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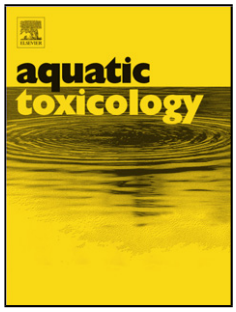

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Lethal and sub-lethal chronic effects of the herbicide diuron on seagrass

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Highlights

We performed chronic exposures of two seagrass species to the herbicide diuron
Diuron affected photosystem II (PSII) at 0.3 µg l^{-1} and growth at 7.2 µg l^{-1}
Biomarkers indicated that carbon-assimilation from photosynthesis dropped following 0.6 µg l^{-1} diuron exposure
Energetic reserves in the seagrass were halved at 1.7 µg l^{-1} after 11 weeks
Chronic exposure to diuron is likely to enhance the impacts of low light stress during flood plumes

Abstract

Photosystem II herbicides from agricultural sources have been detected throughout nearshore tropical habitats including seagrass meadows. While PSII herbicides have been shown to inhibit growth in microalgae at low concentrations, the potential impacts of chronic low concentration exposures to seagrass health and growth have not been investigated. Here we exposed two tropical seagrass species *Halodule uninervis* and *Zostera muelleri* to elevated diuron concentrations (from 0.3 to 7.2 µg l^{-1}) over a 79-day period followed by a 2-week recovery period in uncontaminated seawater. PAM fluorometry demonstrated rapid effect of diuron on photosystem II (PSII) in both seagrass species at 0.3 µg l^{-1}. This effect included significant inhibition of photosynthetic efficiency ($\Delta F/F_m'$) and inactivation of PSII (F_v/F_m) over the 11 week exposure period. Significant mortality and reductions in growth was only observed at the highest exposure concentration of 7.2 µg l^{-1} diuron. However, biochemical indicators demonstrated that the health of seagrass after this prolonged exposure was significantly compromised at lower concentrations. For example, the drop in C:N ratios (0.6 µg l^{-1}) and reduced $\delta^{13}C$ (1.7 µg l^{-1}) in seagrass leaves indicated reduced C-assimilation from photosynthesis. Critically, the energetic reserves of the plants (as measured by starch content in the root-rhizome complex) were approximately halved following diuron exposure at and above 1.7 µg l^{-1}. During the 2-week recovery period, the photosynthetic capacity of the seagrass improved with only plants from the highest diuron treatment still exhibiting chronic damage to PSII. This study shows that, although seagrass may survive prolonged herbicide exposures, concentrations ≥ 0.6 µg l^{-1} diuron equivalents cause measureable impacts on energetic status that may leave the plants vulnerable to other simultaneous stressors. For example, tropical seagrasses have been heavily impacted by reduced light from coastal flood plumes and the effects on plant energetics from light limitation and diuron exposure (highest in flood plumes) are very similar, potentially leading to cumulative negative effects.

1. Introduction

1.1. Developmental pressures on tropical coastal ecosystems

By 2050 more than half of the global population is expected to live in the tropics (State of the Tropics, 2014), increasing the risk of coastal contamination with agrochemicals (Lacher and Goldstein, 1997). The Great Barrier Reef (GBR) is one of the best studied tropical coastal zones extending over 2000 km along the coast of northeast Australia. Its ecological significance at a global scale is acknowledged through its World Heritage listing; however the ecosystem services provided by the GBR are inextricably linked to those of its adjacent catchments (Stoeckl et al., 2011). Pollutants entering the GBR lagoon are predominantly catchment-derived and arise from economically important land-uses, such as grazing and agriculture crops (Bartley et al., 2014; Waterhouse et al., 2012).

1.2. Herbicides and the Great Barrier Reef

Herbicide usage within GBR catchments has increased by up to 7-fold in recent decades (Lewis et al., 2009) and it is estimated that over 30,000 kg of herbicides are introduced each year to the GBR via agricultural run-off (Kroon et al., 2012). Photosystem II (PSII) herbicides including diuron and atrazine are the most frequently detected in the GBR lagoon (Lewis et al., 2009; Shaw et al., 2010; Smith et al., 2012). This class of herbicide acts by binding to the D1 protein of photosystem II inhibiting electron flow, which in turn, limits carbon fixation in plants (Oettmeier, 1992). PSII herbicides are applied in crops and horticulture to target grass weeds often prior to periods of irrigation or rainfall which can in turn lead to concentrations of up to 8.5 $\mu g\ l^{-1}$ diuron in creeks that flush into the GBR (Davis et al., 2013). Concentrations of PSII herbicides within the coastal zone of the GBR lagoon can exceed 1 $\mu g\ l^{-1}$ (Lewis et al., 2009; Lewis et al., 2012), higher than GBR guideline values (GBRMPA, 2010). Since D1 is a highly conserved protein (i.e. it has been retained despite speciation), PSII herbicides also affect non-target organisms including marine phototrophs, such as seagrasses (Flores et al., 2013; Haynes et al., 2000; Macinnis-Ng and Ralph, 2004).

1.3. Seagrasses and herbicides

Highly productive and ecologically important seagrass ecosystems have become threatened globally due to increasing anthropogenic pressures (Orth et al., 2006; Waycott et al., 2009). One of the key pressures on seagrasses and other benthic species in tropical systems is severe light limitation for primary productivity caused by the large amounts of suspended solids delivered during the heavy rainfall periods in summer months (Collier et al., 2012a; Fabricius et al., 2014). PSII herbicides can also limit primary production in seagrass by reducing photosynthetic efficiency and/or causing damage to PSII (Flores et al., 2013; Haynes et al., 2000; Ralph, 2000; Seery et al., 2006). Since herbicide exposure peaks during flood periods (Lewis et al., 2009; Smith et al., 2012) there is mounting concern that persistent low concentrations of PSII herbicides in nearshore waters of the GBR may contribute to seagrass decline (Waterhouse et al., 2012; Waterhouse et al., 2013).

The effects of diuron on the photosynthetic capacity of seagrass can be measured at concentrations of 0.1 to 0.5 $\mu g\ l^{-1}$ (Chesworth et al., 2004; Flores et al., 2013; Haynes et al., 2000; Macinnis-Ng and Ralph, 2004; Ralph, 2000) which can be found in the GBR lagoon (Lewis et al., 2009; Lewis et al., 2012; Smith et al., 2012). The most sensitive indicator for PSII herbicide effects acts are measurements of photosynthetic condition made using pulse amplitude modulation (PAM) fluorometry. In particular the inhibition of effective quantum yield of PSII ($\Delta F/F_m'$) and maximum quantum yield (F_v/F_m) by herbicides that target PSII are

highly responsive and biologically meaningful as they indicate reduction in the photosynthetic capacity and efficiency of the plant which can have flow-on effects to plant energetics and health (Gao et al., 2011; Ralph et al., 2007e). In light-limiting scenarios the effect of PSII herbicides is to severely limit carbon fixation (Johnson and Bird, 1995), while in high irradiance scenarios the blocking of electron transport pathways by PSII herbicides increases oxygen radical formation, causing additional chronic photoinhibition and damage to PSII (Genty et al., 1989; Osmond et al., 1999; Ralph and Burchett, 1995). Limited data also suggests that growth in seagrass seedlings (Gao et al., 2011) and mature plants (Johnson and Bird, 1995; Mitchell, 1987) can be significantly reduced by chronic exposures of atrazine. Plant-scale impacts, such as reductions in growth and abundance, have flow-on effects that could diminish their ecological function as productive sources of food, habitat and as nutrient sinks.

The acute sensitivity of photophysiological processes in seagrass to PSII herbicides is similar to that of tropical microalgae and corals (Flores et al., 2013). While exposure of microalgae to PSII herbicides leads to proportional reductions in $\Delta F/F_m'$ and growth (Magnusson et al., 2008), similar relationships for chronic seagrass exposure require further elucidation. Since the primary impacts of light and PSII herbicide exposure both centre on the function of PSII and the flow-on effects from reduced photosynthetic C incorporation, we assessed the chronic impacts of diuron exposure on seagrass under moderate light conditions expected in flood plumes. A set of biomarkers was assessed to link productivity declines in a long-term experimental exposure of seagrasses to diuron (Table 1). Clarifying the associations between these impacts represents an important step towards understanding the potential chronic effects of PSII herbicides on the health of seagrass meadows. It also provides insight into the likely cumulative effects of the co-occurring stressors, herbicides and low light.

2. Methods

2.1. Experimental approach

In the present study we exposed two tropical seagrass species to four elevated concentrations of diuron. The plants were potted in natural sediments with the exposure conducted under continuous flow-through conditions for 11 weeks, followed by a two week recovery period in uncontaminated water (the sediments were not renewed). The photosynthetic performances of plants were assessed throughout the exposure using PAM fluorometry while other biomarkers and growth were assessed at the end of the exposure period. The biomarkers used to assess the chronic effects of diuron are outlined in Table 1. The stress response caused by low light is described, as well as known or predicted responses to diuron for these biomarkers. Herbicide exposure was expected to affect PSII and pigments in seagrasses in the opposite manner to low light stress. However, as both low light and diuron result in lower energetic surplus, the flow-on effects at the plant-scale could be similar.

2.2. Plant collection

Halodule uninervis Ascherson (Cymodoceaceae) is a tropical seagrass species distributed throughout the Indo-West Pacific and *Zostera muelleri* Irmisch ex Ascherson (Zosteraceae), (syn *Zostera capricorni*) is a tropical to temperate species found in Australia and New Zealand (Waycott et al., 2004). Both occur in north-eastern Australia and the Great Barrier Reef (GBR). *H. uninervis* and *Z. muelleri* were collected from intertidal seagrass beds (<1 m)

from Cockle Bay, Magnetic Island (19° 10.88'S, 146° 50.63'E) and Pelican Banks, Gladstone, Australia (23° 46.005'S, 151° 18.052'E), respectively. *H. uninervis* and *Z. muelleri* plants were collected using a hand trowel, sealed in plastic bags with seawater and placed into 10 cm-diameter plastic pots for transport to the Australian Institute of Marine Science (AIMS) in Townsville, Australia.

