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## Trichloroacetic acid cycling in Sitka spruce saplings and effects on sapling health following long term exposure

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### Abstract

Trichloroacetic acid (TCA,  $\text{CCl}_3\text{COOH}$ ) has been associated with forest damage but the source of TCA to trees is poorly characterised. To investigate the routes and effects of TCA uptake in conifers, 120 Sitka spruce (*Picea sitchensis* (Bong.) Carr) saplings were exposed to control, 10 or 100  $\mu\text{g l}^{-1}$  solutions of TCA applied twice weekly to foliage only or soil only over two consecutive 5-month growing seasons. At the end of each growing season similar elevated TCA concentrations (approximate range 200–300  $\text{ng g}^{-1}$  dwt) were detected in both foliage and soil-dosed saplings exposed to 100  $\mu\text{g l}^{-1}$  TCA solutions showing that TCA uptake can occur from both exposure routes. Higher TCA concentrations in branchwood of foliage-dosed saplings suggest that atmospheric TCA in solution is taken up indirectly into conifer needles via branch and stemwood. TCA concentrations in needles declined slowly by only 25–30% over 6 months of winter without dosing. No effect of TCA exposure on sapling growth was measured during the experiment. However at the end of the first growing season needles of saplings exposed to 10 or 100  $\mu\text{g l}^{-1}$  foliage-applied TCA showed significantly more visible damage, higher activities of some detoxifying enzymes, lower protein contents and poorer water control than needles of saplings dosed with the same TCA concentrations to the soil. At the end of each growing season the combined TCA storage in needles, stemwood, branchwood and soil of each sapling was <6% of TCA applied. Even with an estimated half-life of tens of days for within-sapling elimination of TCA during the growing season, this indicates that TCA is eliminated rapidly before uptake or accumulates in another compartment. Although TCA stored in sapling needles accounted for only a small proportion of TCA stored in the sapling/soil system it appears to significantly affect some measures of sapling health.

Keywords: Enzyme activity; Exposure; Forest damage; Needles; Protein, soil

## 1. Introduction

Trichloroacetic acid (TCA,  $\text{CCl}_3\text{COOH}$ ) is a phytotoxic chemical that has been detected in all environmental compartments (McCulloch, 2002). Although banned as a herbicide in the late 20th century (partly due to its indiscriminate effects on non-target plant species), TCA is still actively forming in the environment today and there is considerable debate about its present-day sources. The major source of TCA in the environment is postulated to be the atmospheric photooxidation of anthropogenically-produced chlorinated C2-hydrocarbons, but natural formation in soils has also been reported (deJong & Field, 1997; Haiber et al., 1996; Hoekstra et al., 1998; Hoekstra et al., 1999a; Hoekstra et al., 1999b and Keppler et al., 2000).

TCA has been widely detected in rural forests, particularly in conifer needles at concentrations of  $<1\text{--}180\text{ ng g}^{-1}$  (Frank, 1991; Frank et al., 1992; Juuti et al., 1996; Norokorpi & Frank, 1995; Plümacher & Renner, 1993; Plümacher, 1994 and Stidson et al., 2004) but the routes of uptake of TCA into tree foliage are poorly quantified. It is not clear if the TCA measured in needles is taken up as TCA (from the atmosphere or soil) or is formed in the plant from C2-chlorocarbon precursors. Frank et al. (1992) suggested that TCA in trees is formed from the needle uptake of chlorinated solvents, such as tetrachloroethene, which are subsequently transformed to TCA in the plant either by photolysis or by detoxification by the P-450 monooxygenase enzyme. According to Blanchard (1954) and Sutinen et al. (1995), TCA can enter plants in soil pore water taken up by the roots and transported to foliage via the transpiration stream. Direct uptake of TCA on needle surfaces from air or water has been thought unlikely since TCA is highly soluble in water and the surfaces of needles are lipophilic. However, if a direct atmospheric route of TCA input to foliage exists there may be significant implications for trees in forests which are regularly exposed to cloudwater.

Many field-based and controlled experiments suggest that exposure to TCA has adverse effects on tree health (Frank et al., 1990; Norokorpi & Frank, 1995; Plümacher, 1994; Sutinen et al., 1997 and Cape et al., 2003). Reported impacts of TCA exposure range from impaired growth to more subtle alterations of tree physiological functioning, such as changes in the structure of chloroplasts (important for photosynthesis) and needle surface waxes (Sutinen et al., 1995). There is particular concern regarding the effects of TCA exposure on coniferous trees because TCA may accumulate in foliage to phytotoxic levels over a number of years. Most experiments to investigate the routes of uptake and effects of TCA on trees have applied TCA in short-term studies that may overlook any chronic effects of TCA on tree health. Here, results are reported from an experiment in which 6–7 year-old saplings of Sitka spruce were exposed to multiple doses of TCA over two full growing seasons at near-realistic long-term application rates. Sitka spruce is an economically-important conifer species in Great Britain, accounting for 49% of the area under conifers in 1995 (Forestry Commission, 2002). The experimental aims were to identify for the saplings: (1) the routes of TCA uptake (atmospheric vs. soil); (2) TCA stores and fluxes within the experimental system; (3) the effects of chronic TCA exposure at near-realistic loading on health (growth, visual damage, needle enzyme activity, needle physical properties).

## 2. Materials and methods

### 2.1. Experimental design

The experiment was conducted on 120 potted saplings of Sitka spruce (*Picea sitchensis* (Bong.) Carr) of Queen Charlotte Island provenance in an unheated greenhouse at the Bush Estate, Scotland (55° 52' N, 3° 12' W). The saplings had been grown according to normal nursery practice and were 6 years old at the start of the 2001 growing season. The potted saplings were placed in plastic saucers and were irrigated identically during the experiment by adding de-ionised water to the saucers. The saplings had, as younger plants, been used for a single-season TCA exposure experiment during 1999 (Cape et al., 2003). This experiment used the same plants in the same experimental design as in 1999 to examine the long-term effects of TCA exposure. No TCA treatments were given in 2000, but plants were maintained in the greenhouse throughout the year, and re-potted in the intervening period.

The saplings were divided into six groups of 20 saplings. One of the six tallest saplings was randomly assigned to each group, one of the second six tallest saplings to each group and so on, until each group contained one of the six smallest saplings. The groups were then divided by height into four blocks (H1, H2, H3, H4) of five plants with H1 containing the tallest saplings and H4 containing the shortest saplings.

During both the 2001 and 2002 growing seasons each group of saplings received the same one of six treatment-level combinations consisting of three TCA concentrations (levels): de-ionised water control (L0), 10  $\mu\text{g l}^{-1}$  (L10), or 100  $\mu\text{g l}^{-1}$  (L100) TCA solution; and two treatment techniques: pouring directly onto the soil (Ts), or spraying a fine mist onto the foliage (Tf). The six groups are subsequently referred to as L0Ts, L0Tf, L10Ts, L10Tf, L100Ts and L100Tf (with height blocks H1 to H4 in each group). The treatments were applied on average every 3.5 days on 44 occasions during the first growing season (3 May–8 October 2001) and on 46 occasions during the second growing season (30 April–4 October 2002). The volume of solution applied per sapling on each occasion was 106 ml in 2001 and 200 ml in 2002, calculated to be equivalent to an estimated canopy interception storage capacity of 2 mm (before throughfall and stemflow occur) from measurements of the average projected sapling canopy surface area at the start of each season. These data yield a total application rate of TCA per growing season of 900 and 9000  $\mu\text{g m}^{-2}$  for the two TCA dose levels, respectively. An annual wet deposition flux of 1000  $\mu\text{g TCA m}^{-2}$  has been measured at an upland Sitka spruce site in Scotland (Heal et al., 2003b). So although the concentrations of individual TCA doses in this experiment are higher than ambient, the chronic exposure rate (particularly at the lower level) is broadly realistic.

