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# Additive toxicity of herbicide mixtures and comparative sensitivity of tropical benthic microalgae

Marie Magnusson [a,b,c], Kirsten Heimann [b], Pamela Quayle [d,1], Andrew P. Negri [e,*]

[a] AIMS@JCU, Australian Institute of Marine Science, James Cook University, Townsville, QLD 4811, Australia
[b] School of Marine and Tropical Biology, James Cook University, Townsville, QLD 4811, Australia
[c] School of Pharmacy and Molecular Sciences, James Cook University, Townsville, QLD 4811, Australia
[d] The University of Queensland, National Research Centre of Environmental Toxicology (EnTox), 39 Kessels Road, Coopers Plains 4108, Australia
[e] Australian Institute of Marine Science, PMB 3, Townsville, MC, QLD 4810, Australia

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ABSTRACT

Natural waters often contain complex mixtures of unknown contaminants potentially posing a threat to marine communities through chemical interactions. Here, acute effects of the photosystem II-inhibiting herbicides diuron, tebuthiuron, atrazine, simazine, and hexazinone, herbicide breakdown products (desethyl-atrazine (DEA) and 3,4-dichloroaniline (3,4-DCA)) and binary mixtures, were investigated using three tropical benthic microalgae; *Navicula* sp. and *Cylindrotheca closterium* (Ochrophyta) and *Nephroselmis pyriformis* (Chlorophyta), and one standard test species, *Phaeodactylum tricornutum* (Ochrophyta), in a high-throughput Maxi-Imaging-PAM bioassay (Maxi-IPAM). The order of toxicity was; diuron > hexazinone > tebuthiuron > atrazine > simazine > DEA > 3,4-DCA for all species. The tropical green alga *N. pyriformis* was up to 10-fold more sensitive than the diatoms tested here and reported for coral symbionts, and is recommended as a standard tropical test species for future research. All binary mixtures exhibited additive toxicity, and the use of herbicide equivalents (HEq) is therefore recommended in order to incorporate total-maximum-load measures for environmental regulatory purposes.

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## 1. Introduction

### 1.1. Herbicide contamination in coastal marine environments

Herbicide contamination of surface water and sediments is now common in coastal habitats of the Great Barrier Reef (GBR) (Haynes et al., 2000a; Lewis et al., 2009; Shaw et al., 2010). Many pesticides are persistent to varying extent and their continued and growing use poses a potential threat to marine communities via chronic exposure, even when environmental concentrations of individual chemicals are not acutely toxic (Faust et al., 2001; McClellan et al., 2008). The effects of agrochemicals on relevant aquatic organisms in tropical regions are insufficiently researched. An exception is the photosystem II (PS(II))-inhibiting herbicide diuron, which has been investigated extensively in laboratory experiments in relation to its effects on seagrasses (Haynes et al., 2000b), mangroves (Duke et al., 2005) and coral reef communities (Cantin et al., 2009; Jones et al., 2003; Negri et al., 2005) in the Great Barrier Reef (GBR), and has subsequently been proposed to cause negative effects at existing environmental concentrations (Lewis et al., 2009). However, very few studies have explored the influence of herbicides on the less conspicuous benthic estuarine communities of microalgae in tropical environments (Magnusson et al., 2008) despite their recognized ecological importance as significant primary producers (Miller et al., 1996; Underwood and Kromkamp, 1999). In particular, the combined effect of multiple pollutants is poorly investigated.

### 1.2. The application of PAM fluorometry for measuring inhibition of photosynthesis in ecotoxicology

Many of the herbicides commonly detected in coastal environments, such as diuron, atrazine and hexazinone function by inhibiting electron transfer through photosystem II (PS(II)), thereby reducing photosynthetic efficiency (Tomlin, 2000). These changes in photochemical efficiency can be estimated using pulse amplitude modulation (PAM) fluorometry. Using this technique, chlorophyll fluorescence measurements are employed to derive the effective quantum yield [*Y*(*II*), see dose-response fluorescence measurements section], a parameter proportional to the photosynthetic efficiency of PS(II) (Genty et al., 1989; Schreiber, 1986). During the last decade, PAM fluorometry has been applied to study the effects of herbicides on a wide range of organisms, including corals

* Corresponding author. Tel.: +61 7 47534322; fax: +61 7 47725852.
*E-mail address:* a.negri@aims.gov.au (A.P. Negri).
[1] Present address: Griffith University, Gold Coast Campus, Edmund Rice Drive, QLD 4222, Australia.

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(Jones and Kerswell, 2003; Negri et al., 2005), crustose coralline algae (Harrington et al., 2005), seagrass (Haynes et al., 2000b) and temperate microalgae (Bengtson-Nash et al., 2005b; Schmitt-Jansen and Altenburger, 2007). The biological relevance of *Y*(*II*) inhibition as a toxicological endpoint (Ralph et al., 2007), has recently been resolved for some microalgae, where a linear 1:1 relationships between inhibition of effective PS(II) quantum yield *Y(II)* and population level responses such as specific growth rate ($\mu$) and biomass increase were established in 3-day batch culture experiments using two species of tropical microalgae (Magnusson et al., 2008).

### 1.3. Chemical mixtures

While regulators and environmental managers derive water quality guideline concentrations for individual pesticides (GBRMPA, 2009), PS(II)-inhibitors are usually detected in complex mixtures in coastal waters of the GBR (Lewis et al., 2009; Shaw et al., 2010). Individual herbicides may be present below observed effect concentrations but there is potential for additivity or synergistic interactions. Herbicides with common modes of action such the PS(II)-inhibitors diuron, tebuthiuron, hexazinone, atrazine and simazine may exhibit additive toxicity (Faust et al., 2001). Strong relationships have been demonstrated between the biological effects of PS(II)-inhibitors in complex mixtures and the expected cumulative toxicity of these mixtures derived by summing the concentrations of individual herbicides from chemical analysis (LC–MS) after applying herbicide potency factors to each component relative to diuron (Bengtson-Nash et al., 2005c; Muller et al., 2008; Shaw et al., 2009). These results highlight the importance of identifying sensitivity and response to herbicide mixtures in ecologically important primary producers.

### 1.4. Aims

A rapid 96-well assay to detect *Y(II)* inhibition in microalgae has been developed using the imaging-PAM (Maxi-IPAM, Heinz Walz, GmbH) (Schreiber et al., 2007). Here, Maxi-IPAM fluorometry was used to determine the acute phytotoxicity of a suite of herbicides commonly detected in the Australian environment to benthic microalgal species isolated from tropical North Queensland. Herbicide breakdown products and binary herbicide mixtures were also included in order to better mimic more complex conditions, common to contaminated waters where multiple toxicants may be present. To enable direct comparisons between the sensitivities of local microalgal species and standard ecotoxicology test species, the commonly used temperate diatom *Phaeodactylum tricornutum* (ISO, 1995) was included in this series of bioassays.

