Development of a miniature seagrass bioassay for PSII herbicides and its application to mixtures and simultaneous stressors

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At the beginning of my degree it was said that I may go a little crazy with only seagrass and herbicides to keep me company and I am inclined to agree to some degree! Nevertheless I have made it through the other side and that is due to many supportive and helpful people!

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Thesis Abstract

Seagrass species are widely distributed along all major coasts of Australia and provide a number of crucial ecological functions in which they grow, but also to surrounding ecosystems. During the monsoon season along the Great Barrier Reef (GBR) increased rainfall causes significant freshwater runoff from adjacent agricultural land, transporting suspended sediment, nutrient and contaminants into nearby rivers and ultimately coastal inshore waters. Photosystem-II (PSII) herbicides are the most commonly applied herbicides along the Queensland coast, and diuron is among the most commonly detected PSII herbicides in this marine environment. Recent risk assessments have highlighted the need for new tools to assess: (i) the toxicity of alternative PSII herbicides to seagrass, (ii) the combined effects of mixtures of PSII herbicides on seagrass and (iii) the combined effects of PSII herbicides in the presence of other stressors.

A miniature bioassay methodology was developed and validated in Chapter 2 to measure effects of photosystem-II herbicide exposure on the seagrass, *Halophila ovalis.* Three exposure types: (i) potted, intact plants, (ii) hydroponic and (iii) isolated leaves, were experimentally exposed to a range of concentrations of diuron for a 24 h experimental period. The effects of diuron on photosynthetic activity in PSII (the target activity of PSII herbicides) was measured, including the inhibition of effective quantum yield ($\Delta F/F_m'$) and maximum quantum yield (F_v/F_m) using pulse amplitude modulation (PAM) fluorometry. Results from the Imaging-PAM were compared directly to the widely used mini-PAM to evaluate consistency between methods. Identical sensitivity was observed for all exposure types and with both instruments ($p > 0.05$). The diuron concentration which inhibited $\Delta F/F_m'$ by 50% (IC_{50}) ranged from 2.1 to 4.3 µg l^{-1} in all cases, which was also similar to previously published values for other seagrass species (IC_{50} = 2.41 – 2.47, Flores et al 2013). Validation of the miniature bioassay technique, which measured acute fluorescence responses in isolated leaves with an Imaging-PAM, enabled confident application of this technique to: 1. assess the comparative toxicity of alternative PSII herbicides and mixtures to *H. ovalis* and, 2. assess the combined effects of herbicides and simultaneous stressors.

Fifteen PSII herbicides are registered for application on farms in the GBR catchment. The miniature bioassay was applied in Chapter 3 to measure the comparative acute toxicity (24-48 h exposure) of 10 of these herbicides to PSII activity in *H. ovalis* in

isolation and in mixtures (binary and complex combinations). Results revealed a wide range of toxicities (IC_{50}), with diuron the most potent (4.3 µg l^{-1}) following 24 h exposure and fluometuron the least toxic (132 µg l^{-1}). This thesis includes the first toxicity data for seagrass for five of these emerging herbicides (metribuzin, fluometuron, ametryn, bromacil and prometryn). PSII herbicides are usually detected in mixtures with other PSII herbicides in the GBR and its catchments. Since all PSII herbicides share the identical mode of action (binding to the D1 protein of PSII, displacing plastoquinone, blocking electron transfer and suppression of ATP production), the combined effect in mixtures were expected to be additive. The model of joint action Concentration Addition (CA) was applied to demonstrate additivity. The effects of both mixture types were largely additive, demonstrating that additive effects models can be used for calculating the risk posed by multiple PSII herbicides to seagrasses.

The exposure of seagrass to herbicides is greatest in summer months in conditions that may include elevated temperature and irradiance stress. In Chapter 2, the miniature seagrass assay was used to examine 28 combinations of diuron from 0 to 100 µg l^{-1} (7 levels) and light from 40 to 400 µmol m^{-2} s^{-1} (4 levels). In Chapter 5, the miniature bioassay was applied to 36 combinations of temperature and PSII herbicides. Temperature ranged from 15 to 40°C (6 levels), and diuron concentrations from 0 to 30 µg L^{-1} (6 levels). As temperature, light and herbicides affect PSII activity (but with different modes of action) the model of Independent Action (IA) was used to assess whether these combined effects were additive, antagonistic or synergistic. High light exposures (200 and 400 µmol photons $m^{-2}s^{-1}$) caused greater PSII inhibition than low light (40 and 100 µmol photons $m^{-2}s^{-1}$), particularly at lower diuron concentrations. However, when the inhibition of $\Delta F/F_m'$ was calculated relative to controls at the same light intensity there was no significant difference in IC_{50} (IC_{50} = 2.2 – 3.5 µg l^{-1}; p = >0.05) among light treatments. When inhibition was calculated relative to the control light intensity (100 µmol photons $m^{-2}s^{-1}$), the impacts on $\Delta F/F_m'$ and F_v/F_m were additive, with the blocking of electron transport by diuron likely compounding the photooxidative stress under high light conditions. Photosynthetic activity in *H. ovalis* leaves as measured in the miniature assay was greatest at ~28°C and declines in $\Delta F/F_m'$ and F_v/F_m were observed for extreme temperatures of 15 and 40°C in the absence of diuron. There was a strong interaction between temperature and diuron on PSII activity with large differences among temperatures at low diuron concentration, and convergence of inhibition at all

temperatures at high diuron concentrations (10 and 30 μg l^{-1}). Comparison of observed inhibition of $\Delta F/F_m'$ versus additive inhibition predicted from the IA model revealed that the majority of combinations of diuron and temperature stress led to sub-additive interactions. The diuron $\Delta F/F_m'$ IC_{50} values were lowest at 30°C indicating greatest toxicity. However, when inhibition of $\Delta F/F_m'$ was calculated relative to 30°C extreme temperature treatments induced greater inhibition at low herbicide concentrations compared to all other treatments.

The miniature bioassay methodology is a robust approach to test seagrass *H. ovalis* sensitivity to PSII herbicides, and combined stressors. It was applied to build upon current ecotoxicological data sets for existing and emerging PSII herbicides. PSII herbicides negatively impact numerous photosynthetic processes in seagrass species. Additive and interactive effects occur when PSII herbicides are encountered simultaneously with other stressors, such as in PSII herbicide mixtures and / or sub-optimal environmental conditions. This work can improve risk assessments of key photosynthetic habitats to PSII herbicides and has the potential to improve ecological guidelines for PSII herbicides, with the aim of marine species protection.

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Glossary of terms

$\Delta F/F_m'$	Effective quantum yield
AIMS	Australian Institute of Marine Science
ANOVA	Analysis of variance
ANZECC	Australian and New Zealand Environmental Conservation Council
AOI	Area of interest
APVMA	Australian Pesticide and Veterinary Medicines Authority
ATP	Adenosine triphosphate
CA	Concentration Addition
Chl *a*	Chlorophyll *a*
CI	Confidence Interval
E_k	Minimum saturating irradiance
F	Light adapted minimum fluorescence
F_o	Dark adapted minimum fluorescence
F_m	Dark adapted maximum fluorescence
F_m'	Light adapted maximum fluorescence
F_v/F_m	Maximum quantum yield
GBR	Great Barrier Reef
GBRMPA	Great Barrier Reef Marine Park Authority
h	Hours
IA	Independent Action
IC_x	The concentration eliciting x % inhibition of the endpoint measured
I-PAM	Imaging-PAM
JCU	James Cook University
K_{ow}	Octanol-water partitioning coefficient
ML	Measuring light
NADPH	reduced form of nicotine adenine dinucleotide phosphate

NOEC	No observed effect concentration
NPQ	Non-photochemical quenching
PAM	Pulse Amplitude Modulation
PAR	Photosynthetically active radiation
PSII	Photosystem II
Qb	Quinone b (Plastoquinone)
qP	photochemical quenching
rETR	Relative electron transport rate
$rETR_{max}$	Maximum electron transport rate (Maximum photosynthetic capacity)
RLC	Rapid light curve
ReP	Relative equivalent Potency
SE	Standard error
TU	Toxic Unit
TU_{sum}	The sum of Toxic units
Y	$\Delta F/F_m'$ or F_v/F_m when calculating % inhibition

Chapter 1. Introduction

1.1. Seagrass in the Great Barrier Reef: significance and threats

Seagrass meadows are located in the shallow and intertidal zones of all major coasts of Australia as well as other marine and estuarine environments (Kirkman, 1997). Seagrass diversity in Australia is high (Coles et al., 2003) with over half of the 72 globally recognised species represented (Kilminster et al., 2015). Twelve of these species are distributed within the nearshore and catchment areas of the Great Barrier Reef (GBR) (Waycott et al., 2007). Approximately 1741 km^2 of seagrass is currently mapped for the GBR region (Waycott et al., 2007); however, this does not include deeper (> 15 m) meadows, which may cover close to 40,000 km^2 (Coles and Long, 2000). As productive seagrass ecosystems provide connectivity between terrestrial, reef and mangrove habitats, their environmental and economic value is great (Björk et al., 2008; Orth et al., 2006). Seagrass meadows form the basis of numerous ecologically significant marine habitats (Björk et al., 2008) and carry out a number of important ecological functions such as providing the primary food source for many species, some of which are endangered (Björk et al., 2008; Kilminster et al., 2015). For example, seagrass represents over 95% of the total food intake of dugongs and adult green sea turtles (Lanyon et al., 1989). Other crucial functions include providing suitable habitat and nursery grounds for other organisms (Björk et al., 2008), sediment stabilisation (Björk et al., 2008; Costanza et al., 1997; Kirkman, 1997) and carbon and nitrogen sequestration (Fourqurean et al., 2012; Kilminster et al., 2015).

Tropical seagrass differ from temperate species as they tend to exhibit faster growth and life histories specifically adapted to dynamic environments with regular disturbance (Waycott et al., 2005). Short-lived small species such as *Halophila ovalis* differ from more robust and long-lived examples like *Enhalus acoroides* (Figure. 1.1). Both strategies provide distinct advantages for responding to and recovering from environmental disturbance (Waycott et al., 2007). Short lived and fast growing species such as *H. ovalis* are considered colonising species (Waycott et al., 2007). This category

is defined by characteristics such as low resilience to disturbance but rapid recovery through fast turnover of biomass (Kilminster et al., 2015; Waycott et al., 2007). These traits enable *H. ovalis* to inhabit areas that are regularly disturbed by biological (e.g. grazing) and non-biological (e.g. wave energy) processes (Waycott et al., 2007). In contrast, longer-lived seagrass species are characterised by greater above- and below-ground biomass, slower growth and larger carbohydrate energy stores (Kilminster et al., 2015; Waycott et al., 2007). Traits such as these allow for enhanced short-term physiological resilience to stress, however, if conditions are unfavourable over the long-term then recovery rate will likely be limited (Waycott et al., 2007). Slow recovery can also be exacerbated by little or no seed bank formation (Kilminster et al., 2015).

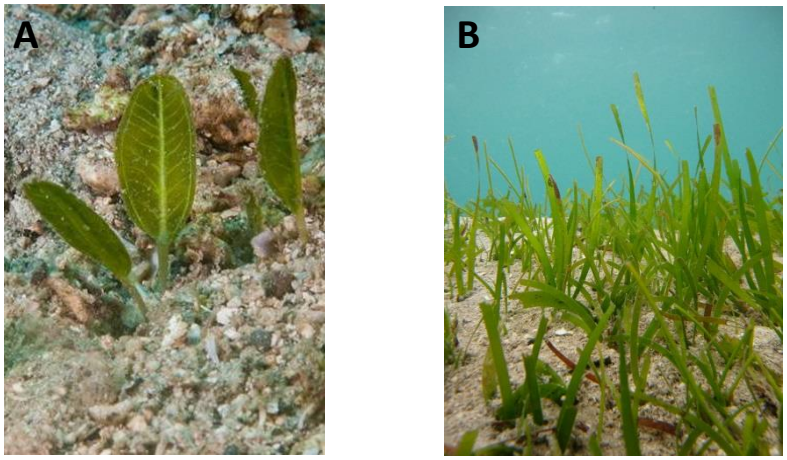

Figure 1.1. Representative photos to highlight physical differences between short-lived, colonising seagrass species (**A**, *Halophila ovalis*) and longer-lived species (**B**, *Cymodocea rotundata*). Photo credit: Dr. Catherine Collier.

Nearshore seagrass habitat distribution and abundance is threatened by a number of anthropogenic stressors including coastal development, dredging, increased boat activity and anchoring (Duarte, 2002; Jackson et al., 2001). Additionally, natural stressors such as increased turbidity and eutrophication also can cause sub-optimal conditions (Björk et al., 2008; Waycott et al., 2007). All seagrass species rely on a number of factors such as appropriate light, temperature, pH, CO_2 and nutrient conditions in order to survive, and loss in biomass occurs when these requirements are limited or disrupted with flow-on consequences for ecological services (Waycott et al., 2007). Community composition is also governed by species-specific adaptability to available environmental

resources (Waycott et al., 2007). For example, light availability dictates seagrass depth distribution and species with lower minimum irradiance requirements are better adapted to survive at deeper depths (Waycott et al., 2004). In general, seagrasses have relatively high minimum light requirements (typically 10 – 20% of surface irradiance) compared to other marine primary producers and terrestrial plants; therefore, small reductions in light can result in negative impacts (Dennison et al., 1993; Duarte, 1991; Dunton, 1994; Fourqurean and Zieman, 1991). There are numerous reported events of seagrass loss associated with deteriorating light quality and availability throughout Australia including the GBR (Bulthuis and Woelkerling, 1983; Collier et al., 2012; Dennison et al., 1993; Gordon et al., 1994; Ralph et al., 2006). Stressors that cause reduced light availability include eutrophication and high turbidity (Devlin and Schaffelke, 2009). During periods of heavy rainfall, river run-off is the largest carrier of suspended particulates, nutrients and chemical contaminants into the coastal zone of the GBR lagoon (Furnas, 2003). An average of 70 km^3 fresh water discharge is transported into the GBR region with the majority of this run-off entering in pulsed flood events (Devlin and Schaffelke, 2009; Furnas, 2003). The large nutrient influx (inorganic nitrogen in particular) increases the likelihood of eutrophication affecting adjacent seagrass communities (Kirkman, 2014) due to light adsorption by epiphytic algae (Shephard et al., 1989; Walker and McComb, 1992). The influx of photosystem II herbicides from adjacent agricultural activity during monsoonal floods also poses threats to seagrass of the GBR (Ralph, 2000; Schaffelke et al., 2005; Waycott et al., 2005). The contribution of herbicide exposure to seagrass loss during flood plume events is not known, but may pose a significant risk (Waterhouse et al., 2012). Seagrass loss from light limitation may be exacerbated by a variety of other simultaneous pressures including herbicide exposure, low salinity and high seawater temperatures (see section below discussing climate effects on seagrass).

1.2. PSII herbicides in the GBR

Herbicides are applied extensively in agricultural land practices with the aim of preventing and reducing the emergence of weeds within the respective crop (Calcino et al., 2008; Davis et al., 2014). Photosystem-II (PSII) herbicides are not highly selective and are applied at doses high enough to prevent the emergence of weeds but with an application method and concentration that is not high enough to kill the crops. PSII

herbicides such as diuron are among the most widely used herbicides in Australia and internationally and are commonly applied to a range of crops including wheat, barley and sugar cane (Holmes, 2014). In Queensland, sugar cane production represents 97% of diuron usage (Holmes, 2014). During the monsoon season nearly a third of the GBR lagoon is exposed to PSII herbicide contamination, with approximately 30,000 kg of PSII herbicide entering the GBR annually (King et al., 2012). While the concentrations of PSII herbicides are highest during the summer flood plume events (Davis et al., 2013; Shaw et al., 2010), PSII herbicides are detected in the GBR catchment area year round (Shaw et al., 2010). There are 15 PSII herbicides registered for use in adjacent catchment areas of the GBR (Davis et al., 2015), with diuron and atrazine representing two of the most commonly detected PSII herbicides in the GBR and its catchments (Lewis et al., 2012; Smith et al., 2012). Other common and emerging PSII herbicides such as hexazinone, tebuthiuron, metribuzin and ametryn are also detected in the GBR and its catchments (Davis et al., 2015; Smith et al., 2012).

PSII herbicides are broad-spectrum residual herbicides applied with the purpose of pre- and post-emergent weed control. The 15 PSII herbicides registered for use within the GBR catchment span a range of different chemical classes including triazine, uracil and triazinone with a common mode of action to reduce photosynthetic efficiency by inhibiting electron transport in PSII (Oettmeier, 1992). Diuron exposure leads to direct displacement of plastoquinone from the electron acceptor region from the D1 protein of PSII (Oettmeier, 1992). This process is reversible; however, if the photosynthetic pathway is interrupted through prolonged exposure, this will lead to starvation and decline in plant fitness (Oettmeier, 1992). Inhibition of electron transport can also lead to oxidative stress and plant death (see below).

Nearshore Marine organisms face the greatest risk of exposure to PSII herbicides (Waterhouse et al., 2012) and since the D1 protein in PSII is conserved among many algae and higher plants, PSII herbicides are capable of affecting non-target nearshore species such as seagrass (Flores et al., 2013; Negri et al., 2015; Ralph, 2000). During the wet season, herbicides entering the GBR lagoon may reach concentrations that threaten numerous keystone primary producers such as seagrass, mangroves and corals, regularly exceeding the derived ecological guideline (0.9 $\mu g\ l^{-1}$) for species protection (Lewis et al., 2012). The combined issue of high potency (toxic impact) and long persistence (duration of environmental presence) of diuron in particular has caused concern for

aquatic and nearshore marine ecosystems including the GBR (Holmes, 2014). A recent review of diuron by the Australian Pesticides and Veterinary Medicines Authority (APVMA) and subsequent restrictions in the use of diuron has led to an increase in a number of emerging and less common PSII herbicides being applied to crops along the GBR catchment area (Davis et al., 2015; Lewis et al., 2012). Although the long-term future registration of diuron for Australian crops is uncertain, its high potency and persistence has led to its common application as a reference compound in ecotoxicology studies and to facilitate comparison in potency between species and with other PSII herbicides (Lewis et al., 2011). The half-life of PSII herbicides may be a significant property affecting seagrass recovery from the combined impact of flood plumes. Diuron for example exhibits a seawater half-life of over 500 d (Mercurio et al., 2015) and if persistent within the seagrass meadows, turnover of above-ground biomass may be inhibited by the presence of lingering diuron.

1.3. Measurement of PSII effects

The effects of PSII herbicides on the photophysiology of PSII in plants can be measured using fluorescence techniques (Ralph et al., 2007b). When PSII blocks the transfer of electrons there is excess excitation energy that can cause cellular damage if it is not dissipated (Genty et al., 1989; Osmond et al., 1999; Rutherford and Krieger-Liszkay, 2001). One pathway of excess energy dissipation occurs via re-emission at a longer wavelength as chlorophyll *a* fluorescence (Ralph and Gademann, 2005; Silva et al., 2009). This fluorescence enables the assessment of excitation activity of photosystems *in situ* using instruments such as pulse amplitude modulation (PAM) fluorometers (Beer et al., 1998; Björk et al., 1999). Common chlorophyll *a* fluorescence measures include the effective quantum yield of PSII ($\Delta F/F_m'$) and the maximum quantum yield of PSII (F_v/F_m) (Beer et al., 2001; Genty et al., 1989; Ralph et al., 2007b; Silva et al., 2009). $\Delta F/F_m'$ can be directly linked with photosynthetic efficiency, under illuminated conditions (Genty et al., 1989; Ralph et al., 2007b). F_v/F_m on the other hand is an indicator of photosystem health as it is measured under dark conditions, allowing re-oxidation of all photosystems (Genty et al., 1989; Osmond et al., 1999). Therefore a reduced F_v/F_m indicates a proportion of inactive (damaged) photosystems. As changes in various chlorophyll *a* fluorescence measurements relate to physiological responses

(including photosynthetic rates in seagrass; Beer et al., 1998) these measurements represent relevant and sensitive biomarkers for stress that can be used to describe ecotoxicological endpoints (Ralph et al., 2007b). This term "end point" is commonly used throughout this thesis and refers to an observed outcome that reflects the effect of the tested stressor or treatment.

The inhibition of $\Delta F/F_m'$ and F_v/F_m can be calculated and plotted against herbicide concentration to develop concentration-response curves (Flores et al., 2013). Such curves can then be used to derive information such as IC_{10} and IC_{50} values (concentrations which inhibit quantum yield by 10 or 50 %). IC_{10} values can be used to describe toxic thresholds while IC_{50}s are commonly adopted for toxicity comparisons between contaminants (Warne et al., 2014). Both quantum yield end points as well as many other parameters can be measured and recorded using pulse modulated amplified (PAM) fluorometry techniques (Ralph et al., 2007b). Chlorophyll *a* fluorescence analysis has been used to measure the effects of contaminants on photosynthetic efficiency and rates in a number of studies, including many test species (Cantin et al., 2007; Faust et al., 2001; Haynes et al., 2000b; Ralph, 2000; Wilkinson et al., 2015). Changes in $\Delta F/F_m'$ represent the most sensitive fluorescence indicator of physiological stress caused by PSII herbicides (Ralph et al., 2007). Inhibition of $\Delta F/F_m'$ by PSII herbicides is also ecologically relevant as this reduction in photosynthetic activity leads to reduced growth, and finally, mortality in seagrasses (Negri et al., 2015; Seery et al., 2006).

PAM fluorometry is a rapid and non-invasive method of testing a number of photosynthetic end points through measuring known processes imperative to photosynthetic activity (Ralph et al., 2007b). PAM fluorometers measure chlorophyll *a* fluorescence excited by a strong but brief light source, both before and during a saturating pulse of >3000 µmol photons m^{-2} s^{-1}. The pulse causes all active photosystem reaction centres to close (to allow electron transfer). Fluorescence emission before and during the pulse is recorded and used to calculate quantum yields (Schreiber et al., 1994). Many PAM fluorometers excite and measure light using a flexible fibre optic probe but in recent years the MAXI imaging-PAM (I-PAM) (WALZ, Germany) has been developed which enables the capture of fluorescence images across a 2-dimensional surface. This function allows visual interpretation of fluorescence across surface (such as seagrass leaves; Ralph et al., 2005) and in response to stressors; for example, herbicide uptake pathways. In addition, the I-PAM enables semi-automatic data recording, whereby, a number of data

points can be recorded simultaneously (Schreiber et al., 2007). Chlorophyll *a* fluorescence measurements using PAM fluorometers are highly effective indicators for measuring PSII herbicide phytotoxicity because changes in some fluorescence responses are directly affected by herbicide binding in PSII (Flores et al., 2013; Negri et al., 2015; Ralph, 2000). One limitation of PAM fluorometry is that photosynthetic processes occurring outside of the PSII cannot be directly measured or inferred (Beer et al., 2001; Ralph et al., 2007b).

1.4. Climate impacts on seagrass

Photosynthesis and PSII are sensitive to many other environmental impacts, which also have flow-on effects for plant health. For example, elevated temperature affects growth rates and physiological processes such as photosynthetic efficiency (Campbell et al., 2006; Collier et al., 2014; Waycott et al., 2007). Water temperature is a limiting factor in seagrass photosynthesis with greater photosynthetic efficiency observed with increasing temperature, up to a threshold (Björk et al., 2008; Pérez and Romero, 1992; Terrados and Ros, 1995). The optimal temperature range for tropical seagrass is between 15 and 33°C (Berry and Bjorkman, 1980; Collier et al., 2011; Pérez and Romero, 1992), however in some seagrass species, photosynthetic efficiency continues to increase up until 35°C (with limited growth rates) (Campbell et al., 2006; Collier and Waycott, 2014). Tropical seagrass species are often considered stenothermal (Den Hartog, 1970) and thus live close to the thermal optimal threshold (Ralph, 1998), so while photosynthetic rate and efficiency increase within the thermal range they can decline sharply when water temperatures exceed this threshold (Campbell et al., 2006; Ralph, 1998).

Ocean acidification is likely to cause an increased level of CO_2 dissolved within seawater as a result of elevated atmospheric concentrations (Guinotte and Fabry, 2008; Koch et al., 2013). Projected climatic shifts in ocean acidification could benefit seagrass production and biomass as current levels of CO_2 availability is sometimes considered a limiting factor for production in seagrass species (Björk et al., 2008; Guinotte and Fabry, 2008). However, increased CO_2 availability may change competition between seagrass and epiphytic algae as increased algal growth may also result (Beer and Koch, 1996). Additionally seagrass have the ability to sequester excess carbohydrates (produced via

carbon fixation) in below-ground stores, further enhancing possible positive responses to rises in oceanic CO_2 concentrations (Koch et al., 2013).

Future rises in water temperatures related to climate change may cause significant impacts to seagrass health and geographical distribution (Table 1.1; Orth et al., 2006; Ralph, 1998; Thorhaug et al., 1978). Within the coastal zone, temperature extremes are frequently experienced at low tide. In shallow habitats during low tide, periods of increased air temperature combined with elevated solar radiation cause short-term temperature increases which far exceed oceanic temperatures (Berkelmans, 2002; Massa et al., 2009; Rasheed and Unsworth, 2011), and optimum thermal thresholds for seagrass (Campbell et al., 2006; Collier and Waycott, 2014; Massa et al., 2009). This phenomenon is natural and rarely lasts longer than a few days (Anthony and Kerswell, 2007). However, climate change is projected to cause changes in barometric pressure (leading to tidal changes) and wind as well as rises in air and water temperatures (Bernstein et al., 2007). Such conditions could potentially transform short-term temperature extremes into highly stressful events, whereby temperatures exceed seagrass thresholds with greater frequency (Bernstein et al., 2007). As air and water temperatures increase so will the frequency of desiccation events due to the rise in exposure frequency (Waycott et al., 2007). Such increase in desiccation events is likely to favour meadows with predominant composition of species such as *H. ovalis* and *Halodule sp.* (Waycott et al., 2007). Small leaf stature and flexibility of such species allow leaves to lie flat against moist sediment, preventing drying out of the leaf surface (Beer et al., 2006; Björk et al., 1999; Collier and Waycott, 2014).

Rainfall patterns are also expected to be affected by climate change, specifically causing more intense rainfall events (Lough and Great Barrier Reef Marine Park, 2007; Waycott et al., 2007). Coastal and estuarine habitats are, and will continue to be, the communities most impacted by flood plume events due to close proximity of meadows to river outlets (Waycott et al., 2007). Within the coastal zone, light availability is the primary limiting factor for seagrass production (Björk et al., 2008; Carruthers et al., 2002; Mellors, 2003; Waycott et al., 2005). Persistent, long term suspension of sediments within the water column from more frequent and intense flood plumes will increase the occurrence of high turbidity events, reducing light penetration to seagrass growing beneath the plume, and reducing overall photosynthetic rates (Table 1.1; Björk et al., 2008; McMahon et al., 2013; Ralph et al., 2007a). A resulting carbon imbalance will

cause reductions in growth (Figure 1.2.) as more carbon will be used in respiration than will be fixed through photosynthetic processes (Collier et al., 2011; Fourqurean and Zieman, 1991; Kerr and Strother, 1985; McMahon et al., 2013). A similar flow-on effect can occur from reduced photosynthetic C-fixation associated with herbicide exposure (See conceptual model, Figure 5 in Negri et al., 2015).

