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Acute and additive toxicity of ten photosystem-II herbicides to seagrass

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Photosystem II herbicides are transported to inshore marine waters, including those of the Great Barrier Reef, and are usually detected in complex mixtures. These herbicides inhibit photosynthesis, which can deplete energy reserves and reduce growth in seagrass, but the toxicity of some of these herbicides to seagrass is unknown and combined effects of multiple herbicides on seagrass has not been tested. Here we assessed the acute phytotoxicity of 10 PSII herbicides to the seagrass *Halophila ovalis* over 24 and/or 48 h. Individual herbicides exhibited a broad range of toxicities with inhibition of photosynthetic activity ($\Delta F/F_m'$) by 50% at concentrations ranging from 3.5 μg l^{-1} (ametryn) to 132 μg l^{-1} (fluometuron). We assessed potential additivity using the Concentration Addition model of joint action for binary mixtures of diuron and atrazine as well as complex mixtures of all 10 herbicides. The effects of both mixture types were largely additive, validating the application of additive effects models for calculating the risk posed by multiple PSII herbicides to seagrasses. This study extends seagrass ecotoxicological data to ametryn, metribuzin, bromacil, prometryn and fluometuron and demonstrates that low concentrations of PSII herbicide mixtures have the potential to impact ecologically relevant endpoints in seagrass, including $\Delta F/F_m'$.

Seagrass is found in coastal habitats globally, including all marine bioregions of Australia, and its distribution and form depends strongly on local environmental and anthropogenic conditions[1]. The estimated total area of seagrass meadows within the Great Barrier Reef (GBR) World Heritage Area is greater than 40,000 km[2], and exceeds that of coral reef[2,3]. This together with their profound ecological importance highlights the significance of monitoring and protecting seagrass habitats[1]. One of the most widespread and common species throughout tropical and subtropical regions of Australia is *Halophila ovalis*[4], with this species inhabiting the shallow zones to depths as great as 50 m[5]. *H. ovalis* is considered a colonising species[1,6] with a high turnover in above-ground material[7] and rapid re-growth when environmental conditions are again favourable[1]. Species such as *H. ovalis* may be considered a sentinel species as its sensitivity to environmental disturbances may provide insights or early warning of environmental stress (both seasonal and anthropogenic)[8].

Photosystem II (PSII) herbicides are applied extensively to crops and the high mobility and persistence[9] of these herbicides can result in elevated concentrations in the marine environment. For example, an estimated 30,000 kg per annum of PSII herbicides are transported through waterways into nearshore waters of the World Heritage listed GBR each year[10]. Herbicide concentrations are highest nearshore and in the vicinity of seagrass meadows[11], which are sensitive to PSII herbicides[12–14]. These coastal seagrasses are also at risk from elevated turbidity from catchment and urban runoff, as well as port development[15]. Agricultural runoff from GBR catchments results in mixtures of PSII herbicides being detected at concentrations exceeding 0.9 μg l^{-1}, the current 99% species protection guideline for the GBR[16–18]. These herbicides block electron transport in PSII of plants by binding to the D1 protein in the thylakoid membrane

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Herbicide	Chemical class	Log K_{ow}	Water solubility (mg l^{-1})	CAS number
Diuron	phenylurea	2.6	37.4	330-54-1
Fluometuron	Phenylurea	2.4	110	2164-17-2
Tebuthiuron	Phenylurea	1.8	2,500	34014-18-1
Atrazine	s-triazine	2.5	29,800	1912-24-9
Ametryn	s-triazine	2.6	200	834-12-8
Metribuzin	s-triazine	1.6	1050	21087-64-9
Simazine	s-triazine	2.1	6.2	122-34-9
Prometryn	s-triazine	3.1	33	7287-19-6
Bromacil	uracil	1.9	807	317-40-9
Hexazinone	triazinone	1.2	33,000	51235-04-2

Table 1. Properties of herbicides tested. Water solubility calculated at > 20 °C. All data from[64].

and displacing plastoquinone, which in turn inhibits the synthesis of ATP and NADPH[19]. The most sensitive indicator of PSII effects on marine organisms is the inhibition in effective quantum yield of PSII ($\Delta F/F_m$') which can be measured using the non-invasive technique of pulse amplitude modulation (PAM) fluorometry (see Methods section)[20]. A reduction in $\Delta F/F_m$' by PSII herbicides is directly linked to reduced photochemical efficiency[21], and subsequently in seagrass to starvation and declines in growth and community fitness[13,20,22]. The ecological relevance of $\Delta F/F_m$' inhibition as an endpoint in marine PSII herbicide toxicity studies is further demonstrated by its strong correlation with growth inhibition in microalgae[23,24] and relationship with reduced reproductive output in corals[25,26].