## 2. Materials and methods

### 2.1. Microalgae – isolation and culture maintenance

The test organisms; *Navicula* sp. Bory (Ochrophyta) (North Queensland Algal culturing and Identification Facility [NQAIF] 110), *Cylindrotheca closterium* (Ehrenberg) Reimann and Lewin [syn. *Nitzschia closterium*] (Ochrophyta) (NQAIF 079), and *Nephroselmis pyriformis* (N. Carter) Ettl. (Chlorophyta) (NQAIF 117) were isolated from tropical Queensland sediments and cultured and maintained as described previously (Guillard f/2 medium at 24 °C, 12:12 h light:dark cycle with an irradiance of 43 μmol photons $m^{-2}$ $s^{-1}$) (Magnusson et al., 2008). *P. tricornutum* Bohlin (Ochrophyta) (CS 29) was obtained from the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Collection of Living Microalgae (CCLM) and is routinely maintained at the NQAIF under the same conditions as the three other species in this study. *Navicula* sp. and *N. pyriformis* were extensively tested in acute toxicity- and mixture experiments using at least five replicate cultures ($n$ = 5), whereas *C. closterium* and *P. tricornutum* were included at within culture replication level ($n$ = 1 culture, eight wells per dose) in the acute toxicity experiments.

### 2.2. Toxicant preparation

The herbicides chosen represent use patterns and environmental contamination in Queensland, Australia (Hamilton and Haydon, 1996; Haynes et al., 2000a; Lewis et al., 2009). These herbicides are, however, common global contaminants (Carafa et al., 2007; Konstantinou and Albanis, 2004; Shaw et al., 2008). Analytical grade diuron, tebuthiuron, atrazine, simazine, hexazinone, 3,4-dichloroaniline [3,4-DCA], and desethyl-atrazine [DEA], were purchased from Sigma Aldrich. Chemical classes and sites of action of these herbicides are summarized in Table 1. Stock solutions were prepared in acetone-rinsed glassware to 10 mg $L^{-1}$ with Milli-Q filtered water using 1% ethanol or, in the case of simazine, DMSO as carrier. Stock solutions were tightly capped and stored dark at 6 °C. Concentration series for dosing were diluted appropriately from stock solutions immediately prior to each experiment. Final ethanol or DMSO concentration was 0.05% (v/v) and appropriate solvent control treatments were included in each experiment. All solvents were HPLC-grade.

The fluorescence response of all diuron positive controls fell within two standard deviations of the average diuron-control response of preceding replicates. Standard guidelines suggest potential loss of toxicant due to adsorption to test vessels is expected at higher hydrophobicities (octanol/water partition coefficient, log $K_{ow}$ > 4) (OECD, 2000), and Riedl and Altenburger (2007) recommend exposure concentrations to be measured for compounds with a log $K_{ow}$ > 3. All herbicides tested here, however, were nonvolatile with low to moderate $K_{ow}$s between 1.2 and 2.6 (Table. 1) (Tomlin, 2000). Therefore binding to polypropylene test plates or stock solution storage glassware was not likely, as shown for atrazine recovery (Bandow et al., 2010; Liyanage et al., 2006). On this basis, nominal concentrations were deemed accurate for the chosen exposure regimes.

### 2.3. Dose-response fluorescence measurements

Photosynthetic efficiency of the microalgae was estimated using a Maxi Imaging-PAM (Maxi-IPAM) (Heinz Walz GmbH, Effeltrich, Germany) in dose-response experiments. Parameters measured were light-adapted minimum and maximum fluorescence, $F$ and $F'_m$, respectively, from which the effective quantum yield, *Y(II)*, was calculated following Eq. (1)

**Table 1**
Herbicides and herbicide breakdown products tested in the bioassay.

| | Chemical class | Site of action | Log $K_{ow}$ |
|---|---|---|---|
| *Herbicides* | | | |
| Diuron | Phenylurea | PS(II) | 2.6 |
| Tebuthiuron | Phenylurea | PS(II) | 1.8 |
| Atrazine | Triazine | PS(II) | 2.3 |
| Simazine | Triazine | PS(II) | 2.1 |
| Hexazinone | Triazinone | PS(II) | 1.2 |
| *Breakdown products* | | | |
| 3,4-dichloroaniline (3,4-DCA) | | na | 2.7 |
| Desethylatrazine (DEA) | | na | 1.51 |

na = not applicable. 3,4-DCA is a breakdown product of diuron, and DEA is a breakdown product of both atrazine and simazine. $K_{ow}$ = octanol/water partition coefficient (Tomlin, 2000).

$$Y(II) = (F'_m - F)/F'_m \quad (1)$$

Toxic effects elicited by the herbicides were quantified by calculating % inhibition of *Y(II)* (photoinhibition) in treatments compared to controls:

$$\%\ \text{Inhibition} = 100 * (1 - Y(II)_{\text{treatment}}/Y(II)_{\text{control}}) \quad (2)$$

Replicate algal cultures ($n = 5$) were sub-cultured to a known cell density three and six days prior to each experiment for *Navicula* sp. and *N. pyriformis*, respectively. Differing growth rates between the organisms necessitated the use of differing culture age of test cultures to maintain testing during exponential growth. By systematically testing suspensions of varying cell densities at different instrument settings, the optimal cell density for maximum sensitivity and reproducibility using the Maxi-IPAM was determined to be to $1.5 \times 10^5$ cells $mL^{-1}$ for *Navicula* sp. The selection criteria were the lowest possible cell density emitting a steady state fluorescence signal between 0.08 and 0.12 units at a low gain setting (Schreiber et al., 2007). Replicate cultures were diluted accordingly before each assay. Due to differences in cellular chlorophyll concentration and size, densities of *N. pyriformis* cultures was adjusted to achieve a similar steady state chlorophyll fluorescence signal, corresponding to $8 \times 10^5$ cells $mL^{-1}$. Densities of *C. closterium* and *P. tricornutum* cultures ($n = 1$) in exponential growth phase were likewise adjusted to $4 \times 10^5$ and $1.9 \times 10^5$ cells $mL^{-1}$, respectively to achieve a steady state chlorophyll fluorescence ($F$) signal of 0.08–0.12. Maxi-IPAM settings were: ML = 12, ML frequency = 8 (corresponding to photosynthetically active radiation [PAR] of 4 µmol photons $m^{-2}\,s^{-1}$), gain = 3 and damping = 2 throughout the experiment.

Herbicide exposures were performed in black polypropylene 96-well-plates (Greiner, Sigma Aldrich). Plates containing microalgae suspensions only were acclimatized to the measuring light (ML) in the Maxi-IPAM for 6 min before initiating five saturation pulses at 90 s intervals to obtain initial $F$ and $F'_m$ values (Schreiber et al., 2007). An appropriate acclimation period following dosing was determined for each toxicant by monitoring inhibition for up to 4 h (using an $IC_{50}$-dose previously estimated during range-finder experiments). To estimate consistency of inhibitory response between the replicate cultures, diuron was included as a positive control at a final concentration 2.4 µg $L^{-1}$ in five wells on each plate in all dose-response experiments. Diuron-control response was deemed consistent if falling within two standard deviations of the average diuron-control response of the preceding replicates (Muller et al., 2008).

