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LOMONOSOV MOSCOW
STATE UNIVERSITY

METHODOLOGICAL GUIDE

PRINCIPLES OF BIODIAGNOSTICS OF WASTE HAZARDS AND SOIL HEALTH

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Principles of Biodiagnostics of Waste Hazards and Soil Health (Methodological Guide). – Moscow: «GEOS», 2024 – 64 p.

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PREFACE

This manual provides brief information on some methods of biotesting and bioindication of environmental objects. It describes the procedure for biotesting and microbial indication of soil and waste quality, requirements for test organisms, a list of necessary materials, equipment, regulatory documents and standards recommended for obtaining results in assessing ecotoxicity, functional activity and biodiversity of the soil microbiome.

These methods are widely used in practice when establishing the hazard class of waste and assessing soil health.

The manual has been prepared within the project "The "Clean Water" project as the most important component of cooperation between the Russian Federation and the countries of the Global South: socio-economic and technological dimensions" supported by the grant from the Ministry of Science and Higher Education of the Russian Federation program for research projects in priority areas of scientific and technological development (Agreement № 075-15-2024-546).

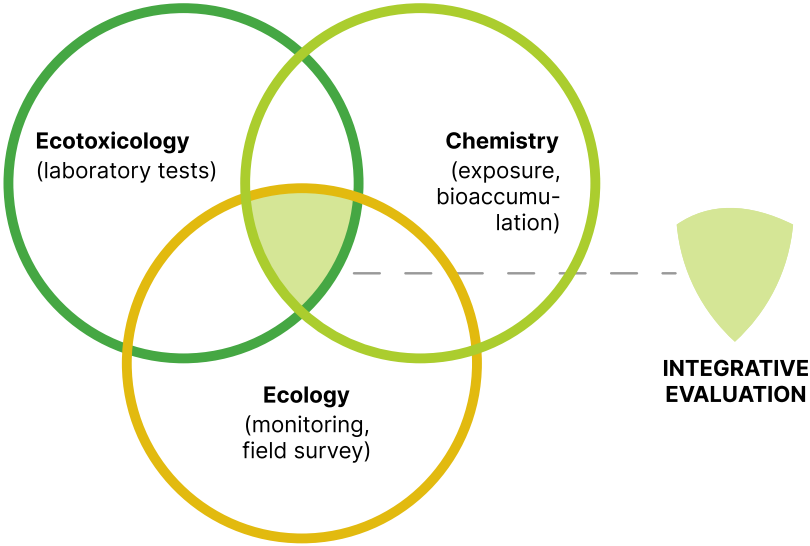
The manual is recommended for participants of trainings and master classes within the framework of international forums, including the International School on Ecology «Ecology: objects of accumulated environmental damage, hazardous waste, Russian and world practice» (26.08.2024 – 04.09.2024) .

A detailed acquaintance with biodiagnostic technologies and their practical application of the described methods is possible within the framework of the educational programs at Lomonosov Moscow State University.

BIODIAGNOSTICS IN ECOLOGICAL ASSESSMENT OF SOILS AND HAZARD WASTE

Biodiagnostic methods include biotesting of ecotoxicity through laboratory rapid analyses and bioindication of the state of biota in field conditions.

The triad of methodological approaches that make up pollution risk assessment



A bioassay is a method to determine the potency or effect of a substance based on its impact on living organisms, cells, tissues or plants.

Biotests are widely used to detect the biological hazard of various substances, assess the risk of environmental pollution and determine the hazard class of waste.

SOILS

The soil is the upper loose cover of the earth's crust and interacts with other components of the environment, including wastes disposed in land. **A bioassay** is a technique for determining soil quality and identifying any harmful effects from wastes (or other resource) on the soil, as well as the presence of bioavailable chemicals in the soil at concentrations high enough to have toxic effect on living organisms. Bioassays are useful for examining soil quality at the different stage of (bio)remediation. This method is simple, economical, and direct, allowing to determine if soil is “healthy” and whether wastes are safe to the surrounding environment.

SOIL-LIKE WASTES

The composition of some wastes is similar to the earth's crust, but certain properties prevent their sustainable recycling and can be defined as soil-like bodies. They can be introduced into environment as a Technosols or transformed into eco-friendly soil-like mixtures. However, chemical composition alone might not be enough to use them without studying their toxicity to the environment, especially soils. Bioassay is the best way to test such objects and exam their possible impact.

WASTES

Biological organisms are used in laboratory tests to assign wastes to hazard classes (I–V), to reveal the waste impact to surroundings, especially soils, to detect soil contamination, as well to assess the quality of soil-like wastes.

Production and consumption wastes are substances or objects formed in the process of production, performance of work, provision of services or in the process of consumption, which are disposed of, intended for disposal or subject to disposal in accordance with the Russian Federal Law.

The code of each type of waste has on 11-digit structure

The first eight characters of the code are used to encode the origin of the type of waste and its composition.

The ninth and tenth digits of the code are used to encode the aggregate state and physical form of the type of waste.

The eleventh character of the code is for coding the hazard class of the type of waste, depending on the degree of negative impact on the environment.

In the 11th character of the code, the digit 0 is used for blocks, types, subtypes, groups and subgroups; for types of waste, the significant digit indicates: 1 - hazard class I (the most hazard); 2 - hazard class II; 3 - hazard class III; 4 - hazard class IV; 5 - Hazard class V (non-hazard).

According to Russian legislation, the waste assignment to hazard class is done by using bioassay of the sample aqueous extract from the waste sample.

The aqueous extract is prepared with a mass ratio of waste and water 1:10.

The hazard class of waste is determined by the dilution of an aqueous extract, in which no harmful effects on living organisms have been detected.

The waste samples that have no harmful effects without any dilution of the initial extract are considered safe (belonging to hazard class V).

Correspondence of the multiplicity of dilution (Md) of hazardous waste aqueous extract to a certain hazard class

The waste hazard class	The multiplicity of dilution (Md) of an aqueous extract from the waste <1>	Waste hazard characteristics
I	Md > 10000	extremely hazardous waste
II	1000 < Md 10000	highly hazardous waste
III	100 < Md 1000	moderately hazardous waste
IV	1 < Md 100	low-hazard waste
V	Md = 1	practically non-hazardous waste

The choice of method for preparing waste samples for bioanalysis depends on the characteristics of the waste, and above all on the state of aggregation and the level of salinity.

Standard or Guideline

- ISO 19204:2017 Soil quality — Procedure for site-specific ecological risk assessment of soil contamination (soil quality TRIAD approach)
- Federal Waste Classification Catalog. Rosprirodnadzor Order No. 242 dated 05/22/2017 (as amended on 01/18/2024) "On Approval of the Federal Waste Classification Catalog"
- On approval of criteria for assigning waste to hazard classes I-V according to the degree of negative impact on

the environment. Ministry of natural resources and ecology of the Russian Federation order dated December 4, 2014 N 536

- Resources saving. Waste treatment. Terms and definitions GOST 30772-2001
- Waste products resulting from the productive process and consumption. Federal Act dated 24.06.1998 N 89-FZ.
- MR 2.1.7.2297-07 Methodological recommendations. 2.1.7. Soil. Cleaning of populated areas. Household and industrial waste. Sanitary protection of the soil. Substantiation of the hazard class of production and consumption waste by phytotoxicity

AQUATIC AND TERRESTRIAL TEST BIOTA

Aquatic and terrestrial test biota are used worldwide in ecotoxicological studies. These bioassays provide qualitative and quantitative data on adverse effects from chemicals or environmental pollutants on living organisms in acute and chronic toxicity tests.

There is a wide range of aquatic and terrestrial test biota that can be used for standardized and culture-independent toxicity testing, bioassay services, or other research applications.

These freshwater, marine and estuarine biota can also be used as starter culture to maintain live stocks or as back-up in the culturing facility of environmental laboratories. The suitability of test cultures for biotesting is assessed by the reaction to a reference toxicant. The reference toxicant is usually potassium dichromate — $K_2Cr_2O_7$.

Many of these test species are identical to the test organisms used in standardized toxicity tests. As an example, the test biota is used to study whole effluent toxicity tests under national permit and regulatory frameworks.

Ecotoxicity tests are bioassays performed to determine whether a potentially toxic compound or an environmental

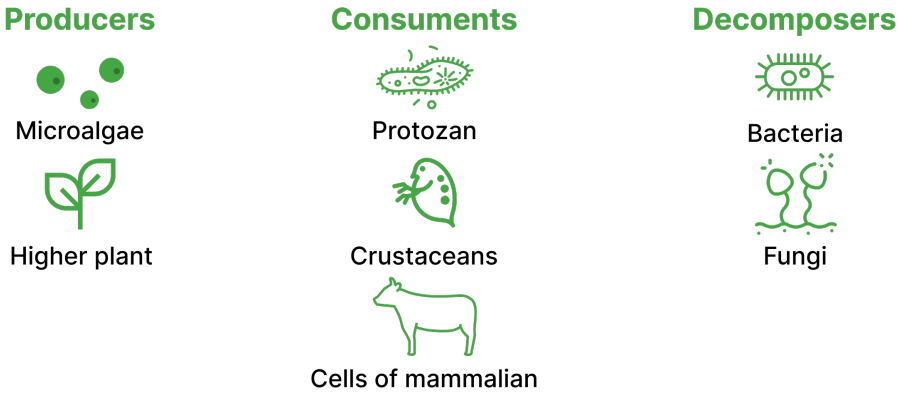
sample (e.g. effluent, leachate, sediment or soil sample) causes a biologically significant response in test organisms (test biota).

