Bull Environ Contam Toxicol (2009) 83:484–487
DOI 10.1007/s00128-009-9826-1

Utility of Delayed Fluorescence as Endpoint for Rapid Estimation of Effect Concentration on the Green Alga *Pseudokirchneriella subcapitata*

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Received: 24 December 2008 / Accepted: 8 July 2009 / Published online: 23 July 2009

Abstract Algal growth inhibition tests for environmental risk assessment require improved efficiency to evaluate large numbers of chemicals. As an endpoint for rapid estimation of the effect concentration of test chemicals, we propose the delayed fluorescence (DF) measurement from an alga 24 h after exposure. Eight chemicals (bifenox, bromoxynil, bensulfuronmethyl, diuron, diflufenican, thiobencarb, *m*-chlorophenylhydrazone and 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone) were tested. The EC_{50} values from the 24 h DF measurement were similar to those from the conventional 72 h growth test for seven tested chemicals excepting thiobencarb. We conclude that 24 h DF measurement is a possible endpoint for rapid estimation of the EC_{50} values obtained in the 72 h growth test for those seven chemicals.

Keywords Delayed fluorescence (delayed luminescence) · Algal growth inhibition test

The algal growth inhibition test is used for toxicity assessment of chemicals on primary producers in aquatic ecosystems (OECD 1984). The test involves estimating the effect concentration (e.g., EC_{50}) based on 72-h growth measurement after exposure to a test chemical. The growth inhibition test, however, is complex and slow, thus limiting throughput when evaluating a large number of chemicals. To improve the efficiency in evaluating the toxicity of a large number of chemicals, it would be helpful to carry out a rapid screening test before the conventional 72 h growth inhibition test.

Recently, delayed fluorescence (DF) techniques have been proposed to achieve rapid bioassay for screening chemical toxicity on algae and plants. DF, also termed delayed luminescence, is photo-stimulated ultra-weak luminescence observed in a wide range of living systems and macromolecules, including algae and plants. DF from algae and plants can be detected for up to a few minutes after light excitation in the dark (Strehler and Arnold 1951). DF is thought to originate from emissions from chlorophyll in a photosystem reaction center chemically excited by charge recombination in a back reaction in the photosynthetic electron transport chain (Jursinic 1986; Schmidt and Senger 1987; Katsumata et al. 2008). Moreover, it has been suggested that DF is also related to trapped electrons in macromolecules in the photosystem, cytoskeleton (Scordino et al. 2000). DF has the potential to serve as an intrinsic probe of the photosynthetic activity and structural state of algae, and its utility in evaluating the effects of toxic chemicals on algae has been reported (Bürger and Schmidt 1988; Scordino et al. 1996; Katsumata et al. 2006; Zrimec et al. 2007). It has been suggested that short-term inhibition of DF emission (i.e., 15 min or 1 h) after exposure to a toxic chemical is correlated with long-term growth inhibition (i.e., 72 h) in green algae (Katsumata et al. 2006; Zrimec et al. 2007) and duckweed (Drinovec et al. 2004).

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In this report, we compare the EC_{50} values of eight test chemicals, obtained from the conventional 72 h growth inhibition test and DF intensity measurement after 24 h incubation, and we discuss the possibility of rapidly estimating the EC_{50} values obtained in the conventional 72 h test by using DF measurement in 24 h.

Materials and Methods

The green alga *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*), strain ATCC22662, was used for all tests. The alga was cultured in a GLP laboratory (JCL Bioassay Corporation, Japan). The alga was incubated with OECD medium: H_3BO_3, 0.185 mg/L; $MnCl_2{\cdot}4H_2O$, 0.415 mg/L; $ZnCl_2$, 0.003 mg/L; $FeCl_3{\cdot}6H_2O$, 0.08 mg/L; $Na_2EDTA{\cdot}2H_2O$, 0.1 mg/L; $CoCl_2{\cdot}6H_2O$, 0.0015 mg/L; $Na_2MoO_4{\cdot}2H_2O$, 0.007 mg/L; $CuCl_2{\cdot}2H_2O$, 0.00001 mg/L; $CaCl_2{\cdot}2H_2O$, 18 mg/L; NH_4Cl, 15 mg/L; KH_2PO_4, 1.6 mg/L; $NaHCO_3$, 50 mg/L; $MgCl_2{\cdot}6H_2O$, 12 mg/L; and $MgSO_4{\cdot}7H_2O$, 15 mg/L.