Diuron is one of the most potent PSII herbicides detected in marine and estuarine waters, inhibiting $\Delta F/F_m$' by 50% (IC$_{50}$) at between 2.1 and 3.5 μg l^{-1} in marine microalgae[23], seagrass[8,12,13,27] and corals[28,29]. The other most commonly detected PSII herbicide atrazine is on average ~8-fold less potent than diuron towards tropical seagrasses, corals, microalgae, foraminifera and crustose coralline algae[12]. The range of potencies among other PSII herbicides is wider still, with tebuthiuron, being approximately 14-fold less toxic than diuron to a similar range of non-target marine species[12]. The differences in potency among the PSII inhibitors is likely due to the diverse stearic and lipophilic properties of the herbicides[30] along with other differences in structural interactions between the herbicides and the binding site in the D1 protein[19]. Changes in the usage patterns of PSII herbicides have led to an increasing diversity of herbicides being detected in the catchments and lagoon of the GBR[31]. While there may be sufficient ecotoxicological data to derive guidelines for some PSII herbicides such as, atrazine, ametryn, diuron, hexazinone and tebuthiuron[18], little is known of the relative toxicity of other PSII herbicides, such as bromacil, prometryn, metribuzin and fluometuron, which have also been detected in the GBR or its catchments[32–36]. Understanding the relative toxicities of these alternative PSII herbicides to tropical marine species, such as seagrass, is important for the sustainability and management of agricultural practices adjacent to the GBR catchment area and other locations where PSII herbicides are detected[27,37–39].

PSII herbicides are generally detected in complex mixtures with other PSII and/or non-PSII herbicides[40–42]. Although the PSII inhibitors are represented by a range of chemical classes (e.g. phenylurea, s-triazine and uracil), all have the same mode of action and their combined effects are considered additive for a variety of freshwater[24,43–45] and estuarine microalgae[23]. The Concentration Addition (CA) model of joint action is valid for multiple PSII herbicides as this combines the concentration and potency of each component to calculate the expected total toxicity of a mixture[46,47]. A common approach to test the applicability of CA is to apply Toxic Unit (TU) values to the herbicide concentrations that induce the equivalent toxicity. For example, the concentration which inhibits $\Delta F/F_m$' by 50% (IC$_{50}$) = 1 TU and is different for each herbicide[23,45]. Concentration-response curves of herbicide mixtures containing a range of TU values can then be used to validate CA for combinations of herbicides in a mixture (see Methods section).

Since the values of the World Heritage listed GBR are based on the fitness and survival of foundation, habitat-forming species such as seagrass, ecotoxicological data for these species should be included in future risk assessments and for the derivation and assessment of future water quality guidelines[48]. Here we assess the acute toxicity of 10 PSII herbicides (Table 1) individually and in mixtures to the tropical seagrass species, *Halophila ovalis*. The acute effects of individual herbicides on photosynthetic performance ($\Delta F/F_m$') of isolated leaves using a miniature bioassay was assessed in concentration-response experiments over a 24 and/or 48 h exposure period(s) in a static system[8]. The miniature bioassay methodology was also applied to examine the toxicity of PSII herbicides in binary and complex mixtures. Data from this study will broaden the relevant ecotoxicity data to include a range of alternative and emerging PSII herbicides and validate the additive toxicity of PSII herbicide mixtures on seagrass for application in monitoring programs and guideline development.
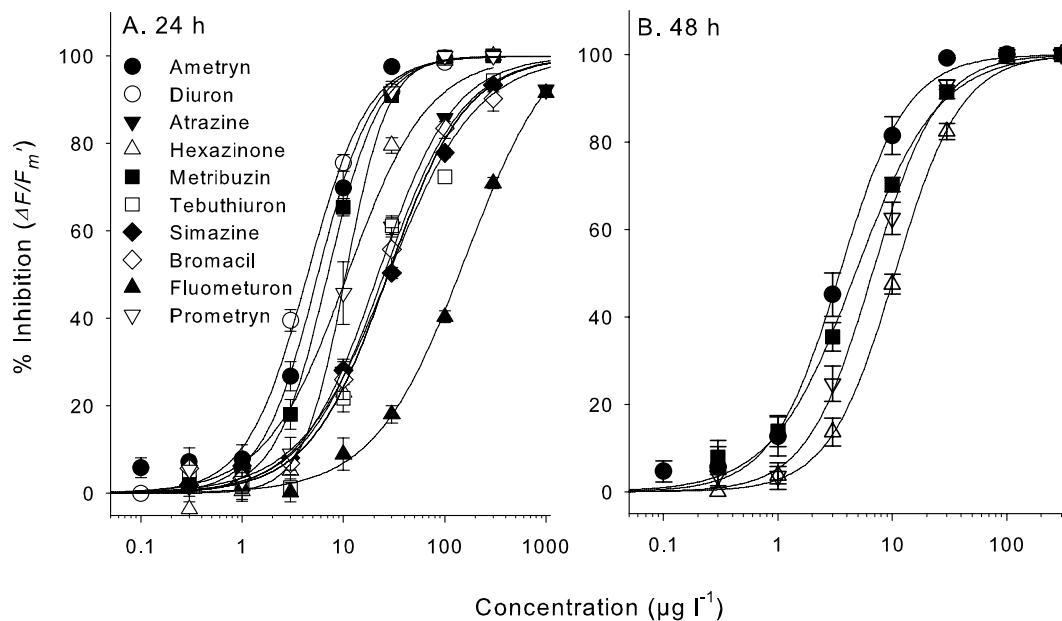
Figure 1. Concentration-response curves for individual herbicides. Concentration response curves for inhibition of $\Delta F/F_m$' measured at (**A**) 24 h and (**B**) 48 h for 10 individual herbicides, relative to each solvent control. The four herbicides in (**B**) did not reach maximum inhibition of $\Delta F/F_m$' within 24 h. Bars represent SE ± n = 9.

