CONTENT

I. Microbes and oxygen:

1. A strictly **aerobic** organism has an absolute requirement for oxygen. Supplementing oxygen (agitation, aeration) will improve growth.

Facts on aerobes and aerobic metabolism:
Aerobic organisms have an efficient metabolism as compared to that of most anaerobes because of the high reduction potential of molecular oxygen, which serves as the Terminal Electron Acceptor (TEA) for respiration. This advantage comes with a price, because both the chemical and the metabolic reduction of oxygen result in the production of highly toxic and reactive oxygen species (1, 2). The univalent reduction product of oxygen, superoxide (O_2^-), reacts with hydrogen peroxide in the presence of transition metals to produce the reactive hydroxyl radical $OH^{\cdot}$, which is most likely responsible for the toxic effects of molecular oxygen (3). Aerobic organisms have developed mechanisms to protect themselves from oxygen toxicity. These involve the enzymes SuperOxide Dismutase (SOD) (Eq. 1) (4), catalase (Eq. 2) (5), and nonspecific peroxidases (Eq. 3) (5, 6).

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (1)$$

$$2H_2O_2 \rightarrow 2H_2O + O_2 \quad (2)$$

$$H_2O_2 + RH_2 \rightarrow 2H_2O + R \quad (3)$$

For normal growth, aerobic organisms require molecular oxygen at near-atmospheric concentrations (21% v/v), whereas anaerobic organisms vary in their responses to oxygen, ranging from the extremely sensitive methanogens (7) to the more aerotolerant, sulfate-reducing *Desulfovibrio* (8), some species of which may be microaerophilic (9, 10). Although most anaerobes inhabit ecosystems that are periodically exposed to air, they are unlikely to contain SOD or catalase, because both enzymes generate molecular oxygen (Eqs. 1 and 2) and thereby potentially propagate the production of reactive oxygen species. Although there are some exceptions (11), SOD and catalase genes are not generally present in anaerobes, as illustrated by their absence from the complete genome sequences now available for the anaerobic organisms *Methanococcus jannaschii* (12), *Archaeoglobus fulgidus* (13), *Pyrococcus horikoshii* (14), *P. abysii* (15), and *Thermotoga maritima* (16), as well as the incomplete genome of *Clostridium acetobutylicum* (17). So what defense mechanism against reactive oxygen species do these organisms possess? Our data suggest that anaerobes contain an enzyme involved in oxygen metabolism: SuperOxide Reductase (SOR).

2. An **anaerobic organism** or **anaerobe** is any organism that does not require oxygen for growth.
   - **Obligate anaerobes** will die when exposed to atmospheric levels of oxygen (even 10 mins is lethal)
   - **Facultative anaerobes** can use oxygen when it is present.

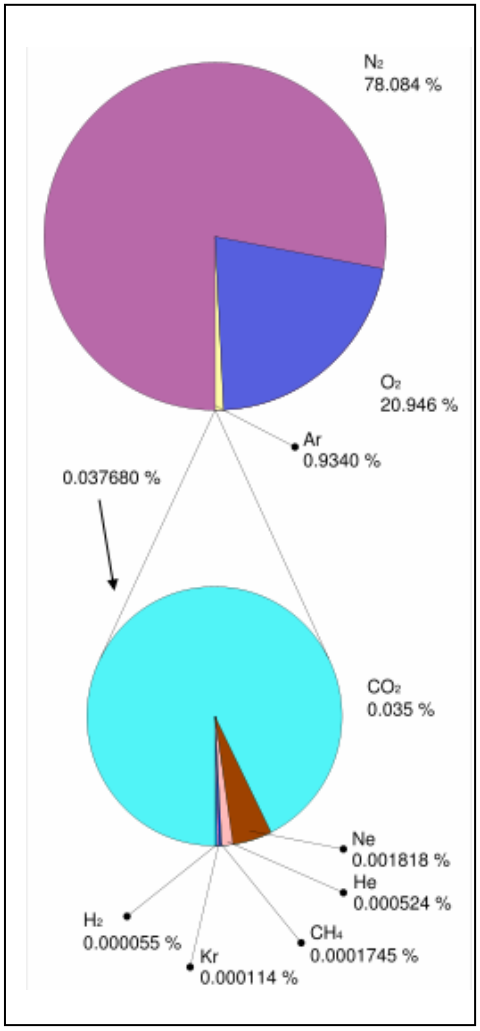
3. An **aerotolerant** organism does not require oxygen, and is also not affected by exposure to air.

4. A **microaerophile** uses oxygen, but only at very low concentrations (low micromolar range) with growth inhibited by normal oxygen concentrations (approximately 200 micromolar).

5. A **nanaerobe** cannot grow in the presence of micromolar concentrations of oxygen, but can grow with and benefit from nanomolar concentrations of oxygen.

II. Air and it's composition:

Earth's atmosphere is a layer of gases surrounding the planet Earth and retained by the Earth's gravity. It contains roughly 78% nitrogen and 21% oxygen, trace amounts of other gases, and water vapour (LHS panel). This mixture of gases is commonly known as **air**. The atmosphere protects life on Earth by absorbing ultraviolet solar radiation and reducing temperature extremes between day and night.



The atmosphere has no abrupt cut-off. It slowly becomes thinner and fades away into space. There is no definite boundary between the atmosphere and outer space (RHS panel). Three-quarters of the atmosphere's mass is within 11 km of the planetary surface. In the United States, persons who travel above an altitude of 50.0 miles (80.5 km) are designated as astronauts. An altitude of 120 km (75 mi or 400,000 ft) marks the boundary where atmospheric effects become noticeable during re-entry. The Karman line, at 100 km (62 mi), is also frequently used as the boundary between atmosphere and space.

