ENVIRONMENTAL TOXICOLOGY

Lecture notes, part 1

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1. Introduction: effects of chemicals on the environment

Chemical substances in the environment can be hazardous to the structure and function of the ecosystem and through that to humans. The aim of **environmental toxicology** is to characterize the adverse effects of chemical substances on the ecosystem and humans, though we cannot measure these effects directly.

Chemical substances in the environment cause global problems. Not only xenobiotics (men made artificial substances), but also natural substances can be harmful when unusually distributed in the environment or if extreme high values enter the element cycle of the ecosystem.

The **hazard** of a chemical substance originates from its chemical structure. A chemical substance is hazardous either found on a laboratory shelf or referred to in the computer of the designer chemical engineer. However, the **risk** of a chemical substance is manifested once it gets into the environment. The risk, among others, depends on the properties of the organisms using the environment.

In the case of humans we cannot measure the effect of chemical substances as we do with testorganisms. It is not possible to examine the effect of chemicals at various concentrations on a group of individuals and different populations to determine the no effect concentration. In the case of ecosystems we cannot test the same ecosystem either. We cannot include in the protocol the testing of all the constituents of the whole system and the interactions between them, as we are not perfectly familiar with the functioning and structure of a healthy and unharmed ecosystem.

Based on the results of ecotoxicity tests we can predict the effect of chemical substances on humans and on the ecosystem. We can extrapolate to the effects on humans from the test results with testorganisms, which have similar metabolism to humans. The effects on the ecosystem can be predicted by ecotoxicity tests performed with testorganisms from different trophic levels and based on various principles.

Ecotoxicity tests may aim at investigating any level of an ecological system: from molecular level through communities to the whole ecosystem.

Ecotoxicity aims at defining the no adverse effect concentration of a chemical substance in the environment. This can be determined from the concentration-response relationship. Environmental quality criteria should also be based on that. In human toxicology the dose of the chemical substance added to the test animal or the contaminant concentration of the inhaled air is the basis of setting the no adverse effect concentration to human (Gruiz *et al.*, 2001).

2. Environmental toxicology

Environmental toxicology is the science and practice of the adverse effects – mainly of chemicals and other man-made agents – in the environment and through the environment. The targeted receptors of these adverse effects may be both the ecosystem and the human (enfo.hu). Environmental toxicology attempts to anticipate where these substances go in the environment (their fate) and what ecological effects they have when they get there (Calow, 1998, 2009).

Environmental toxicology includes the study of chemical substances – potential and actual contaminants – polluting air, water, soil and food, their impacts upon the structure and function of ecological systems, including man as well as the use of these results for decision making and environmental management (enfo.hu).

As an overall assessment of the whole ecosystem is still impossible and also very expensive, environmental toxicology uses typical species selected from the ecosystem or laboratory testorganisms to examine their response to the chemicals. We may extrapolate from these data to the whole ecosystem (Gruiz *et al.*, 2001).

The two main reasons for measuring the ecological effects of chemicals are:

- To anticipate how toxicants are likely to impact ecological system. The hazard of the chemicals originates from their physical and chemical properties. These tests are applied for example to chemicals prior to release and to existing chemicals for which actual effects may still be unknown.
- To assess what changes are taking place in ecological systems under the influence of released substances, so to assess the environmental risks of the chemicals (Calow, 1998, 2009).

Environmental toxicology requires **multidisciplinary** approach of a variety of specialists.
Components of environmental toxicology are:

- Analytical chemistry
- Biology
- Biochemistry
- Biometrics
- Chemistry, chemical engineering
- Ecology
- Evolutionary Biology
- Limnology
- Marine Biology and Oceanography
- Mathematical and Computer Modelling
- Meteorology
- Microbiology
- Molecular genetics
- Pharmacokinetics
- Physiology
- Population biology
- Risk Assessment
- Risk management (Gruiz *et al.*, 2001)

3. *Environmental toxicology in risk management*

The results of environmental toxicology are used in the prediction of hazard and risk of single chemicals and contaminated environment, supporting decision making in environmental management and policy (Figure 1). Environmental toxicology in risk management has its main role in designing monitoring systems, in risk assessment, in establishing risk based environmental quality criteria, in the selection of the appropriate risk reduction measure and in the determination of the target value of the remediation (Gruiz *et al.*, 2001).

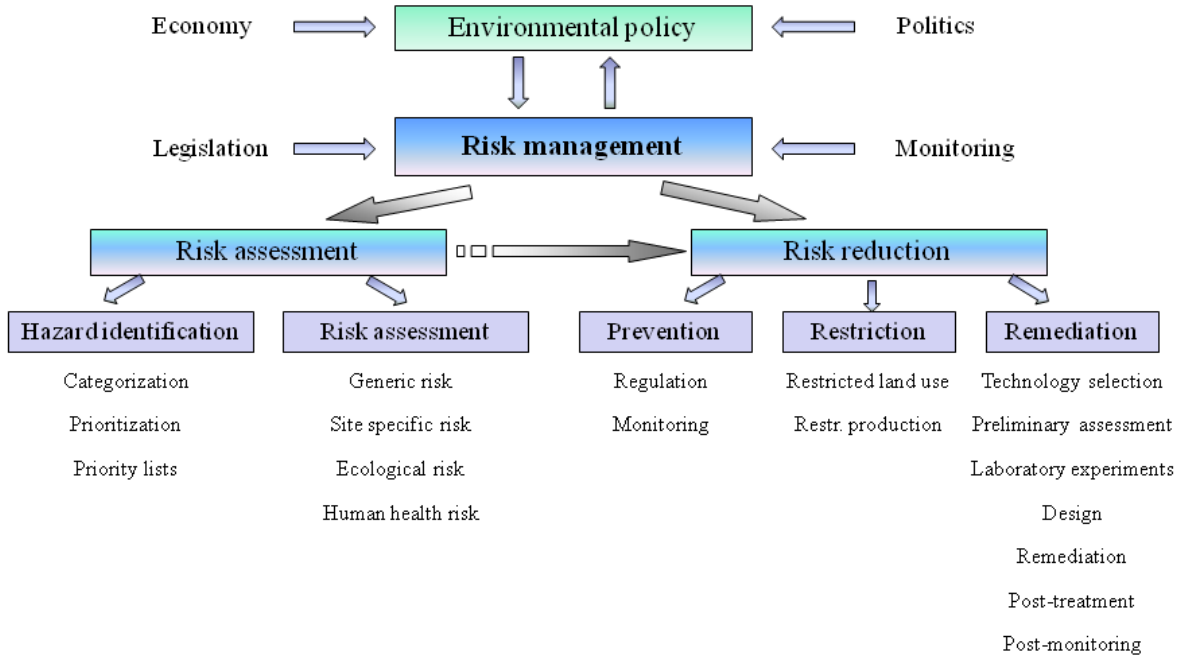


Figure 1 Risk management

4. Interaction of chemicals with the ecosystem

To understand the **effect of chemicals** the process can be broken down into three stages (Figure 2):

1. The chemical substance enters the environment and interacts with it. It spreads, partition occurs between the different phases, it is transformed into other substances, degrades etc. These processes define the environmental concentration of the substance which reaches and affects the members of the biota.
2. The chemical substance interacts with the living organism at an active space at molecular level. This can be an important structural element or a molecule, for example enzyme, nucleotide acid or membrane receptor of the organism.
3. The effect of the interaction at molecular level appears at higher levels such as biochemical and physiological levels, behaviour, population, community or the whole ecosystem.

