Environmental Quality Management

Justyna Rybak, Barbara Kołwzan

Ecotoxicology course in English. Theory and laboratory practice

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Reviewed by:
dr hab. Kazimierz Grabas

CONTENT

PART ONE

1. INTRODUCTION

1.1. ASSESSING THE INFLUENCE OF BIOLOGICAL AND CHEMICAL FACTORS ON THE TOXICITY OF SUBSTANCES

The toxicity of a substance is a result of its physical and chemical properties, structure as well as the biological activity of the living organism. **Physical and chemical factors** are dependent on solubility, dissociation ability and the chemical structure of the substance. **Biological factors** are closely related to the cells, tissues and organ structure and are dependent on the age, sex and the general condition of the organism.

1.1.1. PHYSICAL AND CHEMICAL FACTORS

1. Chemical structure. Compounds with a high chemical activity such as acids, alkalis, aldehydes and unsaturated compounds very easily influence physiological processes. Organic compounds are different in that their toxicity depends on their three dimensional atomic structure in addition to the type of radical and functional groups: – isomers optically active, levorotatory, are more harmful

– toxicity increases with the influence of following groups: ($-NH_2$), (NO_2), (-N=O), (=N-N=O), (-CN), ($-CH=CH_2$), (C_6H_5)
– toxicity decreases with the influence of following groups: (-SH), ($-SO_3H$), (-COOH), (CH_3 –C0-), CH_3O-), (-N=N-), (C_2H_5)
– toxic properties of aromatic compounds increase with the number of substituted groups ($-CH_3$) and (-OH) and isomers in the couple position are the most harmful
– an association of aromatic and aliphatic hydrocarbons with z chlorine gives more solid and more harmful compounds; their toxicity increases with the number of groups substituted with chlorine.

2. Solubility. The solubility of substances in water and lipids is very important. Water is the main substance in most organisms; thus, cell membranes are the barrier limiting the spread of compounds. The real threat is posed only by compounds soluble in water and lipids.

3. Dissociation. Toxicants in non-ionized form could dissolve in the lipids of cell membranes and penetrate through them into cells resulting in a toxic effect.

4. Boiling and evaporation point. Low boiling point, thereby high gas pressure and its saturation ability in the environment are very important physicochemical factors accelerating intoxication.
5. Particles size. Danger of intoxication increases with the decreasing size of aerosol and liquid particles (diameter less than 1 μm).
The mentioned physical and chemical properties of substances influence their susceptibility to – **biotransformation** (metabolic transformations inside living organisms). A limited number of xenobiotics do not transform at all, e.g. highly polar organic compounds (oxalic acid, sulphonic acids, quaternary ammonium bases), fast-removing volatile substances (ethyl ether, cyclo-propane, short –chain alkanes) and highly lipophilic substances (some PCBs).

The biotransformation of xenobiotics takes place with the participation of enzymes with microsomal enzymes being the most important. These enzymes carry out oxidation, reduction and coupling reactions. Metabolites are a result of these reactions which may exhibit different biological activities, i.e. sometimes advanced compared to the initial substance. If the products exhibit a lower toxicity than the initial reactants, such a phenomenon is called detoxication. Procancerogens pose a separate problem and become carncerogenic, as a result of metabolic changes.

Biotransformation also refers to the metabolic activity of microbes in nature. As a result of their enzymatic activity, highly toxic metabolites may be produced. One example of this is the production of highly cancerogenic nitrosamines from nitrites and secondary and tertiary amines by bacteria from the genus *Micrococcus* and *Achromobacter*.

1.1.2 . BIOLOGICAL FACTORS

1. Age. Sensitivity of an organism to intoxication increases when its enzymatic detoxicant system is not fully formulated or active. The period of embryonic development is of great importance because enzymatic system of the foetus is not fully developed; thus, fast dividing cells are highly susceptible to potential damages. In the initial period of postembryonic life (after birth), defence mechanisms of young organism are still imperfect. This condition is usually maintained until maturity is reached when defence and regulatory abilities are at maximum. In aged organisms, the sensitivity to harmful factors increases due to a decrease in the biological activity of tissues and organs.
2. Sex. Differences in the rate of metabolism of alien substances between females and males exist during their entire mature life. The major differences are related to compounds metabolized by microsomal enzymes under the control of sex hormones.
3. Individual factors, diseases. During pregnancy, the reaction of organisms to intoxication could change because many enzymes decrease their activity. Liver and kidney failure directly influence xenobiotic metabolism; thus, heart diseases have an indirect influence. Malnutrition, deficit of vitamins and general bad condition of

the organism lead to a decrease in the activity of many enzymatic systems and an increase in the susceptibility to intoxication.

4. **External environmental factors.** Factors such as atmospheric pressure and temperature also influence blood hemodynamic and cause stress. Daylight also affects the biological rhythm of the organism.

1.2. DEPENDENCE OF DOSE AND EXPOSURE TIME ON TOXICITY

Poison – a substance, after entering into an organism at a small dose, causes some disturbance in the functioning of the living organism or even death due to its toxic and dynamic properties. Up to today, a poison still has the same meaning as described by Paracelsus in 1525: “Everything is a poison and nothing is a poison at the same time. The dose is important only”

Toxicity depends mainly on the **amount and concentration** of toxicant in the living organism. The concentration of a toxicant is very important, particularly for acids and alkalis. These substances
can cause severe damage and intoxication at high concentrations; however, the same amount, but diluted is not toxic. Heavy metals, sulfonamides and others are neutralized in the urinary tract; although if present in small amounts, they could damage the kidneys.

The factor of **time** plays an important role for substances chemically bound to proteins and accumulated inside an organism causing chronic toxicity. Such a situation usually occurs at the final links of trophic chains (for example DDT or PCB). An amount of a substance which is introduced into living a organism in relation to its weight is defined as **dose**.

The following are dose types in relation to their effects:

1. **threshold, minimum dose** (*dosis minima*, DM) – an amount of a substance causing the first observable biological consequences
2. **curative dose** (*dosis curativa*, DC) causes pharma-therapeutical effect and does not disturb physiological processes in the organism
3. **toxic dose** (*dosis toxica*, DT) – causes reversible toxic effects or reversible physiological disturbances in the functioning of the organism.
4. **lethal dose (***dosis letalis*, DL) causes irreversible physiological disturbances in the functioning of the organism and its death

Quantity doses of **xenobiotics** (chemical substances alien for an organism causing a toxic effect(s)) are dependent on their ability to be absorbed into the blood. A certain amount of xenobiotics is chemically bound to the proteins of plasma; the rest is transported by blood circulation and absorbed into tissues and organs causing intoxication.

The most dangerous path of toxicant penetration is through the **respiratory system**. Resorption takes place mainly in pulmonary alveoluses where toxicants directly enter the arterial blood. Toxicants entering the organism through the **alimentary path** are usually resorbed in the small intestine. From there they enter into liver where they undergo complete or partial detoxication. The least significant

path is through the skin. If the skin is undamaged, substances only soluble in lipids are able to penetrate.

The influence of xenobiotics on unicellular or multicellular organisms is varied. Microorganisms are sensitive to all chemical compounds because they can easily penetrate intercellular structures through covering layers. At the same time, however, they exhibit great a great ability to adapt. Microorganisms can adjust their metabolism to environmental changes.

Multicellular organisms, despite having well-developed structures that isolate them from the environment, exhibit a surprisingly high sensitivity to certain toxicants. Some cells and tissues of multicellular organisms posses specific receptors for defined toxicants which enable particle adsorption and diffusion inside. These parts of multicellular organisms are called **sensitive cells or tissues**.

The **transport mechanisms of xenobiotics through biological membranes are** identical for all organisms and include:

1. **Passive transport (passive diffusion)** – the majority of alien substances penetrates into membranes in this way. Passive transport occurs without the use of cell energy in accordance with the concentration gradientor electric potential on both membrane surfaces.
2. **Transport through pores of membranes (convective absorption)** –enables the penetration of ions and hygrophilous substances with a molecular mass up to 200 without the use of energy.
3. **Facilitated transport** – occurs through the creation of protein carrier complexes existing inside membranes with compounds or ions. Facilitated transport takes place without energy use in accordance with the concentration gradients on both sides of the membrane.
4. **Active transport** –occurs against the concentration gradients or potentials. During active transport, energy is required and is made available through ATP disintegration.
5. **Transport by ions pairs** –strongly ionized substances have a tendency to couple with ions from the environment or membranes. The complexes of electrically neutral molecules penetrate through membranes by passive diffusion
6. **Endocytosis and exocytosis** – macromolecules are absorbed and removed from these processes; the cell membrane creates secretory vesicles (endosomes) which transport macromolecules.

Every living organism tries to remove toxicants from its interior using biochemical and physiological mechanisms. Unicellular organisms use active transport, convector absorption and exocytosis. Evolutionary advanced organisms remove xenobiotics through excretions, excrements or exhaled air. In terrestrial mammals, the kidneys play a major role in excretion. Substances easily soluble in water and with low molecular mass are excreted in urine. Macromolecules and compounds that are soluble in lipids are captured in the liver and excreted with bile. Volatile toxicants are released through the lungs by passive diffusion. Thus, in a non-contaminated environment very fast excretion can occur with the exception of substances easily soluble in blood or those that readily couple with

haemoglobin. Less important excretion pathways include through the skin, hair, nails, saliva, sweat and milk.

Biotransformation is another method of eliminating toxicants from an organism. Biotransformation leads to the creation of an intoxicant product or the **accumulation** (gathering) of xenobiotics inside tissues and/or organs where their intoxication status can be changed from acute to chronic.

The basic mechanism of toxicant activity relates to the tendency of joining a reactive cell element, i.e. **a receptor**. Receptors can be the active centre of enzymes, proteins regulating the outer surface of cell membranes as well as nucleic acids. These three structures play a key role in cells activity. For this reason, when toxicants penetrate cells negative biochemical and physiological processes can occur:

– disturbance of cell metabolism
– haemoglobin blockage
– induction of mutation
– induction of cancer

1.3. BIOLOGICAL MONITORING

1.3.1. MONITORING

In order to protect the environment, it is important to have or to obtain current data. Such data enables the forecasting of possible changes in the environment and ecosystem. **Monitoring** is a measurement system that gathers this kind of data **involving observation, assessment and the prognosis of environmental conditions**.

A public monitoring program exists in Poland and was created in 1991 by the Chief Inspectorate for Environmental Protection (Główny Inspektorat Ochrony Środowiska). The program includes the monitoring of air, groundwater, surface water, soil, biocenosis, transformation of the land surface (focused on the mining industry), waste disposal, noise and radiation.

Monitoring is conducted through **a network of measurement points** that takes into consideration local natural conditions (e.g. the presence of protected areas), geographical conditions, distances from pollution sources and population density **among others**.

1.3.2. PHYSICAL AND CHEMICAL MONITORING

Abiotic elements of environment can be analyzed **physically and chemically.** Such analyses are realized in fully automatic, continuously operating measurement stations. The highly developed monitoring of air pollutants is an example of this. One such monitoring station is situated near a large, declining spruce forest in the Izerskie Mountains (Góry Izerskie) between Czerniawa and

Świeradów and is integrated into the European early warning network. This station is equipped with modern measurement equipment that is able to continuously register the concentrations of the air pollutants responsible for the ecological degradation in the Sudetes region (SO_2, NO_2, O_3, dust including PAHs and heavy metals). Pollution data is passed on to the Bilthoven Institute in the Netherlands and to other institutions in Poland.

1.3.3. BIOLOGICAL MONITORING

Data gathered during the monitoring of air, soil and water is very important, but it does not provide information about the influence of pollutants on living organisms. Information concerning the influence of pollutants on living organisms is obtained through the **direct study of the actual condition of living organisms** which involves **biomonitoring or biological/ecological monitoring**. Biomonitoring is conducted based on the assumption that environmental conditions and organisms are bound together in a causal-consecutive way. Living systems (organisms, populations and biocenosis) represent the conditions of where they are located. The condition of organisms does not only reflect the present active factors (e.g. present pollution), but also the factors which were active in the past. Additionally, organisms react simultaneously to all active factors.

Biomonitoring enables the **complex assessment** of ecosystem conditions while physical and chemical monitoring controls only selected indicators of pollution. Moreover, physical and chemical monitoring is only able to register the condition of the studied environmental element at the time when the sample is collected. Since biomonitoring is not able to deliver very precise information about the types and concentrations of pollutants, **both systems are used complementary to each other**.

Three basic scientific techniques are used in biomonitoring: biotests (also called toxicity tests), bioindication and studies of the structure and function of ecosystems.

1.3.4. BIOTESTS

Biotests are conducted on environmental samples such as water and soil. Test organisms are exposed to these samples and the toxicity on these organisms is evaluated. This kind of study allows the assessment of the level of environmental contamination based on the toxic effect. One particular type of biotests includes genotoxic tests as described in the last laboratory instruction. Biotests do not directly indicate the level of risk for living organisms inhabiting a studied ecosystem because they provide only the reaction of selected test organisms under artificial laboratory conditions. However, they do provide an indirect evaluation of the potential influence of pollutants on biocenosis. The reliability of this evaluation depends on the representativeness of certain organisms for the studied site

(laboratory 4). In order to replicate natural conditions as much as possible and be able to observe a chronic toxicity, the duration of the experiment should be extended. Studies should include at least 3 generations of plant and animal representatives.

In our experiment (laboratory 1, exercise 1), a water ecosystem model is influenced by contaminated water. Based on the number and biomass changes of test organisms, we can determine if the pollutant has an influence on the natural ecosystem. Species used in this experiment are *Lemna minor* and *Daphnia magna*. Both of these species are very sensitive to toxicants, are very fertile, have a short life-cycle and are good representatives of water ecosystems.

1.3.5. BIOINDICATION

Bioindication (from Latin *indicare* – indicate) is a very direct way of obtaining information about the condition of the environment. This method is based on studying changes in the physiology and behaviour of living organisms caused by environmental contamination. In this respect, organisms are indicators of contamination and are called bioindicators. Bioindicators can be divided into **2 groups**:

a. organisms that accumulate contaminants in their tissues and organs (bioaccumulation)

b. organisms showing sensitivity to contaminants demonstrated by physiological or biochemical disorder(s)

Species able to accumulate toxicants are particularly useful in biomonitoring because they allow tracing the fate of toxicants that enter into the food chain of biocenosis.

1.3.6. MONITORING OF ECOSYSTEMS

Bioindication and biotests are mainly based on the observation of individuals; thus, it is not possible to assess the condition of the entire ecosystem. In nature, an individual never exists in isolation, an individual organisms are always a part of biocenosis. Even conducting experiments on species assemblages and creating an ecosystem model, it is not possible to reconstruct the sophisticated network of connections exist in the natural ecosystem. Due to this fact, field studies are most reliable because they provide information about the structure and function of the ecosystem. Such monitoring includes the **measurement of basic processes taking place in the ecosystem:**

a. production of organic matter

b. decay and accumulation of organic matter

c. decomposition

Processes are measured with special indicators, e.g. during the monitoring of forest ecosystems the indicator of plant production is the rate of organic fallout

(mainly leaves) and decomposition – a loss of forest bed weight, which is placed in nylon bags and left for one year. In addition to gathering data about the controlling factors and functioning of ecosystems, information is gathered about the structure of biocenosis. Changes in species structure are mainly studied with one method, the **index of species richness:** d=S/N, being based on the analysis of biodiversity. Where, S – number of individuals of a particular species, N – total number of organisms of all species found.

A biocenosis equilibrant consists of a few common species (which are numerous), but of many rare species (which are not abundant). If the biocenosis is influenced by strong contamination, the species structure will change characteristically: the number of rare species decreases whilethe number of common species increases (which were not numerous before) as they are the most adapted to new environment. As a rule, the number of species decreases, but the number of specimens increases resulting in a lower value for the index of species richness. Fig. 1 shows the dependence of the total number of species on the number of specimens.

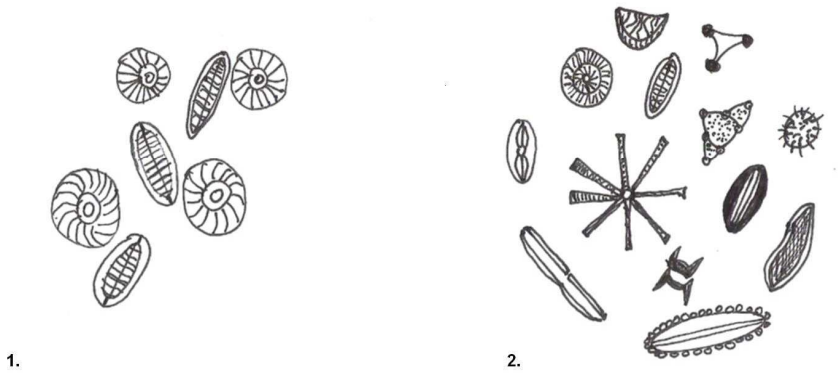


Fig. 1. Species structure of diatom assemblages in water after its contamination (1) and before (2). In the contaminated environment only very tolerant species can survive (low number of species), but they are numerous.

A special technique called **teledetection** is used for assessing the condition of ecosystems, especially forest ecosystems. This method is based on satellite measurements of electromagnetic radiation reflections. Both damaged/dead and healthy conifer needles reflect light in the visible and infrared range, but they have different chlorophyll and water content inside the tissues. The chlorophyll and water content inside tissues are directly connected to the level of damage and can serve as a basis for creating detailed maps of the actual condition of the forest ecosystem.

While ecosystem monitoring provides important information about the real condition of an ecosystem, it also has certain limitations. Many indicators describing the condition of an ecosystem have only comparative, relative value. Tools do not yet exist that allow the assessment of borders of tolerance to harmful factors of ecosystems. Thus, in order to determine if productivity or the species structure is affected by pollution, it is necessary to conduct reference studies in unpolluted areas. Furthermore, ecosystem monitoring does not provide information very quickly. For this reason, biomonitoring is often restricted to biotests and bioindication procedures.

2. METHODS OF ASSESSING WATER TOXICITY

2.1. ASSESSING THE TOXIC EFFECTS OF POLLUTANTS ON AQUATIC ORGANISMS USING LETHAL PHYSIOLOGICAL TESTS

Biomonitoring is conducted through biological studies in which the toxic effects of pollutants on ecosystems are evaluated. Biomonitoring includes toxicological tests conducted under laboratory conditions, model ecosystem studies and field experiments. Biomonitoring covers **bioindication**: the application of selected indicator species in assessing environmental contamination and documenting natural conditions.

Bioindication includes toxicological tests conducted in the laboratory, model studies of micro- and macro – assemblages and field studies. Toxicological studies are the first step in gaining knowledge about the toxicity of different chemicals and their mixtures. From toxicological tests it is possible to assess the potential threat to certain populations, their mortality, growth ratio, reproducibility and functional disturbance disorders.

Toxicological monitoring should be conducted using indicator species (bioindicators) representing all trophic levels which are characteristic of the studied ecosystem. The selection of test methods is of great importance in toxicity studies. A great number of indicator species is used from all over the world. Organisms differ from each other in terms of their sensitivity to certain groups of toxicants; thus, it is recommended to use a battery of tests including different groups of bioindicators.

2.1.1. TEST ORGANISMS

Organisms used in tests should be of the same age and have the same physiological activity; in general, juveniles are commonly used. Some important traits of test organisms are as follows:

Sensitivity to toxicants,

Breeding simplicity.

Representativeness for the studied environment (i.e. aquatic bacteria for the aquatic environment, soil bacteria for the soil environment).
Because of various sensitivities, toxicological studies should be conducted using **representatives of all trophic levels**: decomposers, producers and consumers.
T**he following are commonly used**:
Among bacteria: *Pseudomonas, Bacillus, Sphaerotilus natans*
Algae: *Chlorella* sp., *Scenedesmus* sp., *Ancistrodesmus* sp., *Rhizodorium* sp.
Plants: *Elodea canadensis*, *Lemna minor* (Fig. 2)
Protozoans: *Paramaecium* sp., *Tetrahymena* sp.
Oligochaeta: *Tubifex* sp., *Lumbricus* sp.
Crustaceans: *Daphnia* sp. (Fig. 3), *Cyclops* sp., *Asellus* sp. (Fig. 4), *Gammarus* sp.
Insects: mayflies, dipterans, caddisflies
Mollusks: *Physa* sp., *Planobarius* sp.
Fish: approximately 150 species

Fig. 2. *Lemna minor* – one of the species commonly used in toxicity testing.

Fig. 3. *Dapnia magna* – one of the species commonly used in toxicity testing.

Fig. 4. *Asellus aquaticus* – one of the Crustaceans commonly used in toxicity testing.

Performing laboratory biotests requires taking into account many factors and choosing those of greatest importance. Ambiguous results are always an effect of differences between natural and laboratory conditions.

