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Comparative effects of herbicides on photosynthesis and growth of tropical estuarine microalgae

Marie Magnusson [a,b], Kirsten Heimann [b,*], Andrew P. Negri [c]

[a] AIMS@JCU, Australian Institute of Marine Science, School of Marine and Tropical Biology, James Cook University, Townsville, QLD 4811, Australia
[b] School of Marine and Tropical Biology, James Cook University, Douglas Campus, Townsville 4811, Australia
[c] Australian Institute of Marine Science, PMB 3 Townsville MC, QLD 4810, Australia

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ABSTRACT

Pulse amplitude modulation (PAM) fluorometry is ideally suited to measure the sub-lethal impacts of photosystem II (PSII)-inhibiting herbicides on microalgae, but key relationships between effective quantum yield [*Y*(*II*)] and the traditional endpoints growth rate (μ) and biomass increase are unknown. The effects of three PSII–inhibiting herbicides; diuron, hexazinone and atrazine, were examined on two tropical benthic microalgae; *Navicula* sp. (Heterokontophyta) and *Nephroselmis pyriformis* (Chlorophyta). The relationships between *Y*(*II*), μ and biomass increase were consistent ($r^2 \geqslant 0.90$) and linear (1:1), validating the utility of PAM fluorometry as a rapid and reliable technique to measure sub-lethal toxicity thresholds of PSII-inhibiting herbicides in these microalgae. The order of toxicity (EC_{50} range) was: diuron (16–33 nM) > hexazinone (25–110 nM) > atrazine (130–620 nm) for both algal species. Growth rate and photosynthesis were affected at diuron concentrations that have been detected in coastal areas of the Great Barrier Reef.

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1. Introduction

Extensive use of pesticides in agriculture is accompanied by the risk of environmental contamination. The Queensland coast of Australia has an intensive agricultural industry and over 80% of the Great Barrier Reef (GBR) catchment area supports some form of agriculture (Gilbert and Brodie, 2001), with 40 drainage catchments that discharge directly into the GBR lagoon. Diuron (phenylurea), atrazine (*s*-triazine) and hexazinone (triazonone) are among the most commonly used herbicides in Australian agriculture (Hamilton and Haydon, 1996; Radcliffe, 2002). Recent studies have demonstrated herbicide contamination of water and sediments in a large number of samples taken along the Queensland coast (Haynes et al., 2000b; Mitchell et al., 2005; Shaw and Müller, 2005; Rohde et al., 2006). Coastal pollution from pesticides is also a global issue. For instance, atrazine was present in estuarine sediments of the Adriatic Sea eight years after the implementation of a total ban of its use in Italy (Carafa et al., 2007). Sources of herbicide contamination in estuaries may also include antifouling paints, which often contain diuron and irgarol (triazine) boosters (Konstantinou and Albanis, 2004).

Diuron, atrazine and hexazinone are all photosystem II (PSII) inhibitors and act by competing with plastoquinone at the Q_B binding site of the D1 protein in the PSII reaction centre, thereby inhibiting energy transfer (Oettmeier, 1992). The inhibition of photosynthesis by PSII inhibitors can be estimated using pulse amplitude modulation (PAM) fluorometry, a rapid and non-invasive technique well suited for investigating changes in photochemical efficiency. A proportion of the absorbed light energy in PSII can not be used to drive electron transport and is dissipated as heat or chlorophyll fluorescence (Schreiber, 1986; Genty et al., 1989). PAM fluorometry measures this fluorescence which is then used to derive the effective quantum yield [*Y*(*II*)], a parameter proportional to the photosynthetic efficiency of PSII (Schreiber, 1986; Genty et al., 1989). Inhibition of *Y*(*II*) has been used to examine the sub-lethal toxicity of herbicides towards a variety of microalgae, with some being sensitive to diuron at environmentally relevant concentrations (Schreiber et al., 2002; Bengtson-Nash et al., 2005a,b; Escher et al., 2006). Similar sensitivities were measured using ^{14}C uptake in benthic microalgae in temperate waters

Abbreviations: β-car, β-carotene; chl, chlorophyll; Da, diatoxanthin; Dd, diadinoxanthin; F, light adapted minimum fluorescence; F'_m, light adapted maximum fluorescence; Fx, fucoxanthin; LH, light harvesting pigment; Lut, lutein; M, specific growth rate; Mg-DVP, Mg-2,4-divinyl pheophophyrin a5 monomethyl ester; Nx, neoxanthin; NPQ, non-photochemical quenching (photoprotective) pigment; NQAIF, North Queensland algal culturing and identification facility; OD, optical density; PSII, photosystem II; RETR, relative electron transport rate; RLC, rapid light curve; Siph-der, siphonaxanthin derivative; Vauch, vaucheriaxanthin; Vx, violaxanthin; *Y*(*II*), effective quantum yield; Zx, zeaxanthin.

* Corresponding author. Fax: +61 7 47251570.
E-mail address: Kirsten.Heimann@jcu.edu.au (K. Heimann).

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(Arrhenius et al., 2004). Although $Y(II)$ inhibition is becoming recognized as a valid sub-lethal indicator of photosystem stress (Jones et al., 1999; Schreiber et al., 2002), no studies have directly compared this endpoint to traditional indicators of herbicide phytotoxicity in microalgae. Therefore, the potential relationships between herbicide-induced reductions in $Y(II)$ and effects at the organismal level are largely unknown.

