SEMICONTINUOUS CULTURE SYSTEM FOR *LEMNA GIBBA* BIOASSAY: FUNCTIONING AND THEORY OF OPERATION

M. MKANDAWIRE* – C. BRACKHAGE – B. TAUBERT – E.G. DUDEL
**e-mail: mkanda@forst.tu-dresden.de*

Institute of General Ecology and Environmental Protection, Dresden University of Technology, Pienner Straße 8, D-01737 Tharandt, Germany
(phone: +49-351-463-31393; fax: +49-351-463-31399)
**Corresponding author*

(Received 1st Oct 2004, accepted 28th June 2005)

Abstract. To overcome the problems associated with high concentrations of nutrient and test chemicals in laboratory *Lemna gibba* bioassays, a semi-continuous culture system was adopted, and specific equipment was designed and constructed to simulate steady-state conditions of the most natural aquatic systems. The equipment for *Lemna* semicontinuous culture consists of a growth chamber and a circulation control unit. *Lemna gibba* is kept in the growth chamber in a growth pot (with 1 mm diameter perforation at about 10 cm above the bottom), which retains the plant but allows an efficient exchange of the growth medium. Flow rate and composition of the medium is therefore varied independently of the *L. gibba* population density. The media are kept in circulation, and recharges or discharge of the media are controlled. The system took into consideration chemical processes such as O_2 and CO_2 exchange and maintained a degree of turbulence through continuous shaking of the growth chambers. It is also possible to investigate growth under different supply of O_2 and CO_2, and pH control with dissolved CO_2 or reducing conditions with N_2 gases. Theoretical analysis of growth in the steady state shows that integrated activation time of the dilution pump is proportional to the growth rate of *L. gibba*. Theoretical analysis was also used to determine the minimum flow-rate and nutrient concentration of the medium to cover the requirements of *L. gibba*. Experiments were carried out that demonstrated that the steady state growth could be attained and be controlled by the nutrient concentration, flow rate, and recharge intervals. The cultures could be kept at steady state over 21 days of the *Lemna* test period.
Keywords. *Lemna gibba, steady state, semicontinuous culture, batch culture, nutrient media*

Introduction

The validity of results obtained from laboratory tests performed batch-wise are often questioned because of dissimilarities to natural systems in respect to physical characters as well as process conditions such as high nutrient and test substance concentrations [2, 17]. The need to ensure realistic concentrations in hydroponic culture tests has been recognised for some decades [2, 15]. Various strategies, e.g., recharge or buffer system, have been developed. One of such systems is continuous culture, which became widely used particularly for microbes, algae and cell culture for industrial and research purposes [1, 7, 10, 11, 30, 31]. The early development of the continuous culture system can be traced back to simultaneous works of Novick & Szilard [27] and Monod [25].

Continuous culture systems are described as: (1) steady-state, where nutrients are supplied to the culture at a constant rate, and a constant amount of biomass is maintained in the culture. Steady-state systems have constant metabolic and growth parameters. (2) Turbidostat, where medium is delivered only when the population density of the culture reaches some predetermined point, as measured by the extinction of space in the culture. Fresh medium is, then, added to the culture and an equal volume is removed [32]. (3) Chemostat, where the medium is delivered at a constant rate, which

http://www.ecology.kee.hu ● ISSN 1589 1623

ultimately determines growth rate and density [5, 7, 27]. In order to deliver exactly the same amounts of medium to the cultures and reproducibility of the experiments, a steady-state approach through a semicontinuous culture mode is usually preferred [1, 7, 10]. Semicontinuous culturing differs from batch culture in that fresh medium is added to the culture at a regular rate and spent medium is removed at the same rate. The principal advantage is that tests are conducted with realistic concentrations emulating the nature of the nutrient buffering capacity [15].

In recent years, continuous culture techniques have found their way into the bioassay methods of ecotoxicology and bioremediation. However, their adoption and application for higher plant hydroponic cultures has been so limited because realisation of a system that circulates and re-charges large volumes while maintaining a low realistic concentration proved to be expensive [2, 3, 4]. Consequently, most test procedure like the standard *Lemna* tests use batch culture modes, despite the well publicised difference from natural conditions [12–14, 28, 29]. In *L. gibba* cultures, a semicontinuous culture mode reduces competition between algae and *Lemna* spp., reduces poisoning from metabolic excretion, and reduces large changes of chemical speciation in the solution [20, 33]. To investigate mechanisms that control processes in phytoremediation and ecotoxicity of uranium and arsenic to aquatic macrophytes, laboratory and field trials with the model plant *L. gibba* in a semicontinuous culture were opted. Hence, the semicontinuous culture equipment was designed for *L. gibba*, whose functioning and design are described in this paper.