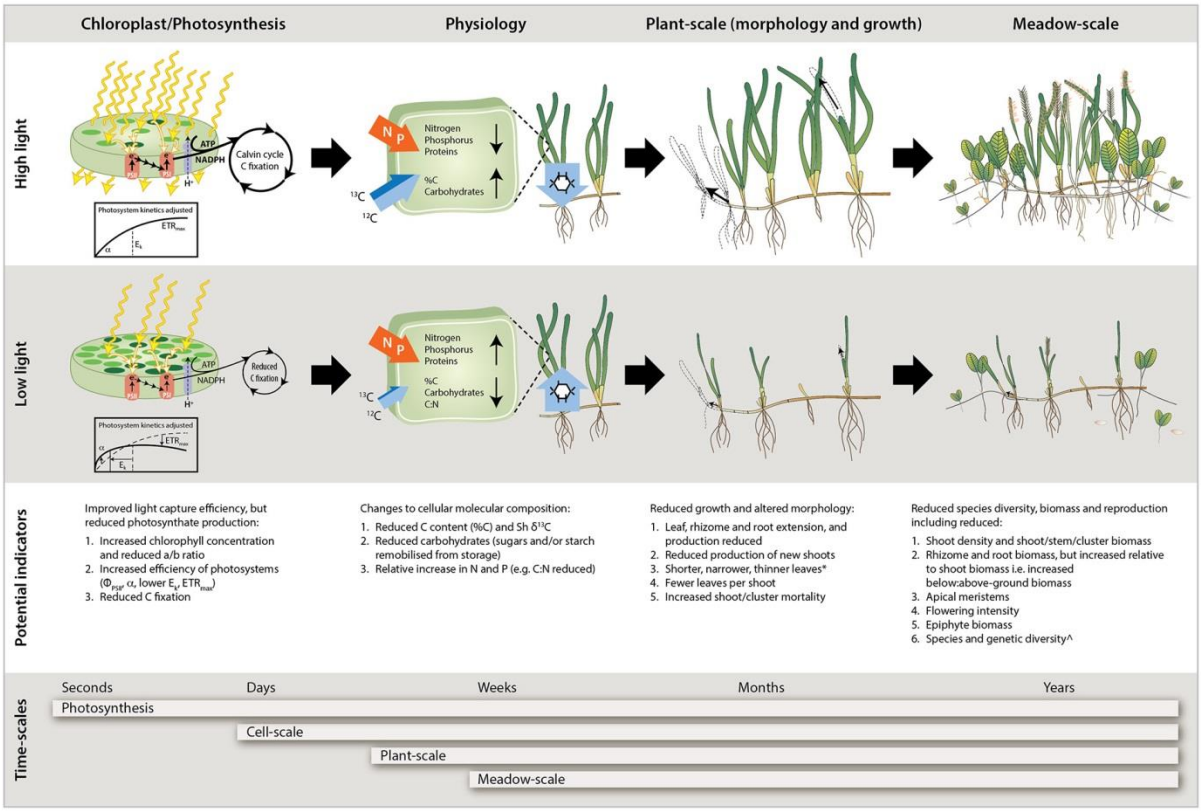

Figure 1.2. Conceptual diagram demonstrating the pathway of seagrass response under low light conditions. Responses are separated by photosynthetic, other physiological, plant-scale (growth and morphology) and meadow-scale variables. The timescales at which the responses occur are indicated in the lower section of the diagram. Diagram from McMahon et al. (2013).

Table 1.1. A summary of stressors discussed within this thesis and how each affects photosystem II in seagrass.

Stressor	Impacted Photosystem region	Mode of Action	Reference
PSII herbicide	D1 protein of PSII	Blocks electron transport via displacement of Q_b electron acceptor from niche	(Oettmeier, 1992)
Light intensity	PSII reaction centre	Over-excitation of reaction centres due to excess energy absorption. May cause damage due to energy build-up	(Ralph and Gademann, 2005)
Elevated temperature	Thylakoid membrane	Lability of the thylakoid membrane is altered causing changes in binding site conformation within PSII	(Ralph, 1998)

1.5. Multiple stressors and mixture toxicity

The toxicities of individual PSII herbicides to marine species are relatively well researched across a range of test species, with particular emphasis on compounds most commonly detected in the GBR and its catchments (diuron, atrazine, ametryn, hexazinone and tebuthiuron) (Flores et al., 2013; Haynes et al., 2000; Jones et al., 2003; Knauert et al., 2008; Magnusson et al., 2010; Negri et al., 2015; Negri et al., 2011; Ralph, 2000; Wilkinson et al., 2015). However, within the coastal waters of the GBR, PSII herbicides are usually detected in mixtures of two or more herbicides (Gilliom et al., 1999; Lewis et al., 2009; Shaw et al., 2010). All registered PSII herbicides used along the GBR exhibit identical modes of action and their combined impact on phototrophic organisms can lead to additive effects (Escher et al., 2008; Faust et al., 2001; Magnusson et al., 2010). Additivity is observed when the combined impact of two or more contaminants is equal to the sum of their individual effects. This poses significant problems for current regulation and management practices whereby ecological trigger values and guidelines

are usually derived from solitary contaminant studies. The composition of herbicide mixtures can include herbicides present in concentrations below the observable effect concentration but the mixture composition may have a significant additive inhibitory effect (Faust et al., 2001; Magnusson et al., 2010). In an attempt to monitor and regulate contaminant pollution, regulators often rely on estimates of total load (mass) of pollutants entering water bodies (Hardy and Koontz, 2008; Raha, 2007). This process assumes that all contaminants affect ecosystems individually and reductions in the load of an individual contaminant would proportionately reduce effects to ecosystem health (Smith et al., unpublished). Perversely, a large reduction in a low toxicity herbicide may have far less of a positive affect than small reductions in load of a high toxicity herbicide (Smith et al., unpublished). To ensure proportional improvement in ecosystem health the relative toxicity of herbicides within a mixture should be considered and applied to assessing the risks of herbicide loads (Smith et al., unpublished).

PSII herbicides are found within coastal waters year round due to high persistence however concentrations peak during flood plume events in the monsoon season along the GBR (Kennedy et al., 2012; Lewis et al., 2012; Shaw et al., 2010). During this period PSII herbicides are likely encountered in the presence of multiple stressors such as other herbicides and pesticides, unfavourable environmental stressors such as low light, elevated temperatures and low salinity. Such stressors may act simultaneously in a cumulative way to increase sensitivity of seagrass to PSII herbicide (Davis et al., 2015).

The effects of multiple stressors including mixtures of the same herbicides are calculated in different ways. For herbicides with the same mode of action the Concentration Addition (CA) model of joint action can be applied (Safe, 1998). This method requires the measurement of the same endpoints for example IC_{50} of quantum yield inhibition for multiple herbicides with the same mode of action. The potencies are then compared relative to the reference herbicide such as diuron to obtain the Relative potencies (ReP) (USEPA, 2008). CA combines the concentration and ReP of each mixture component to calculate predicted overall toxicity of the mixture (Berenbaum, 1985; Porsbring et al., 2010). The miniature bioassay methodology developed in this thesis (Chapter 2) represents an ideal tool for measuring mixture toxicity, and was used to further validate the application of CA as efficient calculation of mixture toxicity (Chapter 5). The Independent Action model on the other hand is suited to calculating the expected effects of stressors with dissimilar (independent) modes of action (Berenbaum,

1985; Bliss, 1939), such as herbicides combined with light and temperature (Chapters 2 and 4 respectively).

1.6. Contribution of ecotoxicology to management and regulation

Governing bodies around the globe follow particular methods and criteria in order to derive ecologically relevant aquatic trigger values and guidelines. These values, when implemented, are often targeted at protecting a certain percentage of species within an ecosystem. For example, if a trigger value (or guideline) is exceeded due to PSII herbicide runoff, management practices are applied to avoid such events being repeated (GBRMPA, 2010). Ecologically relevant guidelines are important for monitoring and protecting the survival and function of key marine habitats, however current guidelines are regularly exceeded during flood plume events along the GBR (Brodie et al., 2011; Lewis et al., 2011). Additionally some PSII herbicide guideline values do not adequately address toxicity to key marine species such as seagrass, with concentrations below guideline values causing significant effects on photophysiology (Flores et al., 2013; this thesis). Current Australian (and New Zealand) guideline development requires data sets to adhere to specific criteria to be deemed reliable. For instance, high reliability values require data from at least five marine species, from four different taxonomic groups (ANZECC and ARMCANZ., 2000; GBRMPA, 2010; Warne, 2002). In contrast, a low reliability rating is any data set that does not meet these stipulations. In the case of low reliability, freshwater values may also be are applied (ANZECC and ARMCANZ., 2000; Batley et al., 2013; GBRMPA, 2010). Two of the five priority herbicides (hexazinone and tebuthiuron) registered for use within the catchments of the GBR are represented by low reliability guidelines and more reliable ecotoxicology data is needed to remedy this situation. Current ecological guidelines are derived by calculating a contaminant concentration, protective of a proportion of a species (99, 95, 90% protection (GBRMPA, 2010)). For high value water-ways such as the World Heritage Listed GBR lagoon 99% species protection is applied (GBRMPA, 2010). Hypothesis testing such as the statistical no observed effect concentration (NOEC) data currently comprise the majority of ecological guideline datasets. Future guideline calculation will favour statistically based

endpoints such as effect concentration (EC_x) and inhibition concentrations (IC_x) as preferred toxicity endpoints (Batley et al., 2013).

A number of key changes are suggested to improve ecological guideline calculations for water quality (including herbicides) within the GBR. Firstly, as the health of the GBR World Heritage Area is predominantly guided by the health of habitat-forming species such as seagrass and coral, ecotoxicological data for such species should be a necessary requirement for a guideline derivation and risk assessments. Secondly, at present, photosynthetic responses are not always accepted for endpoint derivation due to a lack of evidence supporting clear ecological relevance (GBRMPA, 2010). However, recent research demonstrates that suppression of photosynthetic efficiency in seagrass by PSII herbicides can cause reductions in energy storage, growth and survival if prolonged (Negri et al., 2015; Seery et al., 2006). Furthermore, declines in community fitness will almost certainly have a cascading effect on higher trophic level organisms (Haynes et al., 2000; Ralph et al., 2007b; Wahedally et al., 2012). Strong correlations between effects of PSII herbicides on quantum yields in PSII and growth rates in marine microalgae (Magnusson et al., 2008) also support the relevance of this indicator for guideline derivation. Although acute PSII herbicide exposure is predominantly reversible, sub-lethal photosynthetic impacts represent excellent early indicators of declining water quality. Potential additive, synergistic or antagonistic effects in conjunction with contaminant mixtures and environmental stress (such as sub-optimal temperature and light intensity) are poorly understood and thus are not acknowledged in guideline setting (ANZECC and ARMCANZ., 2000; GBRMPA, 2010). Additionally, preferred, large scale, multi-species experiments are logistically difficult and slow to produce adequate data sets for guideline calculation (Batley et al., 2013).

The development of medium-throughput bioassays to test the impacts of PSII herbicides on the photophysiology of seagrass would significantly improve our ability to compare the potencies of multiple herbicides, test their additive effects in mixtures and understand the influence of simultaneous stressors on their impacts. The main hypotheses of this thesis were:

I. An acute seagrass phytotoxicity bioassay for PSII herbicides based on isolated leaves can provide a rapid miniature alternative to whole-plant assays to examine the relative potencies of these herbicides;

II. The effects of multiple PSII herbicides in mixtures on PSII activity in seagrass are additive.

III. Combinations of PSII herbicides and other stressors, including irradiance and extreme temperatures, will interact to impact on PSII activity in seagrass.

1.7. Thesis structure

The following data chapters (2-4) have been written and prepared as published (Chapter 2) or draft manuscripts (Chapter 3 and 4). Each therefore contains brief introduction to the significance of seagrass, herbicide effects and photosystem II function. Additionally, the miniature bioassay methodology has been outlined in varying detail in all data chapters. Again, this is due to manuscript preparation. Chapter 5 brings together the findings of all data chapters to present a comprehensive discussion of the results of the thesis and potential for future research.

Chapter 2. A miniature bioassay for testing the acute phytotoxicity of photosystem-II herbicides on the seagrass *Halophila ovalis*

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2.1. Abstract

Photosystem II (PSII) herbicides have been detected in nearshore tropical waters such as those of the Great Barrier Reef and may add to the pressure posed by runoff containing sediments and nutrients to threatened seagrass habitats. There is a growing number of studies into the potential effects of herbicides on seagrass, generally using large experimental setups with potted plants. Here we describe the successful development of an acute 12-well plate phytotoxicity assay for the PSII herbicide Diuron using isolated *Halophila ovalis* leaves. Fluorescence images demonstrated Diuron affected the entire leaf surface evenly and responses were not influenced by isolating leaves from the plant. The optimum exposure duration was 24 h, by which time the inhibition of effective quantum yield of PSII ($\Delta F/F_m$') was highest and no deterioration of photosystems was evident in control leaves. The inhibition of $\Delta F/F_m$' by Diuron in isolated *H. ovalis* leaves was identical to both potted and hydroponically grown plants (with leaves remaining attached to rhizomes), indicating similar reductions in photosynthetic activity in these acute well-plate assays. The sensitivity of the assay was not influenced by irradiance (range tested 40 to 400 μmol photons m^{-2} s^{-1}). High irradiance, however, caused photo-oxidative stress in *H. ovalis* and this generally impacted in an additive or sub-additive way with Diuron to damage PSII. The bioassay using isolated leaves is more rapid, uses far less biological material and does not rely on specialised aquarium facilities in comparison with assays using potted plants. The development and validation of this sensitive bioassay will be useful to reliably screen and monitor the phytotoxicity of existing and emerging PSII herbicides and contribute to risk assessments and water quality guideline development in the future.

2.2. Introduction

2.2.1. Tropical seagrass significance

Seagrasses provide essential nursery and feeding grounds for commercially important fish, crustaceans and molluscs (Kirkman, 1997) and form, almost exclusively, the diet of a number of macro-grazers, including the endangered dugong and green sea turtles (Kirkman, 1997). Seagrass also promotes sediment stabilisation (Kirkman, 1997; Marbà et al., 2006), nutrient cycling (Touchette, 2007) and carbon sequestration (Fourqurean et al., 2012). As far back as two decades ago, the net total ecosystem services that seagrass provide annually were estimated to be worth \$3.8 trillion yr^{-1} (Costanza et al., 1997). However, seagrass habitats are declining globally with approximately 110 km^2 lost on an annual basis (Orth et al., 2006; Waycott et al., 2009).

2.2.2. Effects of water quality on seagrass populations

The degradation of water quality factors in the dramatic decline of seagrass meadows around the globe (Kirkman, 1997). One of the best-studied examples is the Great Barrier Reef (GBR) which experiences heavy rainfall over the summer wet season, delivering large amounts of suspended solids into near shore habitats, resulting in reductions in light for primary productivity by benthic species (Fabricius et al., 2014). Seagrass loss on the GBR is strongly influenced by long periods of severe light attenuation caused by suspended solids and phytoplankton that peak during these flood events (Collier et al., 2012). Along with increased sediments and nutrients, several agricultural herbicides which target photosystem II (PSII) have been regularly detected in the catchment rivers and estuaries and within nearshore habitats of the GBR lagoon (Haynes et al., 2000b; Kennedy et al., 2012; Lewis et al., 2009).

2.2.3. Herbicides and seagrass

Diuron is one of the most commonly detected PSII herbicides in inshore and coastal waters adjacent to the GBR (Lewis et al., 2009; Lewis et al., 2012; Smith et al., 2012). Diuron has been detected year-round in the GBR though concentrations peak during the wet season when flood plumes deliver herbicides that are washed from the land into waterways during heavy rainfall (Davis et al., 2013; Shaw et al., 2010). Upon delivery to the GBR lagoon, some dilution of herbicides occurs and yet PSII herbicides

have also been detected within river plumes at concentrations up to 1 μg l^{-1} (Lewis et al., 2009). Diuron has been sampled in sub-tidal sediments (up to 10 μg kg^{-1}), within intertidal seagrass specimens (up to 1.7 μg kg^{-1}) (Haynes et al., 2000) and in creeks flowing into the GBR lagoon (up to 8.5 μg l^{-1}) (Lewis et al., 2012). PSII herbicides block electron transport in PSII and a Diuron concentration of 0.5 μg l^{-1} reduces photosynthetic efficiency by 10% in two GBR species (Flores et al., 2013). Longer-term effects from chronic photoinhibiton include plant starvation, reduced growth and finally adverse effects on competitive fitness, likely impacting on higher trophic levels (Haynes et al., 2000b; Ralph et al., 2007b; Wahedally et al., 2012).

2.2.4. Acute seagrass assay development

A highly relevant and sensitive indicator of PSII herbicide effects on seagrasses and other photosynthetic organisms such as corals, is the change in quantum yield of PSII as measured using pulse amplitude modulated (PAM) fluorometers (Jones et al., 2003; Ralph et al., 2007b). The most sensitive parameter measured by PAM fluorometers is inhibition of effective quantum yield ($\Delta F/F_m'$) (Flores et al., 2013; Ralph et al., 2007b) . This is proportional to reduced photosynthetic efficiency under experimental light levels and provides a link to diminished photosynthetic carbon fixation (energy) (Genty et al., 1989; Ralph et al., 2007b). PAM fluorometry has also been used to measure inhibition of the maximum quantum yield (F_v/F_m) which is proportional to damage to PSII due to oxidative stress caused by PSII herbicides blocking electron transport under illuminated conditions (Genty et al., 1989; Osmond et al., 1999). Plotting inhibition of quantum yields against PSII herbicide concentrations yields typical dose-response curves from which herbicide concentrations that inhibit 10 and 50% of $\Delta F/F_m'$ and F_v/F_m (IC_{10} and IC_{50}) can be derived (Flores et al., 2013).

Previous studies have used PAM fluorometers with flexible fibre optic probes to measure fluorescence responses of individual leaves from intact potted plants exposed to herbicides for ~3 days (Flores et al., 2013; Haynes et al., 2000b; Ralph, 2000). While providing precise and relevant ecotoxicolgical data, the whole-plant assays require large experimental aquarium setups and many days of preparation and maintenance. A potentially rapid and sensitive alternative approach is to apply the spatial imaging Maxi-Imaging-PAM (I-PAM) (Walz, GmbH Germany) to individual seagrass leaves in well-plates. This approach has been successfully used to quantify the toxicity of PSII

herbicides on isolated whirls and fronds of multiple macroalgae species in 24-well (Küster and Altenburger, 2007), and in 96-well formats with several species of microalgae (Magnusson et al., 2010; Schreiber et al., 2007). The application of I-PAM to small individual seagrass leaves offers the potential of developing a more rapid and less expensive method to assess and monitor the phytotoxicity of PSII herbicides to seagrass under a range of environmental conditions.

In this study, we quantified the acute phytotoxicity of PSII herbicide, Diuron, on *Halophila ovalis* while validating a 12-well plate fluorescence bioassay using the I-PAM. Fluorescence-derived phytotoxicity endpoints in the isolated leaves were directly compared with potted and unpotted but intact (hydroponic) seagrasses and the influence of light on photosynthetic efficiency and damage to PSII were assessed.

2.3. Materials and Methods

A miniature 12-well plate phytotoxicity assay was developed to assess the exposure of seagrass to PSII herbicides in the following way:-

1. Sample collection and experimental setup. All acute exposures (up to 24 h) were conducted in static conditions using measured concentrations of Diuron.
2. PAM fluorometry (see below) was applied as a specific and sensitive indicator of PSII herbicide toxicity to isolated seagrass leaves and intact plants.
3. Leaves were screened for high levels of photosynthetic efficiency before the start of each experiment.
4. Rapid light curves were used to assess the photosynthetic performance of the seagrass as a function of irradiance and to enable the selection of ambient illumination for the experiments.
5. Fluorescence images were captured using the I-PAM to spatially monitor photosynthetic impact of Diuron in the isolated leaves.
6. The photosynthetic condition of leaves were re-examined by I-PAM regularly over 24 h in the absence of herbicide to test for leaf deterioration over the exposure period. The impact of Diuron on effective quantum yields was also monitored ensure maximum uptake (a steady state of toxic effect) had been reached within 24 h.

7. Dose-response relationships were compared between I-PAM and Mini-PAM data to verify consistency with other studies.
8. Dose-response relationships were compared between isolated leaves in 12-well plates and intact plants (both potted and hydroponic), in flow-through conditions, to validate the sensitivity of the well-plate method.
9. Dose-response relationships were compared using the well plate method at four light levels to (i) test consistency and repeatability under different irradiance conditions and (ii) examine the potential for Diuron to impact on seagrass under varying light conditions.
10. Potential interactions between irradiance and Diuron on effective and maximum quantum yields were explored using the Independent Action (IA) model.

2.3.1. Sample collection and experimental setup

Halophila ovalis is a tropical seagrass species widely distributed throughout the Indo-Pacific and is found in all marine habitats throughout Australia (Waycott et al., 2004) and is one of the target species for *Dugong dugon* foraging (Marsh et al., 2011). It is a rapidly growing species with leaf pairs emerging from the rhizome. It is also highly sensitive to environmental stress (Campbell et al., 2006; Collier et al., 2014; Ralph, 2000; Ralph and Burchett, 1995), responding like an r-strategist by dying off when stressed and re-populating from its seedbank (Inglis, 2000). Its rapid response time to habitat modification could make it an excellent sentinel species, providing early warning of changes to environmental conditions. *H. ovalis* plants were collected from intertidal meadows during low tide from Cockle Bay, Magnetic Island (19° 10.88'S, 146° 50.63'E) under permit MTB41, a permit issued for limited impact research in the GBR Marine Park which was assessed through the Department of Employment, Economic Development and Innovation self-assessable Fisheries Queensland Code MP05 for the removal of marine plants.

A small plug of seagrass with its associated sediment (5-10 cm depth) was removed and placed in plastic plant pots lined with plastic bags. The bag was pulled up over the seagrass and a small amount of water was added to the bag and secured at the top for transport. Plants were taken to the Australian Institute of Marine Science (AIMS) and placed into 1000 l outdoor aquaria within 4 h from collection under 20% ambient

light (maximum of ~ 400 µmol photons $m^{-2}s^{-1}$ under shade cloth) and water temperature conditions (25 – 28°C).

Diuron, also called DCMU or (3-(3,4-dichlorophenyl)-1,1-dimethylurea) is a phenyl-urea group photosystem II herbicide and was obtained from Sigma Aldrich (>95% pure). Stock solutions of 5 mg l^{-1} Diuron in milli-Q water were prepared using 2 ml ethanol (<0.03% v/v solvent carrier in exposures). Individual working solutions of each concentration were then prepared in 0.45 µm-filtered seawater with the same proportion of ethanol carrier. Water samples for herbicide analysis (2 ml) were taken 1 h and 24 h after dosing and pipetted into 4 ml amber glass vials. Samples were then spiked with 20 µl of a surrogate standard, d5-Atrazine (Novachem, Victoria, Australia) and stored frozen. Diuron was analysed as per Flores et al. (2013).

2.3.2. PAM fluorometry

Pulse Amplitude Modulated (PAM) fluorometer measurements were conducted using two different instruments for comparison. Mini-PAM measurements were obtained by placing a 2 mm fibre optic probe perpendicular to the leaf surface, approximately at the centre, offset from the mid-vein. Measurements were made only on young, healthy leaves (second or third leaf pair from the terminal end of the rhizome). Minimal fluorescence (F with illuminated samples and F_0 with 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 and the effective quantum yield of PSII calculated from $\Delta F/F_m' = (F_m' - F) / F_m'$. In order to calculate the maximum quantum yield of PSII (F_v/F_m), seagrass was dark adapted for 30 min and F_0 and F_m measured, as above, from $F_v/F_m = (F_m - F_0) / F_m$. F_v/F_m is a measure of the optimal photosynthetic efficiency and inhibition of F_v/F_m can indicate photooxidative stress and damage to PSII from irradiance stress. $\Delta F/F_m'$, on the other hand, reflects the level of PSII activity under ambient light conditions, providing a more sensitive and realistic assessment of PSII herbicide impacts on photosynthesis (Ralph et al., 2007b).

Imaging-PAM (I-PAM) measurements were conducted in 12-well plates by individually placing each well plate into the imaging chamber and controlled using Data-MAXI software on a desktop computer (Imaging Win, Walz GmbH, Germany). Actinic light was set to 100 µmol photons $m^{-2}s^{-1}$ and a measuring intensity of four was applied

to generate similar quantum yields as observed for Mini-PAM measurements. Following the imaging process, Imaging Win was used to select single area of interest (AOI) of 3 – 5 mm diameter for each leaf in order to maximise the leaf surface area for yield measurements. $\Delta F/F_m$', and F_v/F_m, were calculated as above and were typically measured prior to dosing and at 24 h (end of exposure).

2.3.3. Seagrass leaf screening and general bioassay conditions

Before removing leaves, any epiphyte growth was removed from the leaf surface. Stems were cut to the base of the leaf and pinched closed with forceps to minimise formation of air bubbles in the midrib. Any bubbles on the external surface of the leaf were gently removed to prevent floating. Before each I-PAM measurement, all leaves which were not horizontally orientated were repositioned until flat against the bottom of the well. Care was taken to ensure the leaf was not in contact with the well wall to minimise fluorescence interference. Once in the I-PAM chamber a ~5 sec interval was taken to ensure all water movement ceased before measurements were taken.

To ensure only healthy, reliable leaves were used in the well plate experiments, cut leaves underwent a screening process. Second and third leaf pairs from the terminal, apical end of the rhizome were selected and removed. Single leaves were placed in each well of 12-well plates (Nunclon, Thermo scientific) containing 0.45 µm filtered seawater (5 ml each well). Maximum quantum yield of each cut leaf was measured using the I-PAM and only leaves exhibiting F_v/F_m greater than 0.65 were used to ensure optimal leaf health and to allow for greater reliability. This threshold was chosen based on greater consistency of measurements in leaves taken over 24 h in our pilot experiments when initial F_v/F_m was greater than 0.65. Average leaf length was 10.0 mm ± 2.5 (range of all leaves) and width was 4.8 mm ± 1.2. This screening process was performed immediately prior to running the assays to ensure the leaves were in healthy condition for the experiment.

2.3.4. Irradiance conditions

Prior to experiments, the plants were maintained for at least 48 h upright in aquaria under a constant temperature of 26°C ± 1, salinity of 34 – 36 ppt and light of 280 ± 15 (range under all lights) µmol photons $m^{-2}s^{-1}$ (Aqua Illumination SOL White LED lights) which was equivalent to the mean irradiance at the collection site from 2008 – 2012 (Collier unpub.). For well-plate assays, leaves were placed horizontally in filtered seawater and left for at least 1 hr at 100 µmol photons $m^{-2}s^{-1}$. The effective quantum yield of PSII ($\Delta F/F_m'$) were equivalent (~ 0.60 – 0.65) for vertical leaves at 280 µmol photons $m^{-2}s^{-1}$ and horizontal leaves at 100 µmol photons $m^{-2}s^{-1}$ using the same light source, indicating that the proportion of PSII reaction centres closed due to photosynthetic activity was equivalent under both conditions.

In order to assess the photosynthetic performance of the seagrass as a function of irradiance, rapid light curves (RLC) were used with the I-PAM for leaves in well plates maintained for > 1 h under 100 µmol photons $m^{-2}s^{-1}$ (Ralph and Gademann, 2005). $\Delta F/F_m'$ was measured using the I-PAM software at 12 actinic light intensities and the relative electron transport rate (rETR) was calculated and plotted against irradiance according to the equation $rETR = \Delta F/F_m' \times PAR$ (Ralph and Gademann, 2005). Minimum saturating irradiance (E_k) and maximum photosynthetic capacity ($rETR_{max}$) were also calculated.

2.3.5. Spatial response of seagrass to herbicide uptake

To assess the homogeneity of Diuron uptake in isolated *H. ovalis* leaves, the fluorescence response to 10 µg l^{-1} Diuron was observed using the I-PAM. Leaves were acclimated under 100 µmol photons m^{-2} s^{-1} at 27°C for 2 h in 12-well plates with 5 ml filtered seawater. Leaves were individually exposed to 10 µg l^{-1} Diuron and a solvent control and $\Delta F/F_m'$ and F_v/F_m measured over 24 h.

2.3.6. Exposure durations

The duration of acute exposure for the well-plate assay needed to be long enough to ensure steady-state (maximum) inhibition of $\Delta F/F_m'$ in *H. ovalis* but short enough so that the leaves had not appreciably deteriorated (no substantial decline in photosynthetic capacity F_v/F_m in controls). Leaves were placed in 12-well plates and acclimated horizontally at 100 µmol photons m^{-2} s^{-1}. Leaves (n = 16) were exposed to 10 µg l^{-1} Diuron or solvent control solutions for up to 24 h. $\Delta F/F_m'$ was measured at 0, 2, 4, 6, 12

and 24 h and F_v/F_m at 30 min, 12.5 and 24.5 h, following $\Delta F/F_m'$ measurements and a 30 minute dark adaption period. All subsequent bioassay experiments were performed over 24 hours when >95% of maximum inhibition had been reached at 10 µg l^{-1} Diuron.

2.3.7. Dose-responses from I-PAM and Mini-PAM

Dose response curves were produced for the inhibition of $\Delta F/F_m'$ and F_v/F_m in response to seven elevated Diuron concentrations in well-plate assays under an illumination of 100 ± 10 µmol photons $m^{-2}s^{-1}$. Single leaves, with all stems removed, were placed within each individual well and allowed to acclimate for ~2 h. Leaves were then transferred to 12-well plates so each well plate contained three leaf replicates of the solvent control and three Diuron concentrations (i.e. four treatments × three replicate = 12 wells). Concentrations were randomised across all well plates to minimise any well plate effect. There were a total of nine replicate leaves for each Diuron concentration across multiple plates. The % inhibition of $\Delta F/F_m'$ and F_v/F_m relative to solvent control treatments were calculated and plotted as dose-response curves (see below). The mean measured concentrations used in each dose-response relationship within this study were: 0 (below reporting limit 0.05), 0.08, 0.24, 0.80, 2.2, 8.2, 31 and 113 µg l^{-1}. In order to examine potential differences in Diuron phytotoxicity to *H. ovalis* leaves calculated using Mini-PAM and I-PAM measurements, a direct comparison of dose-responses was conducted following the 24 h Diuron exposure period.