### 2.4. Toxicity of binary mixtures

A toxic unit (TU) approach (often referred to as dose addition or concentration addition (CA) (Faust et al., 1993) was used to examine mixture toxicity, following Pape-Lindstrom and Lydy (1997). In this approach, a value of 1 TU is assigned to the $IC_{50}$ of each toxicant. The sum of the TU contributed by each compound describes the total toxicity of the mixture ($TU_{sum}$) according to Eq. (3)

$$TU_{\text{sum}} = C_{(1)}/\text{IC}_{50(1)} + C_{(2)}/\text{IC}_{50(2)} + \cdots + C_{(i)}/\text{IC}_{50(i)} \quad (3)$$

where $C_i$ is the actual concentration of the $i$th chemical in the mixture. The experimentally derived toxicity of the mixture was compared to the expected (calculated from $TU_{sum}$). If 50% inhibition occurred at a $TU_{sum}$ value of 1, toxicity of the mixtures is considered additive, if 50% inhibition occurred at $TU_{sum}$ values less than 1, toxicity of the mixtures is synergistic, and if 50% inhibition occurred at a $TU_{sum}$ value larger than 1, toxicity of the mixtures is antagonistic.

TU experiments were performed similarly to single-chemical-exposure experiments, with a dilution series of eight (*Navicula* sp.) or nine (*N. pyriformis*) mixtures including 0.06, 0.13, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0 and 4.0 $TU_{sum}$s. In each mixture, the individual components were added at identical proportions of their individual $IC_{50}$ to prevent the response to a more potent chemical from masking the mixture response. The binary mixtures included combinations of the various chemical classes, and were diuron + atrazine (urea + triazine), diuron + tebuthiuron (urea + urea), and atrazine + simazine (triazine + triazine). Replicate cultures ($n = 6$) of both species were tested using binary mixtures following the method above except for within-culture replication being decreased to two wells per concentration. Within-herbicide mixtures, i.e. dose-response to a dilution series of each individual herbicide made up in TU for that particular herbicide, were added to serve as mixture controls (i.e. diuron + diuron, etc.) ($n = 6$). Inhibition-data was plotted against concentration expressed as toxic units.

### 2.5. Statistical analyses

A four parameter sigmoidal regression with the maximum constrained to 100% was fitted to all dose-response inhibition data (Motulsky and Christopoulos, 2003) in SigmaPlot 7.1 (SPSS Inc.) (Eq. (4))

$$Y = \min + (\max - \min)/(1 + (x/\text{EC}_{50})^{\text{Hillslope}}) \quad (4)$$

This equation was solved for $x$ to determine $IC_{50}$ and $IC_{10}$ values in SigmaPlot 7.1. Adjustable parameters were Hillslope, minimum and $EC_{50}$. $IC_{50}$ and $IC_{10}$ values are reported as nominal concentrations based on the initial dosages. Lowest observed effect concentrations (LOEC) were determined for all endpoints and herbicides/species combinations using one-way ANOVA followed by Dunnett's post hoc $t$-test (Statistica 7, StatSoft Inc. Oklahoma, USA). Any significant differences between $TU_{mix}$ and $TU_{control}$ $IC_{50}$s were determined using a one-way ANOVA followed by Tukey's HSD post hoc test (Statistica 7, StatSoft, Inc. Oklahoma, USA).

## 3. Results and discussion

### 3.1. Optimal exposure durations for phytotoxicity assays

Maximum inhibition of *Y(II)* was reached within minutes from dosing for all herbicides in assays with *Navicula* sp. and *N. pyriformis* (Fig. 1), with the exception of hexazinone (Fig. 1 C), which required as long as 3 h to reach maximum phytotoxicity (% inhibition of *Y(II)*) in assays with *N. pyriformis*. The responses of *C. closterium* and *P. tricornutum* to hexazinone were also slow, reaching maximal inhibition at approximately 1–3 h, respectively (data not shown). Therefore, exposure time for hexazinone was chosen as 3 h after which maximum inhibition was consistently reached for all species. 3,4-DCA had no effect on *Y(II)* and was excluded from further testing. Tebuthiuron toxicity to *Navicula* sp. declined slightly between 30 and 150 min after dosing (Fig. 1B). Likewise, the toxicity of desethylatrazine (DEA) remained steady for the first 15 min after dosing in *Navicula* sp., followed by a gradual 20% decrease until 3 h when the experiment was terminated (Fig. 1F). As dosing a full 96-well plate takes approximately 10–15 min, an incubation time after complete dosing is necessary to ensure maximum toxicity is reached for all wells across the plate and to minimize the effect of different exposure times across the plate (Muller et al., 2008). Acute exposure time was therefore chosen as 6 min timed from complete dosing of a full plate for the remaining test species and toxicant combinations.

