylindrical bacteria are called bacilli, from the Latin word meaning —little rod. Among the diseases caused by this type of bacteria are typhoid fever, some kinds of diarrhea, cholera, dysentery and eye infections. **Pseudomonas aeruginosa** and **Salmonella choleraesuis** are examples.
- **Spirilla**, Spirochetes: Spiral, curved or corkscrew shaped bacteria are called Spirilla or Spirochetes. This type of microorganism causes trench mouth and syphilis, among others.



2.2. Morphology of Bacteria

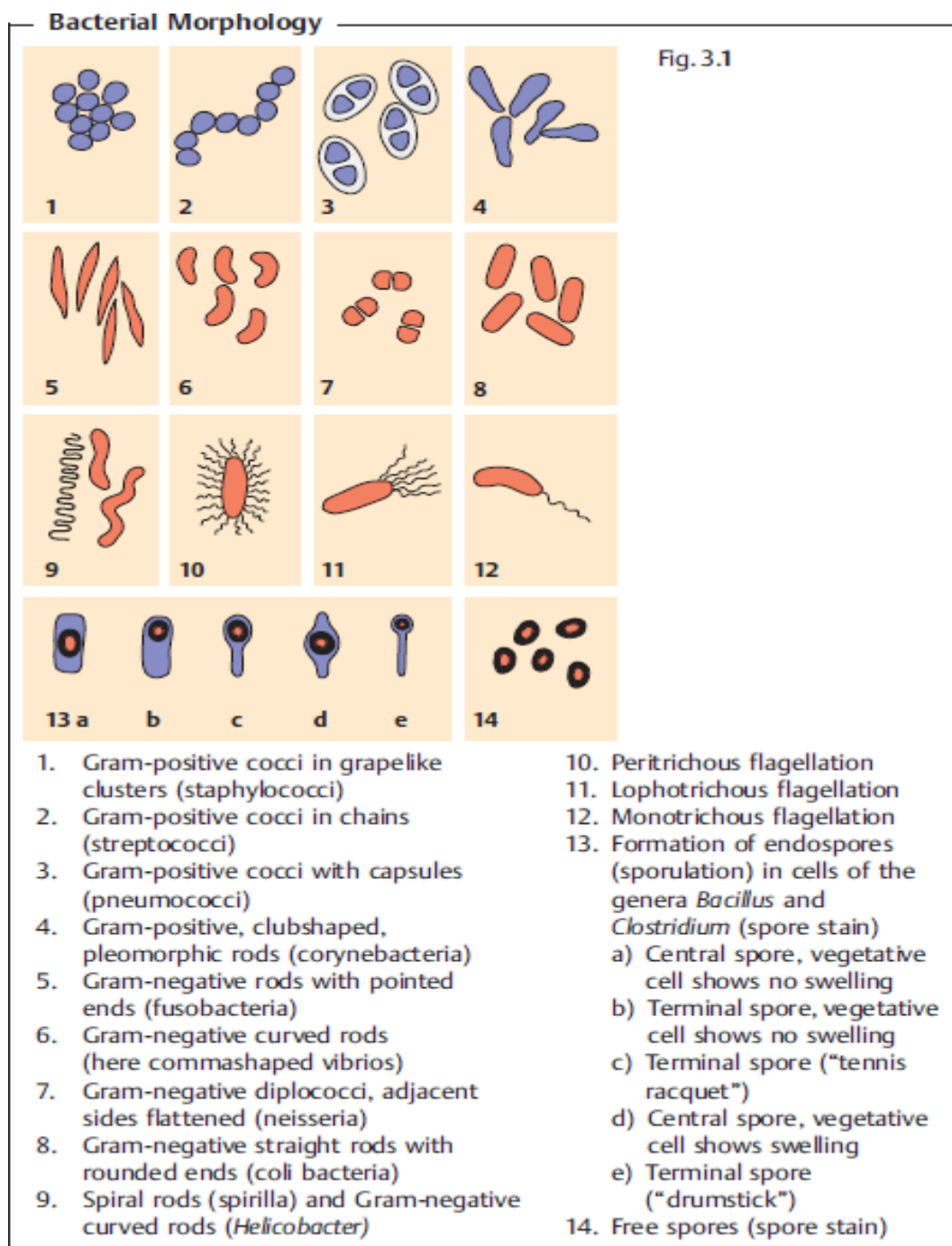


Table 3.1 Morphological Characteristics of Bacteria (see Fig. 3.1 for examples)

Bacterial form	Remarks
Cocci	Occur in clusters (Fig. 3.2), chains, pairs (diplococci), packets
Straight rods	Uniform thickness, rounded ends (Fig. 3.3), pointed ends, club form
Curved rods	Commashaped, spiral (Fig. 3.4), screwshaped
Mycoplasmas	Bacteria without a rigid cell wall; coccoid cells, long threads
Chlamydiae	Two forms: spherical/oval elementary bodies (300 nm); spherical/oval reticulate bodies (1000 nm)
Rickettsiae	Short coccoid rods (0.3–1 μm)

■ **Simple staining.** In this technique, a single staining substance, e.g., methylene blue, is used.

■ **Differential staining.** Two stains with differing affinities to different bacteria are used in differential staining techniques, the most important of which is gram staining. Gram-positive bacteria stain blue-violet, Gram-negative bacteria stain red (see p. 211 for method).

Three basic forms are observed in bacteria: spherical, straight rods, and curved rods (see Figs. 3.1–3.4).

The Morphology and Fine Structure of Bacteria 149

Cocci



Fig.3.2 Cocci are spherical bacteria. Those found in grapelike clusters as in this picture are staphylococci (Scanning electron microscopy (SEM)).

Rod Bacteria

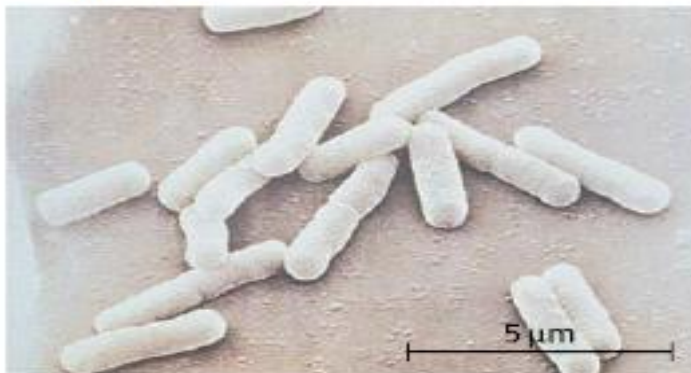


Fig.3.3 The straight rod bacteria with rounded ends shown here are coli bacteria (SEM).

Spirilla

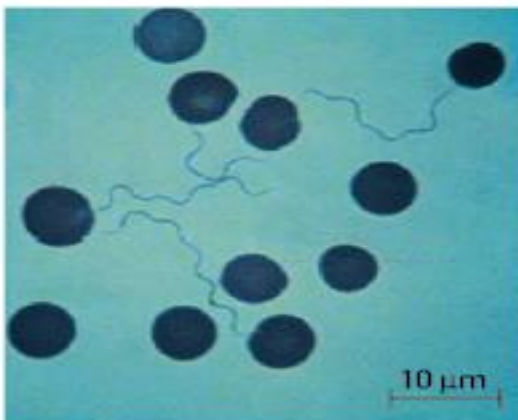
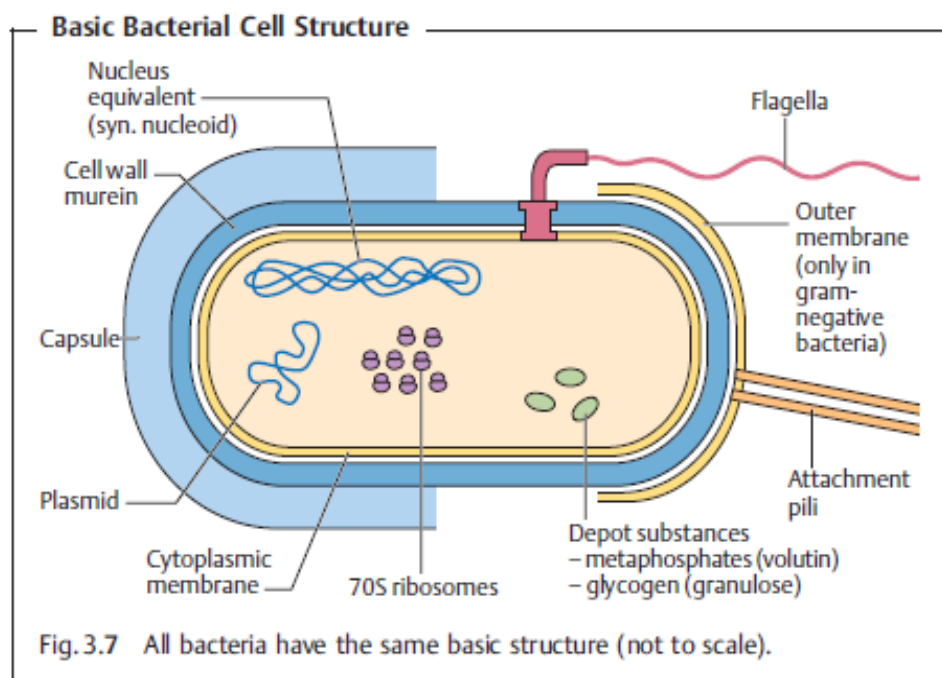


Fig.3.4 Spirilla, in this case borrelia are spiral bacteria (light microscopy (LM), Giemsa stain).

2.3. Structure of the Bacterial Cell



- Beginning from the outermost structure and moving inward, bacteria have some or all of the following structures:

capsule	This layer of polysaccharide (sometimes proteins) protects the bacterial cell and is often associated with pathogenic bacteria because it serves as a barrier against phagocytosis by white blood cells.
Outer membrane	(not shown) This lipid bilayer is found in Gram negative bacteria and is the source of lipopolysaccharide (LPS) in these bacteria. LPS is toxic and turns on the immune system of , but not in Gram positive bacteria.
cell wall	Composed of peptidoglycan (polysaccharides + protein), the cell wall maintains the overall shape of a bacterial cell. The three primary shapes in bacteria are coccus (spherical), bacillus (rod-shaped) and spirillum (spiral). Mycoplasma are bacteria that have no cell wall and therefore have no definite shape.

periplasmic space	(not shown) This cellular compartment is found only in those bacteria that have both an outer membrane and plasma membrane (e.g. Gram negative bacteria). In the space are enzymes and other proteins that help digest and move nutrients into the cell.
plasma membrane	This is a lipid bilayer much like the cytoplasmic (plasma) membrane of other cells. There are numerous proteins moving within or upon this layer that are primarily responsible for transport of ions, nutrients and waste across the membrane.
Pili	This hollow, hair like structures made of protein allow bacteria to attach to other cells. A specialized pilus, the sex pilus, allows the transfer of plasmid DNA from one bacterial cell to another. Pili (sing., pilus) are also called fimbriae (sing., fimbria). Predominant chemical composition is protein.
Flagella	The purpose of flagella (sing., flagellum) is motility. Flagella are long appendages which rotate by means of a "motor" located just under the cytoplasmic membrane. Bacteria may have one, a few, or many flagella in different positions on the cell. Predominant chemical composition is protein.
Ribosomes	Sites of translation (protein synthesis) Predominant chemical composition is protein and RNA
Chromosome	Genetic material of cell chemical composition is DNA
Plasmid	Extrachromosomal element. Predominant chemical composition is DNA.

Capsules

- Bacterial capsules are organized accumulations of gelatinous materials on cell walls, in contrast to slime layers (a water secretion that adheres loosely to the cell wall and commonly diffuses into the cell), which are unorganized accumulations of similar material.
- The capsule is usually thick enough to be seen under the ordinary light microscope (macrocapsule), while thinner capsules (microcapsules) can be detected only by electron microscopy.
- Capsules confer several advantages when bacteria grow in their normal habitat. These include helping to:
 - Prevent desiccation
 - Resist phagocytosis by host phagocytic cells
 - Prevent infection by bacteriophages
 - Aid bacterial attachment to tissue surfaces in plant and animal hosts or to surfaces of solids objects in aquatic environments
- Capsule formation often correlates with pathogenicity.

Flagella

- Many bacteria are motile, and this ability to move independently is usually attributed to a special structure, the flagella (singular: flagellum).
- Depending on species, a cell may have:
 - a single flagellum (monotrichous bacteria; trichous means hair);
 - one flagellum at each end (amphitrichous bacteria; amphi means on both sides);
 - a tuft of flagella at one or both ends (lophotrichous bacteria; lopho means tuft);
 - or flagella that arise all over the cell surface (peritrichous bacteria; peri means around).
- A flagellum is a threadlike appendage extending outward from the plasma membrane and cell wall.
- Flagella are slender, rigid, locomotor structures, and measure about 20 μm across and up to 15 to 20 μm long.

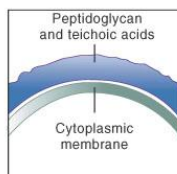
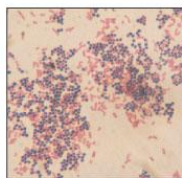
- Flagellation patterns are very useful in identifying bacteria and can be seen by light microscopy, but only after being stained with special techniques designed to increase their thickness.
- The detailed structure of flagella can be seen only in the electron microscope.
- Bacterial cells benefit from flagella in several ways.
 - They can increase the concentration of nutrients
 - Or decrease the concentration of toxic materials near the bacterial surfaces by causing a change in the flow rate of fluids.
 - They can also disperse flagellated organisms to areas where colony formation can take place.
 - The main benefit of flagella to organisms is their increased ability to flee from areas that might be harmful.

Cell Wall

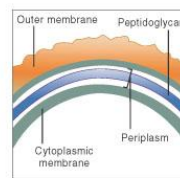
- The main structural component of most prokaryotes is the rigid cell wall.
- Functions of the cell wall include:
 - Providing protection for the delicate protoplast from osmotic lysis (bursting)
 - Determining a cell's shape
 - Acting as a permeability layer that excludes large molecules and various antibiotics and playing an active role in regulating the cell's intake of ions
 - Providing a solid support for flagella
- Cell walls of different species may differ greatly in structure, thickness, and composition.
- The cell wall accounts for about 20 to 40% of a bacterium's dry weight.
- The tasks of the complex bacterial cell wall are to protect the protoplasts from external noxae, to withstand and maintain the osmotic pressure gradient between the cell interior and the extracellular environment (with internal pressures as high as 500–2000 kPa), to give the cell its outer form and to facilitate communication with its surrounding.

Copyright © The McGraw-Hill Companies, Inc. Permission required for reproduction or display.

Table 3.5 Comparison of Features of Gram-Positive and Gram-Negative Bacteria



Gram-Positive



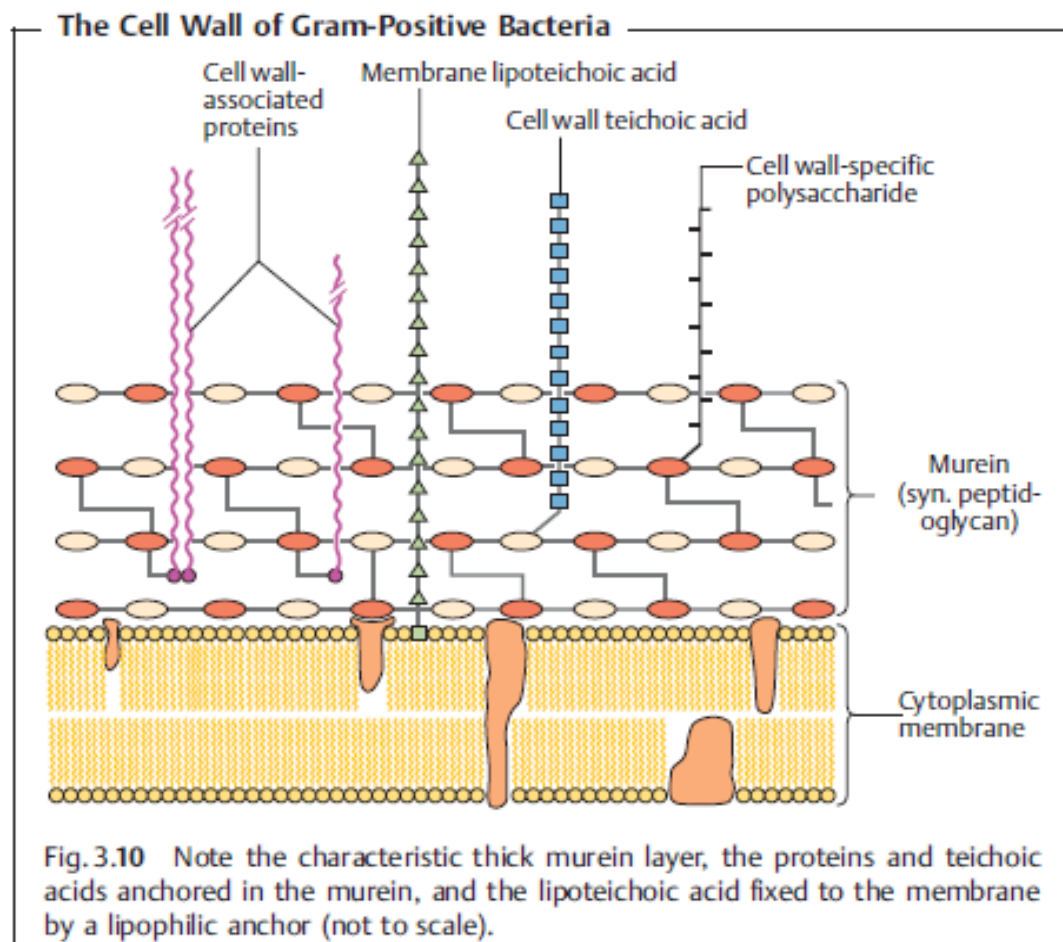
Gram-Negative

Color of Gram-Stained Cell	Purple	Reddish-pink
Representative Genera	<i>Bacillus</i> , <i>Staphylococcus</i> , <i>Streptococcus</i>	<i>Escherichia</i> , <i>Neisseria</i> , <i>Pseudomonas</i>
Distinguishing Structures/Components		
Peptidoglycan	Thick layer	Thin layer
Teichoic acids	Present	Absent
Periplasm	Absent	Present
Outer membrane	Absent	Present
Endotoxin (lipopolysaccharide)	Absent	Present
Porin proteins	Absent (unnecessary because there is no outer membrane)	Present; allow passage of molecules through outer membrane
General Characteristics		
Sensitivity to penicillin	Generally more susceptible (with notable exceptions)	Generally less susceptible (with notable exceptions)
Sensitivity to lysozyme	Yes	No (unless also treated with EDTA)
Form that results from removal of peptidoglycan	Protoplast	Spheroplast

The cell wall of Gram-positive bacteria

- The murein sacculus may consist of as many as 40 layers (15–80 nm thick) .
- Murein (syn. peptidoglycan). The most important structural element of
- the wall is murein 30 % of cell wall mass, a netlike polymer material surrounding the entire cell (sacculus). It is made up of polysaccharide chains crosslinked by peptides. and teichoic acids anchored in the murein, and the lipoteichoic acid fixed to the membrane by a lipophilic anchor).
- Other constituents of gram-positive cell walls include teichoic acids and teichuronic acids. are polyol phosphate polymers, such as polyglycerol phosphate
- **Teichoic acids** are polyribitol phosphate or polyglycerol phosphate.
- Sugars (such as glucose and galactose), amino sugars (such as glucosamine), and the amino acid D-alanine are found in some of these compounds.
- Teichoic acids are attached covalently to the 6-hydroxyl group of muramic acid in the peptidoglycan.

- **Teichuronic acids** are polymers of two or more repeating subunits, one of which is always a uronic acid, such as glucuronic acid, or the uronic acid of an amino sugar, such as aminoglucuronic acid (Figure 4.47). The subunits are linked covalently to peptidoglycan, but the linkage group is unknown. The specific function of these acids is unknown.



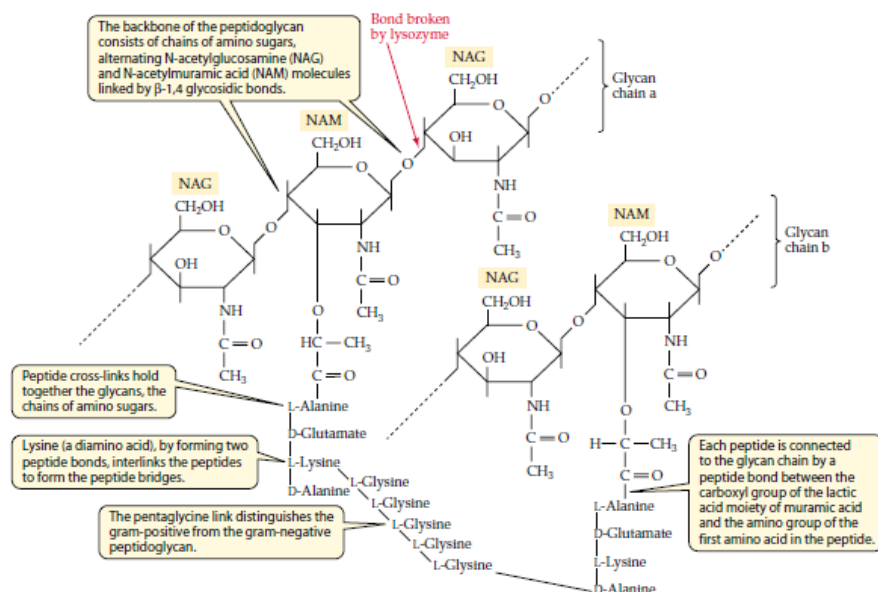


Figure 4.42 Peptidoglycan of a gram-positive bacterium
Chemical structure of the peptidoglycan layer of *Staphylococcus aureus*. Note that some amino acids in the peptide cross-links, alanine and glutamic acid, are in the D-stereoconfiguration. See also Figure 4.44.

The Structure of Murein

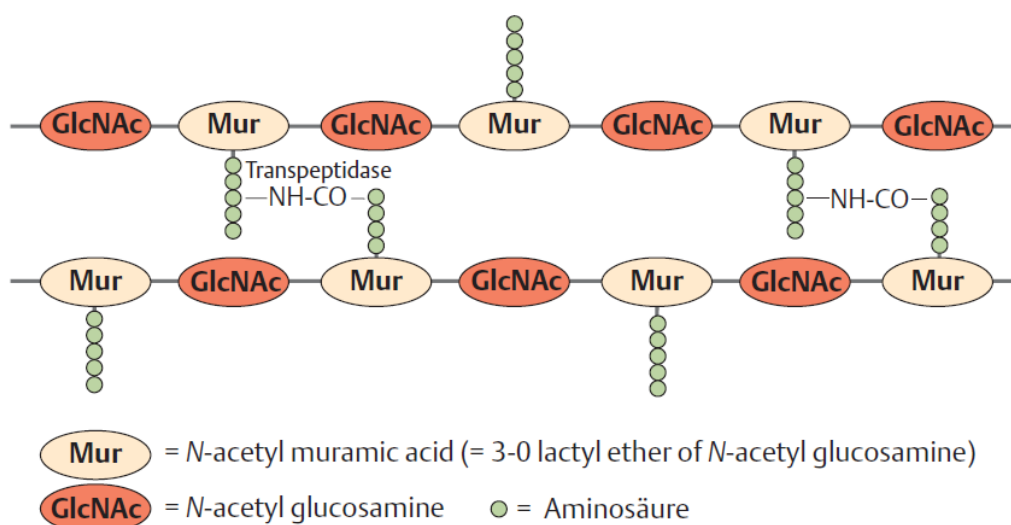


Fig. 3.9 Soluble murein fragments of Gram-negative and Gram-positive bacteria can stimulate excessive cytokine secretion in macrophages by binding to toll-like receptors and CD14. Cytokines cause the clinical symptoms of sepsis or septic shock syndrome (see under Lipid A, p. 156).

The Murein Building Block

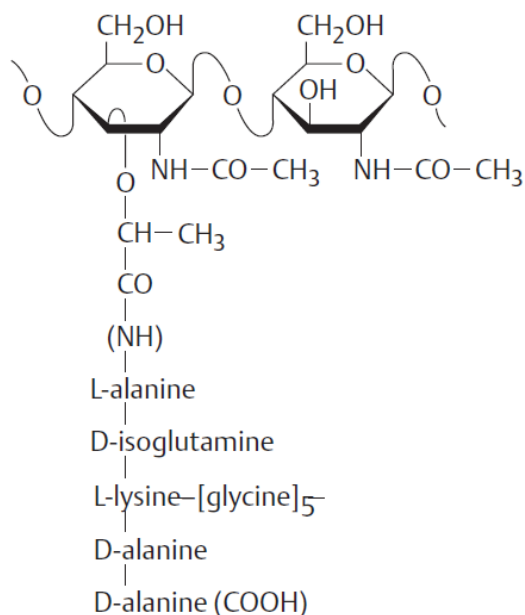
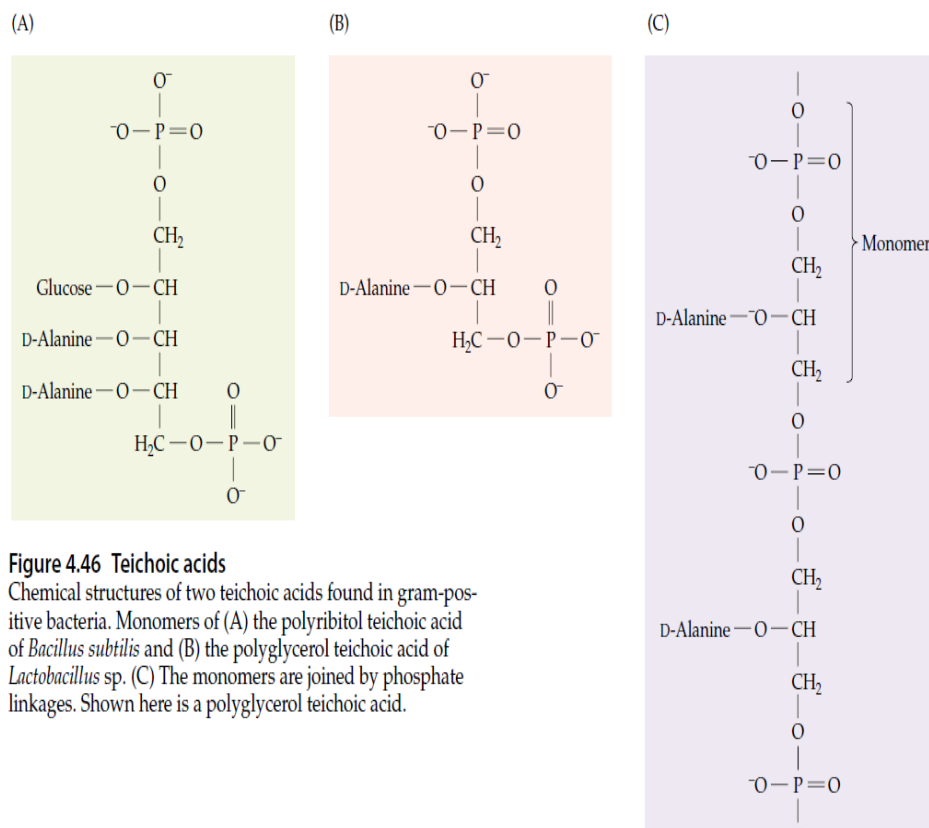


Fig. 3.8 The murein (syn. peptidoglycan) of the cell wall is composed of a series of identical subunits. The terminal D-alanine is split off each time a new crosslink is synthesized. Only in staphylococci is a pentaglycine interpeptide bridge inserted between adjacent peptides.

•



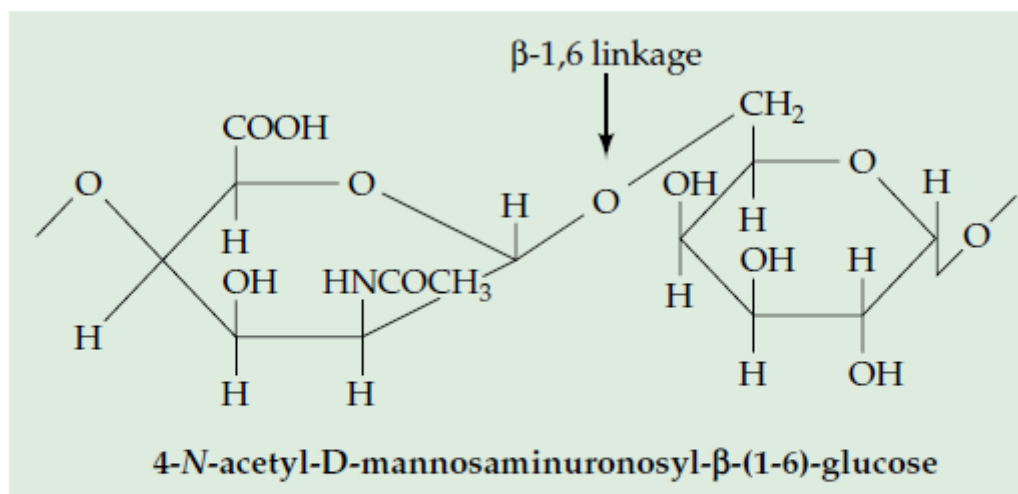


Figure 4.47 Teichuronic acids

A teichuronic acid found in the cell wall of *Micrococcus luteus*.

Cell wall of Gram Negative Bacteria.

- In fact the cell wall of gram negative bacteria is mcplexed multilayered its consites of the following :
- outer membrane layer consists of LPS lipopolysaccharides,Omp outer membrane protines and phospholipid- murein and periplasmic space
- Here, the murein is only about 2 nm thick and contributes up to 10% of the dry cell wall mass (Fig. 3.11).
- The outermembrane is the salient structural element. It contains numerous proteins(50% by mass) as well as the medically critical lipopolysaccharide.

— The Cell Wall of Gram-Negative Bacteria —

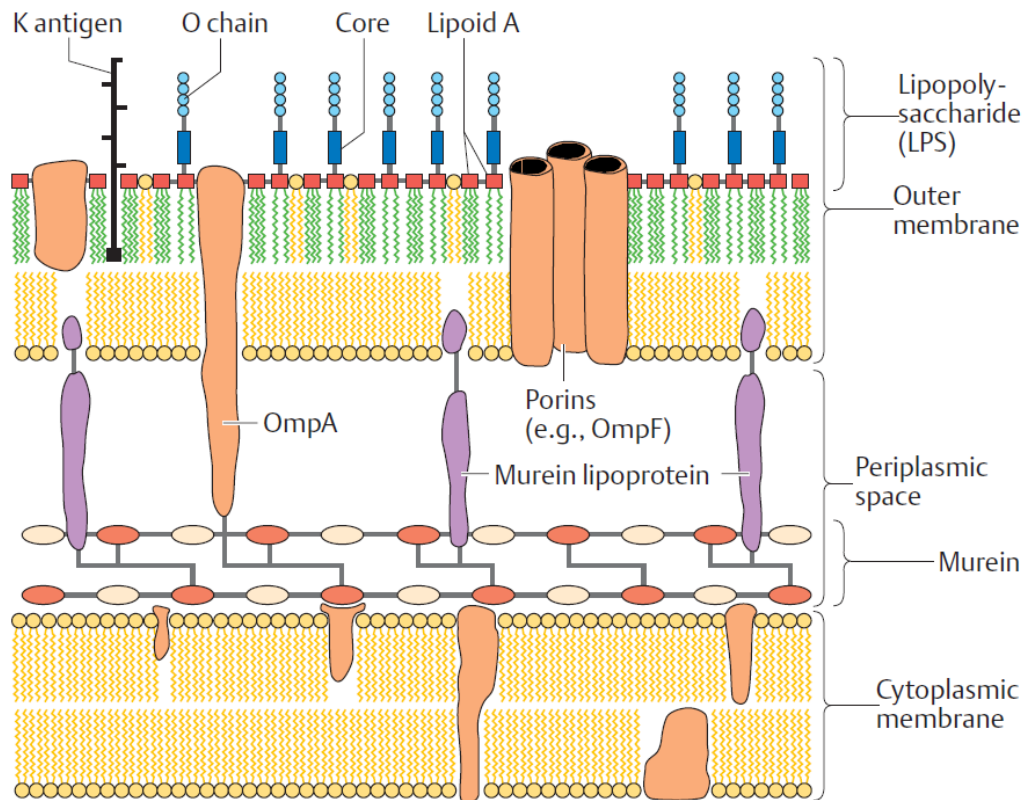


Fig. 3.11 Note the characteristic thin murein layer and the outer membrane connected to it by proteins (OmpA, murein lipoprotein). Many different proteins are localized in the outer membrane. Its outer layer is made up of closely packed lipopolysaccharide complexes (see Fig. 3.12).

Outer membrane proteins:

- OmpA (outer membrane protein A) and the murein lipoprotein form a bond between the outer membrane and murein.
- Porins, proteins that form pores in the outer membrane, allow passage of hydrophilic, low-molecular-weight substances into the periplasmic space.
- Outer membrane-associated proteins constitute specific structures that enable bacteria to attach to host cell receptors.
- A number of Omps are transport proteins.
- Lipopolysaccharide (LPS). This molecular complex, also known as endotoxin, is comprised of the lipid A, the core polysaccharide, and the O-specific polysaccharide chain.