I. Solubility of oxygen in liquids:
Low diffusion rates in water
1. about 10,000x slower than in air
2. consider: air = 20% O but saturation in water at 20C = 10 mg/L (0.001 %)
less Oxygen availability leads to chemically reducing sediments & as time progresses, a cascade of **electron** acceptors (for bacterial metabolism) are depleted

Table 3.5 + O depletion at about +330 **mV Refer to Electron Tower Figure**

REFER TO A MICROBIOLOGY TEXT BOO

Further Reading**:** La Duc, M. T., Satomi, M.and Venkateswaran, K. (2004). *Bacillus odysseyi* sp. nov., a round-spore-forming bacillus isolated from the Mars Odyssey spacecraft. International Journal of Systematic and Evolutionary Microbiology 54, 195-201 (click here).

III. Oxygen, its origin and aerobic life:

Oxygen is a chemical element. In the periodic table it has the symbol **O** and atomic number 8. Oxygen is the second most common element on Earth composing around 49% of the mass of Earth's crust and 28% of the mass of Earth as a whole, and is the third most common element in the universe. On Earth, it is usually covalently or ionically bonded to other elements. Unbound oxygen (usually called molecular dioxygen, O_2, a diatomic molecule; Note: O_3 is a triatomic molecule aka ozone, first appeared in significant quantities on Earth during the Paleoproterozoic era (between 2.5 billion years ago and 1.6 billion years ago) as a product of the metabolic action of early anaerobes (domain *Archaea* and domain *Bacteria*). This new presence of large amounts of free oxygen drove most of the organisms then living to extinction. The atmospheric abundance of free oxygen in later geological epochs and up to the present has been largely driven by photosynthetic organisms, roughly three quarters by phytoplankton (see under Planktons, below) and algae in the oceans and one quarter from terrestrial plants.

Planktons

Planktons are primarily divided into broad functional (or trophic level**,** trophic level is the position that an organism occupies in a food chain - what it eats, and what eats it.) groups:

- **Phytoplankton** (from Greek *phyton*, or plant, planktos meaning wanderer or drifter, drift in water columns) are autotrophic prokaryotic -cyanobacteria- or eukaryotic algae, diatoms, dinoflagellates that live near the water surface where there is sufficient light to support photosynthesis. **PRIMARY PRODUCERS**
- **Zooplankton** (from Greek *zoon*, or animal), small protozoans or metazoans (e.g. crustaceans and other animals) that feed on other plankton. Some of the eggs and larvae of larger animals, such as fish, crustaceans, and annelids, are included here. **CONSUMERS.**
- **Bacterioplankton**, bacteria and archaea, which play an important role in remineralising organic material down the water column (note that many phytoplankton are also bacterioplankton). **RECYCLERS.**

IV. The Winogradsky Column- A Simple Microcosm:

It is best to look at the microbial life to the consequences of the presence and absence of oxygen in an ecosystem with a microcosm- a miniaturized laboratory ecosystem. An animation can be followed using the following links http://www.sumanasinc.com/webcontent/anisamples/microbiology/winogradsky.html

Answer the following questions:
Dissect the physiologies of the various types of microbes present.
Can you identify which is a primary producers, a consumer and a recycler?
Describe how you would proceed to make another type of microcosm.

V. Anaerobe Habitats:

(a) Oral Cavity

(b) Rumen

(c) Human Gastrointestinal Tract

(d) Termite Guts

(e) Bioremediation:

Anaerobic microbial activity can be carried out in the absence of O_2 as a terminal electron acceptor (TEA) in respiration. Alternatives to oxygen in respiration can occur in the presence of $N0_3^-$, FeIII, MnIV, SO_4^{2-}, and even CO_2 in the case of methanogenesis.

The anaerobic microbial guild that will grow depends on the availability and concentrations of most energetically favorable TEA and carbon sources in that particular environment. For example, if sufficient

NO_3^- is present in the absence of O_2, the environment is said to be nitrate reducing and conditions may also exist for an iron reducing, sulfate reducing, or methanogenic environment. In addition, a particular site may have zones with one or more of these conditions prevailing.

The zones are of particular relevance to biodegradation since different organic contaminants, such as benzene, toluene, and chlorinated compounds will have different microbial degradation rates depending on the presence and concentration of the TEA. Rates under nitrate reducing conditions are often faster than under methanogenic conditions as nitrate reduction is more energetically favourable. Similarly, aromatic compounds may biodegrade more readily under nitrate reducing conditions than under sulphate reducing conditions. Some compounds, such as chlorinated compounds and MTBE, may actually biodegrade at higher rates in the presence of TEA than under aerobic conditions. Therefore, in bioremediation it is crucial that an understanding of the anaerobic site conditions be gained before an assessment of bioremediation potential activity can be made. General biodegradation predictions can be made from an introductory site characterization, and realistic microcosm studies can then be set up based upon this information.

Recommended site characterization protocol:
1) A characterization of the presence of the major physiological groups of anaerobes (guild groups) such as iron, Manganese, sulfate reducing bacteria (SRB) using MPNs and plate counting from the site. The presence of total heterotrophic bacteria and hydrocarbon degrading bacteria (anaerobic and aerobic) could also be counted.

2) A concurrent characterization / estimation of the inorganic chemistry (sulfate, nitrate-N, ammonia-N, Ortho-Phosphate, Dissolved Oxygen, redox potential [mV], and pH, ferrous and ferric iron, sulfide, and methane) of the site should be undertaken and will provide data on physicochemical (abiotic) conditions that could support anaerobic biodegradation of target contaminants.

Anaerobic plate counts for hydrocarbon-degraders and total heterotrophs:
The culture assays are similar in principle to aerobic assays, except that they are performed in the absence of oxygen. Alternate terminal electron acceptors to oxygen, such as sulfate, nitrate, and ferric iron are added to the media to meet anaerobic respiration needs. A standard anaerobic agar (DIFCO) can be used for total anaerobic heterotrophic plate counts. For anaerobic hydrocarbon degraders, a combination of diesel and jet fuel can be added to the media as sole carbon sources. A minimal salts mixture and trace elements may be added to meet growth requirements.