PSII inhibitors have been shown to negatively affect photosynthesis in tropical organisms such as seagrasses (Haynes et al., 2000a) and symbiotic dinoflagellates in corals (Jones and Kerswell, 2003; Owen et al., 2003; Negri et al., 2005) at low ($\leqslant 1\ \mu g\ L^{-1}$) concentrations. Despite estuarine sediments recording the highest herbicide loads in tropical marine environments, there have been no studies into the effects of relevant herbicides on microalgal species from these receiving habitats. The microphytobenthic communities of estuaries are key primary producers and may be present in higher biomass than phytoplankton in shallow habitats (Gambi et al., 2003). Regulation of oxygen levels at the water-sediment interface is highly dependent on the photosynthesis of microphytobenthos, and they are also an important food-source for benthic feeders (MacIntyre et al., 1996; Gambi et al., 2003). Microalgal communities thus form an essential functional group in these habitats and exposure to herbicides may therefore carry detrimental effects to the ecosystem level.

This is the first examination of the relationships between the inhibition of photosynthetic efficiency [$Y(II)$] by the PSII inhibitors diuron, atrazine, and hexazinone and the more traditional measures of growth rate (μ) and culture biomass increase in microalgae. The estuarine microphytobenthic *Navicula* sp. (diatom) and *Nephroselmis pyriformis* (green alga) were chosen as common tropical representatives of two important phyla. A possible secondary effect of PSII inhibitors is oxidative stress due to a build-up of excess energy in the photosystem (Rutherford and Krieger-Liszkay, 2001), which can lead to damage of the photosynthetic apparatus. The effects of herbicide exposures on pigment concentrations in both algal species as a further indicator of sub-lethal stress are therefore also examined.

2. Experimental section

2.1. Toxicant preparation

Analytical grade herbicides were purchased from Sigma Aldrich Australia (diuron [CAS 330-54-1], hexazinone [CAS 51235-04-2] and atrazine [CAS 1912-24-9]). Stock solutions were prepared in acetone-rinsed glassware with autoclaved, deionised water using dimethyl sulfoxide (DMSO) as carrier (final DMSO concentration in experimental vessels was 0.01% (v/v)). IC_{50} and IC_{10} values are reported as nominal concentrations from 5 replicate cultures.

2.2. Microalgae – isolation and culture

The test organisms, *Navicula* sp. (Heterokontophyta) (North Queensland algal culturing and identification facility (NQAIF) 110) and *Nephroselmis pyriformis* (Chlorophyta) (NQAIF 117), were isolated from north Queensland sediments using the micro-capillary single cell isolation technique (Anderson et al., 2005b). Both isolates were cultured and sub-cultured at 24 °C in Guillard's (*f*/2) marine medium (Anderson et al., 2005a) prepared using 0.45 μm filtered and autoclaved 35 ppt sea water under sterile conditions. Cultures were established at least six months prior to experimentation and were maintained as batch cultures in exponential growth phase by subculturing fortnightly, and kept under a 12:12 h light:dark cycle with an irradiance of 43 μmol photons $m^{-2}\ s^{-1}$.

2.3. Growth rate, biomass and photosynthetic Y(II) measurements

The optical density at 750 nm (OD_{750}, measured as % transmission in a Varian DMS 90 UV visible spectrophotometer) was validated as a proxy for cell density (counts by haemocytometer) for both species in replicated ($n = 3$) cultures. The OD_{750}s were linearly correlated with cell counts (*Navicula* sp. $r^2 = 0.99$; *N. pyriformis* $r^2 = 0.94$) and were consequently used as an estimation of cell density.

Replicate mother cultures for both organisms ($n = 5$) in exponential growth phase were inoculated in 99 mL *f*/2 into 250 mL Erlenmeyer flasks and dosed with a dilution series of seven concentrations of herbicide and a DMSO carrier control to a final test volume of 100 mL with 0.01% (v/v) DMSO. Initial cell density was selected to ensure an OD_{750} in the linear range at day 3 after herbicide dosing. Three day old mother cultures of *Navicula* sp. were hence used for inoculation to initial cell densities of 3×10^4 cells mL^{-1}, and five day old mother cultures of *N. pyriformis* were used for inoculation to 8×10^4 cells mL^{-1} for each treatment. Experimental flasks for *Navicula* sp. showed no significant lag-phase and were therefore immediately dosed with herbicide or control solution. *Nephroselmis pyriformis* cultures showed a two-day lag-phase and were therefore dosed at day three after inoculation to ensure the cultures had reached exponential growth phase before exposure to herbicides. Carrier controls were performed separately prior to experimentation (addition of *f*/2 medium in a manner identical to addition of DMSO solution, $n = 5$ for each species).

Biomass increase and growth rate (μ) were calculated from OD_{750} measurements taken at days 0, 3, 5 and 10. At each sampling time, a subset of the flasks (including a range of herbicide treatments and controls) was also sampled for microscopic inspection and direct cell counts to ensure that OD_{750} remained a valid proxy for cell density. In order to enable direct comparison of dose response for the three different endpoints (raw data expressed in different units), all responses were converted to % of control. Percent of control biomass from day 0 (t_0) to day i (t_i) was expressed as per Eq. (1). Percent of control growth rate (μ) was calculated as per Eq. (2), where N_i and N_0 = cells mL^{-1} at days i (t_i) and 0 (t_0), respectively.

$$\%\ \text{of control biomass increase} = 100 \times ([OD_{750}]_{ti} - [OD_{750}]_{t0})_{\text{treatment}} / ([OD_{750}]_{ti} - [OD_{750}]_{t0})_{\text{control}} \quad (1)$$

$$\%\ \text{of control growth rate}\ (\mu) = 100 \times (\text{Ln}[N_i/N_0]/[t_i - t_0])_{\text{treatment}} / (\text{Ln}[N_i/N_0]/[t_i - t_0])_{\text{control}} \quad (2)$$

