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Detection of photosynthetic herbicides: Algal growth inhibition test vs. electrochemical photosystem II biosensor

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ABSTRACT

We compared a novel PSII-biosensor assay with a standard algal growth inhibition test for detection of photosynthetic herbicides—diuron, atrazine and isoproturon in liquid samples. To evaluate the convenience and sensitivity, values of the parameters EC50 and LOD and the duration of assays were compared. The biosensor assay was made with an electrochemical biosensor toxicity analyser with immobilised Photosystem II (PSII) complex. Using the PSII-biosensor assay, higher sensitivity (LOD) to herbicides (10^{-8}–10^{-9} M) was achieved as compared to standard algal growth inhibition tests (about 10^{-7} M). The results of both assays showed a good correlation as concerns their EC50 values while the interval of detectable concentrations is about twice wider for PSII-biosensor. A proposed measurement protocol includes the reference standard of phytotoxicity (RSP). The main advantage of the PSII-biosensor assay is that it can be completed in about 1 h and is by 1–2 orders more sensitive than standard algal growth inhibition test, which takes 72 h.

1. Introduction

For several decades, the herbicide contamination of water and soil has been of general concern since it affects drinking water quality and impacts on crop safety. A group of commonly used herbicides are those that are photosynthesis inhibitors (ureas, triazines and phenolics), used in agriculture since 1950s. An undesirable side effect of the agricultural use of 'photosynthesis-inhibiting' herbicides is that they may enter non-target habitats (e.g. freshwater ecosystems) in various ways: by spray drift, leaching, run-off and/or accidental spills. Photosynthetic herbicides are often only weakly degraded by hydrolysis, and so are relatively stable in a water environment. For these reasons, authorities have set environmental criteria that must be met before these chemicals can be legally used to protect crops (e.g. US EPA, 1978; OECD, 1984) with necessary feedback control of water contamination.

The management of environments has led to the development of chemical and biological methodologies that assess water quality. However, an expensive chemical analysis does not in all cases have a direct relation to environmental toxicity due to its complexity in surface water samples (interactions with organic and inorganic compounds) and different pathways of degradation with unknown toxicity of metabolites. Meanwhile, bioassays have become an indispensable tool for evaluating the damage of contaminants on organisms. The detection methods used to assess these compounds' biological toxicity are predominantly based on their photosynthesis-inhibition effect.

Phototrophic microorganisms are sensitive to some herbicides because the targets of these compounds are usually either photosynthesis or energy-transport enzymes. Unicellular microalgal strains, *Selenastrum*, *Desmodesmus* and *Chlorella*, with a cell size of 3–10 μm, have frequently been used in bioassays (estimating the toxicity of liquid samples—surface waters, soil extracts, etc.) due to their fast growth (a doubling time of several hours) in liquid growth media, where tested samples can be easily added.

Abbreviations: Atrazine, 1-chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine; Diuron, 3-(3,4-dichlorophenyl)-1,1′-dimethylurea; Duroquinone, tetramethyl-p-benzoquinone; EC50, effective concentration of compound causing a 50% inhibition; FeCy, potassium ferricyanide; HPLC, high-performance liquid chromatography; Isoproturon, 3-(4-isopropylphenyl)-1,1′-dimethylurea; LED, light-emitting diode; LOD, limit of detection; MES, 2-(N-morpholino) ethanesulphonic acid; PI, phytotoxicity index; PSII, Photosystem II; r.u., relative units; RSP, reference standard of phytotoxicity

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Most photosynthesis-inhibiting herbicides block electron flow in the Photosystem II (PSII) complex (Draber et al., 1991; Huppatz, 1996; Van Rensen, 1989; Böger and Sandmann, 1998). This so-called Hill reaction is a measure of photochemical activity of the PSII complex, where oxygen production is monitored in the presence of an artificial electron acceptor (Bishop, 1958; Moreland et al., 1959). Herbicidal PSII-inhibitors bind to the D1 protein of the PSII core, are competing with the reversibly bound plastoquinone (the Q_B acceptor). Biosensors, based on PSII complex, have been used for the detection of photosynthetic herbicides within the last two decades (for review see Giardi et al., 2001). For example, intact cells, isolated thylakoids or PSII particles can be immobilised on an electrochemical detector, thus enabling the direct detection of their photochemical activity (e.g. Rawson et al., 1989; Carpentier et al., 1991; Pandard et al., 1993; Rouillon et al., 1995, 1999; Touloupakis et al., 2005; Bettazzi et al., 2007). If the activity is inhibited by a herbicide, then this effect can be directly measured. Based on this principle, we have recently developed the electrochemical biosensor using thick-film printed electrodes with immobilised PSII preparations (Koblizek et al., 2002; Maly et al., 2005).