2.1.2. BIOTEST TYPES

Biotoxicological studies are conducted under constant, standardized conditions (pH, temperature, humidity etc.). In biotoxicological studies, a change of toxicity is studied as it depends on the concentration and time of toxicant activity.

There are various methods of assessing the toxicity of contaminants in aquatic and soil organisms. These methods can be divided into two groups according to the experiment duration:

short-term, assessing acute toxicity ,

long-term, assessing chronic toxicity,

Toxicity tests can also be divided according to the influence of the toxicant:

lethal tests based on the observation of organism death (LC_{50} concentration is the dose of a toxic substance or radiation that is required to kill half the members of a tested population after a specified test duration),

physiological tests based on observations of metabolic changes in photosynthesis, respiration, enzymatic activity (EC_{50} –**half the maximal effective concentration** referring to the concentration of a drug, antibody or toxicant which induces a response halfway between the baseline and maximum after some specified exposure time. The EC_{50} represents the concentration of a compound where 50% of its maximal effect is observed.

Biotests do not directly show the level of exposure to toxicants for organisms inhabiting certain ecosystem because they consist of selected test organisms observed under artificial laboratory conditions. They do, however, provide an indirect potential assessment (NOEC – *No Observable Concentration Dose* –no observed effect concentration being the highest concentration of a substance that does not produce a significant ecotoxic effect in an organism or in an organism population; NOEL – *No Observable Adverse Effect Level* – no observable effects limit which is the highest dose at which no effects can be observed. It is used as a measure of chronic toxicity).

The reliability of biotests depends on the level of species representativeness for the particular environment. In order to make laboratory conditions more natural and to determine, if possible, chronic toxicity, the duration of the experiment should be extended so that it lasts at least three subsequent generations of test plants or animals.

2.1.3. TOXICOLOGICAL MONITORING OF WATER

Toxicological monitoring of water has been recently developed. Test methods are based on assessing the influence chemicals on the survival, growth, reproduction and enzymatic activity of water organisms. Aquatic organisms, being a part of water biocenosis, are used as bioindicators in toxicity testing in which the accumulation of chemicals in their tissues and bodies are tested (Table 1). Testing should be conducted for all biocenosis levels: producers, consumers and decomposers.

In Poland, certain standards are applied in toxicological monitoring. For example, commonly used organisms include the following: algae – *Chlorella sp*, crustaceans – *Daphnia magna*, *Gammarus varsoviensis* and fish – *Lebistes reticulatus*. *D. magna* specimens are used in most cases because they are multicellular, fresh water, aquatic invertebrates that have a well defined digestive system. Similar organisms are commonly used in other countries and are recommended by international organizations (ISO standards).

Evaluating the level of water toxicity with the application of biotests is based on selected organisms which are placed in the studied water or water solution containing the studied toxicant. The death and/or functional disturbances or disorders in the selected organisms is observed.

Table 1. Bioindicators commonly used in assessing water toxicity.

Trophic level	Test organism
Decomposer	*Pseudomonas fluorescens*
	Vibrio fischeri
	Candida boidini
Consumers	*Paramecium caudatum* *Brachionus calyciflorus*
	Artemia salina *Thamnocephalus platyurus* *Daphnia magna* *Ceriodaphnia dubia* *Gammarus varsoviensis*
	Dugesia tigrina *Lebistes reticulates* Chironomids
Producers	*Chlorella vulgaris* *Scenedesmus quadricauda* *Selenastrum capricornutum* *Lemna minor*

Today, ready-to-use tests are sold as packages. They can assess the potential toxicity in a very short period of time. Such packages include eggs or larvae, static

forms of invertebrates which can be stored for a long time. The main advantage of these tests is their simplicity of use, high sensitivity of test organisms and reliability of obtained results. Studies with Toxkit include zooplankton representatives *Brachionus calyciflorus* (Rotoxkit F), *Brachionus plicatilis* (Rotoxk it M.), larvae of *Artemia* (Artoxkit M.), crustaceans *Daphnia magna* (Daphtoxkit F), *Ceriodaphnia dubia* (Ceriodaphtoxkit F), *Thamnocephalus platyurus* (Thamnotoxkit F), *Heterocypris incongruens* (Ostracodtoxkit F) and protozoans *Tetrahymena thermophila* (Protoxkit F) and producer representatives as algae *Selenastrum capricornutum* (Algaltoxkit F).

The **IQ Tox-Test** is a new method also used to conduct ecotoxicological research. The IQ-Tox Test technology can provide early detection of contaminants that enter the water environment. This technology has detected chemical and biological agents below the HLDC (human lethal dose concentration). Many animals depend on sugar as a basic source of energy. If their ability to digest sugar is incapacitated due to the effects of chemical or biological contamination, they will die. *Daphnia magna* is used as a test organism for exposure to this kind of toxicants that reduce the ability to ingest and enzymatically cleave a fluorometrically tagged sugar substrate. The enzymatic response technology allows the user to characterize a sample for toxicity by measuring contaminants as related to the suppressions of *D. magna* enzyme activity. The bioassay is detected through fluorescent stimulation (Fig. 5) of a metabolized marker on galactose ingested by *Daphnia magna* that have been exposed to varying concentrations of a chemical that may be a possible environmental contaminant. After exposure to the toxic chemical, the *Daphnia* enzyme system will be rendered nonfunctional. Ordinarily, death would follow in a few hours or several days. This method is rapid, sensitive and simple to perform. The IQ-Tox Test is extremely effective due to the complexity of the test organism. The tests are scored by eyesight, i.e. simply by viewing the tests under long wave ultraviolet light and counting the number of glowing or non-glowing *D. magna* in each exposure chamber cell. The greater the number of organisms adversely affected translates to a greater toxicity. IQ-Tox Test is normally used to study contaminants present in water.

The test system Microtox is also worth mentioning. **Microtox®** is a standardized toxicity test system that is rapid, sensitive, reproducible, ecologically relevant and cost effective. It is recognized and used throughout the world as a standard test for aquatic toxicity testing.

The procedure uses the bioluminescent marine bacterium (*Vibrio fischeri*) as the test organism. The bacteria are exposed to a range of concentrations of the substance being tested. The reduction in intensity of light emitted from the bacteria is measured along with standard solutions and control samples. The change in light output and concentration of the toxicant produce a dose / response relationship. The results are normalized and the EC_{50} (concentration producing a 50% reduction in light) is calculated.

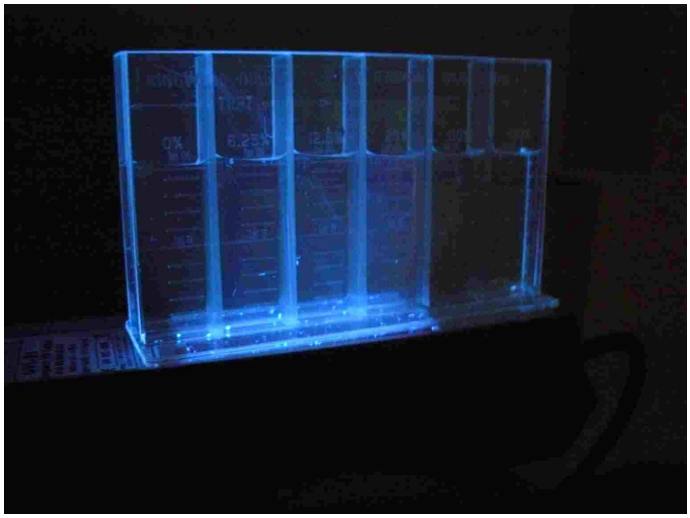


Fig. 5. Glowing *Dahnia magna* used in the IQ-Tox Test

2.1.4. WATER BIOINDICATION WITH THE USE OF BIOTIC INDICES

The search for improved methods of monitoring water quality has led to the development of techniques for rapid bioassessment of rivers and the evaluation of water quality using benthic macroinvertebrates. The purpose of biological assessment is to characterize the status of the water condition associated with anthropogenic perturbation. The Polish BMWP-PL system (Fig. 6, 7, 8, 9, 10, 11), a modified version of the method used by the British Monitoring Working Party (BMWP) is recommended, but rarely used for measuring water quality. All sampling and data processing are done according to the stipulations of BMWP-PL (http://www.eu-star.at/pdf/PolishMacroinvertebrateSamplingProtocol.pdf from the date 24.03.2009). Representative sampling sites are chosen within the studied water body. At each site, macroinvertebrate samples are obtained using the kick sampling technique. A handnet (160 µm mesh size) is used in sampling usually 0.25 m^2 of the substratum at sampling points. Collected macroinvertebrates are preserved with 96% ethanol. All specimens are identified to family level or higher taxon in the case of non-insects according to BMWP-PL. The percentage of macroinvertebrate taxa are calculated from each site for the total sampling period. The Average Score per Taxon (ASPT) is usually determined by dividing the macroinvertebrate score (obtained from BMWP-PL calculations) by the number of taxa at each site. At each site, the minimum physical state of the water should also be examined using the multi–probe method for pH, temperature and conductivity.

Fig. 6. One of the methods applied in the biomonitoring of water (method of biotic indices).

Fig. 7. A hand net used in the biomonitoring of water.

Fig. 8. A mayfly larva from the Baetidae family – a taxon indicating moderate water conditions.

Fig. 9. *Erpobdella octoculata*, – a leech species indicating poor water conditions.

Fig. 10. A caddisfly larva from the Sericostomatidae family -a taxon indicating good water conditions.

Fig. 11. A dipterans larva from the Chironomidae family – a taxon indicating very poor water conditions used in both bioindication with biotic indices and toxicity tests.

2.1.5. TYPES OF TOXICITY

The death of organisms used for lethal tests and functional disturbances disorders for physiological tests are criteria for the level of toxicity of chemical compounds. Special terms are used when defining the lethal or sublethal concentrations of toxicants. **Acute toxicity** describes the adverse effects of a substance which result either from a single exposure or from multiple exposures in a short space of time (usually less than 24 hours). To be described as *acute* toxicity, the adverse effects should occur within 14 days of the administration of the substance. **Sublethal toxicity**, occurring under the influence of a **sublethal concentration**, causes functional disturbance disorders and the time of exposure must be at least 1/10 of the life span of one generation for the studied organism. **Chronic toxicity** occurs when an organism has continuous or repeated exposure to a toxicant that causes toxic effects. Chronic toxicity is often correlated with the accumulation of toxicants in the studied organism.

2.1.6. CHEMICAL CLASSIFICATION OF TOXICANTS

The following are **toxicants**:
1. oxidants – Cl, O_3, $KMnO_4$, H_2O_2
2. gases – NH_4, H_2S SO_2, CO_2
3. salts of heavy metals – Pb, Cr, Cd, Cu, Ni, Hg, Zn, As
4. cyanides

5. aliphatic compounds – chloroform, ether
6. aromatic compounds – phenol, hydrocarbons
7. alkaloids
8. tannins
9. dyes
10. detergents
11. pesticides

2.1.7. TOXIC EFFECTS OF POISON ACTIVITY

Toxic effects of poison activity depend on
a. chemical structure and properties of the poison,
b. conditions of testing: compound concentration, exposure time, pH, temperature, humidity,
c. presence of organic matter in the environment and possibility of complex creation which can act antagonistically or synergistically.

Biotoxic studies are conducted under constant, standardized conditions (pH, temperature, humidity and without the influence of organic matter). Changes in toxicity are studied depending on exposure time and concentration.

The **toxic effect of a poison** depends on the sensitivity of the test organism which is a function of sex, age, weight and the condition of the organism.

2.1.8 BIOTEST RESULTS

In lethal tests, the final result is the concentration causing 50% population mortality after a certain exposure time, t = 24, 48, 72 hours. – **LC_{50}** or **TL_{50}**.
LC_{50} (*Lethal Concentration*) is the concentration causing 50% mortality while TL_{50} is called the mean tolerance limit, i.e. the toxicant concentration at which 50% of organisms can survive. The maximum tolerated concentration **MATC** can be calculated in evaluating the influence of toxicants on organism physiology. In chronic tests, the chronic value **ChV** is the geometric mean of two concentrations: the highest concentration that does not cause any changes in organism function (NOEC) and the lowest concentration causing damage (LOEC). The LC_{50} and MATC concentrations allow the safe concentrations of compounds to be determined.

The **safe concentration** can also be determined based on acute toxicity tests, assuming **coefficients of safety** and multiplying them obtained in our studies by LC_{50}. The value of the coefficient of safety ranges from 0.05 to 0.3 (outside of Poland). In Poland, the coefficient of safety is 0.1 for degradable compounds and 0.01 for accumulative compounds (in the environment).

There is not a real classification system of chemicals in terms of their acute toxicity. Thus, chemicals are classified based on their threshold values. A threshold value is the concentration that does not cause a lethal effect when testing acute

toxicity. Table 2 and 3 shows chemical toxicity based on concentration and LC_{50} values. When assessing water toxicity we commonly use invertebrates and plants (Fig. 12 and 13).

Table 2. Chemical toxicity based on concentration.

Threshold concentration (mg/dm^3)	Toxicity
<1	Very highly toxic
1–9	highly toxic
10–99	Medium toxic
100–500	Little toxic
>500	Very little toxic

Table 3. Chemical toxicity based LC_{50} values.

LC_{50} (mg/dm^3)	Toxicity
<1	Enormously toxic
0.1–1	Very highly toxic
1–10	Highly toxic
10–100	Medium toxic
100–1000	Little toxic
1000–10000	Very little toxic
>10000	Non-toxic

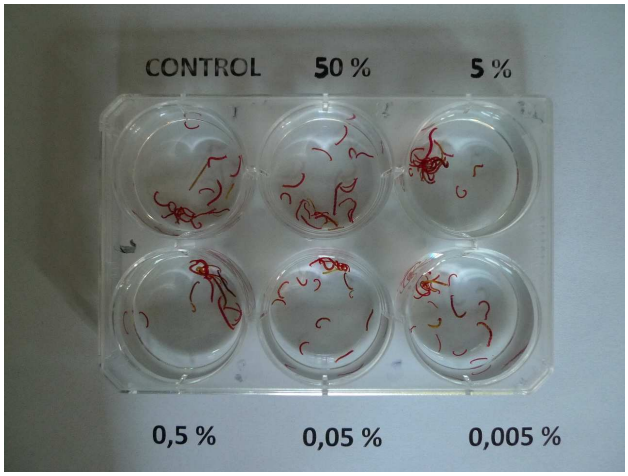


Fig. 12. Toxicity test with Chironomid larvae.

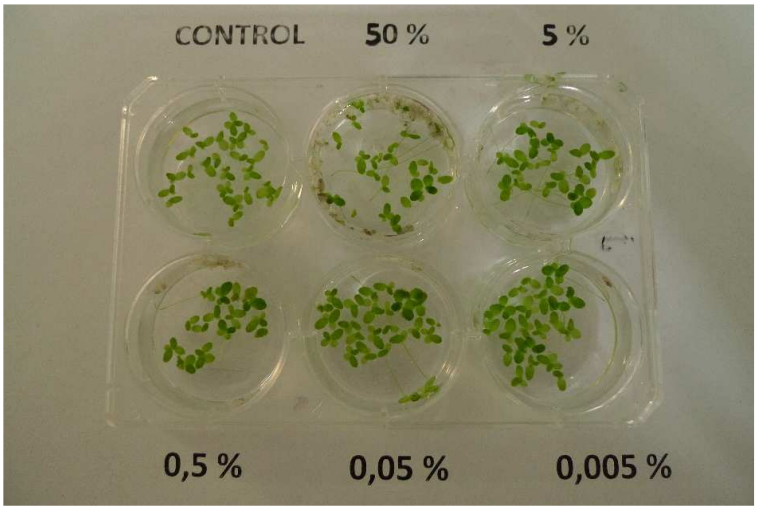


Fig. 13. Toxicity test with *Lemna minor*.

3. METHODS OF ANALYZING SOIL TOXICITY

3.1. ECOTOXICOLOGICAL ASSESSMENT OF CONTAMINATED SOIL

Soil is an active component of each land ecosystem. Processes such as the decomposition of organic matter, part of primary production, take place in the soil. Processes above the ground level take water and minerals directly from the soil. Moreover, soil functions as a filter, buffer and protects the ecosystem against unwanted elements. These soil properties remain unchanged as long as the biogeochemical equilibrium, responsible for the biological activity of soil, is not disturbed.

3.1.1. SOIL DEFINITION

Soil is the surface layer of the earth's crust originating from maternal rock under the influence of **pedogenic factors** such as climate, water and the activity of living organisms. It is comprised of mineral parts (fragments of maternal rock) and organics (living organisms – edaphon and their products). The space between particles is filled with air and/or water.

The pH is a very important factor influencing soil processes such as weathering of rocks; mineralization and humification of organic remains as well as the growth and development of edaphon and plants.

Soil pH can be

- strongly acidic (pH 4.5),

- acidic (pH 4.6 – 5.5),
- slightly acidic (pH 5.6 – 6.5),
- neutral (pH 6.6 – 7.2),
- alkaline (pH >7.2).

Soil is characterized by horizontal **soil levels**. Soil level structure from the surface downward is known as the **soil profile**. Soil has the following levels: bedding, humus level (accumulation), eluvial level (washing out), illuvial level (washing out, consisting from mineral particles) and substrate level (maternal rock). Substances derived from higher levels are washed out by water and are often detained at the illuvial level.

3.1.2. SOURCES OF SOIL CONTAMINATION

The development of civilization has been and still remains a cause of soil degradation which diminishes the activity and productivity of soil. Aggressive factors causing soil degradation include dust and gaseous air contaminants, sludge, industrial and communal waste and chemicals used in agriculture. These pollutants mainly stem from industry, mining, agriculture, transport and households. Some toxic substances are derived directly from the soil as a result of natural processes: chemical transformations and from the metabolism of living organisms.

Motor transport is a source of soil contamination by **petroleum derivates**: lubricants, hydrocarbons (often carcinogenic) and also **lead**. Nitrogen oxides emitted by cars are precursors of carcinogenic nitrosamines arising in soil. Detergents and factories play a major role in causing soil contamination. Industrial activities also emit dust containing a large amount of heavy metals that accumulate in the soil over many years. Acid rain plays a crucial role in soil contamination and stems from the emission of sulphur and nitrogen oxides (SO_2, SO_3, N_x, O_y) into the atmosphere from fossil fuel combustion. Sulphoxides are created from the oxidation of sulphur contained in fuel while nitrogen oxides are created mainly from the oxidation of atmospheric nitrogen during combustion, although they can also from nitrogen present in fuel. SO_2 and reduced nitrogen oxides undergo further oxidation in the atmosphere in both the gaseous and liquid phases. In the atmosphere a mixture of sulphuric and nitric acid is created after reacting with water and finally the mixture falls in precipitation as acid rain (pH < 4.5).

pH reduction of soil can also be a result of natural processes:

a. hydrolysis of salts, e.g. $AlCl_3$

$$AlCl_3 + 3\ H_2O = Al(OH)_3 + 3\ H^+ + 3\ Cl^-$$

b. inorganic and organic acids are natural products of living plants, e.g. root excretions

c. nitrification

d. decomposition of organic matter

e. oxidation of elementary sulphur and sulphides by bacteria (e.g. *Thiobacillus thiooxidans*)

3.1.3. SENSITIVITY OF SOIL TO CONTAMINATION

Various types of soil differ in their sensitivity to contamination which depends on soil sorption properties, thickness and chemical composition layers, environmental conditions (humidity, temperature and pH) and the biocenosis composition.

The process of contamination begins from the topmost layer and progresses towards the bottom of the soil profile in the direction of water infiltration. Thus, greater **soil permeability** leads to deeper contamination in the soil. Humus, particularly chelat complexes of colloid humic acids, forms the **sorption complex of soil.** The presence of aluminium and silicon hydroxides also influences soil sorption and hydration properties. The sorption complex gives the appropriate trophic properties of soil and provides for soil hydration. The sorption complex also offers natural protection against contamination by retaining metal ions and macromolecular toxicants such as root excretions, pesticides, detergents and dust. Heavy metals are bound to humus reducing their toxicity, although humus associated with organic compounds can result in a higher resistance to decomposition by soil enzymes.

The level of acidification depends on the presence of metal carbonates mono and dihydroxides. The presence of calcium carbonate makes the soil resistant to acidification, e.g. as in the reaction with sulphuric acid:

$$CaCO_3 + H_2SO_4 = CaSO_4 + H_2CO_3$$

From this reaction the products are nearly insoluble calcium sulphate and unstable carbonic acid which disintegrates into CO_2 and H_2O. Thus, the H^+ ion is bound to water particles.

