Rapid effects of diverse toxic water pollutants on chlorophyll a fluorescence: Variable responses among freshwater microalgae

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Abstract
Chlorophyll a fluorescence of microalgae is a compelling indicator of toxicity of dissolved water contaminants, because it is easily measured and responds rapidly. While different chl a fluorescence parameters have been examined, most studies have focused on single species and/or a narrow range of toxins. We assessed the utility of one chl a fluorescence parameter, the maximum quantum yield of PSII (Fv/Fm), for detecting effects of nine environmental pollutants from a range of toxin classes on 5 commonly found freshwater algal species, as well as the USEPA model species, Pseudokirchneriella subcapitata. Fv/Fm declined rapidly over <20 min in response to low concentrations of photosynthesis-specific herbicides Diuron® and metribuzin (both <40 nM), atrazine (<460 nM) and terbuthylazine (<400 nM). However, Fv/Fm also responded rapidly and in a dose-dependent way to toxins glyphosate (<90 μM), and KCN (<1 mM) which have modes of action not specific to photosynthesis. Fv/Fm was insensitive to 30–40 μM insecticides methyl parathion, carbofuran and malathion. Algal species varied in their sensitivity to toxins. No single species was the most sensitive to all nine toxins, but for six toxins to which algal Fv/Fm responded significantly, the model species P. subcapitata was less sensitive than other taxa. In terms of suppression of Fv/Fm, within 80 min, patterns of concentration-dependence differed among toxins; most showed Michaelis–Menten saturation kinetics, with half-saturation constant (Kₘ) values for the PSII inhibitors ranging from 0.14 μM for Diuron® to 6.6 μM for terbuthylazine, compared with a Kₘ of 330 μM for KCN. Percent suppression of Fv/Fm by glyphosate increased exponentially with concentration. Fv/Fm provides a sensitive and easily-measured parameter for rapid and cost-effective detection of effects of many dissolved toxins. Field-portable fluorometers will facilitate field testing, however distinct responses between different species may complicate net Fv/Fm signal from a community.

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Abbreviations: Chlorophyll, chl; PAM, pulse amplitude modulated; PSII, photosystem II; Fv/Fm, maximum quantum yield of PSII; ΦPSII, quantum yield of photosystem II.
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1. Introduction

Microalgae are commonly used for aquatic toxicological testing because they are easy to grow and expose to dissolved toxins in culture, they are sensitive to hazardous chemicals or wastewater contaminants, and they have short generation times that allow rapid assessment of growth responses to toxins. United States Environmental Protection Agency (USEPA) and the Organisation for Economic Co-operation and Development (OECD) have standardized tests that measure toxicity of dissolved chemicals as inhibition of growth over 3–4 days in replicate microalgal cultures, typically using model test species, including the green alga *Pseudokirchneriella subcapitata* (Korshikov) Hindak (formerly *Selenastrum capricornutum*) (OECD, 1984; USEPA, 2002). Although microalgae and other model organisms (including vertebrates) are important for characterizing toxin effects under controlled conditions, growth and maintenance of test organisms can be time consuming and expensive, and correct end-point measurements for meaningful comparisons are not easily predicted (Hughes et al., 1988). For detection of environmental contamination, and in water security applications, there is a need to find cheaper and faster testing procedures which provide reliable and ecologically-relevant detection of aquatic toxins (Galloway et al., 2004). This has led to the development of instrumentation for rapid testing including immobilized algae (Bozeman et al., 1989), miniaturized microplate and microbiotest assays using algae (Lukovsky, 1992; Muller et al., 2008), array-chip multi-algal biosensors (Pedola and Melkonian, 2005) and in-line algal biomonitors (recent advances in applications reviewed by Ralph et al., 2007; Buonasera et al., 2011) These new developments offer advantages over the USEPA standard 3–4 day growth bioassays, but can still require extensive set-up and extended incubation periods.

Advances in technologies to measure chlorophyll *a* (chl *a*) fluorescence offer good prospects for toxicity testing using bioassays with algal species (Brack and Frank, 1998; Buonasera et al., 2011). Chl *a* fluorescence provides a sensitive integrated measure of a key photosynthetic process - the efficiency of energy conversion at photosystem II (PSII) reaction centers. Chl *a* fluorescence parameters respond to environmental stresses, including nutrient limitation (Parkhill et al., 2001; Young and Beardall, 2003), high light or UV exposure (Franklin and Forster, 1997) and oxidative stress (Drábková et al., 2007). Rapid changes in chl *a* fluorescence parameters have previously been shown to provide similar information to suppression of photosynthetic C fixation (Dorigo and Leboulanger, 2001), and growth inhibition over 3–4 d (e.g. Fai et al., 2007; Macedo et al., 2008; Ralph et al., 2007; Kviderová, 2009). Pulse Amplitude Modulated (PAM) fluorometers offer one approach to very high sensitivity measurement of chlorophyll *a* fluorescence in cells for use with natural marine or freshwater phytoplankton samples, and are increasingly being applied to toxicity testing (Schreiber et al., 1993, 2002). Studies have frequently focused on a small number of toxins or a single test species (often *P. subcapitata* or other species commonly used in laboratory studies) and have examined responses of chl *a* fluorescence to toxins over periods of 5–72 h (e.g. Brack and Frank, 1998; Juneau et al., 2002; Vallotton et al., 2008; Kviderová, 2009). However, chl *a* fluorescence changes much more rapidly in response to stress (including exposure to toxic chemicals (Rodriguez et al., 2002; Macedo et al., 2008) and so responses over minutes rather than hours, are potentially useful for rapid toxicological assessments (Rodriguez et al., 2002; Schreiber et al., 2002; Rodriguez and Greenbaum, 2009).