The main objective of the present study was to compare the standard algal growth inhibition test (EN ISO 8692:2004) with a novel PSII-biosensor assay for detection of photosynthetic herbicides in liquid samples. We evaluated the time it took to complete an assay, as well as the values of the parameters EC50 and LOD determined by both methods. Here, we also present the set-up of a laboratory biosensor toxicity analyser and the standardized measuring procedure for the detection of photosynthetic herbicides in an aqueous solution based on relative comparison to the reference standard of phytotoxicity.

2. Materials and methods

2.1. Standard algal growth inhibition test (bioassay)

This method of pollutant assay defined by the European Standard (EN ISO 8692:2004) uses cultures of the unicellular green microalga *Desmodesmus subspicatus* (CHOD.) HEGEW et SCHMIDT (formerly *Scenedesmus subspicatus*), which are exposed to various concentrations of herbicides under defined conditions. At the start of the test, 25 mL cultures were inoculated by $10{,}000 \pm 2000$ cells mL^{-1} in an inorganic medium and grown at 23 ± 2 °C, pH 8 ± 1 under the irradiance intensity of 100 μmol photon m^{-2} s^{-1}. The microalgal cultures were incubated for a period of 72 h and mixed manually three-times a day. The cell concentration in each flask was determined at least 24, 48 and 72 h after the start of the test.

In the tests, the algal cultures were exposed to 6 discrete concentrations of the tested herbicide in the range of a 10–90% inhibition, including the control. These were 4.65×10^{-7}, 6.98×10^{-7}, 9.30×10^{-7}, 1.40×10^{-6}, 2.33×10^{-6} and 4.65×10^{-6} M for atrazine; 3.39×10^{-7}, 4.85×10^{-7}, 7.27×10^{-7}, 9.69×10^{-7}, 1.45×10^{-6} and 2.42×10^{-6} M for isoproturon and 4.29×10^{-8}, 8.58×10^{-8}, 1.72×10^{-7}, 2.58×10^{-7}, 3.43×10^{-7} and 4.29×10^{-7} M for diuron.

The mean specific growth rate (μ) for exponentially growing cultures was calculated as

$$\mu = \frac{\ln N_2 - \ln N_1}{t_2 - t_1} \quad (1)$$

where t_1 is the time of the first measurement at the start, t_2 the time of the final measurement at the end of the test, N_2 the number of cells per mL at time t_2 and N_1 the number of cells per mL at t_1. Changes in mean cell density (cells mL^{-1}) were determined in duplicates. The inhibition of the growth rate was determined in relation to the control (culture without herbicide).

Values for the mean growth inhibition of each test substance concentration and the controls were plotted against the concentration (in logarithmic scale) to establish a dose–response curve. The sensitivity of the algal culture in the test cultivation was verified by the addition of potassium dichromate (about 1.2 mg L^{-1}), which represents the reference standard EC50 for this biotest. The parameter EC50 expresses the mean effective concentration of herbicide that results in a 50% reduction in the growth rate relative to the control.

2.2. Biosensor assay

We used the Biosensor Toxicity Analyser (BTA), consisting of the screen-printed sensor Pt:Ag (type AC1.W2.RS), the microflow unit and a control box (BVT Technologies Ltd., Brno, Czech Republic), to detect herbicides in liquid samples. The control box connected to a computer using the Biolyser software provides us with a semiautomatic amperometric measurement as well as data acquisition and processing.