Photoinhibition (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. A dose response curve was plotted using inhibition data at 24 h exposure. 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.

2.3.8. Response of single leaf well plate vs hydroponic and potted plants

The photobiological response of single leaves to Diuron in 12-well plate format exposures was compared with intact potted plants (including sediment to 4.5 cm depth) and hydroponic plants (whole plants in 0.45 µm filtered seawater without sediments or nutrients). While potted plants most closely represent the natural state, hydroponic plants represent an intermediate or bare state where the entire root-rhizome system is intact but not submerged in sediments. Potted plant samples were contained in 500 ml experimental pots (13.5 × 9.8 × 4.5 cm). The hydroponic and potted arrangements were placed in 6 l indoor glass aquaria (Flores et al., 2013). A constant temperature of 26°C ± 1, salinity of 34 – 36 and light of 280 ± 15 (range) µmol photons $m^{-2}s^{-1}$ was maintained. Single leaf well plate exposures were performed as described above.

2.3.9. Effects of irradiance on assay sensitivity and damage to PSII

To assess how irradiance and Diuron affected the photosynthetic efficiency of *H. ovalis*, the seagrass was exposed to 40, 100, 200 and 400 µmol photons m^{-2} s^{-1} with varying concentrations of Diuron (0 - 100 µg l^{-1}). This experiment utilised the well plate bioassay with isolated horizontal leaves and dose-response curves for inhibition of effective and maximum fluorescence yields were developed. Inhibition was calculated in two ways: (i) in comparison to the yield of the solvent control at the relevant irradiance and (ii) in comparison to the yield of the solvent control at the lowest irradiance.

2.3.10. Exploring interactions between irradiance and Diuron on photosynthetic yields

Expected inhibition of both $\Delta F/F_m'$ and F_v/F_m for additivity of effects was calculated by applying the Independent Acion (IA) equation to the inhibition data: $P(L,D)_p = P(L) + P(D) – P(L) \times P(D)$ (Bliss, 1939; van Dam et al., 2012). Here $P(L,D)_p$ is the predicted additive effect of both variables tested; P(L) is the effect of light intensity in the absence of Diuron and P(D) is the effect of Diuron at the control light level, 40 µmol photons m^{-2} s^{-1}. Both P(L) and P(D) are derived from raw data means. Expected combined effect of both light intensity and Diuron exposure were calculated for seven herbicide and four light combinations and plotted against the measured inhibition data relative to the 40 µmol photons m^{-2} s^{-1} light intensity treatment solvent control (greatest yields of all four light treatments).

2.4. Results

2.4.1. Leaf screening and general bioassay conditions

A series of preliminary experiments indicated that consistent maximum quantum yield measurements were only possible following careful pre-screening of the leaves (initial F_v/F_m greater than 0.65). Damaged leaves and/or those exhibiting low F_v/F_m values deteriorated over 24 h in well plates resulting in low and often erratic fluorescence response.

2.4.2. Irradiance conditions

A rapid increase in rETR was observed during the light limiting region of the curve ($\alpha = 0.43$). The minimum saturating irradiance (E_k) of 44 µmol photons m^{-2} s^{-1} was relatively low, indicating that the seagrass maintained in the indoor aquarium at 280 µmol photons $m^{-2}s^{-1}$ for upright leaves followed by horizontal acclimation for the assay at 100 µmol photons $m^{-2}s^{-1}$ were adapted to relatively low light. These measurements allowed us to choose four irradiances for subsequent experiments that represented light limiting (40 µmol photons m^{-2} s^{-1}), saturated (100 µmol photons m^{-2} s^{-1}) and two higher irradiances representing irradiance-stressed conditions at 200 and 400 µmol photons m^{-2} s^{-1} where illumination exceeded the photosynthetic capacity of the seagrass (leading to elevated photoinhibition).

2.4.3. Spatial response of seagrass to herbicide uptake

The isolated *H. ovalis* leaves exhibited an even change in fluorescence across their surface following exposure to Diuron (Figure. 2.1). There was a small area near the cut stem that showed reduced $\Delta F/F_m'$, but Diuron affected the entire surface of the leaves rather than spreading from the stem and the midrib (the stem was therefore not chosen as part of the area of interest for any quantitative experiments).

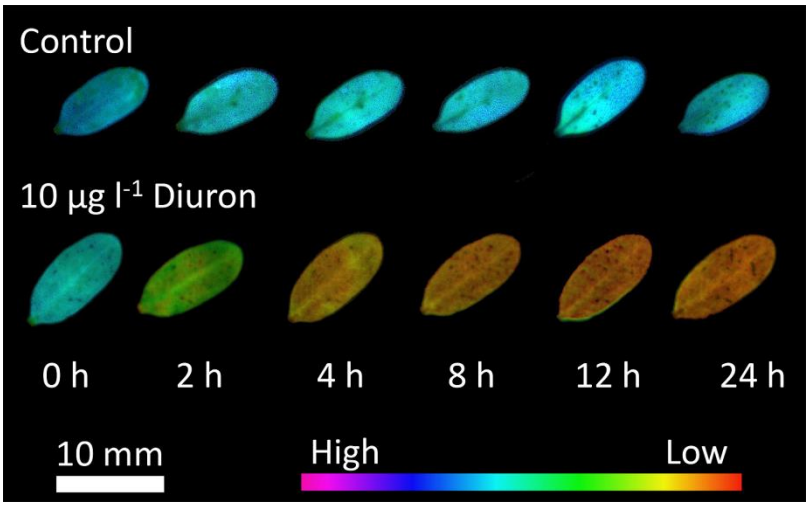


Figure 2.1. $\Delta F/F_m'$ measured using an I-PAM over a 24 h period. One series illustrates a solvent control leaf over 24 h while the other illustrates changes in $\Delta F/F_m$' due to exposure to 10 µg l^{-1} Diuron over the same period. All measurements taken under 100 µmol photons $m^{-2}s^{-1}$.

2.4.4. Time taken to reach maximum toxicity

Leaves selected by pre-screening (above) exhibited little reduction in F_v/F_m from 0.71 ± 0.01 (SE) to 0.69 ± 0.01 (less than 3%) in solvent controls indicating limited deterioration or additional photoinhibitory stress over 24 h. In this experiment 10 µg l^{-1} Diuron inhibited $\Delta F/F_m'$ by 82% ± 2% (SE) after 24 h relative to solvent control leaves. The uptake of Diuron was rapid with 95% maximum inhibition of $\Delta F/F_m'$ (toxicity) reached after 12 h and steady state toxicity between 18 and 24 h (Figure. 2.2), indicating that consistent toxicity should be expected between experiments after exposure periods of 18 h or more. The static nature of the bioassay methodology had potential to effect herbicide uptake due to an undisrupted boundary layer surrounding each individual leaf. However the uptake kinetics (figure 2.2) suggested little impact when compared to the kinetics of a previously conducted flow through assay testing diuron toxicity on a number of seagrass species (including *H. ovalis*) (Flores et al., 2013).

2.4.5. Dose-responses from I-PAM and Mini-PAM

Dose-response curves obtained from different PAM fluorometers indicated that although the inhibition of $\Delta F/F_m'$ by Diuron was similar in each case (Figure. 2.3A) that the mini-PAM was slightly but significantly ($F_{3,24} = 5.40$, $p < 0.05$) more sensitive to Diuron. The Diuron concentrations that inhibited $\Delta F/F_m'$ by 10% and 50% (IC_{10} and IC_{50}) can be found in Table 1. Inhibition of F_v/F_m in *H. ovalis* by Diuron was consistent between techniques (Figure. 2.3B and Table 1, $F_{3,24} = 27.4$, $p > 0.05$). The complete inhibition of F_v/F_m was not reached over this range of exposures potentially due to incomplete binding of Diuron to the binding sites of PSII.

2.4.6. Dose-response relationships for 12-well plates in comparison with potted and hydroponic plants

The effect of Diuron on $\Delta F/F_m'$ in *H. ovalis* leaves in 12-well plates was identical to whole potted or hydroponic plants, with IC_{10} values ranging from 0.53 to 1.0 and IC_{50} values ranging from 3.0 to 3.5 µg l^{-1} (Figure. 2.4A and Table 1, $F_{3,24} = 5.40$, $p > 0.05$). However, Diuron caused more inhibition of F_v/F_m in well plate leaves which exhibited IC_{50} for F_v/F_m of 4.2 µg l^{-1} compared with 11 and 16 µg l^{-1} for potted and hydroponic arrangements respectively (Figure. 2.4B, Table 1, $F_{3,24} = 27.4$, $p < 0.05$).

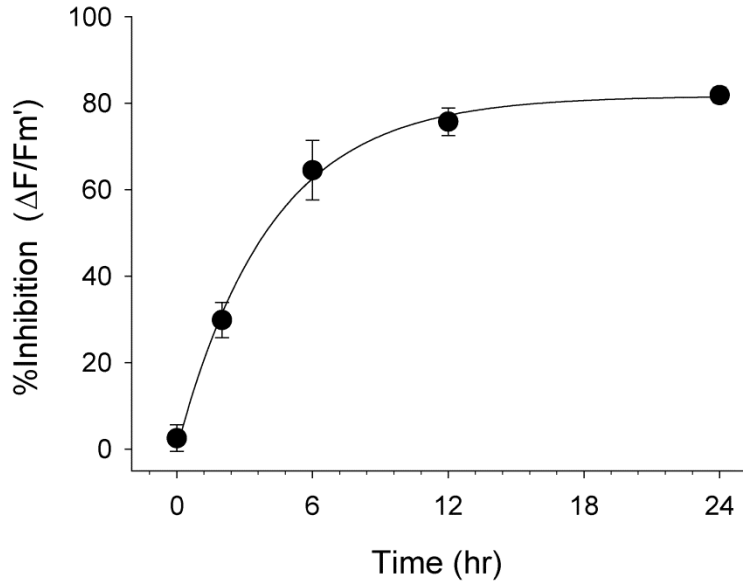


Figure 2.2. Inhibition of effective quantum yield ($\Delta F/F_m'$) in *H. ovalis* by 10 µg l^{-1} Diuron relative to solvent control conditions versus time. Bars ± SE, n = 16. All measurements recorded at 100 µmol photons $m^{-2}s^{-1}$.

2.4.7.Effects of irradiance on assay sensitivity

The inhibition of $\Delta F/F_m'$ relative to solvent controls at the corresponding light intensity was relatively consistent across herbicide exposures (Figure. 2.5A). Although the IC_{10} and IC_{50} at the 200 and 400 µmol photons m^{-2} s^{-1} were numerically lower than those under lower illuminations, the differences were not significant (Table 2.2, $F_{3,24}$ = 2.00 , $p > 0.05$). Similar results were observed for inhibition of F_v/F_m by Diuron when measured against the controls at corresponding light intensities (Figure. 2.5B, Table 2.2), although the IC_{50} for *H. ovalis* exposed at 400 µmol photons m^{-2} s^{-1} was lower than for the other treatments ($F_{3,24} = 5.83$, $p < 0.05$).

The combined inhibition of $\Delta F/F_m'$ relative to the solvent control measurements was far greater under increased illumination compared to those taken under 40 µmol photons m^{-2} s^{-1} (Figure. 2.6A). At low Diuron concentrations there was a clear step-wise inhibition that increased with light due to the increased photosynthetic activity. The inhibition converged at approximately 80% inhibition when the Diuron concentration reached 10 µg l^{-1}.

Table 2.1. Diuron concentrations that inhibit 50% (IC_{50}) and 10% (IC_{10}) quantum yields in different sample arrangements of *H. ovalis*. Different superscripted letters indicate statistically different IC_{50} values ($p < 0.05$).

	$\Delta F/F_m'$				F_v/F_m			
	IC_{50}	**95% CV**	**IC_{10}**	**95% CV**	**IC_{50}**	**95% CV**	**IC_{10}**	**95% CV**
12-well I-PAM	3.5[a]	2.5-4.3	0.78	0.46-1.2	4.3[a]	3.1-6.3	0.66	0.33-1.14
12-well Mini-PAM	2.1[b]	1.5-3.2	0.53	0-1.1	4.2[a]	3.1-5.9	0.44	0.20-0.75
Potted Mini-PAM	3.0[a]	2.6-3.5	0.79	0.59-1.0	12[b]	7-24	0.86	0.27-2.1
Hydroponic Mini-PAM	3.5[a]	3.0-4.0	1.0	0.81-1.3	16[b]	12-22	1.5	1.1-2.1

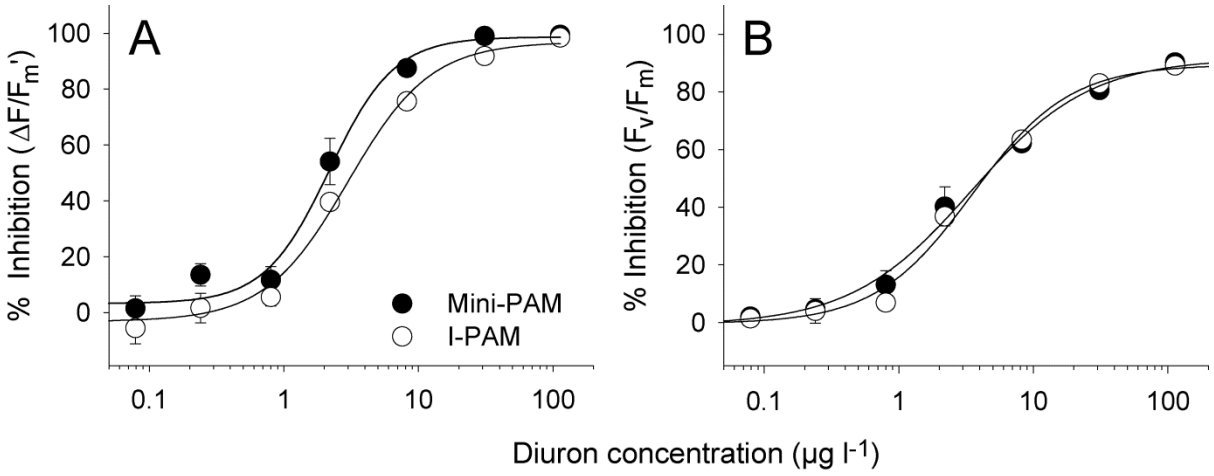


Figure 2.3. Concentration-response curves for Diuron and *H. ovalis* using different PAM fluorometers. Inhibition (%, relative to solvent control) of A) effective quantum yield ($\Delta F/F_m'$) and B) maximum quantum yield (F_v/F_m) of the single leaf, multi-well plate sample orientation. Results from both I-PAM and Mini-PAM following a 24 h exposure period to a series of Diuron concentrations at 100 µmol photons $m^{-2}s^{-1}$. Mean ± SE of nine replicate leaves. Curves exhibited r^2 between 0.98 and 0.99.

2.4.8. Fitting the effects of irradiance and Diuron to the Independent Action model

The inhibition of $\Delta F/F_m'$ was incorporated into the mixture model of Independent Action (IA) to test whether the measured inhibitions were additive, synergistic or sub-additive. A plot of expected combined inhibitions (IA) against measured inhibitions (Figure. 2.7A) revealed a strong agreement with the additive model (most of the data points overlapped the 1:1 line indicating additivity).

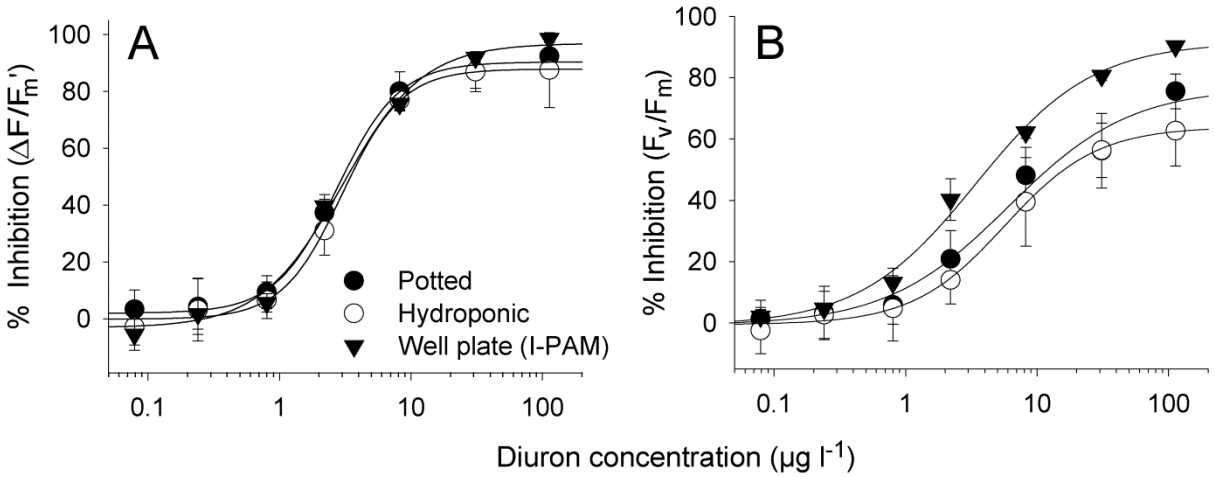


Figure 2.4. Dose-response curves for Diuron and *H. ovalis* using 12-well plate vs potted and hydroponic arrangements. Inhibition (%) of A) effective quantum yield ($\Delta F/F_m'$) and B) maximum quantum yield of PSII (F_v/F_m) after a 24 h exposure period to a series of Diuron concentrations at 100 µmol photons $m^{-2}s^{-1}$. Yield inhibitions were calculated against mean yield values for solvent controls at the corresponding irradiance. n=9 ± SE, curve fits exhibited r^2 between 0.98 and 0.99.

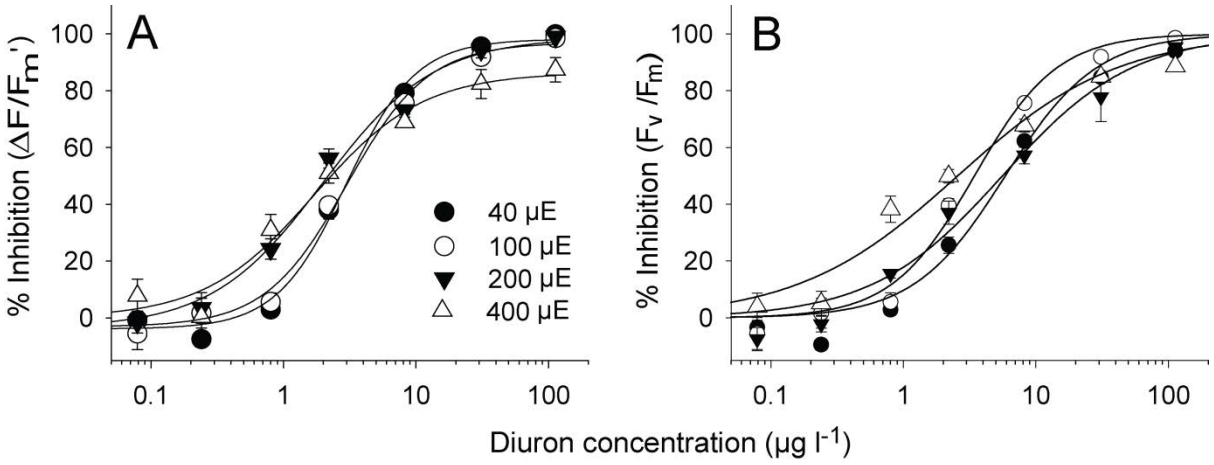


Figure 2.5. Dose-response curves for Diuron and *H. ovalis* at four different irradiances relative to the yields of corresponding irradiances. Inhibition (%) of A) effective quantum yield ($\Delta F/F_m'$) and B) maximum quantum yield of PSII (F_v/F_m) after a 24 h exposure to a series of Diuron concentrations at four irradiances. Yield inhibitions were calculated against mean yield values for solvent controls at the corresponding irradiance. Mean ± SE of nine replicate leaves. All curves fits exhibited r^2 between 0.98 and 0.99.

A similar series of converging dose-response curves were observed for inhibition of F_v/F_m at four light intensities and seven Diuron concentrations (Figure 2.6B). An

inhibition of F_v/F_m of ~20% was observed under 400 µmol photons m^{-2} s^{-1} at low Diuron concentrations indicating light-induced damage to photosystem II at this irradiance. For each combination of light pressure and Diuron concentration, inhibition increased. The IA plots of expected vs observed inhibition for combined herbicide and light stress (Figure. 2.7B) indicated additivity of these pressures (data overlapped the 1:1 line) or sub-additivity (data lay under the line).

2.5. Discussion

An acute 12-well plate phytotoxicity assay using isolated *H. ovalis* leaves was successfully developed and applied to the PSII herbicide Diuron. The assay applied PAM fluorometry (both Mini-PAM and I-PAM), which non-destructively measures change in the efficiency of PSII and will be useful to rapidly and reliably screen exposure to existing and emerging PSII herbicides. Fluorescence images demonstrated that Diuron affected PSII across the entire leaf surface evenly and measurements were not affected by isolating leaves from the plant.

Table 2.2. Diuron concentrations (µg l^{-1}) that inhibit 50% (IC_{50}) and 10% (IC_{10}) of *H. ovalis* leaf quantum yields at different irradiance levels. Yield inhibitions were calculated against mean yield values for solvent controls at the corresponding irradiance (µmol photons m^{-2} s^{-1}). Different superscripted letters indicate statistically different IC_{50} values ($p < 0.05$).

	$\Delta F/F'$				F_v/F_m			
Irradiance	**IC_{50}**	**95%CV**	**IC_{10}**	**95% CV**	**IC_{50}**	**95% CV**	**IC_{10}**	**95% CV**
40	3.3^a	2.4-4.5	0.97	0.57-1.53	5.4^a	3.9-8.0	1.23	0.67-1.97
100	3.5^a	2.5- 4.3	0.78	0.46-1.27	4.3^a	3.1-6.3	0.66	0.33-1.14
200	2.2^a	1.5-3.5	0.32	0.13-0.64	5.3^a	3.1-9.8	0.55	0.19-1.22
400	2.3^a	1.2-4.6	0.25	0.07-1.03	2.7^b	1.3 - 4.4	0.18	0.06-0.49

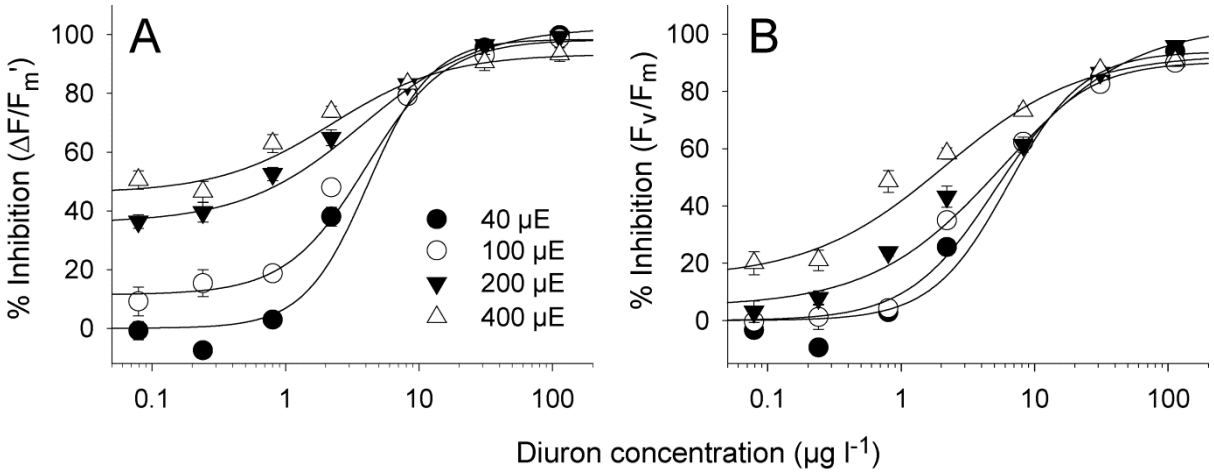


Figure 2.6. Dose-response curves for Diuron and *H. ovalis* at four different irradiances relative to the control yields held under 40 µmol photons m^{-2} s^{-1}. Inhibition (%) of A) effective quantum yield ($\Delta F/F_m'$) and B) maximum quantum yield of PSII (F_v/F_m) after a 24 h exposure period to a series of Diuron concentrations at four irradiances. Yield inhibitions were calculated against mean yield values for solvent controls at the lowest irradiance. Mean ± SE of nine replicate leaves. All curves fits exhibited r^2 between 0.97 and 0.99.

The inhibition of $\Delta F/F_m'$ (but not F_v/F_m) by Diuron in isolated *H. ovalis* leaves was identical to potted and hydroponic plants, following the optimum exposure duration of 24 h. The sensitivity of isolated *H. ovalis* leaves to Diuron was also consistent (i.e. IC_{50} and IC_{10} were similar) with other potted seagrasses (*Halodule uninervis* and *Zostera muelleri*) in a study that proposed revision of the environmental guidelines based on toxicity responses to four herbicides (Flores et al., 2013).

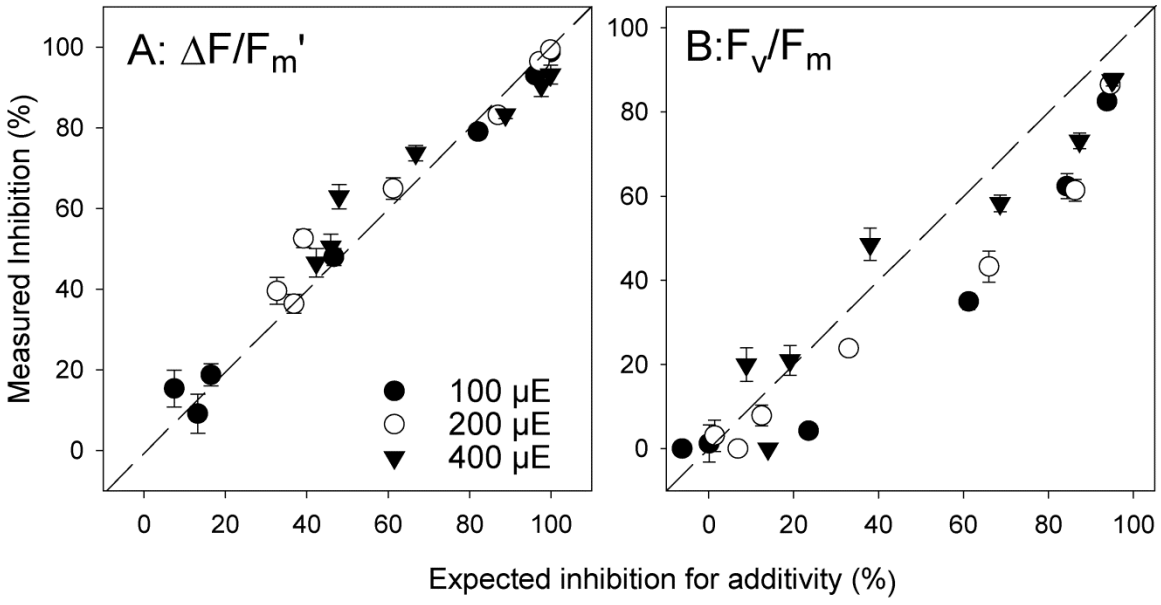


Figure 2.7. Measured vs expected toxicities. Comparison between measured and expected (IA) combined effects of elevated light intensities and Diuron on A) $\Delta F/F_m'$ and B) F_v/F_m of *H. ovalis*. Data points intersecting the zero-interaction line indicate additivity; points below the additivity line suggest sub-additivity; and datapoints above the additivity line indicate synergism. All inhibition calculated relative to 40 µmol photons m^{-2} s^{-1} solvent control mean. Mean ± SE of nine replicate leaves.

This similarity indicates that this rapid bioassay could be suitable for assessment of compliance with water quality guidelines. While the sensitivity of the assay was not influenced strongly by irradiance, high light levels did cause photo-oxidative stress in *H. ovalis*. Irradiance acted in an additive or sub-additive way with Diuron to impact on PSII thus highlighting the need for understanding how interacting environmental factors could influence screening procedures, as well as seagrass sensitivity to herbicides under variable environmental conditions.