The Lipopolysaccharide Complex

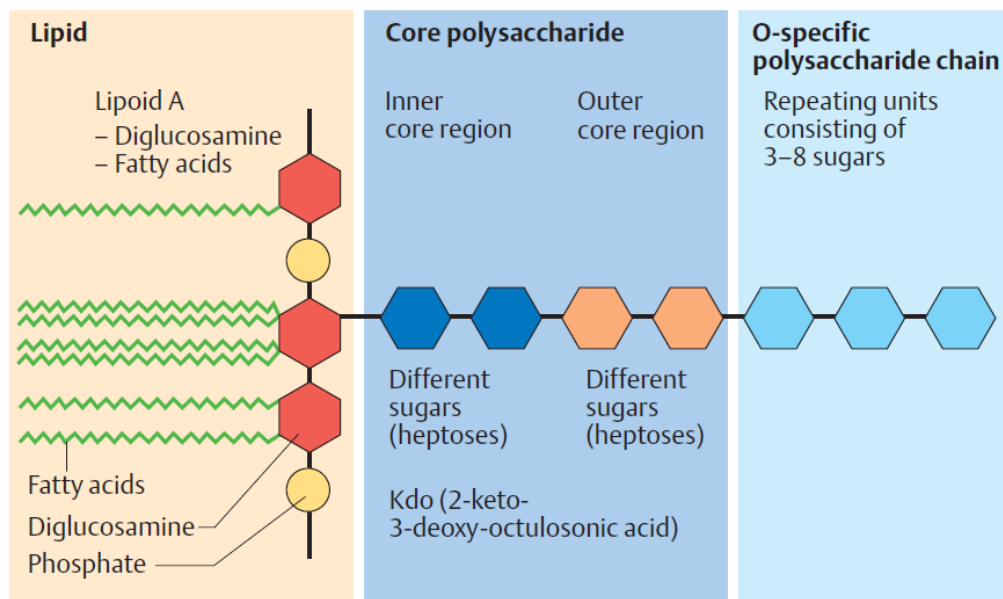


Fig. 3.12 The three-part lipopolysaccharide complex (LPS) of Gram-negative bacteria is anchored in the outer membrane by means of its lipid moiety. LPS is also known as endotoxin.

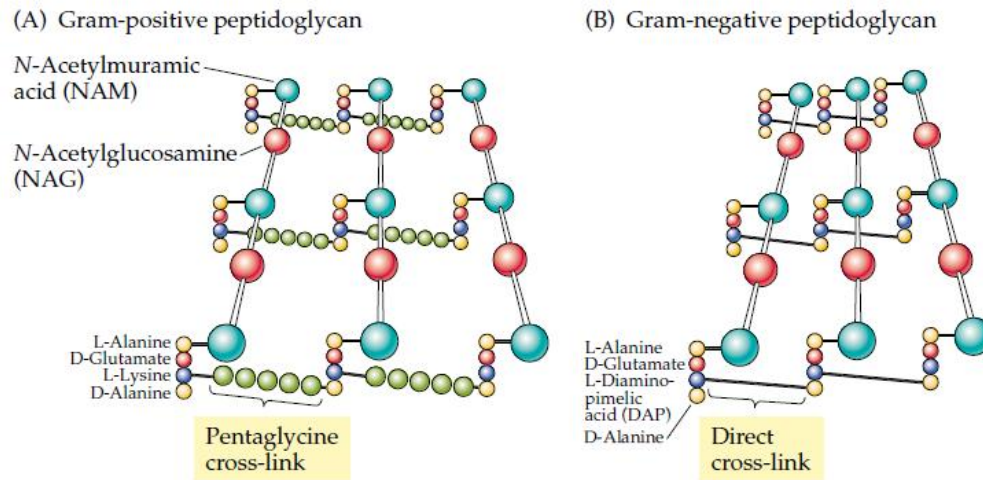
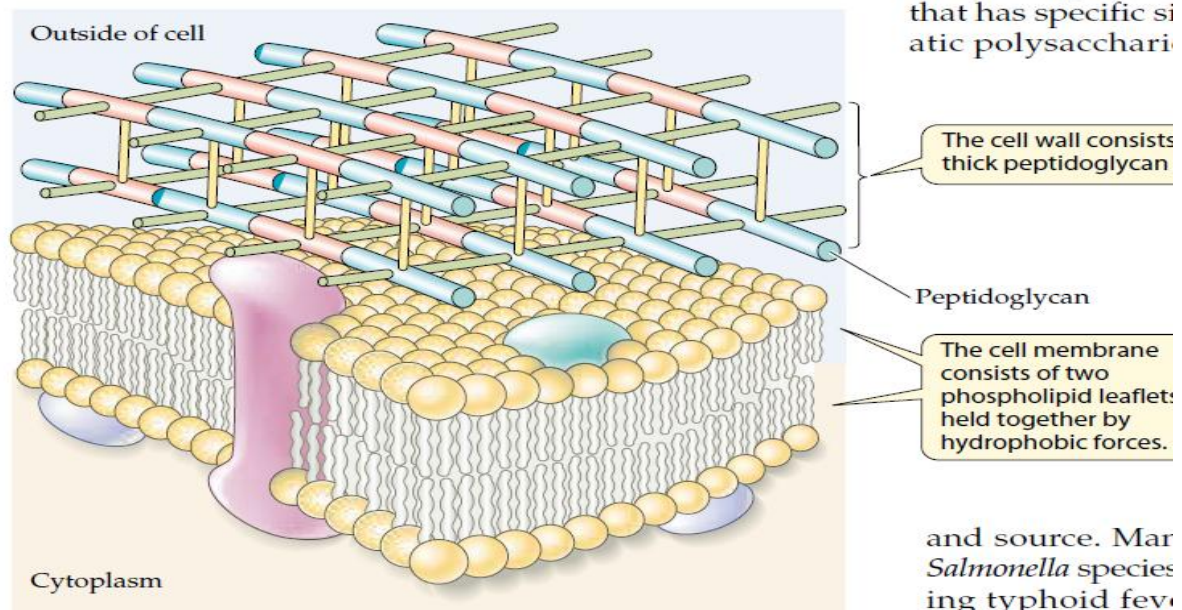


Figure 4.44 Cell walls of gram-positive and gram-negative bacteria

The diagrams show the two-dimensional network of the peptidoglycan sac surrounding (A) a gram-positive and (B) a gram-negative cell. This layer is the major structural component of bacterial cell walls. The *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) are linked to form the amino sugar backbone (glycan). The glycan chains are held together by peptide bridges.

(A) Gram-positive cell envelope



(B) Gram-negative cell envelope

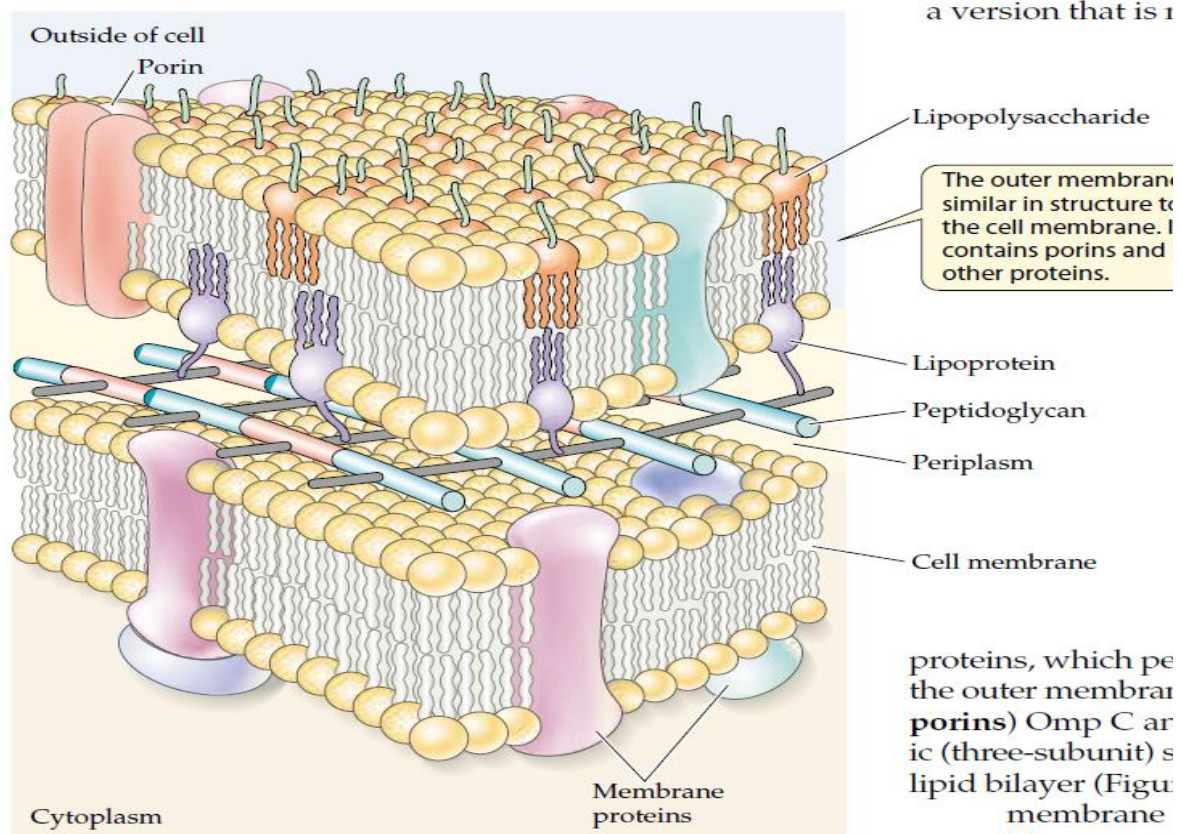


Figure 4.48 Cell envelopes of bacteria

Models of cell envelopes of (A) a gram-positive bacterium, showing the cell membrane and cell wall, and (B) a gram-negative bacterium, showing the cell membrane, peptidoglycan, and outer membrane.

lently to lipid A is that has specific siatic polysacchari

and source. Mar *Salmonella* species ing typhoid fever, bubonic plague), pound. Other gra a version that is i

proteins, which pe the outer membra porins) Omp C ar ic (three-subunit) s lipid bilayer (Fig: membrane ble nutrient well as ino responsible into the cell saccharides

2.7. Plasma Membrane (Cytoplasmic Membrane)

- Surrounded externally by the cell wall and composed of a lipoprotein complex, the *plasma membrane* or cell membrane is the critical barrier, separating the inside from outside the cell (see Figure 11.1).
- About 7 to 8 μm thick and comprising 10 to 20% of a bacterium's dry weight,
- the plasma membrane controls the passage of all material into and out of the cell.
- The inner and outer faces of the plasma membrane are embedded with water-loving (hydrophilic) lips, whereas the interior is hydrophobic.
- Control of material into the cell is accomplished by screening, as well as by electric charge.
- The plasma membrane is the site of the surface charge of the bacteria.
- In addition to serving as an osmotic barrier that passively regulates the passage of material into and out of the cell, the plasma membrane participates in the entire active transport of various substances into the bacterial cell.
- Inside the membrane, many highly reactive chemical groups guide the incoming material to the proper points for further reaction.
- This active transport system provides bacteria with certain advantages, including the ability to maintain a fairly constant intercellular ionic state in the presence of varying external ionic concentrations.
- In addition to participating in the uptake of nutrients, the cell membrane transport system participates in waste excretion and protein secretions.

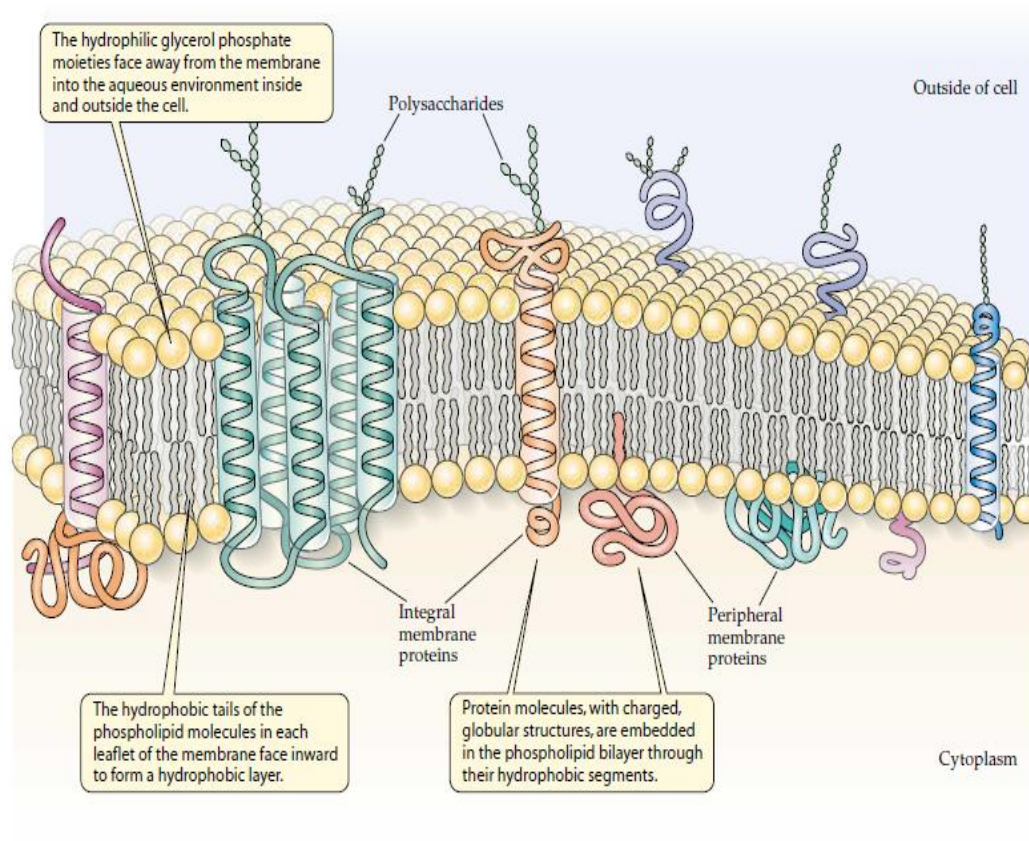


Figure 4.33 Bacterial cell membrane structure
The fluid mosaic model of cell membrane structure. The bilayer structure consists of two phospholipid leaflets.

The Most Important Bacterial Cytoplasmic Membrane Proteins

Permeases	Active transport of nutrients from outside to inside against a concentration gradient.
Biosynthesis enzymes	Required for biosynthesis of the cell wall, e.g., its murein (see under "Cell wall" p. 152). The enzymes that contribute to the final murein biosynthesis steps are for the most part identical with the "penicillin-binding proteins" (PBPs).
Secretion system proteins	Four secretion systems differing in structure and mode of action have been described to date. Proteins are moved out of the cell with the help of these systems. A common feature of all four is the formation of protein cylinders that traverse the cytoplasmic membrane and, in Gram-negative bacteria, the outer cell wall membrane as well. See p. 17 on the special relevance of the type III secretion system to virulence.
Sensor proteins (also known as signal proteins)	Transmit information from the cell's environment into its interior. The so-called receiver domain extends outward, the transmitter domain inward. The transmission activity is regulated by the binding of signal molecules to a receiver module. In two-component systems, the transmitter module transfers the information to a regulator protein, activating its functional module. This regulator segment can then bind to specific gene sequences and activate or deactivate one or more genes (see also Fig. 1.4, p. 19).
Respiratory chain enzymes	Occur in bacteria with aerobic metabolism. Aerobic respiration functions according to the same principles as cellular respiration in eukaryotes.

2.8. Cytoplasm

- Within a cell and bounded by the cell membrane is a complicated mixture of substances and structures called the cytoplasm (see Figure 11.1).
- The cytoplasm is a waterbased fluid containing ribosomes; ions; enzymes; nutrients; storage granules (under certain circumstances); waste products; and various molecules involved in synthesis, energy metabolism, and cell maintenance.

2.9. Mesosome

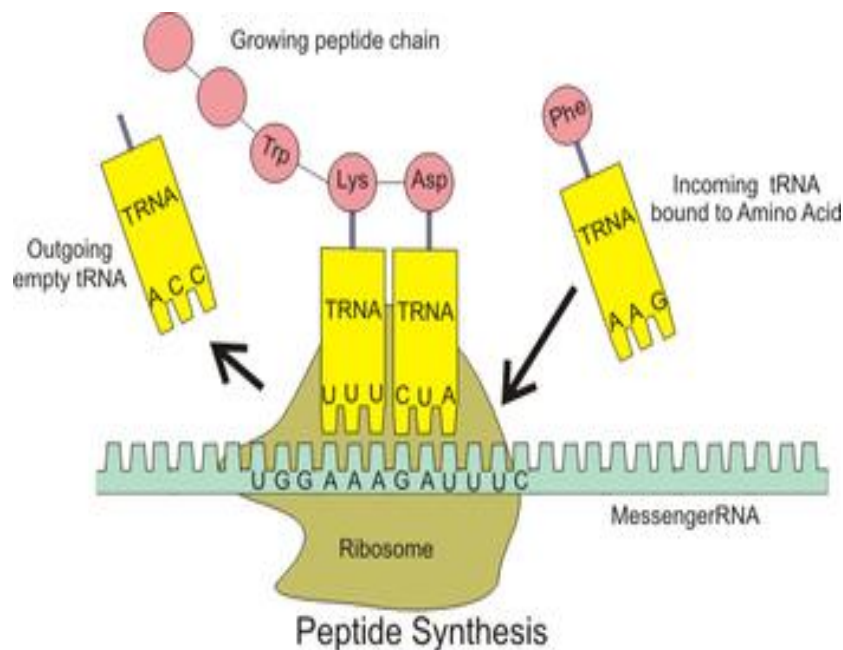
- A common intracellular structure found in the bacterial cytoplasm is the Mesosome (see Figure 11.1).
- Mesosomes are invaginations of the plasma membrane in the shape of tubules, vesicles, or lamellae.
- Their exact function is unknown.
- Currently many bacteriologists believe that mesosomes are artifacts generated during the fixation of bacteria for electron microscopy.

2.10. Nucleoid (Nuclear Body or Region)

- The nuclear region of the prokaryotic cell is primitive and a striking contrast to that of the eucaryotic cell (see Figure 11.1).
- Prokaryotic cells lack a distinct nucleus, the function of the nucleus being carried out by a single, long, double strand of DNA that is efficiently packaged to fit within the nucleoid.
- The nucleoid is attached to the plasma membrane.
- A cell can have more than one nucleoid when cell division occurs after the genetic material has been duplicated.

2.11. Ribosomes

- The bacterial cytoplasm is often packed with ribosomes (see Figure 11.1).
- *Ribosomes* are minute, rounded bodies made of RNA and are loosely attached to the plasma membrane.
- Ribosomes are estimated to account for about 40% of a bacterium's dry weight; a single cell may have as many as 10,000 ribosomes.
- Ribosomes are the site of protein synthesis and are part of the translation process.
- Ribosome: The house of protein synthesis



- Ribosome is an organelle (an internal component of a biological cell) the function of which is to assemble the twenty specific amino acid molecules to form the particular protein molecule determined by the nucleotide sequence of an RNA molecule.
- One of the central tenets of biology, often referred to as the central dogma of molecular biology, is that DNA is used to make RNA, which is used to make proteins.
- The DNA sequence in genes is copied into a messenger RNA (mRNA). Ribosomes then read the information in this mRNA and use it to create proteins.
- This process is known as translation; the ribosome translates the genetic information from the RNA into proteins.
- Ribosomes do this by binding to an mRNA and using it as a template for determining the correct sequence of amino acids in a particular protein.
- The amino acids are attached to transfer RNA (tRNA) molecules, which enter one part of the ribosome and bind to the messenger RNA sequence.
- The attached amino acids are then joined together by another part of the ribosome.
- The ribosome moves along the mRNA, "reading" its sequence and producing a corresponding chain of amino acids.
- Ribosomes are made from complexes of RNAs and proteins.
- Ribosomes are divided into two subunits 30S and 50S.

- The smaller subunit binds to the mRNA, while the larger subunit binds to the tRNA and the amino acids. When a ribosome finishes reading a mRNA, these two subunits split apart.
- Ribosomes have been classified as ribozymes, because the ribosomal RNA seems to be most important for the peptidyl transferase activity that links amino acids together.

2.12. The Structure of Bacterial DNA

- A bacterium's genetic information is stored in its **chromosome** and **plasmids**.
- Each of these structures is made of a single DNA double helix twisted to the right, then additionally twisted to the left about its helical axis .
- Plasmids consisting of linear DNA also occur, although this is rare.
- This DNA topology solves spatial problems and enables such functions as replication, transcription, and recombination.
- Some genes are composed of a mosaic of minicassettes interconnected by conserved DNA sequences between the cassettes .
- **Chromosome.** The chromosome corresponds to the nucleoid
- The chromosomes of E. coli and numerous other pathogenic bacteria have now been completely sequenced.
- Plasmids. The plasmids are autonomous DNA molecules of varying size (3×10^3 to 4.5×10^5 bp) localized in the cytoplasm.
- Large plasmids are usually present in one to two copies per cell, whereas small ones may be present in 10, 40, or 100 copies.
- Plasmids are not essential to a cell's survive.

2.13. Macromolecules that make up cell material

Macromolecule	Primary Subunits	Where found in cell
Protein	amino acids	Flagella, pili, cell walls, cytoplasmic membranes, ribosomes, cytoplasm
Polysaccharides	sugars (carbohydrates)	capsules, inclusions (storage), cell walls
Phospholipids	fatty acids	Membrane
Nucleic Acids (DNA/RNA)	nucleotides	DNA: nucleoid (chromosome), plasmids rRNA: ribosomes; mRNA, tRNA: cytoplasm

3. Microscopic Techniques

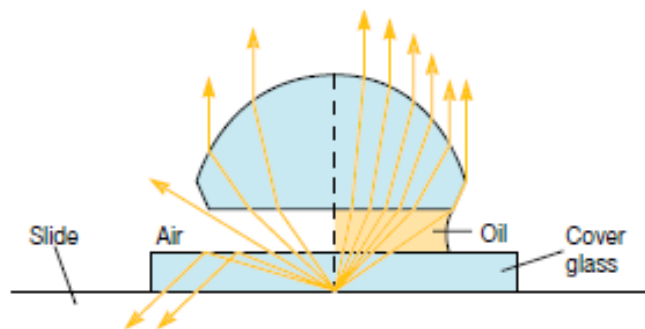
- Microbiologists employ a variety of light microscopes in their work: bright-field, dark-field, phase-contrast, and fluorescence are most commonly used.
- In fact, the same microscope may be a combination of types: bright-field and phase-contrast, or phase-contrast and fluorescence.
- These microscopes and the principles of microscopy extensively used to study the form, structure, staining characteristics, and motility of different microorganisms.
- Therefore, proficiency in using the different microscopes is essential to all aspects of microbiology and must be mastered at the very beginning of a microbiology course.

Bright-Field Light Microscope and Microscopic Measurement of Organisms

- The bright-field light microscope is an instrument that magnifies images using two lens systems.
- Initial magnification occurs in the objective lens.
- Most microscopes have at least three objective lenses on a rotating base, and each lens may be rotated into alignment with the eyepiece or ocular lens in which the final magnification occurs.
- The objective lenses are identified as the low-power, high-dry, and oil immersion objectives.
- Each objective is also designated by other terms. These terms give either the linear magnification or the focal length.
- The latter is about equal or greater than the working distance between the specimen when in focus and the tip of the objective lens.
- For example, the low-power objective is also called the 10×, or 16 millimeter (mm), objective; the high-dry is called the 40×, or 4 mm, objective; and the oil immersion is called the 90×, 100×, or 1.8 mm objective.
- As the magnification increases, the size of the lens at the tip of the objective becomes progressively smaller and admits less light.
- This is one of the reasons that changes in position of the substage condenser and iris diaphragm are required when using different objectives if the specimens viewed are to be seen distinctly.

- The condenser focuses the light on a small area above the stage, and the iris diaphragm controls the amount of light that enters the condenser.
- When the oil immersion lens is used, immersion oil fills the space between the objective and the specimen.
- Because immersion oil has the same refractive index as glass, the loss of light is minimized (figure 1.1).

Figure 1.1 The Oil Immersion Objective. An oil immersion objective lens operating in air and with immersion oil. Light rays that must pass through air are bent (refracted), and many do not enter the objective lens. The immersion oil prevents the loss of light rays.



- The **eyepiece**, or **ocular**, at the top of the tube magnifies the image formed by the objective lens. As a result, the total magnification seen by the observer is obtained by multiplying the magnification of the objective lens by the magnification of the ocular, or eyepiece.
- For example, when using the 10× ocular and the 43× objective, total magnification is $10 \times 43 = 430$ times.

Principles of Microscopic Measurement

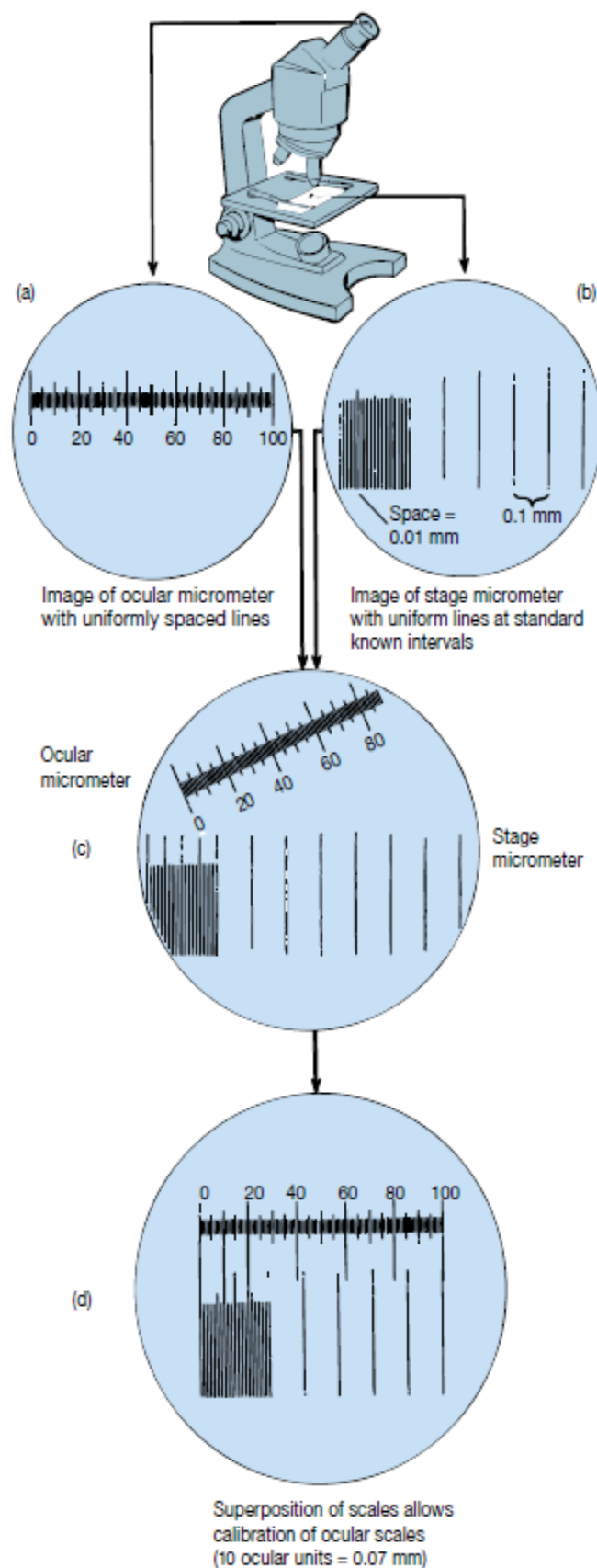
- It frequently is necessary to accurately measure the size of the microorganism one is viewing.
- For example, size determinations are often indispensable in the identification of a bacterial unknown.
- The size of microorganisms is generally expressed in metric units and is determined by the use of a microscope equipped with an ocular micrometer.
- An **ocular micrometer** is a small glass disk on which uniformly spaced lines of unknown distance, ranging from 0 to 100, are etched.

- The ocular micrometer is inserted into the ocular of the microscope and then calibrated against a stage micrometer, which has uniformly spaced lines of known distance etched on it.
- The stage micrometer is usually divided into 0.01 millimeter and 0.1 millimeter graduations.
- The ocular micrometer is calibrated using the stage micrometer by aligning the images at the left edge of the scales.
- The dimensions of microorganisms in dried, fixed, or stained smears tend to be reduced as much as 10 to 20% from the dimensions of the living microorganisms.
- Consequently, if the actual dimensions of a microorganism are required, measurements should be made in a wet-mount.

3.1. Dark-Field Light Microscope

- The compound microscope may be fitted with a darkfield condenser that has a numerical aperture (resolving power) greater than the objective.
- The condenser also contains a dark-field stop.
- The compound microscope now becomes a dark-field microscope.
- Light passing through the specimen is diffracted and enters the objective lens, whereas un diffracted light does not, resulting in a bright image against a dark background (figures 3.1–3.2).
- Since light objects against a dark background are seen more clearly by the eye than the reverse, dark-field microscopy is useful in observing unstained living microorganisms, microorganisms that are difficult to stain, and spirochetes (figure 3.2), which are poorly defined by bright-field microscopy.

Figure 1.4 Calibrating an Ocular Micrometer.



3.2. Phase-Contrast Light Microscope

- Certain transparent, colorless living microorganisms and their internal organelles are often impossible to see by ordinary bright-field or dark-field microscopy because they do not absorb, reflect, refract, or diffract sufficient light to contrast with the surrounding environment or the rest of the microorganism.
- Microorganisms and their organelles are only visible when they absorb, reflect, refract, or diffract more light than their environment.
- The **phase-contrast microscope** permits the observation of otherwise invisible living, unstained microorganisms.
- In the phase-contrast microscope, the condenser has an annular diaphragm, which produces a hollow cone of light; the objective has a glass disk (the phase plate) with a thin film of transparent material deposited on it, which accentuates phase changes produced in the specimen.
- This phase change is observed in the specimen as a difference in **light intensity**.
- Phase plates may either retard (positive phase plate) the diffracted light relative to the undiffracted light, producing **dark-phase-contrast microscopy**, or advance (negative phase plate) the undiffracted light relative to the directed light, producing **bright-phasecontrast microscopy**.

3.3. Fluorescence Microscope

- Fluorescence microscopy is based on the principle of removal of incident illumination by selective absorption, whereas light that has been absorbed by the specimen and re-emitted at an altered wavelength is transmitted.
- The light source must produce a light beam of appropriate wavelength.
- An excitation filter removes wavelengths that are not effective in exciting the fluorochrome used.
- The light fluoresced by the specimen is transmitted through a filter that removes the incident wavelength from the beam of light.

4. Bacterial Staining

- Living bacteria sometimes may be studied directly by bright-field or phase contrast microscopy.
- This is useful when one needs to demonstrate, for example, bacterial motility.
- The first exercise in this part of the manual provides an opportunity for observing bacteria alive and unstained.
- Since living bacteria are generally colorless and almost invisible because of their lack of contrast with the water in which they may reside, staining is necessary in order to make them readily visible for observation of intracellular structures as well as overall morphology.
- The exercises in this part of the manual have been designed to give the student expertise in staining and slide preparation, an appreciation for bacterial morphology, and experience in how to specifically stain some specialized bacterial features such as endospores, capsules, and flagella.

Gram Stain

- The Gram stain procedure was originally developed In 1883-4 by the Danish physician Dr. H. C. GRAM, a physician who was working with R. Koch, to differentiate pneumococci from Klebsiella pneumonia discovered that bacteria fell into two distinct categories when stained sequentially with CRYSTAL VIOLET followed in sequence by a bath in an IODINE SOLUTION produces "purple colored iodine-dye complexes" in the cytoplasm of bacteria ,a wash with a destaining agent and a COUNTER-STAIN

Mechanism of work

- If the cells were bathed following an initial treatment with the crystal violet.
- One group of cells RESISTED the removal of the crystal violet when washed with 95%ETHANOL or ACETONE (DECOLORIZING AGENTS), whereas the second group was readily DECOLORIZED by a brief rinse with these reagents.
- To visualize the decolorized cells, Gram briefly exposed them to a COUNTER-STAIN, or a stain of a different color from the crystal violet.
- Gram settled on the red counter staining dye SAFRANIN.

- The difference between Gram-positive and Gram-negative bacteria is in the permeability of the cell wall to these "purple colored iodine-dye complexes" when treated with the decolorizing solvent.
- Thus cells which resisted decolorization remained DEEP PURPLE or BLUE and came to be referred to a GRAM POSITIVE cells, whereas cells that easily lost the crystal violet dye were red after counter staining. These red cells came to be called GRAM NEGATIVE
- Gram and others drew several important conclusions from these staining results.
- First, they realized that DIFFERENTIAL STAINING of cells and cell components was possible.
- Secondly, they recognized that this staining was DIAGNOSTIC and could be used to IDENTIFY CELLS and SUBSTANCES.
- Thirdly, they reasoned that cells and cell components DIFFERED CHEMICALLY as evidenced by their differential staining.

