ENVIRONMENTAL TOXICOLOGY

Manual of the laboratory practices

Budapest University of Technology and Economics
Department of Applied Biotechnology and Food Science

2013

TABLE OF CONTENT

LABORATORY PRACTICE 1

Respirometric measurement of microbial activity with OxiTop® Control System

Prepared by Orsolya Klebercz

Budapest University of Technology and Economics
Department of Applied Biotechnology and Food Science
2013

The goal of the measurement

The state and activity of soil can be characterized by the continuous measurement of CO_2-production of the adequately prepared soil. With the aid of the respiration measurement we may answer the questions:

- whether the soil was contaminated,
- whether the contamination was toxic and inhibited the microflora of the soil,
- whether the microflora has adapted to the contamination and functioned actively,
- whether the microflora could be activated,
- what were the technological parameters required for the optimal function of the microflora.

The CO_2 production is proportional to the quantity of the degraded hydrocarbons and it is the measure of biological oxidation, so the system is suitable for the survey of the soil's biological state, and for the characterization and monitoring of biodegradation. It is also an appropriate measurement tool of the differences in the type, age and concentration of organic contaminant according to the soil type, and it helps in deciding whether additional nutrients or additives (for ex.: detergents) are needed for the success of biodegradation technology. In the case of carbonate containing soils, the released CO_2 may influence interfere with the measurement.

The respirometric measurement to be performed during the laboratory exercise will examine soil respiration continuously for 5 days in a closed vessel.

1. Measuring principle

Our test is a pressure measurement. If oxygen is consumed in a closed vessel at a constant temperature, while the emerging CO_2 is sequestrated by an absorbent (NaOH), a negative pressure develops. The OxiTop measuring head measures and stores the pressure data during the whole duration of a measurement once started.

To the manometric measurement of oxygen consumption the following conditions must be fulfilled:

- The (biologically active) sample must be contained in an impermeable vessel.
- Above the sample must be enough space to provide satisfactory amount of oxygen for the biological degradation

- The CO_2 absorbing agent must be placed in the vessel so that it could not get in contact with the sample.
- Pressure measurement tool must be placed into the reaction vessel
- The vessel must be stored at constant temperature during the measurement time.

2. The OxiTop Control closed vessel respirometric system

The OxiTop Control device is a tool for the measurement of respiration intensity. The system measures the consumption of oxygen due to the by the respiration of anaerobic microbes in the sample. Soil microbes consume oxygen and produce CO_2, which is sequestrated by NaOH, causing pressure drop in the vessel proportional to the quantity of the sequestrated CO_2.

3. Required tools and materials

- OxiTop-C measuring heads
- OxiTop OC110 Controller
- ACHAT OC PC communicating software
- AK 540/B data transfer wire for RS 232 port
- Measuring vessels
- Sodium hydroxide
- Environmental sample (soil)

Figure 1. shows the parts of the device used, and a system ready for measurement.

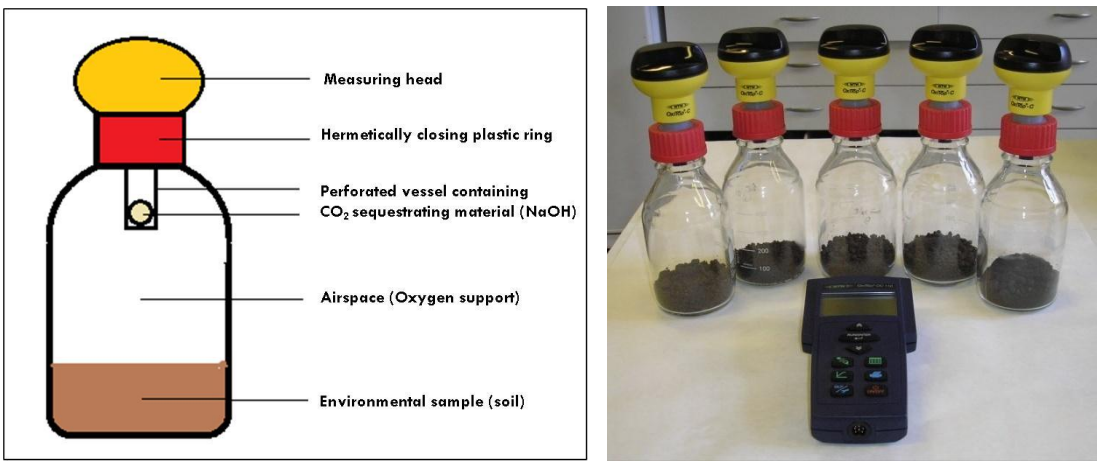


Figure 1. The closed vessel *OxiTop Control* soil respirometric tool

4. Implementation of the measurement

We place 100 g of soil sample into the measuring vessels (250 ml of volume), and other additives if needed. After homogenizing the samples we fasten the perforated pots containing 1–1,5 g NaOH onto the vessels, and screw the OxiTop measuring heads on the top to close the system.

After that we set the measurement parameters on the Control Panel (measurement mode, time, measuring limits), and start the measurement by pointing the Control Panel to the measuring heads within 40 cm distance to achieve infrared connection between the two devices. Flash of red light signals the start of the measurement. Constant temperature and a dark environment are required during the whole runtime, so that external parameters do not disturb the measured pressure values.

The measured data are collected in the measuring head, and after the measurement time expires, we can obtain the whole dataset using the Control Panel, and transfer them into an Excel sheet with the aid of data transfer wire and processing software ACHAT OP PC.

Main parameters of the measurement

Volume of reactor vessel:	250 ml
Soil quantity:	100 g
Moisture content of soil samples:	15–18 %
Temperature:	21.5 ^{o}C
Measurement mode:	pressure change Δp [hPa]
Measuring limit:	300 hPa
Measuring time:	5 days
NaOH quantity:	1.5 g

5. Evaluation of data

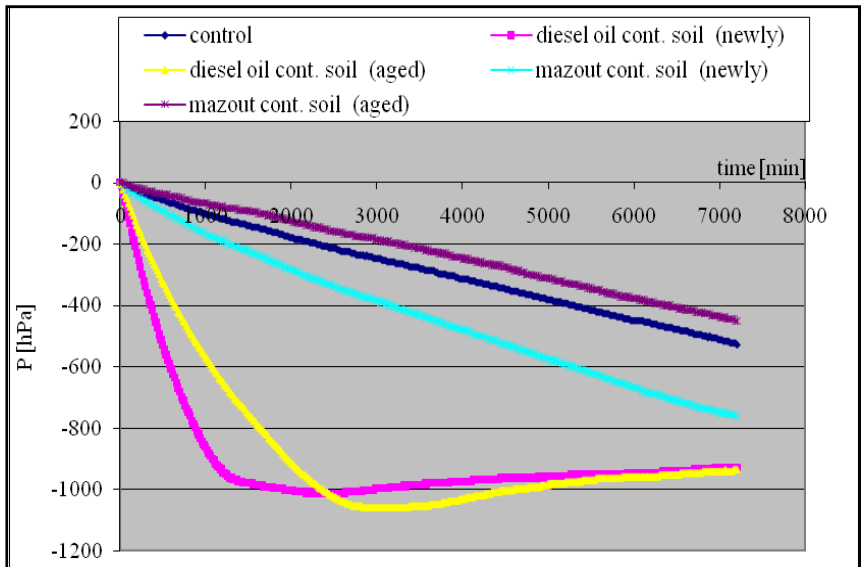


Figure2. Pressure drop in the 5 days OxiTop measurement

The OxiTop measuring head measures and stores the pressure data during five days at every 20 minutes. Since temperature is constant**,** the pressure changes are caused by the soil respiration solely (*Figure 2*). Soil microbes consume oxygen and produce CO_2, which is sequestrated by NaOH, resulting pressure drop in the vessel proportional to the quantity of sequestrated CO_2.