The endpoint(s) observed or measured may include the number of surviving organisms (mortality), size or growth of organisms, number of eggs or offspring produced (reproduction) or any relevant biochemical (e.g. luminescence, etc.) or physiological variable (mobility, behavior, etc.).

In accordance with the Criteria for classifying waste into I - V hazard classes according to the degree of negative impact on the environment (Order of the Ministry of Natural Resources of Russia dated 04.12.2014 N 536):

- The responses of two test-organisms from different taxonomic groups must be considered
- The aqueous extracts from solid samples (wastes or soil like wastes) are used for eco-toxicological control
- More reliable results are obtained in a battery of biotests based on the reactions of representatives of all the main trophic ecosystem levels.

BIO Scanner - was developed as tools for rapid assessment of biosafety components of the environment and man-made objects on the test reaction of the body all tne major trophic levels



TOXICITY TERMS

Acute toxicity is a discernible adverse effect (lethal or sublethal) induced in the test organisms within a short period of exposure to a test material.

EC50 is the median effective concentration (i.e., the concentration estimated to cause a specified non-lethal or lethal effect on 50 % of the organisms). The particular effect must be specified as well as the exposure time (e.g., "96-h EC50 for immobilization").

Endpoint means the variables (i.e., time, reaction of the organism, etc.) that indicate the termination of a test, and also means the measurement(s) or value(s) derived, that characterize the results of the test (LC50, EC50, etc.).

ERA (Environmental Risk Assessment) is a systematic approach for assessing and managing the risk associated with human health and ecological entities caused by an event occurring in the environment

Immobility is defined as the inability to swim during the 15 seconds which follow gentle agitation of the test solution.

LC50 is the medial lethal concentration (i.e., the concentration of material in water that is estimated to be lethal to 50 % of the test organisms).

Lethal means causing death by direct action. Death of daphnids is defined here as the cessation of all visible signs of movement or other activity, including second antennae, abdominal legs, and heartbeat as observed through a microscope.

LT50 is the time (period of exposure) estimated to cause 50 % mortality in a group of organisms held in a particular test solution. The value is best estimated graphically.

Sublethal means detrimental to the organism, but below the level which directly causes death within the test period.

Toxicity is the inherent potential or capacity of a material to cause adverse effects on living organisms.

Toxicity test is a determination of the effect of a material on a group of selected organisms under defined conditions. An aquatic toxicity test usually measures the proportions of organisms affected by their exposure to specific concentrations of chemical, effluent, elutriate, leachate, or receiving water.

TEST ORGANISMS AND BIOTESTING TECHNOLOGIES

The most popular test organisms for determining the hazard class of waste are crustaceans, algae, protozoa, bacteria, seeds of higher plants

CRUSTACEAN

METHOD ASSIGNMENT

The acute toxicity test with crustaceans is one of the most internationally used bioassays for toxicity screening of chemicals and for toxicity monitoring of effluents, contaminated waters, aqueous extracts of waste and soils.

Crustaceans are well characterized, have a rapid parthenogenetic reproductive cycle and show sensitivity to a range of environmental xenobiotics.

THE ESSENCE OF THE METHOD

The toxicity assessment is based on the immobilization and lethality of crustaceans under the influence of the tested water samples, which are measured in the acute tests.

The exposure time for endpoints evaluated in acute toxicity test with crustaceans encompass responses (immobilisation and lethality) - 48-96 hours according to various standard protocols (FR 1.39.2007.03221 FR 1.39.2007.03222 FR 1.39.2006.02505 OECD 202 ISO 6341; DIN 38412-L30).

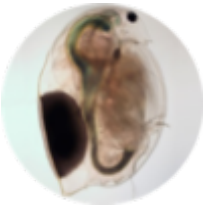
MATERIALS AND INSTRUMENTS

- *Daphnia magna* and *Ceriodaphnia affinis* are a well-established and widely used model organisms for freshwater toxicity testing

- *Artemia salina* cysts - model organisms for estuarine / marine tests toxicity testing
- Live food (microalgae and yeast)
- Cultivated water for control
- Testing sample
- Exposure vessels
- Salinometer
- pH meter
- Climate control camera

PROCEDURE

Freshwater tests



Daphnia magna (FR 1.39.2007.03221)

The stocks crustaceans *Daphnia magna* are cultivated in laboratory conditions on live food (microalgae and yeast).

1. The control and test samples in a volume of 100 ml are poured into separate vessels (3 replicates with control water and 3 replicates with test sample);
2. Juvenile crustaceans *Daphnia magna* aged 24 hours are placed in vessels with control water and a test sample (10 crustaceans per vessel);
3. The vessels are placed on exposure in the climate controller;
4. Endpoints as immobilization and lethality evaluated after 96 hours (count live crustaceans);
5. Calculate the percentage of survivors in the experiment and in control vessels.

Ceriodaphnia affinis (FR 1.39.2007.03222)

1. The stocks crustaceans *Ceriodaphnia affinis* are cultivated in laboratory conditions on live food (microalgae and yeast);
2. The control and test samples in a volume of 20 ml are poured into separate vessels (5 replicates with control water and 5 replicates with test sample);
3. Juvenile crustaceans aged 24 hours are placed in vessels with control water and a test sample (4 crustaceans per vessel);
4. The vessels are placed on exposure in the climate controller;
5. Endpoints as immobilization and lethality evaluated after 48 h (count live crustaceans);
6. Calculate the percentage of survivors in the experiment and in control vessels.

Estuarine / marine tests

Artemia salina cysts (FR 1.39.2006.02505)

Artemia cysts are placed in artificial or natural sea water to obtain juveniles.

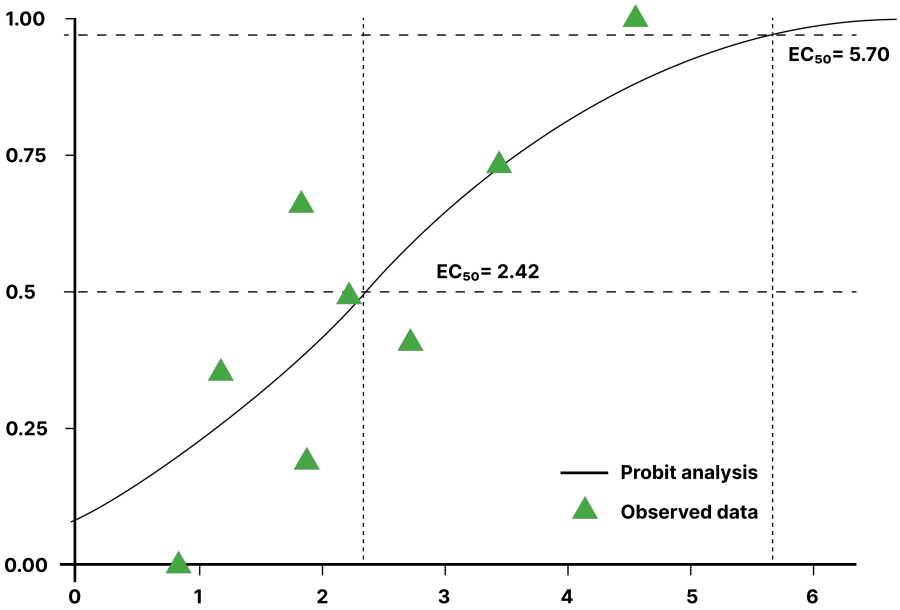
1. Juvenile crustaceans aged 24 hours are placed in vessels with control water and a test sample (4 crustaceans per vessel)

2. The vessels are placed on exposure in the climate controller
3. Endpoints as immobilization and lethality evaluated after 72 hours
4. Calculate the percentage of survivors in the experiment and in control vessels

RESULTS EVALUATION

The acute toxicity effect of the test sample (EC_{50}) is established provided that the crustacean immobilization in the control does not exceed 10%.

The calculation EC_{50} is usually performed using probit analysis.



Probit analysis: on the graph reflecting the relationship between the effect and concentration, a point is determined corresponding to the probit value of 5 (50% effect). A perpendicular line is then dropped from this point onto the concentration axis. The intersection of this perpendicular with the X axis is the desired value of the logarithm of the concentration, EC_{50}.

STANDARD OR GUIDELINE

- Methodology for Determining the Toxicity of Water and Aqueous Extracts from Soils, Sewage Sludge, and Waste Based on Mortality and Changes in Fertility of *Ceriodaphnia.* **(FR.1.39.2007.03221)**
- Methodology for Determining the Toxicity of Water and Aqueous Extracts from Soils, Sewage Sludge, and Waste Based on Mortality and Changes in Fertility of *Daphnia*. **(FR.1.39.2007.03222)**

- Methodology for Determining the Toxicity of Highly Mineralized Surface and Waste Waters, Soils, and Waste Based on Survival of Brackish-Water Crustaceans *Artemia salina* L. (FR.1.39.2006.02505 / PND F 14.1:2.14-06 / 16.1:3.11-06)

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2. Sergeeva, Y. D., Kiryushina, A. P., Calero, V. K., Fedorova, O. A., Terekhova, V. A. Comparison of the efficiency of micro- and nanoparticles of zero-valent iron in the detoxification of technogenically polluted soil. Eurasian Soil Science 56, 2 (2023), 238–246.