Sixty pots of each of *H. uninervis* and *Z. muelleri* were maintained in outdoor aquaria (1000 l) with flow-through filtered seawater (5 µm) under 70% shading (maximum 350 µmol photons m^{-2} s^{-1}), ambient temperature (23-25°C) and salinity at 35-36 PSU. Plants were transferred into indoor flow-through experimental tanks and allowed to acclimate for at least one week before experimentation. Prior to experimentation, *Z. muelleri* and *H. uninervis* plants (with approximately 20 shoots each) were re-planted with a mixture of their original sediment and silica beach sand. *Z. muelleri* were planted into 700 ml plastic pots (10 cm diameter; 9.5 cm deep) modified with 2 cm holes and fitted with pool skimmer socks while *H. uninervis* plants were re-planted into 1000 ml plastic experimental units (16 x 12.5 cm, 5.5 cm depth) and fitted with skimmer socks. The pot sizes reflected surface space needed for growth of each species, while the holes and skimmer socks allowed some water exchange through the sediment.

2.3 Experimental design

The potted plants were placed into 16 l glass aquaria with flow-through 1-µm filtered seawater under gentle aeration. Plants were illuminated over 12 h cycles (Aqua Illumination LED), ramping up for the first two hours to 388 ± 33 µmol photons m^{-2} s^{-1} then down to darkness over the last two hours. Temperature was maintained at 25.5 ± 1.5°C and salinity at 34.6 ± 0.8 PSU.

Diuron stock solutions (5 mg l^{-1}) were prepared in Milli-Q ultrapure water using a < 0.03% w/w solvent carrier ethanol. Seagrasses were exposed to 4 concentrations of diuron, along with a solvent carrier control (see below). Diuron was delivered from 240 l stock tanks to the experimental tanks using a 2400 l h^{-1}pump (Eheim 1260) attached to a timer to pulse treatment concentrations (6 sec every 6 min, approx. 110 ml) into the treatment tanks for a turnover rate of approximately 1.5 times a day.

Four independent replicate tanks were used for each diuron treatment and each tank contained three pots of each seagrass species. The nominal concentrations used for the present study were chosen from results obtained in the study by Flores et al. (2013) whereby diuron concentrations of 0.5, 0.9, 2.4 and 10 µg l^{-1} inhibited chlorophyll fluorescence of *H. uninervis* by ~10, 20, 50 and 80%, respectively. Water samples (2 ml) were taken from within the experimental tanks (n = 4 replicate tanks taken at 3, 7 and 77 days) to quantify treatment concentrations. Herbicide analysis followed the procedure used in Flores et al (2013). Measured diuron concentrations in the five treatment levels (n = 4 replicate tanks taken at 3, 7 and 77 days) were 0 µg l^{-1} (solvent control, below the limit of reporting ~ 0.05 µg l^{-1}), 0.33 ± 0.02 µg l^{-1}, 0.55 ± 0.07 µg l^{-1}, 1.65 ± 0.17 µg l^{-1} and 7.16 ± 0.57 µg l^{-1} corresponding to a control concentration of < 0.2nM and treatments of 1.42 ± 0.09nM, 2.4 ± 0.3nM and 7.1 ± 0.7nm and 31 ± 2nM. Exposure to diuron lasted for 79 days plus a 2 week

recovery where there was a complete exchange of treatment water for clean seawater (no herbicide).

2.4. Chlorophyll fluorescence

Chlorophyll a fluorescence measurements (effective quantum yield, $\Delta F/F'_m$ and maximum quantum yield, F_v/F_m) were taken just prior to the start of exposure and at days 3, 7, 27, 57, 77 of exposure, then after 14 days of recovery in uncontaminated water using a pulse amplitude modulated chlorophyll fluorometer (miniPAM, Walz, Germany). Measurements were obtained by placing a 2 mm fibre-optic probe perpendicular to the surface of the seagrass leaf. One measurement per leaf on 5 leaves per pot (n= 60 replicate leaves per species per treatment) was taken in the middle section of the leaf, approximately 10 to 20 mm upwards from the sheath. Measurements were made only on healthy, young, non-senescent leaves. Minimum fluorescence (F in illuminated samples and F_0 in dark-adapted samples) was determined by applying a weak pulse-modulated red measuring light (650 nm, 0.15 µmol photons $m^{-2}s^{-1}$). To quantify light adapted maximum fluorescence (F_m'), a short pulse (800 ms) of saturating actinic light (>3000 µmol photons $m^{-2}s^{-1}$) was applied. The effective quantum yield in an illuminated plant ($\Delta F/F_m$', Eq. 1) provides an estimate of the efficiency of photochemical energy conversion within PSII under a given light intensity (Genty et al., 1989). The reversible binding of PSII herbicides to the D1 protein in PSII results in an acute and temporary reduction in $\Delta F/F_m$' (Jones and Kerswell, 2003).

$$\Delta F/F_m' = (F_m' - F) / F_m' \qquad \text{Eq. 1}$$

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 (Genty et al., 1989) and reductions in F_v/F_m indicate inactivation and/or photo-oxidative damage to PSII (chronic photoinhibition) (Schreiber, 2004). In the present study, seagrass was 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 as per Eq. 2:

$$F_v/F_m = (F_m - F_0) / F_m \qquad \text{Eq. 2}$$

The inhibition of $\Delta F/F_m'$ and F_v/F_m due to the binding of herbicides or damage to the D1 protein in PSII (Osmond et al., 1999) was calculated according to Eq. 3:

$$\text{Inhibition (\%)} = ((\text{Yield}_{\text{Control}} - \text{Yield}_{\text{Treatment}}) / \text{Yield}_{\text{Control}}) \times 100 \qquad \text{Eq. 3}$$

Rapid light curves (RLC) were measured after 24 days of diuron exposure on one random leaf from each *H. uninervis* and *Z. muelleri* pot (n= 12 replicate leaves per species per

treatment) using a mini-PAM fluorometer, where the actinic irradiance was incremented in eight steps covering 1 - 700 µmol photons m^{-2} s^{-1}. Measurements were made on young, non-senescent leaves, 10-20 mm from the top of the leaf sheath. Relative electron transport rate (rETR) was calculated according to Eq. 4:

$$rETR = \Delta F/F_m' \times PAR \qquad \text{Eq. 4}$$

which is the product of the effective quantum yield by the actinic irradiance (photosynthetically active radiation, PAR) from the internal halogen lamp of the mini-PAM (calibrated with a Li-cor quantum sensor [Li-Cor, Lincoln, NE, USA]). This is considered a relative ETR (rETR) since leaf absorptance was not directly measured. rETR was plotted against irradiance, and photosynthetic rate (α), maximum relative electron transport rate ($rETR_{max}$) and minimum saturating irradiance (E_k) were calculated by fitting the hyperbolic tangent model by Jassby and Platt (1976).

2.5. Biochemistry: Carbon-to-nitrogen ratio, stable isotope ratios and carbohydrates

At the end of the exposure period, at least five shoots per pot per tank of each species were removed, washed free of sediment and epiphytes then placed at -20°C for later analysis. Samples from within each treatment tank were pooled for analysis (n = 4 per treatment). Shoots were then separated into two categories: above-ground (leaves) and below-ground (rhizomes and roots) samples. Samples were freeze-dried for 48 h and ground finely in a mini bead beater (BioSpec Products, Inc, USA). Above-ground and below-ground samples were analysed for carbon-to-nitrogen ratio (C:N), $\delta^{13}C$ and $\delta^{15}N$. Below-ground samples were additionally analysed for carbohydrates.

A stable isotope ratio mass spectrometer (Thermo Electron Corporation Delta V Advantage), operating as continuous-flow coupled to an elemental analyser (FlashEA 1112 HT O/H-N/C) which is interfaced to the mass spectrometer via an intelligence interface (Finnigan Conflo III), was used to analyse for C:N, $\delta^{13}C$ and $\delta^{15}N$. Normalisation was performed based on international standards provided by NIST & IAEA (National Institute of Standards and Technology and International Atomic Energy Agency). Starch was extracted and quantified as per Collier et al. (2012b).

2.6. Pigments

At the end of the diuron exposure one leaf from each pot (n = 12 per treatment) was sampled for pigment analysis. The portion of the leaf that was analysed originated from the top of the sheath to approximately 1 cm in length. This was to ensure the same part of the leaf was analysed for all treatments. Leaves were snap frozen in liquid nitrogen and stored at -80°C for further analysis. Samples were extracted twice in buffered methanol (98:2 MeOH:0.5M tetrabutylammonium acetate pH 6.5) and pigments were separated as per the protocol in Uthicke et al. (2012). Standards were obtained from the International Agency for ^{14}C Determination (DHI, Denmark) and calibration curves were prepared and run at the same conditions. The peaks were identified by comparison of retention times (Wright and Jeffrey

1997) and PDA spectral confirmation and quantified using the calibration curves. Pigment concentrations were normalised to leaf wet weight.

2.7. Growth and mortality

Leaf extension was measured at five time points (days 14, 28, 55, 76, 92), including once during the recovery period. A 25-gauge syringe needle was used to puncture the leaves (3-5 leaves per pot) directly above the leaf sheath of *H. uninervis* and *Z. muelleri* to measure for leaf extension. The total leaf extension (mm) was calculated as the sum of lengths from the initial mark to scars on new leaves from each shoot. The rate of leaf extension for the whole shoot was normalised to daily growth. Leaf extension rates were averaged per tank (n = ~75 ± 9 leaves per treatment *for H. uninervis* plants and 50 ± 14 leaves per treatment for *Z. muelleri* plants).

Shoot count was used as a proxy for mortality. Mortality was calculated by taking the difference in number of shoots per pot at the end of the exposure from the initial number of shoots. Shoot count was also recorded during the recovery period. Shoot count was averaged per tank (n = 4 tanks per treatment).

