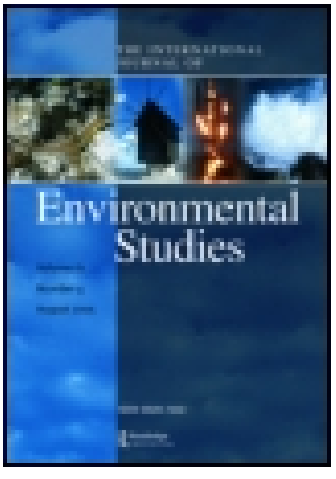

International Journal of Environmental Studies
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/genv20

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Published online: 20 Dec 2014.

To cite this article: Alison Bleaney, Christopher W. Hickey, Michael Stewart, Marcus Scammell & Rye Senjen (2014): Preliminary investigations of toxicity in the Georges Bay catchment, Tasmania, Australia, International Journal of Environmental Studies, DOI: 10.1080/00207233.2014.988550

To link to this article: http://dx.doi.org/10.1080/00207233.2014.988550

International Journal of Environmental Studies, 2014
http://dx.doi.org/10.1080/00207233.2014.988550

Preliminary investigations of toxicity in the Georges Bay catchment, Tasmania, Australia

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North-eastern Tasmania, Australia has been an area of major production for Pacific oysters (*Crassostrea gigas*) for over 25 years. Since the mid-1990s, increased oyster mortality has been observed. The purpose of the present study was to identify the agent causing aquatic toxicity and to investigate whether there is a chemical and/or toxicological link between river foam and monoculture timber plantation forests of exotic eucalypts (*Eucalyptus nitens*) present in the catchment area. Foam samples from the George River catchment demonstrated high toxicity to a freshwater cladoceran and larvae of a marine blue mussel species. After filtration to remove most particulates, foam samples also demonstrated a marked reduction in toxicity to blue mussels, which suggested that the toxicity is particle associated. Foam and leaf extracts of *E. nitens* were then fractionated using HPLC and size exclusion chromatography and the resulting fractions were screened for cladoceran and blue mussel toxicity. Toxicity was detected in fractions common to both the foam and the leaf extracts. This study suggests that there may be a chemical and toxicological relationship between foam and *E. nitens* leaf components.

Keywords: Pollution; Toxicity; Oysters; Eucalypts; Plantations; Water; Tasmania

Introduction and background

Oyster mortality in Georges Bay, Tasmania

Georges Bay, near St. Helens, in north-east Tasmania, Australia, has been an area of major production for Pacific oysters (*Crassostrea gigas*) since the 1970s. From the mid-1990s, however, there has been a continued decline in oyster production through increased oyster mortality, shell deformities and slow growth rates. Much of the mortality and deformities are reported after rainfall (>20 mm) in the George River catchment (see figures 1 and 2) and subsequent river water rises and flooding events, or following oyster-handling procedures in the 2–6 weeks subsequent to these flooding events [1]. Both the catchment tributaries and the George River – which flows into Georges Bay – are relatively short and fast flowing; with water taking approximately 18 h to move from the top of the catchment to the outflow [1,2]. The George River catchment supplies the drinking water for the residents of St Helens and surrounding areas. Human activities in the catchment are predominantly agricultural (mainly dairy with some cropping) [3]. Additionally, since the early

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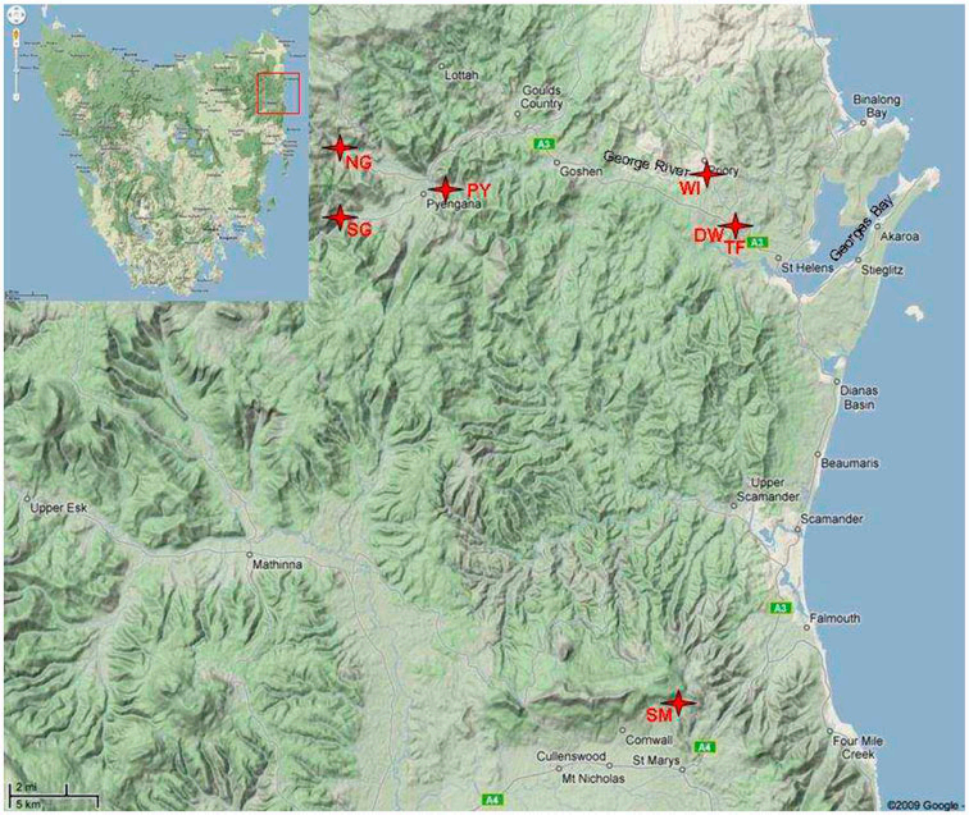

Figure 1. Map of north-east Tasmania showing approximate sampling locations (after [6]). © 2009 Google.

1990s, monoculture fast rotation eucalypt (mainly *Eucalyptus nitens (E.nitens)*) plantations have increased 30-fold in the catchment area [1], with 2934 ha of eucalypt plantations as of 2007 (approximately 7% of the total area) [4].

Although the increase in eucalypt plantation area may appear small, its location in the upper catchment and the topography means a substantial increase in leaf litter being taken by water run-off into the river, causing the potential for increased water contamination [5]. A variety of pesticides, often in combinations, are applied by ground and aerial methods to mitigate damage caused by insects, browsing animals (especially wallabies and possums), weeds and unwanted understorey plants. For instance, simazine contamination of the South George River, caused by forestry practices, was documented in 1994 [1].

In February 2004, 90% mortality of the intertidal oysters in Georges Bay was observed after a significant flood caused by a one in one hundred-year rain event. The largest oyster mortality occurred on 9 February 2004 following the heavy rainfall event that resulted in the highest recorded flooding of the George River [6]. Numerous observations then made by local oyster farmers suggested that a narrow band of surface water may have been associated with the bulk of oyster deaths on intertidal leases. Scammell [6] reported that oyster fishermen and other concerned parties speculated that a pollutant located in the surface water was responsible for this mortality, or at least acted as a contributing factor. Percival recommended in his report into oyster 'ill-thrift' in Georges Bay that activities occurring