Bacteria enumerations by MPN method for anaerobic iron reducers, nitrate reducers and sulfate reducers
Most probable number (MPN) techniques are adaptations of the classical Standard Methods technique originally developed for the enumeration of Coliform bacteria in wastewater. Specialized media are used for each anaerobic group tested for.

Methods Used to Determine Anaerobes in Bioremediation Procedures:

Physiological group	Media used	Growth indicator	Reference
Fe(III) Reducers	FWA medium with ferric citrate as TEA & acetate as sole carbon source.	Color change: brown□ green□ clear	Lovley et al. 1988
Nitrate Reducers	Nitrate Broth	Bray'sNitrate Powder Color Change	Focht & Joseph, 1973
Sulfate Reducers	SRB medium	FeS black precipitate.	Postgate et al.., 1979

Anaerobic microcosm studies to determine biodegradability/ biodegradability potential

Although biodegradation rates can often be inferred by a combination of microbial site characterization data and historical contaminant chemistry data, laboratory biodegradation microcosm studies can often be an effective tool in determining contaminant breakdown rates. Microcosm studies can be designed in which microbial parameters can be measured throughout the assay, or in which specific breakdown products can be quantified. These studies follow the generalized protocol below:

- Using site characterization data, microcosms are designed to mimic the anaerobic site conditions (nitrate-, sulfate-, iron- reducing, or methanogenic).
- Using soil, sediment, and/ or water from the site containing indigenous microbes, microcosms are set up as serum bottle (water or slurry) or flow-through column (soil or sediment).
- The desired contaminant(s) are added.
- Changes in contaminant concentrations through time are monitored to establish breakdown rates.

VI. Anaerobe Diversity & Physiology:

Obligate anaerobes may use fermentation or anaerobic respiration. In the presence of oxygen, facultative anaerobes use aerobic respiration; without oxygen some of them ferment, some use anaerobic respiration. Aerotolerant organisms are strictly fermentative. Microaerophiles carry out aerobic respiration, and some of then can also do anaerobic respiration.

There are many chemical equations for anaerobic fermentative reactions.
Fermentative anaerobic organisms mostly use the lactic acid fermentation pathway:

$C_6H_{12}O_6 + 2\ ADP + 2\ phosphate \rightarrow 2\ lactic\ acid + 2\ ATP$

The energy released in this equation is approximately 150 kJ per mol, which is conserved in regenerating two ATP from ADP per glucose. This is only 5% of the energy per sugar molecule than the typical aerobic reaction generates.

Plants and fungi (e.g., yeasts) generally use alcohol (ethanol) fermentation when oxygen becomes limiting:

$C_6H_{12}O_6 + 2\ ADP + 2\ phosphate \rightarrow 2\ C_2H_5OH + 2\ CO_2 + 2\ ATP$

The energy released is about 180 kJ per mol, which is conserved in regenerating two ATP from ADP per glucose.

Anaerobic bacteria and archaea use these and many other fermentative pathways, e.g., propionic acid fermentation, butyric acid fermentation, solvent fermentation, mixed acid fermentation, butanediol fermentation, Stickland fermentation, acetogenesis or methanogenesis.

Some anaerobic bacteria produce toxins (e.g., tetanus or botulinum toxins) that are highly dangerous to higher organisms, including humans.

Obligate(strict)anaerobes die in presence of oxygen due to the absence of the enzymes superoxide dismutase and catalase which would convert the lethal superoxide formed in their cells due to the presence of oxygen.

Cellular Respiration:

It is a process in which the chemical bonds of energy-rich molecules (eg glucose) are converted into energy usable for life processes. Oxidation* (see oxidizing and reducing agents) of organic material is an exothermic reaction that releases a large amount of energy rather quickly. The equation for the oxidation of glucose is: $C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$ + Energy released (2830 kJ mol^{-1})

Bonfire, is a massive uncontrolled release of energy as light; cellular respiration is the same process but it occurs in gradual steps that result in the conversion of the energy stored in glucose to usable chemical energy in the form of ATP. ATP is known as the universal currency because when the phosphoanhydride bonds in ATP are hydrolysed in an exergonic reaction, the energy yield is 30kJ per mole under standard conditions. Waste products ($CO_2 + H_2O$) are released through exhaled air, sweat and urine in humans.

VII. Anaerobic Sample Collection:

(a) Non-clinical samples- Mix mud and water, and scoop in sterile containers to brim, cap and transport on ice (if mesophilic) or at room temperature (if thermophiles). BOTTLE EFFECT problems

(b) Clinical samples- Samples should be collected contamination-free and protected from oxygen exposure. Anaerobic bacteria cultures should be obtained from an appropriate site without the health care professional contaminating the sample with bacteria from the adjacent skin, mucus membrane, or tissue. Swabs should be avoided when collecting specimens for anaerobic culture because cotton fibers may be detrimental to anaerobes. Abscesses or fluids can be aspirated using a sterile syringe that is then tightly capped to prevent entry of air. Tissue samples should be placed into a degassed bag and sealed, or into a gassed out screw top vial that may contain oxygen-free prereduced culture medium and tightly capped. Samples placed in anaerobic transport fluid and transported on ice. The specimens should be plated as rapidly as possible onto culture media that has been prepared.

VIII. Methods of Culturing Anaerobes:

Even 10 min exposure to air for some anaerobes can be lethal. The methods of obtaining specimens for anaerobic culture and the culturing procedure are performed to ensure that the organisms are protected from oxygen.

