Tasmanian Investigation
Test Number 16

Explanation
Discussion with Professor Winder and Dr Khalil from the University of NSW indicated that testing on human cell lines was possible and that the University had a commercially available test.

It was decided that it would be extremely useful to identify and calibrate impacts on human cell lines against our other test organisms. This would provide a useful complement to tests 1 & 3 (Sea Urchin development tests).

Sampling Details
19 August, 2005.

Locations Sampled
South George.

Purpose
Are the toxicants toxic to human cell lines?

Investigating Laboratory(s)
Chemical Safety and Applied Toxicology Laboratories, University of New South Wales (UNSW)

Sample Provided to the UNSW
A 2ml toxic methanol fraction (covering the 75% to 100% methanol soluble toxicants) containing 2.5 toxic units (can be diluted by a factor of 2.5 and produce 50% mortality) when tested on Cladocerans.

Method
Toxicants in 2ml of methanol were Added Back to sterile water to make up the original concentration. Liver cells, lung cells and skin cells were then exposed to a range of dilutions of the toxicants for 24 hours. Cell mortality was then assessed. Each cell line was repeated three times providing a total of nine dilution curves.

Results
The toxicants killed all cell lines within the 24 hour period. Indeed, initial results at three hours exposure indicated rapid toxicity to liver cells. Liver cells were more sensitive than Cladocerans (Graphs 12 to 14). Skin cells correlated closely with the level of toxicity observed in Cladocerans (Graphs 15 to 17). By comparison, lung cells were the least sensitive cell lines (Graphs 18 to 20).

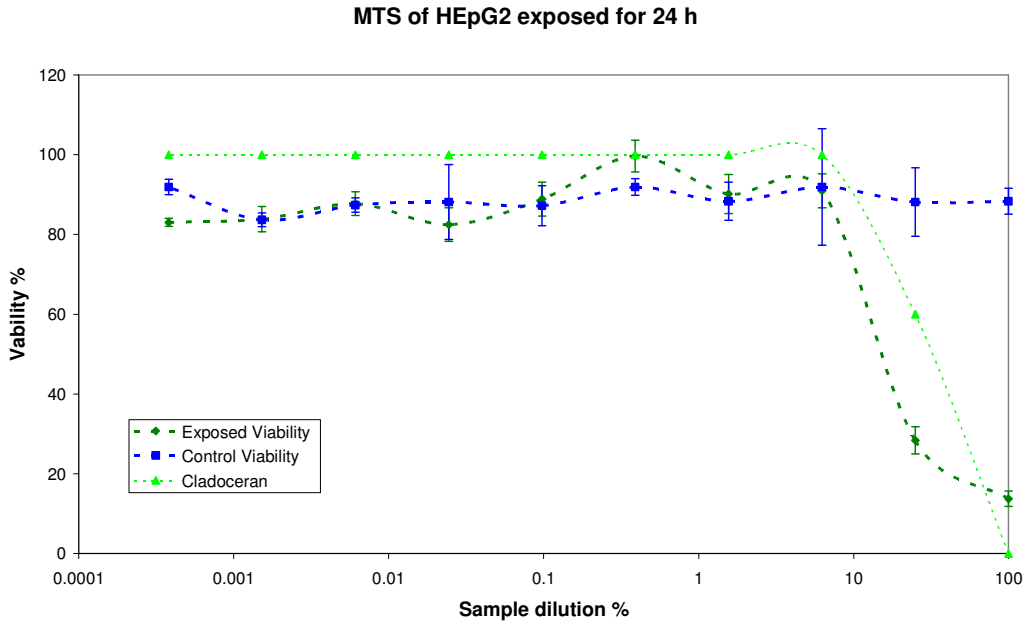
Conclusions

Human liver cell lines are killed by the toxicants at lower concentrations when compared with Cladocerans. Skin cells have similar sensitivity to the toxicants as Cladocerans. Lung cells are the least sensitive cell line. Oysters and Sea Urchins are more sensitive than the Cladocerans.

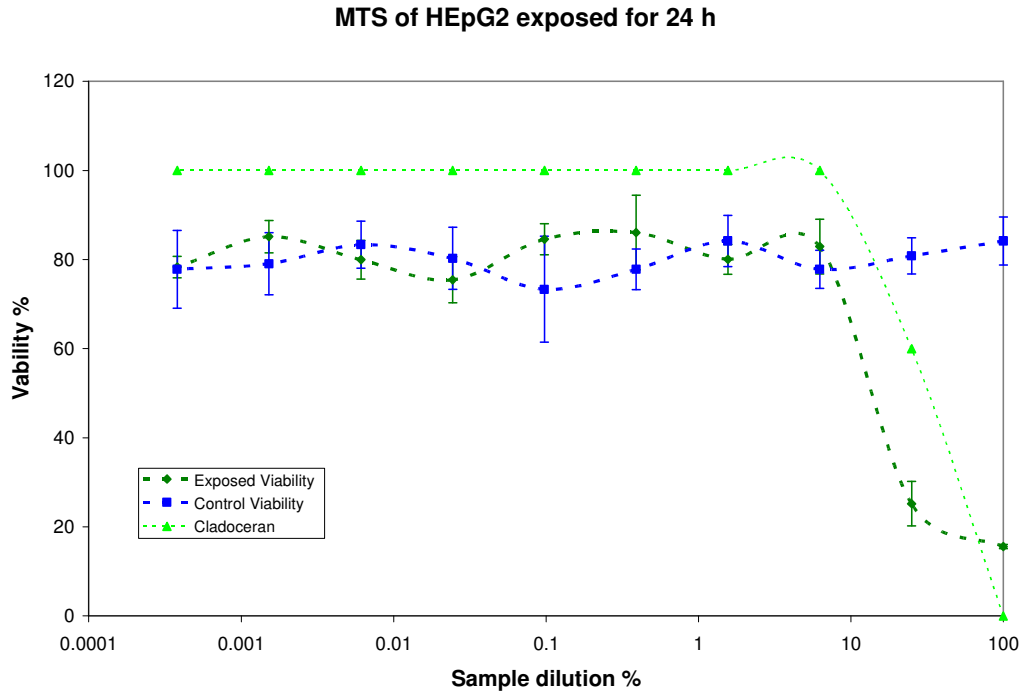
Status

The use of three replicates on three cell lines confirms the result. No further test required. The result also confirms that the prior Sea Urchin development test was an appropriate model for human cell toxicity. According to the scientific literature, the Sea Urchin test is also an appropriate model for human cancer risk.

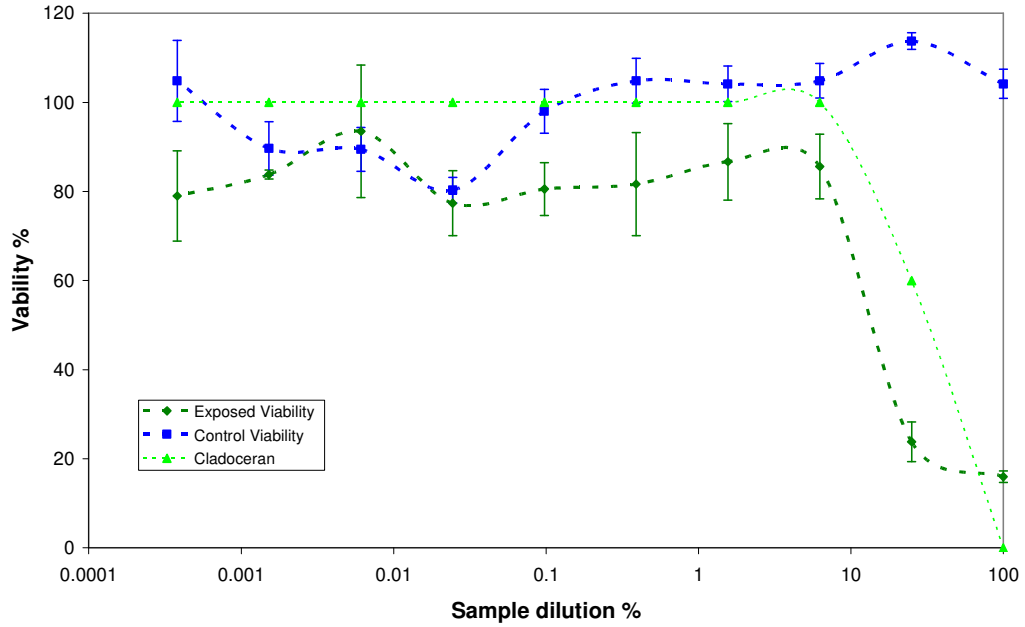
Graph 12: Dilution curves showing toxicity to liver cells, replicate 1, compared with reference cell lines and Cladocerans (note the x-axis is a log scale).



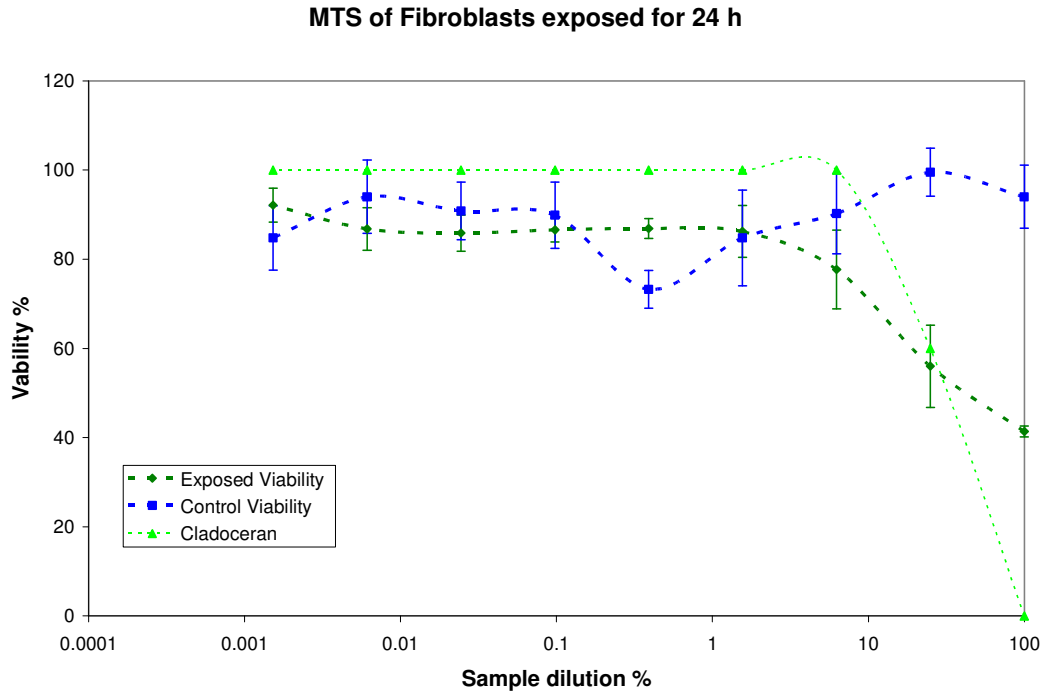
Graph 13: Dilution curves showing toxicity to liver cells, replicate 2, compared with reference cell lines and Cladocerans (note the x-axis is a log scale).