Another factor influencing a soil's resistance to acidification is the **sorption capacity.** The sorption capacity depends on the presence of mineral colloids and humus. The sorption capacity of soil alone is not sufficient for preventing environmental degradation from acidification because part of the sorption capacity can already be filled with H^+ ions.

The level of acidification is also related to the **type of vegetation** (it is commonly known that spruce forests increase the level of soil acidification) and climate (temperature, humidity).

3.1.4. SOIL CONTAMINATED BY HEAVY METALS

The mechanisms influencing the toxicity of heavy metals in soil are well known (Fig. 14). The toxic effects of heavy metals on microorganisms are governed by **the concentration of soluble forms**, not by general their content. The level of free, washed out ions is dependent on the sorption properties of soil.

Metals bound to soil colloids do not easily enter microorganisms making them less toxic.

Anions accompanying cations can be factors increasing the toxicity of metals. It has been found that lead uptake is associated with the presence of the following anions: acetate > NO_3^- > Br^-.

Toxic properties of some metals are dependent on the solubility of the particular salt (the higher solubility, the more toxic the metal). However, even metals which are present in soluble compounds can precipitate in the soil as carbonates.

The pH of the environment determines the **oxidation level of cations**, which is directly related to their toxicity. For example, lead in the acidic environment is mainly present as Pb^{+2} and is strongly toxic, whereas the hydroxide $Pb(OH)_4^{-2}$ appears when soil acidification decreases making the lead lose its toxic effect. Chromium ions having the oxidation state +6, $(CrO_4)^{-2}$ and $(Cr_2O_7)^{-2}$, are more toxic in the neutral environment than Cr^{+3} ions; however, in acidic soil Cr^{+3} ions are more toxic and are commonly derived from soluble chloride and sulfate.

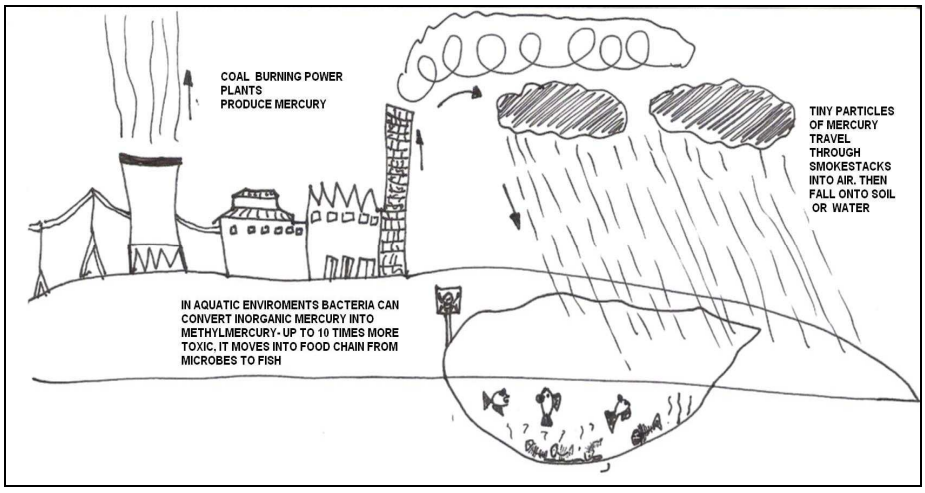


Fig. 14. How one of heavy metal (mercury) contamination spreads.

3.1.5. SOIL ACIDIFICATION

Very acidic soil is inhabited by **fauna of low activity**. Earthworms play a crucial role in soil genesis and are particularly sensitive to acidic conditions. **Plants have various tolerance levels for** acidic soil. *Fabaceae*, which cooperate with the bacterium *Rhizobium* sp. in assimilating nitrogen from atmosphere, are extremely sensitive to soil acidification.

The acidification of the environment is highly damaging to many soil microbe groups. Soil contaminated by sulfate having a pH value lower than 3 does not contain heterotrophic bacteria and Actinomycetales. Notably, fungi are more tolerant to low pH values. Genera *Trichoderma*, *Fusarium*, *Penicillium*, *Spicaria*, *Humicola* and the species *Mycelia sterilla* are present in soil having a pH less than 2.5.

The genus *Bacillus* is quite tolerant of acidification since it produces endospores, although the genus *Arthrobacter* is more sensitive.

In the environment, the activity of bacteria assimilating free nitrogen decreases with increasing acidity. The active assimilation of free nitrogen is determined by the pH value which should be above 5, but it should be noted that bacteria assimilating nitrogen (e.g. genus *Azotobacter*) in soil with a pH less than 5 have been observed. For the majority of the genus *Rhizobium* bacteria (symbiotic) the pH values enabling the assimilation of nitrogen vary between 6.5 – 7.5. Nitrifying bacteria are very sensitive to acidic soil while ammonifying bacteria are less sensitive.

As the soil increases in acidity, cellulose degrading bacteria are increasingly replaced by fungi.

3.1.6. THE EFFECTS OF SOIL CONTAMINATION

The introduction of chemical contamination into the soil negatively influences those organisms inhabiting soil (Fig. 15). In this respect, the chemical contamination causes sensitive species to die off while promoting the excessive development of resistant organisms. This situation leads to the disturbance of matter and energy circulation in soil and thus disrupts normal soil processes.

Soil microorganisms may be able to utilize soil contamination as a building or energetic substrate. If this is the case, they have the ability to partially or completely degrade many toxic compounds. When exposed to pollutants, sensitive strains are eliminated leaving only those strains which are not as affected. One unique aspect of microbes is that they are able to adapt to increasing concentrations of toxic substances with their adaptability varying from species to species. Because of this phenomenon, an overall drop in the number of microorganisms is observed when contamination is first introduced into the system. After some time growth reoccurs, but the population composition changes. The soil organism assemblages are simplified in terms of their diversity making them less resistant to

destabilization. The reduction or elimination of certain groups of microbes because of contamination is caused by the circulation of elements.
Some methods of evaluating the influence of a pollutant on soil microorganisms are based on estimating the ability of microorganisms to mineralize organic compounds of nitrogen and carbon into inorganic forms. The inhibition of organic mineralization causes soil to lose its fertility. The inhibition of organic mineralization also causes a reduction in the amount of mineral salts in the soil, which are essential for plant development. As a result, the remaining elements in the food chain are influenced by the negative consequences of microorganism intoxication.
Both aquatic and terrestrial **plants** are sensitive to chemical contamination. Contamination halts growth, photosynthesis and respiration. These changes are not only the result of direct intoxication, but also reflect physical and chemical alterations in the environment inhabited by soil organisms. The change in physical soil properties leads to the destruction of the soil structure causing particle dispersion. This translates into a decrease in soil filtration resulting in the retention of water in depressions. Disturbances in the flow of water through the soil take place in the root zone of plants which can lead to nutrient deficiency. More serious nutrient deficiency symptoms in plants mean that water consumption is less effective.
Microorganisms utilizing certain organic soil contaminants, for example hydrocarbons, causes anaerobic conditions to prevail in the root zone of plants. Oxygen deficiency and the associated formation of hydrogen sulfide destroy the majority of plant roots including tree roots with very developed root systems.

Changes in the physical, chemical and biological properties of soil limit plant development in contaminated areas. Alterations in the anatomy of roots, shoots and leaves can be attributed to pollution. For example, deformation and plasmolysis of roots commonly occur in contaminated areas and the xylem in transport tissue can become plugged inhibiting the development of new root hair. Inhibition in the growth of roots is a major problem for plants disrupting the transport of water and minerals. Disruptions in water transport are characterized by the accumulation of petroleum compounds around cell walls, primarily around the parenchyma. Leaves and shoots exhibit symptoms of chlorosis in which only leaves of small sizes having a very narrow assimilation surface appear. Shoots of such plants are short, tiny and poorly developed. Dehydration of tissues including the local destruction of dermal tissue and the last layer of the parenchyma are typical disorders in occurring in plants affected by contamination. All of these negative consequences caused by pollution limit cell division and thus the growth of the entire plant causing die offs. The effects of contamination lead to a worsening in the chemical, physical and biological properties of soil resulting in a decrease in the quality and quantity of plant biomass. The complete loss of soil quality parameters is defined as devastation.

Soil contamination can have a direct or indirect impact on animals and humans. Soil contamination can enter the body through various pathways including through the skin, by inhalation and with food (Fig. 14). The air that

people and animals breathe can contain gasses, vapors and dust particles derived from contaminated soil and transported by the wind. Some contaminants are incorporated inside the human body though direct contact with human skin or by the settlement of soil particles on the skin surface. This kind of contamination finds its way together with food into the alimentary tract of animals and humans. Soil is often directly consumed by small children (dirty hands or the consumption of contaminated food).

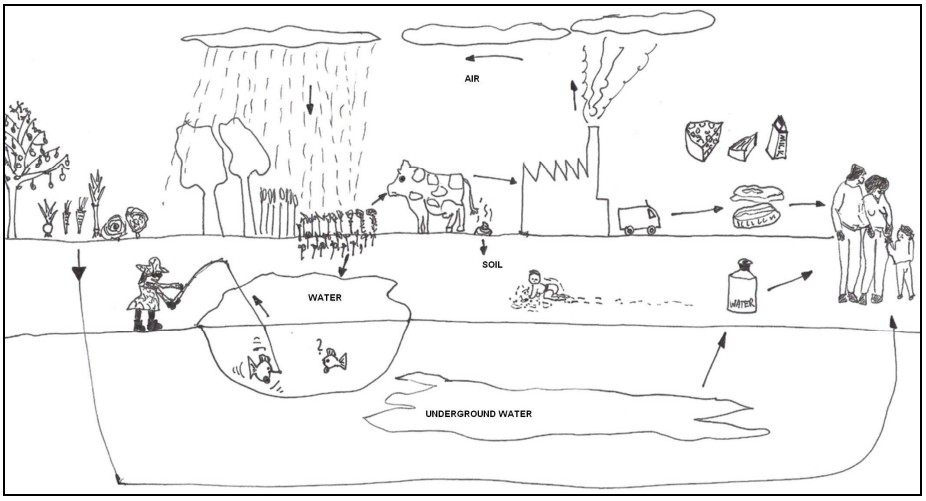


Fig. 15. Pathways of contaminated soil penetration into the human body.

3.1.7. BIOINDICATION OF SOIL

Bioindication is commonly used method in evaluating the level of contamination in soil.

Biological tests should be conducted for soil when

- there is a risk for harmful effects from contaminated soil on living organisms,
- there is a risk of contaminants leaking from the soil into groundwater,
- if the soil has low level contamination,
- if the soil is intended for agricultural or garden use, when it is necessary to evaluate biologically the effectiveness of soil purification on previously contaminated soil.

The selection of bioindication tests depends on the intended use and function of soil (tab. 4). The analysis should be done for contaminated soil or water soil extract.

Direct soil analysis enables the assessment of soil quality as a habitat for microorganisms. The analysis of water extract allows the measurement of contaminants that wash out from soil. In the environment, these contaminants can infiltrate into the soil and reach the groundwater as well as enter water bodies through surface runoff. The tendency of soil contaminants to enter surface water or groundwater is a function of their retention in the soil. Once in water, the contaminants can affect the biocenosis of the aquatic environment.

In conducting a direct toxicity assessment at the producer level, the best representatives are plants whose growth, reproduction and yield are strongly influenced by the presence of xenobiotics

(alien compounds) (*Avena sativa*, *Brassica rapa*, *Lepidium sativum I Phaseolus aureus*, *Secale cerale*, (fig. 16, 17 and 18). *Lepidium sativum* is commonly used as a standard assessment of toxicity for different chemicals in soil. This test is based on the direct observation of the influence of contaminants on its germinated seeds. Cytotoxic substances cause the inhibition of merystemic cell division leading to the inhibition of plant organ function.

Fig. 16. Toxicity test with *Secale cerale* (shoot and root length–inhibition growth test).

Fig. 17. Toxicity test with *Lepidium sativum* (germination test).

Fig. 18. *Lepidum sativum* – a species commonly used in soil toxicity testing.

The reaction of consumers to contamination is studied using the following species in standardized tests: Collembola-springtails (Fig. 19) – *Folsomia candida*, Crustacea*;* Isopoda – Oniscidea, *Oniscus asellus;* and bees. The very characteristic and commonly tested animal is the earthworm, *Eisenia fetida* (Fig. 20, 21 and 22) Its mortality is the basis for assessing the acute toxicity of many compounds. Such tests mainly measure the results of toxicant activity which penetrates the skin. The springtail test measures the influence on reproduction mainly through the intoxication of adult specimens, their eggs and juveniles. The test on Oniscidea measures the influence of chemicals on the metabolism mainly by intoxication through food. Isopods are very important due to the fact that they are very common, widespread and play a crucial role in the physical decomposition of leaves making them more easily degradable by microorganisms. Isopods are also very resistant to starvation.
The test on bees is intended to assess toxic results on useful species.
In addition, *Enchytraeus albidu* and *Panagrellus redivivus* are also used as bioindicators. Table 4. shows some important criteria for biotests in tems of the planned soil use.

Fig. 19. Springtails, a group often used in soil monitoring.

Fig. 20. An earthworm – a very sensitive group used in soil monitoring.

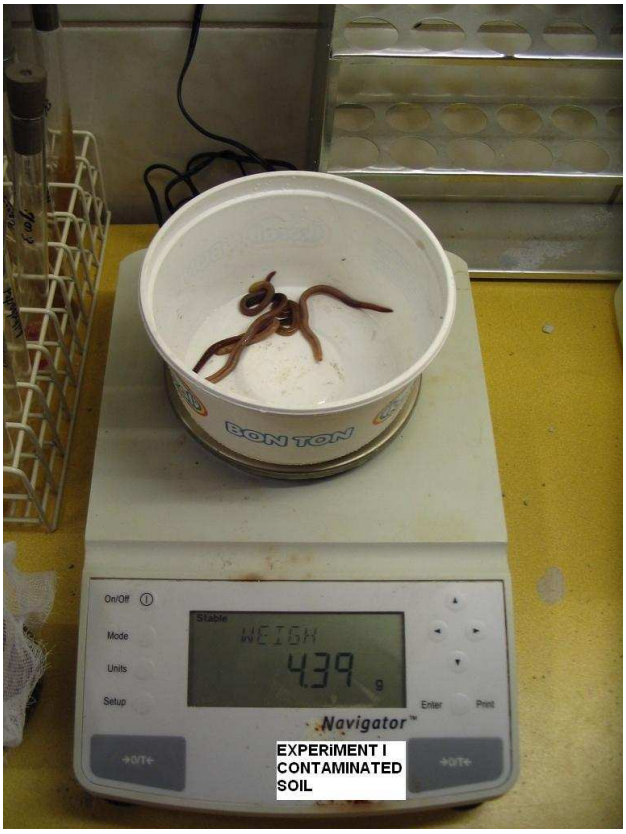


Fig. 21. Earthworms –- assessing their mass during a toxicity test.

Fig. 22. Toxicity test for soil using *Eisenia fetida*.

Table 4. Selection criteria for biotests in tems of the planned soil use.

Intended soil use	**Soil function**		
	Retaining function	**Habitat function**	
		Place of plant growth	**Microhabitat of soil biocenosis**
Type of conducted biotests	**Tests on water organisms**	**Tests on soil organisms**	
Soil below sealed areas	yes	No	No
Industrial unsealed areas	yes	No	No
Soil used for surface covering and fill	yes	(yes)	No
Parks and recreational areas	yes	(yes)	(yes)
Agricultural and horticultural areas	yes	Yes	Yes
Bioindicator types	Luminescent bacteria, Alga, Crustaceans	Vascular plants	Luminescent bacteria, Nitrification and respiration processes, Earthworms

Destruents (decomposers) can be used to estimate the influence of contamination on soil microflora. During testing, this is done by analyzing the number of bacteria belonging to certain physiological groups. Also, the enzymatic activity (dehydrogenase, catalase, esterase) and the rate of respiration in the soil can be measured with soil respiration taking into account oxygen uptake and carbon dioxide emissions.

It is essential for ecotoxicological studies to take into account the retaining function of soil. Soil has a solid structure, although pores filled with air and/or water are situated between soil particles and can take up from 30 to 60 % of soil volume. Particularly during heavy rainfall contaminants can migrate from the soil and become dissolved in water. Once in solution, toxic substances can enter groundwater and surface water where they pose a threat to water biocenosis and the people who use this water, e.g. for drinking water. Bioindicators inhabiting the water environment are also used to evaluate the toxicity of soil extracts. Common bioindicator organisms were described in the previous chapter.

Microbiotests, such as the system Microtox, are also used in toxicological soil studies. Direct contact tests (TBK) enable direct contact between the bioindicators and the studied sample. This allows the toxicity of compounds poorly soluble in water and of substances that create complexes in soil or in bottom sediments to be measured.

4. METHODS FOR ASSESSING THE TOXICITY OF DUST AND GASES

4.1. ANALYZING THE INFLUENCES OF GASES AND DUST ON THE DEVELOPMENT AND FUNCTION OF PLANTS AND ANIMALS

4.1.1. INTRODUCTION

Industry emits a large amount of gases and dust into atmosphere. These emissions are by-products of different technologies, although they mainly stem from fuel consumption. The most commonly emitted gasses include carbon dioxide, carbon monoxide, sulphur dioxide and nitrogen oxides. Anhydrides of very strong acids are among the most harmful to plants and animals. The commonly emitted gasses sulphur dioxide, nitrogen oxides and hydrogen fluoride (emitted by the glass industry) react with water present in the atmosphere, soil, hydrosphere and within organisms. From such reactions strong acids are formed. **Carbon monoxide** is able to bind with haemoglobin and this toxin can cause death in animals including humans since animal organisms use haemoglobin for the transport of oxygen and carbon dioxide. The toxicity of carbon monoxide is significantly lower for plants than the anhydrides of strong acids, although carbon monoxide does act toxically in greater concentrations. **Carbon disulfide** is highly toxic to animals because it affects their central nervous system. **Industrial dust**

mainly includes substances insoluble in water and they can have a mechanical impact on tissues and toxicants such as aromatic hydrocarbons and heavy metals.

4.1.2. BIOLOGICAL CONSEQUENCES OF SOIL ACIDIFICATION

When acid rain reaches soil, gases bind with the capillary water in soil. The presence of acids in soil, mainly sulphuric acid, can cause the following effects: an increase in soil acidification, washout of alkaline nutrients such as Ca, Mg, K, and Na, creation of an ion balance disorder, limiting the influence of edaphon causing changes in species composition and a transformation of toxins into forms more assimilable for plants. For the latter, a higher uptake of toxins can lead to intoxication.

4.1.3. INDUSTRIAL DISEASE OF FORESTS

Industrial forest disease (Fig. 23) is a complicated and long-term phenomenon usually consisting of four stages. **Stage I** is when industrial gases, mainly sulphur dioxide and nitrogen oxides penetrate into stomata and enter the parenchyma cells. Dust creates an isolation layer on the surface of the leaf making respiration and cuticle transpiration impossible while also limiting photosynthesis due to the lack of light. This disruption leads to a lower production of substances that protect the plant and this can make the plant weaker making it more susceptible to, infection by different pathogens such as insects and fungi. At this stage of the disease the assimilation apparatus is destroyed which influences the gradation and succession of pest assemblages. During **stage II** these pests attack treetops with insects feeding on leaves. The pests attacking spruce forests use monosaccharides in needles for their growth. Later, pests which attack lower parts of the trunk appear. The gradation of cambium and phloem pests, which are secondary pests, suggests that **stage III of the disease is occurring**. Pests attack trees already affected with these trees unable to secret sufficient resin to flood the pest microhabitat. Pine, which is quite resistant to physiological disorders, has a large number of characteristic pests. Spruce trees react very quickly to physiological disorders and thus pest assemblages are not that varied. Unlike some conifers, deciduous species do not have varied pest assemblages. **Stage IV** of industrial disease takes place after the forest has thinned out. In this stage, weeds cover the undergrowth or the area changes into more of a wetland.

Effects of forest industrial disease:
1. Treetops become thinned out; conifer trees lose older needles.
2. The tops of trees dry at an advanced stage.
3. Treetops change colors from green into yellow or grey. They fade away.
4. Deciduous species and larches lose their leaves earlier.

5. Needles of pines and firs become yellow from the end; the border between the yellow and green part of needle is very sharp and visible.
6. Needles of larch become brownish or reddish; their color changes gradually into yellow and finally into green at the base of the needle.
7. Needles of spruce become spotted irregularly; these spots are yellow at the beginning, concave, and then turn red and brown; finally they fall down.
8. Leaves of oak become spotted, mainly among nerves; these spots are light green at the beginning; then they turn yellow and reddish with their edges lighter.
9. Leaves of beech also become spotted, but they turn yellow and white at the surface; spots on clone leaves become darker.
10. Reddish and brownish spots appear on the leaves of linden, alder, birch, hornbeam and ash; older trees have white spots.
11. If the concentration of gases is high, leaves are undeveloped with the surface lower and the needles shorter.

