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Wrocław University of Technology

# Environmental Quality Management

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Justyna Rybak

# SANITARY BIOLOGY

Wrocław 2011

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# CONTENT

# Topic 1: Cell and colony morphology

**In order to pass the exercise the student should**
understand the purpose of describing cell and colony morphology,
know the basic morphological types of bacterial cells,
know different kinds of prepared slides and the stages of their preparation,
understand why cells are stained and the basic methods of staining,
understand the procedure of the Gram stain and be able to perform it,
understand the difference between gram-positive and gram-negative bacteria,
know which morphology forms stain positive and which stain negative by the Gram method,
understand why there is a need for immersion oil when using the 100x microscope lens,
understand which properties are used to describe morphology of a colony and be able to describe the colony of choice,
understand the following terms: murein, pleomophism, protoplast, S and R colonies.

**Cell morphology**

Cell morphology is the science that deals with the external appearance of cells and their arrangements. It is necessary to identify microorganisms to understand the species present. It is insufficient to identify precisely the species of bacteria solely based on morphological properties (i.e. biochemical tests are also required for identification). Morphology research, however, delivers initial information that allows the narrowing of the search results. For instance, the statement that cells have a spherical shape allows the exclusion of a large group of cylindrical bacteria.

Bacterial cell size is approximately 1 μm. Their shape can be narrowed down to three basic morphological types:

- spherical (cocci),
- cylindrical (rods, bacilli),
- helical.

Spherical forms also called **cocci** (singular coccus). Cocci can be found as single cells, two-cell arrangements (diplococci that is cocci in pairs), multi-cells arrangements such as the sarcina arrangement (cocci forming a cube of eight), **streptococcus** arrangement (cocci in chains) or **staphylococcus** arrangement (cocci in grape-like clusters).

Cylindrical forms of bacteria are called **rods**. One of the most important sanitary wise bacteria – enteric bacteria are rods. Some of the rods are thicker on one end, are club-shaped and are called **corynebacteria**. The rod-shaped bacteria which are more elongated and then cut off at a 90 degree angle are known as

**bacilli** (singular bacillus). Bacilli have the ability to create endospores inside of cells making them exceptionally resistant to unfriendly environmental conditions.
Some bacteria are comma-shaped (vibrios) or are corkscrew-shaped (spirilla and spirochetes). Some of the bacteria create filamentous forms including forms that are branched out in shape (actinomycetes). Other bacteria exhibit so called **pleomorphism** – the occurrence of two or more forms. For example, the common soil bacteria *Arthobacter* sp., based on the amount of nutrients in the soil, can form rods (when nutrients are plentiful) or cocci (under starvation conditions). Therefore, these forms are called rodococci. Pleomorphism can be caused artificially using penicillin which interferes in the cell wall synthesis. The cells that are created are then are misshaped, frequently branched out and are called **L-forms**. After the cessation of the noxious agent, the cells return to their original shape.

**The staining of microorganisms**

In the natural state bacteria are poorly visible under the microscope. It is necessary to apply various staining techniques to make them visible. Staining is divided into the following categories:

- simple staining – when single dye is used,
- differential staining – when more than one dye is used.

Based on other criteria staining can be divided into the following categories:

- direct staining – when the object is being stained,
- indirect staining – when the background is being stained.

In order to stain bacteria, the preparation has to be performed. Preparation is made on the microscope slide by the transfer of microorganisms on its surface. There are two basic types of preparations:

- a wet mount,
- fixed preparations.

In the first case the microorganisms that are transferred on the microscope slide are in the living state; in the second case the microorganisms are subjected to fixation. The purpose of **fixation** is to increase the cell staining ability and to avoid them being washing away from the glass during the staining process. Fixation can be accomplished by heating the cells over the flame of a Bunsen burner or by treating it with alcohol. As a result, the cells die and their cell walls become permeable for the dyes. Besides this, the internal cell structures become denatured disclosing functional groups of macromolecules reacting with the dyes.
The most common technique of staining in microbiology is the **Gram stain** (named after the Danish scientist). This is differential direct staining in which two contrasting basic dyes are used: violet (crystal violet) and red (fuchsine dye). The actual dying process is followed by heat fixation. This staining consists of the following stages (fig. 1.1):

- staining the fixed preparation by crystal violet,
- fixation of staining by iodine in the form of the Lugol's solution,
- washing off the dye with alcohol,

- staining the preparation with contrasting dye – fuchsine dye.

After the addition of the first dye all the cells turn violet. The addition of Lugol's solution causes the formation of an insoluble crystal violet-iodine complex. This complex is extracted with 95% alcohol. It turns out that the bacteria that have a thick layer of murein (peptidoglycan) in their cell wall do not lose color complex and they stay violet after the alcohol extraction. Those with the thin murein layer, however, lose the dye. In order to make them visible, the second contrasting dye is introduced – a red basic fuchsine.

In the end, some of the cells will dye violet and some will dye red. The violet cells are called **gram-positive bacteria** and the red cells are called **gram-negative bacteria**.

Gram staining is a process of differential staining as it allows the differentiation between two basic groups of bacteria based on which dye they react to. The basis of these differences is the difference in the cell wall structure.

**Gram-positive and gram-negative bacteria**

The significance of the Gram stain is that the differences in bacterial staining are correlated with other more important differences between them. Some of them are as follows.

Gram-positive bacteria:

- have a thick cell wall with a high proportional content of murein,
- are sensitive to penicillin,
- are sensitive to lysozyme which dissolves their cell wall (specifically murein) leaving the protoplasts – the cells without the walls,
- are sensitive to anionic detergents,
- some of them have the ability to create endospores.

Gram-negative bacteria:

- have a thin cell wall with a low content of murein, but with an additional specific outer membrane outside the cell wall,
- are usually resistant to lysozyme,
- are not sensitive to anionic detergents,
- have no ability to create endospores.

Gram-positive bacteria are typically bacilli and cocci, but typical rods (e.g. enteric rods) are gram-negative. Staining by the Gram method is not a permanent property of bacteria. Some bacteria are in principle gram-positive, but in old cultures they stain gram-negative or in a mosaic pattern: part of the cell is violet and part of it is red. For this reason, only credible results can be achieved when the cells are taken from a fresh culture. It is also worth mentioning that not all bacteria are able to stain by the Gram method. For example, mycobacteria belonging to actinomycetes (including the fameous *Mycobacterium tuberculosis* causing tuberculosis in humans) have their wall soaked in lipids and waxes so much so that the dyes cannot penetrate inside the cell. However, fungi (yeasts for example) can be stained by the Gram method and are usually gram-positive.

The Gram staining process described above is a kind of direct staining since the object of the research is being dyed. However, as mentioned before, indirect staining is also used in which the object itself is not being dyed, but instead the background is. The dyes for this have the property of ink such as chinese ink or nigrosine. Indirect staining is used to exhibit bacteria that do not absorb any dyes (for example spirochetal bacterium *Treponema pallidum* that causes syphilis) or in order to make bacterial capsules more visible.

**Colony morphology**

Bacterial cells, after reaching the surface of a solidified culture medium, start to divide (at a different rate depending on environmental conditions and the species) and after a certain time (usually a couple of days) they create a group of cells called a colony (surface colony) observable with the naked eye. There are about $10^9$ cells in one colony. The external appearance of a colony, in other words its morphology, is typical of the specific microorganism which is particularly helpful in its identification.

Some species of gram-negative bacteria create two types of colonies: S and R. **S colonies** have a smooth surface and **R colonies** have a rough surface. The colony types are associated with the differences in the structure of the outside membrane covering the cell wall of the bacteria. The cells forming an R colony exhibit some losses in lipopolysaccharide complex (LPS) creating this membrane. Since the outer membrane is responsible for the pathogenic properties of many gram-negative bacteria (e.g. *Salmonella* rods) strains creating R colonies do not cause infections.

Less unique colonies are created when the cells are not on the surface, but at a depth in a solidified medium. Then so called **embedded colonies** are created having a lens-shape due to the pressure coming from the inside of the medium which impacts the cells that are dividing.

# Laboratory exercises

**Task 1. Getting familiar with a microscope**

1. Become familiar with key microscope parts (fig. 1.2)
2. Become familiar with immersion microscopy (fig. 1.3)

**Task 2. The cell morphology – Gram staining method**

Performing a preparation and fixing it:

1. Degrease the glass microscope slide by rubbing it firmly with a dry soap and remove the residue with a piece of soft, dry cloth.
2. Light up the burner and flame a loop.
3. Cool off the loop and remove the cap of a test tube containing a suspension of bacteria and pass the lip of the tube through the flame.
4. Take a suspension of bacteria with the loop.
5. Burn the entry of the test tube and replace the cap.
6. Put the tube into the test tube rack.
7. Spread the suspension over the entire slide to form a thin film.
8. Put the slide on a staining tray and allow it to completely air dry.
9. Re-sterilize the loop and put it away into the test tube rack.
10. After drying, grab the slide by tweezers and pass it (film side-up) three times over the flame of the burner (heat fixation).

Gram staining method:

1. Wait and pour over the slide with **crystal violet** for 1.5 min.
2. Wash gently with water and pour **Lugol's solution** for 1.5 min.
3. Wash gently with water and decolorize with alcohol for up to 30 seconds (no longer than 30 s).
4. Wash off with water.
5. Pour with **fuchsine** for 30 s.
6. Wash off with water and dry the preparation by covering it gently with blotting-paper.
7. Place the prepared slide on a stage (in the slide holder) and place a drop of immersion oil on the slide.

8. Rotate the oil immersion objective into place and turn gently the knob of coarse focus control until the objective touches the oil.
9. Turn the knob of fine focus control until the specimen comes into focus.

Describe the morphology of the observed cells and do the drawing.
Are the observed bacteria gram-positive or gram-negative?

**Task 3. Morphology of a cell – indirect staining**

1. Take two microscope slides and degrease one of them per instructions in Task 1.
2. Using a sterile pipette, take a small amount of bacterial suspension and put one drop of it on the degreased slide.
3. Using another pipette, take a small amount of nigrosine and place a drop of it next to the drop with bacteria.
4. With the second slide (using the shorter edge) mix up the two drops and spread the mixture across the slide.
5. Allow the prepared slide to completely air dry.
6. Observe the preparation using the oil immersion objective.

Describe the morphology of the observed cells and do the drawing.

**Task 4. Morphology of a colony**

Choose two different colonies grown on an agar Petri dish and describe their morphology according to the schematic diagram (fig. 1.4). Describe the colony using the magnifying glass. Do the drawing.

**Figure 1.1.** Gram stain.

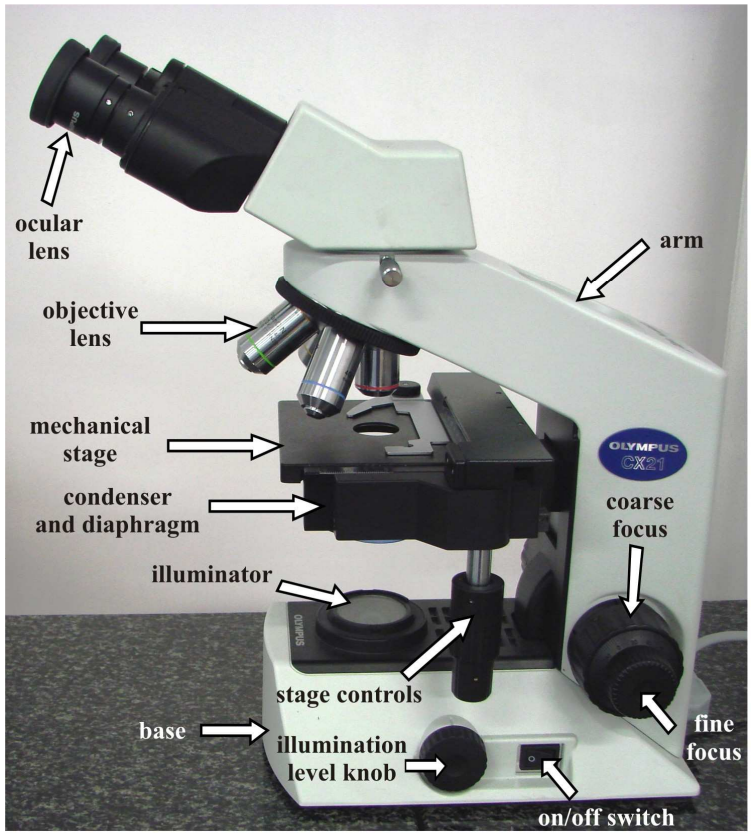


**Figure 1.2.** Components of a microscope.

The **mechanical stage** provides a surface for the placement of a slide (between the slide holder arms).
The **stage controls (slide positioning controls)** are two knobs that enable moving the slide vertically (forward and backward) or horizontally (left and right).
This microscope has two **ocular lenses (eyepieces)** that magnify 10 X and four different **objective lenses** magnifying: 10 X, 20 X, 40 X and 100 X, respectively. The last one is used for immersion microscopy. The objective lenses are situated in the **revolving nosepiece** (a movable turret above the stage).
The **final magnification** of the microscope is the product of ocular lens magnification and objective magnification.
The **coarse focus** (adjustment) **knob** and **fine focus** (adjustment) **knob** enable **focusing**. Turning the knobs causes the rising or lowering of the stage and thereby decreases or increases the distance between the specimen on the slide and the objective lens (called the working distance). The knobs allow bringing the specimen into sharp focus.
The **condenser** is located under the stage and contains lenses that concentrate the light coming from the light source. This component is critical to achieve optimal focus.

The **diaphragm**, located under the condenser, enables adjustment of the amount of light coming through the condenser. It is essential to obtain a proper contrast.

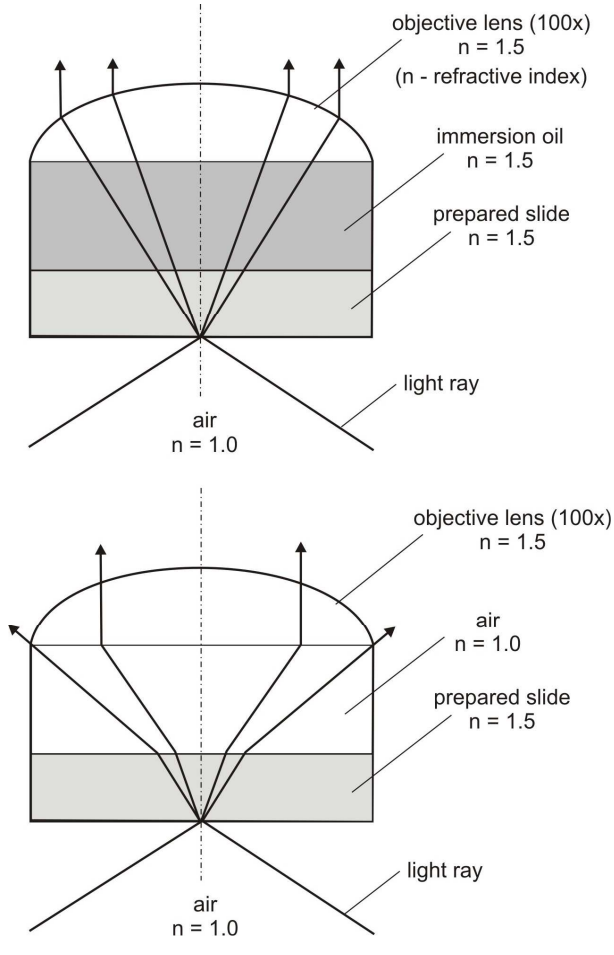


**Figure 1.3.** Principle of immersion microscopy.

On the above illustration, light rays follow a homogenous path because the immersion oil as well as the prepared slide have the same refractive index (n = 1.5). The image is not distorted.
On lower illustration, light rays follow a heterogenous path because the air and the prepared slide have different refractive indexes (1.0 and 1.5, respectively). Because of the lack of immersion oil between the lens and the slide, the rays bend and the image is distorted.

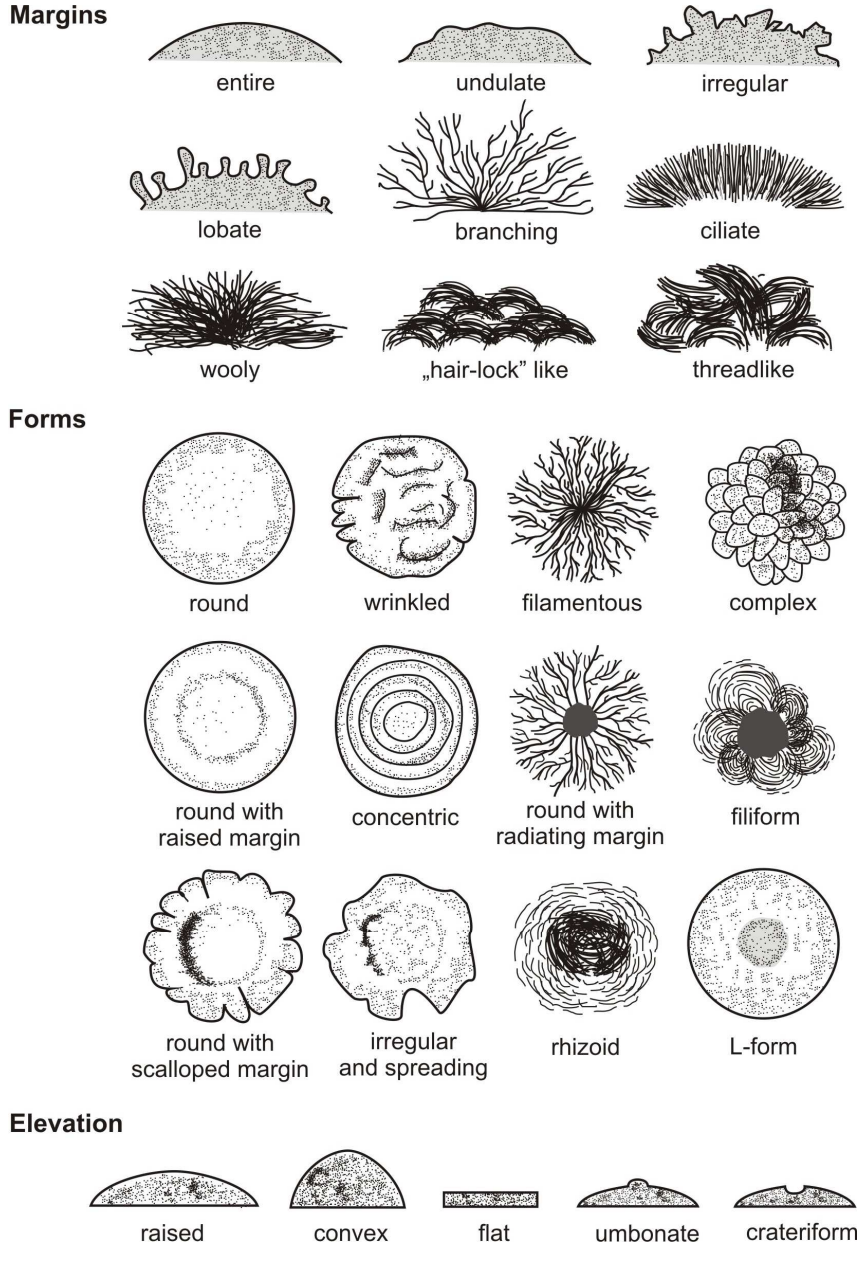


**Figure 1.4.** Colony morphology.

# Topic 2: Sterilization and disinfection

**In order to pass the exercise the student should**
understand the difference between sterilization and disinfection,
know the basic methods of sterilization with their drawbacks and disadvantages focusing especially on steaming under pressure (autoclaving),
be familiar with basic groups of disinfectants,
recognize what factors influence disinfectants,
know what the phenol coefficient is, how to describe it and what influences its value,
know factors that influence ultraviolet radiation,
know how to check sterilization effectiveness (sporals),
understand the concepts: bacteriocidal agent, bacteriostatic agent, temperature coefficient, vegetative, and dormant forms of microorganisms

**Sterilization**

Sterilization means destroying or removing all microorganisms, their **vegetative forms** (showing activity: feeding, respiration, reproduction, etc) as well as their **dormant forms** (endospores of bacilli, spores of fungi). This effect can be achieved by applying physical factors such as temperature, radiation or filtration.

**Steaming under pressure**

This method gives the best effects. It leads to the removal of both vegetative and dormant forms of all microorganisms found on the sterilized materials. This process takes place in **autoclaves**. The steam may come from an outside source, requiring a pressure reducer (most autoclaves have one already built in). An autoclave is a double wall boiler with a heavy lid sealing it tightly. It includes a manometer, thermometer and a safety valve. Water is boiled using an electric heater. The created steam pushes out the air through an opened outlet. Next, the unit is closed and the steam creates high pressure inside the boiler (higher than atmospheric pressure) which then raises the temperature. Increasing the pressure by 0.5 atm causes the temperature to rise to 111.7°C and raising pressure by 1 atmosphere will raise temperature to 121.6°C. Even dormant forms of microorganisms die in these conditions. Microbiological culture media are sterilized in 121°C for 20 minutes. This is not the full work cycle of the unit. Time for air removal, adding steam and cooling off have to be added with the whole process taking about 100 minutes. Materials that steam has difficulty penetrating require higher pressure and extended time for sterilization.

**Decoctation method**

This method refers to sterilization in live steam (temperature of 100°C). This process takes place in a Koch apparatus consisting of a boiler with a lid. It includes

warmer heating water on the bottom of the unit. Above the water is a metal net on which items needing sterilization are placed. Next the boiler is closed off. Released steam removes air and flows through the unit (the unit's pressure equals atmospheric pressure). Decoctation takes between 20 to 30 minutes. It does not cause dormant forms of bacteria, fungal spores, or the hepatitis B viruses (HBV) to die. This method is used to sterilize microbiological culture media and thermo-sensitive items.

**Tyndallization**

This procedure consists of heating materials three times in running steam for 30 minutes in 24 hour periods. First heating causes only vegetative forms to die. In the 24 hour period between heating, samples are cultured (incubated) in a microbiological incubator at room temperature. During that time dormant forms germinate. In the second heating vegetative forms originated from dormant ones during the 24 period and are destroyed. Another 24 period and third heating are implemented to make sure that all spores have already germinated.

**Pasteurization**

This process allows for partial sterilization and consists of a onetime heating of liquid to a temperature of 62°C for 30 minutes. In industrial conditions higher temperatures are used (70–90°C) along with shorter pasteurization times (seconds) and shorter cooling off periods to minimize the deterioration of vitamins and changes in smell or taste. Pasteurized products include milk, juices, beer and wine. This process destroys most bacteria and other microorganisms (protozoans, fungi, algae, viruses). However, bacterial dormant forms, fungal spores, and the hepatitis B viruses (HBV) are not destroyed (HBV is not destroyed even by boiling).

**Hot air (dry heat sterilization)**

This method is used to sterilize items that are high temperature resistant. Sterilization takes place in temperatures ranging from 140 to 180°C. Dry heat sterilizers are used in the process. Items must be wrapped to prevent recontamination (paper or metal containers are used). The dryer work cycle depends on temperature, number of items as well as on heating and cooling off time.

In units that include fans, the average exposure time at 160°C is 60 minutes with the complete cycle time lasting 150 minutes; at 180°C exposure time is 30 minutes.

In older units (without fans) the sterilization time is longer. At 160°C the exposure time is 100–120 minutes and a complete cycle takes 5–6 hours.

**Sterilization by incineration and flaming**

Incineration is used to destroy materials and items contaminated by pathogenic microorganisms. For example, corpses of animals used in experiments are incinerated. Flaming is used to sterilize small objects such as wire loops,

platinum wires and wire needles. In addition, the lips of tubes and flasks are passed through the flame (after removing caps) in order to prevent contamination.

**Sterilization using ionizing radiation**

Two types of radiation are used in sterilization: beta and gamma. Gamma radiation has a high penetration rate; beta is electron radiation with high energy levels. Effective doses of gamma radiation are between 0.1–1 Mrad. When using this type of radiation, it is very important to use the correct dosage. Not enough radiation can cause some dormant forms to survive. This can lead to mutations, which in turn can cause a higher virulence (pathogenic abilities) among surviving cells. The average dosage is between 2.5–3.0 Mrad. It is important to understand that ionizing radiation can cause structural changes in chemical compounds or cause them to break down, which in turn causes changes in chemical features that may affect microorganisms. Moreover, radiation of organic substances can cause the formation of free radicals, peroxides and compounds with double bonding, i.e. mutagenic and cancerogenic products.

**Sterilization by filtration**

In this method, microorganisms are physically stopped by a filter. Filters work like a strainer in which the diameter of the pores has to be smaller than that of the filtered cells. Moreover, some of the microorganisms are stopped on the surface by adsorption forces. Liquids with a high sensitivity to physical factors are subjected to sterilization by filtration. This method allows the removal of not only live cells and dormant forms, but also of dead cells and their parts.