Precautions

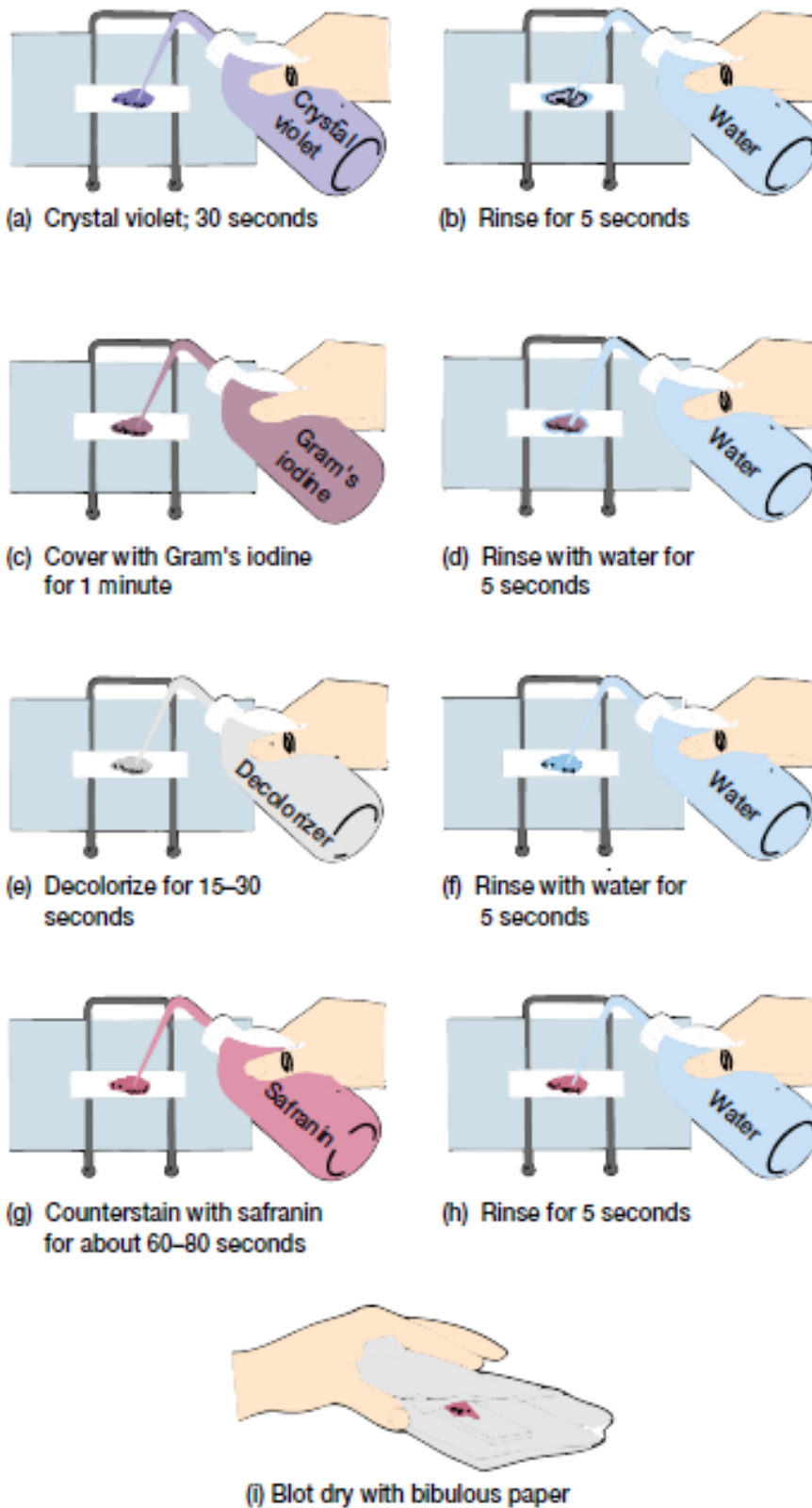
- Performing a good gram stain is easy, but it does require some experience.
- For example, for the correct preparation of the smear.
- Make a thin film of the material on a clean glass slide, using a sterile loop or swab for viscous specimens Air dry, then heat fix the slide by passing it several times through a flame (the slide should not become too hot to touch) Smears that are not properly fixed tend to be washed away during staining and washing resulting in the absence of stained bacteria., Also the age of the culture is important.
- Very young and very old cells often produce poor results, whereas mid-log cells that are healthy and growing optimally, tend to give dependable results.
- The media the cells are grown in and the environmental conditions may also effect the outcome of a gram stain because these ultimately reflect on the chemical nature of the cell. the timing of the various steps, the quality of the reagents and the experience of the person performing the procedure all influence the final results. While many bacteria are "cleanly" G+ or G- a large number are to some degree GRAM VARIABLE.
- Failure to follow these directions may cause staining artifacts and disrupt the normal morphology of bacteria and cells.

- To be visible on a slide, organisms that stain by the Gram method must be present in concentrations of a minimum of 10^4 to 10^5 organisms/ml of unconcentrated staining fluid.
- At lower concentrations, the Gram stain of a clinical specimen seldom reveals organisms even if the culture is positive.

4.1. Staining procedure

- Divide a clean slide into 2 sections. Obtain the cultures indicated by the instructor, including a G+ and a G- control
- Aseptically place loops of the known G+ and G- control cells at either end of the slide.
- Allow the 2 smears to dry and heat fix as mentioned before.
- Flood the 2 smears with the crystal violet solution for 1 min.
- Wash gently with tap water, but don't dry; shake off most of the water.
- Flood the smears with the iodine solution for 1 min. & rinse again with water and shake almost dry.
- Rinse with the decolorizing reagent as demonstrated by the instructor.
- Immediately, rinse the smears with water to remove the last bit of the crystal violet/decolorizing reagent. At this point the G+ cells will still retain their crystal violet and will thus be purple (like your fingers at this stage?), whereas the G- cells will be UNSTAINED. Shake off the excess water.
- Flood the smears for about 30 seconds with the safranin counter stain. Finally wash with water and blot dry.
- Examine the respective G+ and G- smears at 40X until you locate a visible cluster of bacterial cells. Then add immersion oil and switch to 100X.

Figure 8.3 Gram-stain Procedure.

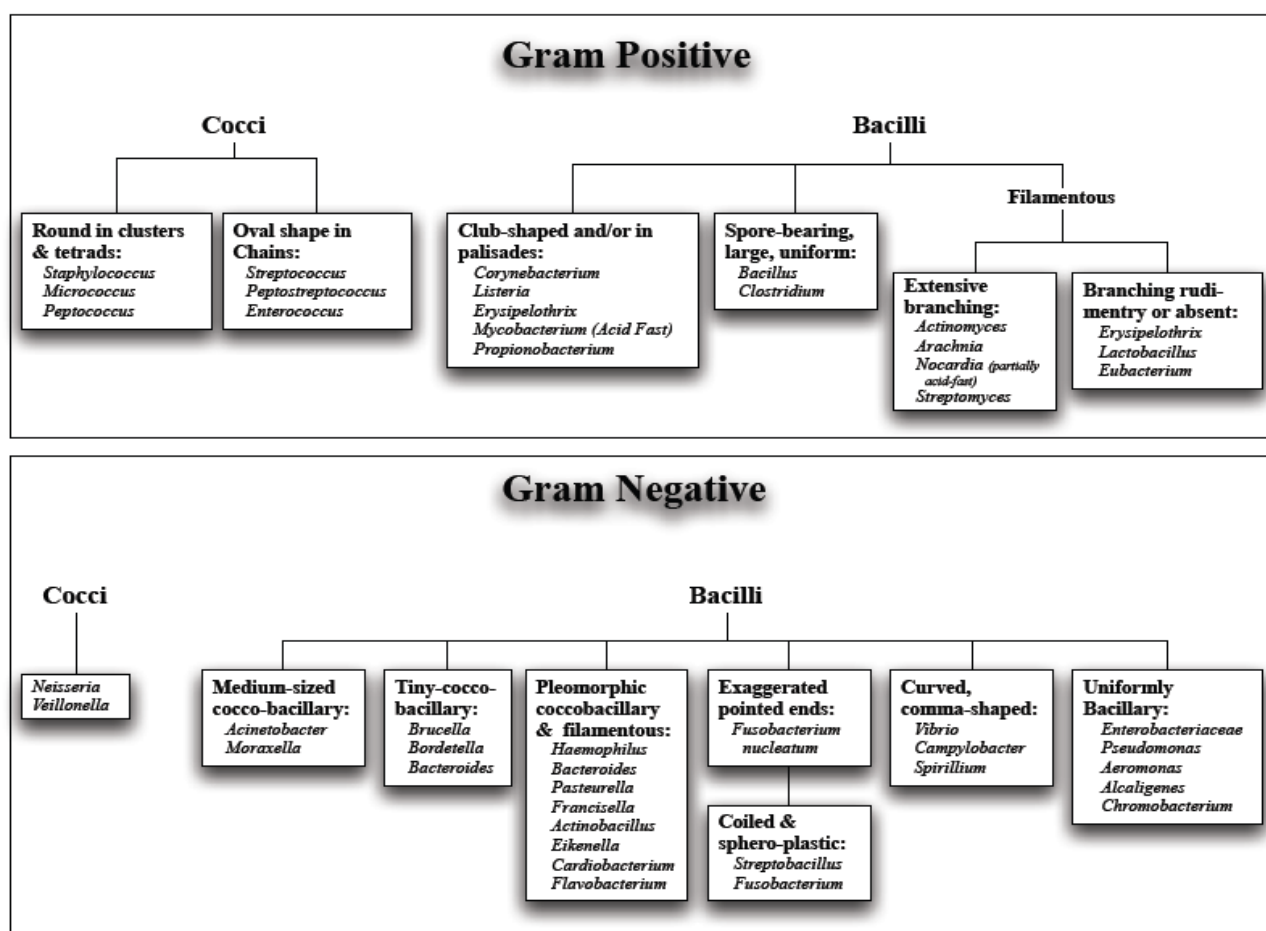


Identification flow charts

Differentiation via Gram stains and cell morphology.

Gram Stain & Morphological Flowchart

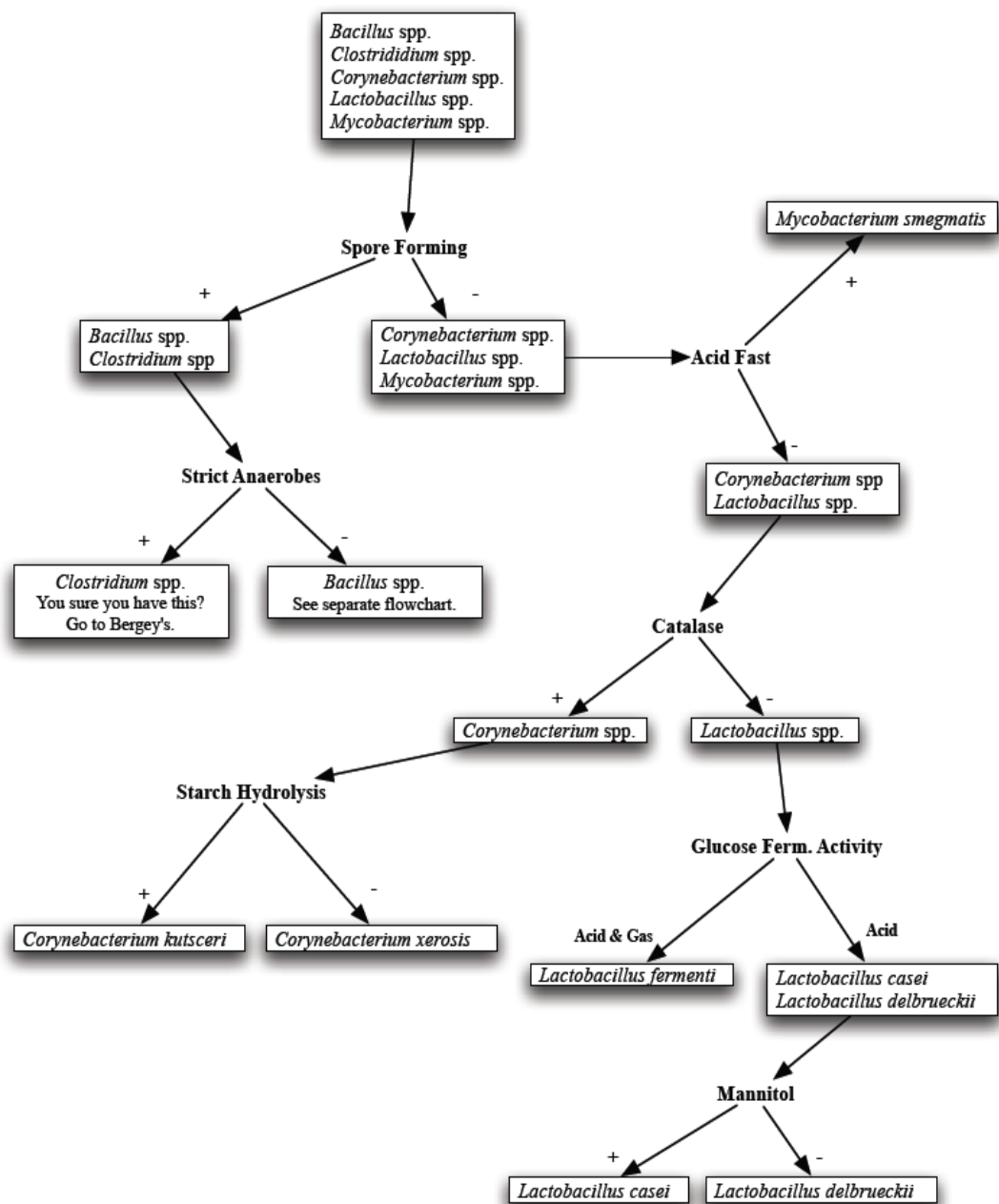
Some Examples



Identification flow charts

Gram Positive Rods ID Flowchart

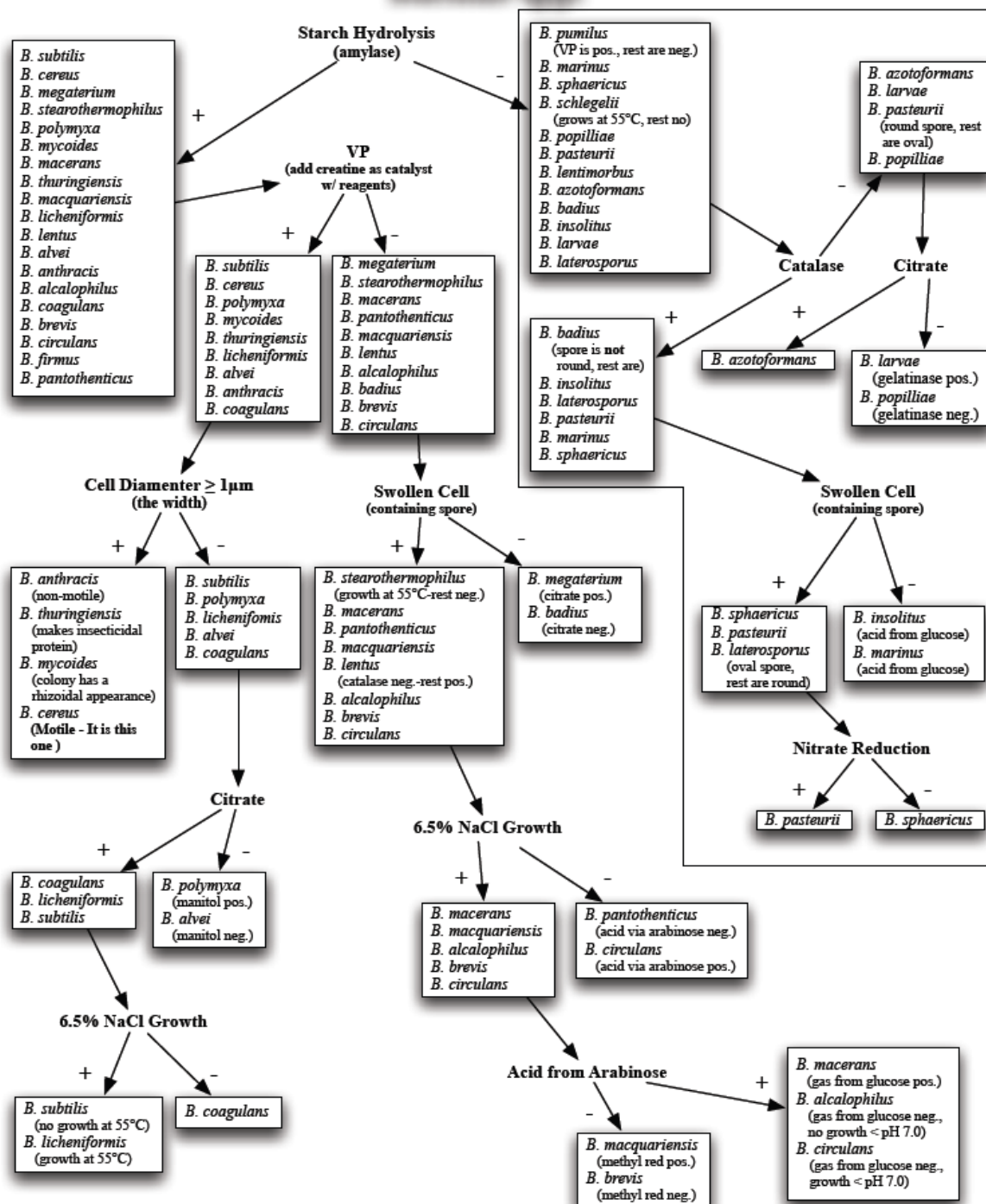
Gram Positive Rods



Identification flow charts

Bacillus spp. ID Flowchart

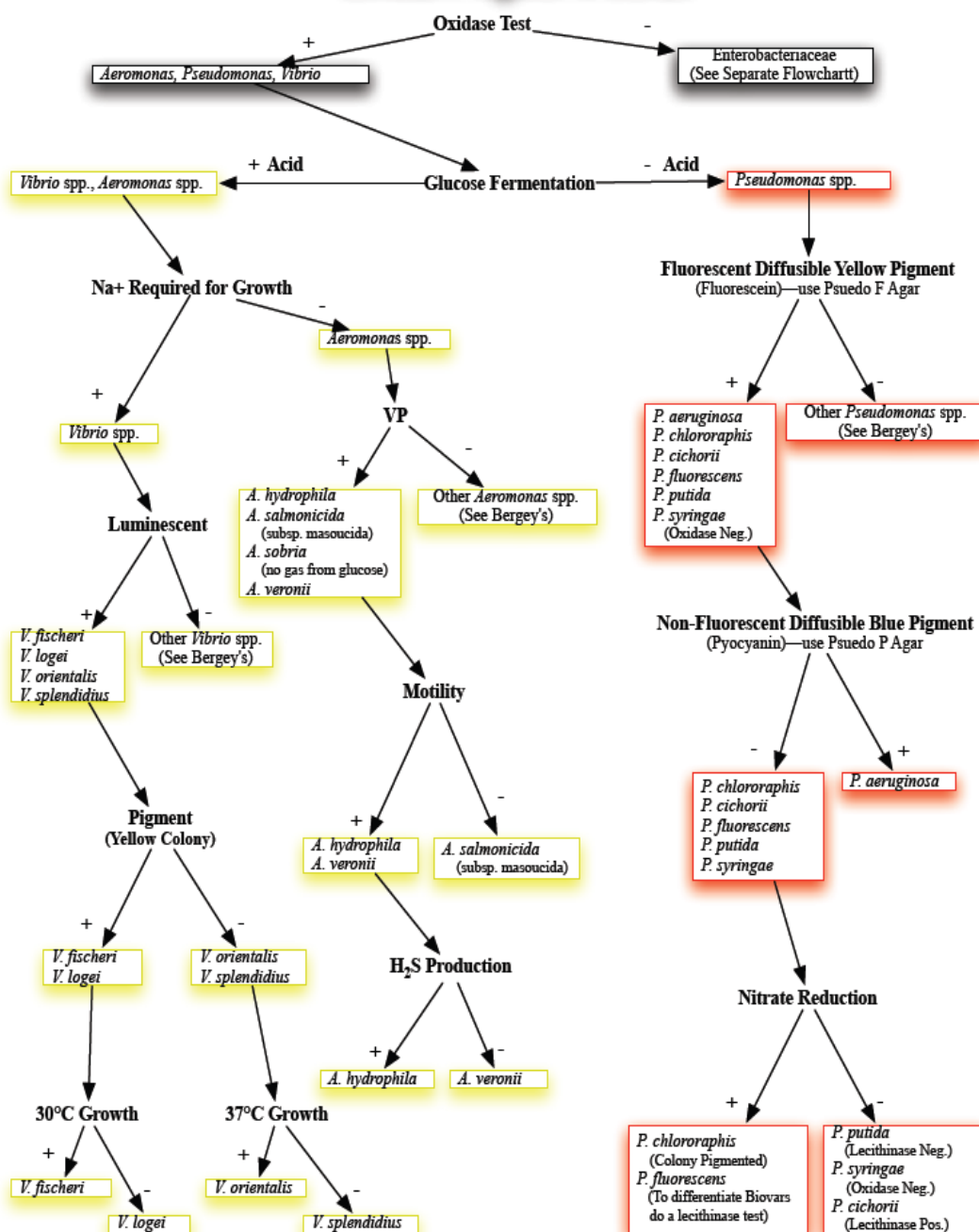
Bacillus spp.



Identification flow charts

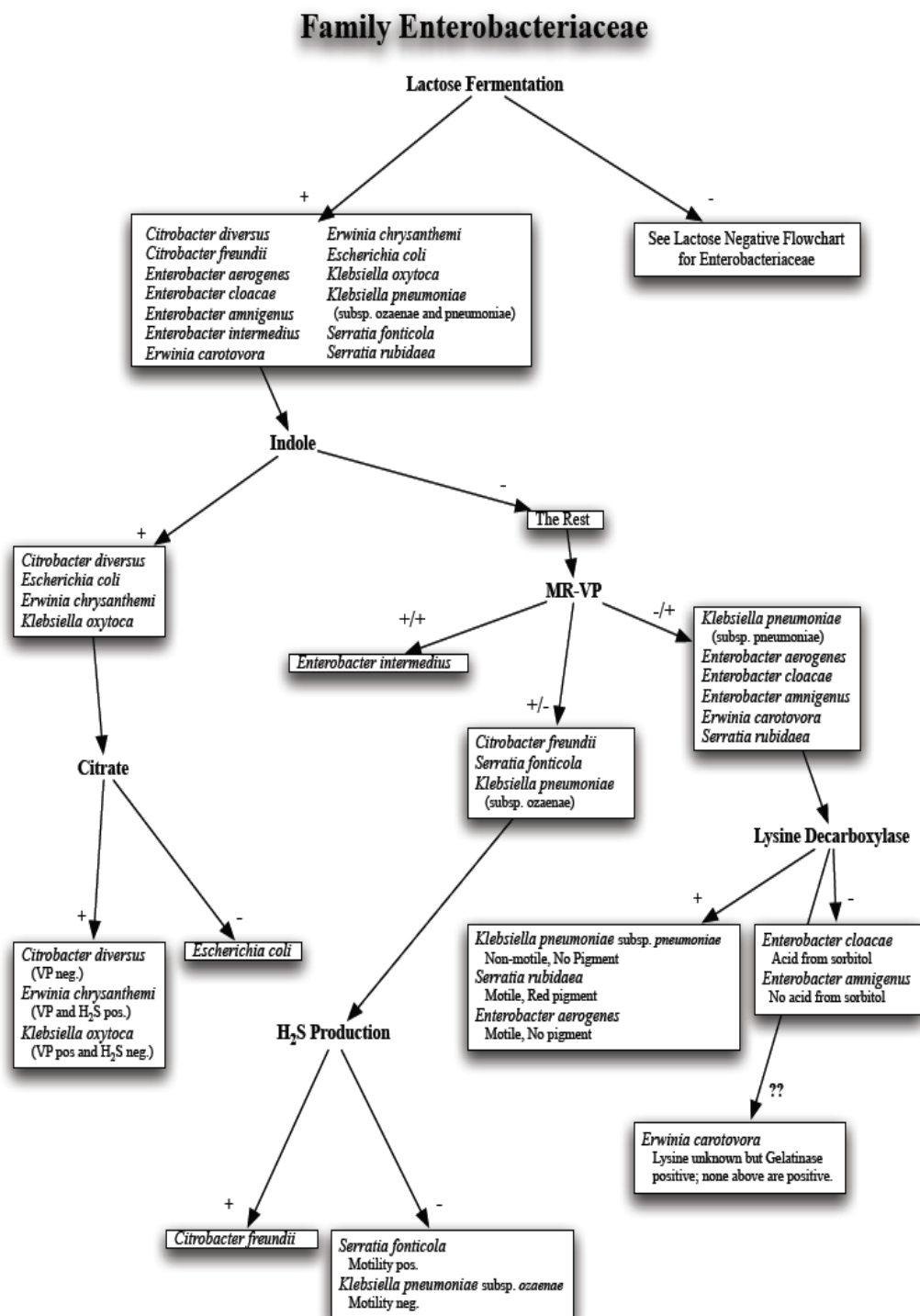
Gram Negative Rods ID Flowchart

Gram Negative Rods



Identification flow charts

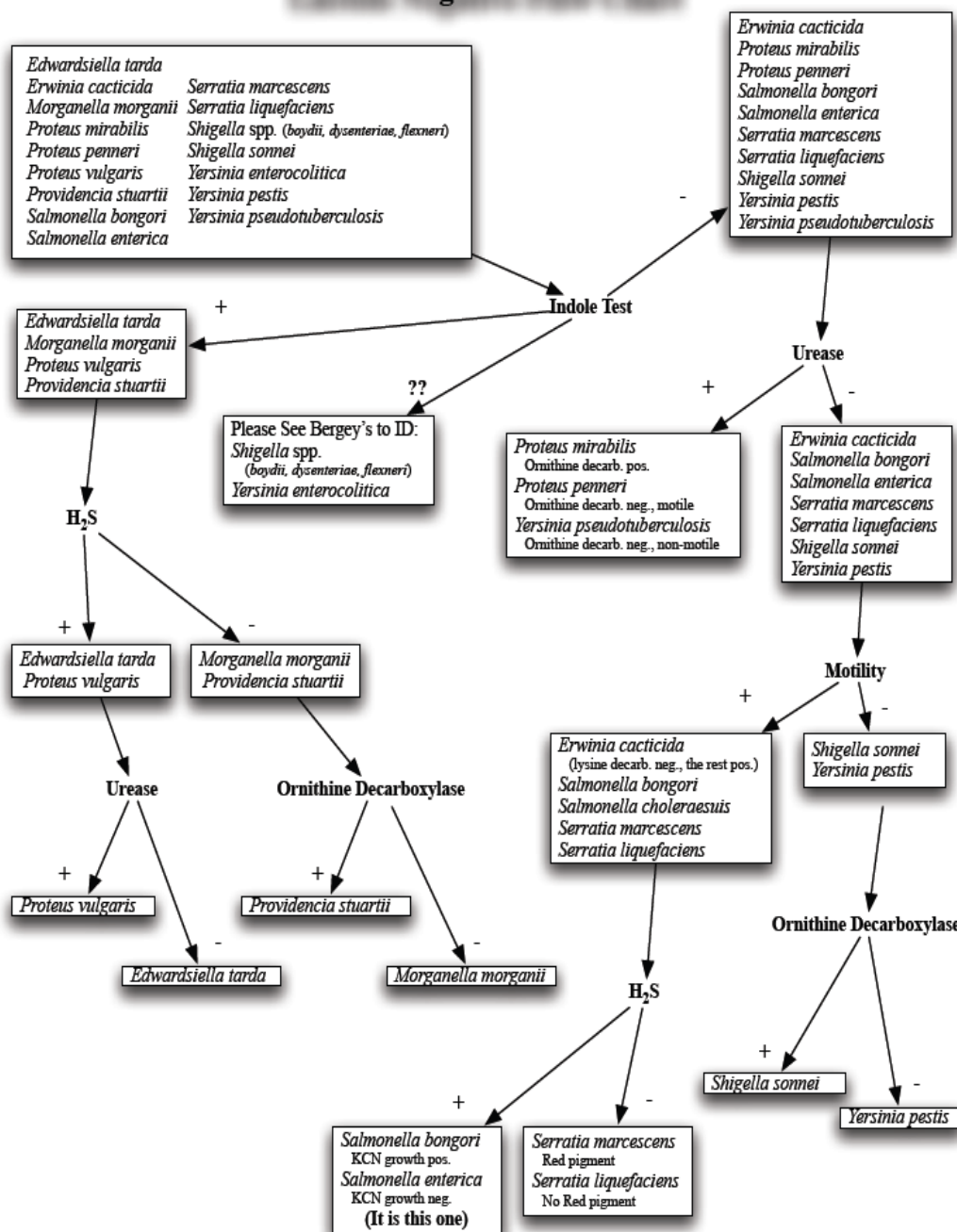
Family Enterobacteriaceae Lactose Positive ID Flowchart



Identification flow charts

Family Enterobacteriaceae Lactose Negative ID Flowchart

Family Enterobacteriaceae Continued
Lactose Negative Flow Chart



5. Bacterial Growth factors and Conditions

- Several factors affect the rate at which bacteria grow, including temperature, pH, and oxygen levels.
- The warmer the environment, the faster the rate of growth. Generally, for each increase of 10°C, the growth rate doubles. Heat can also be used to kill bacteria.
- Most bacteria grow best at neutral pH. Extreme acidic or basic conditions generally inhibit growth, though some bacteria may require acidic and some require alkaline conditions for growth.
- Bacteria are aerobic, anaerobic, or facultative. If aerobic, they require free oxygen in the aquatic environment.
- Anaerobic bacteria exist and multiply in environments that lack dissolved oxygen (DO).
- Facultative bacteria (e.g., iron bacteria) can switch from an aerobic to anaerobic growth or grow in an anaerobic or aerobic environment.
- Under optimum conditions, bacteria grow and reproduce very rapidly.
- As stated previously, bacteria reproduce by binary fission.
- An important point to consider in connection with bacterial reproduction is the rate at which the process can occur.
- The total time required for an organism to reproduce and the offspring to reach maturity is called generation time.
- Bacteria growing under optimal conditions can double their number about every 20 to 30 min.
- Obviously, this generation time is very short compared to that of higher plants and animals.
- Bacteria continue to grow at this rapid rate as long as nutrients hold out — even the smallest contamination can result in a sizable growth in a very short time.

Microbial Nutrition and Cultivation

Element	Form in which usually supplied	Occurrence in biological systems
<i>Macronutrients</i>		
Carbon (C)	CO ₂ , organic compounds	Component of all organic molecules, CO ₂
Hydrogen (H)	H ₂ O, organic compounds	Component of biological molecules, H ⁺ released by acids
Oxygen (O)	O ₂ , H ₂ O, organic compounds	Component of biological molecules; required for aerobic metabolism
Nitrogen (N)	NH ₃ , NO ₃ ⁻ , N ₂ , organic N compounds	Component of proteins, nucleic acids
Sulphur (S)	H ₂ S, SO ₄ ²⁻ , organic S compounds	Component of proteins; energy source for some bacteria
Phosphorus (P)	PO ₄ ³⁻	Found in nucleic acids, ATP, phospholipids
Potassium (K)	In solution as K ⁺	Important intracellular ion
Sodium (Na)	In solution as Na ⁺	Important extracellular ion
Chlorine (Cl)	In solution as Cl ⁻	Important extracellular ion
Calcium (Ca)	In solution as Ca ²⁺	Regulator of cellular processes
Magnesium (Mg)	In solution as Mg ²⁺	Coenzyme for many enzymes
Iron (Fe)	In solution as Fe ²⁺ or Fe ³⁺ or as FeS, Fe(OH) ₃ etc	Carries oxygen; energy source for some bacteria
<i>Micronutrients</i>		
Copper (Cu)	Present as contaminants at very low concentrations In solution as Cu ⁺ , Cu ²⁺	Coenzyme; microbial growth inhibitor
Manganese (Mn)	In solution as Mn ²⁺	Coenzyme
Cobalt (Co)	In solution as Co ²⁺	Vitamin B ₁₂
Zinc (Zn)	In solution as Zn ²⁺	Coenzyme; microbial growth inhibitor
Molybdenum (Mo)	In solution as Mo ²⁺	Coenzyme
Nickel (Ni)	In solution as Ni ²⁺	Coenzyme

- In this section we introduced the major groups of macromolecules found in living cells; the raw materials from which these are synthesized are ultimately derived from the organism's environment in the form of nutrients.
- These can be conveniently divided into those required in large quantities (macronutrients) and those which are needed only in trace amounts (micronutrients or trace elements).
- In the following section we briefly describe the role of each element, and the form in which it may be acquired.
- All microorganisms must have a supply of the nutrients described above, but they show great versatility in the means they use to satisfy these requirements.

- **Carbon** is the central component of the biological macromolecules. Carbon incorporated into biosynthetic pathways may be derived from organic or inorganic sources (see below); some organisms can derive it from CO₂, while others require their carbon in 'ready-made', organic form like sugars.
- **Hydrogen** is also a key component of macromolecules, and participates in energy generation processes in most microorganisms. In autotrophs (see 'Nutritional categories' below), hydrogen is required to reduce carbon dioxide in the synthesis of macromolecules.
- **Oxygen** is of central importance to the respiration of many microorganisms, but in its molecular form (O₂), it can be toxic to some forms. These obtain the oxygen they need for the synthesis of macromolecules from water.
- **Nitrogen** is needed for the synthesis of proteins and nucleic acids, as well as for important molecules such as ATP. Microorganisms range in their demands for nitrogen from those that are able to assimilate ('fix') gaseous Nitrogen (N₂) to those that require all 20 amino acids to be provided preformed. Between these two extremes come species that are able to assimilate nitrogen from an inorganic source such as nitrate, and those that utilize ammonium salts or urea as a nitrogen source.
- **Sulphur** is required for the synthesis of proteins and vitamins, and in some types is involved in cellular respiration and photosynthesis. It may be derived from sulphur containing amino acids (methionine, cysteine), sulphates and sulphides.
- **Phosphorus** is taken up as inorganic phosphate, and is incorporated in this form into nucleic acids and phospholipids, as well as other molecules such as ATP.
- **Metals** such as copper, iron and magnesium are required as cofactors in enzyme reactions. Many microorganisms are unable to synthesize certain organic compounds necessary for growth and must therefore be provided with them in their growth medium.
- These are termed growth factors (Table), of which three main groups can be identified: amino acids, purines and pyrimidines (required for nucleic acid synthesis) and vitamins.
- Vitamins are complex organic compounds required in very small amounts for the cell's normal functioning.
- They are often either coenzymes or their precursors. Microorganisms vary greatly in their vitamin requirements.

- Many bacteria are completely self-sufficient, while protozoans, for example, generally need to be supplied with a wide range of these dietary supplements.
- A vitamin requirement may be absolute or partial; an organism may be able, for example, to synthesize enough of a vitamin to survive, but grow more vigorously if an additional supply is made available to it.