Fig. 23. Industrial forest disease in the Karkonosze and Izerskie Mountains.

4.1.4. THE DIRECT EFFECTS OF INDUSTRIAL ACID EMISSIONS ON PLANTS

Acids in gas form and acids which originate from gaseous acid, mainly sulphur dioxide and its derivatives, influence plants in **three forms**: gases (react with water of the cell plasma creating acids), aerosol of acids, acid solutions in form of acid rain and capillary water in soil. The natural pH of rain is usually in the

acidic range, from 5–6. Rain with a pH <4.5 is considered acid rain while rain with a pH less than 3.6 is very harmful to the environment.
The gaseous acids and the other acid forms influence the following physiological processes of plants:
– transpiration
– photosynthesis
– respiration
– metabolism of free aminoacids
– conversion of monosaccharides into polisaccharides
– permeability of cell membranes

Acid emissions destroy leaves, specifically needles, cuticle (wax) and stomata which leads to an increase in transpiration replicating what is naturally caused by heat. Destruction of the root system diminishes water and nutrient uptake and this damage extends to the water supply system of the plant above ground. This leads to the following effects: needles fall off, growth is limited and there is a decrease in the frost and pest resistance. The assimilation apparatus is reduced and as a consequence the plant suffers from starvation.

Toxic factors in nature usually **act synergistically** during continuous changes of meteorological conditions. This is the main reason why separate analyses of toxic factors under laboratory conditions frequently produce completely different results compared to analyses of the synergistic influence regarding these factors on environment.

The manifestations of **transpiration disorder** depend on the time when the gaseous acid affects the plant. Blocked stomata in the open position increase the amount of transpiration (stomata transpiration), while the dehydration of cell plasma causes resistance to transpiration: dehydrated stomata cells collapse and close the stomata reducing the amount of water lost by the plant. If the osmotic potential (the sucking power of the plant) breaks down, the plant becomes unable to take in a sufficient amount of water and dies from dehydration.

The gaseous acids and other acids cause a reversion in the transpiration process. Usually the upper parts of the treetop transpire much more than the lower parts. Trees under the influence of acidic toxins react in the opposite way meaning they transpire more in the lower parts of treetop. Pine and fir under the influence of sulphur dioxide decrease their intensity of transpiration by a factor of 1.5 to 2.0. In general, trees subject to these toxins vary 1.5 times more in their transpiration intensity compared to trees in good condition.

Soil acidification destroys the rhyzosphere limiting root growth causing dwarfing. This destruction diminishes **water and nutrient uptake** by plants. **Ions of metals** are washed out from the soil by the acidic capillary water and such ions can also have a toxic effect on the roots.

Long term exposure to sulphur dioxide with a concentration of 130–230 $\mu g/m^3$ causes a **decrease of photosynthesis intensity** in coniferous trees**.** Firs exposed to sulphur dioxide for 10 weeks exhibit a 60% drop in photosynthesis intensity. Other gaseous acids such as hydrogen fluoride have a similar influence on photosynthesis.

Plant damage by gaseous acids includes **feofitin** – a pathological product of chlorophyll disintegration. Damage to this causes the chlorophyll content to decrease in the plant changing the proportion of chlorophyll a:b. During this process, sulphur dioxide binds to iron compounds in chloroplasts, the cell content dies and the chlorophyll creates lumps becoming brown losing its color. Changes in the **ultrastructure of the chloroplast** also occur.

The threshold value for sulphur dioxide influencing **respiration** is greater than the threshold value for influencing photosynthesis. For example, a fir specimen without light under the influence of sulphur dioxide exhibits a growth in respiration intensity on the first day of the experiment; after four days the intensity drops to a normal level and later decreases further.

Plants damaged by sulphur dioxide also show

– an increase in the concentration of low molecule saccharides and a decrease in the starch concentration
– disorder in the metabolism of free aminoacids and a decrease in protein content
– growth in the permeability of cell membranes resulting in losses of various valuable compounds, i.e. sugars, proteins and minerals

Different species exhibit a **varied sensitivity** to acids and industrial emissions. Symptoms of chronic intoxication are very often observed in trees and shrubs. Coniferous trees, changing their needles every few years, are usually more sensitive to toxic emissions than deciduous trees which change their leaves every year.

4.2. THE INDIRECT EFFECTS OF INDUSTRIAL ACID EMISSIONS ON PLANTS

Industrial emissions **are not the direct cause** of intoxicated forests. Disturbances in physiological processes lead to a **weakening of trees** making them more sensitive to **natural limiting factors** such as frost, wind, snow, drought, reduction of assimilating apparatus by pests, further weakness caused by microorganisms, fungi and secondary pests. The synergistic influence of these factors very often leads to **starvation and death** of the tree.

4.2.1. THE EFFECTS OF DUST ON PLANTS

Industrial dust includes carbon, volatile ash, silicates, oxides, phosphates and fluorides. Dust containing lead is particularly toxic to plants. **The direct influence of dust** is purely mechanical with dust covering the surface of plants leaves and other parts. The blocking of stomata causes disruptions in the exchange of gases, transpiration, photosynthesis (due to a lack of basic substrate – CO_2), cell respiration and energy exchange. The dust covering the surface of leaves makes cuticle transpiration difficult (cuticle transpiration proceeds through the cuticle layer, not through the stomata). Also, the availability of light to the parenchyma

cells becomes limited influencing the intensity of photosynthesis. When the humidity of air is high, all soluble constituents in dust are dissolved in water and enter the plants. If they happen to be toxic, the soluble constituents can cause the intoxication of the organism. One common industrial dust, cement dust, can even burn through the epidermis. In addition, the disintegration of chlorophyll and the loss of immunity to parasites can occur.

Indirect effects also take place and concern a change in the chemical properties of soil. Such a situation can be favourable to some organisms such as for the calciphilous species growing near a cement plant. Changes in the chemical properties of soil (as with other environmental factors) alter the composition of species in natural or nearly natural biocenosis. This occurs through the over reproduction of some species which out compete other plants thereby changing the biodiversity and homeostasis of the biocenosis.

Industrial dust includes ions of lead which can block enzymes and change the electrolytic homeostasis. The presence of lead causes cell divisions to become limited, the amount of acetylo- Coenzyme A to decrease and the content of organic acids to diminish in roots. The dark phase of photosynthesis is also repressed. Lead negatively influences the germination process of seeds and their growth probably by affecting the uptake of microelements. Lead is accumulated in plants organs, particularly in the roots and leaves. Different species of plants readily accumulate lead or other heavy metals. This ability can be favourable for people and environmental protection. For example, plants able to accumulate a large amount of lead can be planted along motorways under the condition that they are not used later as food for animals or people (grazing cattle near motorways or planting with fruit trees). A contaminated area should be planted with vegetation that can later be used for decoration or in non-food industries. Of course it is possible to plant edible species that are not able to accumulate heavy metals at high level. Examples of the **effects on plants of selected metals** contained in industrial dust are showed in tab. 5.

4.2.2. THE INDIRECT EFFECTS OF DUST ON ANIMALS AND PEOPLE

The long term inhalation of dust including insoluble mineral particles can lead **to pneumoconiosis** in animals and people. Two forms of **pneumoconiosis** are known: carbon and silicone. This disease is common in miners, grinders and stonemasons as an occupational disease. In this disease inhaled dust is deposited on lung tissue causing irritation and inducing the overgrowth of **connective tissue** that forms the scaffolding for the lungs. This overgrowth is a direct cause of respiratory failure. The excess dust is transferred by the lymphatic system as is the case for all alien substances. That is why the dust also accumulates in the lymph nodes inducing changes similar to those occurring in the lungs, i.e. fibrosis and dust incrustation.

Table 5. The influence of selected metals on some of the physiological processes of plants.

Metal	The influence on metabolism
Mercury	Disturbance of the proportion between chlorophyll a and b
Copper	Decrease of respiration intensity, synthesis of chlorophyll, activity of some enzymes
Zinc	Chlorosis and a drop in photosynthesis intensity
Cadmium	Drop of chlorophyll content and necrosis
Arsenic	Inhibition of germination and growth of plants
Chrome	Plant fading, underdevelopment of the root system, low biomass growth
Manganese	Chlorosis
Iron	Disturbance of the metabolism of other elements and growth inhibition

5. USING BIOINDICATORS IN EVALUATING AIR POLLUTION

5. 1. INTRODUCTION

Important features of bioindicators. The definition of bioindicator and the criteria for using different organisms as bioindicators in environmental assessment are ambiguous. Bioindicators become useful when it is possible to assess the exact amount of toxicants in time based on the presence of a bioindicator or its reaction. Also important is that from the reaction of the organism or merely from its presence, it is possible to assess not only the contamination of the environment, but also to detect the changes in the functioning of living organisms caused by toxicants. This is because living organisms are the subject of studies by ecologists and should be the only important criterion when considering environmental protection actions. A reliable bioindicator or biondicative reaction should register changes of toxicant factor intensity in time. In the past, priority was given to features of bioindicators such as rapid reaction, visual changes and low cost of their use. However, improved laboratory methods and equipment coupled with the high cost of physical and chemical analyses led to a change in the important criteria of bioindicators. The application of bioindicators is usually much cheaper than chemical and physical labor intensive methods. The new laboratory methods enable the detection of changes which cannot be perceived by our senses. For indicator species it is crucial that they are common.

Organisms and indicative reactions, bioindication and bioaccumulation. In the biological research of environmental conditions it is important to distinguish between bioindicators, e.g.lichens, mosses and fish, from indicative bioreactions, e.g. photosynthesis, respiration, and chlorophyll content. Although not typically biological, it is common to examine humus and the activity of humus enzymes as well as to conduct an acidification analysis and determine the buffering capacity of bark. Bark and humus are able to accumulate some toxicants and thus they can be applied as biotests. It is also possible to use bioaccumulators which differ significantly from bioindicators. Bioindicators are the organisms which exhibit physiological changes caused by toxicants. Bioaccumulators are able to accumulate toxicants inside their bodies. Bioaccumulation is an organism's defensive mechanism against a disturbance of physiological processes. The same organism could accumulate toxicants while also serving as a bioindicator.

Laboratory conditions versus natural conditions. It is commonly known that acute and chronic intoxication symptoms vary depending on the dose of the toxicant. Usually under laboratory conditions, one well-known factor in constant atmospheric conditions or a stable group of factors acting with well known proportions is investigated. In nature, toxicants usually act synergistically, in groups which are not stable in terms of quality and quantity under changeable atmospheric conditions and under the influence of other factors connected with natural selection that are absent from laboratory conditions. In nature, toxicants can enter the organism in many possible ways. It is very rare that only one part of the environment is contaminated, e.g. only air or food or soil. In contrast, such a situation is likely or even required under laboratory conditions.

Classification. Bioindicators and bioaccumulators exhibit a great variety and thus they can be divided into a few groups. As bioindicators, wild animals can be also used if they are living within the study area. Such organisms exhibit a natural genetic variability. Organisms transplanted into a study area or organisms used in laboratory experiments are selected according to their utility in the experiment, e.g. hereditary attributes are taken into consideration such as race or the variant particularly sensitive to the studied toxicant. Trees growing in the study area can play the role of bioindicators indicating the results of long-term toxicant influence on organisms. The majority of bioindicators exhibit physiological disorders as a reaction to even low toxicant concentrations while at the same time they are resistant enough to physiological disturbances that they can tolerate high concentrations of toxicants. This capability enables bioindication for a wide range of toxicant concentrations. There are also some bioindicators which die or migrate in response to a certain concentration of toxicant or an influence of an environmental factor, e.g. the appearance or decline of the hygrophilous species which indicates changes in water quality for a study area. The segregation of different species according to threshold values of the lethal concentration of a toxicant enables the creation of a scale for assessing the toxicant concentration in the environment. Knowledge concerning the species composition in the study area is sufficient for such studies. A threshold value for the most sensitive species inhabiting the study area is a barrier below which the concentration of a toxicant is

maintained. The lichen scale used for evaluating the sulfur dioxide concentration in the atmosphere can serve as an example.

5.2. LICHENS AS BIOINDICATORS OF SULFUR DIOXIDE IN THE ATMOSPHERE

Lichens (Fig. 24) are organisms consisting of **two components**: alga and fungus. The alga most often belongs to Chlorophyta or Cyanophyta and the fungus to Basidiomycota or Ascimycota. The systematics of lichens is based on the systematic membership of the fungus component. Both components in lichen are living in **symbiosis**, which means they are **pioneering organisms**. Because of this they are able to inhabit places inaccessible to other organisms where there is a lack of existing water and nutrients: rocks, sand, bark of trees, walls of houses and fences. The fungus delivers bases to the alga while taking the shape of a thallus. The fungus also creates cortical protective layers in the thallus of lichen which are built from joined hyphae. The fungus plays an important role protecting the alga from drying and delivering water with mineral salts. In exchange, the fungi obtain organic compounds produced by the alga during the photosynthesis process. Lichens can uptake nutrients not only from the soil, but also from the atmosphere. With such uptake abilities lichens also pick up toxicants which are transmitted to the atmosphere in the form of gasses and dust. They are particularly sensitive to the effects from sulphur dioxide and sulphuric acid (created from sulphur dioxide in the atmosphere). Other toxicants such as fluorine, lead and cadmium do not exhibit such visible effects on lichens.

The **sensitivity of lichens** to environmental pollution was first observed in 1896 in Paris. Coal replaced firewood heating in flats during this year. In 1866, 33 species of arboreal lichens were observed in Parisian parks. In contrast in 1896 there was not even a single species present. A similar effect was observed in other cities in Europe and the United States when wood was replaced by coal containing a high concentration of sulphur. The return of lichens to Parisian parks was observed again in 1990.

The British scientists Hawksworth and Rose ordered lichens growing on the bark of deciduous trees based on their sensitivity to the content of the sulphur dioxide in air. They created the **lichen scale** in 1972 which is commonly used for determining the concentration of atmospheric sulphur dioxide. A team from Wrocław University coordinated by Ewa Bylińska simplified the initial lichen scale developed by British scientists for application by less skilled users. This scale will also be used during these laboratory exercises.

Fig. 24. *Hypogymnia physodes*, one of the bioindicators for sulphur dioxide.

5.3. PLANTS AS BIOINDICATORS OF GASEOUS POLLUTION

Some plants are good bioindicators of gaseous pollutants. As **bioindicators of sulfur dioxide, the** *Fabaceae* **plant family is widely applied**, i.e. clover, lucerne lupine, broad bean tulips, swordtails, croci, begonias and seedlings of the grapevine. In addition, the plovers of *Pinus strobus* pine are good indicators of air pollution from hydrogen fluoride. A vase experiment with the use of different grass species is commonly applied to conduct the bioindication of permanent and gaseous fluorine compound. For the **bioindication of ozone** young tobacco plants (Bel 3 variant) are commonly used.

With gaseous pollution a blotchiness in leaves occurs very often and this kind of symptom is easily observable even for the inexperienced researcher. Blotchiness is a clear symptom of plant intoxication and is used in bioindication. This blotchiness can have the character of chlorosis and of necrosis. Chlorosis (Fig. 25) means in practice a reduced amount of chlorophyll and therefore, the lack of green pigment. **Chlorosis** often takes place only in the parenchyma of the leaf between its nerves or edges of the leaf. **Necrosis** (Fig. 26) simply means the dying out of some parts of plants. Fragments of leaves die out most often forming characteristic stains. Also, entire leaves may dry out starting from the edges or a central part; another possibility is that the growing tops of shoots may die out. Chlorosis and necrosis could also result from a deficiency of some nutrients. The indicators of atmospheric air pollution are also symptoms of industrial forest illness described in the previous chapter.

Fig. 25. Chlorosis caused by pollution.

Fig. 26. Necrosis caused by pollution.

5.4. BIOACCUMULATION AND ITS APPLICATION IN EVALUATING ENVIRONMENTAL POLLUTION

Bacteria accumulate harmful substances in the mucus surrounding their cells, in their cell walls and cell membranes. The penetration of poison into the cell causes intoxication and can result in the death of microbes. An exception to this is when microorganisms are able to include a poison in their metabolic processes. The accumulated toxicant then returns to the environment after the death of the organism causing secondary pollution of the environment.

Plant and fungus cells store their reserve materials and harmful substances in vacuoles. Higher plants accumulate the most harmful substances in garner organs, most often the edible parts of vegetables. Harmful substances can also be found at higher concentrations in roots (due to the uptake from soil with water and mineral salts) and in leaves (from the uptake of air in the process of gas exchange and permeating through the cuticle). The uptake and bioaccumulation of metals by plants depend on the following factors: type and chemical form of the metal, its concentration, plant species and age as well as factors connected with the abiotic environment, i.e. the soil composition, pH moisture content, oxidation-reduction potential and to some extent temperature and light intensity. Oxygen conditions in water are very important in the case of bioaccumulation by aquatic plants. The pH, hardness and type of substances dissolved in water are also very significant.

Vertebrates accumulate harmful substances in the liver, adipose tissue and also in their milk and eggs. Considerable research has been conducted regarding the ability of some lichens and mosses to accumulate heavy metals as well as the tendency of pine and spruce needles to accumulate sulfur compounds.

5.5. BIOREACTIONS AS AN INDICATOR OF ENVIRONMENTAL CONTAMINATION

Apart from easily visible symptoms, **disorders of physiological processes** are used as indicators of environmental pollution. The following processes are studied: intensity of photosynthesis, respiration and seed germination. It is possible to measure the intensity of respiration and photosynthesis by applying of direct methods (the rate of respiration and photosynthesis) or indirect methods (content of chlorophyll, feofitin).

The inhibition of germination is one of the bioreactions used for investigating the toxicity of chimney dusts and soil. This examination consists of determining the ability of seeds to germinate when placed in a humid chamber and subjected to the influence of an extract of dust or soil. Seeds of various species of plants exhibit a diversified sensitivity to examined toxicants. The extracts of low concentrated water-soluble toxicants could even stimulate the germination process. As a rule, the

inhibiting effect of dust or contaminated soil produces a delay in germination and does not completely block this process. Contaminated seedlings in the initial phase of their growth develop more slowly than reference samples. Germination is a physiological process dependant also on natural environmental factors such as humidity of the soil and temperature among others. For example, the presence of light delays germination. Certain species have unique requirements with some requiring over-cooling or drying for a few months in order to gain the ability to germinate.

6. BIODEGRADATION OF XENOBIOTICS AND ITS INFLUENCE ON NATURAL DECOMPOSITION PROCESSES

6.1. INTRODUCTION

Many xenobiotics enter the environment with the majority of them being very harmful to living organisms due to their toxic and mutagenic properties. The biodegradation of organic compounds is already well known as this constitutes an important part of carbon circulation in the biosphere. In comparison, little is known about synthetic organic compounds. Microbiological transformations of xenobiotics often occur with great difficulty and thus pollution caused by these compounds can be permanent. Permanent pollutants are usually taken and accumulated in living organisms. The spreading of pollutants long distances through particular links in a food chain is a dangerous feature of this type of toxicants. However, many synthetic organic compounds undergo biological decomposition thanks to bacteria being able to adapt to utilize a new substrate. Bacteria do this by producing appropriate adaptive enzymes. This bacterial ability plays a major role in the process of detoxifying an ecosystem. Products of metabolic transformations, in contrast with many initial substrates, do not generally exhibit harmful effects on living organisms. However, there are exceptions to this and it is commonly known that dangerous products are created from the metabolism of precursors which do not show such properties. For example, nitrates are precursors of carcinogenic nitro-amines formed in the soil. The ability of microorganisms to degrade xenobiotics depends on the concentration of the xenobiotic in the environment. This concentration should be low enough so that it does not cause lethal effects or disorders of physiological functions in living organisms. When there is an increase in the toxicant concentration, changes in the microorganism population structure may take place and as a consequence, decomposition processes of organic matter can be disturbed.

The susceptibility of organic substances to biological decomposition is also dependant on their chemical structure. Even slight modifications in the structure of molecules can make them available as a substrate for microorganisms. For example, microorganisms do not attack the branched chain of alkyl since it does not match the active centres of their enzymes. However, when the alkyl chain is

straightened, it becomes susceptible to immediate microbiological disintegration. The susceptibility of aromatics to biodegradation is dependent on their amount, type and mutual arrangement of substituents. Chlorinated hydrocarbons are more resistant to biological degradation if they contain more halogen substituents, but this resistance decreases from fluorine to chlorine and it is the lowest when bromine and iodine are present in the ring. The presence of the chlorine atom in the aromatic ring in the meta position makes this ring completely unavailable to microorganisms. This effect is smaller when the halogen atom is placed in the position ortho or para.