Filters composed of diatomaceous earth, sintered glass, and membrane filters are used most often. Because their pores are too small, using gravity is impossible. High pressures or low pressures need to be used.

**Diatomaceous earth filters**

These are known as **Berkefeld filters**. Usually they are filters obtained by sintering diatomaceous earth at 2000°C. They are very efficient in removing both vegetative and dormant forms but they do not stop viruses. The flaw of this filter is its brittleness (leaching into the filtrate). Also, some of the filtrate liquid is absorbed into the filter.

In microbiology, filters with the smallest pores (“W” symbol) are used. Filters are sterilized using an autoclave. During the filtration process, liquid is poured in such a way that the candle is completely submerged in it.

**Sintered glass filters**

They are also known as **Schott filters**. They are obtained by melting powdered glass in adequate temperatures. They are in the form of funnels with a filter layer on the bottom. For sterilizing liquids nr 5 filters are used (smallest pore diameter). This type of filters is characterized by high efficiency and no side effects: no absorption into the filter, leaching, or changes in the pH of the filtered

liquid. Additionally, these filters can be sterilized using temperature. This type of filters stops all types of microorganisms except for viruses.

**Membrane filters**

Membrane filters are made of derivatives of cellulose. The membranes are about 80–150 μm thick with the pore size varying. The correct filter will remove all microorganisms from the filtered liquids. Membrane filters are first sterilized by boiling them in distilled water. The next step is to put them in the proper filtration device. Filtration takes place under high or low pressure depending on the construction of the device. The pH of the filtered liquid does not change. It is not absorbed by the filter and neither are any of the filter components released into the liquid. It is important to remember that the sensitivity of the method depends on the microorganism quantity in a unit of liquid volume. The sensitivity is high when the cell quantity does not exceed 10–500 cells in 100 ml.

**Disinfection**

Disinfection is the process of destroying vegetative forms of microorganisms using chemical and physical agents. It does not destroy all dormant forms. One of the goals is the fast removal of microorganisms, but the main objective is the removal of pathogens from different surfaces: devices, floors, containers, human and animal body parts. Basically, disinfection is used on items that need to be decontaminated, but cannot be sterilized. Disinfectants cause considerable and irreversible changes to the microorganism cell structure as well as to its metabolism. Such changes may inhibit growth or kill the microbes. From this, disinfectants can be divided into two basic groups:

- **Bacteriostatic agents** – they inhibit reproduction (after removing those agents bacteria resume reproduction).
- **Bacteriocidal agents** - they kill microorganisms.

The effectiveness of disinfectants depends on many factors:

- Concentration
- Time
- Temperature
- Humidity
- pH
- Presence of organic substances

The speed of disinfection depends on agent concentration. The higher the concentration, the faster the process progresses. However, it is not a proportional dependence; for example, doubling the phenol concentration will shorten the time to kill all cells in a culture of *Salmonella paratyphi* by a factor of seven.

In order to establish the activity range of a particular disinfectant, the survivability of microorganisms is determined as a function of time for a defined concentration and culture density.

The impact of temperature on disinfection effectiveness is expressed by the **temperature coefficient ($Q_{10}$)** using the formula:

$$\text{Temp. coef. } (Q_{10}) = \frac{\text{time to kill at temp. x}}{\text{time to kill at temp. x + 10}}$$

The coefficient value depends on temperature with a higher temperature resulting in a higher coefficient value.
The presence of organic substances can be of a chemical or physical nature, which in turn can cause different kinds of reactions:

- The disinfectant reacts with organic substances leading to inactivation. When organic substances compete with microorganisms, they can bind completely with the agent. This in turn causes its inactivity. For example, chlorine used to disinfect water binds with compounds polluting water causing the actual chlorine concentration to be lower than the one used.
- The disinfectant can, due to binding with organic substances, transform into insoluble compounds. This results in it precipitating out of solution and thus being eliminated from the environment.
- The disinfective agent can be eliminated from the environment by adsorption to organic substances (which may be in form of a suspension or colloid).
- Fats, phospholipids, some cations and anions can inactivate disinfective agents by eliminating them from the environment.
- Organic substances can create a layer of lower water content around the bacterial cell hindering disinfectant access to the cell.

Humidity has an important influence on the disinfection process because most disinfectants do not operate in a dry environment. The proper humidity allows for the correct condensation of disinfective agent on the cell surface and its penetration.

The concentration of hydrogen cations has not only a direct influence on bacteria, but also on disinfective agents. For example, phenol is most active with a neutral pH. Increasing the pH up to 10 increases its dissociation which in turn lowers its bacteriocidal activity.

An important problem is the formation of microorganisms resistant to disinfectants. This occurs mainly through the application of disinfectants at sublethal concentrations. However, the mechanisms of this resistance vary. One of the reasons could be enzymatic adaptation. Inductive enzymes that take part in the degradation of a disinfective agent are formed only after coming in contact with a certain compound or analogous compound. During adaptation, bacterial growth is halted. Some of the bacteria die, but those that do survive have the ability to accomplish enzymatic biodegradation of the disinfectant. Subjecting large bacterial populations to harmful agents such as disinfectants, results in cell mutations that enhance the resistance to the applied agent compared to the parent cell.

The following are the most commonly used chemical disinfective agents:

1. Acids and alkalis

Acids and alkalis denaturate important biological macromolecules (e.g. proteins) and alter membrane permeability. They are strong disinfectants that destroy the vegetative and dormant forms of microorganisms. The second action is called **germicidal activity** (destroying the endospores). However, their usage is limited because of their corrosiveness.

Examples:

- the milk of lime as 20% suspension of $Ca(OH)_2$,
- lye (NaOH),
- peracetic acid $C_2H_4O_3$ (as a mixture of acetic acid $CH_3COOH$ and hydrogen peroxide $H_2O_2$ in a watery solution).

2. Oxidizing agents (Oxidants)

Oxidants destroy the cell membrane of microorganisms causing cell lysis. The halogenated compounds and their derivatives belong to this group.

Examples:

- sodium hypochlorite NaClO – used to disinfect toilets (e.g. *Domestos*) and, as diluted solutions, in swimming pools and drinking water (this is called "chlorination" of water because the active free chlorine is involved),
- chloramine $NH_2Cl$ – used in water disinfection (is more stable than free chlorine),
- potassium permanganate $K_2MnO_4$ – used for treating the fungal infections of the hands and feet and also to disinfect aquariums,
- hydrogen peroxide $H_2O_2$ – used in hospitals and (as a 3% solution) for skin cuts,
- iodine as Lugol's iodine – commonly used for skin cuts,
- ozone $O_3$ – because of its very high oxidation potential it is used for the disinfection of water in place of chlorine.

3. Heavy metal salts

Heavy metals denaturate proteins. The usage of heavy metals as disinfectants is limited because of their toxic action to higher organisms. Examples are

- merbromin (mercurochrome is the trade name) – organomercuric compound used for minor cuts and scrapes (it acts only bacteriostatically),
- thiomersal – also the organomercuric compound, used mainly as an **antifungal (fungicidal) agent** (it also acts only bacteriostatically),
- $CuSO_4$ (copper sulfate) – fungicidal and algicidal agent,
- $SeS_2$ (selenium sulfide) – a fungicide.

4. Alcohols

These disinfectants cause denaturation of proteins and act most strongly in water solutions (especially as a 70% water solution). The water enables the alcohol to diffuse into the cell (100% alcohol denatures only the surface of the cell membrane and acts weaker than water solutions). The commonly used alcohols are ethanol and isopropanol. A mixture of these alcohols kills vegetative bacteria, inactivates the HIV viruses and other enveloped viruses, but is ineffective against endospores and non-enveloped viruses (ex. enteroviruses).

5. Phenols

Phenols cause denaturation of proteins. Phenol is the oldest known disinfectant, but it is toxic and corrosive to the skin and for these reasons is now rarely used for disinfection purposes. On the other hand, derivatives of phenol (phenolics) are widely used for domestic purposes and in hospitals (primarily in surgical suits) because they are less irritating and destroy viruses, bacteria, fungi and also have germicidal activity. Phenolics are especially useful for killing so called **blood borne pathogens** (e.g. HIV virus, hepatitis B virus – HBV and tuberculosis bacteria – MTB).

Examples:

- *Lysol* – the mixture of cresols (methylphenols) and a grey soap (as 3–10% water solutions) used as a disinfectant household cleaners,
- o-phenylphenol – often used instead of phenol (it is a less corrosive agent),
- chlorhexidine – useful in dental applications as the active ingredient in mouthwash (in preventing tooth decay and dental plaque),
- thymol – it exists in the oil of the herb thyme (*Thymus vulgaris*) and is used to prevent fungal growth in bee colonies (in the past it was used by Ancient Egyptians for embalming).

6. Quaternary ammonium compounds (Quats)

Quats are cationic (positively charged) detergents. They have a very broad range of action, are inexpensive and safe for the user. They disrupt the cell membrane of microbes and are particularly effective when used with anionic (negatively charged) detergents which mechanically remove microorganisms. Yet in contrast to phenols, they demonstrate a limited effectiveness in the presence of organic compounds; some bacteria such as *Mycobacterium tuberculosis* and *Pseudomonas* sp. are resistant to quats.

Examples:

- *Sterinol,*
- *Septolete* (benzalkonium chloride).

7. Aldehydes

They have a wide antimicrobial action including germicidal and fungicidal activity.

Examples:

- formaldehyde – the simplest aldehyde ($CH_2O$), its aqueous solutions are named **formalin** (usually 37%) and are used for animal (human) tissue fixation and as an embalming agent,
- glutaraldehyde – is used to disinfect medical equipment (a 10 hour exposure to 2% solution of this aldehyde is used for **cold sterilization**), but it can cause asthma.

8. Ethylene oxide

This compound causes denaturation of microbial proteins (enzymes). Ethylene oxide is also used for the cold sterilization of heat-labile materials (plastic syringes, Petri dishes). Because of its alkylating activity, the compound is mutagenic and carcinogenic.

# Laboratory exercises

**Task 1. Investigation of UV ray action on airborne microorganisms**

Investigation of the presence of microorganisms in air before turning on the UV lamp:

1. Place two agar plates and two Sabouraud agar plates near the turned off UV lamp and take the covers off for 10 minutes.
2. After 10 minutes put the covers on and remove the plates. Put new plates in their place.

Investigation of the presence of microorganisms in air after the action of the UV lamp:

3. Turn the UV lamp on for at least 30 minutes.

**Caution: do not expose yourself to UV rays!**

4. Turn the UV lamp off after 30 minutes and open the new set of plates for 10 minutes. After 10 minutes close the dishes.
5. Label the dishes accordingly: "before UV" and "after UV" and incubate them at room temperature for a week.

When the incubation time is over, count the colonies on the agar plates and the Sabouraud agar plates, before and after exposure to UV rays. Try to distinguish between bacterial colonies (common convex and colorful or flat and mate) and fungi (common filamenous).

Rate the effectiveness of the UV rays and explain what factors can influence it. Which microorganisms are more sensitive: bacteria or fungi?

**Task 2. Test the effectiveness of selected disinfectants**

1. Using a marker, divide the bottom of a Petri dish containing agar into 5 sectors and label them accordingly: K, -OH, $H_2O_2$, NaCl, M. These mean control (not disinfected), disinfected with alcohol, hydrogen peroxide, physiological solution and soap, accordingly. On the cover write your initials, date and time.
2. Lift the cover and carefully touch your finger to the agar surface in sector K. Close the dish.
3. Using sterile tweezers take sterile cotton wool, moisten it in alcohol and wet your other finger.
4. Touch that finger carefully to the sector OH (like in step 2). Dispose of the used cotton wool.
5. Using the same method, wet you fingers with hydrogen peroxide and physiological solution and touch them to the appropriate sectors. Remember to use a different finger each time.
6. Wash your hands using soap. Dry them and then touch your not previously used finger to the sector M.
7. Incubate the dish at room temperature for 1 week.

When the incubation process is over, compare the growth in each sector taking into account the number of colonies, their morphological differences, but not the growth area (why?). What do these differences mean? Did washing your hands using soap radically remove microorganisms from the surface of your finger? If not, what is the significance of washing your hands for personal hygiene?

**Task 3. Microbiological evaluation of sterilization effectiveness using steam in an autoclave**

Blotting paper discs containing between $10^8$ and $10^9$ endospores of *Bacillus stearotermophilus* (**sporals A**) are used in the evaluation. These endospores are able to take on a vegetative form. Moreover, they have strict survival characteristics during sterilization using steam under pressure.

1. Take sporal A out of the package and place it in different places in the autoclave or inside materials being sterilized.
2. Turn the autoclave on (only the person authorized to do so), sterilize for 20 minutes at a temperature of at least 121°C.
3. After the sterilization, move the sporal (keeping the sterile environment) into the tubes containing sugar broth (use sterile tweezers).
4. Incubate for 2 days at 55°C.
5. In case the sterilization failed, a pad-like growth will be visible on the surface of sugar broth (pellicle growth). If sterilization was successful, the broth will remain clear.

**Task 4. Microbiological evaluation of sterilization effectiveness using hot air**

**Sporal S** containing $10^8$–$10^9$ *Bacillus subtilis* endospores, able to take on vegetative forms, are used in this evaluation. These endospores have strict survival characteristics during sterilization using hot and dry air.

1. Take the sporal S from the package.
2. Sterilize for a minimum of 2 hours at 160°C.
3. After the sterilization, move the sporal (keeping the sterile environment) into the tubes containing sugar broth (use sterile tweezers).
4. Incubate at 37°C for 2 days.
5. In case the sterilization failed, a pad-like growth will be visible on the surface of sugar broth (pellicle growth). If sterilization was successful, the broth will remain clear.

**Task 5. Getting familiar with the basic equipment used in sterilization**

a. autoclave,
b. incubator,
c. filters.

**Task 6. Determining the phenol coefficient**

The phenol coefficient allows for determining the bacteriocidal action of a given disinfectant in comparison to the bacteriocidal action of phenol in the same conditions. Such conditions are strictly determined, i.e. time of disinfectant action, microorganisms used for testing, temperature and time of incubation (fig. 2.1).

1. Prepare phenol stock solution and its dilutions and distribute into tubes (5 ml in each). At the same time prepare the same quantity of tubes containing nutrient broth.
2. Prepare stock solution of tested disinfectant and its dilutions and distribute into tubes (5 ml in each). At the same time prepare the same quantity of tubes containing nutrient broth.
3. At 30 second intervals inoculate each phenol dilution with 0.5 ml of *E. coli* culture and mix it using a vortex mixer.
4. Do the same with dilutions of the tested disinfectant
5. After 10 minutes of incubation, transfer the bacteria using a wire loop from each inoculated dilution of phenol and tested disinfectant into the corresponding tube with nutrient broth.
6. Incubate all tubes for 48 hours at 37°C.
7. After incubation, put the results in a table. Use "+" to show growth indicated by turbidity of the broth and "-" to show the lack of growth (transparent broth).
8. Calculate the phenol coefficient according to the equation:

$$\text{Fenol coefficient} = \frac{\text{Highest dilution of tested agent able to kill bacteria}}{\text{Highest phenol dilution able to kill bacteria}}$$

What does the obtained result mean?

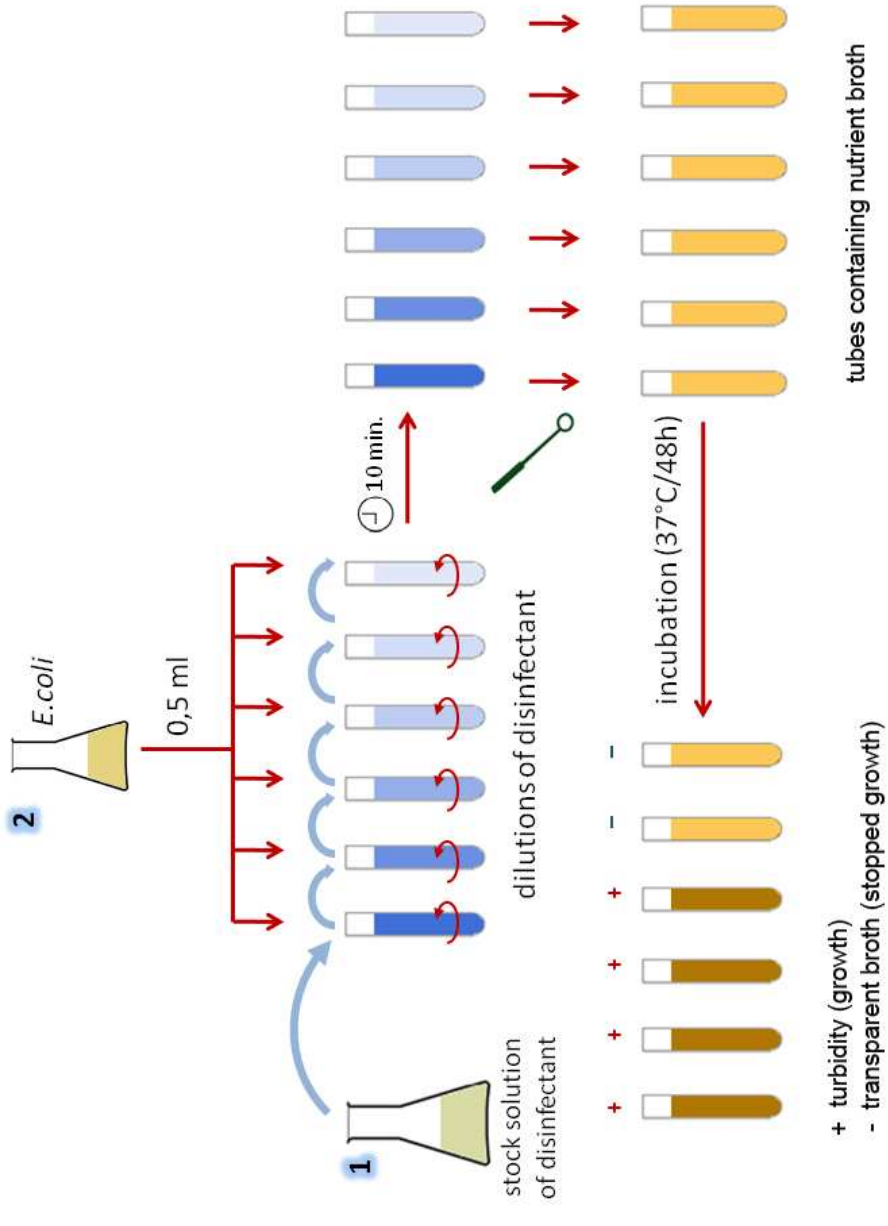


**Figure 2.1.** Scheme of determining the phenol coefficient.

# Topic 3: Methods of inoculation and cultivation of microorganisms

**In order to pass this exercise the student should**
be able to recognize nutritional requirements for autotrophic and heterotrophic microorganisms,
understand the reasons for different types of inoculation,
know the criteria for media division with examples and a brief description,
be able to explain what is nutrient agar, nutrient broth and gelatin,
be able to characterize the microorganisms based on their requirements with respect to pH, atmospheric oxygen and osmotic pressure,
be able to explain the methods for the cultivation of microorganisms under anaerobic conditions,
be able to list basic methods of cultivation, including phases of a batch culture,
understand the difference between batch and continuous cultures,
understand the following terms: inoculation, inoculum, strain, isolation of a strain, microbiological cleanness, colony, incubation, autotrophs, heterotrophs, prototrophs, auxotrophs, psychrophiles, mesophiles, thermophiles, aerobes, anaerobes, facultative anaerobes, microaerophiles, generation time, logarithmic growth, chemostat, colony forming unit (cfu),
be able to perform the basic types of inoculation, including the isolation of a pure strain and the creation of a dilution series.

**Inoculation and cultivation of microorganisms**

With cultivation one can multiply the microorganisms living in their natural habitat by the *in vitro* method. In order to set up a microbiological culture one should introduce it to a sterile medium, that is to **inoculate** the medium. The microorganisms that have been inoculated are called the **inoculum**. The content of the inoculum is not always known, for example when an environmental sample is used. This is due to the presence of the variety of bacteria and fungi (in water, wastewater or soil). The inoculated microorganisms are maintained in a medium (are **incubated**) for a period of time (**incubation time**) at a specific temperature (**incubation temperature**). During incubation, the inoculated cells grow and divide. It is also important to remember that there are microorganisms that do not grow on any media and they cannot be cultivated. Examples of such microorganisms include the spirochaete bacterium *Treponema pallium* causing syphilis or *Mycobacterium leprae*, the causative agent of leprosy.

There are different reasons for inoculation and thus for cultivation:

- Isolation of microorganisms from environmental samples such as water or soil, including obtaining a so called pure microbial strain, i.e. an aggregate of cells derived from a single initial cell. This is used for the further

examination and identification of isolated strains (determining the genus and species name).
- Multiplication of cells from a known and identified strain in order to obtain certain products of its metabolism or investigate its ability to degrade some pollutants.
- Counting the number of cells in a given sample.

The medium on which the cultivation occurs has to sustain the nutritional requirements of the microorganisms. Furthermore, the cultivation has to be maintained in the optimal environmental conditions such as temperature, pH, access to oxygen and osmotic pressure.

**Nutritional needs**

All the bacteria and fungi require a source of energy, carbon, nitrogen and many other elements for their growth and reproduction. Their nutritional needs can be summarized by the easy to remember abbreviation **CHOPKNS** composed of the symbols of the most important chemical elements for microorganisms. Besides the elements above, every media needs to contain certain trace elements in low concentrations, e.g. Co, Mn, Zn, Cu, Mo and Ca.

Special attention is directed to the source of energy and carbon. In the case of **autotrophic organisms** the source of energy is from solar radiation (photoautotrophs) or the oxidation of reduced inorganic compounds (chemoautotrophs). For both groups of organisms carbon dioxide from air is the source of carbon. For that reason the media for autotrophic organism cultivation do not require carbon.

**Heterotrophic organisms** use organic compounds for their source of energy and carbon. There are two groups of microorganisms which can be distinguished based on their need for organic compounds:

- prototrophs, requiring only one kind of carbon compound for growth,
- auxotrophs, requiring more than one kind of carbon compound for growth.

**Prototrophs** are exceptionally adapted with their metabolism as they can synthesize all the necessary organic compounds from very basic, even one-carbon compounds such as methane or formate. They include most of the organisms that live in very poor nutrient environments (soil, water). There are also prototrophs that live in rich environments (e.g. *Escherichia coli* in the human large intestine).

**Auxotrophs** often live in other organisms. For example, numerous pathogenic bacteria and fungi are auxotrophs. There is a wide variation among them when it comes to nutritional requirements. Some of them require only one additional compound (e.g. typhoid rod *Salmonella typhi* requires the amino acid tryptophan). Some others require a number of amino acids, purine and pyrimidine "bases" and vitamins (e.g. lactic acid fermentation bacteria). Different types of media are used in microbiology depending on the purpose of research. Based on their texture, they can be divided into the following:

- liquid,

- solid,
- semisolid.

Liquid media are usually used to multiply microorganisms in order to obtain a high cell biomass and their metabolic products. An example is **nutrient broth**, one of the most frequently used mediums. The broth is a mixture of peptone (product of enzymatic hydrolysis of protein), beef extract (extract containing organic bases and vitamins) and NaCl, added to ensure the proper osmotic pressure. Microorganisms with medium nutritional requirements grow well on broth. For many organisms living in poor in nutrient environments (soil and water), broth is too rich a medium inhibiting their growth. On the other hand, for many pathogenic bacteria broth is too poor and must be enriched (for example with blood or yeast extract). This type of medium is referred to as **enriched medium**.

Solid media are used primarily to isolate pure strains, store them, to study the morphology of a colony, and in quantitative research (determining the number of cells in the sample). Agar and gelatine are used mainly for the solidification of liquid media. In the case of some autotrophic bacteria that are sensitive to higher concentrations of organic compounds, a silica gel is used instead and mineral media are solidified.

**Agar** is a mixture of two polysaccharides: agarose and agaropectin which are the polymers of galactose. It is produced by certain marine algae from the group of red algae as a component of their cell walls. Agar is produced commercially in the form of powder or granules. After mixing with water and heated to a temperature of 95–98°C, agar melts and creates a colloidal suspension called sol. During cooling it retains a liquid form to a temperature of about 45–48°C, when it solidifies and turns into gel. Agar by itself (known as agar-agar) is not a source of carbon for most of the bacteria because they cannot hydrolyze it. It is used solely for the solidification of liquid media (in an amount of 1.5–2%). Nutrient broth with added agar is called a **nutrient agar** (MPA in abbreviation).