Of a range of chl *a* fluorescence parameters which respond rapidly to toxin exposure, the most commonly used is the quantum yield of PSII, a measure of photochemical efficiency (Ralph et al., 2007; Buonasera et al., 2011). Changes in PSII quantum yield could be used to examine toxin exposure in natural freshwater algal assemblages over less than 25 min, with potential applications for real-time testing and water security applications (Schreiber et al., 1993; Rodriguez et al., 2002; Buonasera et al., 2011). However, successful application of chl *a* fluorometry to toxicity testing in natural ecosystems requires much more detailed understanding of the variability of responses of Fv/Fm to both a wider range of toxins and in a wider range of species.

In the present study, we aimed to examine the variability of responses of Fv/Fm in diverse algal species in response to a range of toxins. Our specific objectives were: 1) to assess the range of responses of different algal taxa, particularly how sensitivities of common algal taxa compare with that of laboratory model organisms, 2) to identify which toxins elicit a response in Fv/Fm and over what concentration ranges, and 3) to clarify whether sensitivity of Fv/Fm responses is related to specific toxins via examination of changes in chl *a* fluorescence parameters F0 and Fm. We selected five freshwater algal species representing four broad taxonomic groups commonly found in temperate freshwater ecosystems (chlorophytes, diatoms, chrysophytes and cryptophytes), and included the EPA model chlorophyte species *P. subcapitata* for comparison. We exposed the microalgae to nine environmental pollutants (some with specific effects on photosynthesis, as well as those with other modes of action) with potential for contamination in natural waterways or reservoirs (Gilliom et al., 1999; Rodriguez et al., 2002), and measured very short-term changes in Fv/Fm.

2. Materials and methods

2.1. Test organisms and culture conditions

Freshwater algal taxa tested represented four distinct taxonomic groups — Chlorophyta, Bacillariophyta, Cryptophyta and Heterokontophyta (Wehr and Sheath, 2003). Cultures of the algae were obtained from the Canadian Phycological Culture Collection (CPCC, www.phycoll.ca) and all cultures were maintained axenically in the same growing conditions (18 °C, 100 μmol photons m−2 s−1 on a 12:12 light:dark cycle, gently stirred and bubbled with filtered air). Triplicate cultures of each species were diluted with growth medium every 3–5 days to keep cells in exponential growth phase. The green alga *Chlamydomonas reinhardti* Dang. (CPCC 84 syn. UTEX 89) was grown in DY-V freshwater medium pH 7.0 (Andersen, 2005).
The green alga *P. subcapitata* (Korshikov) Hindak (CPCC 37, formerly known as *S. capricornutum* or *Raphidocelis subcapitata* syn. UTEX 1648), the diatoms *Navicula pelliculosa* (Breb.) Hilsie (CPCC 552 syn. CCAP 1050/3c) and *Asterionella formosa* Hass. (CPCC 605), the cryptophyte *Cryptomonas erosa* Ehrenberg (CPCC 446) and the chrysophyte *Syrnua peterseni* Korshikov (CPCC 442 syn. CCMP 866) were all grown in CHU-10 medium, pH 7.0 (Andersen, 2005). DY-V and CHU-10 are artificial freshwater media with similar constituents but we found *Chlamydomonas* grew better in DY-V than CHU-10; DY-V contains MES or MOPS but CHU-10 does not include an organic pH buffer (Andersen, 2005). Cell growth was monitored by changes in bulk chl a fluorescence measured in a Turner TD-700 fluorometer with red-sensitive PM tube and a chl a optical kit (Turner Designs, Sunnyvale, CA, USA). When cultures reached mid log-phase, 20 mL of cultures were transferred to 50 mL tubes for toxin exposure and chl a fluorescence measurements.