The biosensor consisted of the PSII complex (biosensing element) isolated from the thermophilic cyanobacterium *Synechococcus elongatus* f. *thermalis*, strain KOVROV 1972/8 (Koblizek et al., 1998; Setlikova et al., 1999; Maly et al., 2005). This preparation was immobilised on the surface of the platinum-working electrode located in the centre of the radially oriented three-electrode sensor using a glutaraldehyde–BSA–glycerol matrix. After 12 h polymerisation at 4–8 °C in the dark, the biosensor was inserted into the microflow unit specially designed for monitoring PSII activity (containing a red LED placed in front of the electrode slot). The unit was submerged into a glass-jacketed vessel in 6 mL of a buffered solution (0.6 mL of 150 mM MES buffer, pH 6.5, 1 M NaCl, 50 mM $MgCl_2$+4.5 mL H_2O) containing duroquinone (2×10^{-4} M final concentration; serving as an artificial electron acceptor). The solution is pumped through a 1 mm channel, passing by the working electrode (Loop 1; 10–100 μL min^{-1}), while the other, larger-diameter bypass channel provides mixing of the solution inside the vessel (Loop 2; 1–20 mL min^{-1}).

The measuring protocol consists of three main steps represented by a series of 4 light/dark cycles. In the first step, the response of the PSII-biosensor to the control (negative sample; solution without herbicide) is determined. In the second step, the measured sample (e.g. 6 mL of solution containing herbicide) is added. In the last step, the reference phytotoxicity standard is applied to determine the rate of inhibition in the sample. The test can be completed in about 1 h including washing and sample application (Fig. 1A). One light/dark cycle consists of a short light pulse (5 s), when the signal (measured as current in tens of nA) on the electrode would increase due to the reduction of the artificial electron-acceptor duroquinone by electrons transported through the PSII complex. The light pulse would be followed by a 70 s dark period, when the signal would decrease (due to reoxidation of duroquinone) close to its initial value; hence a peak to baseline difference value would be acquired. If the herbicide was added to the medium, a

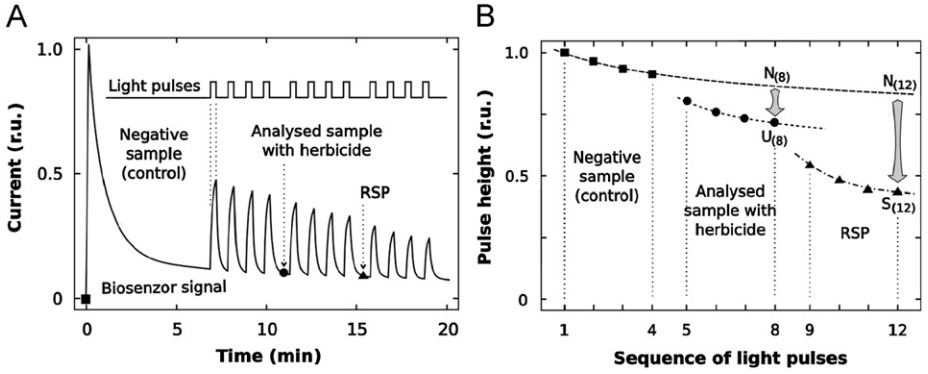

Fig. 1. *Panel A.* The measuring protocol consists of an equilibration period (about 300 s) and 3 series of light/dark (5/70 s) cycles when the negative sample (solution without herbicide), analysed sample (solution containing herbicide) and the reference phytotoxicity standard (RSP; see Fig. 2) are added subsequently (lower curve). The upper curve shows when the LED is switched on and off. The activity is calculated from the peaks' height (r.u.). *Panel B.* Diagram of a smoothed and normalised record for the calculation of the phytotoxicity index (PI). The extent of inhibition in the analysed sample is correlated to RSP.

decrease in signal peaks during the illumination period would be observed in a concentration-dependant manner due to the blocked electron transport between the PSII complex and duroquinone.

The resulting peak values of twelve consecutive measuring cycles were plotted against time. A power-function $y=a\times x^{-b}$ was found to be the most suitable to fit the signal decrease. The ratio between the 8th pulse and the predicted curve of the control (the arrow between the dashed and dotted curves in Fig. 1B) was taken for calculation of the inhibition index $I_U\,[\%]=(1-U_{(8)}/N_{(8)})\times 100$ of a certain herbicide concentration. The value U represents the height of pulse in an unknown sample containing herbicide and N is the value for the control (negative sample) at the same time on the dotted curve (Fig. 1B). In this way, concentration–response curves in the range from 10^{-10} to 10^{-4} M were established for various herbicides. The specific concentrations were 4.64×10^{-10}, 1.39×10^{-9}, 4.64×10^{-9}, 1.39×10^{-8}, 4.6×10^{-8}, 1.39×10^{-7}, 4.6×10^{-7}, 1.39×10^{-6}, 4.6×10^{-6}, 1.39×10^{-5} and 4.6×10^{-5} M for atrazine; 1.45×10^{-10}, 4.85×10^{-10}, 1.45×10^{-9}, 4.85×10^{-9}, 1.45×10^{-8}, 4.85×10^{-8}, 1.45×10^{-7}, 4.85×10^{-7}, 1.45×10^{-6}, 4.85×10^{-6}, 1.45×10^{-5} and 4.85×10^{-5} M for isoproturon and 1.29×10^{-10}, 4.29×10^{-10}, 1.29×10^{-9}, 4.29×10^{-9}, 1.29×10^{-8}, 4.29×10^{-8}, 1.29×10^{-7}, 4.29×10^{-7}, 1.29×10^{-6}, 4.29×10^{-6}, 1.29×10^{-5} and 4.29×10^{-5} M for diuron.