The effect of herbicides on *in vivo* chlorophyll fluorescence was measured as effective quantum yield [$Y(II)$] using a mini-PAM fluorometer (Heinz Walz GmbH, Effeltrich, Germany) equipped with a waterproof fiberoptic probe (active diameter 5.5 mm). Fluorescence measurements were taken by immersing the probe directly into the culture (measuring light intensity = 9, gain = 4) and one measurement was taken per replicate at days 3, 5 and 10. Light adapted minimum fluorescence (F) was determined while applying a weak pulse-modulated red measuring light (ca. 0.2 μmol photons $m^{-2}\ s^{-1}$), and maximum fluorescence (F'_m) was determined after the application of a saturating pulse of actinic light (>10,000 μmol photons $m^{-2}\ s^{-1}$). Effective quantum yield $Y(II) = (F'_m - F)/F'_m = \Delta F/F'_m$ was calculated for controls and treatments, and toxic response in treatments was expressed as a percentage of control values. A more detailed description on the use of PAM fluorometry to monitor photoinhibition in microalgae can be found in (Schreiber et al., 2002; Bengtson-Nash et al., 2005).

A 3-day (72 h) exposure was chosen as the main experimental time frame for endpoint comparison and determination of EC_{50} concentrations following standardized ecotoxicology test guidelines (USEPA, 1996; OECD, 2006). Comparison before 3 days was not feasible as growth rate could not be reliably measured in a single day, and the cultures were too dilute to measure a reliable fluorescence signal with the mini-PAM. Sampling at days 5 and 10 was included to investigate effects beyond the standardized 3-day exposure.

2.4. Pigment analysis

In a separate experiment, algal cultures of both species were dosed with diuron, atrazine, hexazinone at previously determined 3-day IC_{50}-concentrations (biomass) in the same manner as above ($n = 5$). After three days of herbicide exposure, 15 mL algal suspension was gently filtered onto glass–fibre filters (pore size 1.2 μm retention, Millipore, Sigma Aldrich Australia) and immediately placed in a −80 °C freezer. Pigments were extracted sequentially by sonication in 100% acetone (30 W, 20 sec. on ice) within two months. High performance liquid chromatography (HPLC) was used to analyse the extracts on a Waters 600 HPLC, combined with a Waters PDA 996 photodiodearray detector, on a 3 μm, 50 × 4.6 mm C-18 Gemini 110A column (Phenomonex, Australia). A two solvent gradient with a flow rate of 1 mL min^{-1} was used to separate the pigments. Solvent A = 70:30 v/v methanol: 28 mM tetrabutyl–ammonium acetate (TBAA, 1.0 M aq. Sigma–Aldrich, Australia); Solvent B = 50:50 v/v methanol:acetone. The proportion of solvent B was 25% at $t = 0$ min, rising linearly to 100% at 5 min and held at 100% until 10 min when it was linearly reduced to 25% at 11 min and maintained at 25% until 18 min for analysis of the diatom pigments. For sufficient resolution of pigment peaks in *N. pyriformis*, the method was extended: 25% solvent B at $t = 0$ min, rising linearly to 100% at 10 min and held at 100% until 15 min when it was linearly reduced to 25% at 16 min and maintained at 25% until 23 min. The peaks reported were identified by comparison of retention times, absorption spectra and spiking with pigment standards obtained from the International Agency for ^{14}C Determination (DHI, Denmark). When possible, pigments were quantified using response factors of external standards (Jeffrey et al., 1997). As the mixed pigment standard containing lutein (Lut) obtained from DHI was not quantitative, extinction coefficients from (Jeffrey et al., 1997) were used and the response factor estimated based on that of diadinoxanthin (Dd). Likewise, the concentration of Mg-24-divinyl phaeporphyrin a5 monomethyl ester (Mg–DVP) was estimated based on the literature extinction coefficients (Jeffrey et al., 1997) and the response factor of Chlorophyll c_2 (Chl c_2). The concentration of vaucheriaxanthin (Vauch) was estimated based on the response factor of violaxanthin (Vx). The concentrations of siphonaxanthin derivatives (Siph-der) were estimated using the literature extinction coefficient of siphonaxanthin and the response factor of fucoxanthin (Fx) which shares an identical terminal carbonyl function in the chromophore (Jeffrey et al., 1997).

2.5. Data treatment

IC_{50} and IC_{10} concentrations for biomass increase, μ and $Y(II)$ were calculated by fitting a 4-parameter logistic regression to the dose response data in Sigmaplot 2001 (version 7.1, SPSS Inc.). A linear regression was fitted to the endpoint comparison data (Sigmaplot 2001, version 7.1, SPSS Inc.). Mean photosynthetic pigment concentrations and pigment composition ratios were compared across all treatments with a one-way ANOVA ($\alpha < 0.05$). Dunnett's post hoc test was used to identify means significantly different from control means. All statistical analyses were performed using Statistica 7 (StatSoft, Inc. Oklahoma, USA).

3. Results and discussion

Both test organisms consistently exhibited exponential growth in all controls from t_0 to t_3 (*Navicula* sp. $\mu = 0.73 \pm 0.05$, *N. pyriformis* $\mu = 0.83 \pm 0.03$ [average ± SE, $n = 20$]) throughout the experiment, and OD_{750} remained a valid proxy for cell density without any adjustments to the initial equations. In the absence of herbicides $Y(II)$ remained at 0.685 ± 0.003 for *Navicula* sp. and at 0.598 ± 0.005 for *N. pyriformis* (average ± SE, $n = 20$). The DMSO carrier had no significant effect on μ, biomass increase or $Y(II)$ at days 3, 5 or 10 (ANOVA, $p > 0.05$).