Herbicide	EC$_{10}$	95% CI	EC$_{50}$ = (1 TU)	95% CI	R^2	ReP
24 h exposures						
Diuron	1.2	1.0–1.5	4.3	3.9–4.7	0.99	1.0
Fluometuron	17	13–22	132	116 –150	0.99	0.033
Tebuthiuron	3.9	2.9–5.2	28	24–31	0.98	0.16
Atrazine	3.4	2.8–4.0	22	20–24	0.99	0.19
Ametryn	1.4	1.1–1.8	5.6	4.9–6.3	0.99	0.77
Metribuzin	1.9	1.6–2.3	7.0	6.5–7.5	0.99	0.61
Simazine	3.0	2.5–3.6	28	26–30	0.99	0.15
Prometryn	3.7	2.8–4.9	11	9.4–12	0.99	0.41
Bromacil	3.4	2.6–4.6	25	22–29	0.99	0.17
Hexazinone	6.4	5.6–7.3	17	16–18	0.99	0.26
48 h exposures						
Ametryn	0.8	0.6–1.0	3.5	3.0–4.0	0.99	1.23
Metribuzin	0.8	0.6–1.0	4.8	4.3–5.3	0.99	0.90
Prometryn	1.6	1.3–2.0	6.7	6.0–7.5	0.99	0.64
Hexazinone	2.5	2.0–3.0	11	9.7–12	0.99	0.41

Table 2. Phytotoxicity inhibition endpoints for individual herbicides after 24 and 48 h exposures. Inhibition of $\Delta F/F_m$' IC$_{10}$ and IC$_{50}$ data (μg l^{-1}) with 95% confidence intervals. Toxic units for the mixture experiments were assigned as 1 TU = IC$_{50}$ at 24 h. Relative potencies (ReP), relative to diuron (IC$_{50}$ diuron/ IC$_{50}$ herbicide).

Results

Potencies of individual herbicides. All herbicides tested inhibited $\Delta F/F_m$' in *H. ovalis* enabling classical concentration-response relationships to be fitted (Fig. 1) with high levels of confidence (r^2 values = 0.98–0.99). The herbicide concentrations that inhibited $\Delta F/F_m$' by 10% (IC$_{10}$) and 50% (IC$_{50}$) are listed in Table 2. After 24 h, diuron was the most potent of the herbicides, exhibiting the lowest IC$_{50}$ of 4.3 μg l^{-1} (Table 2). Fluometruon with an IC$_{50}$ of 132 μg l^{-1} was the least potent of the herbicides tested
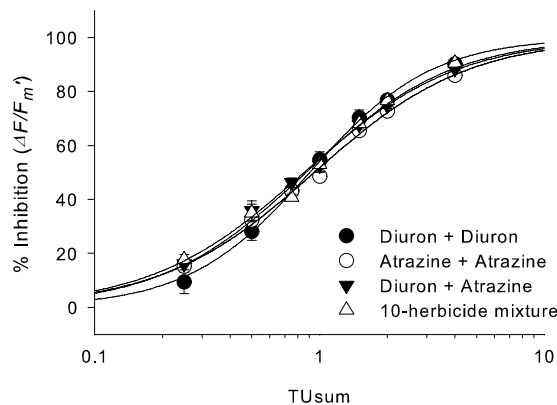
Figure 2. Concentration-response curves for inhibition of $\Delta F/F_m$' by herbicide mixtures. Inhibition was measured at 24 h for binary and complex (10) herbicide mixtures, relative to each solvent control. Bars represent SE ± n = 9.

Herbicide mixture	IC$_{10}$ (TU$_{sum}$)	95% CV	IC$_{50}$ (TU$_{sum}$)	95% CV
Diuron + diuron	0.23	0.20–0.27	0.90[a,b]	0.87–0.94
Atrazine + atrazine	0.17	0.14–0.20	0.95[a]	0.90–1.0
Diuron + atrazine	0.15	0.12–0.18	0.85[b]	0.81–0.90
10-herbicide-mix	0.17	0.14–0.20	0.87[a,b]	0.8–0.95

Table 3. A comparison of additive toxicity of binary and complex mixtures. $\Delta F/F_m$' IC$_{10}$ and IC$_{50}$ data (TU) of all four herbicide mixtures after 24 hr exposures. The proportions of each mixture are equal. For example the binary mixtures contain 50% v/v of each component while the 10-herbicide mix comprises 10% v/v of each herbicide. Different letters in superscript indicate significant differences in IC$_{50}$ (p < 0.05). Note all IC$_x$ values are listed as TU$_{sum}$ values not concentrations.

in the 24 h assays. Maximum inhibition of $\Delta F/F_m$' was reached before 24 h for all herbicides[8] apart from ametryn, metribuzin, prometryn and hexazinone which reached maximum inhibition by 48 h. This additional 24 h of exposure resulted in lower IC$_{50}$ values, reducing them by a further 63% to 69% (Fig. 1B and Table 2). The potencies for each of the herbicides can be evaluated using the Relative Potencies (ReP) compared to the reference herbicide diuron (IC$_{50}$ diuron/IC$_{50}$ herbicide) (Table 2). ReP values > 1 indicate potencies proportionally greater than diuron and ReP values < 1 indicate potencies less than diuron.