ALGAE

METHOD ASSIGNMENT

Algotesting is used to assess water quality, waste hazard class, and soil pollution.

THE ESSENCE OF THE METHOD

Measurement of algotoxicity is based on the assessment of the inhibition of the growth of the algae cell population or fluorescent indicators in the experiment relative to the control over a certain period of time (48 or 72 hours). The control is the nutrient medium without the addition of the test sample.

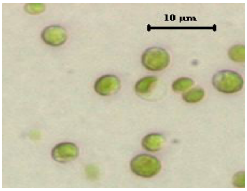
MATERIALS AND INSTRUMENTS

- Inoculum suspension of algae cells
- Flasks
- Cylinders
- Pipette dispensers or pipettes
- Goryaev chamber and cover slips
- Optical microscope with ×200 magnification (for manual cell counting)
- Fluorimeter (for example, Foton-10, Fluorat-02-3M or other)
- EcoTestSoft (ETS)

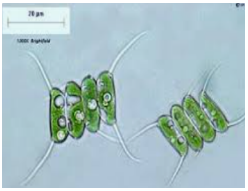
PROCEDURE

Algologically pure culture

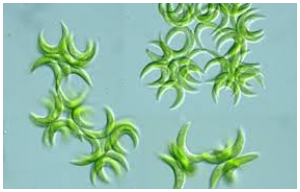
Freshwater algal tests



Chlorella vulgaris



Scenedesmus quadricauda



Selenastrum capricornutum. (Syn *Pseudokirchneriella subcapitata*)

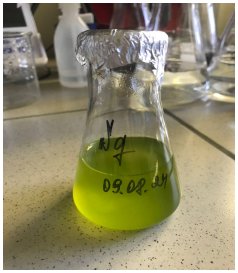
Estuarine / marine tests

Phaeodactylum tricornutum

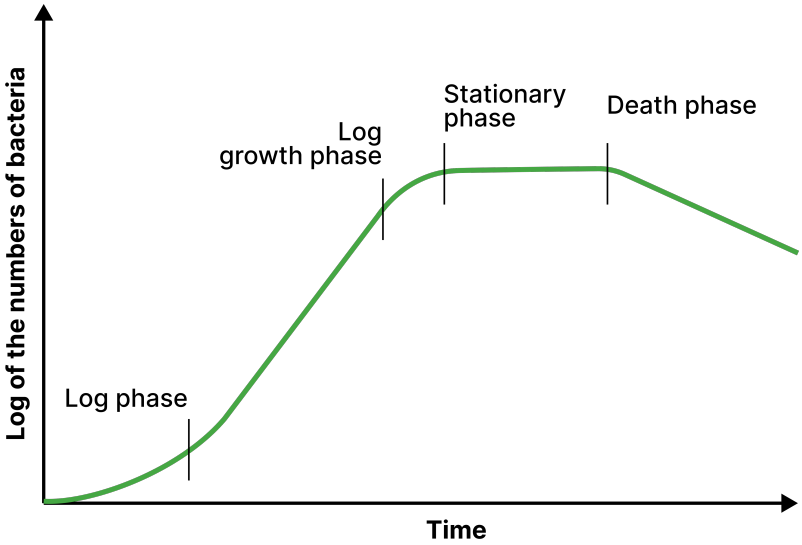
Preparing the stock micro algal culture

1. The stocks micro algal culture for freshwater test is cultivated in laboratory conditions on nutrient medium. The nutrient medium for marine algae is prepared using natural or artificial seawater of the required salinity (usually 20 ‰, which corresponds to the average salinity of seawater in the shelf zone).
2. Media with cell cultures are added to flasks (50-100 cm^3 in volume).

The flasks are placed in a luminostat (3.5 thousand lux) with a day/night cycle (12:12 h) or in special cultivator.



3. To assess toxicity, a culture in the logarithmic growth phase at the age of 3-5 days is used (inoculum).

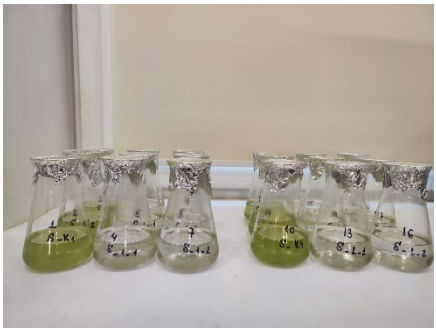


The algae culture with a population of 30-50 thousand cells/cm^3 is sterilely poured into experimental flasks (50 or 100 cm^3) and the volume of the toxicant solution required to create a particular concentration in the sample is added.

The flasks are placed in a termoluminostat (the left picture).

Special devices (the right picture) are also used for exposure of control and experimental samples in smaller volume vessels (up to 10 cm^3)

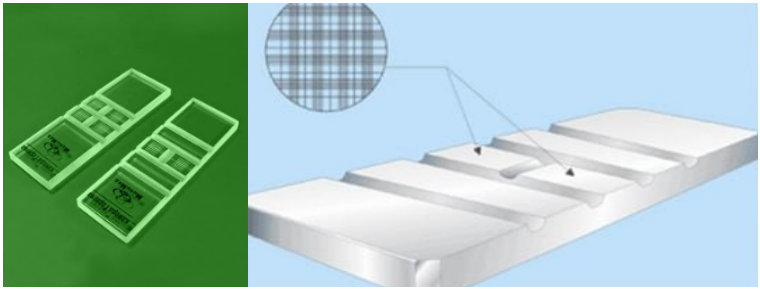
4. After 72 hours, the biotesting is complete. The experiments are carried out in 3-5 replicates to obtain statistically significant results. In each flask, the number of cells is taken into account to determine the presence of acute toxic effect of the water sample.



Microalgae bioassay procedure (to assess toxicity)

1. To compare the growth of the algal cell population in the experiment and in the control a direct cell counting method using microscopy is used

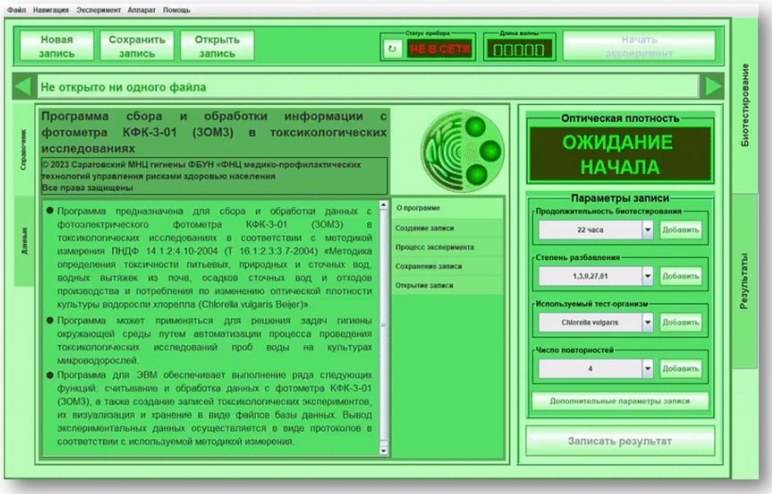
2. The cells in culture calculated after 72 hours using Goryaev chamber.



External appearance and grid of a Goryaev chamber

A fluorimeter is used to compare the fluorescence readings of algae.

To account for the development of algae based on population density, EcoTestSoft (ETS) is used. This software environment facilitates automatic data recognition, processing of measurement results from photometers and calculation of toxicity indices according to formulas provided in the methodological protocols.



RESULTS EVALUATION

The effective concentration, EC_{50} value, for 72 hours can be determined by probit analysis using Excel or graphically.

STANDARD OR GUIDELINE

- FR 1.39.2007.03223 Toxicity Analysis of Water, Water Extracts from Soils, Sediments of Sewage Waters, and Wastes by Dynamics of Chlorophyll Fluorescence Level and Abundance of Algal Cells
- ISO 8692
- OECD 201
- ISO 1025

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CILIATES

METHOD ASSIGNMENT

The bioassay method with freshwater tests Ciliates (protozoan, infusoria) is designed to determine the toxicity of substances present in the studied aquatic environment (sample) with toxicants (heavy metals, pesticides, petroleum products, surfactants, xenobiotics, etc.

THE ESSENCE OF THE METHOD

The method is based on determining the mortality (immobility) of *Paramecium caudatum* exposed to toxic substances present in the studied aquatic sample, compared to the control.

The criterion for acute toxicity is the death of 50% or more paramecia within 24 hours in the studied sample, provided that in the control the death does not exceed 10% of test organisms.