General *Lemna gibba* growth conditions

The most important parameters regulating frond growth are nutrient quantity and quality, light, pH, turbulence, salinity, and temperature [6, 8, 12, 18, 19]. The optimal parameters as well as the tolerated ranges are species-specific, and a broad generalization for the most important parameters for *Lemna* spp. is given in *Table 1*. The *Lemna* semi-continuous culture has been designed to take into consideration as many parameters as possible while its optimum is reached when operated in plant chamber (ecotron).

Table 1. *A generalized set of conditions for culturing L. gibba*

parameters	optimal range	source
Temperature (°C)	18–24 °C	ISO/WD 20079
Salinity (g l^{-1})	20–24 g l^{-1}	ISO/WD 20079
Light intensity (lux)	4200 and 6700 lux 85–125 µE m^{-2} s^{-1} or 400–700 nm	EPA712–C–96–156 OPPTS 850.4400 ISO/WD 20079
Photoperiod	14–16 hr d^{-1}	own unpublished data
pH	5.6–7.5	Mkandawire *et al*. (2002)

Parts and set-up of the culture system equipment

The system is designed to maintain a steady state in the *L. gibba* culture. The system has 12 *Lemna* culture vessels composed of two parts: (1) the outer vessel and (2) an inner growth pot. The medium is circulated to the culture at a constant rate. The flow rate of the medium is adjusted according to the concentration of the medium and growth

http://www.ecology.kee.hu ● ISSN 1589 1623

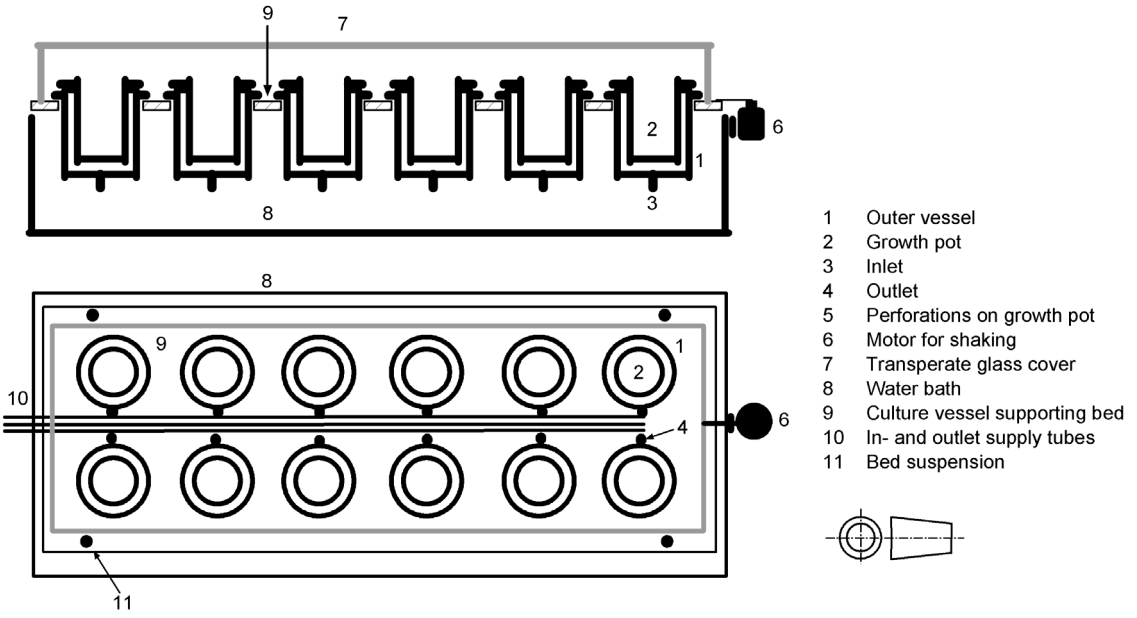


Figure 1. *Frond and plan in 1st angle orthographic projection view of the part set-up of the main semicontinuous culture unit*