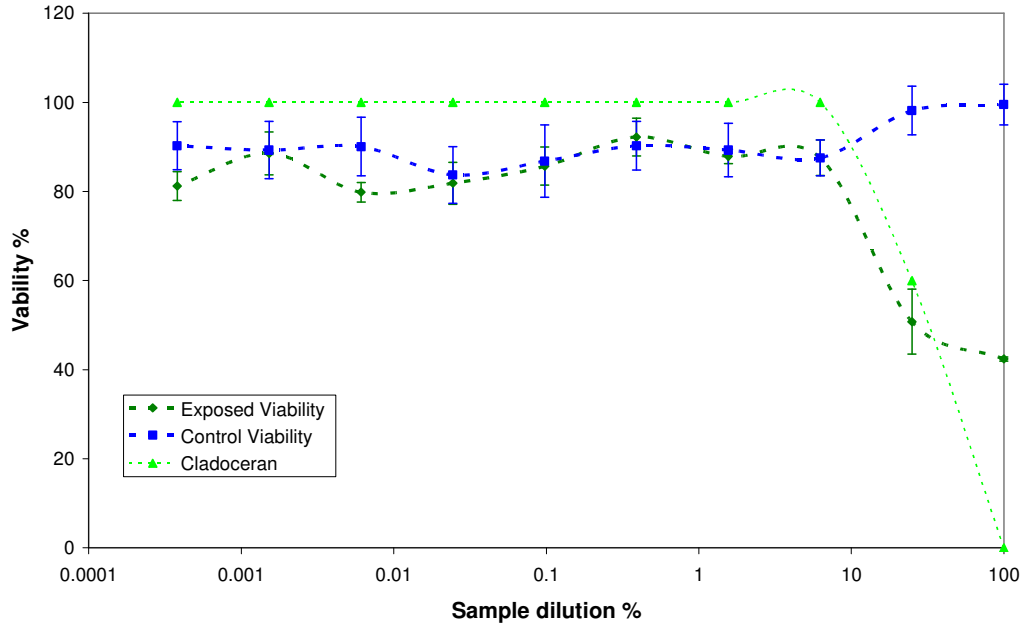
Graph 14: Dilution curves showing toxicity to liver cells, replicate 3, compared with reference cell lines and Cladocerans (note the x-axis is a log scale).



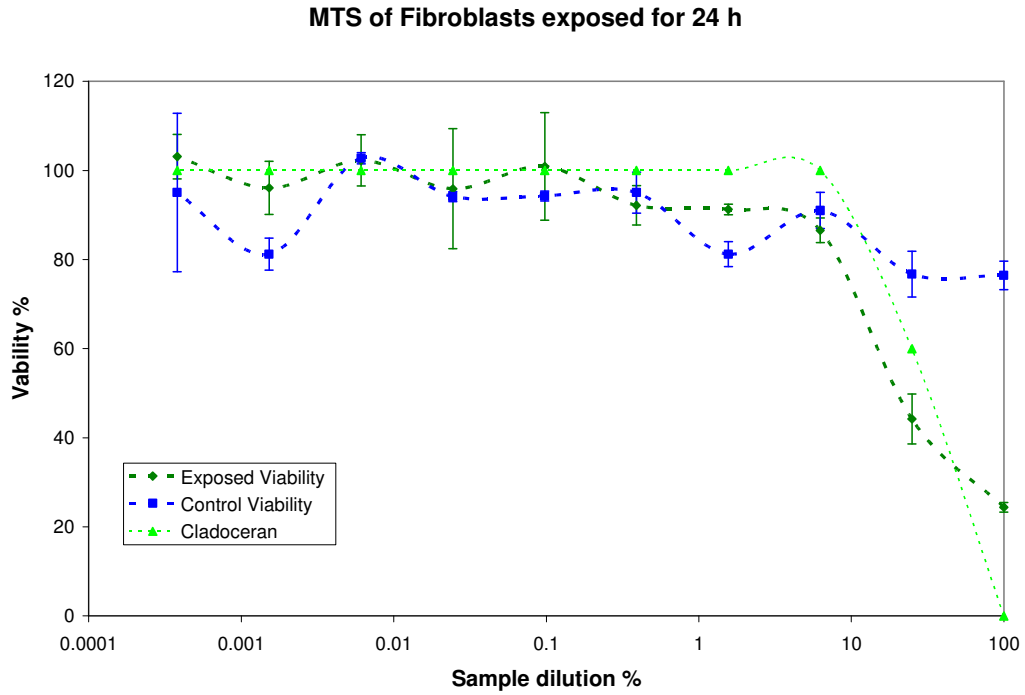
Graph 15: Dilution curves showing toxicity to skin cells, replicate 1, compared with reference cell lines and Cladocerans (note the x-axis is a log scale).



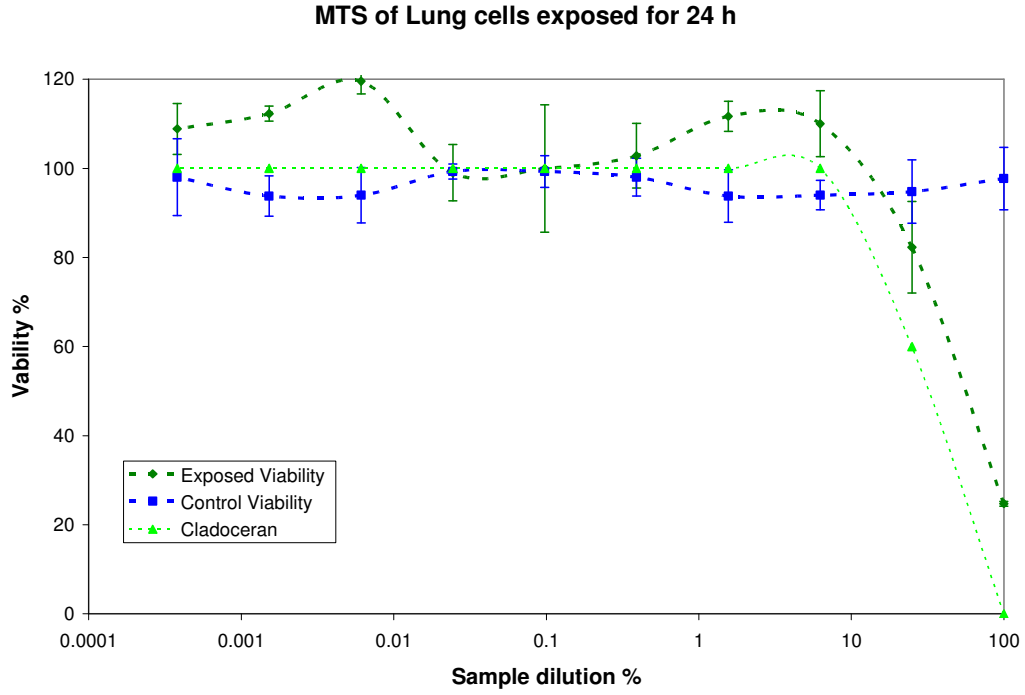
Graph 16: Dilution curves showing toxicity to skin cells, replicate 2, compared with reference cell lines and Cladocerans (note the x-axis is a log scale).



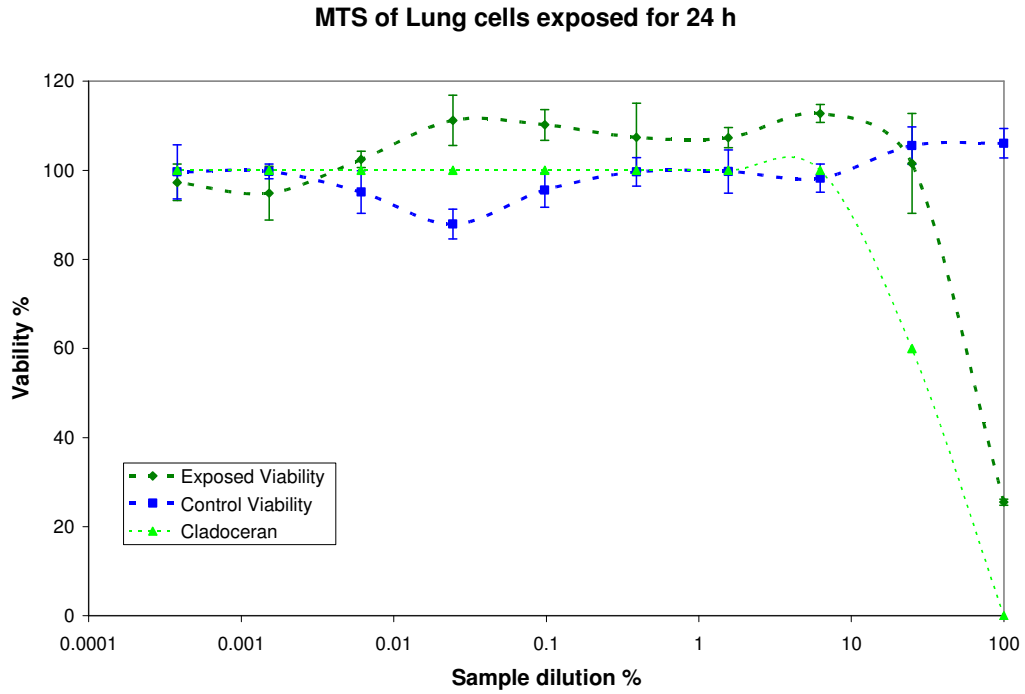
Graph 17: Dilution curves showing toxicity to skin cells, replicate 3, compared with reference cell lines and Cladocerans (note the x-axis is a log scale).