### 2.2 Toxins

We tested nine toxins representing six chemical classes including those with modes of action directly on photosynthetic processes and photosystem II (PSII) (atrazine, metribuzin, terbuthylazine and Diuron\(^\text{a}\)) and others with modes of actions not directly related to photosynthesis (KCN, glyphosate, carbofuran, malathion and methyl parathion) (Table 1). The toxins atrazine, metribuzin, and terbuthylazine (triazine herbicides), carbofuran (a carbamate pesticide), malathion and methyl parathion (organophosphate pesticides) were obtained from SPEX Certiprep (Metuchen, NJ, USA), and glyphosate (a phosphonoglycine herbicide) (Restek, Bellefonte, PA), all supplier-certified as 99% pure, and KCN (Fisher Scientific, Hannover Park, IL, USA) and Diuron\(^{\text{b}}\) (DCMU, a phenyl-urea herbicide) (Sigma–Aldrich Corp. St. Louis, MO, USA), certified as ≥98% pure, were also tested. Toxins were dissolved in water, acetone, ethanol or methanol according to their solubility (see Table 1) and small volumes (<1% of culture volume) of toxin solutions were added directly to the 20 mL culture subsample and gently mixed. Final toxin concentrations tested ranged from 1 nM to 30 mM. To test for effects of the solvent without the toxin, control trials for each species were carried out with solvents alone at the highest volume added (0.2 mL solvent in 20 mL culture). All species-toxin concentration combinations were tested using triplicate cultures.

### 2.3 Chl a fluorescence and cell viability measurements

Chl a fluorescence was measured using a Walz Pulse Amplitude Modulated XE-PAM fluorometer (Schreiber et al., 1993) (Walz GmbH, Effeltrich, Germany). The excitation light was filtered using a 5 mm BG39 band-pass filter (500 nm peak; Schott Elmsford, NY, USA). Fluorescence emission was filtered by a 1 mm R65 long-pass (>650 nm) dichroic filter (Balzers, Zarentem, Belgium). Preliminary trials were carried out to determine the effect of dark acclimation prior to addition of toxins. Dark incubation either increased the quantum yield or had no effect, so for all species the following exposure and measurement protocol was used. Immediately after harvesting from the culture, cells were incubated for 30 min in the dark at 18–22 °C with gentle shaking, prior to initial measurement of chl a fluorescence. In dark-incubated cells, the Xe-PAM fluorometer measured F\(_{0}\) - the minimal chl a fluorescence output, and F\(_{m}\) - the maximal chl a fluorescence output during a saturating pulse, and derived \(\Phi_{\text{max}}\) the maximum quantum yield of PSII (Schreiber et al., 1993; Parkhill et al., 2001; Ralph et al., 2007), hereafter referred to as \(F_{v}/F_{m}\):

\[
\Phi_{\text{max}} = F_{v}/F_{m} = (F_{m} - F_{0})/F_{m} \tag{1}
\]

The percent suppression of \(F_{v}/F_{m}\) over time in response to toxins was calculated as:

\[
\% \text{ suppression} = (F_{v}/F_{m,0} - F_{v}/F_{m,t})/F_{v}/F_{m,0} \times 100 \tag{2}
\]

where \(F_{v}/F_{m,0}\) is the initial \(F_{v}/F_{m}\) at time 0, prior to toxin exposure, and \(F_{v}/F_{m,t}\) is the \(F_{v}/F_{m}\) after exposure time \(t\) (2–80 min) to the toxin. For each culture subsample tested, cell density and PAM settings were adjusted to give similar initial \(F_{0}\) output across trials.

Measurements were made just prior to toxin addition and then at 2, 5, 10, 20, 40, and 80 min after toxin addition. During exposure to toxins, samples were incubated in the dark in a shaking incubator at 18–22 °C, and subsamples were removed for each time-point measurement in a darkened room. The exception was for Diuron\(^{\text{b}}\) which required low irradiance (<5 \(\mu\text{mol}\) photons m\(^{-2}\) s\(^{-1}\)) during the 80 min exposure to be effective. However, the initial \(F_{v}/F_{m}\), prior to Diuron\(^{\text{b}}\) addition, was determined as for other toxins, following 30 min dark incubation.

In order to examine more detailed dose–response relationships of the \(F_{v}/F_{m}\) suppression in response to 6 of the toxins, a sub-set of the toxin–species combinations examined were tested with a finer resolution of toxin concentrations.

When significant inhibitory effects of toxin were detected after 80 min, cell viability was assessed in 1 mL of culture by adding 20 \(\mu\text{L}\) of 1% (w/v) Evans Blue and incubating for 5 min before observing and enumerating cell staining using an Olympus BX-41 microscope. Evans Blue is impermeable to live cells but freely penetrates the membrane of dead cells and stains blue (Crippen and Perrier, 1974), thus blue-stained cells were identified as dead, with heat-killed cultures (80 °C for 10 min) serving as positive controls.