3.1. Relationships between inhibition of photosynthesis and growth

The dose-response curves for PSII inhibitors exhibited very similar shapes and slopes for each endpoint and for both species of benthic microalgae isolated from nearshore tropical habitats in 3-day batch culture experiments (Fig. 1). The concentrations of PSII herbicides that inhibited 50% of μ, biomass increase or $Y(II)$ were also consistent for each herbicide and species (Table 1). The order of toxicity (3-day EC_{50} range for all endpoints) for *Navicula* sp. was; diuron (16–33 nM) > hexazinone (56–110 nM) > atrazine (300–

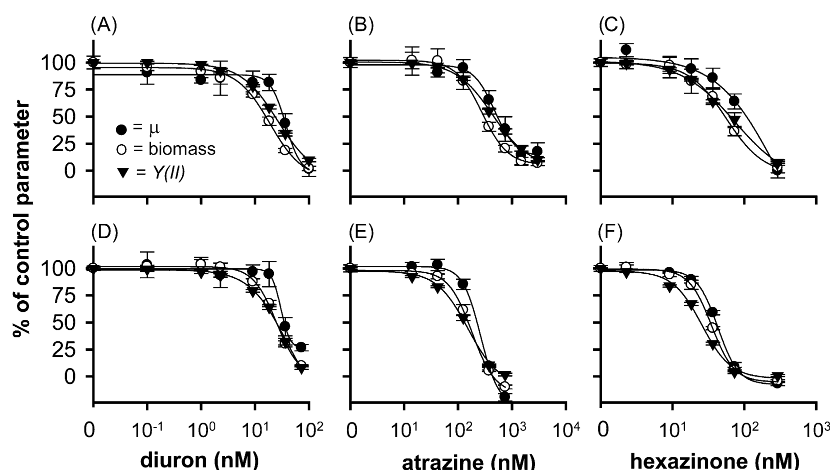

Fig. 1. *Navicula* sp. 3-day growth rate (μ), biomass increase and effective quantum yield [$Y(II)$] response to (A) diuron, (B) atrazine, (C) hexazinone, and *N. pyriformis* 3-day dose response to (D) diuron, (E) atrazine, (F) hexazinone. Mean (±SE), $n = 5$ (both species diuron μ: $n = 3$. *Navicula* sp. atrazine and hexazinone μ; $n = 4$).

Table 1
Summary of 3-day growth rate (μ)-, biomass increase- and effective quantum yield [$Y(II)$] EC_{50} and EC_{10} values in nM (and $\mu g\ L^{-1}$) for *Navicula* sp. and *N. pyriformis* exposed to diuron, atrazine, and hexazinone

	Navicula sp.			*Nephroselmis pyriformis*		
	μ	Biomass	$Y(II)$	μ	Biomass	$Y(II)$
EC_{50} (SE)						
Diuron	33(4) [7.8(1)]**	16(1) [3.7(0.2)]	24(1) [5.5(0.2)]	33(4) [8(1)]**	25(2) [5.8(0.5)]	25(1) [5.9(0.2)]
Atrazine	620(200) [130(40)]*	300(40) [65(9)]	460(30) [99(7)]	230(10) [50(2)]	160(10) [35(2)]	130(6) [28(1)]
Hexazinone	110(20) [27(5)]*	56(4) [14(1)]	62(6) [16(2)]	41(2) [10(0.5)]	33(1) [8.4(0.3)]	25(1) [6.2(0.3)]
EC_{10} (SE)						
Diuron	10(4) [2.4(0.7)]**	2.5(1) [0.5(0.2)]	4.3(1) [1.0(0.2)]	22(9) [5.2(2)]**	9.3(1) [2.2(0.2)]	4.7(1) [1.1(0.2)]
Atrazine	160(70) [35(15)]*	120(20) [26(4)]	86(5) [19(1)]	110(20) [23(4)]	51(7) [11(2)]	31(1) [6.8(0.2)]
Hexazinone	26(10) [6.5(3)]*	13(4) [3.4(1)]	13(1) [3.3(0.3)]	19(1) [4.8(0.3)]	15(2) [3.8(0.5)]	8.1(1) [2.1(0.3)]

Average (SE). $n = 5$, * $n = 4$, ** $n = 3$.

620 nM). For *N. pyriformis*, the order of toxicity and 3-day EC_{50} range for all endpoints was diuron (25–33 nM) > hexazinone (25–41 nM) > atrazine (130–230 nM). Similar patterns were observed for the 3-day EC_{10}s (Table 1). The least sensitive measure of toxicity for the PSII-inhibiting herbicides was μ, which exhibited consistently higher 3-day EC_{50}s than did biomass or $Y(II)$ measurements. Biomass increase proved to be a more sensitive endpoint (lower EC_{50}) than $Y(II)$ for *Navicula* sp. in diuron and atrazine exposures (Table 1). In contrast, $Y(II)$ was slightly more sensitive than biomass increase for *N. pyriformis*.

The inhibition of effective quantum yield [$Y(II)$] proved to be a reliable predictor for inhibition of growth rates (μ) and biomass increase in 3-day batch culture experiments with similar results observed for both species. The relationships between μ, biomass increase and $Y(II)$ at day 3 were linear ($r^2 \geqslant 0.90$) for each species and PSII herbicide dose (Fig. 2, Table 2). The regression slopes were also close to unity for both *Navicula* sp. (0.86–1.2) and *N. pyriformis* (0.71–1.3), indicating good agreement between each endpoint for concentrations spanning 3 orders of magnitude and for two very different organisms. At high herbicide concentrations [low $Y(II)$] the biomass increase or μ response was often negative indicating cell death (Fig. 2D–F).