The blocks were arranged randomly in the unheated greenhouse and were moved around once during each growing season. Cardboard disks were placed on the soil surface around the trunks in all pots to prevent the spray applications from contaminating the soil and to ensure parity of soil moisture retention for all saplings. A couple of the saplings in the H3 and H4 blocks of the L10Ts group could not be distinguished from each other due to fading of labels during the 2001 growing season so samples from both batches were amalgamated for analysis. The L10 treatments

were discontinued in the 2002 growing season after analysis of the 2001 results showed no marked difference from the controls.

## **2.2. Sampling of sapling material and soil**

Needle and soil samples were collected at the start of each growing season before dosing commenced and at the end of each growing season about one week after dosing ceased. The most recent needles (year C) were sampled by cutting a whole shoot from the 2nd or 3rd whorl of every tree. In October 2002 needles from the previous year class (C+1) and branchwood were also sampled from the same shoot. Samples were pooled by height block within each group and either analysed immediately or stored in sealed polythene bags at  $-30^{\circ}\text{C}$  until analysis. Soil was sampled by taking a core (2 cm in diameter, 10 cm in length) from every pot at 4 cm from the base of the sapling stem. On the day of collection soil samples were homogenised and grit removed by passing through a 2 mm sieve and then stored in polythene bags at  $-30^{\circ}\text{C}$  until analysis.

## **2.3. TCA analysis**

TCA was determined in triplicate analyses of all soil and sapling shoot samples following the method of Plümacher and Renner (1993) in which TCA is thermally decarboxylated to chloroform. Full details of the analytical methodology as applied here are given in Cape et al., 2003 and Heal et al., 2003a and only summarised here.

Before analysis the sampled shoots were immersed in de-ionised water, rinsed, and blotted dry to remove any surface TCA. Needles were stripped from the shoot and ground to a powder under liquid nitrogen with a pestle and mortar to ensure complete release of TCA from the needle matrix. Branchwood was prepared in the same manner. Homogenised needles or branchwood (1 g) were weighed into vials, 1 ml of water added, and the capped vials heated to  $100^{\circ}\text{C}$  for 1.5 h to effect decarboxylation of TCA to chloroform. An aliquot of the headspace was transferred by Perkin Elmer HS40 automated headspace sampler and the chloroform detected by GC-ECD. The TCA was quantified against chloroform produced by 1 ml TCA aqueous calibration solutions in vials undergoing the same process. Replicate needle samples were heated and analysed at  $60^{\circ}\text{C}$  only to determine the background chloroform present in the needles. The TCA concentration equivalent to the background-corrected chloroform was corrected using a previously-determined partition ratio of 1.94 to allow for the different partitioning of TCA between headspace and water or needle+water matrices. The sieved fresh soil samples were analysed in the same way as the needles, but using a separate partition ratio.

To correct measured TCA concentrations to dry weight, the moisture content of needle, soil and branchwood samples were determined from weight loss of fresh samples dried to constant weight at  $60^{\circ}\text{C}$ .

## **2.4. Sapling health**

Sapling health following chronic exposure to TCA was assessed in four ways:

1. The height and stem diameter of each sapling was measured at the start and end of each growing season. Sapling height was measured from the rim of the pot to the tip

of the lead shoot. Stem diameter along two perpendicular axes was measured at pot rim height using vernier callipers, and the mean taken.

2. Saplings were visually assessed for signs of needle damage on three separate occasions in September 2001 towards the end of the growing season. On each occasion saplings were assessed in a random order and the extent of damage assigned to a four-value scale of 0 (descriptor: no visible damage), 25, 50 or 75 (descriptor: heavy browning of needles on all branches including youngest shoots, evidence of loss of needles).

3. The activities of peroxidase (POX) and glutathione-S-transferase (GST) enzymes were determined in year C needles at the end of the 2001 growing season as an indicator of sapling stress. Both enzymes have been found to be involved in the detoxification of xenobiotics in plants (Schröder et al., 1997). A shoot was cut from each sapling at the end of the growing season in October 2001 and samples were pooled by height block within each group. Shoots were rinsed with de-ionised water, blotted dry and stored at  $-80^{\circ}\text{C}$  before analysis in triplicate for GST and POX activity. GST activity was determined using the procedure described in Schröder et al. (1997). Needle samples were ground with liquid nitrogen to a powder, and 10 volumes (w/v) of 100 mM Tris/HCl buffer added at pH 7–8 containing 1% PVP K30, 5 mM EDTA and 0.25% Nonidet™ P40. The slurry was homogenised, allowed to stand, centrifuged and the supernatant filtered. GST activity was determined spectrophotometrically in triplicate in the purified extract using substrates of 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB) ( Habig et al., 1974), and dichloromethane (DCM) ( Schröder and Belford, 1996). POX activity was determined from the change in absorbance measured at 420 nm for 5 min of 1 ml assay (910  $\mu\text{l}$  0.05 M potassium phosphate buffer (pH 6.0), 20  $\mu\text{l}$  3.4 M guaiacol as substrate, 20  $\mu\text{l}$  0.9 mM  $\text{H}_2\text{O}_2$ , 50  $\mu\text{l}$  enzyme). Blanks were subtracted and activity was calculated from a molar extinction coefficient of  $26.6\text{ mM}^{-1}\text{ cm}^{-1}$ . Protein content was determined in duplicate in the same needles by the method of Bradford (1976) using bovine serum albumin as a standard.

4. Rates of water loss from needles collected on 17 September 2001, near the end of the first growing season, were measured to assess the effects of TCA application on physical needle properties. Two excised needles per leader shoot of each sapling were pooled by height block, fully hydrated overnight, weighed (at time  $t=0$ ) and then placed in an atmosphere of constant humidity  $>50\%$  for 72 h. The needles were reweighed on 18 occasions during this period and then placed in an oven at  $70^{\circ}\text{C}$  for three days to obtain the dry needle mass. The data were analysed as described by Cape and Percy (1996) using Eq. (1),

$$R(t) = R_{\infty} + (R'_0 - R_{\infty})e^{-kt} \quad (1)$$

where,

$$R(t) = \frac{m(t) - m_d}{m_f - m_d} \quad (2)$$

is the change in relative needle water content with time ( $m(t)$  is mass of needles at time  $t$ , and  $m_f$  and  $m_d$  are the fresh and dry masses, respectively),  $R'_0$  is the extrapolated relative needle water content at  $t=0$ ,  $R_\infty$  is the relative needle water content at infinity, and  $k$  is a first-order rate coefficient corresponding to a measure of needle surface integrity for a given specific needle surface area. Using least squares non-linear regression, Eq. (1) was fitted to the rate of weight loss with time for each sample, for times  $>3$  h, to obtain estimates for  $R'_0$ ,  $R_\infty$  and  $k$  for each group-block combination.

All data were analysed using ANOVA or General Linear Model parametric statistics.