Finally, the reference standard of phytotoxicity RSP (see below) was used to verify the correct function of the biosensor (Fig. 1B). The inhibition index for RSP was calculated as $I_S\,[\%]=(1-S_{(12)}/N_{(12)})\times 100$ where S represents the height of the 12th pulse in the presence of RSP and N is the value for the control corresponding to S (Fig. 1B). Here, note that the calculated I_S value at this point corresponds to the response of the PSII biosensor to the unknown sample plus the RSP that was added subsequently.

2.3. Reference standard of phytotoxicity

When comparing inhibition rates for photosynthetic herbicides as measured with the PSII-biosensor in our experiments with the information from heterogeneous literature, we found it necessary to introduce some reference standard of phytotoxicity—a reference that can be used to define the activity of various photosynthetic herbicides. Another reason was to have a tool for comparing herbicide assays in aqueous samples (surface and ground water, soil extracts) using various bioassay methods. In the past, the term 'atrazine index' had been discussed to correlate some herbicide inhibition to a corresponding concentration of atrazine (Piletskaya et al., 1999). But this herbicide is not suitable as a standard since its use is restricted in many countries.

Looking for a compound whose solution of defined analytical concentration results in a reproducible inhibition of PSII photochemical activity, we have found diuron as the most appropriate compound. It has been widely used as a principal tool in photosynthesis research and its derivatives have been applied as commercial herbicides. This compound is stable in aqueous solutions under normal laboratory conditions (the half-time of hydrolysis at pH 5 is several years) with a relatively low toxicity to animals and humans. We determined a molar concentration of diuron that resulted in a 50% reduction in the PSII-biosensor activity relative to the control (solution without herbicide). This concentration, of about 1×10^{-7} M was taken as the reference standard of phytotoxicity (hereon as RSP) and was tested in our measurements with the PSII-biosensor (Fig. 2). This concentration conforms to the data published by other authors (Conrad et al., 1993; ElJay et al., 1997; Soukupova et al., 1999; Giardi et al., 2005).

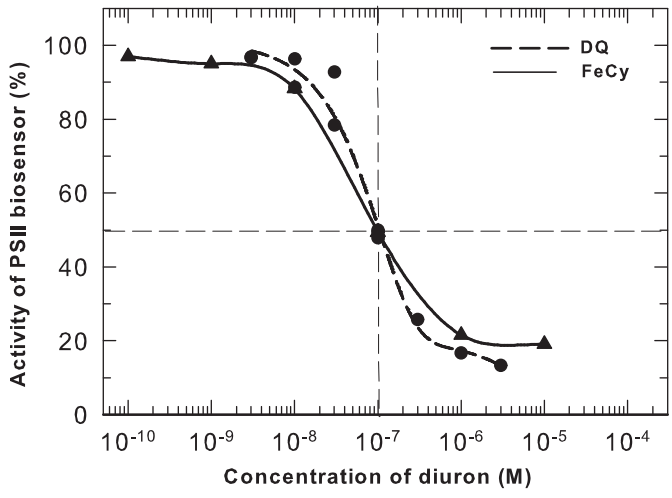

Fig. 2. Determination of the reference standard of phytotoxicity (RSP) from measurements with the PSII-biosensor. A molar concentration of diuron—1×10^{-7} M (~23.3 μg diuron per 1 litre of solution) that resulted in a 50% reduction in PSII-biosensor activity relative to the control (solution without herbicide) was defined as RSP (see dashed lines in the graph). Two electron-transport mediators—0.2 mM duroquinone (DQ; dashed curve with closed circles) and 1 mM potassium ferricyanide (FeCy; solid curve with triangles) were used in the experiments.