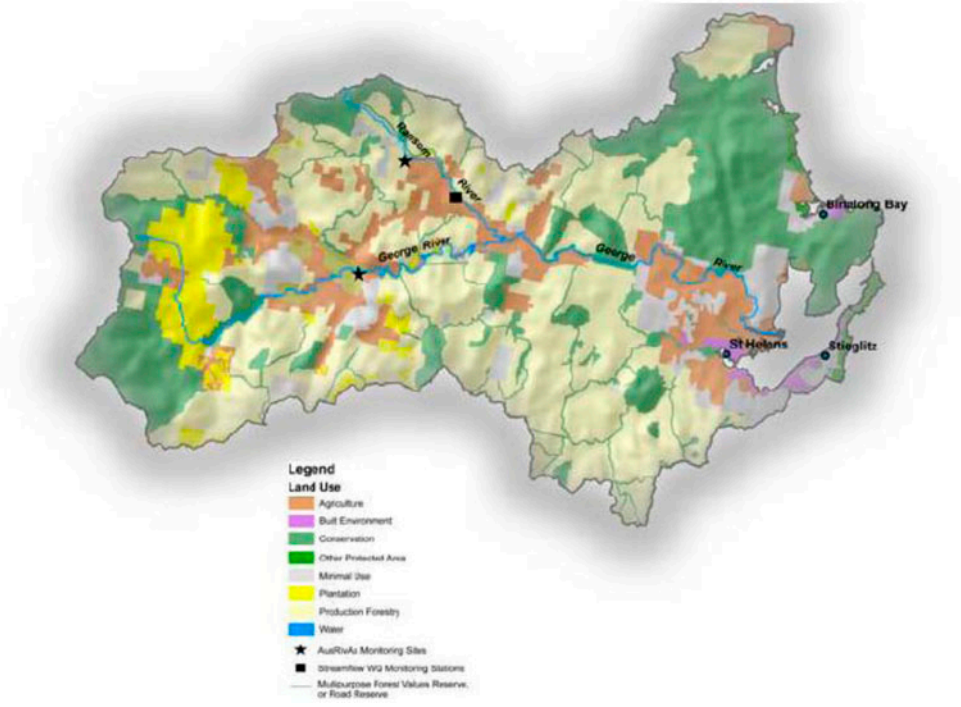

Figure 2. George River catchment, Tasmania [5].

in the catchment needed investigation [7]. Additional community concerns about water quality in the George River catchment arose from the crash of a pesticide spraying helicopter in the catchment in December 2003 and the subsequent finding of insecticide and herbicide residues in the soil close to the crash site [1].

Pilot study

Following the extensive oyster mortality event in 2004, a number of water samples were collected and tested for the presence of anthropogenic chemicals. No chemicals were identified in these analyses [8]. Yet the oysters continued to show signs of disease. In January 2005, further efforts were concentrated on toxicity testing. Two methods of collecting water samples were used: a skimmer box (which accumulates surface foam) to concentrate surface water ingredients, and a grab sample, which collects a representative sample of water from the water column. The two grab samples (collected January 2005) from Pyengana and the North George River (George River tributaries) were both toxic to sea urchin larvae and one was also toxic to oyster larvae. Two further grab samples, collected during a storm event in February 2005 also showed toxicity to oyster larvae. All surface water foam samples obtained using the skimmer box were toxic to oyster and sea urchin larvae. A range of organic compounds were identified in the foam samples. None appeared man-made and the origins of these chemicals remained unknown [8].

Accordingly, a Toxicity Identification and Evaluation (TIE) was commissioned and completed in early 2008 [8]. The key findings included that the toxins: were present in surface

foam during all dry weather samples; had a relatively short half-life; were primarily attached to fine particulate matter, but some remained dispersed or dissolved; were not a chelatable metal; were not volatile; were soluble in methanol; were detected in different fractions of the sample; were not large proteins; were not of blue-green algal origin; were unlikely to be of bacterial or fungal origins; affected multiple test targets (cladocerans, oysters, sea urchins and three human cell lines (as tested by Khalil and Winder [9])) at similar concentrations; and were not found downstream of natural forests.

A chemical analysis of the leaves of the *E. nitens* monoculture plantations present in the catchment was then carried out using an add-back style experiment. The hypothesis was that if the methanol soluble dry weather toxin, always present in foam samples, was from *E. nitens*, then water downstream of *E. nitens* would have an overlapping section of its chemical signature when compared to reference water containing extracts added from *E. nitens* leaves. This overlap would not be present when compared to water downstream of natural eucalypt forests.

A pilot study was conducted [8] using Liquid Chromatography/Mass Spectrometry (LC/MS) analyses comparing South George River (George River tributary) water, *E. nitens* leaf extract and surface water from another North Eastern Tasmanian catchment (Gardiners Creek) downstream of only natural forests and where no toxicity had been detected. The results showed a clear overlapping chemical signature between the toxin in the South George water and the *E. nitens leaf* extract, but not the Gardiners Creek water. This strongly suggested that the *E. nitens* may be the source of the toxin. This pilot study was considered in need of verification and further investigation; hence the present study.

Study objectives

The main objectives were to identify the agent causing aquatic toxicity using a two-phase approach. *Phase 1* involved a toxicity assessment of base-flow multi-site water and foam samples from the Georges River system against the sensitive freshwater (*Ceriodaphnia dubia*) and marine species (*Mytilus galloprovincialis*). *Phase 2* aimed to determine whether there was a chemical and/or toxicological link between the river foam and monoculture timber plantation forests of exotic eucalypts (*E. nitens)* present in the George River catchment area. This was achieved by iterative toxicity-directed fractionation and LC/MS analysis of foam, Tasmanian monoculture *E. nitens* and naturally occurring Victorian *E. nitens,* leaf extracts. Figure 1 shows sampling locations in Tasmania for both phases. Figure 2 shows the George River catchment.

Materials and methods

Bioassays and test species

All tests used ethanol (EtOH) as a carrier solvent at a concentration of 1 mL/L (0.1%), since laboratory experience showed that the ethanol sensitivity of these species was below the no-effect concentration threshold. All tests included a solvent control series.

C. dubia (cladocerans; freshwater species) were tested for 48 h survival and *M. galloprovincialis* (blue mussels; a marine species) were tested for 48 h larval development. Both tests were used on the same water and foam samples. Brine adjustment of salinity occurred prior to the blue mussel testing in order to provide comparative sensitivity measurements.

Screening tests were undertaken using either undiluted or a 10-fold dilution series for extracts at 0.1% ethanol. Definitive tests had a lower dilution factor to obtain greater precision for the toxicity estimates. Toxic units (TU) were calculated on the basis of the 50% effect concentration (i.e. $1/EC_{50}$ or $1/LC_{50}$). The mass-specific toxicity of foams and chemical extracts were assessed after 48–96 h of exposure, following Standard Evaluation Procedure guidelines [10]. These guidelines are used for pesticide toxicity assessment, with the following categorical descriptors: super toxic = <0.01 mg/L; extremely toxic = >0.01–0.1 mg/L; highly toxic = >0.1–1.0 mg/L; moderately toxic = >1.0–10.0 mg/L; and slightly toxic = >10.0–100.0 mg/L.

All toxicity testing for the Phases 1 and 2 measurements were undertaken at the National Institute of Water and Atmospheric (NIWA) laboratories in Hamilton, New Zealand.

Statistical analysis

Statistical measures of toxicity concentration-response exposures were calculated using ToxCalc V5.0.22A (Tidepool Scientific Software 1994).

Phase 1

Sampling

Sampling was undertaken at five river sites during summer low-flow conditions, including a reference site from Gardiners Creek, St Marys (abbreviated SM) on 15 February 2009 (figure 1 and table 1). Both water and foam samples were collected from two George River sites (Drinking Water Intake, abbreviated WI, and South George River). All samples were collected in pre-cleaned polythene sample containers (5–20 L). Foam samples were concentrated using a floating 'skimmer box' and transferred to a wide-mouthed 5 L pre-rinsed plastic bucket with a sealed lid. Although pesticide samples are normally collected in glass, it is assumed that toxicity for these sites is particle associated and hence should not adsorb to the plastic sampling container. Air-freight shipping of large water volumes in plastic is logistically easier. All samples were ice-cooled and placed in insulated containers for air freight to New Zealand (a chain-of-custody protocol was followed). On arrival, all containers were still surrounded by residual ice packs with no leakage of samples. Additional ice was then added to the surrounding packaging and the samples were transferred to laboratory refrigeration and held in ice until the initial series of tests were performed.

Toxicity testing and analyses were initiated on 17 February 2009. All water samples were filtered through 50 μm nylon mesh to remove large particle debris. Foam samples were filtered through a 150 μm nylon mesh because of the large amount of coarse particulate matter in the samples. Sub-samples for pesticide and herbicide analyses were transferred to amber glass bottles. Sub-samples of foam samples were held frozen (−80 °C) prior to use for definitive blue mussel testing (7 and 22 April 2009).

