CRC 151

Cotton Catchment Communities CRC

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22nd August 2011

Luda Kuchieva
Research Office
The University of Sydney NSW 2006

Dear Luda,

Project Title: "Herbicide Test Kits & sustainability indicators extension".
Project Number: 5.01.98.09

I am pleased to advise that the above mentioned project has been reviewed and approved by the Cotton Catchment Communities CRC Ltd at the Company Management Team Meeting on the 27th July 2011.

Funding has been approved for $45,000.00 in 2011/12 and the carry over of unspent funds from Project 2.03.09 and is conditional upon the satisfactory performance against deliverable milestones as listed in the project application attached.

The funding has also been approved on the condition that the following milestone is inserted:
A marketing plan be developed by a consultant and Terms of Reference agreed to by the CEO of Cotton CRC. The marketing plan is to include the distribution of Diuron test kits to the market by June 2012.

Please find attached a Schedule 3 which we require your organisation to sign and return. Payment for 2011/12 will not be made until we receive the signed Schedule 3 Please ensure that this is returned within 30 days of receipt or the Cotton CRC will consider withdrawal of the funding offer. Please note that should your organisation commence activities relating to this project prior to submitting the signed Schedule 3, it does so at its own financial risk.

The Cotton CRC requires that the project leader completes a progress report due by 30th November 2011 in the project Management System "Centric" and the Final Report due by the 31st May 2012 on a template available on request.

Please contact Lynda George if you have any questions.

Yours sincerely,

Philip Armytage
Chief Executive Officer

c.c. Jane Macfarlane, Programme Leader
Dr Angus Crossan, Usyd
Professor Ivan Kennedy, Usyd
Margaret Wheeler, CRDC

Payment - approve
Review.

Cotton Catchment Communities CRC

FINAL REPORT

(due on completion of project)

Part 1 - Summary Details

Cotton CRC Project Number:

Project Title: Herbicide test kits & sustainability indicators

Project Commencement Date: 1/07/2008 **Project Completion Date:** 31/06/2011

Cotton CRC Program: The Catchment

Part 2 – Contact Details

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Supervisor: Professor Ivan Kennedy

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Part 3 – Final Report Guide (due within 3 months on completion of project)

Background

Our previous research (Cotton CRC project 2.3.04) showed that cotton growers considered they lacked the capability to monitor the quality of their farm water. Whilst nutrient monitoring kits were being trialled by growers and environmental officers, pesticide monitoring was too complex and expensive to be considered for adoption in a BMP program or environmental management system (EMS). Also, without the capability for assessment of on-farm water quality for reporting the validation of BMP and CRC milestones 2.3.2 could not be achieved.

However, there is a continuing need for the use of environmentally hazardous herbicides by industry and an increase in regulatory review and environmental pressure with respect to pesticide use.

A clear hypothesis developed: Could rapid and cost effective analytical tools be developed that could be used in the myBMP program and other environmental management systems (EMS).

The research team has a history of successful NRM related projects, particularly in the field of environmental chemistry, analysis and risk assessment to support resource quality research and development of agro-ecosystems. The team also had considerable experience in the development of ELISA technology; Enzyme-Linked Immunosorbent Assay, which relies on the immune response of mammals to detect chemical targets.

This project aimed to build on existing capability and develop rapid portable test kits for pesticides based on ELISA technology (sensitive and cost effective diagnostic tools that). It was envisaged that these simple test kits could be designed for use in BMP systems to monitor target pesticides and ensure on-farm water quality. They would need to cost significantly less than commercial analyses and provide information immediately, thereby enabling cost-effective environmental management. Potential benefits for users include piece-of-mind regarding water quality with respect to conforming to regulation, subsequent uses of water (eg aquaculture), or to verify environmental stewardship (BMP) and improve brand strength. Such tools and their application were also expected to provide the framework to develop sustainability indicators.

Objectives

1. To develop a simple field test kit for priority chemicals to facilitate cost effective environmental management.

   In summary, this "proof-of-concept" project was a success with ELISAs and gold labelled rapid test strips for the determination of diuron, fluometuron, and prometryn developed. This project has delivered commercial ready rapid tests

using the active ester method. The conjugates of hapten 4C-KLH, hapten 6C-KLH, hapten 4C-BSA and hapten 6C-BSA were all used as immunogens. Duplicate male rabbits were immunized with each immunogen and it was found that rabbits immunized with the conjugate of hapten 4C-KLH produced the most specific antibody.

