Tasmanian Investigation
What is the Toxin and what are its characteristics?

Purpose
Toxicity Identification and Evaluation. The purpose of the following series of tests is to identify and physically characterise the toxin(s).

Question 1: Is the toxin(s) stable through time?

Results
Initial tests revealed toxin(s) was present. Toxin(s) dissipated over the next two tests indicating that the toxin(s) breaks down through time (Table 6).

Advanced Analytical ran tests for man-made pesticides, man-made herbicides and general screens. Detection limits were around 1 µg/L (i.e. microgram per litre). No man-made chemicals were detected. According to the literature, detection limits were adequate for most chemicals except pyrethroids.

Conclusions
The toxin(s) break down through time. Tests need toi be run on fresh samples.

Question 2: Do the toxin(s) occur naturally in undisturbed areas?

Location Sampled
Creek feeding into Lake Augusta (a World Heritage Area).

Results
No toxin was identified from this area.

Conclusions
No naturally occurring toxin was identified. However, surrounding vegetation was grasses. Test to be repeated downstream of temperate Eucalypts.

Question 3: Is the toxin(s) a metal (like Copper or Zinc)?

Experiment
Raw water samples were tested on the 4th of March 2005. This test involves the addition of a chelating agent called EDTA. If a metal is present, then EDTA will settle it out of the water column reducing or removing toxicity. This method is best suited to di-valent metals like Copper and Zinc (removing toxicity completely) but will also reduce toxicity associated with tri-valent and mono-valent metals.

Results
Toxicity was not reduced or removed.

Conclusions
The toxin(s) is not a metal.

Question 4: Is the toxin(s) volatile (like petroleum products or fragrant oils)?

Experiment

This test involves bubbling nitrogen through the sample. If a volatile substance is present, then aeration will evaporate it out of the water column reducing or removing toxicity.

Results
Toxicity was not reduced or removed (Table 9).

Conclusions
The toxin(s) is not a volatile substance.

Question 5: Is the toxin(s) dissolved in the water column or attached to particulate matter?

Experiment
Raw water samples were tested on the 4th, 7th and 9th of March 2005. These tests involve filtering the sample and testing the material that is removed as well as the remaining filtered water. If the toxin(s) is attached to particulate matter, then filtration or centrifuge will remove toxicity. If it is dissolved in the water column, filtration will not remove toxicity.

Results
Toxicity was reduced or removed using centrifuge and glass fibre filtration. Various test indicated that the toxin(s) was not attached to coarse (i.e. clearly visible) material but was attached to very fine particulate matter. Filtration reduced toxicity but did not always completely remove it (as indicated by the addition of PBO, discussed in later tests.

Conclusions
The toxin(s) is predominantly attached to very fine particulate matter.

Question 6: Is the toxin(s) an organic chemical?

Experiment
Raw water samples were tested on the 9th of March 2005. These tests involve filtering a centrifuged sample through a C18 column (a type of activated carbon filter) and testing the material that is removed as well as the filtered water. This test could not be run until the appropriate clean up method had been established (Test Number 8). Toxicity was greatly reduced by this time (Test Number 4) so this test will be repeated with a fresh sample.

The methanol extraction of toxin(s) from the C18 column concentrates the toxin(s) from the original sample (usually 1 litre of water) into 2ml of methanol. This methanol is then added back to water allowing the concentration to increase. The add back concentration is the concentration of toxin(s) compared to the amount present in the original sample. Thus “4x” means four times the concentration that was in the original sample.

Results
A toxin(s) was isolated and eluted from the column using methanol. Methanol extraction also removed toxin(s) from the material trapped by glass fibre filtration.

Conclusions
The toxin(s) is methanol soluble. The toxin(s) is probably an organic chemical.

Question 7: Is the toxin(s) enhanced or inhibited by the addition of PBO?

Experiment
Raw water samples were tested on the 4th, 7th and 9th of March 2005. These tests involve the addition of PBO to a variety of filtered samples. If a pyrethroid-like substance is present, PBO dramatically increases toxicity. If an Organo-phosphate is present, PBO removes or reduces toxicity.

Results
Addition of PBO to a variety of samples enhanced toxicity. In the case of C18 methanol extract, toxicity was enhanced by a factor of approximately 16.

