Sensitivity of the algal biotest ISO 10253 to the photosystem 2 herbicides in seawater

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Abstract

The sensitivity of marine algal biotest ISO 10253 to the photosystem 2 (PS2) herbicide diuron (DCMU) was determined. Using the diatom *Phaeodactylum tricornutum*, we found that the algal growth rate was reduced to 50 % of the control value (EC_{50}) for *ca.* 200 nM DCMU. This value is too high to allow a practical application of the biotest for concentrations of the PS2 herbicides found in natural waters. The mechanisms causing the low sensitivity of the biotest to the PS2 herbicide were investigated by measuring parameters of photosynthetic apparatus in the diatom prior and during the biotest. The apparent dissociation constant for DCMU in *P. tricornutum* found by measurements of inhibition of oxygen evolution and of variable fluorescence was in the range 60-90 nM. This should lead to a much higher sensitivity of the biotest than found in our experiments. The low biotest sensitivity is caused by an acclimation to sub-lethal DCMU concentrations. The acclimation is manifested by the chlorophyll content per cell that is increasing with the DCMU concentration. During a prolonged exposure to sub-lethal herbicide concentrations, we observed also a selection of DCMU resistant organisms indicating that also an adaptation may decrease the test sensitivity. The biotest sensitivity may

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Abbreviations: Chl - total chlorophyll ($a+c_1+c_2$); DCMU - 3-[3,4-dichlorophenyl]-1,1-dimethylurea; EC_{50} - effective concentration of the substance resulting in a 50 % reduction in the growth rate; K_{app} - apparent dissociation constant; PS - photosystem; Q_A and Q_B - the primary (A) and the secondary (B) quinone acceptors of photosystem 2; RC - photosystem 2 reaction centre.
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increase when the acclimation and adaptation are limited by shortening of the experiment duration.

Additional key words: bioassay; chlorophyll fluorescence; DCMU; 3-[3,4-dichlorophenyl]-1,1-dimethylurea; diuron; *Phaeodactylum tricurnutum*; quinone acceptors.

Introduction

Herbicides acting on PS2 are widely used for an inexpensive elimination of weeds in agriculture. Some of the PS2 herbicides, most notably 3-(3,4-dichlorophenyl)-1,1-dimethylurea, have been also extensively used as a principal tool of photosynthesis research (see Duysens and Sweers 1963 and Izawa 1977 for reviews) and the molecular mechanism of their action on plants is known. The herbicide molecule binds in the Q_B-binding pocket of the D1-protein of PS2 where otherwise plastoquinone molecules accept electrons from the primary quinone acceptor Q_A (*e.g.*, Duysens and Sweers 1963, Trebst and Draber 1986, Draber *et al.* 1991, Krause and Weis 1991). Thus, the herbicide binding blocks the electron transport from the PS2 to the cytochrome *b/f* complex and prevents the photosynthetic energy conversion.

A fraction of the herbicides applied in agriculture is washed into natural waters and represents a serious pollution problem. Herbicides can be toxic to human and animal health (Stevens and Sumner 1991) and application of some of the herbicides has been restricted by law in the most developed countries (Ware 1986). Yet, the peak concentrations of some restricted-use PS2 herbicides (*e.g.*, atrazine) that are found in surface waters may reach tens or even hundreds in nanomolar concentrations (Huber 1993, Bintein and Devillers 1996, Solomon *et al.* 1996). Typical values are much lower and sensitive gas and high-pressure liquid chromatography have to be applied on samples pre-concentrated either by extraction or in cartridges (Bardalaye and Wheeler 1986, Wells *et al.* 1994, Lacorte *et al.* 1998).

The sensitivity of the algal growth inhibition biotests is not sufficient to detect these levels of the herbicide pollution (El Jay *et al.* 1997) although the corresponding acute inhibition of the photosynthetic reactions may be substantial. Here, we investigate and compare the results of the biotest with measurements of the acute inhibition of photosynthesis in order to identify mechanisms that limit the biotest sensitivity.

Materials and methods

Marine algal growth inhibition test ISO 10253: Sterile artificial sea water prepared according to ISO 10253 (100 cm^3 in 250 cm^3 glass Erlenmeyer flasks) was poisoned with DCMU (*Riedel-de Haën*, Seelze, Germany) and inoculated by *Phaeodactylum tricornutum* Bohlin strain Cough (*CCMP, Bigelow Laboratory for Ocean Sciences*, W. Boothbay Harbor, Maine 04575, USA) to the final concentration of 10 000 cells per cm^3. Two flasks were used for each herbicide concentration. The herbicide was

added in ethanol solution so that the final solvent concentration during the biotest was always 0.01 %. The final concentrations of the herbicide were 0, 0.1, 1, 3, 10, 18, 32, 56, 100, 300, and 1000 nM. The flasks were placed on a thermostated stainless-steel block (20±1 °C, 120×60 cm) in front of two rows of fluorescence tubes (*Tungsram F33*, 36 W, Hungary) that provided incident 100 μmol(photon) m^{-2} s^{-1} of photosynthetically active radiation (measured by *Li-189*; *LiCor*, Lincoln, NE, USA). The growth was measured by counting the cells in 17 % Lugol solution under microscope in a Bürker cell. In parallel, the growth was also monitored by measuring the absorbance (scattering) of the suspension at 750 nm in a 5 mm cuvette (*Spekol EK1, Carl Zeiss,* Jena, Germany).