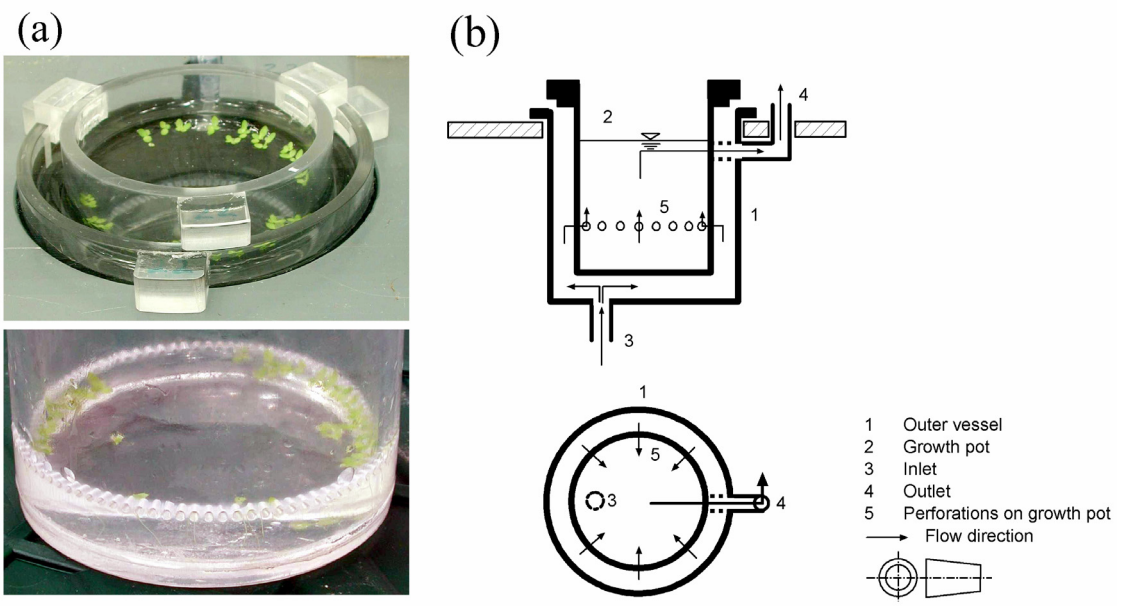


Figure 2. *Picture of growth vessel showing the perforation on the growth pot; and (b) front and plan view of Lemna growth vessels showing the circulation on the medium*

rate of *L. gibba*. If the growth rate is not well calculated or defined, the literature recommends that approximately 20% of culture should be removed as spent and replaced by an equal volume [10, 11, 16]. Each *Lemna* growth vessel has an inlet and outlet connection (3 and 4). The growth pot has perforation at 20 mm from the base and top to enable the flow of solutions. This also allows easy monitoring with minimum disturbances; i.e., once the growth pot is pulled out of the chamber, the *Lemna* specimen is left at a 20 mm depth of the medium, e.g., during growth observations with frond area with graphic image equipment.

http://www.ecology.kee.hu ● ISSN 1589 1623

The growth vessels are deep in a water bath (5) to equilibrate temperatures in the vessels (*Fig. 1*). The bed is suspended (9) and swings with the help of a motor (6), to a required turbulence. The turbulence or the continuous shaking of the growth vessels helps to maintain the solution well mixed, and exchange of gases with the air. The vessels are covered with (7) a transparent glass cover to avoid fallouts from the air.

The medium circulates from the vessel through the tube to filters, which helps reduce the growth of microorganisms like algae and remove dead fronds, and back to the culture vessel. Fresh nutrient medium is stored in a container (11). Opening a valve and closing the clamp on the medium line can add medium to the culture vessel. Fresh medium flows into the culture vessel, and the spent culture flows out into a collecting vessel. Optionally, the system allows working in reducing conditions by allowing a flow of N_2, and different O_2 and CO_2 regimes. As fresh medium is added to the culture vessel, the level of the liquid in the culture vessel rises to the level of the outflow tube and the old medium flows out of the culture vessel into a spent medium flask.

