MINIATURIZED ALGAL GROWTH BIOASSAY

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Abstract

A new miniaturized procedure for the evaluation of algal growth potential (AGP) and the toxicity of surface water and ground water is presented. Unfiltered samples are frozen and stored at -20 °C. After thawing the samples are centrifuged and dispensed into the wells of an immunological plate. The plates, and their contents, are sterilized with uv light followed by inoculation with *Chlorella kessleri*. A simple unit with a capacity of 250 samples (25 plates, 6 replicates per sample) is used for cultivation. Yields are evaluated in situ, in a special plate reader, by nephelometry (absorbency measured at 750 nm). The procedure may be used for testing of toxicity, the assessment of algal growth potential, investigation of biochemical features, etc..

Lukavský, J.: **Miniaturisierter Algenwachstumstest**

Es wird eine neue, miniaturisierte Methode zur Bestimmung des Algenwachstumspotentials (AGP) und der Toxizität, sowohl in Oberflächengewässern als auch im Grundwasser, vorgestellt. Die nicht filtrierten Proben werden eingefroren und bei -20 °C aufgehoben. Nach Auftauen werden die Proben zentrifugiert und die entsprechenden Mengen in die Vertiefungen einer immunologischen Platte verteilt. Die Platte mit den Proben wird durch Abdecken unter einer Germizidlampe sterilisiert und dann mit der Alge *Chlorella kessleri* beimpft. Die einfache Kultivierungsanlage hat eine Kapazität von 250 Proben (25 Platten). Das Wachstum wird nephelometrisch (die Absorbans bei 750 nm) bestimmt. Die Methode eignet sich zur Bestimmung von AGP, von Toxizität verschiedener Stoffe und Extrakte, zu Tests biochemischer Merkmale, usw.

Lukavský, J.: **Miniaturizovaný řasový růstový biotest**

Miniaturizovaná metoda pro stanovení řasového potenciálu (AGP) a toxicity vody, povrchové i podzemní je následující: Nefiltrovaný vzorek je zmrazen a uchováván při -20 °C. Po rozmrazení jsou vzorky odstředěny a odměřeny do jamek sérologické destičky. Destička se vzorky je sterilizována otevřením pod germicidní lampou a potom naočkována řasou *Chlorella kessleri*. Jednoduché kultivační zařízení má kapacitu 250 vzorků (25 destiček). Růst je hodnocen nefelometricky tj. absorbancí při 750 nm. Metoda je vhodná pro stanovení AGP, toxicity látek a výluhů, testy biochemických znaků atd.

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Algal strains for bioassays, as *Chlamydomonas, Chlorella, Cladophora, Selenastrum, Scenedesmus*, and another 650 blue-greens, algae and mosses, for assays of algal growth potential and toxicity, education etc.. Contact the author for details. Fax: +42/333/2391.

INTRODUCTION

In spite of great progress in hydrochemistry, bioassay remains a valuable technique. It enables, using very simple equipment, the monitoring and screening of large sets of samples and the selection of suspicious ones for more detailed analysis.

PRINCIPLES OF GROWTH BIOASSAY

The sample (water, extract, solution of tested substance, etc.) is sterilized, inoculated with the testing organism and cultivated. At the stationary phase of the growth curve, the yield of biomass is evaluated. Its quantity, in comparison to a control sample, is proportional to nutrient charge (AGP) or indirectly proportional to the quantity of toxicants.

Growth bioassay lasts a few generations and it includes reproduction, the most sensitive step in the life cycle. Mutagenic or chronic effects can also be discovered by this method. Twelve million organic, inorganic, synthetic and natural compounds have been described, yet. They can hardly be evaluated in samples by precise, selective but expensive procedures.

The procedure described here (LUKAVSKÝ 1992) is based on the cultivation of a testing alga in a volume of 0.2 ml, in the wells of an immunological plate.

The minicultures can be evaluated directly in the plate by a plate reader, and absorbencies converted into dry weight, cell count, etc..

Another variant is the cultivation of a giant colony on a miniature agar plate in the well of the immunological plate (LUKAVSKÝ 1984). Disadvantages are the presence of agar and boiling, whilst advantages are that the colour or turbidity of the tested sample does not interfere with the result. The colony is growing on a surface and light is coming from above. This procedure is also very simple.

