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Trichloroacetic acid in Norway spruce/soil-system. II. Distribution and degradation in the plant

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Abstract

Independently from its origin, trichloroacetic acid (TCA) as a phytotoxic substance affects coniferous trees. Its uptake, distribution and degradation were thus investigated in the Norway spruce/soil-system using ^{14}C labeling. TCA is distributed in the tree mainly by the transpiration stream. As in soil, TCA seems to be degraded microbially, presumably by phyllosphere microorganisms in spruce needles. Indication of TCA biodegradation in trees is shown using both antibiotics and axenic plants.

Keywords: ^{14}C labeling; Microbial degradation; Phyllosphere; Radio-indicator methods; TCA stability; TCA uptake

1. Introduction

The possible connection between the volatile chlorinated C_2-chlorocarbons (and their atmospheric photooxidation product, TCA) and forest decline symptoms was first mentioned by Frank (1984, 1988, 1989). Attempts to correlate measured TCA concentrations in conifer needles with defoliation of trees or damage of the surface wax layer have been pursued (Frank et al., 1992, 1994; Gullvaag et al., 1995; Norokorpi and Frank, 1995). The probable adverse effects of TCA on forest trees are due to its phytotoxic nature; this has also been exploited by its use as a herbicide, albeit at concentrations much higher (1.12–2.24 $g\,m^{-2}$—Blanchard, 1954) than those observed in environmental samples of soil (0.1–380 $\mu g\,kg^{-1}$ dw—Renner et al., 1990), soil water (0.2–2.8 $\mu g\,l^{-1}$—Frank, 1988; Hoekstra and de Leer, 1993; Plümacher, 1995) or coniferous tree needles (Table 1).

Anthropogenic TCA and its precursors may reach forest ecosystems by long distance air transport followed by wet precipitation or dry deposition while TCA production by microorganisms in soil can be a local phenomenon. TCA mainly enters the terrestrial environment via soil. TCA is degraded in the soil by microorganisms; this process being influenced by TCA concentration and the soil characteristics. Soil humidity, heterogeneity and humic acid content determine the degradation rate of TCA, whereas temperature does not seem to have a substantial effect (Forczek et al., 2001; Matucha et al., 2003; Schröder et al., 2003). Only CO_2 can be found in the atmosphere as a TCA soil degradation product; neither CO, CH_4 nor $CHCl_3$ is detectable. The rapid degradation of low concentrations of TCA spiked to the soil (Forczek et al., 2001) has raised

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Table 1
TCA concentrations in conifer needles according needle age reported in the literature

Reference	Tree species	Sample origin	*n*	Needle age	TCA content in needles (average) [ng g^{-1} FW]
Frank et al., 1989	Norway spruce	S-Germany	n.d.	C + 2	10–300
			1	C	30
Frank et al., 1990	Scots pine	Finland	10	C + 2	33–180 (74)
		Finland	10	C + 2	20–73 (45)
	Scots pine	Germany	15	C + 1	4–96
Juuti et al., 1996	Norway spruce and Scots pine	W-Finland	10	C	3–14 (9)
	Norway spruce and Scots pine	Finland	130	C + 2	1–180 (23) (5 > DCA)
Matucha et al., 2001b	Norway spruce	NW-Czech Republic	10	C	28–110 (63.1)
			10	C + 1	34–127 (77.8)
			10	C + 2	28–144 (67.0)
Coufal et al., 2003	Norway spruce	Czech Republic	29	C	3.4–110 (34.9)
			29	C + 1	1.5–127 (37.9)
			19	C + 2	4.2–144 (42.1)

C—current-year needles; C + 1—one-year-old needles; C + 2—two-year-old needles; n.d.—no data; *n*—number of samples.