Eight chemicals were tested, including six typical herbicides, bifenox (BFN), bromoxynil (BMN), bensulfuronmethyl (BSM), diuron (DCM), diflufenican (DFF), and thiobencarb (TBC), and two photosynthetic inhibitors, CCCP (uncoupler of membrane potential, *m*-chlorophenylhydrazone, CCC) and DBMIB (electron transport inhibitor at cytochrome b_6f complex, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone, DBM). All were obtained from Wako Pure Chemical Industries, Japan. The test chemicals were dissolved in the OECD medium at five different concentrations (Table 1). N,N-dimethylformamide (DMF) or water was used as a vehicle, where necessary. The DMF concentration in the final algal suspension was less than 100 mg/L, in accordance with the OECD Test Guideline 201 (TG201; OECD 1984).

Algal growth inhibition tests were carried out in accordance with TG201 in a GLP laboratory (JCL Bioassay Corporation, Japan). Algal suspensions, inoculated at 10^4 cells/mL in 100 mL of growth medium in an Erlenmeyer flask (300 mL capacity), were exposed to the final concentration of the test chemical ("exposed sample") or were not exposed ("control sample"). These samples were prepared in triplicate for each test concentration. The samples were incubated under continuous illumination from fluorescent lamps (4,000–5,000 lux, $\sim$60 μmol m^{-2} s^{-1}) at a temperature of 23 ± 2°C in an orbital shaking culture (100 rpm). Algal cell density was determined with a particle counter (CDA-500, Sysmex, Japan, detection range 3–12 μm) every 24 h in the 72 h growth test.

Table 1 Concentrations of the test chemicals

Test chemical	Vehicle	Test concentration (μg/L)				
Bifenox (BFN)	DMF	0.2	0.4	1	2.1	4.7
Bromoxynil (BMN)	DMF	100	330	1,100	3,640	12,000
Bensulfuronmethyl (BSM)	DMF	10	25	63	156	390
CCCP (CCC)	DMF	100	180	320	580	1,050
DBMIB (DBM)	DMF	500	900	1,600	2,900	5,200
Diuron (DCM)	Water	0.5	1.5	4.5	13.5	40.5
Diflufenican (DFF)	DMF	0.05	0.12	0.36	0.96	2.6
Thiobencarb (TBC)	DMF	5.1	12.3	29.5	70.8	170

After 24 h incubation, 3.0 mL of algal culture was dispensed in a cuvette (10 × 10 × 40 mm). The DF of the dispensed sample was measured with an ultra-weak luminescence detector system (PMX-310c-prototype, Hamamatsu Photonics, Japan). Figure 1 shows a schematic diagram of the system. The dispensed sample was left to stand in the dark for 60 s and was then illuminated for 1 s with red and near infrared light (50 μmol m^{-2} s^{-1}) from a light emitting diode (L735-04AU, Epitex, Japan) with the shutter closed. After the excitation light was turned off, the shutter was opened, and the DF intensity was detected at 0.1 s intervals from 1 to 60 s by a photomultiplier (PMT). The DF signals were converted to numerical data at a signal integration circuit and a personal computer. The relative DF intensity was expressed as a count. The DF intensities in the 1–60 s period were integrated to obtain the delayed fluorescence intensity (DFI).

Growth rate and 50% effect concentration (EC_{50}) were determined with probit analysis software (Ecotox-Statics Ver. 2.6, The Japanese Society of Environmental Toxicology, Japan). For calculation of the growth rate and EC_{50} from 0 to 72 h (72hRate), we used the cell densities at 0, 24, 48, and 72 h. For calculation of the growth rate and EC_{50} based on DFI from 0 to 24 h (24hDFI), we used the DFI at 0 and 24 h for the endpoint values. The results of

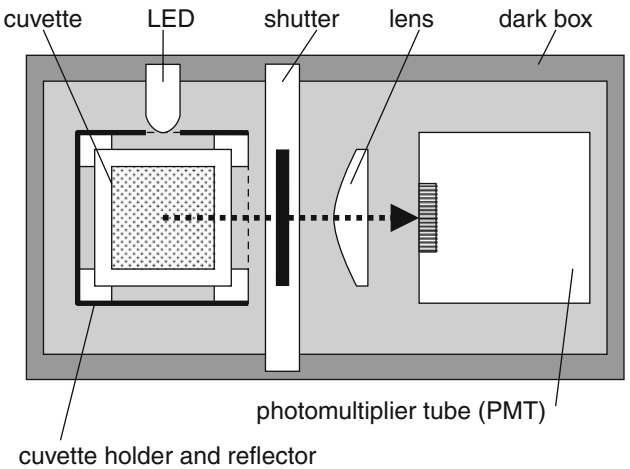

Fig. 1 Schematic diagram of the ultra-weak luminescence detector system

triplicate samples under each exposure condition were used for the calculation.