PROCEDURE

1. Samples (5-10 ml) are frozen and stored at -20 °C. Samples are not filtrated or parallel filtrated and non-filtrated ones are processed. Extreme pH must be parallel tested also in sample adjusted to a pH of about 6.5.

2. Sample is centrifuged to eliminate suspended solids. Extracts are diluted to the desired concentration by nutrient solution e.g. Z8 after STAUB (1961), but without EDTA, e.g. by logarithmic row, 1; 0.3; 0.1; 0.03; 0.01; etc.. Immunological plate (FB-flat bottom, 9x12 cm, 96 wells of 0.25 ml) is filled at 0.2 ml/well. Six replicates are filled into every column with a sample. Marginal wells are filled with distilled water to reduce evaporation.

3. Plates are exposed, uncovered, under an ultra-violet tube for 2 hours for sterilization.

4. Testing alga e.g. *Chlorella kessleri* FOTT & NOVÁK, strain LARG/1 is diluted to 400000 cells/ml. Five µl of the inoculum is dispensed into a well, blanks are the same inoculum injected into distilled water and also into the dilution solution.

5. Plates are cultivated at 30-35 °C in a cultivation unit, under fluorescent tubes, irradiation of 30 $W.m^{-2}$ (6000 lx), in 2% vol/vol CO_2 (Fig.1 and 2).

6. Absorbency is evaluated at 750 nm, as long as stationary phase is reached, at approximately one week intervals and converted into dry weight.

7. Heavy metals can be determined by using a variant spiked with Na_2EDTA (5 µl of solution with 1 mg/ml); limiting nutrients can be evaluated by parallel replicates spiked with P,N, Mg, etc..

Variant with agar plates: the sample is boiled with 2% of agar-agar, wells (FB or bottom) are filled with 0.2 ml. After solidification one giant colony is inoculated to every well using a wire. Colonies are evaluated under the microscope LUKAVSKÝ (1983).

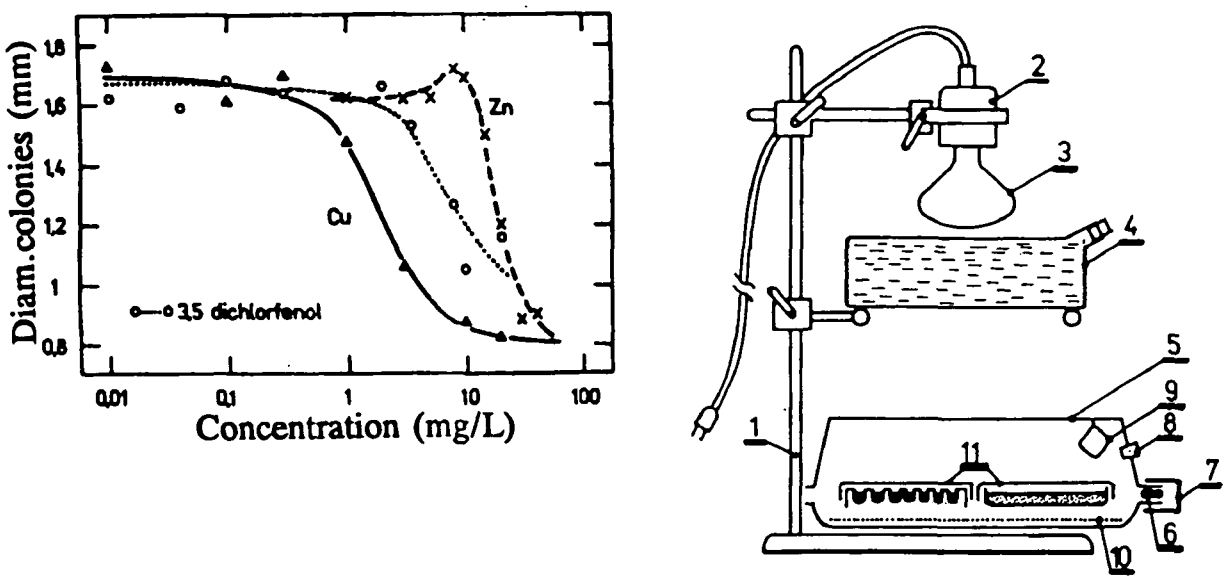

Fig.1.: Assay with agar plates in immunological plate and giant colonies of *Chlorella kessleri*. **Right:** the scheme of a simple cultivation unit: 1-laboratory stand, 2-lamp holder, 3-lamp, 4-heat filter, 5-box, 6-polyurethane tape, 7-clamp, 8-supply of CO_2, 9-CO_2 checking, 10-gauze, 11-Petri dish, immunological plate.