Mixture toxicity. The response of *H. ovalis* to the four mixtures tested (binary and complex) were also plotted as concentration-response curves (Fig. 2). The four curves largely overlapped across the range of Toxic Units (TUs) indicating little difference in the response of $\Delta F/F_m$' between the different mixtures and this was confirmed by the calculated IC$_{50}$ which ranged between 0.85 TU – 0.95 TU (3). For additivity using the Concentration Addition (CA) method, the IC$_{50}$ of each of the mixtures would be expected to be close to 1 TU, which was determined by the individual concentration-responses (Table 2). The reference mixtures of [diuron + diuron] and [atrazine + atrazine] exhibited IC$_{50}$s of 0.90 TU – 0.95 TU indicating slightly more sensitive responses to both herbicides than was observed during the individual herbicide assays. F-test analysis indicated a significant difference within the 4-way mixture comparison ($F_{3,24}$ = 3.21, p < 0.05). The post-hoc analysis indicated that the IC$_{50}$ of [diuron + atrazine] was slightly (11%) but significantly lower (i.e. more potent) than the IC$_{50}$ of [atrazine + atrazine] (Table 3). This indicates a possible synergistic interaction; however, there was no significant difference between the IC$_{50}$ of the [diuron + atrazine] mixture and the other mixtures (Table 3).

Discussion

Phytotoxicity in non-target plants, such as seagrass, has been documented previously for the PSII herbicide diuron in several studies[8,12,13,49] and its effects in chronic exposures lead to both declines in stored energy in the root-rhizome complex and whole-plant effects, including reduced growth and survival[13]. Here we extend the toxic threshold (IC$_{10}$) and comparative toxicity data (IC$_{50}$) for inhibition of photosynthesis ($\Delta F/F_m$') in *H. ovalis* to a further nine PSII herbicides, and this matched dataset includes the first ecotoxicological information for ametryn, metribuzin, bromacil, prometryn and fluometuron for any seagrass species. Confirmation of additive toxicity of binary and complex PSII herbicide mixtures to *H. ovalis* further validates the importance of additive ecotoxicological effects (when the mode of action

is the same) for application in field monitoring, water quality guideline development and in ecological risk assessments.

Herbicide potencies.   The PSII herbicides demonstrated a wide range of potencies with diuron being most toxic ($IC_{50} = 4.3\,\mu g\,l^{-1}$) and all other herbicides exhibiting $IC_{50}s < 30\,\mu g\,l^{-1}$ except fluometuron which was four-fold less toxic than all other herbicides after 24 h (Table 2). All of these herbicides bind to the same site in the D1 protein[19] and differences in potency are likely due to the diverse stearic, and lipophilic properties of the herbicides, where herbicides "fit" and form different covalent attachments with the protein[30]. We previously demonstrated even uptake and binding of diuron through the leaf surface of *H. ovalis* using Imaging-PAM fluorometry and no flooding of the vascular system via the cut stems of isolated *H. ovalis* leaves[8]. Herbicides with different structures and hydrophobicity are likely to be transported through the leaf and to and from the binding site at various rates, potentially contributing to less rapid impacts of ametryn, metribuzin, prometryn and hexazinone (Table 2). PSII herbicides must cross the hydrophobic semi-permeable cell membrane of the cell in order to successfully inhibit photosynthetic function, and absorption may be more difficult for less lipophilic herbicides[19] such as hexazinone. These slow acting herbicides here are all related *s*-triazines or triazinones, but the group exhibits a wide range of water solubilities and lipophilicities (Table 1).

This study provides the first seagrass phytotoxicity data for fluometuron, ametryn, metribuzin, prometryn and bromacil, and builds on limited toxicological data for atrazine, hexazinone, simazine and tebuthiuron to tropical species (Table 4). *H. ovalis* was generally more sensitive to many of these PSII herbicides when compared to other species groups (Table 4), though with some exceptions. Atrazine for example inhibited $\Delta F/F_m$' at similar concentrations in *H. ovalis* as for other seagrass species in 3 day exposures of potted plants[12], but at lower concentrations (i.e. greater sensitivity) than green algae[45,50] or coral[28,51] (Table 4). *H. ovalis* was also more sensitive to simazine than green algae[45], coral[28] and diatoms[52]. Despite differences in sensitivity of $\Delta F/F_m$' inhibition between species to the same herbicide, these differences were usually within an order of magnitude due to the well conserved binding site on the D1 protein in PSII[53]. Differences in experimental conditions including temperature[29], light levels[8] and exposure time[13] between studies are also likely to affect apparent toxicity, highlighting the need for strictly controlled and repeatable experimental procedures in phytotoxicity studies.