Results and Discussion

Figure 2 shows dose–response curves indicating inhibition of the exposed samples relative to the control sample at the two endpoints: algal growth in 72 h and DFI in 24 h (72hRate and 24hDFI). The dose–response curves of the two endpoints are mostly similar for the tested chemicals, except TBC. For TBC, inhibition for the 24hDFI endpoint was lower than for the 72hRate. EC_{50} values of individual test chemicals with 72hRate and 24hDFI were determined from the dose–response curves in Fig. 2. The EC_{50} value determined from the inhibition rate based on the 72hRate endpoint is termed $72hEC_{50}$. Similarly, the EC_{50} value calculated based on 24hDFI is $24hDF_{50}$. These values are shown in Table 2, which also shows the ratio of $24hDF_{50}$ to $72hEC_{50}$. The ratio of the calculated EC_{50} values of the two endpoints showed some variation in each test chemical. Four out of the eight chemicals showed very good toxicity estimation results: BFN (ratio, 1.0), DBM (1.0), DCM (0.8), and DFF (1.2). Two of the other four chemicals showed low ratios, BMN (0.5) and CCC (0.4), and one showed a high ratio, BSM (1.8). Nevertheless, these results may be acceptable for rough toxicity screening. In contrast, the ratio of TBC was very high (1676), meaning that it is difficult to use for estimating toxicity. As shown in the dose–response curves in Fig. 2, the inhibition values of

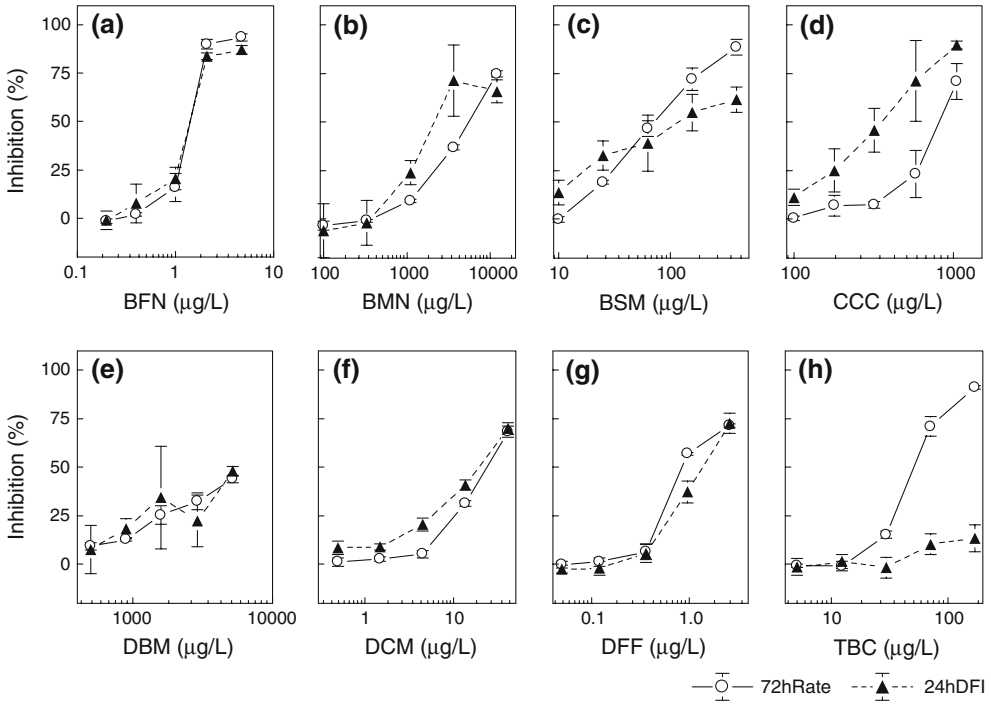

Fig. 2 Dose–response curves of inhibition of the exposed samples relative to the control sample at the two different endpoints (72hRate and 24hDFI)