2.8. Data Analysis

PAM and growth data were pooled from replicate tanks following a nested one-way analysis of variance (ANOVA) which indicated no significant tank effect. Outliers were removed when values exceeded 1.5 times the interquartile range (Hoaglin et al., 1986). A 2-way ANOVA was performed to test the effect of Diuron and time on chlorophyll fluorescence data (repeated measures ANOVA was not appropriate as the same leaves were not measured at each time point). Inhibitions of photosynthetic yields were calculated relative to solvent control and dose-response curves were plotted using inhibition data at 3, 7 and 77 days. A four parameter logistic curve was fitted to each data set separately (Sigmaplot 11.0) (Flores et al., 2013). IC_{50} values and corresponding confidence intervals were determined from each curve by applying standard curve analysis. The probability that midpoints (IC_{50}s) 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 the post-hoc results presented for each comparison in the relevant Results sections. For other parameters, statistical differences between controls and treatments were analysed using one-way ANOVA with Tukey-Kramer post-hoc test at significance of $p < 0.05$. The assumption of equal variances of ANOVA was tested with the Levene's test and ANOVA tests were performed only when data met all ANOVA assumptions. Photosynthetic yields and rapid light curve data did not meet the assumptions of ANOVA even after log transformation therefore non-parametric Kruskal-Wallis tests were used with Bonferroni post-hoc test and significant differences reported in relevant tables. Statistical analyses were performed using Number Cruncher Statistical Software (NCSS 2007, Kaysville, USA).

3. Results

3.1 Photophysiology

The inhibition of effective quantum yields ($\Delta F/F_m'$) by diuron at four elevated concentrations was significant at ≥ 0.3 µg l^{-1} over the 11 week period for both species (Figs. 1A and 1B, electronic supplementary material, ESM Table S1). The highest diuron treatment (7.2 µg l^{-1}) inhibited $\Delta F/F_m'$ by 59 – 78% in both species and during recovery this dropped to 19 – 20% inhibition after 2 weeks in uncontaminated seawater (water was replaced over 20 times during that period in the flow-through system). At 1.7 µg l^{-1}, inhibition of $\Delta F/F_m'$ ranged from 25 to 38% and significant inhibition was also observed for the 0.6 µg l^{-1} (9 - 21%) and 0.3 µg l^{-1} (5- 14%) treatments. In lower diuron concentration treatments (≤ 0.6 µg l^{-1}) the % inhibition of $\Delta F/F_m'$ recovered during the 2 week recovery period and was not significantly different to controls after this period for both species (Figs. 1A and 1B, Table S1). Similar results were observed for the inhibition of maximum quantum yields (F_v/F_m) during the exposure period and plants in the highest diuron treatment exhibited significant chronic photoinhibition of 17 – 20% F_v/F_m following the 2 week recovery period (Figs. 1C and 1D, Table S1).

Fig. **1.** Effect of diuron on quantum yield. Inhibition of effective quantum yield ($\Delta F/F_m'$) in (A) *H. uninervis* and (B) *Z. muelleri* and maximum quantum yield (F_v/F_m) in (C) *H. uninervis* and (D) *Z. muelleri* relative to solvent control over the duration of the experiment.

Dose-response curves were used to determine the concentrations of diuron that inhibit 10% and 50% fluorescence yields (IC_{10} and IC_{50}) after 3, 7 and 77 days of exposure (Fig. S1). The IC_{50} concentrations for inhibition of $\Delta F/F_m'$ reduced over the course of the diuron exposure for *H. uninervis* ($F_{2,12} = 7.19$, $p < 0.05$) but not for *Z. muelleri* ($F_{2,12} = 2.98$, $p > 0.05$) (Table 2). These small but significant changes in the sensitivity of seagrass to diuron over time were also apparent in the two-way ANOVA (Table S1) during the exposure period. Differences in IC_{50}s over time were not tested for F_v/F_m as 50% inhibition was not reached in most cases (Table 2).

Table 2. Concentrations of diuron (µg l^{-1}) that inhibit $\Delta F/F_m'$ and F_v/F_m by 10% (IC_{10}) and 50% (IC_{50}) over time for both seagrass species (95% CI range). Different superscripted letters for $\Delta F/F_m'$ indicate statistically different IC_{50} values ($p < 0.05$).

Species/duration	$\Delta F/F_m'$		F_v/F_m	
	IC_{10}	IC_{50}	IC_{10}	IC_{50}
H. uninervis				
3 day	0.46 (0.25-0.67)	3.5 (2.5-4.6)[a]	0.57 (0.22-1.03)	8.6*
7 day	0.51 (0.28-0.76)	3.8 (2.7-5.1)[a]	0.82 (0.64-1.03)	9.5*
77 day	0.41 (0.28-0.57)	2.8 (2.2-3.4)[b]	0.54 (0.48-0.62)	5.8 (5.4-6.2)
Z. muelleri				
3 day	0.78 (0.10-0.69)	3.3 (1.5-6.4)[a]	0.64 (0.48-0.82)	>10*
7 day	0.18 (0.05-0.41)	2.7 (1.6-4.5)[a]	0.81 (0.63-1.00)	>10*
77 day	0.40 (0.18-	2.4 (1.7-3.5)[a]	0.67 (0.40-1.00)	>10*

.062)

* indicates 50% inhibition was not reached – and the EC_{50} was estimated by extrapolation

Rapid light curves were used to measure photosynthetic performance, including: maximum relative electron transport rates ($rETR_{max}$), the minimum saturating irradiance (E_k), and photosystem efficiency at limiting light levels (α) (Fig. 2). $rETR_{max}$ was significantly reduced in both species at 7.2 µg l^{-1} and at 1.7 µg l^{-1} for *H. uninervis* (Tables 3 and S2). E_k was higher in *Z. muelleri*, than *H. uninervis* and it was more than 2-fold higher in both species exposed to 7.2 µg l^{-1} relative to controls demonstrating that more light was required to saturate photosynthesis in the presence of diuron. This was driven by the shallow slope of the light response curve (i.e. low α) (Table S2). α proved a sensitive RLC parameter, declining significantly ($p<0.05$, Table S3) at 0.6 µg l^{-1} for both species. At the highest diuron concentration (7.2 µg l^{-1}), α was reduced by 74% and 76% relative to the controls in *H. uninervis* and *Z. muelleri*, respectively.

Fig. **2.** Effect of diuron on rapid light curves. Rapid light curve for the exposure of (A) *H. uninervis* and (B) *Z. muelleri* to diuron for 24 d. Mean relative electron transport rate (rETR) ± SE, n = 12 leaves per treatment. Means ± SE.

3.2. Biochemistry: Pigment ratios and concentrations

Seagrass leaves contained the pigments chlorophyll *a*, chlorophyll *b*, alpha and beta carotene, neoxanthin, violaxanthin, lutein and sometimes traces of zeaxanthin. There were no differences in total chlorophylls, carotenoids and xanthophylls among all treatments (Tables 3 and S4). However, there was a significant decrease in Chl *a*:Chl *b* ratios in the two highest diuron treatments for *H. uninervis* (12% and 19% decline at 1.7 µg l^{-1} and 7.2 µg l^{-1}) while a decrease (10%) was observed in the highest diuron treatment for *Z. muelleri* (Tables 3, S4 and S5). In addition, total carotenoids: total chlorophyll ratios were significantly reduced in the highest diuron treatments of *H. uninervis* and *Z. muelleri* representing 20% and 22% declines respectively (Tables 3, S4 and S5).

Table 3. Summary results for the effects of 11 week diuron exposure on multiple markers for seagrass health. Significance at $p < 0.05$ was determined using one-way ANOVAs and Kruskal-Wallis tests (ESM Tables S1-S8).

	H. uninervis				*Z. muelleri*			
Significant effect diuron concentration	0.3 µg l^{-1}	0.6 µg l^{-1}	1.7 µg l^{-1}	7.2 µg l^{-1}	0.3 µg l^{-1}	0.6 µg l^{-1}	1.7 µg l^{-1}	7.2 µg l^{-1}
$\Delta F/F_m'$	-9%	-13%	-38%	-76%	-8%	-14%	-44%	-79%
F_v/F_m	-6%	-11%	-25%	-56%	-3%	-9%	-22%	-43%
α		-16%	-34%	-75%		-19%	-40%	-74%
$rETR_{max}$			-34%	-75%				-43%
E_k				+157 %				+127%
Chl *a*:Chl *b*			-12%	-19%				-10%
Carot:Chl				-20%				-22%
C:N leaves		-13%	-15%	-33%				-18%
C:N roots				-37%				
$\delta^{13}C$ leaves			-24%	-64%			-30%	-46%
$\delta^{13}C$ roots					-8%			
Starch			-43%	-96%			-54%	-53%
Growth				-22%				-23%
Cumulative mortality							-31%	

3.3. Biochemistry: Stable isotope ratios

Carbon-nitrogen (C:N) ratios were reduced in the leaves of both seagrass species in the presence of diuron and the reduction was significant in *H. uninervis* at concentrations as low as 0.6 µg l^{-1} diuron (Tables 3, S6 and S7). Reductions were also observed in the root-rhizome complex compared with the controls but the differences were less pronounced. Significant reductions in $\delta^{13}C$ were also evident in the leaves of both species following diuron exposures of ≥ 1.7 µg l^{-1} for 11 weeks (Tables 3 and S6). There was no difference in $\delta^{15}N$ in the leaves or roots of either species (data not shown).

3.4. Starch content of the root-rhizome complex.

The starch content of the root-rhizome complex of *H. uninervis* and *Z. muelleri* was 21 ± 2% and 10 ± 1% (w/w) respectively (Fig. 3). This starch content declined significantly (Table S7) by 43% and 54% in *H. uninervis* and *Z. muelleri* following 79 d exposure to 1.7 µg l^{-1} diuron. While the starch content in *Z. muelleri* declined no further at the highest diuron concentration, starch content in *H. uninervis* dropped to only 4% of the control content when exposed to 7.2 µg l^{-1} diuron (Fig. 3 and Table 3).