### **3. Results and discussion**

#### **3.1. Routes of TCA uptake into foliage**

##### **3.1.1. TCA in current needles**

TCA concentrations in current needles in October 2001, April 2002 and October 2002 are shown in Fig. 1. (Sampling in April 2002 was prior to fresh needle growth so needles sampled at this time belong to the same cohort sampled in October 2001; needles sampled in October 2002 are the new growth). Needle concentrations are within the range measured in forest foliage (up to  $180 \text{ ng TCA g}^{-1}$  fresh weight). The mean ( $\pm 1$  S.D.) TCA concentration in needles of all control (L0) saplings in October 2001, April 2002 and October 2002 was  $51 (\pm 22) \text{ ng g}^{-1}$  dwt with a range of  $12\text{--}98 \text{ ng g}^{-1}$  dwt arising from variability between saplings. There was no significant difference in needle TCA concentrations between L0 and L10 saplings in October 2001 and April 2002. The control saplings were also subject to background TCA in the de-ionised water (measured on five occasions, mean TCA concentration  $1.5 \mu\text{g l}^{-1}$ ) that was used to water the saplings and make up treatment solutions.

Concentrations of TCA were very significantly greater in needles of L100 saplings than in L0 and L10 saplings on each sampling occasion ( $P < 0.001$  in October 2001 and  $P < 0.01$  in April 2002 and October 2002) for both foliage (Tf) and soil (Ts) applications (Fig. 1). There was no significant difference in needle TCA concentration between L100Tf and L100Ts saplings. These results demonstrate that TCA applied to either foliage or soil is taken up into the sapling needles and that the saplings do not discriminate between TCA from the two sources. TCA uptake from the foliage treatment appears to be particularly efficient compared with direct application to the soil since not all of the TCA solution sprayed onto the foliage will have been intercepted by the canopy. One mechanism that could explain this is that canopy evaporation may increase the effective TCA concentration at the canopy surface, creating a concentration gradient that accelerates TCA movement through the plant cuticle. The evidence of a canopy only uptake route is not due to TCA measured on the external needle surface since the needles were washed prior to analysis to eliminate this possibility.

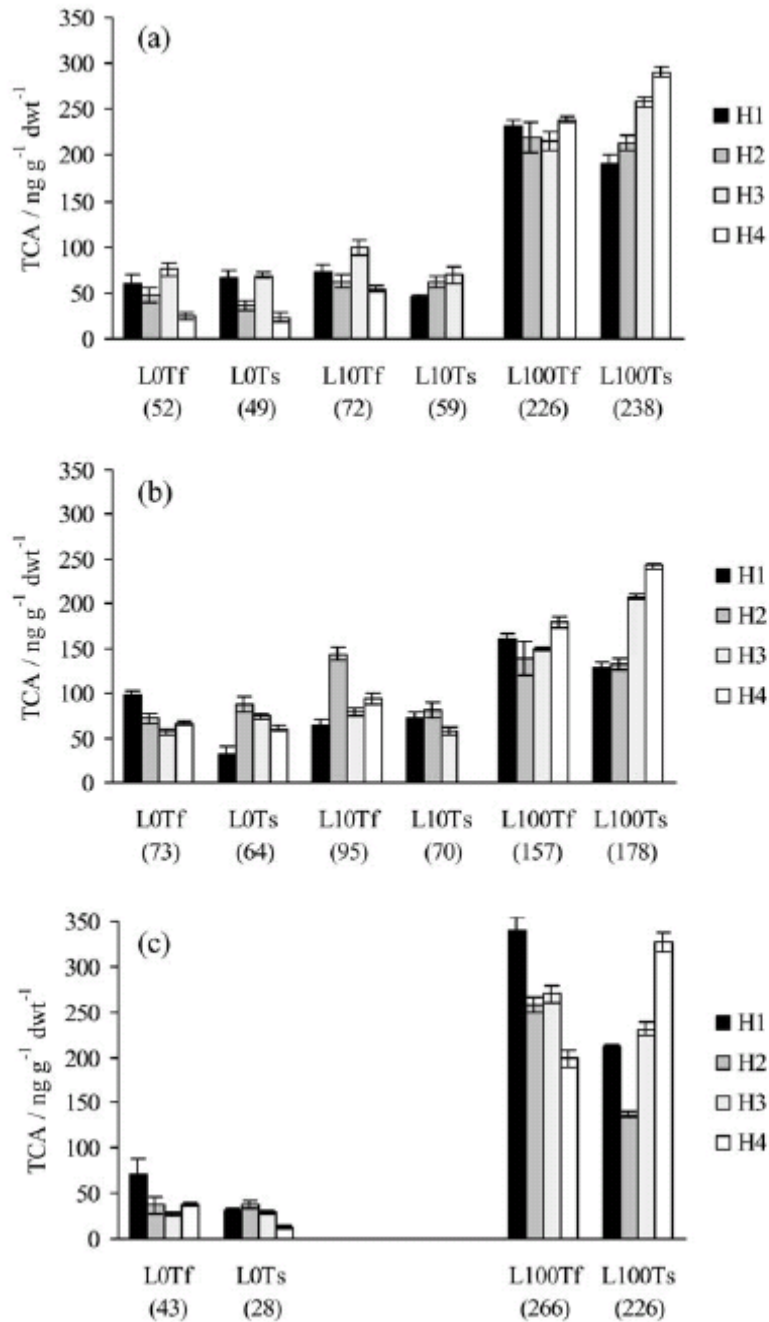


Fig. 1. TCA concentrations in most recent needle class sampled in (a) October 2001, (b) April 2002 and (c) October 2002. Needles sampled in April 2002 are the same cohort as sampled in October 2001. Error bars are 1 S.D. of analytical triplicates of samples pooled by height block. The mean values for each level-treatment group are shown in parentheses below the x-axis.

The saplings were deliberately dosed at approximately the same specific rate (TCA dose per mass of sapling) in each growing season. The resultant similar concentrations of TCA in the fresh (year C) needles of the L100 dosed saplings at the end of both growing seasons (Fig. 1a and c) suggests that uptake and elimination rates remained fairly uniform. The absence of enhanced concentrations in needles of L10 dosed saplings suggests that saplings were able to degrade low level chronic TCA application.



The pathway of TCA uptake into sapling needles when TCA is applied to soil is assumed to be uptake through roots and then movement to the needles in the transpiration stream. The uptake into needles from foliar application of TCA is likely to occur through initial uptake into branchwood; TCA concentrations measured in branchwood sampled in October 2002 were very significantly greater in L100Tf saplings than in L100Ts saplings (Fig. 2). In addition, no uptake into needles was observed in an experiment in which excised Sitka spruce needles were immersed in de-ionised water, 10  $\mu\text{g l}^{-1}$  or 100  $\mu\text{g l}^{-1}$  TCA solutions at pH 7 or 4 for up to 24 h (data not shown). Other ions, e.g.  $\text{SO}_4^{2-}$ , have also been shown preferentially to transfer through branchwood rather than needles in conifer saplings (Percy and Baker, 1989), corroborating the suggestion that TCA from atmospheric sources initially enters saplings via branchwood and subsequently translocates to needles via the transpiration stream.

