CHLOROPHYLL FLUORESCENCE AS A BIOINDICATOR OF EFFECTS ON GROWTH IN AQUATIC MACROPHYTES FROM MIXTURES OF POLYCYCLIC AROMATIC HYDROCARBONS

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(Received 20 April 2000; Accepted 28 August 2000)

Abstract—Chlorophyll-a fluorescence induction is a rapid technique for measuring photosynthetic electron transport in plants. To assess chlorophyll-a fluorescence as a bioindicator of effects of polycyclic aromatic hydrocarbon mixtures, chlorophyll-a fluorescence parameters and plant growth responses to exposure to the wood preservative creosote were examined in the aquatic plants Lemna gibba and Myriophyllum spicatum. Exposure to creosote inhibited growth of L. gibba (EC50 = 7.2 mg/L total polycyclic aromatic hydrocarbons) and M. spicatum (EC50 = 2.6 mg/L) despite differences in physiology. Creosote also diminished maximum PSII efficiency (Fm/Fn) (EC50 = 36 and 13 mg/L for L. gibba and M. spicatum) and the effective yield of photosystem II photochemistry (ΔF/ΔFm). The similarity between growth and chlorophyll-a fluorescence EC50s and slopes of the response curves suggests a close mechanistic link between these end points. The predictive power of chlorophyll-a fluorescence as a bioindicator of whole-organism effects applied to complex contaminant mixtures is discussed.

Keywords—Aquatic plants Chlorophyll fluorescence Polycyclic aromatic hydrocarbons Bioindicator Photosynthesis

INTRODUCTION

Risk assessment requires rapid, sensitive techniques to assess exposure and effects of environmental contaminants on organisms. Bioindicators are rapid physiological or biochemical assays that can often detect effects of contaminants in organisms even when the toxicant is unknown. While an abundance of potentially useful assays seems to be available, the primary impediment to their widespread use is the lack of a demonstrated link between effects at the suborganism level with effects on growth or reproduction at the individual level and with higher levels of biological organization, such as populations or communities [1]. A bioindicator for which there is a defined link to growth of the organism can be used in a predictive manner to assess the potential impacts of a contaminant before detrimental effects to populations occur.

Photosynthetic electron transport is a universal feature of higher plants, algae, and cyanobacteria. This essential process is sensitive to many classes of environmental contaminants, including herbicides [2,3], metals [4], and organic contaminants [5]. Pulse amplitude modulated (PAM) chlorophyll-a fluorescence is a rapid method for measuring photosynthetic electron transport from plants in vivo, and it requires very little sample preparation [6]. Stimulation of fluorescence from chlorophyll-a in photosystem II (PSII) reaction centers under different light conditions produces several parameters, each describing the efficiency of a photochemical reaction or process within the photosynthetic apparatus. The variable fluorescence ratio Fv/Fm, which describes the maximum efficiency of PSII photochemistry [7,8], is calculated from the minimum fluorescence (F0) in the dark and the maximum fluorescence (Fm) on application of a saturating light to the dark-adapted plant. The minimum (F0) and maximum (Fm) fluorescence levels in the light-adapted plant are used to calculate ΔF/ΔFm, the quantum yield of photochemistry in functional PSII reaction centers during steady-state photosynthesis. This parameter represents the efficiency with which excitation energy captured by the chlorophyll antenna is used in electron transport. It is closely related to the quantum yield of carbon assimilation [9] and to the photochemical quenching parameter describing photochemical quenching (qP). The qP term describes the fraction of fluorescence quenched in a light-adapted plant relative to a dark-adapted plant due to induction of photochemistry and estimates the extent to which electron transport is restricted at PSII [6]. Nonphotochemical quenching of fluorescence, described by qN, is due to a number of biophysical and biochemical mechanisms that dissipate excess excitation energy in the pigment antenna to protect PSII from overexcitation [7,10]. Chlorophyll-a fluorescence can potentially detect impacts of contaminants not acting directly on electron transport since chemicals that inhibit any cellular process downstream of PSII, such as carbon assimilation or respiration, or damage membranes or proteins associated with photosynthetic electron transport will lead to excitation pressure on PSII [11,12].

We have previously shown that chlorophyll-a fluorescence can be used to detect inhibition of photosynthetic electron transport in plants exposed to polycyclic aromatic hydrocarbons (PAHs) and photomodified PAHs [5,13,14]. The PAHs consist of aromatic ring compounds and are common contaminants in aquatic environments [15,16]. The toxicity of many PAHs is greatly enhanced in the presence of ultraviolet radiation [15,17]. The observed photoinduced toxicity is generally attributed to generation of singlet oxygen and hydroxyl radicals in the tissue of organisms exposed to PAHs, leading to oxidation of membrane lipids and other biomolecules [18,19]. However, the light-mediated production of oxyPAHs, which often have greater aqueous solubility and toxicity than the...
parent PAH, is also an important mechanism of toxicity [12,20].