Toxicity identification

Toxicity identification assessment was undertaken on one representative site on the George River (WI) and the reference site (SM). The cladoceran toxicity of both unfiltered and

Table 1. Sites and sample identifiers for Georges Bay catchment water samples (15 February 2009).

Sample no.	Site	Sample code	Sample type	Site location	Test species[a]
1	South George (SG)	SG	Freshwater	41 17 56 S 147 57 33 E	CL, BM
2	North George (NG)	NG	Freshwater	41 15 47 S 147 57 02 E	CL, BM
3	Pyengana: SG & NG river waters confluence	PY	Freshwater	41 17 07 S 148 00 54 E	CL, BM
4	Water intake for St Helens drinking water supply	WI	Freshwater	41 17 11 S 148 12 32 E	CL, BM
5	Drinking water at water treatment plant (St Helens)	DW	Freshwater (treated)	41 18 22 S 148 13 08 E	CL, BM
6	Treatment filtrate (St Helens water treatment plant)	TF	Freshwater (treated - residue)	41 18 22 S 148 13 08 E	CL
7	Gardiners Creek St. Mary's	SM	Freshwater (reference site)	41 33 01 S 148 10 46 E	CL, BM
8	Foam at water intake at (St Helens drinking water supply)	DW_F	Foam	41 17 07 S 148 00 54 E	CL, BM
9	South George River Foam	SG_F	Foam	41 17 56 S 147 57 33 E	CL, BM
10	Add back: SM, 1 x 5x	SM-1X, _5X	Freshwater		CL
11	Add back: WI, 1 x 5x	WI_1X, _5X	Freshwater		CL
12	Foam: WI, 0.45 um filtered, x% dilution	WI_F_fx	Foam		CL, BM
13	Foam: SG, 0.45 um filtered, x% dilution	SG_X	Foam		CL, BM

[a]CL = *Ceriodaphnia dubia*, BM = *Mytilus galloprovincialis*.

filtered samples was analysed; an 'add-back' experiment was then undertaken in particle-filtered WI and SM water with a 1x and 5x particle concentrate reconstitution. Water samples were centrifuged (3500 rcf, 4 °C, 30 min, Sorval centrifuge) in Corex glass centrifuge tubes. The supernatant was discarded and the pellet of particulate material was reconstituted with 0.45 μm filtered site water, using a tissue homogeniser to provide approximately a 1x (i.e. original particle concentration) and 5x particle concentration. All dilutions were effected with clean control water (Nanopure™ grade – Barnstead). The levels of particle concentration were validated by Coulter Counter particle counts on all samples and the add-back samples subjected to cladoceran toxicity tests.

Chemical characterisation

A range of general and specific water-quality measurements were undertaken, following established protocols. These included: suspended sediment particulate organic matter (by loss on ignition) on all samples; particle counts for add-back experiments (Coulter particle counter, Beckman Coulter Inc, FL, USA); particle size distribution (using EyeTech, Ankersmid Ltd, Oosterhout, Netherlands, laser: 0.1–300 μm); and trace-level analysis for 89 organonitrogen and organophophorus pesticides (Hill Laboratories, Hamilton, New Zealand; www.hill-laboratories.com).

Foaming capacity of extracts

The standard test procedure was: (i) addition of 10 μL of EtOH extract to 500 μL (2%) of distilled water in a 1.5 mL polyethylene Eppendorf microfuge tube (I.D. 10 mm); (ii) held in a 16 tube high-density foam rack (20 mm thickness) attached to sabre saw; (iii) agitated for 30 s at speed 3 on sabre-saw (Hitachi model CR12 V); (iv) measurement of foam height using electronic calliper. Large bubbles on tube walls were noted as 'trace'. EtOH served as the control and produced no foam or bubbles. All measurements were undertaken at room temperature.

Phase 2

Chemicals

All solvents used in this study were of analytical grade or higher. All water samples used were Nanopure™ grade (Barnstead). Preparative HPLC was performed with gradient-grade acetonitrile and analytical HPLC was performed with hypergrade acetonitrile. Methanol used in LH-20 fractionations was HPLC grade.

Sample collection and pre-processing

Sampling of foam for phase-2 analyses was undertaken on 19 September 2009 from the South George River in high-flow conditions using procedures described previously. Sample site location details were recorded on the chain of custody form. Samples were concentrated using a floating 'skimmer box' and were transferred to wide-mouthed 5 L plastic buckets with sealed lids. An estimated 45 L of foam was collapsed to about 8 L of liquor. All samples were ice-cooled and placed in insulated containers for air freight to Adelaide, South Australia for preprocessing.

The foam samples were preprocessed by filtration through 140 μm nylon mesh to remove large particulate debris. Filtered foam liquor was frozen at −80 °C prior to shipping. All containers were couriered frozen to NIWA Hamilton laboratories and stored at −80 °C until subsequent processing. *E. nitens* leaves were collected from five randomly selected plantation trees in the South George catchment and from old growth Victorian *E. nitens* in Bulleen, Victoria. Leaves from each tree were wrapped in aluminium foil and refrigerated prior to shipping. Leaves were transferred to −80 °C refrigeration and couriered to NIWA Hamilton with the foam samples.

The blue mussel embryo-larval toxicity tests were used for the definitive toxicity assessments for two primary reasons: (i) they were found to be markedly more sensitive to the

foam than cladocerans and (ii) the major site of potential ecological effects contains marine bivalves (oysters) in Georges Bay, and blue mussel larvae provide the best surrogate species for toxicity assessment.

Extraction

Frozen foam samples were thawed and transferred to stainless steel trays covered with foil to a depth of about 5 mm. The foam was then frozen (−20 °C) and freeze dried at a tray temperature of −20 °C and vacuum of 100 mTorr until dry. The dried foam was sealed in a 500 mL bottle and frozen at −20 °C until further use. Tasmanian new growth leaves and Victorian old growth leaves of *E. nitens* were frozen at −20 °C and freeze dried in the dark. Dried leaves were ground to a coarse powder in a stainless steel vessel.

Leaves (5.00 g) and foam (2.00 g) were transferred to a stainless steel accelerated solvent extraction (ASE) cell and triple extraction carried out with ethanol at 2000 psi and 40 °C. All respective extracts were pooled. Each was concentrated by rotary evaporation, transferred to a scintillation vial and made to a final volume of 10 mL with ethanol. To determine extract concentrations, aliquots (500 mL) of each were transferred to a pre-weighed vial and dried under a stream of nitrogen at 40 °C, until a constant weight was achieved.

Toxicity-directed fractionation

All crude ethanol extracts were fractionated by preparative HPLC with photodiode array (PDA) detection and LH-20 size exclusion chromatography. After LH-20, the toxic foam fraction was further fractionated by analytical HPLC (figure 4). All chemical fractions were screened for toxicity after the initial preparative HPLC fractionation (First fractionation series; 18 fractions). The toxic fractions which were common to foam, and *E. nitens* leaf extracts (F7–F9) were bioassayed further to provide definitive toxicity measurements and combined for subsequent purification. The combined common fraction (F7c) was then bioassayed and further fractionated by LH-20 size exclusion chromatography (Second fractionation series; 20 fractions). These were toxicity screened and the common toxic fractions measured for definitive toxicity. This procedure was repeated with an analytical HPLC fractionation of the toxic foam fraction only (Third fractionation series; 14 fractions) applying individual screening toxicity assessment. The toxicity of this third fractionation foam series was subsequently assessed after recombining individual fractions and adjustments to the original volume. To avoid repeated drying and redissolution, the dry weights of the crude extracts and purified fractions were determined after solvent evaporation at the completion of the purification and bioassay procedures. Because this was undertaken at the end of the procedures, some of the samples had insufficient material to obtain reliable dry weight measurements and hence mass-specific measures were not calculated for these samples.