The results can be interpretated as follows:

1. The pressure drop in every vessel shows that the soil samples are active, and the biodegradation of organic contaminants has started.
2. On the two steep curves we can observe that diesel oil can be degraded faster and easier by the microbes than the mazout components of high molecular weight.
3. Biodegradation starts faster in freshly contaminated soils than in the case of older contaminations, since in case of fresh contamination the proportion of easily accessible components is higher, while in the older contamination it is rich in hardly biodegradable components
4. The fact that biodegradation is intense in old diesel contaminated soil indicated also the presence of active microflora adapted to the contamination.

5. In the case of old mazout contamination the activity is even lower than in the control soil. This implies that the hydrocarbons are hardly or not at all available in the soil, therefore re-activation of the microflora is required.
6. Pressure values illustrate well the processes taking place in the soil, and the adaptive capacity of microorganisms. Microbes adapted easily to the easily degradable diesel oil (higher oxygen demand and pressure drop). In these samples we can see that at a certain point the pressure drop stops –due to the saturation in NaOH – for this reason we need more NaOH in the case of such an active respiration.

Enumeration of soil microorganisms
Viable plate counts
Counting of *Escherichia coli* and coliform cells

Prepared by Mária Tolner

Budapest University of Technology and Economics
Department of Applied Biotechnology and Food Science
2013

Microbiology of the soil

The vast majority of the microorganisms are found in the soil under natural conditions. Soil microorganisms are indispensable for several processes, without them the survival and evolution of the biosphere would not be possible. For example, only the soil microorganisms are capable of fixing the atmospheric nitrogen. Microorganisms that can fix nitrogen are prokaryotes (both bacteria and archaea,) and are called diazotrophs. Soil microorganisms play an important role in the nitrogen, sulphur, phosphorus and carbon cycle.

Also the moisture, humus, nitrogen, trace element and mineral content of the soil and the soil particles' size affect the number of microorganisms.

The soil acts as a buffer against the organic matter and waste contaminating the soil, but its ability to degrade and store them is limited. If the existing balance in the soil is disturbed the habitat and the microorganism composition typical to that soil type may be irreversibly altered. Large amounts of mutagen, toxic pollutants in the soil may result total extinction of the soil microflora. Some organisms are not able to adapt therefore they become extinct while others can adapt and proliferate. After contamination generally new microbe populations are formed, which are resistant to the contaminant degrading it while feeding on it.

Measuring the size and composition of the microbial population provides valuable information to assess the quality, activity of the soils. Low microorganism number may indicate that contaminants are present in the soil.

Quantitative analysis of soil microorganisms

Soil may contain up to 10^8–10^{12} bacterial cells per gram of soil. There are some common (basic) procedures used to measure the number of individuals in the soil: the direct microscopic count, the viable count and the MPN method (Most Probable Number).

The **direct microscopic count** involves the enumeration of the total number of microbial cells in a soil sample both living and dead, with the aid of a microscope.

The **viable count** involves counting the number of living cells that form colonies on agar plates or solid media. Each viable microorganism in a suspension will give a single colony after incubation in a suitable media. After incubation the number of colonies is counted.

In this case the viable counts are expressed as colony forming units (CFU)/ml. Determination of CFU can be done with **pour plate method** and **spread plate method**.

Pour plate method

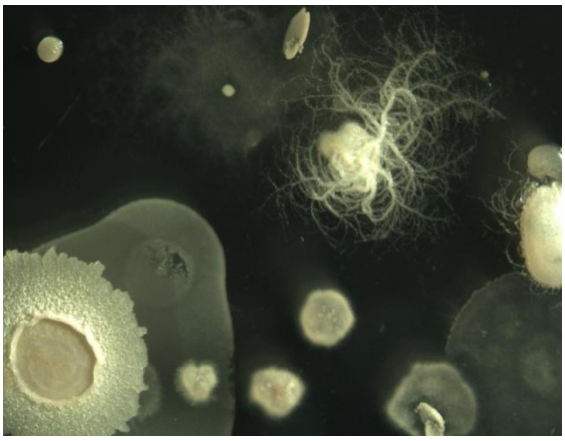


Fig 1. Soil microorganisms on agar plate

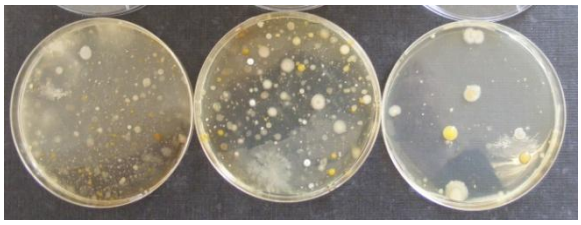


Fig 2. Bacteria colonies on Petri dishes after incubation

- Melt in boiling water bath the sterile agar media contained in the tubes, then cool the medium at 50–55^{o}C.
- Make tenfold dilution series from the soil samples in sterile water (10^{1}–10^{4}).
- Pipette 0.1−0.1 ml from dilutions of the soil suspensions into labelled Petri dishes.
- Pour cooled agar media onto the sample suspension within the Petri dish and swirl the plate carefully to mix the sample with the agar.
- After the plates are solidified place the plates into an incubator at 30–37^{o}C (it depends on the media).
- Incubate the plates for 24–48 hours.
- Count the colonies on plates.

Spread plate method

- Melt in boiling water bath the sterile agar media contained in the tubes.
- Pour cooled agar media into the Petri dishes. Wait until the media is solidified.
- Make tenfold dilution series from the soil samples in sterile water (10^{1}–10^{4}).
- Pipette 0.1−0.1 ml from dilutions of the soil suspensions onto the surface of the solidified agar.
- Using a glass spreader spread the suspension over the whole surface of the agar.
- Place the plates into an incubator at 30–37 ^{o}C (it depends on the media).
- Incubate the plates for 24–48 hours.
- Count the colonies on plates.

The **MPN method** is based on the presence or absence of bacteria using ten-fold dilution in which replicate tubes of general or special (selective) broth are inoculated with 1 ml aliquots of the serial dilution. Growth or positive tests of a specific product (gas formation, acid production etc) are recorded at the end of incubation time. 3–5 parallel tubes per dilution are used, then evaluate the results based on Hoskins table.

Types of growth media

Many types of general and special media have been developed in order to grow microorganisms or select certain organisms and to differentiate them.

General media are containing ingredients, which are allowing that a large proportion of the microorganisms grow well on them. Growth media must provide everything needed for the bacterial culture to live and grow, including water, nutrients, and the proper pH. For example Nutrient media for the bacteria cells, Malt extract media for yeasts.

Elective culture media, due to their composition are able to elect/separate the microorganisms with special micro-nutrient utilization capabilities; for example Rappaport-Vassiliadis (RV) Enrichment Broth for *Salmonella sp.*

Many special media have been developed by microbiologists in order to select certain organisms and to differentiate among them. These media are called selective or differential media.

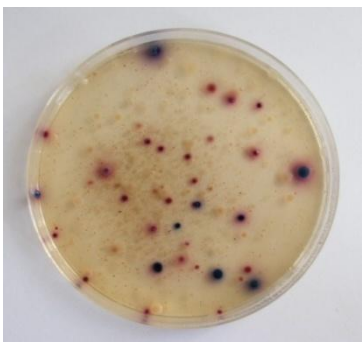


Fig 3. Salmon-red colonies (coliforms) and blue-violet (*Escherichia coli*) colonies in Chromocult agar

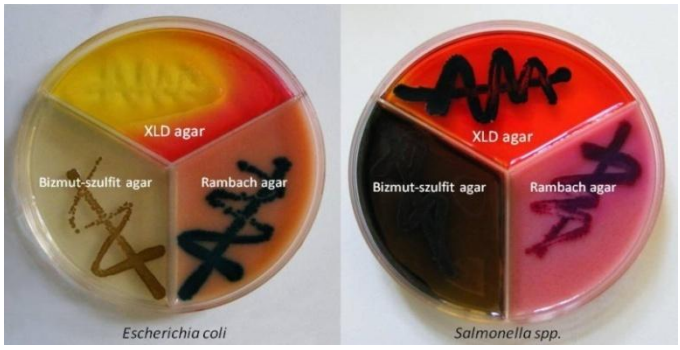


Fig 4. Differential media

Selective media are culture media containing at least one ingredient that inhibits the reproduction of unwanted organisms, but permits the reproduction of specific microorganisms. The ingredients are called selective agents (for example antibiotics, chemicals or dyes).