- **Selected microbial growth factors**

Growth factor	Function
Amino acids	Components of proteins
<i>p</i> -Aminobenzoic acid	Precursor of folic acid, involved in nucleic acid synthesis
Niacin (nicotinic acid)	Precursor of NAD^+ and NADP^+
Purines & pyrimidines	Components of nucleic acids
Pyridoxine (vitamin B ₆)	Amino acid synthesis
Riboflavin (vitamin B ₂)	Precursor of FAD

Nutritional categories:

- Microorganisms can be categorised according to how they obtain their carbon and energy.
- As we have seen, carbon is the most abundant component of the microbial cell, and most microorganisms obtain their carbon in the form of organic molecules, derived directly or indirectly from other organisms.
- Microorganisms which obtain their carbon in this way are described as heterotrophs, and include all the fungi and protozoans as well as most types of bacteria.
- Microorganisms as a group are able to incorporate the carbon from wide range of organic compounds into cellular material.
- A significant number of bacteria and all of the algae do not, however, take up their carbon preformed as organic molecules in this way, but derive it instead from carbon dioxide these organisms are called autotrophs,.
- We can also categorise microorganisms nutritionally by the way they derive the energy they require to carryout essential cellular reactions.
- **Autotrophs** thus fall into two categories:
 - **Chemoautotrophs** obtain their energy as well as their carbon from inorganic sources; they do this by the oxidation of inorganic molecules such as sulphur or nitrite.
 - **Photoautotrophs** have photosynthetic pigments (bacteriochlorophyll) enabling them to convert light energy into chemical energy .
- The great majority of heterotrophs obtain energy as well as carbon from the same organic source.
- Such organisms release energy by the chemical oxidation of organic nutrient molecules, and are therefore termed chemo-organotrophic heterotrophs (most bacteria).
- on the other hand The organisms which are need a molecule to act as a source of electrons acceptor (reducing power) to drive their energy generating systems .
- Those able to use an inorganic electron donor such as H_2S or ammonia are called Chemolithotrophs heterotrophs.

5.1. Factors affecting microbial growth:

- Assuming the nutrients are present in an adequate supply, what other factors do we need to consider in order to provide favorable conditions for microbial growth? As the following, growth may be profoundly affected by a number of physical factors.

Temperature:

- Microorganisms as a group are able to grow over a wide range of temperatures, from around freezing to above boiling point.
- For any organism, the minimum and maximum growth temperatures define the range over which growth is possible; this is typically about 25–30 °C.
- Growth is slower at low temperatures because enzymes work less efficiently and also because lipids tend to harden and there is a loss of membrane fluidity.
- Growth rates increase with temperature until the optimum temperature is reached, then the rate falls again (Figure).
- The optimum and limiting temperatures for an organism are a reflection of the temperature range of its enzyme systems, The optimum temperature is generally closer to the maximum growth temperature than the minimum.
- Once the optimum value is passed, the loss of activity caused by denaturation of enzymes causes the rate of growth to fall away sharply (see also Figures).

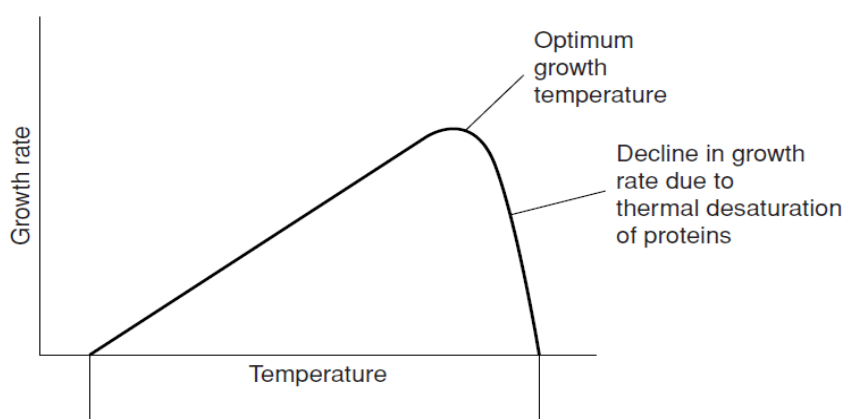


Figure 5.4 Effect of temperature on microbial growth rate. The factors governing the minimum, optimum and maximum temperatures for a particular organism are indicated. The curve is asymmetrical, with the optimum temperature being closer to the maximum than the minimum

FACTORS AFFECTING MICROBIAL GROWTH

97

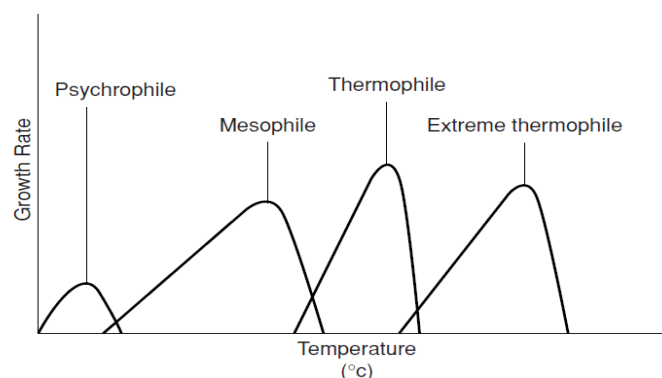


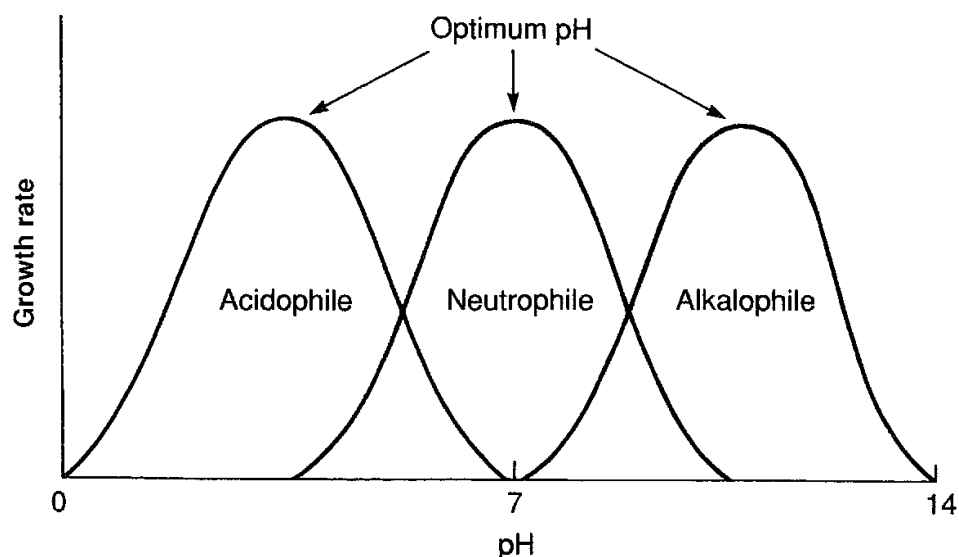
Figure 5.5 Microorganisms can be categorised according to the temperature range at which they grow

- They are three types of microorganisms can be categorised according to the temperature range at which they grow (mesophiles,thermophiles and Psychrophiles).
- The majority of microorganisms achieve optimal growth at ‘middling’ temperatures of around 20–45 °C; these are called mesophiles (Figure 5.5).
- Contrast these with thermophiles, which have become adapted to not only surviving, but thriving at much higher temperatures.
- Typically, these would be capable of growth within a range of about 40–80 °C, with an optimum around 50–65 °C. Extreme thermophiles have optimum values in excess of this, and can tolerate temperatures in excess of 100 °C.
- In 2003, a member of the primitive bacterial group called the Archaea was reported as growing at a temperature of 121 °C, a new world record.
- Psychrophiles occupy the other extreme of the temperature range; they can grow at 0°C, with optimal growth occurring at 15 °C or below. Such organisms are not able to grow at temperatures above 25 °C or so. Psychrotrophs, on the other hand, although they can also grow at 0 °C, have much higher temperature optima (20–30 °C).
- Members of this group are often economically significant due to their ability to grow on refrigerated foodstuffs.

pH

- Microorganisms are strongly influenced by the prevailing pH of their surroundings.
- As with temperature, we can define minimum, optimum and maximum values for growth of a particular type (Figure 5).

- The pH range (between minimum and maximum values) is greater in fungi than it is in bacteria.
- Most microorganisms grow best around neutrality (pH 7).
- Many bacteria prefer slightly alkaline conditions but relatively few are tolerant of acid conditions, and fewer still are acidophilic.
- Fungi, on the other hand, generally prefer slightly acid conditions and therefore tend to dominate bacteria when these prevail.
- The reason for the growth rate falling away either side of the optimum value is again due to alterations in three-dimensional protein structure.
- The pH value of growth media is adjusted to the desired value by the addition of acid or alkali during its preparation.
- The metabolic activities of microorganisms often means that they change the pH of their environment as growth proceeds, so it is important in a laboratory growth medium that a desirable pH is not only set but maintained.
- This is achieved by the use of an appropriate buffer system.
- Phosphate buffers are widely used in the microbiology laboratory; they enable media to minimize changes in their pH when acid or alkali is produced.



Effect of pH on microbial growth rate. Individual species of microorganism occupy a relatively narrow range of pH. Although for most species this is around neutrality, both acidophilic and alkalophilic forms exist. The shape of the curve reflects the properties of a particular organism's enzymes and other proteins.

Oxygen

- Oxygen is present as a major constituent (20 per cent) of our atmosphere, and most life forms are dependent upon it for survival and growth.
- Bacteria are categorized as the following according to their O₂-related behavior:
- **Obligate aerobes.** These bacteria can only reproduce in the presence of O₂
- Aerobic organisms require oxygen to act as a terminal electron acceptor in their respiratory chains.
- **Obligate anaerobes** These bacteria die in the presence of O₂. Their metabolism is adapted to a low redox potential and vital enzymes are inhibited by O₂. They are cultured in special anaerobic chambers, and oxygen excluded from all liquid and solid media
- **Facultative anaerobes** These bacteria can oxidize nutrient substrates by means of both respiration and fermentation. Facultative anaerobes are able to act like aerobes in the presence of oxygen, but have the added facility of being able to survive when conditions become anaerobic
- **Aerotolerant anaerobes.** These bacteria oxidize nutrient substrates without using elemental oxygen although, unlike obligate anaerobes, they can tolerate it.
- **Microaerophiles** require oxygen, but are only able to tolerate low concentrations of it (2–10 per cent), finding higher concentrations harmful. Organisms inoculated into a static culture medium will grow at positions that reflect their oxygen preferences.

100

MICROBIAL GROWTH

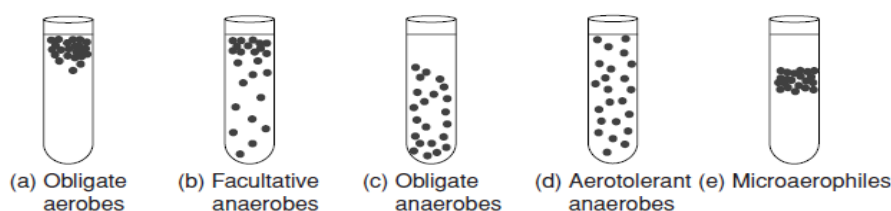


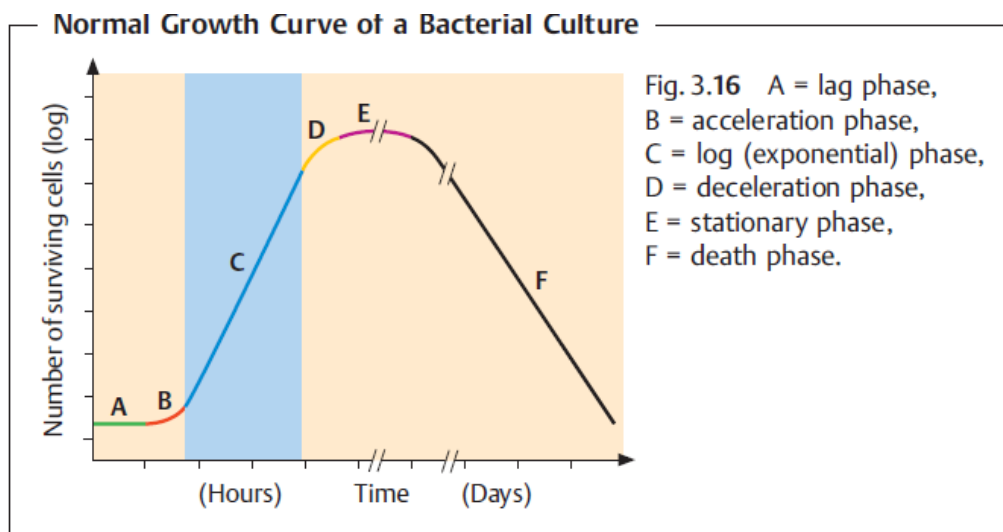
Figure 5.7 Microorganisms have different oxygen requirements. In a static culture, microorganisms occupy different regions of the medium, reflecting their pattern of oxygen usage. (a) Obligate aerobes must grow at or near the surface, where oxygen is able to diffuse. (b) Facultative anaerobes are able to adjust their metabolism to the prevailing oxygen conditions. (c) Obligate anaerobes, in contrast, occupy those zones where no oxygen is present at all. (d) Aerotolerant anaerobes do not use oxygen, but neither are they inhibited by it. (e) Microaerophiles have specific oxygen requirements, and can only grow within a narrow range of oxygen tensions

Osmotic pressure

- Osmosis is the diffusion of water across a semipermeable membrane from a less concentrated solution to a more concentrated one, equalising concentrations.
- The pressure required to make this happen is called the osmotic pressure.
- If a cell were placed in a hypertonic solution (one whose solute concentration is higher), osmosis would lead to a loss of water from the cell (plasmolysis).
- In the opposite situation, water would pass from a dilute (hypotonic) solution into the cell, causing it to swell and burst. The rigid cell walls of bacteria prevent them from bursting; this, together with their minute size, makes them less sensitive to variations in osmotic pressure than other types of cell.
- They are generally able to tolerate NaCl concentrations of between 0.5 and 3.0 per cent. Haloduric ('salt-tolerant') bacteria are able to tolerate concentrations ten times as high, but prefer lower concentrations, whereas halophilic ('salt-loving') forms are adapted to grow best in conditions of high salinity such as those that prevail in the Dead Sea in the Middle East.
- In order to do this without plasmolysis occurring, they must build up a higher internal solute concentration, which they do by actively concentrating potassium ions inside the cell.

Growth curve:

- Bacteria reproduce asexually by means of simple transverse binary fission.
- Their numbers (n) increase logarithmically ($n = 2^G$). The time required for a reproduction cycle (G) is called the generation time (g) and can vary greatly from species to species.
- Fast-growing bacteria cultivated in vitro have a generation time of 15–30 minutes.
- The same bacteria may take hours to reproduce in vivo. Obligate anaerobes grow much more slowly than aerobes; this is true in vitro as well.
- Tuberculosis bacteria have an in-vitro generation time of 12–24 hours. Of course the generation time also depends on the nutrient content of the medium.
- The so-called normal growth curve for bacteria is obtained by inoculating a nutrient broth with bacteria the metabolism of which is initially quiescent, counting them at intervals and entering the results in a semi log coordinate system. Unicellular growth usually occurs in a series of different phases (Figure



1- Lag phase.

- When an inoculum of bacteria is first introduced into some growth medium, it will probably require a period to adapt to its new surroundings –the less familiar these are, the longer the period of adaptation .
- If, for example, the carbon source in the medium is unfamiliar, the cells will need time to synthesise the necessary enzymes for its metabolism.
- The length of the lag phase will also depend on the age and general health of the cells in the inoculum. During this period, there is no net increase in bacterial numbers, however the cells are metabolically active.

2- Log (exponential) phase.

- When the bacteria have acclimatised to their new environment and synthesised the enzymes needed to utilise the available substrates, they are able to start regular division by binary fission.
- This leads to the exponential increase in numbers referred to above.
- Under optimal conditions, the population of cells will double in a constant and predictable length of time, known as the generation (doubling) time.
- The value for the widely used laboratory bacterium *E. coli* is 20 min, and for most organisms it is less than an hour.
- The generation time can only be determined during phase C, either graphically or by determining the cell count (n) at two different times and applying the formula:

$$g = \frac{t_2 - t_1}{\log_2 n_2 - \log_2 n_1} .$$

3- Stationary phase.

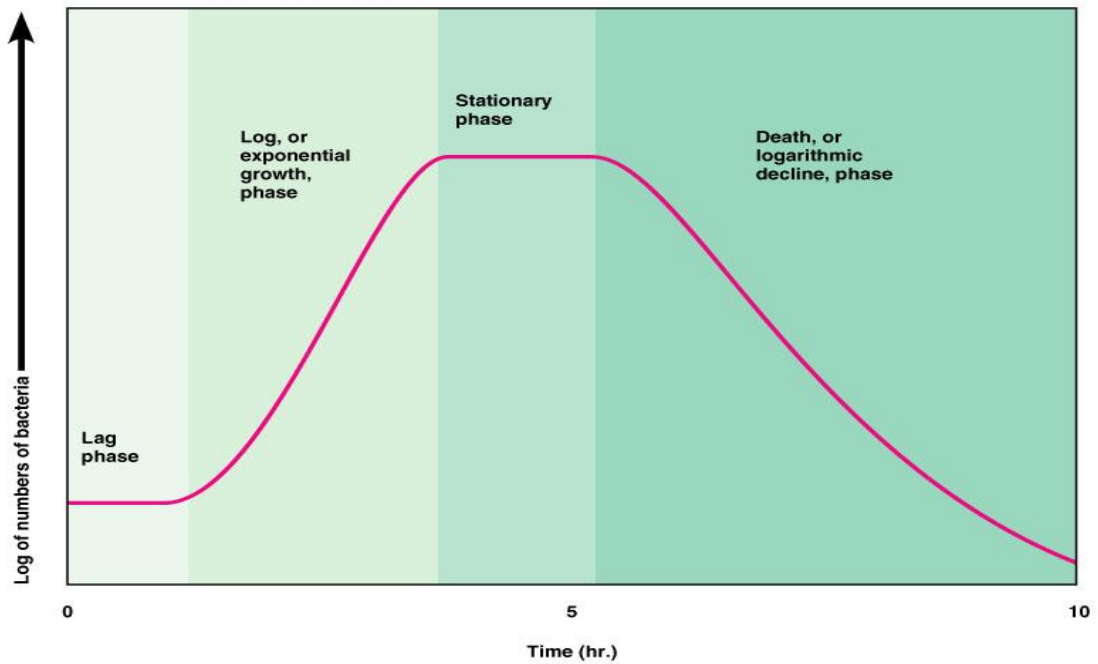
- As discussed above, the exponential phase is limited by environmental factors, and as the rate of growth slows down, the culture enters the next phase.
- The levelling out of the growth curve does not mean that cell division has ceased completely, but rather that the increase due to newly formed cells is

cancelled out by a similar number of cell deaths. Eventually, however, as the

death rate increases, the overall numbers fall and we enter the final phase of growth.

4- Death phase.

- As cells die off and the culture is unable to replace them, the total population of viable cells falls. This is the death (or decline) phase.



6. Bacterial Metabolism

6.1. Types of Metabolism

- Metabolism is the totality of chemical reactions occurring in bacterial cells.
- They can be subdivided into anabolic (synthetic) reactions that consume energy and catabolic reactions that supply energy. In the anabolic, endergonic reactions, the energy requirement is consumed in the form of light or chemical energy—by photosynthetic or chemosynthetic bacteria, respectively. Catabolic reactions supply both energy and the basic structural elements for synthesis of specific bacterial molecules. Bacteria that feed on inorganic nutrients are said to be lithotrophic, those that feed on organic nutrients are organotrophic.

Catabolic Reactions

- Organic nutrient substrates are catabolized in a wide variety of enzymatic
- processes that can be schematically divided into four phases:

1-Digestion.

- Bacterial exoenzymes split up the nutrient substrates into smaller molecules outside the cell.
- The exoenzymes represent important pathogenicity factors in some cases.
- Nutrients can be taken up by means of passive diffusion or, more frequently, specifically by active transport through the membrane(s).
- Cytoplasmic membrane permeases play an important role in these processes.

2- Preparation for oxidation.

- Splitting off of carboxyl and amino groups, phosphorylation, etc.

3- Oxidation.

- This process is defined as the removal of electrons and H^+ ions. The substance to which the H^2 atoms are transferred is called the hydrogen acceptor.
- The two basic forms of oxidation are defined by the final hydrogen acceptor.

Aerobic Respiration.

- Here oxygen is the hydrogen acceptor.
- In anaerobic respiration, the O_2 that serves as the hydrogen acceptor is a component of an inorganic salt like SO_4 , NO_3 Fermentation.
- Here an organic compound serves as the hydrogen acceptor.
- The main difference between fermentation and respiration is the energy yield, which can be greater from respiration than from fermentation for a given nutrient substrate by as much as a factor of 10.
- Fermentation processes involving microorganisms are designated by the final product, e.g., alcoholic fermentation, butyric acid, Lactic acid fermentation, etc.
- The energy released by oxidation is stored as chemical energy in the form of a thioester (e.g., acetyl-CoA) or organic phosphates (e.g., ATP).

Bacterial Oxidation Pathways

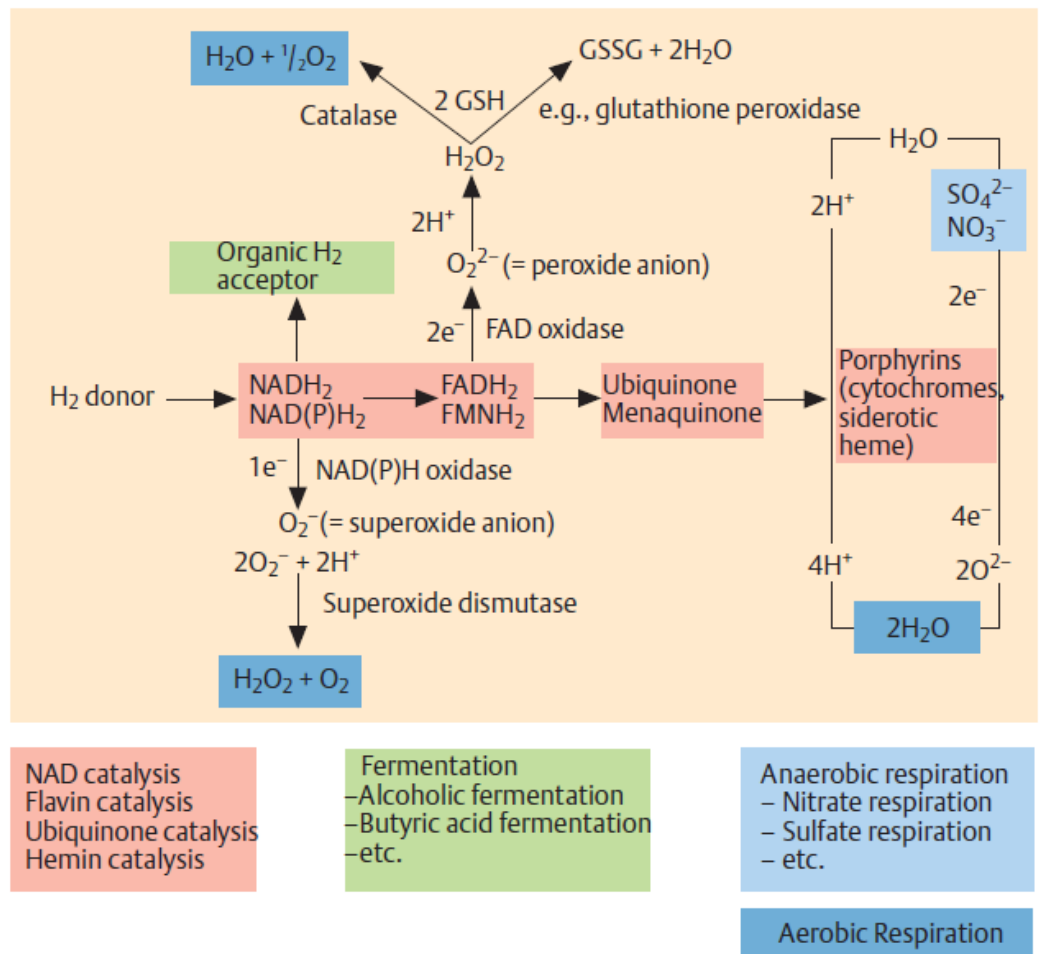


Fig. 3.15 In oxidation of organic nutrient substrates, protons (H^+) and electrons (e^-) are transferred in more or less long chains. The respiration is aerobic when the final electron acceptor is free oxygen. Anaerobic respiration is when the electrons are transferred to inorganically bound oxygen. Fermentation is the transfer of H^+ and e^- to an organic acceptor.

The role of oxygen. Oxygen is activated in one of three ways:

- Transfer of $4e^-$ to O_2 , resulting in two oxygen ions ($2 O^{2-}$).
- Transfer of $2e^-$ to O_2 , resulting in one peroxide anion ($1 O_2^{2-}$).
- Transfer of $1e^-$ to O_2 , resulting in one superoxide anion ($1 O_2^-$).
- Hydrogen peroxide and the highly reactive superoxide anion are toxic and therefore must undergo further conversion immediately by Catalase, Peroxidase and super oxide dismutase enzymes respectively.

Anabolic Reactions:

- **The anabolic** (synthetic) reactions that consume energy to building up the most important macromolecules needed during growth.
- Some bacteria (E. coli) are capable of synthesizing all of the complex organic molecules that they are comprised of, from the simplest nutrients in a very short time.
- These capacities are utilized in the field of microbiological engineering.
- Antibiotics, amino acids, and vitamins are produced with the help of bacteria.
- Some bacteria are even capable of using aliphatic hydrocarbon compounds as an energy source.
- Such bacteria can “feed” on paraffin or even raw petroleum. It is hoped that the metabolic capabilities of these bacteria will help control the effects of oil spills in surface water.
- Bacteria have also been enlisted in the fight against hunger: certain bacteria and fungi are cultivated on aliphatic hydrocarbon substrates, which supply carbon and energy, then harvested and processed into a protein powder (single cell protein).
- Culturing of bacteria in nutrient media based on methanol is another approach being used to produce biomass.

Glycolysis and TCA cycle Example of catabolic reaction:

- The initial sequence of reactions, in which a molecule of glucose is converted to two molecules of pyruvate*, is called glycolysis (Figures).
- In the first phase of glycolysis, glucose is phosphorylated and its six-carbon ring structure rearranged, before being cleaved into two three-carbon molecules.
- In the second phase, each of these undergoes oxidation, resulting in pyruvate to yield energy in form of ATP.
- The pathway, which takes place in the cytoplasm, comprises a series of 10 linked reactions, in which each molecule of the six-carbon glucose is converted to two molecules of the three carbon pyruvate, with a net gain of two molecules of ATP.
- The reactions by which ATP is generated from ADP in the second phase of glycolysis are examples of substrate-level phosphorylation, so-called because the phosphate group is transferred directly from a donor molecule.

- What happens next to the pyruvate produced by glycolysis depends on the organism concerned, and on whether the environment is aerobic or anaerobic; we shall look at these possibilities in due course.
- Respiration is the term used to describe those ATP generating processes, aerobic or anaerobic, by which oxidation of a substrate occurs, with an inorganic substance acting as the final electron acceptor.
- In aerobic respiration, that substance is oxygen; in anaerobic respiration, a substance such as nitrate or sulphate can fulfil the role.

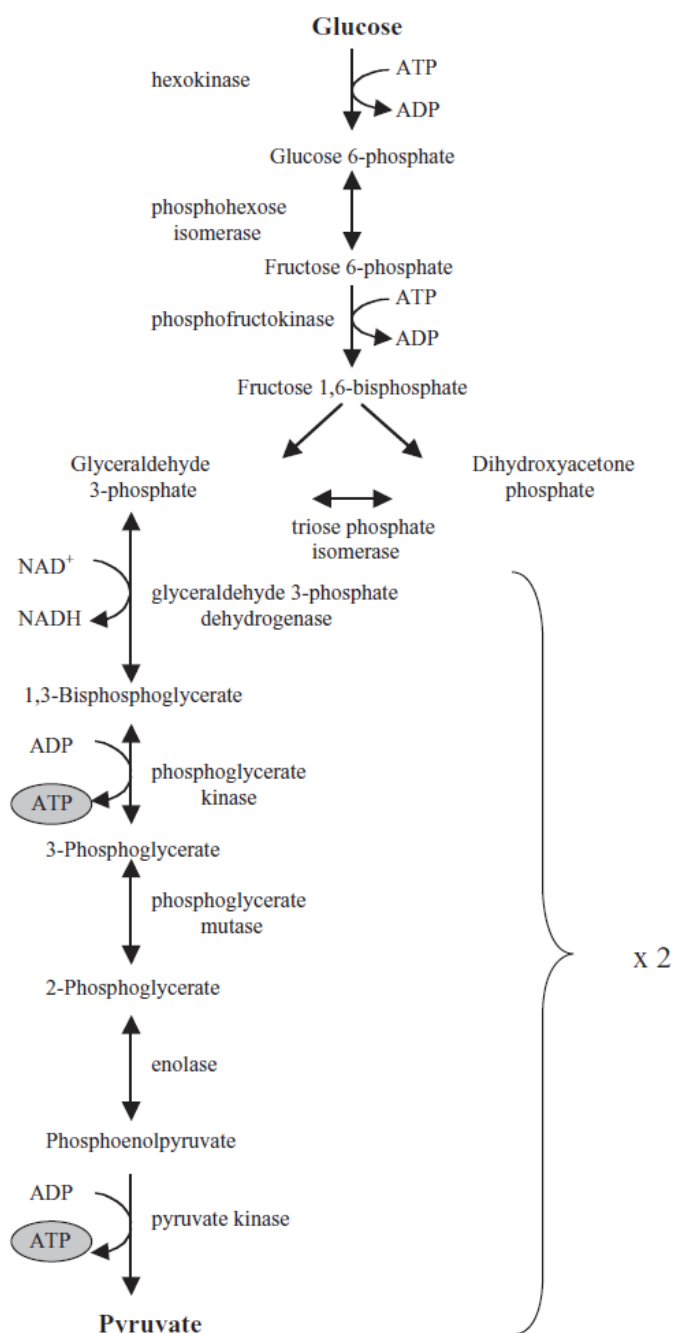
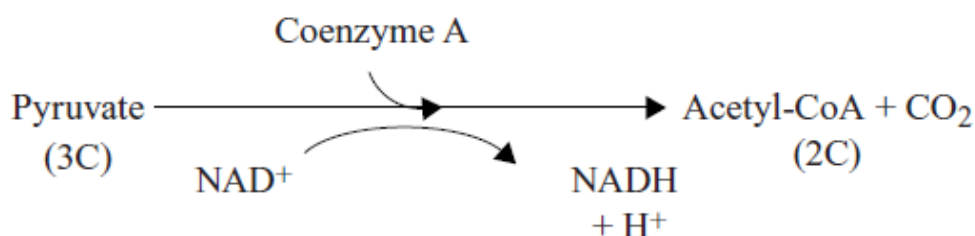


Figure 6.17 Glycolysis: a more detailed look. Glycolysis comprises 10 separate enzyme-catalysed reactions. Of the two 3-carbon compounds formed in the first stage, dihydroxyacetone phosphate cannot directly enter the later part of the pathway, but must first be converted to its isomer glyceraldehyde-3-phosphate. For each molecule of glucose, two molecules of each compound are therefore produced from this point onwards, and the yield of ATP and NADH is likewise doubled

- In most aerobic organisms, the pyruvate is completely oxidised to CO₂ and water by entering the tricarboxylic acid (TCA) cycle, also known as the Krebs cycle or simply the citric acid cycle (Figure 6.20). During this cycle, a series of redox reactions result in the gradual transfer of the energy contained in the pyruvate to coenzymes (mostly NADH). This energy is finally conserved in the form of ATP by a process of oxidative phosphorylation.
- Pyruvate does not itself directly participate in the TCA cycle, but must first be converted into the two-carbon compound acetyl-Coenzyme A:



PRINCIPLES OF ENERGY GENERATION

129

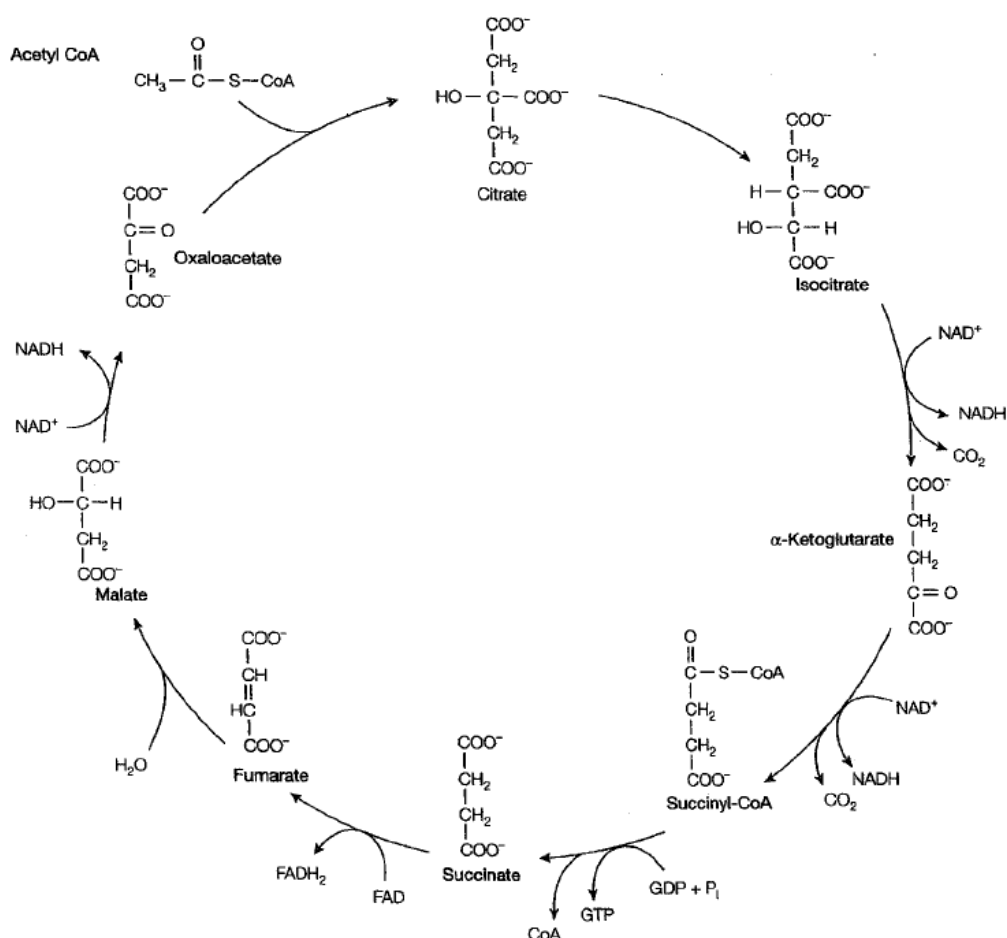


Figure 6.20 The TCA cycle. Acetyl-CoA may derive from the pyruvate of glycolysis or from lipid or amino acid metabolism. It joins with the four-carbon oxaloacetate to form the six-carbon citric acid. Two decarboxylation steps reduce the carbon number back to four and oxaloacetate re-enters the cycle once more. Although no ATP results directly from the cycle, the third phosphate on GTP can be easily transferred to ADP ($\text{GTP} + \text{ADP} = \text{GDP} + \text{ATP}$), thus generating one molecule of ATP per cycle. In addition, substantial reducing power is generated in the form of NADH and FADH₂. These carry electrons to the electron transport chain, where further ATPs are generated

- Several of the intermediate molecules in the TCA cycle also act as precursors in other pathways, such as the synthesis of amino acids, fatty acids or purines and pyrimidines (Anabolic metabolism, below).
- Other pathways regenerate such intermediates for continued use in the TCA cycle.
- Most of the energy originally stored in the glucose molecule is now held in the form of the reduced coenzymes (NADH and FADH₂) produced during glycolysis and the TCA cycle.