Organisms are rarely exposed to single PAHs in the aqueous environment. Samples from contaminated sites contain complex mixtures of PAHs [16] as well as PAH photoproducts (X.-D. Huang, B.J. McConkey, and B. Greenberg, unpublished observations). The PAHs in mixtures often are in greater aqueous concentrations than single PAHs because of cosolubility effects [21]. Therefore, to assess the applicability of chlorophyll-a fluorescence as a bioindicator, we examined the response of various fluorescence parameters in plants exposed to the wood preservative creosote, a mixture composed primarily of PAHs, with minor quantities of oxyPAHs and substituted PAHs [22]. Two aquatic plant species, *Myriophyllum spicatum* (Eurasian Milfoil) and *Lemma gibba* (duckweed), were exposed to a range of creosote concentrations in defined growth medium and lighting conditions. The concentration response of chlorophyll-a fluorescence parameters was compared to conventional growth-based end points.

**MATERIALS AND METHODS**

**Plant culture**

Cultures of *Lemma gibba* L. (G-3) were propagated axenically on half-strength Hutner’s medium fortified with 30 g/L sucrose in large glass flasks under 50 μmol/m2/s continuous photosynthetically active radiation (PAR, 400–700 nm) supplied by cool-white fluorescent lamps.

Whole *Myriophyllum spicatum* L. (Haloragaceae) plants were collected locally from a shallow pond. The meristematic tips were excised and washed in 2% hypochlorite solution to sterilize the plant surface. Plants were cultured axenically in capped 25 × 150-mm quartz tubes containing Andrew’s medium [3] fortified with 15 g/L sucrose. The plants were maintained in a growth chamber at 25°C under 100 μmol/m2/s PAR supplied by cool-white fluorescent lamps on a 16:8-h light:dark cycle.

**Creosote exposure**

*L. gibba* were exposed to marine-grade liquid creosote (Stella Jones, New Westminster, BC, Canada) in 8-d static renewal toxicity tests [23]. The PAH composition of the creosote had been partially characterized previously by high-performance liquid chromatography. The 15 most abundant PAHs detected were found to constitute 38.4% (w/w) of the creosote [24]. Aqueous solutions of creosote were made up at nine concentrations from 0.01 to 100 mg/L. Dimethylsulfoxide at 0.1% (v/v) was sterilized the plant surface. Plants were cultured axenically in capped 25 × 150-mm quartz tubes containing Andrew’s medium [3] fortified with 15 g/L sucrose. The plants were maintained in a growth chamber at 25°C under 100 μmol/m2/s PAR supplied by cool-white fluorescent lamps.

**Leaf chlorophyll concentration**

Chlorophyll was extracted from whole *L. gibba* plants and from the leaves of *M. spicatum*. Weighed plants or leaves were extracted in *N,N*-dimethylformamide (Aldrich Chemicals) for 24 h at 4°C in the dark. Chlorophyll-a and chlorophyll-b concentrations were measured simultaneously by spectrophotometry [27]. Leaf chlorophyll concentrations were calculated on a fresh-weight basis.

**Growth end points**

The number of *L. gibba* fronds was counted every 2 d, and a growth rate was calculated on the basis of the increase in fronds over 8 d according to an exponential growth model. Growth rate was calculated as

\[ \frac{1}{t} \log(F/F_0) = \log(2)^{-1} \]

where \( t \) is the duration of the test in days, \( F_0 \) is the initial number of fronds, and \( F \) is the number of fronds at the end of the test.

Growth measurements were taken from *M. spicatum* at the end of the 12-d exposure. The shoot length and number of leaf nodes of the main shoot as well as side shoots were recorded. The number and length of all roots were recorded. Fresh weight was measured from whole plants after excess water was re-
moved by blotting. Since the aseptic technique precluded weighing the plants at the beginning of the test, the increase in fresh weight over the initial weight was calculated by subtracting the average fresh weight of 10 unexposed shoots 3 cm in length.

Chlorophyll-a fluorescence

Chlorophyll-a fluorescence induction was measured from *L. gibba* after 4 and 8 d of exposure to creosote and from *M. spicatum* after 4, 8, and 12 d of exposure. Plants were removed from the growth chamber and dark adapted for 15 min prior to measurement. Chlorophyll-a fluorescence was measured from whole plants using a PAM fluorometer (PAM-2000, Walz, Effeltrich, Germany). All light sources and fluorescence measurements were directed through a fiber optic light guide. For *L. gibba*, the end of the light guide was positioned 1 mm above plants floating in petri dishes; for *M. spicatum*, measurements were taken directly through the wall of the test tube without removing the plants from the growth medium.