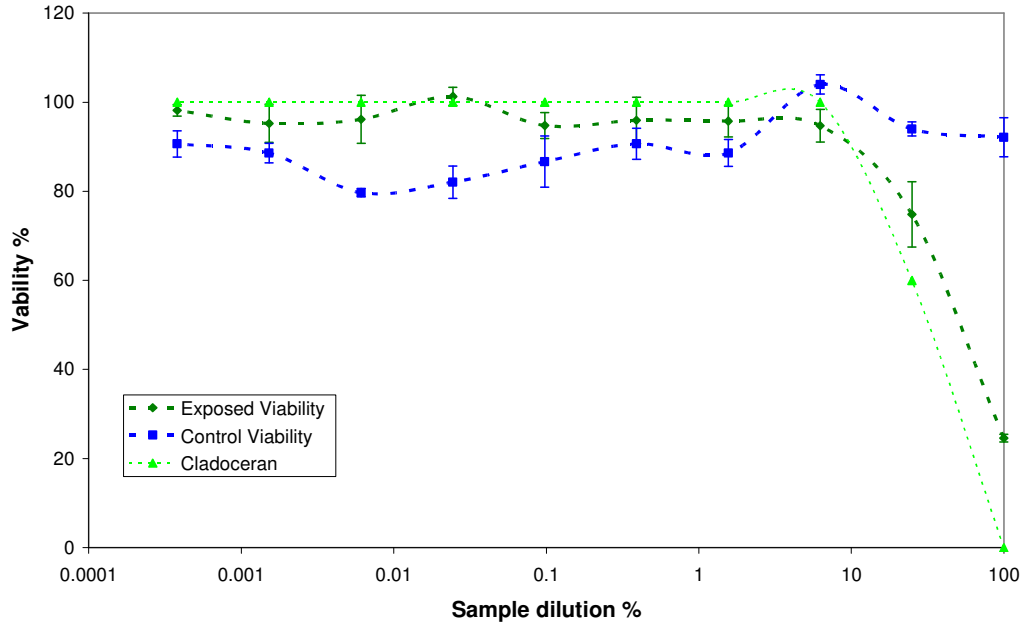
Graph 18: Dilution curves showing toxicity to lung cells, replicate 1, compared with reference cell lines and Cladocerans (note the x-axis is a log scale).



Graph 19: Dilution curves showing toxicity to lung cells, replicate 2, compared with reference cell lines and Cladocerans (note the x-axis is a log scale).



Graph 20: Dilution curves showing toxicity to lung cells, replicate 3, compared with reference cell lines and Cladocerans (note the x-axis is a log scale).



Tasmanian Investigation
Test Number 17

Explanation
Test 5 asked whether the toxicants occur naturally in undisturbed areas. While no toxicant was identified, the area investigated was alpine and therefore only tests production of toxicants by alpine biological communities. It is not known if the bacterial and fungal communities are likely to be substantially different above and below the snow line. However, the types of vegetation are substantially different. Eucalypts are known to have chemical defences against herbivores which include toxic secondary plant metabolites. Alpine grasses do not appear to contain such chemical defences.

The DPIWE hypothesised that Cineole and Pineole (oils from Eucalyptus leaves) were the most likely cause of toxicity. While it has been demonstrated that these oils are not the cause of toxicity because they were not present in any toxic methanol fraction, other secondary plant metabolites may be present. Thus, in addition to Test 5, another surface concentrate needs to be collected downstream of natural stands of Eucalypts.

Sampling Details
10 August 2005

Location Sampled
St Mary's, Tasmania, downstream of a natural Eucalypt Forest.

Purpose
Does the toxicant occur naturally in undisturbed areas?

Investigating Laboratory(s)
Ecotox Services Australia

Sample submitted to Ecotox Services
A skimmer box was placed directly downstream of a natural Eucalypt Forest for 24 hours to allow accumulation of surface waters. Surface foam was submitted to Ecotox Services for immediate testing.

Method
Cladocerans were exposed to concentrated surface sample for 48 hours.

Results
No toxicity was observed.

Conclusions
The toxicants are not present, at sufficient concentrations to be toxic, in surface water concentrates from undisturbed areas.

Status
This second test confirms the finding of Test 5. No further testing required.

Tasmanian Investigation
Test Number 18

Explanation
Discussion with Dr Humpage of South Australian Water led to the suggestion that the toxicants' characteristics were consistent with toxicants produced by blue-green algae. Dr Humpage suggested that they may be microcystins or nodularin, both of which are methanol soluble cyclic peptide hepatotoxicants. This matches the observations to date.

Sampling Details
19 August, 2005

Locations Sampled
South George.

Purpose
Is there a known cyanobacterial toxicant present?

Investigating Laboratory(s)
Australian Water Quality Centre, a business unit of SA Water

Sample submitted to SA Water
2 ml methanol fraction containing approximately 2.5 toxic units confirmed using Cladocerans.

Method
Methanol sample analysed using HPLC with detection by Photo Diode Array and Mass Spectrometry.

Results
The sample did not contain any microcystin or nodularin. Other compounds were present but were not able to be identified using this method.

Conclusions
The methanol soluble toxicant is not of blue green algal origin.

Status
Hypothesis falsified. No further test required.

Tasmanian Investigation
Test Number 19

Explanation
Following from Test 15, where amino acids were identified, another set of tests was run to determine if the toxicants were proteins.

Sampling Details
Methanol extracts from Advanced Analytical Laboratories containing large quantities of toxicants (the same sample used in Test 13 and 15).

Locations Sampled
South George.

Purpose
Is the toxicant a protein?

Investigating Laboratory(s)
Australian Proteome Analysis Facility, Macquarie University, Sydney.

Sample submitted to Macquarie University
The methanol extract submitted for amino acid analysis was used for protein identification.

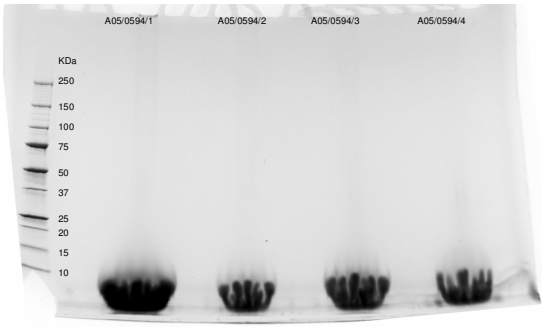
Method
SDS-PAGE gel and stain for protein.

Results
No proteins identified. Sample may be too old.

Conclusions
No protein present in this sample, but the age of the sample may be a factor (six weeks old). Fresh sample to be submitted.

Status
To be confirmed.

Photo 3: Picture of SDS-PAGE gel showing no distinct protein bands.



Tasmanian Investigation
Test Number 20

Explanation
Discussion of results to date with Dr Chariton (CSIRO) led to the hypothesis that the toxicant might be the result of a bacterial or fungal imbalance due to years of chemical applications by silviculture. Under such a situation, a selective advantage may have resulted in a dominance of a toxicant producing micro-organism. If this were the case, one would expect the soil to test positively for the presence of toxicants.

Sampling Details
21 August, 2006

Locations Sampled
River Mouth
George Bay
Georges River Bridge
Forest Plantation, South George
Forest Plantation drainage ditch.

Purpose
Are the soils toxic?

Investigating Laboratory(s)
Ecotox Services Australia and Advance Analytical Services Australia.

Sample submitted to Laboratories
Soil samples were submitted to Ecotox Services Australia which were then sub-sampled for analysis by the two laboratories.

Method
Amphipods were used as the test organism to determine if the soils were leaching toxicants. Soils were also analysed for Simazine and its metabolites, Atrazine and its metabolites and for ten synthetic pyrethroids.

Results
Simazine and its metabolites were identified in soil from the Plantation. Metabolites of Atrazine were identified at all the locations. Despite the presence of contaminants the soils were not toxic to the test organism.

Conclusions
Soil samples were not toxic to amphipods, indicating that no biological or man-made toxicants were present in sufficient quantities to be toxic.

Status
To be confirmed.

Tasmanian Investigation
Test Number 21

Explanation
Following from Test 15 (amino acids were identified) and Test 19 (checking for proteins), another set of tests was run to determine if the toxicants were proteins.

Sampling Details
Methanol extracts from water sampled August 30, 2005.

Locations Sampled
South George.

Purpose
Is the toxicant a protein?

Investigating Laboratory(s)
Australian Proteome Analysis Facility, Macquarie University, Sydney.

Sample submitted to Macquarie University
The methanol extract submitted for amino acid analysis was used for protein identification. The extract contains 2.5 toxic units as determined from Cladoceran tests.