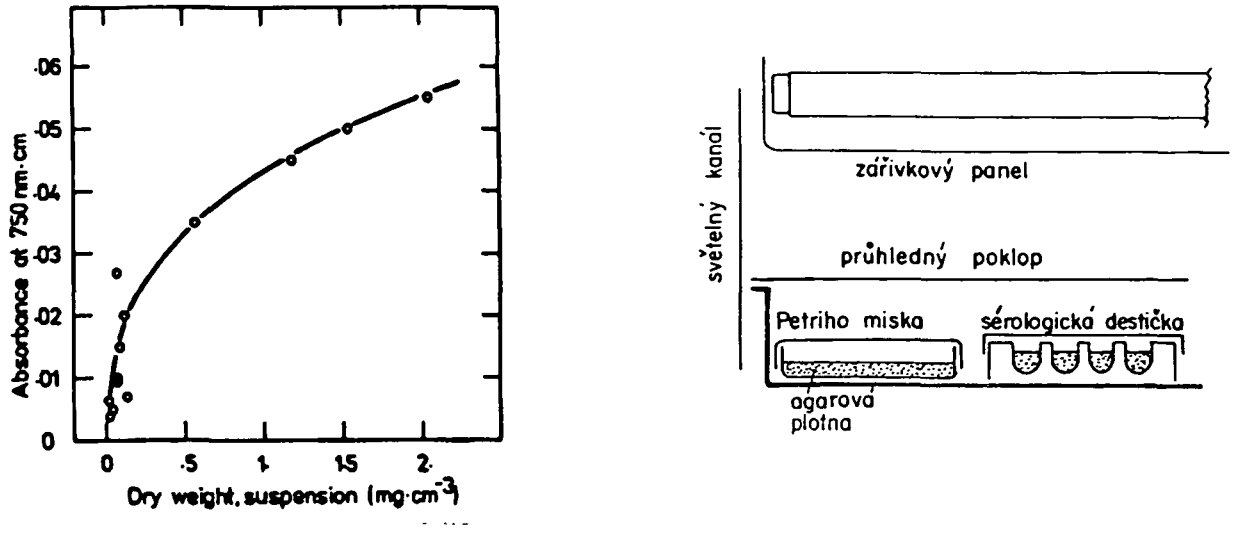

Fig.2: The scheme of cultivation unit for plates and Petri dishes. **Left:** conversion graph absorbency - dry weight for the alga *Chlorella kessleri*.

Agar plates in Petri dishes, inoculated with diluted inoculum resulted in cloned, single-cell colonies. Such colonies can also indicate mutagenic changes according to the presence of yellow,white, lobate, small, etc., colonies. Concentrated inoculum on an agar plate resulted in a continuous growth layer, which can be washed for biochemical evaluation.