### 2.4 Statistical analysis of toxin effects

For each species and toxin concentration, the \(F_{v}/F_{m}\) measured at time zero (immediately before toxin addition), and at 80 min were compared for significant differences using analysis of variance (1-way ANOVA of \(F_{v}/F_{m}\) variables with time as the factor, for each toxin–species combination). When significant declines were observed, the inhibitory effects of different toxins in the six algal species were examined by plotting \(F_{v}/F_{m}\) against time over 80 min. Most plots showed an exponential decline in \(F_{v}/F_{m}\) over time so the time axis was log-transformed to linearize the plots. A linear model was fitted to the decline and the slope of line tested for significant differences from zero. Toxin sensitivity was evaluated as
Table 1 – The toxins tested and solvents used, their chemical names, classes, modes of action and the summary toxicity determined in other studies.

<table>
<thead>
<tr>
<th>Toxin (solvent)</th>
<th>IUPAC chemical name</th>
<th>Chemical class, use, CAS number, EPA toxin class&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mode of action</th>
<th>LC50&lt;sup&gt;b&lt;/sup&gt; aquatic vertebrates µg mL&lt;sup&gt;−1&lt;/sup&gt;</th>
<th>EC50 green algae&lt;sup&gt;b&lt;/sup&gt; µg mL&lt;sup&gt;−1&lt;/sup&gt;</th>
<th>Conc. range used µg mL&lt;sup&gt;−1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diuron&lt;sup&gt;®&lt;/sup&gt; (ethanol)</td>
<td>3-(3,4-dichlorophenyl)-1, 1-dimethylurea (DCMU)</td>
<td>Phenyl-urea herbicide 330-54-1, EPA III</td>
<td>PS II inhibitor</td>
<td>6.7–47</td>
<td>0.005</td>
<td>0–58</td>
</tr>
<tr>
<td>Glyphosate (water)</td>
<td>N-(phosphonomethyl) glycine</td>
<td>Phosphonoglycine herbicide 1071-83-6, EPA II</td>
<td>Aromatic amino acid synthesis inhibitor</td>
<td>5–240</td>
<td>7–400</td>
<td>0–750</td>
</tr>
<tr>
<td>KCN (water)</td>
<td>Potassium cyanide</td>
<td>Inorganic rodenticide 151-50-8, EPA I</td>
<td>Oxidative metabolism inhibitor (mitochond.)</td>
<td>0.065–1.4</td>
<td>0.025–0.045</td>
<td>0–1950</td>
</tr>
<tr>
<td>Atrazine (acetone)</td>
<td>6-chloro-N&lt;sup&gt;2&lt;/sup&gt;-ethyl-N&lt;sup&gt;4&lt;/sup&gt;-isopropyl-1, 3,5-triazine-2,4-diamine</td>
<td>Triazine herbicide 1912-24-9, EPA III</td>
<td>PS II inhibitor</td>
<td>5–50</td>
<td>0.04–1.5</td>
<td>0–10</td>
</tr>
<tr>
<td>Metribuzin (methanol)</td>
<td>4-amino-6-tert-butyl-3-methylthio-1, 2,4-triazin-5(4H)-one</td>
<td>Triazinone herbicide 21087-64-9, EPA III</td>
<td>PS II inhibitor</td>
<td>0.030–0.15</td>
<td>0.043</td>
<td>0–5</td>
</tr>
<tr>
<td>Terbutylazine (methanol)</td>
<td>N&lt;sup&gt;2&lt;/sup&gt;-tert-butyl-6-chloro-N&lt;sup&gt;4&lt;/sup&gt;-ethyl-1,3, 5-triazine-2,4-diamine</td>
<td>Triazine herbicide algaecide, microbiocide 5915-41-3, EPA III</td>
<td>PS II inhibitor</td>
<td>7–8</td>
<td>0.016</td>
<td>0–50</td>
</tr>
<tr>
<td>Methyl parathion (methanol)</td>
<td>O,O-dimethyl O-4-nitrophenoxy-phosphorothioate</td>
<td>Organothiophosphate insecticide 298-00-0, EPA I</td>
<td>Cholinesterase inhibitor</td>
<td>0.8–25</td>
<td>2.9–100</td>
<td>0–10</td>
</tr>
<tr>
<td>Carbofuran (methanol)</td>
<td>2,3-dihydro-2,2-dimethylbenzo-furan-7-yl-methylcarbamate</td>
<td>Carbamate insecticide nematicide 1563-66-2, EPA I/II</td>
<td>Cholinesterase inhibitor</td>
<td>0.1–5</td>
<td>270</td>
<td>0–10</td>
</tr>
<tr>
<td>Malathion (methanol)</td>
<td>Diethyl (dimethoxyphos-phinothiolio) succinate</td>
<td>Organothiophosphate insecticide 121-75-5, EPA III</td>
<td>Cholinesterase inhibitor</td>
<td>0.3–15</td>
<td>0.01</td>
<td>0–10</td>
</tr>
</tbody>
</table>

<sup>a</sup> EPA Toxin classes: I (highly toxic), II (moderately toxic), III (slightly toxic).