(a) Principles & Equipment

(i) Removing oxygen: Boiling (microwaving), cooling under OFN and purging with OFN gas during dispensing. Oxygen is less soluble in liquid media at high temperatures or in viscous liquids

(ii) Chemical Reducing Agents: Sodium thioglycollate, L-cystine, L-cysteine, Titanium(III) citrate, and Dithiothreitol (DTT), each at concentrations of 0.1, 0.05, and 0.01%; sulfide, amorphous iron sulfide

Chemical oxidizing and reducing agents

Oxidation describes the *loss* of an electron (a fundamental subatomic particle that carries a negative electric charge, participates in electromagnetic interactions, and interacts with adjacent nuclei in chemical bonding) by a molecule, atom or ion. Substances that have the ability to oxidize other substances are said to be oxidative and are known as oxidizing agents, oxidants or oxidizers. Put in another way, the oxidant removes electrons from the other substance, and is thus reduced itself. Oxidants are usually chemical substances with elements in high oxidation numbers (e.g., H_2O_2, MnO_4^-, CrO_3, $Cr_2O_7^{2-}$, OsO_4) or highly electronegative substances that can gain one or two extra electrons by oxidizing a substance (O, F, Cl, Br).

Substances that have the ability to **reduce** other substances are said to be **reductive** and are known as **reducing agents**, **reductants**, or **reducers**. Put in another way, the reductant transfers electrons to the substance. Reductants in chemistry are very diverse. *Metal reduction* - electropositive elemental metals can be used (Li, Na, Mg, Fe, Zn, Al). These metals donate or *give away* electrons readily. Other kinds of reductants are *hydride transfer reagents* ($NaBH_4$, $LiAlH_4$), these reagents are widely used in organic chemistry, primarily in the reduction of carbonyl compounds to alcohols. Another useful method is **reductions involving hydrogen gas (H_2) with a palladium** (platinum, nickel) **catalyst**. These *catalytic reductions* are primarily used in the reduction of carbon-carbon double or triple bonds.

The chemical way to look at redox processes is that *the reductant transfers electrons to the oxidant*. Thus, in the reaction, the **reductant** or *reducing agent* looses electrons and is **oxidized** and the **oxidant** or *oxidizing agent* gains electrons and is **reduced**.

(iii) Maintaining anaerobiosis:

Purging with OFN gas during dispensing.
Use of membrane fractions to create anaerobiosis http://www.oxyrase.com/
Mineral oil over heated and cooled broth to prevent re-entry of oxygen
0.5 cm mineral oil overlay of culture medim containing Oxyrase (20 µl/ml)

(iv) Indicators of anaerobiosis: Resazurin (Oxoid), Methylene Blue indicator strip (Becton Dickinson)

(v) Culture vessels (Thick walled Pyrex tubes with rubber butyl rubber bungs, Hungate tubes, Serum vials, Belco Tubes)

(vi) Anaerobic chambers- can be flexible or inflexible

Coy chambers at the URL http://www.coylab.com
Bactron Anaerobic chambers http://www.shellab.com/applications/bactron.html
Forma Scientific chambers http://www.labx.com/v2/adsearch/detail3.cfm?adnumb=293707
Make your own:

Balish, E. et al., (1977). Transparent Plastic Incubator for the Anaerobic Glove Box Appl Environ Microbiol. 33(3): 525–527.
Dickman, M. D. (1979). Compact anaerobic glove box for hospitals and research laboratories. Journal of Clinical Microbioliology 9(2):294-296.

(vi) Pure culture isolation in Agar Roll Tubes

(vii) Other commercial equipment

GasPak® method in anaerobic jars
Brewer anaerobic jars
Oxoid jars
Difco anaerobic jars
Becton Dickinson jars
Make your own- Click Clack jars
Double-tube
Mini-tube
Sandwiched Microtiter Plate
Mitsubishi AnaeroPack
AnaeroGen oxygen-scavenging envelopes (Oxoid)

(b) Liquid Media for enrichment & culturing

(c) Isolation Techniques

IX. Complications of Culturing Anaerobes in a Clinical Setting:

Purpose

Anaerobic bacterial cultures are performed to identify bacteria that grow only in the absence of oxygen and which may cause human infection. If overlooked or killed by exposure to oxygen, anaerobic infections result in such serious consequences as amputation, organ failure, sepsis, meningitis, and death. Culture is required to correctly identify anaerobic pathogens and institute effective antibiotic treatment.

Precautions

It is crucial that the health care provider obtain the sample for culture via aseptic technique. Anaerobes are commonly found on mucous membranes and other sites such as the vagina and oral cavity. Therefore, specimens likely to be contaminated with these organisms should not be submitted for culture (e.g., a throat or vaginal swab). Some types of specimens should always be cultured for anaerobes if an infection is suspected. These include abscesses, bites, blood, cerebrospinal fluid and exudative body fluids, deep wounds, and dead tissues. The specimen must be protected from oxygen during collection and transport and must be transported to the laboratory immediately.

Description

Anaerobes are normally found within certain areas of the body but result in serious infection when they have access to a normally sterile body fluid or deep tissue that is poorly oxygenated. Some anaerobes normally live in the crevices of the skin, in the nose, mouth, throat, intestine, and vagina. Injury to these

tissues (i.e., cuts, puncture wounds, or trauma) especially at or adjacent to the mucous membranes allows anaerobes entry into otherwise sterile areas of the body and is the primary cause of anaerobic infection. A second source of anaerobic infection occurs from the introduction of spores into a normally sterile site. Spore-producing anaerobes live in the soil and water, and spores may be introduced via wounds, especially punctures. Anaerobic infections are most likely to be found in persons who are immunosuppressed, those treated recently with broad-spectrum antibiotics, and persons who have a decaying tissue injury on or near a mucous membrane, especially if the site is foul-smelling.