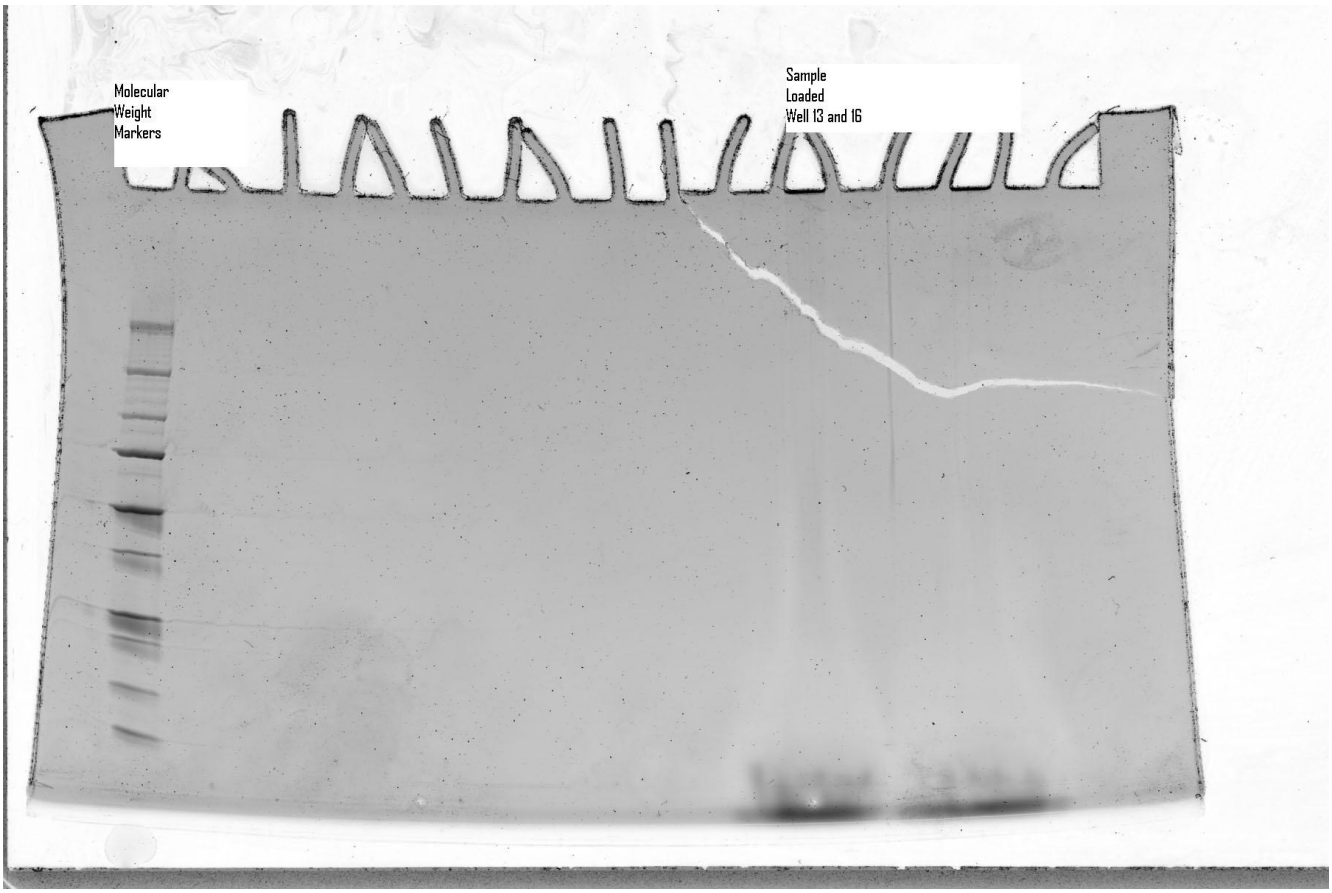
Method
SDS-PAGE gel and stain for protein.

Results
No proteins identified.

Conclusions
No protein present in this sample.

Status
Protein has not been identified. No further testing required. Laboratory is inquiring whether there are alternative tests to obtain the toxicants' identification.

Photo 4: Picture of SDS-PAGE gel showing no distinct protein bands



Tasmanian Investigation
Test Number 22

Explanation
This test repeats Test 20 aimed at determining if a bacterial or fungal toxicant might be present in the soils.

Sampling Details
8 November, 2006

Locations Sampled
River Mouth
Georges Bay
Georges River Bridge
Plantation, South George

Purpose
Are the soils toxic?

Investigating Laboratory(s)
Ecotox Services Australia and Advance Analytical Services Australia.

Sample submitted to Laboratories
Surface soil samples were submitted to Ecotox Services Australia which were then sub-sampled for analysis by the two laboratories.

Method
Amphipods were used as the test organism to determine if the soils were leaching toxicants. Soils were also analysed for Simazine and its metabolites, Atrazine and its metabolites, and for trace elements and other triazines.

Results
Simazine and its metabolites as well as Atrazine were identified in soil from the Plantation. Despite the presence of contaminants, the soils were not toxic to the test organism.

Conclusions
Soil samples were not toxic to amphipods indicating that no biological or man made toxicant was present in sufficient quantities to be toxic.

Status
Confirmed. Biological toxicants are not present in the surface soil at sufficient concentrations to be toxic. No further testing required.

Tasmanian Investigation
Test Number 23

Explanation
Toxicity tests had now ruled out any form of toxicants downstream of natural catchments. Tests had also ruled out methanol soluble blue-green algal toxicants. Toxicity tests ruled out bacterial and fungal toxicants. Tests had ruled out man-made toxicants. Tests had also ruled out other toxicants like metals and volatiles. However, testing indicated that methanol soluble toxicants were present in every surface concentrate sample that had been taken in the George River system. To further complicate the situation, the toxicants have a relatively short half life (4 days), thus the supply of toxicant has to be permanent to be consistent with our results.

Oysters had been successfully farmed in the area since 1980 without any major mortality events. Anecdotal evidence indicates that symptoms first appeared in oysters in the late nineties and were sufficiently numerous to be under scientific investigation by the end of 2000 at the behest of the DPIWE.

The only obvious conclusion is that the toxicants must be coming from an introduced biological organism. The only organism that has been knowingly introduced in very large numbers are the trees being planted in the plantations (of which there are many) in the catchment.

The main species planted is the tree *E.nitens*. These trees have been the subject of intensive selective breeding programs supported by intensive genetic research. The trees are described as having been sourced from "genetically improved" seed. This description is not unique to *E.nitens* as other promoters apply the same term to other eucalypt hardwood species such as *E. globulus*, *E. dunni, E. grandis* and selected hybrids for trees grown in other States of Australia.

The extent to which biotechnological manipulation has occurred to these tree species and possibly others is unknown to the general public.

Locations Sampled
Leaves from a young tree (approximately 6 years old) in a plantation adjacent to South George River, sampled 8 November 2006.

Purpose
To determine if the leaves contain toxicants with similar characteristics to the water contaminant.

Investigating Laboratory(s)
Ecotox Services Australia

Method
Leaf samples were crushed under liquid nitrogen to extract cellular contents. The contents were then checked to see if methanol extractable toxicants were present and a dilution curve was run. Test organisms were Cladocerans.

Results
Methanol soluble toxicants were present in the 70% to 100% range. These toxicants were not PBO synergists. Leaf extract equivalent to 1 gram of leaf contained more than 100 toxic units (the limit of the dilution).

The 70% elution lost toxicity through time.

Conclusions
These initial results appear to be consistent with the characteristics of the toxicants isolated in the TIE with respect to the methanol soluble toxicants observed after PBO synergism dissipated.

Status
To be confirmed.

Tasmanian Investigation
Test Number 24

Explanation
A search of the internet reveals that there has been extensive research effort in the area of Eucalyptus genomics and that a wide variety of biotechnology tools have been successfully applied to Eucalypts. These tools range from genetic marking, gene silencing, within species gene insertion through to between species gene insertion (Genetically Modified Organisms or GMOs). Australian law only seems to recognise GMOs as posing a risk. International Convention (Cartagena Protocol on Biosafety to the Convention on Biological Diversity) recognises that within species manipulations could be equally hazardous. Under this Convention, the UN identify "Living Modified Organisms" (LMOs), a definition which includes the GMOs as well as any organism that has been modified via modern biotechnology such that it is no longer a member of the naturally occurring gene pool.

Given that the tools are available, it is possible that the trees have been manipulated in some way.

The only tests that are readily commercially available are tests to identify GMOs. Even these tests are only available for a handful of indicators. Tests to determine if a plant falls into the broader category of Living Modified Organism were not able to be sourced.

Locations Sampled
Leaves from a young tree (approximately 6 years old) in a plantation adjacent to South George River, same as Test 23.

Purpose
To determine if the leaves contain certain promoters, bacterial toxicant and terminators used in Genetic Modification.

Investigating Laboratory(s)
Genetic ID, Iowa, USA

Method
Leaf samples were sent to Iowa for extraction.

Results
No commonly used promoters, terminators or bacterial toxicant were identified.

Conclusions
These tests have not allowed for the identification of the trees as GMOs.

Status
These trees are not GMOs, however, it cannot be determined if they fall under the broader definition of LMOs.

Tasmanian Investigation
Test Number 25

Explanation
Leaf toxicity testing continued.

Locations Sampled
Leaves from a young tree plantation (approximately 6 years old) adjacent to South George River, same as Tests 23 and 24.

Purpose
To determine if the leaves contain toxicants with similar characteristics to the water contaminant.

Investigating Laboratory(s)
Ecotox Services Australia
Australian Proteome Analysis Facility