Fig. 3. Effect of diuron on starch content. Concentrations of starch (% w/w) in the root-rhizome complex. Mean ± SE of seagrass plants from four replicate tanks exposed to diuron for 11 weeks. * indicates significantly different from controls. Significance at $p < 0.05$ was determined using one-way ANOVAs (ESM Table S7).

3.5. Growth

Growth rates were 2.2 ± 0.3 mm d^{-1} for *H. uninervis* and 5.1 ± 0.7 mm d^{-1} *Z. muelleri* in the absence of herbicides (Fig. 4A). Growth rates decline significantly (Table S8) by 22% and 23% for *H. uninervis* and *Z. muelleri*, respectively, when measured following 11 weeks of exposure to diuron at 7.2 µg l^{-1} (Fig. 4A and Table 3). Growth rates were also measured following 2 weeks of recovery and were not different in any of the diuron treatments in comparison with the controls at that time point (data not shown).

Fig. **4.** Effect of diuron on growth and cumulative mortality. Growth rates (A) and cumulative mortality (B) after 11 weeks, mean and SE of seagrass plants from four replicate tanks. * indicates significantly different from controls. Significance at $p < 0.05$ was determined using one-way ANOVAs (ESM Table S8).

3.6. Cumulative mortality

The number of shoots in control treatments at the end of the 11 week exposure was < 5% different from the original number at the start of the experiment for both species (Fig. 4B). The maximum cumulative mortality was 22% for *H. uninervis* at 7.2 µg l^{-1} diuron and 31% for *Z. muelleri* at 1.7 µg l^{-1} diuron (Fig. 4B and Table 3); however, only cumulative mortality in *Z. muelleri* plants exposed at 1.7 µg l^{-1} diuron were different from controls due to large variability within treatments (Table S8).

4. DISCUSSION

Diuron rapidly reduced the capacity of two seagrass species to harvest sunlight, and over a prolonged period this led to reductions in carbon assimilation and energy storage in the below-ground biomass of plants (Table 3). The whole-plant effects of low concentrations of diuron were similar to those caused by light limitation (Fig. 5) indicating that chronic exposure to diuron may escalate seagrass loss during severe, high-turbidity flood plume events.

4.1 Effects of diuron on photophysiology and pigment composition

Diuron caused a relatively constant decrease in photosynthetic efficiency ($\Delta F/F_m'$) over the duration of the exposure, but a significant decline in the IC_{50} values from 3.8 to 2.4 µg l^{-1} for *H. uninervis* indicated slightly greater sensitivity in this species at 11 weeks. Overall the inhibition of $\Delta F/F_m'$ during the chronic exposures were similar to those previously reported (2.1 – 3.5 µg l^{-1}) for tropical seagrass species exposed to diuron over 1 – 3 days (Flores et al., 2013; Wilkinson et al., 2015). Blocking of electron transport by diuron can also result in oxidative stress and damage to PSII (Schreiber, 2004) and evidence for damage to PSII in both seagrass species was indicated by inhibition of F_v/F_m at ≥0.3 µg l^{-1} diuron. Recovery of the function of PSII following the chronic exposure to low diuron concentrations (≤0.6 µg l^{-1}) was relatively rapid as observed in earlier studies (Macinnis-Ng and Ralph, 2003) but seagrass exposed to the highest diuron treatments (≥1.6 µg l^{-1}) over 11 weeks still exhibited inactivation of PSII and possibly damage (inhibition of F_v/F_m) after 2 weeks in uncontaminated seawater. These results reflect the reversible nature of binding of PSII herbicides (Jones and Kerswell, 2003) enabling rapid recovery at low concentrations and slow repair of damage due to chronic herbicide exposure at higher concentrations.

Table 4. The mean daily irradiance of both seagrass species used in the present study as effectively shaded by diuron. Calculated based on average surface irradiance (SI) assumptions by (Collier et al., 2012b).

Treatment (µg l^{-1} diuron)	0	0.3	0.6	1.7	7.2
% inhibition of $\Delta F/F_m'$	0	6.6	12.7	32	66
Quanta (mol m^{-2} day^{-1})	14.0	13.1	12.2	9.5	4.7
% shading of full SI	54.6	57.6	60.4	69.2	84.6
% SI	45.4	42.4	39.6	30.8	15.4

Rapid light curves in our study revealed that chronic diuron exposure affects the photosynthetic capacity of seagrasses over a wide range of light conditions (Fig. 2) and the comparison of effects caused by chronic diuron exposures (here) and effects resulting from acclimation to different light intensities (Ralph and Gademann, 2005) is useful. For example, both chronic low light conditions (Ralph and Gademann, 2005) and diuron exposure (this study) resulted in reduced $rETR_{max}$. In the case of low light adapted plants this is likely due to adaptation of the photosynthetic apparatus to operate efficiently but in the case of diuron exposure, the herbicide binding to the D1 protein inhibits electron transport resulting in increased florescence and non-photochemical quenching, restricting photochemical potential of the system (Fig. 5). The minimum saturating irradiance (E_k) of low light adapted plants is lower than those adapted to high light (Ralph and Gademann, 2005); however, E_k is higher for diuron-exposed plants (i.e. it takes greater irradiance to reach maximum photosynthetic capacity) (Fig. 2) as the diuron inhibits photosynthetic efficiency ($\Delta F/F_m'$) under light limited conditions (Schreiber, 2004). In the presence of diuron the chlorophyll *a:b* ratio decreased, reflecting an up-regulation of the less abundant chlorophyll b (Table 3). The proportional increase in the production of chlorophyll *b* may improve the capacity of the PSII to absorb a broader range of wavelengths of PAR to increase production without generating extra self-shading (Givnish, 1988) as has been observed in light-limited seagrasses (Collier et al., 2012b) and now for herbicide-exposed plants.

4.2. Effects of diuron on carbon fixation and storage

The energetic status of seagrass relies on the balance between C fixation via photosynthesis or inorganic C uptake and loss through respiration of leaves and the root-rhizome complex (Alcoverro et al., 2001; Fourqurean and Zieman, 1991). Here we demonstrate that chronic

exposure to diuron under conditions of moderate light intensity has similar negative outcomes as light limitation on carbon fixation, assimilation and storage in *H. uninervis* and *Z. muelleri*.

The reduction in C:N ratios in leaves and roots following 11-week diuron exposures indicates a significant flow-on effect from herbicide exposure to seagrass energetic balance. Chronic blocking of electron transport in PSII by diuron over 11 weeks is likely to have reduced photosynthetic C assimilation, resulting in the observed reduction in C:N (Fig. 5). Reduced C:N has been consistently observed in seagrass leaves under light limited conditions (Abal et al., 1994; Collier et al., 2009; Fourqurean et al., 1997; Grice et al., 1996; Peralta et al., 2002), demonstrating a common outcome for C:N balance in seagrasses with chronic diuron exposure. The decline in $\delta^{13}C$ in leaves was significant for both species at 1.7 µg l^{-1} diuron and this has also been consistently observed in seagrass grown under light limitation (Collier et al., 2009; Cooper and DeNiro, 1989; Grice et al., 1996), where the lighter ^{12}C isotope is preferentially assimilated under lower light intensity (reduced photosynthetic carbon assimilation, Fig. 5). Reduced $\delta^{13}C$ has also been correlated with low productivity rates in seagrass gown under light limiting conditions (Grice et al., 1996) and its decline in the presence of diuron provides evidence that this chronic herbicide exposure had a strong effect on the energetic status of the plant.

One of the most important markers of energetic status in seagrass is the amount of non-structural carbohydrates stored in the root-rhizome complex (Alcoverro et al., 2001; Brun et al., 2003). In *H. uninervis* and *Z. muelleri* other soluble sugars represent only approximately a quarter of the total non-structural carbohydrates (data not shown), with the remainder being starch. Starch is mobilized to maintain metabolic processes under conditions of low light and other stressful conditions, allowing regrowth under conditions of negative C balance (Alcoverro et al., 2001). The strong and consistent decrease in below-ground starch content in both seagrass species following diuron exposures (Table 3) represents a third line of evidence to demonstrate similar outcomes of chronic herbicide exposures to light limitation, resulting from reduced photosynthetic carbon fixation (Fig. 5).

Fig. **5.** Conceptual pathway for effects of the PSII herbicide diuron on seagrass, including indicators and responses.

4.3 Comparing herbicide effects on photophysiology with shading

Chronic exposure to PSII herbicides may exacerbate low light impacts on seagrass, as the effects of diuron on photosynthetic efficiency leading to declines in plant health (reduced C:N ratio, $\delta^{13}C$, starch reserves, Table 3) reflect those in light limited plants (Table 1, Fig. 5). The relationship between the effects of herbicide exposure and light limitation *in situ* are likely to be complex as exposures to the contaminant and light are in continual flux. However, the inhibition in photosynthetic efficiency ($\Delta F/F_m'$) by 10% caused by approximately 0.4 µg l^{-1} diuron may represent an effective "shading equivalent" of 10% (Table 4) that adds to physical shading by suspended sediments and algae during flood plumes. The highest diuron concentration caused inhibition of $\Delta F/F_m'$ of 66% in both species over the 11 week period. As the mean daily irradiance in the experiment was ~14 mol m^{-2} day^{-1}, this diuron exposure could cause a shading equivalent (apparent drop of irradiance) by 66% down to ~4.8 mol m^{-2} day^{-1} which is ~15% of normal surface irradiance (SI) (Collier et al., 2012b) (Table 4). This irradiance is similar to the low light treatment in Collier et al. (2012b) and both studies reported reduced leaf extension in *H. uninervis* and *Z. muelleri* under these conditions. Further work is needed to quantify the potential of converting PSII herbicide concentrations to "shading equivalents" and this type of relationship is only likely to be valid in low light conditions as other outcomes such as non-photochemical quenching

will occur under higher irradiance (Ralph and Gademann, 2005). However, this is a potential first step towards understanding how chronic herbicide exposures may increase the risk of losing nearshore seagrass habitats following flood plumes. Other logical extensions of this work would be to link the "shading equivalents" with relevant herbicide guidelines for incorporation into risk assessments for light limitation on seagrass habitats threatened by flood plume events.