the question of TCA uptake with the following translocation, distribution and accumulation in spruce trees. Even though TCA is well degraded in humid soil with high humic acid content, TCA is always detected in trees in forest environment and in controlled experiments with spruce and pine seedlings (e.g. experiments: Juuti et al., 1993; Sutinen et al., 1995, 1997; Schröder and Wolf, 1996; Forczek et al., 2001; Matucha et al., 2001a). The uptake of TCA by the plants follows the previously assumed route: atmosphere → soil → roots → xylem → needles (Frank, 1989; Uhlířová et al., 1996; Forczek et al., 2001; Matucha et al., 2001a). When sprayed as an aqueous solution directly on the spruce foliage, most of the TCA remained physically adsorbed to the surface and could be washed down by any form of wet precipitation; therefore the penetration of the cuticular wax layer or stomatal uptake of coniferous needles is not a probable path of uptake for this compound (Frank, 1991; Sutinen et al., 1995, 1997).

The observed natural concentrations of TCA in needles vary much in dependence on plant in species, individual trees and needle age classes (seasonally and with altitude) (Table 1). Needle samples from Norway spruce trees growing in mountainous habitats of the Czech Republic contained 1.5–144 ng g^{-1} needle FW TCA (Matucha and Uhlířová, 1999; Matucha et al., 2001b; Coufal et al., 2003), which is comparable with results of other studies (Table 1). These naturally occurring TCA levels are the result of continuous uptake, translocation and degradation of TCA.

Localization of radioactively labeled [1,2-^{14}C]TCA enable us to interpret the TCA translocation pathways in spruce needles. Results of Uhlířová et al. (1996) and Schröder et al. (2003) proved that 3 h after the application of [1,2-^{14}C]TCA into the solution with detached spruce shoots, the measured radioactivity in the needles was not yet evenly distributed. The highest concentration of radioactivity was observed in transport tissues, while the lowest concentration of radioactivity was close and within the cuticle of the needles.

The absorbed TCA could lead to tissue and cell damage in spruce. Hence, TCA may have a possible role in forest decline symptoms described during the last decades (e.g. Frank, 1988, 1989, 1991; Frank et al., 1990). Little is known about the chronic and polyfactorial character of TCA effects upon coniferous trees (Coufal et al., 2003). Scots pine seedlings did not exhibit visible symptoms either over a 9 or an 18-week-long experiment, and the structure of the mesophyll cells was mainly similar to that in healthy conifer needles (Sutinen et al., 1995, 1997). However, changes in enzyme activity of both the oxidative metabolism and the xenobiotic degrading systems were observed in the needles (Schröder et al., 1997).

Studies of the degradation and metabolism of TCA in animals and plants date back several decades. Blanchard (1954) did not find any degradation products of TCA in pea and corn plants. Even though the applied amount of radioactivity remained constant in oat and wheat plants 6 weeks after application (Chow, 1976), incorporation of metabolites into the plant tissue could be possible. TCA degradation or mobilization in spruce needles was demonstrated by Frank (1991) and in Scots pine by Sutinen et al. (1995). The half-life of TCA was reported to be about 10 days in spruce needles (Frank, 1991).

After the application of [1,2-^{14}C]TCA, $^{14}CO_2$ formation was almost instantly observed in the soil and in the spruce needles (Matucha et al., 2001a). Apparently TCA is degraded to CO_2 but the rate and course of this degradation reaction are unknown. Up to now, there has not been found or specified any degradation product of TCA detectable in spruce needles or in the atmosphere, and in fact, TCA has been reported to be chemically stable in plants (Blanchard, 1954; Chow, 1976; Schröder et al., 2003). Our present study aims to clarify the distribution, translocation and degradation of TCA in Norway spruce trees. Special attention was paid to the role of microorganisms in TCA degradation in needles.

2. Material and methods

Radioindicator methods were used for the study of translocation, distribution and degradation of TCA within Norway spruce. Radioactively labeled [1,2-^{14}C]TCA (specific activity 3.7 GBq mmol^{-1}, radiochemical purity >98%) was prepared according to Bubner et al. (2001), it enabled us to follow the radioactivity in different parts of plants. Three experiment types were conducted, on potted cuttings [A_1–A_3]; on sterile grown seedlings [B_1–B_2] and on cut shoots [C_1–C_4].

