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## Leachates of *Eucalyptus globulus* in Intermittent Streams Affect Water Parameters and Invertebrates

*key words:* summer pools in streams, leaf leachate in streams, toxicity, zoobenthic fauna

### Abstract

Low order streams running through *Eucalyptus globulus* plantations in Central Portugal are frequently reduced to isolated permanent or temporary summer pools with darkly stained water due to leaf leachates. Here we assess the toxicity of such leachates to the shredder *Sericostoma vittatum*. Leachates resulted in deoxygenated and more acid water, and increased its phenolic content and conductivity. *S. vittatum* exposed to high concentrations of leachates failed to grow and died within 30 days even in the presence of high quality food. Larvae in water with half the leachate concentration, consumed less leaf material, had lower growth rates, did not pupate and died within 100 days. Eucalypt leachates *per se* may affect the viability and ecology of macroinvertebrates in Portuguese streams.

### 1. Introduction

Plantations of *Eucalyptus globulus* LABILL. are known to negatively affect terrestrial (SOUSA, 2003) and aquatic systems (GRAÇA *et al.*, 2002). Several studies in the Iberian Peninsula demonstrate that the replacement of the native deciduous vegetation by eucalypt monocultures led to important changes in stream hydrology, organic matter dynamics and litter quality; all factors have been implicated in the impoverishment of fungal and invertebrate communities in streams (CANHOTO and GRAÇA, 1995; BÄRLOCHER and GRAÇA, 2002; GRAÇA *et al.*, 2002; MOLINERO and POZO, 2004; OTERMIN *et al.*, 2002).

Most studies evaluating the impacts of eucalypt plantations on stream detritivores have focused on direct effects of the low quality and/or on toxicity of the leaf litter on the consumers (e.g., CANHOTO and GRAÇA, 1996, 1999). However, the leaves' impact on stream communities may extend to their release of soluble organic and inorganic compounds by leaching (TUCKEY, 1970). Leaching in *E. globulus* can last for up to 7 days, with mass losses up to 25% of the leaf mass (HART and HOWMILLER, 1975) and a concomitant decrease in leaf caloric value (≈17%) and phenolics (e.g., CANHOTO and GRAÇA, 1996).

Flow in streams draining eucalypt plantations in Central Portugal varies strongly with season and may be intermittent, with high flow in autumn/winter and low flow in summer. During the warmer seasons, some streams may be reduced to a series of standing pools without visible water flow between them. These pools, usually confined to channel depressions or by abundant woody debris (MOLINERO and POZO, 2004), are discrete, highly retentive structures saturated with leaves at different stages of degradation. The tolerance of invertebrates (and decomposers) to the high level of eucalypt solutes in these stagnant waters is not

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known. Moreover, these periods coincide with the peak litter fall of eucalypt (GRAÇA *et al.*, 2002). CHERGUI *et al.* (1997) suggested that large inputs of senescent leaf litter into streams (amplified by thermal stress) are responsible for toxicity and deoxygenation of the water. OTERMIN *et al.* (2002) further suggested that drought events in eucalypt streams are responsible for a drastic reduction in abundance and diversity of the stream macroinvertebrate community. However, the relative importance to the biota of stress associated with the estival flow reduction in eucalypt streams is unclear.

The purpose of this study was to evaluate the (a) physico-chemical characteristics of the summer pools formed in a stream draining an eucalypt plantation in Central Portugal and (b) the effects of eucalypt leaf leachates on the survivorship, feeding and growth of stream invertebrates, using *Sericostoma vittatum* SPENCER (Trichoptera; Sericostomatidae) as a model. This species is a common and abundant shredder in some Central Portuguese low order streams.

## 2. Materials and Methods

### 2.1. Stream Pools

The physico-chemical characteristics of the pools were evaluated in a second order stream, 'Ribeiro Lázaro', Caramulo, draining an eucalypt plantation (40°30'572" N; 8°13'234" W; drainage basin area – 128.2 ha), at the end of August. The studied reach measured ≈50 m and contained 3 isolated pools (water volume: 72.2 l ± 61.2 SE). In each pool we measured *in situ* water temperature, pH, O<sub>2</sub> saturation and conductivity. One litre water samples were brought to the laboratory for phenolic measurements (Folin-Ciocalteu assay; MARTIN and MARTIN, 1982).