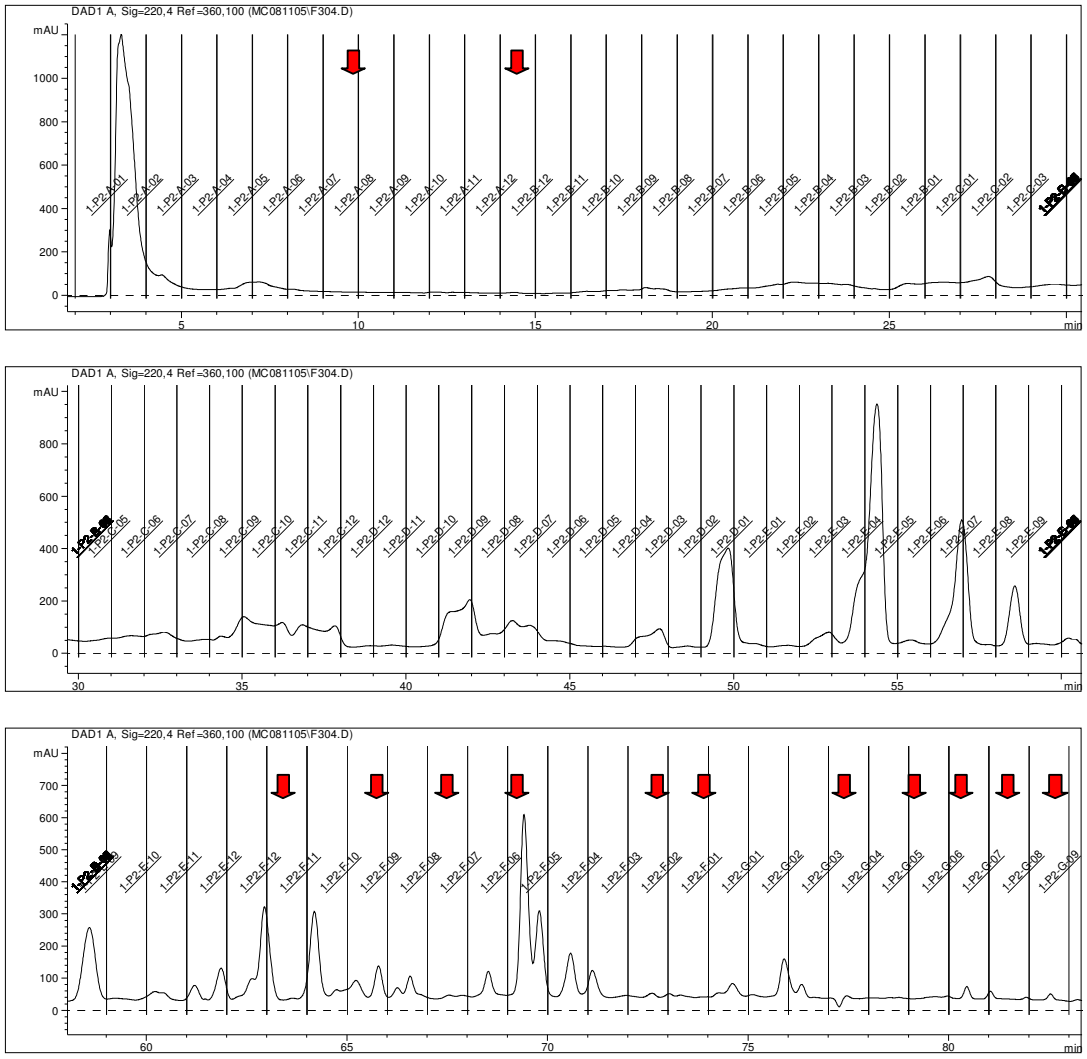
Method
Leaf samples were crushed under liquid nitrogen to extract cellular contents. The contents were then checked to see if methanol extractable toxicants were present and a dilution curve was run. Test organisms were Cladocerans. Methanol extracted toxicants were submitted for HPLC and further analysis. The fractions isolated by the HPLC process were the submitted by Australian Proteome Facility back to Ecotox to identify which fractions contained toxicant.

Results
In all, the leaf contained at least 13 toxicants in the 80% methanol to 100% methanol fractions. Two of these were at the relatively water soluble (i.e. 80%) end of the range and 11 were at the more methanol soluble (i.e. 100%) end of the range. The most toxic of the methanol fractions was resubmitted for amino acid analysis and further testing. Amino acid analysis indicated a range of amino acids were present, however, further analysis using Mass Spectrometry could not identify what this toxicant was.

Conclusions
These results again appear to be consistent with the characteristics identified in the TIE.

Status
Toxicity of the leaf is confirmed. Further characterisation to be undertaken.

Fractions Identified as Toxic by Ecotox Services Australia using Cladocerans



A8	100% mortality	G7	30% mortality
A12	100% mortality	G8	20% mortality
F11	20% mortality	G9	10% mortality
F9	50% mortality		
F7	40% mortality		
F5	50% mortality		
F2	10% mortality		
F1	20% mortality		
G4	20% mortality		
G6	40% mortality		

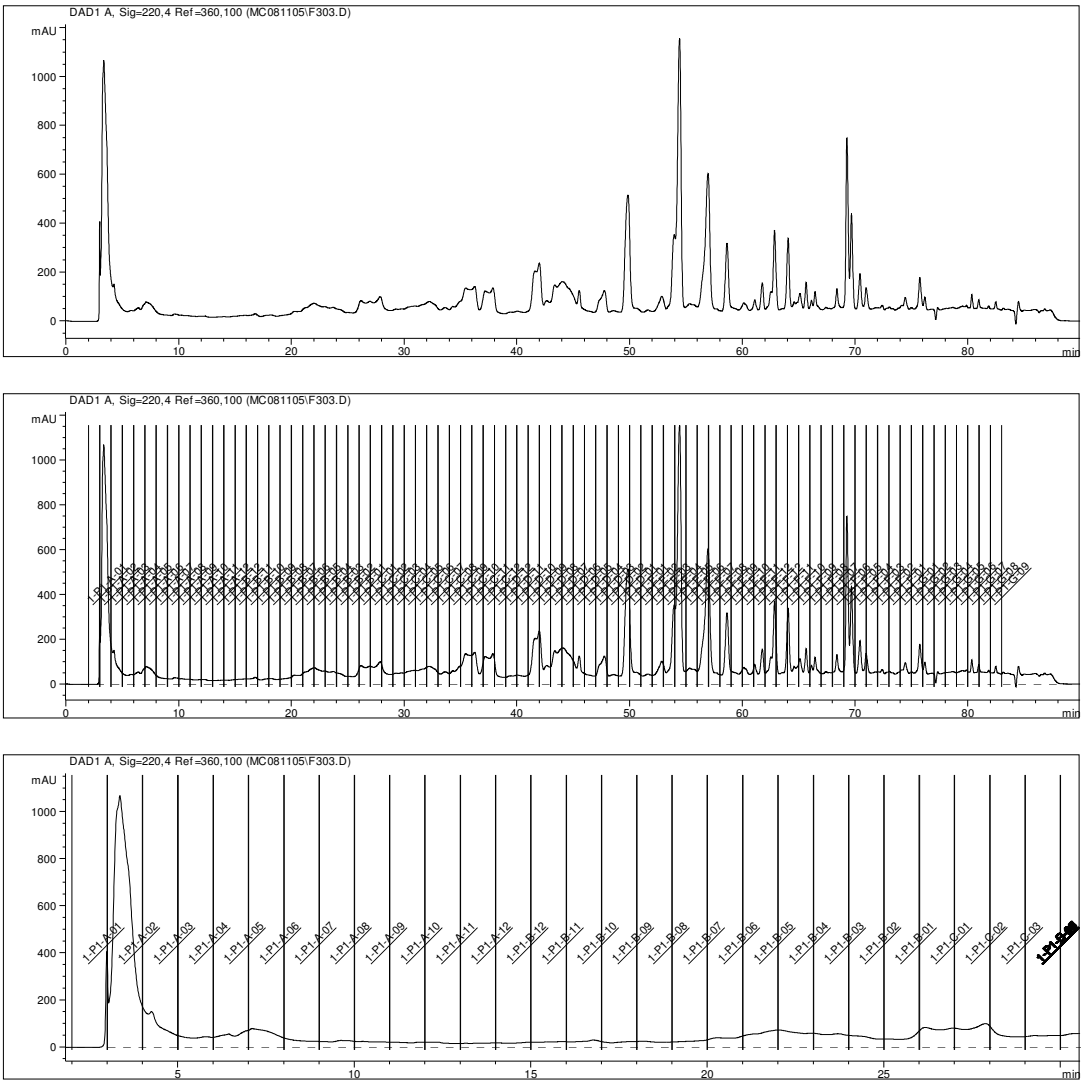
Proteome Laboratory HPLC Results – Peaks represent amino acid bonds

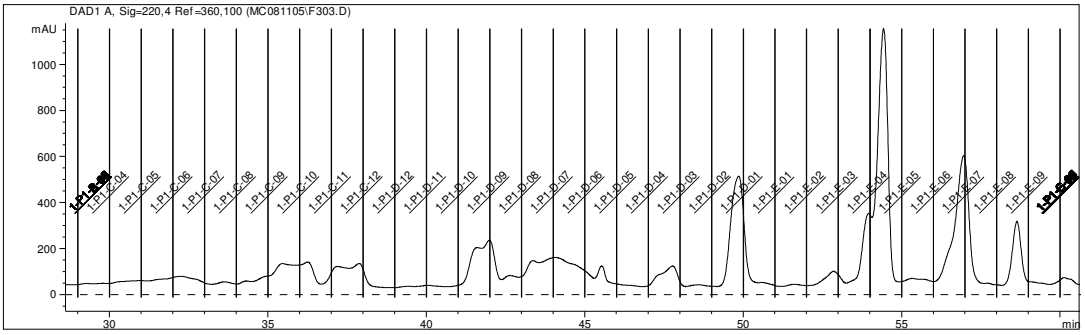
Extraction of SPE

Sample	Volume (mL)	Buffer
Fraction 1	5	Milli Q
Fraction 2	6	50% Methanol
Fraction 3	5	80% (2mL) to 100% Methanol (3mL)
Fraction 4	8	100% Methanol (5mL) and 100% Isopropanol (3mL)

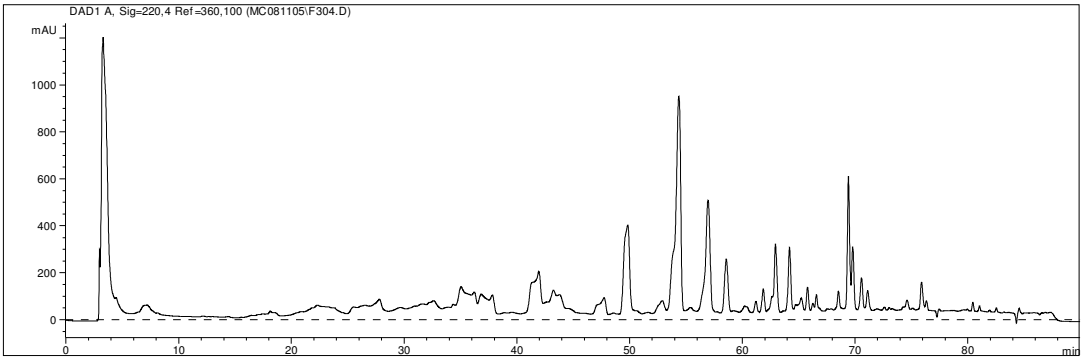
HPLC run
1ml of fraction 3 taken and volume reduced by half in a speedivac. Sample spun at 14.1rcf and 400uL injected.