Application to water quality guidelines.   Ecotoxicity threshold values (ETVs) developed specifically for the GBR are intended to protect 99% of species in the World Heritage Area; however, these were developed from limited toxicity data[18] (Table 5). Inhibition of $\Delta F/F_m$' is directly and quantitatively linked to inhibition of photochemical efficiency[54] and this in turn leads to reduced energy status and/or growth and mortality in seagrass following chronic PSII exposures[13,20,22]. Inhibition of ($\Delta F/F_m$') is also well correlated with reduced growth in microalgae[23,24] and energetics and reproduction in corals[25,26] and can therefore be considered ecologically relevant as a basis from which guidelines can be developed or assessed. Five of the herbicides registered for use in catchments of the GBR and tested here have no current guidelines; therefore, the matched IC_{10} and IC_{50} data (Table 2) provides valuable toxicity data as a contribution to risk assessments, interpretation of water quality monitoring and derivation of future guidelines. For some of the herbicides, greater than 10% inhibition of seagrass photosynthesis occurred at concentrations lower than current and proposed ETVs (Table 5).

Mixture toxicity.   The overlapping concentration-response curves of all PSII herbicide mixtures and similarity between $IC_{50}s$ ($TU_{atr+diu}$ of 0.85 was only 6% and 11% lower than either $TU_{diu+diu}$ or $TU_{atr+atr}$) indicates additivity of herbicide effects on PSII activity in *H. ovalis* (Table 3). The small but significant difference between IC_{50} values for [atrazine + diuron] and [atrazine + atrazine] indicated a potentially weak synergistic effect, but no differences between IC_{50} for the 10-herbicide mixture (TU_{mix}) and either of the controls was evident, supporting overall additivity. These results build upon previous research demonstrating the validity of additive effects of PSII herbicide mixtures on photosynthesis with estuarine microalgae in the laboratory[23] and in microcosms[55], and on cell division in the freshwater green algae *Scenedesmus acuolatus* for multiple complex mixtures of up to 18 *s*-triazines[45]. While Concentration Addition (CA) model of joint action is an appropriate approach for calculating total toxicity in mixtures of toxins with the same mode of action (such as PSII herbicides), alternative approaches should be applied to mixtures containing PSII herbicides and pesticides with other modes of action[56]. Contributions towards total toxicity by multiple PSII herbicides, each acting simultaneously at concentrations below individual guidelines can result in ecologically significant effects on aquatic organisms[45] and water quality guidelines based on single herbicides, even widespread and potent herbicides like diuron, could underestimate the ecological threat posed by herbicide mixtures. Concentration Addition has already been applied to compare the actual and expected additive phytotoxicity of field samples containing more than one PSII herbicide[57–59]. CA has also been applied to calculate total toxicity for complex mixtures of PSII herbicides in the field towards guideline reporting and risk assessments[16,17,33] and the current study validates this approach for PSII herbicides and ecologically important seagrass species. Matched ecotoxicity datasets like this one for multiple PSII herbicides are valuable, not only for comparing toxicities of individual herbicides but are critical for direct application in evaluating the total toxicity and risks posed by mixtures that are commonly observed in the environment such as the GBR and its catchments[16,17,60].

