Attachment 4

Pergamon

Marine Pollution Bulletin Vol. 41, Nos. 7–12, pp. 288–293, 2000

Printed in Great Britain
PII: S0025-326X(00)00127-2
0025-326X/00 $ - see front matter

The Impact of the Herbicide Diuron on Photosynthesis in Three Species of Tropical Seagrass

DAVID HAYNES†*, PETER RALPH‡, JOELLE PRANGE§,†† and BILL DENNISON§
†*Great Barrier Reef Marine Park Authority, 2-68 Flinders Street, P.O. Box 1379, Townsville 4810, Qld, Australia*
‡*University of Technology, Sydney, P.O. Box 123, Broadway 2007, Sydney, Australia*
§*Marine Botany, University of Queensland, St Lucia 4072, Qld, Australia*
††*Griffith University, Nathan 4111, Australia*

The impact and recovery from exposure to the herbicide diuron [DCMU; 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea] was assessed for three tropical seagrasses, maintained in outdoor aquaria over a 10-day period. Photosynthetic stress was detected using chlorophyll *a* fluorescence, measured with a Diving-PAM (pulse amplitude modulated fluorometer). Exposure to 10 and 100 μg l^{-1} diuron resulted in a decline in effective quantum yield ($F/F_{m'}$) within 2 h of herbicide exposure in *Cymodocea serrulata*, *Halophila ovalis* and *Zostera capricorni*. Effective quantum yield also declined over the first 24 h of exposure in *H. ovalis* at even lower diuron concentrations (0.1 and 1.0 μg l^{-1}). Effective quantum yield in *H. ovalis* and *Z. capricorni* was significantly depressed at all diuron concentrations (0.1–100 μg l^{-1}) after 5 days exposure, whereas effective quantum yield in *C. serrulata* was only significantly lower in plants exposed to highest diuron concentrations (10 and 100 μg l^{-1}). Effective quantum yield depression was present 5 days after plants exposed to 10 and 100 μg l^{-1} diuron were returned to fresh seawater. These results indicate that exposure to herbicide concentrations present in nearshore Queensland sediments present a potential risk to seagrass functioning. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: diuron; fluorometry; pollution effects; seagrass; toxicity; water quality.

Introduction

Sugarcane production is the largest intensive agricultural industry carried out in Queensland, Australia. It represents one of Australia's largest export industries and industry sales generated approximately AUS $1.2 billion in 1991 (Anon, 1992). The industry is situated primarily along the coastal fringe and is concentrated in northern Queensland, adjacent to the Great Barrier Reef World Heritage Area. Diuron [DCMU; 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea] is a phenylurea herbicide used extensively in the Queensland cane industry for pre-emergence weed control (Hamilton and Haydon, 1996), and as a consequence, is a common contaminant of sugarcane growing soils (Müller *et al.*, 2000). The herbicide has also been found to be a widespread contaminant of the nearshore Queensland environment adjacent to sugarcane production areas, where it occurs in concentrations of up to 10 μg kg^{-1} (Haynes *et al.*, 2000).

The toxic action of diuron on plant photosynthesis is well understood (van Rensen, 1989). The herbicide inhibits the photoreduction side of photosystem II (PSII) by binding with high affinity at the Q_B-binding site of the PSII photosynthetic complex and preventing Q_B from binding at this location. Exclusion of the Q_B from its binding site blocks electron transfer from Q_A to Q_B, which limits electron flow in PSII (Sandmann and Bölger, 1986). This results in a decrease in measurable variable fluorescence (ΔF), and a concomitant decline in effective quantum yield ($\Delta F/F_{m'}$) from the affected plant.