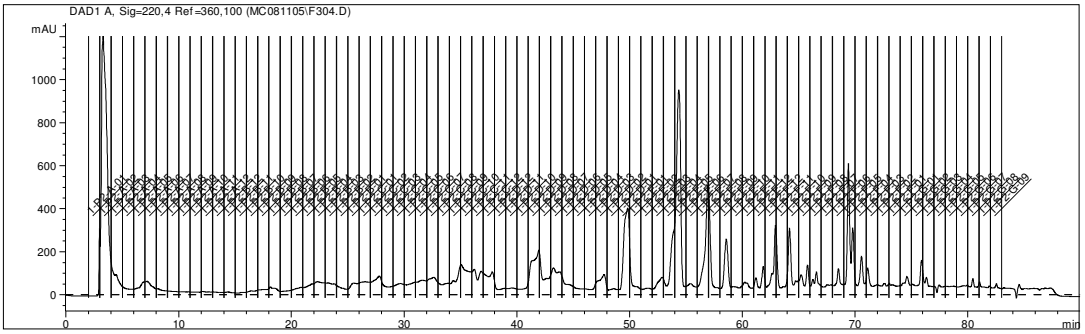
Fraction 3 Injection 1- 220nm

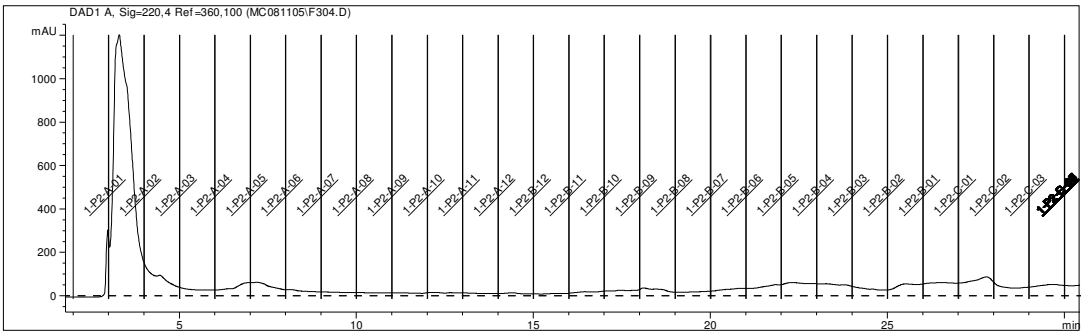


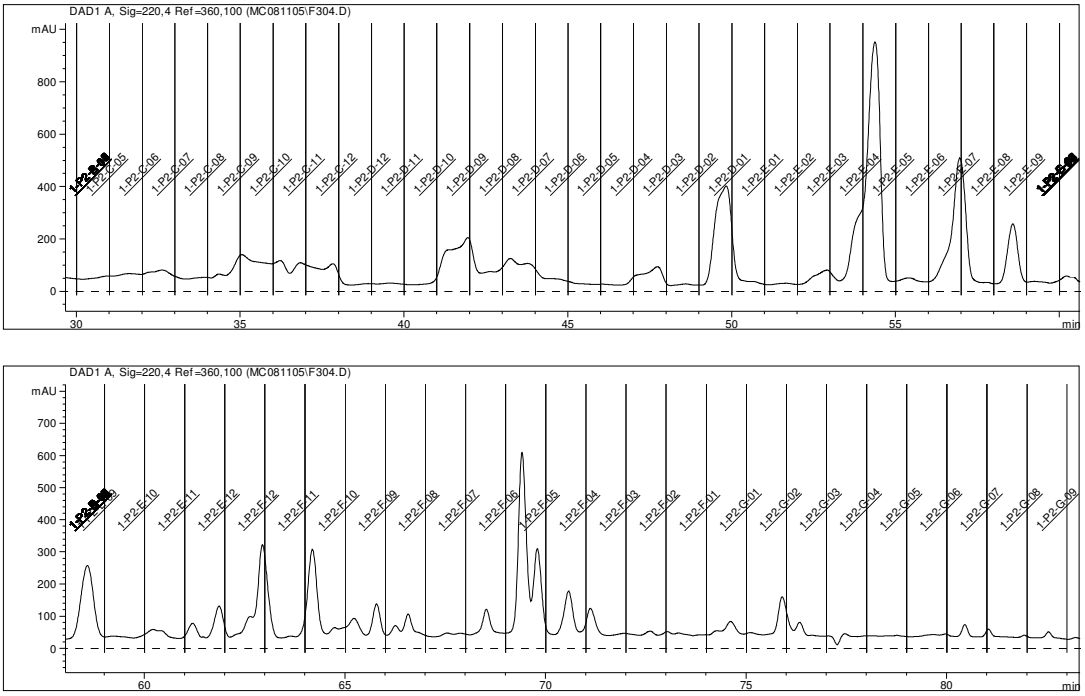


Fraction 3 Injection 2- 220nm

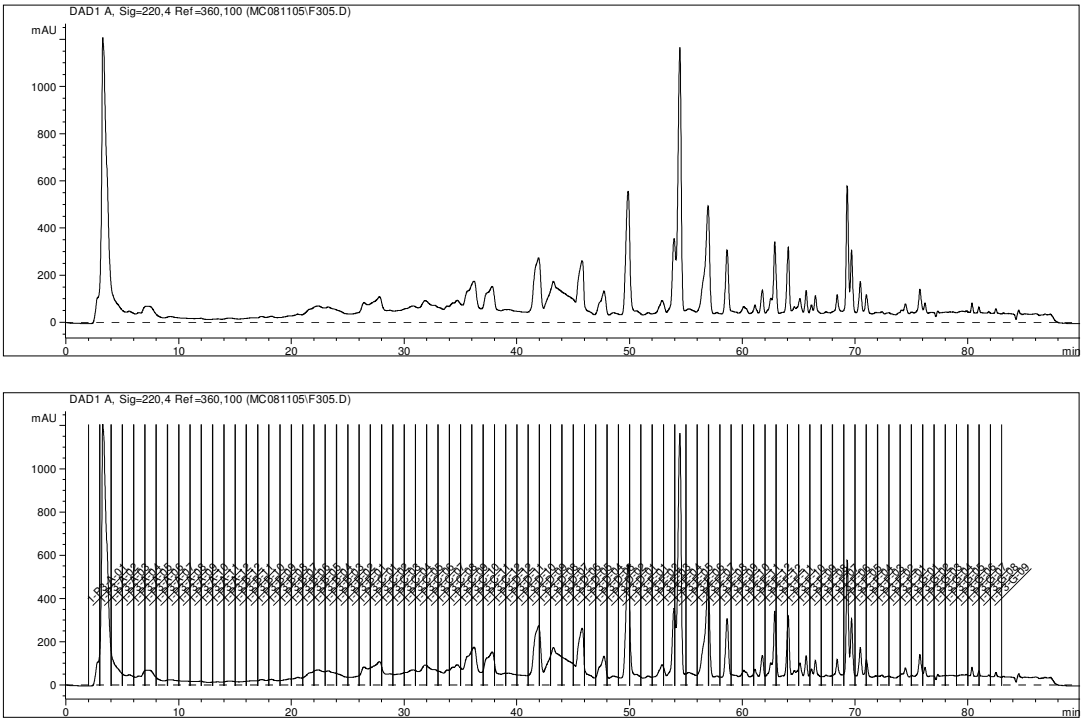


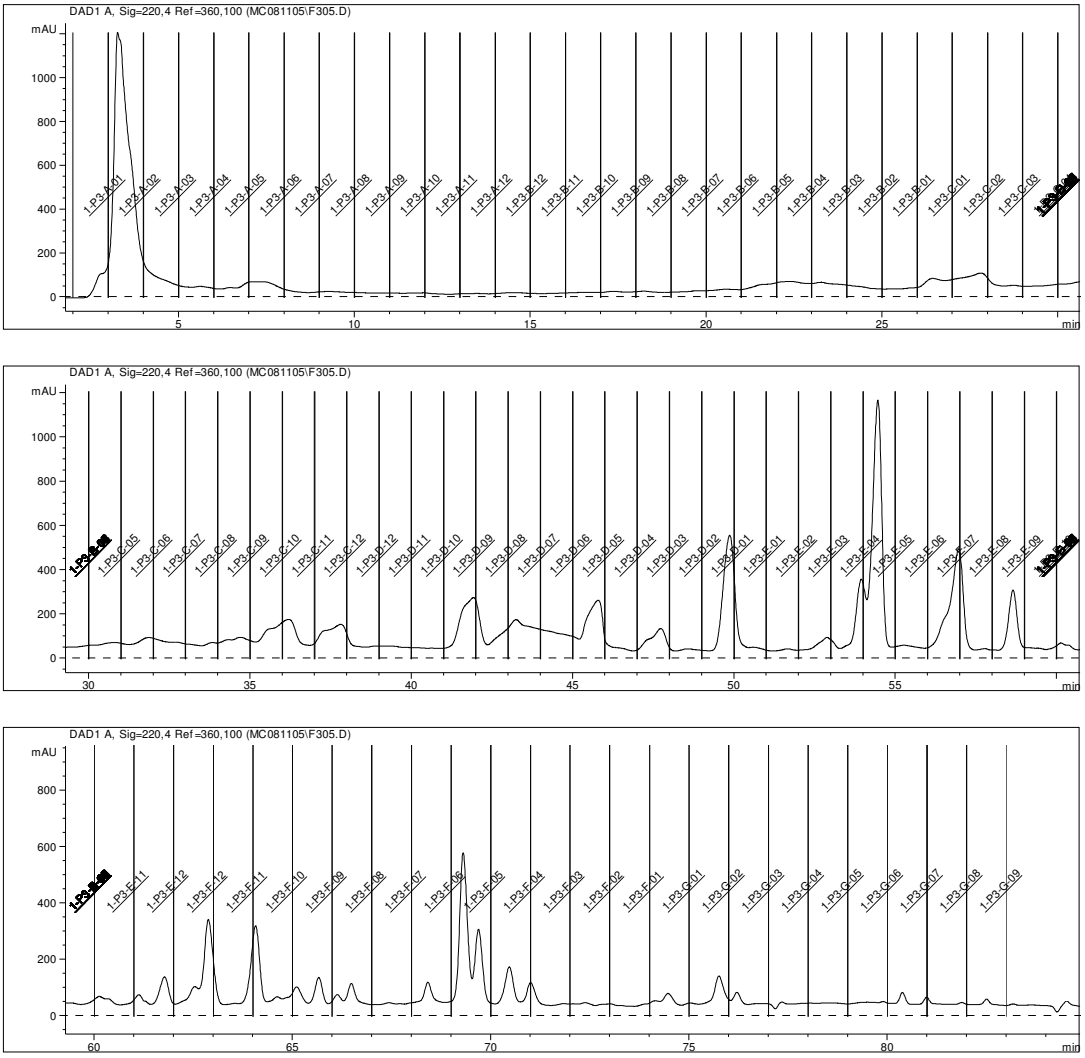




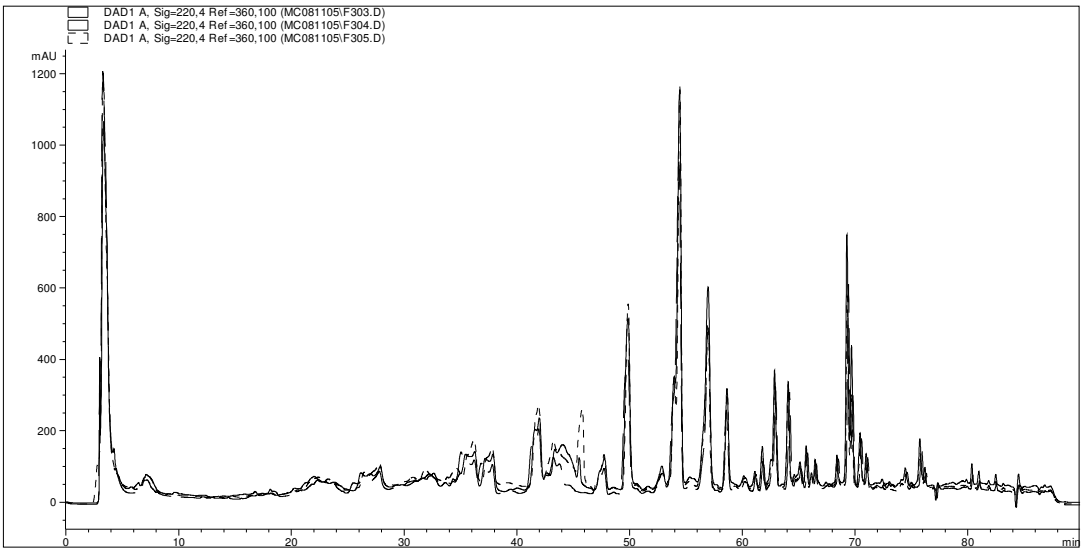


Fraction 3 Injection 3- 220nm





Overlay of the Three Injections – 220nm



Tasmanian Investigation
Test Number 26

Explanation
The only chemicals that have been clearly identified from toxic methanol extracts from both the water and the leaf are amino acids. If the trees are the source of the methanol soluble biological toxicant identified in every surface concentrated water sample taken, then there should be some correlation between the amino acid ratios.

Given that no proteins have been identified, there is no evidence as to how the amino acids are joined together, if at all. Therefore, the assumption has been made that these amino acids may react differently in the water column depending on whether they are Hydrophilic (water loving) or Hydrophobic (water hating).

Purpose
To investigate if correlations exist between the amino acids isolated from toxic methanol fractions extracted from both the water and the leaves.

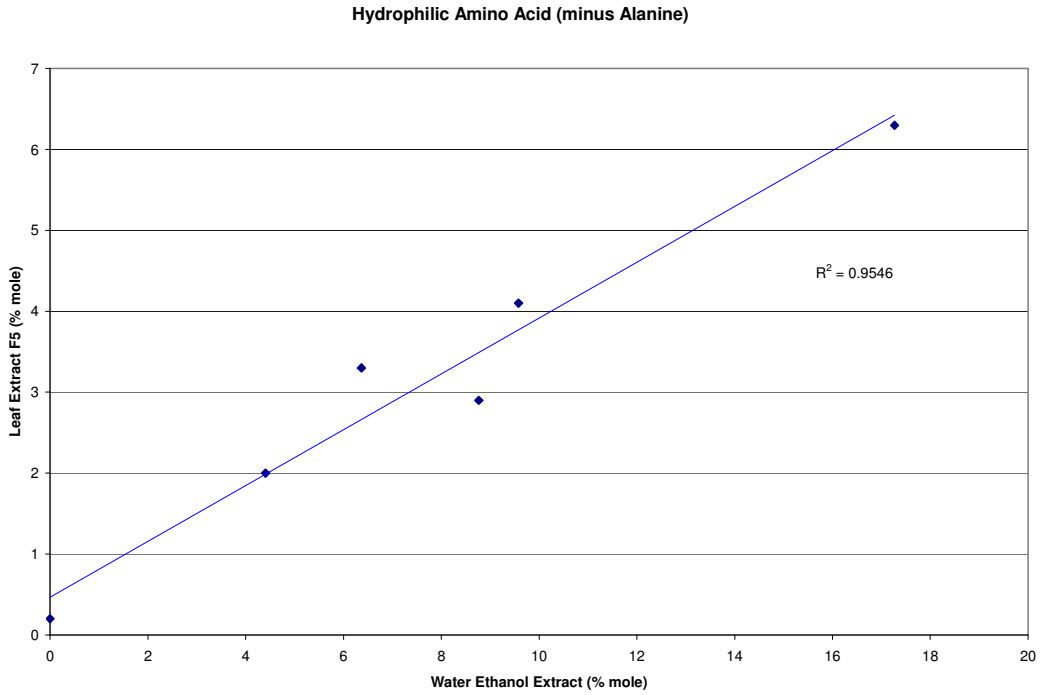
Method
An XY scatter plot of the hydrophilic amino acids identified in the leaf and water samples were plotted using Excel and a linear correlation was run. The same was done for the hydrophobic amino acids.

Results
A good correlation was found between the amino acid ratios in the leaf and the amino acid ratios in the water. This suggests that the trees may be the source of toxicants observed in the water.

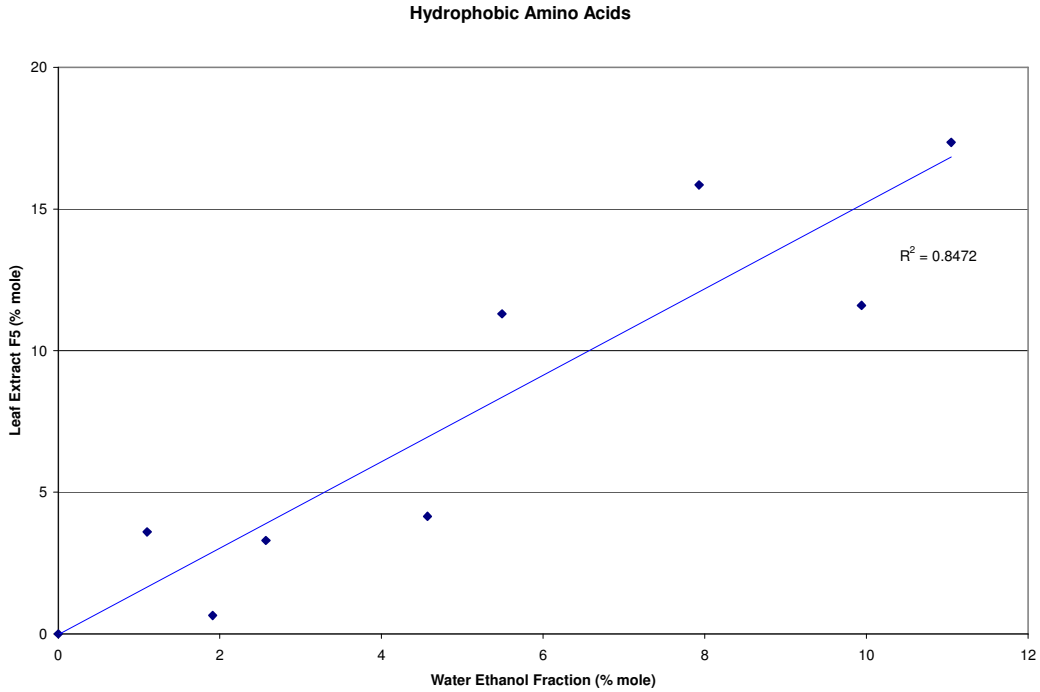
Conclusions