2.5.1. Optimising the assay

The main challenge in developing a well-plate phytotoxicity assay for isolated seagrass leaves was to ensure that the photosystems in the leaves were not degraded during the exposure period and that the exposure was sufficiently long for the leaves to reach steady-state response to the herbicide. The integrity of the leaves was confirmed in two related ways. Firstly, fluorescence images across the leaf surface using I-PAM showed that in uncontaminated seawater there was little difference in fluorescence response over the 24 h (Figure. 2.1). This was quantified by measuring the photosynthetic efficiency (F_v/F_m) in solvent controls and confirming there was <3% decrease over the exposure period. The fluorescence images also demonstrated that Diuron was acting on PSII evenly across leaves. Previous studies have shown very fine-scale differences in absorptivity across seagrass leaves (Ralph et al., 2005) but this was less evident for $\Delta F/F_m'$ (which is minimised as it is based on a ratio of fluorescence emissions). Ralph et al. (2005) also demonstrated differences in leaves of different ages with older leaves and the tips of leaves often exhibiting lower $\Delta F/F_m'$ values due to either chronic oxidative damage or differences in light adaptation over the length of a leaf. In the current study these differences were minimised by choosing young leaves and pre-screening for leaves with high photosynthetic efficiencies. Importantly the images demonstrated that the

herbicide did not flood through the vascular system but entered the leaf evenly across the semi-permeable cell walls. This is consistent with a previous study which demonstrated that the PSII herbicide Atrazine was preferentially transported through the leaves rather than via the root-rhizome complex (Schwarzschild et al., 1994). The uptake of Diuron was relatively rapid and steady state inhibition of $\Delta F/F_m'$ by ~12 h was consistent between the isolated leaf well-plate format and potted *H. uninervis* and *Z. muelleri* (Flores et al., 2013). An optimum exposure was considered somewhere between 12 and 24 h when the herbicide had caused maximum toxicity, but the photosystems of leaves had not appreciably deteriorated. Our previous study using potted *H. uninervis* and *Z. muelleri* showed no additional inhibition of $\Delta F/F_m'$ or F_v/F_m by Diuron between 24 and 72 h (Flores et al., 2013).

2.5.2. Sensitivity in comparison with intact plants and studies

The inhibition of photosynthetic activity ($\Delta F/F_m'$) by Diuron in isolated leaves using the 12-well plates was identical to inhibition in intact plants (both hydroponic and potted). The narrow range of IC_{50} (Table 2.1, 3.0 – 3.5 μg l^{-1}) demonstrated not only the consistency of the method with larger, more time-consuming alternatives, but also supported our case that the isolated leaves are a valid alternative for acute phytotoxicity tests and supported previous studies that herbicide toxicity in seagrass is via leaf, not root-complex uptake (Schwarzschild et al., 1994). The IC_{10} and IC_{50} values for $\Delta F/F_m'$ in isolated *H. ovalis* leaves were also similar to those reported for the effects of Diuron on $\Delta F/F_m'$ in potted *H. uninervis* and *Z. muelleri* over 24 and 72 h (Flores et al., 2013). Although statistically different for $\Delta F/F_m'$, there was good agreement in assay results for the measurement of inhibition of leaves using the two PAM instruments (Table 2.1), which demonstrates that the 12-well plate technique is equally valid for use with a Mini-PAM or Diving-PAM (operationally the same as a Mini-PAM) if an I-PAM is not available. Although there was very good agreement of inhibition of $\Delta F/F_m'$ between well plate, potted and hydroponic methods, the horizontal leaves in well plates were more sensitive to the combination of herbicide and light as demonstrated by the lower F_v/F_m in comparison with potted and hydroponic leaves (Table 2.1). We had determined the most reliable way of ensuring similar photosynthetic activity of the controls in horizontal isolated leaves and intact plants was to adjust the irradiance so that $\Delta F/F_m'$ was similar

for both cases (100 and 280 µmol photons $m^{-2}s^{-1}$ respectively). However, it seems that the horizontal leaves at 100 µmol photons $m^{-2}s^{-1}$ may have been under greater photooxidative pressure (lower F_v/F_m) using these conditions (Ralph and Burchett, 1995). This result does not affect the utility of this assay (and has no effect on the inhibition of $\Delta F/F_m'$), but highlights the influence of irradiance on PSII with herbicides and may differ between isolated leaves and intact plants.

2.5.3. Consistency of endpoints under different irradiance

As discussed above, the sensitivity of aquatic plants to PSII herbicides can be influenced by irradiance (Runcie et al., 2009; Sharon and Beer, 2008). Photosynthetic activity increases with irradiance (light-limited phase) until saturating irradiances are reached and $\Delta F/F_m'$ values are reduced as more PSII reaction centres become inactive (photoinhibited phase) (Figure. 2.6A), and energy is dissipated by fluorescence and non-photochemical quenching (Ralph and Gademann, 2005). Diuron and other PSII inhibitors also decrease the capacity of PSII by blocking electron transport (Genty et al., 1989; Osmond et al., 1999; Rutherford and Krieger-Liszkay, 2001) and $\Delta F/F_m'$ is further reduced (Figure. 2.6A). Indeed the model of Independent Action (Figure. 2.7A) demonstrated that the effect of irradiance and Diuron on photosynthetic activity were largely additive across the conditions tested. In the present study there was little difference in IC_{50} values for $\Delta F/F_m'$ (2.2 – 3.5 µg l^{-1}) under different irradiances (when inhibition was calculated against the $\Delta F/F_m'$ of control leaves at the corresponding irradiance). This result shows that although the irradiance spanned an order of magnitude that the relative inhibition of $\Delta F/F_m'$ was not appreciably influenced, further highlighting the robust nature of this endpoint.

2.5.4. Cumulative impacts of Diuron with high irradiance

High irradiance has the potential to cause photo-oxidative damage to PSII and the rapid light curve indicated 400 µmol photons m^{-2} s^{-1} far exceeded the photosynthetic capacity of these leaves (Ralph and Gademann, 2005) and would have caused stress to the photosystems. This was demonstrated by the high level of inhibition seen in F_v/F_m by 400 µmol photons m^{-2} s^{-1} relative to control values at the lowest illumination, even at low

herbicide concentrations (Figure. 2.6B). The measured inhibition of F_v/F_m by combinations of high irradiance and Diuron was generally additive (close to the expected inhibition according to the IA model, Figure. 2.6B). However, both combinations of light and Diuron that most exceeded a 1:1 ratio (indicating a synergistic effect) were high irradiance treatments. Generally combinations of Diuron and lower light yielded combined impacts on F_v/F_m that were sub-additive. Regardless of the scale of interaction the combined impacts of Diuron and high irradiance was to increase the impact on PSII (Figure. 2.6B). This highlights the need for carefully controlled and consistent environmental conditions during future screening, particularly in relation to compliance assessment. This finding that high irradiance increases the sensitivity of seagrass to herbicides adds to the growing body of evidence that herbicides increase the vulnerability of tropical species, such as corals (Negri et al., 2011) and foraminifera (van Dam et al., 2012), to other pressures such as climate-related high sea surface temperatures. While no other multiple-stress experiments have been performed on seagrass in concert with herbicide exposure, seagrasses are vulnerable (especially photosynthetic efficiency) to changing environmental conditions including osmotic stress (Ralph, 1998), desiccation (Björk et al., 1999; Kahn and Durako, 2009) thermal stress (Collier and Waycott, 2014; Ralph, 1998) and low light (Durako et al., 2003). Herbicides could exacerbate sensitivity of seagrasses to these other environmental factors, and more studies are needed to quantify how they may be protected from climate pressures by improving water quality, and reducing herbicide residue accumulation in the coastal habitats.

2.5.5. Application of the assay

While fluorescence well-plate assays have been used widely and effectively to study the impacts of PSII herbicides on microalgae (Magnusson et al., 2012; Schreiber et al., 2007), only one previous study has attempted to apply I-PAM fluorometry to investigate herbicide toxicity in multicellular aquatic plants in a miniature well plate system. Küster and Altenburger (2007) carried out a similar toxicological experiment uisng macrophyte and macroalgae thallus samples exposed to PSII herbicides in 24-well plates over 24 h. Their results are consistent with our study in demonstrating that the utility and convenience by which macrophyte leaves can be used as relevant biomaterial for acute assasys, as long as the extent of herbicide uptake and leaf health have been assessed as appropriate and compare well with intact plants. The *H. ovalis* well-plate

assay was able to detect effects on photosynthetic capacity at concentrations lower than environmental guidelines for 90% species protection within the GBR (GBRMPA, 2010). These results closely correspond to those presented in previous acute toxicity studies of other tropical seagrass species common along the GBR (Flores et al., 2013). The high sensitivity of the well-plate assay should enable its application as a biomonitoring technique for existing and emerging PSII herbicides in natural waters (Magnusson et al., 2013). *H. ovalis* has proven to be a suitable assay species (Haynes et al., 2000b; Beer et al., 2006; Ralph, 2000), as its ovate leaves are easily isolated with minimal damage, the leaves are small and fit within a micro-assay setup and it is a ubiquitous species, occurring throughout the Indo-Pacific in coastal and estuarine habitats which are at the forefront of exposure to runoff containing herbicides. Similar assays could be developed for other large-leafed species but care should be taken to minimise injury to leaves by cutting and validation that preferential uptake of herbicide via cutting wounds is negligible. This assay for acute effects of PSII herbicides on photosynthetic capacity ($\Delta F/F_m'$) and maximum quantum yield (F_v/F_m) to PSII in seagrass is likely to be applicable to a variety of PSII herbicides under a broad range of conditions such as reduced salinity and temperature extremes. It does not rely on elaborate experimental aquarium systems to maintain potted plants and takes far less time, resources and biological material to deliver reliable ecotoxicology data. Application of this assay has the potential to provide management agencies and regulators with considerably more toxicological data for the development of improved risk assessments and water quality guidelines.

Chapter 3. Acute individual and mixture toxicity of ten photosystem-II herbicides to the seagrass *Halophila ovalis*

3.1. Abstract

Photosystem II (PSII) 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, reduce growth and trigger mortality in seagrass. However, the toxicity of many PSII herbicides found in the GBR and their mixtures to seagrasses is unknown. 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 photosynthesis by 50% (IC_{50} of effective quantum yield, $\Delta F/F_m'$) ranging from 4.3 µg l^{-1} (diuron) to 132 µg l^{-1} (fluometuron). Since all PSII herbicides have the same mode of action, their combined effects in mixtures are expected to be additive. We assessed potential additivity using the Concentration Addition (CA) model 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 $\Delta F/F_m'$.

3.2. Introduction

3.2.1. Tropical seagrass significance

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 (Kilminster et al., 2015). The estimated total area of seagrass meadows within the Great Barrier Reef (GBR) catchment area is greater than 40,000 km^2 (Coles et al., 2009), and exceeds that of coral reef (McKenzie et al., 2010; Morrissey et al., 1998). This together with their profound ecological importance highlights the significance of monitoring and protecting seagrass habitats (Kilminster et al., 2015). One of the most widespread and common species throughout tropical and subtropical regions of Australia is *Halophila ovalis* (Waycott et al., 2004), which occurs within the shallow photic zone to depths as great as 50 m (Lee Long et al., 1996). *H. ovalis* is considered a colonising species (Kilminster et al., 2015; Rasheed, 2004) with a high turnover in above-ground material (Duarte and Chiscano, 1999) and rapid re-growth when environmental conditions are again favourable (Kilminster et al., 2015). 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) (Wilkinson et al., 2015).

3.2.2. PSII herbicides and tropical marine species

Photosystem II (PSII) herbicides are applied extensively to crops and the high mobility and persistence 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 (Kroon et al., 2012). Herbicide concentrations are highest nearshore and in the vicinity of seagrass meadows (Lewis et al., 2013), which are sensitive to PSII herbicides (Flores et al., 2013; Haynes et al., 2000; Negri et al., 2015). These coastal seagrasses are also at risk from elevated turbidity from catchment and urban runoff, as well as port development (Grech et al., 2011). 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 (GBRMPA, 2010; Lewis et al., 2012; Smith et al., 2012).

PSII herbicides block electron transport in Photosystem II (PSII) of plants by binding to the D1 protein in the thylakoid membrane and displacing plastoquinone, which

in turn inhibits the synthesis of ATP and NADPH (Oettmeier, 1992). This results in reductions in carbon fixation required for growth in plants, including seagrass, and ultimately starvation and declines in community fitness (Negri et al., 2015; Ralph et al., 2007b; Wahedally et al., 2012). 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) (Ralph et al., 2007b). A reduction in $\Delta F/F_{m'}$ is directly linked to reduced photochemical efficiency (Schreiber et al., 1994). Prolonged photoinhibition from exposure to PSII herbicides can also result in whole-organism responses in microalgae (Escher et al., 2008; Magnusson et al., 2010) and corals (Cantin et al., 2007; Cantin et al., 2009).

3.2.3. Diversity of PSII herbicides and their toxicity

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 (Magnusson et al., 2010), seagrass (Chesworth et al., 2004; Flores et al., 2013; Negri et al., 2015; Wilkinson et al., 2015) and corals (Jones and Kerswell, 2003; Negri et al., 2011). 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 (summarised in Flores et al., 2013). 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 (Flores et al., 2013). The differences in potency among the PSII inhibitors is likely due to the diverse stearic and lipophilic properties of the herbicides (Haworth and Steinback, 1987) along with other differences in structural interactions between the herbicides and the binding site in the D1 protein (Oettmeier, 1992).

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 (Davis et al., 2014). Diuron and atrazine are two of the most commonly detected PSII herbicides in marine and estuarine waters (Davis et al., 2015; Kennedy et al., 2012; Lewis et al., 2012; Smith et al., 2012; Solomon et al., 1996). While there may be sufficient ecotoxicological data to derive guidelines for some PSII herbicides such as, atrazine,

ametryn, diuron, hexazinone and tebuthiuron (GBRMPA, 2010), little is known of the relative toxicity of other registered PSII herbicides, such as bromacil, prometryn, metribuzin and fluometuron, which have also been detected in the GBR or its catchments (Bainbridge et al., 2009; Davis et al., 1976; Kennedy et al., 2012; Mitchell et al., 2009; Packett et al., 2009). The toxicity of the aforementioned PSII herbicides, plus ametryn, have likely not been tested on seagrass species prior to this study (Table 3.1). In all, there are 15 PSII herbicides registered for use in catchments of the GBR (Davis et al., 2015), though many have only received significant monitoring attention in recent years and little ecotoxicological testing has been undertaken on these herbicides (Davis et al., 2014). 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.

Table 3.1. A summary of published toxicity data for the 10 PSII herbicides tested relevant to the current study.

Herbicide	Duration	Test phylum	Common name	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}	(Wilkinson et al., 2015)
	72 h	Angiospermae	Seagrass	$\Delta F/F_m'$ / F_v/F_m	2.4 – 2.47 µg l^{-1}	(Flores et al., 2013)
	5 day	Angiospermae	Seagrass	$\Delta F/F_m'$ (LOEC)	0.1 µg l^{-1}	(Haynes et al., 2000b)
	4 days	Angiospermae	Seagrass	$\Delta F/F_m'$ (LOEC)	10 µg l^{-1}	(Ralph, 2000)
	77 days	Angiospermae	Seagrass	$\Delta F/F_m'$ (IC_{50})	2.4 – 2.8 µg l-1	(Negri et al., 2015)

	34 h	Dinoflagellate	Coral	$\Delta F/F_m'$ (IC_{50})	2.9 – 5.9 µg l^{-1}	(Jones et al., 2003)
	2 – 3 mo	Dinoflagellate	Coral	$\Delta F/F_m'$ (IC_{50})	1.2 – 5.0µg l^{-1}	(van Dam et al., 2015)
	4 day	Heterokontophceae	Diatom	$\Delta F/F_m'$ (IC_{50})	2.6 – 18 µg l^{-1}	(Magnusson et al., 2010)
	4 day	Chlorophyceae	Green algae	$\Delta F/F_m'$ (IC_{50})	2.1 µg l^{-1}	(Magnusson et al., 2010)
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}	(Sikka and Pramer, 1968)
Tebuthiuron	**24 h**	**Angiospermae**	**Seagrass**	**$\Delta F/F_m'$ (IC_{50})**	**27.6 µg l^{-1}**	**Present study**
	72 h	Angiospermae	Seagrass	$\Delta F/F_m'$(IC_{50})	29.1 – 29.7 µg l^{-1}	(Flores et al., 2013)
	24 h	Dinoflagellate	Coral	$\Delta F/F_m'$ (IC_{50})	175 µg l^{-1}	(Jones and Kerswell, 2003)
	4 day	Heterokontophceae	Diatom	$\Delta F/F_m'$ (IC_{50})	51 – 94 µg l^{-1}	(Magnusson et al., 2010)
	4 day	Chlorophyceae	Green algae	$\Delta F/F_m'$ (IC_{50})	12 µg l^{-1}	(Magnusson et al., 2010)
Atrazine	**24 h**	**Angiospermae**	**Seagrass**	**$\Delta F/F_m'$ (IC_{50})**	**21.8 µg l^{-1}**	**Present study**
	72 h	Angiospermae	Seagrass	$\Delta F/F_m'$ (IC_{50})	13.4 – 18.2 µg l^{-1}	(Flores et al., 2013)
	96 h	Angiospermae	Seagrass	$\Delta F/F_m'$ (LOEC)	10 µg l^{-1}	(Ralph, 2000)
	14 d	Angiospermae	Aquatic plants	$\Delta F/F_m'$ (IC_{50})	22 – 132 µg l^{-1}	(Fairchild et al., 1998)
	18 – 36 h	Chlorophyceae	Green Algae	Respiration	80 µg l^{-1}	(Kratky and Warren, 1971)
	30 min	Chlorophyceae	Green algae	Growth (LOEC)	1 µg l^{-1}	(Behra et al., 1999)
	96 h	Chlorophyceae	Green algae	$\Delta F/F_m'$ (IC_{50})	94 – 176 µg l^{-1}	(Fairchild et al., 1998)
	24h	Chlorophyceae	Green algae	$\Delta F/F_m'$ (IC_{50})	38.8 µg l^{-1}	(Faust et al., 2001)
	2 h	Chlorophyceae	Green algae	$\Delta F/F_m'$ (IC_{50})	103 µg l^{-1}	(Muller et al., 2008)
	2 h	Heterokontophceae	Diatom	$\Delta F/F_m'$ (IC_{50})	45 µg l^{-1}	(Muller et al., 2008)
	24 h	Dinoflagellate	Coral	$\Delta F/F_m'$ (IC_{50})	45 µg l^{-1}	(Jones and Kerswell, 2003)
	34 h	Dinoflagellate	Coral	$\Delta F/F_m'$ (IC_{50})	37 – 88.2 µg l^{-1}	(Jones et al., 2003)
	4 day	Heterokontophceae	Diatom	$\Delta F/F_m'$ (IC_{50})	34 – 77 µg l^{-1}	(Magnusson et al., 2010)
	4 day	Chlorophyceae	Green algae	$\Delta F/F_m'$ (IC_{50})	14 µg l^{-1}	(Magnusson et al., 2010)
Ametryn	**48 h**	**Angiospermae**	**Seagrass**	**$\Delta F/F_m'$ (IC_{50})**	**3.6 µg l^{-1}**	**Present study**
	18 – 36 h	Chlorophyceae	Green algae	Respiration	10 µg l^{-1}	(Kratky and Warren, 1971)
	24 h	Chlorophyceae	Green algae	$\Delta F/F_m'$ (IC_{50})	3.6 µg l^{-1}	(Faust et al., 2001)
	24 h	Dinoflagellate	Coral	$\Delta F/F_m'$ (IC_{50})	1.7 µg l^{-1}	(Jones and Kerswell, 2003)
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}	(Fairchild et al., 1998)
	h - days	Chlorophyceae	Green algae	$\Delta F/F_m'$ (IC_{50})	12.3 – 39.7 µg l^{-1}	(Brock et al., 2004)
	96 h	Chlorophyceae	Green algae	$\Delta F/F_m'$ (IC_{50})	23 – 152 µg l^{-1}	(Fairchild et al., 1998)
Simazine	**24 h**	**Angiospermae**	**Seagrass**	**$\Delta F/F_m'$ (IC_{50})**	**27.8 µg l^{-1}**	**Present study**
	96 h	Angiospermae	Seagrass	$\Delta F/F_m'$ (LOEC)	10 µg l^{-1}	(Ralph, 2000)
	24h	Chlorophyceae	Green algae	$\Delta F/F_m'$ (IC_{50})	56.9 µg l^{-1}	(Faust et al., 2001)
	24 h	Dinoflagellate	Coral	$\Delta F/F_m'$ (IC_{50})	150 µg l^{-1}	(Jones and Kerswell, 2003)
	2 h	Chlorophyceae	Green algae	$\Delta F/F_m'$ (IC_{50})	76 µg l^{-1}	(Muller et al., 2008)
	2 h	Heterokontophceae	Diatom	$\Delta F/F_m'$ (IC_{50})	400 µg l^{-1}	(Muller et al., 2008)
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}	(Faust et al., 2001)
Bromacil	**24 h**	**Angiospermae**	**Seagrass**	**$\Delta F/F_m'$ (IC_{50})**	**25.2 µg l^{-1}**	**Present study**
	18-36 h	Chlorophyceae	Green algae	Respiration	90 µg l^{-1}	(Kratky and Warren, 1971)
	2 h	Phaeophyceae	Macroalgae	$\Delta F/F_m'$ (IC_{50})	8.23 µg l^{-1}	(Seery et al., 2006)
Hexazinone	**48 h**	**Angiospermae**	**Seagrass**	**$\Delta F/F_m'$ (IC_{50})**	**10.6 µg l^{-1}**	**Present study**
	72 h	Angiospermae	Seagrass	$\Delta F/F_m' / F_v/F_m$	4.4 – 6.9 µg l^{-1}	(Flores et al., 2013)
	24 h	Dinoflagellate	Coral	$\Delta F/F_m' / F_v/F_m$	8.8 µg l^{-1}	(Jones and Kerswell, 2003)
	2 h	Chlorophyceae	Green algae	$\Delta F/F_m' / F_v/F_m$	21 µg l^{-1}	(Muller et al., 2008)
	2 h	Heterokontophceae	Diatom	$\Delta F/F_m'$ (IC_{50})	22 µg l^{-1}	(Muller et al., 2008)
	4 day	Heterokontophceae	Diatom	$\Delta F/F_m'$ (IC_{50})	5.7 – 6.9 µg l^{-1}	(Magnusson et al., 2010)
	4 day	Chlorophyceae	Green algae	$\Delta F/F_m'$ (IC_{50})	2.4 µg l^{-1}	(Magnusson et al., 2010)

3.2.4. Toxicity of PSII herbicide mixtures

PSII herbicides are generally detected in complex mixtures with other PSII and/or non-PSII herbicides (Gilliom et al., 1999; Lewis et al., 2009; Shaw et al., 2010). 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 additive for a variety of freshwater (Benitez et al., 2006; Escher et al., 2008; Faust et al., 2001; Gatidou et al., 2015) and estuarine microalgae (Magnusson et al., 2010). The Concentration Addition (CA) mixture model 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 (Berenbaum, 1985; Porsbring et al., 2010). A common approach to test the applicability of CA is to apply Toxic Unit (TU) values to the herbicide concentrations that induce the same toxicity. For example, the concentration which inhibits $\Delta F/F_{m'}$ by 50% (IC_{50}) = 1 TU and is different for each herbicide (Faust et al., 2001; Magnusson et al., 2010). Concentration-response curves of herbicide mixtures containing different 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 survivability 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. Here we apply a recently developed miniature seagrass bioassay (Wilkinson et al., 2015) to assess the potency of 10 PSII herbicides both individually and in mixture compositions. This will broaden the relevant ecotoxicity data to include a range of alternative and emerging PSII herbicides and test the validity of CA of the effects of PSII herbicides to seagrass.

3.3. Materials and Methods

3.3.1. Experimental approach

A miniature bioassay (Wilkinson et al., 2015) was applied to measure acute toxicity responses of tropical seagrass, *Halophila ovalis* to a broad range of photosystem II herbicides. All herbicides were tested according to matched toxicity data set criteria (identical environmental conditions and consistent test species within the same

laboratory). Direct comparison between toxicities can be made using this approach. The effect of individual herbicides on photosynthetic performance ($\Delta F/F_{m'}$) of isolated leaves was assessed in concentration-response experiments over a 24 and/or 48 h exposure period(s) in a static system. In chronic exposures of seagrass to the PSII herbicide diuron, inhibition of $\Delta F/F_{m'}$ was strongly linked to reduced energetic status and whole-plant effects including growth and mortality (Negri et al., 2015). The miniature bioassay methodology was applied to test the toxicity of a number of mixtures (binary and complex). Each mixture contained herbicides of equal proportion (potency and volume). Maximum quantum yield (F_v/F_m) was monitored as an indicator of leaf health throughout the exposure period (See below for further details).

3.3.2. 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). 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.

3.3.3. Herbicides

Photosystem II inhibiting herbicides from four chemical classes (Table 2) 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 (Haynes et al., 2000; Kennedy et al., 2012; Lewis et al., 2009; Shaw et al., 2010; Smith et al., 2012). The herbicide diuron was included as a reference toxicant. 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) which does not affect $\Delta F/F_m'$ (Flores et al., 2013). 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 (Bandow et al., 2010; OECD, 2000). 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.

Table 3.2. Properties of herbicides tested. Water solubility calculated at >20 °C. All data from (Tomlin, 2000).

Herbicide	Chemical class	Log K_{ow}	Water solubility (mg l^{-1})	CAS number
Diuron	phenylurea	2.6	37.4	330-54-1
Fluometuron	Phenylurea	2.38	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	2.63	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	1.88	807	317-40-9
Hexazinone	triazinone	1.2	33,000	51235-04-2

3.3.4. 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 (Wilkinson et al., 2015). 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 (Ralph et al., 2007b; Wilkinson et al., 2015). 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 (Genty et al., 1989). 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).

To quantify $\Delta F/F_m'$, actinic light (100 ± 3 µE) 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.

3.3.5. Screening

A screening process was performed immediately prior to running the assays to ensure the leaves were in optimal condition for the experiment (Wilkinson et al., 2015). 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 (Wilkinson et al., 2015). Average leaf length was 10.0 mm ± 2.5 (range of all leaves) and width was 4.8 mm ± 1.2.

3.3.6. Experimental duration and leaf health

F_v/F_m was measured at 0, 12, 24 and 48 h to assess whether PSII remained intact and active (Wilkinson et al., 2015). 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 (Wilkinson et al., 2015). 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.

3.3.7. Concentration-response curves

Concentration-response curves were plotted by fitting four parameter logistic curves (SigmaPlot 11.0 and Graph Pad Prism V 6.0) to the $\Delta F/F_m'$ inhibition data obtained through averaging $\Delta F/F_m'$ measurements on nine single leaf replicates. 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} was considered different when p <0.05 and post-hoc results are presented for each comparison in the relevant results sections.

3.3.8. 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 TU concentration for each component was based on its IC_{50} (= 1 TU) at 24 h calculated from the individual assays (Table 3.3). 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 3.1).

$$TU_{sum} = C_{(1)}/IC_{50\,(1)} + C_{(2)}/IC_{50\,(2)} + \ldots + C_{(i)}/IC_{50\,(i)} \quad \text{(eq 3.1)}$$

$C_{(i)}$ 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 (Magnusson et al., 2010). 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} values were tested using the F-test in GraphPad V6.0 as previously described.

3.4. Results

3.4.1. Potencies of individual herbicides

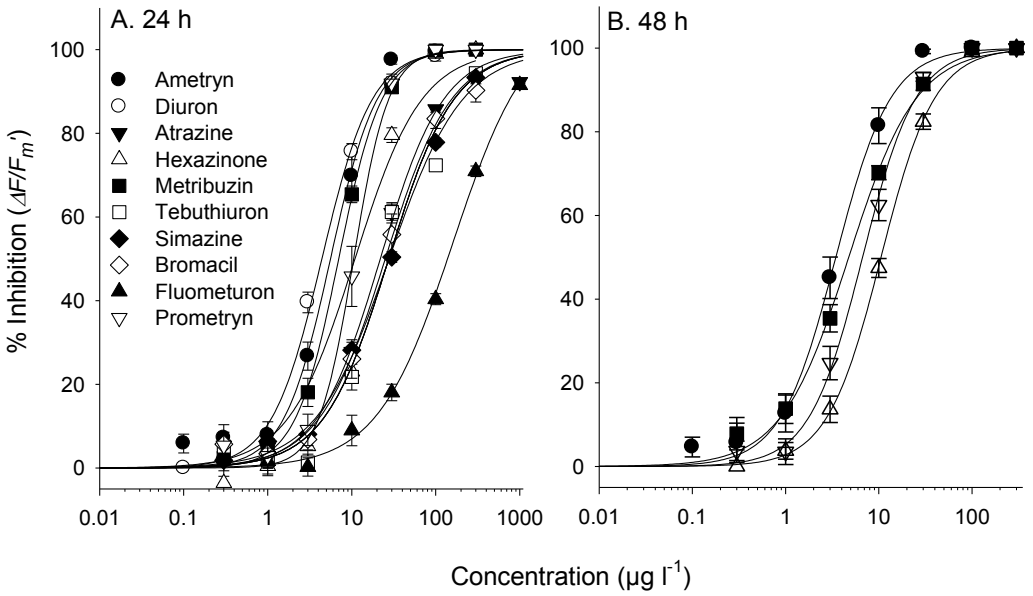


Figure 3.1. Concentration-response curves for individual herbicides. Concentration response curves for inhibition of $\Delta F/F_{m'}$ measured at (A) 24 h and (B) 48 h for 10 individual herbicides, relative to each solvent control. Bars represent SE± n=9. All experiments were conducted under 100 µmol photons $m^{-2}s^{-1}$ irradiance.