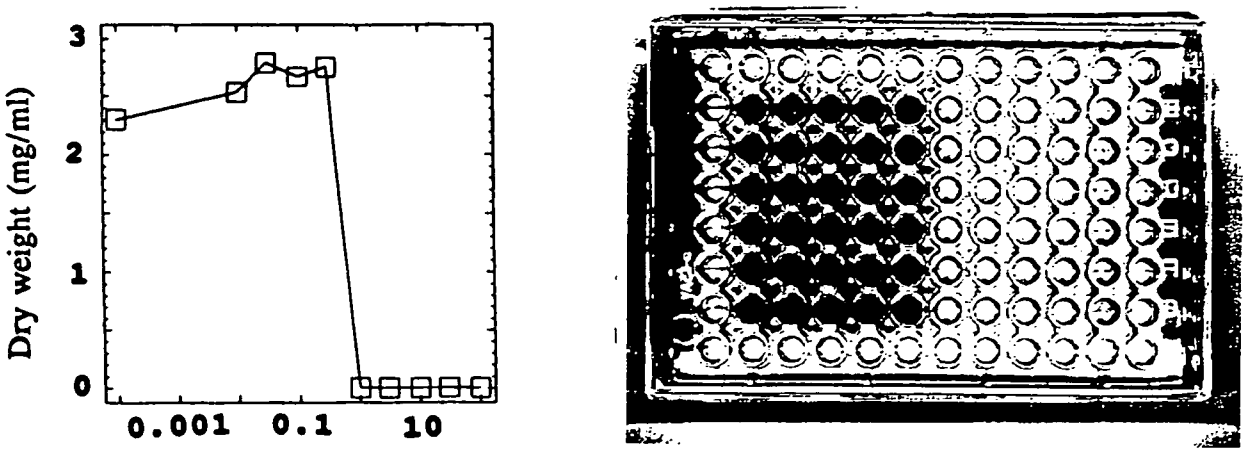

Fig.3. (above) Immunological plate with $K_2Cr_2O_7$, in solution Z8, conc. Cr: 0; 0.01; 0.03; 0.1; 0.3; 1; 3; 10; 30; 100 mg/L; *Chlorella kessleri*. **Left:** absorbency from the same plate.

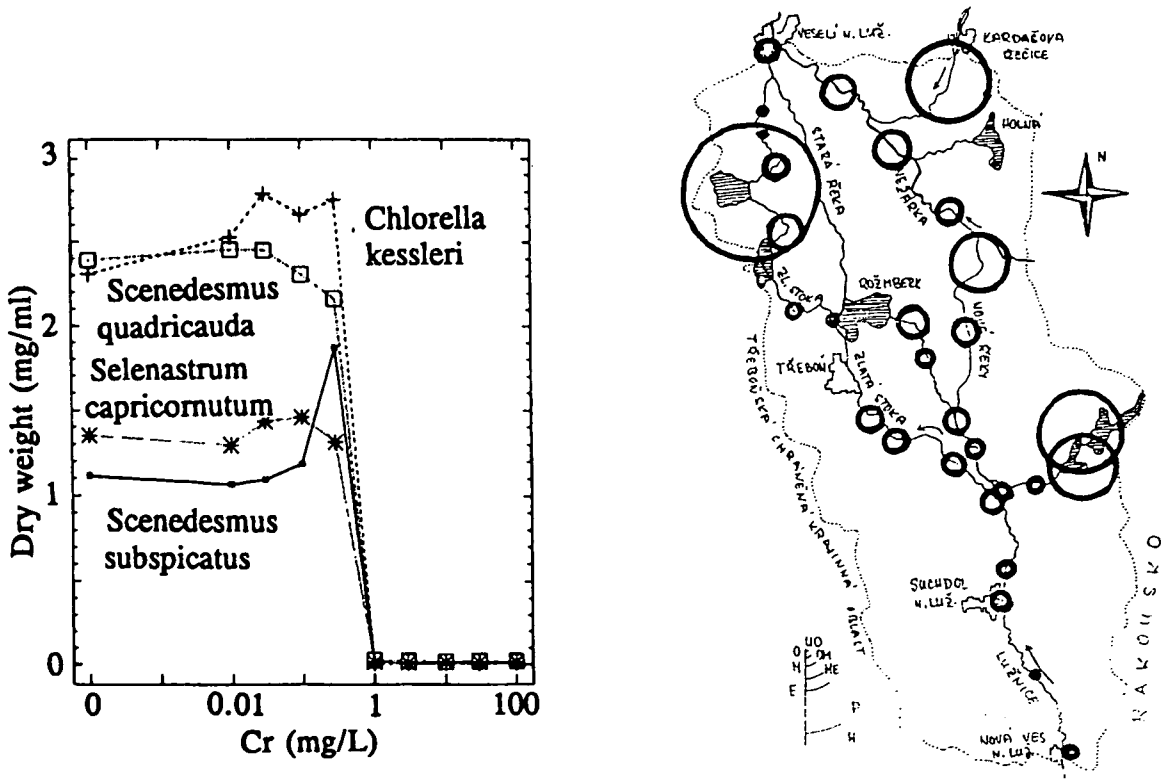

Fig.4. Toxicity of Cr to different strains of *Chlorella, Scenedesmus* and *Selenastrum*. **Right:** An example of monitoring of algal growth potential by miniaturized bioassay, in running waters of Protected Landscape Třeboňsko. Diameters of rings are proportional to trophic status: UO = ultraoligotrophic, O = oligotrophic, M = mesotrophic, E = eutrophic, P = polytrophic, H = hypertrophic. From LUKAVSKÝ (1989).