Inhibition of $Y(II)$ has been successfully applied to obtain EC_{50} values for a number of herbicides, using marine microorganisms in both dual channel ToxY PAM instruments (Waltz, Germany) (Bengtson-Nash et al., 2005a,b), and more recently in 96-well format using I-PAM instruments (Escher et al.,2006). These studies all indicate that $Y(II)$ in microalgae is sensitive towards herbicide exposure, but the current study is the first to directly link inhibition of $Y(II)$ with declines in μ and biomass in microalgae, endpoints that are used routinely by industry and regulators (USEPA, 1996; OECD, 2006). These findings are consistent with a recent report of good agreement between fluorescence based ($Y(II)$, I-PAM) and growth rate (frond biomass increase) estimated EC_{50}s in the freshwater macrophyte *Lemna gibba* (duckweed, Magnoliophyta) exposed to PSII-inhibiting herbicides (Kuster and Altenburger, 2007). Good agreement between both $Y(II)$ and growth (μ or biomass) estimated EC_{50}-values and the slopes of the respective dose response curves was also reported for the macrophytes *L. gibba* and *Myriophyllum spicatum* (Magnoliophyta) exposed to the wood preservative creosote (a mixture of polycyclic hydrocarbons, PAHs) (Marwood et al., 2001). The confirmation of a similar pattern in such different organisms as freshwater angiosperms and, as reported for the first time here, marine unicellular green algae and diatoms is encouraging, supporting our conclusion that $Y(II)$ can be used as a reliable predictor for the effect of PSII herbicides organismal growth rates.

The most consistent 3-day EC_{50} estimations in this study were generally provided by $Y(II)$ (%CV 4–20%) and increase in biomass (%CV 6–29%). Mathematically, μ is expected to generate a less

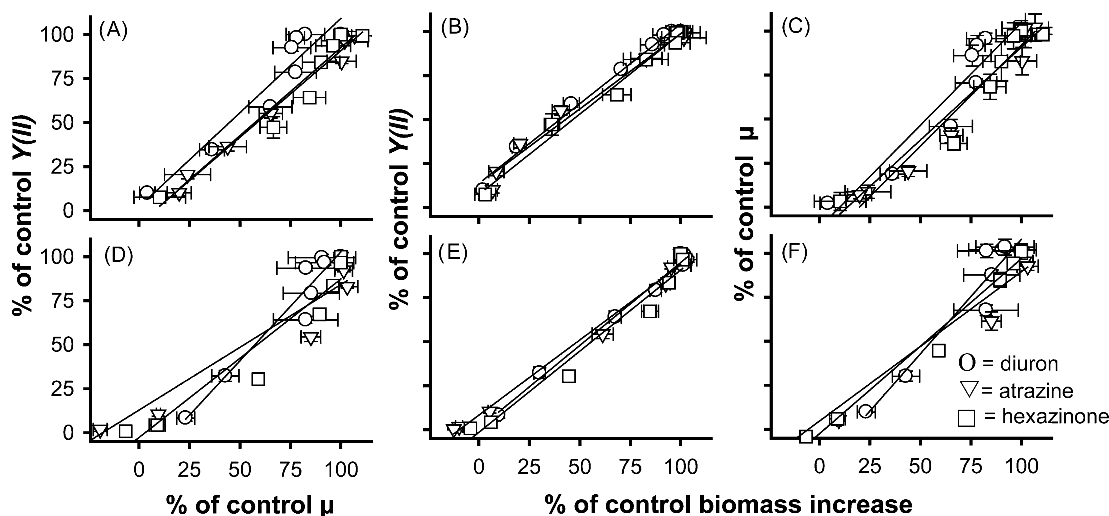

Fig. 2. Relationship between growth rate (μ), biomass increase and effective quantum yield [$Y(II)$] responses to herbicides after 3 days exposure. $n = 5$, Average (±SE). Error bars not visible are smaller than symbol. (A–C) *Navicula* sp. and (D–F) *Nephroselmis pyriformis*.

Table 2
Equations and goodness of fit for linear regressions illustrating relationship between all endpoints measured for *Navicula* sp. and *N. pyriformis* exposed to PSII herbicides for three days

	μ vs. $Y(II)$	Biomass increase vs. $Y(II)$	μ vs. biomass increase
Navicula sp.			
Diuron	$f = 1.1x + 2.1$ $r^2 = 0.92$	$f = 0.91x + 14$ $r^2 = 0.99$	$f = 1.2x - 12$ $r^2 = 0.90$
Atrazine	$f = 1.0x - 7.5$ $r^2 = 0.98$	$f = 0.86x + 13$ $r^2 = 0.98$	$f = 1.1x - 23$ $r^2 = 0.96$
Hexazinone	$f = 0.98x - 7.6$ $r^2 = 0.94$	$f = 0.91x + 7$ $r^2 = 0.98$	$f = 1.1x - 15$ $r^2 = 0.92$
N. pyriformis			
Diuron	$f = 1.2x - 21$ $r^2 = 0.93$	$f = 0.84x + 0.93$ $r^2 = 0.99$	$f = 1.3x - 22$ $r^2 = 0.92$
Atrazine	$f = 0.71x + 13$ $r^2 = 0.93$	$f = 0.85x + 8.3$ $r^2 = 0.99$	$f = 0.85x + 4.6$ $r^2 = 0.97$
Hexazinone	$f = 0.90x - 2.7$ $r^2 = 0.92$	$f = 0.9x - 1.4$ $r^2 = 0.97$	$f = 0.99x - 2.6$ $r^2 = 0.98$