Some specimens from which anaerobes are likely to be isolated are:

- blood
- bile
- bone marrow
- cerebrospinal fluid
- direct lung aspirate
- tissue biopsy from a normally sterile site
- fluid from a normally sterile site (like a joint)
- dental abscess
- abdominal or pelvic abscess
- knife, gunshot, or surgical wound
- severe burn

Some of the specimens that are not suitable for anaerobic cultures include:

- coughed throat discharge (sputum)
- rectal swab
- nasal or throat swab
- urethral swab
- voided urine

Specimen collection

The keys to effective anaerobic bacteria cultures include collecting a contamination-free specimen and protecting it from oxygen exposure. Anaerobic bacteria cultures should be obtained from an appropriate site without the health care professional contaminating the sample with bacteria from the adjacent skin, mucus membrane, or tissue. Swabs should be avoided when collecting specimens for anaerobic culture because cotton fibers may be detrimental to anaerobes. Abscesses or fluids can be aspirated using a sterile syringe that is then tightly capped to prevent entry of air. Tissue samples should be placed into a degassed bag and sealed, or into a gassed out screw top vial that may contain oxygen-free prereduced culture medium and tightly capped. The specimens should be plated as rapidly as possible onto culture media that has been prepared.

Culture

Cultures should be placed in an environment that is free of oxygen, at 95°F (35°C) for at least 48 hours before the plates are examined for growth.

Gram staining is performed on the specimen at the time of culture. While infections can be caused by aerobic or anaerobic bacteria or a mixture of both, some infections have a high probability of being caused by anaerobic bacteria. These infections include brain abscesses, lung abscesses, aspiration pneumonia, and dental infections. Anaerobic organisms can often be suspected because many anaerobes have characteristic microscopic morphology (appearance). For example, *Bacteroides* spp. are gram-negative rods that are pleomorphic (variable in size and shape) and exhibit irregular bipolar staining. *Fusobacterium* spp. are often pale gram-negative spindle-shaped rods having pointed ends. *Clostridium* spp. are large gram-positive rods that form spores. The location of the spore (central, subterminal, terminal, or absent) is a useful differential characteristic. The presence of growth, oxygen tolerance, and Gram stain results are sufficient to establish a diagnosis of an anaerobic infection and begin antibiotic treatment with a drug appropriate for most anaerobes such as clindamycin, metronidazole, or vancomycin.

Gram-negative anaerobes and some of the infections they produce include the following genera:

- *Bacteroides* (the most commonly found anaerobes in cultures; intra-abdominal infections, rectal abscesses, soft tissue infections, liver infection)
- *Fusobacterium* (abscesses, wound infections, pulmonary and intracranial infections)
- *Porphyromonas* (aspiration pneumonia, periodontitis)
- *Prevotella* (intra-abdominal infections, soft tissue infections)

Gram-positive anaerobes include the following:

- *Actinomyces* (head, neck, pelvic infections; aspiration pneumonia)
- *Bifidobacterium* (ear infections, abdominal infections)
- *Clostridium* (gas, gangrene, food poisoning, tetanus, pseudomembranous colitis)
- *Peptostreptococcus* (oral, respiratory, and intra-abdominal infections)
- *Propionibacterium* (shunt infections)

The identification of anaerobes is highly complex, and laboratories may use different identification systems. Partial identification is often the goal. For example, there are six species of the *Bacteroides* genus that may be identified as the *Bacteroides fragilis* group rather than identified individually. Organisms are identified by their colonial and microscopic morphology, growth on selective media, oxygen tolerance, and biochemical characteristics. These include sugar fermentation, bile solubility, esculin, starch, and gelatin hydrolysis, casein and gelatin digestion, catalase, lipase, lecithinase, and indole production, nitrate reduction, volatile fatty acids as determined by gas chromatography, and susceptibility to antibiotics. The antibiotic susceptibility profile is determined by the microtube broth dilution method. Many species of anaerobes are resistant to penicillin, and some are resistant to clindamycin and other commonly used antibiotics.

Diagnosis/Preparation

The health care provider should take special care to collect a contamination-free specimen. All procedures must be performed aseptically. The health care professional who collects the specimen should be prepared to take two samples, one for anaerobic culture and one for aerobic culture, since it is unknown whether the pathogen can grow with or without oxygen. In addition, health care professionals should document any antibiotics that the patient is currently taking and any medical conditions that could influence growth of bacteria.

Aftercare

In the case of vein puncture for anaerobic blood cultures, direct pressure should be applied to the vein puncture site for several minutes or until the bleeding has stopped. An adhesive bandage may be applied, if appropriate. If swelling or bruising occurs, ice can be applied to the site. For collection of specimens other than blood, the patient and the collection site should be monitored for any complications after the procedure.

Risks

Special care must be taken by the health care team obtaining, transporting, and preparing the specimen for anaerobic culture. Poor methodology may delay the identification of the bacterium, may allow the patient's condition to deteriorate, and may require the patient to provide more samples than would otherwise be required. Patients may experience bruising, discomfort, or swelling at the collection site when tissue, blood, or other fluids are obtained.

Results

Negative results will show no pathogenic growth in the sample. Positive results will show growth, the identification of each specific bacterium, and its antibiotic susceptibility profile.

Patient education

A health care team member should explain the specimen collection procedure to the patient. If the patient is seriously ill, the team member should explain the procedure to the patient's family members. The patient and his or her family should understand that because bacteria need time to grow in the laboratory, several days may be required for bacterium identification.

X. Anaerobic Infections.

ANAEROBIC BACILLI

I. INTRODUCTION

Anaerobes are the most primitive organisms in regard to oxidation. The more oxidative an organism (pigments, cytochromes, etc.), the more developed and/or further evolved it is. Anaerobes have a relatively inefficient metabolic system. They ferment anaerobically to derive energy from their substrates. Obligate anaerobes are killed in the presence of molecular oxygen.

II. *Clostridium* species

A. DESCRIPTION

The Clostridia are large anaerobic, Gram-positive, motile rods. Many decompose proteins or form toxins, and some do both. Their natural habitat is the soil or the intestinal tract of animals and humans, where they live as saprophytes.

B. MORPHOLOGY AND IDENTIFICATION

1. Most species are motile and possess peritrichous flagella.
2. *Clostidium* sp. are known for the production of spores that allow survival of the organism under severe nutrient deprivation and dehydration conditions. Spores are even resistant to the actions of common antimicrobial agents and treatments. *Clostridium* spores are usually wider than the diameter of the rods in which they are formed. Placement can be central, subterminal, or terminal.