Herbicide	Duration	Test phylum	Common name	Indicator Endpoint	Response concentration	Reference
Diuron	24 h	Angiospermae	Seagrass	$\Delta F/F_{m'}$ (IC_{50})	4.3 μg l^{-1}	Present study
	24 h	Angiospermae	Seagrass	$\Delta F/F_{m'}$ (IC_{50})	3.5 μg l^{-1}	8
	72 h	Angiospermae	Seagrass	$\Delta F/F_{m'}/F_v/F_m$	2.4–2.47 μg l^{-1}	12
	5 day	Angiospermae	Seagrass	$\Delta F/F_{m'}$ (LOEC)	0.1 μg l^{-1}	65
	4 days	Angiospermae	Seagrass	$\Delta F/F_{m'}$ (LOEC)	10 μg l^{-1}	49
	77 days	Angiospermae	Seagrass	$\Delta F/F_{m'}$ (IC_{50})	2.4–2.8 μg l^{-1}	13
	34 h	Dinoflagellate	Coral	$\Delta F/F_{m'}$ (IC_{50})	2.9–5.9 μg l^{-1}	51
	2–3 mo	Dinoflagellate	Coral	$\Delta F/F_{m'}$ (IC_{50})	1.2–5.0 μg l^{-1}	66
	4 day	Heterokontophceae	Diatom	$\Delta F/F_{m'}$ (IC_{50})	2.6–18 μg l^{-1}	23
	4 day	Chlorophyceae	Green algae	$\Delta F/F_{m'}$ (IC_{50})	2.1 μg l^{-1}	23
Fluometuron	24 h	Angiospermae	Seagrass	$\Delta F/F_{m'}$ (IC_{50})	132 μg l^{-1}	Present study
	30 min + 48 h	Chlorophyceae	Green algae	Growth	2.5–10 ml l^{-1}	67
Tebuthiuron	24 h	Angiospermae	Seagrass	$\Delta F/F_{m'}$ (IC_{50})	28 μg l^{-1}	Present study
	72 h	Angiospermae	Seagrass	$\Delta F/F_{m'}$ (IC_{50})	29.1–29.7 μg l^{-1}	12
	24 h	Dinoflagellate	Coral	$\Delta F/F_{m'}$ (IC_{50})	175 μg l^{-1}	68
	4 day	Heterokontophceae	Diatom	$\Delta F/F_{m'}$ (IC_{50})	51–94 μg l^{-1}	23
	4 day	Chlorophyceae	Green algae	$\Delta F/F_{m'}$ (IC_{50})	12 μg l^{-1}	23
Atrazine	24 h	Angiospermae	Seagrass	$\Delta F/F_{m'}$ (IC_{50})	22 μg l^{-1}	Present study
	72 h	Angiospermae	Seagrass	$\Delta F/F_{m'}$ (IC_{50})	13.4–18.2 μg l^{-1}	12
	96 h	Angiospermae	Seagrass	$\Delta F/F_{m'}$ (LOEC)	10 μg l^{-1}	49
	14 d	Angiospermae	Aquatic plants	$\Delta F/F_{m'}$ (IC_{50})	22–132 μg l^{-1}	50
	96 h	Chlorophyceae	Green algae	$\Delta F/F_{m'}$ (IC_{50})	94–176 μg l^{-1}	50
	24 h	Chlorophyceae	Green algae	$\Delta F/F_{m'}$ (IC_{50})	38.8 μg l^{-1}	45
	2 h	Chlorophyceae	Green algae	$\Delta F/F_{m'}$ (IC_{50})	103 μg l^{-1}	52
	2 h	Heterokontophceae	Diatom	$\Delta F/F_{m'}$ (IC_{50})	45 μg l^{-1}	52
	24 h	Dinoflagellate	Coral	$\Delta F/F_{m'}$ (IC_{50})	45 μg l^{-1}	68
	34 h	Dinoflagellate	Coral	$\Delta F/F_{m'}$ (IC_{50})	37–88.2 μg l^{-1}	51
	4 day	Heterokontophceae	Diatom	$\Delta F/F_{m'}$ (IC_{50})	34–77 μg l^{-1}	23
	4 day	Chlorophyceae	Green algae	$\Delta F/F_{m'}$ (IC_{50})	14 μg l^{-1}	23
Ametryn	48 h	Angiospermae	Seagrass	$\Delta F/F_{m'}$ (IC_{50})	3.6 μg l^{-1}	Present study
	24 h	Chlorophyceae	Green algae	$\Delta F/F_{m'}$ (IC_{50})	3.6 μg l^{-1}	45
	24 h	Dinoflagellate	Coral	$\Delta F/F_{m'}$ (IC_{50})	1.7 μg l^{-1}	68
Metribuzin	48 h	Angiospermae	Seagrass	$\Delta F/F_{m'}$ (IC_{50})	4.8 μg l^{-1}	Present study
	14 d	Angiospermae	Aquatic plants	$\Delta F/F_{m'}$ (IC_{50})	14–36 μg l^{-1}	50
	h - days	Chlorophyceae	Green algae	$\Delta F/F_{m'}$ (IC_{50})	12.3–39.7 μg l^{-1}	69
	96 h	Chlorophyceae	Green algae	$\Delta F/F_{m'}$ (IC_{50})	23–152 μg l^{-1}	50
Simazine	24 h	Angiospermae	Seagrass	$\Delta F/F_{m'}$ (IC_{50})	28 μg l^{-1}	Present study
	96 h	Angiospermae	Seagrass	$\Delta F/F_{m'}$ (LOEC)	10 μg l^{-1}	49
	24 h	Chlorophyceae	Green algae	$\Delta F/F_{m'}$ (IC_{50})	56.9 μg l^{-1}	45
	24 h	Dinoflagellate	Coral	$\Delta F/F_{m'}$ (IC_{50})	150 μg l^{-1}	68
	2 h	Chlorophyceae	Green algae	$\Delta F/F_{m'}$ (IC_{50})	76 μg l^{-1}	52
	2 h	Heterokontophceae	Diatom	$\Delta F/F_{m'}$ (IC_{50})	400 μg l^{-1}	52
Prometyrn	48 h	Angiospermae	Seagrass	$\Delta F/F_{m'}$ (IC_{50})	6.7 μg l^{-1}	Present study
	24 h	Chlorophyceae	Green algae	$\Delta F/F_{m'}/F_v/F_m$	13.2 μg l^{-1}	45
Bromacil	24 h	Angiospermae	Seagrass	$\Delta F/F_{m'}$ (IC_{50})	25 μg l^{-1}	Present study
	2 h	Phaeophyceae	Macroalgae	$\Delta F/F_{m'}$ (IC_{50})	8.23 μg l^{-1}	70
Hexazinone	48 h	Angiospermae	Seagrass	$\Delta F/F_{m'}$ (IC_{50})	11 μg l^{-1}	Present study
	72 h	Angiospermae	Seagrass	$\Delta F/F_{m'}/F_v/F_m$	4.4–6.9 μg l^{-1}	12

Continued

Herbicide	Duration	Test phylum	Common name	Indicator Endpoint	Response concentration	Reference
	24 h	Dinoflagellate	Coral	$\Delta F/F_m$'/F_v/F_m	8.8 µg l^{-1}	68
	2 h	Chlorophyceae	Green algae	$\Delta F/F_m$'/F_v/F_m	21 µg l^{-1}	52
	2 h	Heterokontophceae	Diatom	$\Delta F/F_m$' (IC_{50})	22 µg l^{-1}	52
	4 day	Heterokontophceae	Diatom	$\Delta F/F_m$' (IC_{50})	5.7–6.9 µg l^{-1}	23
	4 day	Chlorophyceae	Green algae	$\Delta F/F_m$' (IC_{50})	2.4 µg l^{-1}	23

Table 4. A summary of relevant toxicity data for the 10 PSII herbicides tested towards a range of species. Lowest observed effect concentration (LOEC).