Free-living ciliates *Paramecium caudatum* are widely used in biotests

MATERIALS AND INSTRUMENTS

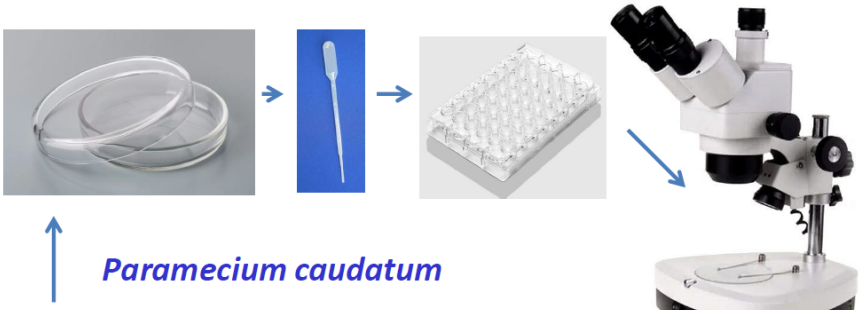
- Soil or waste extract samples or aqueous solutions
- Pipettes 0.1 ml and 1 ml (with appropriate tips)
- Plastic wells 1 ml
- Distilled water
- Concentrated culture of *Paramecium qaudatum*
- Optical microscope
- "BioLat" device
- Metal disk with holes for wells
- Personal computer with pre-installed program "AutoCiliata"

PROCEDURE

Direct counting of ciliates under the microscope

1. *Paramecium caudatum* ciliate culture is transferred with a pipette from a Petri dish into the wells of the plate.

2. Five wells are allocated for one test solution. 10-15 individuals are placed in each well with a pipette.
3. Then the contents of each well with ciliates are counted and recorded in the test protocol.
4. All wells with ciliates are filled with test solutions. The control series of wells are filled with distilled water.
5. Leave for 24 hours, after which they are counted again under a binocular microscope.
6. The results are recorded in the test protocol.



Paramecium caudatum

RESULTS EVALUATION

The sample is considered toxic if after 24 hours the immobility of 50% or more of the infusoria is observed. At the same time, in the control the death of test organisms should not exceed 10%.

If more than 10% of the individuals died in tested sample, then there is a harmful effect, toxicity of the test solution.

Ciliate cell counting using video image analyzer Biolat

Biolat let to make an automatically measurement of ciliates quantity. The effect of the samples on ciliates is assessed by the proportion of surviving individuals after some time.

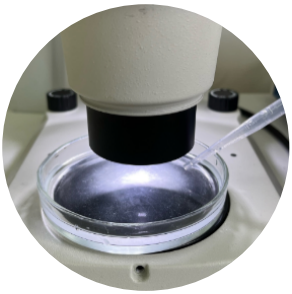
PROCEDURE

Image analyzer counts the number of ciliates in each well containing 0.5 ml of the analyzed solution and 0.1 ml of ciliates cell suspension when scrolling through a metal disc.

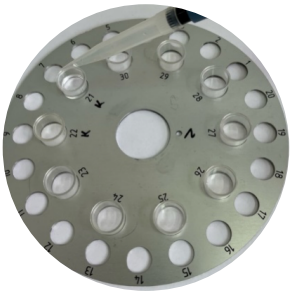
All measurements are automatically entered into an Excel table, where the average value for each well is calculated.

The number of moving objects (ciliates) in the wells is measured in the beginning of the experiment and after the incubation period (24 hours).

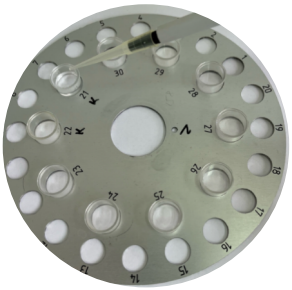
The scheme of the analyses experiment using Biolat



1. Concentrate the initial culture of ciliates using a pipette into a separate container.

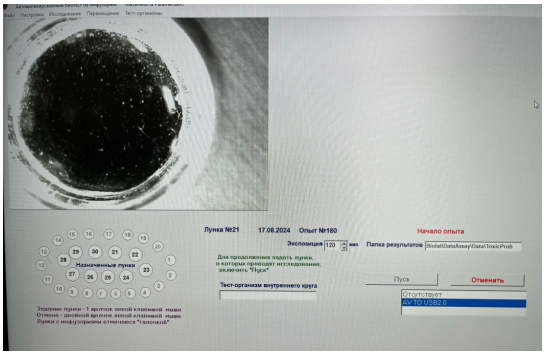


2. Add 0.5 ml of the test solutions to empty wells.



3. Add 0.1 ml of pre-mixed concentrated microorganism culture to the wells.

4. Turn on the computer and the device. Launch the program, select the tabs "Research" - "Toxicity of *Paramecium*" - "Without dilution" - "Start of the experiment" - "Next". Specify the numbers of the wells to be studied and select "Start"



5. After the calculation, save and close the automatically created new Excel sheet with the results.

6. During the incubation period, place the wells with infusoria in a sealed place so that the water does not evaporate

7. After the incubation period, repeat the above procedures, choosing the option "end of experiment"

STANDARD OR GUIDELINE

- Rakhleeva A.A., Terekhova V.A. Method for Assessment of Toxicity of Wastes, Soil, Sewage Sludge, Surface and Ground Water Using Biotesting Techniques with Paramecium caudatum Ehrenberg **(FR. 1.39.2006.02506)** ; Moscow University: Moscow, Russia, 2006.
- Methodology for measuring toxicity by the reaction of ciliates Euplotes to medium-mineralized aqueous extracts of solid waste or wastewater **(FR.1.31.2024.48369)**.
- Terekhova V.A., Gershkovich D.M., Gladkova V.I. et al. Biotesting in environmental control. Moscow: GEOS. 2017
- Standardized protocol for toxicity testing / OECD method

REFERENCES (PRACTICAL APPLICATION)

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2. Kiryushina, A., Bondarenko, L., Dzeranov, A. *et al.* The effect of silica-magnetite nanoparticles on the ecotoxicity of the antibiotic ciprofloxacin. Environ Sci Pollut Res 30, 55067–55078 (2023). https://doi.org/10.1007/s11356-023-26233-9
3. O. Yakimenko, A. Ziganshina, V. Terekhova, I. Panova, M. Gladkova, M. Timofeev, A. Yaroslavov. Ecotoxicity of polyelectrolyte formulations in water and soil matrices. Environmental Science and Pollution Research, (29):65489–65499, 2022.

PLANT SEEDS

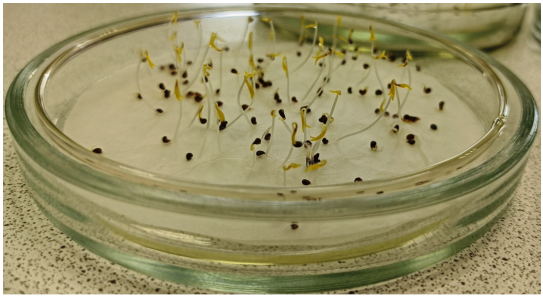
METHOD ASSIGNMENT

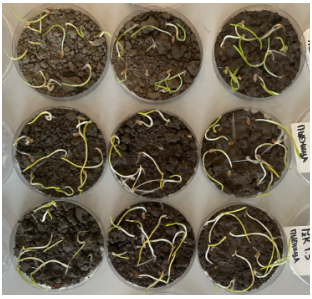
Phytoassay (phytotesting) is widespread method to determine the toxicity of wastes, natural and man-made environments, especially, soils, tehnosols, soil-like wastes and etc.

THE ESSENCE OF THE METHOD

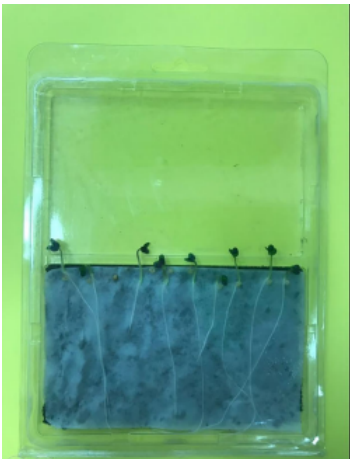
Phytotoxicity is recorded by changes in the growth and morphological parameters of the plant: seed germination inhibition, root growth inhibition, or any other adverse effects on plants.

Phytotoxicity can be carried out using the eluate method (aqueous extract of the sample) or by the direct contact of the test plant seed with the waste/ soil sample - applicative method. For express phytotesting, Petri dishes or plastic tablets are used. Usually, seeds of dicotyledonous plants (white mustard, radish, etc.) and monocotyledonous plants (oats, barley etc.) are used.





Petri dishes



Plastic tablets

MATERIALS AND INSTRUMENTS

- Air-dry soil (or waste) samples
- Plant seeds (*Sinapis alba, Radish sativum, Avéna satíva)*
- *Glass flasks (V=250 cm³)*
- *Glass cups (V=75 cm³)*
- *Funnels*
- *Petri dishes / plastic tablets*
- *Measuring pipettes*
- *Paper filters*
- *Water (distilled)*
- *Measuring ruler (with division value of 1 mm)*
- *Luminostat*

PROCEDURE

The applicative method

1. The control sample (uncontaminated) soil with a similar main characteristic as the contaminated tested soil;
2. The tested soil samples are pre-moistened to 60% of the total moisture capacity;
3. The moisturized samples are applied in Petri dishes or plastic tablets;

4. The seeds in amount 10-30 placed in Petri dishes or plastic tablets in vertical position for 4- 7 days of exposure.
5. At least 3 Petri dishes or plastic tablets for control and 3 for the test sample.