Experiments [A_1] were performed with four-year-old cuttings of Norway spruce (*Picea abies*/L./Karst.), which were obtained from the Forest District Kálek (700 m a.s.l.), Ore Mts., Czech Republic. The potted spruce cuttings were genetically identical, obtained through vegetative propagation (cuttings from mature spruce). Each pot contained one single cutting and approximately 1200 g of soil substrate.

In experiments with whole spruce trees, [1,2-^{14}C]TCA was applied to the roots. The trees were then left in an open field for up to 12 weeks in order to avoid contamination by assimilation of $^{14}CO_2$ when cultivated in a chamber as experienced in previous experiments (Matucha et al., 2001a). After the period of exposure, the spruce trees were harvested, the needles sorted into different age classes (C—current-year needles; C + 1—one-year-old needles; C + 2—two-year-old needles), wooden parts, roots and soil. All of these compartments were sampled and the radioactivity was measured by the combustion method at the ICEM, Prague.

In replanting experiments [A_2], the soil was thoroughly washed away from the roots with water after an initial exposure time (9 and 25 days) of [1,2-^{14}C]TCA. The spruce was replanted into TCA-free soil of the same composition.

In split-root experiments [A_3] the soil was washed away with water at the beginning of the experiment. Approximately one third of the root system was immersed into a radioactively labeled TCA-solution (5 kBq ml^{-1}; total activity 1620 kBq, i.e. 0.22 μg ml^{-1}, total amount 71.37 μg), while the remaining roots were replanted into TCA-free soil. After the treatment time (3 weeks) the cutting was harvested, radioactivity of needles, roots, soil and the remaining solution was measured with the above-mentioned methods.

Eight-week-old Norway spruce seedlings were used in further experiments [B_1] to ascertain the role of microorganisms in the degradation of TCA in Norway spruce. The seedlings were reproduced by somatic embryogenesis and grown in a sterile environment, then placed in a [1,2-^{14}C]TCA solution of 18.5 kBq ml^{-1} (i.e. 0.61 g ml^{-1}, total amount 9.11 μg), in which only the roots were submerged. Care was taken to ensure only roots were available for colonization by soil organisms by planting sterile spruce seedlings into soil through holes of plastic Petri-dishes, while the rest of the seedlings remained in a sterile environment. The separated root and shoot parts of the colonized seedlings were also used for degradation experiments [B_2].

Model experiments with Norway spruce shoots [C_1] were conducted as described earlier for spruce shoot experiments (Uhlířová et al., 1996; Forczek et al., 2001). Cut ends of detached current-year shoots of Norway spruce (2–5 g fresh weight) were placed into 30 ml [1,2-^{14}C]TCA-solution (17 kBq ml^{-1}, i.e. 0.75 μg ml^{-1}) in 500 ml Erlenmeyer flasks for up to 7 days (Table 3). The solution was roughly fractionated by ultrafiltration [C_3] with a membrane disc NMWL 3000 after exposure, and radioactivity of both, the macromolecular and the low-molecular fractions, was measured. Final experiment [C_4]: the shoot was let to take up 185 kBq [1,2-^{14}C]TCA solution in 0.5 ml distilled water and the production of $^{14}CO_2$ was followed in the moistened atmosphere inside of 500 ml Erlenmeyer flasks without additional liquid.

The radioactivity of the atmosphere was measured by liquid scintillation after $^{14}CO_2$ absorption in 1 M KOH-solution. The chloroform formation was checked by its absorption in methylcellosolve, which was applied before the KOH absorbers. The radioactivity of the needles was measured by combustion of the plant samples (Forczek et al., 2001; Matucha et al., 2001a). Radioactivity of liquid samples was measured on a Beckman LS 6500 (Fullerton, CA, USA) liquid scintillation spectrometer. To estimate the abundance of bacteria in the needles, needles were homogenized using an Ultra-Turrax homogenizer (IKA, Staufen i. Br., Germany) and the resulting suspension was used as an inoculum for the dilution plate technique of counting bacterial colony forming units.