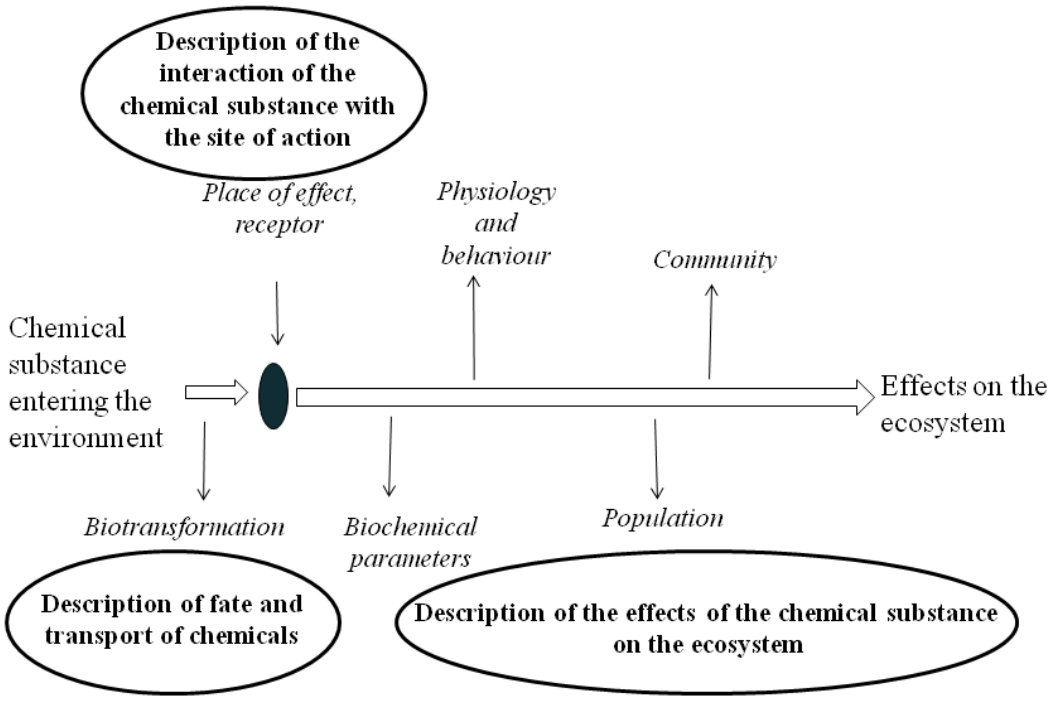


Figure 2 The steps of the interaction of the chemicals with the ecosystem and the main functions of environmental toxicology (based on Landis and Yu, 1999, 2003)

The site of action, meaning the molecular interaction between the chemical substance and the organism determines the final effects. The receptor may be a nucleotide acid, a membrane or a specific protein of a nerve or a non-specific molecule, such as narcotics, which have an overall effect on membranes changing their throughput or other properties, therefore their normal function.

Any of the responses occurring at higher levels, namely at the level of the organism, of the population, of the community or of the whole ecosystem, may be applied as measured parameter in the ecotoxicological test. If we describe well the primary effects on the higher levels, we can predict well the effects on the whole ecosystem from the results of simple measurements or from the structure of the chemical substance However, due to the lack of knowledge, we still have to choose measurement parameters for the higher levels, rather than extrapolating from the results of measurements at lower levels (Gruiz *et al.*, 2001).

Basically only **three basic function** need to be **described by environmental toxicology** (Figure 2):

1. Description of the fate and transport of chemicals in the biosphere and the organism after the release to the environment.
2. Description of the interaction of the material with the site of action.
3. Description of the impact of this molecular interaction upon the function of the ecosystem (Landis and Yu, 1999, 2003).

The **steps of the interaction** of the chemicals with the ecosystem and the **parameters** that can be measured at each step are the following:

0. Chemical and physical-chemical characteristics of the xenobiotic

    The chemical structure of a specific molecule determines the impact of the compound at molecular level. The contribution of the physical-chemical characteristics of a compound to the observed toxicity is called QSAR – Quantitative Structure-Activity Relationship.

1. Introduction of the xenobiotic into the environment – biotransformation / biodegradation / bioaccumulation

- Enzyme induction
- Glutathione S transferases
- Mixed function oxidases
- Hydrolases
- DNA repair enzymes

2. Interaction with the site of action

- DNA/RNA
- Membrane receptors
- Key enzymes
- Biochemical integrity

3. Biochemical parameters (level of molecules)

- Stress proteins
- Metabolic indicators
- Acetylcholine-esterase inhibition
- Adenylate energy change
- Metallothionein production
- Immune-suppression

4. Physiological and behavioural characteristics (level of organisation)

- Chromosomal damage
- Lesion and necrosis
- Carcinogenic effects
- Teratogenic effects
- Reproductive success
- Behavioural alterations
- Mortality
- Compensatory behaviours

5. Population parameters

- Population density
- Productivity
- Mating success
- Alterations in genetic structure
- Competitive alterations

6. Community parameters
    - Structure
    - Diversity
    - Energy transfer efficiency
    - Stability
    - Successional state
    - Chemical parameters
7. Ecosystem parameters
    - Diversity and distribution of species
    - Metabolism
    - Element cycle
    - Landscape changes (Landis and Yu, 1999, 2003; Gruiz *et al.*, 2001)

5. Classification of environmental toxicity tests

There are a large number of toxicological tests that have been developed in environmental toxicology because of the large variety of investigated species and ecosystems. These tests are possible to be classified for example on the basis of the test duration relative to the life span of the organism or according to the number of species, the type of testorganisms, the complexity of the biological community etc. In the following table (Table 1) the main classification parameters and the test types are summarized.

Table 1 Summary of ecotoxicological tests classification

Parameters	**Ecotoxicological test types**
Test duration	Short-term = acute Long-term = chronic
Number of species	Single species Multispecies
Type of the testorganisms	Bacterial cells Algae Fungi Plants Animals Multispecies systems (microcosms, mesocosm, field studies eg. *in situ* biomonitoring etc.)
Tested ecosystems	Aquatic ecosystem Terrestrial ecosystem
Exposure scenarios	Terrestrial ecosystem Whole-body test Feeding studies Injection of a controlled amount (intramuscular, intravenous) Placement of a controlled amount into the stomach by a tube

Environmental toxicity tests are typically classified according to their duration, the number of species involved and are further subdivided along a gradient ranging from acute and chronic basic laboratory bioassays to complex field experiments (*Figure 1*). While more complex tests offer more reliable information in environmental risk assessment based on the results of ecotoxicity tests, they have been used sparingly due to their complexity, cost, and long duration (Römbke and Notenboom, 2002).