4.4. Linking chronic diuron exposure and whole plant effects

Chronic diuron exposure caused whole plant effects including reduced growth and cumulative mortality over the 11-week exposure period but only at higher diuron concentrations (7.2 $\mu g\ l^{-1}$). Growth rates have also been shown to decline in *Z. marina* subjected to PSII herbicides previously, including exposures of 5 $\mu g\ l^{-1}$ diuron over 10 d (Chesworth et al., 2004) and 10 $\mu g\ l^{-1}$ Atrazine over 4 weeks (Gao et al., 2011; Wahedally et al., 2012). Mortality was also reported in *Zostera marina* exposed to $\geq$100 $\mu g\ l^{-1}$ atrazine (Delistraty and Hershner, 1984; Gao et al., 2011; Schwarzschild et al., 1994). Trends linking increases in cumulative mortality were apparent (Table 3, Fig. 5) however there was a high degree of variability in the response of plants across most treatments limiting the precision of this endpoint. Continued growth and survival of seagrass in the presence of diuron was likely due to a combination of: (i) the plants drawing upon energetic reserves from below-ground biomass and (ii) continued fixation of carbon by partially blocked (yet functional) photosystems (Fig. 5). This study shows that, although seagrass may survive prolonged herbicide exposures, concentrations $\geq$ 0.6 $\mu g\ l^{-1}$ diuron equivalents cause measureable impacts on energetic status that may leave the plants vulnerable to other simultaneous stressors.

Results from this study indicate that the major threat of chronic diuron exposure to seagrass (under low to moderate light conditions often encountered in flood plumes), is to contribute cumulatively to the impacts of light limitation by enhancing loss of productivity in the plants and drawing upon energy reserves of plants, finally leading to impacts at a meadow scale. Some seagrass however are subjected to high irradiance and simultaneous exposure to diuron or other PSII herbicides and under these circumstances may increase photo-oxidative stress leading to a second pathway for seagrass loss. The only study to expose seagrass to both potential stressors demonstrated that high irradiance and diuron combined to increase chronic photoinhibition after 24 h in *Halophila ovalis* leaves (Wilkinson et al., 2015) and this combination of pressures is worth investigating over a more prolonged period.

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References

Abal, E., Loneragan, N., Bowen, P., Perry, C., Udy, J., Dennison, W., 1994. Physiological and morphological responses of the seagrass *Zostera capricorni* Aschers, to light

intensity. Journal of Experimental Marine Biology and Ecology 178, 113-129. http://dx.doi.org/10.1016/0022-0981(94)90228-3

Alcoverro, T., Manzanera, M., Romero, J., 2001. Annual metabolic carbon balance of the seagrass *Posidonia oceanica*: the importance of carbohydrate reserves. Marine Ecology Progress Series 211, 105-116. http://dx.doi.org/10.3354/meps211105

Bartley, R., Bainbridge, Z.T., Lewis, S.E., Kroon, F.J., Wilkinson, S.N., Brodie, J.E., Silburn, D.M., 2014. Relating sediment impacts on coral reefs to watershed sources, processes and management: A review. Science of the Total Environment 468, 1138-1153. http://dx.doi.org/10.1016/j.scitotenv.2013.09.030

Boardman, N.K., 1977. Comparative photosynthesis of sun and shade plants. Annual Review of Plant Physiology 28, 355-377. http://dx.doi.org/10.1146/annurev.pp.28.060177.002035

Brun, F.G., Vergara, J.J., Navarro, G., Hernández, I., Perez-Llorens, J., 2003. Effect of shading by Ulva rigida canopies on growth and carbon balance of the seagrass *Zostera noltii*. Marine Ecology Progress Series 265, 85-96.

Chesworth, J.C., Donkin, M.E., Brown, M.T., 2004. The interactive effects of the antifouling herbicides Irgarol 1051 and Diuron on the seagrass *Zostera marina* (L.). Aquatic Toxicology 66, 293-305. http://dx.doi.org/10.1016/j.aquatox.2003.10.002

Collier, C.J., Lavery, P.S., Ralph, P.J., Masini, R.J., 2009. Shade-induced response and recovery of the seagrass *Posidonia sinuosa*. Journal of Experimental Marine Biology and Ecology 370, 89-103. http://dx.doi.org/10.1016/j.jembe.2008.12.003

Collier, C.J., Waycott, M., McKenzie, L.J., 2012a. Light thresholds derived from seagrass loss in the coastal zone of the northern Great Barrier Reef, Australia. Ecological Indicators 23, 211-219. http://dx.doi.org/10.1016/j.ecolind.2012.04.005

Collier, C.J., Waycott, M., Ospina, A.G., 2012b. Responses of four Indo-West Pacific seagrass species to shading. Marine Pollution Bulletin 65, 342-354. http://dx.doi.org/10.1016/j.marpolbul.2011.06.017

Cooper, L.W., DeNiro, M.J., 1989. Stable carbon isotope variability in the seagrass *Posidonia oceanica*: Evidence for light intensity effects. Marine Ecology Progress Series 50, 225-229.

Davis, A.M., Thorburn, P.J., Lewis, S.E., Bainbridge, Z.T., Attard, S.J., Milla, R., Brodie, J.E., 2013. Environmental impacts of irrigated sugarcane production: Herbicide run-off dynamics from farms and associated drainage systems. Agriculture, Ecosystems and Environment 180, 123-135. http://dx.doi.org/10.1016/j.agee.2011.06.019

Delistraty, D.A., Hershner, C., 1984. Effects of the herbicide atrazine on adenine nucleotide levels in *Zostera marina* L. (eelgrass). Aquatic Botany 18, 353-369. http://dx.doi.org/10.1016/0304-3770(84)90056-1

Fabricius, K.E., Logan, M., Weeks, S., Brodie, J., 2014. The effects of river run-off on water clarity across the central Great Barrier Reef. Marine Pollution Bulletin 84, 191-200. http://dx.doi.org/10.1016/j.marpolbul.2014.05.012

Flores, F., Collier, C.J., Mercurio, P., Negri, A.P., 2013. Phytotoxicity of four photosystem II herbicides to tropical seagrasses. PLoS ONE 8, e75798. http://dx.doi.org/10.1371/journal.pone.0075798

Fourqurean, J.W., Moore, T.O., Fry, B., Hollibaugh, J.T., 1997. Spatial and temporal variation in C: N: P ratios, δ15N, and δ13C of eelgrass *Zostera marina* as indicators of ecosystem processes, Tomales Bay, California, USA. Marine Ecology Progress Series 157, 147-157. http://dx.doi.org/doi:10.3354/meps157147

Fourqurean, J.W., Zieman, J.C., 1991. Photosynthesis, respiration and whole plant carbon budget of the seagrass *Thalassia testudinum*. Marine Ecology Progress Series 69, 161-170.

Gao, Y., Fang, J., Zhang, J., Ren, L., Mao, Y., Li, B., Zhang, M., Liu, D., Du, M., 2011. The impact of the herbicide atrazine on growth and photosynthesis of seagrass, *Zostera marina* (L.), seedlings. Marine Pollution Bulletin 62, 1628-1631. http://dx.doi.org/10.1016/j.marpolbul.2011.06.014

GBRMPA, 2010. Water quality guidelines for the Great Barrier Reef Marine Park (Revised). Great Barrier Reef Marine Park Authority, Townsville. Available: http://www.gbrmpa.gov.au/corp_site/key_issues/water_quality/water_quality_guidelines. Accessed March 2015.

Genty, B., Briantais, J.M., Baker, N.R., 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochim Biophys Acta 990, 87-92. http://dx.doi.org/10.1016/S0304-4165(89)80016-9

Givnish, T.J., 1988. Adaptation to sun and shade: a whole-plant perspective. Functional Plant Biology 15, 63-92. http://dx.doi.org/10.1071/PP9880063

Grice, A., Loneragan, N., Dennison, W., 1996. Light intensity and the interactions between physiology, morphology and stable isotope ratios in five species of seagrass. Journal of Experimental Marine Biology and Ecology 195, 91-110. http://dx.doi.org/doi:10.1016/0022-0981(95)00096-8

Haynes, D., Ralph, P., Prange, J., Dennison, B., 2000. The impact of the herbicide diuron on photosynthesis in three species of tropical seagrass. Marine Pollution Bulletin 41, 288-293.

Hoaglin, D.C., Iglewicz, B., Tukey, J.W., 1986. Performance of some resistant rules for outlier labeling. Journal of the American Statistical Association 81, 991-999. http://dx.doi.org/10.1080/01621459.1986.10478363

Johnson, J.R., Bird, K.T., 1995. The effects of the herbicide Atrazine on *Ruppia maritima* L. growing in autotrophic versus heterotrophic cultures. Botanica Marina 38, 307-312. 10.1515/botm.1995.38.1-6.307

Jones, R.J., Kerswell, A.P., 2003. Phytotoxicity of photosystem II (PSII) herbicides to coral. Marine Ecology-Progress Series 261, 149-159. http://dx.doi.org/10.3354/meps261149

Kitajima, K., Hogan, K., 2003. Increases of chlorophyll a/b ratios during acclimation of tropical woody seedlings to nitrogen limitation and high light. Plant, Cell & Environment 26, 857-865.