**Gelatin**, i.e. bone glue is a protein obtained in the process of boiling animal skin and bones containing collagen. The disadvantage of gelatin as a solid medium is that it is already dissolves at approximately 30–35°C, below the incubation temperature of many microorganisms. Some bacteria and fungi can degrade gelatin and liquefy it which is used in identification investigations.

Semisolid medium texture is in between the liquid and solid medium and it contains 0.15–0.2% of agar. Semisolid media are used to determine if the bacteria are motile or non-motile. After the inoculation of semisolid deep tube agar, bacteria capable of motion will move and thanks to the low-density of agar they will inhabit the entire volume of the medium. As the result of many cell divisions, turbidity forms in the entire volume of the agar. If the bacteria are not capable of movement, the inoculated cells divide only along the line of puncture.

Other divisions of media are based on the nutritional requirements of microorganisms:

- basal media,
- enriched media,

- special media,
- selective media,
- selective-multiplying media,
- selective-differential media.

**Basal media** meet the basic nutritional needs of microorganisms. Nutrient broth and nutrient agar fall into this category. Basal media are the foundation for other more complicated media.

**Enriched media** are used to culture more nutritionally demanding pathogenic microbes (e.g. streptococci and staphylococci), which are adapted to live in a nutritionally rich host environment. Several different kinds of enriching components can be added to the basal medium including anticoagulated blood (often from sheep), extracts of animal organs (e.g. liver), yeast extract and hydrolyzed casein – the main protein in cow's milk among others.

**Special media** are necessary for culturing microorganisms with specialized nutritional requirements called fastidious microorganisms. Gonococci (*Neisseria gonorrhoea*) causing gonorrhea, *Mycobacterium tuberculosis* which causes tuberculosis (TB) or *Corynebacterium diphtheriae* causing diphtheria are all examples of fastidious microorganisms. Compared to a basal medium extra nutrients are added (egg yolk, serum, vitamins) to special media.

**Selective media** allow for the growth of only a particular subset of microbes (which we want to isolate) by creating more favorable growing conditions or adding a compound to inhibit the growth of unwanted microorganisms. An example is a medium that allows the isolation from soil of *Azotobacter*, a bacteria that fixes atmospheric nitrogen. In this particular medium there are no compounds containing nitrogen, an essential element for growing all cells. Thus only microorganisms capable of fixing molecular nitrogen ($N_2$) from air are able to grow on the medium. This medium also contains mannitol, a compound which is the favorite source of carbon for *Azotobacter*. Another example of selective media are acidic media used for selecting fungi. The low pH of these media stops the growth of most bacteria which prefer neutral or a slightly basic environment. Fungi are acidophilic microorganisms and grow and divide normally on such media.

**Selective-multiplying media** are often liquid and allow not only the isolation of a particular microorganism (which is usually present in a small amount), but also the multiplication of the microorganism to obtain a high biomass in order to perform further analysis with the microbe (e.g. identification). An example of this is a selenine-cystine broth commonly used for isolating *Salmonella* rods from food products or soil. Selenine is an inhibiting factor which stops the growth of gram-positive bacteria, especially cocci.

**Selective-differential media** allow for not only the growth of selected microbes, but also for the differentiation between them. An example is **Endo agar**, often used for testing sanitary conditions. It allows only gram-negative bacteria to grow; therefore, it is a selective media (the presence of fuxin stain inhibits the growth of gram positive bacteria). The medium also allows for the differentiation of bacteria between those capable and incapable of fermenting lactose. During the

fermentation of lactose, acetaldehyde is produced giving a red color when combined with fuxin (previously colorless because it is reduced by sodium sulfite which is also added to the media). Thus, bacteria that ferment lactose (*Escherichia coli*) create red color colonies with metalic (fuxin) shine. Bacteria unable to ferment this sugar create pink or colorless colonies.
Culture media can be categorized as

- **Synthetic media** or defined culture media (are prepared from chemically pure, defined ingredients and therefore their composition is exactly known),
- **Natural media** or **complex media** (contain natural origin ingredients of unknown composition such as plant or animal tissue extracts, yeast extract, milk, blood, etc.),
- **Semi-synthetic media** (media with a known mineral composition and natural ingredients of an unknown composition).

**Temperature**

Microorganisms exhibit a great diversity of temperature requirements that must be taken into account during culturing. There are three **cardinal temperature points** that are used to categorize microorganisms:

- Minimum growth temperature, below which cell growth and divisions are not present.
- Optimum growth temperature, in which the cells grow and divide the most rapidly.
- Maximum growth temperature, above which growth and divisions do not occur.

Temperatures below the minimum or above the maximum are not always lethal to microorganisms, but these temperatures always inhibit their development. Cardinal temperatures do not always indicate precise temperatures. There may be some, usually narrow, ranges of temperatures depending on other factors such as the pH of the medium. The optimum growth temperature for the cultured microorganism is usually used for culture incubation. In order to achieve the proper temperature during growth, the culture is carried out in **microorganism incubators**. The incubators posses a thermostatic device which ensures a constant, predetermined temperature inside.

Based on the cardinal temperature points, microorganisms are classified into three main groups:

- **psychrophiles**, with an optimum temperature of about 20°C (minimum about –10°C, maximum about 30°C),
- **mesophiles**, with an optimum temperature of 37°C (minimum of 15°C, maximum of 45°C),
- **thermophiles**, with an optimum temperature of 55°C (minimum of 30°C, maximum of 75°C).

The majority of microorganisms living in water or soil are psychrophiles.

Mesophiles are microorganisms living on or inside the bodies of endothermic (warm-blooded) animals (birds and mammals). Among mesophiles are both pathogenic and non-pathogenic (saprophytic) species that make up the microflora of the organism.

Thermophilic microorganisms are mainly gram-positive bacilli and cocci. They occur in fermentating plant debris such as manure, compost, hay stacks, hot springs, etc. There is also a group of prokaryotic microorganisms called archaea or archeons (until recently classified as a bacteria) that live in extreme conditions where the temperature exceeds 100°C, such as the hydrothermal vents on the ocean floor. Microorganisms living in such extremely high temperatures are classified as **extreme thermophiles**.

**pH**

As in the case of temperature and other environmental factors, there is also an optimum, minimum and maximum pH. The appropriate concentration of hydrogen ions (negative logarithm of which represents the pH) has a serious impact on cell growth. Most bacteria prefer a neutral or slightly alkaline pH (around 7–7.5), while fungi grow better in an acidic pH (around 5.2–5.6). During culturing, the pH changes quite rapidly from the formation of metabolic products. In order to maintain the optimum pH, media are prepared using appropriate buffers.

**Oxygenation**

Based on oxygen requirements, microorganisms are classified as:

- aerobes,
- obligate anaerobes,
- facultative anaerobes,
- microaerophiles.

**Aerobes** require an oxygen rich environment where the concentration of oxygen in the atmosphere is 20%. In culture media that are not aerated such as on a liquid medium (like nutrient broth), the bacteria grow only on the surface of the liquid. The bacteria form a pellicle while the rest of the medium remains transparent (e.g. bacteria of the genus *Bacillus* and many fungi). At depth in the liquid medium these microorganisms can grow only with aeration.

**Obligate anaerobes** cannot tolerate oxygen because its presence creates hydrogen dioxide ($H_2O_2$) during metabolic processes. Hydrogen dioxide is a strong oxidizing agent and these organisms do not posses catalase activity (catalase is an enzyme that decomposes $H_2O_2$ to $H_2O$ and $O_2$). An example of obligate anaerobes includes bacilli belonging to *Clostridium* genus, e.g. *C. tetani* – the clostridium causing tetanus. Culturing such bacteria requires the removal of oxygen from the medium which can be achieved by various methods (see below).

**Facultative anaerobic microorganisms** are capable of growing in both aerobic and anaerobic conditions. An example includes *Escherichia coli*, a common inhabitant of the large intestine in humans. In non aerated broth the

bacterium grows in the whole medium (with different levels of oxygenation) and creates a uniform fine turbidity.

**Microaerophiles** are microorganisms that require oxygen for growth, but at low concentrations. In non aerated broth they create turbidity at a certain depth of the medium where the oxygen concentration is appropriately low. Examples include lactic acid fermentation bacteria of the genus *Lactobacillus*.

To ensure good oxygenation of a culture, shaking is applied for liquid cultures and air can be distributed using a diaphragm pump for all types of cultures. Also, a large gas exchange surface will help ensure oxygenation especially in the case of cultures on solid media.

**Anaerobic conditions** in a culture can be achieved by various methods:

- boiling a medium immediately before inoculation,
- placing a sterile piece of paraffin on top of the liquid medium,
- deep inoculation in the solid medium,
- the addition to a medium of reducing substances that lower the oxidation-reduction potential (ascorbic acid, sodium thioglycollate),
- culturing both aerobic and anaerobic bacteria in a tightly sealed container (aerobic bacteria will consume oxygen and create anaerobic conditions),
- culturing in **anaerostats**, in which air is removed for example with a water pump and in its place an inert gas such as nitrogen is introduced.

**Osmotic pressure**

Most microorganisms can grow only at a particular concentration of salt in an **isotonic solution** (salt concentration inside and outside the cell is equal). Cells in a **hypotonic solution** in which the concentration of salt outside the cell is lower and water tends to penetrate into the interior of the cell may burst; although due to the cell wall, it is possible for cells to withstand some water pressure. For this reason, the dilution of microbiological samples with distilled water is not applied. Instead a physiological salt solution is used, which is a 0.85% NaCl solution. In **hypertonic solutions** (the concentration of salt outside the cell is higher) cell survival depends on the ability to resist the flowing out of water and drying. Microorganisms able to withstand higher concentrations of salt (up to 15%) are known as **osmotolerant** (ex. staphylococci), whereas those that can withstand even higher concentrations are known as **osmophiles** or **halophiles** (some archeons).

**Types of cultures**

Cultures can be divided into

- batch cultures,
- continuous flow cultures,
- synchronous cultures.

In **batch cultures** microorganisms inoculated into the medium grow and reproduce in a closed system until the nutrient is depleted and/or until the products of metabolism accumulate to a toxic level. In this type of culture several distinctive phases of bacterial population growth can be observed:

- lag chase,
- log (logarithmic) or exponential chase,
- stationary chase,
- death chase.

These phases can be graphed as the so called growth curve (fig.3.1). In the **lag phase** (I) the inoculated cells adapt to the new environment in what is called the acclimation process. This process relies on the synthesis of required enzymes, synthesis of other proteins, replication of DNA and synthesis of RNA. This ultimately leads to an overall increase in cell size. The length of this phase depends on the size of the inoculum and on the similarity of the previous culture conditions from which the inoculum was transfered to the conditions present in the new culture. More similar conditions result in a shorter lag phase.

In the **logarithmic (exponential) phase** (II, III, IV) cells begin to divide. The length of the cell determines when the division occurs. Every cell divides into two identical cells. After a certain period of growth, the resulting cells again divide into two and hence the number of generated cells (population growth) is given by $2^n$, where n is number of cell divisions equivalent to the number of generations. The time between two successive divisions is called the **generation time**. The generation time depends on culture conditions and the species characteristics of a given microorganism. For a particular culture the generation time is stable. For *Escherichia coli* the generation time is about 30 minutes under optimal conditions for a laboratory culture, but it may amount to even one day in non-laboratory conditions. If the number of cells in the inoculum is $N_0$, the number of cells N after n generations will be $N = N_0 \times 2^n$. The number of microorganisms doubles every generation growing exponentially with time. The phase of the culture is called "logarithmic" because the logarithm of the number of cells is proportional to the time, but the number of cells (without taking the logarithm) is not.

In the **stationary phase** (V) some cells die from the depletion of nutrients, oxygen and the generation of toxic metabolic products. The number of cells dying is equal to the number of cells dividing resulting in no net change of cell number.

Over time, the amount of cells dying is higher than the amount of cells dividing. Thus, the overall number of cells decreases over time and the culture turns into the **death phase** (VI, VII). In this phase, many cells assume unusual shapes (undergo involution) before death and become difficult to identify.

The sequence of events may be different than those described above. If the used medium is removed and replaced with fresh medium, the logarithmic growth phase may be maintained for long periods of time. This type of microbial culture is called a **continuous flow culture**. In contrast to a static culture, it is an open system with a continuous flow of culture medium (used and fresh). It is kept in bioreactors called **chemostats** (because the chemical environment is static) allowing for the control of cell growth by dispensing the appropriate amount of nutrients and regulating the flow rate of the medium. This allows obtaining a steady-state in which the cell concentration is stable. This is important for studies of the physiological processes of microorganisms when stable conditions are necessary.

Continuous flow cultures are used in the food and pharmaceutical industries for the production of certain substances (acetic acid, antibiotics) and biomass (yeast). Biological sewage treatment plants also use a type of continuous flow culture in which bacteria are grown as an activated sludge on the medium which is continuously supplied wastewater.

**Synchronous cultures** are cultures in which the cells divide simultaneously (i.e. synchronously), in contrast to other cultures where cell divisions are uncoordinated. Because of synchronous divisions of cells, changes in the culture are a reflection (manifold increase) of the changes in a single cell. This allows the study of biological processes occurring during the life cycle of a cell that are usually unnoticed due to the insufficient sensitivity of the research methods. Synchronized divisions can be achieved by many ways, including shock induced by low temperature. To achieve low temperature shock, bacteria are moved for about 15–60 minutes to a temperature that is much lower than the optimal. Under these conditions, a synchronization of the physiological state of cells occurs. When moved to an optimum temperature bacteria start cell divisions at the same time. The achieved synchronization is not permanent and disappears spontaneously when it is not sustained.

# Laboratory exercises

**Task 1. Streak-plate inoculation on a solid medium (nutrient agar) – pure culture technique**

The objective of this inoculation is the isolation of pure strains of microorganisms as a colony. A pure strain is the assembly of cells coming from an initial cell (in the biological sense it is a clone). Streak-plate inoculation consists of streaking a suspension of cells on a surface of an agar medium with a loop. As the loop is streaked across the surface of the agar, the number of cells transferred to the medium will be smaller and smaller, until finally a few cells will remain on the loop and will stick to the agar one by one. Each of these separated cells begins to divide and they create a separate colony – an isolated pure strain.

In order to perform the inoculation one should

1. Flame and cool off the loop.
2. Pick the suspension of bacteria (preferably variety of strains) with a loop and spread it gently on the agar surface according to the selected method (fig. 3.2).
3. After the inoculation flame the loop again.
4. Let the inoculated culture incubate for several days at room temperature.

After incubation determine if the pure strains have been successfully isolated and how many strains were present in the inoculated suspension.

**Task 2. Inoculation with a loop on an agar slant**

The cultures on an agar slant are used for storing strains.

In order to perform the inoculation one should

1. Flame and cool off the loop.
2. Pick the suspension of a particular strain with a loop and spread it on the surface of an agar slant beginning at the bottom and leading towards the top of the slope using a Z pattern (fig. 3.3).
3. After the inoculation flame the loop again.
4. Incubate the inoculated culture for several days at room temperature.

After the incubation describe the outcomes of the inoculation (growth intensity, color, luster etc.).

**Task 3. Observations of the types of growth on a liquid medium (nutrient broth)**

The inoculation is used to determine the oxygen requirements for a particular strain of bacteria.

1. Pick the suspension of a particular strain with a loop and transfer it to the test tube with a nutrient broth. Shake the loop slightly after putting it in the broth.
2. The inoculated culture should be incubated for several days at room temperature.

After incubation determine if the particular strain is aerobic, anaerobic or facultative anaerobic based on its growth type (fig. 3.4).

**Task 4. Inoculation by the stubbing method in an agar deep tube with TTC**

This method of inoculation is used to test bacteria motility (not all bacteria can effectively move). If the test strain exhibits motility, it leaves a trail of pink (red) as a result of the enzymatic conversion of the colorless compound tetrazolium chloride (TTC) present in the medium to the pink compound formazan (TF). This reaction is catalyzed by dehydrogenase which is active in the energetic processes of the cell.

1. Pick the suspension of a particular strain of bacteria with the inoculating needle (fig. 3.5).
2. Inoculate the agar deeply by stubbing it with the needle all the way to the bottom of the agar (this can be achieved by holding the test tube upside down and stubbing the agar from the bottom side).

After several days of incubation determine the ability of the inoculated strain to move based on the redness of the media beyond the line of stubbing.

**Task 5. Deep inoculation on Petri dishes**

This type of inoculation is frequently used in quantitative research (determining the number of cells in the studied sample). It is usually required that the dilution is performed before the inoculation.

Perform a dilution (fig. 3.6):

1. Set in series six tubes with 9 ml of physiological solution (the number of tubes is proportional to the expected density of microorganisms in the investigated sample) in the test tube rack.
2. Using an automatic pipette add to the first tube 1ml of bacterial suspension and mix the contents of the tube with a vortex mixer. This way 10-fold (10x) dilution will be achieved.
3. Change the pipette tip (discard the used one to the special jar), pick 1ml of the 10- fold dilution, transfer it to the next test tube with the physiological solution and mix it to achieve the 100-fold ($10^2$x ) dilution.
4. Use the same procedure to make the next dilutions: $10^3$ x, $10^4$ x , $10^5$ x and $10^6$ x.

Performing an inoculation:

1. Set out 6 sterile Petri dishes, each for a different dilution and label them respectively.
2. Starting from the dilution $10^6$ x, take 1ml from each dilution using one pipette tip and pour it into the appropriate Petri dish
3. Into each Petri dish pour 1 tube of melted agar (cooled to a temperature of about 45°C) and mix it with the dilutions done previously. Allow it to solidify.
4. Incubate for several days at room temperature.

After incubation, determine the number of cells in 1 ml of investigated sample. For this purpose, count all the colonies grown on the agar plate on which there are from 30 to 300 colonies (note the two types of colony: surface and deep). Take into account the sample dilution and finally express the bacterial cell concentration as colony forming units per 1 ml of sample (cfu/ml). Compare this with the results obtained using the spread-plate inoculation (Task 6).

**Task 6. Spread-plate inoculation on agar using a bent glass rod**

This type of inoculation is frequently used in quantitative research likewise the deep inoculation method. It has, however, the advantage over deep inoculation in that there is no thermal shock caused by pouring hot agar. Furthermore, this method is better suited in the cultivation of aerobic microorganisms such as fungi.
Before the inoculation perform the dilution as in Task 5.
The inoculation should be performed on a set of agar plates.

Performing an inoculation:

1. Set out 6 agar plates, one for each dilution and label them accordingly.
2. Take 0.1 ml from each dilution starting from the largest one using one pipette tip and transfer it to the surface of the appropriate agar plate.
3. Sterilize the bent glass rod over the flame of a burner and wait until it cools off.
4. Spread the transferred dilutions evenly with a sterile bent glass rod over the entire surface of the agar plate. Start from the plate designed for the largest dilution.
5. Put away the bent rod in the dish with alcohol.
6. Incubate the plates for a week at room temperature.
7. After incubation, determine the number of cells in 1 ml of investigated sample. Count the colonies on the plates for which their number ranges from 30 to 300. Take into account the dilution of the sample and express the bacterial cell concentration as cfu/ml. Compare it with the results obtained using the deep inoculation method (Task 5).

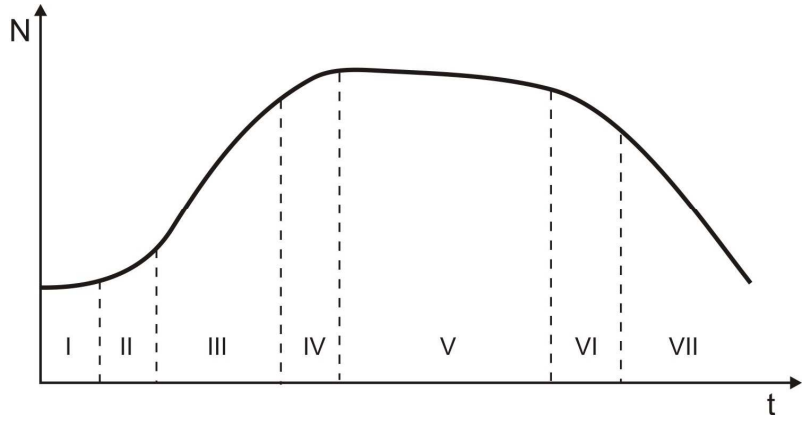


**Figure 3.1.** Batch culture – growth curve
(N – number of cells, t – time of incubation).

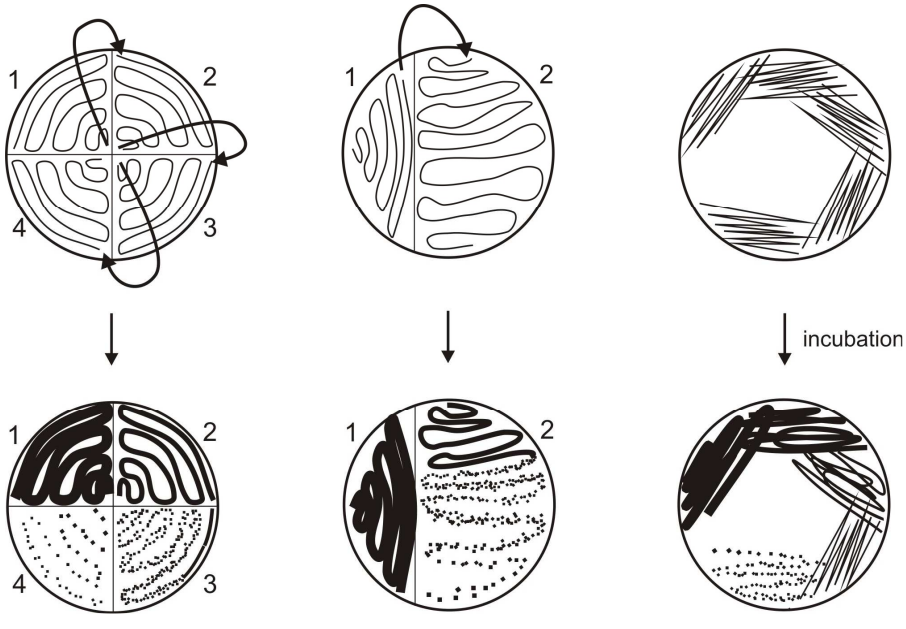


**Figure 3.2.** Streak-plate inoculation on nutrient agar – pure culture techniques.

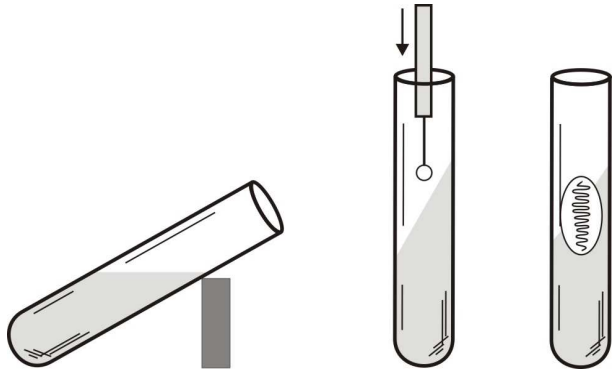

**Figure 3.3.** Inoculation with a loop on agar slant.

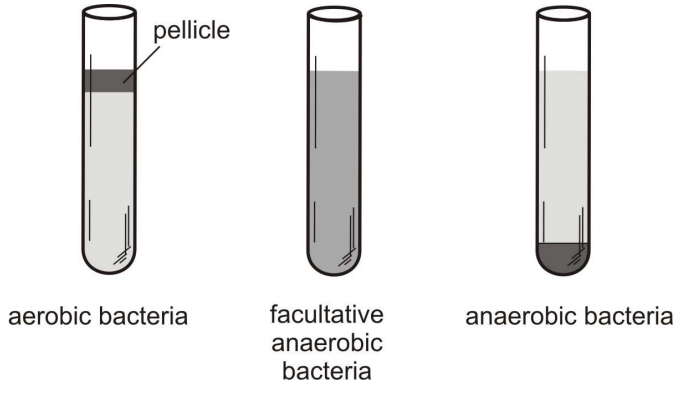


**Figure 3.4.** Types of growth with a nutrient broth.

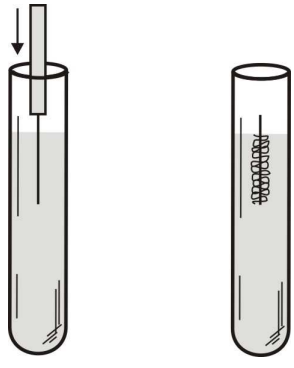

**Figure 3.5.** Inoculation by the stubbing method in an agar deep tube.

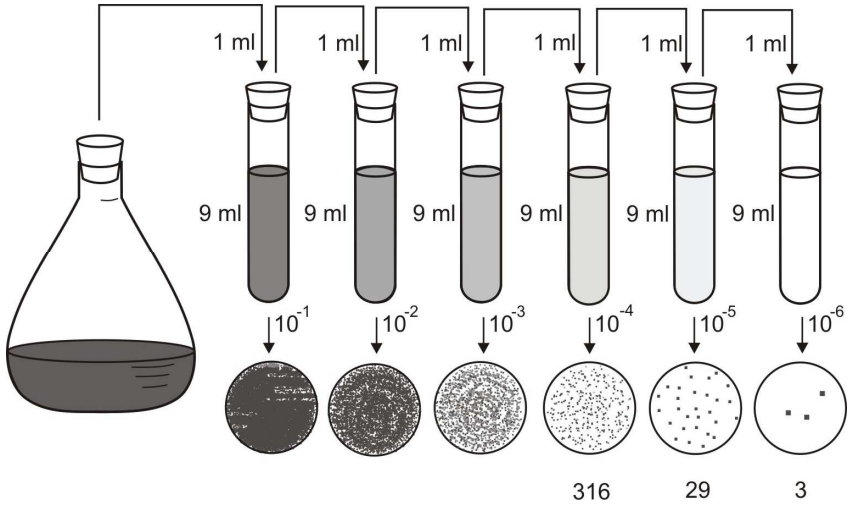


**Figure 3.6.** Plate count dilution procedure.

# Topic 4: Microbiology of water

**In order to pass this exercise the student should**
know the causes of water contamination and the microbiological quality in water should be controlled,
know the indicator microorganisms used for the microbiological analysis of water and be able to characterize their basic features,
know the bacteriological criteria for drinking water,
be able to establish the number of indicator microorganisms in the studied sample of water and know the basic issues and definitions applied when determining the microbiological pollution of water (e.g. MPN).

