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Delayed Fluorescence of *Lemna minor*: A Biomarker of the Effects of Copper, Cadmium, and Zinc

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Duckweeds (family Lemnaceae) are widely used for aquatic toxicity testing (Wang 1990). Growth inhibition tests using duckweed have become standard laboratory phytotoxicity test. Nevertheless, further short-term functional and biochemical assays are being developed to give additional support to understanding of the mechanisms of toxicity. Frequently, rapid chlorophyll fluorescence is studied for the assessment of metal toxicity (Prasad et al. 2001; Appenroth et al. 2001), herbicide exposure (Dewez et al. 2002; Hulsen et al. 2002), polycyclic aromatic hydrocarbon toxicity (Mallakin et al. 2002; Marwood et al. 2001), and their mixtures (Frankart et al. 2002; Babu et al. 2001). Changes in fluorescence due to chemical exposure are rapidly induced and correlate well with whole-organism effects.

In contrast to rapid fluorescence, which is light emission on the nanoseconds time-scale, delayed fluorescence (also termed delayed luminescence or delayed light emission) corresponds to light emission on the seconds time-scale – it is the phenomenon of long-lived light emission by plants after being illuminated with light and put into darkness (Strehler and Arnold 1951). The main source of delayed fluorescence is photosystem II (PSII) (Jursinic 1986). The intensity of delayed fluorescence was reported to be a measure of photosynthetic activity (Schneckenburger and Schmidt 1996). It was also shown that the damage of the thylakoid membrane causes reduction of delayed fluorescence intensity (Yun et al. 1997). Delayed fluorescence originates from a metastable state after charge separation in PSII and is therefore affected by many variables, to which rapid fluorescence is often, but not always, immune (Jursinic 1986).

The aim of this work was to assess delayed fluorescence as a biomarker of copper, cadmium and zinc effects in *L. minor*. Copper and zinc are essential elements, but become toxic at high concentrations, whereas cadmium is nonesential and toxic already at low concentrations. Copper binds to PSII and affects electron transport in photosynthesis, inhibits the Calvin cycle and protein synthesis in chloroplasts (Valvilin et al. 1995). Cadmium has many harmful effects–it inhibits the Calvin cycle and interacts with the water-splitting complex in PSII (Krupa 1999), to name some. The major effects of zinc in higher plants are on the activity of the enzyme RubisCo (Ciscato et al. 1999). To observe the acute effects of these three metals the relative change of delayed fluorescence intensity in 24 h of metal exposure was

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measured. We also compared delayed fluorescence and rapid fluorescence in metal exposed plants, and for copper with the growth inhibition test. We discuss delayed fluorescence as a complementary biomarker to rapid fluorescence measurements and to the growth inhibition test.

MATERIALS AND METHODS

Cultures of *L. minor* were grown from a single plant, collected at a local pond. The fronds were disinfected by immersing in NaOCl (0.01 M) for 30 sec followed by rinsing with distilled water. Stock cultures were maintained at 25 ± 0.2 °C in Petri dishes containing 40 ml of 20X-AAP mineral growth medium (ASTM 1992, pH 7.5) without the chelator $Na_2EDTA{\cdot}2H_20$ (Teisseire et al. 1998). Continuous photosynthetically active radiation (PAR) was supplied by warm-white fluorescent lights with a light intensity of 3000 ± 300 lx (~ 35 μmol m^{-2} s^{-1}). Every 7 days the culture medium was renewed.

The copper, cadmium and zinc solutions were prepared from stock solutions of $CuCl_2{\cdot}2H_2O$, $CdCl_2{\cdot}H_2O$ and $ZnCl_2$. Metal concentrations in stock solutions were measured by flame atomic absorption spectrophotometry (AAnalyst 100, Perkin-Elmer, calibration against Merck standards). Reagent grade chemicals (Kemika, Zagreb; Riedel De-Haen) were used for the mineral growth medium and metal samples. The growth medium contained 0.08 μg/L Cu^{2+} and 31.4 μg/L Zn^{2+}. Metal concentrations were determined in a preliminary study and set to 0.059, 0.1, 0.146, 1.0, 2.17, 6.0 and 10 mg/L of Cu^{2+}, 0.018, 0.1, 0.149, 1.0, 10, 84.5 and 500 mg/L of Cd^{2+}, and 1.0, 10, 75, 100, 500, 4000 and 40000 mg/L of Zn^{2+}. The pH of the medium was set to 7.5 ± 0.2 for testing the effects of copper and cadmium, and to 5.0 ± 0.2 for testing zinc.