LC/PDA/MS analysis

All fractions from each fractionation series were analysed by liquid chromatography/photodiode array/mass spectrometry (LC/PDA/MS). The PDA window was set from 200 to 600 nm and MS window (with positive and negative polarity switching) set from *m/z* 100–2000.

Results

Phase 1

Water and foam toxicity

High toxicity was measured with both cladocerans and blue mussels in both foam samples tested from the Water Intake (WI) and South George River (SG) (see table 2). No toxicity was measured with either cladocerans or blue mussel tests in the water samples from the George River catchment or Gardiners Creek, St Marys (reference site). The foams had markedly higher toxicity for the blue mussels than cladocerans and there was significantly higher toxicity of unfiltered foam compared with filtered foam for blue mussels (table 3). This indicates that the toxicity is largely particle associated.

The low-flow river suspended solid (SS) concentrations in the George River catchment were low, generally <2 g/m^3, with average particle size of 6–8 μm. But high SS concentrations in each of the foam samples were observed, 2600 and 4800 g/m^3, with an average particle size of 14 and 21 μm (table 4).

Table 2. Definitive toxicity for water and foam samples from Georges River catchment, sampled 15 February 2009. Series A, water 50 μm pre-filtered, Series B, water 50 μm + 0.45 μm filtered.

				Concentration (%) for				
Test organism	Sample ID	Test date	Series	LC 50	LC20	NOEC	LOEC	TEC
Cladoceran	SG_F	17/2/09	A	<10.0	<10.0	<10.0	10	<10.0
	WI_F	18/2/09	A	4.4	–[a]	2.2	4.6	3.2
	TF	17/2/09	A	>100	62.7	46	100	67.8
				Concentration (%) for				
				EC 50	EC20	NOEC	LOEC	TEC
Blue Mussel	SG_F	22/2/09	A	0.26	0.1	<0.46	0.46	<0.46
	SG_F	22/2/09	B	4.54	–[a]	1.0	2.2	1.48
	WI_F	22/2/09	A	0.23	0.09	<0.46	0.46	<0.46
	WI_F	22/2/09	B	1.12	0.42	<0.46	0.46	<0.46

Note: NOEC = no observed effect concentration; LOEC = lowest observed effect concentration; TEC = threshold effect concentration (geometric mean of NOEC and LOEC).
[a]Trimmed Spearman-Karber test used due to lack of partial responses no LC_{20} or EC_{20} able to be calculated.

Table 3. Foam suspended solids specific toxicity to cladocerans and blue-mussels.

Site/sample	Code	Ceriodaphnia (CL) LC_{50} (%)	Blue-mussels (BM) EC_{50} (%)	CL Toxic units[a] TU_{50}	BM Toxic units[a] TU_{50}	CL TU/SS[b]	BM TU/SS[b]
Water Intake foam	WI-F	4.4	0.23	23	435	0.0047	0.091
South George Foam	SG_F	<10	0.26	10	385	0.0038	0.15

[a]Toxic unit = 100/EC_{50}.
[b]Suspended sediment data.

Table 4. Particle count and suspended solids measurements for water and foam samples from Georges River catchment, sampled 15 February 2009.

Test no.	Site	Code	Conc. (%)	Particle count (particles/mL) [calculated addback]	Suspend solids (g/m^3)	Inorganic SS (g/m^3)	Organic SS (g/m^3)	Inorganic (%)
1	South George (SG)	SG	100		1.9	1.2	0.7	63
2	North George (NG)	NG	100		1.1	<0.5	0.6	<50
3	Pyengana: SG & NG river waters confluence	PY	100		2.7	0.8	1.9	30
4	Water intake for St Helens drinking water supply	WI	100	21,060	2.6	<0.5	2.1	<20
5	Drinking water at water treatment plant (St Helens)	DW	100		10.9	3.3	7.6	30
6	Treatment filtrate (St Helens water treatment plant)	TF	100		8300	4700	3600	57
7	Foam at water intake (St Helens drinking water supply)	SM	100	15,260	3.4	1.6	1.8	47
8	South George River Foam	WI_F	100	60,150,600	4800	1600	3200	33
8		WI_F	22	6,683,400				
8		WI_F	10	13,043,800				
9	Foam SG	SG_F	100		2600	1100	1500	42
10	ADD-back SM_1x	SM_1x	100	52,213{3.5]				
11	ADD-back SM_5x	SM_5x	100	164,500 [10.8]				
12	ADD-back WI_1x	WI_1x	100	41,440 [2.0]				
12	ADD-back WI_5x	WI_5x	100	144,620 [6.9]				

Samples from the Water Intake (WI) and both foams (WI and SG) were tested with the pesticide and herbicide screen and all analytes were found to be below the analytical detection limit, indicating a low probability that toxic response could be attributable to these chemicals.

The particle-associated toxicity from the foam shows that the river sites are >100 times below the cladoceran toxicity effects threshold (for 50% response), and 3–5 times below the blue mussel effect threshold (low-flow river, no particulate matter in column.

The estimated toxic threshold (based on the TU_{50}) for blue mussels is very low (total SS 6–10 g/m^3). This suggests that shellfish larvae will be particularly vulnerable to adverse effects. Most notably, at high foam concentrations (2.2% unfiltered), near complete disintegration of the blue mussel embryos occurred in both of the foam tests. The level of suspended solids equating to this threshold is 57–105 g/m^3 (from table 4). No embryo disintegration was observed in the filtered foam samples to a markedly higher test concentration (10%). This indicated that the disintegration was a particle-associated phenomenon.

Phase 2

Initial toxicity assessment of foam sample

The foam sample was collected from South George River after a prolonged period of high flow and contained significant quantities of large plant material. This was initially processed by filtration through a 140 μm nylon mesh filter to remove debris, prior to refrigerated storage. An initial sub-sample was screened for toxicity to cladocerans and found to be about 20x more toxic than the previous summer low flow (EC_{50} 1.5%, suspended sediment (SS) = 700 mg/L; TU/SS = 0.095. c.f. 0.0047–0.0038 table 2). This analysis provided confidence that the foam sample was adequately toxic for the comparative analytical procedures to be undertaken.

Chemical and toxicological characterisation of extracts

Table 5 summarises the results of the initial toxicity characterisation of foam and *E. nitens* extracts. The Tasmanian and Victorian leaf extracts are directly comparable, being extracted from the same quantity of leaf material (5.00 g), even though the ethanol extract weights differed (table 5). The Tasmanian foam was extracted from a lower mass of material (2.00 g) with much lower dry weights of ethanol extracts compared to the leaves. The difference in the initial and extract dry weight is the quantity of residual material which was not soluble in ethanol.

Table 5. Toxicity characterisation of foam and *E. nitens* leaf extract.

Sample	Code	EC_{50}	**TU_{50}**	EC_{25}	TU_{25}	Dry weight (mg/mL)	Toxicity[a] (mg/L)	US EPA category[a]	Foam (mm)
Victorian leaves	VOL	1.7	**58.8**	1.3	80	152	2.6	Moderately toxic	4.1
Tasmanian leaves	TNL	4.8	**20.8**	3.4	29.2	231	11.1	Slightly toxic	7.2
Tasmanian foam	TNF	2.8	**35.7**	1.6	62.5	30	0.84	Highly toxic	5.6

Notes: (i) all toxicity measurements to blue-mussel (*M. galloprovincialis*) larvae in 0.1% Ethanol.
(ii) Toxic unit (TU_x) values calculated from corresponding EC_x values (TU = 1/EC; i.e. higher TU is greater toxicity).
[a]US EPA categorisation of toxic potency based on EC_{50} and dry weight [10]. Procedure used for pesticide standard evaluation procedures.

The Victorian leaves were 2.8 times more toxic than the Tasmanian leaves and the extracts contained a lower dry weight of material (66%). By comparison with the Tasmanian foam sample, the Victorian leaf extract was 1.7 times more toxic, with 3.1 times more dry weight of material, but the Tasmanian leaves were 0.6 times as toxic, with 13 times as much dry weight of material.