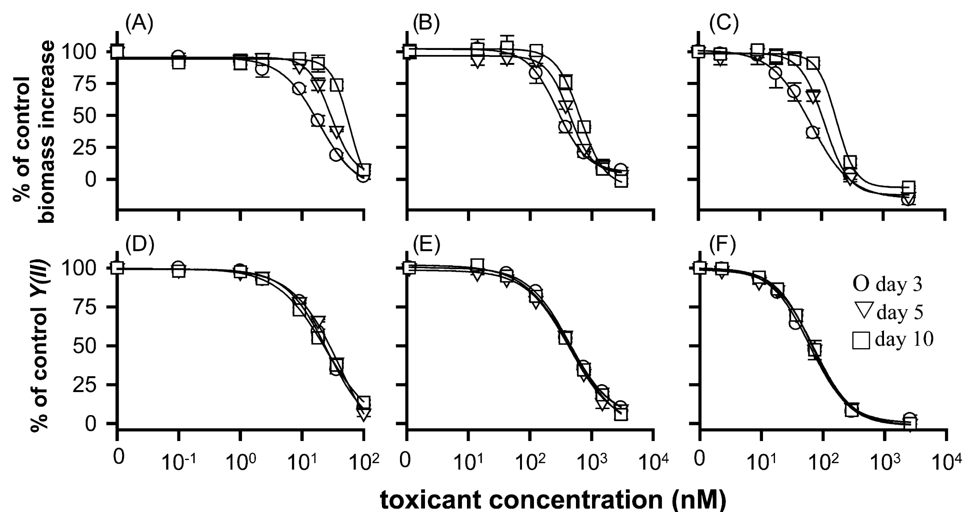

Fig. 3. *Navicula* sp. time-dependent biomass increase response to (A) diuron, (B) atrazine and (C) hexazinone; and time-dependent $Y(II)$ response to (D) diuron, (E) atrazine and (F) hexazinone at days 3, 5 and 10. Average (±SE), $n = 5$.

variable (but higher) EC_{50} estimate than biomass increase as it takes into account absolute growth, different starting values and test duration, and is consequently recommended as the key endpoint measured in various standardized toxicity tests (Eberius et al., 2002). However, fouling organisms such as many diatoms (including *Navicula* sp. in this study) are often difficult to re-suspend completely prior to sampling, which may result in larger variability in μ and biomass estimates between replicates. Complete detachment and re-suspension of cells is not essential for $Y(II)$ measurements, which are calculated from ratios of the fluorescence values ΔF and F'_m and are therefore independent of cell density (Schreiber et al., 2007). The $Y(II)$ parameter exhibits consistent response over time and between replicates, is independent of cell density in the sample chamber, shows a robust relationship with culture growth responses (Fig. 2) and is numerically more sensitive than μ, providing a larger biological safety margin when determining environmentally acceptable threshold concentrations. Recent research also shows that very short (10 min) exposures of microalgae to PSII inhibitors in 96-well plates provide similar results to the 3-day experiments performed here, further simplifying the task of toxicity assessment of PSII inhibitors towards microalgae (Escher et al., 2006; Muller et al., 2007). PAM fluorescence-based techniques can consequently be recommended as a suitable, ecologically relevant tool for assessing toxic impacts.

3.2. Comparative sensitivity of test species to PSII herbicides

Both *Navicula* sp. and *N. pyriformis* were highly sensitive to PSII inhibitors, with 50% inhibition of μ, biomass increase and $Y(II)$ at environmentally relevant concentrations of diuron (Mitchell et al., 2005) after 3 days (Table 1). Using 3-day biomass increase as an endpoint, *Navicula* sp. ($IC_{50} = 16 \pm 1$ nM) was more sensitive to diuron than *N. pyriformis* ($IC_{50} = 25 \pm 1$ nM) (Fig. 1 and Table 1). However, *N. pyriformis* was almost twice as sensitive to the triazine/triazinone herbicides, with 3-day biomass increase IC_{50} values of 160 ± 10 and 33 ± 1 nM for atrazine and hexazinone, respectively, compared to 300 ± 40 and 56 ± 4 nM for *Navicula* sp. Contrary to biomass increase, there was no difference in sensitivity for $Y(II)$ inhibition by diuron between the two species (3-day $Y(II)$ IC_{50} 24 ± 1 nM for *Navicula* sp. and 25 ± 2 nM for *N. pyriformis*) or μ (3-day ± IC_{50} 33 ± 1 nM for both species) (Fig. 1 and Table 1). *Nephroselmis pyriformis* was still more sensitive to atrazine and hexazinone, with estimated $Y(II)$ and μ IC_{50} values being 2.5–3.5 fold lower than those for *Navicula* sp.

The higher sensitivity *N. pyriformis* to some PSII-inhibiting herbicides is consistent with previous studies, which often report chlorophytes to be more sensitive than bacillariophytes when comparing herbicide toxicity across phyla (Guasch et al., 1997; Berard et al., 2003). Sensitivity to herbicides has been linked to

cell-size in microalgae (DeLorenzo et al., 2004), and the much larger surface area:volume ratio in *N. pyriformis* may partly explain its higher sensitivity. Light history has previously been implicated in periphytic (attached) microalgae, with shade-adapted (generally diatom-dominated) communities less susceptible than sun-adapted (chlorophyte-dominated) communities (Guasch and Sabater, 1998). This is however not relevant in our study where both species had identical, environmentally relevant low irradiance, light histories.

3.3. Extended exposure

There is a recovery in biomass increase at days five and ten compared to day three for both species in the presence of PSII inhibitors without any replenishment of nutrients to the cultures, while inhibition of *Y(II)* remained constant over the period (Fig. 3 and Fig. 4). No comparisons were made with μ at days 5 and 10 as control cultures were no longer in exponential growth phase. The recovery in biomass after a prolonged exposure to PSII-inhibiting herbicides implies that the effect is algistatic (delaying growth) at low to medium concentrations. This effect could also be due to slight reductions in herbicide concentrations with time (by growth dilution or degradation). However, consistent inhibition of *Y(II)* in both organisms up to day 10 indicates little appreciable loss of herbicide. It is possible that growth could be rescued by a switch to heterotrophic nutrient acquisition. Heterotrophy has previously been shown in *Nephroselmis*, where cells cultured in medium supplemented with 100 μM glucose showed complete absence of Chls *a* and *b* and no photosynthetic activity, yet exhibited highly increased growth rates after acclimation to the new medium (Lewitus and Kana, 1994). Heterotrophy has also been shown for eight species of freshwater benthic diatoms, where utilization of complex hydrocarbons was activated under light limitation (Tuchman et al., 2006). It is also possible that reduced light harvesting is compensated for by state transitions with cyclic electron flow through PSI providing energy for ATP-synthesis, thus enabling some growth despite low *Y(II)* (Muller et al., 2001).