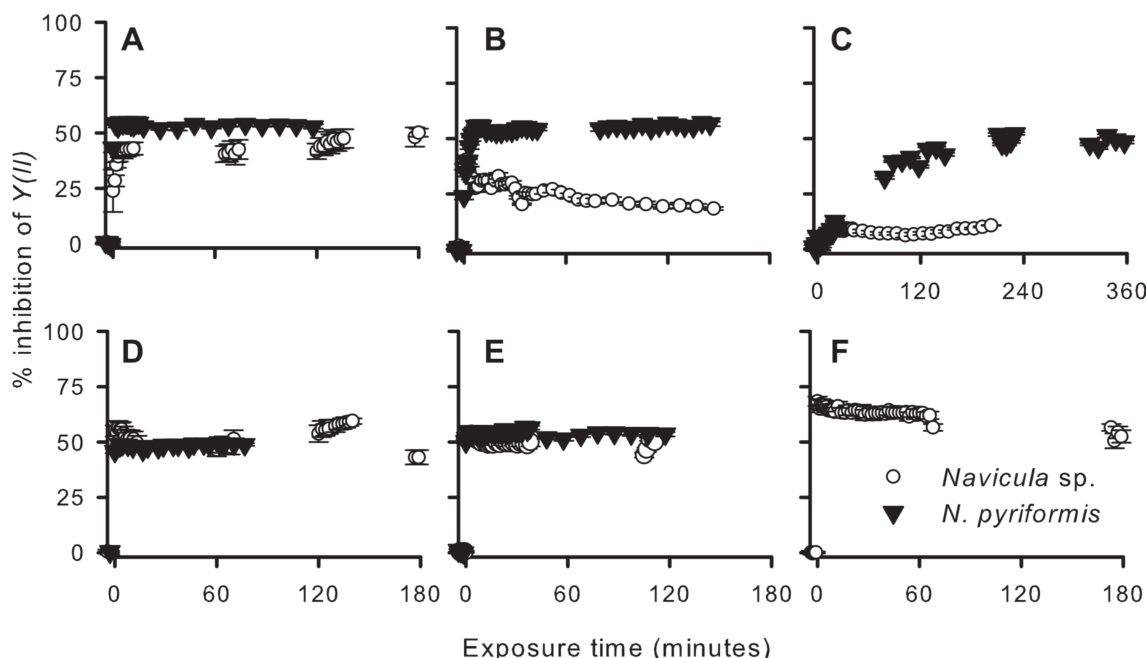


**Fig. 1.** Time-dependent inhibition of Y(II) in *Navicula* sp. (circles) and *N. pyriformis* (triangles) in response to a single-concentration dose of (A) diuron [2.4 μg $L^{-1}$], (B) tebuthiuron [*Navicula* sp.; 35 μg $L^{-1}$, *N. pyriformis*; 14 μg $L^{-1}$], (C) hexazinone [2.3 μg $L^{-1}$], (D) atrazine [*Navicula* sp.; 54 μg $L^{-1}$, *N. pyriformis*;14 μg $L^{-1}$], (E) simazine [*Navicula* sp.; 152 μg $L^{-1}$, *N. pyriformis*; 32 μg $L^{-1}$], and (F) DEA [*Navicula* sp.; 1.0 mg $L^{-1}$] Average ± SE ($n$ = 5 cultures, eight wells/culture).

Other studies have reported maximum PS(II)-inhibition of microalgae to PS(II)-targeting herbicides within minutes (Schreiber et al., 2007). The delayed effect on *Y(II)* of microalgae by hexazinone has also been reported, with toxicity increasing for up to 2 h from dosing in the green alga *Chlorella vulgaris* (Muller et al., 2008). The delayed response of *P. tricornutum* to hexazinone in the present study was not observed by Muller et al. (2008) despite the same origin of both strains (CSIRO, CS29). These within-strain differences in response-kinetics may be due to multiple morphology states of *P. tricornutum,* which includes triradiate, fusiform, and oval forms that differ with respect to their cell surface, valve and cytoplasm components (Round et al., 1990; Tesson et al., 2009). However, the CS29 culture used here and by Muller et al. (2008) stably expressed the fusiform morphotype which makes it less likely that observed differences in response are due to either cell surface chemistry or area:volume ratio. Additional differences and similarities of the experimental design and culturing conditions which may explain differing toxicological response in *P. tricornutum* are discussed further in the section on comparative sensitivity of local, tropical microalgae.

### 3.2. Comparative potency of five herbicides and breakdown products

After maximum inhibition was reached, the order of toxicity was the same for all species tested: diuron > hexazinone > tebuthiuron > atrazine > simazine > DEA > 3,4-DCA [no effect on *Y(II)*] (Table 2). $IC_{50}$-values for the four species ranged from 2.1 to 4.4 μg $L^{-1}$ for diuron, 2.4–6.9 μg $L^{-1}$ for hexazinone, 12–94 μg $L^{-1}$

**Table 2**
Effect concentrations (μg $L^{-1}$) for diuron, tebuthiuron, atrazine, simazine, hexazinone, and DEA to microalgae. Average ± (SE), $n$ = 5 cultures.

| Compound | Effect concentration (μg $L^{-1}$) | *Navicula* sp. | *Nephroselmis pyriformis* | *Phaeodactylum tricornutum*[a] | *Cylindrotheca closterium*[a] |
|---|---|---|---|---|---|
| Diuron | $IC_{50}$ | 2.6 (0.1) | 2.06 (0.06) | 2.71 (0.03) | 4.4 (0.1) |
| | $IC_{10}$ | 0.78 (0.06) | 0.32 (0.01) | 0.42 (0.01) | 0.63 (0.01) |
| | LOEC | 1.2 | 0.1 | 0.1 | 0.3 |
| Tebuthiuron | $IC_{50}$ | 94 (3) | 11.9 (0.2) | 51.4 (0.7) | 76.9 (0.7) |
| | $IC_{10}$ | 16.7 (0.5) | 2.31 (0.07) | 7.6 (0.2) | 10.1 (0.2) |
| | LOEC | 8.7 | 1.1 | 8.7 | 8.7 |
| Atrazine | $IC_{50}$ | 36 (8) | 14.2 (0.4) | 33.6 (0.4) | 76.7 (1.3) |
| | $IC_{10}$ | 5.6 (2) | 1.96 (0.09) | 4.5 (0.1) | 11.1 (0.4) |
| | LOEC | 13.5 | 1.1 | 4.5 | 4.5 |
| Simazine | $IC_{50}$ | 157 (5) | 24.4 (0.6) | 101 (2) | 242 (5) |
| | $IC_{10}$ | 25.3 (1.8) | 3.65 (0.1) | 11.3 (0.4) | 35.1 (0.6) |
| | LOEC | 24.6 | 4.0 | 2.0 | 16 |
| Hexazinone | $IC_{50}$ | 5.7 (0.1) | 2.4 (0.05) | 6.6 (0.1) | 6.9 (0.08) |
| | $IC_{10}$ | 1.41 (0.05) | 0.47 (0.01) | 1.74 (0.05) | 1.7 (0.08) |
| | LOEC | 1.2 | 0.07 | 0.6 | 0.6 |
| DEA | $IC_{50}$ | 633 (9) | 216 (7) | 426 (6) | 1080 (20) |
| | $IC_{10}$ | 111 (6) | 26.6 (0.8) | 46 (1) | 102 (4) |
| | LOEC | 68.1 | 15.6 | 7.88 | 62.5 |

*Abbreviations*: DEA, desethylatrazine; LOEC, lowest observed effect concentration. LOEC determined by ANOVA followed by Dunnetts post hoc $t$-test.
[a] $n$ = 1 culture, average and SE within plate (eight wells).

for tebuthiuron, 14–77 μg $L^{-1}$ for atrazine, and 24–242 μg $L^{-1}$ for simazine. Dichloroaniline (3,4-DCA) is a general toxicant and had no effect on any of the fluorescence parameters measured with the Maxi-IPAM in this assay (results not shown). DEA did induce some photoinhibition, but only at very high concentrations ($IC_{50}$ ranging from 216–1080 μg $L^{-1}$), and is not expected to be phytotoxic at environmentally relevant concentrations. Notably, diuron and hexazinone elicited 26–40% inhibition of photosynthesis at the concentration trigger values for protection of 95% of species set by the Great Barrier Reef Marine Park Authority (GBRMPA) (diuron trigger-value = 1.6 μg $L^{-1}$, hexazinone trigger-value 1.2 μg $L^{-1}$) (GBRMPA, 2009). The $IC_{10}$s and lowest observed effect concentrations (LOECs) commonly were below the 95% species protection triggers proposed for diuron, tebuthiuron, and hexazinone for all the species tested here, particularly so for the green alga *N. pyriformis* (Table 2). Since *Y(II)* inhibition by PS(II)-inhibitors is strongly correlated with reduced growth of this species (Magnusson et al., 2008), current trigger values may not adequately protect sensitive marine primary producers such as *N. pyriformis*.