The crude leaf and foam extracts were categorised by the US EPA standard evaluation procedure for pesticide risk assessment [10]. This procedure categorises the potency to aquatic organisms based on the measured toxicity (EC_{50}) and the quantity of material per litre. Accordingly, the Tasmanian leaves were 'slightly toxic', the Victorian leaves 'moderately toxic' and the Tasmanian foam 'highly toxic' (table 5). This categorisation is relative to weight and thus would be expected to increase 'toxicity', as a crude product is purified to the active chemical(s). The potential for adverse environmental effects will be related to the quantity of toxicant released into the environment.

The foam production ability was also determined on each of these extracts using a standardised foam assay. Figure 3 shows the results; table 5 summarises the foam height. All extracts produced substantial foams with similar fine bubble structure (figure 3), which were stable to decay. The foams showed marked differences in both the production of foam and the rate of decay. The Tasmanian leaves produced the most foam (maximum 7.2 mm) and had the slowest decay rate (approximate half-life 12 h). Tasmanian foam produced the next greatest amount of foam (5.6 mm), with a decay half-life of approximately 2.8 h. Victorian leaves had the lowest foam production (4.1 mm) and a decay half-life of 2.4 h.

The crude extracts were fractionated by a preparative HPLC procedure to produce 18 fractions for use in bioassays (figure 4). Toxicity screening bioassays with the cladoceran and blue mussel were used for the bioassay-directed fractionation procedure. The basis for this approach is that toxic fractions common to foam and leaf extracts are the compounds of interest for further chemical purification and characterisation.

Figures 5 and 6 display the chemical fractionation of crude extracts and superimposed toxicity screening data. These results showed that toxicity occurred in only a few Tasmanian foam and leaf fractions generated by the purification procedures, with highest toxicity for both cladocerans and blue mussel occurring in a single Tasmanian foam fraction (f8). Common toxicity fractions occurred in the f7–f8 fractions in Tasmanian foam and both Tasmanian and Victorian leaves – affecting both cladocerans and blue mussels. But a higher number of toxic fractions occurred in the Victorian leaf extracts compared to the Tasmanian foam or Tasmanian leaf samples. Furthermore, a concentration of toxicity occurred over fractions f12–f14 in both leaf fractions. These fractions were not toxic in the Tasmanian foam. A range of fractions (f7–f9) were toxic and common to foam and both

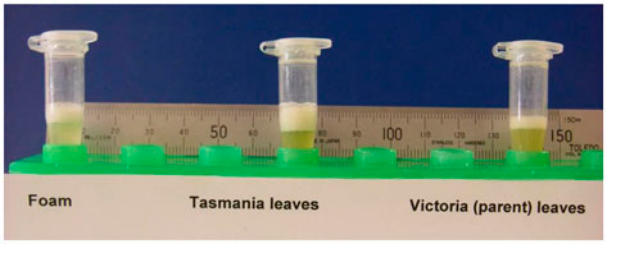

Figure 3. Photograph of results of foam assay with crude extracts.

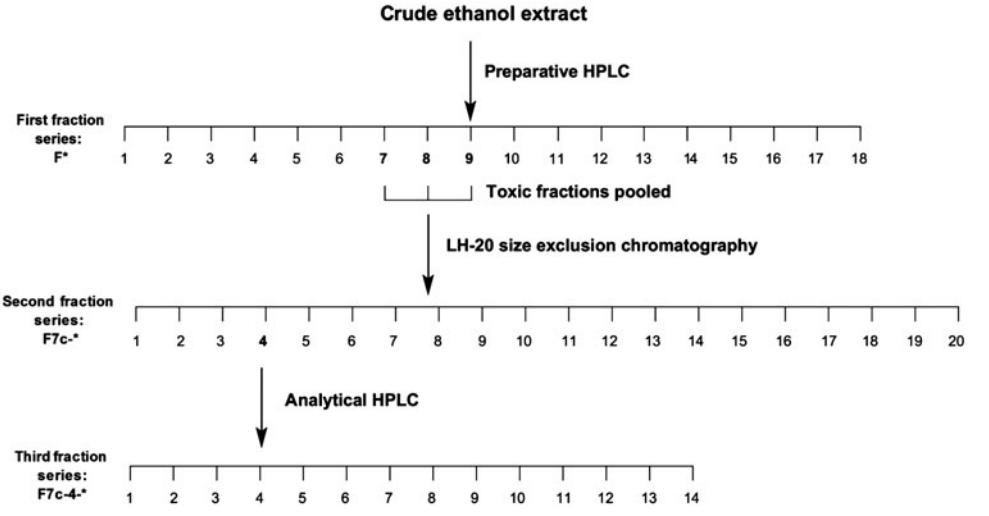

Figure 4. Fractionation scheme used for foam and leaf extracts.

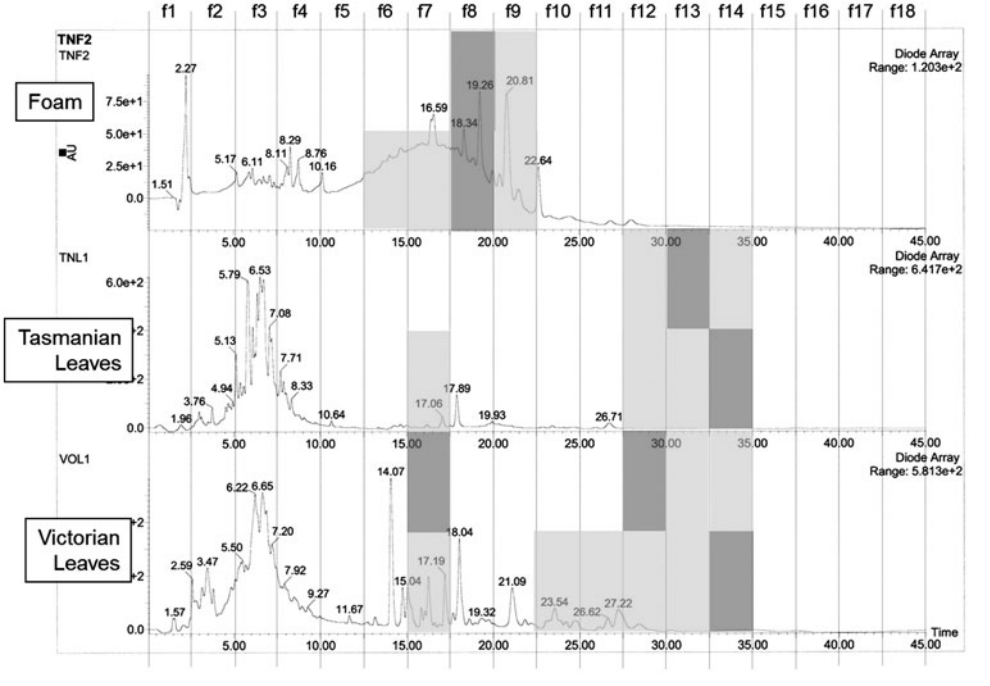

Figure 5. Chemical fractionation (preparative HPLC) and toxicity of Tasmanian foam and Tasmanian and Victorian leaf extracts. Shaded boxes on each trace are toxic fractions for cladocerans (top) and blue mussel (bottom). Darkest shaded box are most toxic fraction(s).

leaf extracts. Foam-forming ability in chemical fractions was not generally concordant with the high-toxicity fractions – with the sum of the foam in the various fractions being markedly less than the total foam production in the crude extract (table 6).