- This is now converted to no less than 34 molecules of ATP per glucose molecule by oxidative phosphorylation in the remaining steps in aerobic respiration (three from each molecule of NADH and two from each of FADH₂).

Oxidative phosphorylation and the electron transport chain

- The purpose of the electron transport is the same for all systems, however, that is, the transfer of electrons from NADH/FADH₂ via a series of carriers to, ultimately, oxygen. Around half of the energy released during this process is conserved as ATP.
- The carrier molecules, which act alternately as acceptors and donors of electrons, are mostly complex modified proteins such as flavoproteins and cytochromes, together with a class of lipid-soluble molecules called ubiquinones (also called coenzyme Q).
- The carriers are arranged in the chain such that each one has a more positive redox potential than the previous one.
- In the first step in the chain, NADH passes electrons to flavin mononucleotide (FMN), and in so doing becomes converted back to NAD⁺, thereby ensuring a ready supply of the latter for the continuation of glycolysis (Figure).
- From FMN, the electrons are transferred to coenzyme Q, and thence to a series of cytochromes; at each transfer of electrons the donor reverts back to its oxidised form, ready to pick up more electrons
- The final cytochrome in the chain transfers its electrons to molecular oxygen,
- which, as we have seen, acts as the terminal oxygen acceptor.
- The negatively charged oxygen combines with protons from its surroundings to form water.
- Four electrons and protons are required for the formation of each water molecule:



PRINCIPLES OF ENERGY GENERATION

131

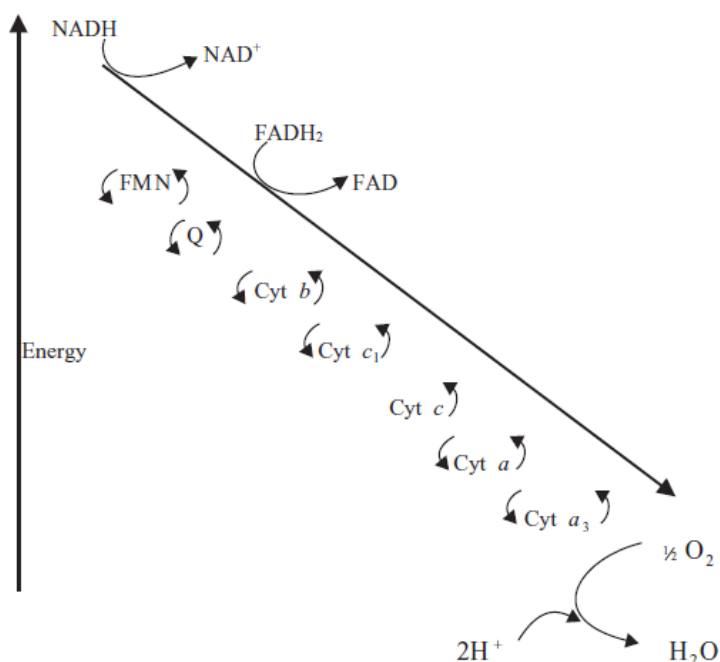
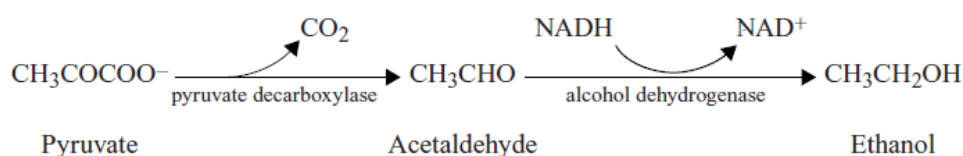


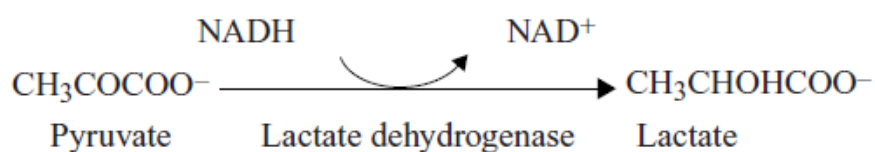
Figure 6.21 The electron transport chain. Electrons from NADH and FADH₂ pass from one electron carrier to another, with a gradual release of energy as ATP by chemiosmosis (see Figure 6.22). The electron carriers are arranged in order of their reduction potential (tendency to gain electrons) and oscillate between the oxidised and the reduced state. FMN = flavin mononucleotide, Q = coenzyme Q.

Fermentation:

- A microbial process by which an organic substrate (usually a carbohydrate) is broken down without the involvement of oxygen or an electron transport chain, generating energy by substrate level phosphorylation.
- Two common fermentation pathways result in the production of respectively lactic acid and ethanol. Both are extremely important in the food and drink industries.
- Alcoholic fermentation, which is more common in yeasts than in bacteria, results in pyruvate being oxidised via the intermediate compound acetaldehyde to ethanol.



variety of microorganisms carry out lactic acid fermentation. Some, such as *Streptococcus*, *E. coli* and *Lactobacillus*, produce lactic acid as the only end product; this is referred to as homolactic fermentation.



Glucogenesis is example of Anabolic Reaction

- Biosynthesis of carbohydrates (photoautotrophic ones) are able to incorporate inorganic carbon as CO_2 or HCO_3^- into hexose sugars, most commonly via the Calvin cycle.
- Heterotrophic organisms are unable to do this, and must convert a range of organic compounds into glucose by a series of reactions called gluconeogenesis (Figure

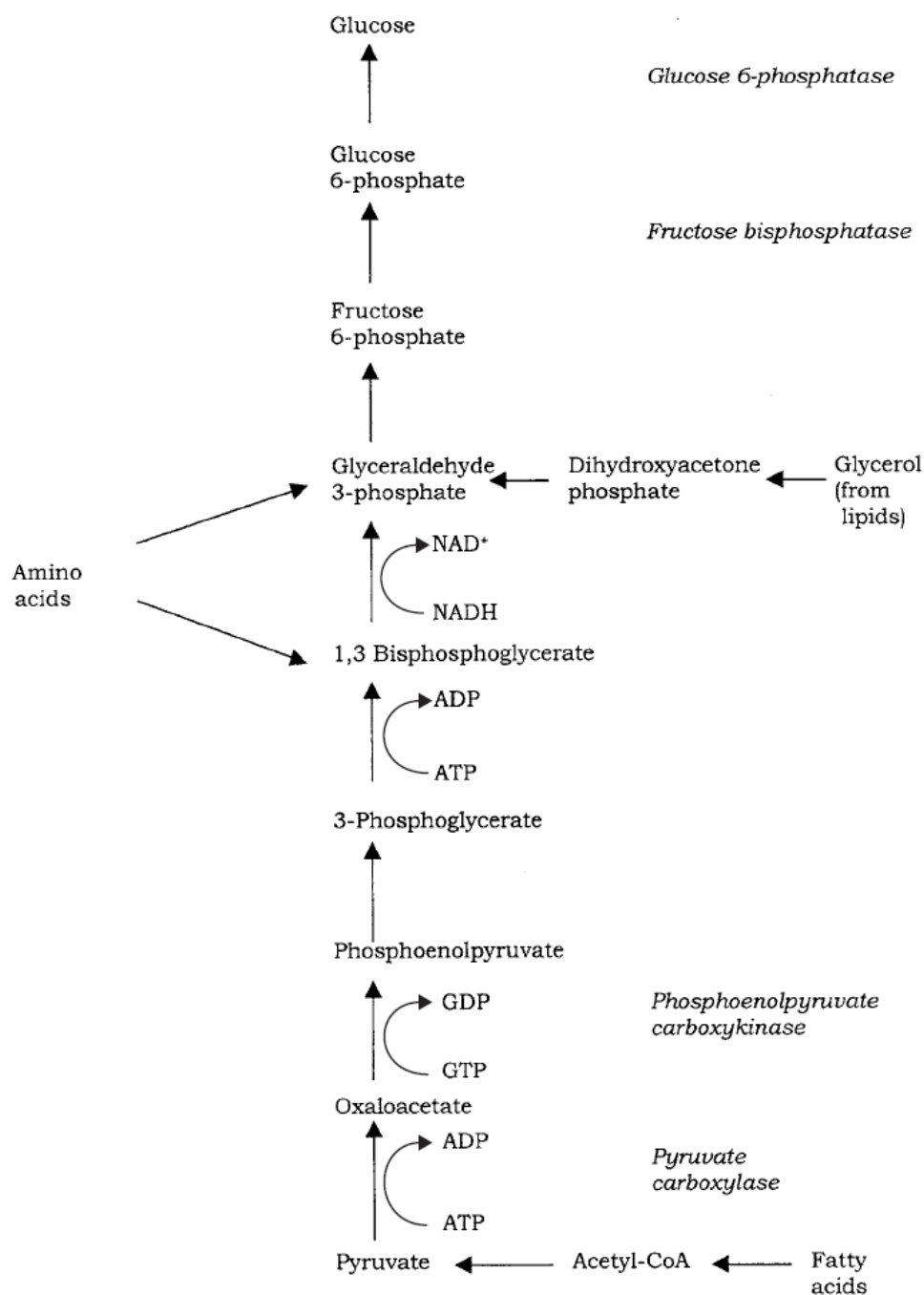


Figure 6.34 Gluconeogenesis. Non-carbohydrate precursors can feed into a pathway that converts pyruvate to glucose in a series of reactions that are mostly the reverse of glycolysis. Enzymes not found in glycolysis are shown in italics

ANABOLIC REACTIONS

1:

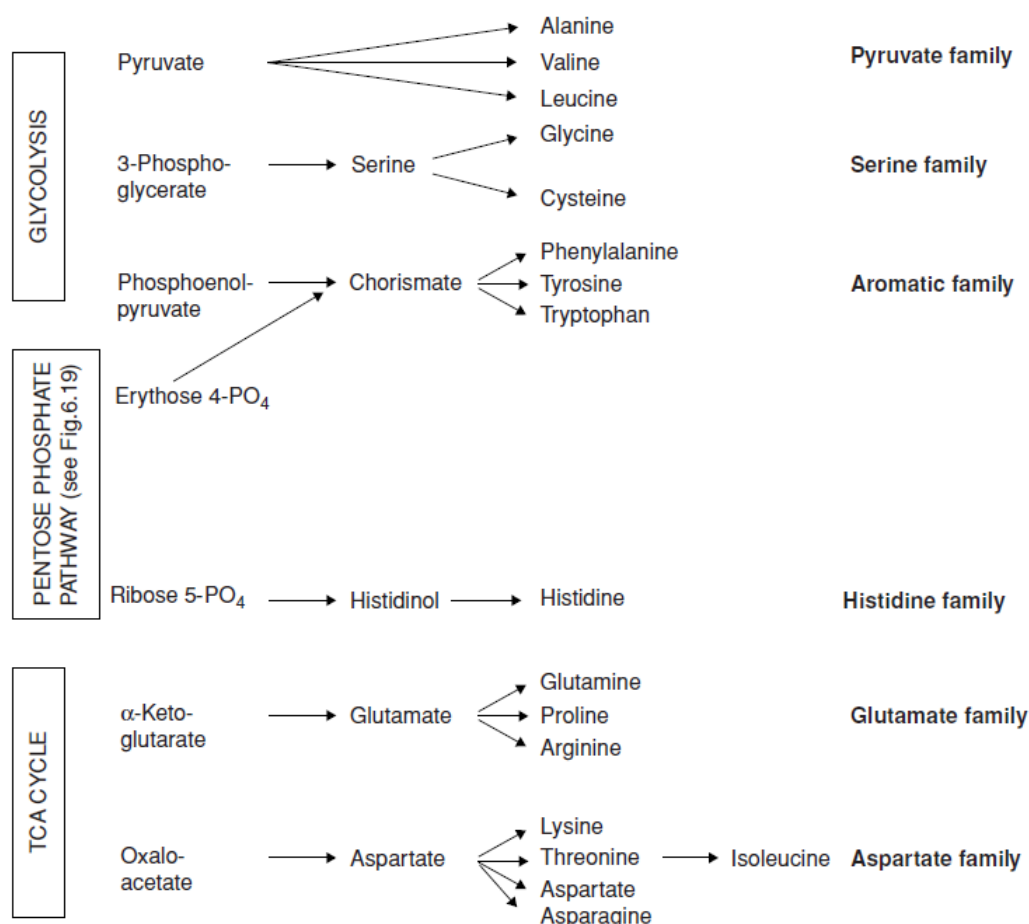
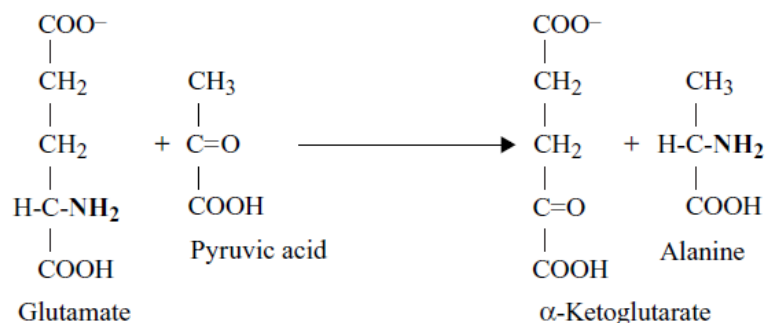


Figure 6.37 Amino acid biosynthesis. The carbon skeleton of amino acids is obtained from a limited number of precursor molecules, mainly intermediates in glycolysis or the TCA cycle. The amino group originally derives from inorganic sources, but can then be transferred from one organic molecule to another

7. Types of media

- We can classify The Media according To Their Action as the Following:
 - **Enrichment media:** designed to increase numbers of desired microbes to detectable levels
 - **Minimal media:** have low nutrients supporting growth of bacteria live in lownutrients environment.
 - **Differential media :** Differentiation of colonies of desired microbes from others.
 - **Selective media:** Suppression of unwanted microbes; encouraging desired microbes.
 - **Reducing media :** Growth of obligate anaerobes.
 - **Transport media:** Highly buffering media used to transport and maintain microorganism in limited time to analysis.
 - **Chromogenic media:**
 - Highly selective media depends on chromo complex compound resulting from specific enzyme reaction which give specific colored colony specific for certain organism and dose not need further identification.
 - **Chemically defined media:** antibiotic assay and vitamin assay.
- We can also classifying the media according to physical state of the media to liquid media, semisolid media, and solid media).

• Media Used In Water

• Media used in HPC:

• R2A:

• Ingredients:

Approximate Formula* Per Liter

Yeast Extract.....	0.5 g
Proteose Peptone No. 3.....	0.5 g
Casamino Acids.....	0.5 g
Dextrose.....	0.5 g
Soluble Starch.....	0.5 g
Sodium Pyruvate.....	0.3 g
Dipotassium Phosphate.....	0.3 g
Magnesium Sulfate.....	0.05 g
Agar.....	15.0 g

- **Mode of Action:**

The low concentration of yeast extract, casein hydrolysate, peptone and glucose allows a wide spectrum of bacteria to grow without the fast-growing bacteria suppressing the slow-growing species, such as would be the case on richly nutritious media like e.g. Plate Count Agar. The content of starch and pyruvate allows particularly the injured bacteria to grow again more quickly.

- **Nutrient agar:**

Approximate Formula* Per Liter

Beef Extract.....	3.0 g
Peptone.....	5.0 g
Sodium Chloride.....	8.0 g
Agar.....	15.0 g

- **Mode of Action:**

Early in the 20th century, the American Public Health Association published the formula for a general purpose medium for the growth of a wide variety of nonfastidious microorganisms. Nutrient Agar consists of peptone, beef extract and agar. This relatively simple formulation provides the nutrients necessary for the replication of a large number of microorganisms that are not excessively fastidious. The beef extract contains water soluble substances including carbohydrates, vitamins, organic nitrogen compounds and salts. Peptones are the principle sources of organic nitrogen, particularly amino acids and long-chained peptides. Agar is the solidifying agent.

- **Media used in detection of total and fecal coliforms:**

- **Lauryl Sulfate Broth**

Approximate Formula* Per Liter

Pancreatic Digest of Casein.....	20.0 g
Lactose.....	5.0 g
Dipotassium Phosphate.....	2.75 g
Monopotassium Phosphate.....	2.75 g
Sodium Chloride.....	5.0 g
Sodium Lauryl Sulfate.....	0.1 g

- **Mode of Action:**

The fermentation of lactose with gas formation is a presumptive test for coliforms.

Sodium lauryl sulfate inhibits organisms other than coliforms.

- **Brilliant Green Bile Broth :**

Approximate Formula* Per Liter

Peptone 10.0 g

Oxgall..... 20.0 g

Lactose..... 10.0 g

Brilliant Green..... 13.3 mg.

- **Mode of Action:**

Brilliant Green Bile Broth 2% contains two inhibitors of both gram-positive and selected gram-negative organisms; i.e., oxgall and brilliant green dye. Organisms, primarily coliforms, which are resistant to the action of the inhibitors and which ferment the lactose, are able to replicate in this medium. Fermentation is detected by gas production. Gas production within 48 ± 3 hours is considered positive evidence of fermentation by coliform bacilli.

- **m-Endo Agar:**

Approximate Formula* Per Liter

Dipotassium Phosphate..... 3.5 g

Peptic Digest of Animal Tissue..... 10.0 g

Agar..... 10.0 g

Sodium Sulfite..... 2.5 g

Basic Fuchsin..... 0.5 g.

- **Mode of Action:**

Endo Agar is a differential and slightly selective culture medium for the detection of coliform and other enteric microorganisms. Selectivity of Endo Agar is due to the sodium sulfite/basic fuchsin combination, which results in the suppression of gram positive microorganisms. Coliforms ferment the lactose, produce pink to rose-red colonies and similar coloration of the medium.

• **m Endo Agar LES(Lawrence Experimental Station).**

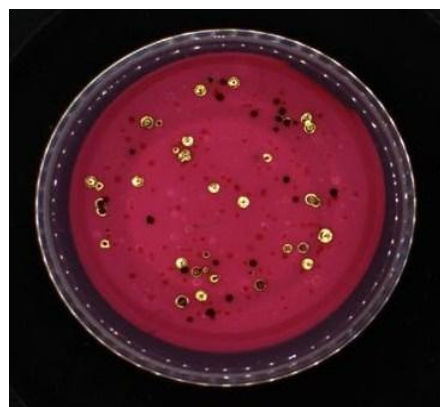
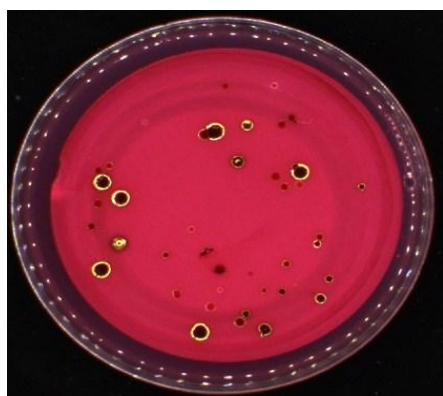
Approximate Formula* Per Liter containing 20 mL of 95% ethanol.

Yeast Extract	1.2 g
Casitone.....	3.7 g
Thiopeptone.....	3.7 g
Tryptose.....	7.5 g
Lactose.....	9.4 g
Dipotassium Phosphate.....	3.3 g
Monopotassium Phosphate.....	1.0 g
Sodium Chloride.....	3.7 g
Sodium Desoxycholate.....	0.1 g
Sodium Lauryl Sulfate.....	0.05 g
Sodium Sulfite.....	1.6 g
Basic Fuchsin.....	0.8 g
Agar.....	15.0 g

• **Mode of Action:**

Sodium desoxycholate and sodium lauryl sulfate are added as inhibitors. Basic fuchsin is a pH indicator. Sodium sulfite is added to decolorize the basic fuchsin solution. Agar is the solidifying agent. Lactose-fermenting bacteria produce acetaldehyde that reacts with the sodium sulfite and fuchsin to form red colonies. The development of a metallic sheen occurs when the organism produces aldehydes with the rapid fermentation of lactose. If the inoculum is too heavy, the sheen will be suppressed. Lactose-non fermenting bacteria form clear, colorless colonies.

Coliforms in m-Endo LES agar



• **m FC Agar WITH 1% ROSOLIC ACID:**

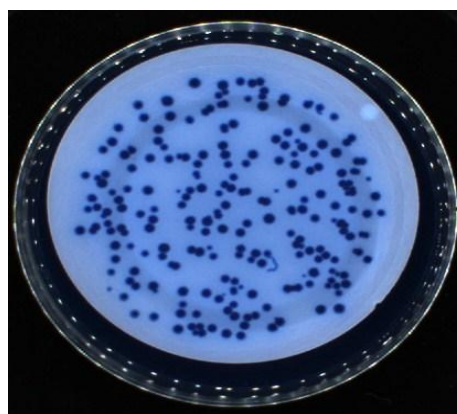
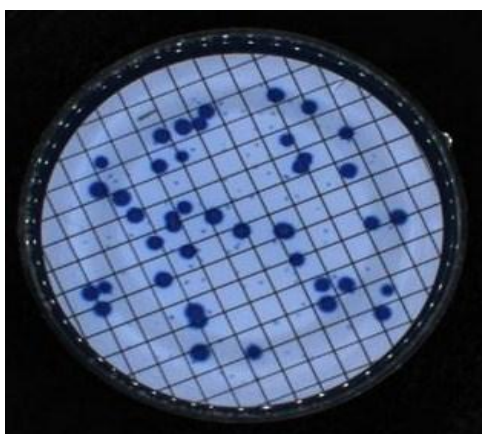
Approximate Formula* Per Liter

Tryptose.....	10.0 g
Proteose Peptone No. 3.....	5.0 g
Yeast Extract.....	3.0 g
Lactose.....	12.5 g
Bile Salts No. 3.....	1.5 g
Sodium Chloride.....	5.0 g
Agar.....	15.0 g
Aniline Blue.....	0.1 g
Rosolic Acid.....	1 g/vial

• **Mode of Action:**

m FC Agar and m FC Broth Base contain peptones as sources of carbon, nitrogen, vitamins and minerals. Yeast extract supplies B-complex vitamins that stimulate bacterial growth. Lactose is a carbohydrate. Bile Salts No. 3 inhibits growth of gram-positive bacteria. m FC Agar contains agar as the solidifying agent. The differential indicator system combines aniline blue and rosolic acid. Colonies of fecal coliforms are blue; non-fecal coliforms and other organisms are gray to cream-colored.

.Fecal coliforms in mfc agar:



- **Media for fecal streptococci:**

- **Azide Dextrose Broth:**

Approximate Formula* Per Liter

Beef Extract.....	4.5 g
Pancreatic Digest of Casein.....	7.5 g
Proteose Peptone No. 3.....	7.5 g
Dextrose.....	7.5 g
Sodium Chloride.....	7.5 g
Sodium Azide.....	0.2 g

- **Mode of Action:**

Azide Dextrose Broth contains beef extract and peptones as sources of carbon, nitrogen, vitamins and minerals. Dextrose is a fermentable carbohydrate. Sodium chloride maintains the osmotic balance of the Sodium azide inhibits cytochrome oxidase in gram-negative bacteria. Group medium. D streptococci grow in the presence of azide, ferment glucose and cause turbidity



• m Enterococcus Agar

Approximate Formula* Per Liter

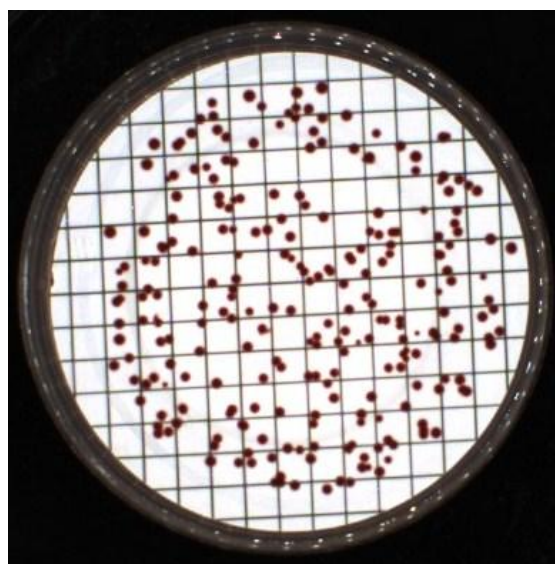
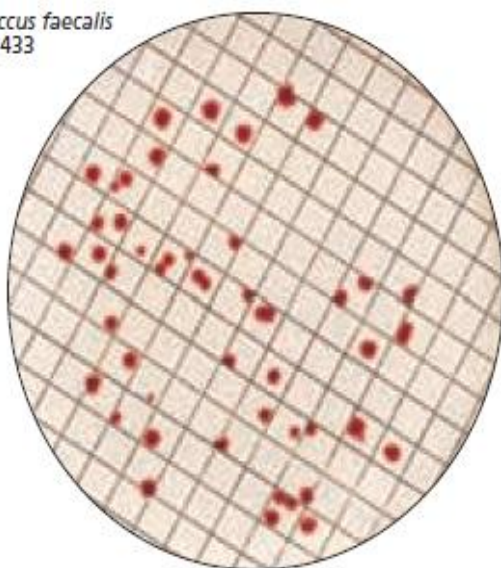
Tryptose.....	20.0 g
Yeast Extract.....	5.0 g
Dextrose.....	2.0 g
Dipotassium Phosphate.....	4.0 g
Sodium Azide.....	0.4 g
Agar.....	10.0 g
2,3,5-Triphenyl Tetrazolium Chloride.....	0.1 g

• Mode of Action:

The enterococcus group is a subgroup of the fecal streptococci that includes *E. faecalis*, *E. faecium*, *E. gallinarum*, and *E. avium*.¹ Enterococci are differentiated from other streptococci by their ability to grow in 6.5% sodium chloride, at pH 9.6 and at 10°C and 45°C. Sodium azide is the selective agent to suppress the growth of gram-negative organisms. Agar is the solidifying agent. Triphenyl tetrazolium chloride (TTC) is the dye used as an indicator of bacterial growth. TTC is reduced to the insoluble formazan inside the bacterial cell, resulting in the production of red colonies.

E. faecalis in m enterococcus agar.

Enterococcus faecalis
ATCC™ 19433



• Bile Esculin Agar:

Approximate Formula* Per Liter

Pancreatic Digest of Gelatin..... 5.0 g

Beef Extract.... 3.0 g

Oxgall..... 20.0 g

Ferric Citrate..... 0.5 g

Esculin..... 1.0 g

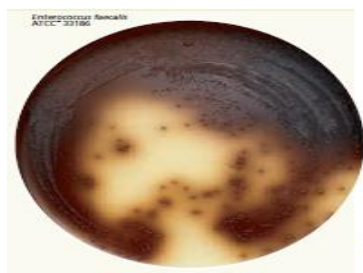
Agar 14.0 g.

• Mode of Action:

The major use of Bile Esculin Agar is to differentiate between enterococci/Group D streptococci and non Group D streptococci. It may also be used for the presumptive identification of other groups of organisms Enterococci/Group D streptococci hydrolyses esculin to form esculetin and dextrose. Esculetin combines with ferric citrate in the medium to form a dark brown or black complex which is indicative of a positive result. Bile salts will inhibit Gram-positive bacteria other than enterococci/Group D streptococci..

Expected Results

Any blackening of the plated medium indicates a positive result; if no blackening occurs the test is negative



• Brain Heart Infusion Broth with 6.5 % Na Cl:

BHI Broth is a nutritious, buffered culture medium that contains infusions of brain and heart tissue and peptones to supply protein and other nutrients necessary to support the growth of fastidious and nonfastidious microorganisms.

In the formulation containing 6.5% sodium chloride, the salt acts as a differential and/or selective agent by interfering with membrane permeability and osmotic and electro kinetic equilibria in salt-intolerant organisms. The broth medium that contains 6.5% sodium chloride is used to differentiate the enterococci from nonenterococcal group D streptococci by the 6.5% salt tolerance test.

8. Control of Microorganisms

Introduction

- Inhibiting the growth of microorganisms is termed antisepsis, while destroying them is called disinfection.
- Disinfection does not mean that all microbial forms are killed.
- The total destruction of all microbial forms is called sterilization.
- However, disinfection does reduce the number of disease-causing organisms to an acceptable number.
- Growing, thriving bacteria are easy to control by disinfection.
- Some bacteria, however, form spores — survival structures — that are much more difficult to destroy.
- It is difficult for the disinfection to penetrate the protective shell of the spore material.

Terms

- **Sterilization** is the killing of all microorganisms and viruses or their complete elimination from a material with the highest possible level of certainty. An object that has been subjected to a sterilization process, and then packaged so as to be contamination-proof, is considered sterile.
- **Disinfection** is a specifically targeted antimicrobial treatment with the objective of preventing transmission of certain microorganisms.
- The purpose of the disinfection procedure is to render an object incapable of spreading infection.
- **Preservation** is a general term for measures taken to prevent microbe caused spoilage of susceptible products (pharmaceuticals, foods). Decontamination is the removal or count reduction of microorganisms contaminating an object.
- The objective of aseptic measures and techniques is to prevent microbial contamination of materials or wounds.

In antiseptic measures, chemical agents are used to fight pathogens in or on living tissue, for example in a wound.

8.1. Principles of Sterilization and Disinfection

- **Sterilization** is defined as the killing or removal of all microorganisms and viruses from an object or product.
- **Disinfection** means rendering an object, the hands or skin free of pathogens.
- The term asepsis covers all measures aiming to prevent contamination of objects or wounds.
- Disinfection and sterilization makes use of both physical and chemical agents. The killing of microorganisms with these agents is exponential.
- A measure of the efficacy of this process is the D value (decimal reduction time), which expresses the time required to reduce the organism count by 90%. The sterilization agents of choice are hot air (180 °C, 30 minutes; 160 °C, 120 minutes) or saturated water vapor (121 °C, 15 minutes, 2.02 × 10⁵ Pa; 134 °C, three minutes, 3.03 × 10⁵ Pa).
- **Gamma rays** or high-energy electrons are used in radio sterilization at a recommended dose level of 2.5 × 10⁴ Gy. disinfection is usually done with chemical agents, the most important of which are aldehydes (formaldehyde), alcohols, phenols, halogens (I, Cl), and surfactant detergents.

Mechanisms of Action

- When microorganisms are killed by heat, their proteins (enzymes) are irreversibly denatured. Ionizing radiation results in the formation of reactive groups that contribute to chemical reactions affecting DNA and proteins.
- Exposure to UV light results in structural changes in DNA (thymine dimers) that prevent it from replicating.
- This damage can be repaired to a certain extent by light (photo reactivation). Most chemical agents (alcohols, phenols, aldehydes, heavy metals, oxidants) denature proteins irreversibly.
- Surfactant compounds (amphoteric and cationic) attack the cytoplasmic membrane.
- Acridine derivatives bind to DNA to prevent its replication and function (transcription).

8.2.

8.3. Physical Methods of Sterilization and Disinfection

Heat

- The application of heat is a simple, cheap and effective method of killing pathogens.
- Methods of heat application vary according to the specific application.& Pasteurization.
- This is the antimicrobial treatment used for foods in liquid form (milk):
- Low-temperature pasteurization: 61.5 8C, 30 minutes; 71 8C, 15 seconds.
- High-temperature pasteurization: brief (seconds) of exposure to 80–85 8C in continuous operation.
- Uperization: heating to 150 8C for 2.5 seconds in a pressurized container using steam injection & Disinfection.
- The guideline values for hot-air sterilizers are as follows: 180 8C for 30 minutes, 160 8C for 120 minutes, whereby the objects to be sterilized must themselves reach these temperatures for the entire prescribed period.& Moist heat sterilization.
- Autoclaves charged with saturated, pressurized steam are used for this purpose:
- — 121 8C, 15 minutes, one atmosphere of pressure (total: 202 kPa).
- — 134 8C, three minutes, two atmospheres of pressure (total: 303 kPa).
- In practical operation, the heating and equilibrating heat up and equalizing times must be added to these, i.e., the time required for the temperature in the most inaccessible part of the item(s) to be sterilized to reach sterilization level.
- When sterilizing liquids, a cooling time is also required to avoid boiling point retardation.
- The significant heat energy content of steam, which is transferred to the cooler sterilization items when the steam condenses on them, explains why it is such an effective pathogen killer.
- In addition, the proteins of microorganisms are much more readily denatured in a moist environment than under dry conditions.