Analysis of chlorophyll fluorescence is a sensitive and early indicator of damage to photosynthetic apparatus (Krause and Weis, 1991; Schreiber *et al.*, 1994) and recent studies utilizing pulse amplitude modulated (PAM) fluorometry have demonstrated the utility of this technique to rapidly measure stress response in marine angiosperms (Dawson and Dennison, 1996; Beer *et al.*, 1998; Ralph and Burchett, 1998a,b; Ralph, 2000). PAM fluorometry involves the application of a saturating pulse of white light which results in a reduction of PSII reaction centres and an increase in fluorescence to maximal levels ($F_{m'}$) as excess light energy is dissipated as heat. The change in fluorescence from background (F) to $F_{m'}$ denotes the variable fluorescence (ΔF) under saturating light conditions. The ratio of variable fluorescence to maximal

*Corresponding author. Tel.: +61-747-500-7004.
E-mail address: d.haynes@gbrmpa.gov.au (D. Haynes).

fluorescence ($\Delta F/F_{m'}$) in illuminated samples provides a measure of the effective quantum yield of electron flow through PSII (Jones *et al.*, 1999).

Although diuron's toxic effect on photosynthetic biochemical pathways has been extensively studied for over 30 years (van Rensen, 1989), the long-term environmental impact of both chronic and acute diuron exposure are essentially unknown for the (Australian) marine environment. Extensive losses of seagrass beds have occurred worldwide in recent years (Walker and McComb, 1992; Preen *et al.*, 1995; Short *et al.*, 1996), and in Australia, the loss of 450 km^2 of seagrass beds over the last 10 years can be attributed to anthropogenic impacts (Kirkman, 1997). The general hypothesis related to these losses is that a decrease in light reaching submerged plants reduces effective photosynthesis, ultimately leading to plant death. However, the possibility exists that the low level diuron contamination of Queensland nearshore environments could affect the competitive fitness of local seagrasses, eventually leading to changes in community structure with major ecosystem flow-on impacts (Haynes *et al.*, 1998, 2000). The objective of this work was to assess the acute toxicity of a range of environmentally relevant diuron concentrations to three tropical seagrass species using chlorophyll fluorescence as a measure of photosynthetic efficiency (Dawson and Dennison, 1996; Jones *et al.*, 1999; Ralph, 2000).

Methods and Materials

Plant collection

Three tropical seagrass species (*Halophila ovalis*, *Cymodocea serrulata* and *Zostera capricorni*) were collected in shallow water (<3 m) from Wanga Wallen Banks, Moreton Bay, Australia, (27°25′S, 153°22′E) in November 1998. Plants were collected using a 105-mm diameter PVC corer, and intact cores (sediment and seagrass) were transferred to 1 l pyrex beakers and sealed in plastic bags to avoid excessive water loss during transportation to the laboratory. Intact cores were transferred to flow-through aquaria and seagrass allowed to acclimate for 24 h prior to experimentation.

Experimental set-up

Specimens of each of the three acclimated seagrass species (and their associated sediment cores) were randomly placed in 10, 50 l glass aquaria and exposed to five replicated diuron treatments (0, 0.1, 1.0, 10 and 100 μg l^{-1}). All glass aquaria used in the trial were cleaned prior to use with anionic detergent and rinsed in 10% nitric acid followed by distilled water. The 50 l aquaria were housed inside larger outdoor flow-through (50 l h^{-1}) aquaria that minimized water temperature variation over the experimental period. There was no water exchange between the experimental and larger aquaria. Aquaria were covered with 50% neutral density shade screens and clear plastic covers to minimize rainwater dilution of experimental diuron concentrations. Aquaria containing seagrass were aerated over the experimental period.

Seagrass were exposed to diuron for a 5-day period, rinsed, and replaced in fresh seawater and monitored for a further 5-day recovery period. Tests were conducted under static conditions, with herbicide exposure a single dose addition to the water at the beginning of the experiment. Experimental diuron concentrations (0, 0.1, 1.0, 10 and 100 μg l^{-1}) were based on concentrations detected in nearshore marine sediments collected from the northern Queensland coast (Haynes *et al.*, 2000). Standard solutions of diuron were prepared from technical grade diuron (98% pure, Sigma). A stock solution (10 mg l^{-1}), was made by dissolving diuron in acetone (2 ml), then diluting in 100 ml seawater, followed by gentle heating to volatilize the acetone (Schwarzschild *et al.*, 1994). Seawater diuron concentrations were verified using high performance liquid chromatography (HPLC) interfaced via a high flow electrospray source to a triple stage mass spectrometer (LSMSMS) following extraction in dichloromethane and hexane. Dosing of the experimental aquaria was accurate, and reproducible, with less than 14% concentration differences between experimental replicates (Table 1). No diuron was detected in control aquaria water and the level of diuron maintained in aquaria during the 5-day exposure period remained stable, with less than 20% loss from the initial dose.