Photosynthetic activity: The oxygen evolution rate was measured by a Clark electrode (*YSI 5331*, Yellow Springs, OH, USA) in a thermostated cuvette (*Gilson Medical*, Middleton, WI, USA). The irradiance from 26 red light-emitting diodes (*Hewlett Packard, HLMP 8103*) was adjusted to drive irradiance-limited photosynthesis [*ca.* 100 μmol(photon) m^{-2} s^{-1}]. The polarizing voltage and signal digitalization and processing was provided by the *OxyCorder* (*P.S. Instruments*, Brno, Czech Republic).

The fluorescence decay kinetics reflecting the reoxidation of the primary quinone acceptor Q_A^- after a single turnover saturating flash was measured using the dual-modulation kinetic fluorometer *FL-100* (*P.S. Instruments*, Brno, Czech Republic; Trtílek *et al.* 1997).

Chlorophyll (Chl) was extracted from the suspension by 90 % acetone. The absorbance of the extract was measured at 720, 664, and 630 nm (A_{720}, A_{664}, A_{630}). The Chl concentration [μM] was calculated from the absorbance using the following equations (modified from Humphrey 1979):

$$\text{Chl } a = 1000[11.47(A_{664} - A_{720}) - 0.40(A_{630} - A_{720})]/893$$
$$\text{Chl } c_1+c_2 = 1000[24.36(A_{630} - A_{720}) - 3.73(A_{664} - A_{720})]/610$$

Results

The growth rate measured by cell numbers and by absorbance at 750 nm was inhibited by 50 % (EC_{50}) at *ca.* 200 nM of DCMU (Fig. 1*A*). The growth measured by cell numbers was not affected (more than by 5 %) by the presence of up to the 56 nM concentration of DCMU.

The mechanisms responsible for the low sensitivity of the biotest were investigated by a titration of photosynthetic activity by the herbicide (Fig. 1*B*). The CO_2-dependent oxygen evolution rate measured in the limiting irradiance of 100 μmol(photon) m^{-2} s^{-1} was inhibited to 50 % at *ca.* 60 nM DCMU.

In parallel to the measurements of oxygen evolution, the kinetics of the reoxidation of the primary quinone acceptor of PS2 (Q_A^-) was followed by fluorescence decay kinetics after a single-turnover saturating flash (20 μs) in samples containing different herbicide concentrations (Fig. 2). The fluorescence always started at a low level corresponding to the oxidized Q_A acceptor. The emission

increased stepwise upon the flash as the Q_A acceptors in all PS2 reaction centres were reduced by the primary photochemistry. The subsequent fluorescence decay approximated the kinetics of the Q_A^- reoxidation. The reoxidation can be resolved into two or three exponential components. The components with the characteristic decay times shorter than 100 ms reflected the reoxidation in reaction centres free of DCMU. The slowest phase of the decay reflected presence of the centres in which the rapid reoxidation was blocked by the herbicide. The relative amplitude of this component was approximately proportional to the percentage of the blocked centres. The amplitude of the slow component was also plotted against the DCMU concentration (Fig. 1*B*). The fluorescence measurements yielded a similar titration curve as obtained in the oxygen evolution experiment. The slightly lower sensitivity of the fluorescence experiment may be explained by the interference of the reaction centre connectivity (Lavergne and Briantais 1996).