1.3 Development of ELISA

1.3.1 Optimisation of ELISA

For the competitive indirect ELISA (CI-ELISA) microtiter plates were coated with 100 μL per well of hapten 4C-OVA (0.1 μg mL^{-1} in 50 mM carbonate buffer, pH 9.6) and stored overnight at 4 °C. Plates were then washed with PBST and blocked with 200 μL per well of 0.5% milk powder diluted in PBS. 50μL per well of diuron and 50 μL per well of rabbit anti-diuron pAb (1 μg mL^{-1}, diluted in PBS) were then added

before the plates were shaken gently for one minute before incubating for 1 h at 37°C.

The plates were then washed with PBST, and 100 μL per well of 10,000-fold diluted

secondary antibody were added and incubated at 37°C for 30 min. After a final

washing step, 150 μL of substrate were added the each well and incubated for 15 min. The enzyme reaction was stopped with 1.5 M H_2SO_4 and the plates were read at 450 nm (reference 650 nm).

For the competitive direct ELISA (CD-ELISA) microtiter plates were coated with 100 μL per well of rabbit anti-diuron pAb (2.0 μg mL^{-1} in 50 mM carbonate buffer, pH 9.6) and stored overnight at 4 °C. The plates were then washed with PBST and blocked with 200 μL per well of 0.5% milk powder diluted in PBS. 50 μL per well of diuron and 50 μL of diuron enzyme-tracer 10,000-fold diluted in PBS was then added. The plates were shaken gently for one minute before incubating for 1 h at

37°C, then washed with PBST. 100 μL of substrate were then added to each well.

After 30 min the reaction was stopped with 50 μL of 1.5 M H_2SO_4 per well, and the absorbance was read at 450 nm (with reference at 650 nm).

1.3.2 Evaluation of ELISA

1.3.2.1 Sensitivity

Assay	LOD (IC_{15}) (μg/L)	Sensitivity (IC_{50}) (μg/L)	Detection range (μg/L)
CI-ELISA	0.05±0.008	0.48±0.09	0.01-100

Water source	Sample No.	Con. detected (µg/L)	Con. Spiked (µg/L)	Con. determined after spiked (µg/L)	Recovery (%)
Gwydir River at Yarraman Bridge	1	0.2	2	2.8	130
	2	0.21	2	2.7	124.5
	3	0.17	2	2.6	121.5
	4	0.2	2	2.6	120
	5	0.25	2	2.8	127.5
Mehi River at Bronte	6	0.18	2	2.5	116
	7	0	2	2.4	120
	8	0.18	2	2.7	126
	9	0.07	2	5	246.5
Tailwater Auscott	10	3.1	2	6	145
	11	3.1	2	6	145
	12	3.1	2	4.5	130
Thalaba Creek Merrywinebone	13	0.14	2	2.6	123
	14	0.14	2	2.76	131
	15	0.17	2	2.8	131.5
Moomin Creek at Iffley	16	0.11	2	2.5	119.5
	17	0	2	2.25	112.5
	18	0	2	2.2	110

1.4 Development of colloidal gold-based immunochromatographic (ICG) strip

1.4.1 Summary of optimised parameters of colloidal gold-based immunochromatographic (ICG) strip:

- Diameter of colloidal gold: 17 nm
- pH of the colloidal gold solution: 9.0
- Amount of antibody conjugated to 1 mL of colloidal gold: 20 µg
- Nitrocellulose (NC) membrane: Millipore 180 sec/4 cm
- Dilution buffer: 1×PBS
- Coating antigen: hapten 6C-BSA conjugate
- Treatment of sample pad: soaked in the solution containing 5% (w/v) PVP, 1% BSA (w/v) and 0.1% (v/v) Tween-20, then dried for 2h at 50 °C.
- The conjugate pad and NC membrane were not treated.

1.4.2 Evaluation of colloidal gold-based immunochromatographic (ICG) strip

1.4.2.1 Sensitivity

	16	+	+	0.11
Moomin Creek at Iffley	17	+	+	nd
	18	+	+	nd

Note: +: an obvious red band was observed (below the limit of detection); ±: a faint band was observed; -: no band was observed (above the target concentration); nd: not detected.