<sup>b</sup> Summary LC50 (median lethal dose) and EC50 (half maximum effective concentration) values from EcotoxNet (2010) and PAN (2010).
percent suppression of $F_v/F_m$ at 80 min relative to $F_v/F_m$ values just prior to toxin addition. To examine toxin sensitivity over a finer range of toxin concentrations, the dose–response relationships of selected species to 6 of the toxins were analyzed as % suppression of $F_v/F_m$, and the relationships were modeled with non-linear regression, using a rectangular hyperbola (for Michaelis–Menten kinetics), or, in one case where the data clearly did not follow saturation kinetics, an exponential model. All statistical analyses used Sigmastat (v. 3.1, Systat Software Inc, Chicago, IL).

3. Results and discussion

3.1. Rapid toxin detection using $F_v/F_m$

The chl $a$ fluorescence parameter $F_v/F_m$ of algal cells provided an extremely rapid method to detect responses to several aquatic toxins in the EPA model test species $P. subcapitata$, and a range of common freshwater algal taxa. In each case, for the controls with solvent-only additions, the slope of regression lines for $F_v/F_m$ over time was not significantly different from zero ($P > 0.05$ ANOVA). However, following exposure to 6 of the 9 toxins tested, $F_v/F_m$ declined significantly over < 80 min ($p < 0.05$ ANOVA) (Fig. 1, Table 2). In response to atrazine, metribuzin (Fig. 1) and terbuthylazine, $F_v/F_m$ declined rapidly over ~10 min but thereafter remained stable. $F_v/F_m$ decline in $S. petersenii$, $N. pelliculosa$, $A. formosa$ and $C. erosa$ in response to KCN or glyphosate continued almost throughout the full 80 min (Fig. 1). Most of the suppression of $F_v/F_m$ was observed within 20 min following toxin addition (Fig. 1), and 80 min certainly could serve as an appropriate duration for a bioassay based on algal $F_v/F_m$ suppression. $F_v/F_m$ was not significantly suppressed in most algal species in response to the cholinesterase inhibitors methyl parathion, carbofuran and malathion (Table 2) demonstrating that these pesticides, which inhibit nerve function in insects, will not readily induce rapid responses in $F_v/F_m$ in microalgae.

Despite strong toxin effects on $F_v/F_m$, there was little evidence of cell mortality within the 80 min exposure for any species. Only in cultures exposed to very high glyphosate concentrations (400 μM) did Evans Blue staining indicate compromised cell membranes in approximately 30–50% of cells of each species. The rapid responses of $F_v/F_m$ over 80 min therefore represent sub-lethal effects on algal cells.

3.2. Algal species differences

To address our objective of determining algal species sensitivity to toxins, we found that there clearly were different sensitivities between algal species, but no single algal taxon was the most sensitive to all nine toxins (Fig. 1; criteria defined in Table 2). For example, based on percent suppression of $F_v/F_m$ in response to the toxins glyphosate, KCN, atrazine, metribuzin and terbutylazine, the EPA model species $P. subcapitata$ was not the most sensitive algal taxon (Fig. 1, Table 2). Therefore, we predict that use of data on toxin suppression of $F_v/F_m$ from the model species $P. subcapitata$ will result in underestimating the sensitivities of photosynthesis in commonly found freshwater species or natural assemblages.

Fig. 1 – A range responses of $F_v/F_m$ to exposure to toxins in six microalgal species. Suppression of $F_v/F_m$ is shown over 80 min following addition at 0 min of A) glyphosate (440 μM), B) KCN (1 mM), C) atrazine (460 nM) and D) metribuzin (40 nM) Species symbols shown in panel D are the same for all plots. Points are means ± standard error of measurements in three replicate trials.
Differences in algal sensitivity to a range of toxins have been reported (Juneau et al., 2002; Fairchild et al., 1998) and may relate to genetically-determined mechanisms for assimilation and detoxification or to variable toxin uptake kinetics and capacity between algae (Weiner et al., 2004) which in turn depend on the presence of other ions such as Cl (Lee et al., 2005). Rapid screening methods for toxin detection using algal Fv/Fm responses could readily incorporate a wider range of the most sensitive or ecologically important species.

Broad taxonomic group was not a good indicator of toxin sensitivity (Fig. 1, Table 2). The two chlorophytes, P. subcapitata and C. reinhardtii showed divergent responses of Fv/Fm to toxin exposure. P. subcapitata was highly sensitive to KCN, relatively insensitive to glyphosate and atrazine while C. reinhardtii was relatively insensitive to KCN but highly sensitive to glyphosate and atrazine (Fig. 1, Table 2). The two diatoms, A. formosa and N. pelliculosa also showed different sensitivity to toxins. Both species were highly sensitive to glyphosate but while A. formosa was less sensitive to atrazine, it was more sensitive to methyl parathion (Table 2). In conventional multi-day algal growth bioassays, N. pelliculosa growth was more sensitive to atrazine than growth of a marine green alga Dunaliella tertiolecta (Hughes et al., 1988) but for Fv/Fm, N. pelliculosa was less sensitive than C. reinhardtii (Table 2). The differences in responses to toxins between groups suggest that no one species is likely to provide the ideal, most sensitive test organism for all the toxin types tested. However, the recommended ideal, to include a suite of test species (Fairchild et al., 1998), becomes more practical with such rapid screening methods based on Fv/Fm, while this would be more expensive and less practical for standard 3–4 day growth inhibition assays.

