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Validation of duckweed microbiological test for assessing hazardous substances

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Abstract

For this study, we used modified EN ISO 20079. The modification consists in application of polystyrene macroplates, with the advantage of requiring low sample volumes for the test (10 ml). *Lemna minor* was cultivated as a monoculture by various doses of chemicals for seven days. The aim of our work was to compare the acute toxicity ($168hEC_{50}$) obtained from conventional testing (100 ml) and from microbiological tests (10 ml) using reference toxicants: potassium chloride and 3.5 – dichlorphenol. The resulting value $168hEC_{50}$ for potassium chloride using conventional test was 9.78 $g.l^{-1}$ and for 3.5 – dichlorphenol it was 5.71 $mg.l^{-1}$. The resulting values from microbiological tests were 8.69 $g.l^{-1}$ and 4.24 $mg.l^{-1}$, respectively. The comparison of measured values of acute toxicity from conventional tests and microbiological tests indicates that microbiological tests are an appropriate alternative to today commonly used ecotoxicological biotests, for measuring and assessing of biological effects of toxic substances in the aquatic environment.

Keywords: duckweed (*Lemna minor*), microbiological tests, potassium chloride, 3.5 – dichlorphenol

Introduction

Due to the enormous number of potentially polluting substances contained in waste waters from municipal and environmental sources, here grows a necessity of providing the information about water quality. A chemical-specific approach is insufficient to provide the complex information about water quality. Therefore, it is essential to use biological test systems with living cells or organisms that give a global response to the quantum of micropollutants present in the sample.

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Settled life style of plants makes them an organism constantly exposed to the pollution. Also plants are the major nutriment source for nearly all higher organisms and as such play an active role in transferring contaminants to higher trophic levels (Radic et al. 2010).

Duckweed (*Lemna minor*) is used in quality studies to monitor heavy metals and other aquatic pollutants. The plants posses physiological properties (small size, rapid growth between pH 5 – 9, and vegetative propagation), that make duckweed an ideal test system. Among the developmental parameters, the most commonly assessed in ecotoxicological test systems, are growth parameters (Wang and Freemark 1995).

In EN ISO 20079 Water quality – Determination of the toxic effect of water constituents and waste water on duckweed (*Lemna minor*) – Duckweed growth inhibition test are tested 100 ml sample volumes. Using microvolumes (10 ml) in microbiological tests can be a good tool to include in a battery of tests for phytotoxicity screening of a wide range of chemicals and environmental samples, with the advantage of allowing large numbers of samples to be tested, and generating low volumes of waste (Paixao et al. 2008).

The organisms commonly employed in microbiological tests are bacteria, protozoa, invertebrates, fish and tissue cultures etc. But standard microbiotest using duckweed as a test organism has not been created yet. The aim of this work is to validate a miniaturized duckweed test using macrotitration plates as a suitable alternative to conventional ecotoxicological biotests.

Materials and methods

Growth inhibition tests, with duckweed, were performed according to the standard EN ISO 20079. The 7-day toxicity test conducted at 24 ± 2°C in test vessels containing a minimum of 100 ml of test solution and 3-frons plants. Our suggested modification requires lower volumes of test solutions and reduced amount of plants. We employed a static test, where the test solutions are not renewed during the test. For the comparison of sensitivity and validation of the accuracy and reliability of the results of this method were performed examinations with the reference toxicants.

We investigated the effect of various doses of two defined referent toxicants: potassium chloride (3; 4; 7; 10 and 15 $g.l^{-1}$) and 3.5 – dichlorphenol (1.5; 2.1; 3; 4.2 and 5.9 $mg.l^{-1}$) on *Lemna minor*.

Plant material was obtained from a culture collection of the ecotoxicological laboratory of the University of Veterinary and Pharmaceutical Sciences Brno and was adapted for the test. The stock culture of duckweed is cultivated in SIS medium so we also used SIS medium with pH 6.5 for preparation of the concentration row (OECD 2004). In the situation when the validation requirements are fulfilled it is allowed using e.g. SIS medium (EN ISO 20079 2007).

We tested samples of 100 ml volume (conventional test: 150 ml beaker) and of 10 ml volume (Microbiological test) in the same time. Polystyrene macroplates used for the microbiological test consist of six dimples of maximum volume 15 ml with flattened bottom and with the cover. Test vessels in conventional test were covered by the foil to minimize evaporation and accidental contamination. All applied vessels avoided shadowing or changes in the spectral characteristics of light.

In 100 ml volume the initial number of fronds was nine. In microbiotest the number was reduced to five. The vessels with referent toxicants in three replicates, and the control in six replicates, were incubated during seven days under a continuous warm fluorescent lightning and with the temperature of 24±2°C.

For the test to be valid, the doubling time of frond number in the control must be less than 2.5 days, corresponding to approximately a seven-fold increase in seven days. The toxic influence of reference toxicants was evaluated on a basis of growth inhibition expressed as number of fronds and comparison of growth rates (Fig. 1 and 2) (EN ISO 20079 2007).

Formula 1:

$$\mu_{i-j} = \ln (N_j) - \ln (N_i) / t_{j-i}$$

Average specific growth rate
μ_{i-j} : average specific growth rate from moment time i to j
N_i : number of fronds observed in the test or control vessel at time i (the end of the test)
N_j : number of leaves observed in the test or control vessel at time j (the start of the test)
t_i : moment time for the start of the period
t_j : moment time for the end of the period

Formula 2:

$$\% I_r = [(\mu_c - \mu_T) / \mu_c] \times 100$$

Percent inhibition of growth rate
$\% I_r$: percent inhibition in average specific growth rate
μ_c : mean value for μ in the control
μ_T : mean value for μ in the treatment group

Results and discussion

Our conventional tests and also the microbiological tests were able to fulfill the validity requirements. EN ISO 20079 specifies ranges of resulting $168hEC_{50}$ values for both toxicants. The values of $168hEC_{50}$ for potassium chloride (using APHA medium) has to lie in the range of 2.2 – 3.8 $mg.l^{-1}$ and for 3.5 – dichlorphenol (modified Steinberg medium) in 5.5 – 10.0 $g.l^{-1}$. But the values of acute toxicity for both referent toxicants using SIS media are not known. The resulting values of $168hEC_{50}$ for potassium chloride were corresponding with the declared range, for conventional test it was 9.78 $g.l^{-1}$ and for microbiotest it was 8.69 $g.l^{-1}$. For 3.5 – dichlorphenol resulting values of $168hEC_{50}$ (5.71 $mg.l^{-1}$ and 4.24 $mg.l^{-1}$, respectively) were higher in both cases of testing (Table 1).

Table 1: Comparison of the resulting values of $168hEC_{50}$ for potassium chloride and 3.5 – dichlorphenol

$168hEC_{50}$	KCl	3.5 - dichlorphenol
Conventional test	9.78 $g.l^{-1}$ 95% interval of reliability = 9.16 – 10.40	5.71 $mg.l^{-1}$ 95% interval of reliability = 5.52 – 5.90
Microbiotest	8.69 $g.l^{-1}$ 95% interval of reliability = 8.18 – 9.20	4.24 $mg.l^{-1}$ 95% interval of reliability = 4.15 – 4.33

Conclusion

From the resulting values of 168 hours lasting growth inhibition test using water bioindicator *Lemna minor* ($168hEC_{50}$) for two referent toxicants we can observe a good correlation between conventional (100 ml) test and microbiotest (10 ml). In our microbiotest we also carried out the validity requirements. So it can be seen that microbiological tests for assessing toxic effect of chemicals or other hazardous substances are a suitable alternative to commonly used ecotoxicological biotests.

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