The eluate method

1. Embed filter paper into Petri dishes or plastic tablets;
2. Add 8-5 cm^3 of the tested extract or its dilutions and add 8-5 cm^3 of distilled water to the control
3. The seeds in amount 10-30 placed into Petri dishes or in amount 10 into plastic tablets for 4- 7 days of exposure at 20 - 23°C

RESULTS EVALUATION

Phytotesting in Petri dishes

The number of germinated seeds and the length of the roots (the sprouts) of seedlings in control and experimental samples are calculated. The inhibition effect of "germination" and "root length" is calculated:

$$E_{eff}=(L_c-L_{exp})/L_c*100$$

E_{eff} - the inhibition effect (%);
L_{exp} - the average length of the roots in the experiment (mm);
L_c - the average length of the roots in the control (mm).

An indicator of the toxic effect of the tested samples (wastes, soils) is a decrease in seed germination and/or a decrease in the length of roots of seedlings compared to the control. Alternative innovative way (makes the test more operational, reliable, and free from researcher errors):

- use a camera, take photos of seedlings, and then transfer them to a computer; digital photos are processed automatically using computer image analyzers;
- the data is automatically archived and saved on the computer;
- access the data at available time.

(Terekhova V.A., Yakimenko O.S., Voronina L.P., Kydralieva K.A. The method of measuring the biological activity of humic substances by the Phytoscan phytotesting method. 2014.)

STANDARD OR GUIDELINE

- **MR 2.1.7.2297-07 Methodological recommendations. 2.1.7. Soil.** Cleaning of populated areas. Household and industrial waste. sanitary protection of the soil. Substantiation of the hazard class of production and consumption waste by phytotoxicity

- FR .1.31.2024.48371 Guidelines for measuring toxicity average mineralized water media and waste by changing the length of the roots of seedlings of white mustard seeds *Sinapis alba L.*
- ISO 11269-1:2012. Soil quality — Determination of the effects of pollutants on soil flora — Part 1: Method for the measurement of inhibition of root growth. https://www.iso.org/standard/51388.html Accessed March 2012
- ISO 11269-2:2012 Soil quality — Determination of the effects of pollutants on soil flora — Part 2: Effects of contaminated soil on the emergence and early growth of higher plants. https://www.iso.org/standard/51382.html Accessed January 2012

REFERENCES (PRACTICAL APPLICATION)

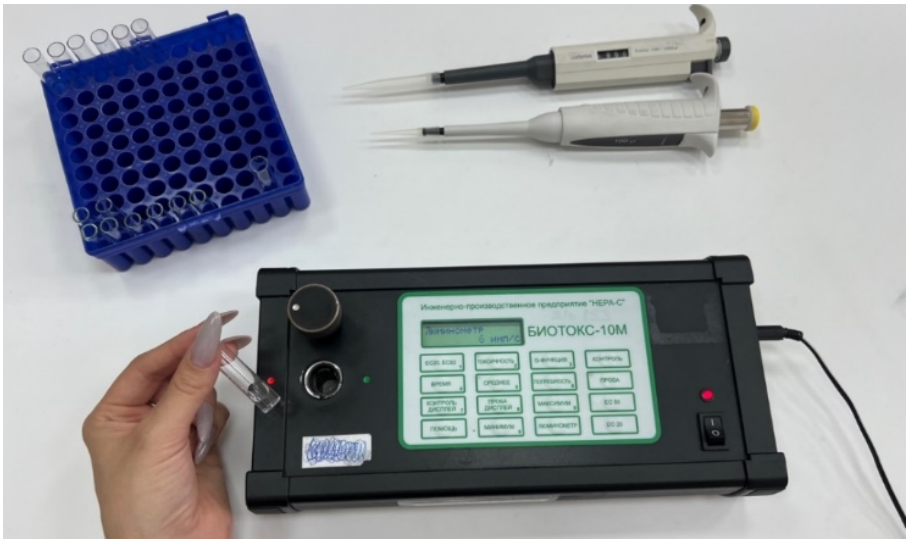
1. Terekhova V.A., Prudnikova E.V., Kiryushina A.P., Karpukhin M.M., Plekhanova I.O., Yakimenko O.S. Phytotoxicity of Heavy Metals in Contaminated Podzolic Soilsof Different Fertility Levels. 2021. Eurasian Soil Science. V 54, № 6, c. 964-974 DOI: 10.1134/S1064229321060132
2. Kovaleva E.I., Perebasova P.M., Avdulov D.A., Ladonin D.V., Trofimov S.Y. A The efficacy of ameliorants for remediation of soils contaminated with heavy metals, in a lab experiment //Moscow University Soil Science Bulletin. 2024. V 79. № 2, pp. 177-189
3. Kovaleva Ekaterina I., Guchok M.V., Terekhova V.A., Demin V.V., Trofimov S.Ya Drill cuttings in the environment: possible ways to improve their properties. 2021. Journal of Soils and Sediments V 21, pp. 1974-1988 DOI: 10.1007/s11368-020-02787-w

LUMINESCENT BACTERIA

METHOD ASSIGNMENT

The main applications of the luminescent bacterial test are:

- Continuous monitoring of drinking water, bodies of water, soil, air, materials, and products for toxic effects;
- Rapid control of industrial waste and discharges;
- Real-time monitoring of technological processes;
- Determination of the toxicity level of new products;
- Medicine and pharmaceuticals, monitoring the toxic effects of materials and drugs;
- Food industry, ensuring the safety of food products.



THE ESSENCE OF THE METHOD

Experiments involve measuring the **decrease in luminescence intensity** in the presence of toxic substances.

The schematic diagram of the functioning of the bioluminescent bacterial test is presented below.

Is the sample toxic?	
yes	no
Luminescence fade	No luminescence fade
Toxic compounds are present in testing sample.	Concentration of toxic compounds un the testing sample is too low or toxic compounds are not present in the testing sample.

The main measured parameter is **bioluminescence** in the visible spectrum. The test is **fast, integral, sensitive, and objective** for assessing environmental pollution.

The response of luminescent bacteria to toxic substances fully **correlates** with the response of other biological organisms. **Luminometer "Biotox-10"** is a specialized device for toxicological analysis using the "Ecolum" test system.

MATERIALS AND INSTRUMENTS

- Soil extract samples

- Pipette 0,1 ml and 0,9 ml (with according tips)
- Glass cuvettes
- Distilled water
- “Ecolum” bacterial test system
- Luminometer "Biotox-10"

PROCEDURE

Principle of integral toxicity determination

Luminescence Intensity depends on external factors and requires comparison with a control. Control measurement always precedes the experimental measurement. Optimal Temperature for this testing is 15-25°C.

Biotesting procedure and results processing

1. Suspension Preparation: Rehydrate lyophilized bacteria by adding 10 cm^3 of cooled distilled water (pH 6.8-7.4) and allow to sit for 30 minutes, stirring occasionally. The working suspension should be used within 8 hours and can be stored at room temperature.

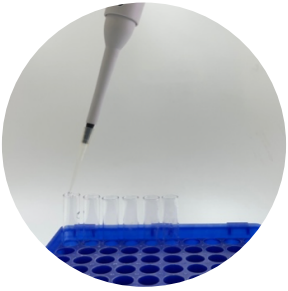
2. **Biotesting:** Conduct using aqueous extracts of the sample in various dilutions (100, 1000, 10000).
3. **Control and Test Samples:** Add 0.9 cm^3 of distilled water to control cuvettes and 0.9 cm^3 of the sample to test cuvettes, then add 0.1 cm^3 of the bacterial suspension to each.

4. **Bioluminescence Measurement:** Measure with the "Biotox" device after 30 minutes (standard method) or 5 minutes (rapid method).
5. **Toxicity Calculation:** Calculate the toxicity index T as the arithmetic mean of three measurements:

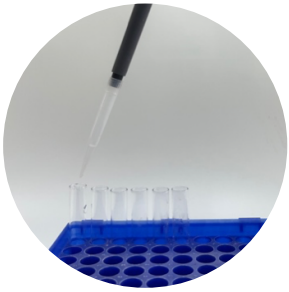
$$T = (T_1 + T_2 + T_3)/3$$

6. **Cuvette Cleaning:** Rinse with 10% nitric acid, tap water, and distilled water.
7. **Quality Control of Culture:** Conduct quarterly by checking sensitivity to a model toxicant (zinc sulfate).

The scheme of the analysis experiment



1. Add 0,1 ml of bacterial suspension to cuvettes



2. Add 0,9 ml of sample extracts and wait for 30 minutes

3. Measure bacterial luminescence using the Biotox

RESULTS EVALUATION

Toxicity Calculation. The "Biotox" device calculates toxicity using the formula $T = (i_0 - i)/i_0$, where i_0 and i are the luminescence intensities of the control and experimental samples, respectively.

Test System Parameters:

EC_{50} (50% reduction in luminescence)

EC_{20} (20% reduction in luminescence).

Toxicity index **T**	Toxicity level
T <20	Acceptable level of sample toxicity
50 >**T** ≥ 20	Sample is toxic
T > 50	Acute toxicity of the sample

STANDARD OR GUIDELINE

- PND F T 14.1:2:3:4.11-04. Measuring Toxicity of Surface, Marine, Ground, Drinking, and Waste Waters, Water Extracts of Soils, Wastes, and Sediments of Sewage Waters by Change of Intensity of Ecolum Bacterial Bioluminescence Test System (Nera-S, Moscow, 2010)
- ISO 11348-3 and ISO 21338
- ISO 15799:2003: Soil Quality—Guidance on the Ecotoxicological Characterization of Soils and Soil Materials (International Organization for Standardization, Geneva, 2003).