3.4. Effects on pigment content

Pigment concentrations were measured from controls and herbicide-treated cultures after 3 days exposure. The major pigments extracted from *Navicula* sp. were identified as the light harvesters (LH) chlorophyll *a* (chl *a*), chlorophyll c_2 (chl c_2) and fucoxanthin

Table 3
Pigment composition/cell (fg cell^{-1}) after three days in microalgae treated with 3-day growth IC_{50}-concentrations of diuron, atrazine and hexazinone

Pigment	Control	Diuron	Atrazine	Hexazinone
Navicula sp.				
chl *a* (LH)	2350 (90)	2320 (75)	2290 (110)	2660 (350)
Fx (LH)	1190 (40)	1230 (33)	1240 (49)	1400 (170)
chl c_2 (LH)	240 (3)	255 (14)	254 (19)	286 (36)
Dd (NPQ)	267 (11)	248 (8)	258 (10)	284 (40)
Da (NPQ)	26 (2)	16.3 (0.5)*	19 (1)*	20 (3)
β-car (NPQ)	13.2 (0.4)	12.1 (0.3)	12.4 (0.5)	14 (2)
Nephroselmis pyriformis				
chl *a* (LH)	403 (3)	450 (10)*	442 (8)*	464 (13)*
chl *b* (LH)	115 (2)	135 (7)*	132 (2)*	136 (3)*
Siph-der (LH)	44.4 (1.1)	57.0 (2.5)*	57.5 (1.6)*	61.1 (2.1)*
Mg–DVP (LH)	19.1 (0.1)	23.3 (0.9)*	22.4 (0.5)*	23.5 (0.6)*
β-car (NPQ)	61.5 (0.6)	48.7 (1.2)*	46.4 (0.8)*	48.0 (1.6)*
Lut (NPQ)	34.1 (2.2)	21.8 (0.5)*	21.9 (0.7)*	23.2 (1.0)*
Vx (NPQ)	31.6 (0.3)	25.5 (0.5)*	23.6 (0.3)*	24.9 (1.1)*
Vauch (NPQ)	17.3 (0.8)	18.8 (0.7)	19.2 (0.7)	18.9 (0.5)
Nx (NPQ)	14.7 (0.6)	16.3 (0.8)	16.2 (0.7)	18.7 (1.3)*

Average (SE), $n = 5$ cultures. *Significantly different from control (one-way ANOVA, $p < 0.05$). Abbreviations: = light harvesting pigment (LH), non-photochemical quenching (photoprotective) pigment (NPQ). chlorophyll (chl), fucoxanthin (Fx), diadinoxanthin (Dd), diatoxanthin (Da), β-carotene (β-car), siphonaxanthin derivative (Siph-der), Mg-2,4-divinyl pheophophyrin a_5 monomethyl ester (Mg–DVP), lutein (Lut), violaxanthin (Vx), vaucheriaxanthin (Vauch), neoxanthin (Nx).

(Fx), and the protective carotenoid (NPQ) pigments diadinoxanthin (Dd), diatoxanthin (Da), and β-carotene (β-car) (Table 3). In *N. pyriformis*, the major LH pigments were: chl *a*, chlorophyll *b* (chl *b*), Mg-2,4-divinyl phaeporphyrin a5 monomethyl ester (Mg–DVP) and siphonaxanthin derivatives (Siph-der), while the major NPQ pigments were: neoxanthin (Nx), violaxanthin (Vx), lutein (Lut), vaucheriaxanthin (Vauch) and β-car.

In *N. pyriformis*, there were significant differences in cellular pigment concentrations between controls and diuron, atrazine or hexazinone EC_{50} treatments for all pigments except for Vauch (no significant difference) and Nx (concentration of Nx in hexazinone treatments larger than in controls) at day 3 (Table 3). In general the cellular LH pigment content of *N. pyriformis* increased to more than 113% of controls (ANOVA, $F_{3,16} = 9.334$, $p < 0.01$), while the NPQ pigments decreased to 80–84% of controls (ANOVA, $F_{3,16} = 34.81$, $p < 0.01$). Da was the only pigment in *Navicula* sp. that exhibited any significant changes in the presence of herbicides at EC_{50} concentrations compared with controls (ANOVA,

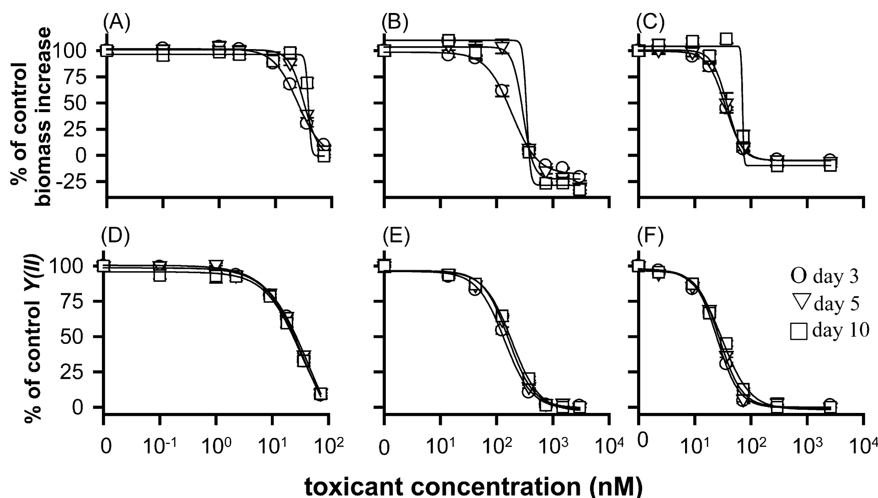

Fig. 4. *Nephroselmis pyriformis* time-dependent biomass increase response to (A) diuron, (B) atrazine and (C) hexazinone; and time dependent *Y(II)* response to (D) diuron, (E) atrazine and (F) hexazinone at days 3, 5 and 10. Average (±SE), $n = 5$.