Kroon, F.J., Kuhnert, P.M., Henderson, B.L., Wilkinson, S.N., Kinsey-Henderson, A., Abbott, B., Brodie, J.E., Turner, R.D.R., 2012. River loads of suspended solids, nitrogen, phosphorus and herbicides delivered to the Great Barrier Reef lagoon. Marine Pollution Bulletin 65, 167-181. http://dx.doi.org/10.1016/j.marpolbul.2011.10.018

Lacher, T.E., Goldstein, M.I., 1997. Tropical ecotoxicology: status and needs. Environmental Toxicology and Chemistry 16, 100-111. http://dx.doi.org/10.1002/etc.5620160111

Lee, K.-S., Park, S.R., Kim, Y.K., 2007. Effects of irradiance, temperature, and nutrients on growth dynamics of seagrasses: A review. Journal of Experimental Marine Biology and Ecology 350, 144-175. http://dx.doi.org/doi:10.1016/j.jembe.2007.06.016

Lewis, S.E., Brodie, J.E., Bainbridge, Z.T., Rohde, K.W., Davis, A.M., Masters, B.L., Maughan, M., Devlin, M.J., Mueller, J.F., Schaffelke, B., 2009. Herbicides: A new threat to the

Great Barrier Reef. Environmental Pollution 157, 2470-2484. http://dx.doi.org/10.1016/j.envpol.2009.03.006

Lewis, S.E., Schaffelke, B., Shaw, M., Bainbridge, Z.T., Rohde, K.W., Kennedy, K., Davis, A.M., Masters, B.L., Devlin, M.J., Mueller, J.F., Brodie, J.E., 2012. Assessing the additive risks of PSII herbicide exposure to the Great Barrier Reef. Marine Pollution Bulletin 65, 280-291. http://dx.doi.org/10.1016/j.marpolbul.2011.11.009

Macinnis-Ng, C.M., Ralph, P.J., 2003. Short-term response and recovery of *Zostera capricorni* photosynthesis after herbicide exposure. Aquatic Botany 76, 1-15. http://dx.doi.org/10.1016/S0304-3770(03)00014-7

Macinnis-Ng, C.M.O., Ralph, P.J., 2004. In situ impact of multiple pulses of metal and herbicide on the seagrass, *Zostera capricorni*. Aquatic Toxicology 67, 227-237. http://dx.doi.org/10.1016/j.aquatox.2004.01.012

Magnusson, M., Heimann, K., Negri, A.P., 2008. Comparative effects of herbicides on photosynthesis and growth of tropical estuarine microalgae. Marine Pollution Bulletin 56, 1545-1552.

McMahon, K., Collier, C., Lavery, P.S., 2013. Identifying robust bioindicators of light stress in seagrasses: a meta-analysis. Ecological Indicators 30, 7-15. http://dx.doi.org/doi:10.1016/j.ecolind.2013.01.030

Mitchell, C.A., 1987. Growth of *Halodule wrightii* in culture and the effects of cropping, light, salinity and atrazine. Aquatic Botany 28, 25-37. http://dx.doi.org/doi:10.1016/0304-3770(87)90054-4

Oettmeier, W., 1992. Herbicides of photosystem II, in: Barber, J. (Ed.), The Photosystems: Structure, Function and Molecular Biology. Elsevier, Amsterdam, pp. 349-408.

Orth, R.J., Carruthers, T.J.B., Dennison, W.C., Duarte, C.M., Fourqurean, J.W., Heck, K.L., Hughes, A.R., Kendrick, G.A., Kenworthy, W.J., Olyarnik, S., Short, F.T., Waycott, M., Williams, S.L., 2006. A global crisis for seagrass ecosystems. BioScience 56, 987-996. http://dx.doi.org/10.1641/0006-3568(2006)56[987:AGCFSE]2.0.CO;2

Osmond, C.B., Anderson, J.M., Ball, M.C., Egerton, J.J.G., 1999. Compromising efficiency: the molecular ecology of light resource utilisation in terrestrial plants, in: Scholes, C., Baker, M. (Eds.), Advances in physiological plant ecology. Blackwell, Oxford, pp. 1-24.

Peralta, G., Pérez-Lloréns, J., Hernández, I., Vergara, J., 2002. Effects of light availability on growth, architecture and nutrient content of the seagrass *Zostera noltii* Hornem. Journal of Experimental Marine Biology and Ecology 269, 9-26. http://dx.doi.org/10.1016/S0022-0981(01)00393-8

Ralph, P., Durako, M., Enriquez, S., Collier, C., Doblin, M., 2007a. Impact of light limitation on seagrasses. Journal of Experimental Marine Biology and Ecology 350, 176-193. http://dx.doi.org/10.1016/j.jembe.2007.06.017

Ralph, P.J., 1999. Photosynthetic response of *Halophila ovalis* (R. Br.) Hook. f. to combined environmental stress. Aquatic Botany 65, 83-96. http://dx.doi.org/10.1016/S0304-3770(99)00033-9

Ralph, P.J., 2000. Herbicide toxicity of *Halophila ovalis* assessed by chlorophyll a fluorescence. Aquatic Botany 66, 141-152. http://dx.doi.org/10.1016/S0304-3770(99)00024-8

Ralph, P.J., Burchett, M.D., 1995. Photosynthetic responses of the seagrass *Halophila ovalis* (R. Br.) Hook. f. to high irradiance stress, using chlorophyll *a* fluorescence. Aquatic Botany 51, 55-66. http://dx.doi.org/10.1016/0304-3770(95)00456-A

Ralph, P.J., Gademann, R., 2005. Rapid light curves: a powerful tool to assess photosynthetic activity. Aquatic Botany 82, 222-237. http://dx.doi.org/10.1016/j.aquabot.2005.02.006

Ralph, P.J., Smith, R.A., Macinnis-Ng, C.M.O., Seery, C.R., 2007e. Use of fluorescence-based ecotoxicological bioassays in monitoring toxicants and pollution in aquatic systems: Review. Toxicological & Environmental Chemistry 89, 589 - 607. http://dx.doi.org/10.1080/02772240701561593

Ruban, A.V., Berera, R., Ilioaia, C., van Stokkum, I.H.M., Kennis, J.T.M., Pascal, A.A., van Amerongen, H., Robert, B., Horton, P., van Grondelle, R., 2007. Identification of a mechanism of photoprotective energy dissipation in higher plants. Nature 450, 575-578. http://www.nature.com/nature/journal/v450/n7169/suppinfo/nature06262_S1.html

Schreiber, U., 2004. Pulse-amplitude (PAM) fluorometry and saturation pulse method: an overview. In: Papageorgiou, G., Govindjee, (Eds.), Chlorophyll fluorescence: A Signature of Photosynthesis. Advances in Photosynthesis and Respiration Series. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Schwarzschild, A.C., MacIntyre, W.G., Moore, K.A., Laurence Libelo, E., 1994. *Zostera marina* L. growth response to atrazine in root-rhizome and whole plant exposure experiments. Journal of Experimental Marine Biology and Ecology 183, 77-89. http://dx.doi.org/10.1016/0022-0981(94)90158-9

Seery, C.R., Gunthorpe, L., Ralph, P.J., 2006. Herbicide impact on *Hormosira banksii* gametes measured by fluorescence and germination bioassays. Environmental Pollution 140, 43-51. http://dx.doi.org/10.1016/j.envpol.2005.07.001

Shaw, M., Furnas, M.J., Fabricius, K., Haynes, D., Carter, S., Eaglesham, G., Mueller, J.F., 2010. Monitoring pesticides in the Great Barrier Reef. Marine Pollution Bulletin 60, 113-122. http://dx.doi.org/10.1016/j.marpolbul.2009.08.026

Smith, R., Middlebrook, R., Turner, R., Huggins, R., Vardy, S., Warne, M., 2012. Large-scale pesticide monitoring across Great Barrier Reef catchments – Paddock to Reef Integrated Monitoring, Modelling and Reporting Program. Marine Pollution Bulletin 65, 117-127. http://dx.doi.org/10.1016/j.marpolbul.2011.08.010

State of the Tropics, 2014. State of the Tropics 2014 report. James Cook University, Cairns, Australia. http://stateofthetropics.org/the-report.

Stoeckl, N., Hicks, C.C., Mills, M., Fabricius, K., Esparon, M., Kroon, F., Kaur, K., Costanza, R., 2011. The economic value of ecosystem services in the Great Barrier Reef: our state of knowledge. Annals of the New York Academy of Sciences 1219, 113-133.

Uthicke, S., Vogel, N., Doyle, J., Schmidt, C., Humphrey, C., 2012. Interactive effects of climate change and eutrophication on the dinoflagellate-bearing benthic foraminifer *Marginopora vertebralis*. Coral Reefs 31, 401-414. http://dx.doi.org/10.1007/s00338-011-0851-2

Wahedally, S., Mamboya, F., Lyimo, T., Bhikajee, M., Björk, M., 2012. Short-term effects of three herbicides on the maximum quantum yield and electron transport rate of tropical seagrass *Thalassodendron ciliatum*. Tanzania Journal of Natural and Applied Sciences 3, 458-466.

Waterhouse, J., Brodie, J., Lewis, S., Mitchell, A., 2012. Quantifying the sources of pollutants in the Great Barrier Reef catchments and the relative risk to reef ecosystems. Marine Pollution Bulletin 65, 394-406. http://dx.doi.org/10.1016/j.marpolbul.2011.09.031

Waterhouse, J., Maynard, J., Brodie, J., Randall, L., Zeh, D., Devlin, M., Lewis, S., Furnas, M., Schaffelke, B., Fabricius, K., Collier, C., Brando, V., McKenzie, L., Warne, M., Smith, R., Negri, A., Henry, N., Petus, C., da Silva, E., Waters, D., Yorkston, H., 2013. Assessment of the risk of pollutants to ecosystems of the Great Barrier Reef including differential risk between sediments, nutrients and pesticides, and among NRM regions: Part A in Waterhouse et al. Assessment of the relative risk of water quality to ecosystems of the Great Barrier Reef. Department of the Environment and Heritage Protection, Queensland Government, Brisbane.