Hexazinone was the only toxicant that consistently elicited a maximum response of 100% inhibition of *Y(II)* in the assay (Fig. 2). Even diuron, the most potent of the herbicides tested, generally only reached 97–98% inhibition, and no more than 84% inhibition was reached in *C. closterium*. Additionally, increasing the maximum diuron concentration from 15 to 20 μg $L^{-1}$ did not increase maximum inhibition reached in *C. closterium*. This trend has been observed previously, and may reflect heterogeneity in PS(II), with a fraction of PS(II) reaction centres not connected *via* the secondary acceptor $Q_B$ to the plastoquinone pool (non-B type PS(II)) (Muller et al., 2008; Schreiber et al., 2007). Muller et al. (2008) also suggested that differences in toxicant-resistant PS(II) activity between test compounds may be due to inhibition of non-B-type electron transport by some herbicides (for example hexazinone).

### 3.3. Comparative sensitivity of local, tropical microalgae

All four species were sensitive to the herbicides tested; however, the order of species sensitivity differed between the herbicides (Table 2). The locally isolated green alga *N. pyriformis* was always the most sensitive species in the test-battery, particularly to simazine, where the difference between it and the least sensitive species (*C. closterium*) was an order of magnitude (simazine $IC_{50}$ 24.4 μg $L^{-1}$in *N. pyriformis* compared to 242 μg $L^{-1}$in *C. closterium*) (Fig. 2 and Table 2). For the remaining herbicides, *N. pyriformis* was generally 2–5 fold more sensitive compared to the three other species tested. The two local diatom species (*Navicula* sp. and *C. closterium*) showed sensitivities generally comparable to the standard test-species *P. tricornutum*.

The higher sensitivity *N. pyriformis* to PS(II)-inhibiting herbicides is consistent with previous studies, which often report chlorophytes to be more susceptible to herbicides compared to ochrophytes (diatoms) (Bérard et al., 2003; Guasch et al., 1997). Smaller cell-size in microalgae has been reported to increase sensitivity to toxicants (DeLorenzo et al., 2004; Weiner et al., 2004), and the much larger surface area:volume ratio in *N. pyriformis* compared to the three other species tested may partly explain its higher sensitivity. However, sensitivity did not correlate with cell-size amongst the diatoms tested in this study but varied with the herbicide tested. It is possible that morphological cell surface characteristic differences between the three diatom species can explain the observed responses to some extent. There is direct access of the pollutant to the plasma membrane due to the absence of a frustule in this morphotype of *P. tricornutum,* and *via* pores in the silica frustule in *Navicula* sp., compared to restricted access in *C. closterium* which has a poreless, weakly silicified frustule (Graham and Wilcox, 2008; Round et al., 1990).

All herbicide $IC_{50}$-estimates fell within the lower range of previously published ranges for similar organisms and test-systems (Bengtson-Nash et al., 2005a; Escher et al., 2006; Schreiber et al., 2002). For example, the acute $IC_{50}$ for *P. tricornutum* exposed to diuron in the current study was 2.7 μg $L^{-1}$. This is similar to the acute $IC_{50}$ for *P. tricornutum* exposed to diuron ($IC_{50}$ = 3.3 μg $L^{-1}$) obtained using a ToxY-PAM fluorometer (Schreiber et al., 2002). Acute $IC_{10}$s (inhibition of *Y(II)*, ToxY-PAM) for *P. tricornutum* exposed to PS(II)-inhibitors were 0.74 μg $L^{-1}$ (diuron), 2.7 μg $L^{-1}$ (hexazinone), 4.4 μg $L^{-1}$ (atrazine), and 29 μg $L^{-1}$ (simazine) (Bengtson-Nash et al., 2005a), values similar to those for the least sensitive species in the current study (Table 2). Contrasting this, diuron 1-h $IC_{50}$s were reported to be 18 μg $L^{-1}$ for *P. tricornutum*

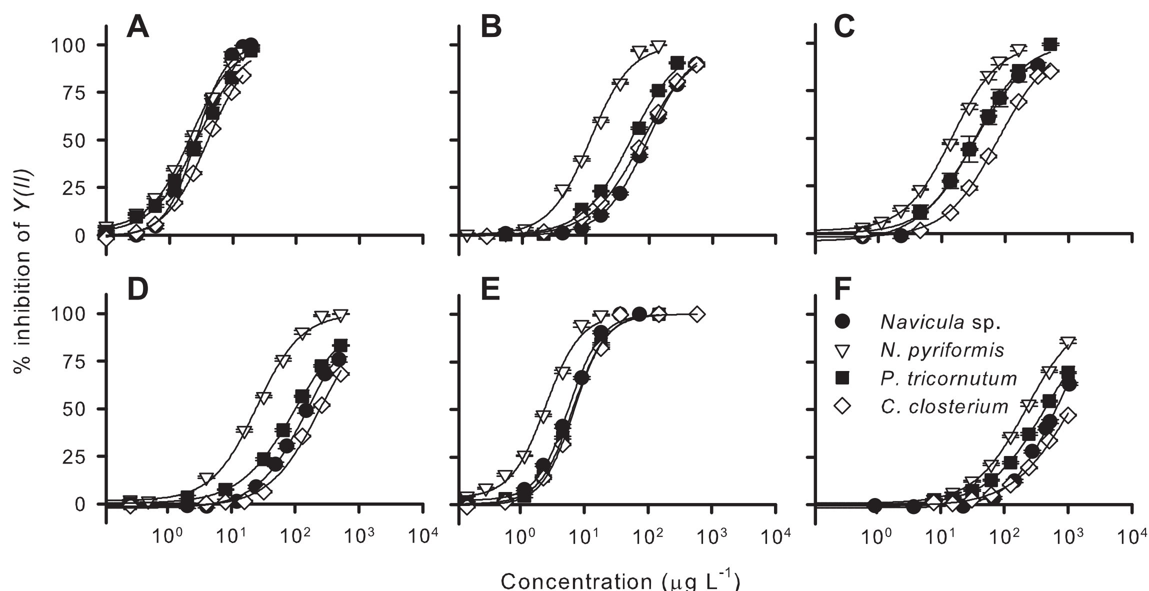


**Fig. 2.** Inhibition of *Y(II)* in four species of microalgae in herbicide dose-response experiments. (A) diuron, (B) tebuthiuron, (C) atrazine, (D) simazine, (E) hexazinone and (F) DEA. Average ± SE (*n* = 5 cultures) (*C. closterium* and *P. tricornutum* *n* = 1 culture, eight wells within culture replication).

and 20 $\mu g L^{-1}$ for *C. vulgaris*, respectively in a Maxi-IPAM study similar to the current study (Muller et al., 2008). In the same study, hexazinone immediately exerted maximal inhibitory response in *P. tricornutum* (Muller et al., 2008) as opposed to the 3 h delay reported here. The test and culturing conditions were nearly identical in the two studies (Maxi-IPAM measuring light (ML) = 10 with an exposure time of 1 h, algae cultured in Guillard f/2 medium at 23 °C under a 12:12 light:dark cycle, 50 μmol photons $m^{-2} s^{-1}$ in the study by Muller et al. (2008)). Actinic light and exposure duration has only a small effect on *Y(II)* inhibition caused by diuron (Schreiber et al., 2007) and is not likely to explain observed difference between the two studies. Likewise, both studies employed cultures in logarithmic growth at similar cell densities (corresponding to steady state fluorescence, *F*, 0.08–0.12). With such large within-strain variability in both sensitivity and response time to common agricultural contaminants under very similar test- and culturing conditions, *P. tricornutum* cannot be recommended as a standard test organism in ecotoxicological studies.