Since the number of leaves inside the pools were difficult to quantify due to their degradation, we estimated this value from the density of leaves in the riparian area and stream banks. This number was 894 leaves ± 246 (SE). We calculated that up to 32 g of leaf dry mass may accumulate per 1 l of pond water.

### 2.2. Effects of Eucalypt Immersion in Water

To assess the direct effect of leachates in the water, we submerged 32 sets of 8.00 g (± 0.01 SE) of air-dried senescent eucalypt leaves in 250 ml of artificial pond water (APW – ca. 80 mg l<sup>-1</sup>; Cl, 145 mg l<sup>-1</sup>; Mg, 12 mg l<sup>-1</sup>; Na, 18 mg l<sup>-1</sup>; K, 3 mg l<sup>-1</sup>; pH 7.9; CANHOTO and GRAÇA, 1999). The flasks were placed in a non-illuminated room, at 20 °C. After 2, 4, 6, 8, 12, 48 and 168 hours, 4 bottles were randomly taken to measure oxygen, pH, conductivity, water phenolic contents and leaf mass loss. One-way ANOVA, with time as a factor, was used to evaluate the changes in each parameter due to leaves leaching (ZAR, 1996).

### 2.3. Larvae Survival, Consumption and Growth

To assess the effects of eucalypt leaf extracts on invertebrate, leaf leaching (32 g l<sup>-1</sup>) was stimulated under laboratory conditions (15 °C; 12 : 12 h light/dark photoperiod), in strongly aerated APW. The solute (L100%) was collected, decanted and frozen until needed. Eucalypt leachates with half the concentration (L50%) were obtained from the initial solution using APW (1 : 2).

For chemical characterization, the eucalypt extracts were obtained as above, but in aerated distilled water. Leachates were then analyzed for total phenolics (Folin-Ciocalteu assay; MARTIN and MARTIN, 1982), total lipids (sulphophosphovanillin assay; GESSNER and NEUMANN, 2005), organic nitrogen (Kjeldahl method; WILDE *et al.*, 1972), terpenes (steam distillation followed by analytical gas chromatography; SALGUEIRO *et al.*, 2003), dissolved organic carbon – DOC (Standard Methods Evaluation for Water and Wastewater – 5310C) – and the concentration of Na, K, Mg, Ca, F, Cl, CH<sub>3</sub>CO<sub>2</sub>, NO<sub>3</sub>, PO<sub>4</sub>, SO<sub>4</sub> ions (ion analyzer; Dionex DX – 120).

Larvae of *S. vittatum* ( $6.78 \text{ mg} \pm 2.9 \text{ SE}$ ) were collected in 'Ribeira de S. João', Lousã ( $40^{\circ}05'59''\text{N}$ ;  $8^{\circ}14'02''\text{W}$ ; drainage basin area –  $18 \text{ km}^2$ ), a 4<sup>th</sup> order stream in Central Portugal. Individuals were acclimatized to laboratory conditions ( $15^{\circ}\text{C}$ ; 12:12 h light/dark photoperiod) for 1 week. One hundred and eight larvae were individually allocated to plastic containers filled with 200 ml of aerated APW, L50% or L100%. The bottom of the containers was covered with a layer of fine, ashed ( $500^{\circ}\text{C}$ ; 8 h) stream sand. Each individual was provided with two pre-weighted alder disks (*Alnus glutinosa* L.) cut from leaves conditioned in the same stream for 3 weeks. Leaf disks without larvae were used as controls.

The leaf disks were replaced every two days. The aqueous media (APW, L100% or L50%) and sand were replaced weekly for up to 102 days. Survival, consumption and specimens mass were estimated every two days. The maximum length of the anterior opening of the caddis case (Lc) was used to determine the larvae dry mass (Dw) according to the expression (CANHOTO, 1994):

$$\ln Dw = (Lc - 1.664)/0.599 .$$

Mean specific growth rates were determined as a % of dry mass gained per day (SUTCLIFFE *et al.*, 1981).