2.4. Chemicals and solutions

Stock solutions of herbicides were prepared from analytical standards (grade PESTANAL, Fluka/Riedl van Haen)—diuron no. 45463, isoproturon no. 36137 and atrazine no. 45330 dissolved in dimethylsulphoxide at a concentration of 1×10^{-2} M. Then, we prepared diluted solutions (substandards) of 1×10^{-4}, 1×10^{-6} and 1×10^{-8} M from the stock solutions using deionised water and precious Hamilton syringes. The concentrations were controlled using HPLC. Range of final concentrations was obtained by dilution of 100–1000 times concentrated amount of herbicides and was specific for the type of herbicide and bioassay (based on former results) with the aim to cover full range of response curves.

Duroquinone (Aldrich) was dissolved in ethanol at concentration 1.64 g L^{-1} (10^{-2} M) and potassium ferricyanide (Aldrich) was dissolved in deionised water. The measuring buffer consisted of 150 mM MES (pH 6.5), 1 M NaCl and 50 mM $MgCl_2$ and was diluted 10 times for assay.

3. Results

We used two methods of herbicide assay—the standard algal growth inhibition test and the PSII-based biosensor—to detect herbicides in liquid samples. Dose–response curves (concentration vs. inhibition activity) were compared for both methods and three herbicides—atrazine (triazine-type), isoproturon and diuron (urea-type).

3.1. Dose–response curves determined by algal growth inhibition test (biotest)

The algal cultures were exposed to 6 doses of tested herbicides in the range of a 10–90% inhibition ranging between 10^{-8} and 10^{-5} M, as well as the control. The dose–response curves for herbicides were established as the correlation between the logarithm of herbicide concentration and the inhibition of the growth rate (measured as cell number change). The patterns of dose–response curves for atrazine, isoproturon and diuron were similar—showing sigmoidal trends in a narrow inhibition range within one order of concentration between 10^{-7} and 10^{-6} M (Fig. 3). The algal cultures were more sensitive to diuron as compared to atrazine and isoproturon.

3.2. Dose–response curves determined by PSII-biosensor

Compared to the dose–response curves in the biotest experiments, the curves measured with the PSII-biosensor were not as steep, spanning 4–5 orders of concentration in the range of a 10–90% inhibition (Fig. 3). In all the cases, the PSII-biosensor assay had much higher sensitivity to low concentrations of herbicides than did the biotest. The inhibition curves measured with the PSII-biosensor showed a higher sensitivity to atrazine and diuron compared to isoproturon (Fig. 3).

In each PSII-biosensor measurement, RSP was added subsequently to the sample with herbicide (see Fig. 3C). This step allows an estimation of the extent of inhibition in the samples containing herbicides and verifies the activity of the PSII-biosensor. At low herbicide concentrations, the addition of RSP caused an inhibition of the PSII-biosensor activity, which corresponded to EC50. When the concentration of herbicides in the sample increased (close to EC50 and above), the effect of the RSP addition was small since it did not significantly change the total inhibition value. This range was between 10^{-7} and 10^{-6} M depending on the herbicide used. In practise, when any sample