For delayed fluorescence measurements a computer-assisted photon-counting luminometer was built. The apparatus included an illuminator for photo-excitation, a thermostated sample holder and a light detector. A 20 W halogen lamp was used as the illuminator, providing light intensity of 2000 lx (~ 25 μmol m^{-2} s^{-1} PAR) at the sample position. The sample holder for 5 ml cuvettes was thermostated to 25 ± 0.1 °C. For light detection, a red-light-sensitive photomultiplier tube (Hamamatsu R1104) with a Hamamatsu C3866 Photon Counting Unit for signal conditioning and amplification was used, coupled to a personal computer for data collection and storage. Delayed fluorescence of *L. minor* was measured after a 3 sec illumination pulse. Average background noise was then subtracted from the luminescence data. The delayed fluorescence intensity (*DFI*) was calculated as the sum of measured photon counts between 1-60 seconds after sample illumination. Individual samples consisted of a single plant with 3 or 4 fronds in 5 ml cuvettes containing 1.5 ml of growth medium. Samples were incubated in a growth chamber for 24 hours before testing. The initial *DFI* of individual samples was measured before the addition of 0.5 ml of metal solution or distilled water for the controls. Samples were then returned to the growth chamber and *DFI* was measured again 24 hours later. The relative change of delayed fluorescence intensity (ΔDFI) was calculated as the quotient of delayed fluorescence intensity at the end and at the start of the test. Each

experimental set was composed of 12 individual samples – a control sample and 3 treated samples in triplicate. The metal solutions were assigned to individual samples using random block design. The results presented were pooled together from 21 experimental sets to get 6-12 points per metal concentration.

Rapid chlorophyll fluorescence measurements were performed on a Plant Stress Meter mark II (BIOMONITOR S.C.I. AB, illumination time 5 sec, light intensity 250 μmol m^{-2} s^{-1}). The maximum quantum yield of PS II photochemistry (*F*v/*F*m ratio, reviewed in Roháček 2002) was measured on dark adapted samples (at least 6 min in dark). Individual samples were prepared in the same manner as for the *DFI* measurements. Rapid fluorescence was measured after plants had been incubated in the growth chamber for 24 hours. The experimental set was composed of 7 metal concentrations of each tested metal (Cu, Cd, and Zn) and a control. The controls and each metal concentration were done in 6 replicates.

The growth inhibition test with *L. minor* was conducted following the protocol in ASTM (1992) standard for the duckweed toxicity test, with modifications. Briefly, four plants (12 fronds) were placed in Petri dishes with growth medium (20X-AAP without $Na_2EDTA{\cdot}2H_20$) supplemented with copper. Three replicate samples at each concentration (0.1, 1.0, 10 mg/L, and 0.08 μg/L for control) were incubated in growth chambers at 25 ± 0.2 °C under 3000 ± 300 lx (~ 35 μmol m^{-2} s^{-1} PAR). At the end of 7 d, green fronds were counted, and the increase in frond number (Δ*N*) was used as a test endpoint. The entire experiment was repeated three times.

The concentration response for each endpoint (Δ*DFI*, *F*v/*F*m, and Δ*N*) was described using a logistic model (Haanstra and Doelman 1985):

$$\%\ \text{Inhibition} = \left[\left(1+\frac{P}{100-P}\right)\left(\frac{C}{EC_P}\right)^b\right]^{-1}$$

where *C* is the metal concentration, *b* represents the slope of the concentration response curve, and *P* is set to 50 for EC_{50} estimation or to 10 for EC_{10} estimation. The data were fitted using Microcal Origin 6 software with the Levenberg-Marquardt algorithm. Student's *t* test with a significance level of 0.05 was used to detect differences between control and treatment mean values of test endpoints.