Since chlorophyll-a fluorescence parameters vary considerably under different measurement conditions, fluorescence induction curves were optimized for each plant by incrementally adjusting the light levels on the PAM fluorometer. A weak measuring light (<1 μmol/m²/s) was used to determine *F₀*, the minimum fluorescence obtained in the dark-adapted state. Maximum fluorescence (*F₉₅*) was determined for the dark-adapted state by applying a saturating pulse of white light (4,000 μmol/m²/s, 600-ms duration) from a halogen lamp. Pulses of white light at greater fluence rates did not change *F₉₅*, indicating that the fluence rate used was saturating to the dark-adapted plants. Actinic light from red diodes (655 nm) was used to induce electron transport. An actinic fluence rate was used to induce electron transport. An actinic fluence rate from red diodes (655 nm) was used to induce electron transport.

Maximum quantum efficiency of PSII photochemistry was calculated as

\[ \frac{F_{92}}{F_{m}} = \frac{F_{m} - F_{o}}{F_{m}} \]  

(2)

The effective quantum yield of PSII photochemistry was calculated as

\[ \frac{\Delta F}{F_{m}} = \frac{F_{m} - F_{o}}{F_{m}} \]  

(3)

Photosynthetic and nonphotosynthetic quenching of fluorescence parameters were calculated as

\[ qP = \frac{F_{m} - F_{o}}{(F_{m} - F_{o})} \]  

and

\[ qN = 1 - \frac{F_{m} - F_{o}}{(F_{m} - F_{o})} \]  

(4)

(5)

Because nonphotosynthetic quenching generally increases in plants under stress, the parameter 1 - qN was used as an index for nonphotosynthetic quenching.

Statistical analysis

Chlorophyll-a fluorescence data often do not exhibit a Gaussian (normal) distribution [28]. Common descriptors of variance, such as standard deviation, which assume normal distribution, are inappropriate for this type of data. Thus, we have presented concentration-response curves of chlorophyll-a fluorescence data using box plots with median, 25th, and 75th percentiles to indicate the distribution of the data. For comparison, plots of leaf chlorophyll concentration and growth end points are also presented in this manner. The response data were plotted on the basis of ΣPAH, the sum concentration of the 15 predominant PAHs at each creosote concentration [24], which was calculated by multiplying the nominal creosote concentration by the fraction of PAHs in the creosote we used (38.4%). Chlorophyll-a fluorescence measurements and the shape of the concentration-response curves were similar at each time point; therefore, only curves from the final measurement (8 d for *L. gibba* and 12 d for *M. spicatum*) are shown.

The concentration response for each end point was described using a trimmed logit model suitable for continuous response data [29]. Iterative regression minimizing χ² adjusted for degrees of freedom used to fit the function

\[ \% \text{ Inhibition} = 100(1 + e^{(x-\mu)/\beta})^{-1} \]  

(6)

to the measured data, where x is the logarithm of the ΣPAH concentration, μ is the logarithm of the EC50, and β represents the slope of the concentration-response curve [20,29]. The model used only data from the steepest part of the response curve by trimming data for which the mean response fell outside 1% of the predicted response (1 < % Inhibition < 99). In this way, the regressions are driven by the data in the center of the distributions. The regression provided estimates of variance for μ and β that were used to calculate 95% confidence intervals for the EC50 and slope. Only EC50s are presented in this study, but any level of inhibition (e.g., 20%) could be determined by substituting that value into the equation and solving for the concentration x.

RESULTS

Creosote effects on plant growth

The growth rate of *L. gibba* was inhibited by creosote in a concentration-dependent manner (Fig. 1). Plants in higher-creosote treatments were smaller than controls, and colonies consisted of one or two fronds rather than three or four fronds, as in control plants. There was almost no new frond production
after 8 d at the highest concentration tested, and remaining plants were very small and chlorotic.

All growth-based end points in \textit{M. spicatum} were also diminished by exposure to creosote (Fig. 2). Growth in the highest-creosote treatment was completely inhibited, resulting in nearly zero accumulation of biomass (fresh weight) (Fig. 2A). Increase in shoot length was inhibited by creosote (Fig. 2B) because of diminished shoot elongation, fewer nodes, and fewer new shoots (data not shown). However, low creosote concentrations stimulated shoot elongation up to 125\% of control values. Root production and elongation were inhibited by very low creosote concentrations and were completely suppressed at concentrations greater than 1 mg/L \textgreek{Sigma}PAH (Fig. 2C).

In general, growth end points had high experimental variability. In addition, the shoot length and fresh-weight end points exhibited hormesis; that is, these responses were stimulated rather than inhibited in plants exposed to low creosote concentrations. Hormesis in these end points resulted in reduced fit of the regression curve (lower $R^2$) but had little effect on the EC50 because the curve-fitting procedure is driven primarily by data close to the EC50 and is not susceptible to outliers in the tails of the data set [29]. Estimated EC50s for inhibition of growth were higher for \textit{L. gibba} (7.2 mg/L \textgreek{Sigma}PAH; Table 1) than for \textit{M. spicatum} (0.14–2.6 mg/L \textgreek{Sigma}PAH, depending on the end point; Table 2). Although the growth end points in \textit{M. spicatum} were more sensitive than growth in \textit{L. gibba}, higher variability existed in the former, especially shoot length and root length, resulting in poorer regression coefficients.