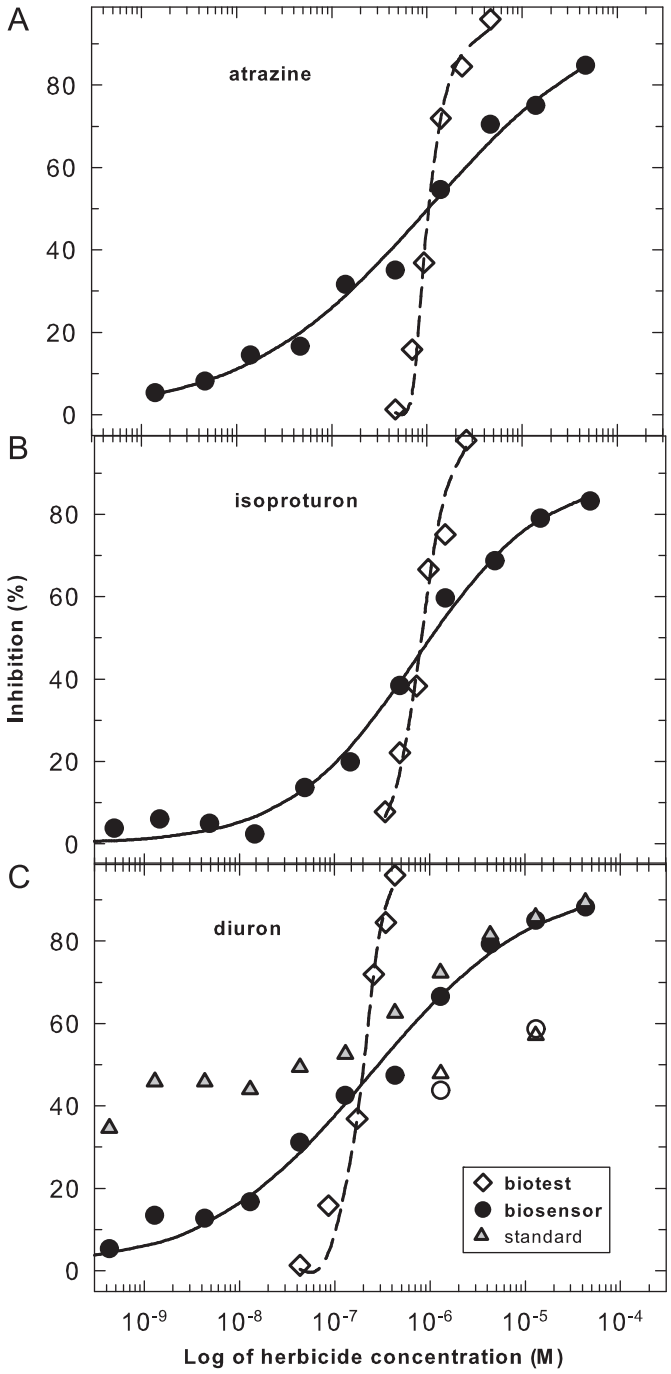

Fig. 3. Dose–response curves (concentration vs. inhibition rate) for the herbicides atrazine (panel A), isoproturon (panel B) and diuron (panel C) measured by the biosensor toxicity analyser (close circles) in comparison with the standard algal growth inhibition test (EN ISO 8692:2004) (open diamonds). RSP was added subsequently (grey triangles). EC50 and LOD values were calculated from the inhibition curves for both the methods.

contains a high concentration of a pollutant, exceeding EC50, it would be necessary to dilute it several times, to outweigh its effect against RSP.

If some biosensors were not functioning properly, giving misleading values, it was possible to recognise this from the response to RSP. For example, the two samples showing the inhibition rate between 45% and 60% (in Fig. 3C, open circles vs. open triangles) were suspicious since the addition of RSP had such a small effect. This situation might have occurred if the inhibition by the sample had been higher ($>80\%$) due to high diuron concentration (between 10^{-6} M and 10^{-5} M)—and then the addition of RSP would not have significantly changed total inhibition rate.

3.3. Determination of EC50 and LOD

The dose–response curves were analysed by fitting the experimental points to a logistic dose–response relationship for inhibition of PSII activity or growth rate (Streibig, 1988)

$$I_X = C + \frac{D-C}{1+\exp\{b[\log(X)-\log(EC50)]\}} \quad (2)$$

The inhibition (I_X) to a dose (X) is restricted between the lower and upper asymptote, D and C. EC50 denotes the concentration required for 50% inhibition, halfway between C and D and b is the slope of the curve close to EC50.

The limit of detection (LOD) of the assay was calculated from the response and its standard deviation at zero concentration (Rodbard, 1978)

$$Y_{min} = Y_0 - t \times S\sqrt{\frac{1}{n_0}+\frac{1}{n_1}} \quad (3)$$

Y_0 denotes the mean of the control, t is the percentage from Students t-distribution at the chosen fractal and the appropriate number of degrees of freedom (n_1-2), S the standard deviation of the control and n_0 and n_1 are the number of replicates for Y_0 and the unknown sample, respectively. The concentration values were calculated in moles of herbicide per litre (mol L^{-1} = M), or μg L^{-1}.