Radiation

- Non ionizing radiation. & Ultra-violet (UV) rays (280–200 nm) are a type of Non ionizing radiation that is rapidly absorbed by a variety of materials.
- UV rays are therefore used only to reduce airborne pathogen counts (surgical theaters, filling equipment) and for disinfection of smooth surfaces.
- Ionizing radiation. Two types are used:

- Gamma radiation consists of electromagnetic waves produced by nuclear disintegration (e.g., of radioisotope ^{60}Co).
- Corpuscular radiation consists of electrons produced in generators and accelerated to raise their energy level.
- Radio sterilization equipment is expensive.
- On a large scale, such systems are used only to sterilize bandages, suture material, plastic medical items, and heat-sensitive pharmaceuticals.
- The required dose depends on the level of product contamination (bioburden) and on how sensitive the contaminating microbes are to the radiation.
- As a rule, a dose of 2.5×10^4 Gy (Gray) is considered sufficient. One Gy is defined as absorption of the energy quantum one joule (J) per kg.

Filtration

- Liquids and gases can also be sterilized by filtration.
- Most of the available filters catch only bacteria and fungi, but with ultrafine filters viruses and even large molecules can be filtered out as well.
- With membrane filters, retention takes place through small pores.
- The best-known type is the membrane filter made of organic colloids (e.g., cellulose ester).
- These materials can be processed to produce thin filter layers with gauged and calibrated pore sizes.
- In conventional depth filters, liquids are put through a layer of fibrous material (e.g., asbestos).
- The effectiveness of this type of filter is due largely to the principle of adsorption.
- Because of possible toxic side effects, they are now practically obsolete.

8.4. Chemical Methods of Sterilization and Disinfection

Ethylene oxide.

- This highly reactive gas (C_2H_4O) is flammable, toxic, and a strong mucosal irritant. Ethylene oxide can be used for sterilization at low temperatures (20–60 °C).
- The gas has a high penetration capacity and can even get through some plastic foils.
- One drawback is that this gas cannot kill dried microorganisms and requires a relative humidity level of 40–90% in the sterilizing chamber.
- Ethylene oxide goes into solution in plastics, rubber, and similar materials, therefore sterilized items must be allowed to stand for a longer period to ensure complete desorption.

Aldehydes.

- Formaldehyde ($HCHO$) is the most important aldehyde.
- It can be used in a special apparatus for gas sterilization.
- Its main use, however, is in disinfection. Formaldehyde is a water-soluble gas. Formalin is a 35% solution of this gas in water.
- Formaldehyde irritates mucosa; skin contact may result in inflammations or allergic eczemas.
- Formaldehyde is a broad-spectrum germicide for bacteria, fungi, and viruses. At higher concentrations, spores are killed as well.
- This substance is used to disinfect surfaces and objects in 0.5–5% solutions.
- In the past, it was commonly used in gaseous form to disinfect the air inside rooms (5 g/m³).
- The mechanism of action of formaldehyde is based on protein denaturation.
- Another aldehyde used for disinfection purposes is glutaraldehyde. With 2 % Alcohols.
- The types of alcohol used in disinfection are ethanol (70 %), propanol (60 %), and isopropanol (70%–80 %). Alcohols are quite effective against bacteria and fungi, less so against viruses.
- They do not kill bacterial spores. Due to their rapid action and good skin penetration, the main areas of application of alcohols are surgical and hygienic disinfection of the skin and hands.

- One disadvantage is that their effect is not long-lasting (no depot effect). Alcohols denature proteins.

Phenols.

- Lister was the first to use phenol (carbolic acid) in medical applications. Today, phenol derivatives substituted with organic groups and/or halogens (alkylated, arylated, and halogenated phenols), are widely used.
- One common feature of phenolic substances is their weak performance against spores and viruses. Phenols denature proteins.
- They bind to organic materials to a moderate degree only, making them suitable for disinfection of excreted materials.

Halogens.

- Chlorine, iodine, and derivatives of these halogens are suitable for use as disinfectants.
- Chlorine and iodine show a generalized microbicidal effect and also kill spores.
- Chlorine denatures proteins by binding to free amino groups; hypochlorous acid (HOCl), on the other hand, is produced in aqueous solutions, then disintegrates into HCl and $\frac{1}{2}$ O₂ and thus acts as a powerful oxidant.
- Chlorine is used to disinfect drinking water and swimming-pool water (up to 0.5 mg/l). Calcium hypochlorite (chlorinated lime) can be used in nonspecific disinfection of excretions.
- Chloramines are organic chlorine compounds that split off chlorine in aqueous solutions.
- They are used in cleaning and washing products and to disinfect excretions. Iodine has qualities similar to those of chlorine.
- The most important iodine preparations are the solutions of iodine and potassium iodide in alcohol (tincture of iodine) used to disinfect skin and small wounds.
- Iodophores are complexes of iodine and surfactants (e.g., polyvinyl pyrrolidone).
- While iodophores are less irritant to the skin than pure iodine, they are also less effective as germicides.

Oxidants.

- This group includes ozone, hydrogen peroxide, potassium permanganate, and peracetic acid.
- Their relevant chemical activity is based on the splitting off of oxygen. Most are used as mild antiseptics to disinfect mucosa, skin, or wounds.

Surfactants.

- These substances (also known as surface-active agents, ten sides, or detergents) include anionic, cationic, amphoteric, and nonionic detergent compounds, of which the cationic and amphoteric types are the most effective.
- The bactericidal effect of these substances is only moderate.
- They have no effect at all on tuberculosis bacteria (with the exception of amphotensides), spores, or non encapsulated viruses.
- Their efficacy is good against Gram-positive bacteria, but less so against Gram-negative rods.
- Their advantages include low toxicity levels, lack of odor, good skin tolerance, and a cleaning effect.

Summary of mechanisms of antibacterial action of antiseptics and disinfectants

Target	Antiseptic or Disinfectant	Mechanism of action
Cell envelop (cell wall ,outer membrane)	Glutaraldehyde EDTA, other permeabilizers	Cross linking of proteins Gram-negative bacteria : removal of Mg^{2+} and Ca^{2+} release of some LPS
Cytoplasmic (inner) membrane	QACs Chlorhexidine Diamines PHMB, alexidine Phenols and chlorinated phenol	Generalized membrane damage involving phospholipids bilayer Low concentration affect membrane integrity, high concentration cause congealing of cytoplasm Induction of leakage of amino acids Phase separation and domain formation of membrane lipids

		Leakage ; some cause uncoupling
Cross-linking of macromolecules	Formaldehyde Glutaraldehyde	Cross linking of protein, RNA & DNA. Cross linking of protein in cell envelop & elsewhere in the cell
DNA intercalation	Acridines	Intercalation of an acridines molecules between two layer of base pairs in DNA
Interaction with thiol group	Silver compound	Membrane-bound enzyme (Interaction with thiol group)
Effects on DNA	Halogens Hydrogen peroxides, Silver ions	Inhibition of DNA Synthesis DNA strand breakage
Oxidizing agent	Halogens Peroxygens	Oxidation of thiol group to sulfides, sulphoxides & disulphoxides Hydrogen peroxides : activity due to from formation of free hydroxyl radicals ($\cdot\text{OH}$) which oxidize thiol group in enzyme and proteins : PAA :disruption of thiol groups in proteins & enzymes

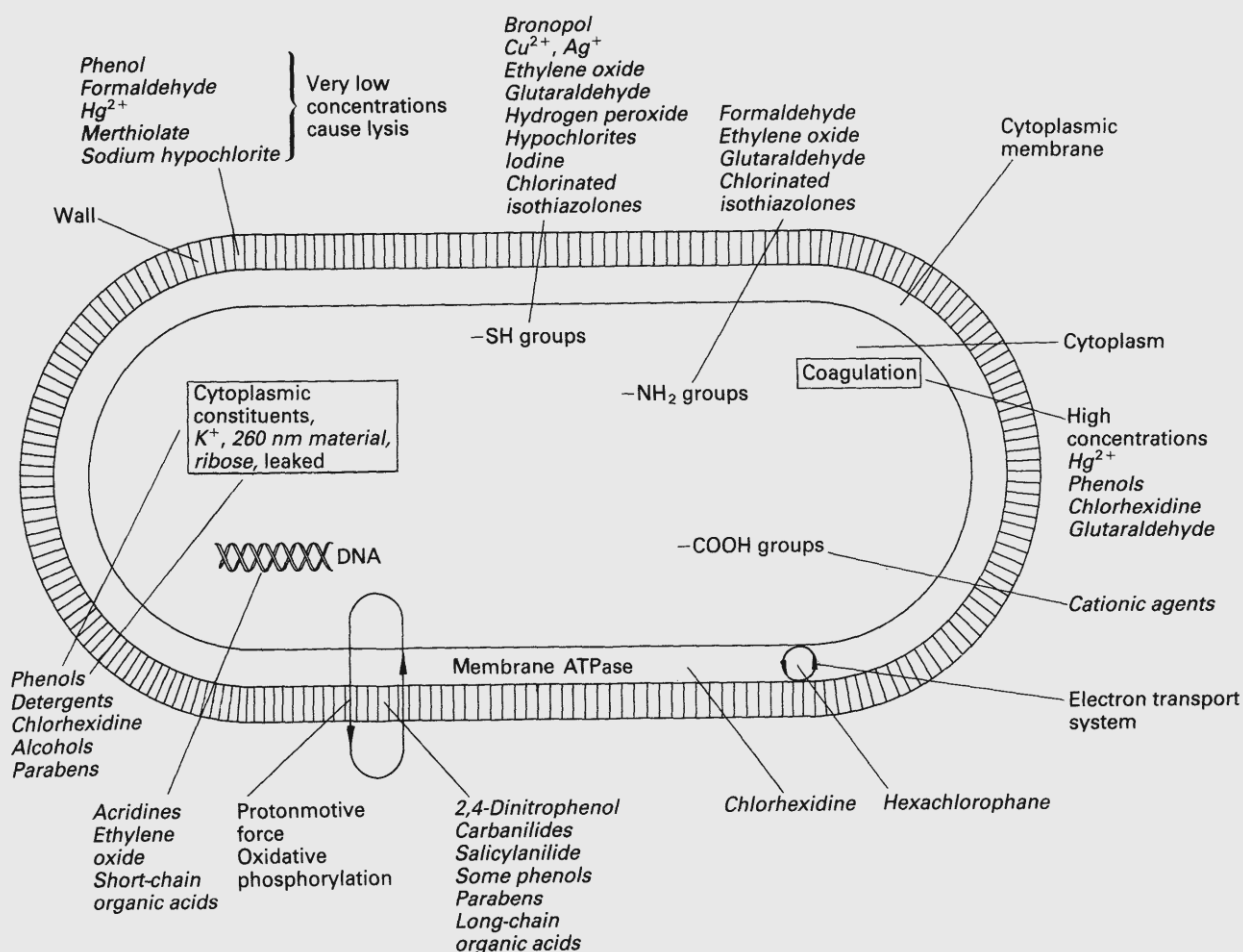


Fig. 12.1 Diagram showing main targets for non-antibiotic antibacterial agents.

Intrinsic bacterial resistance mechanisms to antiseptics and disinfectants (Gilbert and Brown 1995):

Type of resistance	Examples	Mechanism of resistance
Impermeability		
Gram-negative bacteria	QACs, triclosan, diamines	Barrier presented by outer membrane may prevent uptake of antiseptic or disinfectant, glycocalyx may also be involved
Mycobacteria	Chlorohexidine, QACs, Glutraldehyde	Waxy cell wall prevent adequate biocides entry Reason for resistance of <i>M. chelonae</i>
Bacterial Spores	Chlorhexidine, QACs, Phenolics	Spores coat (s) & cortex present a barrier of antiseptics and disinfectant
Gram-positive bacteria	Chlorhexidine	Glycocalyx / mucoexo -polysaccharide may be reduced diffusion of antiseptic.

Table 10.5 Properties of commonly used disinfectants and antiseptics

Class of compound	Effect of organic matter	pH optimum	Toxicity and OES*	Other factors
Alcohols Ethanol	Slight		Avoid broken skin, eyes OES: 1000 ppm/1900 mg m ⁻³ , 8 h only	Poor penetration, good cleansing properties
Aldehydes Glutaraldehyde	Slight	pH 8	Respiratory complaints and contact dermatitis reported Eyes, sensitivity OES: 0.2 ppm/0.7 mg m ⁻³ , 10 min only	Non-corrosive, useful for heat sensitive instruments
Biguanides Chlorhexidine	Severe	pH 7–8	Avoid contact with eyes and mucous membranes Sensitivity may develop	Incompatible with soap and anionic detergents Inactivated by hard water, some materials and plastic
Chlorine compounds Hypochlorite	Severe	Acid/neutral pH	Irritation of skin, eyes and lungs OES: 1 ppm/3 mg m ⁻³ , 10 min; 0.5 ppm/1.5 mg m ⁻³ , 8 h	Corrosive to metals
Iodine preparations Iodophors	Severe	Acid pH	Eye irritation OES: 0.1 ppm/1 mg m ⁻³ , 10 min only	May corrode metals
Phenolics Clear soluble fluids	Slight	Acid pH	Protect skin and eyes Very irritant	Adsorbed by rubber/plastic
Black/white fluids	Moderate/severe		Sensitivity. May irritate skin	Greatly reduced by dilution
Chloroxylenol	Severe		OES: 10 ppm/38 mg m ⁻³ , 10 min; 5 ppm/19 mg m ⁻³ , 8 h	Adsorbed by rubber/plastic
QACs Cetrimide and benzalkonium Chloride	Severe	Alkaline pH	Avoid contact with eyes	Incompatible with soap and anionic detergents Adsorbed by fabrics

* From the *Control of Substances Hazardous to Health (COSHH) Regulations* (1988).
OES, occupational exposure standard; QAC, quaternary ammonium compound.

Table 10.6 Examples of the main antimicrobial groups as antiseptics, disinfectants and preservatives

Antimicrobial agent	Antiseptic activity		Disinfectant Activity		Preservative activity	
	Concentration	Typical formulation/ application	Concentration	Typical formulation/ application	Concentration	Typical formulation/ application
Acids and esters, e.g. benzoic acid, parabens						
Alcohols, e.g. ethyl or isopropyl	50–90% in water	Skin prep.	50–90% in water	Clean surface prep., thermometers		
Aldehydes, e.g. glutaraldehyde	10%	Gel for warts	2.0%	Solution for instruments		
Biguanides, e.g. chlorhexidine† (gluconate, acetate etc)	0.02% 0.2% 0.5% (in 70% alcohol)	Bladder irrigation Mouthwash Skin prep.	0.05%	Storage of instruments, clean instrument disinfection (30 min)	0.0025% 0.01%	Solution for hard contact lenses Eye-drops
	1.0%	Dusting powder, cream dental gel	0.5% (in 70% alcohol)	Emergency instrument disinfection (2 min)		
	4.0%	Pre-op. scrub in surfactant				
Chlorine, e.g. hypochlorite	≤0.5% avCl ₂	Solution for skin and wounds	1–10%	Solution for surfaces and instruments		
Hydrogen peroxide	1.5% 3–6%	Stabilized cream Solution for wounds and ulcers, mouthwash	3.0%	Disinfection of soft contact lenses		

Second Part

Water Microbiology

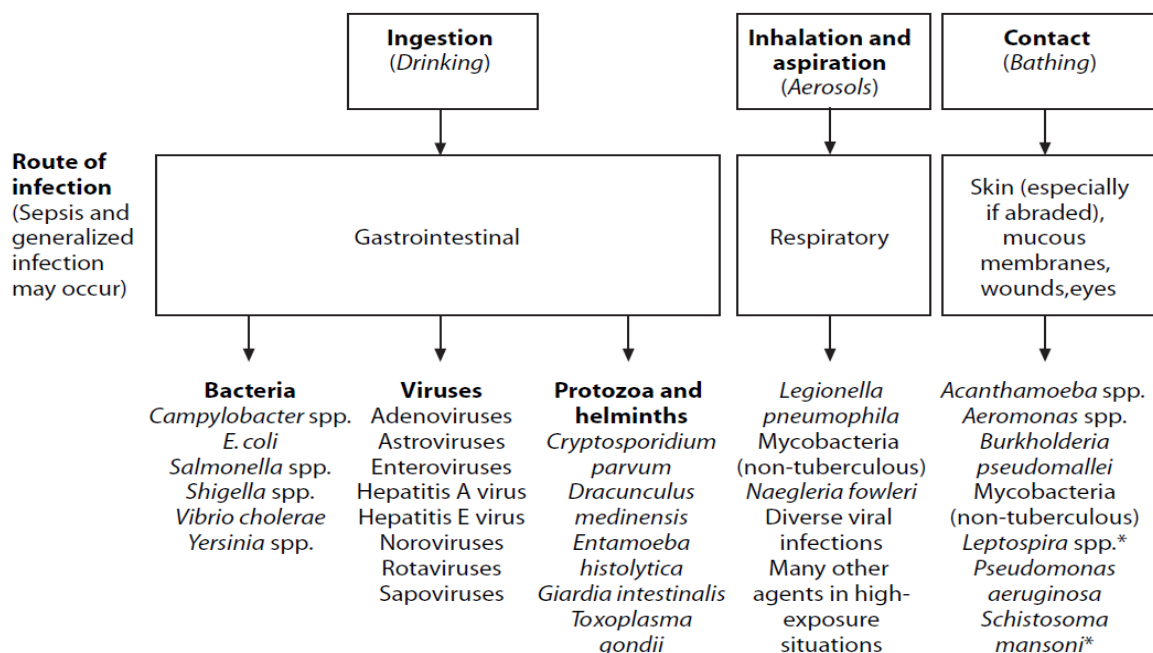
1. History of Water Microbiology:

- Water treatment isn't a new concept. Evidence of decontamination efforts of water dates back to before 2000 BC in Sanskrit writings advising that water should be boiled and filtered.
- Hippocrates wrote of waters contributing to ill health and the need to boil or strain water in 400 BC.
- There are many reports in the early 18th century describing the use of a variety of treatment methods such as filtering stones, acids and oils to purify water. It may be surprising to note that as early as 1732, the practice of sedimentation and sand filtration was popular among individual households.
- Municipal water supplies began widespread use of sand filtration in 1832.
- Dr. John Snow, also known as the Father of Epidemiology, advanced this theory most notably following the famous Broad Street Pump incident in 1852. Snow realized from maps of the London area that cases of cholera were isolated to regions served by the Southwark and Vauxhall Company, using the lower Thames River as its water source and not in a neighboring section of the city served by the Lambeth Company, whose water came from the upper Thames.
- Removal of the Broad Street pump handle supplying water from the Southwark and Vauxhall Company was largely responsible for the cholera epidemic in the region.
- Several years later, Louis Pasteur defined the "germ theory" of disease, stating that infectious disease wasn't spontaneous but a result of the presence and/or growth of harmful microorganisms.
- In 1883 Robert Koch published an article entitled: About Detection Methods for Microorganisms in Water. In that historic paper that marked the introduction of the application of microbial indicators for surveillance of water hygiene,
- Koch described for the first time the methodology for HPC measurement in water, and showed its value as a measure of water treatment technology performance.

2. Waterborne Diseases:

8.5. Waterborne infections:

- The pathogens that may be transmitted through contaminated drinking-water are diverse. The spectrum changes in response to variables such as:
 - Increase in human and animal populations.
 - Escalating use of wastewater.
 - Changes in lifestyles and medical interventions.
 - Population movement and travel.
 - Selective pressures for new pathogens and mutants.
 - Recombination of existing pathogens.
- The immunity of individuals also varies considerably, whether acquired by contact with a pathogen or influenced by such factors as age, sex, state of health and living conditions.
- For pathogens transmitted by the faecal–oral route, drinking-water is only one vehicle of transmission. Contamination of food, hands, utensils and clothing can also play a role, particularly when domestic sanitation and hygiene are poor. Improvements in the quality and availability of water, in excreta disposal and in general hygiene are all important in reducing faecal–oral disease transmission.



* Primarily from contact with highly contaminated surface waters.

3. Bacterial Waterborne Diseases:

8.6. Introduction

- There are three main types of microorganisms that can be found in drinking water: bacteria, viruses, and protozoa. These can exist naturally or can occur as a result of contamination from human or animal waste. Some of these are capable of causing illness in humans. ⁽¹⁾ Most bacterial pathogens potentially transmitted by water infect the gastrointestinal tract and are excreted in the faeces of infected humans and other animals.
- The greatest risk from microbes in water is associated with consumption of drinking-water that is contaminated with human and animal excreta, although other sources and routes of exposure may also be significant.
- The human bacterial pathogens that can be transmitted by consuming contaminated drinking water, and that present a serious risk of disease, include *Salmonella* spp, *Shigella* spp, enterovirulent *E. coli*, *Vibrio cholera*, *Yersinia enterocolitica*, *Campylobacter jejuni* and *C. coli*.
- After being excreted in faeces from the body of their host, bacterial pathogens gradually lose viability and the ability to cause infection. The rate of decay varies with different bacteria; it is usually exponential, and after a certain period a pathogen will become undetectable.
- The most common waterborne pathogens are those that are highly infectious or highly resistant to decay outside the body. Pathogens with a low persistence (i.e. those that do not survive long outside the host) must rapidly find a new host and are more likely to be spread by person-to-person contact or by poor personal or food hygiene than by drinking water. If drinking water is fecally contaminated, bacterial pathogens are likely to be widely and rapidly dispersed. Outbreaks of waterborne disease are therefore frequently characterized by infection across a whole community. ⁽¹⁾
- However, there are also some waterborne bacterial pathogens, that occur naturally in the environment may cause disease opportunistically in humans. Those most at risk are people with impaired local or general defense mechanisms, such as the elderly, the very young, people with burns, people who have undergone recent surgery or who have suffered serious injury, and people with severely compromised immune systems. In

such individuals, if water used for drinking or bathing contains large numbers of opportunistic pathogens it can occasionally produce infections of the skin, and of the mucous membranes of the eye, ear, nose and throat. Examples of such opportunistic agents are *Pseudomonas aeruginosa*, species of *Klebsiella*, *Aeromonas*, *H.pylori*, and *Legionella*.

- The main goal of drinking water treatment is to remove or kill these organisms to reduce the risk of illness. Although it is impossible to completely eliminate the risk of waterborne disease, adopting a multi-barrier, source-to-tap approach to safe drinking water will reduce the numbers of microorganisms in drinking water.

Waterborne pathogens and their significance in water supplies					
Pathogen	Health significance	Persistence in water supplies	Resistance to chlorine	Relative infectivity	Important animal source
Bacteria					
<i>Burkholderia pseudomallei</i>	Low	May multiply	Low	Low	No
<i>Campylobacter jejuni</i> , <i>C. coli</i>	High	Moderate	Low	Moderate	Yes
<i>Escherichia coli</i> – Pathogenic	High	Moderate	Low	Low	Yes
<i>E. coli</i> – Enterohaemorrhagic	High	Moderate	Low	High	Yes
<i>Legionella</i> spp.	High	Multiply	Low	Moderate	No
<i>Non-tuberculous mycobacteria</i>	Low	Multiply	High	Low	No
<i>Pseudomonas aeruginosa</i>	Moderate	May multiply	Moderate	Low	No
<i>Salmonella typhi</i>	High	Moderate	Low	Low	No
<i>Other salmonellae</i>	High	May multiply	Low	Low	Yes
<i>Shigella</i> spp.	High	Short	Low	Moderate	No
<i>Vibrio cholerae</i>	High	Short	Low	Low	No
<i>Yersinia enterocolitica</i>	High	Long	Low	Low	Yes

- To protect public health, it is important to have accurate, reliable, and scientifically defensible methods for determining when water is contaminated by pathogens and to what extent. Furthermore, recent and forecasted advances in microbiology, biology, and analytical chemistry make it timely to assess the current paradigm of relying predominantly or exclusively on traditional bacterial indicators for waterborne pathogens in order to make judgments concerning the microbiological quality of water to be used for recreation or as a source for drinking water supply.

- However, an increased understanding of the diversity of waterborne pathogens, their sources, physiology, and ecology has resulted in a growing understanding that the current indicator approach may not be as universally protective as was once thought. In this regard, several limitations of bacterial indicators for waterborne pathogens have been reported and are discussed throughout this material.

8.7. Bacterial Pathogens:

3.2.1. *Escherichia coli*

General Description

- *Escherichia coli* is a bacterium found exclusively in the digestive tract of warm-blooded animals, including humans.⁽¹⁾ where it generally causes no harm. However, in other parts of the body, *E. coli* can cause serious disease, such as urinary tract infections, bacteraemia and meningitis. A limited number of enteropathogenic strains can cause acute diarrhea
- Several classes of enteropathogenic *E. coli* have been identified on the basis of different virulence factors, including enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC). More is known about the first four classes named; the pathogenicity and prevalence of EAEC and DAEC strains are less well established.

Human health effects

- EHEC serotypes, such as *E. coli* O157:H7. Although it is not usually a concern in treated drinking water, outbreaks involving consumption of drinking water contaminated with human sewage or cattle faeces have been documented.
- *E. coli* serotype O157:H7 causes abdominal pain, bloody diarrhea, and hemolytic uraemic syndrome (HUS).
- This bacterium produces potent toxins (verotoxins) related to *Shigella* toxins. The incubation period is 3–4 days, and the symptoms occur for 7–10 days. It is estimated that 2–7% of *E. coli* O157:H7 infections result in HUS, in which the destruction of erythrocytes leads to acute renal failure.
- Studies have shown that the dose required to produce symptoms is lower than that for most other enteric pathogenic bacteria.⁽¹⁾ The infectivity of EHEC strains is substantially higher than that of the other strains. As few as 100 EHEC organisms can

cause infection.⁽²⁾ The probability of becoming ill depends on the number of organisms ingested, the health status of the person, and the resistance of the person to the organism or toxin. Children and the elderly are most susceptible to HUS complications. Evidence suggests that the incidence of *E. coli* O157:H7 infections and HUS has increased since the serotype was first recognized.

- ETEC produces heat-labile or heat-stable *E. coli* enterotoxin or both toxins simultaneously, and is an important cause of diarrhea in developing countries, especially in young children.
- Symptoms of ETEC infection include mild watery diarrhea, abdominal cramps, nausea and headache. Infection with EPEC has been associated with severe, chronic, non-bloody diarrhea, vomiting and fever in infants. EPEC infections are rare in developed countries, but occur commonly in developing countries, with infants presenting with malnutrition, weight loss and growth retardation. EIEC causes watery and occasionally bloody diarrhea where strains invade colon cells by a pathogenic mechanism similar to that of *Shigella*.

Source and occurrence

- Enteropathogenic *E. coli* are enteric organisms, and humans are the major reservoir, particularly of EPEC, ETEC and EIEC strains. Livestock, such as cattle and sheep and, to a lesser extent, goats, pigs and chickens, are a major source of EHEC strains. The latter have also been associated with raw vegetables, such as bean sprouts. The pathogens have been detected in a variety of water environments.

Routes of exposure

- Infection is associated with person-to-person transmission, contact with animals, food and consumption of contaminated water. Person-to-person transmissions are particularly prevalent in communities where there is close contact between individuals, such as nursing homes and day care centers.

Significance in drinking-water

- Waterborne transmission of pathogenic *E. coli* has been well documented for recreational waters and contaminated drinking-water. A well publicized waterborne outbreak of illness caused by *E. coli* O157:H7 (and *Campylobacter jejuni*) occurred in the farming community of Walkerton in Ontario, Canada. The outbreak took place in

May 2000 and led to 7 deaths and more than 2300 illnesses. The drinking-water supply was contaminated by rainwater runoff containing cattle excreta

Treatment technology

- Similar to the non-pathogenic strains of *E. coli*, *E. coli* O157:H7 is susceptible to disinfection. In addition, a multi-barrier approach based upon source protection (where possible), and effective treatment and a well-maintained distribution system will reduce the levels of *E. coli* O157:H7 in drinking water to non detectable or to levels that have never been associated with human illness.
- Conventional testing for *E. coli* (or, alternatively, thermotolerant coliform bacteria) provides an appropriate index for the enteropathogenic serotypes in drinking-water. This applies even though standard tests will generally not detect EHEC strains.

Assessment

- Studies have shown that the survival rate of *E. coli* O157:H7 approximates that of typical *E. coli* in the aquatic environment. Also, although routine examination methods for generic *E. coli* will not detect *E. coli* O157:H7, the former will always occur in greater concentration in faeces than the pathogenic strains, even during outbreaks. *E. coli* O157:H7 will also never occur in the absence of generic *E. coli*. As a result, the presence of *E. coli* can be used as an indicator of the presence of *E. coli* O157:H7.

3.2.2. *Salmonella*

General description

- *Salmonella* spp. belongs to the family Enterobacteriaceae. They are motile, Gram negative bacilli that do not ferment lactose, but most produce hydrogen sulfide or gas from carbohydrate fermentation.

Human health effects

- *Salmonella* infections typically cause four clinical manifestations: gastroenteritis (ranging from mild to fulminant diarrhoea, nausea and vomiting), bacteraemia or septicaemia (high spiking fever with positive blood cultures), typhoid fever / enteric fever (sustained fever with or without diarrhoea) and a carrier state in persons with previous infections. In regard to enteric illness, *Salmonella* spp. can be divided into two fairly distinct groups: the typhoidal species/serovars (*Salmonella typhi* and *S. Paratyphi*) and the remaining non-typhoidal species/serovars.

- Symptoms of nontyphoidal gastroenteritis appear from 6 to 72 h after ingestion of contaminated food or water. Diarrhoea lasts 3–5 days and is accompanied by fever and abdominal pain. Usually the disease is self-limiting. The incubation period for typhoid fever can be 1–14 days but is usually 3–5 days. Typhoid fever is a more severe illness and can be fatal. Although typhoid is uncommon in areas with good sanitary systems, it is still prevalent elsewhere, and there are many millions of cases each year.

Source and occurrence

- *Salmonella* spp. are widely distributed in the environment, but some species or serovars show host specificity. Notably, *S. typhi* and generally *S. Paratyphi* are restricted to humans, although livestock can occasionally be a source of *S. Paratyphi*. A large number of serovars, including *S. Typhimurium* and *S. Enteritidis*, infect humans and also a wide range of animals, including poultry, cows, pigs, sheep, birds and even reptiles. The pathogens typically gain entry into water systems through faecal contamination from sewage discharges, livestock and wild animals. Contamination has been detected in a wide variety of foods and milk. Numerous outbreaks linked to contaminated drinking water have been reported. In most cases, the drinking water was not treated or was improperly treated prior to consumption.

Routes of exposure

- *Salmonella* is spread by the faecal–oral route. Infections with non-typhoidal serovars are primarily associated with person-to-person contact, the consumption of a variety of contaminated foods and exposure to animals. Infection by typhoid species is associated with the consumption of contaminated water or food, with direct person-to person spread being uncommon.

Significance in drinking-water

- Waterborne typhoid fever outbreaks have devastating public health implications.
- However, despite their widespread occurrence, non-typhoidal *Salmonella* spp. rarely cause drinking-water-borne outbreaks. Transmission, most commonly involving *S. Typhimurium*, has been associated with the consumption of contaminated groundwater and surface water supplies. In an outbreak of illness associated with a communal rainwater supply, bird faeces were implicated as a source of contamination.

Treatment technology

- Salmonella survival characteristics in water and its susceptibility to disinfection has been demonstrated to be similar to those of coliform bacteria. In addition, a multi-barrier approach based upon source protection, effective treatment, and a well-maintained distribution system will reduce the levels of Salmonella in drinking water to none detectable or to levels that has never been associated with human illness.

Assessment

- The absence of *E. coli* during routine verification should be an adequate indication of the absence of Salmonella. However, instances have been reported in which these pathogen was isolated from drinking water in the absence of Coliform suppression by elevated HPCs and poor recovery of stressed coliforms seem to be the most plausible explanations for these discrepancies. Total coliform and *E. coli* recoveries are not affected by elevated HPCs and environmental stress in the newer defined-substrate methods.(1)

3.2.3. *Shigella*

General description

- *Shigella* spp. are Gram-negative, non-spore-forming, non-motile, rod-like members of the family Enterobacteriaceae, which grow in the presence or absence of oxygen. Members of the genus have a complex antigenic pattern, and classification is based on their somatic O antigens, many of which are shared with other enteric bacilli, including *E. coli*. There are four species: *S. dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei*.

Human health effects

- *Shigella* spp. can cause serious intestinal diseases, including bacillary dysentery. Over 2 million infections occur each year, resulting in about 600 000 deaths, predominantly in developing countries. Most cases of *Shigella* infection occur in children under 10 years of age. The incubation period for shigellosis is usually 24–72 h. Ingestion of as few as 10–100 organisms may lead to infection, which is substantially less than the infective dose of most other enteric bacteria. Abdominal cramps, fever and watery diarrhoea occur early in the disease.
- All species can produce severe disease, but illness due to *S. sonnei* is usually relatively mild and self-limiting. In the case of *S. dysenteriae*, clinical manifestations may proceed to an ulceration process, with bloody diarrhea and high concentrations of neutrophils in the stool. The production of Shiga toxin by the pathogen plays an important role in this

outcome. *Shigella* spp. seem to be better adapted to cause human disease than most other enteric bacterial pathogens.

Source and occurrence

- Humans and other higher primates appear to be the only natural hosts for the shigellae. The bacteria remain localized in the intestinal epithelial cells of their hosts. Epidemics of shigellosis occur in crowded communities and where hygiene is poor. Many cases of shigellosis are associated with day care centres, prisons and psychiatric institutions. Military field groups and travellers to areas with poor sanitation are also prone to infection.

Routes of exposure

- *Shigella* spp. are enteric pathogens predominantly transmitted by the faecal–oral route through person-to-person contact, contaminated food and water. Flies have also been identified as a transmission vector from contaminated faecal waste.

Significance in drinking-water

- A number of large waterborne outbreaks of shigellosis have been recorded. As the organisms are not particularly stable in water environments, their presence in drinking-water indicates recent human faecal pollution. Available data on prevalence in water supplies may be an underestimate, because detection techniques generally used can have a relatively low sensitivity and reliability. The control of *Shigella* spp. in drinking-water supplies is of special public health importance in view of the severity of the disease caused. *Shigella* spp. is relatively sensitive to disinfection.