Termination	50 μL of 2.5 M H_2SO_4 /well	50 μL of 2.5 M H_2SO_4 /well

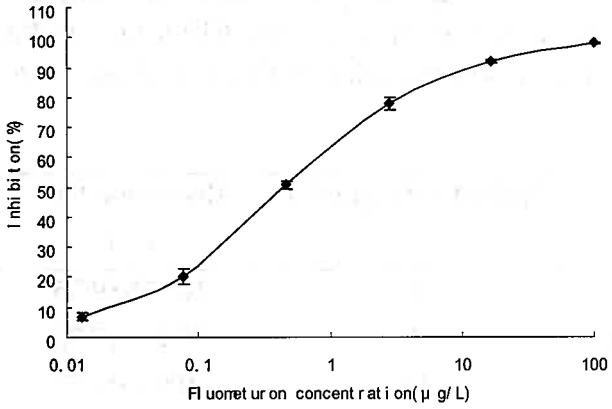
2.3.2 Evaluation of ELISA

2.3.2.1 Sensitivity

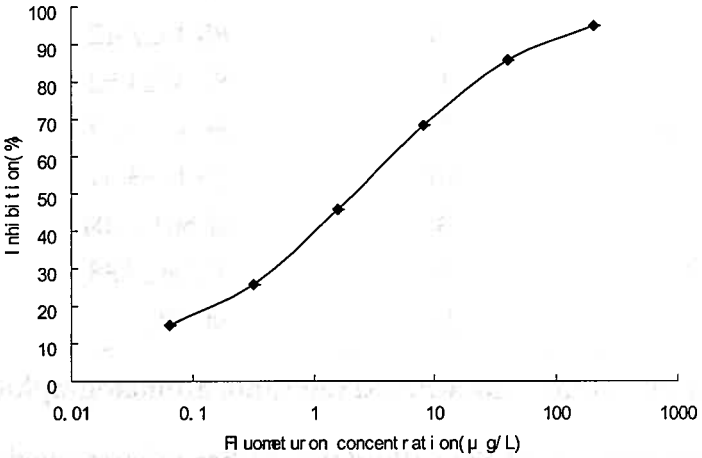
Assay	LOD (IC_{15}) (μg/L)	Sensitivity (IC_{50}) (μg/L)	Detection range (μg/L)
CI-ELISA	0.07±0.082	2.04±0.022	0.064-200
CD-ELISA	0.02±0.028	0.58±0.011	0.012-100

It can be observed that the CD-ELISA was more sensitive and was therefore chosen for further study.

The standard curve of CD-ELISA:



The standard curve of CI-ELISA:



2.3.2.2 Specificity

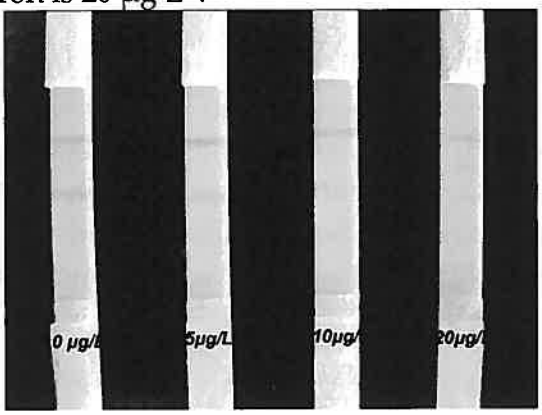
Cross-reactivity (CR) was test used CD-ELISA. The results are shown in the table below. Among all other phenylurea herbicides tested, only neburon showed a high CR of 69.4%. It was expected because of the presence of the butyl side chain which appears to mimic the spacer arm of the immunogen. Diuron and linuron showed CRs of 19.9% and 3.91%, respectively. The remaining phenylurea herbicides tested did not shown a response (<0.01%).

- The sample pad was pretreated by soaking in the solution containing 5% PVP, 1% BSA and 0.1% Tween-20
- The NC membrane was blocked with 1% BSA for 1 h at RT, washed three times with 10 mM PBST, dried at 37 °C oven.
- The conjugate pad wa not treated.