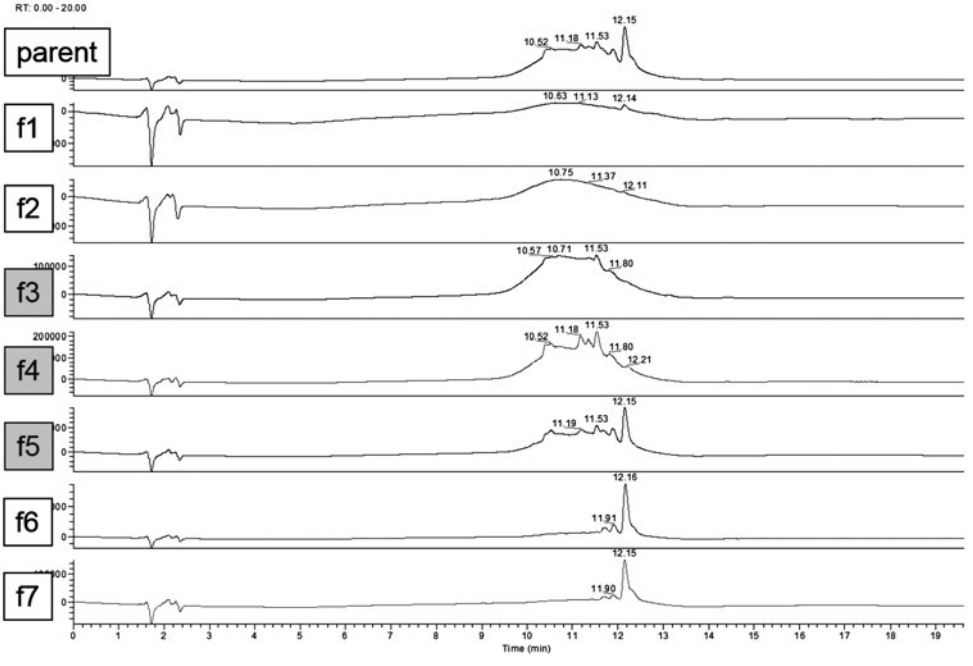

Figure 6. LC/PDA analysis of first 7 foam fractions from LH-20 chromatography on toxic preparative HPLC fractions. Toxic fractions are shaded grey.

The preparative HPLC separation of the Tasmanian and Victorian leaf extracts showed similar UV absorption characteristics in the early eluting non-toxic fractions (f2–f4, figure 5), but there was less similarity in the common toxicity fractions (f7), where the Victorian leaves showed a greater number of resolved peaks. Notably, the highest toxicity occurred in fractions with minimal UV absorption (f12–f14), indicating that the compounds causing the toxicity were characterised by weak UV adsorption. The chemical fractionation of the foam showed poor resolution of the region with toxicity common with the leaves. Foam fractions f6–f9 included a large number of poorly resolved peaks (figure 5). The large number of unresolved peaks observed in the foam appeared to be concurrent with toxicity.

Attempts to resolve these compounds by reverse-phase HPLC proved unsuccessful and a second technique of LH-20 size exclusion chromatography was used, affording 20 fractions (figure 4). Toxicity bioassays were carried out against the more sensitive blue mussel larvae. Only the fractions f7c-3, f7c-4 and f7c-5 from foam proved toxic (table 6). Higher volumes of leaf extracts were fractionated by the same procedure and toxicity was retested. These more concentrated leaf fractions also showed toxicity in the same f7c-3 to f7c-5 foam fractions, indicating common toxic fractions between foam and leaves. Fractions f7c-6–f7c-8 also contained toxic fractions for leaves – which were not present in the foam samples (data not shown).

All toxic foam fractions from the LH-20 fraction series were analysed by LC/MS to provide molecular weight determinations. The toxic foam fractions (f7c-3–f7c-5) appeared to be partially overlapping with the large number of unresolved compounds (figure 6).

Table 6. Toxicity summary for HPLC and LH-20 fractionation (Preparative HPLC Fractions).

	Code	EC_{50}	TU_{50}	EC_{25}	TU_{25}	TU_{50} (% total)	Dry weight (mg/mL)	Toxicity[a] (mg/L)	US EPA category[a]	Foam (mm)
VOL	V7	31	3.2	15.2	6.6	5.5	ND	ND	ND	0.5
	V11	>100	0.6	71.8	1.4	1.1	2	NT	Negligible	0
	V12	12.5	8.0	7.3	13.7	14	6	0.75	Highly toxic	0
	V13	43.4	2.3	33.5	3.0	3.9	10	4.3	Moderately toxic	1.5
	V14	5.2	**19.2**	3.5	28.6	33	2	0.10	Highly toxic	0
	Totals		33.4		53.2	57				
TNL	T7	56.7	1.8	3.5	28.6	8.5	ND	ND	ND	0
	T11	69.2	1.4	6.6	15.2	6.9	1	0.69	Highly toxic	0
	T12	14.4	6.9	2.3	43.5	33	6	0.86	Highly toxic	0
	T13	23.6	4.2	9.9	10.1	20	6	1.42	Moderately toxic	0
	T14	8.4	**11.9**	3.6	27.8	57	4	0.34	Highly toxic	0
	Totals		26.3		125	126				
TNF	F6	80	1.3	2.7	37.0	3.5	1.53	1.22	Moderately toxic	0.5
	F7	18.5	5.4	6.4	15.6	15	1.71	0.32	Highly toxic	1.6
	F8	8.6	**11.6**	3.6	27.8	33	ND	ND	ND	t
	F9	11.8	8.5	4.3	23.3	24	ND	ND	ND	0
	F10	100	1.0			2.8	0.71	0.71	Highly toxic	0
	Totals		27.8		104	78				
LH20 chromatography of fractions 7–9 composite (parent)										
Concentrates[b]		EC_{50}	TU_{50}	Concentration factor	TU_{50} (adjusted)					
f7–9 parent	VOL	0.27	370	5	74.1	100	ND	ND	ND	0
	TNL	0.39	256	5	51.3	100	ND	ND	ND	1.0
	TNF	2	50	3	16.7	100	5.1	0.10	Highly toxic	1.5

(*Continued*)

Table 6. (*Continued*).

	Code	EC_{50}	TU_{50}	EC_{25}	TU_{25}	TU_{50} (% total)	Dry weight (mg/mL)	Toxicity[a] (mg/L)	US EPA category[a]	Foam (mm)
LH20 fractions						TU_{50} (% adjusted total)				
VOL	F3	100	1			1.4	ND	ND	ND	0
	F4	6.4	15.6			21	ND	ND	ND	0
	F5	2.6	**38.5**			52	4	0.10	Highly toxic	0
	Totals		55.1			74				
TNL	F3	70.3	1.4			2.7	5	3.52	Moderately toxic	t
	F4	8.7	11.5			22	5	0.44	Highly toxic	t
	F5	1.7	**58.8**			115	2.5	0.04	Extremely toxic	0
	Totals		71.7			140	ND	ND	ND	
TNF	F3	24.7	4			24	1.75	0.43	Highly toxic	1.0
	F4	18.8	**5.3**			32	1.75	0.33	Highly toxic	0
	F5	38.2	2.6			16	1.25	0.48	Highly toxic	t
	Totals		11.9			71				

Notes: (i) all toxicity measurements to blue-mussel (*M. galloprovincialis*) larvae in 0.1% Ethanol; (ii) Toxic unit (TU_x) values calculated from corresponding EC_x values (TU = 1/EC; i.e. higher TU is greater toxicity); (iii) TU as percentage of total is calculated for respective HPLC and LH-20 extracts. Bold indicates highest toxicity for each fractionation.

Abbreviations: NT = not toxic; ND = not determined; t = trace of foam.

[a]US EPA categorisation of toxic potency based on EC_{50} and dry weight [19] (Toxicity descriptors: super toxic = <0.01 mg/L; extremely toxic = >0.01–0.1 mg/L; highly toxic = >0.1–1.0 mg/L; moderately toxic = >1.0–10.0 mg/L; & slightly toxic = >10.0–100.0 mg/L). Procedure used for pesticide standard evaluation procedures.

[b]Concentrated by stated concentration factor. TU calculations adjusted for this value.