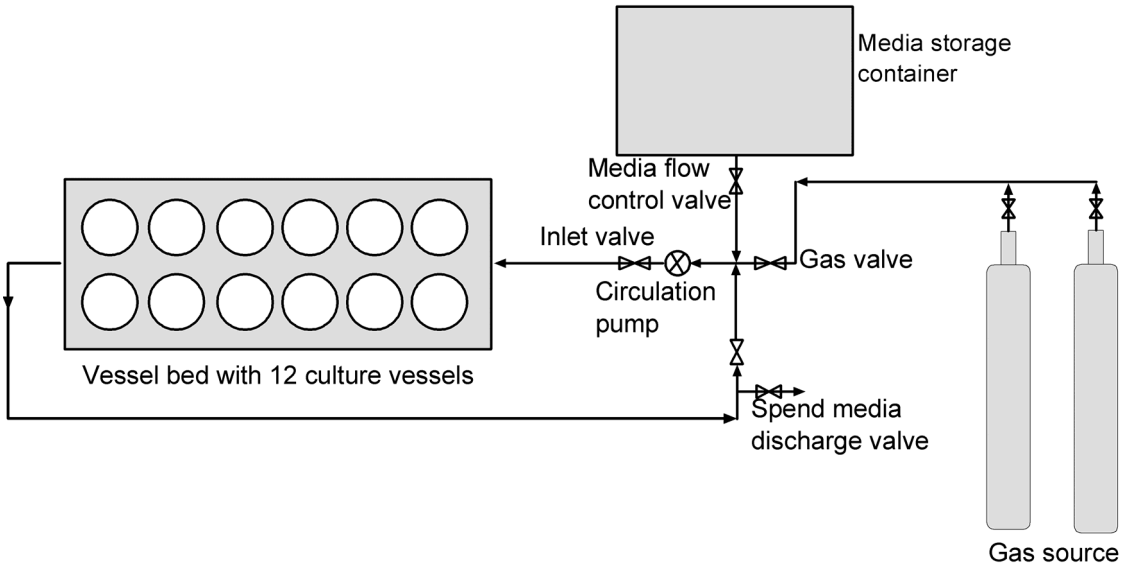


Figure 3. *Principle sketch of the functioning of the Lemna semicontinuous culture system*

Functioning of the *Lemna* semicontinuous culture system equipment

In a semi-continuous system, the fresh medium is delivered to the culture all at once, by simply opening a valve in the medium delivery line. Fresh medium flows into the culture vessel, and the spent culture flows out. Once the required medium has entered the culture, the valve is closed, and the culture is allowed to grow for a given period, when the procedure is repeated. The natural system is at steady state because there is continuous exchange of chemical elements and compounds between the geo- and hydrosphere. In preliminary *Lemna* bioassay studies with a batch system, it was found that the pH decreases as *L. gibba* grows [20, 24]. This happens partially because of release of CO_2 from plants respiration at night, exudation and the resources are depleted in the medium [20, 26]. Shaking creates turbulence in the system that helps release CO_2 from the culture, while at the same time there is dissolution of oxygen. The space between the inner growth pots at the outer vessel in figure 4.3 is for this purpose, particularly when the pot is fully covered by an *L. gibba* mat.

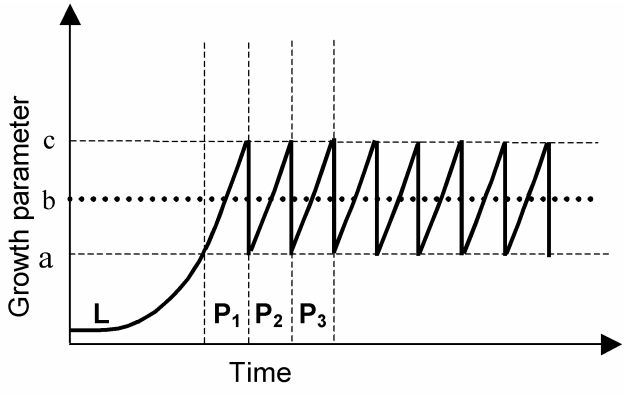
http://www.ecology.kee.hu ● ISSN 1589 1623

Function and operation theory

Experiments with *L. gibba* were conducted to parameterise the processes in the equipment as described elsewhere [20–23]. Performance of *L. gibba* changes in the culture physicochemical condition in batch and semicontinuous cultures have been reported in Mkandawire *et al.* [24]. The data read to the general theory as below.

Under steady state *L. gibba* growth, the growth is expected to be constant throughout the study, but practically the growth rate fluctuates between *a* and *c* around this arbitrary constant growth rate *b* in *Fig. 5*. The length is modulated by the concentration of the medium, which also determines the recharge interval P_n. From the experiment (data not shown), it was observed that the curves smooth when the recharge rate approaches the continuous; i.e., when the gaps from one nutrient recharge into the culture are close to mathematical zero.