Oxygen saturation ( $\approx 100\%$ ) and media pH ( $\approx 7$ ) were systematically monitored and phenolic contents of the leaves offered to the larvae were also measured throughout the experiment (Folin-Ciocalteu assay; MARTIN and MARTIN, 1982).

The slopes of larval survivorship and growth over time were compared by Analysis of Covariance followed by Tukey's test (ZAR, 1996). The median lethal time (LT50) values (from the relationship probit transformation of effect percentage vs. log time) were obtained by probit analysis (FINNEY, 1971). Individual consumption was calculated as the difference between the initial and final leaf dry mass, corrected by controls, and expressed per mg dry mass of consumer per day. The mean feeding rates over 52 days of larvae in APW and larvae in L50% were compared by a *t*-test (ZAR, 1996).

One-way ANOVA, followed by Tukey's Test, was used to compare phenolic content of the leaves offered to the individuals between the 3 testing groups (ZAR, 1996).

### 3. Results

#### 3.1. Stream Pools

All stream pools had a large number of eucalypt leaves in several stages of degradation (Fig. 1). The water was dark, acidic (pH of  $5.2 \pm 0.2$ ), the temperature was  $19.0^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$  (SE). Oxygen saturation was  $21.0\% \pm 0.6\%$ , the conductivity  $178 \mu\text{S cm}^{-1} \pm 40 \mu\text{S cm}^{-1}$  and concentration of phenolics was  $0.12 \text{ mg ml}^{-1} \pm 0.01 \text{ mg ml}^{-1}$ .

#### 3.2. Effects of Eucalypt Immersion in Water

Immersing leaves in the laboratory (48 hours) reduced oxygen and pH, and increased dissolved phenolics and conductivity of the solution (in all cases, ANOVA,  $P < 0.001$ ; Fig. 2).

Leaf mass decreased exponentially ( $k \text{ day}^{-1} = 0.003$ ;  $r^2 = 0.85$ ); in 48 h, the leaf material had lost 14% of its initial mass. All parameters, except phenolics, changed sharply within the first 6 to 8 hours, followed by a smoother change (Tukeys' test,  $p < 0.001$ ). Phenolics increased linearly ( $y = 0.0147x + 0.0108$ ;  $r^2 = 0.99$ ) during the same period. Parameters evaluation after 7 days were similar to the values obtained after 48 h; bacterial colonies started to appear in the aqueous extracts during this period.

#### 3.3. Larvae Survival, Consumption and Growth

Leaching in strongly oxygenated water resulted in a mass loss of 29% and a release of organic and inorganic compounds into the water (Table 1).



Figure 1. Summer pool in the streambed of a low order stream draining an eucalypt (*Eucalyptus globulus*) plantation.

Significantly higher survivorship was observed in individuals maintained in APW (97%) than in the specimens reared with leachates (ANCOVA,  $P < 0.05$ ; Fig. 3). In the presence of leachate, all larvae died by 30 (L100%) and 100 days (L50%). This corresponded to a LT50 of 18.5 days in the 100% leachate (95% CL: 17.7–19.2) and LT50 of 31.1 days in 50% leachate (95% CL: 16.9–45.4).