TABLE 1

Aquaria water column diuron concentration at beginning (day 0) and the end (day 5) of the experimental period.

Treatment	Replicate No.	Measured initial concentration (μg l^{-1})	Measured final concentration (μg l^{-1})
Control	a	<0.1	<0.1
Control	b	<0.1	<0.1
Diuron (μg l^{-1})			
0.1	a	0.1	0.1
0.1	b	0.1	0.2
1.0	a	0.9	0.9
1.0	b	0.8	1.0
10	a	8.1	7.4
10	b	8.4	7.2
100	a	86	74
100	b	100	80

TABLE 2
Aquaria water quality variation during diuron exposure and recovery periods.

Exposure period	Minimum temperature (°C)	Maximum temperature (°C)	Salinity (ppt)	pH
Day 1	20.0	35.0	37.2	8.42
Day 2	20.0	31.0	34.2	8.15
Day 3	22.5	32.0	38.5	8.21
Day 4	22.5	31.5	39.0	8.24
Day 5	22.0	31.0	39.4	8.26
Day 6	22.5	25.0	37.7	8.17
Day 7	23.0	31.0	38.2	8.13
Day 8	23.0	26.0	35.9	8.16
Day 9	23.0	25.0	32.6	8.18
Day 10	23.5	31.0	33.2	8.22

Aquaria salinity and pH were measured daily in both the flow-through and experimental aquaria with a water quality probe (Horiba U-10). Minimum and maximum temperatures were recorded daily from a min/max thermometer placed in one of the flow-through aquaria. Aquaria temperature ranged from 20°C to 35°C. Forty eight hours of rainfall over the experimental period resulted in a slight decrease in salinity in the test aquaria, and this alteration was reflected in a change in seawater pH (Table 2).

Fluorescence measurements

The effect of diuron on seagrass photosynthesis was assessed by measuring change in chlorophyll fluorescence using a diving PAM fluorometer (Walz, Germany) (Schreiber *et al.*, 1994; Jones *et al.*, 1999). Chlorophyll fluorescence analysis was performed underwater, and was measured instantaneously using special clips to ensure a constant distance between the instruments fibre optic head and the seagrass leaf surface. The fluorescence signal was sampled at a standard position on the leaf (approximately in the middle of the adaxial surface on the second leaf from the plant meristem) (Ralph, 2000). Specimens were measured daily (*ca.* 1000 h).

Statistical analysis

Seagrass fluorescence data were analysed using a repeated measures analysis of variance (ANOVA) model. Two effective quantum yield ($\Delta F/F_{m'}$) measurements were taken from separate leaves from two plants per experimental aquaria at each measurement interval. These values were averaged as the samples were contained within the same aquaria, and were not independent. The averaged values from two independent tanks were used in the repeated measures ANOVA, where there were three seagrass species, five treatment levels (diuron concentration) and 12 sample times (day 0, at 2 h following diuron exposure, and then day number 1 to day number 10). Two-way ANOVA was used to compare effective quantum yield between seagrass species and diuron exposure concentrations at day 5 (the end of the diuron exposure period) and at day 10 (after 5 days recovery in uncontaminated seawater). The Tukey multiple comparison procedure with an experiment-wise type I error probability of 0.05 was used to locate any significant differences in effective quantum yield between seagrass species and diuron concentrations. A significant interaction was present for the day 5 analysis, and these data were re-analysed using a one-way ANOVA of effective quantum yield for each species separately. Dunnett's test was used to assess significance ($\alpha = 0.05$) of differences in effective quantum yield between control and diuron exposed plants for these analyses. Data were assessed for gross deviations from normality and, where necessary, transformed ($\log_{10}$) prior to analysis. All statistical computations were carried out using the SYSTAT V6 software package (Wilkinson, 1996).