It is apparent that the process of biodegradation depends on the presence of other easily available nutritional substrates in the environment. For example, it was observed that fertilizing the soil in a contaminated location increases the adaptive ability of microorganisms to use the xenobiotics as a source of carbon. The most toxic and mutagenic xenobiotics are polycyclic aromatic hydrocarbons (e.g. 3,4 – benzopyrene or 1,2 - benzanthracene Fig. 27) as well as many phenol derivatives (chlorine derivatives of phenols). These substances are created from the incomplete combustion of organic substances. They can infiltrate into the water from asphalt roads, combustion gases, petroleum and coal treatment plants (coking plants, gasworks). Aromatic amines are also very dangerous, e.g. benzidine (4,4'– aminobiphenyl) or B - naphtylamine (Fig. 28). They are nearly insoluble, toxic and carcinogenic; thus, their removal from water requires special treatment technology. They enter into the environment from dye factories, synthetic rubber and other plastic factories as well as from pharmaceutical plants. Chlorinated biphenyl derivatives are compounds that are nearly insoluble in water, permanent or very long-lasting and are usually not biodegradable. In addition, the thermal decomposition of these compounds is possible only at temperatures greater than 1573°K. These compounds are used as dielectric liquids in condensers and transformers, as hydraulic fluids, insulating materials in electronics or as additions to paints and varnishes. Thus, they can migrate through food chain stages in the entire biosphere. These substances have been found in many food products, in the adipose tissue of mammals and also in people, e.g. in the breast milk of women. Some pesticides, especially polichlorine hydrocarbons such as DDT (Fig. 29), readily accumulate in some tissues and in links of the food chain.

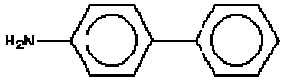
DDT is toxic even after a period of 14 years and is readily accumulated in the fatty tissue of birds and mammals. Detergents are equally dangerous. The ecological aspects of detergent use are in general poorly understood. Some of them are carcinogenic; others are easily degradable, but cause eutrophication; and yet others cause normally insoluble, frequently carcinogenic substances to dissolve in water. The most dangerous are detergents containing quartenary carbon and along hydrocarbon chain. The possibilities for biodegredation consequently diminish as the hydrocarbon chain becomes longer. Also, a longer chain leads to a greater toxicity(e.g. alkylbenzene sulphonate Fig. 30).

Petroleum spills pose a very serious threat to the environment. Soil contaminated with oil is biologically and chemically degraded and petroleum creates a danger for the secondary contamination of water. Petroleum-derived compounds consist

mainly of aliphatic hydrocarbons and a small number of aromatic and cyclic hydrocarbons. Their toxicity and mutagenic character is commonly known. Self – purification of soil and water from petroleum-derived products under natural conditions is a multistage process which can last many years. A long time is required mainly due to the presence of branched alkanes which are less vulnerable to attack of microbes.

Heavy metals are an example of long-lasting pollutants. They are accumulated in sediments and soil, enter the food chain and trigger dangerous effects for biocenosis and human health. Metals in the form of elements are not toxic to living organisms since they are practically insoluble. Only pairs can penetrate into the human body through the respiratory tract and the skin. Therefore, mercury, which evaporates easily, is a poison. The strongest toxic properties occur in the inorganic compounds of metals. They are easily soluble and strongly dissociating and thus they enter easily through cell membranes. In general, heavy metals are found in the soil in an insoluble form. However, they may undergo different transformations from inactive forms into soluble active forms first negatively influencing plants and soil microorganisms and later animals and humans. Such transformations are associated with the physicochemical properties of the soil, especially with pH. For example, lead in an acidic environment occurs mainly in the Pb^{+2} form which is strongly toxic, while with lowering acidification it hydroxides to $(PbO_4)^{-2}$, a form which is less toxic because of its low solubility.

Fig. 27. Policyclic aromatic hydrocarbons, typical contaminants of water.



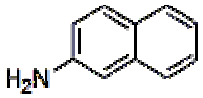


Fig. 28. Aromatic amines.

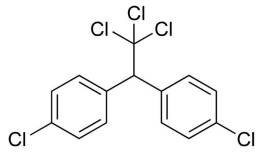


Fig. 29. DDT (dichlorodiphenyltrichloroethane).

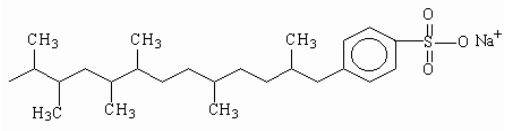


Fig. 30. Detergent with quarternary carbon and a long alkyl chain.

Microorganisms exhibit the ability to accumulate heavy metals from the surrounding environment. Microbiological accumulation processes of metals can be divided into three groups: the sorption of metal ions on the surface of cells, intracellular uptake of metals and a chemical transformation as a result from the activity of biological factors. The transport of metal ions into cells is often accompanied by the secretion of microbial metabolites and the formation of complexes with the metabolites and the metal. Such neutralized metals may be transported to the inside of the cell. Next, ions of metals can undergo metabolic transformations leading to their precipitation in the form of hardly soluble compounds, the creation of chelat or a transformation into volatile compounds. The accumulation of heavy metals in the cell leads to a higher concentration compared to the concentration in water. After the death of the cell, the accumulated compounds are again released into the water.

6.2. BIODEGRADATION OF SELECTED COMPOUNDS

6.2.1. BIODEGRADATION OF AROMATIC HYDROCARBONS

The degradation of aromatics in aerobic conditions occurs in two stages. The first stage is when the transformation of substituents, side chains or entire aromatic rings takes place. The second stage involves the transformation of single aromatic rings. The transformation of phenols consists of incorporating an oxygen atom into the C-H bond of the ring and building a single hydroxylic group in the substrate. Another atom of atmospheric oxygen is reduced to the water molecule. This process is conducted by enzymes belonging to monooxygenases from the group of hydrolases. This is how many aromatics are transformed to the indirect crucial metabolite - pyrocatechin (Fig. 31). This compound can undergo further biochemical transformations with the participation of oxidases or oxygenases. The

oxidation of pyrocatechin by oxidases leads to the formation of quinines which undergo polymerization or condensation creating humine compounds or melanins.

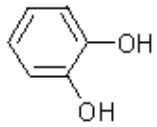


Fig. 31. Pyrocatechin – a crucial metabolite in the degradation of many aromatic compounds.

The oxidation of pyrocatechin by dioxygenase triggers the incorporation of two atoms of oxygen directly into the ring of pyrocatechin followed by its disruption. Pyrocatechin undergoes a further transformation depending on the produced enzyme. The ring cleavage is catalyzed by dioxygenases and the disjunction takes place between two neighboring hydroxyl groups (*ortho* cleavage) or between a hydroxylated and non-hydroxylated carbon atom (*meta* cleveage). The breakdown of the aromatic ring in the ortho position causes the creation of acetyl-CoA which is involved in the citric acid cycle. The meta fission of the aromatic ring leads to semialdehyde 2- hydroksymuconate or its analogues to acetaldehyde and pyruvic acid which are included in the citric acid cycle.

6.2.2. BIODEGRADATION OF CHLORINATED PHENOLS

The biodegradation of chlorinated aromatics relies on the possibility of transforming a coal skeleton into indirect metabolites with the simultaneous transformation of an organic halogen into a mineral form. The removal of the haloid substituent in the metabolic pathway is of crucial importance. This removal may occur differently with different microorganisms. It could take place in the early stage of biodegradation. The fission of the aromatic ring at the beginning followed by the detachment of the haloid substituent is an alternative. Dechlorination in the early stage, which relies on the replacement of the halogen in the aromatic ring by hydrogen, has been observed only in anaerobic conditions.
The other mechanism of dechlorination relies on the replacement of halogen by a hydroxyl group. In this reaction, water is a donator for the hydroxyl group, not molecular oxygen. Dioxygenases participate in reactions in which the cleavage of the aromatic ring occurs first. If a halogen is built into the aromatic ring, biodegradation takes place via the ortho pathwa only. The meta pathway is used when the removal of the halogen from the ring takes place first. This is because meta detachment produces a product which is an inhibitor of this metabolic pathway.

6.2.3. BIODEGRADATION OF ALIPHATIC HYDROCARBONS

The degradation of aliphatic hydrocarbons in aerobic conditions usually occurs with the production of an appropriate alcohol, aldehyde or fatty acid which then undergoes the process of β-oxidization.

6.2.4. THE BIODEGRADATION OF DETERGENTS

Surfactants belong to the group of detergent chemicals and thus they have the ability to reduce the surface tension appearing on the border of two phases. A molecule of surface-active compound consists of a long chain (aliphatic chain straight or branched or alkylacrylic chain which means condensed with an aromatic core) and a polar hydrophilic group (e.g. OH, COOH, NH_2, SO_3). It is possible to divide the biological degradation of detergents into two stages. In the first stage of biodegradation, the detergent is decomposed to straighter elements. As a result of this process, detergents lose their surface-active properties. In the next stage of degradation, the compound disintegrates into CO_2, H_2O, mineral salts and other products from the metabolism of the bacterium. The adsorption of detergent molecules on the surface of bacteria cells is a preliminary stage of detergent removal. The adsorbed molecule through diffusion penetrates into the cell where it undergoes biodegradation allowing space for the adsorption of the next molecule on the surface.

7. TOXIC COMPOUNDS OF NATURAL ORIGIN

7.1. INTRODUCTION

Eight hundred plant species are commonly known as plants which possess prussic acid. Prussic acid mainly occurs in seeds, leaves or bark of the following plant families: *Rosaceae*, *Linaceae*, *Juncaginaceae* and *Caprifoliaceae*. Plants from the genus T*riglochin* belonging to the family *Juncaginaceae* contain a large amount of HCN. It is possible to come across these plants in meadows, bogs and peat bogs. A well-known cyanogenic glicoside, amygdalin, occurs in the pits of plants from the *Rosaceae* family (e.g. cherry, plum, bitter almond or apple). In immature flax seeds (*Linum usitatissimum*) linamarin can be found (in concentrations of about 0.3 %) and immature fruits of *Sambucus nigra* contain sambunigrin. Compounds such as amygdalin and prunazin are found in the leaves and seeds of stone fruits (almonds, peaches, apricots, plums and cherries). The content of amygdalin in 1 g of pits is as follows: in cherries – 1.7 mg, in almonds – 4.5 mg. Consuming a large amount of bitter almonds can cause lethal poisoning. A single bitter almond contains about 1 mg of HCN. Cases of fatal poisonings have

been well documented after consuming 50–60 bitter almonds, whereas a child could even die after just eating 10 bitter almonds. A source of hydrocyanic acid is cyanohydrins which are formed from saccharides and amino acid derivatives (e.g. phenylalanine and tyrosine). Glicoside compounds produce poisonous hydrocyanic acid during their enzymatic disintegration. The mechanism of toxic effect occurs through the inhibition of the oxidation and fermentation processes, the blocking of oxygen transfer (with haemoglobin) and as a result the paralysis of the respiratory system and suffocation occurs.

7.2. DETECTION OF CYANOGENIC GLICOSIDES

Cyanogenic glicosides

Cyanogenesis is a set of processes for the synthesis of cyanogenic compounds, compounds containing the CN group. As a result of enzymatic or acidic hydrolysis, these compounds produce hydrocyanic acid (prussian acid) –HCN. The majority of cyanogenic compounds exhibit a character of glicoside – nitrylozoid. Among 50 cyanogenic compounds produced by plants are amygdalin (almonds), prunazin (blackthorn bush, bird cherry, cherry), sambunigrin (elder, elder herb, elder coral), linamarin (flax) and lotaustralin (clover). During enzymatic hydrolysis (enzyme emulsine) nitrile (cyanohydrins) and free glucose are secreted at the beginning. After this stage, HCN is freed and finally ketone or aldehyde are produced. Cyanogenic glicosides are synthesized from amino acids, e.g. linamarin is derived from valine and amygdaline – from phenylalanine and tyrosine. As is commonly known, hydrocyanic acid and its salts (cyanides) are among the strongest toxins. They demonstrate a strong affinity to the iron-porphyrine system of respiratory enzymes. Thus, they block the function of respiratory enzymes. Hydrocyanic acid joins hemoglobin creating cyanohaemoglobin, which is not able to dissociate to hemoglobin. Some farm animals exposed to hydrocyanic acid from plants (e.g. clover, numerous grasses) exhibit an interesting mechanism of detoxification: the rhodanse enzyme converts the cyanide ion into thiocyanate with the help of sulfur which is disconnected from acid beta-mercapto-pirogronic acid $HSCH_2COO_2H$. This detoxicating reaction is also used during the emergency treatment of people poisoned with cyanide. These patients are intravenously given 50 ml of 30 % sodium thiosulfate. This compound (25% sodium thiosulfate) is also used for gastric rinsing in case of nutritional poisoning. A lethal dose of HCN for a man is about 1 mg/kg of body weight. Therefore, the single consumption of 70 or more almonds, apple stones or fruits of the mandarin tree where cyanogenic glicosides are present in the amount of 0.5–1 mg is dangerous and risky. Cyanogenic glicosides present in herbs together with other elements demonstrate synergism and have a calming effect. They are also relaxing, cholagogic, distolic, anti-inflammatory, cough relieving and antiseptic. They stimulate the respiratory center, although only in healing doses. Water from the seeds of the bitter almond tree *Aqua Amygdalae amarae* were first applied in healthcare many years ago.

It contains about 0.1% of HCN. It was taken in the dose of 2 g, 3 times a day against cough, sleeplessness, nervous excitement and catarrh of the lungs.

7.3. DETECTION OF CAFFEINE

Caffeine (Fig. 32) is a main alkaloid from the seeds of the coffee bush *Coffea arabica*. It also occurs in other plants from the *Theace* and *Sterculiaceae* families. Caffeine is a substance which exhibits multidirectional activity. It has a stimulating effect on the central nervous system; it is an analeptic of the respiratory center and is commonly used in curing some poisonings. It can also act as a psychoanaleptic improving association processes in brain as well as reducing fatigue and sleepiness. Caffeine also widens the cerebral and coronary vessels, and increases diuresis. It intensifies the secretion of stomach acids; thus, people with the gastrointestinal ulcers should strictly avoid caffeine. The mechanism of caffeine activity in the central brain is still poorly known. It is believed that caffeine blocks receptors which are sensitive to adenosine. Caffeine is applied in healthcare as an analeptic for strengthening heart action, in migraines, states of tiredness and in drug and alcohol poisonings. In larger doses(above 0.5 g) caffeine causes excitement, an increased heartbeat and even muscle spasms. Poisoning causes the inhibition of respiratory center action; thus, injections containing caffeine in doses of 100 – 250 mg are applied.
Caffeine is found in the following materials:
Semen Coffeae- seed of coffee (0.3 – 2.5 % caffeine)
Folium Theae – tea leaf (1.2 – 4.5 % caffeine)
Embrio Colae –- embryo cola (0.6 – 3.0 % caffeine)
Semen Cacao –- Seed of cacao tree (0.3 % caffeine)

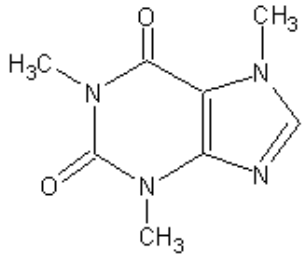


Fig. 32. Caffeine (1.3.7 trimethyl 2 . 6 dioxypurin).

8. INDUCTION OF MUTANTS WITH UV RAYS

8.1. INTRODUCTION

The damaging effects of some contaminants are based on their interaction with the genetic material of organisms causing changes in the genetic structure called mutations. Mutations in well developed organisms, including people, may cause the initiation of cancerous processes, disorders in pregnancy development or inherited genetic diseases often connected with mental impairment. There are many factors causing mutations and these factors are called mutagens. Generally, they are divided into physical and chemical categories. Chemical mutagens and their detection are discussed in the next chapter. Ultraviolet radiation (UV) is considered here as being a physical mutagen. Ultraviolet radiation is electromagnetic radiation at wavelengths from 180 to 380 nm. Because of its character it does not exhibit any interaction with matter and is defined as a non-ionizing in contrast to α,β, and x rays which cause ionization (also mutagenic).

The effects of UV rays form the basis of many important biological phenomena such as the synthesis of vitamin D (against rachitis), the synthesis of melanin – skin pigment (sunbathing), the triggering of different types of skin cancer and finally the mutagenic character of UV which can be fatal for micro and macroorganisms.

Ultraviolet radiation was one of the main factors initiating the chemical reactions necessary for the emergence of life on Earth 3–4 billion years ago. Many potentially harmful phenomena caused by Ultraviolet radiation are minimized thanks to its absorption by the ozone layer in the stratosphere (mainly at 25 km in altitude) and dispersion in the atmosphere. Therefore, rays reaching the Earth's surface are mainly rays at wavelengths from 380–320 nm (called UVA) which act milder. Rays with shorter wavelengths (below 320 nm called UVB) carry more energy and thus act more strongly on the organism. These higher energy rays are readily absorbed by ozone. Air pollution by nitric oxides and freons destroys the ozone layer causing an increase in the intensity of ultraviolet radiation in the dangerous wavelength range. It has been calculated that an ozone loss of about 1% increases the intensity of radiation by about 2%.

8.2. REACTION OF ORGANISMS

Organisms react differently to UV radiation depending on their organizational level. Microorganisms are the most sensitive (bacteria, algae, protozoans) along with viruses. Higher organisms consisting of many cells are more immune because of the relatively low penetrability of this radiation through tissues.

The selective absorption by DNA at the wavelength 260 nm is the basis of the mutagenic character of ultraviolet radiation. An effect of this activity is the creation of characteristic changes in the nucleic acid structure. Covalent connections are formed among pirimidine bases, particularly among thymines, and they are called dimers (from Greek: di-two, mera-part). If a cell does not remove these changes, it will die because of DNA replication disorders. This occurs when a large dose of rays causes too great of changes in the DNA structure and the cell is unable to repair all the damages. Based on its antibacterial properties, ultraviolet radiation is used for the sterilization of air, laboratory equipment and water. It is also possible that the enzymatic repair system initiated by the cell alone will remove all existing damage; thus, the original DNA structure can be reconstructed or it may be reconstructed with mistakes in which case a mutation takes place. Cells can exhibit different repairing systems acting with various precisions. As a rule, the system called photoreactivation acts perfectly. It starts only when light is present and is based on the disintegration of bonds linking dimmers by the special enzyme photolyase. Therefore, in practice to trigger mutational changes the bacterium should be protected from light after exposure to ultraviolet radiation. However, there are also other repairing mechanisms which can remove changes, but they are not able to precisely reconstruct the initial structure. In this respect they cause mutations, especially **transition** or **transversion** types.

In summary, UV radiation influencing genetic material does not usually cause mutagenesis directly (the formation of thymines dimmers it is not a mutation because the sequence of nucleotides in DNA does not change). Mutations appear only during the process of repairing damage caused by UV radiation since only then changes in the sequence of nucleotides occur.

It is not possible to predict the effect of a mutation. The mutation effect depends on the place in the genome (gene) where the mutation occurs. From experience, the bacterium *E. coli B* is sensitive to streptomycin (str^s, s = sensitive). The sensitivity of the bacterium to this antibiotic is determined by the gene coding ribosomal proteins. Streptomycin unites with them disturbing the process of protein biosynthesis (the antibacterial effect of this antibiotic is based on it). As bacteria multiply in gigantic numbers, there is a large chance that the mutation of exactly this gene will happen in some of them. This mutation will cause a change in the structure of the coded ribosomal protein and as a consequence it will prevent the streptomycin from connecting and disrupting the process of translation. The mutagenic bacterium will become immune to the streptomycin and this feature will be transferred to cells which appear later as a result of division. From this, a strain of mutagenic bacteria will appear that is immune to streptomycin (str^r, r = resistance).

9. APPLYING THE AMES TEST AND "REC ASSAY" IN ASSESSING THE POTENTIAL MUTAGENIC AND CANCEROGENIC PROPERTIES OF ENVIRONMENTAL CONTAMINATION

9.1. INTRODUCTION

Currently there are over four million chemicals. Among them about 60000 compounds are in common use. New substances are still being produced with many of them exhibiting mutagenic properties. Some of these compounds have a chemical structure with unusual properties causing them to interact directly with the genetic material of cells. This interaction leads to changes in the genetic structure of cells. Such substances are defined as **direct mutagens**. Among them are mainly alkylating compounds (they join - CH_3 or – C_2H_5 groups to nucleotide bases). An example is mustard gas (battle stinging agent) or epoxy compounds included in many varnishes and glues.