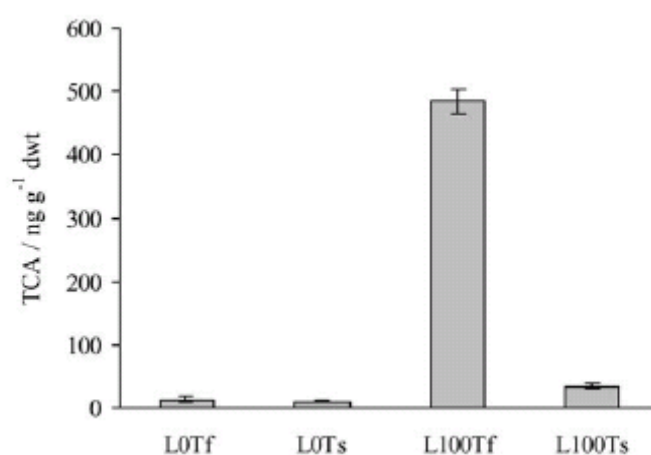


Fig. 2. TCA concentrations in sapling branchwood sampled in October 2002. Error bars are 1 S.D. of analytical triplicates of samples pooled by height block.

There was no significant effect of sapling height on needle TCA concentration across treatment method or dose level. However, in the L100 treatments, needle TCA concentrations decreased significantly with height in the Ts saplings ( $P < 0.05$ ) but not in the Tf saplings. This suggests that there may be differences in TCA uptake and metabolism mechanisms in saplings between TCA applied to foliage or to soil. TCA applied to the soil may be taken up readily into the needles of all saplings via the transpiration stream, but slower growing saplings could be less efficient at metabolising TCA in the needles. In the saplings exposed to foliar TCA applications, TCA movement into plant tissue may be controlled by the properties of the plant surfaces which are independent of sapling height.

### 3.1.2. Temporal changes in needle TCA concentrations

Fig. 3 shows the change in time of TCA concentrations in needles of the same age cohort in the L100 saplings during the experiment (i.e. year C in October 2001 and year C+1 in April and October 2002). In both foliage and soil-treated saplings, needle TCA concentrations were significantly lower at the start of the 2002 growing season in April 2002 than at the end of the growing seasons in October 2001 and October 2002 ( $P < 0.01$  and  $P < 0.005$  for October 2001 and October 2002 comparisons,

respectively). On average, the needle TCA concentrations in L100 dosed saplings in April 2002 were 69 and 75% of the needle concentrations at the end of the 2001 growing season for the Tf and Ts treatments, respectively. (Mean needle concentrations decreased from 226 to 157 ng g<sup>-1</sup> dwt for the L100Tf group and from 238 to 178 ng g<sup>-1</sup> dwt for the L100Ts group). However, needle TCA concentrations in the L100 saplings in April 2002 remained significantly greater than in needles of the control saplings (by a mean of 84 ng g<sup>-1</sup> dwt for Tf saplings and 114 ng g<sup>-1</sup> dwt for Ts saplings). A similar pattern of TCA uptake and elimination in needles was observed in Sitka spruce seedlings by Cape et al. (2003).

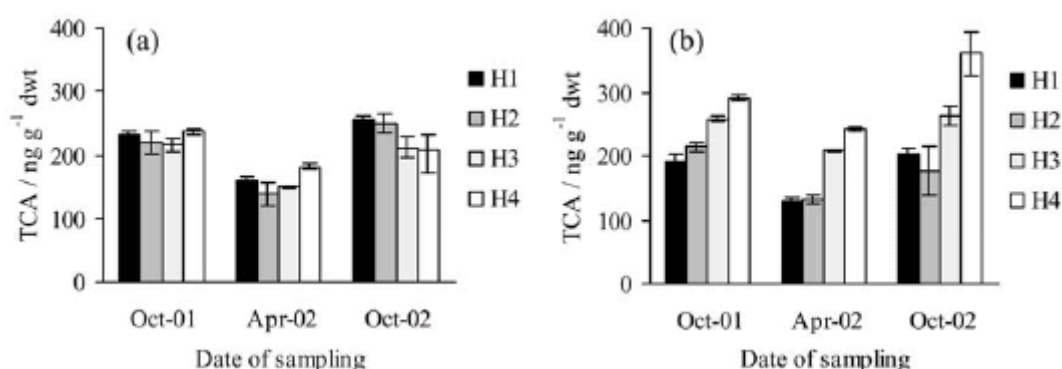


Fig. 3. TCA concentrations in the same cohort of needles established in the 2001 growing season, sampled in October 2001, April 2002 and October 2002, for 100 µg TCA l<sup>-1</sup> (a) foliage-dosed saplings (L100Tf) and (b) soil-dosed saplings (L100Ts). Error bars are 1 S.D. of analytical triplicates of samples pooled by height block.

The loss of TCA from needles during the winter between treatment periods could be due to elimination within the needles or relocation within the sapling. Alternatively the "loss" may arise from dilution of TCA by the plant growth observed over this period from sapling height and stem diameter measurements. Using relationships for sapling biomass (see Section 3.2), the needle mass of the L100 saplings was estimated to have increased by a median value of 7 g between October 2001 and April 2002 which could theoretically account for 45% of the observed decrease in needle TCA concentration over this period, assuming no TCA elimination or relocation in the saplings or addition of TCA through plant watering.

The above data show that TCA accumulated in the needles in the high TCA treatments was eliminated only slowly over winter when saplings are less metabolically active. A net effective loss of 25–30% of TCA in the 6 months from October 2001 to April 2002 corresponds to a half-life of 350 days for needle TCA elimination during winter. As expected, this is considerably longer than a half-life of 50 days for needle TCA elimination in actively growing Sitka spruce saplings derived from an experiment applying a single dose of TCA near the start of the growing season (Heal et al., 2003a).

Fig. 3 also shows that needle TCA concentrations in both L100 Tf and Ts saplings in October 2002 were not significantly different from concentrations detected in the

same cohort of needles at the end of the previous growing season in October 2001. The net TCA additional concentration that accumulated in C+1 needles in the 2002 growing season was  $74 (\pm 37) \text{ ng g}^{-1} \text{ dwt}$  in L100Tf and  $73 (\pm 33) \text{ ng g}^{-1} \text{ dwt}$  in L100Ts, compared to  $266 (\pm 58) \text{ ng g}^{-1} \text{ dwt}$  and  $226 (\pm 79) \text{ ng g}^{-1} \text{ dwt}$ , respectively, accumulated in the C needles of the same treatments over the same period (Fig. 1c). These results show that TCA in sapling needles is not linearly cumulative with dose during prolonged exposure to atmospheric or soil TCA.

### 3.1.3. TCA concentrations in needles of different ages

There was no significant difference in TCA concentrations of needles of different age classes (C and C+1) from L100 saplings sampled in October 2002 (Fig. 4). In contrast, field measurements and some other laboratory experiments have found that needle TCA concentration increases with needle age in conifers (Frank et al., 1990; Plümacher, 1994; Juuti et al., 1996; Sutinen et al., 1997; Hafner et al., 2002 and Stidson et al., 2004). These apparently contradictory results can be reconciled if it is assumed that TCA is taken up rapidly into current needles [as has been indirectly demonstrated through the application of [1,2- $^{14}\text{C}$ ]TCA to Norway spruce seedlings by Forczek et al. (2001)], perhaps due to a faster transpiration stream. After needle uptake, TCA may be eliminated at a faster rate in current needles than in older needles, resulting in the measurement of greater net TCA concentrations in older needles. At the higher TCA treatment level applied in this experiment (relative to environmental concentrations) the rate of TCA application and uptake may have exceeded the rate of metabolic elimination of TCA within the needles, masking the effect of needle age on TCA concentration.