RESULTS AND DISCUSSION

Exposure to copper, cadmium and zinc had distinct and concentration-dependent effects on delayed and rapid fluorescence in *L. minor*.

The effect of metals on the relative change in delayed fluorescence intensity (Δ*DFI*) is shown in Figure 1 (a, b, c). An increase in *DFI* after 24 hours of incubation was observed in all three control groups (Cu experiment and Cd experiment for 10 %, Zn experiment for 2 %). This was ascribed to plant growth during the incubation. Δ*DFI* was smaller in plants where the medium pH was 5 (Zn experiment). The

increase in *DFI* in the control groups served to validate the experimental set-up and provided control values to which test measurements were compared. In contrast to ΔDFI, *Fv/Fm* is not related to plant growth, so absolute values are given in the figures.

The relative change of delayed fluorescence intensity (ΔDFI) was dose dependent and was adequately described by the logistic curve in the tested range of copper, cadmium and zinc concentrations. The estimated EC_{50} values were 2.0 ± 0.2 mg/L for copper, 57 ± 13 mg/L for cadmium and 981 ± 175 mg/L for zinc. The slope of the exponential part of the dose-response curve was similar for copper and zinc, whereas the slope of the cadmium dose-response curve was less steep.

The response of the rapid fluorescence parameter *Fv/Fm* (Figure 1d,e,f) to elevated metal concentrations was less pronounced than the ΔDFI response for all tested metals. The maximal effect of copper was an 11 % reduction in *Fv/Fm*, and a 19 % reduction in the case of cadmium. Only the response to zinc exceeded 50 % inhibition, yealding an EC_{50} of 2234 ± 217 mg/L.

Table 1. Responses of rapid fluorescence, delayed fluorescence and growth parameters of *L. minor* to copper, expressed as % of control (mean ± SD).

Cu conc. [mg/L]	Rapid fluorescence 24h *Fv/Fm*	Delayed fluorescence 24h ΔDFI	Growth inhibition test 7 day ΔN
0.1	99 ± 5	85 ± 10	47 ± 10
1.0	100 ± 2	61 ± 5	5 ± 3
10	89 ± 8	34 ± 4	2 ± 3

The 24-hour response of delayed and rapid fluorescence to copper was then compared to the results of a 7-day growth inhibition test (Table 1). Although the EC_{50} for Cu in the growth inhibition test is lower compared to the EC_{50} for Cu obtained in delayed fluorescence measurements, this does not mean that the growth inhibition test is intristically more sensitive. Delayed fluorescence was affected already after 24 hours of exposure to Cu, whereas growth inhibition cannot be accurately determined in such a short period of exposure.

The measurement of delayed fluorescence is nondestructive, simple and fast compared to the growth inhibition test, and allows quick monitoring of physical or chemical factors that affect photosynthesis. Many other toxicity endpoints in higher plants require 1-2 weeks of metal exposure before significant changes are detected. The 24-hour ΔDFI measurement also proved to be more sensitive than the widely used *F*v/*F*m response in rapid fluorescence measurements.

The main difference between delayed and rapid fluorescence is in the mechanism for generation of the excited state of the chlorophyll molecules. Delayed fluorescence originates from the repopulation of excited states of chlorophyll *a* from stored energy after charge separation (Jursinic 1986), whereas rapid fluorescence reflects the radiative de-excitation of excited chlorophyll molecules before charge separation. Delayed fluorescence is therefore affected by more