Reduced growth rates (primary production) of benthic microalgae in response to PS(II)-inhibitors are of great concern for coastal habitats (Magnusson et al., 2008) and nearshore corals may also be affected by these pollutants (Jones and Kerswell, 2003). Inhibition of *Y(II)* in the symbiotic microalgae (*Symbiodinium* spp.) of corals is correlated to reduced uptake of energy by the coral host (Cantin et al., 2009) and can lead to reduced reproductive output and coral bleaching and mortality (Cantin et al., 2007). The sensitivity of microalgae tested here to PS(II)-inhibitors is generally greater than the sensitivity of *Symbiodinium* spp. in corals (Jones and Kerswell, 2003; Jones et al., 2003; Negri et al., 2005). For example, isolated symbionts have a reported acute $IC_{50}$ for inhibition of *Y(II)* of 5 $\mu g L^{-1}$ diuron (Jones et al., 2003), and early life history stages of hard corals exhibit a LOEC of 1 $\mu g L^{-1}$ for diuron (Negri et al., 2005) compared with 0.1–1.2 $\mu g L^{-1}$ for microalgale in this study. The algal symbionts within the scleractinian corals *Acropora formosa*, *Montipora digitata* and *Porites cylindrica* also showed similar sensitivity to atrazine as the diatoms tested studied here, with 10-h $IC_{50}$ (inhibition of *Y(II)*, PAM fluorometry) of 37, 88 and 67 $\mu g L^{-1}$ for symbionts within the three species of hard coral, respectively (Jones et al., 2003), compared to 36 $\mu g L^{-1}$ (*Navicula* sp.) and 77 $\mu g L^{-1}$ (*C. closterium*) reported here. A strong 1:1 correlation ($r^2 = 0.97$) of $EC_{50}$s for *Navicula* sp. (this study) and *Symbiodinium* spp. (Jones et al., 2003; Jones and Kerswell, 2003) within corals exposed to the herbicides diuron, hexazinone, atrazine and simazine indicates equal sensitivity for these species to the listed herbicides (Fig. 3A, solid line). In contrast, *N. pyriformis* was consistently ($r^2 = 0.97$) 6.7-fold more sensitive towards the same PS(II)-inhibitors compared with *Symbiodinium* spp. (Fig. 3B, solid line). Tebuthiuron was comparatively much more toxic to both microalgae in this study than to *Symbiodinium* spp., as illustrated in the decreased slope of the regressions when including this herbicide in the analysis (Fig. 3A and B, dashed lines).

While *Symbiodinium* spp., (*in hospite* or as isolates) are the most common test organism for herbicide exposure studies in tropical organisms, benthic green algae such as the prasinophyte *N. pyriformis* may be significantly more sensitive and are more likely to be exposed to higher concentrations of herbicides within estuarine waters (Lewis et al., 2009). *Nephroselmis pyriformis* has previously been shown to be very sensitive compared to other microalgae in ecotoxicological studies, for example to ammonia ($NH_3$) in water treatment plant effluent (Källqvist and Svensson, 2003). This species is found in both temperate and tropical waters (Guillou et al., 2004; Magnusson et al., 2008; Moestrup, 1983) and is temperature and halo-tolerant, occurring in salinities from 2 to 36 ppt (Moestrup, 1983). Furthermore, prasinophytes can contribute up to 20% of the biomass in estuarine phytoplankton (Carreto et al., 2003), thus representing a significant proportion of the microalgal assemblage. Based on these properties, *N. pyriformis* can be recommended for inclusion as a standard species in test-batteries for future ecotoxicological research, suitable for both tropical and temperate conditions.

### 3.4. Binary mixtures

All binary mixtures tested exhibited additive toxicity, with $IC_{50}$-estimates overlapping 1 TU (which is the requirement for of additive toxicity using the toxic unit approach (Pape-Lindstrom and Lydy, 1997)), or not being significantly different from the response in control mixtures (ANOVA, $p > 0.05$) (Fig. 4, Table 3).

Both dose-responses to each of the individual herbicides in a mixture, and dose-responses to individual herbicides made in a concentration series corresponding to the same TUs used in the binary mixtures were tested simultaneously on one 96-well plate as described in the methods section. Despite the very low between-culture variability in response and $IC_{50}$-estimates of the single herbicide dose-response experiments (Fig. 2, Table 2), it proved crucial to correct the $TU_{mix}$ against simultaneously determined

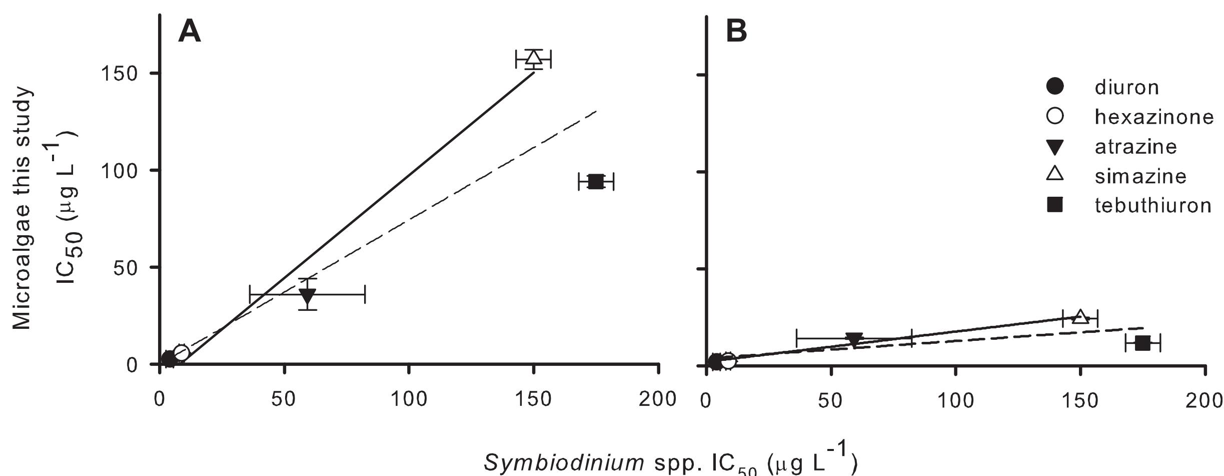


**Fig. 3.** The sensitivity (expressed as $IC_{50}$) of (A) *Navicula* sp., and (B) *Nephroselmis pyriformis* compared to *Symbiodinium* spp. (coral symbiont) to all five herbicides (dashed regression line) and to diuron, atrazine, simazine, and hexazinone (solid line). Average ± stdev, $n = 5$ from this study, variable for *Symbiodinium* spp. where values are adapted from Jones and Kerswell (2003) and Jones et al. (2003). Equations for the regressions are (A) dashed line: $y = 0.74x - 0.06$, $r^2 = 0.80$; solid line: $y = 1.06x - 8.44$, $r^2 = 0.97$, (B) dashed line: $y = 0.09x + 3.90$, $r^2 = 0.58$; solid line: $y = 0.15x + 2.13$, $r^2 = 0.97$.