2.4.2 Evaluation of colloidal gold-based immunochromatographic (ICG) strip

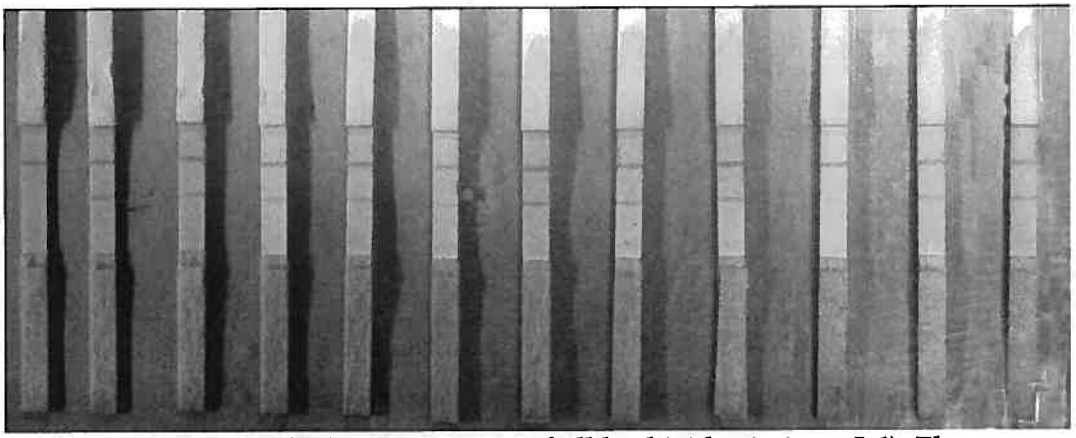
2.4.2.1 Sensitivity

In this study, various concentrations of fluometuron standard (0, 5, 10 and 20 μg L^{-1}) were prepared in PBS. As shown followed, when the concentration of fluometuron is above 20 μg L^{-1}, there is no band on the test line. Therefore, the visual limit of detection for fluometuron is 20 μg L^{-1}.



2.4.2.2 Specificity

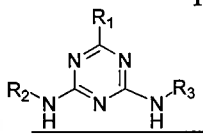
A series of herbicides were used to test the specificity of the assay. The results are shown below. It is observable that there was no interference of the other herbicides except chlortoluron and neburon, even at the concentration as high as 1mg L^{-1}.



Note: from left to right (concentration of all herbicides is 1 mg L^{-1}): Fluometuron, Thidiauron, Fenuron, Monolinuron, Chlorbromuron, Neburon, Difenoxuron, Metobromuron, Monuron, Chlortoluron, Linuron and Buturon

3 Prometryn

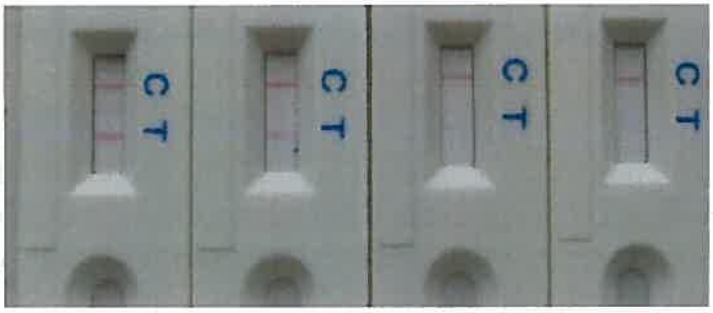
analytes containing isopropylamine(s) were higher than 10%, and the three analytes with two isopropylamines on both R_2 and R_3 groups (prometryn, propazine, and prometon) provided considerable cross reactivities (higher than 50%). Furthermore, since the R_1 group of the hapten was used to link to the protein to form the immunogen, it was less exposed to the antibodies during the process of immunization. Accordingly, it was considered that R_1 group had little contribution to the antibody recognition during the immunoassay. However, the result indicated that the antibody recognize analytes with methylthio groups on the R_1 position more than the analytes with other groups. This is probably because the three-carbon-atom spacer arm of the hapten exposed the methylthio group to the antibodies, and formed corresponding paratopes.



Analyte	R_1	R_2	R_3	IC_{50} (ng/mL))	cross-reaction (%)
prometryn	S-CH_3	$CH(CH_3)_2$	$CH(CH_3)_2$	0.3	100
Propazine	Cl	$CH(CH_3)_2$	$CH(CH_3)_2$	0.42	71.4
Prometon	O-CH_3	$CH(CH_3)_2$	$CH(CH_3)_2$	0.6	50
Ametryn	S-CH_3	CH_2CH_3	$CH(CH_3)_2$	1	30
atrazine	Cl	$CH(CH_3)_2$	CH_2CH_3	1.5	20
Terbumeton	O-CH_3	CH_2CH_3	$CH(CH_3)_3$	3	10
Desmetryn	S-CH_3	CH_3	$CH(CH_3)_2$	3	10
simazine	Cl	CH_2CH_3	CH_2CH_3	6.3	4.7
Simetryn	S-CH_3	CH_2CH_3	CH_2CH_3	6	5
Terbuthylazine	Cl	CH_2CH_3	$C(CH_3)_3$	5.5	5.4
Terbutryn	S-CH_3	CH_2CH_3	$C(CH_3)_3$	14	2.4
Cyanazine	Cl	$CH(CH_3)_2$	$CCN(CH_3)_2$	100	<0.3