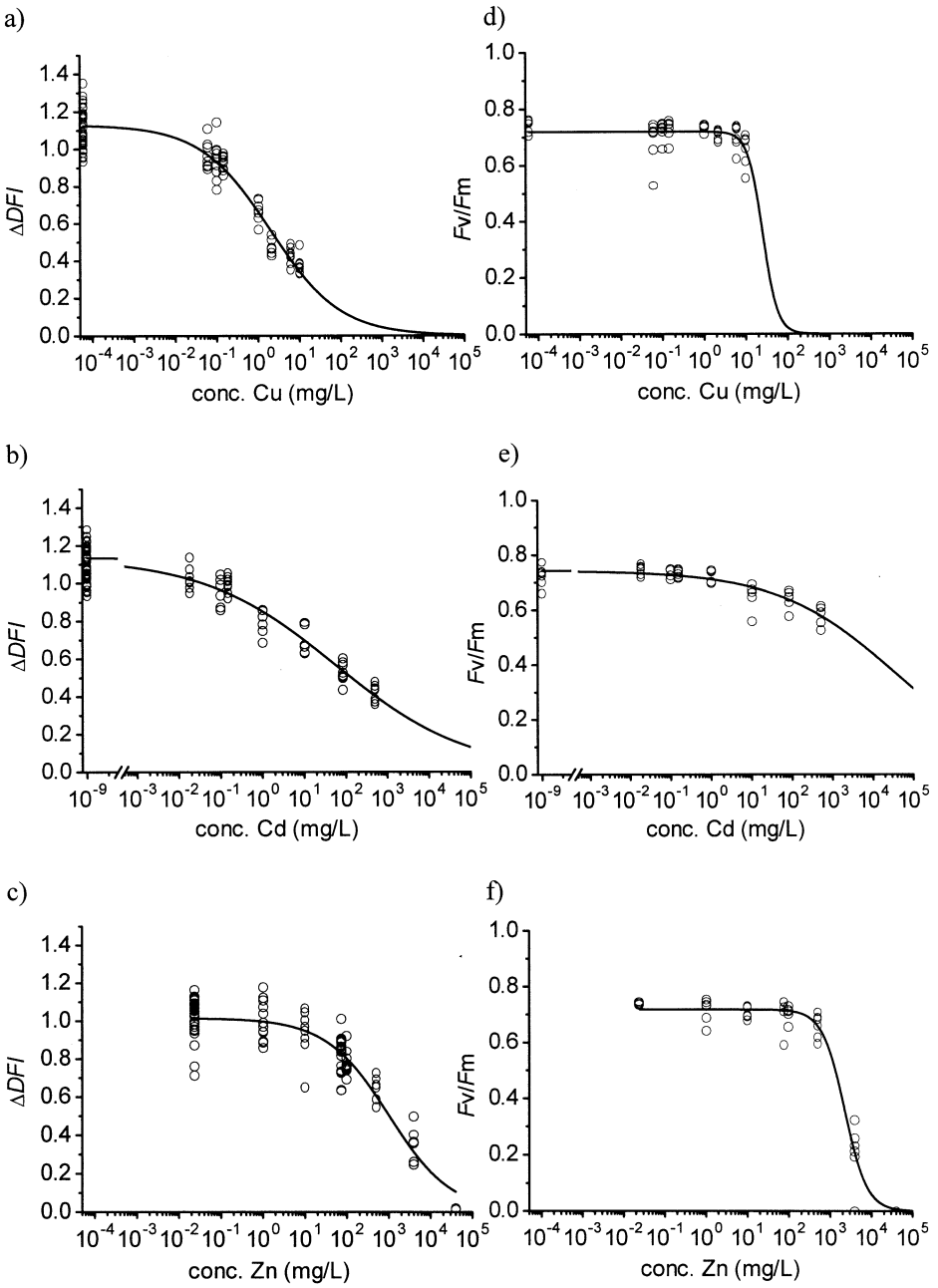

Figure 1. Effects of copper (a,d), cadmium (b,e) and zinc (c,f) on the delayed fluorescence parameter ΔDFI (relative change of delayed fluorescence intensity) and rapid fluorescence parameter *Fv/Fm* (maximum quantum yield of PS II photochemistry) in *L. minor* after 24-hour exposure to different metal concentrations. The circles represent individual measurements and the solid line the estimated mean response using a logistic model.

physical and chemical factors of endogenous or exogenous origin than rapid fluorescence. Thus, delayed fluorescence is a sensitive indicator of the many reactions that compose photosynthesis (Jursinic 1986), and is best used in conjunction with other experimental techniques to understand the physiological mechanisms of toxicity.

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We conclude that *DFI* response is a promising nonspecific biomarker of metal effects either on its own or as a complementary biomarker of metal induced stress in plants. It is currently being investigated in other higher plants and algae (Milani et al. 2003).

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