REFERENCES (PRACTICAL APPLICATION)

1. O. Yakimenko, Aliya Ziganshina, V. Terekhova, I. et al. Ecotoxicity of polyelectrolyte formulations in water and soil matrices. Environmental Science and Pollution Research, (29):65489–65499, 2022.

BIOINDICATION OF SOIL HEALTH

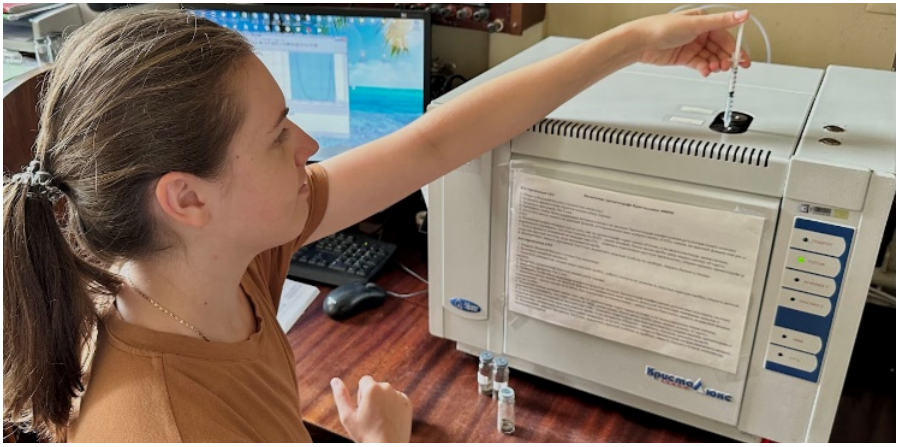
Microbial characteristics of soils - sensitive indicators of soil health. The abundance and diversity of microorganisms really determine the high metabolic potential of soils. Changes in the structural and functional parameters of microbial communities are used to normalize soil quality and soil health.

Bioindication of soil quality (some approaches)

KINETIC RESPIRATION ANALYSIS USING GAS CHROMATOGRAPHY

METHOD ASSIGNMENT

This type of bioindication allows us to identify the effects of negative impacts by studying the functional responses of the microbial community. To adapt to changes in environmental conditions, microorganisms are able to transform their energy consumption strategies by changing their carbon utilization preferences. Therefore, microbial respiration, as an integral indicator of soil biological activity, is a sensitive measure of soil contamination and soil health.



THE ESSENCE OF THE METHOD

The method is based on determining the amount of carbon dioxide released by a sample of soil or waste over a certain time. **Basal respiration (BR)** is determined in native soil (or waste) without the addition of any nutrients after 24 hours of incubation. **Substrate-induced respiration (SIR)** is measured by adding readily available substrate (for example, glucose) to the soil (or waste) for 3 hours of incubation. Based on the results of the SIR assessment, the **microbial biomass carbon content (C_{mic})** in the soil (or waste) can be calculated. It is assumed that the respiration response (CO_2 emission) is directly proportional to the amount of microbial biomass, and in the first few hours after the addition of an easily accessible substrate, no growth of microbial biomass occurs. C_{mic} is calculated using a conversion factor. Based on BR, SIR and C_{mic} data, microbial metabolic quotients are calculated: *q* CO_2, equal to the ratio of BR to C_{mic}, and *QR*, equal to the ratio of BR to SIR.

MATERIALS AND INSTRUMENT

- Air-dry soil (or waste) samples
- Technical scales
- Glass vials

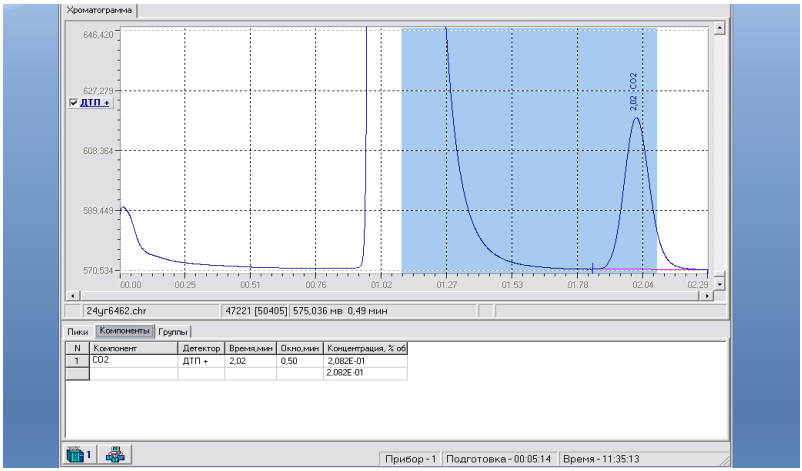
  It is allowed to use vials of different volume from 15 to 100-120 ml, it is necessary to observe the ratio of soil volume and gas phase of the vial not less than 1:10.
- Rubber caps for vials, parafilm
- Thermostat
- Distilled water
- Glucose
- Syringe 1 mL
- Gas chromatograph for CO_2 quantification

PROCEDURE

1. Pre-incubation of samples: air-dry soil (or waste) is sieved through a 2 mm sieve, and then weighed 1 g, placed in a

vial and moistened with distilled water to 60% of maximum water-holding capacity, incubated 3-5 days at 22°C.

2. Determination of BR: after pre-incubation, close the vials, take a sample of air from the vial with a syringe and measure the initial concentration of CO_2 on a gas chromatograph, after 24 hours, repeat the measurement.



Chromatogram (CO_2 peak at the chart) shows the CO_2 concentration measurement results in the Netchrom program

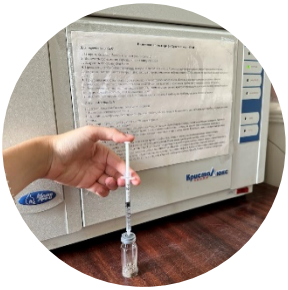
3. SIR determination: open the vials, ventilate them, add 0.1 ml of glucose solution with a concentration of 10 mg/g dry soil (105 °C), close the vials, take an air sample from one of the vials with a syringe, measure the initial CO_2 concentration, incubate for 3 hours, and then measure the final CO_2 concentration.

The scheme of SIR analysis experiment

1. Open vial with soil (or waste)

2. Addition of glucose solution

3. Closed vial with soil (or waste)



4. Air sampling with a syringe

5. Inserting a sample into gas chromatograph

4. Determination of the volume of the gas phase of the vial: weigh the vial with soil, add distilled water, weigh the vial again. Calculate the volume of the gas phase by finding the difference in mass between the vial with soil and water and the vial with just soil.

5. Calculate BR and SIR rates in μg/g dry soil per hour. C_{mic} content in μg/g dry soil. Metabolic quotient qCO_2 is expressed as mg C-CO_2 released per hour per gram of microbial biomass carbon [Anderson, Domsh, 1978; Ananyeva et al., 2008]. QR is a dimensionless value.

Anderson T.H., Domsh K.H. A physiological method for the quantitative measurement of microbial biomass in soil // Soil Biol. Biochem. 1978. № 10. P. 215-221.

Ananyeva N.D, Susyan E.A., Chernova O.V., Wirth S. Microbial respiration activities of soils from different climatic regions of European Russia // European Journal of Soil Biology. 2008. V.44. Pp. 147-157.

RESULTS EVALUATION

The criterion for assessing the ecological state of soils using the indicators BR, SIR, C_{mic}, qCO_2 is the increase or decrease in values relative to the soils of background territories or uncontaminated model objects.

Gradations of microbial community change have been developed for the QR indicator [Ananyeva, 2003]:

QR	QR with pollutant/QR without pollutant	Degree of disruption of soil microbial community
0.10-0.20	1.0	Absent (sustainable and undisturbed communities)
0.20-0.30	1-2	Weak
0.30-0.50	2-5	Average
0.50-1.00	5-10	Strong

0; >1.00	0; >10	Catastrophic (irreversible degree of degradation of microbial community, intensive decomposition of organic matter)

Ananyeva N.D. Microbiological aspects of self-purification and sustainability of soils. Moscow, 2003, 223 p.

STANDARD OR GUIDELINE

ISO 16072 Soil quality — Laboratory methods for determination of microbial soil respiration

ISO 14240-1 Soil quality — Determination of soil microbial biomass. Part 1: Substrate-induced respiration method

REFERENCES (PRACTICAL APPLICATION)

1. Terekhova V.A., Prudnikova E.V., Kulachkova S.A., Gorlenko M.V., Uchanov P.V., Sushko S.V., Ananyeva N.D. Microbiological indicators of heavy metals and carbon-containing preparations applied to agrosoddy-podzolic soils differing in humus content // Eurasian Soil Science. 2021. V. 54. № 3. P.448-458.
2. Terekhova V.A., Kulachkova S.A., Morachevskaya E.V., Kiryushina A.P. A Soil Biodiagnostics Methodology and Features of Some Bioindication and Biotesting Methods (Review) // Moscow University Soil Science Bulletin, 2023, Vol. 78, No. 2, pp. 102–111.
3. Kulachkova S. A., Derevenets E. N., Korolev P.S., Pronina V.V. The Effect of Mineral Fertilizers on Soil Respiration in Urban Lawns // Moscow University Soil Science Bulletin, 2023, Vol. 78, No. 3, pp. 281–291.