4.3.2.3 Accuracy and precision

Three samples spiked with prometryn were analysed over a 3 day period with 3 assays per day, respectively. Spiked river water was diluted 10-fold with PBS before analysis. The soil sample (1 g) was mixed with 2 mL PBS containing 30% methanol and vortexed for 5 min. The mixture was then centrifugated at 5000 rpm for 5 min, afterward the supernatant was removed and diluted with PBS 10-fold before analysis by ELISA. The plant sample (1 g) was mixed with 2 mL of PBS containing 40% methanol and vortexed vigorously for 5 min. The mixture was then centrifugated at 5000 rpm for 5 min, afterward the supernatant was diluted with PBS 10-fold before analysis by ELISA. The coefficients of variation (CV) were all below 15%. The results are shown below.



Note: spike concentration of prometryn (from left to right) is 0, 5, 10, 20 μg L^{-1}

3.4.2.2 Specificity

A serial of triazine pesticides were used to test the specificity of the assay. The cross-reactivities of prometryn, atrazine, propazine and prometon were all nearly 100%, while the cross-reactivities of terbumeton, terbuthylazine, ametryn, and terbutryn were relatively small. While simetryne, simazine, desmetryne and cyanatryne showed minimal indication of cross reaction with the antibody. These results show successful proof-of-concept and would be further characterised during the manufacture and associate development and quality testing.

Outcomes

5. Describe how the project's outputs will contribute to the planned outcomes identified in the project application. Describe the planned outcomes achieved to date.

   The development of these three rapid analytical tests provides the basis for on-farm or ecological water quality testing for herbicides. These tests can be used to begin establishing baselines for farms or for testing adjacent ecological areas to provide a robust ecological management framework.

   The main outcome is that such management is now feasible because of much lower analytical costs and immediacy of data collection to enable effective management. For example, site specific information can be used to monitor chemical use patterns, identify if remediation is required, or to document good practice and certify water quality for other uses such as aquaculture or releasing to local ecosystems.

   The project team is in the process of developing the commercial plan which involves the trialling of the diuron test in Queensland and building capacity of QuickTest Technolgies Pty Ltd, a new spin-off company focuses on development of rapid test technology and its applications.

   It is envisaged that the outputs from this project will therefore be adopted to aid environmental management in other Australian agricultural industries and also begin to return royalties to the Cotton CRC and its stakeholders. The use of the technology is available to improve the management of herbicide residues by identifying and documenting good practice and "hotspots" where investment to minimise risk can be focused. This robust information could be used to provide