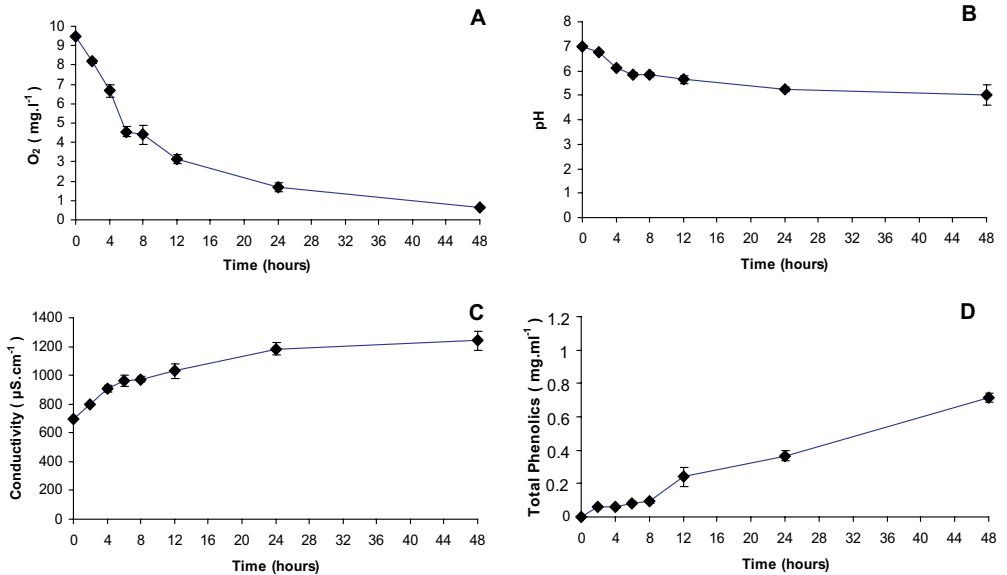


Figure 2. Changes in (A) oxygen, (B) pH, (C) conductivity and (D) total phenolic contents in water, after eucalypt (*Eucalyptus globulus*) leaves immersion, for 48 hours. (Means  $\pm$  SE).

Table 1. Inorganic and organic compounds present in eucalypt leaf extracts. Leaching was induced in laboratory (7 days; 15 °C) by immersion of the leaves in aerated distilled water (32 g l<sup>-1</sup>).

Inorganic compounds	(mg l <sup>-1</sup> )
Na <sup>+</sup>	14.17
K <sup>+</sup>	15.71
Mg <sup>2+</sup>	5.07
Ca <sup>2+</sup>	9.19
F <sup>-</sup>	1.00
CH <sub>3</sub> CO <sub>2</sub> <sup>-</sup>	6.39
Cl <sup>-</sup>	23.94
NO <sub>3</sub> <sup>-</sup>	0.9
PO <sub>4</sub> <sup>2-</sup>	0.41
SO <sub>4</sub> <sup>2-</sup>	5.16
Organic compounds	
DOC	1800 mg l <sup>-1</sup>
Total lipids	0.03 mg ml <sup>-1</sup>
Volatiles*	<0.1% (v/v)
Total phenolics	0.52 mg ml <sup>-1</sup> ;
Total nitrogen	0.01 mg ml <sup>-1</sup>
Conductivity	1 mS cm <sup>-1</sup>
pH	6

\* 2-hydroxycineol (72%), Benzoic alcohol (4.9%), 2-phenylethanol (16.7%), unknown compounds (6.4%)

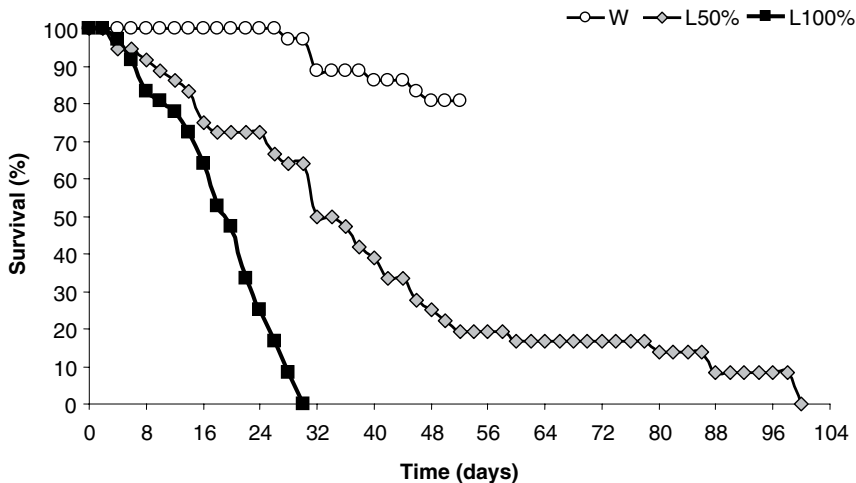


Figure 3. Survival (%) of *S. vittatum* larvae ( $n = 36$ ) maintained in Artificial Pond Water (W), and leachate media (L50% or L100%). Control larvae started to pupate, and eclosed, after 52 days.