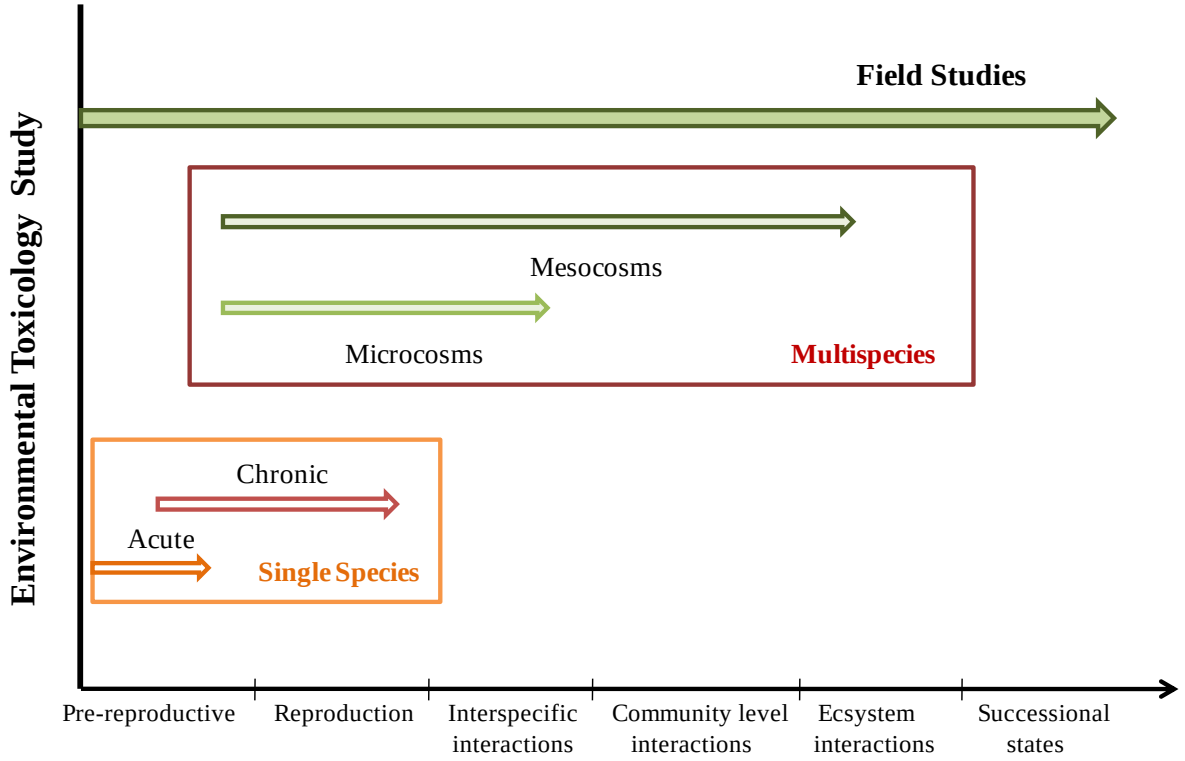


Figure 3 Classification of toxicity tests in environmental toxicology concerning system complexity (Landis and Yu, 1999, 2003)

Acute toxicity tests cover a relatively short period of an organism's lifespan. In the case of fish, daphnia, rats, and birds, usually periods of 24 to 48 h have been used. Even in the case of the short-lived *Daphnia magna*, a 48-h period is just barely long enough for it to undergo its first molting. Vertebrates with generally longer life spans undergo an even smaller portion of their life during these toxicity tests. A common misconception is that those toxicity tests of similar periods of time using bacteria, protists, and algae also constitute acute toxicity tests. Many bacteria can divide in less than 1 h under optimal conditions. Most protists and algae are capable of undergoing binary fission in less than a 24-h period. A 24-h period to an algal cell may be an entire generation (Landis and Yu, 1999, 2003).

Chemicals (contaminants) can elicit acute toxicity by many mechanisms. Cholinesterase inhibition, narcosis, physical effects are example mechanisms that are particularly relevant to the types of chemicals that are commonly responsible for acute toxicity in the environment (Leblanc, 2004).

The ***chronic tests*** may last over one or more generations. Duration of chronic tests involving longer life span test-organisms takes significant proportion of their life, including gestational period of females and spermatogenesis of male test-organisms.

Generally, chronic and sublethal toxicity tests last for a significant portion of an organism's life expectancy. Reproductive tests often examine the reproductive capabilities of an organism. By their nature, these tests must include: (1) the gestational period for females and (2) a significant portion of the time for spermatogenesis for males. Growth assays may include an accounting of biomass produced by protists and algae or the development of newly hatched chicks. (Landis and Yu, 1999, 2003).

Sublethal endpoints including reproductive, immune, endocrine, and developmental dysfunction are generally associated with chronic toxicity. Nevertheless, chronic exposure also can result in direct mortality not observed during acute exposure. For example, chronic exposure of highly lipophilic substances can result in the ultimate bioaccumulation of the chemical to concentrations that are lethal to the organisms. (Leblanc, 2004)

In principle ecotoxicity tests can be carried out at any level in the biological hierarchy of systems, ranging from molecules to ecosystems. The methods range from single species studies through multispecies studies to tests which attempt to measure and determine the effect of chemicals in the field in natural ecosystems.

Single species toxicity tests apply one single species for testing the effect of chemicals. These species are well known organisms, deriving from controlled cultures. Single species are used in most of the laboratory bioassays and toxicological tests.

Multispecies toxicity tests, as their name implies, involve the inclusion of two or more organisms and are usually designed so that the organisms interact. The effects of a toxicant upon various aspects of population dynamics are a goal of these tests.
In the field of microbiology the competition test of two bacterial species uses the competing bacterial strains grown together in the test-medium. A special relation is tested in the prey–predator tests. Food chain effects can be tested using the members of the food chain.

Multispecies tests are for example the microcosms and mesocosms, where – similar to the real ecosystem – any ecosystem-characteristic can be measured or monitored. In microcosms we can measure the number of organisms, number of species, relative distribution of species, respiration or any other metabolic activity of the whole microcosm, independent of the contribution of the individual species or organisms. There is no clear definition of what volume, acreage, or other measure of size constitutes a microcosm. Mesocosms are bigger in size and longer in time, than microcosms, consequently sampling and monitoring has less limitations, than is case of microcosms, therefore diversity and its changes. Often mesocosms are outside and subject to the natural variations in rainfall, solar intensity, and atmospheric deposition. Microcosms are commonly thought of as creatures of the laboratory. (Landis and Yu, 1999, 2003).

The most difficult, costly, and controversial level of toxicity testing is the ***field study.*** Field studies can be observational or experimental. Field studies can include all levels of biological organization and are also affected by the temporal, spatial, and evolutionary heterogeneity that exist in natural systems (Landis and Yu, 1999, 2003).
For example *in situ* biomonitoring means the following of indicator-organisms existing naturally (passive biomonitoring) or placed by the assessor into the environment (active monitoring). The measured endpoints from molecular to population level are optional.

However assessment or monitoring in the field is accompanied by uncertainties due to various environmental circumstances, which are uncontrolled and uncontrollable by the assessor. Moreover the continuous transport and fate processes of the chemical substance, the interactions between substances, the interactions of a substance and matrix with the biota must be taken into account by evaluation.

One of the major challenges in environmental toxicology is the ability to translate the toxicity tests performed under controlled conditions in the laboratory or test site to the structure and function of real ecosystems. This inability to translate the generally reproducible and repeatable laboratory data to effects upon the systems that environmental toxicology tries to protect is often called the ***lab-to-field dilemma***. Comparisons of laboratory data to field results are (scale up) an ongoing and important part of research in environmental toxicology. (Landis and Yu, 1999, 2003).

The characteristics of multispecies systems (microcosms, mesocosms and field studies) will be discussed more detailed in the following chapters.

Aquatic toxicology is based on the response of aquatic ecosystem (marine and freshwater). Aquatic ecotoxicology is the pioneer in environmental toxicology. The very first developments and applications are dated between 1960 and 1970, when industrial and agricultural activities endangered surface waters.