Results

All three species of seagrass exhibited a rapid fluorescence response to diuron exposure (Fig. 1). Significant main effects as well as significant time × species and time × treatment (diuron concentration) interactions were recorded in the repeated-measures analysis of variance (Table 3) indicating the presence of significant variability in seagrass species response to diuron concentration over time. The effective quantum yield of all three seagrass species declined within 2 h of exposure to the most concentrated diuron treatments (10 and 100 µg l^{-1} diuron). Effective quantum yield from *H. ovalis* declined over the first 24 h of the experiment in plants exposed to even lower diuron concentrations (0.1 and 1.0 µg l^{-1} diuron).

Depression of effective quantum yield was maintained in *H. ovalis* over the rest of the 5-day exposure period. Effective quantum yield was also depressed in *Z. capricorni* at all diuron concentrations after 5 days of herbicide exposure (Fig. 2). Depressed effective quantum yield was only exhibited at the two highest diuron concentrations (10 and 100 µg l^{-1}) in *C. serrulata* after 5 days of herbicide exposure. Effective quantum yield at the end of the diuron exposure period (day 5) was significantly lower in plants exposed to highest diuron concentrations (10 and 100 µg l^{-1}) in all seagrass species (Dunnet's test, $\alpha = 0.05$) (Figs. 2 and 3; Tables 4 and 5). Effective quantum yield was still significantly depressed at the end of the recovery period (day 10) in all three seagrass species in plants that had been exposed to the highest (100 µg l^{-1}) diuron concentration (Fig. 3; Table 4).

Discussion

All concentrations of diuron tested showed some degree of toxicity to one or more of the exposed seagrass species, as indicated by a decline in effective quantum yield over the exposure period. This is in agreement with an earlier study which demonstrated that exposure of *H. ovalis* to diuron concentrations of 10 and 100 µg l^{-1} for

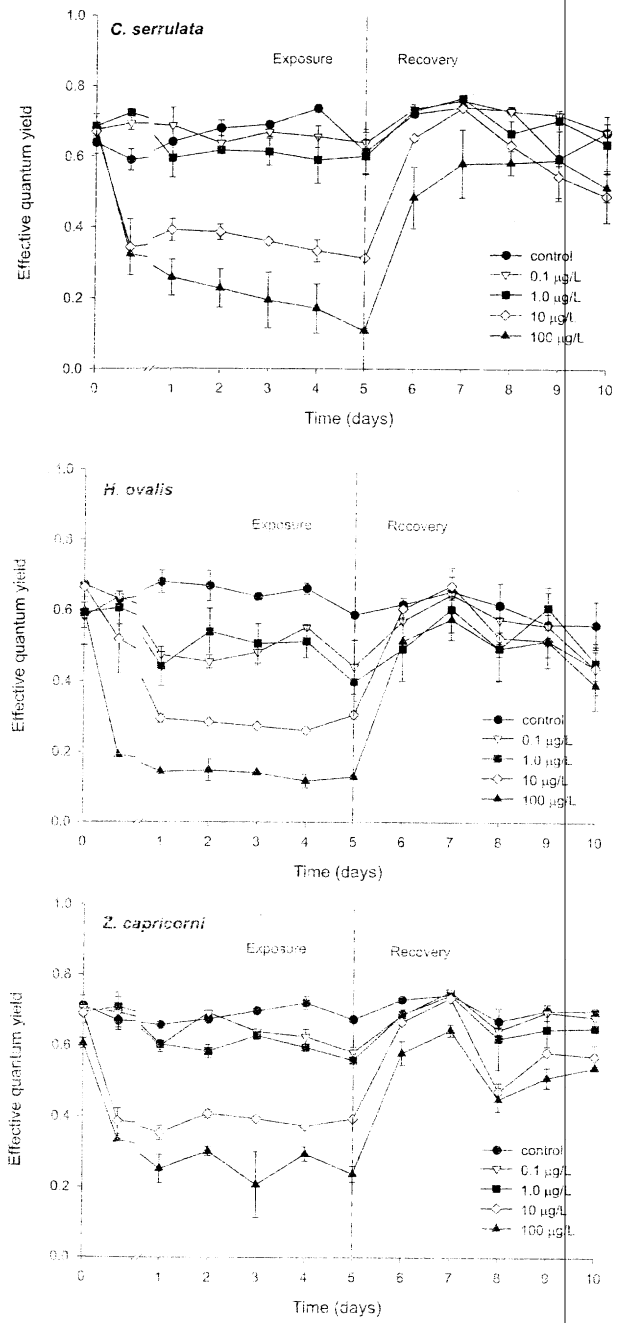

Fig. 1 Effective quantum yield response of *C. serrulata*, *H. ovalis* and *Z. capricorni* to diuron exposure. Units of effective quantum yield are arbitrary ($n = 2$, error bars = 1 SEM).