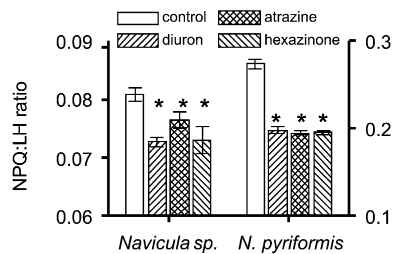

Fig. 5. Mean NPQ: LH pigment ratio in *Navicula* sp. and *N. pyriformis* after three days herbicide exposure at IC_{50}-concentrations. $n = 5$, error bars = SE. * = significantly different from control (ANOVA, $p < 0.01$). Abbreviations: non-photochemical quenching (photoprotective) pigment (NPQ), light harvesting pigment (LH). NB different scales on *y*-axes.

$F_{3,16} = 5.539$, $p < 0.01$), decreasing to 63% and 71% of control cellular concentrations in diuron and atrazine treatments, respectively at day 3 (Table 3).

The xanthophyll and carotene pigments play an important function in non-photochemical quenching (NPQ), dissipating excess energy that might otherwise harm algae under high light stress (Muller et al., 2001). The reversible de-epoxidation of Vx to zeaxanthin (Zx) in green algae, and the similar de-epoxidation of Dd to Da in diatoms, leads to the emission of excess energy as heat, thus limiting possible oxidative damage caused by the formation of triplet chlorophyll ($^{*}Chl^{3}$) or reactive oxygen species (ROS) (Muller et al., 2001). Oxidative stress can also occur as a secondary effect of PSII inhibitors (Rutherford and Krieger-Liszkay, 2001). In the present study the ratio of Da:Dd decreased significantly in all PSII-inhibitor exposures for *Navicula* sp. compared to controls (ANOVA, $F_{3,16} = 18.334$, $p < 0.01$) (Table 3). The ratios of total NPQ:LH pigments in *Navicula* sp. also decreased significantly to 90 ± 1, 94 ± 2 and 90 ± 3% of controls (ANOVA, $F_{3,16} = 8.40$, $p < 0.01$) in diuron, atrazine and hexazinone treatments, respectively (Fig. 5). More pronounced depressions of LPQ:LH ratios (to around 70% of controls) were observed for herbicide treated *N. pyriformis* after day 3 (ANOVA, $F_{3,16} = 128.37$, $p < 0.01$) (Fig. 5, Table 3). Decreases, rather than increases in the Da:Dd (*Navicula* sp.) and NPQ:LH ratios (both species) suggests that neither species suffered from oxidative stress following PSII inhibitor exposure under the illumination used in the current experiment. On the contrary, both species exhibited light limited behaviour in herbicide treatments, attempting to compensate for decreased photosynthesis by up-regulating the concentration of light harvesting pigments at 3 days. Control cultures, however, did not experience low-light stress, as preliminary rapid light curves (RLC) performed with a Maxi Imaging–PAM (Heinz Walz GmbH, Effeltrich, Germany) using stock cultures showed a maximum relative electron transport rate (rETR) around the light intensity used throughout the experiment for both species (data not shown).

3.5. Environmental relevance

The consistent relationship between inhibition of *Y*(*II*) and growth rates is critical as it reveals that inhibition of photosynthesis in PSII can directly translate to reduced growth rates and biomass of tropical benthic microalgae.

Importantly, this is the first study in tropical Australia investigating herbicide effects on marine microalgae other than symbiotic dinoflagellates associated with hard corals. Our results show that tropical benthic microalgae are highly sensitive to herbicide contamination at environmentally relevant concentrations (Mitchell et al., 2005), with growth and photosynthetic efficiency reduced by 10% at diuron concentrations as low as 0.5–2 $\mu g\ L^{-1}$ and at hexazinone concentrations from 2 to 4 $\mu g\ L^{-1}$. The high sensitivity of the microalgal species in this study to PSII inhibitors is of concern. Maximum detected environmental concentrations of diuron (>8 $\mu g\ L^{-1}$ (Mitchell et al., 2005)) are higher than those required for a reduction of growth rates by 50%. Further, the more commonly detected lower concentrations are still high enough to cause 10% inhibition in *Y*(*II*) and biomass. Although atrazine and hexazinone are generally detected at lower concentrations, their presence in the environment may exacerbate the effect of diuron on microalgal communities as PSII inhibitors can act additively (Faust et al., 2001). Inhibition of electron transport by PSII inhibitors at low concentrations is likely to lead to reduced growth rates and biomass of critical benthic primary producers in estuarine habitats. Reductions in microalgal biomass may change the composition of the microorganism community, thus altering food availability and quality for benthic feeders. Changes in pigment and protein content may also change the nutritional value of the microalgae. Even if the effects of low concentrations of PSII inhibitors were merely algistatic, possible accompanying changes in energy acquisition pathways to heterotrophy or cyclic electron flow through PSI may still impart detrimental effects to the system as a whole due to changed primary productivity and oxygenation of the sediment.

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