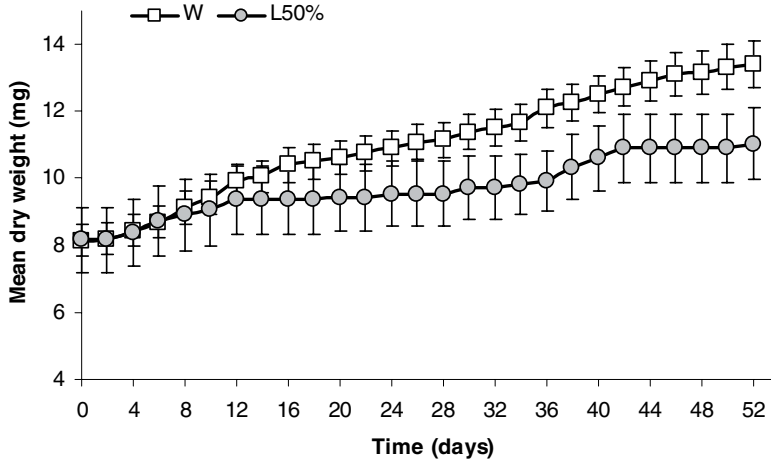


Figure 4. Growth of *S. vittatum* ( $n = 36$ ) maintained in Artificial Pond Water (W) and leachate half concentrated (L50%), for 52 days. (Means  $\pm$  SE).

Control larvae consumed  $0.14 \text{ mg mg}^{-1} \text{ day}^{-1} \pm 0.02 \text{ SE}$  and growth rate was  $1.24\% \text{ day}^{-1}$ . Individuals started to pupate after 52 days. Larvae reared in L50% consumed significantly less ( $0.11 \text{ mg mg}^{-1} \text{ day}^{-1} \pm 0.02$ ;  $t$ -test,  $P < 0.05$ ) and had a lower growth rate ( $0.68\% \text{ day}^{-1}$ ; ANCOVA,  $P < 0.05$ ). None of them was able to pupate and all died at the larval stage (Fig. 4). At the highest leachate concentration (L100%), there was no consumption or growth (Table 2).

Table 2. Mean consumption rates ( $\text{mg alder eaten mg animal dry mass}^{-1} \text{ day}^{-1} \pm \text{SE}$ ) of *S. vittatum* ( $n = 36$ ) maintained in Artificial Pond Water (W) or in eucalypt leachate half concentrated (L50%). No consumption was observed in highly concentrated leachate (L100%).

Media	Consumption rates ( $\text{mg mg animal dry mass}^{-1} \text{ day}^{-1}$ )	SE
W	0.138	0.016
L50%	0.107	0.020
L100%	–	–

Table 3. Phenolics concentrations in terms of tannic acid equivalents observed for alder leaf circles ( $\pm \text{SE}$ ), offered to the *S. vittatum* larvae ( $n = 36$ ), after 2 days immersion in Artificial Pond Water (W) and leachate media (L100% or L50%). Values with the same letter are not significantly different ( $P > 0.05$ ).

Media	Total Phenolics (% of leaf dry mass $\pm$ SE)
W	$0.63 \pm 0.29^a$
L50%	$1.12 \pm 0.29^b$
L100%	$1.78 \pm 0.62^b$

Alder disks used for feeding experiments and exposed to leachates almost doubled (L50%) and tripled (L100%) their initial phenolic content to 1.1% and 1.8%, respectively, of leaf dry mass (ANOVA,  $P < 0.001$ ; Table 3). No significant differences were found in phenolic contents between the leaves immersed in the two leachate media (Tukeys' test,  $P > 0.05$ ).

#### 4. Discussion

In summer, when water flow is reduced or ceased altogether in streams running through eucalypt forests in Central Portugal, the water in small pools receives large amounts of leaves from the riparian vegetation. In contrast to their counterparts running through deciduous forests, eucalypt streams receive the highest inputs in the hot season (ABELHO and GRAÇA, 1996). Summer pools may constitute aquatic refuges in the stream channel for many stream invertebrates. However, we have shown that thermal, chemical and physical conditions in such pools may not be ideal for these invertebrates.