\textbf{Creosote effects on leaf chlorophyll concentrations}

Plants exposed to the highest creosote concentration were bleached after only 2 d, indicating very low chlorophyll concentrations. Total chlorophyll extracted from \textit{L. gibba} at the end of the 8-d exposure confirmed that chlorophyll was diminished in a concentration-dependent manner (Fig. 3A) from approximately 0.7 \textmu g/mg fresh weight in controls to less than 0.1 \textmu g/mg in the highest-creosote treatment. Concentrations of both chlorophyll-\textit{a} and chlorophyll-\textit{b} were diminished (data not shown).

Leaf chlorophyll concentrations in unexposed \textit{M. spicatum} were approximately 1 \mu g/mg fresh weight; this value is somewhat higher than the concentration in unexposed \textit{L. gibba}. Exposure of \textit{M. spicatum} to creosote resulted in diminished chlorophyll at concentrations greater than 1 mg/L \textgreek{Sigma}PAH (Fig. 3B). Although moderate variability in this end point was seen, regressions produced good fits to the data set. The EC50s for chlorophyll concentration for \textit{L. gibba} and \textit{M. spicatum} were similar: 3.4 and 5.6 mg/L \textgreek{Sigma}PAH, respectively.

\textbf{Creosote effects on photosynthesis}

Exposure to creosote had strong, distinct effects on chlorophyll-\textit{a} fluorescence scans in \textit{L. gibba}. Variable fluorescence in the dark-adapted ($F_o = F_{adj}$) and light-adapted ($F_v = F_{adj}'$) states was diminished substantially compared to control plants because of diminishment of both $F_o$ and $F_v$ (Fig. 4). At the highest concentration (38 mg/L \textgreek{Sigma}PAH), the increase in fluorescence induced by the saturating light under steady-state photosynthesis ($F_{adj}'$) was nearly indistinguishable from back-

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|c|c|}
\hline
Measurement end point & Exposure (d) & EC50 (95\% CI) (mg/L \textgreek{Sigma}PAH)$^a$ & $\beta$ (95\% CI) & $\chi^2$ & $R^2$ \\
\hline
Growth & 8 & 7.2 (3.8, 13.8) & $-2.2$ ($-3.3$, $-1.1$) & 0.259 & 0.966 \\
Leaf chl conc. & 8 & 3.4 (2.0, 6.0) & $-2.2$ ($-3.1$, $-1.3$) & 0.020 & 0.997 \\
$F_o/F_v$ & 4 & >38 & $-1.8$ ($-3.1$, $-0.49$) & 0.037 & 0.989 \\
& 8 & 36 (4.8, 270) & $-2.0$ ($-4.2$, 0.31) & 0.192 & 0.988 \\
$\Delta F/F_v'$ & 4 & 22 (0.4, 53) & $-2.1$ ($-3.2$, $-0.94$) & 0.092 & 0.988 \\
& 8 & 13 (7.3, 22) & $-2.8$ ($-4.2$, $-1.5$) & 0.597 & 0.963 \\
$qP$ & 4 & >38 & $-1.4$ ($-6.4$, 3.6) & 0.079 & 0.931 \\
& 8 & >38 & $-0.62$ ($-2.0$, 0.75) & 0.386 & 0.297 \\
$1 - qN$ & 4 & >38 & $-0.69$ ($-1.3$, $-0.06$) & 0.144 & 0.774 \\
& 8 & >38 & $-1.0$ ($-2.5$, $-0.37$) & 0.380 & 0.329 \\
\hline
\end{tabular}
\caption{Regression terms describing concentration–response curves for growth, leaf chlorophyll (chl) concentration, maximum efficiency of photochemistry ($F_o/F_v$), quantum yield of photochemistry ($\Delta F/F_v'$), photochemical quenching ($qP$), and nonphotochemical quenching ($1 - qN$) from \textit{Lemna gibba} exposed to creosote. The median response concentration (EC50) and slope of the curve ($\beta$) were estimated by fitting the mean response from three experiments to a nonlinear function. The deviation of the data from the prediction ($\chi^2$) and the correlation factor of the predicted curve ($R^2$) are shown.}
\end{table}

*Estimates are based on the sum concentration of the 15 most abundant polycyclic aromatic hydrocarbons in creosote.
Table 2. Regression terms describing concentration–response curves for growth, leaf chlorophyll (chl) concentration, maximum efficiency of photochemistry ($F_v/F_m$), quantum yield of photochemistry ($\Delta F/F_m^\infty$), and photochemical quenching ($qP$), from Myriophyllum spicatum exposed to creosote. The median response concentration (EC50) and slope of the curve ($\beta$) were estimated by fitting the mean response from three experiments to a nonlinear function. The deviation of the data from the prediction ($\chi^2$) and the correlation factor of the predicted curve ($R^2$) are shown. Curves could not be fit to the nonphotochemical quenching ($1-qN$) parameter.