These media are necessary for growing pure cultures (for example MacConkey's agar is used to cultivate Gram-negative bacteria Endo agar for *Escherichia coli*, Chromocult agar for *Escherichia coli* and coliforms).

Differential media are special formulation designed to differentiate microorganisms or microorganism groups that are growing in the medium. Differential media usually contain ingredients that are modified only by certain microorganisms.
These are characterised by giving visible reactions with the metabolism product of the microorganisms (for example Endo agar for *Escherichia coli*, Chromocult agar for *Escherichia coli* and coliforms).
Special media are developed for special procedures or tests.

References

Colome, Jaime S. & Cano, Raul J. & Kubinski, A. Mark & Grady, David V: (1986) Laboratory Exercises in Microbiology. West Publishing Company/St. Paul, Minnesota

MSZ 21470/77-1988 - Környezetvédelmi talajvizsgálatok, Mikrobiológiai vizsgálatok

MSZ 448/44-1990 - Ivóvízvizsgálat, Bakteriológiai vizsgálat

MSZ ISO 9308-2:1993 - A coliform, a termotoleráns coliform baktériumok és a feltételezhetően *Escherichia coli* kimutatása és számlálása vízben

Gruiz Katalin, Horváth Beáta, Molnár Mónika (2001) Környezettoxikológia, Műegyetemi Kiadó, Budapest

Dr. Horváth Sándor (1980) Mikrobiológiai praktikum Tankönyvkiadó, Budapest, 1980

Kassem Alef and Paolo Nannipierri (1995) Methods in Applied Soil Microbiology and Biochemistry, Academic Press, London, UK

Lányi Béla (szerk) (1980) Járványügyi és klinikai bakteriológia Országos Közegészségügyi Intézet, Budapest

Szegi József (1979) Talajmikrobiológiai vizsgálati módszerek, Mezőgazdasági Könyvkiadó. Budapest

Puskás Aurél (szerk) (1990) Ipari mikrobiológiai gyakorlatok, Műegyetemi Kiadó

LABORATORY PRACTICE 2

Aliivibrio fischeri bioluminescence inhibition test

Prepared by Ujaczki Éva, Dr. Mónika Molnár, Dr. Beáta Horváth

Budapest University of Technology and Economics
Department of Applied Biotechnology and Food Science
2013

Biotesting of environmental samples by

Aliivibrio fischeri testorganism

Introduction

There are bacteria living in the oceans that actually produce light. These microbes are easily found in seawater, marine sediments, in the guts of marine animals, and on the surface of decomposing fish. There are fish and squid that have evolved the ability to harness the power of these light-producing microbes. These animals have specialized organs that provide bioluminescent bacteria with both a safe place to live and a source of food. In return the animals can use the light that is produced by the microbes either as a means of camouflage, as an aid in hunting, or even as a way of attracting mates.

Several biochemical, physiological, behavioral, population, community parameters and ecosystem effects can function as measurement endpoint. The ecotoxicity testing aims to give the dose-response (concentration-response) relation between hazardous chemical substances and the ecosystem.

Luminescence methods have been extensively used in environmental toxicology for measuring of chemical substances. Bioluminescence is the emission of light from living cells. The mechanism of bioluminescence: the enzyme luciferase catalyses the reaction between luciferin (substrate), adenosine triphosphate (ATP), and oxygen which leads to the emission of light and produces the oxyluciferin.

$$FMNH2 + O2 + RCHO \rightarrow FMN + RCOOH + H2O + Light$$

Development of the applications of bioluminescence to environmental monitoring, remedial investigations, toxicity assessments, and field methods are priority research field. Bioluminescence has been observed in various insects, fish, and bacteria. A bacterial bioluminescence assay are the most widely used for assessing the toxicity of contaminated site. The phenomenon of bioluminescence is known for a long time. Already in ancient times philosophers and scientists studied the light production and emission by living organisms. The *Aliivibri fischeri* bioluminescence inhibition test is a OECD standardized test methods.

1. Investigation of liquid phase environmental samples

1.1. Preparation of the laboratory culture of the testorganism

The testorganism grows in nutrient solution, and is inoculated countinously. The composition of the nutrient solution is as follows:
Aliivibrio fischeri nutrient solution (calculated for 1000 cm^3):

30g	NaCl
6.1g	$NaH_2PO_4x\ H_2O$
2.75g	K_2HPO_4
0.204g	$MgSO_4x7H_2O$
0.5g	$(NH_4)_2HPO_4$
5g	pepton
0.5g	yeast extract
3 cm^3	glicerin
pH=7.2	

Sterilise by autoclaving at 121 ^{0}C, for 10 minutes.

1.2.Cu standard solutes

Since the sensitivity of the method is dependent on the initial light intensity as well as on the temperature, Cu standards should be used besides the environmental samples. Thus the results of investigation carried out at different times can be compared.
The concentration of Cu-standards are as follows: 20, 40, 80, 120, 160, 200, 400 ppm.
The measured Cu-quantities in the cuvettes are as follows : 1, 2, 4, 6, 8, 10, 20 μg Cu.
Use 2% of NaCl for dilution.

1.3.Dilutions of the environmental samples

Dilution	Dilution level	Sample volume	Dilution agent volume
1:1.	1	1	1
1:2	2	1	2
1:3	3	1	3
1:4	4	1	4
1:6	6	1	6
1:8	8	1	8
1:12	12	1	12
1:16	16	1	16
1:24	24	1	24
1:32	32	1	32

In case of environmental samples such as surface and ground water it is useful to measure the sample without dilution at first to screen the toxic samples.

1.4. Determination of luminescence light emission by luminometer

Steps

1) Place 0.2-0.2 cm^3 of cell suspension (prepared according to 1.1. section) into the cuvettes of luminometer

2) Measure immediately the intensity of luminescence light. (I_0)

3) Add a. 0.05-0.05 cm^3 of sample dilution

b. 0.05 - 0.05 cm^3 of Cu-standards

c. 0.05 cm^3 of 2% NaCl (control sample) into the cuvettes.

4) Wait for 30 minutes. Measure the intensity of luminescent light intensity. (I_{30})

1.5.Data evaluation

Evaluation of the data is carried out according to Table 1.

Table 1. Data evaluation in case of liquid phase samples

Sample	**I_0**	**I_{30}**	**$f=I_{30c}/I_{0c}$**	**$Ical=f*I_0$**	**$H\%=100*(Ical-I_{30})/Ical$**
control	I_{0c}	I_{30c}			
Cu_1	I_{0Cu1}	I_{30Cu1}			
Cu_2	I_{0Cu2}	I_{30Cu2}			
Cu_3 /mg/	.	.			
Cu_4	.	.			
Cu_5	.	.			
$sample_1$	I_{0s1}	I_{30s1}			
$sample_2$	I_{0s2}	I_{30s2}			
. /ml/	.	.			
.	.	.			
.	.				

I_0 - Intensity of luminescence at t=0 (cell suspension in the cuvettes)

I_{30} - Intensity of luminescence after 30 minutes contact time

f -Quotient of I_{30} and I_0 of the control sample

Ical - Intensity of luminescence after 30 minutes if environmental samples are not added

H% -Percentage decrease of intensity.