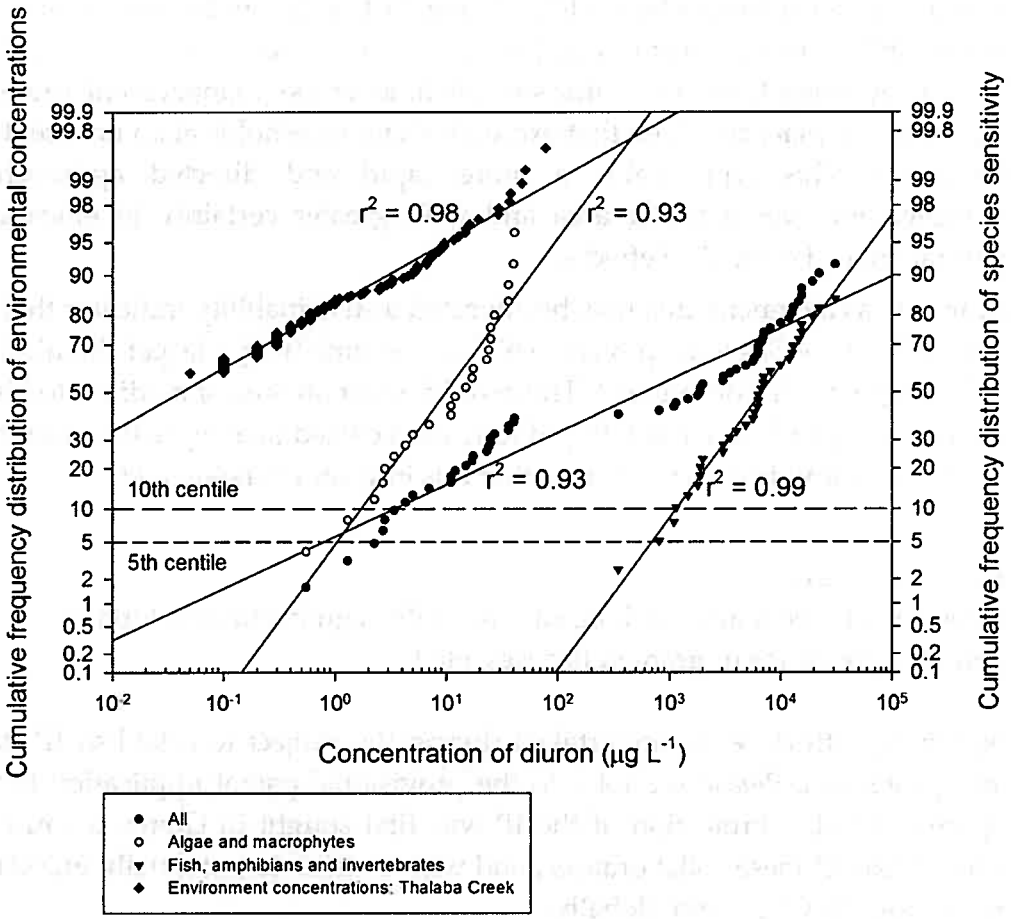


Figure 1 Example of relating distribution of diuron environmental concentrations (µg L^{-1}) at Thalaba Creek, Merrywinebone (Gwydir River catchment, NSW) (closed diamonds) with species sensitivity distributions of All (closed circles); Fish, amphibians and invertebrates (closed triangles); and algae and macrophytes (open circles). Source: Burns 2011

The task of generating exposure distributions required in ERA using conventional grab (e.g. El-Kabbany *et al.*, 2000; Comoretto *et al.*, 2007) and passive sampling (e.g. Hyne *et al.*, 2004; Petty *et al.*, 2004) techniques are an expensive undertaking (Suter II, 2007). These methods for characterising exposure are further limited in their ability to 1) characterise the full range in exposure at a specific location, and 2) characterise the duration of exposure that should translate in to the defined effects.

Rapid tests have the capacity to address these issues in a number of ways:

(a) They may more directly be used to generate distributions of frequency of concentration threshold exceedence that can readily translate into risk. For example the current configuration of the diuron test, a negative sample indicates protection of at least 95% of all species;

(b) They may also be used to characterise exposure pulses by rapidly providing an indication of how long an exposure exceeding the toxicity threshold may last and can clarify the relevance of exposure with respect to acute and chronic exposure durations. These are more relevant for choosing ecotoxicity data in the development of species sensitivity distributions, especially where compounds have shown significant recovery when exposed to the most sensitive organisms (see for example Burns, 2011); and

The potential impact for the cotton industry is high and will depend on successful adoption of the technology into myBMP. Farm runoff, local streams and rivers, and if required, field runoff water can now all be easily analysed (within five minutes), providing ecological robustness not yet observed in the global market. For example, Australian cotton growers will be able to provided compelling evidence (in the form of reproducible data) that their activities do not pose risks to the ecosystem. This project has provided the industry with two key management tools, rapid tests and a framework for their application to provide a sustainability indicator.

Application of these tools this will strengthen the Australian cotton brand, offering global leadership and associated promotional benefits, and also provide a strategic edge for future regulatory reviews on industry practice (or the pesticides required for economic sustainability).

Extension Opportunities

8. Detail a plan for the activities or other steps that may be taken:
   (d) to further develop or to exploit the project technology.

   The project team is currently undertaking a further year of commercially focussed activity to better define the potential for the project technology. These activities are focused on sugar cane catchments in Queensland, where concerns on the use of diuron have been raised recently. These catchments flow into the sensitive Great Barrier Reef Marine Park and we believe the rapid tests can assist by identifying areas of ecological concerns and then be used to confirm management responses adjacent to these areas.

   QuickTest Technologies is continuing to building its capacity to ensure the project technology is exploited within a highly focused and sustainable commercial entity. Other targets are being developed to provide a broader range of testing capability, with the aim of offering a "multi-residue" kit. This would include the targets developed in this project and could reach a much broader market.

   (e) for the future presentation and dissemination of the project outcomes.

   Once IP strategies are finalised, the details of the project are expected to be published to assist dissemination of the technology and demonstrate the robustness of the products.

   (f) for future research.