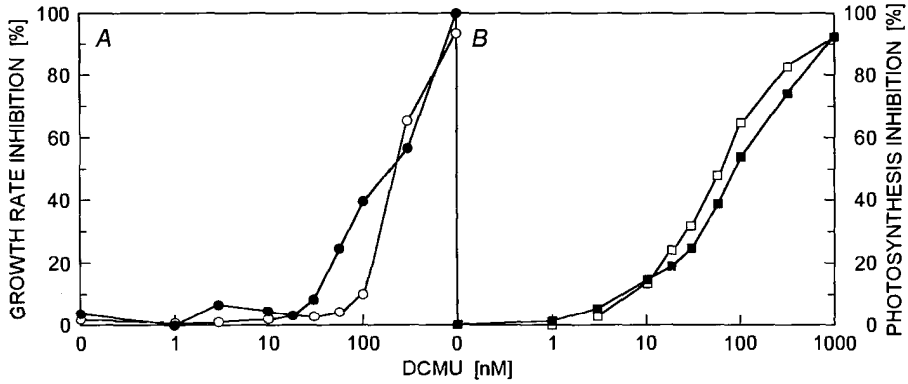

Fig. 1. Inhibition of the growth rate (*A*) and of the photosynthetic capacity (*B*) in *Phaeodactylum tricornutum* by DCMU. The growth rate (*A*) was measured as defined in ISO 10253 norm using the apparent absorbance at 750 nm (●), and cell counts (○). The photosynthetic capacity (*B*) was assessed by measurement of the oxygen evolution in subsaturating irradiance of 100 μM m^{-2} s^{-1} (○) and the amplitude of the slow component of the fluorescence decay following a single-turnover, saturating flash (■). The dependence of the photosynthesis capacity and the herbicide concentration was fitted by the least-square procedure using the equation presented in the Results. The best fit was obtained for [Chl]/[RC] ≈ 250 and K_{app} ≈ 60 nM for the oxygen data and K_{app} ≈ 90 nM for the fluorescence values.

The experimental curves in Fig. 1*B* were fitted by a theoretical curve represented by the following equation

$$I = 100\frac{([RC]+[DCMU]+K_{app})-\sqrt{([RC]+[DCMU]+K_{app})^2-4[RC][DCMU]}}{2[RC]}$$

where [RC] is total concentration of PS2 reaction centres, [DCMU] is total concentration of the herbicide, and K_{app} is the apparent dissociation constant that

characterizes the ability of the herbicide to occupy the thylakoid membrane and the ability to bind to PS2. The higher K_{app}, the less potent is the herbicide. The best fits were obtained for [RC] ≈ 12 nM in the culture of *P. tricornutum* with the concentration of Chl = 3 μM, for K_{app} ≈ 60 nM for the oxygen values, and for K_{app} ≈ 90 nM for the fluorescence values. The K_{app} was much lower than EC_{50} found in the growth inhibition bioassay (≈200 nM). The two values are expected to be identical at least in the initial phase of the biotest. The discrepancy must be caused by processes that take place in the later phase of the biotest.

In a search for acclimation signs, we measured Chl concentration in the biotest algal suspensions containing different herbicide concentrations. The total Chl concentration after 9 d of the growth under the biotest conditions was only slightly variable [0.65–0.95 μM(Chl)] in the suspensions containing less than 18 nM DCMU (Fig. 3). In this range, no inhibition of growth (Fig. 1*A*) and less than 20 % inhibition of photosynthesis (Fig. 1*B*) were found. The total Chl concentration in the suspension was increasing with increasing herbicide content and reached a peak around 56 nM DCMU. The subsequent decline in total Chl was caused by lesser growth in the high DCMU suspensions. The acclimation to the sub-lethal DCMU concentrations is demonstrated further by the Chl content per cell shown also in Fig. 3. The Chl content of the cells monotonously increased up to the DCMU concentrations that substantially inhibit the growth (300 nM). The cells grown in sub-lethal DCMU concentrations can compensate the partial inhibition of the photosynthetic capacity by an increased photosynthetic antenna that can bring more excitation to the remaining functional reaction centres of PS2. This acclimation process lowers the sensitivity of the algal growth to the sub-lethal concentrations of the herbicide applied in light-limited conditions of the biotest. Koenig (1990) showed a similar process of increasing phycocyanin antennae in *Anacystis* sp. in the presence of sub-lethal concentration of DCMU.

Adaptation processes may be another factor lowering the biotest sensitivity. Indeed, the suspensions exposed to a selection pressure of high herbicide concentration frequently started, after a significant lag phase, a slow growth. In order to test if the growth was reflecting an adaptation process, the capacity of the original wild-type algae to bind DCMU was compared with the algae that grew previously for

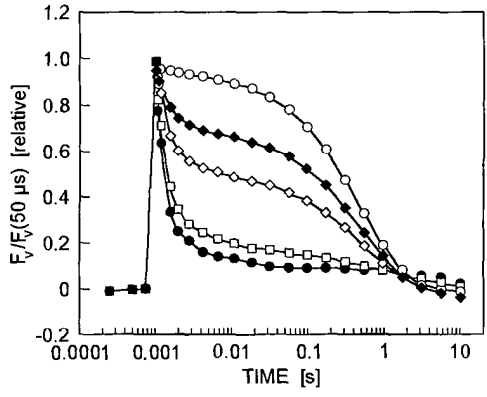

Fig. 2. Fluorescence emission transient elicited in *Phaeodactylum tricornutum* (1 μM Chl) by a single-turnover, saturating flash (20 μs, 620 nm). Variable fluorescence, F_v, is normalized to its value measured 50 μs after the flash trigger, F_v(50 μs). The algae were poisoned by DCMU at 0 nM (●), 1 nM (□), 100 nM (◊), 300 nM (♦), and 3000 nM (○).