1.5.1. Determination of EC20 and EC50

Using the data calculated according to Table 1. prepare H% - log (ml sample) diagram. EC20 and EC50 are determined according to Figure 1.

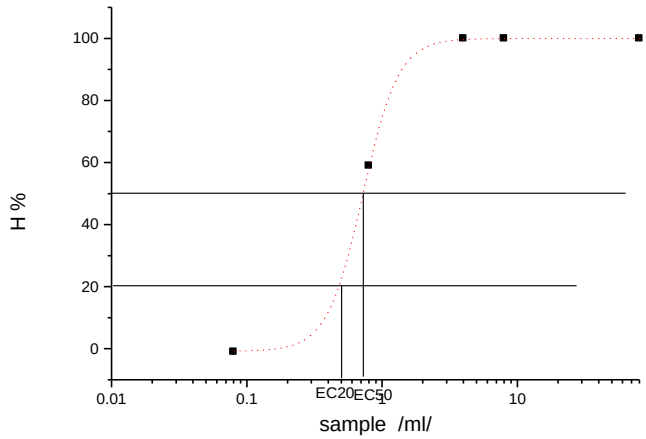


Figure 1. Determination of EC20 and EC50 values

In case of Cu standards the H% - log (mg Cu) diagram is drawn and the $EC20_{Cu}$ and $EC50_{Cu}$ are determined similarly to Figure 1.

1.5.2.Determination of inhibition in Cu equivalent. **($\sum_{Cu}$)**

$\sum_{Cu}$ 20=Inhibition 20= ($EC20_{Cu}/EC20_{sample}$)*10^6 [mg Cu / dm3 sample]
$\sum_{Cu}$ 50=Inhibition 50= ($EC50_{Cu}/EC50_{sample}$)*10^6

2. Investigation of solid phase samples

Introduction

Compared to the liquid phase samples the investigation of solid phase samples has several problems. Extraction of the solid phase sample dilutes to a large extent the sample and it is difficult to find the right extracting agent.

On the other hand, contact biotest has technical problems, because it produces turbidity. Therefore, the detection of light intensity gives false result. The solution may be to use as control an uncontaminated solid sample having the same physico-chemical, biological properties as the investigated sample. However in most cases this uncontaminated solid phase control sample is not available.

2.1. Preparation of the samples

The samples are dried at room temperature, then pulverised.

2.2. Preparation of the culture of testorganism

The testorganism grows in nutrient solution, and is inoculated countinously. The composition of the nutrient solution is as follows:

Aliivibrio fischeri nutrient solution (calculated for 1000 cm^3):

30g	NaCl	0.5g	$(NH_4)_2HPO_4$
6.1g	$NaH_2PO_4x\ H_2O$	5g	pepton
2.75g	K_2HPO_4	0.5g	yeast extract
0.204g	$MgSO_4x7H_2O$	3 cm^3	glicerin

pH=7.2

Sterilise by autoclaving at 121 ^{0}C, for 10 minutes.

2.3.Cu standard solutes

Since the sensitivity of the method is dependent on the initial light intensity as well as on the temperature Cu standards should be used besides the environmental samples. Thus the results of investigation carried out at different times can be compared.
The concentration of Cu-standards are as follows: 20, 40, 80, 120, 160, 200, 400 ppm.
The measured Cu-quantities in the cuvettes are as follows : 1, 2, 4, 6, 8, 10, 20 µg Cu.
Use 2% of NaCl for dilution.

2.4. Dilution of the samples

A) soil, sediment

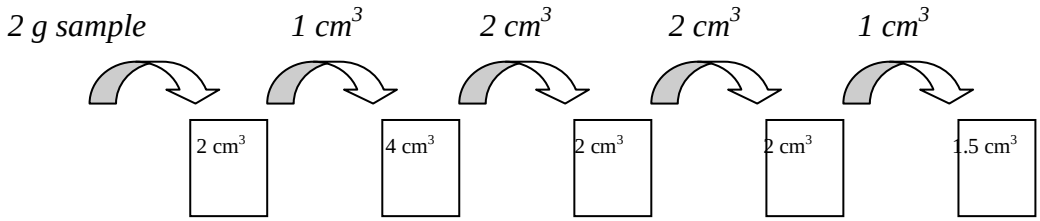


Figure 2. Dilution in case of soil and sediment

The measured soil or sediment quantities in the cuvettes are as follows : 1, 2.5, 5, 10, 50 m. The dilution is carried out using 2% of NaCl.

2.5. Determination of luminescence light emission by luminometer

Steps

1) Place 0.2-0.2 cm^3 of cell suspension (prepared according to 1.1. section) into the cuvettes of luminometer
2) Measure the intensity of luminescence light immediate. (I_0)
3) Add
   a. 0.05-0.05 cm^3 of sample dilution (mix well before adding)
   b. 0.05 - 0.05 cm^3 of Cu-standards
   c. 0.05 cm^3 of 2% NaCl (control sample) into the cuvettes.

3.a. Uncontaminated solid phase control samples are not available

Add the samples according to Figure 3. Measure the light intensity immediately (I_1). I_1 serves as control - assuming, that there is no immediate inhibition of the contaminants in the samples.

3.b. Uncontaminated solid phase control samples are available

Add the dilution series of control sample to the cell suspension in parallel with the contaminated sample. The same dilution series should be used both for the control and for the contaminated sample. There is no need for immediate detection of light inhibition.

4. Wait for 30 minutes. Measure the luminescent light intensity (I_{30}).

2.6. Data evaluation

The calculation is carried out according to Table 2 (3.a) and 3. (3.b).

2.6.1. Determination of EC20 and EC50

Using the data calculated according to Table 2.,3. prepare H% - log (mg dried sample) diagram. EC20 and EC50 are determined according to Figure 1.
In case of Cu standards the H% - log (mg Cu) diagram is drawn and the $EC20_{Cu}$ and $EC50_{Cu}$ are determined similarly to Figure 1.

2.6.2.Determination of inhibition in Cu equivalent. ($\sum_{Cu}$)

$$\sum_{Cu} 20 = \text{Inhibition } 20 = (EC20_{Cu}/EC20_{sample})*10^6 \quad \text{[mg Cu /kg sample]}$$
$$\sum_{Cu} 50 = \text{Inhibition } 50 = (EC50_{Cu}/EC50_{sample})*10^6$$

According to the calculated Inhibition20 and Inhibition50 the samples are classified as follows:

Inhibition 20 [mg Cu/kg soil]	**Inhibition 50 [mg Cu/kg soil]**	**Classification**
<80	**<120**	non toxic
80-250	**120-300**	slightly toxic
250-400	**300-500**	toxic
>400	**>500**	very toxic

Evaluation of the 3.a. case

Sample	**I_0**	**I_1**	**I_{30}**	**$Ical_0=f_0*I_0$**	**$Ical_1=f_1*I_1$**	**$H\%=100*(Ical_c-I_{30}) / Ical_c$**
control						
Cu_1 Cu_2 Cu_3 Cu_4 Cu_5						
1 $sample_1$ 1 $sample_2$ 1 $sample_3$ 1 $sample_4$ 1 $sample_5$						

Evaluation of the 3.b. case

Sample	**I_0**	**I_{30}**	**f_s, f_0**	**Ical**	**H%=100*(Ical-I_{30})/Ical**
controll					
Cu_1 Cu_2 Cu_3 Cu_4 Cu_5			$f_0=I_{30c}/I_{0c}$	$Ical=f_0*I_{0Cu}$	
solid $control_1$ s $control_2$ s $control_3$ s $control_4$ s $control_5$			$fs_1=I_{30sc1}/I_{0sc1}$ $fs_2=I_{30sc2}/I_{0sc2}$ $fs_3=I_{30sc3}/I_{0sc3}$ . .		
1 $sample_1$ 1 $sample_2$ 1 $sample_3$ 1 $sample_4$ 1 $sample_5$				$Ical_1=fs_1*I_{01s1}$ $Ical_2=fs_2*I_{01sam1}$ . . .	