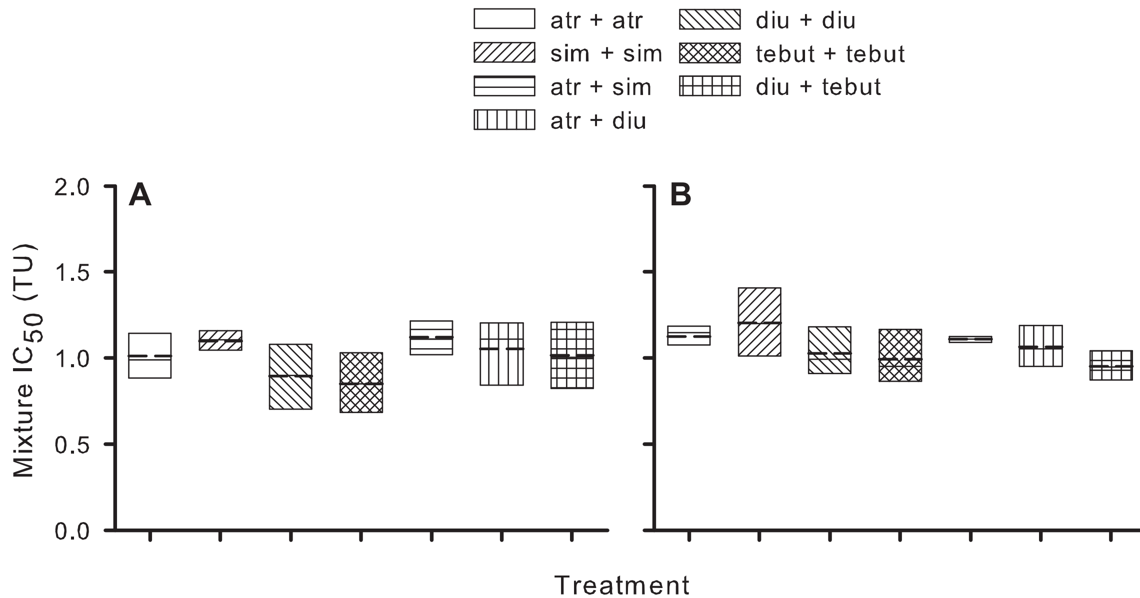


**Fig. 4.** $IC_{50}$s expressed in toxic units (TU) for (A) *Navicula* sp. and (B) *N. pyriformis* exposed to binary herbicide mixtures. Values above 1 denote antagonistic mixture behaviour, values overlapping 1 denote additive mixture behaviour, and values below 1 denote synergistic mixture behaviour. *Abbreviations*: atr, atrazine; diu, diuron; sim, simazine and tebut, tebuthiuron. Dotted lines represents average ($n = 6$), boxes denote 5**th** and 95**th** percentiles.

**Table 3**
ANOVA univariate results comparing mean $TU_{mix}$ $IC_{50}$ for *Navicula* sp. and *N. pyriformis* to the mean $TU_{control}$ $IC_{50}$ of respective species for all mixtures.

| | Effect | df | SS | MS | F | *p* |
|---|---|---|---|---|---|---|
| *Navicula sp.* | | | | | | |
| Atr + Atr, Sim + Sim, Atr + Sim | Intercept | 1 | 20.92 | 20.92 | 1893 | <0.05 |
| | Treatment | 2 | 0.041 | 0.022 | 1.853 | >0.05 |
| | Error | 15 | 0.166 | 0.011 | | |
| | Total | 17 | 0.207 | | | |
| Atr + Atr, Diu + Diu, Atr + Diu | Intercept | 1 | 17.567 | 17.57 | 564.6 | <0.05 |
| | Treatment | 2 | 0.0789 | 0.039 | 1.269 | >0.05 |
| | Error | 15 | 0.4667 | 0.031 | | |
| | Total | 17 | 0.546 | | | |
| Diu + Diu, Tebuth + Tebuth, Diu + Tebuth | Intercept | 1 | 15.285 | 15.29 | 390.2 | <0.05 |
| | Treatment | 2 | 0.086 | 0.043 | 1.093 | >0.05 |
| | Error | 15 | 0.588 | 0.039 | | |
| | Total | 17 | 0.673 | | | |
| *Nephroselmis pyriformis* | | | | | | |
| Atr + Atr, Sim + Sim, Atr + Sim | Intercept | 1 | 23.676 | 23.676 | 1466 | <0.05 |
| | Treatment | 2 | 0.032 | 0.016 | 0.979 | >0.05 |
| | Error | 15 | 0.242 | 0.016 | | |
| | Total | 17 | 0.273 | | | |
| Atr + Atr, Diu + Diu, Atr + Diu | Intercept | 1 | 20.722 | 20.721 | 1396 | <0.05 |
| | Treatment | 2 | 0.028 | 0.014 | 0.959 | >0.05 |
| | Error | 15 | 0.223 | 0.015 | | |
| | Total | 17 | 0.251 | | | |
| Diu + Diu, Tebuth + Tebuth, Diu + Tebuth | Intercept | 1 | 17.706 | 17.706 | 1085 | <0.05 |
| | Treatment | 2 | 0.0172 | 0.009 | 0.528 | >0.05 |
| | Error | 15 | 0.245 | 0.016 | | |
| | Total | 17 | 0.262 | | | |

*Abbreviations*: Atr = atrazine, Sim = simazine, Diu = diuron, and Tebuth = tebuthiuron.

$IC_{50}$s. Additionally, 1 TU of control mixtures did not always elicit 50% inhibition (i.e. the $IC_{50}$ varies sufficiently between the exposures to slightly alter the TU ratios), which shows that the incorporation of mixture controls are required to draw reliable conclusions from this type of experiment. As 1 TU of a compound is by definition equal to the $IC_{50}$ of the compound (Pape-Lindstrom and Lydy, 1997), additivity was here re-defined as when the mean $IC_{50}$ of the binary mixture was not significantly different to the average control-mixture $IC_{50}$ response. For example, the response of *N. pyriformis* to the binary mixture of atrazine + simazine was deemed additive, as the determined TU $IC_{50(atr+sim)}$ was not significantly different from the control mixtures TU $IC_{50(atr+atr)}$ or TU $IC_{50(sim+sim)}$ (ANOVA, $F_{2,15} = 0.979$, $p > 0.05$, Table 3).