The EC50 values found in both assays – PSII biosensor and biotest – ranged between 7.2–8.2 $\times 10^{-7}$ and 2.0–2.3 $\times 10^{-7}$ M for isoproturon and diuron, respectively (Table 1). EC50 for atrazine determined by the PSII biosensor (8.9×10^{-7} M) was higher than that for the biotest (2×10^{-7} M), but still of the same order. These results show that the EC50 values determined by the two methods are rather similar.

On the other hand, the LOD values calculated from the dose–response curves in the PSII-biosensor assay showed much higher sensitivity – of about 1–2 orders of magnitude lower – as compared with the biotest (Table 2). The lowest LOD value was found by the PSII-biosensor assay for diuron (1.0×10^{-9} M), which is 2 orders lower than the value found by the biotest (1.1×10^{-7} M). The values of LOD calculated for isoproturon and atrazine had similar relations—the limit of detection was nearly 2 orders lower in magnitude for the PSII-biosensor as compared with the biotest.

3.4. Phytotoxicity index

If an unknown sample is used, the extent of inhibition can be correlated to RSP according to the following formula, where PI is the phytotoxicity index, I_U is the inhibition caused by an unknown

Table 1
Values of effective concentration causing 50% inhibition (EC50) for the herbicides—diuron, isoproturon and atrazine—calculated from experiments with the PSII-biosensor and algal growth inhibition test.

EC50	Biosensor		Biotest	
	[μg L^{-1}]	[M]	[μg L^{-1}]	[M]
Atrazin	192	8.9×10^{-7}	41	2.0×10^{-7}
Isoproturon	149	7.2×10^{-7}	169	8.2×10^{-7}
Diuron	54	2.3×10^{-7}	46.3	2.0×10^{-7}

Table 2
Values of the limit of detection (LOD) for the herbicides—diuron, isoproturon and atrazine—calculated from experiments with the PSII-biosensor and algal growth inhibition test. The confidential limit for LOD determination is shown in brackets.

LOD	Biosensor		Biotest	
	[$\mu g\ L^{-1}$]	[M]	[$\mu g\ L^{-1}$]	[M]
Atrazine	0.34 (0.14)	1.6×10^{-9} (6.4×10^{-10})	21.5 (18.7)	1.0×10^{-7} (8.7×10^{-8})
Isoproturon	2.04 (1.13)	9.9×10^{-9} (5.5×10^{-9})	81.7 (69.1)	4.0×10^{-7} (3.4×10^{-7})
Diuron	0.24 (0.11)	1.0×10^{-9} (4.6×10^{-10})	25.0 (21.9)	1.1×10^{-7} (9.4×10^{-8})

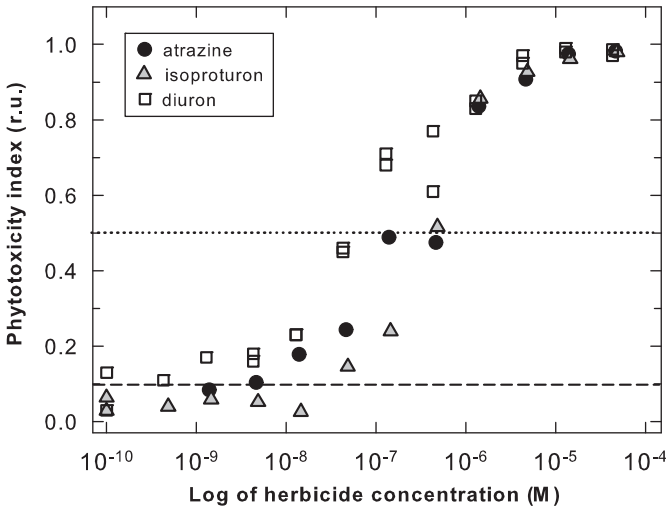

Fig. 4. Correlation between the phytotoxicity index (PI) and concentrations of the herbicides atrazine, isoproturon and diuron calculated from the PSII-biosensor assays. PI expresses the degree of inhibition by herbicide concentration calculated according to the formula (4). The values close to or above 0.1 (the dashed line), which corresponds to a 10% inhibition of PSII-biosensor activity, are considered to be phytotoxic. The dotted line indicates the phytotoxicity values that indicate 50% inhibition, corresponding to the relative phytotoxicity standard (RSP).

sample and I_S is the inhibition after addition of the RSP.

$$\mathrm{PI} = \frac{I_U}{2\times(I_S - I_U) + I_U} \tag{4}$$

PI expresses the degree of inhibition by a certain herbicide concentration. From the PSII-biosensor assays the correlation between the PI value and concentration of herbicides was calculated (Fig. 4). The highest phytotoxicity values were found for diuron, followed by atrazine and isoproturon (Fig. 4). Values above 0.1 (dashed line in Fig. 4), which corresponds to more than 10% inhibition of the PSII-biosensor activity, might be considered as phytotoxic. If the PI value is close to 0.5 or above ($>50\%$ inhibition), corresponding to the RSP, the dilution of samples is recommended to increase the precision of measurements.