Conclusions
The toxin(s) is probably an organic pyrethroid-like substance.

Question 8: Is the toxin(s) an organic chemical?

Experiment
Raw water samples were tested on the 24th of March 2005. These tests involve filtering a centrifuged sample through a C18 column and testing the material that is removed as well as the filtered water.

Results
Toxicity was removed by filtration through the C18 column. Addition of PBO to the filtered water did not enhance toxicity. Filtration through glass fibre also reduced toxicity, but, addition of PBO revealed toxin(s) had passed through the glass fibre filter.

Conclusions
The toxin(s) is an organic chemical.

Question 9: Is the toxin(s) enhanced or inhibited by the addition of PBO?

Results
Addition of PBO to all samples enhanced toxicity. Toxicity is enhanced by a factor of approximately 6.

Advanced Analytical did not identify any pyrethroids at a detection limit of 1 microgram per litre.

Conclusions
The toxin(s) is probably a pyrethroid-like substance. Chemical detection limits need to be improved.

Question 10: Is the toxin(s) a pyrethroid?

Experiment
Six litres of concentrated surface water were collected for immediate extraction onto C18 columns. A sub sample of this concentrated water was put to one side to be checked on arrival in Sydney. Tests on these sub samples indicated that a large

amount of toxin(s) was captured. The EC50 (i.e. the concentration at which 50% of test organisms die) was 9.4% for the South George sample and 5.7% for the Upstream of the Town Water Intake sample. This means the samples could be diluted by a factor of 11 and 17 respectively and still be toxic.

To isolate and concentrate the toxin(s) further, a technique called methanol fractionation was used. The toxin(s) that was taken out of the water by passing it through C18 columns in the field (the toxin(s) sticks to the carbon in the column) was then subjected to this isolation method. The toxin(s) was then removed from the C18 column by passing various dilutions of methanol through the column (organic chemicals are methanol soluble). Initially, 25% methanol mixed with clean water was passed through the column. The total volume of each methanol dilution (fraction) was 2ml. Next, 50% methanol was passed through the column; then 75% methanol; then 80%; 85%; 90%; 95% and finally 100%.

A toxin will usually be isolated in one or two methanol fractions. This concentrates the toxin by a factor of 250 (if isolated in two fractions) or 500 (if isolated in one fraction). Thus, by the time the toxin is submitted to the laboratory, the concentration factor is several thousand times the concentration initially in the raw water column (the initial concentration factor, 11 to 17, multiplied by the concentration factor associated with methanol extraction, 250 to 500), which should make it very easy to identify.

Results
Toxic methanol extracts were identified and will be discussed in the next section. The methanol extracts were submitted to Advanced Analytical Australia and screened. Advanced Analytical reported that no chemicals, either natural or man-made, were present in the methanol extract.

Conclusions
A discussion was then held between Dr Scammell (one of the clients), Dr Krassoi (Ecotox Services), Dr Elkhart (Advanced Analytical Australia) and Dr Tottszer (Advanced Analytical Australia) to determine which types of chemicals could not be detected by their equipment. Dr Elkhart advised that non-polar chemicals like proteins, peptides and amino sugars would stick to the glass in part of the equipment and therefore be missed.

Question 11: What methanol fraction can the toxin(s) be isolated in?

Results
Add Back revealed that toxin(s) was not present in the 25% or 50% methanol fractions. Some toxin(s) was present in the 75% fraction, while toxin(s) was clearly present in the 80%; 85%; 90%; 95% and 100% fraction. Adding PBO did not result in enhanced toxicity for either site. These results suggest that multiple methanol soluble toxins are present.

Conclusions
The pyrethroid-like substance observed previously was now gone but a complex methanol soluble group of toxins remain, as evident by toxicity being spread over six methanol fractions.

Question 12: Are the toxins non-polar molecules?

Sample Tested by Proteomics Laboratory
Toxic Methanol fractionation SG A05/0594/4

Results
Measurable quantities of many water soluble and water insoluble amino acids were found.

Conclusions
The toxins are potentially proteins, peptides or amino sugars. Many organisms produce toxic peptides and proteins including blue green algae, bacteria and fungi.

Although chemical identification may not be possible, the source of the biological toxins may be able to be identified making management of the toxins source possible.