3. Results and discussion

3.1. TCA distribution

The pilot study [A_1] showed that TCA concentrations in various tree parts were highly variable, dependent on

Table 2
TCA uptake into different tree parts [A_1]

	Applied TCA		t (days)	C	C + 1	C + 2	Branches	Wood	Roots
	(kBq)	(μg)		(Bq g^{-1} FW)					
Spruce 1	1665	76	19	520	350	330	280	110	280
Spruce 2	1665	76	12	1180	880	720	n.d.	33	210
Spruce 3	2220	700	66	5180	2930	1430	n.d.	10	160

Notes. Exposure seasons were different. Spruce 1–19 days (November 24–December 13), Spruce 2–12 days (May 26–June 8), Spruce 3–66 days (September 9–November 14). As the form of the TCA-derived radioactivity is not known, one cannot conclude whether the radioactivity comes from TCA and/or degradation product(s).
t—sampling time after application; C—current-year needles; C + 1—one-year-old needles; C + 2—two-year-old needles; n.d.—not determined.

different factors such as timing of experiment and sampling or applied amount of TCA. The highest uptake was detected in current-year needles (Table 2). After application of [1,2-^{14}C]TCA there was a radioactivity gradient in the spruce crown which increased towards the current-year needles, since the mean C:(C + 1):(C + 2) ratio of TCA-derived radioactivity was found to be in average 6:4:3. TCA is translocated from the roots to the needles, where it accumulates. TCA concentrations in needles in the natural environment are found to be higher in older needles because of TCA certain stability in needles. The amount of TCA taken up by the cutting is dependent on the applied concentration and the duration of TCA exposure. It is apparent that trees affected by TCA during their active growth accumulate a higher proportion of TCA than trees affected in their dormant state (compare Spruce 1 and Spruce 2 in Table 2).

Applied TCA is not uniformly distributed throughout the tree. Relative autonomy of the branches can be for example responsible for the fact that variable TCA concentrations in the soil water are reflected in the corresponding branches. As shown by Matucha et al. (2001a), TCA is transported upwards in xylem. The TCA then accumulates in mesophyll cells (Schröder et al., 2003). In experiments with cut shoots [C_1] a rapid uptake takes place because of the exclusion of the root system. Within 3 h the radioactivity is detectable in the needles as is demonstrated by using radioluminography (Schröder et al., 2003). After 6 days, a more or less homogenous distribution of TCA was observed in the individual needles (Forczek et al., 2003). Uptake via roots can be significantly slower because of selective transport mechanisms, but the distribution pattern probably remains the same, the measured radioactivity always being higher in the apical part of the needles.

Redistribution of TCA also may take place in a tree. If radioactively labeled TCA was applied to one separate part (approximately one third) of a root system [A_3] (1620 kBq, i.e. 73.6 μg TCA), radioactivity was found in the other part of the roots (0.1–0.5%) and in the soil (0.8–8.1%) as well. A similar distribution pattern was found in replanting experiments [A_2]. Radioactivity was detected in the replaced soil (13.61 kBq ≈ 0.8% of the originally applied TCA in non-specified form). Similar amounts of radioactivity were detected in the needles as previously (3.3–3.6%). These experiments prove that after initial uptake by spruce roots the TCA is transported to the needles, where it is (partly) degraded. The TCA and/or its degradation products (measured as [1,2-^{14}C]TCA-derived radioactivity) are partly redistributed to the roots and with root exudates, they are also released into the soil. The uptake probably takes place via the xylem, while redistribution can take place via the phloem.