Taxa differences in Fv/Fm sensitivities to toxins suggests that, in mixed algal species assemblages, suppression of Fv/Fm in more sensitive species could potentially be masked by chl a emission from less sensitive species (see discussion in Franklin et al., 2009). This, along with effects of nutrient limitation, temperature variability and photoacclimation or photoinhibition processes in natural conditions (Holmes et al., 1989; Franklin and Forster, 1997; Chalifour and Juneau, 2011), may complicate application of this method to natural phytoplankton assemblages (e.g. Rodriguez and Greenbaum, 2009). We initially screened three cyanobacterial taxa in our experiments, but optimizing Fv/Fm in cyanobacteria using PAM is problematic (Campbell et al., 1998). We tried different filters (620–630 nm band-pass filters) to optimize the excitation and emission wavelengths to increase Fv/Fm values from cyanobacterial cultures, but were not satisfied with the results so we restricted our measurements to eukaryotes. Lower and more variable Fv/Fm values in cyanobacteria could also complicate interpretation of net Fv/Fm responses to toxins in natural populations which include cyanobacteria.

One ecological consequence of variable toxin sensitivity is that sub-lethal effects of toxin contamination of natural waters may influence competition and stimulate changes in species composition of natural phytoplankton assemblages (Seguin et al., 2002; Zananski et al., 2010). In contaminated waters where species composition of natural populations has acclimated to chronic toxin exposure, Fv/Fm may not
change in response to experimental toxin exposure as dramatically as in individual test taxa with no previous exposure history.

3.3. Toxin effects on $F_0$ and $F_m$

As $F_v/F_m$ ratios are derived from measured parameters $F_0$ and $F_m$ (eq. (1)), changes in $F_v/F_m$ can involve changes in one or both parameters (Table 3). In response to DCMU, both $F_0$ and $F_m$ increased in all species. This is the basis for Diuron\textsuperscript{a} (DCMU) use in measuring maximum chl $a$ fluorescence emission (Parkhill et al., 2001), but responses of $F_0$ and $F_m$ to other toxins have been less well documented. Changes in $F_0$ vs $F_m$ varied between algal species (Table 3). For example, in response to KCN and glyphosate, in all species, $F_v/F_m$ declines involved decreases in $F_m$. In contrast, in response to glyphosate, $F_0$ declined in all species except $A$. formosa, but the $F_0$ increased, decreased or was unchanged in response to KCN in different species (Table 3). Between closely related Chlorophyta species, in response to atrazine, $F_0$ and $F_m$ both increased in $P$. subcapitata, but $F_0$ was almost stable and $F_m$ declined in $C$. reinhardtii (Fig. 2). In responses to metribuzin, $F_v/F_m$ declines involved increases in $F_0$ in all species, but no consistent changes in $F_m$ between species including the two green algae.

Differences in contribution of $F_0$ vs $F_m$ to $F_v/F_m$ suppression reflect different toxin modes of action. Differences between even closely related taxa may indicate that responses to specific toxin action on photosynthesis vary. Short-term changes in chl $a$ fluorescence emission over $<$80 min (i.e. responses not dependent on de novo protein synthesis), reflect changes in energy flux through chlorophyll harvesting antennae (LHCII), specific inhibition of photosynthetic electron transport, or changes in use of ATP and NADPH products of electron transport. Increases in $F_0$ could relate to specific inhibition of electron transport (PSII, plastoquinone pool), induced by the herbicides. These expected $F_0$ increases were observed with all sensitive species with Diuron\textsuperscript{a}, terbutylazine and metribuzin but only with $P$. subcapitata and $C$. erosa in response to atrazine. With glyphosate, $F_0$ declines could relate to impaired light absorption or uncoupling of electron transport, while decreases in $F_m$ observed consistently with both glyphosate and KCN, could relate to uncoupling of LCHII from PSII via energy-dependent non-photochemical quenching (qN) (Holmes et al., 1989; Young and Beardall, 2003). However, as incubations were in the dark (except Diuron\textsuperscript{a}), any qN changes will not relate to actinic light-dependent energy responses, but may reflect direct chemical effects on thylakoid pH gradient dissipation, possibly via perturbations of ATP and NADPH use (Holmes et al., 1989; Young and Beardall, 2003). Quenching parameters have been specifically examined to investigate toxin modes of action (Brack and Frank, 1998; Juneau and Popovic, 1999; Juneau et al., 2002, 2005; Fai et al., 2007). Our data suggest that as both $F_0$ and $F_m$ can change, suggesting that steady state F output also varies during incubation, quenching parameters may be complicated to interpret between taxa (see discussion in Juneau and Popovic, 1999; Juneau et al., 2005). Therefore, for rapid toxicological assessment using a range of species, maximum quantum yield may be a more straightforward parameter than any of the quenching parameters.