<table>
<thead>
<tr>
<th>Measurement end point</th>
<th>Exposure (d)</th>
<th>EC50 (95% CI) (mg/L ΣPAH)$^a$</th>
<th>$\beta$ (95% CI)</th>
<th>$\chi^2$</th>
<th>$R^2$</th>
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<tbody>
<tr>
<td>Fresh weight</td>
<td>12</td>
<td>2.6 (1.4, 4.9)</td>
<td>-3.2 (-3.5, -1.1)</td>
<td>1.53</td>
<td>0.953</td>
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<tr>
<td>Shoot length</td>
<td>12</td>
<td>1.6 (1.0, 2.7)</td>
<td>-1.8 (-2.3, -1.3)</td>
<td>3.54</td>
<td>0.814</td>
</tr>
<tr>
<td>Root length</td>
<td>12</td>
<td>0.14 (0.06, 0.30)</td>
<td>-3.0 (-5.4, -0.68)</td>
<td>0.849</td>
<td>0.894</td>
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<tr>
<td>Chl content</td>
<td>12</td>
<td>5.6 (2.9, 10.7)</td>
<td>-1.8 (-2.6, -0.94)</td>
<td>0.160</td>
<td>0.986</td>
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<tr>
<td>$F_v/F_m$</td>
<td>4</td>
<td>&gt;38</td>
<td>-1.5 (-2.4, -0.47)</td>
<td>0.202</td>
<td>0.968</td>
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<tr>
<td></td>
<td>8</td>
<td>36 (12, 113)</td>
<td>-1.8 (-2.9, -0.76)</td>
<td>0.218</td>
<td>0.953</td>
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<td></td>
<td>12</td>
<td>13 (6.0, 28)</td>
<td>-2.8 (-4.6, -1.0)</td>
<td>0.151</td>
<td>0.987</td>
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<tr>
<td>$\Delta F/F_m^\infty$</td>
<td>4</td>
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<td>8</td>
<td>34 (8.5, 140)</td>
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<td>0.302</td>
<td>0.961</td>
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<td></td>
<td>12</td>
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<td>0.964</td>
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<tr>
<td>$qP$</td>
<td>4</td>
<td>&gt;38</td>
<td>NA $^b$</td>
<td>NA</td>
<td>NA</td>
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<tr>
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<td>&gt;38</td>
<td>-1.4 (-17, 14)</td>
<td>0.494</td>
<td>0.772</td>
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</table>

$^a$ Estimates are based on the sum concentration of the 15 most abundant polycyclic aromatic hydrocarbons in creosote.

$^b$ NA = not applicable; the data could not be fit to the model because of a lack of response.

ground fluorescence, and little recovery of $F_m$ occurred on the removal of the actinic light (Fig. 4D). Chlorophyll fluorescence scans from plants treated with lower creosote concentrations appeared similar to controls.

Photosynthetic parameters were diminished in L. gibba only at the upper range of creosote concentrations tested (>10 mg/L ΣPAH) (Fig. 5). Greater concentrations were not tested because the highest concentration of creosote in this experiment was above the aqueous solubility limits of most PAHs, and attempts to suspend greater concentrations of creosote in the media were unsuccessful. Thus, it was not possible to obtain complete concentration–response curves for all fluorescence parameters, but accurate predictions of the EC50 were possible. In general, toxicity was greater after longer exposures to creosote. Exposure to creosote diminished some chlorophyll fluorescence parameters in a concentration-dependent manner but not others. The $F_v/F_m$ and $\Delta F/F_m^\infty$ values were diminished at higher creosote concentrations (Fig. 5A and B), but a clear concentration response was not observed with $qP$ or $1-qN$ (Fig. 5C and D). At the highest creosote concentration, the quenching parameters $qP$ and $1-qN$ could not be measured accurately from the fluorescence induction scan because of a lack of signal from low chlorophyll concentrations. However, it cannot be assumed that these parameters were zero because both the numerator and the denominator approach zero when the fluorescence signal is low.