Other compounds are inactive biologically and they convert into mutagenic compounds only after entering an organism. These compounds are defined as **indirect mutagens** or **promutagens**. Many tissues and organs (placenta, kidney, lung, epithelium of digestive tract) have the ability to activate promutagens which enter the organism exhibit, although the liver is the most important organ participating in the metabolism of these compounds. This organ, with the help of enzymes from the group of oxidases, is able to metabolize almost every alien substance causing its **detoxification**, i.e. the deprival of its toxic properties. In the course of this process, inactive promutagens change into highly reactive metabolites which connect with neutral elements (e.g. with bile acids or glucoronic acid). Then, such complexes are excreted with urine or faeces. However, during the process of detoxification, a certain part of reactive metabolites can deviate from the natural path of transformation and interact with genetic material causing mutations. The most well-known promutagens include polycyclic aromatic hydrocarbons (e.g. benzo (a) piren), aflotoxin (a strong poison produced by the yellow mold *Aspergillus flavus*), CCl_4 and vinyl chloride among others.

Some mutagens can act both directly as well as after activation. Examples of this are ions of metals, e.g. Cr. A distinct relationship between the ability to provoke a mutation and the carcinogenic potential of the substance exists. It should be assumed that substances or factors which exhibit mutagenic properties are also potential **carcinogenic** substances (factors). Often, both definitions (mutagens, carcinogens) are used interchangeably. The idea of **cancerogenic transformation** (healthy cells into cancerogenic ones) is a change in the genetic apparatus of a normal cell, especially within so-called **oncogenes** which allows the possibility of uncontrolled divisions of cells. As the number of diverse substances entering the

environment rises, studies of their mutagenic/carcinogenic properties become increasingly justified. Biological tests play this role.
Studies on live animals are expensive and time-consuming. One sample requires the use of at least 600 animals (mainly mice and rats) and lasts about 2 years. Because of this, monitoring of **genotoxic contamination** is carried out using bacterial tests which are simple, fast and inexpensive. The findings obtained by bacterial models may not be applied to people without significant error; however, in general a high correlation exists between cancerogenicity in direct studies on animals and mutagenicity obtained in bacterial tests.

9.2. AMES TEST

The **Ames test** is the most widely applied test. It was invented in the USA in the 1970s and is continually being improved. It uses specially altered strains of ***Salmonella typhimurium***. This bacterium triggers infectious diseases in animals and food intoxication in people. It also causes intoxication after egg consumption (especially duck eggs).
The test (Fig. 33) uses several strains of the bacterium that carry mutations in genes involved in histidine synthesis i.e. it is an auxotrophic mutant, so that they require histidine for growth. The variable being tested is the mutagen's ability to cause a reversion to growth on a histidine-free medium. Strains used in the test are mutagenic in a few places of the genome and are less virulent (an ability to trigger illness). Since non–mutagenic strains still exhibit this ability, they are considered wild. Therefore, wild strains cannot grow on medium culture without histidine (his-). However, under the influence of the mutagenic factor, bacteria can undergo the **return mutation (reversion)** which will bring back their initial ability to synthesize the amino acid (his+) and enable them to grow on a medium culture devoid of this compound (bacteria synthesize it from the glucose present in the medium culture). Therefore, the growth of test strains in the presence of the studied compound on medium culture without histidine suggests the mutagenic character of this compound.

The mutation usually appears during the replication of DNA accompanying cell divisions. Thus, if a tested sample is going to cause a mutation, a trace amount of histidine in the culture medium is needed. This enables the bacteria to perform a few divisions. The formation of a colony barely visible with the naked eye is an effect of this limited growth. Because of histidine usage further growth is limited only to cells where the reversion occurred (his+ mutants). They create clearly visible, countable colonies. The more strongly mutagenic the studied compound is, the more **revertant** colonies will grow.

In order to properly assess the mutagenicity of a tested sample, a control sample should be prepared where bacteria grow under the same conditions, but without contact to the studied toxic compound. Then, a small amount of mutagenic cells will also appear because there are always some indiscernible mutagenic factors (e.g. UV rays, culture medium components and others) in the environment.

They induce so-called **spontaneous mutations** which could also restore the ability of bacteria to synthesize histidine and enable growth on the test culture medium. The number of reverse colonies occurring spontaneously is a point of reference taken into account when reading test results. It is common practice that in order to classify a studied sample as mutagenic (carcinogenic), a number of revertants on the studied plates should be at least twice as much comparing with test plates without the tested compound.

In order to increase the sensitivity of bacteria to mutagens, test strains posses destroyed cell walls making them more permeable for large particles, e.g. benzo (a) piren (is not able to go through an undamaged wall). Moreover, strains themselves have a limited capacity for repairing DNA damage which increases significantly their sensitivity to mutagens. As mentioned in the introduction of this chapter, bacteria (and other organisms) posses various repairing systems which differ from each other in their ability to precisely reconstruct the initial structure. The function of the repairing system of strains used in the Ames test is reduced and does not act correctly or precisely (repair of damaged fragments cutting out and inserting correct fragments). Although the strains have a repairing system, it makes mistakes (SOS). In order to check whether applied strains exhibit an appropriate sensitivity, they undergo the so-called **positive control**. It relies on examining the size of the provoked reversion by mutagens with a known activity. In the practical laboratory exercise the mutagen daunomycin is used which acts a anti-cancer antibiotic produced by the *Actinomycetales*. It is a direct mutagen.

Many compounds reveal their mutagenic (carcinogenic) properties only after their transformations inside the body, mainly in the liver. In order to create similar conditions inside the human body, a studied sample is subjected to activation with the help of an enzymatic fraction; this fraction is obtained from the liver of a rat.

The Ames test is suitable for studying a broad spectrum of compounds and also for examining environmental samples such as water, soil, air, plant mass and urine among others. Materials taken from the environment before testing are extracted using appropriate solvents or absorbed on special resins. Extracts or eluents are then evaporated, filled with appropriate solvent and entered into the test. This test is not very sensitive towards pesticides and metal compounds with the exception of Cr^{+6}.

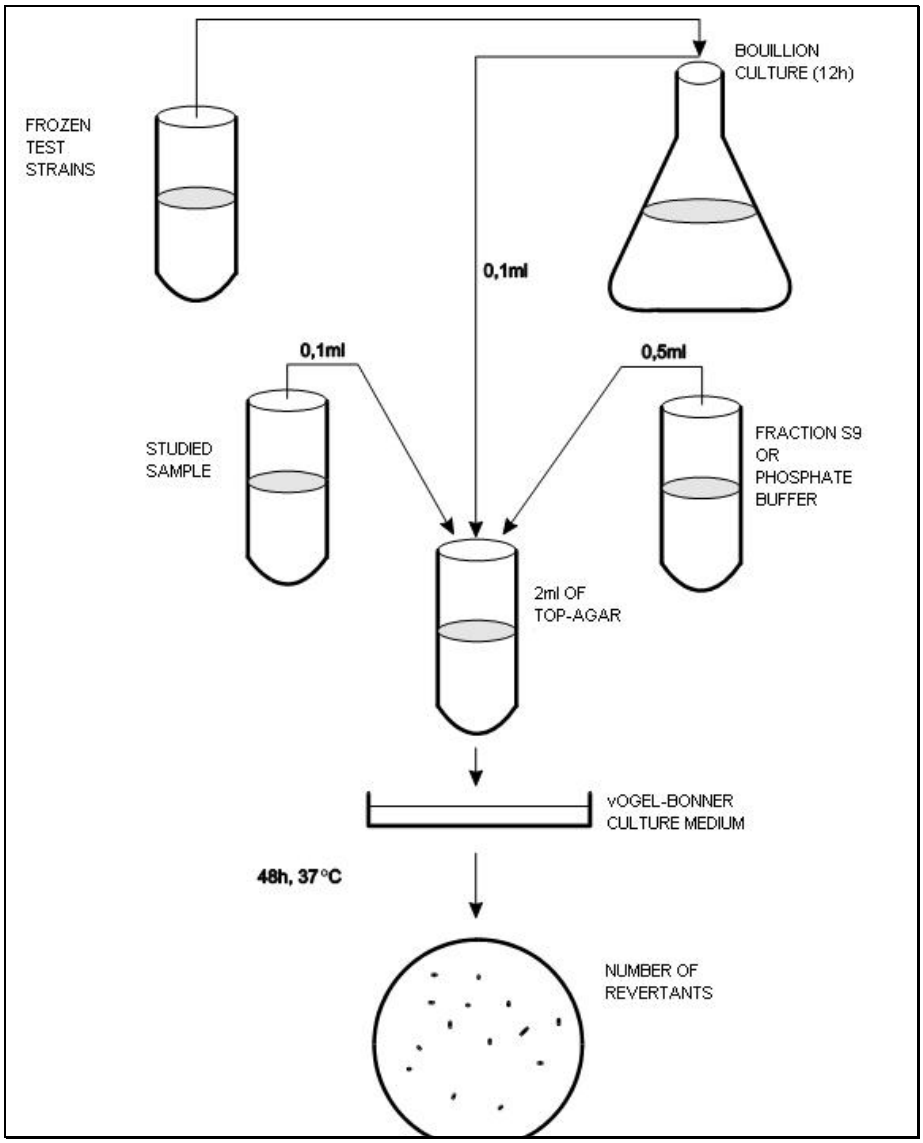


Fig 33. Scheme of the Ames test.

9.3. “REC ASSAY” TEST

Another test often applied is the bacterial test "rec-assay" (from English: recombination assay test). This method is based on examining the **increased lethality** (**fatality**) of mutagenic compounds on cells of mutants of the ***Bacillus subtilis*** bacterium. These mutants do not exhibit the ability to repair damages in DNA with the help of **recombination** (rec-) in contrast to wild strains (rec+). **Recombination repairing** is still the second method (apart from photoreactivation, repairing by cutting out and SOS which are already known mechanisms) of repairing damage to cells without this mechanism while having a remaining repair systems weakened. Therefore, even relatively small DNA damage could cause their growth inhibition. Thus, in contrast with the Ames test, in the rec-assay the studied substance with mutagenic properties does not cause the mutation in test cells, but evokes DNA degradation inhibiting further growth (the cell is not able to repair damage). However, the wild strain is able to grow until the potency of the mutagen does not exceed the repairing abilities of cells. The mutagenic properties of the sample are assessed by comparing the length of the growth line of both strains. The “rec-assay” is a sensitive test particularly useful when examining metal compounds for which the Ames test is less sensitive.

PART TWO

LABORATORY PRACTICE

1. LABORATORY 1

AIM: Evaluating the dependence of toxicity on the type of tested organism

Exercise 1.

Materials:
This exercise should be done with the use of a few species of tested organisms: producers and consumers inhabiting the water environment. The influence of selected disinfectant (commonly used in households) on the mortality of tested organisms will be studied. The aim of this exercise is to assess the sensitivity of tested organisms to studied/ selected disinfectants.

Procedure:
- Prepare the following solutions of disinfectants: 10x, 100x and 1000x.
- Select 10 specimens from each tested organism (producers and consumers) and introduce them into 5 plates with a diameter of 5 cm.
- Remove excess water from the plates with a Pasteur pipette and add prepared solutions of disinfectants; the sample with water is only the control
- After 10 minutes count the number of dead and alive specimens in all samples.

Results:
Place obtained results in table form. Asses the dependence of sensitivity of selected organisms on selected disinfectants on the basis of obtained results.

2. LABORATORY 2

AIM: Evaluating toxicity dependence on type, concentration and time of disinfectant activity.

Exercise 1.

Materials:
Four different types of disinfectants which are usually used in households.
The experiment should be conducted with the tested organism chosen on the basis of results from previous experiments. The most sensitive organism to disinfectant activity should be chosen.

Procedure:

- Prepare half- solutions of disinfectants which derive from their original solution. It is necessary to prepare at least 5 solutions from each of the selected disinfectants.
- Chose 10 specimens from each tested organism (producers and consumers) and introduce them into 6 plates with a diameter of 5 cm.
- Remove excess water from the plates with a Pasteur pipette and add prepared solutions of disinfectants (5 ml); the sample with water is for the control
- Count the number of dead and alive specimens after 10, 20, 30, 40 minutes in all samples.

Results:
Place obtained results in table form. Draw a chart of mortality dependency on concentration and time exposure for each disinfectant. Indicate the most toxic disinfectant on the basis of the results.

3. LABORATORY 3

AIM: Evaluating the influence of contaminants on river plankton biocenosis with the use of the species biodiversity assessment method.

Exercise 1.

Materials:
Microscopes, slides, samples of river biosestone obtained above and below sludge discharge.

Procedure:
Mix the sample. Prepare microscope slides from both sites. Using a lens with the magnitude 10 x define the number of species (without identifying them) and the number of specimens per 30 randomly chosen fields for each sample.

Results:
On the basis of obtained data create a chart for the dependence of species number on the number of specimens for each sample. Axis x: range 1–2 specimens, 2–4, 4–8, 8–16 , etc. in geographic progress; axis y: one species. Calculate the index of species richness for both samples. Compare charts and draw conclusions.

4. LABORATORIES 4, 5, 6

GENERAL AIM: During these three classes we are going to focus on the toxicity of selected disinfectants on water biocenosis. This includes the performance of lethal and physiological tests with the use of indicator species at different food chain levels.

Exercise 1

Lethal test for the acute toxicity assessment of household chemicals based on consumers.

Aim: To be familiar with the common use of lethal tests and with the method of calculating LC_{50}.

Materials:
Results of exercise 1 from laboratory 1 (this is the basis for the calculation of LC_{50} -according to the Reed method). Lethal tests are the main tool which allows for assessing the influence of toxicity on water species. Methods of calculating the toxicity are various: Reed method, probit method, method of graphic interpolation.

All observations should be placed in a table in order to calculate the LC_{50} by the Reed method. This method is based on the presumption that the studied species survived the exposure of a certain concentration; they would also survive in lower concentrations. If they died at a certain concentration, they would also die at higher concentrations. Thus, by cumulating the number of survived, alive and studied we can calculate the "cumulative death-rate percentage" (P).

Table 1. The example of results for the calculation of LC_{50} by the Reed method.

Concentration	Number of animals		Number of animals after cumulation			Percentage of mortality
mg/dm^3	dead	alive	dead	alive	studied	P=m•100/b
1.0	2	8	2	10	12	16.6
2.0	8	2	10	2	12	83.33
4.0	10	0	20	0	20	100

Calculation of LC_{50} : $\log LC_{50} = \log x + k \bullet \log i$
$k=(50–P1)/(P2–P1)$
x – concentration causing the nearest 50% of mortality
i – quotient of geometric progression (1.1 ... 2.0)
k – deviation of coefficient

P1 –cumulative % of mortality lower than 50%
P2 – cumulative % of mortality higher than 50%

Results:
The results should be compared for all tested organisms (compare all results of LC_{50}); then conclusions should be drawn.

Exercise 2
Test the growth inhibition assessing the influence of household chemicals on destruents: *Pseudomonas fluorescens* and *Escherichia coli* (rapid test on bacteria). The aim is to become familiar with the commonly used physiological tests on bacteria.

Materials:
Distilled water, sterile physiological salt, chemicals, suspension of bacteria: *Pseudomonas fluorescens*, *Escherichia coli*, sterile filter paper, agar, automatic pipettes, methylated spirits, pincers, millimeter paper, bent glass rod

Procedure:
Prepare 4 of the half-concentrations (10%, 5%, 2,5%, 1,25%) and do a carpet inoculation of 100 μl of homogenic bacteria suspension with extinction E=0.1 on agar. Put 5 sterile rollers on the inoculated base. Put 10 μl of the study compound (4 concentrations), prepare a control by adding 10 μl physiological salt. Numerate and label rollers on the bottom of the plates. Incubate for 24 or 48 hours with a temperature of 20°C.

Results:
After incubation, measure the width of inhibition zones around the rollers and compare with the control sample. The results should be illustrated in the table using "+" or " – " signs when assessing bacterial growth.

Exercise 3
Growth test assessing the influence of various household chemicals on the producer representative *Lemna minor*

Lemna minor is a plant. Because of its small size and easy growth it is commonly used in toxicology. The influence of a toxicant on *Lemna* sp. is assessed on the basis of its physiological condition (biomass, chlorophyll amount), its morphology, size, leaf shape, their color (chlorosis), root length.

Materials:
Water for dilution, chemicals (1%, 0.5%, 0.25%, 0.125%), *Lemna minor*, nutrient for *Lemna*, pipettes, loops, measuring cylinder, jars.

Procedure:
A maternal culture should be prepared in Erlenmayer flasks (200 cm^3) and filled with up to 100 cm^3 of nutrient for *Lemna* under the light of 2500 lux and a temperature of 20°C. After 8–10 days 2–4 plants (consisting of 3 parts) should be transplanted into a new Erlenmayer flask filled with fresh culture medium.
Prepare half-concentrations of the study compounds using water for dilution in order to obtain final concentrations after introducing culture medium. The test should be done in 5 concentrations and in 3 repetitions for each concentration (including the control sample). Introduce five plants from the maternal culture into the nutrient. Such prepared samples should be kept under the same conditions as the maternal culture. Incubate them for 7 days; mix the culture once a day.

Results:
After 7 days: observe the morphological changes of plants, determine their weight and measure the length of their roots. The results should be put in a table with conclusions drawn.

Exercise 4
Growth test assessing the influence of various household chemicals on algae representatives: *Chlorella* sp. or *Scenedesmus*.

Materials:
Liquid maternal culture of algae: *Chlorella* or *Scenedesmus*, chemicals (1%, 0.5%, 0.25%, 0.125%), nutrient for algae, pipettes, measuring cylinders, slides, filter paper, Fuchs-Rosenthal counting chamber.

Procedure:
Prepare the half-concentrations of studied compounds using water for dilution in order to obtain final concentrations after introducing the culture medium. The test should be done in 5 concentrations and with 3 repetitions for each concentration (including the control sample).
Introduce prepared concentrations into test tubes (9 cm^3) add 1 cm^3 of algae from the maternal culture. The prepared samples should be kept under the lighting of 2500 lux and a temperature of 20°C. Incubate them for 7 days. After 7 days, determine the number of algae in cultures. The results should be put in a table with conclusions drawn.

Calculating the number of algae in the Fuschs-Rosenthal counting chamber
The chambers are ruled with the Fuchs-Rosenthal pattern. This consists of 16 one square millimeter areas orientated by triple lines and each area is sub-divided into 16 squares. It is generally recommended to count randomly 16 one square millimeter areas preferably 8 in each chamber. Each sample should be diluted and transferred with the use of a pipette into a chamber which has a 3.2 mm^3 volume.

Results:
The number of algae in 1ml of suspension should be calculated using the following formula:

$$X = (a * 2)* 1000 /3.2*R$$

where:
x – number of cells/ ml,
a – the number of cells counted in 8 squares,
1000 – conversion factor which enables a value into the volume of 1 cm^3 (1 ml),
3.2 mm^3 – internal volume of the counting chamber,
R – dilution.

Exercise 5
Physiological test assessing the influence of various household chemicals on the photosynthesis rate in algae: *Chlorella sp.* or *Scenedesmus sp*.

Materials:
Liquid nutrient of algae: *Chlorella* or *Scenedesmus*, chemicals (1%, 0.5%, 0.25%, 0.125%), culture medium for algae, pipettes, measuring cylinders, slides, filter paper, Fuchs-Rosenthal counting chamber.

Procedure:
Prepare the half-concentrations of studied compounds using water for dilution in order to obtain final concentrations after introducing the culture medium. The test should be done in 5 concentrations and with 3 repetitions for each concentration (including the control sample).
Introduce prepared concentrations into bottles (45 cm^3), add 5 cm^3 of algae from maternal culture and close the bottles tightly. The prepared samples should be kept under the lighting of 2500 lux and a temperature of 20ºC. Incubate them for 7 days. After 7 days, determine the amount of oxygen using an oxygen electrode. Determine the amount of chlorophyll also by assessing the extinction of acetone extracts which should be obtained by the filtration of the culture, wave length λ = 652 nm (chlorophyll a) and 645 nm (chlorophyll b).

Results:
The chlorophyll a and b levels should be obtained from the standard curve.
Results should be placed in a table with conclusions drawn.

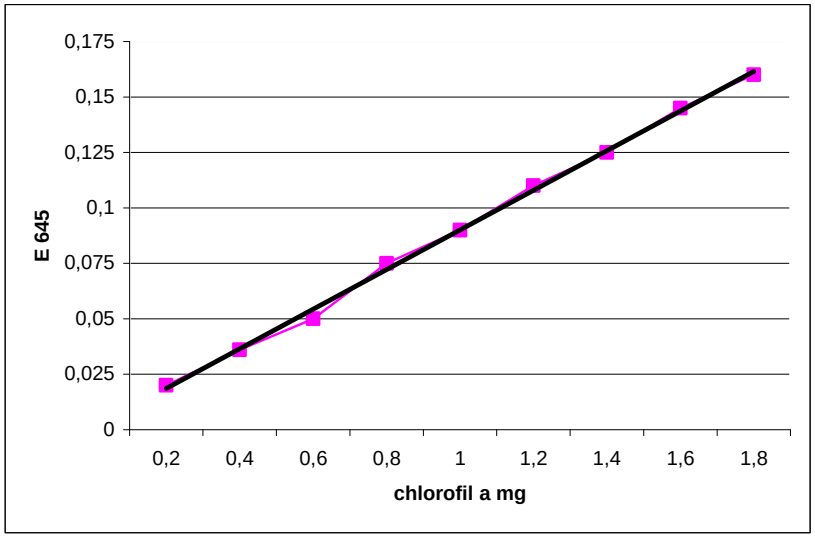


Fig. 1. Standard curve for chlorophyll a.