3.4. Toxin sensitivity - dose-responses

There were clear differences in the dose-responses of suppression of $F_v/F_m$ among different toxins tested (Fig. 3) and minimum toxin concentrations required for a significant decline in $F_v/F_m$ over 80 min in each species (Table 2; selected species in Fig. 3). Although concentrations tested were not always comparable between toxins (due to different detection ranges, modes of action and solubility limitations), generally, $F_v/F_m$ in the test algae was less sensitive to suppression by organo(nitro)phosphate or carbamate pesticides than to the triazine herbicides and phenyl-urea herbicides, that specifically inhibit PSII and electron transport. Some of the effects of specific PSII toxins (atrazine, metribuzin, terbutylazine, Table 1) on chl $a$ fluorescence parameters in microalgae, have been reported in some algal taxa (Brack and Frank, 1998; Fai et al., 2007; Vallotton et al., 2008; Dorigo and Leboulanger, 2001). However, this study also shows significant dose-dependent suppression of $F_v/F_m$ by toxins KCN and glyphosate, which are not known to directly inhibit PSII. To address the objective of determining the concentration ranges over which different toxins affect $F_v/F_m$, each toxin is discussed below.

3.4.1. Diuron\textsuperscript{a}

The specific inhibition of electron transport from PSII to plastoquinone by Diuron\textsuperscript{a} (DCMU) resulted in extremely high sensitivities of $F_v/F_m$ in all species to Diuron\textsuperscript{a} (minimum effect

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**Table 3** Summary of toxins effects on $F_0$ and $F_m$ for each species for which there were significant suppression of $F_v/F_m$ during 80 min exposure (see Table 2). $+$ = increase, $0/+ = $ slight increase, $0 = $ no change, $- = $ decrease, $0/- = $ slight decrease. Blank spaces indicate non-significant responses of $F_v/F_m$ (see Table 2).

<table>
<thead>
<tr>
<th>Algal species</th>
<th>Diuron\textsuperscript{a}</th>
<th>Glyphosate</th>
<th>KCN</th>
<th>Atrazine</th>
<th>Metribuzin</th>
<th>Terbutylazine</th>
<th>Methyl parathion</th>
<th>Carbofuran</th>
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<tr>
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half maximal inhibitory concentration (IC50) concentrations. Maximum suppression in response to metribuzin and terbuthylazine was only 35–42% (Figs. 1 and 3E,F) in contrast to suppression in response to Diuron® which approached 80% of initial values. This might suggest that binding of these herbicides may be less effective than for Diuron® or that toxic effect is limited by cell uptake capacity (Weiner et al., 2004).

3.4.4. Glyphosate
Glyphosate had strong effects on Fv/Fm in all species except P. subcapitata, resulting in almost 100% suppression in N. pelliculosa (Fig. 1). Glyphosate inhibits aromatic amino acid synthesis derived from the shikimate pathway (Funke et al., 2006) rather than PSII directly, so the rapid and dramatic inhibitory effect of glyphosate on Fv/Fm (Fig. 1, Table 1) may involve disruption of integrated carbon and nitrogen assimilation which serves as a sink for photochemically derived energy (Holmes et al., 1989; Young and Beardall, 2003). The consistent effects of glyphosate on Fm (declined in all but A. formosa) and Fm (declined in all species) (Table 3) also suggests some consistent mechanism leading to suppression of Fv/Fm in all algal species by glyphosate. Fv/Fm in the EPA model species P. subcapitata was the least sensitive to glyphosate while Fv/Fm in A. formosa was the most sensitive in response to glyphosate (threshold <90 μM (15 mg L⁻¹)). In A. formosa, suppression of Fv/Fm by glyphosate was not saturated over 0–440 μM but increased exponentially (Fig. 3B), the only toxin to show this dose–response relationship. In comparison to the specific PSII inhibitors (Diuron®, atrazine, metribuzin, terbuthylazine), which saturated after ~10 min (Fig. 1C,D), the decline in Fv/Fm with glyphosate continued over a longer time period in the most sensitive species (Fig. 1A), suggesting that glyphosate effects on PSII might result from cumulative toxic effects on other cellular processes. The threshold concentrations for effects of glyphosate on Fv/Fm in the algae (90–440 μM (15–75 μg mL⁻¹)) were similar to typical median lethal doses (LC50) over 96 h for fish (Fig. 3B, Tables 1and 2) so Fv/Fm responses in microalgae may be suitable to detect rapid and sensitive responses to glyphosate. Glyphosate-based herbicides (e.g. Roundup®) are the most widely used herbicides globally for agricultural and urban weed control and are readily transported into aquatic systems via surface runoff (Byer et al., 2008). The variable sensitivity observed among algal taxa and known insensitivity to glyphosate in some cyanobacteria (Powell et al., 1991) suggests that glyphosate contamination in freshwater ecosystems may promote changes in species dominance.