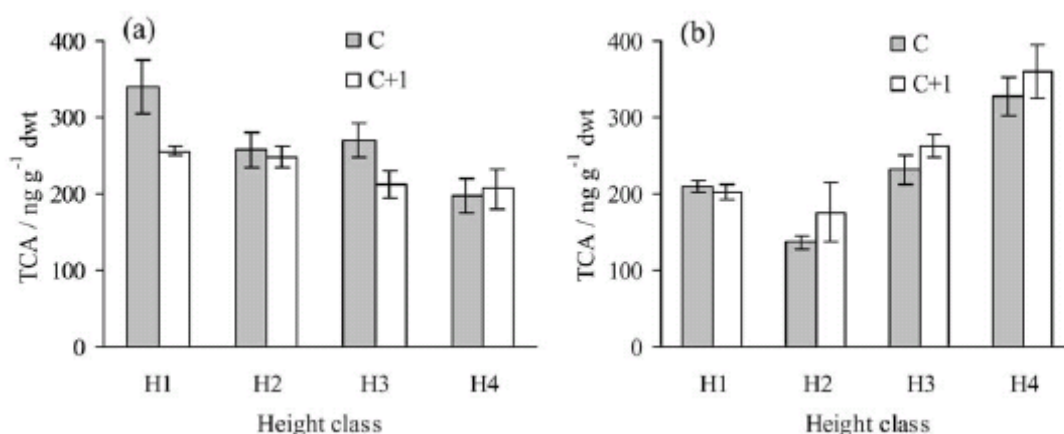


Fig. 4. TCA concentrations in C and C+1 needles sampled in October 2002 for  $100 \mu\text{g}$  TCA l-1 (a) foliage-dosed saplings (L100Tf) and (b) soil-dosed saplings (L100Ts). Error bars are 1 S.D. of analytical triplicates of samples pooled by height block.

### 3.2. TCA behaviour in the sapling/soil system

Soil from the sapling pots was sampled before and after treatment in the 2001 growing season and analysed for TCA to determine the proportion of applied TCA that could be accounted for in the soil. Soil TCA concentrations (Fig. 5) ranged from 8 to  $53 \text{ ng g}^{-1} \text{ dwt}$  (mean= $19 \text{ ng g}^{-1}$ , S.D.= $18 \text{ ng g}^{-1}$ ) in April 2001, and 10–51  $\text{ng g}^{-1}$

dwt (mean=18 ng g<sup>-1</sup>, S.D.=8 ng g<sup>-1</sup>) in October 2001. Soil TCA concentrations were not significantly different either between level-treatment groups at the end of the 2001 growing season or between the start and end of TCA applications. These data show that TCA applied directly to the soil for six months in the Ts treatments did not accumulate in the sapling soil, indicating that the applied TCA was broken down within the soil, and/or was irreversibly leached from the soil into the saucer, and/or was taken up into the sapling by the roots.

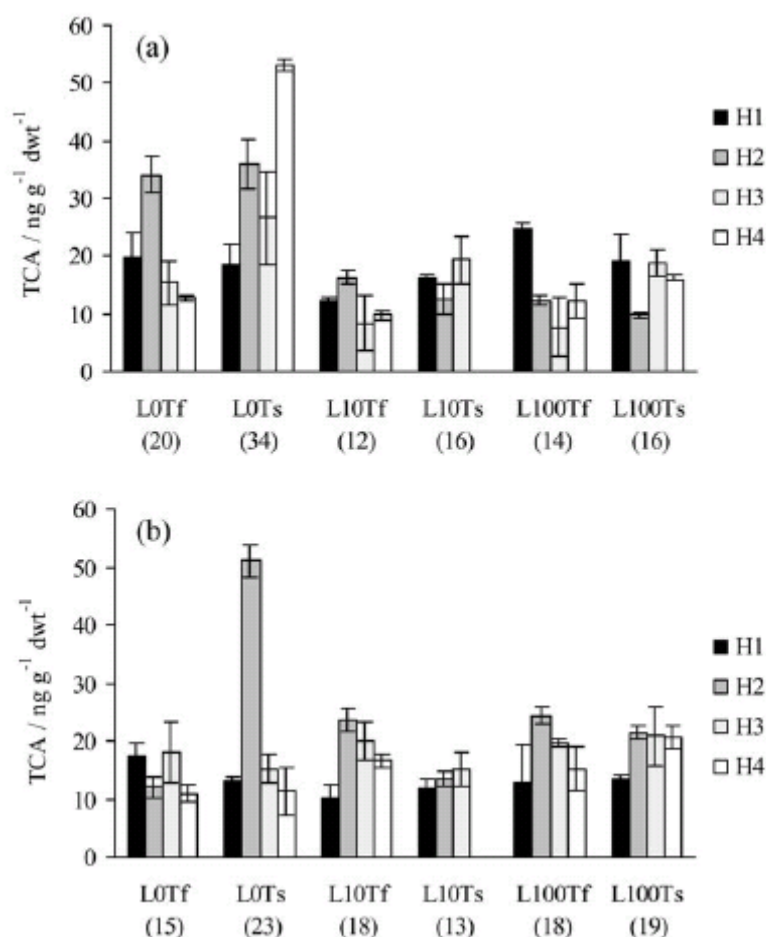


Fig. 5. TCA concentrations in soil samples collected from sapling pots at the (a) start, and (b) end, of the 2001 growing season. Error bars are 1 S.D. of analytical triplicates of samples pooled by height block. The mean values for each level-treatment group are shown in parentheses below the x-axis.

The masses of TCA stored within the different compartments of the experimental system were estimated to improve understanding of TCA uptake and storage in the sapling-soil system. The stemwood mass for each sapling was estimated by multiplying the stemwood specific gravity of Sitka spruce saplings [from Cannell et al. (1983)] by the fresh stemwood volume (derived from the sapling height and diameter measurements and assuming that the stem is a cone). Needle and branchwood masses were then estimated from the dry matter mass ratio for stem:branches:needles of 37:29:34 measured in Sitka spruce saplings of the same age by Cannell et al. (1983). Good agreement was found between the measured dry mass (232 g) of branches and needles of a sapling harvested in February 2003 and the estimated dry mass (261 g), showing that this method provided reasonable estimates

of sapling dry mass. The soil mass in each pot was estimated by multiplying the pot volume by the mean of six measurements of soil bulk density and is therefore the same for all treatments. Soil dry mass was assumed to remain constant throughout the experiment whilst foliage masses were recalculated for every occasion on which sapling height and stem diameter were measured. Table 1 shows the mean estimated mass of needles, branchwood, stemwood and soil per sapling in the different treatments in October 2002. The soil accounts for up to 95% of the soil-sapling system mass.