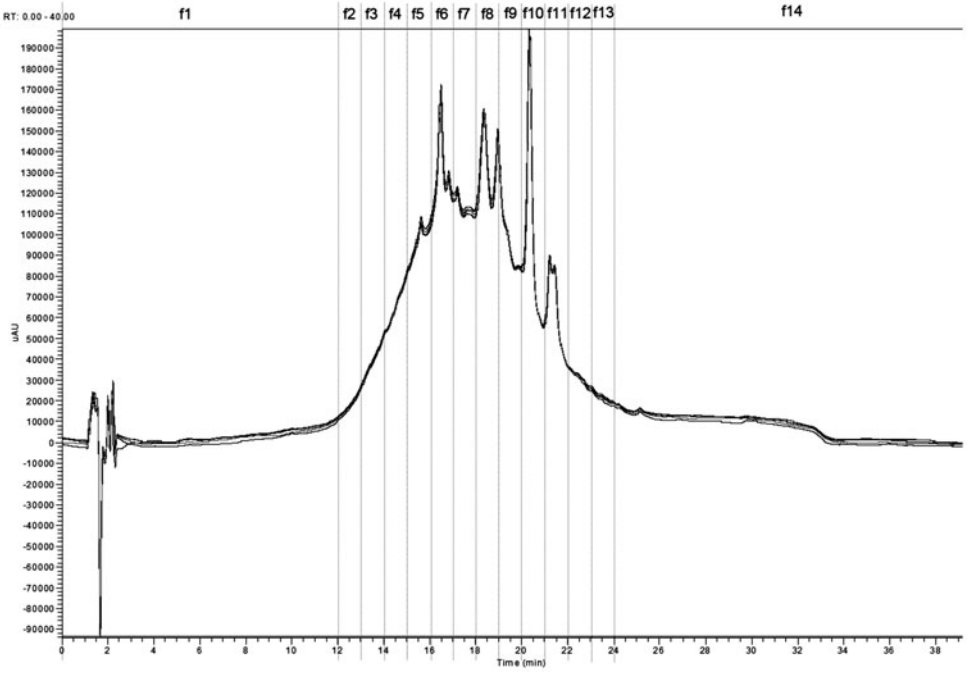

Figure 7. Analytical HPLC fractionation of Toxic Foam Fraction TNF2-1-7C.

To assess whether toxicity in the foam was concurrent with the unresolved mixture of peaks, a third fractionation was carried out on foam only. Fractionation of a toxic second fraction series foam fraction (TNF2-1-7c-4) by analytical HPLC (figure 7) afforded 14 fractions, designated third fraction series (figure 4). No toxicity was observed against blue mussel larvae (data not shown). Pooling all the fractions restored the toxicity – indicating that multiple components of very similar molecular weight were responsible for the observed toxicity.

Quantified toxicity associated with chemical fractionation procedures

Quantitative measures of toxicity were made using the blue mussel bioassay on the crude extracts, common toxicity fractions and composited fractions used for molecular weight fractionation and characterisation (table 6). Toxic units (TU) were calculated relative to the crude extracts and composites used for the molecular weight (LH-20) fractionation. The HPLC-fractionated foam samples accounted for 78% of the total initial crude extract toxicity, with 92% of the foam toxicity found in fractions common with leaf extracts (i.e. f7–f9). Notably, the toxic leaf fraction (f7) common with the foam was a low percentage of the total toxicity (5.5% for Victorian; 8.5% for Tasmanian). The total toxicity recovered from leaf extracts differed between the two *Eucalyptus* sources. The preparative HPLC fractionations recovered 57% of the crude extract toxicity for the Victorian leaves and 126% for the Tasmanian leaves (table 6).

The composite f7–f9 fraction TU values were higher than expected in the plant leaf fractions (f7-9 TU = 51.3, c.f. TNL f7 TU = 8.5, table 6), but the foam composite was

35% less that the corresponding individual fractions (i.e. f7-9 TNF TU = 16.7, c.f. TNF f7 + f8 + f9 of HPLC, TU = 25.5). Similar differences were observed for the molecular weight fractionation (LH-20) where the sum of the toxicity for the fractionated Tasmanian leaves was greater than the original toxicity; the Victorian leaves showed a toxicity reduction (−24%). The cause of these differences in toxicity between crude and fractionated extracts is unknown. A contributory factor with the leaves would be that definitive toxicity on only f7 was determined in the HPLC fractionation (i.e. not f6 and f8). The consistent differences between Victorian and Tasmanian leaves, however, suggest that the differing chemical fractions may have synergistic and antagonistic interactions, in addition to the simple additive toxicity assessed in the toxicity summary (table 6). The nature of these potentially complex interactions was not established; but this effect is also reflected in the final-phase toxicity assessment of the chemical fractionation (Third fraction series, figure 4).

The 'Toxicity' classifications for the chemical fractions follow the US EPA procedure for pesticide categorisation [10]. These classifications are based on the measured toxicity (EC50) and the dry weight of material causing the toxicity. Unfortunately, some of the more highly toxic fractions could not be categorised because the fractionated volume was too low to allow reliable dry weight determination, since most of the material had been used for the chemical analyses and toxicity bioassays. Most of the foam fractions could be categorised and were 'highly toxic' in both preparative HPLC and the subsequent molecular weight fractionations (table 6). Common leaf fractions from the LH-20 were 'highly toxic' for the one Victoria leaf fraction categorised (f5), and 'moderately toxic' to 'extremely toxic' for the three Tasmanian leaf fractions (f3–f5) (table 6).

Chemical characterisation of toxic foam fractions

As the unresolved foam compounds were closely related to toxicity, close analysis of LC/MS data from these fractions was carried out. Time slices through this mass of peaks revealed a common series of compounds in the toxic fractions with molecular weight range between 400 and 500 daltons. This places these compounds in the 'small molecule' range of chemicals (i.e. generally considered below 1000 daltons), as opposed to common biopolymer compounds (proteins, carbohydrates, etc.) that are measured in kilodaltons. As further fractionation of this active foam band (Third series, table 6) resulted in loss of toxicity in these fractions, this indicates that the foam toxicity is the result of multiple components in this limited molecular weight range.

Common Eucalyptus *metabolites*

To assess which common *Eucalyptus* metabolites might be present in the toxic leaf fractions, expected molecular ions were extracted from LC/MS data for those known to be present in *E. nitens*. Validation of sideroxylonal elution and characterisation was based on a previously published study [11].The chemical fractionation procedures in this study were able to reproduce the expected absorption spectra and molecular weight determinations for these compounds.

Table 7. Preliminary evaluation of known *Eucalyptus* metabolites in HPLC fractions from foam and leaf extracts.

Metabolite group	Simple FPCs[a]		Monoterpene euglobals		Macrocarpals[b]	Sideroxylonals[c]	
Molecular weight (MW)	266	252	386		472	500	
Extracted *m/z* (−ve mode)	265	251	385		471	499	
HPLC Retention Time[d]	Not detected	9.9 min	10.4 min	12.6 min	13.5 min	11.5 min	12.1 min	12.8 min
Sample[d,e]								
Tasmanian Leaves (crude extract)	−	+	+	+	++	++	++	−
F7	−	++	++	−	−	+	−	−
F12	−	−	−	−	++	−	−	−
F13	−	−	−	−	−	−	++	−
F14	−	−	−	−	−	−	−	−
Victorian Leaves (crude extract)	−	++	++	+	++	++	++	+
F7	−	++	++	−	−	++	++	−
F10	−	−	+	+	−	+	++	++
F11	−	−	++	++	++	+	++	++
F12	−	−	−	−	++	−	++	−
F13	−	−	+	−	−	−	++	−
F14	−	−	++	−	−	+	++	−
Tasmanian Foam (crude extract)	−	−	−	−	−	−	−	−
F6	−	−	−	−	−	−	−	−
F7	−	−	−	−	−	−	−	−
F8	−	−	−	−	−	−	−	−
F9	−	−	−	−	−	−	−	−
F10−F14	−	−	−	−	−	−	−	−

[a]Includes jensenone (MW 266) and grandinol/homograndinol (MW 252).
[b]Includes 12 macrocarpals with MW 472.
[c]Sideroxylonals A–C (MW 500).
[d]Only toxic fractions included, with the exception of F10–F14 for foam, which are included for comparison.
[e]Most toxic fraction highlighted in bold.