Effects of creosote on chlorophyll-a fluorescence in M. spicatum were similar to effects on L. gibba. Responses of $F_v/F_m$ and $\Delta F/F_m^\infty$ to creosote exposure were clearly concentration dependent (Fig. 6A and B). While no inhibition of these parameters was seen at concentrations below 1 mg/L ΣPAH, the highest concentration diminished $F_v/F_m$ to 20% of control values after 12 d. This parameter in control plants and plants exposed to low creosote concentrations had little variability, but at higher PAH concentrations moderate variability was seen. Similar to L. gibba, the $qP$ and $1-qN$ parameters were not diminished by creosote, even after 12 d of exposure (Fig. 6C and D). At high creosote concentrations, $qN$ was diminished rather than increased, resulting in $1-qN$ values greater than one. This response was greatest in plants exposed for 12 d.

The logit function fit to the response data adequately described the concentration responses of $F_v/F_m$ and $\Delta F/F_m^\infty$, with $R^2 > 0.94$. The estimated EC50s for these end points were approximately an order of magnitude greater than the growth-based end points. The model estimated 8-d EC50s for $F_v/F_m$ and $\Delta F/F_m^\infty$ of 36 and 13 mg/L ΣPAH, respectively, in L. gibba (Table 1). The EC50s in M. spicatum were similar to L. gibba after 8 d but decreased after 12 d of exposure to 13 and 15 mg/L ΣPAH for $F_v/F_m$ and $\Delta F/F_m^\infty$, respectively (Table 2). Meaningful curves could not be fit for the quenching parameters $qP$ and $1-qN$ because of the lack of a positive (diminished) response.

The slopes of the response curves (estimated by the $\beta$ term in the model) were similar for $F_v/F_m$ and $\Delta F/F_m^\infty$ at the end of the exposure in both L. gibba (Table 1) and M. spicatum (Table 2). In addition, the slopes for $F_v/F_m$ and $\Delta F/F_m^\infty$ were similar to the slopes estimated for inhibition of chlorophyll concentration and growth end points. In L. gibba, the slopes for $F_v/F_m$, $\Delta F/F_m^\infty$, growth, and chlorophyll concentration were all
Chlorophyll fluorescence as bioindicator of creosote toxicity

**Fig. 4.** Effect of creosote on chlorophyll fluorescence in *Lemna gibba*. Chlorophyll-a fluorescence scans were measured from plants exposed for 8 d to creosote at (A) 0, (B) 10, (C) 30, or (D) 100 mg/L creosote. Minimum ($F_m$) and maximum ($F_m'$) fluorescence levels were determined from dark-adapted plants at the beginning of the scan. Plants were brought to steady-state photosynthesis using a red actinic light as indicated. Steady-state ($F_s$) and light-adapted maximum ($F_m'$) fluorescence was measured after approx. 8 to 10 min. Minimum fluorescence under far-red light ($F_o$) was measured immediately after the actinic light was removed. Signals were normalized to $F_o$ for comparison.

**Fig. 5.** Chlorophyll-a fluorescence parameters from *Lemna gibba* exposed to creosote. Maximum efficiency of photochemistry ($F_v/F_m$), quantum yield of photochemistry ($\Delta F/F_m$), photochemical quenching ($q_P$), and nonphotochemical quenching ($1-q_N$) were measured from plants after 8 d of exposure. The missing data at high concentrations represent severely chlorotic plants from which measurements could not be obtained.

within the range $-2.8 < \beta < -2.0$ (Table 1). The slopes of responses measured in *M. spicatum* after 12 d were somewhat more variable, but all fell within the range $-3.0 < \beta < -1.8$ and were not statistically different (Table 2). The response for root production had the steepest slope but also had higher variability in the data, resulting in a large confidence interval.

**DISCUSSION**

A major impediment to ecotoxicological assessments is a lack of validated bioindicators that can be used to predict contaminant effects in natural ecosystems. There are numerous physiological or biochemical assays that detect effects of toxicants on various cellular processes, but the applicability of these assays as predictive bioindicators is often compromised by a lack of any quantitative relationship with higher-level end points [1]. The ability of a bioindicator to predict effects at higher biological levels can be determined by comparing sub-organism and whole-organism responses. If the response is tested over the entire toxicant concentration range rather than simply contrasting point estimates, such as EC50s, mechanistic relationships between different biological levels can be better postulated [14]. Bioindicators for which correspondence exists between both the slope of the response curve and the median toxicity end point (e.g., EC50) have a close mechanistic relationship with effects at higher levels of organization and are more valuable as predictive tools.