Treatment	Mean dry mass of material/g per sapling			
	Needles	Stemwood	Branchwood	Soil
L0Tf	74 (11)	80 (11)	63 (9)	3500 (700)
L0Ts	75 (4)	82 (4)	64 (3)	3500 (700)
L100Tf	67 (4)	72 (4)	57 (4)	3500 (700)
L100Ts	66 (11)	72 (12)	57 (9)	3500 (700)

Table 1. Mean dry masses of needles, stemwood, branchwood and soil per sapling for each level-treatment group in October 2002, derived as described in the text. Standard deviations shown in parentheses for needles, stemwood and branchwood are of the four height blocks within each group. Standard deviation shown in parentheses for soil is of the soil density determinations

The mass of TCA stored in each compartment was calculated by multiplying the dry mass by the measured TCA concentrations in soil, needles and branchwood. Soil TCA concentrations measured in 2001 were used for the 2002 calculations. The stemwood TCA concentration was assumed to be zero since Sitka spruce stemwood has been found to contain negligible TCA (Stidson et al., 2004). Table 2 shows the mean TCA mass per sapling in each compartment at the end of the experiment in October 2002 for each level-treatment group. The soil TCA store is markedly greater than the foliage TCA store, accounting for 93–97% of the total TCA in the sapling-soil system in control groups, and 59 and 80%, respectively, of total TCA store in L100Tf and L100Ts groups. The difference for the L100Tf group is due to the extra TCA in the branchwood of saplings in this group (Fig. 2).

Treatment	Mean TCA mass/ $\mu\text{g}$ per sapling			
	Needles	Stemwood	Branchwood	Soil
L0Tf	3.3 ( $\pm 2.0$ )	0	0.8 ( $\pm 0.1$ )	52 ( $\pm 13$ )
L0Ts	2.1 ( $\pm 0.9$ )	0	0.6 ( $\pm 0.03$ )	81 ( $\pm 67$ )
L100Tf	17.8 ( $\pm 4.5$ )	0	27.5 ( $\pm 1.7$ )	64 ( $\pm 18$ )
L100Ts	14.7 ( $\pm 4.7$ )	0	1.9 ( $\pm 0.3$ )	68 ( $\pm 13$ )

Table 2. Mean TCA mass in needles, stemwood, branchwood and soil per sapling for each level-treatment group in October 2002, calculated from the TCA concentrations and masses of sapling material. Standard deviations shown in parentheses are for the four height blocks within each group. Stemwood TCA concentration was assumed to be zero. Soil TCA concentrations were assumed to be the same as those measured in October 2001

TCA inputs to, and changes in TCA storage within, the sapling-soil system for each treatment over the 2001 and 2002 growing seasons are shown in Table 3 and Table 4, respectively. The data take into account measured changes in sapling biomass during the growing season. TCA inputs include the background TCA in the de-ionised water used for watering and in the treatment solutions. Allowing for within-group variability, there was no difference in TCA present in the saplings between the start and end of the growing seasons for L0 saplings and (for the 2001 growing season) L10 saplings. This indicates that the saplings were able to degrade whatever proportion of the L0 and L10 TCA dose was taken up into the saplings. From these observations a half-life of a few 10s days for within-sapling degradation during the growing season can be very approximately estimated, in general agreement with the half-life of 50 days determined separately by Heal et al. (2003a).

Treatment	Change in sapling TCA storage during season/ $\mu\text{g}$	Input of TCA during season/ $\mu\text{g}$	Proportion of input TCA present at end of season/%	Mass of input TCA unaccounted for at end of season/ $\mu\text{g}$
L0Tf	-0.9 ( $\pm 1.4$ )	6.9	0	
L0Ts	0.0 ( $\pm 1.2$ )	6.9	0	
L10Tf	-1.2 ( $\pm 3.7$ )	54	0	
L10Ts	-0.6 ( $\pm 1.9$ )	54	0	
L100Tf	24.0 ( $\pm 2.4$ )	475	5.1 ( $\pm 0.4$ )	451 ( $\pm 2.4$ )
L100Ts	7.5 ( $\pm 0.7$ )	475	1.6 ( $\pm 0.2$ )	468 ( $\pm 0.7$ )

Table 3. Mean changes in TCA storage of the sapling system between May and October 2001 compared with the mass of TCA applied (input). The standard deviations shown in parentheses are for the 4 height blocks within each group

Treatment	Change in sapling TCA storage during season/ $\mu\text{g}$	Input of TCA during season/ $\mu\text{g}$	Proportion of input TCA present at end of season/%	Mass of input TCA unaccounted for at end of season/ $\mu\text{g}$
L0Tf	-1.3 ( $\pm 0.7$ )	13	0	
L0Ts	-1.0 ( $\pm 1.3$ )	13	0	
L100Tf	17.3 ( $\pm 4.1$ )	934	1.9 ( $\pm 0.4$ )	917 ( $\pm 4.1$ )
L100Ts	7.4 ( $\pm 3.6$ )	934	0.8 ( $\pm 0.4$ )	926 ( $\pm 3.6$ )

Table 4. Mean changes in TCA storage of the sapling system between April and October 2002 compared with the mass of TCA applied (input). The standard deviations shown in parentheses are for the 4 height blocks within each group

For the L100 dosed groups, change in TCA stored in the saplings was  $<6$  and  $<2\%$  of total mass of TCA applied for the 2001 and 2002 growing seasons, respectively (Table 3 and Table 4). Combined with estimated within-sapling degradation rates, this suggests that only a small proportion ( $<15\%$ ) of the TCA applied to these high dose groups is taken up into the saplings. In the soil treatment the TCA unaccounted for could result from rapid degradation of TCA in the soil and/or rapid uptake into the



sapling, followed by elimination in the plant tissue. In the foliage treatment, possible pathways for TCA loss include degradation of TCA on the needle surface or adsorbed TCA which was washed off during sample preparation for analysis. Alternatively, TCA could be accumulating in a compartment not accounted for, such as the sapling roots. Forczek et al. (2001) indirectly also recovered only 22% of [1,2-<sup>14</sup>C]TCA applied to the soil in a Norway spruce seedling experiment and suggested that the TCA loss resulted from degradation in the soil and absorption by bark, roots and litter. However, although the proportion of TCA processed with the sapling may be comparatively small, it remains the case that TCA is efficiently biomagnified in the sapling which may have important consequences on sapling health.

### 3.3. Sapling health

#### 3.3.1. Sapling growth

The cumulative growth over two seasons was assessed in order to test for association between long-term, or chronic, exposure to TCA and sapling growth. The mean percentage increases in sapling stem diameter, height and stem volume (approximated as a cone) during the experiment were 51.2, 63.2 and 311%, respectively. No significant differences were found for absolute or relative changes in sapling growth either between treatment level (L0 or L100) or application method (Tf or Ts), apart from for the affect of application method on relative change in sapling height ( $P < 0.01$ ,  $T_f > T_s$ ) (Fig. 6). Therefore in this experiment sapling growth was not affected by application of TCA over two growing seasons, although spraying solution on sapling foliage appeared to increase sapling height compared with applying solution to the soil.

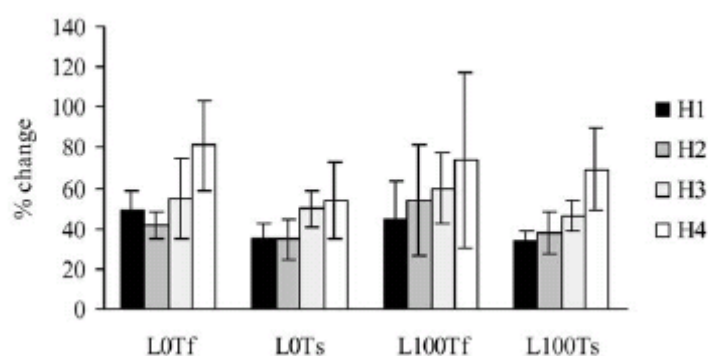


Fig. 6. Percentage changes in stem height of L0 and L100 saplings between April 2001 and October 2002. Error bars are 1 S.D. for the five replicate saplings in each height block.