Waycott, M., Duarte, C.M., Carruthers, T.J.B., Orth, R.J., Dennison, W.C., Olyarnik, S., Calladine, A., Fourqurean, J.W., Heck, K.L., Hughes, A.R., Kendrick, G.A., Kenworthy, W.J., Short, F.T., Williams, S.L., 2009. Accelerating loss of seagrasses across the globe threatens coastal ecosystems. Proceedings of the National Academy of Sciences USA 106, 12377-12381. http://dx.doi.org/10.1073/pnas.0905620106

Waycott, M., McMahon, K.M., Mellors, J.E., Calladine, A., Kleine, D., 2004. A guide to tropical Seagrasses of the Indo-West Pacific. James Cook University, Townsville

Wilkinson, A.D., Collier, C.J., Flores, F., Mercurio, P., O'Brien, J., Ralph, P.J., Negri, A.P., 2015. A miniature bioassay for testing the acute phytotoxicity of photosystem II herbicides on seagrass. PLoS ONE 10(2), e0117541. http://dx.doi.org/10.1371/journal.pone.0117541

Yaakub, S.M., Chen, E., Bouma, T.J., Erftemeijer, P.L., Todd, P.A., 2013. Chronic light reduction reduces overall resilience to additional shading stress in the seagrass *Halophila ovalis*. Marine Pollution Bulletin 83, 467-474. http://dx.doi.org/10.1016/j.marpolbul.2013.11.030

Figures And Tables:

Electronic Supporting Material

Table S1. Results from 2-way ANOVA on the effective quantum yields, $\Delta F/F_m'$, and maximum quantum yields, F_v/F_m, of two seagrass species exposed to varying concentrations of diuron (times 3, 7, 27, 57 and 77 d) and during the recovery period (absence of herbicide; times 7 and 13 d).

Species	Factor	DF	SS	F	p
Exposure period					
H. uninervis	Time	4	0.97	70.90	< 0.01*
$\Delta F/F_m'$	Treatment	4	40.00	2919	< 0.01*
	Time x Treatment	16	0.39	7.11	< 0.01*
	Error	1421	4.87		
H. uninervis	Time	4	1.38	194.66	< 0.01*
F_v/F_m	Treatment	4	23.34	3284.32	< 0.01*
	Time x Treatment	16	0.19	6.69	< 0.01*
	Error	1416	2.52		
Z. muelleri	Time	4	1.79	96.3	< 0.01*
$\Delta F/F_m'$	Treatment	4	33.15	1783.27	< 0.01*
	Time x Treatment	16	0.48	6.49	< 0.01*
	Error	1425	6.62		
Z. muelleri	Time	4	1.27	248.95	< 0.01*
F_v/F_m	Treatment	4	13.00	2551.16	< 0.01*
	Time x Treatment	16	0.17	8.30	< 0.01*

	Error	1386	1.77		
Recovery period					
H. uninervis	Time	1	0.10	126.60	< 0.01*
$\Delta F/F_m'$	Treatment	1	0.10	126.60	< 0.01*
	Time x Treatment	4	3.69×10^{-2}	11.49	< 0.01*
	Error	542	0.43		
H. uninervis	Time	1	0.13	56.52	< 0.01*
F_v/F_m	Treatment	4	1.09	116.51	< 0.01*
	Time x Treatment	4	7.86×10^{-2}	8.37	< 0.01*
	Error	561	1.32		
Z. muelleri	Time	1	0.21	66.79	< 0.01*
$\Delta F/F_m'$	Treatment	4	0.97	75.93	< 0.01*
	Time x Treatment	4	5.88×10^{-2}	4.60	< 0.01*
	Error	541	1.73		
Z. muelleri	Time	1	0.12	100.16	< 0.01*
F_v/F_m	Treatment	4	0.70	143.57	< 0.01*
	Time x Treatment	4	3.85×10^{-2}	7.90	< 0.01*
	Error	552	0.67		

Table S2. Summary of rapid light curve parameters: maximum relative electron transport rate ($rETR_{max}$), and minimum saturating irradiance E_k, (mean of 12 replicates ± SE) photosynthetic rate in the light-limiting region (α) by species and treatment at 24 d. Different superscripted letters indicates statistically different IC_{50} values ($p < 0.05$, ESM Table S3).

	Treatment ($\mu g\ l^{-1}$ diuron)	$rETR_{max}$	E_k	α
H. uninervis	0	64 ± 8^{a}	61 ± 8^{a}	1.06 ± 0.02^{a}
	0.3	71 ± 5^{a}	$72 \pm 5^{a,b}$	$0.99 \pm 0.03^{a,b}$
	0.6	66 ± 6^{a}	$74 \pm 8^{a,b}$	$0.89 \pm 0.02^{b,c}$
	1.7	41 ± 5^{b}	52 ± 3^{a}	$0.70 \pm 0.04^{c,d}$
	7.2	40 ± 1^{b}	151 ± 6^{c}	0.26 ± 0.01^{d}
Z. muelleri	0	81 ± 4^{a}	88 ± 7^{a}	0.96 ± 0.04^{a}
	0.3	95 ± 3^{a}	101 ± 6^{a}	0.90 ± 0.02^{a}
	0.6	81 ± 8^{a}	104 ± 10^{a}	0.78 ± 0.02^{b}
	1.7	$69 \pm 17^{a,b}$	$127 \pm 30^{a,b}$	0.58 ± 0.03^{c}
	7.2	48 ± 4^{b}	200 ± 20^{b}	0.25 ± 0.01^{d}

^{x} denotes significantly different ($p < 0.05$)

Table S3. Results from Kruskal-Wallis tests of photosynthetic efficiencies and light curve parameters of *H. uninervis* and *Z. muelleri* after exposure to elevated concentrations of diuron.

Species	Indicator	df	H	p
H. uninervis	$\Delta F/F_m'$	4	240.83	< 0.01
	F_v/F_m	4	246.09	< 0.01
	α	4	53.78	< 0.01
	$rETR_{max}$	4	32.97	< 0.01
	E_k	4	31.70	< 0.01
Z. muelleri	$\Delta F/F_m'$	4	221.43	< 0.01
	F_v/F_m	4	235.90	< 0.01
	α	4	53.47	< 0.01
	$rETR_{max}$	4	25.14	< 0.01
	E_k	4	24.97	< 0.01

Table S4. Concentrations of pigments and their ratios in seagrass leaves after 11 week exposures to four elevated diuron concentrations. Chl = total chlorophylls, Carot = total carotenoids, Xan = total xanthophylls (n = 4 tanks ± SE). * indicates significantly different from controls. Significance at $p < 0.05$ was determined using one-way ANOVAs (ESM Table S5).

Diuron Treatment	Total Chl (µg/g wet)	Total Carot (µg/g wet)	Total Xan (µg/g wet)	Chl *a*:Chl *b* (mol:mol)	Carot:Chl (mol:mol)	Xan:Chl (mol:mol)
H. uninervis						
0 µg l^{-1}	1454 ± 123	157 ± 11	282 ± 18	2.70 ± 0.03	0.11 ± 0.00	0.20 ± 0.01
0.3 µg l^{-1}	1199 ± 279	122 ± 23	229 ± 48	2.59 ± 0.08	0.10 ± 0.00	0.19 ± 0.00
0.6 µg l^{-1}	1308 ± 103	143 ± 11	275 ± 16	2.53 ± 0.04	0.11 ± 0.00	0.21 ± 0.01
1.7 µg l^{-1}	1458 ± 158	152 ± 11	302 ± 26	2.37 ± 0.09*	0.11 ± 0.01	0.21 ± 0.01
7.2 µg l^{-1}	1810 ± 118	157 ± 11	378 ± 26	2.20 ± 0.04*	0.09 ± 0.00*	0.21 ± 0.01
Z. muelleri						
0 µg l^{-1}	1707 ± 116	118 ± 9	256 ± 16	2.80 ± 0.05	0.07 ± 0.00	0.15 ± 0.00
0.3 µg l^{-1}	1789 ± 360	127 ± 26	259 ± 53	2.71 ± 0.03	0.07 ± 0.00	0.14 ± 0.00
0.6 µg l^{-1}	1757 ± 212	123 ± 14	262 ± 25	2.65 ± 0.07	0.07 ± 0.00	0.15 ± 0.00
1.7 µg l^{-1}	1819 ± 244	118 ± 15	282 ± 34	2.64 ± 0.09	0.07 ± 0.00	0.16 ± 0.01
7.2 µg l^{-1}	2250 ± 161	122 ± 12	328 ± 24	2.53 ± 0.02*	0.05 ± 0.00*	0.15 ± 0.00

Table S5. Results from one-way ANOVAs of pigments and their ratios of two seagrass species exposed to elevated concentrations of diuron over 11 weeks.

Species	Indicator	df between	df within	F	p
H. uninervis	Total Chl	4	15	1.87	0.17
	Total Carot	4	15	1.08	0.40
	Total Xan	4	15	3.73	< 0.05
	Chl *a*:Chl *b*	4	15	10.78	< 0.01
	Carot:Chl	4	15	7.74	< 0.01
	Xan:Chl	4	15	0.81	0.54
Z. muelleri	Total Chl	4	15	0.88	0.50
	Total Carot	4	15	0.05	0.99
	Total Xan	4	15	0.83	0.53
	Chl *a*:Chl *b*	4	15	2.97	0.05
	Carot:Chl	4	15	24.03	< 0.01
	Xan:Chl	4	15	1.21	0.35

Table S6. Results for the effects of 11 week diuron exposure on C:N ratios and $\delta^{13}C$ and $\delta^{15}N$ in the leaves and root-rhizome complex of two species of seagrass (n = 4 tanks ± SE). *indicates significantly different from controls. Significance at $p < 0.05$ was determined using one-way ANOVAs (ESM Table S7).