3.4.5. KCN
Suppression of Fv/Fm by KCN in P subcapitata was virtually 100%, and shown to saturate at ~2 mM in P subcapitata with a Km of 330 μM (Fig. 3C). Rapid declines in Fv/Fm in natural algal assemblages have been shown in response to 2 mM (130 μg mL⁻¹) KCN (Rodriguez et al., 2002) but in this study, five algal species showed threshold effect concentrations of less than 1 mM (65 μg mL⁻¹) (Table 2). Cyanide directly inhibits cytochrome oxidase in mitochondrial electron transport so KCN may affect PSII and thus chl a fluorescence indirectly via a disruption of cellular energy metabolism which links mitochondrial and chloroplast processes (Holmes et al., 1989).

Compared to the specific PSII inhibitors which had fast effects on $F_v/F_m$ (mostly within 10 min of addition, Fig. 1 C,D) suppression by KCN continued over the full 80 min in most sensitive species (Fig. 1B), which may relate to delayed effects via inhibition of non-photosynthetic processes, resulting in escalating toxic effects on cell function over time. It is possible that in some algal species, cyanide could have more direct effects on chl a fluorescence via inhibition of photosynthesis enzymes Rubisco (observed in spinach by Wishnick and Lane (1969)) and a chlororespiration oxidase (observed in a xanthophyte alga by Buchel and Garab (1995), but if so, KCN should have induced an increase in $F_m$, but it declined in all species tested (Table 3). Possible toxic effects in chloroplasts may remain throughout the experiment, but partial recovery of $F_v/F_m$ observed in N. pelliculosa, C. reinhardtii and P. subcapitata (Figs. 1B and 4) suggests induction of a mitochondrial

Fig. 3 – The maximum suppression of $F_v/F_m$ after 80 min, relative to initial values prior to exposure to a range of concentrations for toxins A) Diuron, B) glyphosate, C) KCN, D) atrazine, E) metribuzin and (F) terbuthylazine. Plots are shown for species for which the widest range of doses was examined. Each point is a single determination. Lines were fitted by non-linear regression, using a rectangular hyperbola (Michaelis–Menten) (A, C-F) or an exponential (B), model. For the Michaelis–Menten models, estimates of maximum suppression (Max) and half-saturation constants ($K_m$) are shown on the graphs. A–C are on the same y axis scale, and D–F on the same y axis scale. $r^2$ values for non-linear models are asymptotic values.
alternative oxidase pathway, which is widespread in algal taxa (Eriksen and Lewitus, 1999). The relatively lower sensitivity of C. reinhardtii to KCN (Table 2) may relate to more efficient induction of this alternative pathway. Despite these clear dose–response relationships, the algae were relatively insensitive to KCN, requiring much higher concentrations of KCN to examine effects on Fv/Fm than for the other toxins, especially the PSII inhibitors (Fig. 3). For comparison, model fish species showed a minimum toxic dose during 2–4 days of cyanide exposure three orders of magnitude lower (0.15–0.3 μM, 0.01–0.02 μg mL$^{-1}$) than for multi-day exposure in algae (USEPA Acquire database, 2011), suggesting that algae are not sensitive test organisms for studies of cyanide.

4. Conclusions and significance

- Diverse freshwater algal taxa showed variable sensitivity of Fv/Fm suppression in response to nine toxins; there was no one taxon which was the most sensitive, and lower sensitivity of the laboratory model organism P. subcapitata than other taxa suggest that for studies on these toxins, using a suite of algal species will give more ecologically representative responses.
- Rapid suppression in Fv/Fm offers considerable time-saving advantages over traditional 3–4 day growth bioassays, allowing a larger number of algal species, toxins and toxin concentrations to be tested in the laboratory, with fewer resources.
- While PSII inhibitor toxins were expected to have strong effects on chl a fluorescence parameters, rapid Fv/Fm suppression in response to glyphosate and KCN, which do not directly target PSII, suggest that Fv/Fm changes can reflect whole cell physiological stress.
- Bioassays based on rapid Fv/Fm changes offer most promise in laboratory testing of toxins, where multiple species selection can be customized to the ecosystem or taxa of interest; field-portable fluorometers will facilitate field testing, but distinct responses between species will complicate interpretation of net Fv/Fm signals from a community.

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References


Fig. 4 – Changes in Fv/Fm in P. subcapitata in response to KCN illustrating partial recovery of Fv/Fm following rapid initial suppression. Each point represents measurements in subsamples of three replicate cultures exposed to KCN ± standard error. Dose of KCN (0–30 mM) shown next to each response plot.