Herbicide	IC_{10}	IC_{50}	ANZECC[71] ETV 99%,95%,90%	Proposed ANZECC[72] ETV 99%,95%,90%	GBRMPA[18] ETV 99%,95%,90%
Diuron	1.2	4.3	**1.8, 1.8, 1.8**	0.08, 0.3, 0.4	0.9, **1.6, 2.3**
Fluometuron	17	132	NA	NA	NA
Tebuthiuron	3.9	28	0.02, 2.2, **20**	**4.3, 8.8, 12.0**	0.02, 2, **20**
Atrazine	3.4	22	0.7, **13, 45**	2.8, **3.8, 4.6**	0.6, 1.4, 2.5
Ametryn*	0.8	3.5	NA	0.02, 0.1, 0.3	0.5, **1.0, 1.6**
Metribuzin*	0.8	4.8	NA	NA	NA
Simazine	3.0	28	NA	NA	0.2, **3.2, 11**
Prometryn*	1.6	6.7	NA	NA	NA
Bromacil	3.4	25	NA	NA	NA
Hexazinone*	2.5	11	**75, 75, 75**	0.9, 1.2, 1.5	1.2, 1.2, 1.2

Table 5. Comparison between IC_{10} and IC_{50} values and relevant ecological guidelines. All concentrations in µg l^{-1}. Ecotoxicity threshold values (ETVs µg l^{-1}) formulated to protect 99%, 95%, 90% of phototropic species. Current ANZECC guidelines are freshwater. NA not available. ETVs in bold are not protective of PSII activity in *H. ovalis* at the IC_{10} threshold. *indicates 48 h IC_{50} values.

Methods

Seagrass collection and preparation. *H. ovalis* plants were collected at low tide in intertidal meadows of Cockle Bay, Magnetic Island (19°10.88′S, 146°50.63′E) under Permit MTB41 (Department of Employment, Economic Development and Innovation). Small plugs of seagrass with 5–10 cm (depth) of associated sediment was extracted and placed in plastic plant pots lined with plastic bags. The bag was pulled up over the seagrass with a small amount of water retained and secured for transport. Plants were taken to the Australian Institute of Marine Science (AIMS), Townsville, Queensland and placed into 60 l aquaria within 4 h from collection under moderate illumination (270–300 µmol photons m^{-2}s^{-1}). Water temperature conditions (25–28 °C) and salinity (34–36 ppt) were maintained throughout the acclimation phase.

Herbicides. Photosystem II inhibiting herbicides from four chemical classes (Table 1) were tested individually and in combination for their toxicity to seagrass. This selection of herbicides was based on application rates as well as contamination data in Queensland catchments adjacent to the GBR[14,16,33,41,42]. The herbicide diuron was included as a reference toxicant[24]. All herbicides were purchased in the purest available analytical form (>95%) from Sigma Aldrich. Individual herbicide solutions were prepared in 0.2 µm filtered seawater using ethanol as a carrier (<0.03% v/v). Nominal concentrations are reported as the herbicides are non-volatile, water solubility >30 mg l^{-1} and octanol-water coefficient (log K_{ow}) <4 making loss to adsorption on test vessels unlikely[61,62]. The measured seawater pH, salinity and oxygen concentrations in tests were 8.1, 34–36 psu and 7.0–8.5 mg l^{-1} respectively.

Miniature seagrass leaf assay. Assays were conducted in 12-well plates (Nunclon, Thermo Scientific), each containing 5 ml herbicide solution. Herbicide concentrations were randomized across all plates to minimise well cluster and potential plate effects[8]. Experimental light intensity was 100 ± 7 µE (14:10 h light:dark cycle) and temperature maintained at 26 ± 2 °C for all assays. Fluorescence measurements were made with a MAXI Imaging-PAM (I-PAM) (Walz, Germany).

Two fluorescence parameters were used to assess impacts of PSII herbicides on the seagrass leaves[8,20]. The effective quantum yield in an illuminated plant ($\Delta F/F_m$') provides an estimate of the efficiency of photochemical energy conversion within PSII under a given light intensity[54]. The maximum quantum yield (F_v/F_m) is equivalent to the proportion of light used for photosynthesis by chlorophyll when all

reaction centres are open[54] and reductions in F_v/F_m indicate inactivation and/or photo-oxidative damage to PSII (chronic photoinhibition)[63].