Waters of high fertility and also highly polluted surface waters are abundant in saprophytic and parasitic allochthonous bacteria among which the following are predominant: gram-negative intestinal rods (*Escherichia coli*, *Proteus* sp., *Klebsiella* sp., *Enterobacter* sp.), *Pseudomonas aeruginosa* and *Arthrobacter* sp. Moreover, gram-positive rods (bacilli) of the *Bacillus* sp., *Corynebacterium* sp. and *Clostridium* sp., which are washed out from the soil and get into bodies of water during heavy rainfalls, also belong to the allochthonous bacteria.

Municipal wastes are the main source of pathogenic bacteria. Moreover, from infiltration and surface run-off, soil bacteria find their way into water bodies as well. The role of air in water contamination is significant in the densely populated areas of cities and industrial regions. Since water is a basic human need, the health risks associated with the consumption of contaminated water are of great importance.

**Water transmitted pathogenic microorganisms**

The group of **obligate pathogenic bacteria** (table 4.1), which occur in polluted surface waters, contain rods causing typhoid fever (*Salmonella typhi*) as well as other bacteria of the *Salmonella* genus that are the cause of various infections of the digestive tract. Bacterial dysentery caused by gram-negative rods of the *Shigella* genus is not as common as those above. In surface waters of tropical countries, bacteria of the *Vibrio cholerae* species frequently occur. Moreover, *Mycobacterium tuberculosis* causing tuberculosis and bacteria of the *Leptospira genus* can also be found in polluted waters. The latter bacteria cause bacterial jaundice.

Besides the obligate pathogenic bacteria, there are also numerous gram-negative bacteria in surface waters which are described as **opportunistic microorganisms** (facultative pathogenic). These bacteria belong to the *Pseudomonas, Aeromonas, Klebsiella, Flavobacterium, Enterobacter, Citrobacter, Serratia, Acinetobacter, Proteus* and *Providencia* genera. All of the rods are part of the usual flora of the intestine and are not typically pathogenic as long as they

occur in human or animal digestive tracts. In some cases though, these bacteria find their way into other organs and become a potential cause of different illnesses such as inflammation of the urinary and respiratory systems and also sepsis which is a general infection of all internal organs.

**Table 4.1.** Waterborne bacterial infections.

| Disease type | Species or genus |
|---|---|
| Typhoid fever | *Salmonella typhi* |
| Paratyphoid fever | *Salmonella paratyphi* |
| Animal salmonellosis | *Salmonella* sp. |
| Bacterial dysentery | *Shigella* sp. |
| Cholera | *Vibrio cholerae, Vibrio cholerae type eltor* |
| Stomach and intestine catarrhs | Enteropathogenic *Escherichia coli, Klebsiella pneumoniae, Aeromonas hydrophila, Aeromonas* (*Plesiomonas*) *shigelloides, Pseudomonas aeruginosa, Vibrio parahaemolyticus, Campylobacter* (*Vibrio*) *fetus* subsp. *jejuni, Clostridium perfringens, Bacillus cereus* |
| Yersiniosis | *Yersinia enterocolitica* |
| Tularemia | *Pasteurella* (*Francisella*) *tularensis* |
| Leptospirosis | *Leptospira* sp. |
| Skin infections | *Pseudomonas aeruginosa, Mycobacterium* (*M. balnei, M. phlei, M. marinun, M. kansasii, M. fortuitum, M. cholonei, M. gorgonae*) |
| Bacteremia conjunctivitis, ear and upper-respiratory system infection | *Psudomonas aeruginosa, Pseudomonas cepacia* |
| Fever (pyrogens) | Gram-negative water rods (*Pseudomonas, Achromobacter, Xantomonas, Moraxella, Acinetobacter*) |
| Legionnaires disease | *Legionella pneumophila* |

Besides pathogenic bacteria of surface waters, into which municipal and industrial sewage is disposed, such waters may also contain significant amounts of other pathogenic microorganisms including the polio virus, which causes Heine-Medina disease (polio). Enteroviruses, which cause intestinal infections, occur even in slightly polluted rivers (table 4.2).

Infections of the digestive tract caused by protozoa may also come from contaminated water (table 4.3). Most parasitic protozoa produce cysts which are able to survive outside their host in unfavorable conditions. When the conditions improve, the cysts transform into so called trophozoits, the vegetative form occurring in humans.

**Table 4.2.** Intestinal viruses which may be transmitted by water and diseases caused by them.

| Viruses | Number of types | Forms and disease complexes |
|---|---|---|
| *Poliovirus* | 3 | Palsies, meningitis, fever |
| *ECHO* | 34 | Meningitis, respiratory system diseases, rash, diarrhea, fever |
| *Coxsackie A* | 23 | Herpangina, respiratory system diseases, meningitis, fever |
| *Coxsackie B* | 6 | Cardiac muscle inflammation, innate heart defects, rash, fever, meningitis, respiratory system diseases, pleurodinia |
| *Enteroviruses* | 4 | Meningitis, encephalitis, respiratory system diseases, acute hemorrhage conjunctivitis, fever |
| *Hepatitis virus, type A* | 1 | Hepatitis type A |
| *Norwalk virus* | 1 | Epidemic diarrhea, fever |
| *Parvovirus* | 3 | Accompany the respiratory system diseases |
| *Adenoviruses* | 41 | Respiratory system disease, eye infections, diarrhea |
| *Rotaviruses* | 4 | Epidemic diarrheas (mainly among children) |
| *Reoviruses* | 3 | Respiratory system diseases |

**Table 4.3.** Waterborne diseases caused by Protozoa.

| Pathogenic protozoons | Disease | Symptoms |
|---|---|---|
| *Giardia lamblia* (*flagellates*) | Giardiasis | Chronic diarrhea, stomach cramps, flatulence, weight loss, fatigue. |
| *Cryptosporidium parvum* (*apicomplexans*) | Cryptosporidiosis | Stomach aches, loss of appetite, watery diarrhea, weight loss. |
| *Entamoeba histolytica* (*rhizopods*) | Amoebiosis (amoebic dysentery) | Anywhere from slight to acute diarrhea, fever with shivers (rigor) |
| *Acanthamoeba castellani* (*rhizopods*) | Amoeboid meningoencephalitis | Symptoms from the central nervous system |
| *Naegleria gruberi* (*rhizopods*) | Amoebiosis meningoencephalitis | Gets into the brain of swimming people through the nose, causes acute symptoms of meningitis and encephalitis ending in death |
| *Balantidium coli* (*ciliates*) | Balantidial dysentery | Hemorrhage diarrhea caused by an ulceration of the large intestine |

In polluted surface waters parasitic fungi can also be present, for example *Microsporum* sp., *Trichophyton s*p. and *Epidermophyton* sp. They are dermatophytes causing ringworm and other cutaneous infections.

Human parasites are not usually included in the scope of microbiological research, although along with other pathogens (viruses, bacteria, protozoa), they pose a serious threat to human health (table 4.4). They occur in sewage and may find their way into waters from soils as a result of infiltration and surface run-off. The most infectious forms of the parasitic worms are their eggs. The eggs are excreted in great numbers outside the host body along with faeces and spread through sewage, soil or food. The worm eggs are very resistant to external factors and thus are difficult to eliminate from sewage by chlorination.

**Table 4.4.** Parasitic worms in the human body.

| Parasite | Symptoms |
|---|---|
| Human ascarid – *Ascaris lumbricoides* (Nematoda) | Ascariasis. Nematode larva causes inflammation reactions in various parts of the body. Sometimes it breaks up the pulmonary alveolus. If the intestines contain a lot of ascarids it may cause intestine obstruction or a puncture causing damage to abdomen lining. |
| Whipworm – *Trichuris trichiura* (Nematoda) | Trichuriasis disease is caused by nematodes living in human caecum and the large intestine. It creates changes in the mucosa and at high infestation a serious loss of mucous membrane. Sometimes appendicitis may occur. |
| Spiny-headed worms (Acanthocephala) | Ascanthocephaliasis disease is caused by the invertebrates which incorporate only parasitic forms. The parasites live in the intestines of all vertebrate representatives. In the water environment their hosts are usually crustaceans. The disease manifests itself by inflammations of the digestive system and its physical damage. |
| Tapeworms – *Taenia saginata, T. solium* (Platyhelminthes) | Parasites develop inside the intermediate host until reaching the larva stage called the cysticereus and infect humans who are its final host. Tapeworms live in the small intestine causing nausea, chronic dyspepsia, stomach aches and weight loss. |
| Distomas – *Schistosoma mansoni* (Platyhelminthes) | Schistosomatosis- caused by *Schistosoma mansoni* manifests itself by the ailment of the digestive system, intestine mucosa inflammation and cirrhosis of the liver. |

**Water health standards**

The possibility of infection by water imposes a constant need to control the hygienic-sanitary quality not only of drinking water, but also of swimming pools and surface waters. Water becomes infected by pathogenic bacteria excreted by ill people and carriers (people who keep excreting pathogens with faeces long after they have suffered from an illness). Pathogenic microorganisms are present in sewage and surface waters in lower numbers than other microorganisms. Therefore, they are more difficult to detect than the plentiful saprophytic bacteria. Consequently, much more complex diagnostic methods need to be used in order to detect them.

**The indicator microorganisms**

Current norms are based on the indirect inference about the presence of pathogenic microorganisms relying on the number of indicator microorganisms, which permanently live in human and animal digestive tracts as saprophytes. Their presence indicates that the water is polluted with faecal matter and consequently, there is a danger of contamination with pathogenic microorganisms.

Bacteria which serve as sanitary indicators should meet the following conditions:

- they must be constantly present in the human digestive tract so that they allow the detection of water contaminated with faecal matter,
- the number of indicator bacteria within the intestine and faeces should be high,
- among them should be non-spore-forming bacteria as they enable the detection of 'fresh' faecal-matter water pollution,
- their identification must be possible with readily available methods,
- their life span in the external environment should be longer than that of pathogenic bacteria,
- they should not be able to reproduce in a water environment under natural conditions.

In routine laboratory work, which concerns sanitary-epidemiological supervision, it is impossible to constantly monitor water for all pathogenic and potentially pathogenic microorganisms that may be found in water. Therefore, routine monitoring concentrates mainly on detecting bacteria that indicate faecal contamination of water (table 4.1). The sanitary quality of water may be checked by utilizing the saprophytic microflora that occupies the human large intestine.

The following indicators of water contamination are used:

- *Escherichia coli,*
- total coliforms,
- faecal streptococci,
- bacilli of *Clostridium* genus (sulphite-reducing bacteria)

and in some instances:

- staphylococci, coagulase-positive ones,
- *Pseudomonas aeruginosa.*

***Escherichia coli***

*Escherichia coli* (*E. coli*) is a rod-shaped gram-negative bacteria commonly found in the gastrointestinal tract and feces of warm-blooded animals. Most *E. coli* strains are harmless, but some can cause serious food poisoning in humans. *E. coli* can cause illnesses such as meningitis, septicemia, urinary tract and intestinal infections. A recently discovered strain of *E. coli* (*E. coli* 0157:H7) can cause severe disease and may be fatal in small children and the elderly. *E. coli* are not always confined to the intestine and their ability to survive for brief periods outside the body makes them an ideal indicator organism to test environmental samples for fecal contamination.

**Coliforms**

Bacteria of the coli group (coliforms) are mainly made up of strains of *Escherichia coli* as well as the genera: *Enterobacter, Citrobacter* and *Klebsiella*. They are detected on media containing lactose at 37°C.

**Faecal coliforms (thermotolerant)** are mainly strains of *Escherichia coli* and only some of the strains of *Enterobacter, Citrobacter* and *Klebsiella* which have an ability to ferment lactose at 44°C.

The presence of coliforms in a water sample indicates relatively recent contamination of water with faecal matter, sewage, soil or with decaying plants.

**Faecal streptococci**

While in a water environment, faecal streptococci are characterized by a slightly longer period of survival and stronger resistance to most disinfecting products compared to the coliforms. Faecal streptococci include *Enterococcus* and *Streptococcus* genera, which belong to the serological group of Lancefield D. The detection of faecal streptococci in a test sample that significantly exceeds the coli group bacteria may suggest water contamination with animal faecal matter or sewage from animal farms.

**Bacilli of *Clostridium* genus**

The detection of sulfite reducing bacteria (mainly strains of *Clostridium perfringens*) may suggest less recent contamination with faecal matter; their endospores are able to survive for many years in unfavorable conditions. Sulfite reducing clostridia are a good indicator of properly conducted water treatment processes – coagulation, sedimentation, and filtration. Endospores of these bacteria as well as the cysts of parasitic protozoa (*Cryptosporidium parvum, Giardia lamblia*) should be eliminated in these stages of water treatment because they are especially resistant to disinfecting agents.

Conducting analysis of a water sample in order to detect bacteria of the *Clostridium* genus is technically less complicated than searching for parasitic protozoa and this also ensures that the treated water is free from protozoa and from the eggs of pathogenic worms (helminthes).

***Pseudomonas aeruginosa***

Currently, the detection of *Pseudomonas aeruginosa* bacteria in drinking water, swimming pools and surface waters is recommended in addition to the above elements of sanitary analysis. They are gram-negative rods that do not produce spores. Their characteristic trait is the ability to produce a blue-green pigment – pyocyanin as well as a fluorescent pigment – fluorescein.

Representatives of this species were isolated from human faeces and in cases of infection - from urinary tracts, inner ear, suppurating wounds etc. These bacteria pose a potential pathogenic danger for both humans and animals. In addition, they are widely distributed in surface waters and soil. It is also important to note that the species may live in chlorinated water because it is, to some extent, resistant to disinfection.

**Staphylococci**

The *Staphylococcus* genus is mainly used to assess the sanitary quality of swimming pools. Recreational waters are the cause of infections of respiratory tracts, the skin and eyes. For this reason, microbiological analysis based on standard indicators (coliforms) is insufficient. Some researchers have recommended *Staphylococcus aureus* to be used as an additional indicator of sanitary quality of recreational waters because its presence is associated with human activity in these waters.

**Total number of bacteria**

Non pathogenic water bacteria grow mainly at lower temperatures (psychrophilic bacteria), although gram-negative bacteria in water produce lipopolysaccharides in their cell wall which can be toxic – such as the endotoxins of pathogenic bacteria. Because of this, their numbers in water should be constantly monitored.

An increase in the total number of bacteria in water samples can also be proof of the development of microorganisms on inner surfaces, especially on pipe-joints, seals and of the creation of a biofilm layer . Biofilms of microorganisms are of concern because of the potential protection of pathogens from the action of residual disinfectant in the water and the regrowth of indicator bacteria such as coliforms.

A large increase in their number is evidence of easily available organic compounds in the water. Theoretically, the presence of 0.1 mg organic carbon in water can result in an increase of bacteria up to 108 cfu in 1 ml.

Phosphorus is also a factor which stimulates the growth of microorganisms. Adding even small amounts of this element (<50 mg/l) causes 10 times the acceleration of bacterial growth in a water treatment plant.

**Drinking water criteria in Poland**

The assessment of the bacteriological quality of drinking water in Poland is based on the level of groups of indicators such as *Escherichia coli*, coliforms, total coliforms, *Enterococci* and *Clostridium perfringens*. In 100 ml of water present in

a water-pipe network, not a single bacterial cell considered as an indicator can be present (table 4.5 and 4.6).

**Table 4.5.** Tap water quality criteria in Poland.

| **Indicator** | **Highest acceptable value of indicator** | |
|---|---|---|
| | **Number of bacteria [cfu]** | **Volume of sample [ml]** |
| *Escherichia coli* | 0 | 100 |
| Enterococci (feacal streptococci) | 0 | 100 |

**Table 4.6.** Additional microbiological requirements for tap water.

| **Indicator** | **Highest acceptable value of indicator** | |
|---|---|---|
| | **Number of bacteria [cfu]** | **Volume of sample [ml]** |
| Total coliforms | 0 | 100 |
| Total number of psychrophilic bacteria (22°C) | Without anomaly changes | |
| Sulfite reducing clostridia (*Clostridium perfringens*) | 0 | 100 |

# Laboratory exercises

**Enumeration of coliforms in water**

Typically, tests for coliforms and *E. coli* use two methods:

- a **most-probable-number (MPN) multiple-tube fermentation** based on lactose fermentation with the production of acid and gas within 48 h and
- a **membrane filtration method** also based on lactose fermentation.

If a water sample yields presumptively positive results for coliforms, confirmation taking an extra 24 to 48 h of incubation time is required. *E. coli* is detected with these same methods, but often by using elevated temperature, different medium formulations and a test for indole production with the multiple-tube fermentation method.

**Task 1. Multiple-tube Fermentation Technique for coliforms**

Coliform bacteria are detected by placing an aliquot of the sample in LPB medium containing lactose and bromocresol purple as an indicator of pH. The coliform bacteria are those which will grow in this medium producing gas by fermenting the lactose at 37°C within 48 hours (gas is collected in a Durham tube). The coliform bacteria also change the pH of the medium. A change in color to yellow indicates increasing acidity and the possible presence of lactose fermenting bacteria.

Several fermentation tubes are used for the analysis of each sample of water. Treated water samples may be tested by adding 10 ml samples to ten tubes containing double strength LPB. Surface water and effluent samples are subjected to tubes in dilution series (each of two decimal dilutions). The most frequently used series involves 10 ml, 1.0 ml and 0.1 ml aliquots of sample.

An estimation of the coliform bacterial density can be determined from the number of positive tubes in each series. This estimation is referred to as the **Most Probable Number (MPN)** and is reported as the number of coliform group organisms per 100 ml of sample. For the MPN determination special tables are used.

**Presumptive test (fig. 4.1)**:

1. Arrange the fermentation tubes with LPB media in a test tube rack. The number of tubes and the sample volumes depend upon the quality and character of the water to be examined. For potable water use ten 10 ml portions; for nonpotable water use two tubes per dilution (of 10, 1, 0.1 ml, etc.).
2. Shake sample and dilutions vigorously.
3. Inoculate each tube in a set of two with replicate sample volumes (in increasing decimal dilutions).
4. Mix the test portions in the medium by gentle agitation.

5. Incubate inoculated tubes at 37°C.
6. After 24 +/- 2h swirl each tube gently and examine it for heavy growth, gas and an acidic reaction (shades of yellow color). If no gas or acidic growth has formed, reincubate and reexamine at the end of 48 +/- 3h. Record the presence or absence of heavy growth, gas and acid production. Production of gas or acidic growth in the tubes within 48 +/- 3h constitutes a positive reaction.
7. Submit the tubes with a positive presumptive reaction to the confirmation test.

**Confirmation test** (fig. 4.2):

1. Use brilliant green lactose bile broth fermentation tubes and the Endo agar plates for the test. Submit all primary tubes showing heavy growth, any amount of gas, or acidic growth within 24 h of incubation to the test. If active fermentation or acidic growth appears in the primary tube earlier than 24 h, transfer it to the confirming media, preferably without waiting for the full 24 h period to elapse. If additional primary tubes show active fermentation or acidic growth at the end of a 48 h incubation period, submit these to the confirmation test.
2. Gently shake or rotate the primary tubes showing gas or acidic growth to resuspend the microorganisms.
3. With a sterile metal loop 3 mm in diameter, transfer one loopful of culture to a fermentation tube containing brilliant green lactose bile broth. In addition, perform the streak-plate inoculation on Endo agar. Repeat the inoculations for all other positive presumptive tubes.
4. Incubate the inoculated media for 48 +/- 3 h at 37°C. The formation of gas of any amount in the inverted vial of the brilliant green lactose bile broth fermentation tube at any time within 48 +/- 3 h constitutes a positive result. The colonies developing on Endo agar are defined as typical (pink to dark red with a green metallic surface sheen); atypical (pink, red, white, or colorless colonies without sheen) after 24 h incubation; or negative (all others).
5. Calculate the MPN value from the number of positive results.

**Completed test** (fig. 4.3):

1. From each Endo plate pick one or more typical, well-isolated coliform colony or, if no typical colonies are present, pick two or more colonies considered most likely to consist of organisms of the coliform group and transfer them to Andrade fermentation broth and on an agar slant.
2. Incubate at 37°C for 48 h (the first medium) and for 24 h (the second one). The acidification of the Andrade broth (pink color) and production of gas means a positive result (secondary lactose fermentation), i.e. the tested bacteria are coliforms.

3. Perform the Gram stain and the oxidase test on bacteria grown on agar slants. A negative result for the oxidase test (without blue coloration) and a negative Gram stain (pink coloration of bacteria) means that the tested bacteria are coliforms.

**Task 2. Membrane Filtration method for *Escherichia coli***
(fig. 4.4 and 4.5)

1. Using a flamed forceps, place a membrane filter 0.45 μm on top of the filter holder base.
2. Attach the funnel to the base of the filter unit, taking care not to damage or dislodge the filter. The membrane filter is now located between the funnel and the base and the receiver flask.
3. Measure an appropriate volume (100 ml for drinking water) or dilution of the sample with a sterile pipette or graduated cylinder and pour it into the funnel.
4. Turn on the water vacuum pump.
5. After filtration of the tested water detach the pump.
6. Remove the funnel from the base of the filter unit.
7. Holding the membrane filter at its edge with a flamed forceps, gently lift and place the filter grid-side up on the LES Endo agar plate. Slide the filter onto the agar using a rolling action to avoid trapping air bubbles between the membrane filter and the underlying agar. Run the tip of the forceps around the outside edge of the filter to be sure the filter makes contact with the agar.
8. Incubate the plates (inverted) at 44°C for 18–24 h (If the incubation is performed at 37°C **coliforms** can be identified – see fig. 4.6).

The colonies developing on LES Endo agar are defined as *typical* (pink to dark red with a green metallic surface sheen); *atypical* (pink, red, white, or colorless colonies without sheen) after 24 h incubation; or *negative* (all others).

**Confirmation tests:**

In order to confirm that the colonies belong to the **thermotolerant coliforms**:

1. Pick off with a loop part of a typical colony and inoculate the peptone water with lactose.
2. Incubate at 44°C for 24 h.
3. If a gas appears in the Durham tube after incubation, this testifies that the bacteria are thermotolerant coliforms.

In order to confirm that the colonies belong to ***E. coli***:

1. Pick off with a loop part of the typical colony and inoculate the tryptophan broth.
2. Incubate at 44°C for 24 h.
3. After incubation add 0.2 ml of the Kovacs reagent.
4. If a red ring is formed (which testifies that indole was produced), perform the oxidase test or the β-glucuronidase test.

If the first test shows a negative result or the second one shows a positive result, this means that the bacteria are *Escherichia coli*.
Calculate *E. coli* final values using the formula:

$$\textit{E. coli}/100\ \text{ml} = \frac{\text{Number of colonies}}{\text{Volume of sample filtered (ml)}}$$

Report the results as *E. coli* per 100 ml of water.

**Task 3. Membrane Filtration method for enterococci**

Using the same membrane filtration method, test the water for enterococci. However, after filtration place a membrane filter on the Slanets and Bartley (SB) agar and incubate at 37°C for 24 h. If enterococci are present in the water red colonies develop on the SB agar.
Calculate enterococci final values using the formula:

$$\text{enterococci}/100\ \text{ml} = \frac{\text{Number of colonies}}{\text{Volume of sample filtered (ml)}} \times 100$$

Report results as enterococci per 100 ml of water.