Treatment technology

- *Shigella* survival characteristics in water and its susceptibility to disinfection has been demonstrated to be similar to those of coliform bacteria. In addition, a multi-barrier approach based upon source protection, effective treatment, and a well-maintained distribution system will reduce the levels of *Shigella* in drinking water to none detectable or to levels that has never been associated with human illness.

Assessment

- The absence of *E. coli* during routine verification should be an adequate indication of the absence of *Shigella*. However, instances have been reported in which these pathogen was isolated from drinking water in the absence of Coliform suppression by elevated HPCs and poor recovery of stressed coliforms seem to be the most plausible

explanations for these discrepancies. Total coliform and *E. coli* recoveries are not affected by elevated HPCs and environmental stress in the newer defined-substrate methods.(1)

3.2.4. *Vibrio*

General description

- *Vibrio* spp. are small, curved (comma-shaped), Gram-negative bacteria with a single polar flagellum. Species are typed according to their O antigens. There are a number of pathogenic species, including *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. *Vibrio cholerae* is the only pathogenic species of significance from freshwater environments. While a number of serotypes can cause diarrhoea, only O1 and O139 currently cause the classical cholera symptoms in which a proportion of cases suffer fulminating and severe watery diarrhoea.
- The O1 serovar has been further divided into “classical” and “El Tor” biotypes. The latter is distinguished by features such as the ability to produce a dialysable heat-labile haemolysin, active against sheep and goat red blood cells. The classical biotype is considered responsible for the first six cholera pandemics, while the El Tor biotype is responsible for the seventh pandemic that commenced in 1961.
- Strains of *V. cholerae* O1 and O139 that cause cholera produce an enterotoxin (cholera toxin) that alters the ionic fluxes across the intestinal mucosa, resulting in substantial loss of water and electrolytes in liquid stools. Other factors associated with infection are an adhesion factor and an attachment pilus. Not all strains of serotypes O1 or O139 possess the virulence factors, and they are rarely possessed by non-O1/O139 strains.

Human health effects

- Cholera outbreaks continue to occur in many areas of the developing world. Symptoms are caused by heat-labile cholera enterotoxin carried by toxigenic strains of *V. cholerae* O1/O139. A large percentage of infected persons do not develop illness; about 60% of the classical and 75% of the El Tor group infections are asymptomatic. Symptomatic illness ranges from mild or moderate to severe disease. The initial symptoms of cholera are an increase in peristalses followed by loose, watery and mucus-flecked “rice-water” stools that may cause a patient to lose as much as 10–15 litres of liquid per day. Decreasing gastric acidity by administration of sodium bicarbonate reduces

- The infective dose of *V. cholerae* O1 from more than 10⁸ to about 10⁴ organisms. Case fatality rates vary according to facilities and preparedness. As many as 60% of untreated patients may die as a result of severe dehydration and loss of electrolytes, but well established diarrhoeal disease control programmes can reduce fatalities to less than 1%. Non-toxigenic strains of *V. cholerae* can cause self-limiting gastroenteritis, wound infections and bacteraemia.

Source and occurrence

- Non-toxigenic *V. cholerae* is widely distributed in water environments, but toxigenic strains are not distributed as widely. Humans are an established source of toxigenic *V. cholerae*; in the presence of disease, the organism can be detected in sewage.
- Although *V. cholerae* O1 can be isolated from water in areas without disease, the strains are not generally toxigenic. Toxigenic *V. cholerae* has also been found in association with live copepods as well as other aquatic organisms, including molluscs, crustaceans, plants, algae and cyanobacteria. Numbers associated with these aquatic organisms are often higher than in the water column. Non-toxigenic *V. cholerae* has been isolated from birds and herbivores in areas far away from marine and coastal waters. The prevalence of *V. cholerae* decreases as water temperatures fall below 20 °C.

Routes of exposure

- Cholera is typically transmitted by the faecal–oral route, and the infection is predominantly contracted by the ingestion of faecally contaminated water and food. The high numbers required to cause infection make person-to-person contact an unlikely route of transmission.

Significance in drinking-water

- Contamination of water due to poor sanitation is largely responsible for transmission, but this does not fully explain the seasonality of recurrence, and factors other than poor sanitation must play a role. The presence of the pathogenic *V. cholerae* O1 and O139 serotypes in drinking-water supplies is of major public health importance and can have serious health and economic implications in the affected communities. *Vibrio cholerae* is highly sensitive to disinfection processes.

Treatment technology

- Control measures that can be applied to manage potential risk from toxigenic *V. cholerae* include protection of raw water supplies from human waste, adequate treatment and protection of water during distribution.

Assessment

- *Vibrio cholerae* O1 and non-O1 have been detected in the absence of *E. coli*, and this organism (or, alternatively, thermotolerant coliforms) is not a reliable index for *V. cholerae* in drinking-water.

3.2.5. *Yersinia*

General description

- The genus *Yersinia* is classified in the family Enterobacteriaceae and comprises seven species. The species *Y. pestis*, *Y. pseudo-tuberculosis* and certain serotypes of *Y. enterocolitica* are pathogens for humans. *Yersinia pestis* is the cause of bubonic plague through contact with rodents and their fleas. *Yersinia* spp. are Gram-negative rods that are motile at 25 °C but not at 37 °C.

Human health effects

- *Yersinia enterocolitica* penetrates cells of the intestinal mucosa, causing ulcerations of the terminal ileum. Yersiniosis generally presents as an acute gastroenteritis with diarrhoea, fever and abdominal pain. Other clinical manifestations include greatly enlarged painful lymph nodes referred to as “buboes.” The disease seems to be more acute in children than in adults.

Source and occurrence

- Domestic and wild animals are the principal reservoir for *Yersinia* spp.; pigs are the major reservoir of pathogenic *Y. enterocolitica*, whereas rodents and small animals are the major reservoir of *Y. pseudotuberculosis*. Pathogenic *Y. enterocolitica* has been detected in sewage and polluted surface waters. However, *Y. enterocolitica* strains detected in drinking-water are more commonly non-pathogenic strains of probable environmental origin. At least some species and strains of *Yersinia* seem to be able to replicate in water environments if at least trace amounts of organic nitrogen are present, even at temperatures as low as 4 °C.

Routes of exposure

- *Yersinia* spp. are transmitted by the faecal–oral route, with the major source of infection considered to be foods, particularly meat and meat products, milk and dairy products. Ingestion of contaminated water is also a potential source of infection. Direct transmission from person to person and from animals to humans is also known to occur.

Significance in drinking-water

- Although most *Yersinia* spp. detected in water are probably non-pathogenic, circumstantial evidence has been presented to support transmission of *Y. enterocolitica* and *Y. pseudotuberculosis* to humans from untreated drinking-water. The most likely source of pathogenic *Yersinia* spp. is human or animal waste. The organisms are sensitive to disinfection processes.

Treatment technology

- Control measures that can be used to minimize the presence of pathogenic *Yersinia* spp. in drinking-water supplies include protection of raw water supplies from human and animal waste, adequate disinfection and protection of water during distribution..

Assessment

- Owing to the long survival and/or growth of some strains of *Yersinia* spp. in water, *E. coli* (or, alternatively, thermotolerant coliforms) is not a suitable index for the presence/absence of these organisms in drinking-water.

3.2.6. *Campylobacter*

General description

- *Campylobacter* spp. are microaerophilic (require decreased oxygen) and capnophilic (require increased carbon dioxide), Gram-negative, curved spiral rods with a single unsheathed polar flagellum. *Campylobacter* spp. is one of the most important causes of acute gastroenteritis worldwide. *Campylobacter jejuni* is the most frequently isolated species from patients with acute diarrheal disease, whereas *C. coli*, *C. lariidis* and *C. fetus* have also been isolated in a small proportion of cases. Two closely related genera, *Helicobacter* and *Archobacter*, include species previously classified as *Campylobacter* spp.

Human health effects

- An important feature of *C. jejuni* is relatively high infectivity compared with other bacterial pathogens. As few as 1000 organisms can cause infection. Most symptomatic

infections occur in infancy and early childhood. The incubation period is usually 2–4 days. Clinical symptoms of *C. jejuni* infection are characterized by abdominal pain, diarrhoea (with or without blood or faecal leukocytes), vomiting, chills and fever. The infection is self-limited and resolves in 3–7 days. Relapses may occur in 5–10% of untreated patients. Other clinical manifestations of *C. jejuni* infections in humans include reactive arthritis and meningitis. Several reports have associated *C. jejuni* infection with Guillain-Barré syndrome, an acute demyelinating disease of the peripheral nerves.

Source and occurrence

- *Campylobacter* spp. occur in a variety of environments. Wild and domestic animals, especially poultry, wild birds and cattle, are important reservoirs. Pets and other animals may also be reservoirs. Food, including meat and unpasteurized milk, are important sources of *Campylobacter* infections. Water is also a significant source. The occurrence of the organisms in surface waters has proved to be strongly dependent on rainfall, water temperature and the presence of waterfowl.

Routes of exposure

- Most *Campylobacter* infections are reported as sporadic in nature, with food considered a common source of infection. Transmission to humans typically occurs by the consumption of animal products. Meat, particularly poultry products, and unpasteurized milk are important sources of infection. Contaminated drinking-water supplies have been identified as a source of outbreaks. The number of cases in these outbreaks ranged from a few to several thousand, with sources including unchlorinated or inadequately chlorinated surface water supplies and faecal contamination of water storage reservoirs by wild birds.

Significance in drinking-water

- Contaminated drinking-water supplies have been identified as a significant source of outbreaks of campylobacteriosis. The detection of waterborne outbreaks and cases appears to be increasing. Waterborne transmission has been confirmed by the isolation of the same strains from patients and drinking-water they had consumed.

Treatment technology

- The findings indicated that conventional water treatment and chlorination will probably destroy *C. jejuni* and in drinking water. In addition, a multi-barrier approach based upon

source protection (where possible), effective treatment, and a well-maintained distribution system will reduce the levels of *Campylobacter* in drinking water to none detectable or to levels that have never been associated with human illness.(2)

Assessment

- Studies have shown no correlation between indicator organisms (e.g., *E. coli*, thermotolerant coliforms) and the presence of *Campylobacter* in raw surface water supplies. Thus, coliforms may not be adequate indicators of the presence of both *C. jejuni*

1.1.1. Opportunistic Pathogen

3.2.7. *Helicobacter pylori*

General description

- *Helicobacter pylori*, originally classified as *Campylobacter pylori*, is a Gram-negative, microaerophilic, spiral-shaped, motile bacterium. There are at least 14 species of *Helicobacter*, but only *H. pylori* have been identified as a human pathogen.

Human health effects

- *Helicobacter pylori* is found in the stomach; although most infections are asymptomatic, the organism is associated with chronic gastritis, which may lead to complications such as peptic and duodenal ulcer disease and gastric cancer. Whether the organism is truly the cause of these conditions remains unclear. The majority of *H. pylori* infections are initiated in childhood and without treatment are chronic. The infections are more prevalent in developing countries and are associated with overcrowded living conditions. Interfamilial clustering is common.

Source and occurrence

- Humans appear to be the primary host of *H. pylori*. Other hosts may include domestic cats. There is evidence that *H. pylori* is sensitive to bile salts, which would reduce the likelihood of faecal excretion, although it has been isolated from faeces of young children. *Helicobacter pylori* has been detected in water. Although *H. pylori* is unlikely to grow in the environment, it has been found to survive for 3 weeks in biofilms and up to 20–30 days in surface waters. In a study conducted in the USA, *H. pylori* was found in the majority of surface water and shallow groundwater samples. The presence of *H. pylori* was not correlated with the presence of *E. coli*. Possible contamination of the

environment can be through children with diarrhoea or through vomiting by children as well as adults.

Routes of exposure

- Person-to-person contact within families has been identified as the most likely source of infection through oral–oral transmission. *Helicobacter pylori* can survive well in mucus or vomit. However, it is difficult to detect in mouth or faecal samples. Faecal–oral transmission is also considered possible.

Significance in drinking-water

- Consumption of contaminated drinking-water has been suggested as a potential source of infection, but further investigation is required to establish any link with waterborne transmission.

Treatment technology

- Some work has been carried out on the relative sensitivities of *H. pylori* and *E. coli* to currently used drinking water treatment methods. Similar to other bacteria, a proportion of the *H. pylori* present in the source water will be removed using physical methods, such as coagulation, sedimentation, and filtration.
- This organism is also susceptible to disinfectants commonly used in drinking water treatment. In laboratory disinfectant testing, *E. coli* proved to be more sensitive to chlorine and ozone than *H. pylori*. However, there was little difference between the effectiveness when monochloramine was used. Although *E. coli* is easier to inactivate than *H. pylori* with some disinfectants, the typical water treatment plant is sufficient to inactivate *H. pylori* in the finished water. However, if *H. pylori* do enter the distribution system, potentially through a break in treatment or infiltration into the system, the disinfectant residuals found in the distribution system are probably not sufficient for inactivation.

Assessment

- Currently, there are no regulations governing the presence of *H. pylori* in drinking water, either nationally or internationally. The U.S. EPA has included it on their list of candidate contaminants for possible regulation in drinking water. Further studies are needed to confirm that *H. pylori* are present in drinking water in a viable state and that they can be transmitted by this medium.

3.2.8. *Klebsiella*

General description

- *Klebsiella* spp. are Gram-negative, non-motile bacilli that belong to the family Enterobacteriaceae. The outermost layer of *Klebsiella* spp. consists of a large polysaccharide capsule that distinguishes the organisms from other members of the family. Approximately 60–80% of all *Klebsiella* spp. isolated from faeces and clinical specimens are *K. pneumoniae* and are positive in the thermotolerant coliform test. *Klebsiella oxytoca* has also been identified as a pathogen.

Human health effects

- *Klebsiella* spp. have been identified as colonizing hospital patients, where spread is associated with the frequent handling of patients (e.g., in intensive care units). Patients at highest risk are those with impaired immune systems, such as the elderly or very young, patients with burns or excessive wounds, those undergoing immunosuppressive therapy or those with HIV/AIDS infection. Colonization may lead to invasive infections. On rare occasions, *Klebsiella* spp., notably *K. pneumoniae* and *K. oxytoca*, may cause serious infections, such as destructive pneumonia.

Source and occurrence

- *Klebsiella* spp. are natural inhabitants of many water environments, and they may multiply to high numbers in waters rich in nutrients, such as pulp mill wastes, textile finishing plants and sugar-cane processing operations. In drinking-water distribution systems, they are known to colonize washers in taps. The organisms can grow in water distribution systems. *Klebsiella* spp are also excreted in the faeces of many healthy humans and animals, and they are readily detected in sewage-polluted water.

Routes of exposure

- *Klebsiella* can cause nosocomial infections, and contaminated water and aerosols may be a potential source of the organisms in hospital environments and other health care facilities.

Significance in drinking-water

- *Klebsiella* spp are not considered to represent a source of gastrointestinal illness in the general population through ingestion of drinking-water. *Klebsiella* spp. detected in drinking-water is generally biofilm organisms and are unlikely to represent a health risk.

Treatment technology

- The organisms are reasonably sensitive to disinfectants, and entry into distribution systems can be prevented by adequate treatment. Growth within distribution systems can be minimized by strategies that are designed to minimize biofilm growth, including treatment to optimize organic carbon removal, restriction of the residence time of water in distribution systems and maintenance of disinfectant residuals.

Assessment

- *Klebsiella* is a coliform and can be detected by traditional tests for total coliforms.

3.2.9. *Legionella*

General description

- The genus *Legionella*, a member of the family Legionellaceae, has at least 42 species. Legionellae are Gram-negative, rod-shaped, non-spore-forming bacteria that require L-cysteine for growth and primary isolation. *Legionella* spp. are heterotrophic bacteria found in a wide range of water environments and can proliferate at temperatures above 25 °C.

Human health effects

- Although all *Legionella* spp. are considered potentially pathogenic for humans, *L. pneumophila* is the major waterborne pathogen responsible for legionellosis, of which two clinical forms are known: Legionnaires' disease and Pontiac fever. The former is a pneumonic illness with an incubation period of 3–6 days. Host factors influence the likelihood of illness: males are more frequently affected than females, and most cases occur in the 40- to 70-year age group. Risk factors include smoking, alcohol abuse, cancer, diabetes, chronic respiratory or kidney disease and immunosuppression, as in transplant recipients. Pontiac fever is a milder, self-limiting disease with a high attack rate and an onset (5 h to 3 days) and symptoms similar to those of influenza: fever, headache, nausea, vomiting, aching muscles and coughing. Studies of seroprevalence of antibodies indicate that many infections are asymptomatic.

Source and occurrence

- *Legionella* spp. are members of the natural flora of many freshwater environments, such as rivers, streams and impoundments, where they occur in relatively low numbers. However, they thrive in certain human-made water environments, such as water cooling devices (cooling towers and evaporative condensers) associated with air conditioning

systems, hot water distribution systems and spas, which provide suitable temperatures (25–50 °C) and conditions for their multiplication.

- Devices that support multiplication of *Legionella* have been associated with outbreaks of Legionnaires' disease. *Legionella* survive and grow in biofilms and sediments and are more easily detected from swab samples than from flowing water. *Legionellae* can be ingested by trophozoites of certain amoebae such as *Acanthamoeba*, *Hartmannella* and *Naegleria*, which may play a role in their persistence in water environments.

Routes of exposure

- The most common route of infection is the inhalation of aerosols containing the bacteria. Such aerosols can be generated by contaminated cooling towers, warm water showers, humidifiers and spas. Aspiration has also been identified as a route of infection in some cases associated with contaminated water, food and ice. There is no evidence of person-to-person transmission.

Significance in drinking-water

- *Legionella* spp. are common waterborne organisms, and devices such as cooling towers, hot water systems and spas that utilize mains water have been associated with outbreaks of infection.

Treatment technology

- Owing to the prevalence of *Legionella*, the potential for ingress into drinking-water systems should be considered as a possibility, and control measures should be employed to reduce the likelihood of survival and multiplication.
- Disinfection strategies designed to minimize biofilm growth and temperature control can minimize the potential risk from *Legionella* spp. The organisms are sensitive to disinfection. Monochloramine has been shown to be particularly effective, probably due to its stability and greater effectiveness against biofilms.
- Water Temperature is an important element of control strategies. Wherever possible, water temperatures should be kept outside the range of 25–50 °C. In hot water systems, storages should be maintained above 55 °C, and similar temperatures throughout associated pipe will prevent growth of the organism. However, maintaining temperatures of hot water above 50 °C may represent a scalding risk in young children, the elderly and other vulnerable groups. Where temperatures in hot or cold water distribution systems cannot be maintained outside the range of 25–50 °C, greater

attention to disinfection and strategies aimed at limiting development of biofilms are required.

Assessment

- Unlike the case with gastrointestinal pathogens, where *E. coli* can be used to indicate potential presence, no suitable indicators have been identified to signal increasing concentrations of *Legionella* spp. in a building's plumbing system.
- There is some evidence that increasing *Legionella* concentrations are accompanied by, or preceded by, an increase in other bacteria, resulting in an elevated HPC measurement (i.e., >100 CFU/mL). Hence, elevated HPCs may indicate the presence of *Legionella*. However, the correlation between HPC and *Legionella* is not consistent. This may partially result from the accompanying chlorination of the water, since HPC bacteria are more readily killed than legionellae.
- The ubiquitous nature of legionellae in water ensures that water supplies, regardless of their source, may contain *Legionella* spp. in low quantities. On a daily basis, the population at large is exposed to these low levels with no reaction or with asymptomatic production of antibodies.

3.2.10. *Pseudomonas aeruginosa*

General description

- *Pseudomonas aeruginosa* (*P. aeruginosa*) is a member of the family Pseudomonadaceae and is a polarly flagellated, aerobic, Gram-negative rod. When grown in suitable media, it produces the non-fluorescent bluish pigment pyocyanin. Many strains also produce the fluorescent green pigment pyoverdine. *P. aeruginosa*, like other fluorescent pseudomonads, produces catalase, oxidase and ammonia from arginine and can grow on citrate as the sole source of carbon.
- They are capable of growth in low nutrient situations and can grow in water in distribution systems if they gain access and on materials used in domestic plumbing situations. They may colonize taps and grow on surfaces, such as plastic pipes in drink vending machines

Human health effects

- *P. aeruginosa* can cause a range of infections but rarely causes serious illness in healthy individuals without some predisposing factor. (2) In human volunteer studies, an oral

dose of 10^6 cfu/ml was required to colonize the gut, but none of the volunteers experienced any disease symptom.

- It predominantly colonizes damaged sites such as burn and surgical wounds, the respiratory tract of people with underlying disease and physically damaged eyes. From these sites, it may invade the body, causing destructive lesions or septicaemia and meningitis.
- Cystic fibrosis and immunocompromised patients are prone to colonization with *P. aeruginosa*, which may lead to serious progressive pulmonary infections. Water-related folliculitis and ear infections are associated with warm, moist environments such as swimming pools and spas. Many strains are resistant to a range of antimicrobial agents, which can increase the significance of the organism in hospital settings. (2) In which, it seems likely to be the cause of 10– 20% of nosocomial infections (4)

Source and occurrence

- *P. aeruginosa* is a common environmental organism and can be found in faeces, soil, water and sewage. It can multiply in water environments and also on the surface of suitable organic materials in contact with water. *P. aeruginosa* is a recognized cause of hospital-acquired infections with potentially serious complications. It has been isolated from a range of moist environments such as sinks, water baths, hot water systems, showers and spa pools.

Routes of exposure

- The main route of infection is by exposure of susceptible tissue, notably wounds and mucous membranes, to contaminated water or contamination of surgical instruments. Cleaning of contact lenses with contaminated water can cause a form of keratitis. Ingestion of drinking-water is not an important source of infection.

Significance in drinking-water

- *P. aeruginosa* is predominantly an environmental organism, and fresh surface water is an ideal reservoir. It proliferates in water piping systems and even more in hot water systems and spa pools. As a consequence of contemporary lifestyle, *P. aeruginosa* reaches relatively high numbers in food and on moist surfaces. Daily, substantial numbers of the species are ingested with our food, particularly with raw vegetables, while our body surfaces also are in continuous contact with the organism. (4)*P. aeruginosa* can be significant in certain settings such as health care facilities; there is no

evidence that normal uses of drinking-water supplies are a source of infection in the general population. However, the presence of high numbers of *P. aeruginosa* in potable water, notably in packaged water, can be associated with complaints about taste, odour and turbidity.

- It has been reported in up to 3% of drinking-water samples at a concentration of up to 2300 cfu/ml(4). *P. aeruginosa* is sensitive to disinfection, and entry into distribution systems can be minimized by adequate disinfection.

Treatment technology

- Control measures that are designed to minimize biofilm growth, including treatment to optimize organic carbon removal, restriction of the residence time of water in distribution systems and maintenance of disinfectant residuals, should reduce the growth of these organisms.

Assessment

- *Pseudomonas aeruginosa* is detected by HPC, which can be used together with parameters such as disinfectant residuals to indicate conditions that could support growth of these organisms. However, as *P. aeruginosa* is a common environmental organism, *E. coli* (or, alternatively, thermotolerant coliforms) cannot be used for this purpose.

3.2.11. *Aeromonas*

General description

- *Aeromonas* spp. are Gram-negative, non-spore-forming, facultative anaerobic bacilli belonging to the family Vibrionaceae. They bear many similarities to the Enterobacteriaceae.
- The genus is divided into two groups. The group of psychrophilic non-motile aeromonads consists of only one species, *A. salmonicida*, an obligate fish pathogen that is not considered further here. The group of mesophilic motile (single polar flagellum) aeromonads is considered of potential human health significance and consists of the species *A. hydrophila*, *A. caviae*, *A. veronii* subsp. *sobria*, *A. jandaei*, *A. veronii* subsp. *veronii* and *A. schubertii*. The bacteria are normal inhabitants of fresh water and occur in water, soil and many foods, particularly meat and milk.

Human health effects

- *Aeromonas* spp. can cause infections in humans, including septicaemia, particularly in immunocompromised patients, wound infections and respiratory tract infections. There have been some claims that *Aeromonas* spp. can cause gastrointestinal illness, but epidemiological evidence is not consistent. Despite marked toxin production by *Aeromonas* spp. in vitro, diarrhea has not yet been introduced in test animals or human volunteers.

Source and occurrence

- *Aeromonas* spp. occur in water, soil and food, particularly meat, fish and milk.
- *Aeromonas* spp. is generally readily found in most fresh waters, and they have been detected in many treated drinking-water supplies, mainly as a result of growth in distribution systems. The factors that affect the occurrence of *Aeromonas* spp. in water distribution systems are not fully understood, but organic content, temperature, the residence time of water in the distribution network and the presence of residual chlorine have been shown to influence population sizes.

Routes of exposure

- Wound infections have been associated with contaminated soil and water-related activities, such as swimming, diving, boating and fishing. Septicaemia can follow from such wound infections. In immunocompromised individuals, septicaemia may arise from aeromonads present in their own gastrointestinal tract.

Significance in drinking-water

- Despite frequent isolation of *Aeromonas* spp. from drinking-water, the body of evidence does not provide significant support for waterborne transmission. Aeromonads typically found in drinking-water do not belong to the same DNA homology groups as those associated with cases of gastroenteritis.

Treatment technology

- The presence of *Aeromonas* spp. in drinking-water supplies is generally considered a nuisance. Entry of aeromonads into distribution systems can be minimized by adequate disinfection. Control measures that can limit growth of the bacteria in distribution systems include treatment to optimize organic carbon removal, restriction of the residence time of water in distribution systems and maintenance of disinfectant residuals.

Assessment

- *Aeromonas* spp. are detected by HPC, which can be used together with parameters such as disinfectant residuals to indicate conditions that could support growth of these organisms. However, *E. coli* (or, alternatively, thermotolerant coliforms) cannot be used as an index for the presence/absence of *Aeromonas* spp.

8.8. Viral Waterborne Diseases:

3.2.12. Introduction:

- Viruses are among the smallest of all infectious agents. In essence they are molecules of nucleic acid that can enter cells and replicate in them. The virus particle consists of a genome, either ribonucleic acid (RNA) or deoxyribonucleic acid (DNA), surrounded by a protective protein shell, the capsid. Frequently this shell is itself enclosed within an envelope that contains both protein and lipid.
- Viruses replicate only inside specific host cells, and they are absolutely dependent on the host cell's synthetic and energy yielding apparatus for producing new viral particles.
- The viruses of most significance for drinking water are those that multiply in the human intestine and are excreted in large numbers in the faeces of infected individuals (Enteric viruses).
- Although they cannot multiply outside the tissues of infected hosts, some enteric viruses can survive in the environment and remain infective for long periods. Human enteric viruses occur in water largely as a result of contamination with sewage and human excreta. The numbers of viruses present and their species distribution will reflect the extent to which they are being carried by the population.
- As sewage mixes with receiving water, viruses are carried downstream; the length of time they remain detectable depends on temperature, their degree of adsorption to particulate matter, penetration of sunlight into the water and other factors. Consequently, enteric viruses can be found at the intakes to water treatment plants if the water is polluted by sewage. However, proper treatment and disinfection should produce drinking water that is essentially virus free.
- Isolation of viruses from water indicates that a hazard exists, but it does not prove beyond doubt that water is a vehicle for transmission of disease.
- With the exception of hepatitis E, humans are considered to be the only source of human infectious species. Enteric viruses typically cause acute disease with a short

incubation period. Water may also play a role in the transmission of other viruses with different modes of action.

Waterborne pathogens and their significance in water supplies					
Viruses					
Adenoviruses	High	Long	Moderate	High	No
Enteroviruses	High	Long	Moderate	High	No
Hepatitis A virus	High	Long	Moderate	High	No
Hepatitis E virus	High	Long	Moderate	High	Potentially
Noroviruses and sapoviruses	High	Long	Moderate	High	Potentially
Rotaviruses	High	Long	Moderate	High	No

3.2.13. Viruses:

Enteroviruses

General description

- The genus *Enterovirus* is a member of the family Picornaviridae. This genus consists of 69 serotypes (species) that infect humans: poliovirus types 1–3, coxsackievirus types. Members of the genus are collectively referred to as Enteroviruses. Other species of the genus infect animals other than humans – for instance, the bovine group of Enteroviruses.
- Enteroviruses are among the smallest known viruses and consist of a single-stranded RNA genome in a non-enveloped icosahedral capsid with a diameter of 20–30nm. Some members of the genus are readily isolated by cytopathogenic effect in cell cultures, notably poliovirus, coxsackievirus B, echovirus and Enterovirus.

Human health effects

- Enteroviruses are one of the most common causes of human infections.(2) Within temperate climates most major epidemics occur during the later summer months, whereas in the tropics, disease can occur throughout the year.(1)
- They have been estimated to cause about 30 million infections in the USA each year. The spectrum of diseases caused by enteroviruses is broad and ranges from a mild febrile illness to myocarditis, meningoencephalitis, poliomyelitis, herpangina, hand-foot-and-mouth disease and neonatal multi-organ failure. The persistence of the viruses in chronic conditions such as polymyositis, dilated cardiomyopathy and chronic fatigue syndrome has been described. Most infections, particularly in children, are

asymptomatic, but still lead to the excretion of large numbers of the viruses, which may cause clinical disease in other individuals.

Source and occurrence

- Enteroviruses are excreted in the faeces of infected individuals. Among the types of viruses detectable by conventional cell culture isolation, enteroviruses are generally the most numerous in sewage, water resources and treated drinking-water supplies. The viruses are also readily detected in many foods.

Routes of exposure

- Person-to-person contact and inhalation of airborne viruses or viruses in respiratory droplets are considered to be the predominant routes of transmission of enteroviruses in communities. Transmission from drinking-water could also be important, but this has not yet been confirmed. Waterborne transmission of enteroviruses (coxsackievirus A16 and B5) has been epidemiologically confirmed for only two outbreaks, and these were associated with children bathing in lake water in the 1970s.

Significance in drinking-water

- Enteroviruses have been shown to occur in substantial numbers in raw water sources and treated drinking-water supplies.⁽²⁾ They occur in water either through faecal contamination or by discharge of sewage effluents (Dahling 1989). While waterborne transmission is probable, it has not been proven. The part played by low-level transmission has also been suspected but not proven. There is a suggestion that small numbers of viruses present intermittently or continuously in drinking water cause symptomless infections, and that these are spread by person-to-person contact to cause outbreaks of disease that have no apparent connection with water.
- The virus can also be spread on unwashed foods, particularly in areas where raw sewage is used as fertiliser, or it may be transmitted on the feet of vectors such as houseflies. Infants, with their faeces contained in diapers, also provide a major route of dissemination, particularly in day-care centres.⁽¹⁾
- In view of their prevalence, drinking-water represents a likely, although unconfirmed, source of enterovirus infection. The limited knowledge on the role of waterborne transmission could be related to a number of factors, including the wide range of clinical symptoms, frequent asymptomatic infection, the diversity of serotypes and the dominance of person-to-person spread.

- Enteroviruses have been detected in drinking-water supplies that met accepted specifications for treatment, disinfection and conventional indicator organisms. Within a WSP, control measures to reduce potential risk from enteroviruses should focus on prevention of source water contamination by human waste, followed by adequate treatment and disinfection. The effectiveness of treatment processes used to remove Enteroviruses will require validation. Drinking-water supplies should also be protected from contamination during distribution. Owing to the higher resistance of the viruses to disinfection, *E. coli* (or, alternatively, thermotolerant coliforms) is not a reliable index of the presence/absence of enteroviruses in drinking-water supplies.⁽²⁾

Hepatitis A virus

General description

- HAV is the only species of the genus *Hepatovirus* in the family Picornaviridae. The virus shares basic structural and morphological features with other members of the family, as described for enteroviruses. Human and simian HAVs are genotypically distinguishable.
- HAV cannot be readily detected or cultivated in conventional cell culture systems, and identification in environmental samples is based on the use of PCR techniques.

Human health effects

- HAV is highly infectious, and the infecting dose is considered to be low. The virus causes the disease hepatitis A, commonly known as “infectious hepatitis.” Like other members of the group enteric viruses, HAV enters the gastrointestinal tract by ingestion, where it infects epithelial cells. From here, the virus enters the bloodstream and reaches the liver, where it may cause severe damage to liver cells. In as many as 90% of cases, particularly in children, there is little, if any, liver damage, and the infection passes without clinical symptoms and elicits lifelong immunity. In general, the severity of illness increases with age.
- The damage to liver cells results in the release of liverspecific enzymes such as aspartate aminotransferase, which are detectable in the bloodstream and used as a diagnostic tool. The damage also results in the failure of the liver to remove bilirubin from the bloodstream; the accumulation of bilirubin causes the typical symptoms of jaundice and dark urine. After a relatively long incubation period of 28–30 days on average, there is a characteristic sudden onset of illness, including symptoms such as

fever, malaise, nausea, anorexia, abdominal discomfort and eventually jaundice.

Although mortality is generally less than 1%, repair of the liver damage is a slow process that may keep patients incapacitated for 6 weeks or longer. This has substantial burden of disease implications. Mortality is higher in those over 50 years of age.

Source and occurrence

- HAV occurs worldwide, but the prevalence of clinical disease has typical geographically based characteristics. HAV is excreted in faecal material of infected people, and there is strong epidemiological evidence that faecally contaminated food and water are common sources of the virus. In areas with poor sanitation, children are often infected at a very early age and become immune for life without clinical symptoms of disease. In areas with good sanitation, infection tends to occur later in life.

Routes of exposure

- Person-to-person spread is probably the most common route of transmission, but contaminated food and water are important sources of infection. There is stronger epidemiological evidence for waterborne transmission of HAV than for any other virus.
- Food borne outbreaks are also relatively common, with sources of infection including infected food handlers; shellfish harvested from contaminated water and contaminated produce. Travel of people from areas with good sanitation to those with poor sanitation provides a high risk of infection. Infection can also be spread in association with injecting and non-injecting drug use.