LABORATORY PRACTICE 3

Testorganisms for the testing of water toxicity

Prepared by: Zsuzsanna Kunglné Nagy

TUB, Department of Agricultural Chemical Technology
Department of Applied Biotechnology and Food Sience
2013

Aim of the laboratory exercise

Our purpose is to provide the students some information about the most interesting aquatic ecotoxicological test methods and test organisms. During the laboratory exercise students will study the characteristics of the test organisms from three different trophic levels, following to get an unknown suspension to identify the unicellular test organisms. After identification, students will use Bürker's chamber to determine the algae cell concentration of the suspension.

Introduction of the test organisms

Pseudokirchneriella subcapitata

This is a microalga. It looks like a sickle; it has curved and twisted appearance. Green algae are photosynthetic organisms; their colour is due to the green pigment chlorophyll. The cells are typically presented in a solitary form. It is 8-14 μm long and 2-3 μm wide. This species is quite sensitive to the presence of toxic substances. It can be used as bioindicator species to assess the levels of nutrients or toxic substances in freshwater environments.[1][2]

Chlorella vulgaris

Chlorella is a genus of single-cell green algae. Its shape is spherical with a diameter of 2-10 μm. It has no flagella. *Chlorella* contains the green photosynthetic pigments chlorophyll-a and -b in its chloroplast. Through photosynthesis, it multiplies rapidly, requiring only carbon dioxide, water, sunlight, and a small amount of minerals to reproduce.[3]

Scenedesmus subspicatus (new name: Desmodesmus subspicatus)

It is a large freshwater green alga. It is non-motile. Green algae are photosynthetic organisms, their colour is due to the green pigment chlorophyll. *Scenedesmus subspicatus* can produce two different forms: colony or unicell, depending on the environmental conditions. *Scenedesmus* may be a useful bioindicator of nutrient conditions in lakes and as a model organism in scientific research.[4]

[1] http://silicasecchidisk.conncoll.edu/LucidKeys/Carolina_Key/html/Selenastrum_Main.html
[2] http://www.sciencedirect.com/science/article/pii/S1532045602000777
[3] Scheffler, John (3 September 2007). "Underwater Habitats". Illumin 9 (4)
[4] http://www.nhm.ac.uk/nature-online/species-of-the-day/scientific-advances/industry/desmodesmus-subspicatus/index.html

Tetrahymena pyriformis

Tetrahymena is free-living ciliate protozoa. They are common in freshwater ponds. *Tetrahymena* species are used as model organisms in biomedical research.[5] They also represent the eucaryote cell in environmental toxicology, cell biology and genetic research. It has a size of 25−90 μm, is pear-shaped and the cell is covered with cilia.[6]

Lemna minor

It is a floating freshwater aquatic plant, with one, two or three leaves each with a single root hanging in the water. As more leaves grow, the plants divide and become separate individuals. The root is 1-2 cm long. The leaves are oval, with length of 1-8 mm and width of 0.6-5 mm. It propagates mainly by division, and flowers are rarely produced. Flowers are about 1 mm diameter, with a cup-shaped membranous scale containing a single ovule and two stamens. The seed is 1 mm long, ribbed with 8-15 ribs.[789]

Heterocypris incongruens

Ostracods, or ostracodes, are a class of the Crustacea (class Ostracoda), sometimes known as seed shrimp. They are small crustaceans; their size can vary from 0.25 to 8 mm. The freshwater species rarely exceed 3 mm. Their bodies are flattened from side to side and protected by a bivalve-like, chitinous or calcareous valve or "shell". When disturbed, the shell valves close tightly, leaving nothing on the outside. When they outgrow the shell, they molt it and grow a new one. They can be found in shallow sediment of freshwater. Ostracods can be part of the zooplankton or (most commonly) are part of the benthos, living on or inside the upper layer of the sea floor. They have a wide range of diets, and the group includes carnivores, herbivores, scavengers and filter feeders. They can reproduce both digenic or monogenic.[10]

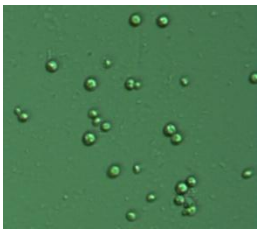
Daphnia magna

Daphnia is a genus of small, planktonic crustaceans, between 0.2 and 5 mm in length. *Daphnia* are one of the several small aquatic crustaceans, commonly called water fleas because of their saltatory swimming style. They live in various aquatic environments ranging from acidic swamps to freshwater lakes, ponds, streams and rivers.[11]

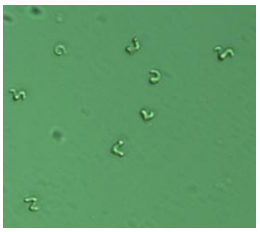
---

[5] Alfred M. Elliott (1973). "Biology of Tetrahymena". Dowen, Hutchinson and Ross Inc. ISBN 0-87933-013-9.
[6] Mónika Molnár and Viktória Feigl (2013) Environmental toxicology lecture notes, part 1, Budapest University of Technology and Economics
[7] Flora of NW Europe: *Lemna minor*
[8] Flora of North America: *Lemna minor*
[9] Plants of British Columbia: *Lemna minor*
[10] http://en.wikipedia.org/wiki/Ostracod
[11] http://en.wikipedia.org/wiki/Daphnia

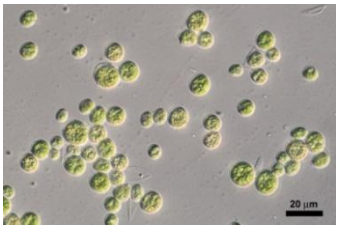
Daphnids can eat bacteria and yeast beside algae. *Daphnia* reproduce parthenogenetically (born only females). Usually 4–10 juvenile animals are nurtured in the brood pouch inside the carapace. Newly hatched *Daphnia* must molt several times before they are fully grown into adults, usually after about two weeks. The reproduction process continues while the environmental conditions continue to support their growth. In harsh environmental conditions, production of new female generations ceases and parthenogenic males are produced.



1. Figure: *Chlorella vulgaris*



3. Figure: *Pseudokirchneriella subcapitata*



6. Figure: *Scenedesmus subspicatus*

2. Figure: *Tetrahymena pyriformis*

4. Figure: *Lemna minor*

7. Figure: *Heterocypris incongruens*

5. Figure: *Daphnia magna*

Running of the laboratory exercise

Survey the aquatic test organisms with two types of microscopes

Microscopes are instruments used to magnify objects that cannot be seen by the unaided eye.[12] There are many categories of microscopes.

[12] Jaime S. Colomé, A. Mark Kubinski, Raúl J. Cano, David V. Grady: Laboratory Exercises in MICROBIOLOGY, West Publishing Company, 1986

During the laboratory exercise we are going to work with two kinds of microscopes:

- Compound binocular light microscope
- Stereo microscope

Surveying with the compound binocular light microscope

We are going to observe the characteristics of three different freshwater green algae and a ciliate protozoa.