TABLE 3

Summary of the repeated-measures ANOVA of seagrass effective quantum yield over the 10-day experimental period ($n = 2$).

Component	*F*-value	*p*
Species	21.676	< 0.001
Treatment	68.274	< 0.001
Species × treatment	1.232	0.346
Constant (time only)	100.866	< 0.001
Species × time	3.080	< 0.001
Treatment × time	19.042	< 0.001
Species × treatment × time	1.404	0.032

72 h depressed effective quantum yield by 25% and 50%, respectively (Ralph, 2000).

Concentrations of 10 and 100 µg l^{-1} diuron reduced effective quantum yield in all three tested species of seagrass by 50–75% after a 5-day exposure period. Lower concentrations of diuron (0.1 and 1.0 µg l^{-1}) reduced effective quantum yield by 10% and 30% in *H. ovalis* and *Z. capricorni*, respectively, whereas effective quantum yield was essentially unaffected in *C. serrulata* exposed to the lower diuron concentrations at the end of the 5-day exposure period.

Recovery of photosynthetic ability was initially rapid in all three tested seagrass species following return to clean seawater. However, recovery was not necessarily

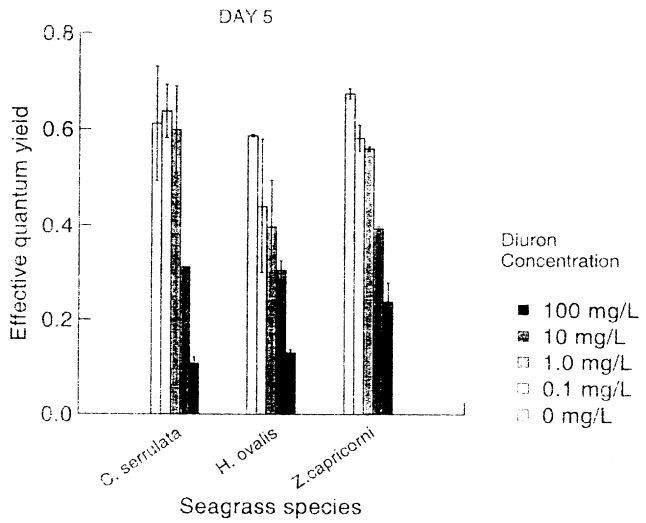

Fig. 2 Effective quantum yield response of *C. serrulata*, *H. ovalis* and *Z. capricorni* after 5-day exposure to diuron. Units of effective quantum yield are arbitrary ($n = 2$, error bars = 1 SEM).

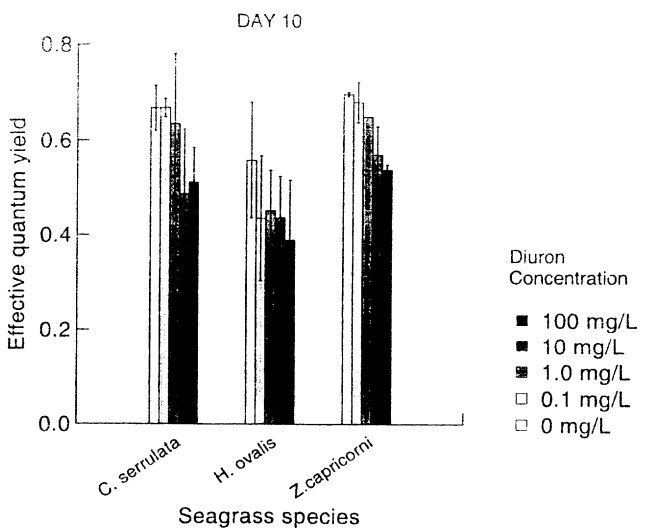

Fig. 3 Effective quantum yield response of *C. serrulata*, *H. ovalis* and *Z. capricorni* after 5-day recovery from diuron exposure. Units of effective quantum yield are arbitrary ($n = 2$, error bars = 1 SEM).