As generally recognized, following leaf immersion, a large array of solutes is released into the water resulting in a rapid loss of mass (GESSNER *et al.*, 1999). In our experiment, eucalypt immersion in standing water at 20 °C caused a 14% decrease in mass within 48 to 168 hours. Published data report eucalypt leaching losses, in a low order stream of 18%, within a week, at 11 °C (e.g., CANHOTO and GRAÇA, 1996). It seems that high water temperatures may have compensated for the absence of flow. Previous work has suggested an important effect of temperature on leaching rates (TUKEY, 1970; CHERGUI and PATTEE, 1990). On the other hand, when immersed in aerated water (15 °C), eucalypt leaves lost about 29% of their mass; strong turbulence and the absence of mesh bags (which may limit the contact of the leaves with the water) may explain these results.

Despite being tough and having a thick cuticle, eucalypt leaves seem to be quite susceptible to leaching, even in comparison to soft deciduous leaves (e.g. *Alnus glutinosa*, *Castanea sativa*; CANHOTO and GRAÇA, 1996). It has been stated that this species-specific characteristic may play an important role in the deleterious ecological effects of leaf litter inputs on lentic or low flow aquatic systems (TRÉMOLIÈRES, 1988). In the case of eucalypt, the behaviour of the watersoluble compounds was probably defined not only by the leaf inherent chemical composition but also by its cuticular structure. Although not monitored, the solubilization of ions is likely to occur before that of the larger organic components. The cuticle, not impervious to ions (MARTIN and JUNIPER, 1970), may retard the release of large molecules (see CANHOTO and GRAÇA, 1999). The dynamics of dissolved phenolics and of conductivity tend to support this assumption (present work, CANHOTO and GRAÇA, 1996).

In most decomposition studies, the dynamics of leaching are usually obscured by low sampling frequency. Here we showed that leaching fits a negative exponential curve or a two-phase process. According to previous works with other leaf species (TRÉMOLIÈRES, 1988; CHERGUI *et al.*, 1997), this pattern may be the result of an initial phase of intense endogenous enzymatic oxidation of polyphenolic substrates, followed by a more gradual microbial deoxygenation and associated acidification. High phenolic and tannin contents are known to inhibit microbial activity (BÂRLOCHER *et al.*, 1995; CANHOTO and GRAÇA, 1999) and to play a key role in the species specific biochemical oxygen depletion effect (TRÉMOLIÈRES, 1988). Whether these leaves have phenoloxidase activity is not known but seems likely, resulting in the intense short-term effects observed soon after immersion. The reduction of dissolved oxygen in the water may have been accentuated by organic compounds oxidation – lipids and carbohydrates were abundant in the leachate media. In open systems, and at later stages of decomposition, the formation and precipitation of phytomelanins polycondensates, clearly responsible for the black color of the stream water pools, may contribute to a continued deoxygenation, and toxicity, of the aquatic environment (TRÉMOLIÈRES and CARBIENER, 1982).