- *Pseudokirchneriella subcapitata* (freshwater green algae)
- *Chlorella vulgaris* (freshwater green algae)
- *Scenedesmus subspicatus* (freshwater green algae)
- *Tetrahymena pyriformis* (ciliate protozoa)

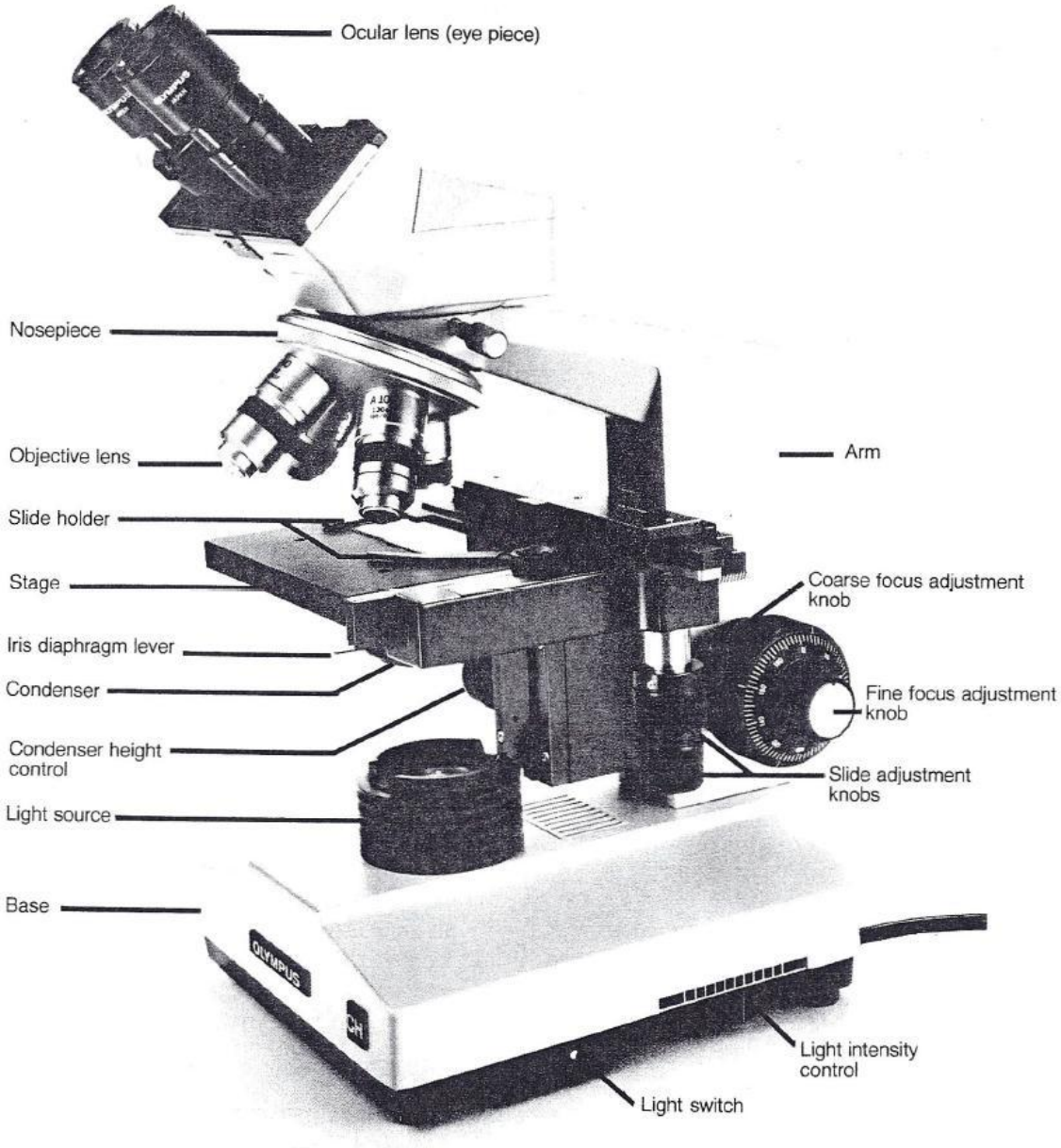
Surveying with the stereo microscope

We are going to have a look at the characteristics of three test organisms from a higher organization level.

- *Lemna minor* (common duckweed)
- *Heterocypris incongruens* (freshwater ostracod)
- *Daphnia magna* (freshwater water flea)

Light microscope[12]

Light microscopes may be **simple or compound**. A simple microscope uses one lens between the eye and the object. It's like a hand-held magnifying glass. On the other hand a compound microscope combines two or more lenses in series between the eye and the object.
In the compound microscope the lens nearest to the eye is called **ocular lens** while the lens nearest to the object is referred to as **objective lens**. In most compound microscopes more objective lenses (usually these objectives magnify 4x, 10x, 40x and 100x) are attached to the rotatable nosepiece. The 100x objective is referred to as the oil immersion lens. It must always be immersed in special oil when in use.
Compound microscopes that have two eye pieces are referred to as **binocular** microscopes, while those which have only one eye piece are called **monocular** microscopes.



8. Figure: Parts of a compound microscope[1]

The overall magnification of a microscope is easy to be calculated:

$$M_{microscope} = M_{objective} \times M_{ocular}$$

M - magnification

For example if the 100x objective lens is in use and the microscope has a 10x ocular lens, the overall magnification of the microscope is 100x10=1000x.

How to use a light microscope

In this microscope the **light source** shines into the objective lens. The field of view is generally bright. The specimen may be darker or brighter than the background depending upon how the **condenser** is adjusted.

Steps procedure of using a microscope:

1. Make an aqueous slide.
2. Plug in the microscope and turn on the **light switch**.
3. Position the slide over the light hole in the **stage** so that light coming through the stage from below hits the specimen. The **slide holder** is positioned by using the **slide adjustment knobs** attached to the stage and just below it.
4. Set the **light intensity control** to the upper-middle range.
5. At first, rotate the **nosepiece** (lens turret) to get the lower power (10x) objective.
6. Adjust the distance between the objective lens and the slide to the closest station using the **coarse focus adjustment knob**. ALWAYS position the lens by viewing the procedure from the side.
7. Before looking through the microscope, make sure that you know which way to turn the **fine focus adjustment** so the distance between the specimen and the lens increases when you turn the fine focus adjustment.
8. Look through the ocular lens and focus the image using the coarse focus knob by increasing the distance between the specimen and the objective lens.
9. When you found the image, use the fine focus knob to sharpen the image.
10. Scan the field by moving the slide and draw what you see.
11. To use the high dry objective lens (40x), abduct the slide holder from the lens firstly, then simply rotate the nosepiece.

Stereo microscope[13]

The stereo microscope is an optical microscope variant designed for low magnification observation of a sample, typically using light reflected from the surface of an object rather than transmitted through it. The instrument uses two separate optical paths with two objectives and eyepieces to provide slightly different viewing angles to the left and right eyes. This arrangement produces a three-dimensional visualization of the sample.

The stereo microscope should not be confused with a compound microscope equipped with double eyepieces and a binoviewer.

[13] Wikipedia: http://en.wikipedia.org/wiki/Stereo_microscope

In such a microscope, both eyes see the same image, with the two eyepieces serving to provide greater viewing comfort. However, the image in such a microscope is no different from that obtained with a single monocular eyepiece.
Unlike a compound light microscope, illumination in a stereo microscope most often uses reflected illumination rather than transmitted (diascopic) illumination, that is, light reflected from the surface of an object rather than light transmitted through an object. Use of reflected light from the object allows examination of specimens that would be too thick or otherwise opaque for compound microscopy. Some stereo microscopes are also capable of transmitted light illumination as well.
Great working distance and depth of field are important qualities for this type of microscope. Both qualities are inversely correlated with resolution: the higher the resolution, the smaller the depth of field and working distance.
The typical magnification in the case of stereo microscopes is under 100x.

Qualitative and quantitative measurement of an unknown sample

All students will get a numbered sample of a microbe-suspension to identify it qualitatively and quantitatively. 2−4 kinds of unicellular microorganisms (*Pseudokirchneriella subcapitata*, *Chlorella vulgaris*, *Scenedesmus subspicatus*, *Tetrahymena pyriformis*) might be found in the samples. After the identification, students will count the number of **algae cells** found in 1 ml of the unknown sample with Bürker's chamber.