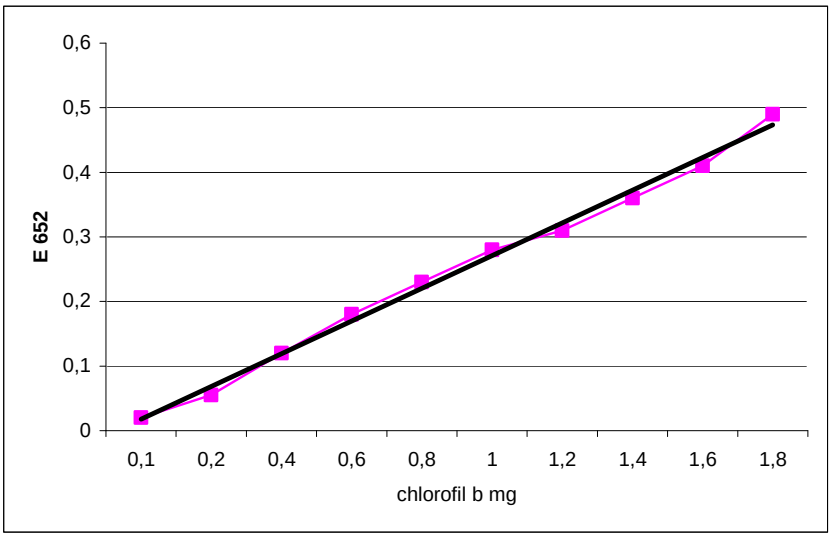


Fig. 2. Standard curve for chlorophyll b.

Exercise 6

Physiological test assessing the influence of various household chemicals on the enzymatic activity of bacteria. The aim is to become familiar with the influence of selected chemicals on the dehydrogenase activity of bacteria.

Materials:

Sterile physiological salt, chemicals, suspension of bacteria: *Pseudomonas fluorescens*, *Escherichia coli*, sterile filter paper, agar, automatic pipettes, methylated spirits, pincers, millimeter paper,

Procedure:

Add 9 cm^3 of bacteria suspension or activated sludge. Add the study compound so that its concentration will be 10%, 5%, 2.5% and 1.25%. The cultures should be incubated in the dark with a temperature of 37°C for 10 minutes; then determine the dehydrogenase activity by the TTC test method.

The determination of dehydrogenase activity

Triphenyltetrazolium chloride (TTC), as an acceptor of hydrogen and electrons, changes into water-insoluble red triformozan (TF) as a result of its reduction. The color intensity relates to the dehydrogenase activity of the studied material.

- Add 2 ml of homogenic suspension of bacteria or activated sludge into a flask

- Add also: 2 ml of buffer TRIS (pH 8.4) and 0.4 ml of glucose solution (1.5%) and TTC (0.2%)
- Incubate at 37°C (no light) for 35 minutes until a distinct red color develops,
- At the same time prepare a control sample (blank), which is the same mixture as the study samples, but without any bacteria. Instead of the bacteria suspension add 2 ml of distilled water into a flask. Incubate also at the same temperature 37°C, but for only 5 minutes.
- After incubation, decant and add a drop of 96% H_2SO_4 in order to inhibit the reaction,
- Take 4 ml with the pipette from the content of the flasks and put this to separate plates; then add 5 ml of buthanole (acetone),
- Put in a water bath (temperature 90°C) for 6 minutes,
- Correct the volume of buthanole and rotate 6 000 rotation per minute,
- Read the extinction of the buthanole fraction for the wavelength 485 nm.

Results:

On the basis of extinction read the amount of TF in mg from the standard curve below.

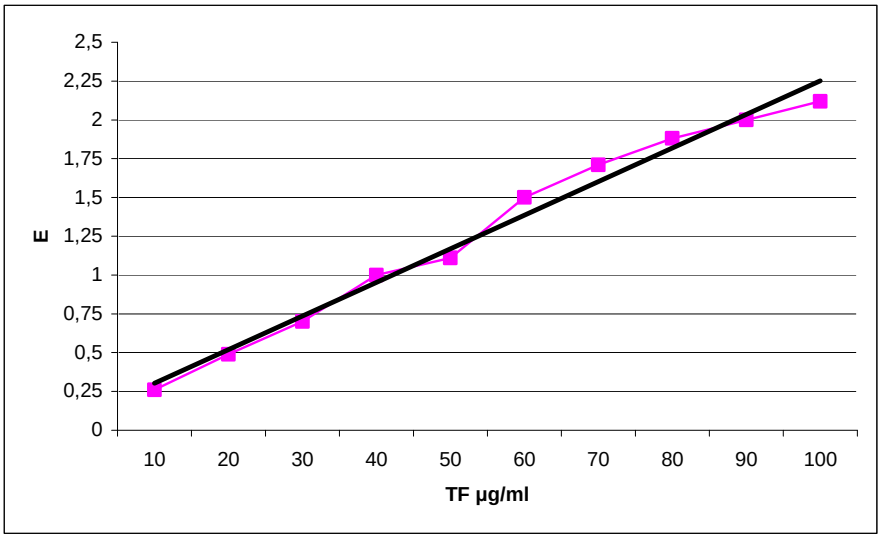


Fig. 3. Standard curve for TF.

5. LABORATORIES 7, 8

GENERAL AIM: During these three classes we are going to focus on the ecotoxicological assessment of contaminated soil. This includes the performance of lethal and physiological tests with the use of indicator species at different food chain levels.

Exercise 1
Inhibition test for the following plants: barley (*Horodeum vulgare*), rye (*Secale cereale*), oats (*Avena sativa*), corn (*Zea mays L. V. saccharata*), white charlock (*Sinapsis alba*), black charlock (*Brassica nigra*), lupin (*Lupinus angustifolius*).

Materials:
Germinated seeds (36–48 h in temperature of 20ºC - incubation in darkness), vases or jars, soil (contaminated by heavy metals, PCB, fuel oil and petroleum) and the control.

Procedure:
Prepare 5 half-concentrations of contaminated soil using the control. Fill up vases with prepared samples (50 g of soil per sample). Put 10 germinated seeds per one vase filled with contaminated soil, 1.5 cm beneath soil surface level. Incubate samples in a phytotron for 7 days. The level of soil humidity should be kept at 80% WHC (the whole water capacity). Apply both artificial and natural light (16 h/d), dividing into day and night. The experiment should be conducted at a temperature of 20±2°C.
At the end of the experiment the plants should be taken out and washed; the following parameters should be measured:

- count the number of plants
- weigh the mass of the plants
- measure the shoot length
- measure the root length
- determine the longest root and shoot

Results:
Determine the average for all parameters separately for each soil type and compare with the results obtained for the control. The report should include charts illustrating the dependence of the number of plants, plants mass, shoot and root length on the concentration of toxicant. Calculate the IC_{50} for each parameter.

Exercise 2
Inhibition test of root length for *Lepidium sativum*

Materials:
Germinated seeds (36–48 h in temperature of 20ºC – incubation in darkness), Petri dishes, soil (contaminated by heavy metals, PCB, fuel oil and petroleum), control. Seeds of cress (*Lepidium sativum*) are used for the exercise. At the beginning, the seeds should be sieved and moistened by distilled water tissue paper, then placed inside plates. The incubation should last from 17 to 24 hours at a temperature of 25°C. Seeds whose roots are about 1mm length should be chosen for further study.

Procedure:
Prepare 5 half-concentrations of contaminated soil using the control. Fill up plates (of diameter 9 cm) with prepared samples (60 g of soil per sample). Prepare the control. Put 25 germinated seeds per one plate filled with contaminated soil. Incubate samples in darkness for 24 hours. Count the number of germinated seeds (%) and measure the length of roots. Growth inhibition should be assessed with the following formula:

$$I\% = \frac{Lk - Lt}{Lk} * 100\%$$

Lk – the average length of roots in control samples,
Lt – the average length of roots in studied samples.

Results:
The report should include charts illustrating the dependence of the percentage of germinated seeds N [%] on the concentration of toxicant C [%], the dependence of the growth inhibition I [%] on the concentration of toxicant C[%].

Exercise 3
The acute toxicity test on the earthworm *Eisenia fetida*

Materials:
Animals for experiments – earthworm *Eisenia fetida*, vases or jars, soil (contaminated by heavy metals, PCB, fuel oil and petroleum) and the control.

Procedure:
Each soil sample should be sieved with the help of a sieve with a mesh diameter of 0.5 mm in order to obtain a homogenous granulation. Then, fill up jars of 1.5 dm^3 volume with prepared samples (800 g of soil per sample). Select animals according to their weight and size. All specimens should have a similar weight. The average

mass of animals should be between 250–350 mg. The Animals should be washed, dried and weighed before the beginning of the experiment. Put the animals into jars filled with contaminated soil (5 concentrations and control). The level of soil humidity should be kept at 60% WHC (the whole water capacity). Apply natural light only (800 lx), dividing into day (8 h) and night (16 h). The experiment should be conducted at a temperature of 20±2ºC. The exposition of animals on different concentrations of studied soil should last 14 days.

Results:
After 7 and 14 days count the number of all animals in jars, weigh and measure them. Calculate $LC_{50.}$

Exercise 4.
The assessment of contaminant influence on the dehydrogenase activity in soil

Method description:
Triphenyltetrazolium chloride (TTC), which is transparent in water extract, as an acceptor of hydrogen and electrons changes into water-insoluble red triformozan (TF) as a result of its reduction (it takes over hydrogen and electrons from TTC dehydogenases. The color intensity reflects dehydrogenase activity of the studied material.

Materials:
Solutions of contaminated soil (contaminated by heavy metals, PCB, fuel oil and petroleum) and control test tubes, corks, $CaCO_3$

Procedure:
Mix 6 g of soil with $CaCO_3$, put in test tubes, add 1 ml of 3% TTC water solution and 2.5 ml of H_2O. Incubate samples 24 h at a temperature of 37°C in the darkness. After incubation add 5–6 ml of acetone and filtrate using filter paper. Read the extinction. The content of TF in samples should be read from a standard curve. Draw a chart illustrating the dependence of the dehydrogenase activity of soil on the concentration of the toxicant. Calculate EC_{50}.

6. LABORATORIES 9, 10

GENERAL AIM: During these two classes we are going to focus on the ecotoxicological assessment of contamination caused by air pollution.

Exercise 1.
The acute intoxication of plants caused by acidic gases: sulfur dioxide and nitrogen oxides
Cases of acute intoxication of plants caused by air contamination from industrial emissions do not happen very often; it usually occurs as a result of an ecological catastrophe caused by accidents. The intoxication may also be the result of acid rain.

Materials:
Eccsicators, crystallizators with hydroponic culture of cress, weighing dish, metal spoon for combustion, concentrated nitric acid, copper filings, sulfur

Procedure:
Prepare three eccsicators. Put the crystallizators with the hydroponic culture of cress inside the eccsicators. Close the first eccsicator with the control culture. Introduce a spoonful of burning sulfur into the second eccsicator. Close it immediately after burning the sulfur and remove the spoon. Put the weighing dish with concentrated nitric acid into the third eccsicator. Pour copper filings into the weighing dish and close the third eccsicator.
As a result of combustion, sulfur dioxide appears. Copper reacts with concentrated nitric acid and as a result transparent nitrogen oxide is produced. Nitrogen oxide reacts with atmospheric oxygen; thus, brownish nitrogen dioxide appears:

$$3\ Cu + 8\ HNO_3 = 3\ Cu(NO_3)_2 + 4\ H_2O + 2\ NO$$
$$2\ NO + O_2 = 2\ NO_2$$

Results:
Observe and note the reaction of plants (including time).

Exercise 2.
The influence of acidic gases on transpiration intensity.

Materials:
Young tomatoes, flasks, corks with pores, black canvas, device for pH measurement, chemicals for the preparation of Knopp culture medium, concentrations of sulfuric acid and sodium base for pH measurement

Procedure:
Place ten young tomato plants in flasks and cover with corks (with pores). The flasks should be filled with culture medium for a hydroponic culture (the recipe is described below). Half of the culture medium should be filled with sulfuric acid with a pH up to 3.5; the rest should be filled with a sodium base with a pH up to 6.6. Five tomatoes should be planted in the culture medium with the acidic pH and the next five with the culture medium with the neutral pH. Mark the level of culture medium in all flasks. Refill evaporated water with a pipette after two days of starting the experiment; note the amount of water used for refilling. Repeat this step weekly and as needed.

Results:
Draw a chart illustrating the obtained results (the dependence of the amount of transpired water on time).

The preparation and procedure of hydroponic culture:
Knopp culture medium: $Ca(NO_3) \cdot 4\ H_2O$ – 1.5 g; KNO_3 – 0.25 g; $MgSO_4 \cdot 7\ H_2O$ – 0.25 g; KCl – 0.12 g; KH_2PO_4 – 0.25 g; $FeCl_3$ 5% – 5 drops; microelements – 1 cm^3; distilled water 1 dm^3;
A modified concentration of microelements according to Arnon: H_3BO_3 – 2.86 g; $MnCl_2 \cdot 4\ H_2O$ – 1.81 g; $ZnSO_4 \cdot 7\ H_2O$ – 0.222 g; $CuSO_4 \cdot 5\ H_2O$ – 0.079 g; H_2MoO_4 – 0.084 g; distilled water – 1 dm^3
Preparation of seedlings for the water culture: put seeds into sterilized, clear quartz sand 10–14 days before the laboratory. Cover the pot with glass. Fill the coaster with water and later refill it as often as needed (the sand should be moist enough). When the seedlings grow up to 2–3 cm and the first leaves appear, the plants should be removed from the sand and placed in a beaker with the culture medium: sand should be removed with seedlings and put into crystallizators filled water; shake slightly in order to wash out sand with water. If an endosperm is big, the seeds should be cut without damaging the roots.
The seeds can be put on stretched gauze covering the crystallisator, filled with water, which is placed in a larger crystallizator also filled with water and covered. The water should be refilled as needed. The seedlings should be left in the crystallizator until the development of the first two leaves. When the seedlings develop the first two leaves, they should be put in the pores of corks with the help of cotton wool.
Water cultures: Jars (flasks), painted outside black or covered by black canvas, should be refilled with culture medium up to 0.5–1.0 cm from the cork. Seedlings are placed in the pores of corks and sealed by cotton wool. The roots should be immersed in culture medium up to the root neck so as not to soak the cotton wool. The water culture should be placed in intense light and ventilated every day by pouring the culture medium into spare dish and again to previous dish. The lost culture medium should be refilled regularly.

Exercise 3.
The influence of soil on plant development

The acidification of capillary water in soil causes the destruction of the rhyzoshere and the underdevelopment of the root system affecting the overall condition of plant. The acidification of capillary water in soil leads to the washing out of nutrients from the soil. As a consequence, such nutrients are lacking and toxic ions of metals (for example Al) are taken up by the plant in large amounts leading to intoxication. Acidic gases and rain as well as natural limiting factors have a large influence on plant growth in acidic soil.

Materials:
Small pots, young tomato plants, distilled water, 0.5 n solution of sulphuric acid, scissors, laboratory scale, pH meter.

Procedure:
Plant 10 young tomato plants per pot. Half of the plants should be watered with distilled water (similar in composition to rainwater) while the rest of the plants should be watered with sulphuric acid (pH=2.0) based on distilled water. The plants should be dug out, cut and weighed (shoots and roots separately) after a few weeks.

Results:
Calculate the decrease of mass growth of roots, shoots and whole plants (%) in relation to the control.

Exercise 4.
Observation of symptoms of industrial forest disease

Materials:
Specimens and photos.

Procedure:
Observe specimens and photos depicting the symptoms of industrial forest disease. Make the following drawings.
1. Thinning out of the treetop of a coniferous tree
2. Drying at the top of the tree
3. Needles becoming yellowish and brownish
4. Spots on leaves
5. Deformations and underdevelopment of leaves

Exercise 5.
Microscopic observation of slides of alveolus and lymph nodes of humans with pneumoconiosis and healthy humans

Dust inhaled by humans accumulates in lung tissues (the pathway of entry), and in lymph nodes (the way of transport in order to excrete it from the organism). For a medical diagnosis a histopathologic examination of lymph nodes is applied. It is much easier to take samples for analysis from lymph nodes than from lungs.

Materials:
Microscopic slides of alveolus and lymph nodes of humans with pneumoconiosis and healthy humans

Procedure:
Conduct microscopic observations and drawings. Pay attention to the overgrowth of connection tissue and dust incrustation.

Exercise 6
Determining the amount of SO_2 in air with the lichen scale

Materials:
Lichen scale

Procedure:
Determine the SO_2 concentration in the air of the study area by using the lichen scale. Important principles of using the lichen scale:

1. Examine photographs of lichens and algae, pay the attention to their shape, size and color;
2. Search for lichens on the bark of deciduous trees growing in the field of study;
3. Compare these lichens with those in the photographs;
4. Read out and note the maximum SO_2 concentration at which one can still find lichens, give the number of the pollution zone;
5. Repeat the observations of lichens on other trees growing nearby – it will allow the more precise determination of the level of air pollution from sulfur dioxide in study area.

Exercise 7
Bioindication of sulfur dioxide with red clover (*Fabaceae* representatives)

This method is applied for determining the concentration of sulfur dioxide in atmospheric air. Sulfur dioxide reacts on plants in the form of a gas (it makes the connection with water in cells creating sulfuric acid), acid rain and acidic capillary water in the soil. In this exercise, for practical reasons, the spraying of a solution of

sulfuric acid was applied as the equivalent of acid rain also causing acidification of the capillary water in soil.

Materials:
Seeds of a red clover, containers with garden soil, sprayer, distilled water, sulfuric acid, pH meter

Procedure:
Plant the clover in 4 containers. Spray the plants with distilled water (with composition similar to rainwater) in one container; spay plants with sulfuric acid for the pH: 2.5, 3.0, 3.5 in the rest of the

containers. Repeat this activity for a few weeks with frequency to prevent the drying of the soil. Observe necroses of the leaves. Measure the surface of the leaves and necrotic stains.

Results:
Calculate % of leaf surface covered with necrotic changes.

Exercise 8
The reduction of germination capacity of seeds as an indicator of the toxicity of chimney dusts and contaminated soil

Germination is a one of biorections used when examining environmental pollution. Water-soluble toxicants have an inhibiting effect on this physiological process.

Materials:
Seeds, cellulose tissue, chimney dust or toxic soil, 6 large Petri dishes, 6 beakers of 100 cm^3 capacity, laboratory scale.

Procedure:
Prepare the row of soil extracts or chimney dust suspected of toxicity with the following concentrations: 2%, 4%, 8%, 16%, 32%. Put cellulose wadding on 6 large plates. Moisten the cellulose wadding with distilled water (control) or with an extract of examined soil or chimney dust. Put 100 seeds on the cellulose wadding in every plate (10 rows for 10 seeds). Cover the plates. Incubate them in darkness at room temperature for 1 week. In case the cellulose wadding becomes dry, moisten it again with distilled water (control) or with the extract having the respective concentration. Count the number of germinated seeds after every 24 hours.

Results:
Depict the number of germinated seeds in the form of a graph as a function of germinated seeds depending on the germination time. Pay attention to differences

in the progress level of seed development cultivated in the presence of extracts depending on different concentrations.

Exercise 9
Bioaccumulation of lead by bacteria
Materials:
Flasks with LB culture medium without agar, with the acetate of lead, containing 0.00, 0.05, 5.00 and 500.00 Pb mg/1 dm^3, *Pseudomonas fluorescens* suspension, rotator, redistilled water, small containers for scale, desiccator, small flasks of 50 cm^3, 1 n HNO_3, analytical balance.

Procedure:
Bacterial culture medium. Prepare 4 flasks containing 100 cm^3 of LB culture medium without agar. One flask should contain the nutrient for control without the toxicant. Add the acetate of lead to three remaining flasks in such amounts that the concentration of lead is 0.05, 5.00 and 500.00 mg/dm^3. Inoculate all flasks with a homogenic suspension of *Pseudomonas fluorescens* by adding 1 cm^3 with a pipette. Incubate for 1 week in room temperature under static conditions.
Centrifugation of bacteria. Spin bacteria with the speed of 12 000 rpm for 15 minutes. Gently pour off the supernatant. Rinse three times with redistilled water of 100 cm^3·Evaporate the water at a temperature of 105°C.