Aim of aquatic toxicology is to understand, how chemical substances cause stress on aquatic ecosystem, to measure the effect of chemical substances on different trophic levels of the a ecosystem, to know differences between species within the community and the development of suitable methods for screening, monitoring, legislation, decision making and environmental management (Landis and Yu, 1999, 2003).

As ecotoxicology developed firstly for aquatic environments the rise of internationally standardized methods and bioassays with invertebrates, fish, and algae generated a large database of toxicity for aquatic organisms (Van Straalen, 2002).

Terrestrial ecotoxicology

Terrestrial ecotoxicology has been defined as the subfield of ecotoxicology which uses tests to assess study, evaluate and quantify the adverse effects of toxic substances on the diversity and function in soil-based plants and animals (Garcia, 2004).

The soil is a spatially heterogeneous, complex system compared to water and air. Various soil compartments and constituents have a great capacity to retain contaminants due to this the soil is a net sink for pollutants (Calow, 1993, 2009). Increasing complexity leads to increasing problems concerning the evaluation of the contaminant effects. Consequently soil pollution studies, soil ecotoxicity tests must not only consider the effects of contaminants on isolated species, but also changes in community structure due to interactions. Another complication is that soil not only acts as substrate for organisms, but also as recipient medium for chemicals (contaminants). So the physico-chemical properties of this medium are crucial in determining the bioavailability of these chemicals to the organisms of the soil ecosystem.

Most ecotoxicological studies of soils are based on invertebrates and focus on worms, collembolans, or enchytraeids as bioindicators. The use of these groups has become standard

because they are widely distributed, play important ecological roles, live in permanent contact with soils, reproduce quickly, and are easily maintained in laboratories (Cardoso and Alves, 2012). Even so, the number of standardized tests available for the soil component of terrestrial systems is still lower nowadays than that available for aquatic systems.

Exposure scenario is determined by the conditions of the contact between the toxicant and the body of the testorganisms.

During a whole-body test the test-organism is immersed into the tested water, sediment or soil and there is a direct contact between the pollutant/contaminated environment and the testorganism. In such cases the whole body, the skin and all dermal surfaces, eye, gill, hair, etc. and in many cases the inner mucosal surfaces in the mouth, trachea, the digestive system, the eye, etc. are exposed to the pollutant/contaminated environment.

Feeding studies aim at the eating of food or toxicants mixed into food or drinking water to model the uptake and the effect through the digestive system. The problem of food-testing is that, the test-animals eat much less or not at all when they feel the presence of toxic material in the food. According to proper test methods the amount of the drunken water and eaten food should be measured individually. This requires special drinkers and feeders.

Using a tube to place a controlled amount of food or water into the stomach makes the method more precise and controllable. .

Injection of a controlled amount (intramuscular, intravenous) of toxicant into body-tissues or blood forces the contact with the cell membranes and the uptake by the metabolic apparatus of the cells.

Human toxicology aims to give the dose-response relation between hazardous chemical substances and human responses. Human toxicity of a chemical is mainly based on the results of animal toxicity tests, the scale of toxic effect on human is always an estimate. Extrapolation from animal data to human is possible assuming that the properly selected animal species' response is analogous to human body's response and that the test-method, the applied test scenario perfectly models the real human exposure.

The main methodology for extrapolation – for example from rat to man – applies a safety factor, based on experience.

The default for the interspecies safety factor is EC50 (human)/EC50 (animal) = 0.1, because drugs and toxic chemical substances are generally ten times more potent in humans based on existing pharmacological and toxicological data.

Animal data are suitable to establish the dose or the concentration of the chemical substance that would cause adverse effect, damage or death of 10, 20, 50, 90% of the treated animals, or determine the lowest effect and the highest no effect concentrations or doses, which are manageable limit values.

Animal testing has many subclasses, according to the applied animal taxon (fish, bird, mouse, rat, dog, monkey, etc.), the type of exposure (acute, repeated or chronic exposure), exposure routes (inhalation, per oral, cutaneous, mixed routes), aim of the test method (toxicity, mutagenicity, reprotoxicity, neurotoxicity testing) and according to the measured endpoint such as death, immobilization, changes in behaviour, irritation, corrosion, organ-toxicity (cardiac-, ophthalmic-, cutaneous-, muscle-, bone-, or hepatotoxicity), cellular toxicity (cell death, mitochondrial, peroxisome, cellular tight junctions, reactive oxygen species, glutathione and glutathione-transferase, metabolomics, DNA-changes, chemokines, etc.) toxicity on endocrine system, immuntoxicity, phototoxicity, photoallergy.

6. *Evaluation of the results of environmental toxicity test - endpoints in environmental toxicology*

There are several methods available for the estimation of toxic endpoints.

Endpoint of the measurement

Biochemical, physiological, behavioural, population, community parameters and ecosystem effects can function as endpoint in the ecotoxicological tests. These measurement-endpoints are the detected characteristic of the organism.

The most commonly measured endpoints are listed below:

- Toxicity tests: growth (cell number, mass production, root lengths, chlorophyll content), survival, mortality, immobilisation, respiration: O_2 consumption, CO_2 production, enzyme activities, ATP production, reproduction, luminescence etc.
- Mutagenicity tests: number of mutants, number of revertants, chromosome abnormalities
- Carcinogenicity tests: tumors
- Teratogenicity tests: reproductive success, cytogenetic characteristics
- Biodegradation tests: consumption of O_2, substrates, production of endproducts, CO_2
- Bioaccumulation tests: chemical analysis of accumulated substances.

When planning a toxicity test all these indicators may be used to determine biomarkers and measured endpoints.

Endpoint of the test evaluation

The test-endpoints are the results of the environmental toxicity test following statistical evaluation.

The well-known and widespread chemical models used for the management of the environment are based on the association between the dose/concentration of the chemical substance and its effect. A growing concentration series of the tested substance (or a growing dose of the contaminated soil) shows increasingly stronger effect on the testorganisms (fish, or plant or crustacean living in that water etc).
The graph describing the response of an enzyme, organism, population, or biological community to a range of concentrations of a chemical substance is the concentration-response curve. Enzyme inhibition, DNA damage, death, behavioural changes, and other responses can be described using this relationship (Landis and Yu, 1999, 2003).

The graphical presentation of the concentration/dose and response data enables for an environmental toxicologist to readily determine important concentration-response (dose-response) relationships. Furthermore, the graphs enable different toxicants to be compared.

Any of the measurable biomarkers relevant for the effect, can be plotted against the dose or concentration; we get the same shape of curve: an S-shaped sigmoid, which is shown in *Figure 4.* This figure presents typical response over concentration of a chemical.

In this figure the measured endpoint is the immobilization of the test-organism, the crustacean *Daphnia magna* showing a concentration dependent response by immobilization in the presence of toxic chemicals, as compared to the control The number of immobile animals was determined after 24, 48, 72 and 96 hours. In the graph, the toxic chemical substance is nicotine, and the number of the immobile animals was determined in the presence of different nicotine-concentrations between 2.5–2500 ppb.

Percentage of inhibition was calculated compared to the control sample (without nicotine).

After plotting the percentage inhibition values function of the nicotine concentration, we fitted the curve to the measuring points with the help of a statistical method, using software able to find the statistical optimum of the fitting.