TABLE 4

Summary of two-way ANOVA of effective quantum yield, day 5 (end of diuron exposure) and day 10 (end of recovery period).[a]

Time	Factor	*F* ratio	*Post hoc* comparison
Day 5 (final exposure day)[b]	Species	24.171[c]	See Table 5
	Treatment	179.711[c]	
	Interaction	6.691[d]	
Day 10 (final recovery day)	Species	18.184[c]	*H. ovalis* *C. serrulata* *Z. capricorni*
	Treatment	5.923[d]	0 0.1 1.0 10 100
	Interaction	0.571	

[a] Concentrations or species joined by a horizontal line were not significantly different as determined by *post hoc* comparision.
[b] Log_{10} transformed prior to analysis.
[c] $p < 0.001$.
[d] $0.001 < p < 0.01$

TABLE 5

Summary of one-way ANOVA of effective quantum yield, by species, day 5 (end of diuron exposure) and *post hoc* comparison (all data log_{10} transformed prior to analysis).

Time	Species	ANOVA *F* ratio	Dunnet's test ($\alpha = 0.05$)
Day 5	*C. serrulata*	119.738[a]	Control $\gg$ 10, 100 µg l^{-1}
	H. ovalis	32.609[b]	Control $\gg$ 10, 100 µg l^{-1}
	Z. capricorni	89.844[a]	Control $\gg$ 10, 100 µg l^{-1}

[a] $p < 0.001$.
[b] $0.001 < p < 0.01$.

sustained, with all species exhibiting fluctuations in effective quantum yield over the 5-day recovery period. The overall decline in effective quantum yield in *H. ovalis* and to a lesser extent in *C. serrulata* at most diuron concentrations is of particular concern. *C. serrulata* and *Z. capricorni* are significant contributors to Queensland seagrass abundance along the majority of the Queensland coast (from Cape York to Hervey Bay) (Lee Long *et al.*, 1993; Kirkman, 1997). *H. ovalis* is also widely distributed over this region and *Cymodocea* and *Halophila* are both important food resources for dugong (*Dugong dugon*; Marsh *et al.*, 1982).

The immediate toxicity of diuron to seagrass and its potential ongoing impact is significant, as monitoring of diuron contamination in the nearshore environment along the Queensland coast has detected diuron at concentrations of 1–10 µg kg^{-1} (Haynes *et al.*, 2000). Highest concentrations of diuron were detected in subtidal sediments from the wet tropics, adjacent to the mouths of the Herbert and Johnstone Rivers. Highest northern Queensland agricultural usage (sugarcane) of the herbicide occurs in these two river catchments (Hamilton and Haydon, 1996). Partitioning models indicate that overlying water concentrations of diuron can reach 1 µg l^{-1} at sediment concentrations of 10 µg kg^{-1} (Haynes *et al.*, 2000), and this is within the range shown here to inhibit tropical seagrass photosynthesis.

The assistance of Ben Longstaff (University of Queensland) in collection of seagrass samples is gratefully acknowledged. The use of the outdoor aquaria facilities at CSIRO, Cleveland, is also gratefully acknowledged. Diuron analyses were carried out by the staff of Queensland Health and Scientific Services.

Anon. (1992) *The Australian Sugar Industry*. Australian Government Publishing Service. Canberra.

Beer, S., Vilenkin, B., Weil, A., Veste, M., Susel, L. and Eshel, A. (1998) Measuring photosynthetic rates in seagrass by pulse amplitude modulated (PAM) fluorometry. *Marine Ecological Progress Series* **174**, 293–300.

Dawson, S. P. and Dennison, W. C. (1996) Effects of ultraviolet and photosynthetically active radiation on five seagrass species. *Marine Biology* **125**, 629–38.