Qualitative investigation of the unknown sample – Identify the unicellular test organisms

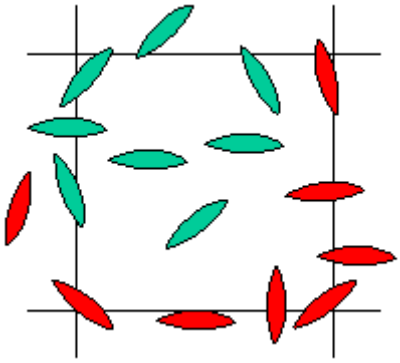
Students will review the micro-morphological characteristics of the test organisms found in the sample to identify them with a compound binocular light microscope. The following test organisms might be in the samples:

- *Pseudokirchneriella subcapitata*
- *Chlorella vulgaris*
- *Scenedesmus subspicatus* (new name: *Desmodesmus subspicatus*)
- *Tetrahymena pyriformis*

Quantitative measurement of an unknown sample – Direct microscopic count with Bürker's chamber

The direct microscopic count is a rapid method for enumerating microorganisms in suspension. This method determines the total number of microorganisms in a suspension by counting the number of cells in a known volume of fluid contained inside a counting chamber[12].

The Bürker's chamber is a specially formed slide with two separated parts. The parts are constructed with known divisions. Two kinds of square sizes are formed on the slide. The surface of the small square is 1/400 mm^2, the surface of the big square is 1/25 mm^2. The depth after covering the slide with coverslip is 0,1 mm. To get **the number of the cells in 1 ml of the suspension**, cells must be counted inside at least 10-10 squares (in the upper and the lower part), diagonally. Cells on the lines of the square may be counted as part of one square and as part of the other. Therefore you may count as part of the square only those cells that are on the top and left lines but not those on the bottom or right lines of the squares (Figure 9).



9. Figure: Count as part of the square only those cells that are on the top and left lines (Source: http://evolution.unibas.ch/ebert/lab/counting.htm)

Calculating the algae concentration from the counted numbers can be done in two ways. One possible way is to calculate with the exact formula:

The exact formula[14]: # algae cells/ml = $(X*1000\ mm^3)/(Y*w^2*d)$

- X = # algae cells counted
- Y = # smallest squares counted
- d = depth of counting chamber
- w = width of 1 square unit

The other way is to work with a specific factor which can be calculated with the characteristics of the chamber and the following formula

Specific factor from the short cut: Factor = $(1000\ mm^3)/(w^2*d)$

- (w^2*d) is the volume of liquid over one square

So this way we can calculate the factor that can simplify the determination of the algae concentration of the suspension in the case of the small and big square of the Bürker's chamber:

[14] http://evolution.unibas.ch/ebert/lab/counting.htm

Factor for small square= $1000/(1/400*0,1) = 4*10^6$
Factor for big square= $1000/(1/25*0,1) = 2.5*10^5$

After having counted the cells inside the square, **calculate the average value** of algae cells found in the 20 squares. Then **multiply the average number by the specific factor** used in the case of the small or big square of the Bürker's chamber.

Requirements for the report

The report must contain the following chapters:

- Aim of the laboratory exercise
- Applied instruments
- Steps of the laboratory exercise
- Types and used magnification of the microscopes
- Characteristics of the examined test organisms – observations
- Peculiarity of sampling
- Number of the samples
- Name of the identified microbes
- Calculation
- Final conclusions

LABORATORY PRACTICE 4

Folsomia candida (Collembola) mortality and *Lepidium sativum* root and shoot growth inhibition test

Written by Ildikó Fekete-Kertész 2013

TUB, Department of Agricultural Chemical Technology
Department of Applied Biotechnology and Food Sience

Introduction

The evaluation of a contaminated site requires more different environmental toxicology tests at the same time, because only one testorganism can hardly represent the whole ecosystem. It means, that generally the use of at least three testorganisms from different trophic levels is necessary. Different trophic levels can be: bacterium, fungi, plant/alga, herbivorous and carnivorous animals.

Environmental toxicology tests applying plants:

In case of plant testorganisms we can distinguish unicellular plants (algae) and higher plants. Higher plants are important members of the ecosystem, because they can transform light into bioavailable energy. Green plants form a protective layer on the top of the soil preventing soil erosion. The endpoints of higher plant toxicity tests are inhibition and growth, photosynthetic and metabolic enzyme activity. The plants used for toxicity tests are chosen by easy maneuverability in laboratory. Standard protocols propose annual plants and grasses.

Environmental toxicology tests applying soil animals:

Although bacteria are most abundant/dominant in soil, the fungi as a group also play an essential role in the decomposition of organic residues. The soil protozoans include flagellates, amoebae and ciliates. Among the ciliates are some genera used in toxicity tests (e.g. *Colpoda* and *Tetrahymena*). There is a tremendous variety of multicellular animals in the soil. Nematodes are the most abundant. Larger metazoans do disturb the soil by ingestion or burrowing. These include mites, springtails (Collembola), isopods, molluscs, millipeds, earthworms. The species used for toxicity tests often have a high reproductive output, as this makes them manageable in cultures. When considering ecotoxicological tests for assessing the risk of soil contaminants, test species should be selected on the basis of their representativeness to the soil community. The set of test species should include the various ways in which soil organisms may be exposed to contamination, that is, through soil pore water, by soil ingestion, ingestion of organic matter, feeding on fungi, through soil air, etc.

References:

K. Gruiz, B. Horváth, M. Molnár: Environmental toxicology, Műegyetemi Kiadó, 2001. (in Hungarian)

Calow, P. (1993) Handbook of Ecotoxicology. Blackwell Science Ltd.

Collembola (*Folsomia candida*) mortality test

Summary:

Type of the test: one species laboratory, animal, acute toxicity and chronic test. It can be used in a microcosm test.

Feasible for: testing the soil directly, or the soil extract should be poured onto standard soil.

Testorganism: *Folsomia candida*, springtail Collembola, sensitive to organic contaminants, mainly to the volatile contaminants and to chemicals absorbed through the skin. Not sensitive enough for heavy metals.

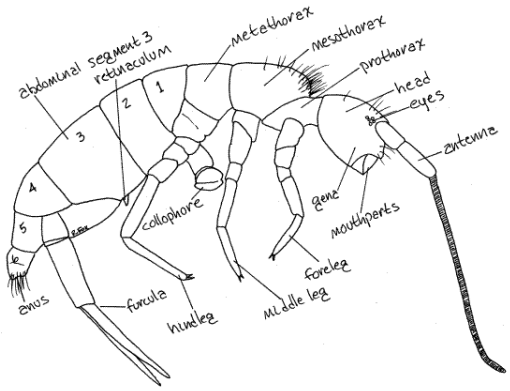
Endpoint: number of animals; lethality; from dilution: EC_{20}, EC_{50}, and ED_{20}, ED_{50}; based on reproductivity test: NOEC.

Necessary instrument: cytoplast microscope or visual.

Duration of the test: acute: 5-10 days, reproductivity test: 20 days.

Standard protocol: ISO 11267:1999, Soil quality -- Inhibition of reproduction of Collembola (*Folsomia candida*) by soil pollutants; OECD/OCDE 232, OECD Guidelines for testing chemicals: Collembolan reproduction test in soil (2009)

Comment: highly reproducible, easily performed test.



Testorganism:

The *Folsomia candida* species belongs to the subclass Collembola (Springtails).
Tiny (max. 3-4 mm) white animals with a furcula (tail-like appendage, that is folded beneath the body to be used for jumping. It is held under tension by a small structure called the retinaculum and when released, snaps against the substrate, flinging the springtail into the air. All of this takes place in as little as 18 milliseconds.). They live in the soil, in forests, there can be 100,000 Collembola in 1 m^2 soil. They respirate with a ventral tube (collophore), that is why they are sensitive to soil vapours. Collembolas reproduce by epimorphosis. In case of suitable humidity environment of 20 °C the eggs hatch within 10–15 days and the animals mature within 10–15 days.