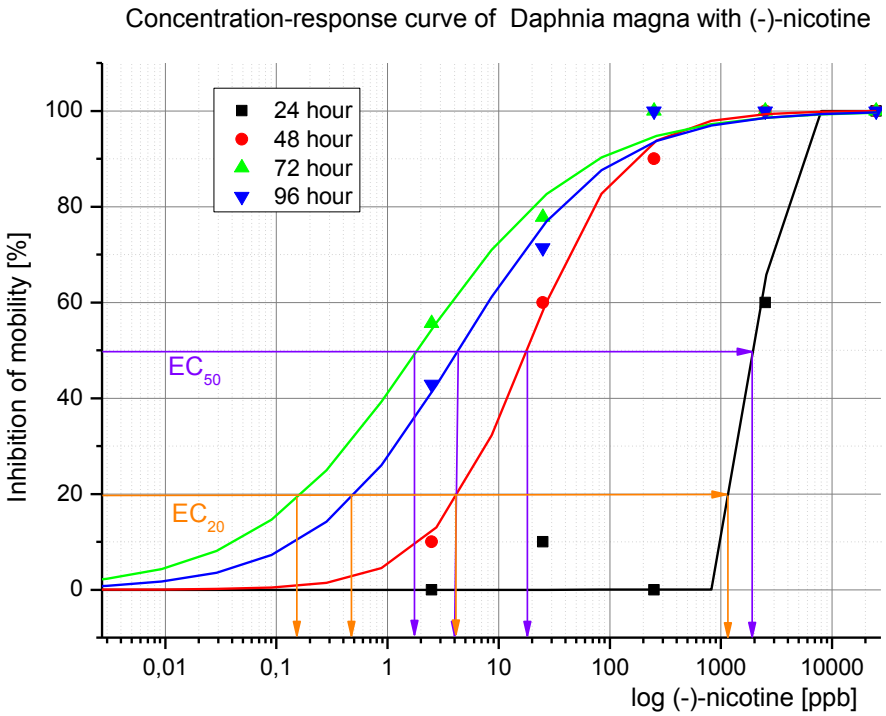


Figure 4 Graphical determination of EC_{20} and EC_{50} values from the "S" curve fitted to the measurement points

The endpoint of the measurement may be all the formerly listed biochemical, physiological, behavioural, population or community parameters depending on the test-organism and its response.

The toxicity test endpoints are function of the duration of the test. Acute (short term) tests use the EC/ED and LC/LD 20 and 50, sometimes 10 or 90 as endpoint.

- EC_{20} and EC_{50}: the concentration that has 20 % and 50 % decrease effect in the measured endpoint, for example the inhibition of shoot growth, or respiration rate, enzyme-activity, etc. in other cases. The abbreviation EC means: effective concentration. These values are always estimated by graphical or computational means.
- ED_{20} and ED_{50}: the dose that has as effect 20 % and 50 % decrease in the measured endpoint-value. The abbreviation means: effective dose. The difference to EC is, that the amount of the effective material is given is dose. This is the case when animal tests are used and the toxicant given to the test-animal (rat, mice, rabbit) is measured, used and plotted on the graph in mass unit, such as μg, mg or g.

  We also use dose, when we do not know the amounts of or even the identity of the contaminants in an environmental sample. In this case we can determine the mass of the water or soil, which results 20 or 50% inhibition in growth, respiration, light emission, etc.
- LC_{20} and LC_{50}: the concentration that causes 20 and 50 % mortality of the testorganisms – estimated by graphical or computational means. The difference to the previously introduced EC_{20} and EC_{50} is, that the measured endpoint is not optional, but fixed: it is lethality and LC is lethal concentration.
- LD_{20} and LD_{50}: the dose that causes 20 and 50 % mortality/lethality of testorganisms – estimated by graphical or computational means.

  Lethal dose is the amount of the effective chemical substance or environmental sample given in mass unit (µg, mg or g).

NOEC/NOEL and LOEC/LOEL values are applied for long term (chronic) tests.

- NOEC and NOEL: No Observed Effects Concentration and Level. This is the highest applied concentration or dose in the test, which did not show any effect when tested, compared to the non-treated control.
- NOAEC / NOAEL: No Observed Adverse Effects Concentration or Level, the highest applied concentration or dose in the test, which did not show adverse effect. It is to emphasize, that stimulation of an effect is excluded from the evaluation.
- LOEC and LOEL: Lowest Observed Adverse Effects Concentration or Level, the lowest applied concentration, which has already caused and effect. When our concentration or dose series is at too large scale, the difference between LOEC and NOEC can be significant.
- MATC: Maximum allowable toxicant concentration, determined by graphical or statistical methods from NOEC and LOEC: NOEC < MATC < LOEC

These endpoints of the test are uniformly used for the quantification of adverse effects in environmental toxicology. These are objective measures, and can directly be used in environmental management and applied for decision-making.

Curve fitting applied for evaluation uses a variety of regression models. Each model has its own set of data specifications in order to be successful. *Probit and Logit* method are the most popular statistical tools for the evaluation of typical "S"-shaped concentration-response curves. (Landis and Yu, 1999, 2003).

In the ***Probit method*** the original data are processed by probit-transformation. Probit is a binary response model that employs a probit link function. This model is most often estimated using standard maximum likelihood procedure, such an estimation being called a probit regression

Logit transformation of data and fitting the curve based on maximal likelihood method can also be applied for calculating EC or LD values. Similar to probit method in case of the lack of partial kill data some assumptions are required for the calculations.

In the case of ***chronic toxicity tests*** the standard method for analyzing chronic toxicity data is ANOVA, which is the abbreviation of Analysis of Variance. It determines the concentrations that are significantly different in effect of the untreated control.

In ANOVA the observed variance in a particular variable is partitioned into components attributable to different sources of variation. In its simplest form ANOVA provides a statistical test of whether or not the means of several groups are all equal, and therefore generalizes *t*-test to more than two groups.

The fixed-effects model of analysis of variance applies to situations in which the experimenter applies one or more treatments to the subjects of the experiment to see if the response variable values change. This allows the experimenter to estimate the ranges of response variable values that the treatment would generate in the population as a whole.

In case of chronic toxicity the equivalence of the control and treated is tested. Analyses of variance is performed on the treatment group. By multiple comparisons between the treatment groups we can identify those groups, which are different from control.

In the first step ANOVA calculates the distance between all treated and control groups. If the F-score is statistically not significant, the treatment have the same effect, there is no difference between the groups. If the F-score is significant, data are examined in a second step to find the groups which are different from the control. With multiple comparisons we can find the groups different from each other.

The goal of the ANOVA-evaluation to find the LOEC or NOEC values, namely the lowest contaminant concentration which is different from control showing a significant adverse effect or the highest no effect concentration which is identical with the control but different from LOEC. This is a so called hypothesis testing model.

7. Single species toxicity test

Organisms which give a response to toxicants/contaminants are of wide ranges: bacteria, fungi, algae plants and animals (such as crustaceans, fishes, clams, insects, rodents, or other mammals). The selection of the testorganism often is determined with the historical expertise of the laboratory or organization.
Practically all organism-types can be used as testorganism, but there are a number of criteria towards organisms to be applicable for environmental toxicity testing, suitable and feasible for getting the answer in form of organism's response to the questions of environmental toxicologists.

One of the most crucial aspects of a toxicity test is the suitability and health of the test organisms or, in the case of multispecies toxicity tests, the introduced community. It is also important to define clearly the goals of the toxicity test. If the protection of a particular economic resource such as a salmon fishery is of overriding importance, it may be important to use a salmonid and its food sources as test species.