Hamilton, D. and Haydon, G. (1996) *Pesticides and fertilisers in the Queensland Sugar Industry – Estimates of Usage and Likely Environmental Fate*. Department of Primary Industries, Qld, Australia.

Haynes, D., Slater, J., Devlin, M. and Makey, L. (1998) Great Barrier Reef water quality monitoring and dugong protection areas. *Reef Research* **8**, 10–15.

Haynes, D., Müller, J. and 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**, 279–281.

Jones, R. J., Kildea, T. and Hoegh-Guldberg, O. (1999) PAM chlorophyll fluorometry: a new *in situ* technique for stress assessment in scleractinian corals, used to examine the effects of cyanide from cyanide fishing. *Marine Pollution Bulletin* **38**, 864–874.

Kirkman, H. (1997) Seagrasses of Australia. State of the Environment Australia Technical Paper Series. Department of Environment, Canberra.

Krause, G. H. and Weis, E. (1991). Chlorophyll fluorescence and photosynthesis: the basics. *Annual Review of Plant Physiology and Plant Molecular Biology* **42**, 313–49.

Lee Long, W. J., Mellors, J. E. and Coles, R. G. (1993) Seagrasses between Cape York and Hervey Bay, Queensland, Australia. *Australian Journal of Marine and Freshwater Research* **44**, 19–31.

Marsh, H., Channells, P. W., Heinsohn, G. and Morrissey, J. (1982) Analysis of stomach contents of dugongs from Queensland. *Australian Wildlife Research* **9**, 55–67.

Müller, J. F., Duquesne, S., Ng, J., Shaw, G. R., Krrishnamohan, K., Manonmanii, K., Hodge, M., Eaglesham, G. K. (2000) Pesticides in sediments from irrigation channels and drains in Queensland. *Marine Pollution Bulletin* **41**, 294–301.

Preen, A. R., Lee Long, W. J. and Coles, R. G. (1995) Flood and cyclone related loss, and partial recovery, of more than 1000 km^2 of seagrass in Hervey Bay, Queensland, Australia. *Aquatic Botany* **52**, 3–17.

Ralph, P. J. (2000) Herbicide toxicity of *Halophila ovalis* assessed by chlorophyll *a* fluorescence. *Aquatic Botany* **66**, 141–52.

Ralph, P. J. and Burchett, M. D. (1998a) Impact of petrochemicals on the photosynthesis of *Halophila ovalis* using chlorophyll flouresence. *Marine Pollution Bulletin* **36**, 429–36.

Ralph, P. J. and Burchett, M. D. (1998b) Photosynthetic response of *Halophila ovalis* to heavy metal stress. *Environmental Pollution* **103**, 91–101.

van Rensen, J. J. S. (1989) Herbicides interacting with photosystem II. In *Herbicides and Plant Metabolism*, ed. A. D. Dodge, pp. 21–36. Cambridge University Press, Cambridge.

Sandmann, G. and Bölger, P. (1986) Sites of herbicide action at the photosynthetic apparatus. In *Encyclopedia of Plant Physiology*, eds. L. A. Staehelin and C. J. Arntzen, Vol. 19, pp. 595–602. Springer, Berlin.

Schreiber, U., Bilger, W. and Neubauer, C. (1994). Chlorophyll fluorescence as a nonintrusive indicator for rapid assessment of *in vivo* photosynthesis. In *Ecophysiology of photosynthesis*, eds. E. D. Schulze and M. M. Caldwell, pp. 49–70. Springer, Berlin.

Schwarzschild, A. C., MacIntrye, W. G., Moore, K. A. and Libelo, E. L. (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.

Short, F. T., Burdick, D. M., Granger, S. and Nixon, S. W. (1996) Long-term decline in eelgrass, *Zostera marina* L., linked to increased housing development. In *Seagrass Biology*, pp. 291–298. Proceedings of the Second International Seagrass Workshop, Rottnest Island, Western Australia.

Walker, D. I. and McComb, A. J. (1992) Seagrass degradation in Australian coastal waters. *Marine Pollution Bulletin* **25**, 191–95.

Wilkinson, L. (1996) *Systat 7.0 for Windows: Statistics*. Microsoft, Chicago, IL, USA.