All herbicides tested inhibited $\Delta F/F_m'$ in *H. ovalis* enabling classical concentration-response relationships to be fitted (Figure. 3.1) 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 3.3. After 24 h, diuron was the most potent of the herbicides, exhibiting the lowest IC_{50} of 4.3 µg l^{-1} (Table 3.3). Fluometruon with an IC_{50} of 132 µg l^{-1} was the least potent of the herbicides tested in the 24 h assays. Range-finding experiments demonstrated that maximum inhibition of $\Delta F/F_m'$ was only reached between 24 and 48 h for ametryn, metribuzin, prometryn and hexazinone (data not shown). This additional 24 h of exposure resulted in lower IC_{50} values by between 63% and 69% (Figure. 3.1B and Table 3.2). The potencies for each of the herbicides can be compared using the Relative potencies (ReP) relative to diuron (IC_{50} diuron/IC_{50} herbicide) (Table 3.3).

Table 3.3. 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).

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	16.7	12.5 – 22.4	132	116 –150	0.99	0.033
Tebuthiuron	3.9	2.9 – 5.2	27.6	24.3 – 31.4	0.98	0.16
Atrazine	3.4	2.8 – 4.0	21.8	20.2 – 23.5	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	27.8	25.7 – 30.2	0.99	0.15
Prometryn	3.7	2.8 – 4.9	10.5	9.4 – 11.8	0.99	0.41
Bromacil	3.4	2.6 – 4.6	25.2	21.9 – 29.1	0.99	0.17
Hexazinone	6.4	5.6 – 7.3	16.7	15.5 – 17.9	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	10.6	9.7 – 11.5	0.99	0.41

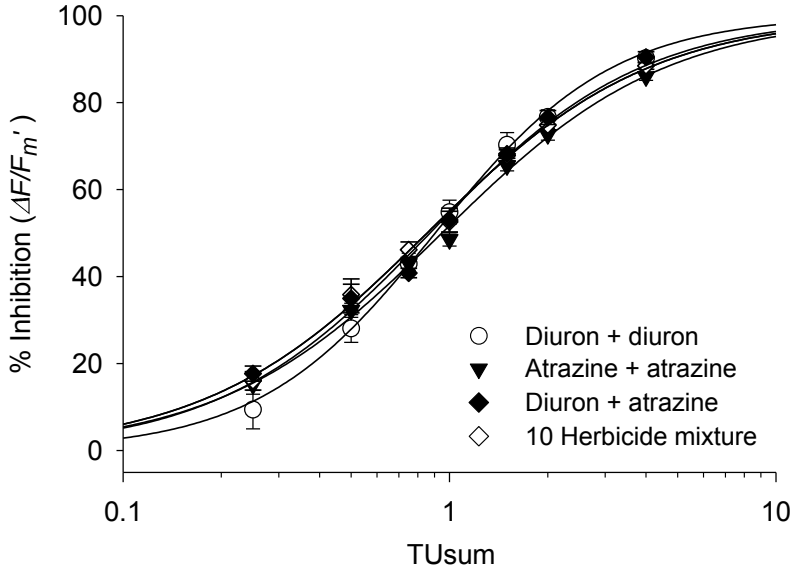


Figure 3.2. Concentration response curves for inhibition of $\Delta F/F_m'$ measured at 48 h for binary and complex herbicide mixtures, relative to each solvent control. Bars represent SE± n=9. All experiments were conducted under 100 μmol photons $m^{-2}s^{-1}$ irradiance.

3.4.2. Mixture toxicity

The response of *H. ovalis* to four mixtures tested (binary and complex) were also plotted as concentration-response curves (Figure. 3.2). The four curves largely overlapped across the range of 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}s which ranged between 0.85 TU – 0.95 TU (Table 3.4). For additivity the IC_{50}s of each of the mixtures would be expected to be close to 1 TU which was determined by the individual concentration-responses (Table 3.3). 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] were slightly but significantly lower (i.e. more potent) than the IC_{50} of [atrazine + atrazine] (Table 3.4). This indicates a possible synergistic interaction; however, there was no significant difference between the IC_{50} of the [diuron + atrazine] and the other mixtures (Table 3.4).

Table 3.4. 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.

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

3.5. Discussion

Phytotoxicity in non-target plants, such as seagrass, has been documented previously for the PSII herbicide diuron in several studies (Flores et al., 2013; Negri et al., 2015; Ralph, 2000; Wilkinson et al., 2015) 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 (Negri et al., 2015). 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 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 Chemical Addition (CA) method for application in field monitoring, water quality guideline development and application and for ecological risk assessments.

3.5.1. Herbicide potencies

The PSII herbicides demonstrated a wide range of potencies with diuron being most toxic (IC_{50} = 4.3 μg l^{-1}) and all other herbicides exhibiting IC_{50} < 30 μg l^{-1} except fluometuron which was four-fold less toxic than all other herbicides after 24 h (Table 3.3). All of these herbicides bind to the same site in the D1 protein (Oettmeier, 1992) 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 (Haworth and Steinback, 1987). 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 (Wilkinson et al., 2015). 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 accounting for less rapid impacts of ametryn, metribuzin, prometryn and hexazinone (Table 3.3). 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 (Oettmeier, 1992) such as hexazinone. The slower inhibition kinetics of hexazinone has been reported in multiple aquatic species including seagrass and microalgae (Flores et al., 2013; Magnusson et al., 2010; Muller et al., 2008). 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 3.2).

3.5.2. Sensitivity of *H. ovalis* to other PSII herbicides

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. *H. ovalis* was more sensitive to many of these PSII herbicides when compared to other species groups (Table 3.1), though with some exceptions. Atrazine inhibited *H. ovalis* $\Delta F/F_m'$ at lower concentrations (i.e. greater sensitivity) in the present study than green algae (Fairchild et al., 1998; Faust et al., 2001) or coral. *H. ovalis* was more sensitive to simazine than green algae (Faust et al., 2001), coral (Jones and Kerswell, 2003) and diatoms (Muller et al., 2008) and earlier studies found even greater sensitivity in *H. ovalis* (Ralph, 2000). Differences in toxicity among studies on the same species is likely due to specific experimental conditions including population-specific differences in sensitivity to the environment (Reusch et al., 2005), light levels (Wilkinson et al 2015) and exposure time. These discrepancies highlight the need for tightly controlled and repeatable experimental procedures in phytotoxicity studies.

3.5.3. Mixture toxicity

The concentration addition (CA) model was comparable to measured responses to herbicide mixtures. The toxic units IC_{50} for $TU_{atr+diu}$ of 0.85 was only 5.5% and 10.5% lower than either $TU_{diu+diu}$ or $TU_{atr+atr}$ (Table 3.4). A small but significant difference between the 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 CA additivity for PSII herbicides on photosynthesis with estuarine microalgae in the laboratory (Magnusson et al., 2010) and in microcosms (Knauert et al., 2008), and on cell division in the freshwater green algae *Scenedesmus acuolatus* for multiple complex mixtures of up to 18 *s*-triazines (Faust et al., 2001). While CA is an appropriate approach for calculating total toxicity in mixtures of toxins with the same mode of action (such as PSII herbicides), other approaches such as ms-PAF (de Zwart and Posthuma, 2005) should be applied to mixtures containing PSII herbicides and pesticides with other modes of action.

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 (Faust et al., 2001). CA has already been applied to compare the actual and expected additive phytotoxicity of field samples containing more than one PSII herbicide, with high levels of agreement reported (Bengtson Nash et al., 2005; Magnusson et al., 2013; Shaw et al., 2012). CA has also been used to calculate total toxicity for complex mixtures of PSII herbicides in the field towards guideline reporting and risk assessments (Kennedy et al., 2012; Lewis et al., 2012; Smith et al., 2012) and the current study further validates this approach.

3.5.4. Relevance to environmental 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 (GBRMPA, 2010) (Table 3.5). 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 3.3) provides valuable toxicity threshold data as a contribution to risk assessments, interpretation of water quality monitoring and derivation of future guidelines. For some of the herbicides, the 95% or 90% ETVs would cause greater than 10% inhibition of seagrass photosynthesis when occurring in isolation (Table 3.5). Inhibition of $\Delta F/F_m'$ is directly and quantitatively linked to inhibition of photochemical efficiency (Genty et al., 1989) and this in turn leads to reduced energy status and/or growth and mortality in seagrass following chronic PSII exposures (Negri et al., 2015). Therefore, this endpoint ($\Delta F/F_m'$) can be considered ecologically relevant as a basis from which guidelines can be developed. Moreover, the concentration of herbicide mixtures (using models such as CA based on TU_{sum}), should always be used for calculating risk and deriving guidelines rather than considering each herbicide in isolation.

Table 3.5. Comparison between IC_{10} and IC_{50} values with relevant ecological guidelines. All concentrations in µg l^{-1}. Ecotoxicity threshold values (ETVs µg l^{-1}) formulated to protect 99%, 95%, 90% and 80% of phototropic species. NA not available. ETVs in bold are not protective of photosynthesis in *H. ovalis* at the IC_{10} threshold. * indicates 48 h IC_{50} values.

Herbicide	IC_{10}	IC_{50}	ANZECC ETV 99%,95%,90% (ANZECC and ARMCANZ., 2000)	Proposed ANZECC ETV 99%,95%,90% (Smith et al., In prep)	GBRMPA ETV 99%,95%,90% (GBRMPA, 2010)
Diuron	1.2	4.3	**1.8, 1.8, 1.8**	0.08, 0.3, 0.4	0.9, **1.6, 2.3**
Fluometuron	16.7	132	NA	NA	NA
Tebuthiuron	3.9	27.6	0.02, 2.2, **20**	4.3, **8.8, 12.0**	0.02
Atrazine	3.4	21.8	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	27.8	NA	NA	0.2
Prometryn*	1.6	6.7	NA	NA	NA
Bromacil	3.4	25.2	NA	NA	NA
Hexazinone*	2.5	10.6	**75, 75, 75**	0.9, 1.2, 1.5	1.2, 1.2, 1.2

Chapter 4. Combined effects of temperature and diuron on photosystem II activity in *Halophila ovalis*

4.1. Abstract

Tropical seagrasses are at the highest risk of exposure to PSII herbicides when elevated rainfall and runoff transports herbicides into estuarine and coastal habitats in summer, coinciding with periods of elevated temperature. Temperature extremes impair photosynthetic efficiency and may lead to irreversible damage to photosystems. Therefore, the miniature bioassay developed in chapter 2 for isolated leaves of *H. ovalis* was applied to 36 combinations of temperature and PSII herbicides. Temperature treatments ranged from 15 to 40°C, and diuron concentrations from 0 to 30 µg L^{-1}. Exposure treatments lasted for 24 h prior to leaves being thoroughly washed in uncontaminated seawater and placed in 30°C (control temperature) for a 24 h recovery phase. Thermal optimum for effective quantum yield ($\Delta F/F_m$') was 28°C and maximum quantum yield (F_v/F_m) was 28°C. Diuron exposure resulted in typical concentration-response curves at all temperatures (except at 40°C). The diuron concentrations which inhibited $\Delta F/F_m$' by 50% (IC_{50}) were lowest at 30°C (Table 1), indicating greatest toxicity. However, when inhibition of $\Delta F/F_m$' was calculated relative to 30°C controls a different picture emerged. Low inhibition was observed for the 30°C and 35°C treatments at low diuron concentrations but inhibition was much higher at low herbicide concentrations for all other temperatures. Convergence of inhibition at all temperatures was observed at high diuron concentrations (10 and 30 µg l^{-1}) and 2-way ANOVA demonstrated significant interaction ($p < 0.01$) from simultaneous exposure to temperature and diuron. The majority of combined effects were considered sub-additive when the Independent Action (IA) model was applied. At higher diuron concentrations, irrespective of temperature, additive interactions were observed. Recovery of leaves was lower following exposures to high diuron and/or high temperature. This study demonstrates that interactive stressors should be considered in the development and application of water quality guidelines.

4.2. Introduction

4.2.1. Seagrass significance

Coastal seagrass meadows are important nearshore habitats globally and are located along all coasts of Australia (Kirkman, 1997). As primary producers, seagrasses form the basis of food webs and almost exclusively comprise the diet of important macro-grazers such as dugongs (*Dugong dugon*) and green sea turtles (*Chelonia mydas*). Additionally, seagrass beds act as nursery grounds for many commercial fish and crustaceans, providing protection from predators and food for juveniles (Kilminster et al., 2015; Waycott et al., 2007). In the tropics, seagrasses also aid the primary production of adjacent coral reef ecosystems through energetic and material subsidies (Heck et al. 2008). Furthermore, the extensive network of roots and rhizomes below ground facilitate sediment stabilization (Kirkman, 1997; Marbà et al., 2006) and the strap-like leaves of most seagrass species act to filter suspended organic matter from the water column (Touchette and Burkholder, 2000), improving coastal water quality.

4.2.2. Temperature stress and seagrass

The growth rates of seagrass follow seasonal trends, with the greatest growth and productivity observed during the warmest months of summer (Dunton, 1990; Orth and Moore, 1986). It has been inferred that these distinct growth patterns are regulated by a number of seasonally varying environmental conditions including water temperature (Dunton, 1996; Lee and Dunton, 1996). Furthermore, water temperature can govern species-specific geographical distribution (Collier et al., 2011; Lee et al., 2007), and future increases in temperature will result in seagrasses losses, particularly near the edge of their distribution ranges (Björk et al., 2008). In shallow habitats, water temperatures can considerably exceed daily or seasonal averages during low tide events (for a few hours), whereby water is heated beyond that of the ocean and this thermal stress may be exacerbated by high solar irradiance (Berkelmans, 2002; Massa et al., 2009; Rasheed and Unsworth, 2011). Several studies have described the effects of elevated water temperature on the photosynthetic performance of seagrass (Beer et al., 2006; Bulthuis, 1987; Collier et al., 2011; Collier and Waycott, 2014; Pearcy et al., 1977; Ralph, 1998). Photosynthetic efficiency increases with temperatures approaching the thermal optimum for photosystem

II (PSII) activity, and rapidly declines when a threshold is crossed (Bulthuis, 1987; Campbell et al., 2006; Ralph, 1998). Heat induced decline in photosynthesis is related to disruption of the stability of the chloroplast, due to the significant lability of the thylakoid membrane to heat stress (Campbell et al., 2006; Pearcy et al., 1977; Thebud and Santarius, 1982). In conjunction with membrane sensitivity, a number of sites within the photosynthetic apparatus are damaged by high temperatures. PSII, in particular, has been identified as one of the most heat-sensitive photosynthetic units in higher plants (Becker et al., 1990; Georgieva and Yordanov, 1994; Havaux, 1994). Thermally-induced photosystem damage can cause reduced oxygen evolving capacity, inhibition of PSII reaction centre function and the detachment of the light harvesting complex from the PSII reaction centre (Schreiber and Armond, 1978).

Positive primary production in seagrass is intricately linked to the balance between photosynthesis and respiration (Collier et al., 2011; Fourqurean and Zieman, 1991; Silva et al., 2009). Exposure to elevated temperatures increases respiration requirements by seagrass and (often in combination with suppressed photosynthesis) leads to reductions in photosynthesis–respiration ratios (P:R ratio) (Marsh et al., 1986; Pérez and Romero, 1992), limiting growth and eventually resulting in meadow decline (Lee et al., 2005; Marsh et al., 1986). This carbon balance can be alleviated by an increasing carbon fixation through increased irradiance-driven photosynthetic activity (Bulthuis, 1987). Optimal photosynthetic rates in tropical species likely ranges from 15 to 33 °C (Campbell et al., 2007; Collier and Waycott, 2014; Lee et al., 2007), but this estimate is based on information from just a few species and locations. While photosynthetic efficiency may be considered optimal at a specific temperature, photosynthetic carbon fixation may be sub-optimal at the same temperature due to respiratory demand (suggesting different optimal thresholds for both processes) (Bulthuis, 1983; Marsh et al., 1986). Therefore, the thermal optima for seagrass growth is generally lower than that of photosynthesis (Marsh et al., 1986). PSII activity is a suitable measure of singular impacts and interactive effects (e.g. temperature and herbicides), as reduced PSII activity and function subsequently affects plant energetics and survivability (e.g. (Collier et al., 2011; Negri et al., 2015).

4.2.3. Herbicides and seagrass

The summer monsoon season in the tropics results in frequent flood plume events and increased coastal turbidity which can exacerbate the impact of thermal stress on seagrasses in the inshore GBR lagoon (Collier and Waycott, 2014). In addition to light limitation, flood plumes in the GBR are responsible for transporting agricultural pesticides from the catchments into the lagoon (Haynes et al., 2000b; Kennedy et al., 2012; Lewis et al., 2009). The most common pesticides detected on the GBR are the photosystem II (PSII) herbicides including diuron and atrazine which occur at some inshore sites year round (Shaw et al., 2010), but in general peak in concentration during the summer monsoon season (Lewis et al., 2009). PSII herbicides bind reversibly to the D1 protein of the chloroplast and therefore directly supress electron transport in PSII (Davis et al., 1976; Jones et al., 2003). PSII herbicides are designed to inhibit function and damage PSII in terrestrial weeds but, since the D1 protein in PSII is conserved among plants, non-target species such as seagrasses and corals are equally affected (Flores et al., 2013; Negri et al., 2015; Ralph, 2000). Plant death occurs from chronic starvation (long-term electron transport rate inhibition) under moderate irradiance (Negri et al., 2015) or oxidative stress under higher irradiance (Ralph and Gademann, 2005).

Chlorophyll fluorescence methods have often been used to measure the effects of herbicides such as diuron on electron transport in PSII in seagrasses (Flores et al., 2013; Negri et al., 2015; Ralph, 2000; Wilkinson et al., 2015). In illuminated plants, photochemical quenching (transferral of electron energy, ultimately aiding ATP production) takes place when PSII reaction centres are functioning optimally. To protect against photo-oxidative damage photo-protective processes dissipate excess energy as either heat or chlorophyll *a* fluorescence (Ralph and Gademann, 2005). This fluorescence can be measured using Pulse Amplitude Modulated (PAM) fluorometry, a non-invasive technique applied to measure photochemical efficiency (Björk et al., 1999; Flores et al., 2013; Ralph et al., 2007b) and impacts on electron transport and damage to PSII can therefore be inferred. The two most common and sensitive end points which are tested regularly in photophysiology studies are effective quantum yield and maximum quantum yield (F_v/F_m) (Genty et al., 1989; Ralph et al., 2007b). In the presence of PSII herbicides, such as diuron, $\Delta F/F_m'$ inhibition indicates reduced photosynthetic efficiency (Genty et al., 1989; Ralph et al., 2007b), which can lead to a decline in stored energy and consequently seagrass mortality if exposure is persistent (Negri et al., 2015). Reduced F_v/F_m is proportional to increased photosystem damage (Genty et al., 1989; Ralph et al.,

2007b). Other studies have demonstrated PSII herbicide exposure reduces seagrass survival (Delistraty and Hershner, 1984; Gao et al., 2011), growth (Behra et al., 1999; Gao et al., 2011) and germination (Seery et al., 2006).

4.2.4. Simultaneous impacts of thermals stress and herbicides on tropical phototrophs

Herbicide concentrations peak during the summer monsoon season within the nearshore Great Barrier Reef (GBR) (Haynes et al., 2000; Lewis et al., 2011; Smith et al., 2012). This also coincides with peak summer temperatures, and temperature extremes in shallow seagrass meadows (McKenzie et al., 2015). Thus, thermal stress can occur simultaneously with herbicide exposure during the summer monsoon; however, there have been no studies examining their combined effects or potential interactions on seagrass physiology and health. Recent research indicates that the presence of PSII herbicides increases the vulnerability of symbiotic organisms such as corals (Negri et al., 2011) and foraminifera (van Dam et al., 2012) to thermal stress. Since both stressors act on the processes and/or integrity of PSII (see above), their combined effects can be readily assessed using chlorophyll fluorescence techniques, with PAM fluorometry having been used to demonstrate additive and sometimes synergistic interactions in both coral (Negri et al., 2011) and foraminifera (van Dam et al., 2012). While tropical corals and foraminifera live close to their thermal thresholds, tropical seagrasses are more thermally tolerant (Waycott et al., 2007) but far more likely to be exposed to herbicides in coastal runoff (Lewis et al., 2009). Combinations of other pressures on the photosystems of seagrass have been tested including diuron and high light intensity on the isolated leaves of the seagrass species *Halophila ovalis* (Wilkinson et al., 2015) (chapter 2). In that study I demonstrated that the simultaneous acute effects were largely additive for $\Delta F/F_m'$ and F_v/F_m according to the model of Independent Action (IA) which assumes stressors have different modes of action but impact on the same process (i.e. function of PSII) (Bliss, 1939; van Dam et al., 2012).

Here I test the hypothesis that acute exposures of the seagrass *H. ovalis* simultaneously to thermal stress and diuron affect PSII to a greater extent than either stressor in isolation. Understanding how these effects combine additively, antagonistically or synergistically will improve our understanding of whether local

management of pollution (such as herbicides) can reduce the vulnerability of seagrasses to annual thermal stress events and global change.

4.3. Methods

4.3.1. Approach

A miniature bioassay (Wilkinson et al., 2015) was applied to measure acute toxicity responses of tropical seagrass, *Halophila ovalis* to 36 combinations of diuron concentrations (0 – 30 µg l^{-1}) and temperatures (15 – 40°C). The effect of thermal stress coupled with diuron exposure on photosynthetic performance ($\Delta F/F_m'$) of isolated leaves was assessed in concentration-response experiments over a 24 h exposure period in a static system. Recovery of PSII activity (F_v/F_m) was assessed after a further 24 h at 30°C (control temperature) for all treatments following a thorough wash of leaves in uncontaminated sea water. The 24 h exposure period was applied as previous studies observed no significant decline in photosystem health of uncontaminated leaves after this period, while still allowing time for diuron concentrations to induce maximum inhibition in isolated leaves (Wilkinson et al., 2015). Potential combined effects were calculated by applying the Independent Action (IA) model to predict additive outcomes for comparison with actual measured effects.

4.3.2. Herbicides

Diuron is one of the most commonly detected PSII herbicides within the lagoon and nearshore waters of the GBR (Lewis et al., 2009; Lewis et al., 2012; Smith et al., 2012), and is a persistent contaminant in seawater with a half-life of approximately 550 days (Mercurio et al in press). Also known as DCMU or (3-(3,4-dichlorophenyl)-1,1-dimethylurea), diuron is a phenyl-urea photosystem II herbicide and was obtained from Sigma Aldrich (>95% pure). Working solutions of diuron were prepared in 0.45 µm-filtered seawater using equal volumes of ethanol carrier (<0.03% v/v). A series of five diuron concentrations (0.3, 1, 3, 10, 30 µg l^{-1}) were tested as well as a solvent control treatment (0 µg l^{-1}). Nominal concentrations are reported in this study as diuron is non-volatile and has a water solubility >40 mg l^{-1} and octanol-water coefficient (log K_{ow}) < 3 making loss to adsorption on test vessels unlikely (Bandow et al., 2010).

4.3.3. Sample collection

H. ovalis is a tropical colonising seagrass species in all marine habitats throughout Australia (Waycott et al., 2004). It is a rapidly growing species with leaf pairs emerging from the rhizome and is considered sensitive to environmental stress (Kilminster et al., 2015; Waycott et al., 2007). *H. ovalis* plants were collected haphazardly across intertidal meadows during low tide from Cockle Bay, Magnetic Island (19° 10.88'S, 146° 50.63'E) under permit MTB41, a permit issued for limited impact research in the GBR Marine Park which was assessed through the Department of Employment, Economic Development and Innovation self-assessable Fisheries Queensland Code MP05 for the removal of marine plants. A small core of seagrass with its associated sediment (5 – 10 cm depth) was removed and placed in plastic plant pots lined with plastic bags. Water was added to the bag and secured at the top to minimise loss of humidity and for transport purposes. Plants were taken to the Australian Institute of Marine Science (AIMS), Townsville, Queensland, and placed into 100 l aquaria within 4 h from collection under moderate light intensity (270 – 300 μmol photons $m^{-2}s^{-1}$) and water temperature conditions (26 ± 2°C).

4.3.4. Screening

To ensure only healthy, reliable leaves were used in the well plate experiments, cut leaves underwent a screening process (Wilkinson et al., 2015). Second and third leaf pairs from the terminal, apical end of the rhizome were selected and removed. Single leaves were placed in labelled wells of 12-well plates (Nunclon, Thermo scientific) containing 0.45 μm filtered seawater (5 ml each well). Maximum quantum yield (following 1 h dark adapted period) of each cut leaf was measured using the I-PAM and only leaves exhibiting F_v/F_m greater than 0.65 were used (Wilkinson et al., 2015). Average leaf length was 10.0 mm ± 2.5 (range of all leaves) and width was 4.8 mm ± 1.2. Each leaf was transferred to well-plates with the different diuron concentrations in the wells. For incubation purposes whole well-plates were used for specific temperatures. Therefore randomisation of temperature was unable to be applied on an individual well scale but rather a well-plate unit scale

4.3.5. Chlorophyll a fluorescence

Chlorophyll *a* fluorescence was measured on the screened leaves using an Imaging-PAM (I-PAM, Walz, Germany). Imaging-PAM (I-PAM) measurements were conducted in 12-well plates by individually placing each well plate into the imaging chamber and controlled using Data-MAXI software on a desktop computer (Imaging Win, Walz GmbH, Germany). The treatment temperatures were maintained during measurements by placing plates in a custom heating block/water bath system. Following the imaging process, Imaging Win was used to select single area of interest (AOI) of 3 – 5 mm diameter for each leaf in order to maximise the leaf surface area for fluorescence measurements. Minimum fluorescence (F with illuminated samples and F_0 with dark-adapted samples) was initiated and recorded 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 and the effective quantum yield of PSII calculated from $\Delta F/F_m' = (F_m' - F)/F_m'$. Actinic light was set to 100 ± 4 µmol photons $m^{-2}s^{-1}$ to generate a moderate level of photochemical quenching (preliminary trials indicated that a steady state $\Delta F/F_m'$ ~0.5 in control samples was reached in < 8 min). Effective quantum yield reflects the level of PSII activity under ambient (actinic) light conditions and is proportional to photosynthetic efficiency (Genty et al., 1989). $\Delta F/F_m'$ provides the most sensitive assessment of PSII herbicide impacts on photosynthesis at a given light intensity (Ralph et al., 2007b). In order to calculate the maximum quantum yield of PSII (F_v/F_m), seagrass was dark adapted for 30 min and F_0 and F_m measured, as above, from $F_v/F_m = (F_m - F_0) / F_m$. F_v/F_m is a measure of the optimal photosynthetic efficiency and inhibition of F_v/F_m can indicate photo-oxidative stress and damage to PSII from irradiance or thermal stress (Genty et al., 1989).

4.3.6. Temperature and diuron co-exposure experiments

Bioassays were conducted in incubators across a range of six temperatures (15 – 40°C ± 0.5 °C) with 5°C increments and at a light intensity of 100 ± 4 µmol photons $m^{-2}s^{-1}$ over a 14:10 h diurnal cycle. Incubators were full enclosed units with the ability to

house up to 12 well-plates. Each incubator contained a LED light source. The specified light intensity was chosen from previous validation conditions of the miniature bioassay (chapter 2). Previous studies (Campbell et al., 2006; Collier et al., 2011; Ralph, 1998) as well as preliminary experiments were also conducted to range-find experimental temperature treatments (data not shown) Results of preliminary studies and previous findings (Collier et al., 2011)These led to 30°C being selected as the 'control' temperature for calculations of inhibition because were generally highest at this temperature. Experimental findings further confirmed that 30°C approximated the optimum temperature for $\Delta F/F_{m'}$ and F_v/F_m (described further in results). Prior to applying the experimental treatments, isolated leaves were pre-screened ($\Delta F/F_{m'}$) at 30°C and then they were returned to dark conditions for 30 min before conducting F_v/F_m measurements in uncontaminated conditions to measure initial health of photosystems of each isolated leaf.