Table 7 shows common metabolites and the likelihood of their occurrence in fractions from this study. None of these common metabolites were detected in any foam fractions. Sideroxylonals are the most abundant *E. nitens* metabolites [12] and are detected in all toxic Victorian *E. nitens* fractions, but only in two Tasmanian toxic leaf fractions. In general, Victorian leaves had higher levels of metabolites than Tasmanian leaves. Notably, for Tasmanian leaves, f14 is the most toxic fraction from this series, but no metabolites were detected in this fraction, suggesting toxicity (in this case) is not caused by common metabolites.

Several metabolites were detected in the common toxic leaf fractions, including simple formylated phloroglucinol compounds (FPCs), macrocarpals and sideroxylonals (table 7). No known *Eucalyptus* metabolites were detected in the Tasmanian foam sample, indicating that the toxic metabolites were unknown compounds of MW about 400–500 as established from the LC/MS analyses. Toxicity tests were undertaken to determine the sensitivity of blue mussels to the sideroxylonal standards supplied by Foley (personal communication). These tests were to a maximum concentration of 1.0 mg/L in bioassay (i.e. using a 1.0 mg/mL stock) and found no detectable toxicity to this species. This stock solution concentration of sideroxylonal was comparable with the concentration measured in the Victorian leaves. Assuming that these purified sideroxylonals are representative of those occurring in the Tasmanian and Victorian leaves, the likely contribution of the sideroxylonals to the blue mussel toxicity is low.

Discussion

The results show a high-toxicity response to foam samples from the George River catchment for both freshwater cladoceran and marine blue mussel species, but no detectable toxicity in river water column samples at the time of low water flow sampling. A marked reduction in toxicity to blue mussels occurred in both foam samples after filtration to remove most particulates. This suggests that particle-associated toxicity was a significant contributor to the foam toxicity.

Comparison of the threshold particulates' (suspended solids [SS]) values with the George River catchment values, averaging about 2 g/m^3 (table 3), shows that the river sites are >100x below the cladoceran toxicity effects threshold, and 3–5x below the blue mussel effect threshold. This strongly suggests that storm river flow and other rainfall events may readily exceed the threshold for blue mussel effects, but only occasionally exceed the threshold for cladoceran effects.

The cladocerans and blue mussel embryos showed marked differences in sensitivity to the foam contaminants. The cladoceran mortality was 19–38 times less sensitive than the blue mussel embryo-larval development (comparison of TU_{50} values, table 3). Although a growth and reproduction test with cladocerans might be expected to be more sensitive than survival (by 2–10 times), the magnitude of this difference suggests that the marine species are inherently more sensitive to this toxicant.

The toxicity results for the Tasmanian foam sample, which was collected from the South George River after a prolonged period of high flows, confirmed the high toxicity of the previous measurements. Mass-specific toxicity to cladocerans was indicatively 20 times more toxic on this occasion than the previous summer low-flow sample. This confirms the persistence of toxic foam in the river system. The chemical fractionation and toxicity measurements on the organic extracts showed that common toxicity fractions occurred in the

Tasmanian foam and both Tasmanian and Victorian leaves – affecting both cladocerans and blue mussels. An unknown mixture of compounds on HPLC and molecular weight ranges of 400–500 daltons were present in the toxic foam fractions. There were differences in Tasmanian *E. nitens* leaves as compared with Victorian *E. nitens* leaves, both in chemical characteristics of common toxic fractions and in the markedly higher foaming ability of the Tasmanian leaf extracts. Specific chemical identification techniques found none of the common *Eucalyptus* metabolites in the toxic foam fractions, and eliminated the possible toxic contribution of sideroxylonals to foam or leaf extracts based on toxicity measurement of standards.

It is highly probable that there is a relationship between foam toxicity and the toxicity from *E. nitens* leaf extracts (both Victorian and Tasmanian) based on the co-occurrence of common toxic fractions. Toxicity classifications for the Tasmanian foam and *Eucalyptus* leaf chemical fractions, following the US EPA procedure for pesticide categorisation, classified the original foam material as 'highly toxic' [10].

This study shows that a chemical and toxicological relationship between foam and *E. nitens* leaf components appears highly likely.

The literature indicates that *Eucalyptus* leaf extracts are toxic and contain pharmacologically active compounds. These may affect neurological pathways [13] and are neurotoxic in human beings [14]. Cyanogenic glycosides cause animal and aquatic acute toxicity. FPCs cause toxicity to liver and serotonin systems in insects, mammals and marsupials via gut and skin absorption [14–16]. The most potent molluscicidal agents from *Eucalyptus* sp. appear to be present in the leaves [17].

The toxicological and chemical data on the Tasmanian foam provide measures of high-potential hazard, but the extent and magnitude of ecological adverse effects has not yet been investigated. The foam constituents generating the toxicity in this study are multi-component and composed of as yet uncharacterised chemicals. The relationship of common chemical fractions in foam to the *E. nitens* leaves is based on the co-occurrence of toxicity in the common fractions in two sequential chromatographic procedures. The chemical nature of the toxicant in the common fractions from Tasmania foam and Tasmanian and Victorian leaves is as yet unknown. Common metabolites, however, have been eliminated because they either were not detected in these fractions or have been shown to have low toxicity (e.g. sideroxylonals). The highly lipophilic nature of the toxin provides the potential to produce an increased and unpredictable toxic effect when mixed with other substances.

There is a need to clarify, for all water users (human, animal, aquatic), the role of the amount and characteristics of the particulates in the foam and water column in the determination of water quality in the George River at any one time. Sediments in short flowing rivers, such as the George River and its tributaries, with intermittent flood or rain events can accumulate and concentrate toxicants with no degree of any certainty of establishing equilibrium between the sediments and the water column [18]. The foam in the George River was found to be relatively stable and may well serve as an accumulator and vector for toxicants. This needs further investigation.

The nature, composition and characteristics of the foam are particularly important for potential environmental adverse effects. Because the foam complex is highly toxic, particularly to marine mussel larvae, there is a risk that toxic material may be transported undiluted to the drinking water intake pipe for St Helens on the way to Georges Bay. Thus, these contaminants pose a risk of environmental and human harm.

Conclusion

These findings indicate the potential for contamination of water catchments and associated estuaries from toxic chemicals and metabolites from monoculture plantations of *E. nitens*. The extent and magnitude of ecological risk posed by growing large acreages of monoculture *E. nitens* in water catchments has yet to be established, not only with regard to the George River and Georges Bay ecosystems, but for all other water catchments and ecosystems associated with *E. nitens* monoculture plantations. Water pollution not only directly affects aquatic organisms, but also may adversely impact environmental and human health through bio-accumulation and food-chain pathways [19].

Further research into the contamination of water catchments by chemicals present in monoculture plantations will require characterisation of sediments, water and leaf, and leaf litter and the potential of these to cause adverse effects in ecosystems. Other pathways by which toxicants may enter the soil and groundwater (e.g. tree roots) also need investigating. A first necessary and urgent step will be to investigate the effects of complex mixtures of toxicants in water systems on immune systems including cytokines using human cell lines and aquatic species. The generation of stable foams is considered an important potential mechanism for transport of contaminants to downstream ecosystems, with potential effects over larger spatial scales than may be anticipated.

The research required must include the role of monoculture plantations, especially those with modifications to the original genetic material (enhanced selective breeding included) of tree seedlings and any consequences to bio-system functioning. There is a need to apply a living risk assessment paradigm, incorporating emerging information and recalibrating risk as new information emerges.

Acknowledgements

We are very grateful for the support of many who have coordinated efforts and worked beyond normal expectations to complete this project. For logistics and organisation: Pete Long, Kate Tobin (Long Howland Lawyers & Advisors). For a weekend collection effort undertaken by: John Franceschinni, Mike Bleaney, Alison Bleaney, Jim Harris, Ian Coatsworth and others. For sample processing and facilities, Fiona Young. For a sample courier flight: Mike Bleaney; Laboratory toxicity testing: Mike Martin, Glenys Croker, Marieke van Kooten, Anathea Albert, Helen Brider, Karen Thompson, David Bremner (NIWA). Particle size assessment: Ron Ovenden (NIWA). For constant encouragement: the Editor.

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