To quantify $\Delta F/F_m$', actinic light ($100 \pm 3\,\mu E$) was applied within the I-PAM chamber for five minutes prior to the activation of the saturating pulse. Minimum fluorescence (F with illuminated samples) was determined by applying a weak modulated blue measuring light (ML setting of 5; 650 nm, 0.15 μmol photons m^{-2}s^{-1}). Light adapted maximum fluorescence (F_m') was determined using a short pulse (800 ms) of saturating actinic light (>3000 μmol photons m^{-2}s^{-1}) and the effective quantum yield of PSII calculated from $\Delta F/F_m' = (F_m' - F)/F_m'$. To quantify F_v/F_m, leaves were dark adapted for 30 min and F_0 and F_m measured in the same fashion as F and F_m' to derive maximum quantum yields $F_v/F_m = (F_m - F_0)/F_m$. F_v/F_m. Inhibition of quantum yields (% inhibition relative to solvent control) was calculated from treatment data as Inhibition (%) $= [(Y_{control} - Y_{sample})/Y_{control}] \times 100$, where Y is $\Delta F/F_m$' or F_v/F_m.

Screening. A screening process was performed immediately prior to running the assays to ensure the leaves were in optimal condition for the experiment[8]. Second and third leaf pairs from the terminal, apical end of the rhizome were transferred to wells containing uncontaminated seawater. Leaves were dark adapted for 30 min and F_v/F_m was measured. Only leaves exhibiting F_v/F_m greater than 0.65 (indicating intact and efficient photosystem II apparatus) were used in the subsequent herbicide assays[8]. Average leaf length was 10.0 mm $\pm$ 2.5 and width was 4.8 mm $\pm$ 1.2.

Experimental duration and leaf health. F_v/F_m was measured at 0, 24 and 48 h to assess whether PSII remained intact and active[8]. The maximum fluorescence yield (F_v/F_m) in uncontaminated solvent controls reduced by less than 8.5% over 24 and 48 h durations in all experiments, confirming that PSII remained intact and functional over the assay duration (one-way ANOVA p $= < 0.05$). Maximum inhibition of $\Delta F/F_m$' in *H. ovalis* leaves by diuron is observed in less than 24 h[8]. Here, range finding exposures were performed for all other herbicides to determine whether maximum inhibition of $\Delta F/F_m$' would be achieved following 24 or 48 h exposures. Leaves were exposed to high concentrations of each herbicide and the exposure duration to reach 95% steady state inhibition was recorded. Maximum inhibition was reached between 12 and 24 h exposure for all herbicides except hexazinone, metribuzin, prometryn and ametryn, which were reached within 48 h.

Concentration-response curves. Concentration-response curves were plotted by fitting four parameter logistic curves to the $\Delta F/F_m$' inhibition data from nine replicate leaves for each concentration (SigmaPlot 11.0 and Graph Pad Prism V 6.0). Herbicide concentrations inhibiting $\Delta F/F_m$' by 10 and 50% (IC_{10} and IC_{50}) were determined from each curve by applying standard curve analysis. The probability that midpoints (IC_{50}) generated by the logistic curves were statistically different was tested by applying the F test in Graph Pad Prism V 6.0. IC_{50}s were considered different when p < 0.05 and post-hoc results are presented for each comparison in the relevant results sections.

Mixture toxicity. Concentration addition (CA) was tested for (i) a binary mixture of [diuron and atrazine] (each 50% v:v) and (ii) a mixture of all [10 herbicides] (each 10% v-v). The Toxic Units (TU) concentration for each component was based on its IC_{50} ($= 1$ TU) at 24 h calculated from the individual assays (Table 3). The bioassay was prepared and conducted in an identical way to solitary herbicide assays (see above). TU_{sum} was calculated from corresponding TU values within the mixture (see Eq 1).

$$TU_{sum} = C_{(1)}/IC_{50(1)} + C_{(2)}/IC_{50(2)} + \dots + C_{(i)}/IC_{50(i)} \qquad \text{(Eq 1)}$$

$C_{(i)}$ refers to the concentration of the *ith* herbicide in the mixture. Expected mixture toxicity is derived from TU_{sum} data and compared directly to experimental data. If 50% inhibition $\Delta F/F_m$' of the mixture was reached at 1 TU (IC_{50}) the effect is additive. If 50% inhibition is obtained at a < 1 TU_{sum} the effect is considered synergistic and if it is reached at > 1 TU_{sum} the mixture toxicity is classified as antagonistic[23]. A TU dilution series was applied to all herbicide mixtures (0, 0.25, 0.5, 0.75, 1, 1.5, 2 and 4 TU). The binary mixtures of (i) [diuron $+$ atrazine] and (ii) [10 herbicides] were compared against duplicate reference mixtures of [diuron $+$ diuron] (50% v/v mixture) and [atrazine $+$ atrazine] (50% v/v mixture) to confirm additivity. Additivity was considered true when the observed mean IC_{50} TU_{sum} was close to unity and not significantly different to the average IC_{50} of the mixture control response. Differences between IC_{50}s were tested using the F-test in GraphPad V6.0.

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A.D.W., C.J.C., F.F. and A.P.N. designed and undertook the experiments, A.D.W., C.J.C., F.F. and A.P.N. analysed the results and A.D.W., C.J.C., F.F. and A.P.N. wrote the manuscript. All Authors reviewed the manuscript.

Additional Information

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