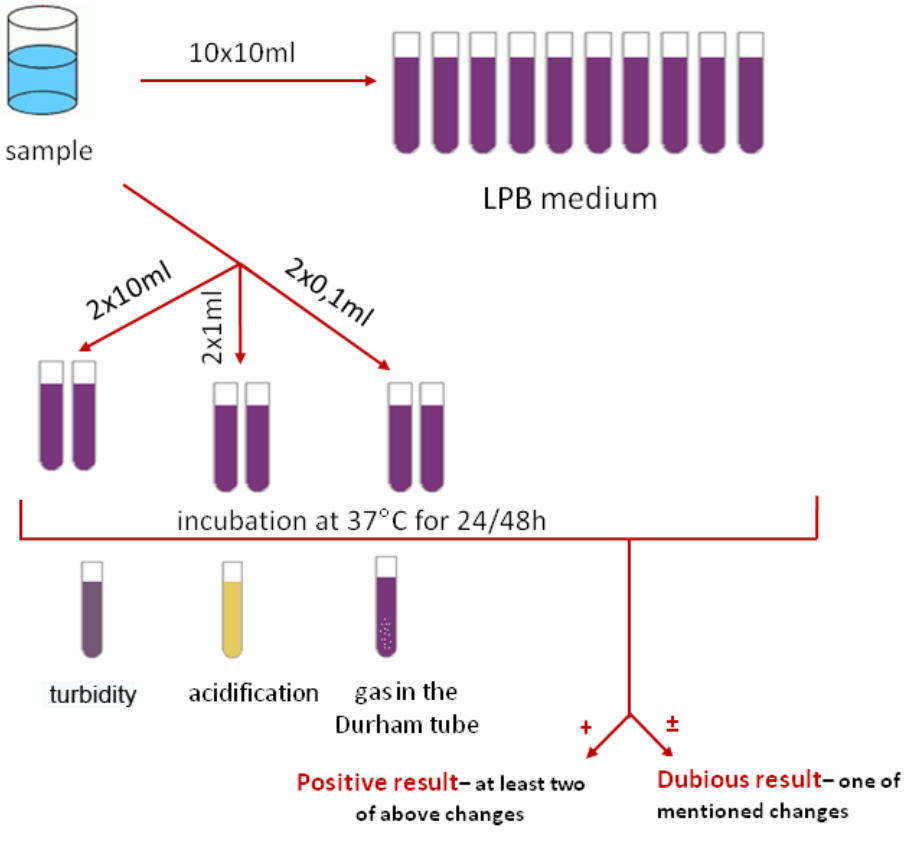


**Figure 4.1.** Multiple-tube Fermentation technique – presumptive test for coliforms.

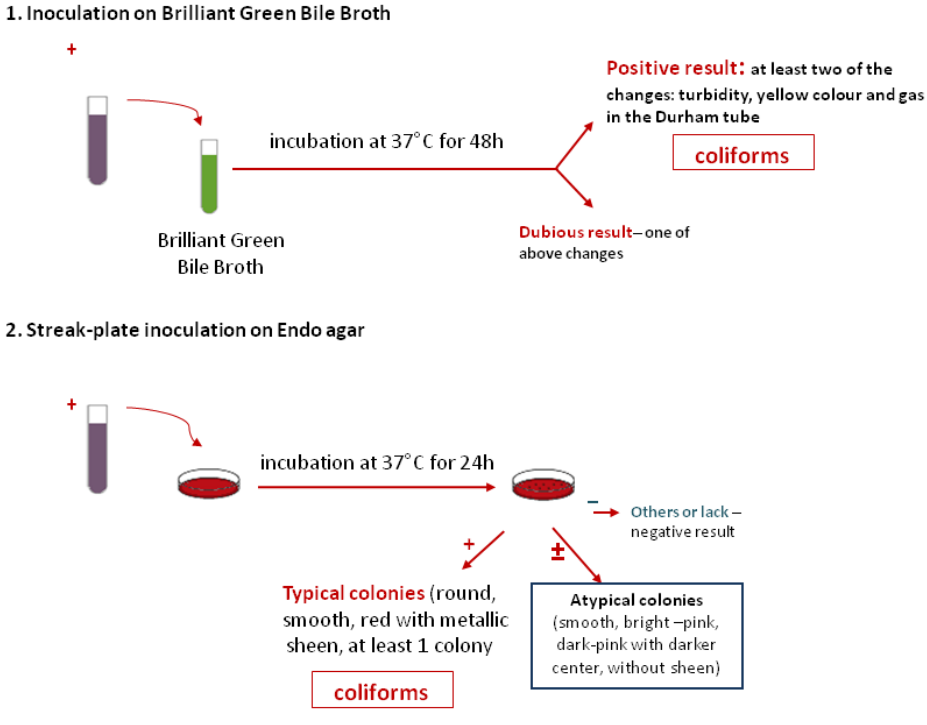


**Figure 4.2.** Multiple-tube Fermentation technique – confirming test for coliforms.

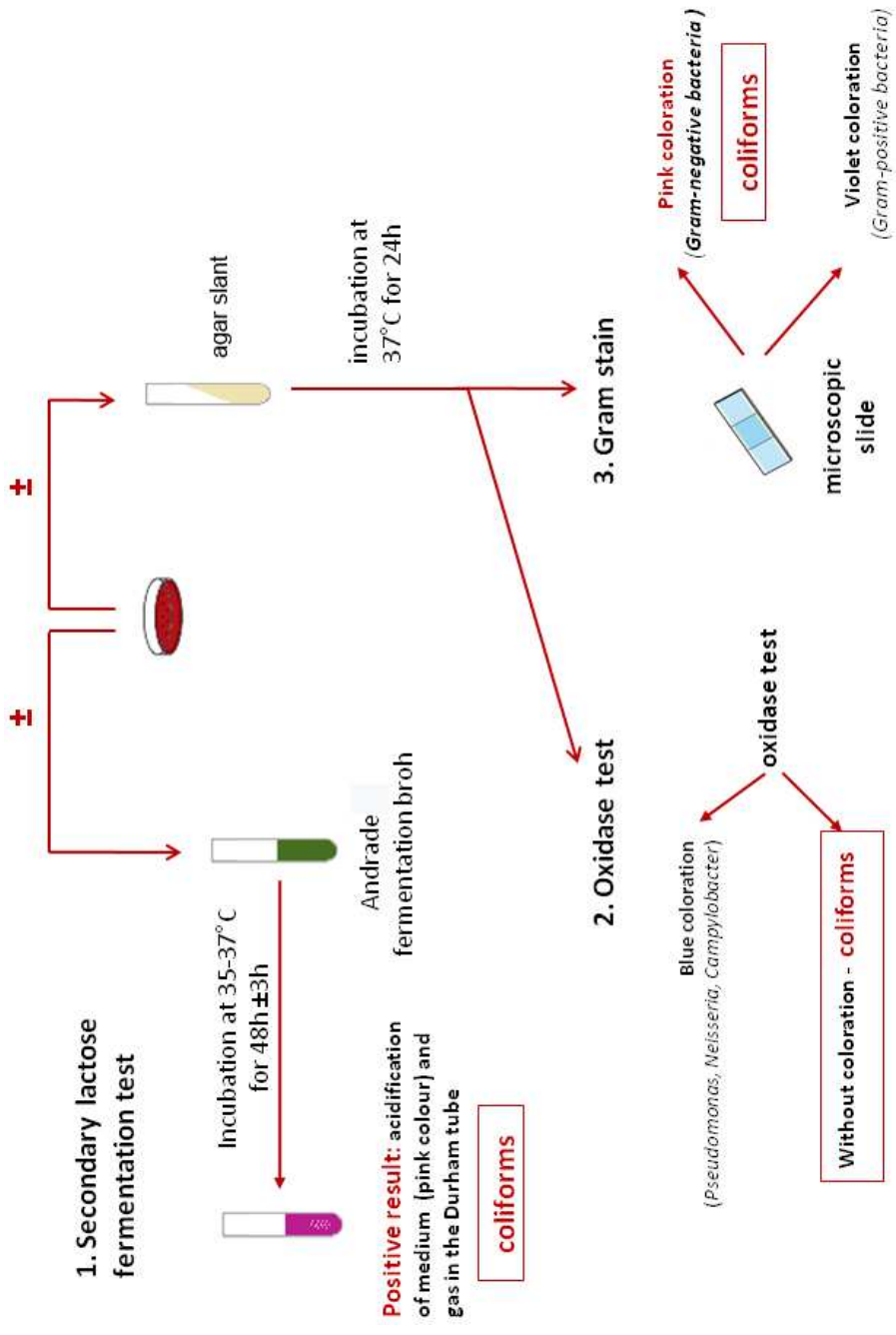


**Figure 4.3.** Multiple-tube Fermentation technique – completed test for coliforms.

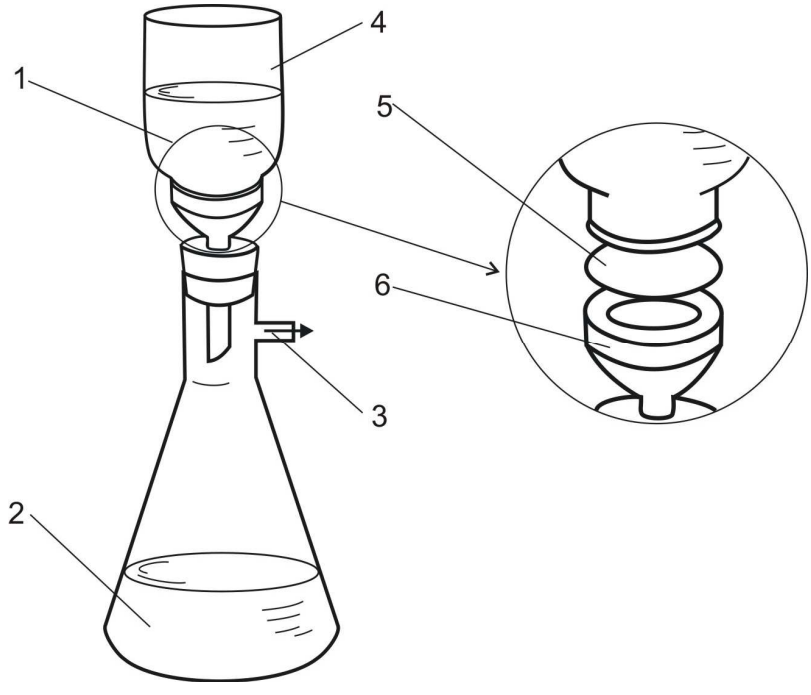


**Figure 4.4.** Membrane filtration system**:** 1 – filtered water, 2 – receiver flask, 3 – connection with water vacuum pump (outlet to vacuum pump), 4 – funnel, 5 – membrane filter, 6 – filter holder base.

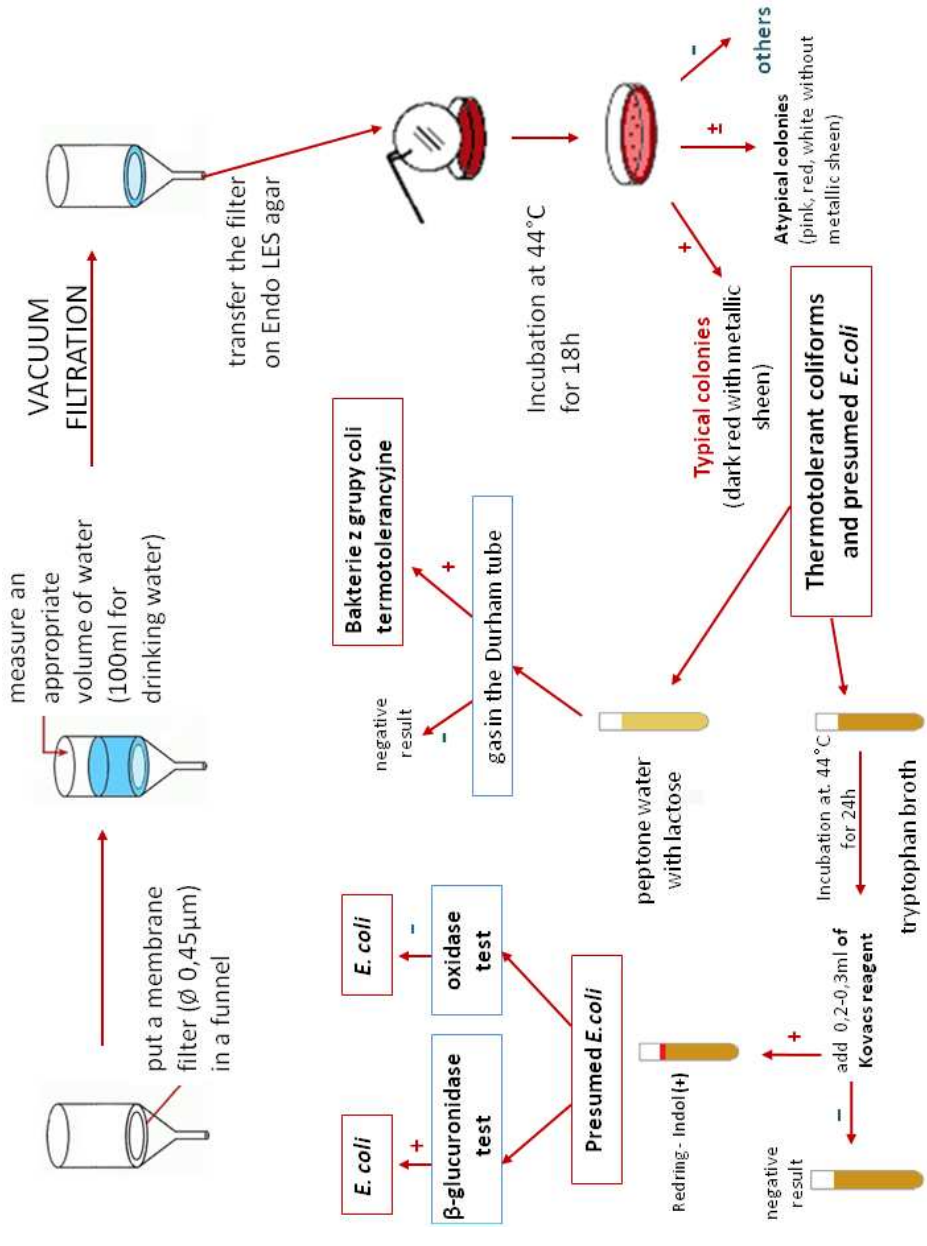


**Figure 4.5.** Membrane Filtration method for thermotolerant coliforms and *Escherichia coli*.

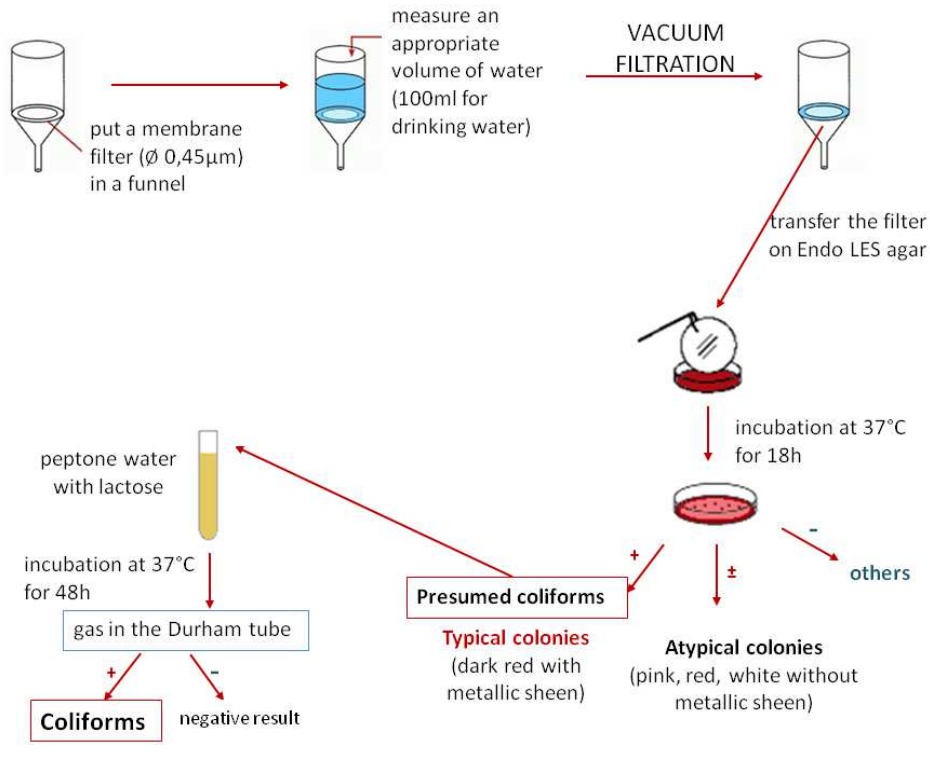


**Figure 4.6.** Membrane Filtration method for coliforms.

# Topic 5. Microbiology of soil

**In order to pass this exercise student should**
know what soil is, what the edaphon is,
know what microorganisms are found in soil and what they are characterized by,
be able to characterize soil microorganisms and discuss their role in the soil,
be able to characterize the autochthonous and zymogenic microflora of soil,
know the following definitions: edaphon, rhizosphere, pathogen, fitoedaphone, zooedaphone, helminths,
know the scope of sanitary microbiological analysis of soil,
be able to assess the number of bacteria in soil, also the number of fungi and eggs of parasitic worms.

**Soil characteristics**

Soil: the top layer of the Earth's lithosphere, formed from weathered rock that has been transformed by living organisms. The process of soil formation starts from the host rock, soil base component and may follow a different course depending on the following soil formation factors:

- climate,
- water,
- living organisms,
- surface configuration,
- human activity and,
- time (soil age).

Soil is a complex formation that allows the functioning of soil ecosystems:

- It takes part in primary biomass production and allows for the anchoring of plants, supplying them with water as well as essential mineral products.
- The decomposition processes of organic matter and the accumulation of humus take place in soil.
- Due to its chemical composition and physical properties, soil forms a habitat for massive amounts of microorganisms and other living organisms.
- Within the habitat soil serves various filtration and buffering functions which protect the ecosystems against the excess flow of unwanted substances from other biosphere elements.

**Soil composition**

Soil is composed of mineral and organic solid particles, air, soil solution and living organisms called the edaphon. The proportions of particular components within soil stay more or less at the same level for the given kind of soil.
Mineral compounds

- They occur in soil in the form of particles of various sizes.

- The smallest fraction consists of mineral colloids built from the aluminosilicates, hydrated silica, aluminum and iron hydroxide.
- Soil colloids strongly absorb oxygen, water and crucial nutrients while they also create a habitat for microorganisms. The colloids are a soil component that determines the water-air relationship.

**Organic substances**

- Soil organic substances are created by the residue of dead plants, animals and microorganisms that are decomposed by the soil-inhabiting microorganisms.
- The decomposition of organic substances consists of different microbiological and physical-chemical processes called **humification** and its end-products are humic substances (humus) which are partially in a colloidal state.
- The organic colloids are a source of food for the microorganisms.
- Moreover, in connection with silty particles they give soil an adequate structure. Humus favors the growth of higher plants due to the ability to absorb water as well as the adsorption and exchange of mineral compounds.

**Soil solution**

The solution of soil consists of water with dissolved organic and mineral substances as well as gases. Water is held in soil due to the capillary forces acting within its aggregates.

The chemical composition of the soil solution constantly changes depending on, among other things, the temperature fluctuations and the amount of water which either dilutes or concentrates the soil solution. Although the chemical composition is constantly changing, the microorganisms that live there have constant access to ammonium, phosphate and potassium salts as well as to nitrates. Moreover, easily available organic compounds such as monosaccharides and amino acids are found in the soil solution. Soil water provides favorable conditions for various organisms (not only for microorganisms, but also for plants):

- it transports building and energy substances along the capillaries,
- it also influences aeration, the amount and the quality of nutrients, the osmotic pressure and the pH of the soil solution.

**Soil atmosphere**

- Soil atmosphere is the air in soil that fills out water-free spaces between the solid particles. Air saturates the soil colloids.
- The amount of air in soil varies between 8–35% of the soil volume.
- Gasses that are always present in the air are $N_2$, $O_2$, and $CO_2$. The transient gasses are $NH_3$, $H_2$, CO, $NO_x$, $SO_2$, $H_2S$, $CH_4$ ,$C_2H_6$ as well as other volatile organic substances (butyric acid, alcohol, esters).

- Soil air is usually saturated with water vapor and contains 10 times more $CO_2$ than air in the atmosphere.
- The change from the oxygen to the oxygen-free metabolism (the reduction of sulfate, denitrification) occurs in soil when the concentration of $O_2$ falls below 1%. As a result, the growth of anaerobic microorganisms is observed.

**Soil edaphon**

- The organisms living in soil create a community called the edaphon. These are bacteria, fungi, unicellular algae, vascular plants and animals especially invertebrates that occur in the surface layer of soil.
- Due to the variety of their metabolic abilities, the soil microorganisms ensure the permanence (continuity) cycles of elements in nature.
- The effect of their activities is not only the mineralization of organic compounds, but also changes in mineral compounds that have a large impact on the development of green plants.
- Edaphon constitutes about 1–10% of the dry mass of the organic matter in soil.

Based on size, the organisms living in soil may be categorized into three groups:

- **Microbiota** (not visible with the naked eye):
  - viruses, bacteria, fungi, protozoa, algae.
- **Mesobiota** (0.2–2 mm):
  - nematodes (earthworms), mites, myriapods, wingless insects, snails, and some small plants.
- **Macrobiota** (>2 mm):
  - earthworms, larger insects, moles, rodents such as field mice, and roots of large plants and trees.

**The characteristics of soil microorganisms**

**Viruses**

- Viruses lead a strictly parasitic existence – they reproduce within bacteria, plant, animal and human cells.
- The most important role of viruses in the soil environment is played by the viruses living in bacteria cells – bacteriophages (phages).
- The role of phages in the soil environment depends on their ability to eliminate some populations of bacteria and on selecting the microorganisms both in a negative and positive way. An example of their negative influence includes the phages that attack the root nodule bacteria (*Rhizobium*) which are the cause of the decline of papilionaceous plant crops.

**Bacteria**

Bacteria constitute the basic mass of soil microorganisms. They are characterized by high metabolic activity.

- Most soil bacteria are characterized by the ability to adhere to surfaces of the mineral molecules and to soil colloids.
- Especially high numbers of bacteria gather around the residue of plant and animal tissues as well as in animal droppings that find their way into the soil. The environment that is especially suitable for the development of bacteria is the plant roots and their underground parts.
- The largest group of soil bacteria is represented by the actinomycetes and rod-coccus bacteria that belong to the *Arthrobacter* genus.
- The number of bacteria varies from a couple of million to a couple of billion cells per 1 g of soil. The highest number of bacteria occurs in a layer of cultivable soil at the depth of up to about 30 cm. In deeper layers their numbers quickly decrease. In the cultivable layer of soil about 30 cm thick there may be anywhere from several hundred kg up to a few tons of bacterial mass per 1 ha.
- In the vicinity of roots and on their surface bacteria find increased amounts of organic compounds such as organic acids, amino acids and vitamins that are excreted by plants. Therefore, in the layer around roots, called the rhizosphere, the number of bacteria is several times higher than in soil far from the roots.
- More bacteria live in soils rich in organic compounds, usually in 1 g of cultivable soil there may be between 0.5–5.0 billion bacteria (1.5–15 tons/ha).
- Acidic soils contain a relatively low number of bacteria and a large number of fungi.

**Actinomycetes (actinobacteria)**

The Actinomycetes are (chemo-) organotrophic bacteria. They form elongated, branched out mycelium-like threads that contain a large number of prokaryotic cells. The width of the threads is 1–5 μm. They mainly live in soil or on decomposing plants. Most of them lead a saprophytic type of existence, and some are pathogenic to plants and animals (for example *Streptomyces somaliensis*, *Actinomyces israelii* and *Nocardia asteroides* cause the subcutaneous infections of feet called mycetoma). About 90% of all actinomycetes isolated from soil are *Streptomyces.*

Their growth abilities in temperatures of 40–50°C give them a wide range decomposition potential for various substances. The Actinomycetes degrade steroids, lignin, chitin, hydrocarbons, fatty and humic acids, which are not easily decomposed by other bacteria. During the decomposition of these substances they produce aromatic compounds. The characteristic smell of freshly plowed soil, especially in spring, comes from the actinomycetes bacteria. The smell is caused by the substance called **geosmin** (*1,10-dimetylo-9-dekalol*), which is produced by

*Streptomyces griseus.* They are an aerobic bacteria, whereas a small group has the ability to conduct metabolic processes in anaerobic conditions (*Actinomyces, Micromonospora*).