The results of this study show that chlorophyll-a fluorescence in aquatic plants is a bioindicator that is somewhat predictive of whole-plant toxicity. Inhibition of photosynthesis in *L. gibba* and *M. spicatum* exposed to PAHs can be linked with inhibition of plant growth. The chlorophyll-a fluorescence parameters describing the quantum efficiency of PSII ($F_v/F_m$) and the quantum yield of PSII photochemistry ($\Delta F/F_m$) were diminished at PAH concentrations that also diminished leaf chlorophyll concentration and inhibited growth of *L. gibba* (frond production) and *M. spicatum* (shoot growth, root growth, and fresh weight). Although growth end points were
generally more sensitive, EC50s for inhibition of chlorophyll-
a fluorescence and EC50s based on growth end points were
within an order of magnitude, and the $\Delta F/F_m$ parameter in $L.$
gibba was almost as sensitive as frond production. In addition,
the slopes of the concentration–response curves were similar
for the growth and chlorophyll-$a$ fluorescence end points,
implying that toxicity from PAHs can be associated primarily
with effects on the photosynthetic apparatus in these plants.
Some components of the mixture apparently affected nonpho-
tosynthetic processes (e.g., respiration and cell division), re-
sulting in diminished growth at low concentrations of creosote.

Chlorophyll-$a$ fluorescence has the ability to be a powerful
bioindicator of PAH effects despite the lower sensitivity of
these end points compared to growth end points. Even a mod-
erate diminishment of chlorophyll-$a$ fluorescence will translate
to deleterious effects on photosynthetic energy production be-
cause of the tight relationship between these two processes.
Thus, a plant in which chlorophyll-$a$ fluorescence is dimin-
ished by even 50% will eventually suffer severely diminished
plant growth. The measurement of plant growth as a toxicity
end point is not practical in the field since time-zero mea-
surements usually cannot be taken. Chlorophyll-$a$ fluores-
cence, alternatively, is ideal for rapidly assessing instantaneous
and/or continuous effects of contaminants in the field. Thus,
although chlorophyll-$a$ fluorescence parameters were not di-
ninished at exactly the same concentrations as growth for the
contaminants used in this study, this assay has the ability to
predict effects at the whole-organism level. Our results reveal
that when impacts on chlorophyll-$a$ fluorescence are observed,
effects such as diminished growth are most likely occurring
as well. We have found in previous studies with phytoplankton
[30] and in unpublished work with aquatic plants that this
technique is an elegant method for detecting contaminant im-
plants in the field.

Of the chlorophyll-$a$ fluorescence parameters examined in
this study, $\Delta F/F_m$ was the most sensitive end point to PAH
toxicity. The $\Delta F/F_m$ parameter is strongly correlated with
photosynthetic C uptake and O$_2$ evolution [9,31]. Mechanically,
$\Delta F/F_m$ is a function of the fraction of open reaction centers
and the quantum efficiency of electron transport through PSII
[9,32]. Thus, this parameter integrates the effects of many
processes that contribute to inhibition of electron transport
either by decreasing the quantum yield of photochemistry or
by increasing the yield of nonphotochemical (thermal) exci-
tation decay processes. The sensitivity to PAH toxicity and
relevance to energy production make $\Delta F/F_m$ the most suitable
chlorophyll-$a$ fluorescence parameter to use as a bioindicator
of toxicity in plants.

Diminished $F_v/F_m$ and $\Delta F/F_m$ can indicate a large propor-
tion of inactive PSII reaction centers due to oxidation or degra-
dation of D1 proteins [8]. Severely reduced pigment concen-
trations in reaction centers would also diminish these param-
eters, which would be consistent with the diminished chlor-
ophyll content of plants exposed to creosote. Diminished PSII
efficiency can also indicate inhibition of electron transport at
other sites in the photosynthetic apparatus downstream of PSII.
Because PSII is the first step in photosynthetic electron trans-
port, inhibition of electron transport anywhere within the elec-
tron transport chain exerts excitation pressure on PSII, which
is most likely cytochrome $b_{6f}$. In the respiratory electron transfer
pathway, oxygen-consuming PAHs specifically inhibit cytochrome $b_{6f}$,
which ana-
gous to cytochrome $b_{6f}$ in the photosynthetic pathway [35].

The lack of response of the chlorophyll-$a$ fluorescence
parameter describing photosynthetic energy quenching ($qP$)
suggests that PAHs had no direct impact on the primary pho-
tosynthetic reactions of PSII. Some quinones are known to in-
hhibit PSII directly because of structural similarity with the $Q_A$-
binding niche in PSII [36]. It has been demonstrated that
quinones are the primary photoproducts produced on exposure
of many PAHs to light [20]. In a mixture of PAHs exposed
UV radiation in an aquatic matrix, quinones capable of
binding the $Q_A$ site were almost certainly present. However,
the lack of effect on $qP$ suggests that quinones capable of
binding PSII were not present in significant concentrations
within the thylakoid membranes.