C. CULTURE AND PHYSIOLOGY

1. Anaerobic culture in which the agar plates or tubes are placed in an airtight jar in which a nitrogen/10% CO2 atmosphere is generated.
2. Fluid media are put in deep tubes containing chopped, cooked meat or 0.1% agar and a reducing agent such as thioglycolate.
3. Large raised colonies with entire margins (*C. perfringens*), or smaller colonies that extend in a meshwork of fine filaments (*C. tetani*). Many show hemolysis on blood agar (hemolysin production).
4. Possible explanations for the obligate anaerobic requirement include:
    - a. Obligate anaerobes lack superoxide dismutase: they have no mechanism to destroy the toxicity due to superoxide anion formation.
    - b. In the presence of oxygen, superoxide anions form peroxides, oxidizing agents lethal to obligate anaerobes (lacking catalase and peroxidase).
    - c. Obligate anaerobes need a reduced environment. Reduction is evaluated using oxygen and hydrogen electrodes. The hydrogen electrode is the most highly reduced (-480 mV potential: a good anaerobic environment). Oxidation to between +50 and +75 mV results in no growth.
    - d. The need for anaerobic culture conditions makes it difficult to isolate anaerobes from patients. A reduced environment can be maintained artificially using reducing agents or biologically by aerobic organisms.

The *Clostridium* species are not as sensitive to oxygen as some anaerobic species; therefore they are a little easier to isolate and identify in the lab. They produce serious disease including:

1. BOTULISM
2. TETANUS
3. GAS GANGRENE
4. PSEUDOMEMBRANOUS ULCERATIVE COLITIS (Antibiotic-induced colitis)
5. FOOD POISONING

III. *Clostridium botulinum*

A. **MORBIDITY/MORTALITY**

Botulism is fortunately not a serious disease in terms of incidence, but it is serious in terms of its potential mortality.

1. In the mid-1970s there was an increase in botulism due to increased home canning.
2. Infant botulism was first recognized in the 1970s.

B. **EXTRACELLULAR PRODUCTS**

1. Both *C. botulinum* and *C. tetani* involve potent neuroexotoxins (exotoxins that specifically affect neuronal tissue).
    - a. *C. botulinum* toxin acts upon peripheral nerves. There are eight serologica l types of toxin. Types A, B, and E cause most human disease.
    - b. *C. tetani* toxin (tetanospasmin) acts centrally at the level of the anterior horn cells of the spinal cord.
2. In the case of botulism, the toxin is ingested in a preformed state (ie in home-canned vegetables). Soil spores germinate and produce toxin. The botulinum toxin is heat labile. If improperly canned vegetables are boiled for 10 minutes, botulism poisoning is no longer a problem. In contrast, Staphyloenterotoxin is heat stable.
3. The toxin is activated by proteases in the gastric fluid and by gastric acidity. The toxin is absorbed in the intestine and is transported systemically via the bloodstream. Botulinus toxin is quick acting with signs and symptoms of intoxication apparent within 12 hours after ingestion.
4. Once the toxin is fixed to the tissue, its actions are very difficult to reverse with antitoxin treatment. Antitoxin is only effective if it binds to the toxin before the toxin binds the neuromuscular junction (within 12 hours after ingestion). Prognosis is poor for patients diagnosed after this time period.
5. Botulinus toxin is a medium-sized (150 kDa) AB type protein toxin. There is a binding component and a toxic component. The toxin inhibits acetylcholine release at the level of the presynaptic terminals at the neuromuscular junction. This leads to flaccid paralysis and potentially, respiratory failure.

C. **SYMPTOMS**

1. Visual disturbances (eye muscles not coordinated, double vision)
2. Inability to swallow and speech difficulties occur within 18-24 h after ingestion.
3. GI involvement is not usually prominent.
4. No fever is apparent.

D. **TREATMENT**

There is no reason to give antibiotics; normally there are no organisms, except in infant botulism (found in infants under 6 months of age). A potent trivalent (A,B,E) antitoxin is available from the CDC; it must be promptly administered IV. Rapid antitoxin treatment has reduced the mortality rate from 65% to below 25%.

E. **INFANT BOTULISM**

Between birth and 6 months there is a unique susceptibility to ingested spores that germinate in the intestine and multiply, producing toxin locally. Toxin is taken up by the bloodstream. Symptoms are milder than in adult botulism and mortality is not as high. The amount of toxin produced is less than would typically be ingested by an adult. After the age of 6 months, the permeability of the intestinal mucosa changes dramatically, contributing to the narrow age range for susceptibility to infant botulism.

IV. *Clostridium tetani*

A. **MORBIDITY/MORTALITY**

1. Tetanus is a disease of low incidence in the U.S. and is a disease that is entirely preventable with toxoid immunization that gives essentially 100% protection. The toxoid booster confers protection for more than 10 years.
2. Tetanus is a serious clinical disease because it is difficult to reverse, with a mortality rate of approximately 50%. The toxin produces spastic paralysis due to uncontrolled repetitive firing. A sardonic smile is characteristic of early stages of tetanus, because the masseter muscles are very sensitive.
3. Tetanus is different from botulism in that the organism grows in the host. There is a host-parasite interaction. The bacteria remain localized at the site of introduction. Disease is almost entirely a neurotoxemia.

B. **EXTRACELLULAR PRODUCTS**

1. Tetanspasmin is a heat labile AB-type toxin, approximately 150 kDa. Subunit "A" binds to the target tissue and subunit "B" has the toxic effect.
2. *C. tetani* also produce one other virulence factor called tetanolysin. It may or may not be important in local infection. The toxin is a hemolysin.