Many types of actinomycetes produce antibiotics such as erythromycin, neomycin, streptomycin, tetracycline plus others as the by-products of metabolism.

**Rod-coccus bacteria**

Club-shaped bacteria that belong to the *Arthrobacter* genus are dominant in numbers representative of the autochthonous soil microflora that make up 2–60% of the whole population. They are characterized by their tendency to form branching and coccus forms. The bacteria are polymorphic. In new bacterial cultures the bacteria grow in the form of long irregular rods whereas in old cultures, they create coccus forms. They are characterized by a high resistance to environmental factors during the vegetative stage. They are capable of surviving in dry soil for a few months, whereas most of the other bacteria that do not produce resting spores die out.

The bacteria have the ability to utilize a wide spectrum of organic compounds as a food substrate. They conduct biodegradation of not easily accessible compounds and may utilize many metabolites of other microorganisms including various polymers, growth factors and the amino acids produced by microorganisms. The bacteria (*Cellulomonas*) which utilize cellulose also belong to the club-shaped forms.

**Fungi**

- Fungi belong to a group of eukaryotic organisms which are the absolute heterotrophs. Most belong to the group of aerobes or fermenting fungi. They take carbon and energy to build their own cells from the decomposition of organic compounds. Fungi do not have chlorophyl. In contrast to bacteria, the fungal cell wall contains chitin, glucans and other polysaccharides.
- They occur mostly in the upper layers of soil; however, they can be found as deep as 1 m.
- They enter into symbiotic relationships with algae, insects and higher plants. Many species of fungi are pathogenic to humans, plants and animals.
- Their vegetative forms create thread-like shreds that are more or less branched out and usually multicellular with thick weaves forming mycelium or thallus. The individual cells are the size of about 10 μm.
- The most common soil fungi are the genera of *Penicillium, Aspergillus, Trichoderma, Verticillium, Fusarium, Rhizopus, Mucor, Zygorhynchus, Chaetomium.*
- Fungi grow best in acidic soils and have a crucial influence on changing the pH.

**The role of bacteria and fungi**

- Both are the co-creators of soil structure as they create humus – the most important component of soil that greatly influences its structure, sorption qualities and the richness in organic compounds.
- They have a significant effect on the creation of the crumb texture and spongy structure of soil by producing mucous capsules and as with filamentous bacteria and fungi by the form of their growth.

**Soil phytoedaphon**

Phytoedaphon consists mainly of algae and to a lesser extent of higher plants. Algae are the main component of phytoedaphon. They are most numerous on the surface of soil reaching deeper through plowing, percolating water, animal activities and their ability to migrate. Two groups are distinguishable: algae colonies that live upon the surface, epiphytoedaphon, and those that live in deeper layers, endophytoedaphon.

- Soil algae are obligatory photoautotrophs; however, the soil algae living in deeper layers probably feed heterotrophically.
- They play a major role in the soil ecosystem and influence its qualities and stability. Through extracellular secretion they fertilize the soil and take part in nutrient discharge into the environment.
- Some blue-green algae (cyanobacteria) are capable of fixing atmospheric nitrogen (*Nostoc, Anabaena, Scytonema, Tolypothrix*). Soil inhabited by these microorganisms contains 26–400 times more nitrogen. Due to the ability of nitrogen ($N_2$) and carbon dioxide ($CO_2$) assimilation, they may be the first to colonize the nitrogen and organic carbon-free base.
- About 2000 species of algae occur in soil. The main species include
  - Blue-green algae *Nostoc, Anabaena, Scytonema, Tolypothrix, Microcoleus, Schizothrix*
  - Green algae – *Ankistrodesmus, Chlorella, Chlorococcum, Chlamydomonas, Characium, Klebsormidium*
  - Diatoms – *Achnanthes, Cymbella, Eunotia, Fragilaria, Hantzschia, Navicula, Nitzschia, Pinnularia*
  - Yellow-green algae *Botrydiopsis, Heterothrix, Heterococcus, Pleurochloris*
  - Euglenoids *Euglena, Peranema*
  - Red algae – *Porphyridium*
- Among the macrobiotic plant organisms that inhabit the soil environment higher plants dominate making up the basic element of biocenoses of all the land ecosystems.

**Fauna of soil**

The soil **microfauna** is represented by protozoans, which mainly feed on bacteria. Their role is to conduct selection and rejuvenate the population of soil bacteria. Amoebae and flagellates dominate among them.

**Mesofauna** is represented by nematodes (eelworms), snails, insects, myriapods, mites and others. They feed on dead organic matter contributing to the formation of humus.

**Macrofauna** is represented by earthworms, moles and rodents. These organisms break up soil material and carry it down to a significant depth. Earthworms play the most important role among the invertebrates by feeding on dead organic matter and absorbing it along with the mineral part of the soil. The non-digested residue mixed with mineral soil and the metabolites are excreted in the form of lumps (coprolites) contributing to the formation of the crumb texture of soil and its loosening. During the period of one year, earthworms in an area of one hectare are able to pass 7 thousand kg of soil through their digestive tracts.

Due to animal mobility and activities, soil undergoes constant mechanical mixing which in turn allows for better aeration, oxygenation and water flow.

**Rhizosphere**

- The rhizosphere is the layer of soil around the roots where, among others, in great concentrations live bacteria, fungi, protozoa, nematodes, mites and springtails, which usually form groups of species characteristic to a given plant.
- The rhizosphere is occupied by a large variety of forms; however, the *Pseudomonas* and *Achromobacter* as well as the denitrifiers are the most numerous. Less numerous are the *Arthrobacter and Bacillus* forms. The above organisms utilize nutrients released by the roots. The increased number of microorganisms is accompanied by a higher activity of soil fauna, especially for those organisms that feed on roots and microorganisms.
- The number of bacteria in the rhizosphere may even be 1000 times higher than outside the rhizosphere. The ratio of bacteria in the rhizosphere to the number of bacteria outside it is called the **rhizosphere effect** and is marked with the R/S symbol (R – rhizosphere, S – soil).
- Microorganisms of the rhizosphere also have a large influence on plants. They lead to a constant breakdown of organic and mineral compounds, which then become available to plants. Moreover, they produce organic and non-organic acids influencing the dissolution of mineral salts and protecting the plants against phytopathogens.
- An unfavorable effect is due to the metabolic activity of microflora that causes the depletion of the oxygen required for the development of denitrifiers. As a consequence, phytotoxic substances may be produced such as alcohols, antibiotics and phenol compounds.

**The symbiosis of fungi and plants**

**Mycorrhiza** is the interaction of fungi with vascular plants. In this type of interaction both organisms benefit. Fungi grow into plant roots. They penetrate its cells and stimulate their growth by producing the auxin hormone. Due to mycorrhiza, plants obtain a larger absorbing surface and better access to nutrients being broken down and absorbed by fungi. Plants supply fungi with organic substances in the form of assimilation products transported from leaves to roots. Mycorrhiza is a wide-spread phenomenon; it concerns not only the roots of trees, bushes and species of flowers, but also cultivated plants such as grains and potatoes.

There are two types of mycorrhiza: ectotrophic and endotrophic.

**Pathogenic organisms in soil**

Soil bacteria can be subdivided into two groups: those that always occur in every soil type (autochthonous) and the bacteria that grow only after a large amount of organic matter is discharged into the soil (zymogenous).

The **autochthonous** bacterial population is uniform and constant in soil since their nutrition is derived from organic matter in native soil. The largest group of soil bacteria is represented by microorganisms which use humic substances as a carbon source. Among them there are aerobic, non sporulating bacteria from the *Arthrobacte*r genus and rod-shaped bacteria from the *Mycobacterium* genus. The stable habitants of soil are actinomycetes from *Streptococcus* and *Nocardia* genus as well as mucous bacteria (*Myxobacteriales*). Most common in soil are aerobic spore-forming bacteria belonging to the genera *Bacillus* and anaerobic bacteria from the *Clostridium* genus. Characteristic for soil are autotrophic bacteria which utilize $CO_2$ as a carbon source and derive their energy either from sunlight (*Chromatium, Chlorobium, Rhodopseudomonas*) or from the oxidation of simple inorganic substances (*Nitrosomonas, Nitrobacter, Thiobacillus*).

The **Zymogenous** bacterial population in soil is low as they require an external source of energy. The population of zymogenous bacteria increases gradually when a specific substrate is added to the soil. The source of soil contamination can be sewage containing large amounts of faecal matter, plant and animal waste. It is known that soil is a recipient of solid wastes able to contain pathogens in high concentrations. Among them there are animal pathogens such as *Clostridium (Clostridium tetani* – causes tetanus, *Clostridium botulinum* – causes botulism)*, Bacillus (Bacillus anthracis* – anthrax, *Bacillus thuringiensis* – insect pathogen)*, Coxiella* and *Streptococcus*. Plant pathogens belonging to the genera *Agrobacterium, Erwinia, Corynebacterium, Pseudomonas* and *Xanthomonas* are also encountered.

Many of the diseases caused by agents from soil have been well characterized. Gastrointestinal infections are the most common diseases caused by enteric bacteria. Some examples include salmonellosis (*Salmonella* sp.), cholera (*Vibrio cholerae*), dysentery (*Shigella* sp.) and other infections caused by

*Campylobacter jejuni*, *Yersinia* sp., *Escherichia coli* O157:H7 and many other strains.

Viruses are the most hazardous and have some of the lowest infectious doses of any of the enteric pathogens. Hepatitis A, hepatitis E, enteric adenoviruses, poliovirus types 1 and 2, multiple strains of echoviruses and coxsackievirus are enteric viruses associated with human wastewater.

Among the most commonly detected protozoa in sewage are *Entamoeba histolytica*, *Giardia intestinalis* and *Cryptosporidium parvum*.

Soil-transmitted helminthic infections are of two types: hookworms, which undergo a cycle of development in the soil (the larvae being infective) and a group of nematodes that survive in the soil merely as eggs that have to be ingested in order for the cycle to continue.

The most common pathogenic helminths likely to be found in soil are *Ascaris lumbricoides* (human roundworm), *Ascaris suus* (pig roundworm), *Trichuris trichiura* (human whipworm), *Taenia saginata* (beef tapeworm), *Taenia solium* (pork tapeworm) and *Toxacara canis* (dog roundworm). The most important from the sanitary point of view are three of these: *A. lumbricoides*, *Trichuris trichiura* and *Toxocara canis*. All of these worms are nematodes. Their life cycles are presented in fig. 5.1–5.3.

***Ascaris lumbricoides*** **(common roundworm)** as an adult form live in the human small intestine and can reach 30 cm in size. The female can produce even 200 000 eggs per day. The eggs pass in feces and develop in the external environment – in soil. They are very resistant to environmental factors owing to their heavy protective tuberculated shell. In 2–4 weeks (depending on the soil conditions) the eggs embryonate and become infectious. After ingestion, the **infectious eggs** (by eating unwashed vegetables contaminated with soil) pass into the small intestine and larvae hatch there. The larvae penetrate the blood vessels and migrate with the blood stream to the lung and then again reach the small intestine where they mature and mate. The worm and also the other two mentioned previously are especially dangerous for children. They may cause malnutrition and growth retardation. The disease caused by *Ascaris* sp. is named **ascariasis**.

***Trichuris trichiura*** is called **whipworm** because its shape resembles a whip. As an adult form the nematodes measure about 30–50 mm and live in the large intestine. The female produce very characteristic barrel-shape eggs with double plugs. The eggs pass in feces and develop in soil for 2–3 weeks. Infection occurs mainly via ingestion of food (especially vegetables) contaminated with soil containing infectious eggs. When they reach the small intestine, the eggs hatch and the larvae develop. Then, the young worms move to the large intestine, penetrate the mucosa and mature. Whipworms may cause diarrhea or constipation, anemia, vomiting and also an inflamed appendix.

***Toxocara canis*** is mainly a parasite of dogs and its adult form lives in the intestine of young dogs. In humans the adult form does not occur (the development of the worm is stopped at the larva stage). Humans are infected by the ingestion of food contaminated with faeces of puppies containing infectious eggs. Particularly vulnerable are children playing in sandboxes. Therefore, all dogs should be routinely treated against this worm to reduce the risk of infection. The eggs hatch in the small intestine, larvae penetrate the blood vessels and migrate through the bloodstream to different human organs, most frequently to the liver or eyes and then they remain there. This can lead to the malfunction of these organs.

# Laboratory exercises

**Task 1. Examination of the "total" number of saprophytic, spore–forming and thermophilic bacteria**

The examination is conducted with the use of deep inoculation on Petri dishes. Analyses include the assessment of

a) "total" number of bacteria on nutrient agar medium at 28°C for the incubation period of 48 ± 2 h,
b) number of thermophilic bacteria on nutrient agar medium at 55°C for the incubation period of 24 ± 2h,
c) number of endospore forming bacteria on nutrient agar medium at 28°C for the incubation period of 48 ± 2 h, – soil dilutions should be warmed in a water bath for 15 minutes at 80°C in order to remove the bacterial flora which does not form endospores.

1. Use 1 ml of suspensions from the following dilutions:
   – sandy soil: $10^{-1}$–$10^{-3}$,
   – garden soil and compost of municipal wastes: $10^{-4}$–$10^{-6}$.
2. Count the number of grown colonies after the incubation period. For calculating the number of colonies, choose plates with 30–300 colonies.
3. Calculate a dry mass of 100 g of a soil sample that was previously dried at a temperature of 105°C in order to obtain a constant weight.
4. The final results should be expressed as the number of bacteria appearing in 1 g of dried mass of soil in regard to the "total number of bacteria". Give also the percentage content of endospore-forming bacteria.

**Task 2. Detection of *Clostridium perfringens***

This detection is conducted by the deep inoculation of samples into the sulfite-iron medium according to Wilson-Blair at 37°C for 18–24 ± 2 h (detection of *Clostridium perfringens* is also possible on solid medium).

- Use 1 ml of suspension from the following dilutions:
  – sandy soil: $10^{-1}$–$10^{-4}$,
  – garden soil and compost of municipal wastes: $10^{-1}$–$10^{-6}$.
- Soil dilutions should be warmed in a water bath for 15 minutes at 80°C in order to remove the bacterial flora which does not form endospores,

The appearance of black colonies deep inside agar after the first 18 h of incubation signifies the presence of *Clostridium perfringens*. Black colonies can often rip/tear off the medium as a result of gas production. Black coloring of the colony is a result of the reduction of sulfite to sodium sulfide, which altogether with iron chloride forms the black colored iron sulfide. The final results should be given as the ***Clostridium perfringens* titer**.

**Task 3. Identification of *Salmonella* sp. - demonstration** (fig. 5.4)

The examination is conducted in 2 stages:

- **Stage I – presumptive test** – culturing on a liquid selective – a multiplying medium with acid sodium selenine where certain pathogenic rods multiply, but the growth of others is prevented to a certain extent – relies on the inoculation (introduction) of the appropriate amount of soil to the multiplying medium and incubation at 37°C for 18–24 h.
  A **positive result** of the presumptive test is characterized by
  – turbidity of the medium,
  – appearance of a brick-red color.
  A **negative result** of the presumptive test is characterized by
  – a lack of turbidity and color of the medium.
  A **dubious result** of the presumptive test is characterized by
  – the appearance of very weak turbidity and at the same time weak coloring of the medium (brick-red color),
  – lack of turbidity and brick-red color

Cultures recognized as dubious after 18–24 h of incubation must undergo the confirmation test.

- **Stage II – confirmation test** – it is performed in order to confirm the presence of *Salmonella* genus bacteria taking into account all positive and dubious cultures of the presumptive test obtained after 24 or 48 h. Positive results are confirmed in order to exclude the so-called positive false results. Examinations confirming the presence of *Salmonella* genus bacteria are performed at 37°C on solid selective-differential media. Such media, depending on the biochemical properties of microorganisms, can demonstrate differences concerning colony morphology that may be useful when identifying microorganisms. This kind of media includes the ability of enteric rods for the fermentation of lactose demonstrated as the change in coloring of the colony or as the ability to change the redox potential during the growth of microorganisms:
- Mc Conkey's agar (streak-plate inoculation).
- SS agar (*Salmonella-Shigella*) (streak-plate inoculation).
- Kligler's agar slant (Triple Sugar Iron Test) (stubbing and streak inoculation).

A **positive result** of the confirmation test is characterized by

- on Mc Conkey's agar – the presence and the growth of colonies not fermenting lactose, i.e. the colonies of pink coloring,
- on SS agar (*Salmonella-Shigella*) – the presence and the growth of smooth, colorless, colonies with black centers,
- on Kligler's slant: – color change of the lower part of slant to yellow while the oblique area remains without changes (so it has a red color, the same as the initial medium), - darkening (blackening) of the medium suggests the appearance of hydrogen sulfide which is distributed inside the agar in the form of bubbles.

In detecting *Salmonella* sp. media allowing for the detection of the biochemical properties of isolated microorganisms are used, e.g. fermentation of sugars, of alcohol and the production of indol. For the inoculation 4 x of 25 g of soil are used so that at least100 g of soil is examined.

**Task 4. Detection of living eggs of intestinal worms – demonstration** (fig. 5.5)
Three species of intestinal worms are detected during the sanitary investigation of soil:

- human roundworm (*Ascaris lumbricoides*),
- toxocara (*Toxocara* sp.),
- whipworm (*Trichuris trichiura*).

Detecting real eggs of intestinal parasites is performed in 3 stages:

- **Stage I** relies on the "ungluing" of parasite eggs from the base (lumps of earth and deposits). This stage lasts 1 h and is conducted using a solution of 5% sodium hydroxide.
- **Stage II** includes
  – flotation- this process is conducted with a 5% zinc sulfate solution and by centrifugation of the soil solution (from 1 to 5 times).
  – thickening to a small amount with the use of a pipette, eggs of parasites float on the surface of the soil solution.
- **Stage III** relies on observation and the identification of parasite eggs with the microscope.

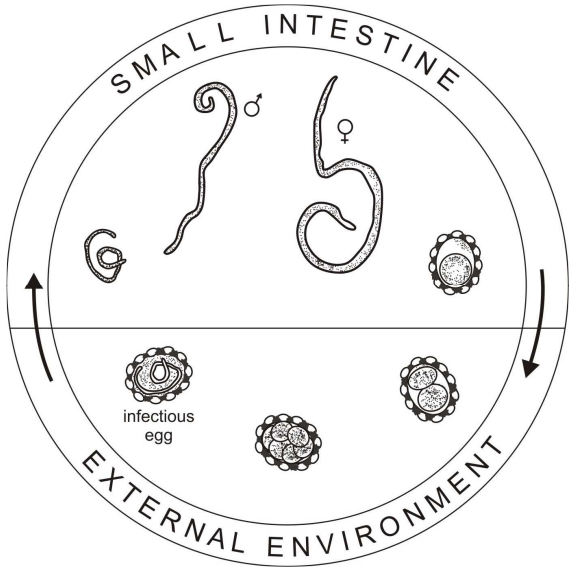


**Figure 5.1.** Life cycle of *Ascaris lumbricoides*.

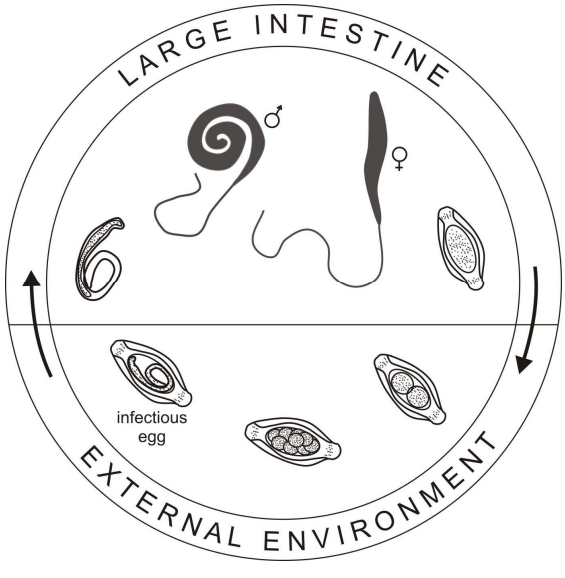


**Figure 5.2.** Life cycle of *Trichuris trichiura*.

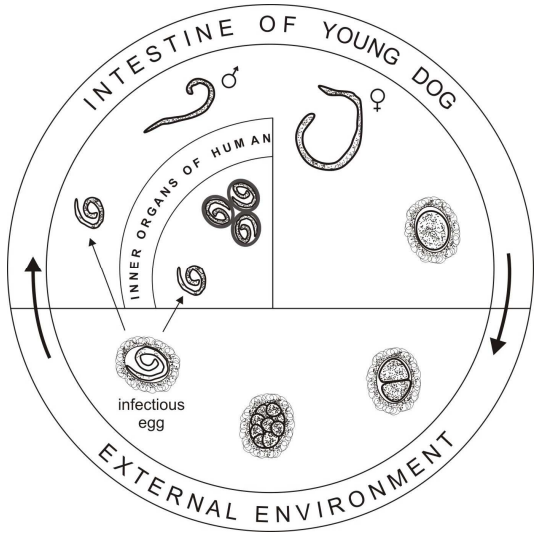


**Figure 5.3.** Life cycle of *Toxocara canis*.

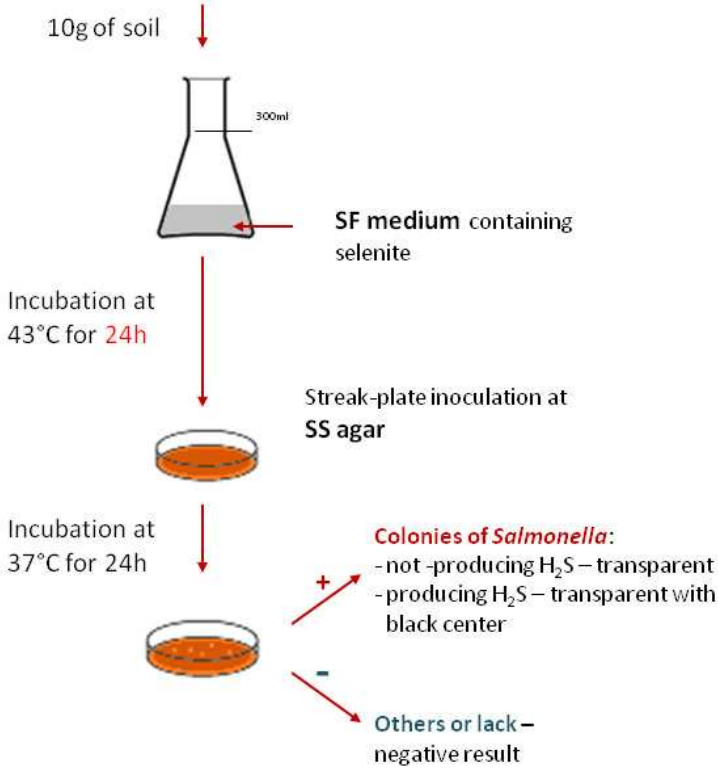


**Figure 5.4.** Isolation and identification of *Salmonella* sp. in soil.

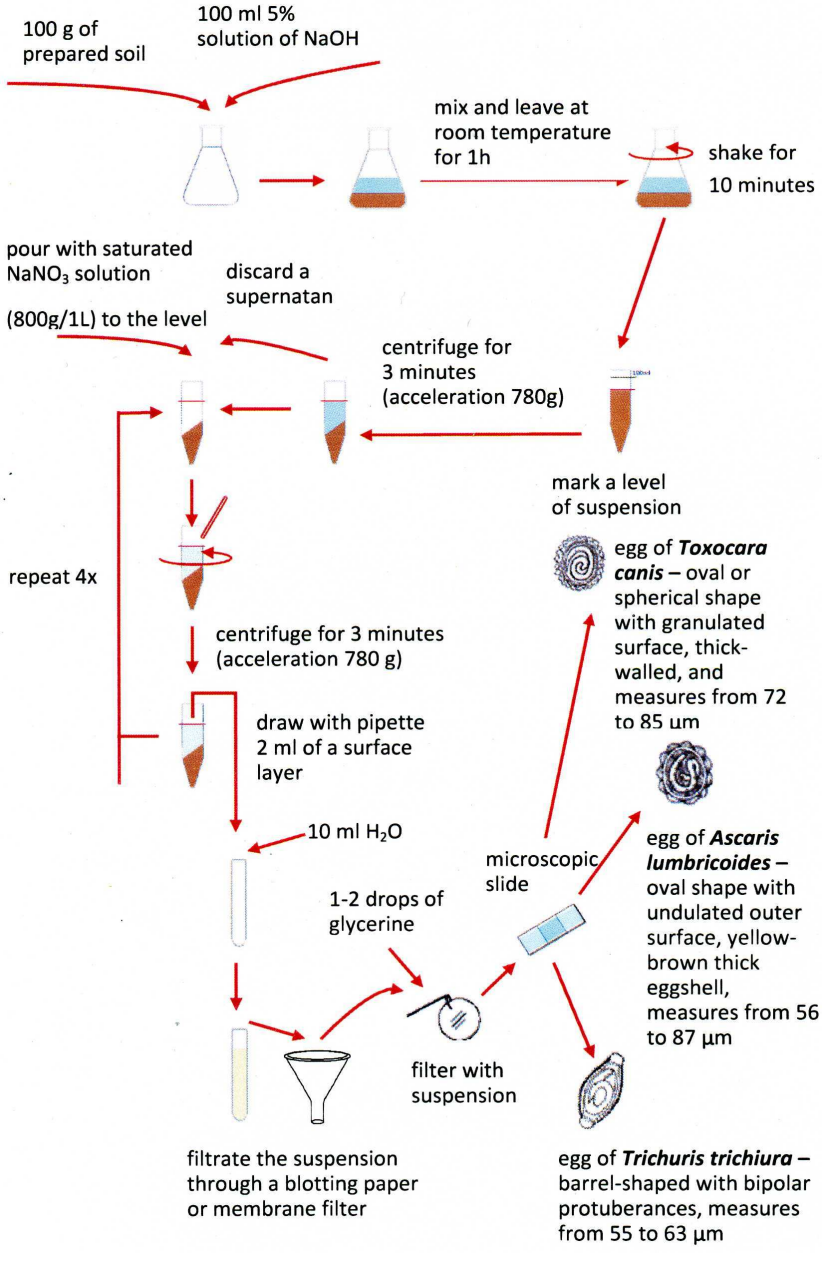


**Figure 5.5.** Isolation and identification of intestinal worm eggs in soil.

# Topic 6: Microbiology of air

**In order to pass this exercise the student should**
know the biological objects or factors found in air and how they influence or interact with air,
know what types of threats are connected with the presence of microorganisms in air and be able to characterize the main sources of emissions of bioaerosols and factors affecting their concentration and spreading,
know the advantages and disadvantages of particular survey methods for microbiological pollutants of air,
know what microorganisms and what types of culture media are a part of the sanitary analysis of air,
know the following definitions: respirable fraction, haemolysis, indicator microorganisms, opportunistic pathogens,
be able to calculate the concentration of microorganisms in air (as cfu/$m^3$) on the basis of the number of growing colonies on culture medium and to assess the level of pollution of studied air.