The results reported here confirm the general assumption that concentration addition (CA) predicts the effect of a mixture of similarly acting chemicals. In multi-component mixtures of 18 different s-triazines in ratios of their respective $IC_{50}$ and $IC_{10}$ values, Faust et al. (2001) showed that even when all herbicides were present at below the no observed effect concentration (NOEC) for each herbicide, a total effect of 47% inhibition of growth in

synchronized cultures of *Scendesmus vacuolatus* was recorded (Faust et al., 2001). This fitted very well with the response predicted by a CA model. It was concluded that there is no environmentally safe threshold-concentration for an individual chemical, as the additive effect may still be significant if similarly acting chemicals are present simultaneously (Faust et al., 2001).

### 3.5. Environmental relevance and recommendations

The current results strongly support the adoption by regulators of Herbicide Equivalent (HEq) units, where the concentrations and potencies of PS(II)-inhibitors can be added together to reliably estimate the potential impacts of mixtures in the field (Bengtson-Nash et al., 2005a; Escher et al., 2006; Schreiber et al., 2002). The Relative Equivalent Potency ($REP_i$) of an individual herbicide compared with diuron can be calculated according to Eq. (5) where $i$ is a second PS(II)-inhibiting herbicide.

$$REP_i = [IC_{50}\ \text{diuron}]/[IC_{50}\ \text{herbicide}\ i] \quad (5)$$

HEqs are then calculated according to Eq. (6) where $C_i$ is the concentration of PS(II)-inhibitor $i$.

$$HEq = \sum (C_i \times REP_i) \quad (6)$$

HEqs can thus be derived for PS(II)-inhibitors in a mixture, resulting in a single concentration with a known effect-estimate. The REPs for tebuthiuron, atrazine, simazine and hexazinone (relative to diuron) for tropical microalgae (Magnusson et al., 2008, present study) are listed in Table 4.

To illustrate the use of HEqs, a combined presence of the marine trigger-value concentrations for protection of 95% of species in the GBR marine park (GBRMPA, 2009) (Table 5), would correspond to HEq = 2.78 $\mu g\ L^{-1}$ of diuron for local microalgae. This would be sufficient to cause more than 50% inhibition of photosynthesis in the majority of the microalgae tested here. Similarly, the combined maximum concentrations of PS(II)-inhibitors during flooding in waterways draining into the GBR (listed in Table 5, data from Lewis et al. (2009)) corresponds to HEq = 26 $\mu g\ L^{-1}$ diuron, enough to almost completely stop photosynthesis in microalgae. While this may be considered a more extreme example, even the more moderate median herbicide concentrations detected in floodwaters (Lewis et al., 2009) correspond to up to HEq = 1.5 $\mu g\ L^{-1}$, potentially giving rise to up to 40% inhibition of photosynthesis in tropical microalgae. While these high concentrations are transient, elevated concentrations are still present (at approximately halved concentrations) nine days after the flooding event (Lewis et al., 2009), with lower chronic concentrations persisting through the year (Shaw et al., 2010; Shaw and Müller, 2005) (Table 5). These chronic concentrations, equivalent to HEqs of up to 0.35 $\mu g\ L^{-1}$ diuron can potentially exert a selection pressure for more tolerant algal species, approximately eliciting a 10% decline in photosynthetic efficiency in *N. pyriformis* which corresponds to a 10% decline in growth (Magnusson et al., 2008).

Considerable information on the sensitivity of tropical microalgae to herbicides under environmentally relevant conditions (low concentrations, inclusion of herbicide breakdown products, and mixtures) was derived through this study. The general pattern of relatively high sensitivity in the tropical benthic microalgae tested here compared to temperate and standard test species, stresses the value of including locally isolated organisms in a test-battery, as these may not be protected by guidelines derived from other species/regions. Additionally, the additive toxicity of the herbicide mixtures emphasise that there may be no environmentally safe threshold-concentration for similarly acting chemicals, as they may still pose a risk when present together in mixtures. This stresses the importance of incorporating total-maximum-load measures (i.e. integrating HEq-values for local species) when establishing guidelines for environmental protection purposes, instead of treating each contaminant in isolation.

**Table 4**
Relative potency of PS(II)-inhibitors (REP) to inhibit $\Delta F/F_{m'}$ compared with diuron. Higher values represent higher potency (data are from Magnusson et al., 2008[1]; Present study[2], $IC_{50}$s expressed in molar concentrations were used to derive HEqs).

| Herbicide | *Navicula* sp. | | *Nephroselmis pyriformis* | | *Phaeodactylum tricornutum* | *Cylindrotheca closterium* | *Mean REP* |
|---|---|---|---|---|---|---|---|
| | [1] | [2] | [1] | [2] | [2] | [2] | |
| Diuron | 1 | 1 | 1 | 1 | 1 | 1 | 1.00 |
| Tebuthiuron | | 0.03 | | 0.17 | 0.05 | 0.05 | 0.08 |
| Atrazine | 0.05 | 0.07 | 0.19 | 0.13 | 0.07 | 0.05 | 0.09 |
| Simazine | | 0.01 | | 0.07 | 0.02 | 0.02 | 0.03 |
| Hexazinone | 0.39 | 0.50 | 1.0 | 0.93 | 0.44 | 0.67 | 0.66 |
| DEA | | 0.00 | | 0.01 | 0.01 | 0.00 | 0.01 |

**Table 5**
The 95% species protection trigger values for diuron, tebuthiuron, atrazine, simazine and hexazinone ($\mu g\ L^{-1}$ and HEqs) set by GBRMPA (2009), the range of chronic herbicide concentrations detected in the Queensland marine and near-shore environment using time-integrating passive samplers and in grab-samples from flood plumes.

| Herbicide | GBRMPA 95% protection trigger value ($\mu g\ L^{-1}$) | GBRMPA 95% protection trigger value HEq[a] ($\mu g\ L^{-1}$) | Environmental concentrations chronic[b] ($\mu g\ L^{-1}$) | Environmental concentrations flood plumes[c] ($\mu g\ L^{-1}$) |
|---|---|---|---|---|
| Diuron | 1.6 | 1.6 | nd – 0.35 | nd – 22 |
| Tebuthiuron | 2.0 | 0.16 | nd | nd – 1.5 |
| Atrazine | 1.4 | 0.13 | nd – 0.0003 | nd – 8 |
| Simazine | 3.2 | 0.10 | nd– 0.0025 | nd – 0.18 |
| Hexazinone | 1.2 | 0.79 | nd – 0.00045 | nd – 5 |
| DEA | na | na | nd | nd – 0.11 |
| SUM HEq | | 2.78 | Max ~0.35 | Median ~1.52<br>Max ~26.2 |

na = not applicable, there are no guidelines for herbicide breakdown products. nd = analysed for, but not detected.
[a] HEq is the herbicide equivalent concentration relative to diuron ($H_{eq} = REP_i \times C_i$) where $C_i$ is the trigger concentration and $REP_i$ is the herbicide potency from Table 4).
[b] Collated from Shaw and Müller (2005) and Shaw et al. (2010).
[c] Estimated from Fig. 2, Lewis et al. (2009).

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