Isolated leaves were then transferred into well-plates containing diuron-treated contaminated seawater (See Herbicides section). Each diuron concentration was represented by 9 single leaf replicates randomly distributed across a set of nine well plates (per temperature treatment). The well plates were then transferred into incubators at each temperature (diuron exposures and temperature exposures were staggered by 50 min to enable enough time for measurement of quantum yields after fixed periods). $\Delta F/F_m'$ measurements were taken at 24 h after the exposure by transferring well plates onto a heating block maintained under the I-PAM at each treatment temperature. Eight minute actinic light acclimation was carried out for each plate prior to the recording of $\Delta F/F_m'$ (see above). Samples were then dark adapted in respective incubators (which could have lights switched off independently of other temperature treatments) for 30 min and F_v/F_m measurements were recorded. After 24 h exposures to both temperature and diuron, all leaves were washed in large volumes of uncontaminated seawater and finally placed back into 12-well recovery plates in 30°C incubators under the same illumination as described above. After a further 24 h, the recovery $\Delta F/F_m'$ and F_v/F_m measurements were recorded as described above.

4.3.7. Data analysis

Temperature only effects to $\Delta F/F_m'$ and F_v/F_m were calculated from solvent control leaf samples following 24 h exposure and a further 24 h recovery period. A three-

parameter, log normal peak curve was fitted to the data. Inhibition of quantum yields (% relative to solvent control) were calculated (Eq. 4.1) from treatment data. Inhibition was separately calculated relative to two temperatures: (i) the solvent control at the relevant treatment temperature and (ii) the solvent control at 30°C. Concentration-response curves were fitted using four-parameter logistic curves in GraphPad Prism v6 (San Diego, USA) using inhibition data at 24 h and after an additional 24 hours recovery in uncontaminated water at 30°C. Diuron 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 in GraphPad Prism. The probability that midpoints (IC_{50}) generated by the logistic curves were statistically different was tested by applying the F-test in GraphPad Prism. IC_{50} values were considered different when $p < 0.05$. The Tukey's post-hoc results are presented for each comparison in the relevant results sections.

$$\text{Inhibition [\%]} = 100 - ((100/Y_{control}) * Y_{sample}) \quad \text{(Eq 4.1)}$$

Where $Y_{control}$ is $\Delta F/F_m'$ or F_v/F_m of the solvent control and Y_{sample} is the $\Delta F/F_m'$ or F_v/F_m of the treatment samples.

Predicted additive inhibition of $\Delta F/F_m'$ was calculated by applying the Independent Action (IA) equation (Eq. 4.2) to the data (Bliss, 1939; van Dam et al., 2012). The predicted inhibiton for additive responses from the IA model were plotted against the actual inhibition data for each combination of diuron and temperature relative to the 30 °C treatment in uncontaminated water (solvent control).

$$P(T,D)_p = P(T) + P(D) - P(T) * P(D) \quad \text{(Eq 4.2)}$$

Where $P(T,D)_p$ is the predicted additive effect of both variables tested; P(T) is the effect of temperature in the absence of diuron and P(D) is the effect of diuron at the control temperature, 30°C. Both P(L) and P(T) are derived from raw data means. If the experimental data falls on the 1:1 line (observed:predicted) then the combined effect of tempeature is considered additive. If the experimental data falls above the line, the effect is synergystic and if the data falls below the line the data is antagonistic or sub-additive. The combined effects of temperature and diuron on inhibition of $\Delta F/F_m'$ was also tested uing a 2-way ANOVA on arcsin transfomed % inhibiton data (NCSS V7, Kaysville,

USA). Contour plots were used to visually represent the effects of the 36 temperature and diuron combinations on both $\Delta F/F_m'$, and F_v/F_m.

4.4 Results

Temperature alone had a strong effect on effective quantum yield ($\Delta F/F_m'$) after 24 h exposures, with $\Delta F/F_m'$ data following a parabolic pattern ($r^2 = 0.91$, maximum $\Delta F/F_m'$ at 28°C). The fitted maximum of $\Delta F/F_m'$ was 0.47 at 28°C. The plotted $\Delta F/F_m'$ of control (30°C) was 0.50, dropping to 0.18 at 15°C and 0.31 at 40°C. After 24 h recovery in uncontaminated water at 30°C, all $\Delta F/F_m'$ recovered to > 0.45 apart from photosystems exposed to the two highest temperatures. A similar relationship was observed for F_v/F_m across the temperature range after 24 h exposure ($r^2 = 0.99$, maximum F_v/F_m at 27.2°C). The 30°C control temperature recorded F_v/F_m of 0.75 while the maximum (fitted) of 0.75, was also at 27°C, dropping to 0.59 at 15°C and 0.66 at 40°C. After 24 h recovery period at 30°C all treatment F_v/F_m (including two extremes) recovered to > 0.65.

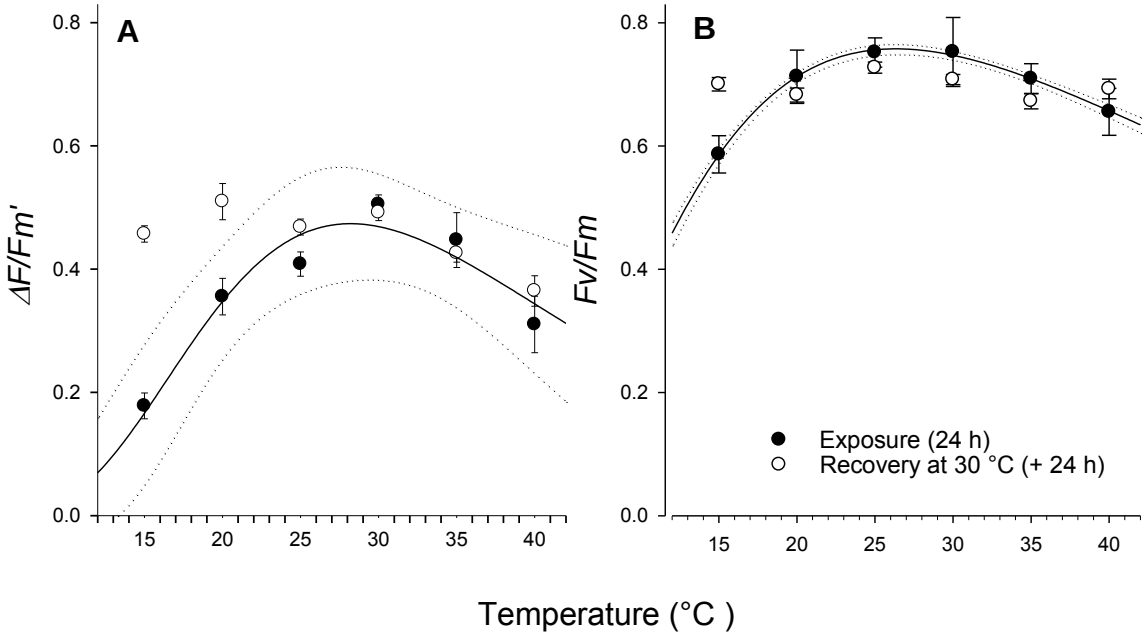


Figure 4.1. Effect of temperature on A) $\Delta F/F_m'$ and B) F_v/F_m of *H. ovalis*. Solvent control yield at six different temperature treatments following 24 h exposure and 24 h recovery at 30 °C. All

$\Delta F/F_m'$ measurements were conducted under 100 µmol photons $m^{-2}s^{-1}$ irradiance. Mean ± SE of nine replicate leaf samples. Dashed lines represent ± 95% CI.

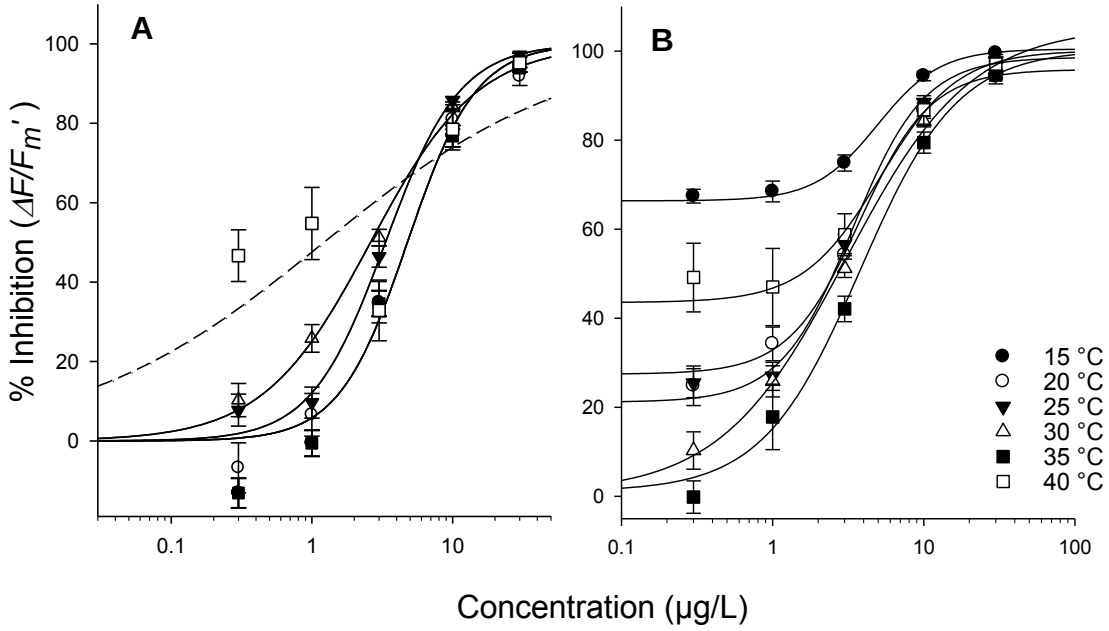


Figure 4.2. Concentration-response curves ($\Delta F/F_m'$) for diuron and *H. ovalis* at six different temperatures. (A) $\Delta F/F_m'$ inhibition relative to solvent controls at the respective temperatures (B) $\Delta F/F_m'$ inhibition relative to 30 °C solvent control. All $\Delta F/F_m'$ measurements were conducted under 100 µmol photons $m^{-2}s^{-1}$ irradiance. Mean ± SE of nine replicate leaf samples.

Classic concentration-response curves for inhibition of $\Delta F/F_m'$ in PSII by diuron were observed at each temperature, except at 40°C (Figure. 4.2). In Figure 4.2A the inhibition of $\Delta F/F_m'$ was calculated relative to the solvent controls at each exposure temperature (e.g. the $\Delta F/F_m'$ values in Figure. 4.1A). While these curves often overlapped, the diuron concentrations (plotted) which inhibited $\Delta F/F_m'$ by 50% (IC_{50}) were lowest at 30°C (Table 4.1). Post-hoc analysis of the F-test (F value = $5.270_{(5, 301)}$, $p < 0.05$) revealed significantly different IC_{50} values for all temperature treatments in comparison with 30°C, except 40°C which had a wide confidence interval range.

Table 4.1. Phytotoxicity inhibition endpoints for individual temperature treatments exposed to a range of diuron concentrations after 24 h exposure presented in figure 2A. Inhibition of $\Delta F/F_m'$ IC_{10} and IC_{50} data (µg l^{-1}) with 95 % confidence intervals. Different letters in superscript indicate significant differences in IC_{50} ($p < 0.05$).

Temperature (°C)	IC_{10}	95 % CI	IC_{50}	95 % CI	R^2
15	1.4	0.9 – 2.3	4.5^{a}	3.5 - 5.7	0.87
20	1.2	0.8 – 1.9	$4.4^{a,b}$	3.5 - 5.5	0.86
25	0.9	0.7 – 1.2	3.3^{c}	2.9 - 3.8	0.94
30	0.4	0.3 – 0.5	2.6^{d}	2.3 - 3.0	0.95
35	1.2	0.7 - 2.0	$4.6^{a,b}$	3.6 - 5.9	0.93
40	0.8	0.3 – 2.3	$4.3^{a,b,c,d}$	2.7 - 6.9	0.56

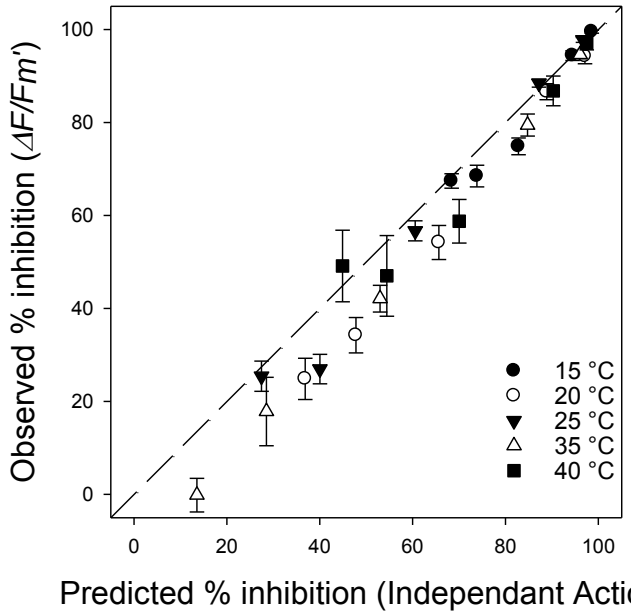
When inhibition of $\Delta F/F_m'$ was calculated relative to 30°C control $\Delta F/F_m'$ a different picture emerged (Figure. 4.2B). Low inhibition was observed for the 30°C and 35°C treatments at low diuron concentrations but inhibition was much higher at low herbicide concentrations for all other temperatures (Figure. 4.2B). Convergence of inhibition was observed at high diuron concentrations (10 and 30 µg l^{-1}). A 2-way ANOVA analysis of this data revealed significant effects of temperature and diuron independently and a strong interaction between temperature and diuron (Table 4.2). This interaction indicated the combined impacts of temperature and diuron on inhibition of $\Delta F/F_m'$ was not additive.

Table 4.2. Results from 2-way ANOVA on the effective quantum yields, $\Delta F/F_m'$ of *H. ovalis* exposed to varying concentrations of diuron at different temperatures after 24 h.

Factor	DF	SS	F	p
Temperature	5	5.372	60.75	< 0.01*
Diuron	5	31.12	351.9	< 0.01*
Temp x Diuron	25	1.789	4.05	< 0.01*
Error	323	43.37		

Figure 4.3. Comparison between predicted (Independent Action) and observed inhibition of $\Delta F/F_m'$ in response to combinations of temperature and diuron. Data points intersecting the zero-interaction line (1:1) indicate additivity; points below the additivity line suggest sub-additivity; and data points above the additivity line indicate synergism. All inhibition calculated relative to 30°C solvent control mean. Mean ± SE of nine replicate leaves.

A plot of $\Delta F/F_m'$ inhibition expected for additivity (according to the IA model) vs



observed inhibition indicated that many of the data points lay below the 1:1 line expected for additive impacts (Figure. 4.3). This graph demonstrates that the interaction between temperature and diuron is sub-additive or antagonistic for most of the combinations of stressors at low inhibition levels. At high inhibition levels above 80% most of the data lies on the 1:1 line, indicating additivity (see also convergence in Figure. 4.4).

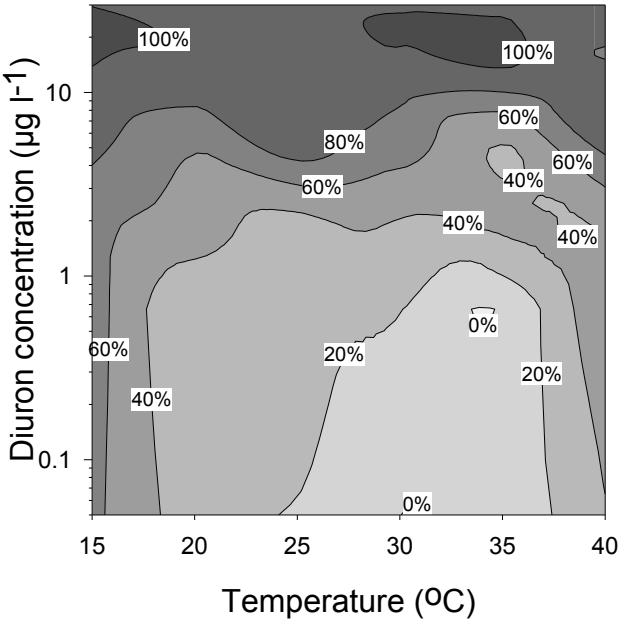


Figure 4.4. Contour plot for the relative $\Delta F/F_m'$ inhibition of *H. ovalis* in response to 36 combinations of temperature and diuron after 24 h. Data is plotted as contours (% inhibition relative to 30°C) with shading gradient to separate distinct % inhibition outcomes relative to inhibition.

A contour plot of $\Delta F/F_m'$ inhibition in *H. ovalis* for all combinations of temperature and diuron over 24 h revealed the strong influences of both stressors on photosynthetic efficiency (Figure. 4.4). The effect of diuron was lowest at temperatures closest to the plotted thermal optimum of 30°C, while diuron caused greater impacts on photosynthetic efficiency at the temperature extremes.

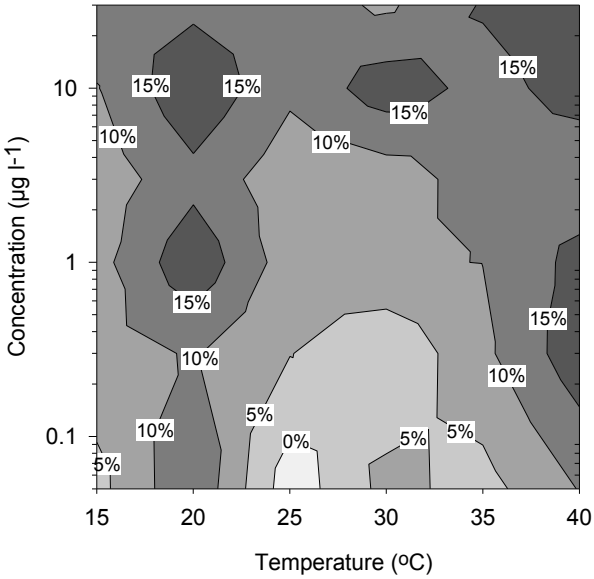


Figure 4.5. Contour plot of inhibition of F_v/F_m following 24 h exposure to 36 combinations of temperature and diuron and a subsequent 24 h recovery period. Inhibition was calculated as the % difference in F_v/F_m measured at the end of the 48 h experiment with h[illegible]ge to PSII following the 24 h exposure period and the additional 24 h recovery period.

Recovery of PSII function in the leaves following exposure to 36 combinations of temperature and diuron were assessed by plotting a contour curve of the % difference in F_v/F_m in each leaf from just prior to the exposures and at the end of the 48 h experiment (Figure. 4.5). This graph revealed that recovery was mainly driven by temperature with poorest recovery observed at ~20°C and at > 35°C for all herbicide concentrations (high % inhibition contours). The strongest recovery was observed at the thermal optimum between 25 and 30°C (low % inhibition contours), although this recovery was reduced at higher diuron concentrations.

4.5. Discussion

This study represents the first investigation into the combined acute effects of simultaneous temperature and herbicide pressures on the photosystem of seagrass. The well-plate approach enabled the effects of 36 combinations of these stressors to be assessed across a 25°C temperature range. The thermal optimum for photosynthetic efficiency ($\Delta F/F_m'$) in *H. ovalis* was ~28°C (fitted) while low and high temperatures

inhibited $\Delta F/F_m'$ as did all elevated concentrations of diuron. There were significant interactions between the effects of temperature and diuron, with a majority of the combinations of these stressors causing sub-additive (antagonistic) effects. While the combined stress was generally less than additive, photosystems were more sensitive to diuron at temperatures outside the expected natural range of 20 – 35°C.

4.5.1. Temperature effects on photosystem II processes

The geographic distribution of seagrasses is influenced by species-specific thermal optima; with long-term survival of tropical seagrass species generally occurring at a thermal range from 15°C to 33°C (Berry and Bjorkman, 1980; Collier and Waycott, 2014; Masini and Manning, 1997). Here the thermal optimum for photosynthetic performance of PSII in *H. ovalis* (maximum $\Delta F/F_m'$ and F_v/F_m at 28 and 27°C respectively, Figure. 4.1) was consistent with the recent thermal history of the plants which were collected and cultured at 26 ± 2 °C. Reduced activity in PSII ($\Delta F/F_m'$ and F_v/F_m) as well as photochemical and non-photochemical quenching outside the normal thermal range in temperate *H. ovalis* were also reported over 4 d exposures (Ralph, 1998), and in repeated 4 h exposures to ≥ 35°C in three tropical Australian species, including *H. ovalis* (Campbell et al., 2006). Campbell et al. (2006) also demonstrated that four other species, which have distribution more restricted to warmer sub-tropical to tropical waters, were more tolerant to thermal stress. *H. ovalis*, with a distribution from temperate to tropical regions is likely to live close to its thermal tolerance in the tropics and intertidal plants may therefore be more vulnerable to spikes in during summer low-tide events where water temperatures can increase by 10°C over short periods to 40°C (Collier and Waycott, 2014; McKenzie and Campbell, 2004). High irradiance, partial desiccation and herbicide exposure may combine with periodic (summer/tidal) or chronic (global change) thermal stress to impact on seagrass meadows (Seddon and Cheshire, 2000; Waycott et al., 2006).

The sensitivity of photosynthesis to thermal stress is likely due to the heat liable nature of the photosystem II apparatus (Berry and Bjorkman, 1980; Rokka et al., 2000; Weis and Berry, 1987). Impacts may include protein denaturing (Bruggemann et al., 1992; Lee et al., 2007; Prasil et al., 1992), alteration of thylakoid membrane conformation (Pearcy et al., 1977; Ralph, 1998) and disassociation of the light harvesting complex from

PSII (Schreiber and Armond, 1978). PSII may therefore experience heat-stress prior to many other indicators (Massa et al., 2009). Impacts on photosynthetic processes in turn disrupt carbon balance, where increased respiration, relative to photosynthetic output results in reduced carbon production (Bulthuis and Woelkerling, 1983; Marsh et al., 1986; Masini and Manning, 1997; Pérez and Romero, 1992; Ralph, 1998). Temperatures around 40°C may represent important thermal thresholds as reduced photosynthetic capacity and limited recovery suggests irreversible damage to photosystems (Campbell et al., 2006; Ralph, 1998) .

Recovery of $\Delta F/F_m'$ in *H. ovalis* (>0.4) following exposure across a 25°C range in uncontaminated seawater was generally consistent across the temperature range, but was lowest for leaves exposed to 40°C (< 0.4) (Figure. 4.1). In all other treatments strong recovery of quantum yields indicate little irreversible damage, and this is especially notable for the lowest temperature of 15°C which caused the greatest reductions in $\Delta F/F_m'$ and F_v/F_m over 24 h but recovered photosynthetic capacity after a further 24 h at 30°C (Figure. 4.1). Less is known about chilling effects on PSII in seagrass; however Ralph (1998) demonstrated similar results with temperate *H. ovalis* exposed to low temperatures of 10 and 12.5°C. In that study, steady F_o in *H. ovalis* under chilling conditions may have been associated with thermal deactivation of PSII reaction centres. Longer studies that acclimate seagrass to these lower temperatures are required to determine whether this effect on photophysiology is likely to be encountered in the field.

4.5.2. Effects of diuron alone on photosystem II processes

Diuron inhibited $\Delta F/F_m'$ in *H. ovalis* by 50% at 2.6 µg l^{-1} at the control temperature of 30°C (Figure, 2A, Table 1). This result was consistent with previous studies examining effects on isolated leaves of *H. ovalis* (3.5 µg l^{-1}) (Wilkinson et al., 2015) and potted *H. ovalis* (3.0 µg l^{-1}) , *H. uninervis* (2.4 – 2.8 µg l-1) and *Z. muelleri* (2.4 – 2.5 µg l^{-1}) (Flores et al., 2013; Negri et al., 2015). Inhibition of $\Delta F/F_m'$ by PSII herbicides including diuron is one of the most sensitive indicators of these herbicides on seagrasses and other plants as the change in fluorescence signal (increase in F_o) represents a measure of the closure of the electron transport mechanism by the binding of herbicide molecules to the D1 protein (Oettmeier, 1992; Ralph et al., 2007b). The 24 h exposures to diuron here: (i) enabled maximum uptake through the leaves and impact on PSII

(Wilkinson et al., 2015) (ii) allowed enough time to measure impacts of thermal stress (Figure. 1) (iii) and was short enough so to allow for an additional set of recovery measurement to be made at 48 h without leaf and PSII deterioration (Figure. 4.1). While the effects of this short exposure to diuron caused the maximum impact on $\Delta F/F_m'$, the toxicity of diuron (IC_{50}) remained consistent over much longer exposures of 11 weeks (Negri et al., 2015), flow-on effects caused by reduced photosynthetic capacity including reduced carbon storage in the root-rhizome, reduced growth, and survival are only observed following chronic exposures (Negri et al., 2015; Seery et al., 2006). The inhibition of $\Delta F/F_m'$ by 10% at 0.4 µg l^{-1} represents an environmentally relevant scenario (Lewis et al., 2009) and the influence of co-stressors such as temperatures outside the normal thermal range of *H. ovails* (see above) may influence this sensitivity.

4.5.3. Interactive effects of simultaneous temperature and diuron exposure

The combined effects of temperature and diuron in 36 combinations over 24 h on the performance of PSII in *H. ovalis* was assessed using a variety of methods. From an ecotoxicological perspective the effects of temperature on the concentration-response curves demonstrated a small but significant shift in sensitivity when inhibition of $\Delta F/F_m'$ was calculated by applying controls (uncontaminated seawater) for each curve at each treatment temperature (Figure. 4.2A, Table 4.1). Using this method diuron at 30°C inhibited $\Delta F/F_m'$ at a lower concentrations (IC_{50} = 2.6 µg l^{-1}) than at most other temperatures. Leaves exposed to the more extreme temperatures of 15 and 35°C exhibited IC_{50}s of 4.5 and 4.6 µg l^{-1} respectively, relative to controls at each of those temperatures (Figure. 4.2A, Table 4.1). This result indicates relatively consistent effects of diuron at each temperature; however this comparison masks the combined impacts of thermal stress and herbicide exposure as results are normalised relative to controls at each temperature. The combined effects of thermal stress and diuron exposure on $\Delta F/F_m'$ are more effectively represented in Figure. 4.2B, where the inhibition is calculated relative to that measured in leaves in uncontaminated water at 30°C (which exhibited the highest $\Delta F/F_m'$ of all controls, Figure. 4.1A). When assessed against controls at 30°C, inhibition of $\Delta F/F_m'$ by temperature extremes was evident for low diuron concentrations and, as the diuron concentration rose, this inhibition further increased (Figure. 4.2B). The concentration-response curves were not parallel, and this convergence demonstrates

interactions between temperature and diuron on $\Delta F/F_m'$. This was confirmed by the 2-way ANOVA revealing strong interactions ($p < 0.01$) between these stressors (Table 4.2). A comparison of the observed against the predicted effects of combinations of temperature and diuron according to the independent action equation revealed most of the combinations caused less inhibition of $\Delta F/F_m'$ than would be expected if the responses were additive (Figure. 4.3). This represents an antagonistic interaction across most of the stressor combinations, but since stressful temperatures and diuron exposure both caused negative responses and the sum of the responses was generally greater than that caused by temperature or diuron alone (Figure. 4.3), we consider "sub-additivity" to be a more appropriate description for this interaction (some other forms of antagonism can reverse or cancel individual effects) (Crain et al., 2008). The parabolic nature of the contour plot (Figure. 4.4) demonstrated well how combinations of temperature extremes and herbicide exposure cause the greatest inactivation of PSII. Previously the effects of the herbicide diuron and thermal stress has been shown to cause additive effects on $\Delta F/F_m'$ in symbiotic corals (Negri et al., 2015) and their isolated symbionts (van Dam et al., 2015) and in foraminifera (van Dam et al., 2012). Although outcomes for PSII activity in seagrass was also worse for combinations of temperature stress and herbicide exposure, the sub-additivity reported here may reflect a greater flexibility in PSII function across a wider thermal range as seagrasses are generally considered far more tolerant of thermal stress than corals and foraminifera (Negri et al., 2011; van Dam et al., 2012). To assess whether the photosystems remained intact after the exposure, the maximum quantum yields (F_v/F_m, a direct measure of photosystem health) were taken in uncontaminated water at 30°C prior to the 24 h temperature and diuron exposures and again following recovery in uncontaminated water for a further 24 h. The % inhibition of F_v/F_m over this period was generally less than 20% but was elevated at both temperature extremes (Figure. 4.5). The recovery of PSII in leaves exposed to higher herbicide concentrations near the thermal maximum was not as great as those exposed to less diuron, indicating potential chronic damage to PSII at diuron concentrations above 3 $\mu g\ l^{-1}$. However, in general, temperature had a greater influence on the recovery of function in PSII.