C. **PATHOGENESIS**

1. *C. tetani* grows in traumatized tissue where the blood supply is cut off and ischemia results, producing an anoxic, anaerobic environment, with low redox potential. Acidity increases with inflammation. The more acidic the environment, the more reduced it tends to be. A puncture wound can also cause spores to be injected deeply into the tissue.
2. It takes 2-3 days for the numbers of *C. tetani* to increase enough so that toxin production will be effective. Tetanus toxin travels intraaxonally to reach the CNS. Gangliosides in spinal cord and brain stem (anterior horn cells) are bound by tetanospasmin; only a small amount of toxin is needed to produce change. Tetanospasmin blocks the inhibitory pathway. Repeated firing at the synapse causes contraction with no relaxation, resulting in generalized muscular spasms, hyperreflexia and seizures result.

D. **TREATMENT**

1. *C. tetani* infection can be treated with penicillin to inhibit the organism's growth and stop further toxin production.
2. Surgical debridement of the necrotic tissue is essential.
3. Muscle relaxants, sedation and assisted ventilation are supportive therapy.
4. Antitoxin is administered to neutralize toxin before it has bound to tissue.

V. *Clostridium perfringens*

Gas gangrene is associate with *C. perfringens* infection, but in almost every case mixed infections are responsible: *Staphylococcus, E. coli,* and *Bacteroides* may also be present (microbial synergism). The counterpart to gas gangrene in muscle is Fusospirocheatel disease in the oral cavity (trench mouth).

A. **MORBIDITY/MORTALITY**

C. perfringens is the most common organism associated with anaerobic invasive disease. The perfringens enterotoxin is a common cause of food poisoning. Disease is not usually fatal if treated promptly.

B. **EXTRACELLULAR PRODUCTS**

1. Protease and toxin activity are apparent in gangrenous wounds. One of the most important virulence factors is the alpha-toxin, (a.k.a. phospholipase C).
2. Theta toxin is produced. It has hemolytic and necrotizing effects.
3. DNAse, hyaluronidase and collagenase are spreading factors.
4. Some strains produce a powerful enterotoxin that causes an intense, self-limiting diarrhea in 6-18 h.

C. **PATHOGENESIS**

1. *C. perfringens* grows in traumatized tissue with low redox potential. Exotoxins cause necrosis and toxemia.
2. *C. perfringens* produces proteolytic metabolic enzymes that break down tissue and proteins. Branch chain amino acids are converted to branch chain volatile acids: isobutyric acid, isovalenic acid, and propanoic acid.

D. **TREATMENT**

1. Passive anti-alpha toxin therapy is fairly effective.
2. Hyperbaric oxygen is another form of wound treatment.
3. Drainage and debridement are used to expose tissues to aerobic conditions.

VI. *Clostridium difficile*

C. difficile produces pseudomembranous ulcerative colitis as a result of oral clindomycin therapy (up to 25% of antibiotic-associated cases of diarrhea).

DIAGNOSIS OF *C. difficile* PSEUDOMEMBRANOUS ULCERATIVE COLITIS

PROCTOSCOPY: Multiple, raised, 2-5 cm plaques adherent to (Direct observation) edematous, friable colonic mucosa.

CULTURE: Anaerobic culture of stool specimen

CYTOTOXIN: Demonstration of cytotoxin in stool sample using a specific cytotoxic assay

A. **EXTRACELLULAR PRODUCTS**

1. Toxin A is a potent enterotoxin and cytotoxin. It binds to the intestinal brush border membranes at specific receptor sites (MW 440-500 kDa). The *C. difficile* cytotoxin causes cell death and tissue necrosis. Some strains of *C. difficile* also produce a second enterotoxin.
2. Toxin B is a potent cytotoxin found in the stools of patients with pseudomembranous colitis (MW 360-470 kDa).

B. **PATHOGENESIS**

1. Both ampicillin and clindamycin can result in drug-resistant *C. difficile*.
2. Binding of the difficile toxin to the gut brush border cells produces watery or bloody diarrhea associated with abdominal cramps, leukocytosis and fever.

C. **TREATMENT**
Discontinue the offending antibiotic. Most strains are susceptible to vancomycin. Supportive therapy is also provided for the colitis.

VII. ENDOGENOUS ANAEROBIC INFECTIONS

The majority of anaerobic infections seen clinically are from **endogenous** microbes. Anaerobes outnumber aerobes on most mucosal surfaces. Aerobes carry out oxidation reactions, leaving the environment in a reduced state that favors anaerobic growth.
Anaerobic infections result from overgrowth by endogenous opportunistic bacteria that occurs with compromised host defense, particularly when tissue pO_2 is reduced. Injury is the most common source of entry of anaerobes into normally sterile body sites.

Ratio of anaerobes to aerobes on body surfaces	
Colon	1000:1
Gingival Crevice	1000:1
Mouth and Upper Resp. Tract	5:1
Skin	100:1

A. CLINICAL SYMPTOMS SUGGESTING ANAEROBIC INFECTION

1. Putrid odor (volatile acidic metabolic byproducts from fermentation, i.e. gangrene)
2. Infection adjacent to a mucosal surface
    - anaerobic lung abscess - can originate with oral flora
    - anaerobic peritoneal abscess - can be caused by gut flora
3. Presence of gas within tissue (H2S gas is present with gas gangrene)
4. Infection in necrotic, avascular tissue or at a site suggestive of anaerobic infection.
5. Bowel perforation
6. Gram stain showing mixed, pleomorphic bacterial flora
7. Negative cultures after a positive Gram stain of clinical samples

B. **CLINICAL SPECIMEN COLLECTION** The following factors are important to keep in mind for anaerobic specimen collection. Most errors in diagnosis occur at the level of collection and transport.

- DO culture aspirated pus.
- DO culture infected tissue.
- DO NOT culture sites normally inhabited by a rich normal flora (such as mucous membranes and GI tract).
- DO NOT culture swabs-most swabs are aerobic and some are treated with substances inhibitory to anaerobes
- RAPID TRANSPORT TO THE LAB: -possibly the most important item on the list

Other factors to bear in mind are temperature, desiccation, oxidation, antimicrobials, and possible overgrowth by facultative anaerobes or aerobes in the specimen.