4



5

Figures 4 and 5. 4: *The main part of the semicontinuous culture system for L. gibba bioassay.* 5: *Periodic fluctuation in steady state in L. gibba semicontinuous culture. L represents pre-culture period; P1, P2, ..., Pn are nutrient recharge and biomass removal intervals also shown as* $t_1, \ldots, t_n$ *in the text.*

The rate of flow of medium into a semicontinuous culture system is sometimes referred to as the dilution rate [9, 15]. When the number of fronds in the culture vessel remains constant over time, the dilution rate is equal to the rate of multiplication in the culture, because a known number of *L. gibba* fronds, removed in relation to the discharge of medium, are replaced by an equal number through multiplication in the culture The recharging interval time (t_n) (or the length of the period between the inflows of nutrients) in a semi-continuous culture is inversely related to the concentration of the nutrient medium. Assuming the undiluted solution medium (N) is the standard used in batch culture, plotting the dilution factor (N) against t_n shows that as t_n approaches zero, the system gradually becomes continuous. The relationship is a linear line regression as:

$$N = -kt_n + c\,, \qquad \text{(Eq. 1)}$$

http://www.ecology.kee.hu ● ISSN 1589 1623

where k is the regression slop constant; c is a constant, x in N_m are integers 0, 1, 2, 3,…, m; and similarly n in t_n are 0, 1, 2, 3,…, n. Therefore, k becomes the flow rate (F) and at t_0 $N = N_m$ it can be rewritten as:

$$N = -Ft_n + N_m . \quad \text{(Eq. 2)}$$

For simplicity, points on the regression are assumed to be the lowest concentration at which a plant is capable to attain its maximum specific growth rate (μ_{max}). The rate of flow (f) of medium into a semicontinuous culture system is directly related to the dilution rate (D), whereas D is inversely related to the volume of the medium (v_{med}):

$$D = \frac{f}{v_{med}} . \quad \text{(Eq. 3)}$$

In this equipment D and f are not the same. Increase in the number of *L. gibba* fronds in the culture is proportional to the specific growth rate (μ) and the yield rate (E), i.e., the amount of *L. gibba* to be removed to maintain a steady state. When the steady state condition are met, the relationship in the dynamic equilibrium becomes as follows:

$$\frac{dx}{dt} = \mu X - DX = 0 \quad \text{(Eq. 4)}$$

and

$$\frac{dx}{dt} = \mu X - E = 0 , \quad \text{(Eq. 5)}$$

where x is the growth parameter, and t is the time. It follows that $\mu X = E$, and further analysis shows that X is constant, resulting in E being directly proportional to μ. This depends on the volume of the culture vessel and on the concentration (S) and further depends on the nutrient uptake by *L. gibba*, and Monod model can be adopted:

$$\mu = \mu_{\max} \frac{s}{s + k_g} ; \quad \text{(Eq. 6)}$$

k_g is the saturation constant at which $\mu = \frac{1}{2}\mu_{\max}$. At D, $S = S_0$ which is theoretically valid even when considering single growth-limiting nutrient elements like N or P. For a small dilution rate, let the *L. gibba* volume ratio *(Z)* be expressed by

$$Z = \frac{X}{V} . \quad \text{(Eq. 7)}$$

This allows that uptake

$$A_s = D(S_0 - S) . \quad \text{(Eq. 8)}$$

Then,

$$\frac{\Delta D}{-\Delta S} = \frac{dx}{ds} = y , \quad \text{(Eq. 9)}$$

where y is the economic quotient. In relationship to changes in the substrate concentration when steady state conditions are fulfilled,

http://www.ecology.kee.hu ● ISSN 1589 1623

$$\frac{dS}{dt} = D(S_0 - S) - \mu_{max} \frac{X}{y} \frac{S}{k_s - S} = 0 . \quad \text{(Eq. 10)}$$