Diuron Treatment	C:N		$\delta^{13}C$		$\delta^{15}N$	
	Leaves	Roots	Leaves	Roots	Leaves	Roots
H. uninervis						
0 µg l^{-1}	21.0 ± 0.4	59.1 ± 3.4	-11.9 ± 0.4	-10.9 ± 0.3	2.0 ± 0.2	-1.4 ± 1.9
0.3 µg l^{-1}	19.7 ± 0.4	61.0 ± 3.6	-12.0 ± 0.3	-10.8 ± 0.1	1.9 ± 0.2	1.1 ± 0.1
0.6 µg l^{-1}	18.2 ± 0.5*	63.6 ± 2.3	-11.6 ± 0.5	-10.7 ± 0.2	1.7 ± 0.0	1.7 ± 0.3
1.7 µg l^{-1}	17.8 ± 0.3*	48.3 ± 2.1	-14.8 ± 0.2*	-11.2 ± 0.3	2.2 ± 0.1	-0.7 ± 1.3
7.2 µg l^{-1}	14.1 ± 0.6*	37.5 ± 2.0*	-19.5 ± 0.2*	-10.6 ± 0.2	2.0 ± 0.4	1.0 ± 0.1
Z. muelleri						
0 µg l^{-1}	16.9 ± 0.7	39.1 ± 1.5	-12.4 ± 0.4	-10.0 ± 0.1	3.0 ± 0.1	2.2 ± 0.1
0.3 µg l^{-1}	16.8 ± 0.7	45.2 ± 4.2	-13.2 ± 0.2	-10.9 ± 0.1*	2.8 ± 0.3	1.9 ± 0.2
0.6 µg l^{-1}	17.1 ± 0.3	46.9 ± 2.7	-12.8 ± 0.5	-10.5 ± 0.2	3.1 ± 0.2	2.5 ± 0.3
1.7 µg l^{-1}	15.3 ± 0.3	31.8 ± 1.4	-16.1 ± 0.6*	-10.4 ± 0.1	2.4 ± 0.3	2.1 ± 0.3
7.2 µg l^{-1}	13.9 ± 0.4*	34.7 ± 1.2	-18.1 ± 0.4*	-10.3 ± 0.3	3.2 ± 0.1	2.2 ± 0.1

Table S7. Results from one-way ANOVAs on C:N ratios and $\delta^{13}C$ and $\delta^{15}N$ in the leaves and root-rhizome complex of two seagrasses after an 11 week exposure to diuron.

Indicator	Plant	df between	df within	F	p
C:N	*H. uninervis* leaves	4	15	33.13	< 0.01
	H. uninervis roots	4	15	15.35	< 0.01
	Z. muelleri leaves	4	14	5.68	< 0.01
	Z. muelleri roots	4	14	6.30	< 0.01
$\delta^{13}C$	*H. uninervis* leaves	4	15	88.65	< 0.01
	H. uninervis roots	4	15	0.97	0.45
	Z. muelleri leaves	4	14	29.16	< 0.01
	Z. muelleri roots	4	14	3.52	< 0.05
$\delta^{15}N$	*H. uninervis* leaves	4	15	0.78	0.55
	H. uninervis roots	4	15	1.73	0.20
	Z. muelleri leaves	4	14	2.06	0.14
	Z. muelleri roots	4	14	1.13	0.38
Starch	*H. uninervis* roots	4	15	37.09	< 0.01
	Z. muelleri roots	4	14	4.94	< 0.05

Table S8. Results from one-way ANOVA on the growth rate and mortality of seagrass exposed to diuron after 11 weeks.

Species	Indicator	df between	df within	F	p
H. uninervis	Growth	4	392	5.54	< 0.01
	Cumulative mortality	4	52	1.44	0.23
Z. muelleri	Growth	4	274	8.94	< 0.01
	Cumulative mortality	4	52	6.32	< 0.01

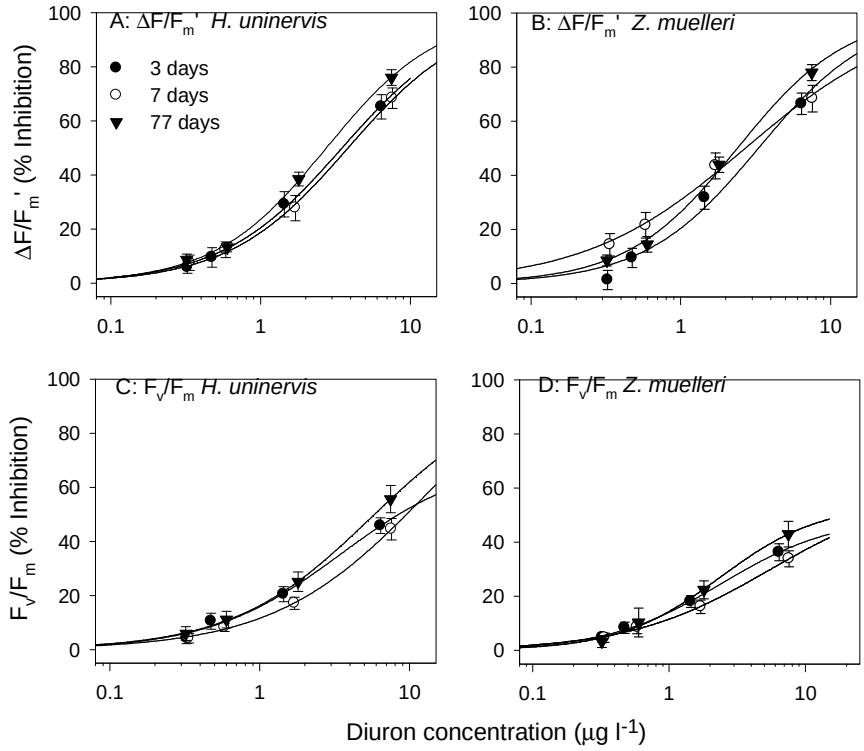

Fig. S1. Dose-response curves for the inhibition of $\Delta F/F_m'$ in (A) *H. uninervis* and (B) *Z. muelleri* and maximum quantum yield (F_v/F_m) in (C) *H. uninervis* and (D) *Z. muelleri* relative to solvent control at three time points. Means ± SE.

Table 1. Potential markers for stress in seagrass used in the present study. These markers are known to be sensitive to stress caused by low light levels but many are untested for herbicide exposure. The known seagrass responses to light as well as the known or hypothesised (H_o) effect of diuron are described. Biomarker responses to low light and diuron were hypothesised to be responding in opposite (≠), or similar (≈) directions

Response variable	Response to low light intensity	Similarity	Expected response to diuron	Expected response timeframe
Photophysiology				

Effective Quantum Yield	$\Delta F/F_m'$	$\Delta F/F_m'$ rises, as the charge separation rate at PS II reaction centers is decreased	≠	$\Delta F/F_m'$ is reduced as diuron binds to the D1 protein of PS II, inhibiting re-oxidation of QA and decreasing photochemical quenching	Hours
Maximum Quantum Yield	F_v/F_m	F_v/F_m increases as photooxidative damage to PSII is decreased.	≠	F_v/F_m is decreased as a fraction of PS II centers are inactivated or damaged.	Hours
The minimum saturating irradiance	E_k	E_k is lower in plants conditioned to low light	≠	E_k may be higher as reduced PSII efficiency increases the light level needed to saturate PSII (H_o)	Hours to D
Relative electron transport rate	$rETR_{max}$	The maximum photosynthetic capacity $rETR_{max}$ is reduced in seagrass grown under light limitation	≈	Inactivation and oxidative damage to PSII reduces electron flow, including maximum potential $rETR_{max}$ (H_o)	Hours to D
Photosynthetic efficiency	α	The rise of the rapid light curve at limiting light intensity (less than E_k) is proportional to efficiency of light capture ($\Delta F/F_m'$) and often increases under light limitation	≠	Efficiency of light capture may be reduced in the presence of diuron as for $\Delta F/F_m'$ (H_o)	Hours to d
Biochemistry					
Total chlorophyll	Chl a	Chl a can increase in shaded plants to increase photon capture	?	Light harvesting pigments may reduce (potentially due to oxidative damage under high light) or increase under low light (to compensate for reduced photosynthetic efficiency)	Weeks
Total xanthophylls ∑neoxanthin + violaxanthin + zeaxanthin + lutein)	Xan	Xanthophylls are harvesters and involved in photoprotection. They can increase under high light stress or herbicide exposure, but reduce in seagrasses in low light conditions	?	As with chlorophylls, the xanthophylls may increase or decrease depending on light intensity.	Days to we
Pigment ratios	Chl *a*:Chl *b*	Chlorophyll *a:b* ratios are lower in shade-adapted plants as Chl b is synthesised to harvest a wider range of wavelengths	≠	Chlorophyll *a:b* ratios increase	Hours to d
	Xan:Total Chl	Can increase to protect the photosystems under conditions causing PSII stress (e.g. high light and/or herbicide), but reduce in low light	≠	Photoprotective pigments increase relative to light harvesting pigments to protect photosystems from oxidative stress	Hours to d
C:N ratio	C:N	Can decline following light limitation as photosynthetic assimilation of C decreases relative to more stable uptake of N.	≈	Can decline following light limitation as photosynthetic assimilation of C decreases relative to more stable uptake of N (H_o)	Weeks
$\delta^{13}C$	$\delta^{13}C$	Under light limitation and reduced C fixation, ^{12}C isotope is assimilated preferentially: $\delta^{13}C$ is reduced	≈	Under reduced C fixation, C is less limiting to photosynthesis and the lighter ^{12}C isotope is assimilated preferentially: $\delta^{13}C$ is reduced (H_o)	Weeks
Starch content		Starch represents the major energy store in many seagrasses and these are remobilised (reduced) in the root-rhizome complex may indicate sub-optimal light conditions	≈	Under reduced photosynthetic quenching and C fixation, starch will be remobilised to supplement growth and respiration (H_o)	Weeks
Whole plant effects					
Leaf growth	mm day^{-1}	Reduce when light limited reflecting less energy for growth; however, can initially increase in an attempt to capture more light.	≈	May reduce due to lower energetic surplus (H_o)	Weeks
Cumulative mortality		As energetic balances are reduced in low light, shoot mortality increases, and new shoot production declines: cumulative mortality rises. Chronic shading can affect seagrass resilience and lead to decreased shoot density and leaf biomass	≈	As energetic balances are reduced shoot mortality increases, and new shoot production declines: cumulative mortality rises (H_o)	Weeks to m