Some of the ***general requirements for choosing a test species*** for use in a toxicity test are listed and discussed below (Landis and Yu, 1999, 2003).

- Availability: the testorganism should be widely available in the nature or in the commerce.
    - Laboratory cultures are the most widely used test-organisms, because under controlled conditions the stability and good quality of the test-organisms can be ensured. The culturing lab can be the same as the testing one is or it can be a specialized lab for culturing the test-organism guarantying quality.
    - Other culture facilities may also occur, e.g. hatcheries for crustacean, fishes, clams or water plants. Test-organs, tissues and blood can be collected from slaughterhouses.
    - Collection from the field may also be a good solution, mainly in those cases, when testorganisms are not easy to culture or even maintain in laboratory: marine organisms, plankton, freshwater clams and higher water-plants, terrestrial species such as insects, or mites.

- Maintenance of the test-cultures in the laboratory can be successful, when we know the species requirement according to food, space and stress well. It is very important to keep the sensitivity and all the required properties of the test-organism, and have a sufficient supply for testing.
- Genetics of the test-organism and history of the culture is very important, to be able to follow the changes and reach the required statistical quality of the test. The genom of *Escherichia coli, Saccharomyces* fungi, *Aliivibrio fischeri* bacterium, *Tetrahymena* the single cell animal, *Drosophyla* or some of the *Nematoda*, are fully mapped. The culture collections give the main genetic and physiological character of the species, subspecies or strain, and this controlled origin may ensure the keeping of these characteristics on the long term by going back to the original culture. Amongst the higher test-organisms there are many species/subspecies which are used for a long time and are known well from genetic and physiologic point of view: rat, mice, guinea-pig, birds or rabbits.
- Sensitivity of the test-organism is an important issue, because it is closely related to the aim of the testing.
    - Relative sensitivity means that a test-organism shows different sensitivity for different toxicants/contaminants. The user should have this information, otherwise any additional chemical substance, its metabolites or contaminants may cause an effect, which is comparable with the main toxicants' effect, and makes the response of the test-organism unduly high and non-linear with the concentration. The other reason for the need of this information is the better matching of test-organism to the substance or problem to test.
    - Special sensitivity for one or a few toxicants
    - Sensitivity for a broad number of toxicants
- Representation of the ecosystem of ecosystem constituents and information needed for the planning of the environmental testing.
    - Sensitivity should be representative for a class or phyla to protect certain taxons, in this case additional information is needed on which families or phyla are represented by the test-organism.
    - Representation of the most sensitive ecosystem member, for early warning

- Being more but not much more sensitive, as the big average of the ecosystem maybe beneficial when integrating its result into a conservative risk management system.
- "Average" sensitivity is beneficial for the risk managers, having a response close to the whole ecosystem, without implementing more complicate and costly monitoring.
- Less sensitive, than the average test-organisms can be used for screening hot spots or the most risky elements of a complex system.
- Some families or phyla, or the minor components of a complex ecosystem are generally not represented by any of the test-organism types.

- Concentration/dose–response relation has multiple requirements:
  - Existing association between the amount of the toxicant/contaminant and the response of the test-organism.
  - Proportional response to the concentration/dose of the toxicant
  - The effective concentration/dose range should be as broad as possible
- Reproducibility, statistics: it is one of the most important requirements when environmental testing is intended to be integrated into a quantitative risk assessment procedure, where objective and quantitative data are needed, and evaluated together with physico-chemical analytical data. In understanding this integrated application it becomes evident, that the quality of environmental toxicity data cannot be of lower quality than the physico-chemical ones, otherwise the complex procedure goes down to a lower level of statistics and validity.

8. Standardization of environmental toxicity test (Landis and Yu, 1999, 2003)

Over the years a variety of test methods have been standardized. These protocols are available from the American Society for Testing and Materials (ASTM), the Organization for Economic Cooperation and Development (OECD), ISO (International Organization for Standardization), the National Toxicology Program (NTP), and are available as U.S. EPA publications, the Federal Register, moreover often from the researchers that developed the standard methodology.

There are distinct advantages to the use of a standard method or guideline in the evaluation of the toxicity of chemicals or mixtures:

- Test results are uniform and comparable.
- Allows replication of the result by other laboratories.
- Provides criteria as to the suitability of the test data for decision making.
- Logistics are simplified, with little or no developmental work.
- Data compiled can be combined with that of other laboratories for use when large data sets are required. Examples are quantitative structure activity research and risk assessment.
- The method establishes a defined baseline from which modifications can be made to answer specific research questions.

Over the years numerous protocols have been published. Usually, a standard method or guide has the following format for the conduct of a toxicity test using the ASTM methods and guides as an example.

- The scope of the method or guide is identified.
- Reference documents, terminology specific to the standards organization, a summary, and the utility of the methodology are listed and discussed.
- Hazards and recommended safeguards are now routinely listed.
- Apparatus to be used are listed and specified. In aquatic toxicity tests the specifications of the dilution water are given a separate listing, reflecting their importance.
- Specifications for the material undergoing tests are provided.

- Test organisms are listed along with criteria for health, size, and sources.
- Experimental procedure is detailed. This listing includes overall design, physical and chemical conditions of the test chambers or other containers, range of concentrations, and measurements to be made.
- Analytical methodologies for making the measurements during the experiment are often given a separate listing.
- Acceptability criteria are listed by which to judge the reliability of the toxicity test.
- Methods for the calculation of results are listed. Often several methods of determining the EC50, LD50, or NOEL are referenced.
- Specifications are listed for the documentation of the results.
- Appendices are often added to provide specifics for particular species of strains of animals and the alterations to the basic protocol to accommodate these organisms.

9. *Typical testorganisms and ecotoxicity test methods*

It is crucial to understand the test procedures in environmental toxicology. In every interpretation of the test results (EC50 and NOEC) there should be a clear understanding of the test method used to obtain the estimate. The understanding should include the strengths and weeknesses of the method and the vagaries of the testorganisms (Landis and Yu, 1999, 2003).

In this chapter we give a short description of a few important ecotoxicity tests applying a single testorganism. Quite often it is the standard method that is modified by researcher to answer more specific questions about the effect of xenobiotics.

The tests are grouped into two main categories:

1. Testing of aqueous solutions, water samples and sediment samples. These tests apply water and sediment living organisms.
2. Testing of soil. These tests apply soil living organisms and water living organisms in some cases. Either the eluates of the soil or the soil itself (direct contact with the testorganism) can be tested.

Testing of aqueous solutions, water samples and sediments

Daphnia magna testorganism

Daphnia magna (water flea) is a crustacea widely used for environmental toxicology evaluation of different chemical substances in water samples (Ohe *et al.*, 2011). This crustacean is an accepted testorganism and indicator species of ecosystem health and exhibiting consistent responses to toxins (OECD 202, 2004 and OECD 211, 2008). *Daphnia* sp. live in biotopes like big lakes as also in small ponds with standing water areas.

Daphnia magna is maintained in a 5 litres volume beaker in a 21.5±1 ºC thermostat with 16:8 h light: dark cycle. The adults are approx. 10 days old female individuals, fed every second day with alga suspension of *Scenedesmus subspicatus*. Boiled and cooled, aerated tap water is used as growth medium, of <500 mS cm^{-1} electric conductivity value (Hart *et al.*, 1999).