Considering the optimal range of substrate concentration where maximum growth rate is attained, uptake A_s can be given as

$$A_s = \mu_{max} \frac{X}{Y} = C , \quad \text{(Eq. 11)}$$

where, C is a constant. Hence,

$$D(S_0 - S) = \mu_{max} \quad \text{(Eq. 12)}$$

and

$$S = S_0 - \frac{\mu_{max} \frac{x}{y}}{D} . \quad \text{(Eq. 13)}$$

In the suboptimal range, the substrate concentration depends on D; and, likewise,

$$D = \mu_{max} \frac{S}{K_s + S} . \quad \text{(Eq. 14)}$$

Conclusion

The principal advantage of semicontinuous culturing is that the rate of dilution controls the growth rate (μ) via the concentration of the growth-limiting nutrient in the medium, found in *L. gibba* cultures to be phosphorus [21, 23]. As long as the dilution rate is lower than the maximum growth rate (μ_{max}) attainable by *L. gibba*, the frond density will increase to a point at which the frond multiplication rate exactly balances the removal and death rate. This steady-state frond density is also characterised by a constancy of all metabolic and growth parameters. On the other hand, if the dilution rate exceeds the maximum growth rate, fronds are removed faster than they are produced, and a total decrease in the entire frond population eventually occurs.

Acknowledgements. The study was supported by BMBF (Federal Ministry of Education and Research) under grant No. 02WB0222. The Dresden University of Technology Academic Workshop constructed the equipment. Dmitry Tychinin of the Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences assisted in improving the quality of the language.

REFERENCES

[1] Allan, V.J.M., Callow, M.E., Macaskie, L.E. & Paterson-Beedle, M. (2002): Effect of nutrient limitation on biofilm formation and phosphatase activity of a *Citrobacter* sp. – Microbiology-Sgm 148: 277–288.

[2] Asad, A., Bell, R.W., Dell, B. & Huang, L. (1997): Development of a boron buffered solution culture system for controlled studies of plant boron nutrition. – Plant and Soil 188: 21–32.

[3] Asher, C.J. & Blamey, F.P.C. (1987): Experimental control of plant status using programmed nutrient addition. – Journal of Plant Nutrition 10: 1371–1380.

[4] Asher, C.J. & Edwards, D.G. (1983): Modern solution culture techniques. – In: Lauchli, A. & Bieleski, R.L. (eds.): Encyclopedia of plant physiology. New series, Inorganic plant nutrition. Springer Verlag, Berlin, pp. 94–119.

http://www.ecology.kee.hu ● ISSN 1589 1623

[5] Baird, M.E., Emsley, S.M. & McGlade, J.M. (2001): Modelling the interacting effects of nutrient uptake, light capture and temperature on phytoplankton growth. – Journal of Plankton Research 23: 829–840.
[6] Beck, T. & Feller, U. (1991): Ammonium-stimulated potassium release from *Lemna minor* L. grown on a medium containing nitrate as N-source. – Aquatic Botany 39: 255–266.
[7] Biebl, H. (2001): Fermentation of glycerol by *Clostridium pasteurianum* – batch and continuous culture studies. – Journal of Industrial Microbiology & Biotechnology, 27: 18–26.
[8] Bottomley, W.B. (1920): The growth of *Lemna* plants in mineral solution and their medium. – Annals of Botany: 343–452.
[9] Dässler, H.-G., Kästner, W. & Ranft, H. (1968): Waldbaulich-ertragskundliche Auswertung eines Anbauversuches im akuten Einflußbereich einer Zinkhütte bei Freiberg. – Arch. Forstwes. 17: 145–158.
[10] Davidson, K. & Gurney, W.S.C. (1999): An investigation of non-steady-state algal growth. II. Mathematical modelling of co-nutrient-limited algal growth. – Journal of Plankton Research 21: 839–858.
[11] de Oliveira, L.A., Jean Blain, C., Durix, A., Komisarczuk Bony, S. & Durier, C. (1996): Use of a semi-continuous culture system (RUSITEC) to study the effect of pH on microbial metabolism of thiamin (vitamin B-1). – Archives of Animal Nutrition-Archiv Fur Tierernahrung 49: 193–202.
[12] DIN (2001): Water quality – Duckweed growth inhibition: Determination of the toxic effect of water constituents and wastewater to duckweed (*Lemna minor*). Deutsches Institut für Normung e.V. durch Beuth Verlag, Berlin.
[13] EPA (1996): Aquatic plant toxicity test using *Lemna* spp., Tiers I and II. United States Environmental Protection Agency Prevention, Pesticides and Toxic Substances Unit: New York.
[14] EPA (2002): Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. Fourth edition. U.S. Environmental Protection Agency, Office of Water (4303T), Washington DC, 350 pp.
[15] Erismann, K.H. & Finger, A. (1968): Lemnaceaen in kontinuierlicher kultur. – Ber. schw. Bot. Ges. 78: 5–15.
[16] Fabregas, J., Patino, M., Morales, E., Cordero, B. & Otero, A. (1996): Optimal renewal rate and nutrient concentration for the production of the marine microalga *Phaeodactylum tricornutum* in semicontinuous cultures. – Appl. Environ. Microbiol. 62: 266–268.
[17] Kohl, K.I. (1997): Do *Armeria maritima* (Mill.) Willd ecotypes from metalliferous soils and non-metalliferous soils differ in growth response under Zn stress? A comparison by a new artificial soil method. – Journal of Experimental Botany 48: 1959–1967.
[18] Landolt, E. (1980) (ed.): Biosystematic investigations in the family of duckweeds (Lemnaceae). – Veröff. Geobotanisches Institut ETH, Zürich.
[19] Landolt, E. & Kandeler, R. (1987) (eds.): The family Lemnaceae: A monographic study. Vol. 2. Phytochemistry, physiology, application, bibliography. – Veröff. geobot. Inst. ETH Stif.
[20] Mkandawire, M., Dudel, G.E. (2002): Uranium attenuation from tailing waters by floating macrophyte *Lemna gibba* L. – In: Merkel, J.B., Planer-Friedrich, B. & Wolkersdorfer, C. (eds.): Uranium in the aquatic environment. Vol. III. Springer, Berlin, pp. 623–630.
[21] Mkandawire, M., Lyubun, Y.V., Kosterin, P.V. & Dudel, E.G. (2004): Toxicity of arsenic species to *Lemna gibba* L. and influence of phosphate on arsenic bioavailability. – Environmental Toxicology 19: 26–35.
[22] Mkandawire, M., Taubert, B. & Dudel, E. G. (2003): Effects of phosphate and ammonium amendments on arsenic and uranium immobilisation by *Lemna* sp. from tailing waters. – In: Kalogerakis, N. (ed.): Second European Bioremediation Conference. Technical University of Crete, and European Community (High-level Scientific Conference Programme), Chania, Creta, Greece, pp. 405–408.