The variable and inconsistent response of the $1-qN$ pa-
parameter also suggests PAHs had no direct effect on electron
transport. The parameter $qN$ represents the loss of fluores-
cence yield due to nonphotochemical quenching of chlorophyll ex-
citation, which dissipates excess energy as heat. These mech-
anism are induced by excessive excitation pressure on PSII
from strong light [8], low temperature [11], or blocked electron

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Fig. 6. Chlorophyll-$a$ fluorescence parameters from Myriophyllum
spicatum exposed to creosote. Maximum efficiency of photochemistry
($F_v/F_m$), quantum yield of photochemistry ($\Delta F/F_m$), photochemical
quenching ($qP$) and nonphotochemical quenching ($1-qN$) were mea-
sured from plants after 12 d of exposure.
transport [37]. While \(1 - qN\) tended to decrease slightly at higher concentrations of creosote in \(L.\ gibba\), the lack of a strong response implies that electron transport at PSII was not blocked to a great extent in plants exposed to creosote. The diminished \(qN\) (elevated \(1 - qN\)) observed in \(M.\ spicatum\) may have been caused by chlorosis or by disruption of the thylakoid membrane proton gradient required for nonphotochemical quenching processes [10].

The different responses of the various chlorophyll-a fluorescence parameters, when taken together, support a mechanism of PAH toxicity in which photosynthetic efficiency was impaired as a result of general damage to essential cellular components, possibly from reactive oxygen species. For instance, the presence of excited chlorophyll pigments in thylakoid membranes in which electron transport has been blocked leads to production of singlet oxygen [7]. Alternatively, free radicals may be produced in the chloroplast via photosensitization reactions by PAHs or oxyPAHs on absorbance of UV light [18,19]. The most abundant photoproducts of PAHs are anthraquinones, which are very efficient redox cyclers, producing appreciable quantities of superoxides in the light when a reducing agent is present [38]. Regardless of the source, the presence of radical species in the chloroplast leads to oxidation of membranes, proteins (e.g., the D1 protein of PSII), and pigments. Based on the loss of leaf chlorophyll in plants exposed to low PAH concentrations, destruction of chlorophyll pigments is apparently an important mechanism of PAH toxicity in plants and may precede damage to the proteins of the photosynthetic apparatus. However, the different PAH components in creosote likely have multiple effects at the biochemical level, such as disruption of electron transport by oxyPAHs that act as electron acceptors [36] or inhibit electron transport [34], or loss of the thylakoid proton gradient due to membrane oxidation.

The results of this study using the PAM fluorescence technique support previous studies of PAH effects on electron transport. During the early events of the chlorophyll-a fluorescence induction curve, the fluorescence ratio \(F/F_m\) is a sensitive indicator of electron transport in \(L.\ gibba\) exposed to photomodified anthracene, which inhibits electron transport downstream of PSII [33]. The half-time required to reduce the plastoquinone pool \((t_m)\) in \(L.\ gibba\) exposed to creosote has also been shown to be a sensitive end point to PAH toxicity (EC50 = 11.6 ppm creosote) [14]. Gensemer et al. found inhibition of frond production (EC50 = 10.7 ppm creosote) and \(F/F_m\) (EC50 = 16.6 ppm creosote) were also sensitive end points [14]. The EC50s for growth and \(F/F_m\) were somewhat lower than those found in this study, but the creosote used in that study was weathered (stirred in air) prior to use and likely contained a different assortment of PAHs.

The hormesis response of the \(M.\ spicatum\) growth end point to values greater than those measured in unexposed plants is a potentially confounding factor in bioindicator applications. When hormesis responses occur, toxicity estimates such as EC50s can be based on the response relative to the controls, the greatest value observed, or the low concentrations pooled with the real control response [39]. With respect to calculation of EC50s in this study, hormesis effects were ignored because of the small relative stimulation response observed. The trimmed logit model described the data reasonably well, with only a minor deviation produced by the stimulation response. This statistical model resists shifts in the logit function due to stimulation by trimming responses from the data set that lie outside specified limits during the iterative regression. Thus, only those points close to the midpoint in the concentration response curve were used in the EC50 estimate. Other statistical approaches exist for describing larger stimulation responses, such as nonlinear functions that can accommodate hormesis responses.

The two plant species used in this study responded similarly to creosote exposure. For responses after 4 or 8 d, \(F/F_m\) and \(\Delta F/F_m\) were inhibited at nearly the same 2PAH concentrations in both plant species. Growth of \(L.\ gibba\) and \(M.\ spicatum\) was inhibited at similar concentrations, even though different measurement end points were examined. The PAHs apparently had similar effects on electron transport in both plant species. The similar responses of \(F/F_m\) and \(\Delta F/F_m\) to PAHs in these two plant species and in phytoplankton [30] is encouraging. These parameters may be appropriate bioindicators that can be measured in different plants in the field exposed to PAHs. An important aspect of this assay is that unlike growth-based endpoints, chlorophyll-a fluorescence can be used to rapidly assess contaminant effects in the field at any time, independent of the duration of the exposure.

Acknowledgement—This study was supported by equipment and grants from Environment Canada through the Canadian Network of Toxicology Centres and by the Natural Sciences and Engineering Research Council grants to B.M. Greenburg.

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