Good correlations were observed between the amino acid ratios in the toxic methanol fraction derived from leaf and the amino acid ratios in the toxic methanol fraction derived from the water.

Status
This offers some additional support that the leaf is possible source of the toxicant(s).

Graph 21: Correlation between hydrophilic amino acid ratios extracted from well F5 (HPLC on Leaf) and methanol extract from water.



Graph 22: Correlation between hydrophobic amino acid ratios extracted from well F5 (HPLC on Leaf) and methanol extract from water.



Tasmanian Investigation
Test Number 27

Explanation
Ongoing confirmation of leaf toxicity.

Locations Sampled
Fresh leaf samples collected from the same location as the previous leaf tests.

Purpose
To identify toxic methanol fractions.

Investigating Laboratory(s)
Ecotox Services Australia

Method
Cell contents extracted as previously described and the extracts were put through methanol fractionation and a dilution curve as previously described.

Results
Leaf extract equivalent to 1 gram of leaf tissue contained in excess of 1,000 toxic units. Methanol extracts indicated toxicity in all methanol fractions. Notably, toxicity was observed in the 75%, 80%, 85%, 90%, 95% and 100% fractions.

Conclusions
This third round of leaf toxicity tests indicates that the leaves are toxic and that the toxicants range over the same fractions observed in raw water samples.

Status
All tests involving the leaf match the toxicants characterised by the TIE. Weight of evidence would suggest that the trees themselves are the most likely source of the methanol soluble biological toxicant observed during all sampling events over the two years of this study. It is not known to what extent biotechnology has been involved in the production of these monocultures.