C. SPECIMEN COLLECTION EQUIPMENT FOR ANAEROBES

1. Sterile applicator sticks and syringes (perhaps the quickest, easiest way to collect)
2. O2-free/CO2-filled tubes
3. Anaerobic culturettes

4. Gas-Pak jars: a packet produces hydrogen and CO2 in the presence of a palladium catalyst and water)
5. Anaerobic chambers (all tests can be done in an anaerobic environment)
6. Cannula systems
7. Gas chromatograph (identification through metabolic by-products from spent medium. Analysis time is reduced to a few days, and is very accurate).

D. **GENERAL ANAEROBE INFECTION CONSIDERATIONS**

1. Some anaerobic infections form abscesses that are difficult to treat and must be removed surgically or drained (debridement).
2. Some important antimicrobials for anaerobic organisms are clindomycin and metronidazole. (Clindomycin: secondary complications include *C. difficile* resistance in the colon).
3. The largest concentration of anaerobes in the abdomen is in the large intestine
4. Primary pathogens
    - *Bacteroides fragilis*
    - *Clostridium perfringens*
    - *Peptostreptococcus*-anaerobic genus of streptococcus
5. Clinical signs include intense local pain, chills, fever, tachycardia, sweating, rigid abdominal wall, rebound, tenderness, shock
6. Causes of anaerobic infection
    - malignant tumors
    - inflammatory changes
    - perforations (trauma, surgery, or ulcers)
7. Diagnosis - if the wound is open, the first procedure is Gram staining. A syringe aspirate of exudates is preferred. A mixed flora usually indicates anaerobic infection.

E. **FEMALE PELVIC REGION**

1. Primary pathogens
    - *Bacteroides fragilis* - most significant
    - *Clostridium perfringens*
    - *Peptostreptococcus*
    - *Bacteroides melaninogenicus* and *Fusobacteria*
2. Clinical signs: foul smelling discharge, lower abdominal pain, fever
3. Causes of anaerobic infection
    - anaerobic flora may ascend through fallopian tubes and enter the pelvis
    - anaerobic flora may pass through tissue and places of injury and enter the pelvis
4. Diagnosis - malodorous discharge; but lack of an odor does not rule out an anaerobic infection. Vaginal smears are unreliable as commensal anaerobes are abundant. Specimens are aspirated through the vaginal wall.

F. **THORAX** (lung is the most frequent site - aspiration pneumonia and abscesses)

1. Primary pathogens
    - *Bacteroides melaninogenicus*
    - *Fusobacterium nucleatum*
    - *Peptostreptococcus*
2. Clinical signs - weakness, listlessness, fever, dull chest percussion
3. Causes of anaerobic infection include aspiration of normal flora of mouth and upper respiratory tract (can also cause chronic infections of the sinuses, middle ear, and abscesses of the brain). Anaerobes hide in the crevices of the mucous membranes, especially the gingivi (responsible for the fetid breath of gingivitis).
4. Diagnosis-transtracheal aspirate is needed. This bypasses contamination of specimen by upper respiratory tract (expectorated sputum will not do). X-rays will also show characteristic shadows or cavitations within the consolidated infiltrate.

5. Alcoholics, drug addicts, and anesthesia patients can aspirate upon losing consciousness and develop aspiration pneumonia
6. Anaerobes can also cause empyema (thick, foul-smelling pus)

G. **CONCLUSION**

1. Anaerobic bacteria are found as normal flora in many places. Infections are caused when they gain access to sterile environments.
2. Anaerobic infections are common in the thorax, abdomen and female pelvis.
3. Abscess formation; high mortality is associated with this type of infection.
4. Diagnosis depends on clinical observation, Gram staining and culture.
5. Effective treatment should be started promptly with selected antibiotics.
6. Antibiotics have been developed that are consistently active against anaerobes. Mixed treatment must sometimes be used to cover anaerobes and aerobes at once.

IMPORTANT ANAEROBES TO REMEMBER

Gram postive spore-forming rods

Clostridium - obligate anaerobes normally exogenous however *C. difficile* and
C. perfringins may be found in the bowel flora
C. botulinum-produces neurotoxin which acts on pre-synaptic cholinergics
C. tetani - neurotoxin that inhibits glycine (an inhibitor itself). Also produces the hemolysin tetanolysin.
C. perfringins-toxin phospholipase C (or lecithinase); egg yolk medium is
the assay for lecithinase or lipase activity
C. difficile - cytotoxin is responsible for ulcerative colitis

Gram negative rods

Bacteriodes fragilis - known for its polysaccharide capsule which is anti-phagocytic
B. melaninogenicus - utilizes hemin in blood-containing media and deposits a black pigment (which is not melanin). It is most frequently found in the oral cavity and works synergistically in fusospirochetal disease and advanced peridontitis; produces enzymes and chronic inflammation.
Fusobacterium necrophorum- distinguished from *Bacteroides* on the basis of end products - also produces enzymes that allow it to be invasive.

Gram positive cocci

Peptostreptococcus - frequently involved in respiratory infections

Gram positive non-spore-forming rods-- not necessarily obligate anaerobes, but may be capnophilic (utilizing carbon monoxide)
Actinomycetes - produces the actinomycoses, involved in chronic infections, particularly localized respiratory infections

Gram negative cocci

Veillonella (not usually pathogenic)

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Organisations

The American Society for Microbiology. 1752 N St. N.W., Washington, DC 20036. (202) 737-3600. <http://www.asmusa.org>.

National Center for Infectious Disease, Centers for Disease Control and Prevention. 1600 Clifton Road NE, Atlanta, GA 30333. (800) 311-3435. <http://www.cdc.gov>.

Other

National Institutes of Health. <http://www.nlm.nih.gov/medlineplus/encyclopedia.html>.

*2006 references for anaerobic techniques can be downloaded using End-Note Reference Manager from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=pubmed&cmd=Display&itool=abstractplus&dopt=pubmed_pubmed&from_uid=4565349