3.2. TCA degradation

The detached spruce shoots [C_1] readily converted the taken up [1,2-^{14}C]TCA to $^{14}CO_2$ (curve B in Fig. 1; Fig. 2). The degradation proceeded most rapidly during the first two days. The product of degradation ($^{14}CO_2$) was observable within an hour. The degradation was most intensive around 6–15 h from $t = 0$ (130–470 Bq h^{-1} g^{-1}

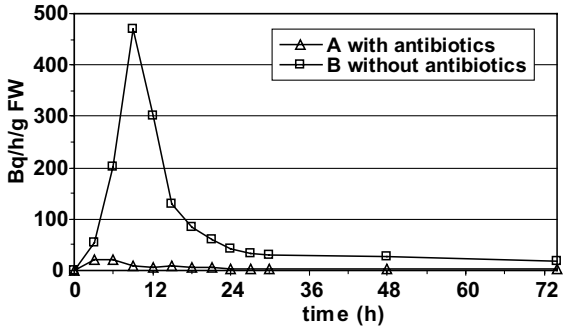

Fig. 1. The rate of [1,2-^{14}C]TCA degradation in detached spruce branches. The cut edge of branches was immersed in [1,2-^{14}C]TCA solution (518 kBq, i.e. 22.82 μg TCA; 0.76 μg ml^{-1} [1,2-^{14}C]TCA solution, with [C_2] and without antibiotics [C_1], curves A and B, respectively) and $^{14}CO_2$ radioactivity release was measured in the atmosphere. Curve B is one representative curve from 20 experiments.

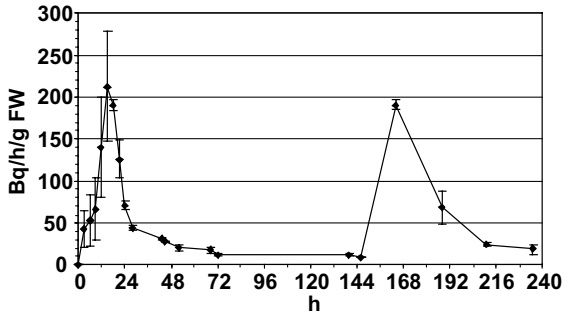

Fig. 2. $^{14}CO_2$ evolution in cut spruce branches after repeated addition of [1,2-^{14}C]TCA [experiments C_1; $n = 2$]. The cut edge of branches was immersed in [1,2-^{14}C]TCA solution (518 kBq, i.e. 22.82 μg TCA; 0.76 μg ml^{-1}) and $^{14}CO_2$ radioactivity release was measured in the atmosphere. Additional 413.6 kBq [1,2-^{14}C]TCA was added to the solution at 140 h. The error bars are standard deviation of analytical duplicates.

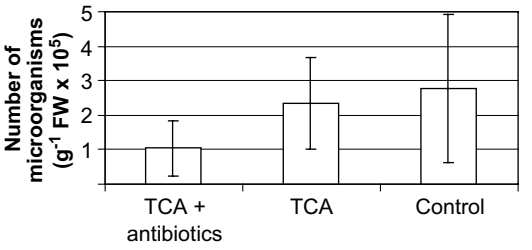

Fig. 3. Number of microorganisms $\times 10^5$ g^{-1} needle FW (mean ± S.D.; $n = 5$) after 4 days of exposure to 835 ng ml^{-1} TCA [C_2]. A mixture of 0.1 mg ml^{-1} of each antibiotic (neomycin, streptomycin, rolitetracycline) was added to the medium.

needle FW, i.e. 3.09–11.18 ng h^{-1} g^{-1} needle FW), after which it rapidly decreased. In the experiments [C_1] 2–3% of the [1,2-^{14}C]TCA-solution (13.93 kBq ± 4.33 kBq of 555 kBq; $n = 16$) was degraded to $^{14}CO_2$ in 48 h. After removing the detached spruce shoots from the [1,2-^{14}C]TCA-solution, $^{14}CO_2$ continued to evolve at 5–20 Bq h^{-1} (i.e. 0.12–0.48 ng h^{-1} relative to 1 g FW) until the termination of experiment (4 days longer). The spruce shoots put into water released back into the water 13–16%, and degraded 0.9–1.5% of the taken up [1,2-^{14}C]TCA daily. The measurements of $^{14}CO_2$ evolution in an environment without a liquid [experiment C_4] revealed a significant degradation by non-sterile plant biomass (4.4 ± 0.7 Bq h^{-1} g^{-1} needle FW). This result indicates that the TCA-degradation activity observed in experiments C_1–C_3 can be attributed at least to considerable extent to the plant.