Test protocol:

The *Folsomia candida* species is feasible for acute and chronic tests, the duration of the acute test is 5–10 days, the measuring endpoint of the test is the percentage of the surviving animal. With this test the EC_{20}, EC_{50} or the ED_{20} and ED_{50} can be determined from the dilution of the soil sample.

Necessary tools

- 370 ml volume glasses
- portable balance with tare button
- automatic pipette and tips (5 ml)

Necessary materials:

- tapwater
- granulated yeast
- standard OECD soil. The composition of the OECD soil: peat (10%), kaolinite clay, min. 30% kaolinite content (20%), quartz sand (70%).

The test:

The test is carried out with 20-20 g of air-dried soil sample in 370 ml jars. The samples are prepared by diluting with OECD ground. OECD soil is used as a control. The dilutions used:

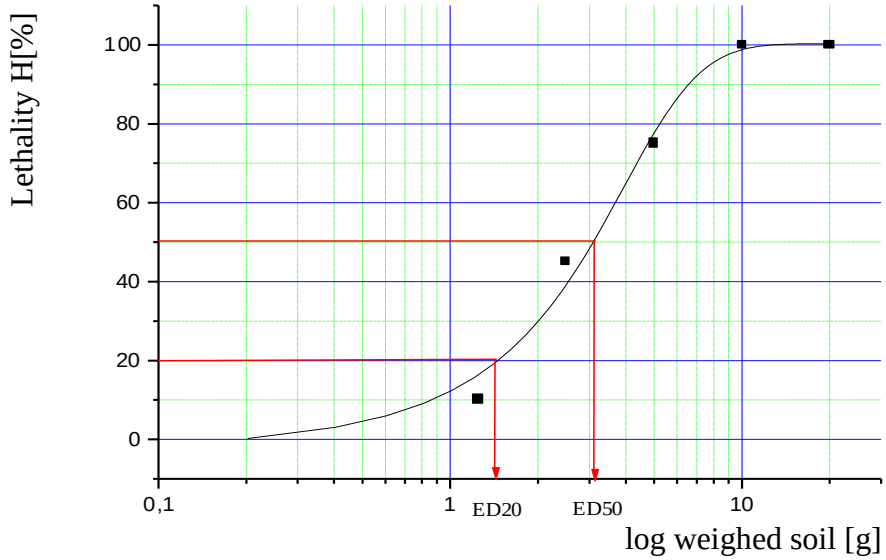
Sample, g	**20**	**10**	**5**	**2,5**	**1,25**
OECD, g	0	10	15	17,5	18,75

- The samples are moistened with 5-5 ml of water and 2-2 mg yeast is added.
- 10 test animals are given into the sample jar.
- The test containers are kept in dark at 20-25 ° C for 7 days.

Evaluation of the test:

- The soil in test jars is suspended in water.
- The suspended material is gently mixed for the soil clots should disintegrate. This way the surviving animals can swim up onto the surface of water.
- Surviving animals are counted on the surface.

- From the number of the surviving and dead animals toxicity can be counted comparing to the toxicity of the control OECD soil, given in mortality percentage. The mortality percentages of the different dilutions are plotted according to the amount of the contaminated soil by Software Origin. From the resulting sigmoid curves ED20 and ED50 values can be determined.



Graphic determination of ED20 and ED50 values

Based on the ED_{20} and ED_{50} values the examined samples are compared for toxicity.

Characterization of the toxicity of the soil samples based on the Folsomia candida mortality test.

ED_{20} [g]	**ED_{50} [g]**	**Characterization**
> 20	> 20	Non toxic
12-20	16-20	Slightly toxic
2-12	4-16	Toxic
< 2	< 4	Very toxic

References:

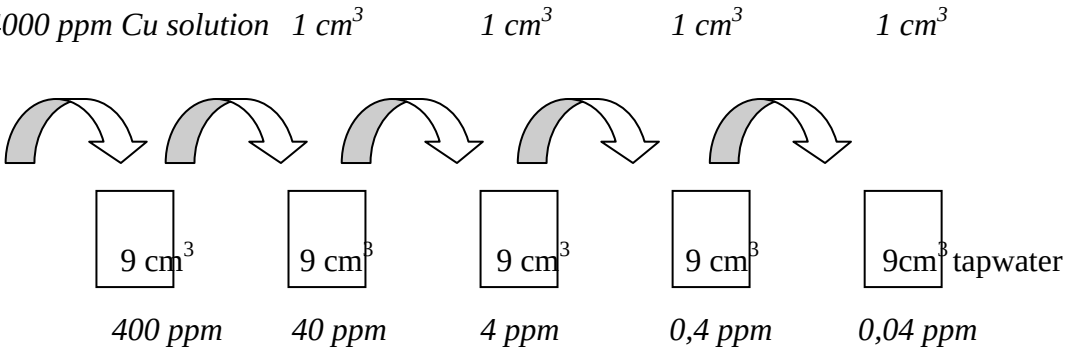
K. Gruiz, B. Horváth, M. Molnár: Environmental toxicology, Műegyetemi Kiadó, 2001. (in Hungarian)

Garden cress (*Lepidium sativum)* seed germination, root and shoot elongation test

The contact garden cress test is based on disciplines of the Hungarian Standard MSZ 21976-17/1988 and OECD 208 protocol. Solid phase samples (soil) and water samples are directly tested. Soil extracts or elutriates are not prepared. The water content of examined soil samples is adjusted. We use uncontaminated brown forest soil and tapwater as negative control sample. The testorganism (garden cress, *Lepidium sativum*) is purchased in seed-shop. Germination rate of seeds must be 95 %.

Test methodology

A 5 member ten-fold dilution series of copper solution is tested during the laboratory practice. The applied stock solution is 4000 mg/L (4000 ppm) concentration.



The water samples:

- $CuSO_4$ solution: 400 ppm; 40 ppm; 4 ppm; 0,4 ppm; 0,04 ppm
- Tap water control sample

1. put a filter paper disc into a Petri-dish.
2. pour 5 cm^3 of the members of the dilution series of the copper solution samples onto the filter paper.
3. 16 garden cress seeds are deposited on the surface of the filter paper.
4. The test is carried out in dark place at 21,5 ^{0}C for 3 days.
5. Evaluate the result of the test after 7 days (measuring root and shoot length with the help of a ruler).

Soil sample:

1. put 5 grams of soil into a Petri-dish.
2. Pour 3,5 cm^3 tapwater.
3. 16 garden cress seeds are deposited on the surface of the soil.
4. The test is carried out in dark place at 21,5 ^{0}C for 7 days.
5. Evaluate the result of the test after 72 hours (root and shoot elongation).

Necessary materials:

- 4000 ppm copper solution
- tap water
- garden cress seeds

Necessary tools:

- Test tube rack and test tubes
- ▫ balance and spoon (when measuring soil sample)
- ▫ petri dish of 9 cm diameter
- ▫ filter paper discs
- ▫ automatic pipettes and pipette tips
- ▫ Al-foil
- ▫ ruler
- ▫ 21.5 ° C thermostat

Data evaluation

The inhibition of root and shoot elongation is given with respect to the control sample in %.

$$H = (C - S / C) * 100$$

H: inhibition of root and shoot elongation in %

C: average length of the root and shoot of the seeds in the control medium [mm]

S: average length of the root and shoot of the seeds in the examined Cu samples [mm]

The same evaluation is applied for each dilution series and soil samples. **X-ppm $CuSO_4$ solution sample** figure is drawn. ED_{20} and ED_{50} values are identified from the figure with the help of Origin 6.0 software.

References

OECD GUIDELINE FOR THE TESTING OF CHEMICALS, Terrestrial Plant Test: 208: Seedling Emergence and Seedling Growth Test http://www.oecd.org/chemicalsafety/testing/33653757.pdf