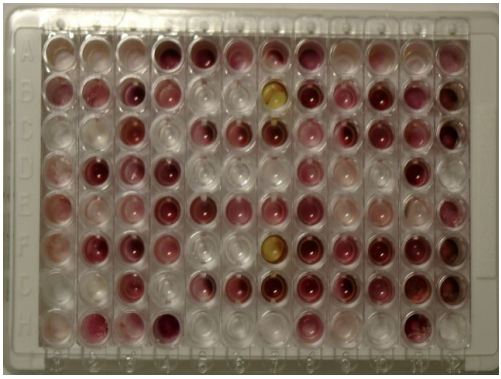
MULTISUBSTRATE TESTING (MST)

METHOD ASSIGNMENT

The multisubstrate testing (MST) method is required to assess the functional diversity of the microbiome, enabling the analysis of community-level physiological profiling (CLPP). Appling MST analysis allows to achieve the wide range of ecological monitoring goals and addresses various scientific problems that have never been resolved without employing MST technology.

THE ESSENCE OF THE METHOD

The principles of the method are similar to wide used Biolog^{tm} microplate assays. It is based on obtaining the substrate utilization spectra-the metabolic fingerprints of natural microbial community. **We developed the original "Eco-log" microplates**, that contains set of 47 sole carbon substrate (including sugars, amino acids, carbonic acids etc.), mineral salts and tetrazolium salt, as indicator of microbial growth. The color development in each substrate-containing well indicates the substrate utilization rate.



"Eco-log" microplates with differently colored substrates in wells

The overall 47-dimensional data vector represents the substrate utilization spectra. It is suitable for further statistical and mathematical analysis to obtain the functional biodiversity indices and to compare samples.

The following indices are used to assess the functional diversity of the microbiome:

N is the functional diversity parameter, which quantifies the number of consumed substrates in the range from 0 to 47;

W is the average metabolic work, calculated by summing the optical densities of cells with consumed substrates and dividing by their number;

d is the rank distribution of the substrate utilization spectrum shape coefficient, which serves as a measure of system destabilization or disturbance, with values ranging from 0.01 to 2.00.

MATERIALS AND INSTRUMENTS

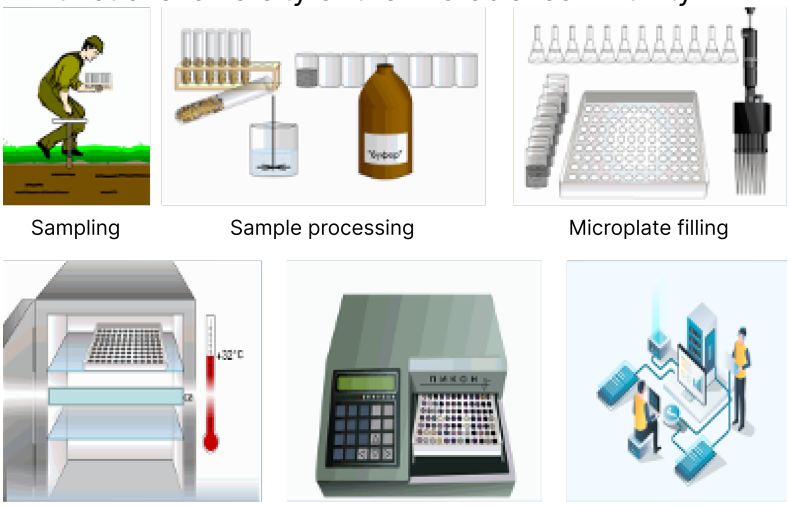
- Sample of air-dried waste or soil
- Distilled water
- Shaker
- Centrifuge
- Indicator triphenyltetrazolium
- "Eco-log" microplates
- Thermostat
- Hardware and software complex "Eco-Log", including a photometer, a personal computer with a program for processing measurement results

PROCEDURE

1. We obtain an extract from soils and waste: place the sample in a centrifuge beaker, add distilled water, shake on a shaker to separate microorganism cells from solid

particles, centrifuge the suspension and separate the supernatant, add triphenyltetrazolium indicator to an aliquot of the supernatant

2. An aliquot of the supernatant with an indicator is placed in 96 cells of an “EcoLog” plate with 47 test substrates (in 2 replicates).
3. We incubate the plates for 72 hours at 28°C until a visually detectable red color appears in the cells.
4. We measure the optical density of each cell in the range of 510 nm using the Eco-Log software and hardware complex.
5. Based on optical density, we calculate indicators of the functional diversity of the microbial community



MST sample preparation

Gorlenko M.V., Kozhevin P.A. Multisubstrate testing of natural microbial communities. M.: MAKS Press, 2005. 88 p.

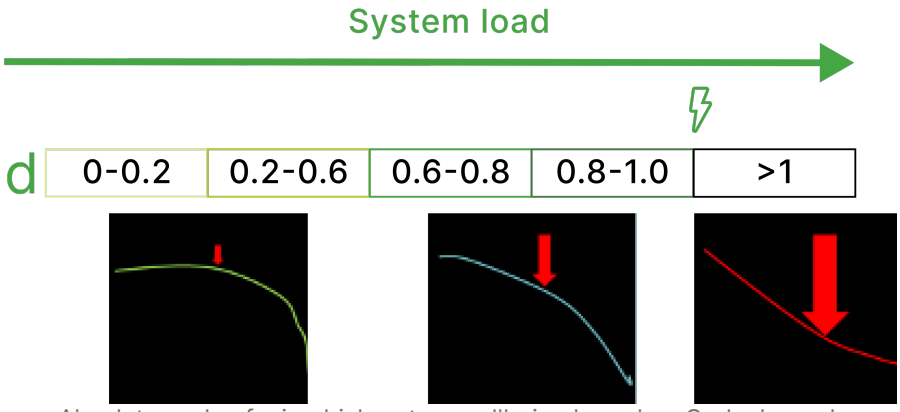
RESULTS EVALUATION

In the microbial systems studied, the **d coefficient** serves as a valuable indicator of the health and stability of the soil microbial

community, with lower values indicating a thriving and stable community.

Conversely, higher values of the coefficient indicate the presence of a disruptive factor affecting the microbial system. Based on the *d* coefficient, microbial communities can be classified as either good (*d* = 0.01-0.4) depressed (*d* = 0.4-0.8), stressed (*d* = 0.8-1) or damaged (*d* >1) according to the classification proposed by Gorlenko in 2005.

$$F(n)=E_0 - b\,\frac{e^n}{d^n}$$



Absolute scale of microbial system wellbeing based on Gorlenko rank distribution shape coefficient **d**.

STANDARD OR GUIDELINE

- FR.1.37.2010.08619 Methods for measuring the intensity of consumption of test substrates by microbial communities of soils and soil-like objects by the photometric method
- Gorlenko M. V., Kozhevin P. A., Terekhov A. S., RF Patent RU2335543 C2 Method of multisubstrate testing of microbic communities and its application. 2008.

REFERENCES (PRACTICAL APPLICATION)

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2. Terekhova V.A., Prudnikova E.V., Kulachkova S.A., Gorlenko M.V., Uchanov P.V., Sushko S.V., Ananyeva N.D. Microbiological indicators of heavy metals and carbon-containing preparations applied to agrosoddy-podzolic soils differing in humus content. // Eurasian Soil Science. 2021. V. 54. № 3. P.448-458

STRUCTURE OF MICROBIOMES FOR SOIL QUALITY ASSESSMENT

METHOD ASSIGNMENT

Traditional and new approaches to obtaining and analyzing data on the structure of the microbiome make it possible to characterize the sustainability of ecosystem functioning and soil quality.

THE ESSENCE OF THE METHOD

Classical sowing of soil suspension on nutrient media and metagenomic analysis are used to analyze the structure of the soil microbiome.

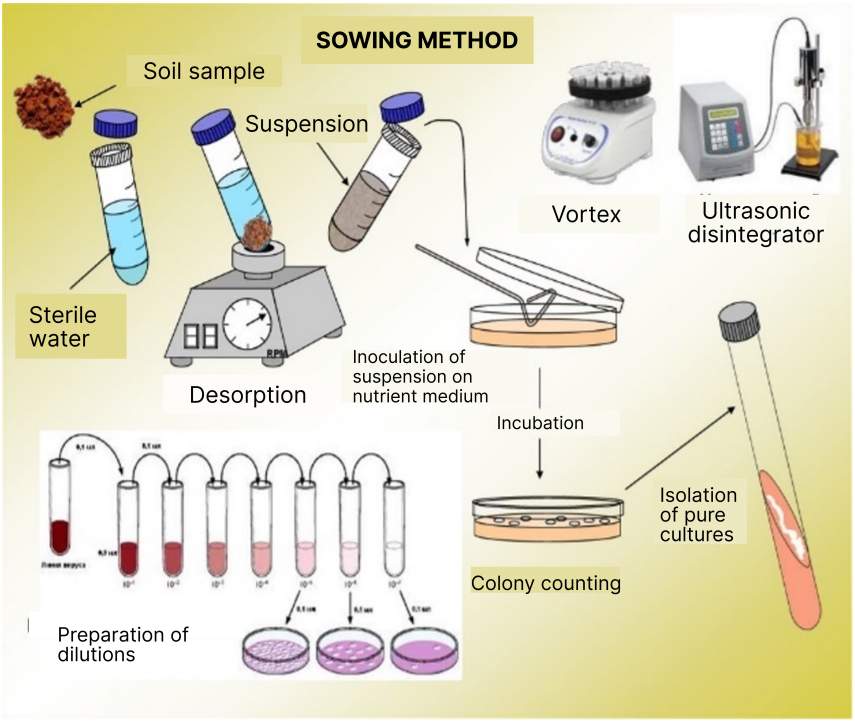
Both methods allow determining indices of the biodiversity of microorganisms - fungi, bacteria, archaea, total and specific biomass, sensitivity and resistance of species и phyla to negative impacts, etc.