Daphnia magna immobilization test

Water flea immobilization bioassay is performed in triplicate for each water sample. 10–10 neonates (<24h old) are gently placed in 150 ml beakers (8 cm high, 6 cm I.D.) containing 50–50 ml of water sample. The test organisms are not fed during the test. The test vessels are maintained for 72 h in a thermostat. The examined endpoint is 24, 48 and 72 h immobilization, where an individual is considered to be immobile if it did not move after 15 sec of gentle agitation. The means and the standard deviation are calculated for each sample and then the inhibition percent is calculated. The test validity requirements are set by the International Organization for Standardization (ISO 6341, 1996), as the mortality in the control beakers cannot exceed 20% at the end of the test.

Daphnia magna heart rate test

The test was developed by Fekete-Kertész *et al.* (2013) based on the work of Villegas-Navarro *et al.* (2003) and Dzialowski *et al.* (2006) with minor modifications. The appropriate testorganisms in 200 µL tested solution are placed onto a single cavity microscope slide with the help of a special fabric spoon, and then the heartbeat rate of each test animal is measured three times for 10 seconds. After the measurement 10 test animals are placed into 50 mL of each test solution and into a control container with the culturing medium. After 24 and 48 hours the heartbeat rate of the test animals is counted again as explained above.

Daphnia magna partial life cycle toxicity test (chronic test)

Water flea chronic test attempts to look at growth and reproductive success of the test organism (Landis and Yu, 1999, 2003). 10–10 neonates (<24h old) are gently placed in 100 ml beakers containing 80 ml of water sample. The test organisms are fed during the test with combinations of green algae. The test vessels are maintained for 21 days in a thermostat at 20 °C with 16 h light and 8 h dark cycles. The examined endpoint is the survival (number of animals alive), growth (length or mass) and reproduction (numbers of offspring derived from each animal) of the testorganisms.

Heterocypris incongruens movement test

Heterocypris incongruens is a freshwater ostracod, its body is covered with calcareous shell. Their reproduction can occur both by fertilized and virgin eggs. *H. incongruens* is a bottom-dwelling animal and feeds on mostly algae and small aquatic organisms. *H. incongruens* can be sensitive to water dissolved and sediment-bound contaminants, this way the total toxicity of the tested medium can be measured.

The culture of *Heterocypris incongruens* is maintained in a 0.5 litre volume beaker kept in a 21.5±1 ºC thermostate with 16:8 h light: dark cycle. The test adults are approx. 10 days old female individuals, fed every second day with alga suspension (a mixture of *Chlorella vulgaris, Pseudokirchneriella subcapitata* and *Scenedesmus subspicatus* species). Standard water (0.6 g $MgSO_4$; 0.96 g $NaHCO_3$; 0.04g KCl; 0.6 g $CaSO_4 \cdot 2\ H_2O$; in 10 L distilled water; pH=6,4) is used as growth medium. A five-step ten-fold dilution series is prepared from the samples.

Three testorganisms are placed into a 2 ml volume (h=15 mm; d=12 mm) glass container and the movement of the testorganisms is registered by a digital microscope camera and Image-Pro Plus 7.0 software. The video record contains 150 images. The test containers are illuminated from the bottom. The movement of the selected coloured points are followed by

the software. From several options (eg. acceleration, angular velocity) the average speed and total travelled distance are the basis of comparison.

For the initial (t=0 min) measurements test-animals are placed into 1 ml of the culturing medium, which is also used as the control solution, then video records are made at t=0 min and after a 30 minutes contact time in the test-solution. After that the test-animals are put into a thermostate (21.5±1 ºC) for 24 and 48 hours in 10 ml of the test-solutions with the same composition used for the 30 minutes contact time test.

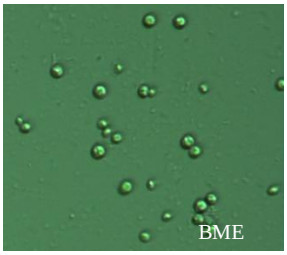
Tetrahymena pyriformis reproduction inhibition test

*Tetrahymena*s represent the eucaryote cell in environmental toxicology, cell biology and genetic research. *Tetrahymena pyriformis* is water living one cell animals. It is a protozoa belonging to the phylum of Ciliophora. It has a size of 25−90 μm, is pear-shaped and the cell is covered with cilia. The test based on the inhibition of reproduction caused by toxic substances was developed by Gruiz and Leitgib (2006).

Tetrahymena pyriformis is maintained in TP broth containing peptone and triptone. 100 μl of cell suspension is inoculated to 5 ml fresh broth weekly.

30 ml TP broth, 468 μl mixture of antibiotics, 600 μl cell suspension and the tested sample is measured into a 100 ml Erlenmeyer-flask. The flasks are shaken in the dark at room temperature. The cell number is counted in Bürker-chamber with microscope at 24, 48 and 72 hours. For the counting the cells they are fixed with 1% formaldehyde solution. The inhibition of reproduction is compared to an uncontaminated control and given in inhibition percentage. The EC20 and EC50 values are determined from the various concentration of the sample.

Algal growth inhibition test



The 96 hours algal growth test examines the toxic effect of chemical substances to primary producers in water. The test uses one cell freshwater algae species. The green algae used in the test are: *Selenastrum capricornutum, Scenedesmus subspicatus, Chlorella vulgaris*.

The one cell algaes are maintained on agar or in mineral salt medium. Their growth is ensured by 10:14 hours dark and light cycles and 21.5 °C. A five-step dilution series is prepared from the sample and a proper amount of algae inoculums (2–3 days old, with 10^4 cell/ml). The test solution is shaken and stored at 21.5 °C. The cell number is determined at 24, 48, 72 and 96 hours. The maximum change in pH during the test is 1.

The growth or the decrease in growth rate is compared to a control kept at same circumstances. The reproduction curve is drawn and the growth inhibition is calculated as follows:

1. From the area underneath the reproduction curve:

$$A = \frac{N_1 - N_0}{2} t_1 + \frac{N_1 + N_2 - 2N_0}{2} (t_2 - t_1) + \cdots + \frac{N_{n-1} + N_n - 2N_0}{2} (t_n - t_{n-1})$$

t_1 – time of the first sampling [h]

t_n – time of sampling no. n [h]

N_0 – initial cell number

N_1 – cell number at time t_1

N_n – cell number at time t_n

Inhibition is calculated as follows:

$$H = 100 * \frac{A_{control} - A_{sample}}{A_{sample}} \qquad [\%]$$

H – reproduction inhibition (%)

$A_{control}$ – calculated area of the control

A_{sample} – calculated area of the sample

2. From growth rate [h^{-1}]

$$\mu = \frac{ln(N_n) - ln(N_0)}{t_n} \qquad [h^{-1}]$$

t_n – time of the last sampling [h]

N_0 – initial cell number

N_n – final cell number

Inhibition is calculated as follows:

$$H = 100 * \frac{\mu_{control} - \mu_{sample}}{\mu_{control}} \qquad [\%]$$

H – growth inhibition (%)

$\mu_{control}$ – growth rate of control [h^{-1}]

μ_{sample} – growth rate of sample [h^{-1}]

Lemna minor reproduction inhibition test

Lemna minor (common duckweed) is cultured in a 20x30x7 cm glass container kept in a 21.5±1 ºC thermostat with 16:8 h light: dark cycle. For the test healthy, two-leaf *L. minor* individuals are used, cultivated in Hoagland's nutrient medium. A five-step ten-fold dilution series is prepared from the samples.