Obtaining the dry mass. Dry the clean small scale container at the temperature of 105°C for 2 hours. After 30 minutes of cooling in the exsiccator with calcium chloride or silica gel, weigh it on the analytical balance with an accuracy of 0.0001 g. Repeat this step in order to get a fixed mass. Pour the suspension of bacteria obtained by centrifugation. Evaporate water at a temperature of 105°C for 6 hours. Cover the small container with a lid, weigh it on the analytical balance with the accuracy 0.0001 g. After weighing, uncover it leaving the lid leaned vertically against the small container. Dry at a temperature of 105°C for 6 hours. After drying, move it with pliers to the exsiccator and after cooling (30 minutes), weigh it again. Drying and weighing should be repeated in order to get a dry mass. The difference between the two last weights should not exceed 0.0003 g.
Mineralization of the bacteria. Mineralize a dry mass of bacteria in the silit stove at a temperature of 550°C for 6 hours. Dissolve 1 n HNO_3 in 10 cm^3. Filter it into small flasks of 50 cm^3 and refill to the line with redistilled water.
The assessment of lead content. Assess the total content of lead in examined bacteria with the atomic absorption method by using the atomic spectroscope at the wavelength 217 nm. In this method the absorption of radiation by atoms of radioactive elements with the characteristic wavelength is used. The higher concentration of examined element is in solution, the higher absorption of radiation at the characteristic wavelength occurs.

Exercise 10
Bioaccumulation of lead by higher plants

Materials:
Plants deriving from the area, grinder, redistilled water, small scale containers, exsiccator, small flasks of 50 cm^3, 1 n HNO_3, analytical balance.

Procedure:
Sample collecting. When taking plant samples their naturalness in the studied area should be considered. When mixed samples are taken consisting of various plant species which are found in the studied area, the proportion among these species should be kept. When various parts from different plant species are collected, a natural proportion among them should be also kept. Selecting plants e.g. according to their age, size, etc. should be avoided. The time of sample collection is dependent on the life cycle (annual) of the examined plants.
Preparation for analysis. The gathered plant material should be cleaned, grinded down and dried off. An initial phase of drying should take place at a temperature of about 30°C; later the temperature can rise gradually to 60°C. The dried material should be crushed into a uniform dust with a grinder. Such prepared material should be kept in a tightly closed container and used for analysis when needed.
Obtaining the dry mass. Dry the clean small scale container at a temperature of 105°C for 2 hours. After 30 minutes of cooling in the exsiccator with calcium chloride or silica gel, weigh it on the analytical balance with the accuracy of 0.0001 g. Repeat this activity in order to get a fixed mass. Place 2–4 g of well mixed plant material into the small container with a teaspoon or spatula and cover with a lid; weigh this on the analytical balance with the accuracy of 0.0001 g. After weighing, uncover it leaving the lid leaned vertically against the small container. Dry at a temperature of 105°C for 6 hours. After drying cover with the lid, move it with pliers to the exsiccator and, after cooling down (30 minutes), again weigh it. Drying and weighing should be repeated in order to obtain a dry mass. The difference between two last weights should not exceed 0.0003 g.
Mineralization of the plant material. Mineralize a dry mass of plants in the silit stove at a temperature of 550°C for 6 hours. Dissolve 1 n HNO_3 in 10 cm^3. Filter it into small flasks of 50 cm^3 and refill to the line with redistilled water.
The assessment of lead content. Assess the total content of lead in the examined material with the atomic absorption method by using the atomic spectroscope at the wavelength 217 nm.

7. LABORATORY 11

GENERAL AIM: During this class we are going to focus on the microbiological degradation of xenobiotics in order to understand the selected methods of determining the decomposition level of these substances and to assess the impact of chemical contamination by phenol, lead acetate and potassium dichromate on the ability of microorganisms to degradate saccharides, fats and proteins.

Exercise 1
Degradation of phenol by *Pseudomonas fluorescens* (a bacteria strain).

Materials:
Suspension of *Pseudomonas fluorescens*, flat-bottomed flasks of 250 cm^3 with the minimal culture medium of 80 cm^3, 0.1 % solution of phenol, small measuring flasks of capacity 50 and 100 cm^3, 1 N solution of Na_2CO_3, solution of two-phases p-nitroaniline.

Procedure:
Add 1 cm^3 of 0.1 phenol solution to the flat-bottomed beaker of 250 cm^3 containing 80 cm^3 of sterile minimal culture medium and inoculate it with *Pseudomonas fluorescens*. Prepare simultaneously a control set containing a sterile minimal culture medium and phenol in the same amount as in the sample with bacteria. Place the beakers on a shaker and incubate them at room temperature for 72 h. After this time, take 80 cm^3 of culture and spin it for 10 minutes at a speed of 10000 rpm and then asses the phenol content in *w* 1 cm^3 of supernatant. Simultaneously estimate the phenol content in the control sample.
Phenol identification: Introduce 1 cm^3 of sample into the measuring small flask of 50 cm^3capacity, add 20 cm^3 of 1 N solution of Na_2CO_3 and 10 cm^3 of p-nitroaniline. Fill it up to 50 cm^3 volume with distilled water, mix and read the absorbance for the wavelength 460 nm after 5 minutes. On the basis of absorbance, read the content of the phenol in 1 cm^3 of the examined sample. Estimate the phenol loss in the examined sample and compare it with the the control.
Preparation of the standard curve: Transfer 1, 2, 5 and 10 cm^3 of phenol solution containing 0.01 mg phenol in 1 cm^3 (100x diluted of 0.1% phenol solution) into four small flasks of 50 cm^3 volume, add 20 cm^3 of 1 N Na_2CO_3 solution and 10 cm^3 of p-nitroaniline solution, fill it up with distilled water to the line and read the absorbance for the wavelength 460 nm after 5 minutes. To zero the device use a blind sample containing distilled water instead of the phenol solution.

Results:
Draw a graph illustrating the dependence of the absorbance on the concentration of phenol in the sample.

Exercise 2
Degradation of a surface-active compound (organophosphates).

Materials:
Solution of surface-active compound containing 5 mg P/dm^3, sodium salt of mononononylophenylphosphorane, suspension of *Pseudomonas fluorescens*, flat-bottomed beakers of 250 cm^3 with the minimal culture medium of 80 cm^3, ordinary and measuring test tubes, funnels and filters, saline solution, distilled water, 10% trichlorineacetic acid TCA, 10n H_2SO_4, ammonium molybdenum, sodium salt of acid 1,2,6-amine-napht-sulfonic, solution of the potassium phosphate (KH_2PO_4) 80 μg P/cm^3.

Procedure:
Add 10 cm^3 of surface-active compound to the flat-bottomed beaker of 250 cm^3 containing 80 cm^3 of sterile minimal culture medium and inoculate it with *Pseudomonas fluorescens*. Prepare simultaneously control sets containing a sterile minimal culture medium and a surface-active compound and a sterile minimal culture medium inoculated with *Pseudomonas fluorescens*. Place the beakers on a shaker and incubate them at room temperature for 24 h. After this time, add 10% TCA solution in order to stop the reaction and estimate the amount of freed phosphorus in samples which means that the hydrolysis of the surface-active compound took place. In order to assess the phosphorus content, spin the samples for 5 minutes at the speed of 10000 rpm and then asses the phosphorus content in supernatant.

Identification of phosphorus according to Fiske-Subbarow method: Introduce 2.5 cm^3 of examined sample into the test tube and add 0.25 cm^3 of 10 n H_2SO_4, 0.4 of ammonium molybdenum and 0.2 cm^3 of sodium salt of acid 1,2,6-amine-napht-sulfonic. Fill the samples with distilled water up to 5 cm^3. Incubate them at a temperature of 37°C for 10 minutes. Read the absorbance for the wavelength 660 nm, zeroing with a blind sample containing 2.5 cm^3 of distilled water instead of 2.5 cm^3 of the tested sample. From the standard curve read the content of phosphorus in every sample. Take into account the values obtained for the control samples. Calculate the amount of freed phosphorus.
This method is based on the fact that in an acidic environment orthophosphate and ammonium molybdenum create ammonium phosphoreomolybdenum. Phosphoreomolybdenic acid under the influence of reducing factors is reduced to mixed molybdenum oxides, called molybdenum blue ($Mo_2O_5 \cdot MoO_3$). Sodium salt of acid 1,2,6-amine-napht-sulfonic is used as a reducing factor.

Preparation of the standard curve: The solution of potassium phosphate (80 µ g P/$cm^{3)}$ should be diluted in order to obtain in 1 cm^3: 8, 16, 24, 32 and 40 µg of phosphorus. Then add distilled water, 10 n H_2SO_4, 2.5% ammonium molybdenum and sodium salt of acid 1,2,6-amine-napht-sulfonic in amounts given by the instructor. Mix the prepared samples and place them into a bath at a temperature of 37°C for 10 minutes. After this time measure the value of absorbance for every sample at the wavelength 660 nm, zeroing with the blind sample.

Results:
Draw a graph illustrating the dependence of the absorbance value on the concentration of phosphorus in the sample.

Exercise 3
The influence of chemical contamination on the decomposition of sugars, fats and proteins.

Procedure:
Preparation of bacterial culture: prepare 4 beakers containing 200 cm^3 of bouillon. Inoculate the bouillon with a dense suspension of the bacteria (*Pseudomonas fluorescens*). Add 5 cm^3 of saturated solutions of phenol, chloride nickel and potassium dichromate into the 3 flasks. Place the beakers on the magnetic mixer for 24–48 h. After this time, spin the content of the flasks, pour liquid out from suspension, wash the suspension twice with NaCl solution. Use such prepared bacterial suspensions for further tasks (a, b, c).

a. Decomposition of strach

Materials:
1% solution of starch, liquid of Lugol, solution of amylase (5 mg/cm^3), physiological salt solution, ordinary and measuring test tubes, funnels and filters

Procedure:
Method 1
Samples consisting of 2 cm^3 of bacterial suspension and 2 cm^3 of 1% solution of starch should be incubated 30 minutes in a bath with a temperature of 38°C. At the same time prepare a blind sample containing 2 cm^3 of water instead of the bacterial suspension. After incubation cool the samples and add 0.4 cm^3 of Lugol liquid. Filter it and read out the absorbance at the wavelength 560 nm. Determine the amylase activity on the basis of the standard curve. In order to prepare the standard curve, measure 1.0, 0.9, 0.8,. down to 0.1, 0.0 cm^3 of amylase solution (5 mg/cm^3) and fill it with 0.9% NaCl up to 1 cm^3. Add 1 cm^3 of starch to each of these solutions. Incubate 30 minutes at a temperature of 38°C, then cool, add 0.2 cm^3 of Lugol liquid and read out the absorbance at the wavelength 560 nm. Draw the standard curve.

Method 2
Introduce 1 cm^3 of saline solution into 9 test tubes. Add 1 cm^3 of bacterial suspension to the first test tube after contact with the toxic compound. Next, do the partial dilutions. Add 1 cm^3 of 0.1% solution of starch into each test tube. Incubate them at a temperature of 37°C for 30 minutes. After this time, add a drop of iodine into each test tube. Note the highest dilution of bacterial suspension in the sample where the tint (coloring) with iodine does not occur. Amylase activity should be assessed on the basis of the highest concentration of bacterial suspension which could decompose 1 mg of starch to products not giving a tint with iodine after 30 minutes of incubation at a temperature of 37°C.

b. Decomposition of fats

Materials:
The culture medium with tributrine (5 g of peptone, 3g of yeast extract, 10 cm^3 of tributrine, 25g of agar-agar, 1dm^3 of distilled water with pH 7.5), bent glass rods, physiological salt solution.

Realization: Carry out a carpet inoculation on the basis of dilutions $10^{-2, -4, -6, -8}$ of examined bacterial suspension on the culture medium with tributrine. Incubate the inoculated plates in a thermostat at a temperature of 25°C. Look for colonies with light zones after 72 h; this suggests the usage of tributrine.

Results:
Count these colonies and refer the results to all colonies per plate.

c. Decomposition of proteins

Materials:
Casein culture medium (10 g of peptone, 0.3 g of K_2HPO_4, 0.5 g of NaCl, 3 g of $CaCO_3$, 1g of $MgSO_4 \cdot 7 H_2O$, 10 g of casein, 25g of agar, 1 dm^3 of distilled water, pH – 7.5), TCA, physiological salt solution, bent glass rods.

Procedure:
Carry out carpet inoculations on the basis of dilutions $10^{-2, -4, -6, -8}$ of examined bacterial suspension on the casein culture medium. Incubate the inoculated plates in a thermostat at a temperature of 28°C. After 48 h, pour 6% of TCA on plates. Look for colonies with clear zones called proteolytic. These zones are devoid of proteins which were digested by TCA.

Results:
Count these colonies and refer the results to all colonies per plate.

8. LABORATORY 12

GENERAL AIM: During this class we are going to focus on the detection of toxic compounds of natural origin.

Exercise 1.
The detection of cyanogenic glicosides

Materials:
Nutcracker, grater, hammer, kitchen board, mortar, small slops, funnel, filter, stand for test tubes water bath for 4 workplaces, 5% $FeSO_4$, 5% NaOH, 10% HCl, H_2O, following fruits:
a) shelled almonds –origin California, USA (result of test -, or poorly positive)
b) almonds flakes – country of origin Spain (result of test – definitely negative)
c) dried plum stones – country of origin Poland (result of test + positive)
d) cherry stones – country of origin Poland (result of test + positive)

Procedure:
After peeling the nutshells, grind the grains and grate them with a porcelain mortar and add 3 ml of distilled water. Put the homogenate into a thermostat at 30^oC or into a water bath for 30 minutes. Next, filter the content with filter paper. Add a few drops of 5% solution of $FeSO_4$, then 1-2 drops of 5% NaOH. Dissolve the formed sludge by adding a few drops of 10 % HCl. If the solution becomes blue (Prussian blue) it means that the CN- group is present.

Results:
Draw conclusions.

Exercise 2.
The detection of caffeine

Materials:
Cafe, mortar, ethanol, small vaporizers, funnel, filter, stand for test tubes, water bath for 4 workplaces and the following items:
a) “Nescafe” instant coffee
b) blended ground “Tchibo” coffee
c) tea
d) instant coffee from “Biedronka ”
As discussed in the presentation, it is possible to show colorful reactions of caffeine by preparing two portions of instant coffee, powdered in the mortar and combined with 5 ml of ethanol (caffeine is well soluble in ethanol and poorly in water), heat up to 50^{o}C for 10 minutes in a water-bath. Next, filter it out with a filter, place the filtrate into the water bath, into two small vaporizers and conduct the characteristic reactions described below.

Procedure:
We can detect caffeine by the two following methods:
1. **Murexid reaction**
A tested sample in the following amount (instant coffee and ground coffee: 1/2 of teaspoon, tea: 1/2 of teabag) should be grinded down with the mortar and divided in two equal parts. Place the first part in the vaporizer and add 10 drops of 3% H_2O_2 solution; add also 1–2 drops of 10% HCl. Evaporate the content to dryness in the water bath and moisten it cautiously with a few drops of ammonia. A characteristic purple coloring may appear.
2. **Reaction with Nessler reagent**
Pour a few milliliters of water over the tea and ground coffee in the vaporizer, cover with a watch glass and warm it for a few minutes evaporating part of the water; next, add 2 ml of Nessler reagent. When caffeine is present, a dark brown sediment will appear. Pour 2 ml of Nessler reagent over the instant coffee and warm it in a water bath. When caffeine is present, a dark brown sediment will appear.

Results:
Draw conclusions.

9. LABORATORY 13

GENERAL AIM: During this class we are going to focus on the mutagenic influence of ultraviolet radiation on organisms.

Materials:

Strain of *E. coli B* sensitive to streptomycin (str^S), 30 cm^3 of $MgSO_4$ solution (00.1 mol/dm^3), 30 cm^3 of bouillon, test tubes with physiological liquid, 10 plates with nutritious agar, 10 plates with nutritious agar + 50 μg/cm^3 of streptomycin, 4 small Petri dishes, ice bath, UV lamp, centrifugal test tubes, pipettes.

Procedure:

Spin 30 cm^3 of all-night culture of the *E. coli* B str^S strain. Pour the supernatant off. Suspend sediments into 30 cm^3 of $MgSO_4$ and put it in an ice bath. Dilute the suspension 10^{-5}, 10^{-6} and inoculate 0.1 cm^3 per every agar plate. Pour out 5 cm^3 of bacterium suspension to 4 small Petri dishes. Irradiate the open plates with a UV lamp for 30, 60, 90 and 120 seconds. Gently mix the content of the plates while irradiating. After the given exposure time, remove the plates one by one from the UV radiation. Protect them from the light. Estimate the titer of every suspension by sieving of 0.1 cm^3 from appropriate dilutions on nutritious agar plates. In order to determine the number of mutants resistant to streptomycin (str^r) from every irradiated suspension, sieve 0.1 cm^3 on two agar plates with streptomycin. Sieve the initial (not irradiated) suspension also to two agar plates with the streptomycin. All cultures should be conducted with the carpet method. Incubate the plates for 24–48 h/37°C.

Results:

Calculate the titer of the bacterium (on plates with nutritious agar) before and after UV irradiating. Calculate the N/N_o log, in which the N_o is the titer of bacterium before irradiating, N – titer of bacterium after irradiating (time t). Draw a curve of the relation N/N_o log for the time t. Calculate the titer of mutants resistant to streptomycin in all samples. Calculate the frequency of the mutation (h) str^S - str^r, for every exposure time: h = M/N

M – titer of mutants in sample

N – titer of all mutants in sample

Draw the curve and estimate the relation h from the exposure time t.

10. LABORATORY 14

GENERAL AIM:
Purpose: During this class we are going to focus on the microbiological methods of estimating the mutagenicity of substances.

Exercise 1
Assessment of the mutagenic effect of chromium with the Ames test

Materials:
Strain of *Salmonella typhimurium* TA 98, Petri dishes with minimal culture medium, small flasks with top-agar containing trace amounts of histidine and biotin, Oxoid bouillon, phosphate buffer, solution of daunomycin, $K_2Cr_2O_7$ (50 mg/cm^3) solution, sterile test tubes, pipettes.

Procedure:
Prepare the following concentrations of $K_2Cr_2O_7$ 50 mg/cm^3, 5 mg/cm^3, 0.5 mg/cm^3. Fill 0.5 cm^3 of phosphate buffer in a sterile test tube. With the use of the automatic micropipette enter 0.1 cm^3 of all-night culture of TA 98 strain into the test tube. Add 0.1 cm^3 of the study compound (one of three concentrations). Place the test tube in a thermostat at a temperature of 37°C for 20 minutes (preincubation). After the incubation, add 2 cm^3 of top-agar, mix it quickly and then pour it out on a plate with minimal culture medium and distribute it regularly. Repeat the previous steps when preparing the positive control; add 0.1 cm^3 of daunomycin solution instead of the examined compound (attention! It is a strongly mutagenic compound). Prepare the control of the spontaneous mutation repeating the steps again, but without adding the examined compound. Prepare all samples in three replicates. The plates should be incubated at a temperature of 37°C for 48 h. After the incubation period, count the emerged colonies for all the plates taking the average of the replicates.

Results:
Assess whether chromium Cr^{+6} exhibits mutagenic properties.

Exercise 2
Assessment of the mutagenic effect of chromium with the "Rec-assay" test.

Materials:
Strain of *Bacillus*: H17 (Rec^+) i M 45 (Rec^-), test tubes with bouillon, petri dishes with nutritious agar, sterile filtration discs (16 mm of the diameter), tweezers, methylated spirit, KCr_2O_7 (5 mg/cm^3) solution.

Procedure: With the help of sterile tweezers put the disc of the filter paper on the surface of the plate with nutritious agar at a distance of 1cm from the edge of the plate. Take the inoculum from the all-night culture of the H 17 strain and spread it in the form of the strip from the edge of the disc. Inoculate in a similar way the M 45 strain at the same plate at a 90° angle in relation to the previous culture. Put two drops of KCr_2O_7 solution into the disc. Incubate for 24 hours at a temperature of 37°C. After the incubation, measure the length of the growth inhibition zones for both strains (from the edge of the disc to the beginning of the growth).

Results:
Compare the studied growth of both strains and assess whether the examined compound exhibits mutagenic properties. The examined substance could be toxic and mutagenic, toxic but non-mutagenic, non-toxic but mutagenic, nontoxic and non-mutagenic.

PART THREE

LITERATURE

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