Study of cultured microorganisms (Sowing method)

The method of sowing a soil suspension on nutrient media is used to isolate and analyze the cultivated species. Counting and species identification are carried out in Petri dishes. An important advantage of this labor-intensive approach is the ability to select individual species and isolate strains important for biotechnology.

PROCEDURE

Basic steps of sowing method:



https://ppt-online.org/213981 (in modification)

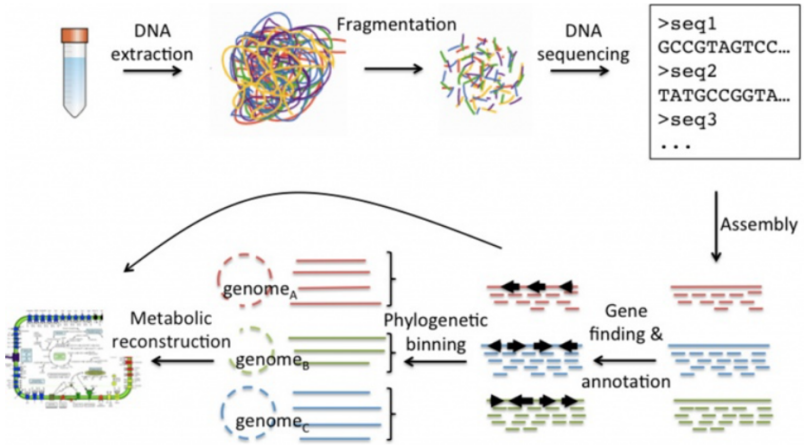
Metagenomic study of the microbiome

Metagenomic sequencing is a molecular tool used to analyze mixed genomic materials extracted from environmental

samples, providing detailed information on species diversity and abundance, population structure, phylogenetic relationships, functional genes, and correlation networks with environmental factors.

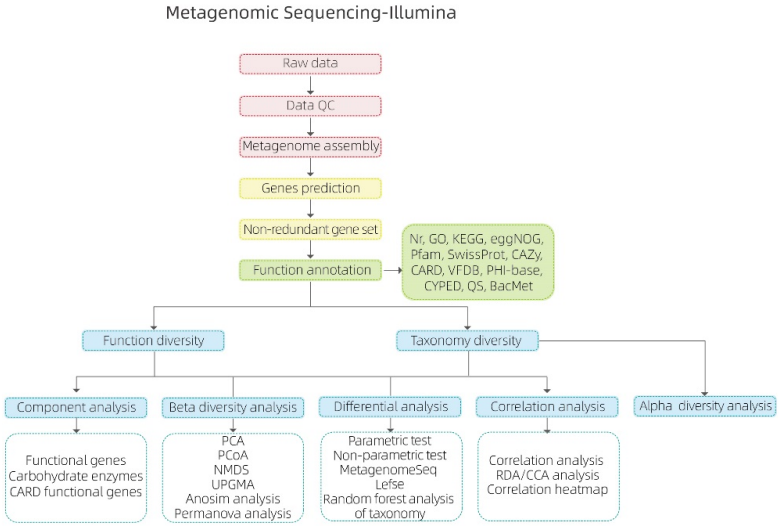
PROCEDURE

Basic steps in a metagenomic study of the microbiome



https://slideplayer.com/slide/12790885/77/images/

Bioinformatics analysis; Analysis of OTU data; Species diversity analysis; Genetic function diversity analysis.; Intergroup analysis; Association analysis with influencing factors**.**



STANDARD OR GUIDELINE

- ISO 15799:2003: Soil Quality—Guidance on the Ecotoxicological Characterization of Soils and Soil Materials (International Organization for Standardization, Geneva, 2003).

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ABOUT US

The laboratories of the Soil Science Faculty of Lomonosov Moscow State University assess the quality of natural environments, preparations for the reclamation of disturbed soils, and the safety of industrial waste.

The Laboratory of Ecotoxicological Analysis of Soils (LETAP) has been state-accredited since 2002. The laboratory evaluates the safety of natural environments (soils, waters) and man-made objects (waste, soil-like substrates, preparations for soil reclamation, including humic, nanocomposite sorbents of toxic substances, natural and artificial polymers used to improve soil structure). The laboratory employs modern biotesting methods for implementing of research projects on biodiagnostics of waste safety, ecological quality of soils, soil substrates and adjacent environments, also develops methods for measuring the toxicity

The laboratory implements educational programs, implements advanced training programs for ecologists on technologies for biotesting the ecological safety of waste, soils, preparations for the reclamation of disturbed lands. The Faculty of Soil Science regularly holds master classes on biodiagnostics of environmental quality, all-Russian and international schools for young scientists.

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Программы дополнительного образования
«ТЕХНОЛОГИИ БИОТЕСТИРОВАНИЯ
В ЭКОЛОГИЧЕСКОМ КОНТРОЛЕ
ПРИРОДНЫХ СРЕД И ТЕХНОГЕННЫХ ОБЪЕКТОВ»
«БИОТЕСТИРОВАНИЕ В АККРЕДИТОВАННОЙ ЛАБОРАТОРИИ:
СТАНДАРТИЗАЦИЯ ТЕСТ-КУЛЬТУР»
ЛЭТАЛ

TOXICITY MEASUREMENT TECHNIQUES DEVELOPED BY LETAP

The registration codes are indicated in the Federal Information Fund for Ensuring the Uniformity of Measurements of the Russian Federation

- **FR.1.39.2006.02506.** PND F T 14.1:2:3.13-06 (PND F T 16.1:2.3:3.10-06) Guidelines for determining the toxicity of waste, soils, sewage, surface and groundwater sediments by biotesting using *Paramecium caudatum Ehrenberg* equidistant ciliate
- **FR.1.39.2006.02505.** PND F T 14.1:2.14-06 (PND F T 16.1:3.11-06) Guidelines for determination of the toxicity of highly mineralized surface and wastewater, soil and waste on the survival of brackish-water crustaceans *Artemia salina L.*
- **FR.1.39.2007.04104.** PND F T 16.3.12-07 Guidelines for determining the toxicity of ash and slag waste by biotesting based on the survival of paramecia and ceriodaphnia
- **FR.1.31.2009.06301** (PND F 14.1:2:4:15-09 16.1:2:2.3:3.13-09) Guidelines for determination of toxicity of soils, waste, sewage sludge, wastewater, surface and groundwater by biotesting based on reactions of mammalian cells *in vitro*
- **FR.1.31.2012.11560** Guidelines for measuring the biological activity of humic substances by phytotesting
- **FR.1.39.2014.18039** Guidelines for measuring soil toxicity by enchitroid reactions
- **FR.1.31.2020.38716** Guidelines for measuring the biological activity of soils, plant substrates, humic substances by biotesting. Phytoscan
- **FR.1.31.2024.48369** Guidelines for measuring toxicity by the reaction of euplotes infusoria to medium-mineralized aqueous extracts of solid waste or wastewater
- **FR.1.31.2024.48371** Guidelines for measuring toxicity average mineralized water media and waste by changing the length of the roots of seedlings of white mustard seeds *Sinapis alba L.*

ABOUT THE EUROPOLITEST COMPANY

"EUROPOLYTEST" is a Russian company established in 2009. It begins with team of like-minded individuals who are dedicated to developing Russian instrument engineering for applied toxicology and related fields.

The range of equipment offered by the company includes the following main groups:

- Equipment for biotesting of own production EUROPOLYTEST® (state registration № 920209);
- Laboratory fluorimeter "EFMA" of own production EUROPOLYTEST ® (state registration № 920209);
- Instrumental-computational complex "BioLaT-3.2" (Russian government standard 31674-2012, (BIOLAT® (state registration № 647817) EUROPOLYTEST).

Compact set of devices "Laboratory for Water Biotesting" - automation of the main stages of biotesting in establishing the integral toxicity of samples of natural waters, soils, bottom sediments, waste classification, wastewater, and water at the outlet of treatment facilities, extractions and washes from waste.

The company is a leader in providing laboratories with equipment and comprehensive biotesting solutions in ecotoxicology and toxicology in the compound feed industry.

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METHODOLOGICAL GUIDE

PRINCIPLES OF BIODIAGNOSTICS OF WASTE YAZARDS AND SOIL HEALTH

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