#### 3.3.2. Observation of foliage damage

There was no significant effect of TCA concentration on apparent damage. However sapling height did have a significant effect, with the blocks of shortest saplings (H4) showing significantly less damage (mean 29%) than the two blocks of tallest saplings (H1: mean 41%,  $P < 0.05$ ; and H2: mean 43%,  $P < 0.005$ ; Tukey test). Significantly more damage was observed in the foliage-dosed (Tf) saplings than the soil-dosed saplings (Ts) (means of 39 and 33%, respectively,  $P < 0.05$ ), suggesting that spraying

solution on foliage, regardless of TCA content, caused the visible damage. Interestingly, the foliage-dosed saplings also had significantly greater height growth rates compared with the soil-dosed saplings (Fig. 6), implying that the stimulation of sapling growth may be linked to needle browning and loss.

A highly significant interaction existed between TCA concentration and application method ( $P < 0.005$ ) in that there was no difference in visible sapling damage between the foliage and soil applications in the control and  $10 \mu\text{g l}^{-1}$  TCA treatments, but in the  $100 \mu\text{g l}^{-1}$  TCA treatment considerably more damage was observed in the foliage-dosed saplings than the soil-dosed saplings. This result suggests that the exposure route of conifers to TCA is an important control on the extent of visible damage as well as the solution concentration, with trees whose foliage is regularly exposed to TCA solution (e.g. via cloudwater) being particularly vulnerable. Recent measurements of TCA in rainwater and cloudwater for an upland forest in south east Scotland showed that TCA concentrations were slightly enriched in cloudwater by a mean factor of 1.2, compared to rainwater, and that cloudwater deposition accounted for 13% of total TCA deposition to the forest (Heal et al., 2003b). Greater enhancement of TCA has been reported in fogwater (Römpf et al., 2001). Furthermore, needles of Sitka spruce saplings are retained for a relatively long period of time [6–8 years compared with 2–3 years for Scots pine (Cannell, 1987)] so TCA may accumulate in the needles of trees exposed to TCA in cloudwater over this time.

### 3.3.3. Enzyme activity in needles

No significant effect of TCA concentration, application method or sapling height was found for peroxidase (POX) activity (data not shown), in contrast to experiments on 2-year old Scots pine seedlings in which greater POX activity was measured in  $0.1 \text{ mg l}^{-1}$  TCA treatments, a considerably greater specific dose than in these experiments (Schröder et al., 1997). The GST enzyme activity results differed for the three xenobiotic substrates used (DCNB, CDNB and DCM). For DCNB (data not shown) no significant effects were observed of TCA concentration, application method or sapling height on GST activity.

When CDNB was used as a substrate (Fig. 7a), there was no significant difference in GST enzyme activity in needles with TCA concentration in soil-treated (Ts) plants, but in foliage-treated plants, activity was significantly greater in plants that had received the  $10 \mu\text{g l}^{-1}$  doses than either the control or the  $100 \mu\text{g l}^{-1}$  doses ( $P < 0.005$ ). With DCM as GST substrate (Fig. 7b), enzyme activity again was only enhanced in plants where foliage was treated, and in this case the effect was statistically significant for both  $10 \mu\text{g l}^{-1}$  ( $P < 0.005$ ) and  $100 \mu\text{g l}^{-1}$  ( $P < 0.05$ ) treatments. An explanation for these observations is that GST activity may be induced at the  $10 \mu\text{g l}^{-1}$  TCA dose to detoxify the added TCA, but become inhibited at higher TCA doses. These results are consistent with the observation of greater visible damage in the foliage-dosed saplings compared with the soil-dosed saplings in October 2001. Cape et al. (2003) report a similar result for enhanced needle GST activity in Sitka spruce seedlings exposed to TCA by foliage routes, but with DCNB as a substrate. In that work, a foliage-wetting effect was apparent since needle GST activity was also enhanced by application of the control dose to the foliage. Although there are broadly consistent trends in all these data, the observed significant differences in needle GST activity for some substrates between trees exposed to TCA by different routes suggests that enzyme activity



measurements should be interpreted with care as indicators of tree stress in experiments of this nature.

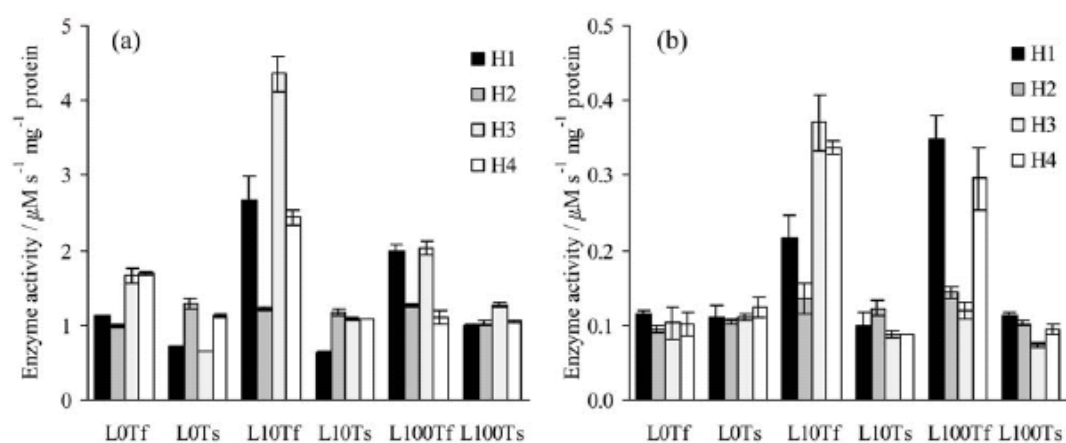


Fig. 7. GST enzyme activities in sapling needles in October 2001 with substrates of (a) CDNB and (b) DCM. Error bars are 1 S.D. of analytical triplicates of samples pooled by height block.

As with GST activities, TCA exposure route had a significant effect on needle protein content (Table 5) ( $P < 0.001$ ) with lower protein contents measured in foliage-dosed saplings than soil-dosed saplings. Of particular note in the needle protein content results was the significant interaction between TCA concentration and application method ( $P < 0.001$ ). In the L10 and L100 treatments considerably less needle protein occurred in the foliage-dosed saplings than in the soil-dosed saplings suggesting that TCA solution applied to the foliage is more potent to sapling needles than TCA applied to the soil. The observation of significantly lower needle protein content in TCA dosed plants compared with control plants (Table 5,  $P < 0.001$ ) is consistent with previous measurements in Norway spruce (Plümacher and Schröder, 1994) and Sitka spruce (Cape et al., 2003). Lower protein contents are probably due to the protein precipitating property of TCA, which results in an associated reduction of the capacity of protein to conjugate and detoxify xenobiotics. Sapling height also had a significant effect on needle protein content ( $P < 0.05$ ) with higher protein contents generally occurring in the shorter saplings. Again this result is consistent with the observation of less visible damage in the shorter saplings.