On the first day 10 healthy and two-leaf *L. minor* individuals are placed into 50 ml of each dilution member of the test solutions. The experiment is carried out with three parallels in 150 cm^3 beakers. Hoagland's nutrient medium is applied as control. The beakers are covered with a translucent plastic film to avoid evaporation and concentration of the test solutions during the experiment. The assembled test systems (beakers) are incubated in a 21,5 ± 1 ºC thermostat for 7 days under the following light conditions: 16:8 h light: dark cycle. On the seventh day *L. minor* individuals are removed from the test-solutions, then dried with filter paper. The wet weight of *L. minor* biomass is determined in case of each sample. The dried biomass is placed into ground-necked test tubes containing 5 ml of 96% ethanol. After 24

hours the optical density of the samples is determined spectrophotometrically at 470, 649 and 664 nm wavelength values.

From the measured optical density values the total chlorophyll content is determined using the following formula (Lichtenthaler, 1987):

$$C_{a+b} = \frac{5{,}24 \cdot A_{664} + 22{,}24 \cdot A_{649}}{sample}$$, in which

C_{a+b}: total chlorophyll content of the sample (mg/sample)

A_{664}: absorbance values at 664 nm wavelength

A_{649}: absorbance values at 649 nm wavelength

From the total chlorophyll values an inhibition percentage is calculated as compared to the values of the control sample using the following formula:

$$H\% = \frac{C - S}{C} \times 100$$, in which

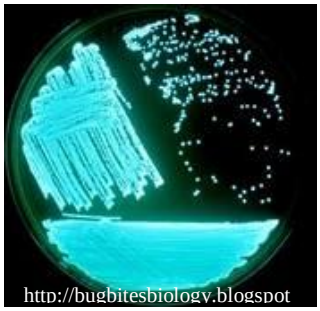
H%: inhibition percentage

C: total chlorophyll content values of the control sample

S: total chlorophyll content values of the sample

Testing of soil and sediment samples

Aliivibrio fischeri bioluminescence inhibition test

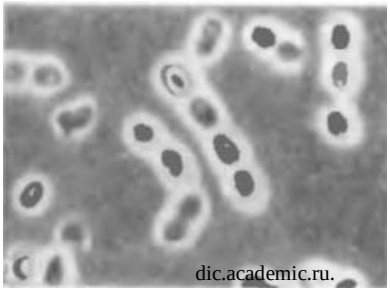


Aliivibrio fischeri (former names: *Vibrio fischeri, Photobacterium phophoreum*) is a marine bacterium that emits light under favourable conditions. In the presence of toxic substances the luminescence is inhibited (Bulich and Isenberg, 1981). The light production of the test bacterium can be measured by a luminometer. The method was developed to direct contact with soil modifying the US EPA Microtox® for aqueous systems standard method by Gruiz *et al.* (2001).

Two grams of air dried soil is suspended in 2 ml 2% NaCl solution and a five-step two fold dilution series is prepared. After the measurement of the reference luminescence intensity, 50 µl of the dilution series was added to 200 µl test medium (16−24 hours old inoculums, shaken at 125 rpm at 22 °C in dark, composition: 30 g NaCl, 6.1 g $NaH_2PO_4.H_2O$, 2.75 g K_2HPO_4, 0.204 g $MgSO_4.7H_2O$, 0.5 g $(NH_4)_2HPO_4$, 5 g peptone, 0.5 g yeast extract, 3 ml glycerol, 1 l water; pH=7.2; sterilized for 20 min at 121 °C). The luminescence intensity is repeatedly measured after 30 min exposure time with a luminometer. The toxicity is characterised by ED50 values (dose producing 50% luminescence inhibition) calculated from the dose-response curve.

Azomonas agilis dehydrogenase activity inhibition test

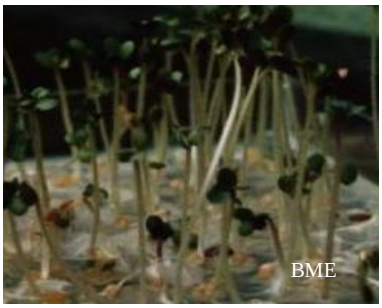
Azomonas agilis (former name: *Azotobacter agile*) bioassay is based on the dehydrogenase activity inhibition caused by toxic effect of the sample. The method was developed to direct contact with soil modifying the Hungarian Standard 21978/30 for aqueous systems standard method by Gruiz *et al.* (2001).



100 ml sterile medium is supplemented with 1 ml 1% 2,3,5-triphenyl tetrazolium chloride (TTC) as an artificial electron acceptor and with the test bacteria previously incubated on a rotary shaker at 28 ± 2 °C for 72 h. The stock solution is injected into the tubes that contained the dilution series of the sample (dilution factor 2). The serial dilutions are incubated at 28 ± 2 °C for 72 h in the dark. TTC is reduced by microbial activity to red-coloured formasan, which is determined visually.

Sinapis alba root and shoot growth inhibition test

In the presence of toxic substances the germination rate, the growth of root and shoot of *Sinapis alba* (white mustard) plants are inhibited (OECD, 2006).



Five grams of air dried soil is measured into a Petri-dish, wetted to its water holding capacity and 20 seeds were placed on top. The samples are incubated at 23°C for three days in the dark. The length of roots and shoots are measured manually with ruler. The data evaluation is based on the root and shoot length in mm units after 3 days of incubation. The growth inhibition is assessed by any noted length changes of mean root and shoot length, relative to the control: $I(\%)=(C–L)/C\times100$, where I, inhibition %; C, length of shoot or root in the control (OECD soil); L, length of shoot or root in the sample. Before the test the germination potential of the seeds was examined at 25±1°C in darkness, and germination over 90% guarantee the proper feasibility of the test.

Panagrellus redivivus nematode reproduction inhibition test

The *Panagrellus redivivus* nematode reproduction inhibition test investigats eluates of the contaminated soils in conventional 96-well microtiter plate. Eight parallel wells are filled with 0.8 ml of liquid nutrient medium and two 12-day-old nematodes were added. The test container is covered and incubated in the dark at 20 ± 2 °C for one week. 0.2 ml of the soil eluate and nutrient medium is added into the wells, when the first generation appears in the most of the test wells. After further one-week incubation, the second generation can be investigated in the wells including the soil extracts. The cell density can be observed with microscope (4-fold magnification).

Folsomia candida (Collembola) mortality test

Folsomia candida is a soil living hexapod from the class of springtails (Collembola) which is sensitive to toxic substances (Wiles and Krogh, 1998). In the acute test the number of animals survived after 1 week contact time is counted. The method was developed to direct contact with soil based on the ISO/TC 190 SC4 WG2 (Biological Methods - Effects on soil fauna) by Gruiz *et al.* (2001).

A two-fold dilution series is prepared from the contaminated soil samples with OECD soil (content: 70% sand, 20% clay, 10% peat; OECD Guideline 207, 1984) at final concentrations from 100% to 6.25%. Ten pieces of fourteen-day-old springtails from a synchronized culture are transferred into the test flasks (250 ml) containing 20 g wet mass of the soil mixtures. The soil mixtures are moistened with 9 ml of water. The springtails are fed with commercial lyophilised bakers'yeast. Test flasks are incubated 20 ± 2 °C in the dark for 7 days. At the end of the incubation period, each soil in the test flask are flooded with distilled water and the floating, living animals are evaluated by counting. The lethality is calculated: I(%)=(C–S)/C×100, where I, inhibition %; C, number of animals survived in the control (OECD soil); S, number of animals survived in the sample.

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