Eucalypt leachates *per se* may affect the ecology of macroinvertebrates in summer pools. Larvae survival, consumption, growth and completion of the life cycle were all negatively affected by eucalypt solutes in the water. Among the chemical panoply that constitutes the leaves leachates, phenols may have a determinant effect on the invertebrates consumption behaviour and performance. The presence of phenolic compounds in the media or adsorbed to alder leaves surface, lowering their nutritional value, may have been responsible for reducing or suppressing (according to the leachate concentration) consumption. Similar inhibition of feeding behaviour was registered by CAMERON and LAPOINT (1978) in experiments with *Asellus militaris* and *Cranonyx schoemackerii* reared in leachates of *Sapium sebiferum*. Phenols are also known to affect protein assimilation by forming complexes with leaf proteins or with larval digestive enzymes (GRAÇA and BÄRLOCHER, 1998; CANHOTO and GRAÇA, 1999). If this was the case, reduced assimilation rates may have been additionally responsible for lower growth rates and reduced ability to complete the life cycle. However, previous works indicates that these larvae can consume and survive on a diet of leaves (e.g., *Castanea sativa*) with higher phenolic contents (FEIO and GRAÇA, 2000) and short term tolerance of invertebrates to media with high phenol concentrations ( $\leq 980 \text{ mg l}^{-1}$  phenol) were reported (GREEN *et al.*, 1985). This suggests that these deleterious compounds are not the sole factor responsible for the toxic effect of the eucalypt leachates. In fact, eucalypt oils, mainly consisting of cineol and pinene, are one of the most prominent characteristics of eucalypt leaves. Their deterrence/toxicity to stream invertebrates, even at low concentrations, has already been demonstrated (TRÉMOLIÈRES, 1988; CANHOTO and GRAÇA, 1999). Eucalypt leachate analysis revealed the presence of small amounts of derivatives of these compounds in the solute. Although the use of intact leaves generally prevents vesicles disruption and release of their content, the loss of terpenes to the aqueous medium is unequivocal. We hypothesize that these compounds may contribute to the observed lethal effects mainly by larvae post-ingestive physiological and toxic responses. In open systems, the deleterious effects of the eucalypt oils are probably higher as most leaves have their cuticle disrupted, facilitating the loss of vesicles or oils to the environment.

Although phenols and oils are obvious candidates to explain the toxicological effect of eucalypt leachates there is a strong possibility that other compounds (e.g., saponosides) may be also be involved. Similar conclusions were obtained by TRÉMOLIÈRES (1988) in a study performed with mayflies using conifer extracts. *Eucalyptus calmdulensis* leachates were also responsible for high mortality rates on golden perch larva (GEHRKE *et al.*, 1993).

Our results suggest that eucalypt leachates may be toxic for reasons other than oxygen depletion. They may be responsible for the deleterious effects promoted by discrete leaf accumulations, as observed in the eucalypt summer pools, or high inputs into the streams. Additive and/or synergistic effects promoted by combined chemical and physical factors are also likely to occur in the warmer season. For instance, it is largely recognized that short-term tolerance of phenols by invertebrates often decreases with increasing water temperatures (GREEN *et al.*, 1985).

Eucalypt summer pools may constitute an unsuitable environment for most benthic macroinvertebrates. Consequences probably vary with environmental factors, leachate concentration and invertebrate species. Physiological tolerance of physico-chemical conditions in the pools and digestive capacity allowing processing of the available food resources may allow invertebrates to overpass the warmer season in such aquatic environments (BOULTON *et al.*, 1991; CANHOTO and GRAÇA, 1999; OTERMIN *et al.*, 2002).

Conditioned eucalypt leaves are usually considered as a poor quality food for shredders. However, the development of leaf biofilms, stimulated by high leachate leaf contents (MCNAMARA and LEFF, 2004), may become an alternative relevant energy source for summer aquatic invertebrates (see FRANKEN *et al.*, 2005). On the other hand, we hypothesize that eucalypt leaves microbial decomposition may be dominated by bacteria (*vs.* fungi) in this season changing leaf quality to potential consumers. The potential of bacteria to cope with



low oxygen/hypoxia and their capacity to use DOC and/or tolerate recalcitrant compounds – terpenes, phenols, cutine – as a source of energy, may raise their importance (*vs.* aquatic hyphomycetes), as microbial processors of eucalypt during summer (BUTTIMORE *et al.*, 1984; BOONE and JOHNSTONE, 1997; FINDLAY *et al.*, 2002; MCNAMARA and LEFF, 2004). Whether this is true and has a significant effect on stream invertebrates feeding, assimilation and growth, is still unknown and needs to be further investigated. A gradient in the patterns and budgets of energy and nutrients as eucalypt streams reduce and resume their flow may be expected. Such cyclical pattern is also likely to contribute to the observed seasonal changes in the eucalypt stream community structure (OTERMIN *et al.*, 2002) and differences found between deciduous and eucalypt streams (see GRAÇA *et al.*, 2002 for a review).

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