30 d with 300 nM DCMU. Both suspensions were harvested, resuspended for 30 min in new artificial seawater with 300 nM DCMU, and washed free of DCMU by three subsequent centrifugation/resuspension cycles. Finally, both algal samples were poisoned by 100 nM DCMU (close to K_{app}) and the fraction of the blocked reaction

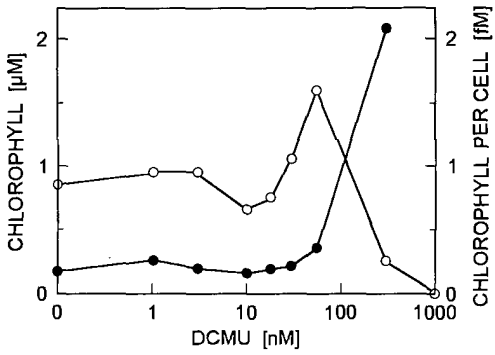

Fig. 3. The total chlorophyll (Chl) concentration (○) and Chl concentration per cell (●) of the *Phaeodactylum tricornutum* suspension grown for 9 d in the presence of DCMU. The algae were grown as defined in the ISO 10253 standard.

centres was assayed by the Q_A-reoxidation kinetics (as in Fig. 2). Fig. 4 shows Q_A^- reoxidation kinetics in the original inoculum and in algae that were exposed for 30 d to 300 nM DCMU. The algae grown under the selection pressure of the herbicide were inhibited by 100 nM DCMU much less than the wild-type strain as shown by the lower amplitude of the slow component of fluorescence decay in the curve compared to the wild-type curve.

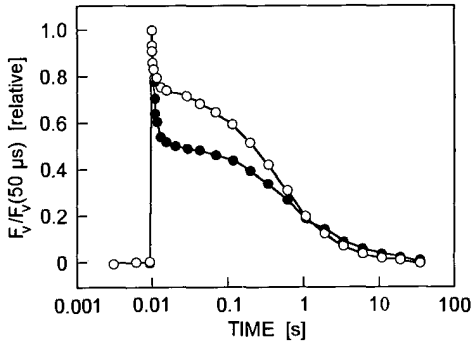

Fig. 4. The fluorescence transient, F_v/F_v(50 μs), elicited in algae poisoned by 100 nM DCMU. The algae were either of wild-type that was previously not exposed to the herbicide (○) or algae that were kept for 30 d under the selection pressure of 300 nM DCMU (●).

Discussion

We show here that the processes of acclimation and adaptation may decrease the sensitivity of the biotest. The photosynthetic capacity of algae was decreased by as much as 30 % in 30 nM DCMU while no growth inhibition was detected in the ISO 10253 test. We propose that a shortening of the biotest duration may increase the biotest sensitivity. New methods allowing quantitation of the algal growth in very diluted suspensions are required to shorten the biotest. The presently dominant methods, cell counts and measurements of absorbance at 750 nm, are not sensitive

enough for this purpose. We propose that fluorescence may be used for the quantitation of the growth in algal biotest suspensions as a sensitive alternative (Lorenzen 1966). Moreover, as shown in Fig. 1*B*, fluorescence may serve to diagnose an acute inhibition of PS2 (see also Arsalane *et al.* 1993, Conrad *et al.* 1993, Ruth 1994, El Jay *et al.* 1997). The dual-modulation fluorometer used in this work is able to measure Q_A^- reoxidation or fluorescence induction in algal suspension starting from Chl concentrations as low as 130 pM (Dijkman *et al.* 1999). This concentration corresponds to the cell density of *ca.* 50 000-70 000 *P. tricornutum* cells per cm^3. This technique is expected to allow significant shortening of the biotest duration.

In general, the low algal density in the biotest may also result in an increase of the biotest sensitivity by increasing the pollutant/algal biomass ratio. However, it is not the case here with the herbicide DCMU. By titrating the oxygen evolution rate, we found (not shown) that in our experimental organism *P. tricornutum*, the concentration of the reaction centres [RC] may be approximately calculated from the Chl concentration by using the ratio [Chl]/[RC] $\approx$ 250. Under the biotest conditions, the starting Chl concentration was *ca.* 1.5 nM Chl while the maximal Chl concentration in the stationary phase was *ca.* 1.0 μM Chl. Thus, the corresponding range of concentrations of reaction centres was *ca.* 6 pM–4 nM. This value was much lower than the apparent dissociation constant found here ($K_{app} \approx$ 60-90 nM) and the inhibition approximated by the above equation depends on the algal density only in a very limited way. Nevertheless, a shortening of the biotest duration remains the proper way to increase its sensitivity and fluorescence.

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