Significance in drinking-water

- The transmission of HAV by drinking-water supplies is well established, and the presence of HAV in drinking-water constitutes a substantial health risk. Within a WSP, control measures to reduce potential risk from HAV should focus on prevention of source water contamination by human waste, followed by adequate treatment and disinfection.
- The effectiveness of treatment processes used to remove HAV will require validation. Drinking-water supplies should also be protected from contamination during distribution. Owing to the higher resistance of the viruses to disinfection, *E. coli* (or, alternatively, thermotolerant coliforms) is not a reliable index of the presence/absence of HAV in drinking-water supplies.

Hepatitis E virus

General description

- HEV consists of a single-stranded RNA genome in a non-enveloped icosahedral capsid with a diameter of 27–34nm. HEV shares properties with a number of viruses, and classification is a challenge. At one stage, HEV was classified as a member of the family Caliciviridae, but most recently it has been placed in a separate family called hepatitis E-like viruses.
- There are indications of antigenic variation, and possibly even differences in serotypes of the virus, whereas human HAV consists of only one clearly defined serotype. HEV cannot be readily detected or cultivated in conventional cell culture systems, and identification in environmental samples is based on the use of PCR techniques.

Human health effects

- HEV causes hepatitis that is in many respects similar to that caused by HAV. However, the incubation period tends to be longer (average 40 days), and infections typically have a mortality rate of up to 25% in pregnant women. In endemic regions, first infections are typically seen in young adults rather than young children. Despite evidence of antigenic variation, single infection appears to provide lifelong immunity to HEV.
- Global prevalence has a characteristic geographic distribution. HEV is endemic and causes clinical diseases in certain developing parts of the world, such as India, Nepal, central Asia, Mexico and parts of Africa. In many of these areas, HEV is the most important cause of viral hepatitis. Although seroprevalence can be high, clinical cases and outbreaks are rare in certain parts of the world, such as Japan, South Africa, the United Kingdom, North and South America, Australasia and central Europe. The reason for the lack of clinical cases in the presence of the virus is unknown.

Source and occurrence

- HEV is excreted in faeces of infected people, and the virus has been detected in raw and treated sewage. Contaminated water has been associated with very large outbreaks.
- HEV is distinctive, in that it is the only enteric virus with a meaningful animal reservoir, including domestic animals, particularly pigs, as well as cattle, goats and even rodents.

Routes of exposure

- Secondary transmission of HEV from cases to contacts and particularly nursing staff has been reported, but appears to be much less common than for HAV. The lower level of

person-to-person spread suggests that faecally polluted water could play a much more important role in the spread of HEV than of HAV. Waterborne outbreaks involving thousands of cases are on record.

- These include one outbreak in 1954 with approximately 40 000 cases in Delhi, India; one with more than 100 000 cases in 1986–1988 in the Xinjiang Uighar region of China; and one in 1991 with some 79 000 cases in Kanpur, India. Animal reservoirs may also serve as a route of exposure, but the extent to which humans contract HEV infection from animals remains to be elucidated.

Significance in drinking-water

- The role of contaminated water as a source of HEV has been confirmed, and the presence of the virus in drinking-water constitutes a major health risk. There is no laboratory information on the resistance of the virus to disinfection processes, but data on waterborne outbreaks suggest that HEV may be as resistant as other enteric viruses. Within a WSP, control measures to reduce potential risk from HEV should focus on prevention of source water contamination by human and animal waste, followed by adequate treatment and disinfection.
- The effectiveness of treatment processes used to remove HEV will require validation. Drinking-water supplies should also be protected from contamination during distribution. Due to the likelihood that the virus has a higher resistance to disinfection, *E. coli* (or, alternatively, thermotolerant coliforms) is not a reliable index of the presence/absence of HEV in drinking-water supplies.

Rotaviruses and orthoreoviruses

General description

- Members of the genus *Rotavirus* consist of a segmented double-stranded RNA genome in a non-enveloped icosahedral capsid with a diameter of 50–65nm. This capsid is surrounded by a double-layered shell, giving the virus the appearance of a wheel – hence the name rotavirus.
- The diameter of the entire virus is about 80nm. *Rotavirus* and *Orthoreovirus* are the two genera of the family Reoviridae typically associated with human infection. Orthoreoviruses are readily isolated by cytopathogenic effect on cell cultures. The genus *Rotavirus* is serologically divided into seven groups, A–G, each of which consists of a number of subgroups; some of these subgroups specifically infect humans, whereas

others infect a wide spectrum of animals. Groups A–C are found in humans, with group A being the most important human pathogens. Wild-type strains of rotavirus group A are not readily grown in cell culture, but there are a number of PCR-based detection methods available for testing environmental samples.

Human health effects

- Human rotaviruses (HRVs) are the most important single cause of infant death in the world. Typically, 50–60% of cases of acute gastroenteritis of hospitalized children throughout the world are caused by HRVs. The viruses infect cells in the villi of the small intestine, with disruption of sodium and glucose transport. Acute infection has an abrupt onset of severe watery diarrhoea with fever, abdominal pain and vomiting; dehydration and metabolic acidosis may develop, and the outcome may be fatal if the infection is not appropriately treated.
- The burden of disease of rotavirus infections is extremely high. Members of the genus *Orthoreovirus* infect many humans, but they are typical “orphan viruses” and not associated with any meaningful disease.

Source and occurrence

- HRVs are excreted by patients in numbers up to 10¹¹ per gram of faeces for periods of about days. This implies that domestic sewage and any environments polluted with the human faeces are likely to contain large numbers of HRVs. The viruses have been detected in sewage, rivers, lakes and treated drinking-water. Orthoreoviruses generally occur in wastewater in substantial numbers.

Routes of exposure

- HRVs are transmitted by the faecal–oral route. Person-to-person transmission and the inhalation of airborne HRVs or aerosols containing the viruses would appear to play a much more important role than ingestion of contaminated food or water. This is confirmed by the spread of infections in children’s wards in hospitals, which takes place much faster than can be accounted for by the ingestion of food or water contaminated by the faeces of infected patients.
- The role of contaminated water in transmission is lower than expected, given the prevalence of HRV infections and presence in contaminated water. However, occasional waterborne and foodborne outbreaks have been described. Two large outbreaks in China in 1982–1983 were linked to contaminated water supplies.

Significance in drinking-water

- Although ingestion of drinking-water is not the most common route of transmission, the presence of HRVs in drinking-water constitutes a public health risk. There is some evidence that the rotaviruses are more resistant to disinfection than other enteric viruses. Within a WSP, control measures to reduce potential risk from HRVs should focus on prevention of source water contamination by human waste, followed by adequate treatment and disinfection.
- The effectiveness of treatment processes used to remove HRVs will require validation. Drinking-water supplies should also be protected from contamination during distribution. Due to a higher resistance of the viruses to disinfection, *E. coli* (or, alternatively, thermotolerant coliforms) is not a reliable index of the presence/absence of HRVs in drinking-water supplies.

4. Water Quality Indicators

8.9. Introduction:

- The ultimate objective for determining the microbiological quality of water is to identify and then minimize the public health risk from consuming water intended for drinking and from exposure to recreational water. Health effects assessments for waterborne pathogens can be based on a number of approaches. Each approach has strengths and weaknesses and all have been or are being used to document and quantify the health risks from microbes in water.
- Bacteria in water are, in general, not present individually, but as clumps or in association with particulate matter. When enumerating bacteria in water it is not the number of individual bacteria present which are counted, but the number of clumps of bacteria or the particles and their associated bacteria. Each clump or particle may have many bacteria associated with it. In the same time, it is impractical to attempt the routine isolation of pathogens because:
 - Pathogens are discrete and not in solution
 - Pathogens often in clumps or adhere to suspended solids
 - Cannot predict likelihood of infectious dose from average concentration
 - Infection and disease development dependent on invasiveness, virulence and immunity
 - Dose-response not cumulative
 - Present in relatively small numbers compared with other types of micro-organism.
 - Many types of pathogen are present and each requires a unique microbiological isolation technique.
- The approach that has been adopted is to analyze for indicator organisms that inhabit the gut in large numbers and are excreted in human faeces. The presence of these indicator organisms in water is evidence of faecal contamination and, therefore, of a risk that pathogens are present.
- If indicator organisms are present in large numbers, the contamination is considered to be recent and/or severe. There is no single indicator organism that can universally be used for all purposes of water quality surveillance. Each of the wide variety of indicators available for this purpose has its own advantages and disadvantages, and the challenge is

to select the appropriate indicator, or combination of indicators, for each particular purpose of water quality assessment. Indicators most commonly used are of faecal or sewage origin, and the following are some of the most important requirements of such indicators

- Should be present when there is a risk of contamination by pathogens
- Should not multiply in environmental conditions under which pathogens cannot multiply
- The indicator population should correlate with the degree of faecal contamination.
- The survival time in unfavorable environmental conditions should exceed that of pathogens
- Should be easy to enumerate and identify by simple methods
- Should have stable characteristics and give consistent reactions in these analyses
- Present in the same or higher numbers than pathogens.
- Specific for faecal or sewage pollution.
- At least as resistant as pathogens to conditions in natural water environments, and water purification and disinfection processes.
- Non-pathogenic.
- Ideally, various other properties are desirable, such as counts which are directly related to those of pathogens. However, the fundamental and most important requirement is that pathogens should be absent or inactivated whenever indicators are absent or inactivated.
- Organisms that fit these criteria include the coliform bacteria, fecal streptococci (enterococci) and the sulfite-reducing clostridia (i.e., *Clostridium perfringens*).

8.10. Characteristics of indicator organisms

4.2.1. Total Coliforms

- Coliform bacteria belong to the family Enterobacteriaceae but are further defined by functional characteristics rather than systematic genus and species.
- Coliforms are gram-negative nonsporing rod-shaped bacteria, oxidase-negative, capable of aerobic and facultative anaerobic growth in the presence of bile salts or other surface-active agents with similar growth-inhibiting properties. They are able to ferment lactose with the production of acid within 48 hours at 35–37°C. Fermentation by these organisms begins with the cleavage of lactose into galactose and glucose by the enzyme β -galactosidase.
- As an indicator group, the number of viable coliform organisms present does not always correlate properly with the presence of pollution. Elevated total coliform counts can occur from the presence of *Klebsiella*, *Citrobacter*, and other organisms of non-fecal origin that do not necessarily reflect the health risks associated with consumption of the water.
- Coliforms have been used extensively as a basis for regulating the microbial quality of drinking-water. Initially total coliforms were used as indicators of faecal contamination and hence of the possible presence of enteric pathogens.
- However, many species of bacteria in the total coliform group survive and grow in the environment, and their value as an indicator of faecal contamination has been questioned by many regulatory agencies.
- Strains of total coliform bacteria may colonize surfaces within systems and become part of a biofilm. The environmental conditions that favor this process are water temperatures greater than 15°C, neutral pH and adequate concentrations of assimilable organic carbon (AOC).
- In temperate climates, growth events typically occur during the summer months, but in tropical or subtropical climates they may occur year-round. Their ability to thrive in the environment or in a drinking-water distribution system makes total coliforms an unreliable index of faecal contamination.
- However, total coliforms can be used in operational monitoring as a measure of deterioration of water quality through distribution systems. Detection of these organisms can reveal microbial growth and possible biofilm formation, as well as

ingress of foreign material including soil. In extreme cases, a high count for the total coliform group may be associated with a low, or even zero, count for thermotolerant coliforms.

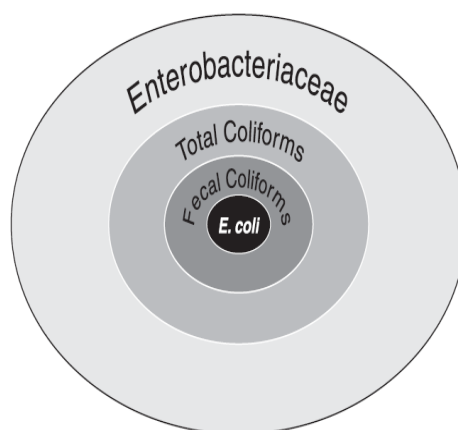
- Such a result would not necessarily indicate the presence of faecal contamination. It might be caused by entry of soil or organic matter into the water or by conditions suitable for the growth of other types of coliform.

The guidelines use total coliforms as an indicator because:

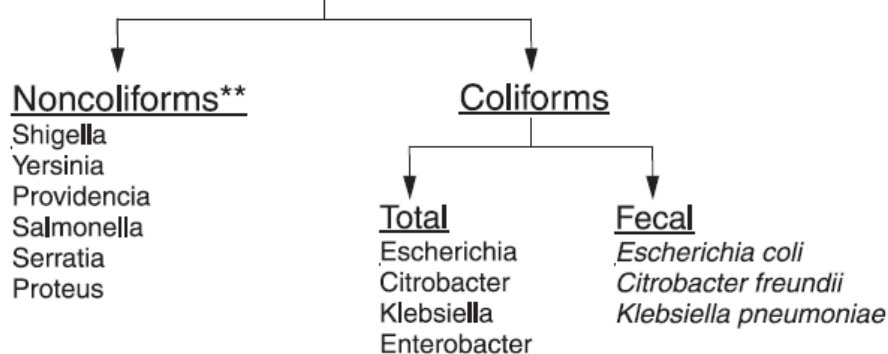
- as a group, they include thermotolerant coliforms which are a direct indicator of faecal contamination; their high numbers and their ability, along with some other more faecal pathogens, to survive longer in natural water than *E. coli* enable them to indicate less recent or more remote incidents of faecal pollution;
- their detection can indicate that conditions may favor the presence of free living opportunistic pathogens such as *Aeromonas*, *Pseudomonas* and *Legionella*;
- Their relative abundance and hardiness makes them a useful indicator of the efficiency of water treatment and disinfection processes, and their absence is a good indication that the barriers to contamination are working.

Methods of Identification and Detection

- Total coliforms can be quantified in water by using membrane filtration (MF) for concentration of the organisms from water, followed by growth in enrichment/selective media or multiple tube dilution (most probable number – MPN) procedures.
- Specific secondary tests are used with both MF and MPN procedures to confirm the identification of coliform organisms.
- Alternatively, the presence of coliform bacteria can be detected by testing for the production of the enzyme β -galactosidase. Enzyme substrate tests incorporate Chromogenic substrates such as ortho-nitrophenyl- β -D-galactopyranoside (ONPG) or chlorophenol red- β -Dgalactopyranoside (CPRG).
- When the substrates are hydrolyzed, a color change is produced. Test methods may also include a substrate such as 4-methylumbelliferyl- β -D-glucuronide (MUG), which is hydrolyzed by the enzyme β -glucuronidase, produced by most *E. coli*, to form the fluorogenic metabolite 4-methylumbelliferyl.
- It has been reported that more coliform bacteria may be detected using enzyme substrate based methodology in comparison to MF based methodology.



Enterobacteriaceae Family*



* Structure adapted from *Bergey's Manual of Systemic Bacteriology*, Vol. 1, edited by Noel Krieg, Williams and Wilkins, 1984.

** Represent main genera.

4.2.2. Escherichia coli and thermotolerant coliforms

- In 1885, T. Escherich discovered a bacterial species that was present almost universally in human fecal material. These bacteria not only occurred in high densities but frequently were associated with the pathogenic typhoid bacillus (*Salmonella typhi*). Escherich's bacteria were named later as *Escherichia coli*
- *Escherichia coli* (*E. coli*) is the faecal indicator of choice used in WHO Guidelines for Drinking-water Quality (WHO, 2004) and several countries are including this organism in their regulations as the primary indicator of faecal pollution. Current data suggest that *E. coli* is almost exclusively derived from the faeces of warm-blooded animals. In human and animal feces, 90 to 100% of the coliform organisms isolated are *E. coli*. In sewage and contaminated water samples, the percentage drops to 59%
- Its presence in drinking-water is interpreted as an indication of recent or substantial post-treatment faecal contamination or inadequate treatment.

- Thermotolerant coliforms, including *E. coli*, can ferment lactose (or mannitol) at $44.5 \pm 0.2^\circ\text{C}$ with the production of acid within 24 hours. Thermotolerant coliforms that produce Indole from tryptophan at $44.5 \pm 0.2^\circ\text{C}$ are regarded as being *E. coli*. *E. coli* also give a positive result in the methyl-red test and a negative Voges-Proskauer test and cannot use citrate as the sole source of carbon. Also, most *E. coli* produce the enzyme β -glucuronidase.
- Thermotolerant coliforms include *E. coli* and also some types of *Citrobacter*, *Klebsiella* and *Enterobacter*. Although thermotolerant species other than *E. coli* can include environmental organisms, populations of thermotolerant coliforms detected in most waters are predominantly composed of *E. coli*. As a result, thermotolerant coliforms are regarded as a less reliable but acceptable indicator of faecal pollution.
- In using *E. coli* or thermotolerant coliforms as an indicator of faecal pollution, a number of issues need to be considered. First, although *E. coli* does not readily grow outside the gut of warm-blooded animals in temperate regions, there is some evidence to suggest that it may grow in the natural environment in tropical region. However, in most cases, *E. coli* would be out competed by other environmental bacteria; therefore, whether growth occurs in nature is questionable. If such growth were to be found in certain tropical regions, then regulations would have to be based upon alternative indicators of post-treatment faecal contamination in storage and distribution systems, such as intestinal enterococci and *Clostridium perfringens* spores.
- Second, *E. coli* is extremely sensitive to disinfection. Its presence in a water sample is a sure sign of a major deficiency in the treatment or integrity of the distribution system. However, its absence does not by itself provide sufficient assurance that the water is free of risks from microbes.
- Many viral and protozoan pathogens are significantly more resistant to disinfection and may survive exposure to disinfectant that inactivates *E. coli*. Ingress of sewage into distribution system conveying water with a disinfectant residual might not be detected using *E. coli* alone: these bacteria might be inactivated while other pathogens remained viable.

Methods of Identification and Detection

- *E. coli*, (or thermotolerant coliforms) numbers can be determined using membrane filtration (MF) for concentration of the organisms from water, followed by growth in

enrichment/selective mediator multiple tube dilution (most probable number – MPN) procedures. Specific secondary tests are used with both MF and MPN procedures to confirm the identification of thermotolerant coliforms.

- Alternatively, the presence of *E. coli*, can be detected by testing for the production of the enzyme β -glucuronidase. Test methods include the enzyme substrates such as 4-methylumbelliferyl- β -D-glucuronide (MUG) which is hydrolyzed by β -glucuronidase to produce the fluorogenic metabolite 4-methylumbelliferyl. Media based on hydrolysis of MUG are commercially available under names such as "Colilert". Such complex sets of tests for the final confirmation of *E. coli* are not recommended as a routine.

4.2.3. Faecal streptococci

- Faecal streptococci are species of Gram-positive cocci belonging to two genera, *Enterococcus* and *Streptococcus*. The relevant species are linked by common biochemical and antigenic properties and are found in the faeces of humans and other animals. Many will grow in 6.5% sodium chloride and at 45°C.
- Enterococci: All faecal streptococci that grow at pH 9.6, 10° and 45°C and in 6.5% NaCl. Nearly all are members of the genus *Enterococcus*, and also fulfill the following criteria: resistance to 60°C for 30 min and ability to reduce 0.1% methylene blue. The enterococci are a subset of faecal streptococci that grow under the conditions outlined above.
- They occur regularly in faeces, but not in such numbers or so invariably as *E. coli*, and certain species of *Enterococcus* are not reliably associated with the gut. Thus, while the specificity of this indicator is acceptable, it is less sensitive than thermotolerant coliforms. It is, however, more persistent in water than *E. coli*, and so may be a better mirror of the presence of certain pathogens which also die off slowly (e.g. viruses).
- The presence of faecal streptococci is evidence of faecal contamination. Faecal streptococci tend to persist longer in the environment than thermotolerant or total coliforms and are highly resistant to drying. It is, therefore, possible to isolate faecal streptococci from water that contains few or no thermotolerant coliforms as, for example, when the source of contamination is distant in either time or space from the sampling point.

Methods of Identification and Detection

- Faecal streptococci are detectable by practical techniques, such as membrane filtration using m-enterococcus agar, at a temperature of 37-44 °C. More recently enterococci can be directly identified as micro-organisms capable of aerobic growth at $44\pm 0.5^{\circ}\text{C}$ and by the ability to hydrolyse 4- methyl-umbelliferyl- β -D-glucoside (MUD) in the presence of thallium acetate, nalidixic acid and 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) resulting in release of the fluorogen which in liquid media is readily detectable under ultraviolet light. or the hydrolysis of aesculin. Routine methods may give “false positives” and additional confirmatory tests may be required.

4.2.4. Clostridium perfringens

- C. perfringens which is Gram-positive anaerobic bacteria has been successfully used as fecal indicator for sewage-contaminated streams, ocean environments and sea water. As the majority of clostridia population forms spore, they are extremely resistant to the environmental stress and persist for longer time than other indicator bacteria (e.g., fecal coliforms and fecal streptococci) and most of pathogens do.
- Temperature and predators did not significantly affect the survival rates of this microorganism. Therefore, spores of C. perfringens represent one of the most conservative indicators of fecal pollution. Spore-forming bacteria are especially useful to determine the ultimate fate of sewage or storm water released into water body. C. perfringens may be ideal microorganisms to evaluate the completeness of disinfection in drinking water treatment processes
- A criticism of proposed C. perfringens usage is that they have extended viability and wide distributions in aquatic sediments. Spores of C. perfringens can be detected even in long distance from contamination sites, indicating remote or old fecal pollution. Additionally, their concentrations vary among different animal species. Feces of cattle, horse and sheep contain less C. perfringens than human feces do. Similar to many alternative fecal indicators, C. perfringens standards have not yet been evaluated based on epidemiological studies on the acceptable risk associated with fecal pollution.

5. WASHING AND STERILIZATION

- Sterilize all contaminated laboratory ware before cleaning to prevent potential contamination to personnel handling contaminated material.
- Remove all markings before initiating washing sequence. If material has dried on glassware, a pre-soaking may be needed.
- If mechanical laboratory washers are used, equip them with influent plumbing of stainless steel or other nontoxic material. Do not use units designed for home use. Ensure that the spray from water jets reaches all parts of deep vessels. Do not use copper plumbing to distribute water. Use stainless steel or other nontoxic material for the rinse-water system. Consistently follow the same washing procedures whether using an automated system or washing by hand.
- Cleanse all glassware and plasticware thoroughly with a suitable detergent and warm water. To remove all traces of residual washing compound, rinse five to ten times with cold water after bubbles/foam are gone. In addition, rinse two to three times with reagent-grade water. If desired, dry glassware before use by placing in a drying oven at 100°C for 10 to 15 min.
- Sterilize glassware, except when in metal containers, by dry heat for 2 to 4 h at a temperature of 170°C. Alternatively, add a small amount of distilled water (to prevent airlock) and autoclave at 121°C for at least 30 min.
- Sterilize glassware in metal containers at 170°C for not less than 2 h.
- For all bottles, loosen caps before autoclaving.
- Perform toxicity test, using the bromthymol blue test, on each batch of washed glassware before initial use of a washing compound and whenever a new formulation or washing procedure is used. If the bromthymol blue test is not done consistently, run the toxicity test on a per-lot or annual basis, whichever is more frequent.
- **Glassware**
 - Retain records and manufacturer certificates of analysis, purity, or tolerance level, if supplied, for all laboratory supplies.
 - The term “glassware” refers to both borosilicate glass and heat-resistant plastic materials. Volumetric glassware, pipettes, graduated cylinders, and beakers with calibration marks should be accurate to the specified volumetric tolerances. Volumetric

glassware is generally either Class A or Class B (undesignated). Class A is the more precise volumetric glassware.

- Determine tolerance once per lot or at a set percentage, e.g., 1 to 4%.
- Before each use, examine glassware and discard items with chipped edges or etched inner surfaces. Particularly examine screw-capped dilution bottles and flasks for chipped edges that could leak and contaminate the sample, analyst, and area.
- Inspect glassware after washing for excessive water beading, stains, and cloudiness and rewash if necessary. Replace glassware with excessive writing if markings cannot be removed. Either covers glassware or store glassware with its bottom up to prevent dust from settling inside it. Perform the following tests for clean glassware:
- **Toxicity Test**
 - pH check—Because some cleaning solutions are difficult to remove completely, spot check batches of clean glassware for pH reaction, especially if soaked in alkali or acid. To test clean glassware for an alkaline or acid residue add a few drops of 0.04% bromthymol blue (BTB) or other pH indicator and observe the color reaction. BTB should be blue-green (in the acceptable neutral range).

6. PREPARATION OF CULTURE MEDIA

- **Reagent-grade water**
- To prepare culture media and reagents, use only distilled or demineralized reagent-grade water that has been tested and found free from traces of dissolved metals and bactericidal or inhibitory compounds. Toxicity in distilled water may be derived from fluoridated water high in silica. Other sources of toxicity are silver, lead, and various unidentified organic complexes.
- Where condensate return is used as feed for a still, toxic amines or other boiler compounds may be present in distilled water. Residual chlorine or chloramines also may be found in distilled water prepared from chlorinated water supplies. If chlorine compounds are found in distilled water, neutralize them by adding an equivalent amount of sodium thiosulfate or sodium sulfite. Distilled water also should be free of contaminating nutrients. Such contamination may be derived from flashover of organics during distillation, continued use of exhausted carbon filter beds, deionizing columns in need of recharging, solder flux residues in new piping, dust and chemical fumes, and

storage of water in unclean bottles. Store distilled water out of direct sunlight to prevent growth of algae. Aged distilled water may contain toxic volatile organic compounds absorbed from the atmosphere if stored for prolonged periods in unsealed containers. Good housekeeping practices that minimize the presence of airborne particulates usually will eliminate nutrient contamination.

TABLE 9020:II. QUALITY OF REAGENT WATER USED IN MICROBIOLOGY TESTING

Test	Monitoring Frequency	Maximum Acceptable Limit
Chemical tests:		
Conductivity	Monthly*	$<2 \mu\text{mhos/cm}$ ($\mu\text{siemens/cm}$) at 25°C
Total organic carbon	Monthly	$<1.0 \text{ mg/L}$
Heavy metals, single (Cd, Cr, Cu, Ni, Pb, and Zn)	Annually†	$<0.05 \text{ mg/L}$
Heavy metals, total	Annually†	$<0.10 \text{ mg/L}$
Total chlorine residual	Monthly or with each use	$<0.1 \text{ mg/L}$
Bacteriological tests:		
Heterotrophic plate count (See Section 9215)	Monthly	$< 500 \text{ CFU/mL}$
Use test [(see 5/2)]	For a new source	Student's $t \leq 2.78$
Water quality test [see 5/1)]‡	Annually	0.8–3.0 ratio

* Monthly, if meter is in-line or has a resistivity indicator light; otherwise with each new batch of reagent water.

† Or more frequently if there is a problem.

‡ This bacteriological quality test is not needed for Type II water or better, as defined in *Standard Methods* (18th and 19th Editions), Section 1080C, or medium-quality water or better, as defined in *Standard Methods* (20th, 21st, and Online Editions), Section 1080C.

7. SAMPLES

- **Sample Collection**
- **Containers:**
- Collect samples for microbiological examination in clean, sterile, noncreative borosilicate glass or plastic bottles or Presterilized plastic bags appropriate for microbiological use.
- **Dechlorination:**
- Add a reducing agent to containers intended for the collection of water having residual chlorine or other halogen unless they contain broth for direct incubation of sample.
- Sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) is a satisfactory dechlorination agent that neutralizes any residual halogen and prevents continuation of bactericidal action during sample transit. The examination then will indicate more accurately the true microbial content of the water at the time of sampling.
- For drinking water samples, the concentration of dechlorination agent may be reduced: 0.1 mL of a 3% solution of $\text{Na}_2\text{S}_2\text{O}_3$ in a 120-mL bottle will neutralize up to 5 mg/L

residual chlorine. See the next Table for preparation of sodium thiosulfate solutions. Where possible, determine normal residual chlorine before sampling at a new site, e.g., pool water may contain a higher chlorine level than normal, to enable laboratory to prepare an adequate amount of dechlorination agent per sample bottle. Discard turbid (bacterial growth) 10% sodium thiosulfate stock solutions.

Solution Strength and $\text{Na}_2\text{S}_2\text{O}_3$ Form	Weight of Compound Required
3%, anhydrous	3 g/100 mL
3%, pentahydrate	4.6 g/100 mL
10%, anhydrous	10 g/100 mL
10%, pentahydrate	15.21 g/100 mL

• Sampling Procedures

- Maintain consistent sampling procedures. When the sample is collected, leave ample air space in the bottle (at least 2.5 cm) to facilitate mixing by shaking, before examination.
- Reject sample bottles that are overfilled and request re-sampling or, alternatively, add overfilled samples to a larger sterile sample bottle in the laboratory to assure adequate mixing.
- Keep sampling bottle closed until it is to be filled.
- Remove cap or stopper, if used, as a unit.
- Do not place cap down on any surface.
- Avoid external contamination during sample collection and do not contaminate inner surface of stopper or cap and bottle neck.
- Fill container without rinsing, replace stopper or cap immediately, and secure hood, if used, around neck of bottle.
- Systematically plan to collect samples that are representative of the water being tested. Sampling frequency and the number of samples to be collected will depend on ultimate data usage needs.
- **a. Potable water:**
 - Select a tap that is supplying water from a service pipe directly connected with the main, and is not, for example, served from a cistern or storage tank.

- Remove from the tap any attachments, such as filters, aerators, flow directors, or screens.
- Open cold water tap fully and let water run to waste for 2 or 3 min, or for a time sufficient to permit clearing the service line.
- Reduce water flow to permit filling bottle without splashing. If tap cleanliness is questionable, choose another tap. If a questionable tap is required for special sampling purposes, disinfect the faucet by flaming before sampling; let water run for additional 2 to 3 min after treatment.
- Do not sample from leaking taps that allow water to flow over the outside of the tap. If sampling from a mixing faucet cannot be avoided, run hot water for 2 min, then cold water for 2 to 3 min, and collect sample as indicated above.
- If the sample is to be taken from a well fitted with a hand pump, pump water to waste for about 5 to 10 min or until water temperature has stabilized before collecting sample.
- In drinking water evaluation studies, collect samples of finished water from distribution sites selected to assure systematic coverage during each month.
- Carefully choose distribution system sample locations to include dead-end sections to demonstrate bacteriological quality throughout the network and to ensure that localized contamination does not occur through cross-connections, breaks in the distribution lines, or reduction in positive pressure.
- Sample locations may be public sites (police and fire stations, government office buildings, schools, bus and train stations, airports, community parks), commercial establishments (restaurants, gas stations, office buildings, industrial plants), private residences (single residences, apartment buildings, and townhouse complexes), and special sampling stations built into the distribution network. Preferably avoid outdoor taps, fire hydrants, water treatment units, and backflow prevention devices. Establish sampling program in consultation with state and local health authorities.
- ***b. Raw water supply:***
 - In collecting samples directly from a river, stream, lake, reservoir, spring, or well, obtain samples representative of the water that is the source of supply to consumers. It is undesirable to take samples too near the bank or too far from the point of draw off, or at a depth above or below the point of draw off.

- To monitor stream and lake water quality, establish sampling locations at critical sites. Sampling frequency may be seasonal for recreational waters, daily for water supply intakes, hourly where waste treatment control is erratic and effluents are discharged into shellfish harvesting areas, or even continuous.
- Take samples from a river, stream, lake, reservoir, or pool by holding the bottle near its base in the hand (use gloves) and plunging it, neck downward, below the surface. Turn bottle until neck points slightly upward and mouth is directed toward the current. If there is no current, as in the case of a reservoir, create a current artificially by pushing bottle forward horizontally in a direction away from the hand. When sampling from a boat, obtain samples from upstream side of boat. If it is not possible to collect samples from these situations in this way, attach a weight to base of bottle and lower it into the water. In any case, take care to avoid contact with bank or stream bed; otherwise, water fouling may occur.
- **Sample Volume**
- The volume of sample should be sufficient to carry out all tests required. For potable water samples collect a minimum of 100 ± 2.5 ml. Larger volumes may be needed for bacterial pathogen, protozoan, and viral analyses.
- **Identifying Data**
- Accompany samples by complete and accurate identifying and descriptive data such as name of system or site; sample type; collection location; sampling depth, date, and time; sampler's name; analyses to be performed; chlorine residual; and reducing agents if used, e.g., sodium thiosulfate and EDTA.
- Record abnormalities or departures from specified sample collection, handling, or receipt procedures.
- Where possible or required for compliance, sample receipt information should indicate chain of custody and shipment handling at temperatures $<8^{\circ}\text{C}$, but not frozen. Do not accept for examination inadequately identified samples.
- **b. Preservation and Storage**
- **Holding Time and Temperature**
- **a. General:**
- Start microbiological analysis of water samples as soon as possible after collection to avoid unpredictable changes in the microbial population. Do not analyze samples

submitted to the laboratory with chlorine residual or with leakage. In such cases, request re-sampling.

- For most accurate results, ice samples during transport to the laboratory if they cannot be processed within 1 h after collection. Maintain samples in the dark and keep cool with ice or blue ice at $<8^{\circ}\text{C}$ but not frozen.
- Samples arriving quickly at the laboratory may not have reached this temperature. Verify and record sample temperature upon receipt either through the use of a control water sample bottle or infrared thermometer.
- ***b. Drinking water for compliance purposes:***
- For coliform *E.coli* analyses, the holding time from collection to analysis is 30 h. While there is no regulatory preservation temperature, attempt to keep samples at $<8^{\circ}\text{C}$ during transport to the laboratory.
- Maintain samples for heterotrophic plate count analysis at $<8^{\circ}\text{C}$ and do not exceed 8 h holding time. Do not freeze. Record sample receipt time and temperature in sample receipt file.
- Analyze samples on day of receipt whenever possible and refrigerate overnight if arrival is too late for processing on same day, as long as holding time conditions can still be met.
- ***c. Non potable water***
- Hold source water, stream pollution, recreational water, and wastewater samples at $<8^{\circ}\text{C}$ during a maximum transport time of 6 h. Do not freeze. Record sample receipt time and temperature in sample receipt files. Refrigerate these samples upon receipt in the laboratory and process within 2 h. When transport conditions necessitate delays in delivery of samples longer than 6 h, consider using either field laboratory facilities located at the site of collection or delayed incubation procedures.