**The air as an untypical environment for microorganisms**

Air is merely a place microorganisms temporarily occupy and use for displacement. There are no metabolic connections occurring between cells in air (such as in soil or water). As a result, they form only a random collection of microorganisms, not a microbiocenosis. Microorganisms get into air as a consequence of wind movement which sweeps them away from various habitats and surroundings (soil, water, waste, plant surfaces, animals, and others), or are introduced during the processes of sneezing, coughing, or sewage aeration.
There are 3 elementary limiting factors in the air:

- a lack of adequate nutrients,
- frequent deficit of water, threat of desiccation,
- solar radiation.

Air contains some organic substances, but they are usually poorly decomposed and there is not enough of them to be utilized as food.

Microorganisms contained in air are constantly subjected to drying, which stops all processes. Some bacteria are especially sensitive to water deficits which may cause bactericidal effects (e.g. gonococci or spirochete which die as soon as they enter the air). Many organisms, however, can successfully cope with water deficits and although they cannot function properly, their dried up forms can survive months and even years as for example endospores and fungal spores.

Solar radiation is also damaging to microorganisms suspended in air as it causes mutations and desiccation (in water and soil solar radiation is usually very weak or simply does not exist).

**Adaptation of microorganisms to the air environment**

There are 3 main groups of microorganisms that occur in air (fig. 6.1):

- viruses,
- bacteria,
- fungi.

The atmosphere can be occupied for the longest time by those forms which, due to their chemical composition or structure, are resistant to desiccation and solar radiation. They can be subdivided into the following groups:

- bacterial resting forms,
- bacterial vegetative forms which produce carotenoid dyes or special protective layers (capsules, special structure of the cell wall),
- spores of fungi,
- viruses with envelopes.

**Resting forms of bacteria**

**Endospores** are the best known resting forms. These structures evolve within cells and are covered by a thick multi-layer casing. Consequently, endospores are unusually resistant to most unfavorable environmental conditions and are able to survive virtually endlessly in the conditions provided by atmospheric air. They are only produced by some bacteria, mainly by the *Bacillus* and *Clostridium* genera. Because each cell produces only one endospore, these spore forms cannot be used for reproduction.

Another type of resting form is produced by very common soil bacteria, the actinomycetes. Their special vertical, filiform cells of the so-called air mycelium undergo fragmentation producing numerous ball-shaped formations. Due to the fact that their production is similar to the formation of fungal spores, they are also called conidia. Contrary to endospores, the conidia are used for reproduction.

**Resistant vegetative cells of bacteria**

The production of carotenoid dyes provides cells with solar radiation protection. Carotenoids, due to the presence of numerous double bonds within a molecule (-C=C-), serve as antioxidants because as strong reducing agents they are oxidized by free radicals. Consequently, important biological macromolecules are protected against oxidation (DNA, proteins etc.). Bacteria devoid of these dyes quickly perish due to the photodynamic effect of photooxidation. That explains why the colonies of bacteria, which settle upon open agar plates, are often colored. The ability to produce carotenoids is possessed especially by cocci and rod-shaped actinomycetes. Mycobacteria, e.g. *Mycobacterium tuberculosis*, besides being resistant to light also demonstrate significant resistance to drying due to a high content of lipids within their cell wall.

High survival rates in air are also a characteristic for the bacteria which possess a capsule, e.g. *Klebsiella* sp. that may cause respiratory system illnesses.

**Fungal spores**

**Spores** are special cells used for asexual reproduction. A very common type of spores found in air is that of conidia. **Conidia** (*gr*. *konia* - dust) are formed in the end-sections of vertical hyphae called conidiophores and are dispersed by the wind. The spores of common molds such as *Penicillium* and *Aspergillus* are examples of the above.

**Resistant viruses**

Besides cells, the air is also occupied by viruses. The highest resistance is demonstrated by those with enveloped nucleocapsids, such as the influenza viruses. Among viruses without enveloped nucleocapsids, enteroviruses are relatively resistant to factors acting in the air environment.

Of course, besides the previously mentioned resistant forms, the air is also occupied by more sensitive cells and viruses, although their survival is much shorter. It is believed that among vegetative forms, gram-positive bacteria demonstrate greater resistance than gram-negative (especially for desiccation) mainly due to the thickness of their cell wall. Viruses are usually more resistant than bacteria.

**Biological aerosols**

Microorganisms in air occur in the form of a colloidal system or the so-called bioaerosol. Every colloid is a system where inside its dispersion medium particles of the dispersed phase occur with a size halfway between molecules and particles visible with the naked eye. In the case of biological aerosols, it is the air (or other gases) that has the function of the dispersion medium, whereas microorganisms are its dispersed phase. However, it is quite rare to have microbes independently occurring in air. Usually, they are bound with dust particles or liquid droplets (water, saliva etc.); thus, the particles of the bioaerosol often exceed microorganisms in size and may occur in two phases:

- dust phase (e.g. bacterial dust) or
- droplet phase (e.g. formed as the result of water-vapor condensation or during sneezing).

**The size of bioaerosol particles**

The average size of the particles ranges from about 0.02 μm to 100 μm. The sizes of certain bioaerosol particles may change under the influence of outside factors (mainly humidity and temperature) or as a result of larger aggregates being formed. By using size criterion, the biological aerosol can be subdivided into the following:

- fine particles (less than 1μm) and
- coarse particles (greater than 1μm)

Fine particles are mainly viruses, endospores and cell fragments. Coarse particles consist mainly of bacteria and fungi, usually associated with dust particles or with water droplets.

**Respirable fraction**

Particles capable of penetrating the pulmonary alveoli are referred to as the respirable fraction. This name refers to all types of particles, not only those of the bioaerosol. It is commonly believed that the respirable fractions consist of particles with diameters less than 10 μm. The contribution of the respirable fraction to the bioaerosol is a measure of its harmfulness as it illustrates the part of the bioaerosol that may penetrate the lungs. That is why methods to determine the size of the respirable fractions are most critical when determining the level of microbiological air pollution.

**Survival and spread of bioaerosols**

There are several factors which influence the ability of a bioaerosol to survive in air:

- particular resistance of a given microorganism (*morphological characteristics)*,
- meteorological conditions (air humidity, solar radiation),
- air pollution,
- the length of time in air.

**Resistance of microorganisms**

It is a species dependent feature which relies on the microorganism's morphology and physiology. The ability of microorganisms to survive in air may be expressed by the time of half-decay $t_{1/2}$, e.g. for many *Enterobacteriaceae* bacteria that time is just 8 seconds.

**Relative humidity**

The content of water in air is one of the major factors determining the ability to survive. At a very low humidity and high temperature, cells face dehydration, whereas high humidity may give cells protection against the solar radiation.

Forms of resting spores with thick envelopes (e.g. bacterial endospores) are not particularly susceptible to humidity variations. Gram-negative bacteria and enveloped viruses (e.g. influenza virus) deal better with low air humidity which is in contrast to gram-positive bacteria and non-enveloped viruses (e.g. enteroviruses) that have higher survival rates in high air humidity.

**Temperature**

Temperature can indirectly affect cells by changing the relative air humidity (the higher the temperature, the lower the relative humidity). It can also have direct effects causing, in some extreme situations, cell dehydration and protein denaturation (high temperatures) or the crystallization of water contained in cells (at temperatures below 0°C). Therefore, it can be concluded that low (but greater than 0°C) temperatures are optimal for bioaerosols (although according to some researchers the optimal temperatures are above 15°C).

**Solar radiation**

Solar radiation, i.e. visible, ultraviolet (UV) and infrared radiation, has a negative influence on the survival rate of the bioaerosol due to the following factors:

- it causes mutation,
- leads to the formation of free radicals which damage important macromolecules,
- creates a danger of dehydration.

**Air pollution**

Pollutants in air, especially hydrocarbons, ozone and nitric oxides, activated by solar radiation (especially UV) create various highly reactive secondary pollutants commonly described as photochemical oxidizers (e.g. peroxiacetyl nitrate – PAN). They are toxic to all living forms including air-suspended microorganisms.

In contrast, non-toxic and non-biological aerosols (dust, fog) disperse and absorb solar radiation which consequently increases the survival of bioaerosols.
It should be noted that the above factors often work simultaneously and are related, e.g. solar radiation increases the temperature whereas high humidity weakens the radiation.

**Concentration of the bioaerosol**

The concentration depends on the following factors:

- amount of emitted microorganisms, depending on the emitter,
- distance from the source of emission,
- wind speed,
- microorganism survival rate, depending on the factors discussed above,
- precipitation.

The amount of emissions and quality content of the emitted bioaerosol depends on the source of the emission. For an aeration tank at a biological treatment plant the factors include the concentration of microorganisms in sewage and the method of aeration. The newly created bioaerosol disperses in a similar way to the non-biological one (e.g. suspended dust) except that the microorganisms die out with time. Blowing wind dilutes the aerosol and causes its concentration to drop as it moves away from the place of emission. In addition, a concentration decrease is caused by gravitational sedimentation and as a result of precipitation.

**Biological aerosols as a human hazard**

There are three types of dangers connected with the presence of microorganisms in air:

- infectious diseases (viral, bacterial and fungal),
- allergic diseases,

- poisoning (endotoxins, mycotoxins).

**Infectious airborne diseases**

The mucous membrane of the respiratory system is a specific type of 'gateway' for most airborne pathogenic microorganisms. Susceptibility to infections is increased by dust and gaseous air-pollution, e.g. $SO_2$ reacts with water that is present in the respiratory system creating $H_2SO_4$ which irritates the layer of mucous. Consequently, in areas of heavy air pollution, especially with smog, there is an increased rate of respiratory diseases.

Bioaerosols may, among other things, carry microbes that penetrate organs via the respiratory system. After settling, microbes from the air may find their way onto the skin or be carried by hands and get into the digestive system (from there, they can be carried by blood to other systems, e.g. the nervous system). Fungi that cause skin infections, intestinal bacteria that cause digestive system diseases or nervous system attacking enteroviruses are all examples of infectious airborne diseases.

**Allergic diseases**

An allergy is a changed, hypersensitive reaction of a person or animal to some substances called **allergens** (gr. *allos*-other, *ergon*-action). Actually, it's an immunologic reaction in which a needless production of antibodies by B lymphocytes occurs as a hypersensitive response to the penetration of antigens (called the allergen).

The strongest allergens are molds, thermophilous actinomycetes as well as gram-negative rods. The strength of allergenic bioaerosols depends not only on the type of microorganisms, but also on their concentration.

**Poisoning**

Poisoning/intoxication is caused by toxins that are produced by some microorganisms. Endotoxins and mycotoxins are the most significant types of toxins in polluted air.

Endotoxins are the components of Gram-negative bacterial cell walls (a lipid fragment of lipopolysaccharides LPS outer membrane). They demonstrate toxic (and allergenic) effects on mammals. After being inhaled into the lungs, they cause acute inflammation of the lungs.

Mycotoxins are produced by various mold fungi. The most common are aflatoxins produced by *Aspergillus flavus*.

**Basic sources of bioaerosol emission**

There are two basic sources of bioaerosols:

- natural,
- related to human activities.

Natural sources are mainly soil and water from which microorganisms are lifted up by the movement of air, and also organisms such as fungi that produce

gigantic amounts of spores that are dispersed by the wind. Therefore, there are always a given number of microorganisms in the air as a natural background concentration. It is estimated that the air is considered to be clean if the concentration of bacteria and fungi cells does not exceed 1000/$m^3$ and 3000/$m^3$, respectively. If the concentration of microorganisms in the air exceeds the above values or contains microorganisms dangerous to humans, then such air is considered to be microbiologically polluted. From the hygienic point of view, living sources of bioaerosols related to human activity are more important than the natural sources. The most important sources of bioaerosol emissions are

- agriculture and the food industry,
- sewage treatment plants,
- waste management.

**Agriculture and the food industry**

The most important components of the above-mentioned aerosols are

- mold fungi (*Aspergillus, Penicillium, Cladosporium, Alternaria*),
- gram-negative rods, mainly *Erwinia* sp.,
- thermophilic acitnomycetes,
- dust of biological origin (particles of skin, feathers, droppings, plant dust).

Contact with such aerosols may often bring about chronic diseases of the respiratory system, e.g. allergic inflammation of pulmonary alveoli *alveolitis allergica.* It is assumed that there is an exceptional health risk when over 50% of the aerosol belongs to the respirable fraction and the concentration of bioaerosols exceeds $10^3$ cfu/$m^3$.

**Wastewater treatment**

In mechanical-biological sewage treatment plants employing the activated-sludge method, the biggest emission of microorganisms can be observed in the mechanical section where raw sewage is introduced (drainage area, grill, sand trap) as well as in the vicinity of aeration tanks and sludge drying beds. Moreover, a strong emission may also be observed during sewage purification in soil and in sprinkling beds. The following are some of the characteristic microorganisms present in bioaerosols and sewage:

- enteric bacteria (*Enterobacteriaceae*), including those of the *coli* group,
- hemolytic bacteria, mainly *Streptococcus* sp. and *Staphylococcus* sp.,
- bacteria *Pseudomonas* sp.,
- yeast such as *Candida albicans and Cryptococcus,*
- intestinal viruses: enteroviruses and reoviruses.

From the stated microorganisms, the intestinal bacteria and viruses are the most specific to sewage bioaerosols and usually do not occur in the down-wind side of the plant. Consequently, they are considered to be indicator microorganisms that are helpful in determining the effect of the sewage-treatment plant on the surrounding environment.

**Waste management**

Various forms of waste management are additional sources of bioaerosol emissions:

- waste storage and
- composting.

**Waste storage**

The air around storage yards contains bacteria found commonly in nature and saprophytic fungi of soil and water origin, some of which are opportunistic pathogens. This means that under favorable conditions (weakening of the immune system, penetrating the body in large numbers) they may invoke various diseases in humans. The following are the dominating genera of bacteria: *Bacillus, Pseudomonas* and *Enterobacter.* The last two genera are Gram-negative bacteria which produce endotoxins and their presence is often observed around waste dumps. In close proximity to dumps, the concentration of microorganisms often exceeds $10^3/m^3$. It is believed that within dump sites and similar communal facilities (e.g. composting site), the total number of bacteria in air should not exceed $10^4/m^3$ and of Gram-negative bacteria $10^3/m^3$. The range of bioaerosols spread by waste storage facilities is usually greater than that of sewage-treatment plants and may often exceed 1000 meters.

**Composting**

Composting also emits large amounts of microorganisms - especially bacteria. Particularly significant air pollution is created during waste sorting when the concentration of bacteria often exceeds the limit of $10^5$ cfu/$m^3$. Among these, there are gram-negative bacteria potentially harmful to humans. Due to the high temperatures (65–70°C) of the composting process, most of the above bacteria usually get neutralized; however, their endotoxins demonstrate a certain degree of thermostability and thus when released into air they can cause poisoning.

The common mold fungus, *Aspergillus fumigatus,* whose spore concentration in air can equal $10^6/m^3$, is a good indicator of the effect of composting facilities on the surroundings.

**Investigation of microbiological air pollutions**

Methods detecting the presence of microbes in air can be subdivided into

- microscopic methods,
- culture methods.

**Microscopic methods**

They consist of

- letting air through a membrane filter or placing a glass coated with a sticky substance (e.g. vaseline) in the air path,
- staining of the trapped microorganisms,
- microscopic testing consisting of cell counting.

The advantage of the above methods is that it allows the detection of live and dead microbes in air as well as those that do not grow on culture media. Because of this, the number of microbes determined is usually higher by one order of magnitude than in culture methods. In addition, it is possible to detect and identify other biological objects, e.g.: plant pollen, allergenic mites, abiotic organic dust (fragments of skin, feathers, plants, etc.). However, the methods have a serious drawback: the inability to determine the species of microbes.

**Culture methods**

These methods consist of transferring microbes from air onto the surface of the appropriate culture medium. After a period of incubation at optimal temperature, the formed colonies are counted and the result is given as cfu/$m^3$ of air (colony forming units). Because a colony can form not only from a single cell, but also from a cluster of cells, the air may contain more microbes than suggested by the cfu result. Besides this, the method allows only the detection of the cells that are viable and those which are able to grow on the medium used. Testing of viruses differs significantly from the methods utilized for other organisms because

- they may develop only in living cells; therefore they require tissue cultures (e.g. the epithelium of the human trachea) or in the case of bacteriophages, bacterial cultures,
- species identification of detected viruses is difficult.

There are three basic ways of sampling the air for use in culture methods:

- Koch's sedimentation method,
- filtration method (also used in microscopic methods),
- impact methods.

**Sedimentation method**

It is the simplest method that consists of a cell settling from the air onto the open Petri dishes filled with the appropriate culture media. The gravitational force affecting the particles of the bioaerosol have significance only in relation to bigger particles, as the smaller ones hit the exposed culture medium as a result of air movement. After a particular period of exposure (usually 10–30 min) the plate is incubated and the resultant colonies counted. The benefit of this popular method, besides its simplicity, is its inexpensiveness. However, it only gives a rough estimate of the number of microbes in air as it contains a series of problems:

- no knowledge about the air volume to which the number of cfu can be related,
- inability to detect the smallest particles of bioaerosol which make up the respiratory fraction, that settle very slowly or never undergo the process of sedimentation (low output),
- high inaccuracy caused by the movement of air that changes the conditions of sedimentation.

The first problem, stated above, can be partially compensated for by utilizing the empirical conversion formula based on the assumption that cells contained in 10 $dm^3$ of air settle upon a 100 $cm^2$ area in 5 minutes. The formula is as follows:

$$x = \frac{a \cdot 5 \cdot 10^4}{\pi r^2 \cdot t}$$

where: x – number of microbes in air (w cfu/$m^3$), a – number of colonies on the Petri dish, $\pi r^2$ – surface of Petri dish ($cm^2$), t – time of exposure (minutes).
In order to limit the disturbance connected with the movement of air, it is recommended to conduct the testing in low wind speeds.

**Filtration methods**

These methods are also rather inexpensive and not complicated; they possess two significant advantages over the sedimentation methods:

- the volume of the air tested is known,
- it is possible to detect very small aerosol that creates the respiratory fraction (nevertheless, it is still impossible to determine its size). The methods consist of using an aspirator to suck in a given volume of air, passing it through a sterile absorbing substance (liquid or solid) and transferring the filtered microbes onto the appropriate culture medium. After a pre-determined time of incubation, the resulting colonies are counted. Most often, a membrane filter or a physiological solution (0.85% NaCl) is utilized for the filtration of air.

Filtration using liquids (sometimes classified as the impact method) is one of the most often used and highly valued techniques of sampling bioaerosols (fig. 6.2). It results in a high output of microbe isolation (including the respirable fraction) as well as significant survival of the filtered microbes.

The filtration process through membrane filters allows the utilization of both culture methods (filters containing microbes are placed directly upon the culture media) as well as microscopic methods (filters are stained and observed under a microscope). However, the disadvantage of this method is that it has a significantly low output because the process of passing the air through pores of the filter creates resistance. That is why the method is not recommended for microbe testing, but is routinely put to use in detecting endotoxins in air.

**Impact methods**

These methods consist of using an aspirator to suck in a pre-determined amount (volume) of air which collides with nutrient agar at high speed (fig. 6.3). This causes the microbes in the air to stick to the surface and after a specific time of incubation they form colonies. The impact methods are the most highly valued and most often used methods of detecting microbes in air. Their biggest advantage is the possibility of detecting and determining the respiratory fraction of the bioaerosol, in other words, determining the size distribution of the particles. This is a very important feature as the size of particles determines the degree of respiratory system penetration.
A disadvantage for the impact method is a decline in the microbe viability caused by the shock of the sudden collision with nutrient agar and also a possibility of the

nutrient culture getting overgrown in cases of high air pollution. The above stated methods are usually quite costly.

**Microbiological evaluation of air pollution**

According to the norms assumed in Poland, atmospheric air is clean when the concentration of bacterial cells does not exceed 1000 cfu/$m^3$ and fungi 3000 cfu/$m^3$.

Inside a building, the total number of bacteria should not exceed 2000 cfu/$m^3$ and fungi 300 cfu/$m^3$. When the concentration of microbes exceeds the above norms, such air is considered to be microbiologically polluted.

Norms for other rooms depend on their pre-determined use, e.g. a surgery room may not contain any fungus and the number of bacteria cannot exceed 100 cfu/$m^3$, whereas in a pigsty there may be 200 000cfu/$m^3$ of bacteria and 10 000 cfu/$m^3$ of fungi.

Very important from a hygienic point of view is the knowledge about the particle size distribution of the bioaerosol. The greater the proportion of small particles in the bioaerosol, which can enter the alveoli (size about 1μm), the greater the health hazard of the air. The inhalation of such air may cause allergies, poisoning and dust diseases.

Qualitative examinations must be limited to indicator microorganisms, as the identification of pathogenic microbes is usually strenuous and expensive. The indicator species need not be pathogenic, but their occurrence points indirectly to a potential threat due to disease-causing microorganisms.

The following indicator microorganisms are used for the microbial analysis of air:

- hemolytic staphylococci,
- mannitol fermenting and non-fermenting staphylococci,
- actinomycete bacteria,
- *Pseudomonas fluorescens*.

Staphylococci are one of the most common bacteria in nature. They are not all pathogenic; many of them appear on human skin and the mucous membranes and do not cause diseases. Pathogenic staphylococci show a high metabolic activity, which can be used to differentiate them from the non-pathogenic ones.

Pathogenic staphylococci cause

- hemolysis of red blood cells (erythrocytes) on blood agar medium,
- acid fermentation of mannitol on mannitol salt agar medium (Chapman medium).

Hemolysis consists of the destruction of erythrocytes by certain toxins produced by bacteria resulting in the formation of a characteristic zone of clearing around the colony (fig. 6.4).

The Chapman medium contains 10% NaCl which ensures that mainly staphylococci grow on this medium (they are resistant to high concentrations of salt). The presence of mannitol in the medium is used to differentiate the mannitol-fermenting staphylococci from the non-fermenting ones. The determination of

haemolysis and of the fermentation of mannitol increases the probability that the detected staphylococci are pathogenic.

Actinomycetes are typical soil bacteria. Their presence in the air can point to the soil environment as the source of pollution.

*Pseudomonas fluorescens* is a common water bacterium. Its presence in the air can point to the water environment as the source of pollution.