Physical conditions such as temperature affect membrane characteristics (including binding site configuration and conformation) and rates of diffusion (Holmstrup et al., 2010; Jones and Kerswell, 2003). As a result, sub-optimal temperatures (both low and high) may cause reduced binding efficiency of diuron and thus change sensitivity to

PSII herbicides. From another perspective, the presence of diuron (or other PSII herbicides) may narrow the optimal temperature range for seagrass, resulting in stress responses at lower maximum temperatures, as reported for corals and foraminifera (Negri et al., 2011; van Dam et al., 2012; van Dam et al., 2015). Throughout the GBR lagoon and catchment area seasonally high sea surface temperatures coincide with the monsoonal flood plume events and the highest herbicide concentrations are detected near seagrass habitats under these conditions (Lewis et al., 2009). The potential for inshore and estuarine seagrasses to simultaneously experience thermal stress and herbicides (as well as irradiance and osmotic stress) are high during this period and while this is recognised by management agencies and regulators (Batley et al., 2013; GBRMPA, 2010), more work like the current study is needed to quantify these interactions. This will enable more effective assessment of risks posed by these stressors to seagrass meadows and will enable adaptation of future water quality guidelines to fully protect tropical species.

Chapter 5. Thesis discussion

5.1. General discussion

Photosystem-II (PSII) herbicides transported from adjacent agricultural land during frequent flood plume events occur in concentrations that can directly impact inshore seagrass communities (Devlin and Schaffelke, 2009; Devlin et al., 2012; King et al., 2012; Lewis et al., 2011). Although concentrations reach a peak during the monsoon season (Lewis et al., 2009), long persistence (Mercurio et al., 2015) explains the presence of such contaminants in the marine environment year round (Shaw et al., 2010). PSII within higher plants including seagrass species is the target site of action for all PSII herbicides (Oettmeier, 1992) and chronic exposures which impact seagrass photosynthesis can cause further impacts on growth and survivability (Negri et al 2015). The hypotheses for this thesis were: i) Seagrass phytotoxicity bioassay for PSII herbicides based on isolated leaves can provide a rapid miniature alternative to whole-plant assays to examine the relative potencies of these herbicides. Experiments in Chapter 2 demonstrated that results from the miniature assay matched those for potted plants and thus represents a valid alternative to measuring PSII herbicide effects on seagrass (Figure. 2.3, Table. 2.1); ii) The effects of multiple PSII herbicides in mixtures on PSII activity in seagrass are additive. Experiments in Chapter 3 demonstrated no interaction between herbicides on photosynthetic activity confirming additivity, (Figure. 3.2, Table. 3.4). iii) Combinations of PSII herbicides and other stressors, including irradiance and extreme temperatures, will interact to impact on PSII activity in seagrass. Experiments in Chapter 2 demonstrated combinations of PSII herbicides and light intensity was additive (Figure. 2.7, Table. 2.2), while in Chapter 4 herbicides in combination with extreme temperatures had interactive effects on photosystem function (Figure. 4.3, Table. 4.2).

A miniature bioassay technique applying Pulse Amplitude Modulated (PAM) fluorometry was successfully developed for the seagrass *Halophila ovalis* (Chapter 2). The validated methodology was then applied to measure the toxicity of 10 PSII herbicides registered for use in catchments of the Great Barrier Reef (Chapter 3). Five of these herbicides were tested on seagrass for the first time during the work outlined in this thesis. Finally, the established bioassay was applied to measure potential combined impacts of the potent PSII herbicide diuron and thermal stress (Chapter 4).

5.2. Miniature bioassay development

The miniature bioassay methodology was successfully implemented as a convenient and rapid alternative to traditional ecotoxicological methods which generally require far more aquarium apparatus, potted plants and large volumes of contaminants (Flores et al., 2013). Success was demonstrated through i) Consistency in toxicity among sample arrangements (Chapter 2); and ii) Repeatability and consistency of the assay in subsequent studies (Chapters 3 and 4). Under standard environmental conditions (26 ± 2°C and 100 µmol photons $m^{-2}s^{-1}$) diuron toxicity (IC_{50}) ranged from 3.5 – 4.3 µg l^{-1} (Table 5.1). This indicates consistent sensitivity of isolated *H. ovalis* samples to diuron when exposed in the miniature bioassay. Repeatability is essential if these ecotoxicological data are to be applied for comparisons between species and herbicides and to the development of water quality guidelines and (GBRMPA, 2010; Warne et al., 2014). These data were also consistent with toxicity to other seagrass species in potted systems (Table 5.1), including toxicity over a broad range of experimental periods. For example Negri et al. (2015) measured a 50% inhibition of $\Delta F/F_m'$ in different species at concentrations (IC_{50}) ranging from 2.4 – 2.8 µg l^{-1} for diuron exposures of 3 – 7 d. The current thesis further establishes inhibition of $\Delta F/F_m'$ as a reliable and consistent indicator of PSII herbicide toxicity among diverse species groups including seagrass (Flores et al., 2013; Negri et al., 2015), coral symbionts (Negri et al., 2011) and marine microalgae (Magnusson et al., 2010).

Table 5.1. Diuron toxicity measured in studies included in this thesis as well as results from relevant studies conducted with different species.

Duration	Test species	IC_{50} (µg l^{-1})	Reference
24 h	*H. ovalis*	3.5	This Thesis (Chapter 2)
24 h	*H. ovalis*	4.3	This Thesis (Chapter 3)
24 h	*H. ovalis*	3.9	Herbicide mixture control, This Thesis (Chapter 3)
48 h	*H. ovalis*	2.6	30°C treatment, This Thesis (Chapter 4)
72 h	*H. uninervis*	2.4	(Flores et al., 2013)
72 h	*Z. muelleri*	2.5	(Flores et al., 2013)
77 d	*H. uninervis*	2.8	(Negri et al., 2015)
77 d	*Z. muelleri*	2.4	(Negri et al., 2015)
24 h	*A. millepora*	2.9	(Negri et al., 2011)
4 d	*Nevicula sp*	2.6	(Magnusson et al., 2010)
4 d	*P. tricornutum*	2.7	(Magnusson et al., 2010)

Through the development and validation process (Chapter 2), uptake of diuron across the isolated leaf surface was observed with no additional uptake via vascular system flooding. The ability to capture 2D fluorescence images using the Imaging-PAM (I-PAM), allowing uptake to be visualised, provides further confidence in the application of the miniature bioassay as an experimental approach for use in future research. Exposing single, isolated leaves in separate wells allowed for rapid measurement of applied endpoints, and was further aided by the semi-automatic data recording capability of the imaging-PAM technology. Rapidity enables the generation of large datasets in relatively short periods of time which would be advantageous for *in vitro* application. The miniature bioassay was designed and implemented with the aim to provide a universal technique for testing toxicity traits of PSII inhibiting contaminants. Minimum space is required to carry out experiments due to the small size of the apparatus, allowing the assay to be applied to situations which may require relatively rapid ecotoxicological testing in a limited area (such as on-board a research vessel out in the field). The non-destructive and non-invasive technique of PAM fluorometry, coupled with medium throughput exposure systems is an ideal partnership for measuring acute PSII effects on ecologically relevant species and endpoints (Ralph, 1998).

5.3. Application to other PSII herbicides and PSII herbicide mixtures

A total of 15 photosystem-II herbicides are registered for agricultural application in areas adjacent to the Great Barrier Reef (Davis et al., 2015), and 10 of these were tested for their toxicity to *H. ovalis* in Chapter 3 (Table 5.2). Five of these are commonly detected and are considered priority contaminants; and therefore, receive the most attention in ecotoxicological studies. However, there are a number of emerging herbicides which are of comparable potency and remain understudied. For instance, half of the PSII herbicides tested throughout Chapter 3 of this thesis had not previously been applied to seagrass and the results in Chapter 3 provide the first data for direct seagrass toxicity comparisons for these herbicides. Additionally, PSII herbicides monitored on the GBR are rarely found in isolation but rather as part of complex mixtures of herbicides with identical modes of action (Lewis et al., 2009; Shaw et al., 2010). The combined effects of PSII herbicide mixtures had not previously been tested on seagrass species, prior to the

research outlined in this thesis (Chapter 3). To ensure sustainable management of agricultural practices, emerging contaminants should be extensively studied on important keystone phototrophic species such as seagrass and coral symbionts. To expect accurate ecological guidelines for registered PSII herbicides, with species protection as the aim, potential combined effects of herbicide (and other contaminants) mixtures are imperative. The miniature bioassay methodology developed in Chapter 2 is an ideal technique to be applied to address these current data gaps.

Table 5.2. Comparison of equivalent potency values for PSII herbicides. In all studies diuron was used as the reference contaminant with a relative equivalent value (ReP) of 1.0. ReP = IC_{50} (diuron) / IC_{50} (PSII herbicide) .

Reference	This thesis	(Flores et al., 2013)	(Magnusson et al., 2010)	(Jones and Kerswell, 2003)
Test species	*H. ovalis*	*Halodule uninervis*	*Nephroselmis pyriformis*	*Acropora Formosa*
Herbicide				
Diuron	1.0	1.0	1.0	1.0
Fluometuron	0.033	-	-	-
Tebuthiuron	0.16	0.080	0.17	0.01
Atrazine	0.19	0.13	0.13	0.05
Ametryn	1.23	-	-	1.35
Metribuzin	0.90	-	-	-
Simazine	0.15	-	0.07	0.02
Prometryn	0.64	-	-	-
Bromacil	0.17	-	-	-
Hexazinone	0.41	0.35	0.93	0.26

The toxicity tests conducted for 10 herbicides were "matched" with identical experimental conditions applied to the same population of *H. ovalis* under identical conditions (same laboratory and aquarium, LED light source and 12-well plate model). This approached enabled the establishment of a matched toxicity dataset which allows for confident comparison of herbicide potencies and thus for the calculation of relative equivalence values (ReP) (Table 5.2.). Values <1 indicate a lower toxicity potential than the reference herbicide diuron while values >1 demonstrate greater toxicities. Equivalency (potency) values are required for measurement of the combined contribution of mixture constituents (Escher et al., 2008; Magnusson et al., 2010). Variations in the sensitivity of different test species across different phyla, was observed in Table 5.1. The potency of atrazine and hexazinone for instance is lower in the coral *Acropora formosa*

than other species. It can be inferred that differences in uptake pathways of each species will alter the potency of such contaminants (Oettmeier, 1992). While PSII herbicides must cross a number of membranes and host animal tissues before reaching the photosynthetic functions of coral symbiotic zooznathellae (Jones and Kerswell, 2003), the uptake of PSII herbicides in *H. ovalis* occurs directly across leaf surface (Chapter 2).

In addition to the derivation of ReP values, concentration addition (CA) was applied to the mixture bioassay data set in order to measure potential additive impacts of PSII herbicide mixture exposure (Chapter 3). CA combines the concentration and potency of each individual constituent to measure the expected overall toxicity of the mixture (Berenbaum, 1985; Porsbring et al., 2010) and overall additivity was evident in *H. ovalis*. The research applying CA to herbicide mixtures builds on previous findings whereby mixture additivity was demonstrated in estuarine microalgae (Magnusson et al., 2010) and green algae (Faust et al., 2001). The miniature bioassay technique along with concentration addition theory should be applied to a large range of PSII herbicides to measure joint toxicity to many different test species. Regulators could benefit from including mixture toxicity data in derivation of ecological guidelines for both common and emerging PSII herbicides.

5.4. Miniature bioassay application to test impacts of multiple stressors

Seagrass are sensitive to a number of environmental stressors but this thesis focused particularly on light and temperature (in addition to PSII herbicide exposure) as both are limiting factors, causing potential additional impact during persistent flood plume events (Table 5.3). Low salinity associated with flood plumes was not considered a priority stressor for assessment of additive impacts in this thesis as *H. ovalis* has relatively broad salinity tolerance, surviving extremes that exceed exposure (Collier et al., 2014; Hillman et al., 1995). Photosystem-II is considered the most sensitive photosynthetic apparatus to thermal stress (Becker et al., 1990; Georgieva and Yordanov, 1994; Havaux, 1994) and sub-optimal light conditions (high light) lead to reduction in photosynthetic efficiency (Ralph and Gademann, 2005; Wilkinson et al., 2015). Photosystem damage may occur when temperatures exceed optima or if light-stressful conditions are encountered (Ralph, 1998; Ralph and Gademann, 2005). Thermally

induced damage occurs when PSII binding affinity is impacted by alterations to thylakoid membrane fluidity or irreversible protein denaturing at extreme temperatures (Pearcy et al., 1977; Ralph, 1998; Thebud and Santarius, 1982). The findings of Chapter 4 support this as limited recovery to F_v/F_m was observed following exposure to 40°C (Figures 4.1B and 4.4). Reductions in photosynthetic efficiency (i.e. reduced quantum yields) are also caused by increased reaction centre inactivity at saturating light intensities and chronic photo-oxidative damage will occur when extended periods at high irradiances are encountered (Ralph and Gademann, 2005). When such stressful conditions are present in combination with diuron (or any PSII herbicide) potential additive effects are observed (Chapters 2 and 4).

Table 5.3. A summary of interactive effects of all tested stressors, when combined with PSII herbicide exposure.

Stressor	Mode of action	Additivity?
Mixtures	Identical	Additive
Light	Independent	Additive
Temperature	Independent	Sub-additive

The miniature bioassay methodology suits application to examining multiple stressors because the small scale approach enables large data sets across a number of treatments to be conducted. Assays can be carried out consecutively with minimum constraints from limited samples and space. As PSII herbicides, temperature and light all invoke inhibition of PSII function via different modes of action, the Independent Action (IA) model was applied to the bioassay to measure combined effects (Bliss, 1939). IA is similar to concentration addition in that predicted inhibition of $\Delta F/F_m'$ is calculated from the observed effects of individual pressures. The observed results are then compared against the predictions, with additivity the hypothesised outcome. In this thesis combined impacts to photosynthetic efficiency ($\Delta F/F_m'$) and/or photosystem damage (F_v/F_m) were measured in the presence of diuron combined with light and temperature (in separate Chapters). Additivity was observed in the all light treatments but was only evident at higher diuron concentrations in temperature treatments (sub-additivity was observed in all other treatments). This finding was in contrast to that of van Dam et al. (2015) and

Negri et al. (2011), whereby overall additivity was observed in coral symbionts when temperature and herbicide exposure was tested. Differences in the responses of PSII between corals and seagrass may be due to greater thermal tolerance in seagrass generally (Chapter 4). The miniature bioassay application along with IA theory can aid regulators gain greater understanding of the potential impacts of herbicide exposure during periods where photosynthetic processes may already be compromised due to unfavourable climatic conditions. Testing the combined sensitivity of a number of important ecosystem-forming primary producers will allow for derivation of robust ecological guidelines which will provide greater species protection during such stressful conditions.

5.5. Further application of the Miniature bioassay

Research throughout this thesis has quantitatively outlined an effective ecotoxicological technique with broad application potential to a number of scenarios, test species and contaminants. The miniature bioassay is a reliable method which has the ability to rapidly measure ecologically relevant photosynthetic endpoints which can be analysed to provide insight into photosystem II activity and health in marine primary producers (Wilkinson et al., 2015). Setup is quick and requires minimum space to test contaminants with excellent reproducibility for generating reliable data sets. Consecutive application of the bioassay is an ideal way to produce numerous single-species toxicity data sets currently used to calculate guideline and trigger values (GBRMPA, 2010). Validation of this methodology allowed for substitution of the widely used mini-PAM device with the I-PAM, however if an I-PAM is unavailable the mini-PAM can be applied with the same confidence, further demonstrating the versatility of this technique. Additionally, an exposure period of 48 h was measured with little decline in photosystem health as a result of isolation of leaf samples. Any contaminants which may require longer than 24 h exposure (i.e. hexazinone and metribuzin) in order to reach maximum inhibition can therefore be included in acute toxicity testing (GBRMPA, 2010). Future application tests with this method are encouraged to test the miniature bioassay potential and reliability at experimental periods exceeding the current parameters (>48 h). The miniature bioassay was applied here to measure complex interactions of a number of individually acting variables as well as toxicity of PSII herbicide mixtures. Such effects were previously untested in seagrass prior to this research (Table 3.1). In addition,

combined inhibitory outcomes have been measured in flood plume-relevant conditions (low light and elevated water temperature). Data sets from all chapters in this thesis build on current knowledge of the toxicity of PSII herbicides detected along the GBR. Future ecological guideline derivation will likely accept mixture toxicity and relevant combined stressors (Batley et al., 2013; GBRMPA, 2010) and research such as that presented in the current thesis should prove a valuable future resource for regulators and managers.

To expand on the growing data pool on PSII herbicide toxicity to marine environments further application of the miniature bioassay technique would be beneficial. Phototrophic test species other than *H. ovalis* can be exposed to similar herbicide concentration and environmental factors to allow for a more complete understanding of flood plume exposure during the monsoon season. For instance, modified testing containers, similar to the 12-well plate would allow application of all other aspects of the miniature bioassay to strap-like leaves of other seagrass species. Testing of PSII herbicide effects in combination with other environmental conditions known to impact seagrass (and other organisms) photosynthesis, such as salinity stress, is also recommended. Another benefit of the miniature bioassay technique is that it can be applied to measure any contaminant which directly impacts photosynthetic functioning of photosystem-II. Preliminary trials measuring potential photosynthetic effects of heavy metals were conducted during the current studies. Inhibition of $\Delta F/F_m'$ recorded for copper however limited effects were encountered when *H. ovalis* was exposed to arsenic. Therefore, thorough application of the miniature bioassay to further heavy metal testing is recommended in order to identify contaminants likely to cause significant adverse effects to PSII function. Data sets recorded through application of this technique can be used in the calculation process of ecological guidelines. Matched toxicity data sets can be readily conducted to allow for reliable comparison of toxicity of a number of herbicides. Such a universal approach will greatly increase the reliability of derived guideline values.

References

Anthony, K.R.N., Kerswell, A.P., 2007. Coral mortality following extreme low tides and high solar radiation. Marine Biology 151, 1623-1631.

ANZECC, ARMCANZ., 2000. Australian and New Zealand guidelines for fresh and marine water quality. Australian and New Zealand Environment and Conservation Council and Agriculture and Resource Management Council of Australia and New Zealand.

Bainbridge, Z.T., Brodie, J.E., Faithful, J.W., Sydes, D.A., Lewis, S.E., 2009. Identifying the land-based sources of suspended sediments, nutrients and pesticides discharged to the Great Barrier Reef from the Tully–Murray Basin, Queensland, Australia. Marine and Freshwater Research 60, 1081-1090.

Bandow, N., Altenburger, R., Brack, W., 2010. Application of nd-SPME to determine freely dissolved concentrations in the presence of green algae and algae-water partition coefficients. Chemosphere 79, 1070-1076.
Batley, G.E., Chapman, D.R., Fox, C.W., Hickey, J.L., van Dam, S.R., Warne, M.J., 2013. Revision of the Method for Deriving Water Quality Guideline Trigger Values for Toxicants. Prepared for the Council of Australian Governments Standing Council on Environment and Water (SCEW), Canberra.
Becker, D., Bain, G., Norman, J., Moholt-Siebert, M., 1990. Two Sites of Heat-Induced Damage to Photosystem II, in: Baltscheffsky, M. (Ed.), Current Research in Photosynthesis. Springer Netherlands, pp. 3499-3502.
Beer, S., Björk, M., Gademann, R., Ralph, P., 2001. Measurements of photosynthetic rates in seagrasses. Global seagrass research methods. Elsevier, Amsterdam, 183-198.
Beer, S., Koch, E., 1996. Photosynthesis of marine macroalgae and seagrasses in globally changing CO_2 environments. Marine Ecology Progress Series 141, 199-204.
Beer, S., Mtolera, M., Lyimo, T., Björk, M., 2006. The photosynthetic performance of the tropical seagrass *Halophila ovalis* in the upper intertidal. Aquatic Botany 84, 367-371.
Beer, S., Vilenkin, B., Weil, A., Veste, M., Susel, L., Eshel, A., 1998. Measuring photosynthetic rates in seagrasses by pulse amplitude modulated(PAM) fluorometry. Marine Ecology Progress Series 174, 293-300.
Behra, R., Genoni, G.P., Joseph, A.L., 1999. Effect of atrazine on growth, photosynthesis, and between-strain variability in scenedesmus subspicatus (Chlorophyceae). Archives of Environmental Contamination and Toxicology 37, 36-41.
Bengtson Nash, S.M., McMahon, K., Eaglesham, G., Müller, J.F., 2005. Application of a novel phytotoxicity assay for the detection of herbicides in Hervey Bay and the Great Sandy Straits. Marine Pollution Bulletin 51, 351-360.
Benitez, F.J., Real, F.J., Acero, J.L., Garcia, C., 2006. Photochemical oxidation processes for the elimination of phenyl-urea herbicides in waters. Journal of Hazardous Materials 138, 278-287.
Berenbaum, M.C., 1985. The expected effect of a combination of agents: the general solution. Journal of Theoretical Biology 114, 413-431. http://dx.doi.org/10.1016/S0022-5193(85)80176-4
Berkelmans, R., 2002. Time-integrated thermal bleaching thresholds of reefs and their variation on the Great Barrier Reef. Marine Ecology Progress Series 229, 73-82.
Bernstein, L., Bosch, P., Canziani, O., Chen, Z., Christ, R., Davidson, O., Hare, W., Huq, S., Karoly, D., Kattsov, V., Kundzewicz, Z., Liu, J., Lohmann, U., Manning, M., Matsuno, T., Menne, B., Metz, B., Mirza, M., Nicholls, N., Nurse, L., Pachauri, R., Palutikof, J., Parry, M., Qin, D., Ravindranath, N., Resinger, A., Ren, J., Riahi, K., Rosenweig, C., Rusticucci, M., Schneider, S., Sokona, Y., Solomon, S., Stott, P., Stouffer, R., Sugiyama, T., Swart, R., Tirpak, D., Vogel, C., Yohe, G., 2007. Climate change 2007: Synthesis report, in: Allali, A., Bojariu, R., Diaz, S., Elgizouli, I., Griggs, D., Hawkins, D., Hohmeyer, O., Jallow, B., Kajfez-Bogataj, L., Leary, N., Lee, H., Wratt, D. (Eds.), Intergovernmental Panel on Climate Change Working Group four IPCC. Intergovernmental Panel on Climate Change
Berry, J., Bjorkman, O., 1980. Photosynthetic response and adaptation to temperature in higher plants. Annual Review of Plant Physiology 31, 491-543.

Björk, M., Short, F., Mcleod, E., Beer, S., 2008. Managing seagrasses for resilience to climate change. IUCN.
Björk, M., Uku, J., Weil, A., Beer, S., 1999. Photosynthetic tolerances to desiccation of tropical intertidal seagrasses. Marine Ecology Progress Series 191, 121-126.
Bliss, C.I., 1939. The toxicity of poisons applied jointly. Annals of Applied Biology 26, 585-615.
Brock, T.C.M., Crum, S.J.H., Deneer, J.W., Heimbach, F., Roijackers, R.M.M., Sinkeldam, J.A., 2004. Comparing aquatic risk assessment methods for the photosynthesis-inhibiting herbicides metribuzin and metamitron. Environmental Pollution 130, 403-426.
Brodie, J.E., Devlin, M., Haynes, D., Waterhouse, J., 2011. Assessment of the eutrophication status of the Great Barrier Reef lagoon (Australia). Biogeochemistry 106, 281-302.
Bruggemann, W., Van der Kooij, T., van Hasselt, P.R., 1992. Long-term chilling of young tomato plants under low light and subsequent recovery. II. Chlorophyll fluorescence, carbon metabolism and activity of ribulose-1, 5-bisphosphate carboxylase/oxygenase. Planta (USA).
Bulthuis, D., 1983. Effects of temperature on the photosynthesis-irradiance curve of the Australian seagrass, Heterozostera tasmanica. Marine Biology Letters, 47–58.
Bulthuis, D.A., 1987. Effects of temperature on photosynthesis and growth of seagrasses. Aquatic Botany 27, 27-40.
Bulthuis, D.A., Woelkerling, W.J., 1983. Biomass accumulation and shading effects of epiphytes on leaves of the seagrass, Heterozostera tasmanica, in Victoria, Australia. Aquatic Botany 16, 137-148.
Calcino, D., Schroeder, B., Hurney, A., Allsopp, P.G., 2008. SmartCane plant cane establishment and management. Technical Publication TE08010, BSES Limited, Indooroopilly, Australia.
Campbell, C., Atkinson, L., Zaragoza-Castells, J., Lundmark, M., Atkin, O., Hurry, V., 2007. Acclimation of photosynthesis and respiration is asynchronous in response to changes in temperature regardless of plant functional group. New Phytologist 176, 375-389.
Campbell, S.J., McKenzie, L.J., Kerville, S.P., 2006. Photosynthetic responses of seven tropical seagrasses to elevated seawater temperature. Journal of Experimental Marine Biology and Ecology 330, 455-468.
Cantin, N.E., Negri, A.P., Willis, B.L., 2007. Photoinhibition from chronic herbicide exposure reduces reproductive output of reef-building corals. Marine Ecology Progress Series 344, 81-93.
Cantin, N.E., van Oppen, M.J.H., Willis, B.L., Mieog, J.C., Negri, A.P., 2009. Juvenile corals can acquire more carbon from high-performance algal symbionts. Coral Reefs 28, 405-414.
Carruthers, T.J.B., Dennison, W.C., Longstaff, B.J., Waycott, M., Abal, E.G., McKenzie, L.J., Long, W., Lee, J., 2002. Seagrass habitats of Northeast Australia: models of key processes and controls. Bulletin of Marine Science 71, 1153-1169.
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.
Coles, R., Long, L., 2000. Stratification of seagrasses in the Great Barrier Reef World Heritage Area, Northeastern Australia, and the implications for management. Biologia marina mediterranea 7, 345-348.

Coles, R., McKenzie, L., Campbell, S., 2003. The seagrasses of eastern Australia. World atlas of seagrasses. University of California Press, Berkeley, 119-133.
Coles, R., McKenzie, L., De'ath, G., Roelofs, A., Lee Long, W., 2009. Spatial distribution of deepwater seagrass in the inter-reef lagoon of the Great Barrier Reef World Heritage Area. Marine Ecology Progress Series 392, 57-68.
Collier, C.J., Uthicke, S., Waycott, M., 2011. Thermal tolerance of two seagrass species at contrasting light levels: implications for future distribution in the Great Barrier Reef. Limnology and Oceanography 56, 2200-2210.
Collier, C.J., Villacorta-Rath, C., van Dijk, K.-j., Takahashi, M., Waycott, M., 2014. Seagrass proliferation precedes mortality during hypo-salinity events: a stress-induced morphometric response. PLoS ONE 9, e94014.
Collier, C.J., Waycott, M., 2014. Temperature extremes reduce seagrass growth and induce mortality. Marine Pollution Bulletin 83, 483-490.
Collier, C.J., Waycott, M., McKenzie, L.J., 2012. Light thresholds derived from seagrass loss in the coastal zone of the northern Great Barrier Reef, Australia. Ecological Indicators 23, 211-219.
Costanza, R., d'Arge, R., de Groot, R., Farber, S., Grasso, M., Hannon, B., Limburg, K., Naeem, S., O'Neill, R.V., Paruelo, J., Raskin, R.G., Sutton, P., van den Belt, M., 1997. The value of the worlds ecosystem services and natural capital. Nature 387, 253-260.
Crain, C.M., Kroeker, K., Halpern, B.S., 2008. Interactive and cumulative effects of multiple human stressors in marine systems. Ecology letters 11, 1304-1315.
Davis, A., Lewis, S., Smith, R., Negri, A., 2015. Advancing our understanding of the source, management, transport and impacts of pesticides on the Great Barrier Reef 2011-2015, in: Devlin, M., Lewis, S. (Eds.), Synthesis of Pesticide Research on the Great Barrier Reef. James Cook University, Townsville.
Davis, A.M., Lewis, S.E., Brodie, J.E., Benson, A., 2014. The potential benefits of herbicide regulation: A cautionary note for the Great Barrier Reef catchment area. Science of The Total Environment 490, 81-92.
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 & Environment 180, 123-135.
Davis, D.E., Pillai, C.G.P., Truelove, B., 1976. Effects of prometryn, diuron, fluometuron, and MSMA on Chlorella and two fungi. Weed Science 24, 587-593.
de Zwart, D., Posthuma, L., 2005. Complex mixture toxicity for single and multiple species: Proposed methodologies. Environmental Toxicology and Chemistry 24, 2665-2676.
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.
Den Hartog, C., 1970. The seagrasses of the world. North-Holland Publishing Company, Amsterdam.
Dennison, W.C., Orth, R.J., Moore, K.A., Stevenson, J.C., Carter, V., Kollar, S., Bergstrom, P.W., Batiuk, R.A., 1993. Assessing water quality with submersed aquatic vegetation. BioScience 43, 86-94.
Devlin, M., Schaffelke, B., 2009. Spatial extent of riverine flood plumes and exposure of marine ecosystems in the Tully coastal region, Great Barrier Reef. Marine and Freshwater Research 60, 1109-1122.