4. Discussion

Here, we compared the results of the biotest (growth inhibition) with measurements of photosynthetic electron transport inhibition using the PSII biosensor in order to identify the advantages/disadvantages of both the methods. An explanation for the differently shaped dose–response curves (Fig. 3), as measured by the biotest and PSII-biosensor, probably lies in the use of different biorecognition elements—an intact algal cell as opposed to a macromolecular chlorophyll–protein complex. In the intact cell, which contains the complete plant metabolism, the inhibition effect of low concentrations of herbicides may be masked and diminished by various mechanisms.

One explanation of low biotest sensitivity gives an acclimation to sub-lethal herbicide concentrations during the test although the acute inhibition may be substantial, which is in agreement with some reports for algae (ElJay et al., 1997) as well as for diatoms (Soukupova et al., 1999). In the latter, using *Phaeodactylum* in standard biotests (ISO 10253, 1995), the growth rate was shown to be unaffected by up to 5.6×10^{-8} M diuron (LOD); it was reduced to 50% (EC50) by the addition of 2×10^{-7} M diuron. As compared to the growth rate, the photosynthetic oxygen evolution was decreased to one half in the presence of 6×10^{-8} M diuron, which conforms to the data presented in this work. The mechanisms causing the biotest's low sensitivity probably include the processes of adaptation to sub-lethal herbicide concentrations (Soukupova et al., 1999). The cells grown in sub-lethal diuron concentrations (up to 3×10^{-7} M) can compensate the partial inhibition of the photosynthetic capacity by an increased photosynthetic antenna size that brings more excitation to the remaining functional PSII reaction centres. A similar process of increasing phycocyanin antennae was shown in *Anacystis* sp. in the presence of sub-lethal concentrations of diuron (Koenig, 1990). The sensitivity of the algal growth inhibition tests might be increased when the adaptation processes are limited by making the procedure shorter.

In general, the detection limit of the PSII-biosensor is given by the binding constant of the herbicide, which in turn is determined by its chemical structure and by the architecture of the Q_B pocket (Trebst and Draber, 1986; Sobolev and Edelman, 1995). In the case of the PSII-complex immobilised on a printed electrode of biosensor, photosynthetic herbicides can directly approach the binding site and inhibit electron transport through the complex. Such a set-up allows a high concentration of the PSII complexes to be placed in the microenvironment around the electrode, which in turn, results in a well-measurable signal. The efficacy of this approach has made it possible to use low amounts of PSII ($\sim10^{-11}$ mol) for the biosensor. In the PSII-biosensor, even low concentrations of photosynthetic herbicides are detected as a decrease in electron transport activity, which is crucial to achieve low detection limits. However, at very low herbicide concentrations, the number of molecules in the sample starts to be limiting (5 mL of 1 nM herbicide corresponds to 5×10^{-12} mol).

5. Conclusions

In this work we have introduced the phytotoxicity index PI as a measure of herbicide toxicity—correlating the effect of an unknown sample and the reference standard of phytotoxicity. For practical purposes, our measurements suggest that a value above 0.1 (10% inhibition) can be considered phytotoxic corresponding to the herbicide concentrations in the range between 10^{-9} and 10^{-8} M, which can be found in natural samples (Maly et al., 2005).

We concluded that: (i) the EC50 values determined in the algal growth inhibition test and by the PSII-biosensor were similar; (ii) in the PSII-biosensor assay the sensitivity (LOD) to

photosynthetic herbicides was 1- or 2-orders higher compared to the algal growth inhibition test; (iii) the response time of the PSII-biosensor is immediate and the whole test procedure can be completed in 1 h while the standard procedure of the algal growth inhibition test takes 72 h; (iv) the reference standard of phytotoxicity was introduced, which corresponds to the EC50 for diuron and represents a tool for toxicity assessment using the biosensor toxicity analyser.

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