Notes: Immunological plates consist of polystyrene, and can be sterilized by use of an ultra-violet lamp or vapours of peracetic acid. Polystyrene is highly permeable to CO_2 in the cultivation box. The optimal concentration of CO_2 (2%) is checked with a small polyethylene bag with buffer (0.42g $NaHCO_3$ + 7.46g KCl + 30mg bromthymol blue in 1 L distilled water). A change of colour from blue, green to yellow indicates concentrations: <3, 2 and >3% vol/vol of CO_2. This method is simple and does not interfere with the state of metals in contrast to spike of hydrocarbonate and controlling pH above 8, according to ISO 8692.
Immunological plates are commonly used for ELISA assays, and there is a wide spectrum of related equipments.

Ad 1. Freezing is lethal for algae but not for spores of bacteria. Optimally γ irradiation should be used. Ultra-violet, however is simple and it also mineralizes some organics. Thermosensitive compounds can be sterilized by filtration, too.
Ad 4. Sensitivity to toxins should be strain-specific; a standard strain of the testing organism ought to be used. The most commonly used strains are *Raphidocoelis subcapitata (Selenastrum capricornutum), Scenedesmus subspicatus, S. quadricauda, Chlamydomonas reinhardi, Chlorella kessleri.* Surprisingly four tested strains of algae showed very similar sensitivity to Cr (Fig. 4).
Absorbency is a very complicated function of size distribution, concentration and optical properties of the cells of the different algae. See Fig. 2 for a conversion graph.
An inoculum of 1000 cells/ml is usually recommended. Our tests indicated that 10000 cells/ml is optimal. Another increase of inoculum can preceed the assay, but also resulted in the increment of absorbency of blank. The concentration of the inoculum is very important, as poisons are effective in accordance with dry weight not volume of culture!

Ad 5. A simple cultivation unit consists of fluorescent tubes (4 pcs at 40W, 120 cm) over a box (60x120x10 cm) covered with a glass sheet on polyurethane foam sealing (Fig.2). The unit has a capacity of 25 plates i.e. 250 samples, every in 6 replicates (6 wells per a column of the plate, see also Fig. 3). Two to three boxes can be placed under a laboratory table, with a total capacity of 750 samples. Temperature stabilizes, without any controlling, about 30 °C, near optimum for *Chlorella kessleri.* The lethal temperature (37 °C) must be avoided.
Ad 6. Serial port RS-232C enables on-line processing of the data using a PC. Software has been developed for BASIC and more sophisticated software for T-PASCAL with statistical evaluation based on K-statistics.
This miniaturized procedure is more precise (the coefficient of variation is about 8%) than the classical procedures, in volume of a quarter of litre (about 24%).

The relationships among absorbency, cell count, and dry weight are well defined and convertible (Fig.2). E.g. Dry weight = 3.31 + 179.45 * $Abs._{750\ nm}$ + 617.45 * Abs^2, [mg/L, for the alga *Chlorella kessleri*].
The sample is diluted with nutrient solution without EDTA. If diluted sample variants are growing better than the original ones, toxicity is probable. Better growth in variants with EDTA-Na_2 indicate toxicity resulting from heavy metals which are fixed firmly by the chelate. Application of EDTA-Na_2 is recommended as 5 µl from a solution of 100 mg of EDTA-Na_2/L of distilled water, which results in a final concentration of EDTA-Na_2 of 1mg/L.
The threshold of toxicity can be guessed also without measurement, by eye (Fig.3).

Standardization: When introducing the assay as well as the inner standard, a model sample with $K_2Cr_2O_7$ in solution Z8 is recommended. The levels of the suspension in individual wells are not constant, because of evaporation. Algae are sedimented in

the bottom, and reading the plate without shaking is preferable.

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