Table 2 Comparison of EC_{50} values from different measurement methods

EC_{50} of test chemical (µg/L)					Ratio
	$72hEC_{50}$	(95% confidence limit)	$24hDF_{50}$	(95% confidence limit)	$24hDF_{50}/72hEC_{50}$
BFN	1.5	(1.4–1.6)	1.4	(1.3–1.6)	1.0
BMN	5,407	(4,711–6,273)	2,902	(2,251–3,653)	0.5
BSM	75	(65–85)	134	(105–179)	1.8
CCC	815	(745–906)	335	(308–365)	0.4
DBM	6,845	(5,216–10,146)	6,954	(5,169–10,824)	1.0
DCM	24	(21–27)	19	(15–24)	0.8
DFF	1.2	(1.0–1.3)	1.4	(1.3–1.6)	1.2
TBC	56	(51–61)	93,290	(1×10^{-5}–2×10^{7})	1,676

24hDFI of TBC were less than 25%. In this case, the EC_{50} values of the TBC-exposed samples were estimated by extrapolation. Moreover, the 95% confidence interval of $24hDF_{50}$ in TBC is extremely wide (1×10^{-5} to 2×10^{7}). The extremely high value of the ratio of TBC was probably caused by extrapolation error. The results suggest that, for seven of the tested chemicals, excepting TBC, the relation between 24hDFI ($24hDF_{50}$) and 72hRate ($72hEC_{50}$) may be sufficient to employ this approach in rough toxicity testing. Of course, to examine the efficacy of this method in evaluating real water toxicity, we need to test it with a wider variety of chemicals, including river or waste water, instead of the test chemicals used here.

We conclude that the DF intensity (DFI) in 24 h incubation is a possible endpoint for rapid estimation of the EC_{50} values obtained with the conventional 72 h growth inhibition test for seven of the tested chemicals. The technique is expected to be useful for toxicity screening of chemicals on algae.

Acknowledgments We are deeply grateful to Ayame Maihara and Sachiko Tanaka (JCL Bioassay Corporation, Japan) for their valuable work on the growth inhibition tests. This research was supported in part by the Environmental Technology Development Fund of the Ministry of the Environment of Japan.

References

Bürger J, Schmidt W (1988) Long-term delayed luminescence: a possible fast and convenient assay for nutrition deficiencies and environmental pollution damages in plants. Plant Soil 109:79–83

Drinovec L, Drobne D, Jerman I, Zrimec A (2004) Delayed fluorescence of *Lemna minor*: a biomarker of the effects of copper, cadmium, and zinc. Bull Environ Contam Toxicol 72:896–902

Jursinic PA (1986) Delayed fluorescence: current concepts and status. In: Govindjee, Amesz J, Fork DC (eds) Light emission by plants and bacteria. Academic Press, Orlando, pp 291–328

Katsumata M, Koike T, Nishikawa M, Kazumura K, Tsuchiya H (2006) Rapid ecotoxicological bioassay using delayed fluorescence in the green alga *Pseudokirchneriella subcapitata*. Water Res 40:3393–3400

Katsumata M, Takeuchi A, Kazumura K, Koike T (2008) New feature of delayed luminescence: preillumination-induced concavity and convexity in delayed luminescence decay curve in the green alga *Pseudokirchneriella subcapitata*. J Photochem Photobiol B 90:152–162

Organization for Economic Cooperation and Development (OECD) (1984) Guideline for testing of chemicals no. 201. Alga growth inhibition test, Paris

Schmidt W, Senger H (1987) Long-term delayed luminescence in *Scenedesmus obliquus* I. Spectral and kinetics properties. Biochim Biophys Acta 890:15–22

Scordino A, Triglia A, Musumeci F, Grasso F, Rajfur Z (1996) Influence of the presence of atrazine in water on the in vivo delayed luminescence of *Acetabularia acetabulum*. J Photochem Photobiol B 32:11–17

Scordino A, Triglia A, Musumeci F (2000) Analogous features of delayed luminescence from *Acetabularia acetabulum* and some solid state systems. J Photochem Photobiol B 56:181–186

Strehler BL, Arnold W (1951) Light production by green plants. J Gen Physiol 34:809–820

Zrimec MB, Drinovec L, Zrimec A, Tišler T (2007) Delayed fluorescence in algal inhibition tests. Cent Eur J Biol 2(2):169–181