Tasmanian Investigation
Test Number 28

Explanation
To determine if the dry weather toxicant observed in the South George surface water is from plantation leaves.

Location Sampled
Fresh leave collected from the same plantation as the previous leaves tested. Surface water collected from the South George River and from the St Mary's control site.

Purpose
To add toxic leaf fractions back to St Mary's water to identify chemically if the contamination of the South George River is from plantation leaves. This is an add back experiment.

Investigating Laboratory(s)
Ecotox Services Australia
Advanced Analytical

Method
Toxicant was extracted from leaf samples and added back to a control water sample to produce water containing approximately five toxic units. This water was then tested for toxicity against a contaminated South George Water sample and against the reference St Mary's sample. These water samples were analysed using LCMS run in full scan mode, to determine if the organic contamination of the South George sample matched the organic contamination added to the control water sample (leaf spike) and if that was different to the reference sample from St Mary's.

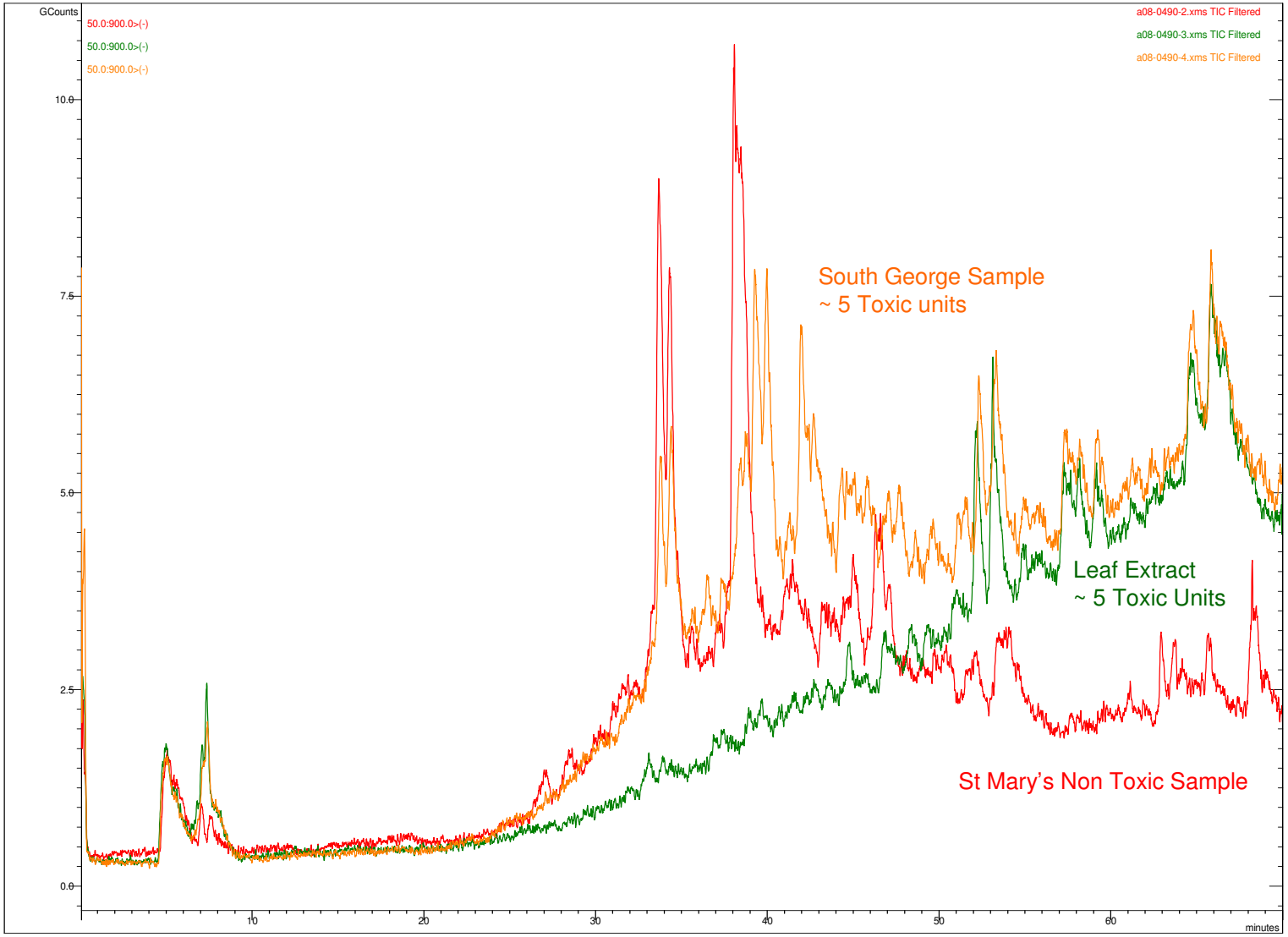
Results
The toxicant extracted from the 1 gram of leaf into methanol was slightly less toxic than in investigation number 27 with 5 toxic units equivalent to a 2% add back to the control water sample.

The St Mary's reference sample was not toxic. The control water sample plus 2% leaf extract was toxic at approximately 5 toxic units. The South George sample was similarly toxic at approximately 5 toxic units.

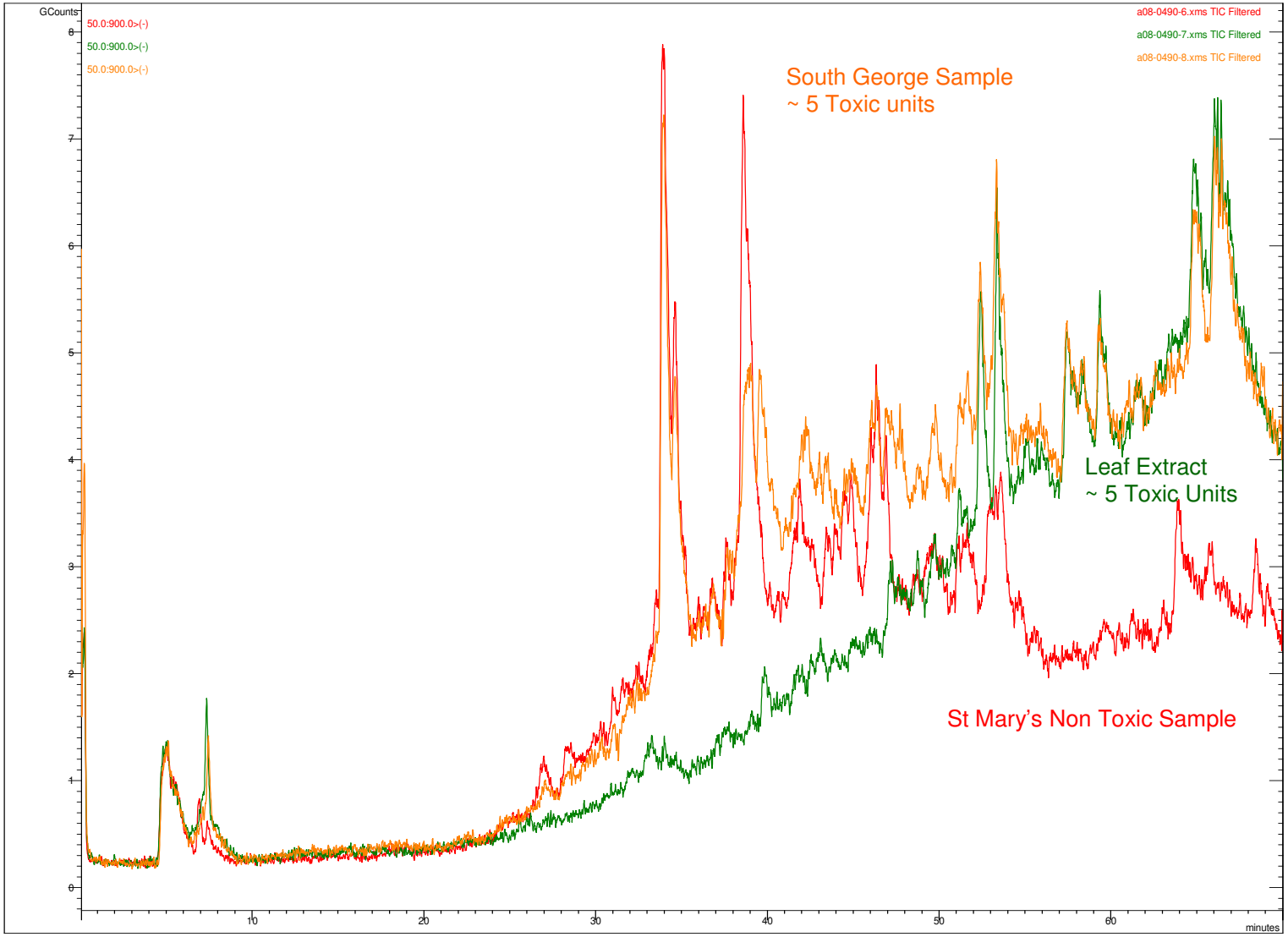
LCMS of the samples showed a clear overlay of the St Mary's organic chemicals and the South George organic chemicals for the first part of the scan but a clear departure through the second half of the scan with Leaf extract and South George water showing clear correlation. St Mary's did not have organic chemicals which were similar to the Leaf extract.

Two graphs of these results follow (one showing negative LCMS and the other showing positive).

Overlaid Chromatogram Plots



Overlaid Chromatogram Plots



Conclusion
The positive and negative LCMS provide further support for the hypothesis that the dry weather toxicant(s) that appears to be permanently present in the George River system originates from plantation leaf, plant material. Isolation and identification of molecular weights and concentrations are still required before a firmer conclusion can be drawn.

Status
This experiment will need to be confirmed and NIWA (New Zealand equivalent of CSIRO) has been approached for a quote to do this.

Once this has been confirmed and the toxicant isolated, a series of calibration experiments will need to be run to determine the significance of these contaminants. These experiments have only demonstrated that contaminated foam is significant to oysters. The extent of contamination of the water column is yet to be determined and it is intended that this will be determined under the guidance of NIWA.