http://www.ecology.kee.hu ● ISSN 1589 1623

[23] Mkandawire, M., Taubert, B. & Dudel, E.G. (2003): Potential of *Lemna* sp. for phytoremediation of uranium and arsenic from mine tailing waters. – In: Turkovskaya, O.V. & Ignatov, V.V. (eds.): International symposium on Biochemical Interaction and Microorganisms and Plant with Technogenic environmental Pollutants. Vol. 1. Saratov University Publishing House, Saratov, 56 pp.
[24] Mkandawire, M., Taubert, B. & Dudel, E.G. (*in press*): Capacity of *Lemna gibba* L. (duckweed) for uranium and arsenic phytoremediation in mine tailing waters. – International Journal of Phytoremediation.
[25] Monod, J. (1950): La technique de culture continue, theorie et applications. – Annales Institute Pasteur 79: 390–410.
[26] Mueller, P., Feller, U. & Erismann, K.H. (1997): Influence of different CO_2-concentration as growth and composition of *Lemna minor* L. grown on nitrate or ammonium.
[27] Novick, A. & Szilard, L. (1950): Experiments with the chemostat on spontaneous mutations of bacteria. – Proc. Natl. Acad. Sci. 36: 708–719.
[28] OECD (1984): OECD guidelines for testing of chemicals: alga, growth inhibition test. Organisation for Economic Corporation and Development, Paris, 12 pp.
[29] OECD (2002): OECD guidelines for the testing of chemicals: *Lemna* sp. growth inhibition test. Organisation for Economic Corporation and Development, Berlin, 22 pp.
[30] Rhee, G.Y. (1973): A continuous culture study of phosphate uptake, growth rate and polyphosphate in *Scenedesmus* spp. – J. Phycol. 9: 495–506.
[31] Rhee, G.Y. (1989): Use of continuous culture in algal biassay. – Hydrobiologia 188/189: 247–257.
[32] Skipnes, O., Eide, I. & Jensen, A. (1980): Cage culture turbidostat: a device for rapid determination of algal growth rate. – Applied and Environmental Microbiology 40: 318–325.
[33] Taubert, B. (2003): Uran Immobilisierung in *Lemna* sp. (Wasserlinsen) in Abhängigkeit von den Milieubedingungen, In Insitut für Allgemeine Ökologie. Technische Universität Dresden, Dresden.

http://www.ecology.kee.hu ● ISSN 1589 1623