Devlin, M.J., McKinna, L.W., Álvarez-Romero, J.G., Petus, C., Abott, B., Harkness, P., Brodie, J., 2012. Mapping the pollutants in surface riverine flood plume waters in the Great Barrier Reef, Australia. Marine Pollution Bulletin 65, 224-235.
Duarte, C.M., 1991. Seagrass depth limits. Aquatic Botany 40, 363-377.
Duarte, C.M., 2002. The future of seagrass meadows. Environmental Conservation 29, 192-206.
Duarte, C.M., Chiscano, C.L., 1999. Seagrass biomass and production: a reassessment. Aquatic Botany 65, 159-174.
Dunton, K., 1996. Photosynthetic production and biomass of the subtropical seagrass halodule wrightii along an estuarine gradient. Estuaries 19, 436-447.
Dunton, K.H., 1990. Production ecology of Ruppia maritima L. s.l. and Halodule wrightii Aschers, in two subtropical estuaries. Journal of Experimental Marine Biology and Ecology 143, 147-164.
Dunton, K.H., 1994. Seasonal growth and biomass of the subtropical seagrassHalodule wrightii in relation to continuous measurements of underwater irradiance. Marine Biology 120, 479-489.
Durako, M.J., Kunzelman, J.I., Kenworthy, W.J., Hammerstrom, K.K., 2003. Depth-related variability in the photobiology of two populations of *Halophila johnsonii* and *Halophila decipiens*. Marine Biology 142, 1219-1228.
Escher, B.I., Bramaz, N., Mueller, J.F., Quayle, P., Rutishauser, S., Vermeirssen, E.L., 2008. Toxic equivalent concentrations (TEQs) for baseline toxicity and specific modes of action as a tool to improve interpretation of ecotoxicity testing of environmental samples. Journal of Environmental Monitoring 10, 612-621.
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.
Fairchild, J.F., Ruessler, D.S., Carlson, A.R., 1998. Comparative sensitivity of five species of macrophytes and six species of algae to atrazine, metribuzin, alachlor, and metolachlor. Environmental Toxicology and Chemistry 17, 1830-1834.
Faust, M., Altenburger, R., Backhaus, T., Blanck, H., Boedeker, W., Gramatica, P., Hamer, V., Scholze, M., Vighi, M., Grimme, L.H., 2001. Predicting the joint algal toxicity of multi-component s-triazine mixtures at low-effect concentrations of individual toxicants. Aquatic Toxicology 56, 13-32.
Flores, F., Collier, C.J., Mercurio, P., Negri, A.P., 2013. Phytotoxicity of four photosystem II herbicides to tropical seagrasses. PLoS ONE 8, e75798.
Fourqurean, J.W., Duarte, C.M., Kennedy, H., Marba, N., Holmer, M., Mateo, M.A., Apostolaki, E.T., Kendrick, G.A., Krause-Jensen, D., McGlathery, K.J., Serrano, O., 2012. Seagrass ecosystems as a globally significant carbon stock. Nature Geoscience 5, 505-509.
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.
Furnas, M.M., 2003. Catchments and corals: terrestrial runoff to the Great Barrier Reef. Australian Institute of Marine Science and CRC Reef, Townsville, Australia.
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.
Gatidou, G., Stasinakis, A.S., Iatrou, E.I., 2015. Assessing single and joint toxicity of three phenylurea herbicides using Lemna minor and Vibrio fischeri bioassays. Chemosphere 119, Supplement, S69-S74.

GBRMPA, 2010. Water quality guidelines for the Great Barrier Reef Marine Park (Revised). Great Barrier Reef Marine Park Authority, Townsville. .
Genty, B., Briantais, J.-M., Baker, N.R., 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochimica et Biophysica Acta (BBA) - General Subjects 990, 87-92.
Georgieva, K., Yordanov, I., 1994. Temperature Dependence of Photochemical and Non-Photochemical Fluorescence Quenching in Intact Pea Leaves. Journal of Plant Physiology 144, 754-759.
Gilliom, R.J., Barbash, J.E., Kolpin, D.W., Larson, S.J., 1999. Testing water quality for pesticide pollution. Environmental Science & Technology 33, 164A-169A.
Gordon, D.M., Grey, K.A., Chase, S.C., Simpson, C.J., 1994. Changes to the structure and productivity of a Posidonia sinuosa meadow during and after imposed shading. Aquatic Botany 47, 265-275.
Grech, A., Coles, R., Marsh, H., 2011. A broad-scale assessment of the risk to coastal seagrasses from cumulative threats. Marine Policy 35, 560-567.
Guinotte, J.M., Fabry, V.J., 2008. Ocean Acidification and Its Potential Effects on Marine Ecosystems. Annals of the New York Academy of Sciences 1134, 320-342.
Hardy, S., Koontz, T., 2008. Reducing Nonpoint Source Pollution Through Collaboration: Policies and Programs Across the U.S. States. Environmental Management 41, 301-310.
Havaux, M., 1994. Temperature-Dependent Modulation of the Photoinhibition-Sensitivity of Photosystem II in Solanum tuberosum Leaves. Plant and Cell Physiology 35, 757-766.
Haworth, P., Steinback, K.E., 1987. Interaction of herbicides and quinone with the Q(b)-Protein of the diuron-resistant *Chlamydomonas reinhardtii* mutant Dr2. Plant physiology 83, 1027-1031.
Haynes, D., Muller, J., Carter, S., 2000. Pesticide and herbicide residues in sediments and seagrasses from the Great Barrier Reef World Heritage Area and Queensland Coast. Marine Pollution Bulletin 41, 279-287.
Haynes, D., Ralph, P.J., Prange, J., Dennison, B., 2000b. The impact of the herbicide diuron on photosynthesis in three species of tropical seagrass. Marine Pollution Bulletin 41, 288-293
Hillman, K., McComb, A.J., Walker, D.I., 1995. The distribution, biomass and primary production of the seagrass Halophila ovalis in the Swan/Canning Estuary, Western Australia. Aquatic Botany 51, 1-54.
Holmes, G., 2014. Australia's pesticide environmental risk assessment failure: The case of diuron and sugarcane. Marine Pollution Bulletin 88, 7-13.
Holmstrup, M., Bindesbøl, A.-M., Oostingh, G.J., Duschl, A., Scheil, V., Köhler, H.-R., Loureiro, S., Soares, A.M.V.M., Ferreira, A.L.G., Kienle, C., Gerhardt, A., Laskowski, R., Kramarz, P.E., Bayley, M., Svendsen, C., Spurgeon, D.J., 2010. Interactions between effects of environmental chemicals and natural stressors: A review. Science of The Total Environment 408, 3746-3762.
Inglis, G.J., 2000. Variation in the recruitment behaviour of seagrass seeds: implications for population dynamics and resource management. Pacific Conservation Biology 5, 251.
Jackson, J.B.C., Kirby, M.X., Berger, W.H., Bjorndal, K.A., Botsford, L.W., Bourque, B.J., Bradbury, R.H., Cooke, R., Erlandson, J., Estes, J.A., Hughes, T.P., Kidwell, S., Lange, C.B., Lenihan, H.S., Pandolfi, J.M., Peterson, C.H., Steneck,

R.S., Tegner, M.J., Warner, R.R., 2001. Historical Overfishing and the Recent Collapse of Coastal Ecosystems. Science 293, 629-637.
Jones, R.J., Kerswell, A.P., 2003. Phytotoxicity of Photosystem II (PSII) herbicides to coral. Marine Ecology Progress Series 261, 149-159.
Jones, R.J., Muller, J., Haynes, D., Schreiber, U., 2003. Effects of herbicides Diuron and Atrazine on corals of the Great Barrier Reef, Australia. Marine Ecology Progress Series 251, 153-167.
Kahn, A.E., Durako, M.J., 2009. Photosynthetic tolerances to desiccation of the co-occurring seagrasses *Halophila johnsonii* and *Halophila decipiens*. Aquatic Botany 90, 195-198.
Kennedy, K., Schroeder, T., Shaw, M., Haynes, D., Lewis, S., Bentley, C., Paxman, C., Carter, S., Brando, V.E., Bartkow, M., Hearn, L., Mueller, J.F., 2012. Long term monitoring of photosystem II herbicides – Correlation with remotely sensed freshwater extent to monitor changes in the quality of water entering the Great Barrier Reef, Australia. Marine Pollution Bulletin 65, 292-305.
Kerr, E.A., Strother, S., 1985. Effects of irradiance, temperature and salinity on photosynthesis of Zostera muelleri. Aquatic Botany 23, 177-183.
Kilminster, K., McMahon, K., Waycott, M., Kendrick, G.A., Scanes, P., McKenzie, L., O'Brien, K.R., Lyons, M., Ferguson, A., Maxwell, P., Glasby, T., Udy, J., 2015. Unravelling complexity in seagrass systems for management: Australia as a microcosm. Science of The Total Environment.
King, J., Alexander, F., Brodie, J., 2012. Regulation of pesticides in Australia: The Great Barrier Reef as a case study for evaluating effectiveness. Agriculture, Ecosystems & Environment 180, 54-67.
Kirkman, H., 1997. Seagrasses of Australia, State of the Environment Technical Paper Series (Estuaries and the Sea). Department of the Environment, Canberra, p. 36.
Kirkman, H., 2014. Impact of proposed port of Hastings expansion on seagrass, mangroves and salt marsh. Report to Victorian National Parks Association.
Knauert, S., Escher, B., Singer, H., Hollender, J., Knauer, K., 2008. Mixture toxicity of three photosystem II inhibitors (atrazine, isoproturon, and diuron) toward photosynthesis of freshwater phytoplankton studied in outdoor mesocosms. Environmental Science & Technology 42, 6424-6430.
Koch, M., Bowes, G., Ross, C., Zhang, X.-H., 2013. Climate change and ocean acidification effects on seagrasses and marine macroalgae. Global Change Biology 19, 103-132.
Kratky, B.A., Warren, G.F., 1971. A rapid bioassay for photosynthetic and respiratory inhibitors. Weed Science 19, 658-661.
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.
Küster, A., Altenburger, R., 2007. Development and validation of a new fluorescence-based bioassay for aquatic macrophyte species. Chemosphere 67, 194-201.
Lanyon, J., Limpus, C., Marsh, H., 1989. Dugongs and turtles: grazers in the seagrass system. Biology of Seagrasses. Elsevier, Amsterdam 610.
Lee, K.-S., Dunton, K.H., 1996. Production and carbon reserve dynamics of the seagrass Thalassia testudinum in Corpus Christi Bay, Texas, USA. Marine Ecology Progress Series 143, 201-210.

Lee, K.-S., Park, S., Kim, J.-B., 2005. Production dynamics of the eelgrass, Zostera marina in two bay systems on the south coast of the Korean peninsula. Marine Biology 147, 1091-1108.
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.
Lee Long, W., Coles, R.G., McKenzie, L., 1996. Deepwater seagrasses in Northeastern Australia-How deep, how meaningful, Seagrass Biology: Proceedings of an International Workshop, Rottnest Island, Western Australia, pp. 25-29.
Lewis, S., Smith, R., Brodie, J., Bainbridge, Z., Davis, A., Turner, R., 2011. Using monitoring data to model herbicides exported to the Great Barrier Reef, Australia, 19th International Congress on Modelling and Simulation, Perth, Australia, pp. 2051-2056.
Lewis, S., Smith, R., O'Brien, D., Warne, M., Negri, A., Petus, C., da Silva, E., Zeh, D., Turner, R., Davis, A., Mueller, J., Brodie, J., 2013. Assessing the risk of additive pesticide exposure in Great Barrier Reef ecosystems" in: al., W.e. (Ed.), 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.
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.
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.
Lough, J.M., Great Barrier Reef Marine Park, A., 2007. Chapter 2: Climate and climate change on the Great Barrier Reef. The Great Barrier Reef Marine Park Authority, Townsville.
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.
Magnusson, M., Heimann, K., Quayle, P., Negri, A.P., 2010. Additive toxicity of herbicide mixtures and comparative sensitivity of tropical benthic microalgae. Marine Pollution Bulletin 60, 1978-1987.
Magnusson, M., Heimann, K., Ridd, M., Negri, A.P., 2012. Chronic herbicide exposures affect the sensitivity and community structure of tropical benthic microalgae. Marine Pollution Bulletin 65, 363-372.
Magnusson, M., Heimann, K., Ridd, M., Negri, A.P., 2013. Pesticide contamination and phytotoxicity of sediment interstitial water to tropical benthic microalgae. Water Research 47, 5211-5221.
Marbà, N., Holmer, M., Gacia, E., Barròn, C., 2006. Seagrass beds and coastal biogeochemistry, in: Larkum, A.W.D., Orth, R.J., Duarte, C.M. (Eds.), Seagrasses: Biology, Ecology and Conservation. Springer Dordrecht.
Marsh, H., O'Shea, T.J., Reynolds III, J.E., 2011. Ecology and conservation of the Sirenia: dugongs and manatees. Cambridge University Press.
Marsh, J., Dennison, C., Alberte, S., 1986. Effects of temperature on photosynthesis and respiration in eelgrass (Zostera marina L.). Journal of Experimental Marine Biology and Ecology 101, 257-267.

Masini, R.J., Manning, C.R., 1997. The photosynthetic responses to irradiance and temperature of four meadow-forming seagrasses. Aquatic Botany 58, 21-36.
Massa, S.I., Arnaud-Haond, S., Pearson, G.A., Serrão, E.A., 2009. Temperature tolerance and survival of intertidal populations of the seagrass *Zostera noltii* (Hornemann) in Southern Europe (Ria Formosa, Portugal). Hydrobiologia 619, 195-201.
McKenzie, L., Campbell, S.J., 2004. Surviving the summer heat: seagrass and corals. Seagrass-Watch news 19, 1.
McKenzie, L., Yoshida, R., Grech, A., Coles, R., 2010. Queensland seagrasses. Status 2010-Torres Strait and East Coast. Fisheries Queensland (DEEDI), Cairns 6.
McKenzie, L.J., Collier, C., Waycott, M., 2015. Reef Rescue Marine Monitoring Program: Inshore seagrass, annual report for the sampling period 1st June 2012 - 31st May 2013. Centre for Tropical Water and Aquatic Ecosystem Research (TropWater), Cairns.
McMahon, K., Collier, C., Lavery, P.S., 2013. Identifying robust bioindicators of light stress in seagrasses: A meta-analysis. Ecological Indicators 30, 7-15.
Mellors, J.E., 2003. Sediment and nutrient dynamics in coastal intertidal seagrass of north eastern tropical Australia. PhD thesis. James Cook University, Townsville, Australia.
Mercurio, P., Mueller, J., G, E., Flores, F., Negri, A., 2015. Herbicide persistence in seawater simulation experiments. PLoS ONE 10.
Mitchell, A., Reghenzani, J., Faithful, J., Furnas, M., Brodie, J., 2009. Relationships between land use and nutrient concentrations in streams draining a 'wet-tropics' catchment in northern Australia. Marine and Freshwater Research 60, 1097-1108.
Morrissey, J.I., Oliver, J., Wachenfeld, D.R., 1998. State of the Great Barrier Reef World Heritage Area 1998. Great Barrier Reef Marine Park Authority, Australia.
Muller, R., Schreiber, U., Escher, B.I., Quayle, P., Bengtson Nash, S.M., Mueller, J.F., 2008. Rapid exposure assessment of PSII herbicides in surface water using a novel chlorophyll a fluorescence imaging assay. Science of The Total Environment 401, 51-59.
Negri, A.P., Flores, F., Mercurio, P., Mueller, J.F., Collier, C.J., 2015. Lethal and sub-lethal chronic effects of the herbicide diuron on seagrass. Aquatic Toxicology 165, 73-83.
Negri, A.P., Flores, F., Röthig, T., Uthicke, S., 2011. Herbicides increase the vulnerability of corals to rising sea surface temperature. Limnology and Oceanography 56, 471-485.
OECD, 2000. Guidance document on aquatic toxicity testing of difficult substances and Mixtures. , in: OECD (Ed.), OECD Series on Testing and Assessment
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.
Orth, R.J., Moore, K.A., 1986. Seasonal and year-to-year variations in the growth of Zostera marina L. (eelgrass) in the lower Chesapeake Bay. Aquatic Botany 24, 335-341.

Osmond, C., Anderson, J., Ball, M., Egerton, J., 1999. Compromising efficiency: the molecular ecology of light-resource utilization in plants. Physiological plant ecology, 1-25.
Packett, R., Dougall, C., Rohde, K., Noble, R., 2009. Agricultural lands are hot-spots for annual runoff polluting the southern Great Barrier Reef lagoon. Marine Pollution Bulletin 58, 976-986.
Pearcy, R.W., Berry, J.A., Fork, D.C., 1977. Effects of growth temperature on the thermal stability of the photosynthetic apparatus of Atriplex lentiformis (Torr.) Wats. Plant physiology 59, 873-878.
Pérez, M., Romero, J., 1992. Photosynthetic response to light and temperature of the seagrass Cymodocea nodosa and the prediction of its seasonality. Aquatic Botany 43, 51-62.
Porsbring, T., Backhaus, T., Johansson, P., Kuylenstierna, M., Blanck, H., 2010. Mixture toxicity from photosystem II inhibitors on microalgal community succession is predictable by concentration addition. Environmental Toxicology and Chemistry 29, 2806-2813.
Prasil, O., Adir, N., Ohad, I., 1992. Dynamics of photosystem II: mechanism of photoinhibition and recovery processes, in: Barber, J. (Ed.), The photosystems: Structure, Function, Molecular Biology. Elsevier Amsterdam, pp. 295-348.
Raha, D., 2007. Paradigm shift in water environment protection in New South Wales. Australian Journal of Water Resources 11, 67.
Ralph, P., Tomasko, D., Moore, K., Seddon, S., Macinnis-Ng, C.O., 2006. Human Impacts on Seagrasses: Eutrophication, Sedimentation, and Contamination, in: Larkum, A.W.D., Orth, R.J., Duarte, C.M. (Eds.), Seagrasses: Biology, Ecology and Conservation Springer The Netherlands, pp. 567-593.
Ralph, P.J., 1998. Photosynthetic response of laboratory-cultured *Halophila ovalis* to thermal stress. Marine Ecology Progress Series 171, 123.
Ralph, P.J., 2000. Herbicide toxicity of *Halophila ovalis* assessed by chlorophyll fluorescence. Aquatic Botany 66, 141-152.
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.
Ralph, P.J., Durako, M.J., Enríquez, S., Collier, C.J., Doblin, M.A., 2007a. Impact of light limitation on seagrasses. Journal of Experimental Marine Biology and Ecology 350, 176-193.
Ralph, P.J., Gademann, R., 2005. Rapid light curves: a powerful tool to assess photosynthetic activity. Aquatic Botany 82, 222-237.
Ralph, P.J., Macinnis-Ng, C.M.O., Frankart, C., 2005. Fluorescence imaging application: effect of leaf age on seagrass photokinetics. Aquatic Botany 81, 69-84.
Ralph, P.J., Smith, R.A., Macinnis-Ng, C.M.O., Seery, C.R., 2007b. Use of fluorescence-based ecotoxicological bioassays in monitoring toxicants and pollution in aquatic systems: Review. Toxicological and Environmental Chemistry 89, 589-607.
Rasheed, M.A., 2004. Recovery and succession in a multi-species tropical seagrass meadow following experimental disturbance: the role of sexual and asexual reproduction. Journal of Experimental Marine Biology and Ecology 310, 13-45.
Rasheed, M.A., Unsworth, R.K., 2011. Long-term climate-associated dynamics of a tropical seagrass meadow: implications for the future. Marine Ecology Progress Series 422, 93-103.

Reusch, T.B.H., Ehlers, A., Hämmerli, A., Worm, B., 2005. Ecosystem recovery after climatic extremes enhanced by genotypic diversity. Proceedings of the National Academy of Sciences of the United States of America 102, 2826-2831.
Rokka, A., Aro, E.-M., Herrmann, R.G., Andersson, B., Vener, A.V., 2000. Dephosphorylation of Photosystem II Reaction Center Proteins in Plant Photosynthetic Membranes as an Immediate Response to Abrupt Elevation of Temperature. Plant physiology 123, 1525-1536.
Runcie, J.W., Paulo, D., Santos, R., Sharon, Y., Beer, S., Silva, J., 2009. Photosynthetic responses of Halophila stipulacea to a light gradient: I–in situ energy partitioning of non-photochemical quenching. Aquatic Biology 7, 143-152.
Rutherford, A.W., Krieger-Liszkay, A., 2001. Herbicide-induced oxidative stress in photosystem II. Trends in Biochemical Sciences 26, 648-653.
Safe, S.H., 1998. Hazard and risk assessment of chemical mixtures using the toxic equivalency factor approach. Environmental Health Perspectives 106, 1051.
Schaffelke, B., Mellors, J., Duke, N.C., 2005. Water quality in the Great Barrier Reef region: responses of mangrove, seagrass and macroalgal communities. Marine Pollution Bulletin 51, 279-296.
Schreiber, U., 2004. Pulse-Amplitude-Modulation (PAM) Fluorometry and Saturation Pulse Method: An Overview, in: Papageorgiou, G., Govindjee (Eds.), Chlorophyll a Fluorescence. Springer pp. 279-319.
Schreiber, U., Armond, P.A., 1978. Heat-induced changes of chlorophyll fluorescence in isolated chloroplasts and related heat-damage at the pigment level. Biochimica et Biophysica Acta (BBA) - Bioenergetics 502, 138-151. http://dx.doi.org/10.1016/0005-2728(78)90138-X
Schreiber, U., Bilger, W., Neubauer, C., 1994. Chlorophyll fluorescence as a non intrusive indicator for rapid assessment of in vivo photosynthesis, in: Schulze, E.D., Caldwell, M.M. (Eds.), Ecophysiology of Photosynthesis, pp. 49-70.
Schreiber, U., Quayle, P., Schmidt, S., Escher, B.I., Mueller, J.F., 2007. Methodology and evaluation of a highly sensitive algae toxicity test based on multiwell chlorophyll fluorescence imaging. Biosensors and Bioelectronics 22, 2554-2563.
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.
Seddon, S., Cheshire, A., 2000. Photosynthetic response of Amphibolis antarctica and Posidonia australis to temperature and desiccation using chlorophyll fluorescence. Marine Ecology Progress Series, 119 - 130.
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.
Sharon, Y., Beer, S., 2008. Diurnal movements of chloroplasts in Halophila stipulacea and their effect on PAM fluorometric measurements of photosynthetic rates. Aquatic Botany 88, 273-276.
Shaw, C.M., Brodie, J., Mueller, J.F., 2012. Phytotoxicity induced in isolated zooxanthellae by herbicides extracted from Great Barrier Reef flood waters. Marine Pollution Bulletin 65, 355-362.
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.

Shephard, S., McComb, A., Bulthuis, D., Neverauskas, V., Steffensen, D., West, R., 1989. Decline of seagrasses, in: Larkum, A.W.D., McComb, A.J., Shephard, S.A. (Eds.), Biology of seagrasses : a treatise on the biology of seagrasses with special reference to the Australian region. Elsevier Science Publication, Amsterdam, The Netherlands, pp. 346-393.

Sikka, H., Pramer, D., 1968. Physiological effects of fluometuron on some unicellular algae. Weed Science 16, 296-299.

Silva, J., Sharon, Y., Santos, R., Beer, S., 2009. Measuring seagrass photosynthesis: methods and applications. Aquatic Biology 7, 127-141.

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. 10.1016/j.marpolbul.2011.08.010

Smith, R., Warne, M., Mengersen, K., Turner, R., unpublished. Toxicity-based pollutant loads: A multispecies toxic equivalency approach.

Smith, R.A., Warne, M.S.J., Delaney, K., Turner, R.D.R., Seery, C., Pradella, N., vardy, S., Rogers, B., Arango, C., Edge, K., Julli, M., In prep. Proposed guideline values for six priority pesticides of the Great Barrier Reef and its adjacent catchments. Department of Science, Information Technology, Innovation and the Arts, Queensland Government, Brisbane.

Solomon, K.R., Baker, D.B., Richards, R.P., Dixon, K.R., Klaine, S.J., La Point, T.W., Kendall, R.J., Weisskopf, C.P., Giddings, J.M., Giesy, J.P., Hall, L.W., Williams, W.M., 1996. Ecological risk assessment of atrazine in North American surface waters. Environmental Toxicology and Chemistry 15, 31-76.

Terrados, J., Ros, J.D., 1995. Temperature Effects on Photosynthesis and Depth Distribution of the Seagrass Cymodocea nodosa (ucria) Ascherson in a Mediterranean Coastal Lagoon: the Mar Menor (SE Spain). Marine Ecology 16, 133-144.

Thebud, R., Santarius, K.A., 1982. Effects of high-temperature stress on various biomembranes of leaf cells in situ and in vitro. Plant physiology 70, 200-205.

Thorhaug, A., Blake, N., Schroeder, P.B., 1978. The effect of heated effluents from power plants on seagrass (Thalassia) communities quantitatively comparing estuaries in the subtropics to the tropics. Marine Pollution Bulletin 9, 181-187.

Tomlin, C., 2000. The pesticide manual: A world compendium (12th edition). British Crop Protection Council, Farnham, Surrey, UK, 1250.

Touchette, B.W., 2007. Seagrass-salinity interactions: Physiological mechanisms used by submersed marine angiosperms for a life at sea. Journal of Experimental Marine Biology and Ecology 350, 194-215.

Touchette, B.W., Burkholder, J.M., 2000. Overview of the physiological ecology of carbon metabolism in seagrasses. Journal of Experimental Marine Biology and Ecology 250, 169-205.

USEPA, 2008. Framework for Application of the Toxicity Equivalence Methodology for Polychlorinated Dioxins, Furans, and Biphenyls in Ecological Risk Assessment. Office of the Science Advisor, Risk Assessment Forum, United States Environmental Protection Agency

van Dam, J.W., Negri, A.P., Mueller, J.F., Altenburger, R., Uthicke, S., 2012. Additive pressures of elevated sea surface temperatures and herbicides on symbiont-bearing foraminifera. PLoS ONE 7, e33900.

van Dam, J.W., Uthicke, S., Beltran, V.H., Mueller, J.F., Negri, A.P., 2015. Combined thermal and herbicide stress in functionally diverse coral symbionts. Environmental Pollution 204, 271-279.
Wahedally, S.F., Mamboya, F.A., Lyimo, T.J., 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*. TAJNAS 3, 458-466.
Walker, D.I., McComb, A.J., 1992. Seagrass degradation in Australian coastal waters. Marine Pollution Bulletin 25, 191-195.
Warne, M.S.J., 2002. Derivation of the Australian and New Zealand water quality guidelines for toxicants. Australasian Journal of Ecotoxicology 7, 123-136.
Warne, M.S.J., Batley, G.E., Braga, O., Chapman, J.C., Fox, D.R., Hickey, C.W., Stauber, J.L., Van Dam, R., 2014. Revisions to the derivation of the Australian and New Zealand guidelines for toxicants in fresh and marine waters. Environmental Science and Pollution Research 21, 51-60.
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.
Waycott, M., Collier, C., McMahon, K., Ralph, P., McKenzie, L., Udy, J., Grech, A., 2007. Vulnerability of seagrasses in the Great Barrier Reef to climate change. Great Barrier Reef Marine Park Authority and Australian Greenhouse Office.
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 106, 12377-12381.
Waycott, M., Longstaff, B.J., Mellors, J., 2005. Seagrass population dynamics and water quality in the Great Barrier Reef region: A review and future research directions. Marine Pollution Bulletin 51, 343-350.
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
Waycott, M., Procaccini, G., Les, D.H., Reusch, T.B.H., Larkum, A.W.D., Orth, R.J., Duarte, C.M., 2006. Seagrass evolution, ecology and conservation: A genetic perspective, Seagrasses: Biology, Ecology and Conservation. Springer, pp. 25-50.
Weis, E., Berry, J.A., 1987. Quantum efficiency of Photosystem II in relation to 'energy'-dependent quenching of chlorophyll fluorescence. Biochimica et Biophysica Acta (BBA) - Bioenergetics 894, 198-208.
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, e0117541.