Group	Height 1 (tallest)	Height 2	Height 3	Height 4 (shortest)	Group mean	Level mean	Treatment mean
L0Tf	2.21	1.75	1.74	2.13	1.96	1.79 <sup>a</sup>	Tf: 1.04 <sup>a</sup>
L0Ts	1.83	0.71	1.61	2.36	1.62		
L10Tf	0.49	0.69	0.45	0.80	0.61	1.22 <sup>b</sup>	
L10Ts	1.79	1.64	1.94	1.94	1.83		
L100Tf	0.55	0.80	0.56	0.34	0.56	0.98 <sup>b</sup>	Ts: 1.62 <sup>b</sup>
L100Ts	1.40	1.08	1.48	1.63	1.40		
Height mean	1.38 <sup>ab</sup>	1.11 <sup>a</sup>	1.29 <sup>ab</sup>	1.53 <sup>b</sup>		Level-treatment interaction <i>P</i> <0.001	

Table 5. Protein content (mg (ml extract)<sup>-1</sup>) of sapling needles sampled in October 2001. Values with the same superscript in a column or row are not significantly different. Interaction between level and treatment was significant at  $P < 0.001$

In summary, although measured needle enzyme activities differ between tree TCA exposure experiments and with substrate used and experimental artefacts may also exist (the stimulation of enzyme activity by spraying solution on foliage), in this experiment the needle enzyme activity and protein content results indicate that stress occurs in conifers exposed to TCA and that the degree of stress is influenced by the route of TCA exposure. Greater stress was apparent in the needle biochemistry of foliage-dosed Sitka spruce saplings than in soil-dosed saplings, in agreement with the visible damage observations. However, it should be cautioned that observations of associations do not necessarily imply a cause-effect relationship.

### 3.3.4. Needle physical properties

An example fit of Eq. (1) to the relative needle water loss for one of the needle samples is shown in Fig. 8. Three variables,  $R'_0$ ,  $k$  and  $\Omega$ , were derived from the curve fitted to each needle sample.  $k$  and  $R'_0$  are as defined earlier and  $\Omega = R_\infty (mf - md)/md$ , where  $mf$  is the fresh needle mass at  $t=0$  and  $md$  is the oven-dry needle mass. There was no significant effect of TCA concentration, application method or sapling height on values of  $k$  and  $\Omega$  (data not shown), nor on fresh mass/dry mass ( $mf/md$ ) ratios. However, there was a significant interaction between TCA concentration and application method ( $P < 0.05$ ) for values of  $R'_0$  (relative water content at  $t=0$ ) (Table 6). For most treatments,  $R'_0$  exceeded 80%, but it was significantly lower (65%) for the  $100 \mu\text{g l}^{-1}$  TCA foliage treatment. Main effect differences in  $R'_0$  were significant for TCA concentration ( $P < 0.05$ ) and application method ( $P < 0.05$ ). Since an estimate of the proportion of initial needle water lost through stomata is given by the quantity  $(1 - R'_0)$ , saplings with lower  $R'_0$  values may be more susceptible to water loss through the stomata in dry conditions, resulting in yellowing and droughting of needles. This is again consistent with both the damage survey and protein content results, providing further evidence that the application of TCA solution to tree foliage has an adverse effect on sapling functioning, in this case through water control in the needles.

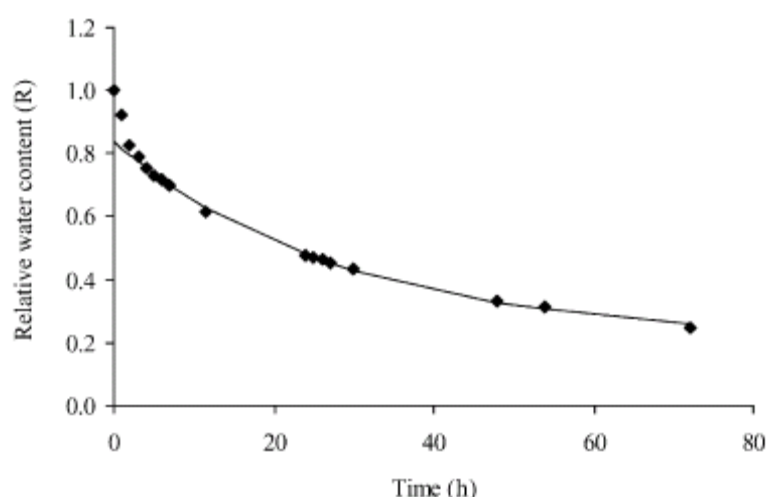


Fig. 8. Rate of water loss from pooled needles (sampled in September 2001) from saplings of height block 1 dosed with  $10 \mu\text{g l}^{-1}$  TCA to foliage (L10TfH1). Eq. (1) is fitted to the data for  $t > 3$  h.

TCA dose level	Treatment method		Level mean
	Foliage (Tf)	Soil (Ts)	
Control	80.9	80.0	80.4 <sup>ab</sup>
10 $\mu\text{g l}^{-1}$	81.9	85.6	83.7 <sup>a</sup>
100 $\mu\text{g l}^{-1}$	64.7	82.7	73.7 <sup>b</sup>
Treatment mean	75.8 <sup>a</sup>	82.7 <sup>b</sup>	Level-treatment interaction $P < 0.05$

Table 6. Mean R<sup>0</sup> values (%) for sapling needles sampled in September 2001, derived from fitting Eq. 1 to measurements of needle weight loss against time at constant humidity. Values with the same superscript in a column or row are not significantly different. Interaction between level and treatment was significant at  $P < 0.05$

#### 4. Conclusions

Exposure of 6–7 year old Sitka spruce saplings to 100  $\mu\text{g l}^{-1}$  TCA over two 5-month growing seasons showed that TCA applied either to the soil or foliage was detected in current year needles at similar concentrations in both growing seasons (up to 340 ng  $\text{g}^{-1}$  dwt), providing further evidence for both above- and below-ground routes of TCA uptake in conifers. The main pathway of needle uptake of foliage-applied TCA is most probably through branchwood and stemwood rather than through the needle cuticle since high TCA concentrations were measured in branchwood of foliage-treated saplings. TCA in sapling needles was eliminated slowly between the two growing seasons suggesting that TCA in conifer needles persists after exposure to TCA from above or below-ground routes. Estimated TCA budgets for the experimental sapling-soil system showed that the vast majority of the applied TCA was unaccounted for, probably due to degradation in the soil or on foliage surface, or metabolism within the sapling. TCA dosing had no measurable effect on sapling growth during the experiment. However the survey of visible damage and assays for needle enzyme activities and protein content at the end of the first growing season showed that there is a significant interaction between TCA concentration and exposure route. Repeated exposure of foliage to TCA in solution causes measurable changes in needle enzyme activity, protein content and physical properties at concentrations as low as 10  $\mu\text{g TCA l}^{-1}$ , whereas no health effects were detected in saplings exposed to the same TCA concentrations via the soil. There may be other effects of TCA on sapling health that only become apparent over a longer time period than in this experiment, e.g. the adaptability of saplings to environmental stresses such as frost, drought, nutrient deficiencies and disease. Overall, TCA in needles only accounted for <16% of TCA stored in the sapling-soil system, but the evidence indicates this TCA may have significant effects on sapling health. Although the results from these controlled experiments may not be directly applied to forest ecosystems, tree saplings (taller than 1 m) are good analogues for investigating routes of TCA uptake and cycling in trees.

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