In addition to the investigation of the microbiological air pollution emitter, one should investigate the typical species from this emission source. This can determine the impact of the emitter on the state of pollution in the air – the occurrence of the indicator microorganisms will mark the border of the impact zone.

# Laboratory exercises

**Task 1. Investigation of microbiological pollution of air using the sedimentation method**

1. Put at four fixed investigation locations (indoor and outdoor) the Petri dishes containing nutrient agar, Sabouraud agar, Chapman agar, Pochon agar and King B agar. For sampling, remove the lids from the plates for the following periods of time:
   10 min. for agar plates and Sabouraud agar plates,
   30 min. for the other plates.
2. Close the plates and incubate them under the following conditions:
   37°C for 48 h (agar plates and Chapman agar plates)
   26°C for 7 days (the other plates).
3. After the incubation period count the grown colonies on the plates.
   On agar plates count all colonies except mold (wooly) ones.
   On Sabouraud agar plates count all colonies.
   On Chapman agar plates count separately colonies with yellow zones (positive mannitol-reaction, man+) and without such zones (negative mannitol-reaction, man-).
   On the Pochon medium – only the characteristic colonies of actinomycetes (flat, opaque, mossy in the middle) and on King B agar – count exclusively colonies that show yellow-green fluorescence under UV light.
4. In order to calculate the concentration of microorganisms in the volume unit of air (as cfu/$m^3$), the obtained values (number of colonies) should be substituted into the formula:

$$x = \frac{a \cdot 5 \cdot 10^4}{\pi r^2 \cdot t}$$

where: x – concentration of microorganisms in air (as cfu/$m^3$), a – number of colonies on plate,
$\pi r^2$ – surface area of Petri dish (~ 100 $cm^2$), t – time of exposure (min.).

Calculated the concentrations write them in the table:

| **Location** | **Nutrient agar – total number of bacteria** | **Sabouraud agar – total number of fungi** | **Chapman agar – staphylococci** | | **Pochon agar – actinomyces** | **King B agar – *Pseudomonas fluorescens*** |
|---|---|---|---|---|---|---|
| | | | **man+** | **man-** | | |
| 1 | | | | | | |
| 2 | | | | | | |
| 3 | | | | | | |
| 4 | | | | | | |

**Task 2. Investigation of microbiological pollution of air using the impact method**

Perform the experiment at the same locations as in Task 1. Compare the data obtained using the impact method and the sedimentation method.

**Task 3. Statement of the degree of microbiological pollution at different distances from the emission source (the case of a composting plant, wastewater treatment plant or landfill site)**

1. Count the colonies grown on plates exposed at different distances from the emitter.
2. Calculate and estimate the concentration of microorganisms in 1 $m^3$ of air from each sampling place.
3. State the emitter impact zone by comparing the bioaerosol concentration at a given sampling place with the concentration at a background (control) sampling place (fig. 6.5).

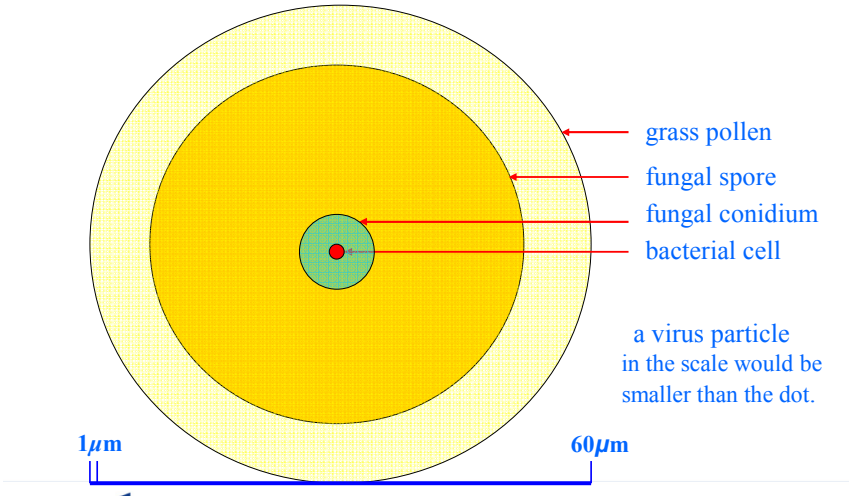


**Figure 6.1.** Biological objects occurring in the air (with the size proportions).

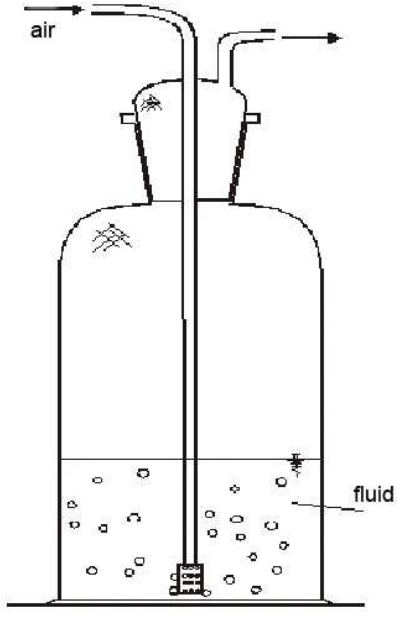


**Figure 6.2.** Washer bottle for bioaerosol absorption with the filtration method.

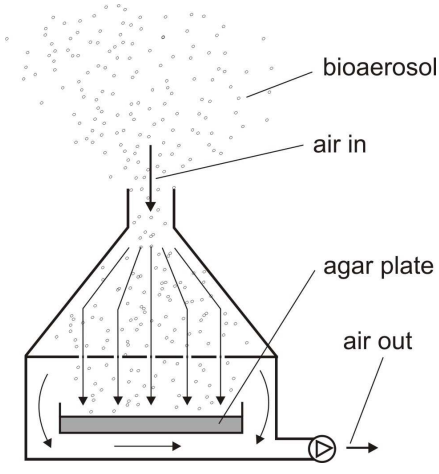


**Fig. 6.3.** Principle of the impact method.

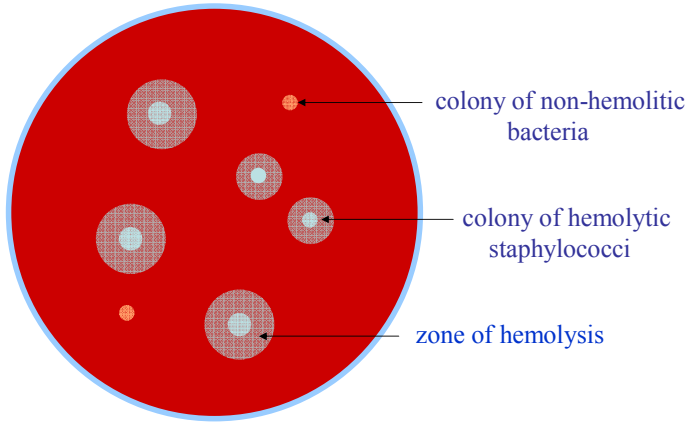


**Figure 6.4.** Colonies of hemolytic staphylococci on blood agar

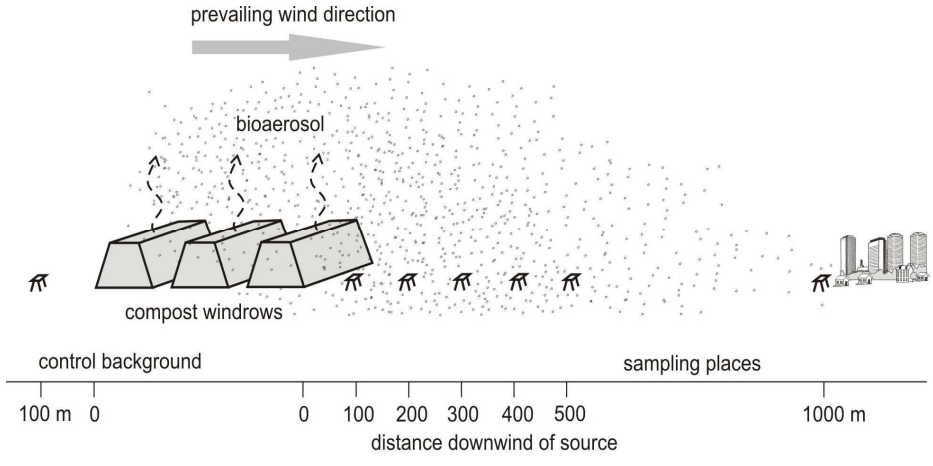


**Figure 6.5.** Investigation of the bioaerosol concentration near a composting plant.

# Topic 7: Biology of wastewater treatment processes

**In order to pass this exercise the student should**
know the classification of methods of wastewater treatment,
know what activated sludge is and what flocculation is, and be able to characterize the morphology of the activated sludge floc (size, shape, structure, filamentousness),
be able to describe the succession stages of microorganisms in activated sludge,
know the role of particular groups of microorganisms in wastewater treatment and know their indicative meaning in the assessment of the condition of activated sludge,
know what the swelling of sludge is and what the two types of swelling (filamentous and non-filamentous) depend on,
know the term: sludge volume index.

Sewage is a water which includes harmful liquids, solids or gaseous substances introduced into waters or soil that may lead to the contamination of surface or underground waters. Sewage also includes used liquids, solutions, colloids, suspensions, radiocontaminated waters, saline waters, heated cooling waters, precipitation waters or waters which contain various impurities from urban and rural areas.

The elimination of contamination from industrial and municipal wastes, prior to their reintroduction to a receiving body of water, is necessary for the proper management of the water supply, environmental protection and to ensure adequate sanitary conditions. The introduction of pollutants, depending on the watercourse, may decrease the water's physical, chemical and sanitary conditions or even cause disturbances in the biological balance.

The objectives of the sewage purification process include

- lowering the content of organic carbon including compounds which are difficult to biodegrade as well as toxic, mutagenic and carcinogenic substances,
- reduction of biogenic substances: nitrogen and phosphorus,
- elimination or inactivation of pathogenic microorganisms and parasites.

Depending on the type of pollutants, there are different methods of purification used prior to reintroduction into a receiving water body. The methods are classified as follows:

- mechanical – in this method only non-soluble pollutants are removed by utilizing the following processes: gravitational and centrifugal sedimentation, flotation, source filtration, separation in hydrocyclones, which allows the removal of organic and mineral suspensions as well as floating bodies,

- physical-chemical – utilizes the following operations and processes: coagulation, co-precipitation, sorption, ion exchange, electrolysis, reverse osmosis, ultrafiltration,
- chemical – utilizes neutralization, oxidation, reduction,
- biological – consists of sewage purification (elimination of organic pollutants as well as biogenic and some refraction compounds) during biochemical processes of mineralization conducted naturally by microorganisms in a water habitat (e.g. sprinkling of wastewater onto agricultural lands), or in special devices (on trickling filters or with activated sludge).

A typical process of sewage purification consists of four stages:

- mechanical (stage I of purification),
- biological (stage II of purification),
- elimination of biogenic compounds (stage III of purification),
- water renovation (stage IV of purification).

**Biological methods of sewage purification**

In biological methods of sewage purification, bacteria which form zoogleal clusters in sewage play a crucial role. Biological purification methods of sewage consist of inducing the enzymatic processes of saprophytic microorganisms that include the partial oxidation of organic substances (sources of carbon) contained in sewage as well as their partial assimilation by microorganisms. As a result of these processes, an increase in cell mass of the active microorganisms occurs. Microorganisms flourish when the ratio of three basic elements **C:N:P is 100:10:1**.

The biological processes of purification can be divided into natural and artificial, depending on where the processes take place – whether they occur in natural conditions or are intentionally triggered in specially designed artificial equipment.

Biological purification can be conducted in oxygen-rich, oxygen-poor or oxygen-free conditions. Biological purification is a process of oxidation and mineralization of the organic compounds present in sewage using micro- and macroorganisms.

During the process of biological purification the following phenomena take place:

- breakdown of organic substances to $CO_2$, $H_2O$, $NH_3$ (dependent on pH),
- nitrification (oxidation of $NH_3$ by *Nitrosomonas* bacteria down to nitrites and then by *Nitrobacte*r bacteria down to nitrates),
- denitrification (transformation of nitrates to gaseous nitrogen $N_2$).

**Trickling filters**

Sewage treatment by trickling filters is conducted in reservoirs filled with loose, grainy and porous material. Sewage is sprayed on the upper layer of the bed with sprinklers and then left to seep through its contents. A mucous **biological film** forms on the content of the bed and is composed of microorganisms such as

bacteria, protozoa and fungi. The filter helps ensure a constant supply of sewage and a proper flow while maintaining contact with the biological film. While flowing, the sewage undergoes mineralization as a result of aerobic decomposition by microorganisms.

The biological film is initially composed of zoogleal bacteria which produce mucous sheets. With time, the composition of species of the mucous membrane changes due to their succession. Besides bacteria, the following appear: fungi, protozoa, annelida and fly larvae. Depending on the amount of treated sewage, the trickling filters may be subdivided into percolating and flushing filters.

Depending on the organic load, the following types of biofilters are distinguished:

- **Low loaded filters** – may be filled with natural or artificial material. The supplied organic material is less than 0.4 kg $BZT_5/m^3d$. In percolating filters the film is more developed and the biological process of decomposition is almost complete. In the final phase of purification, intensive nitrification processes occur leading to an increase of nitrate in the outflow to the secondary settling tank.
- **Mid-loaded filters** – are filled with natural-synthetic material and work with a load between 0.4–0.65 kg $BZT_5/m^3d$. In order to ensure an adequate concentration of the supplied sewage, a part of the purified sewage recirculated. The reduction of organic compounds on these filters is adequate and the nitrification processes partially occur. The implementation of additional purification processes is not necessary.
- **High loaded (flushed) filters** – are filled with natural-synthetic material; the filter is loaded with 0.65–1.6 kg $BZT_5/m^3d$. In flushing filters, the intensity of the sewage flow is greater; however, the biofilm is composed almost entirely of bacteria and does not develop as much as in the above stated case. The flowing sewage washes out the used and dead biological material from the filter. The washed out material is then transported in the form of a floc-filled sediment. Only the partial mineralization of organic compounds occurs on this type of filter and the nitrification process is inhibited. A low content of nitrates in the effluent from the filters testifies to the partial mineralization of organic compounds. In complex systems, after these types of filters, re-purification is implemented as the quality of the purified sewage does not usually meet the required standards.

**Activated sludge**

The process of activated sludge relies on sewage purification by freely suspended matter. It consists of producing 50–100 μm flocs with highly developed surface areas. The floc is made up of a brown or beige mineral nucleus while on its surface it contains heterotrophic bacteria within the mucous envelopes. The method of activated sludge requires the delivery of oxygen into the substrate for the bio-oxidation of organic pollutants. The oxygen concentration should be more than 0.5 $mg/dm^3$ in order to ensure proper oxygen conditions for the bacteria.

Treating sewage with activated sludge consists of mineralizing organic compounds which is conducted mainly by bacteria following the same biochemical processes as observed in self-purification. However, the speed of the process is much greater. This results from the fact that the conditions of intensive aeration, triggered during sewage flow through aeration tanks, are conducive to the development of impurity-decomposing bacteria.

Agglomerates (flocs), which consist of heterotrophic bacteria coagulated with mucus, form during the process of aeration in aeration tanks (flocculation). The floccules absorb impurities contained in sewage, whereas microorganisms in the flocs decompose the absorbed substances.

Activated sludge has a spongy, loose structure, made of small openings of various shapes. Undisturbed floccules easily settle and thus allow the separation of the activated sludge from sewage.

The biocenosis of activated sludge is, for the most part, composed of heterotrophic bacteria. In small percentages – and only under particular conditions and in some arrangements – it is made up of chemolithotrophic bacteria, especially nitrifying bacteria. The most common species of activated sludge are *Zooglea ramigera, Pseudomonas fluorescens, Pseudomonas putida* as well as bacteria of *Achromobacter, Bacillus, Flavobacterium and Alcaligenes* genera. The process of selection occurs naturally. The conditions in an aeration tank, especially the pH air conditions, are the determining factors for the diversity of the bacterial complex.
In unfavorable conditions (overloading of aeration tanks with easily available substrates, high oxygen deficit), an excess development of flocs occurs causing the so-called **active-sludge swelling**.

There are two distinguishable types of swelling: **fibrous and non-fibrous swelling**.

Fibrous swelling is caused by excess filiform bacteria (*Sphaerotilus natans, Beggiatoa alba* or *Thiothrix nivea*) or fungi development. Non-fibrous swelling is caused by bacterial development that produces excess amounts of mucus.

**Activated sludge biocenosis** is made up of not only bacteria, but also of protozoa, nematodes and rotifers. Even though these microorganisms do not play a major role, their presence is important.

Protozoa feed on bacterial cells forcing them to reproduce quickly, which essentially make them an important renewal and reactivating factor of the activated sludge. The most common protozoa are *Vorticella, Carchesium* and *Opercularia* as well as *Anthophysa, Oxytricha, Stylonychia* and *Lionotus.*

There is an inverse relationship between flagellates and ciliates within activated sludge. While a large number of flagellates indicate an overload of sludge, the presence of ciliates shows it is functioning properly. During the course of sewage purification with activated sludge, a characteristic succession of biocenosis is observed.

In sludge development, the floc microlife community that stops succession is called the climax community – a stable, diverse assemblage of microorganisms that are highly efficient in metabolizing waste stream organics. At the start-up of a

plant, aerobic bacteria begin to increase in number with the aeration of the organic waste stream. They begin to colonize substrates and begin laying down a slime film. Amoebae generally are the first protozoa to appear in large numbers and they graze on the developing biofilm. Simultaneously, thin mixed liquor develops with motile rods,spiral bacteria and flagellates appearing. With the rapid proliferation of bacteria, small, spherical floc particles (bacteria and organics) begin to appear in the activated sludge process. Swimming and crawling ciliates appear to feed on bacteria "lawns" within floc particles in the biofilm and on bacteria in the open liquor. Various ciliates, with growing diversity in numbers of species, appear to feed on bacteria and flagellates. Other microlife forms such as rotifers - again with growing diversity of species-begin to appear to feed on swimming and crawling ciliates. Also, stalked ciliates appear on the biofilm feeding on bacteria and smaller flagellates and ciliates.

Within months, a stable biofilm with a diverse community of bacteria, protozoa and micro-invertebrates appear in the mature flocks.

In summary, from performing analyses it is possible to obtain information regarding the quality and quantity as well as the age of activated sludge.

# Laboratory exercises:

**Task 1. Sludge volume index (SVI)**
Determine the SVI of the tested activated sludge.

The aqueous level of activated sludge is assessed by the sludge volume index or Mohlman index. Sludge volume index is the volume (in ml) occupied by 1 g of dried mass of sludge.
SVI = sludge volume [ml]/dried mass of 1000 ml of sludge [g].
Sludge volume is the volume of suspension after 30 min. of settling in an Imhoff funnel.

**Task 2. Dehydrogenase activity of sludge**

The TTC test is used for the assessment of bacterial activity in the activated sludge. Dehydrogenase activity is assessed in this method. TTC plays the role as an artificial hydrogen and electron acceptor. The aqueous solution of this compound is colorless, but after the acceptation of hydrogen and electrons from dehydrogenses, TTC is reduced into colorful (reddish) TF (formazone). The color intensity of this solution shows the level of dehydrogenase activity of bacteria.

1. Pour, using the pipette, 1 ml of Tris buffer of 8.4 pH and 1 ml of homogenized activated sludge into two test tubes.
2. Add 0.4 ml of 40% glucose to the first test tube (substrate sample); do not add glucose to the second test tube (the endogenic sample for the activity towards stored substances).
3. Prepare the blind sample without adding activated sludge.
4. Place 0.4 ml of TTC solution with the pipette into all test tubes, mix them and close with a cork.
5. Put them in a thermostat at a temperature of 37°C and incubate in darkness for 30 minutes.
6. Stop the reaction after 30 minutes of incubation by adding 0.05 ml of sulfuric acid to all test tubes. Mix them.
7. Add 10 ml of acetone to each test tube and to shake as long as required for the entire formazone to dissolve in acetone.
8. Put the samples into the thermostat for 5 minutes at a temperature of 90°C.
9. Rotate them for 15 minutes at 1000 rpm.
10. Measure the absorption of the clear solution at the wavelength $\lambda = 490$ nm.

Determine dehydrogenase activity of 1 ml of activated sludge under studied conditions by using a calibration curve for TF (product of TTC reduction) and results of spectrophotometric measurements for the studied samples. Use μgTF/mg of dry mass of activated sludge for expressing the dehydrogenase activity of activated sludge.

**Task 3. Total number of heterotrophic bacteria**

The procedure recommended in Standard methods is employed in the analysis for the total number of heterotrophic bacteria in water. Serial dilutions in sterile saline solutions ($10^{-1}$ to $10^{-10}$) of samples are prepared. Triplicate, 1 ml volumes from the each dilution are placed on standard methods agar (SMA) using the pour plate technique. Plates are incubated at 10°C for 14 days and only those plates showing between 30 and 300 colonies are enumerated. Counts are expressed as colony-forming units, cfu/ml of the wastewater sample.

**Task 4. Microscopic analysis of activated sludge**

The microscope is used for live microscopic investigations (Olympus CX 41-RF-5 microscope; bright field, phase contrast). All the quantitative analyses are done directly after sampling on a Fuchs-Rosenthal counting chamber (subsamples of 20 µl). For species identification, a 10 µl (2 µl) subsample is fixed with Bouin's solution (15 vol. saturated picric acid, 5 vol. buffered formaldehyde 37%, 1 vol. glacial acetic acid; final concentration 50%). Ciliate populations are divided into the four functional groups: swimming, crawling, stalked ciliates, and the predatory suctoria. Metazoa such as rotifers, and nematodes are investigated in an additional subsample of 10 µl. Results of the macroscopic and microscopic observation of activated sludge put into table 7.1.

Legend -explanation of abbreviations used in activated sludge evaluation sheets.

1. Estimated results of macroscopic and microscopic observation of activated sludge
   - Smell
     - characteristic, substrate-like,
     - sallow,
     - rotten,
     - none.
   - Supernatant
     - clear,
     - cloudy.
   - Shape of floc
     - circular,
     - irregular,
     - agglomerates.
   - Structure
     - loose,
     - tight.
   - Cohesion
     - poor,
     - good.

**Table 7.1.** Results of the macroscopic and microscopic examination of activated sludge.

| **Macroscopic and floc structure properties of activated sludge** | | |
|---|---|---|
| color | | |
| smell | | |
| supernatant | | |
| sedimentation | | |
| shape of floc | | |
| structure of floc | | |
| cohesion | | |
| size | | |
| number of inorganic particles | | |
| filament abundance | | |
| **Microscopic properties of activated sludge** | | |
| **Bacteria** | | |
| Free swimming bacteria | | |
| Zoogleal bacteria | | |
| Filamentous bacteria | | |
| Spirillum | | |
| **Name and quantity of Protozoa and Metazoa found in activated sludge** | | |
| Flagellates | | |
| Rhizopoda | *Amoeba* sp. | |
| | *Arcella* sp. | |
| Ciliates | free swimming | |
| | crawling | |
| | stalked | |
| Rotifers | | |
| Tardigrads | | |
| Nematods | | |
| **The total number of bacteria** | | |
| Dehydrogenase activity | | |
| The general description of the activated sludge activity | | |

a) Floc size
   - big – diameter >500 μm,
   - medium – between 100–500 μm,
   - small <100 μm.

b) Number of inorganic particles, organic filaments, zoogleal bacteria
   - (-) – absent,
   - (±) – rare, observed occasionally,
   - (+) – observed regularly (5–10 particles or cell per sample),
   - (++) – frequent (numerous cells or particles, above 10–15 per sample).

2. Estimated results of microscopic analysis of activated sludge

c) The number of free swimming bacteria
   - (-) – rare,
   - (+) – a few dozen in vision field,
   - (++) – hundreds in vision field.

d) Filamentous bacteria
   - category 0 – nearly total lack of filamentous organisms,
   - category 1 – filamentous organisms are rather rare,
   - category 2 – medium number,
   - category 3 – very common,
   - category 4 – abundant.

   *) Filament shape
   - straight,
   - bent,
   - ball.

   Spirillae
   - (-) – absent,
   - (±) – accidentally,
   - (+) – 5–10 per sample,
   - (++) – above 10–15 per sample.

e) Invertebrates
   - sporadically – less than 1, or 1 organism per 3 samples,
   - rare – 1–3 organisms per sample,
   - not numerous – 3–5 organisms per sample,
   - rather numerous – 6–20 organisms per sample,
   - numerous – 2–5 organisms in vision field,
   - abundant – 6–15 organisms in vision field,
   - massive – above 15 organisms in vision field.

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