Potentially interesting is the similar variability of the number of microorganisms (Fig. 3), the radioactivity released into atmosphere and the radioactivity in the shoot (see Table 3). The very high standard deviations reflect evidently the intrinsic inhomogeneity of the distribution of phyllosphere microorganisms.

To evaluate the state of deactivated TCA in the solution after the experiment [C_1], fractionation was conducted by ultrafiltration [C_3]. It showed that >98% of the radioactivity was bound to species of molecular weight lower than 3000. Hydrolysis of the remaining solution with ammonium hydroxide led to the release of TCA once again.

It is evident from this study that antibiotics influence the degradation of TCA in spruce needles. A significantly lower concentration of $^{14}CO_2$, the only degradation product detected in this experiment, was found in the flasks treated with antibiotics [C_2] (20.5 Bq h^{-1} g^{-1} FW, i.e. 0.49 ng h^{-1} g^{-1} needle FW) than in the non-treated flasks [C_1] (470.6 Bq h^{-1} g^{-1} FW, i.e. 11.19 ng h^{-1} g^{-1} needle FW) (Fig. 1). The number of microorganisms in needles was also significantly reduced by treatment with antibiotics (Fig. 3). No radioactivity was found in methylcellosolve, which was applied before the KOH absorbers [C_1]; it indicates that no chloroform was formed.

The delay in the maximum degradation rate at the beginning of the experiments C_1 (i.e. 0–12 h of TCA degradation, Fig. 1, curve B) is probably caused by the uptake time of TCA, and by the time needed for induction of the degrading enzymes. The amount of released $^{14}CO_2$ rapidly declined after the initial peak of degrading activity, but not because of the decrease of the

Table 3
Radioactivity measured in Norway spruce shoots [C_1 and C_2]

Applied TCA (μg)	n	t (days)	Radioactivity measured in the compartment (%)			RA_{Tot}
			Air [$^{14}CO_2$]	Shoot	Solution	
			$\bar{x}$ ± S.D.			$\bar{x}$ ± S.D.
20.71–24.45	16	2–8	3.1% ± 0.9%	4.3% ± 4.4%	90.4% ± 4.3%	97.8% ± 5.9%
3.52–5.95	4	2–3	2.9% ± 0.5%	4.2% ± 4.0%	79.4% ± 15.2%	86.4% ± 18.8%
Antibiotics 3.57–20.71	2	3	0.5% ± 0.2%	1.9% ± 0.2%	97.6% ± 6.0%	100.0% ± 6.4%

The shoots were exposed for 2–8 days to 30 ml of [1,2-^{14}C]TCA-solution in Erlenmeyer flasks. $^{14}CO_2$ development practically ceased in all cases after 48 h. n—number of samples, t—exposure time, RA_{Tot}—radioactivity balance.

enzyme activity. A consecutive addition of the same amount of TCA produced the same peak as shown in Fig. 2. Differences were found in timing of peaks (including the second peak) and their maximum of $^{14}CO_2$ release, thus one representative curve is presented in Fig. 1. It can be assumed that TCA was chemically deactivated during the degradation process and therefore became unavailable for further degradation by microorganisms. E.g. addition of 19 μg L-lysine or 19 μg L-aspartic acid into the [1,2-^{14}C]TCA-solution [C_1] which contained 19 μg TCA, produced a similar effect as antibiotics—no $^{14}CO_2$ peak was detected. The rate of degradation was 9.7 ± 4.7 Bq h^{-1} g^{-1} needle FW 0.23 ± 0.10 ng h^{-1} g^{-1} needle FW. In conclusion, the TCA became unavailable to microbial degradation, it was probably in a deactivated, but not degraded form.

Sterile spruce seedlings showed a very low TCA-degrading activity [B_1]. About 1 g of sterile material produced only 1/20 of radioactive $^{14}CO_2$ (4.1 ± 2.0 Bq h^{-1} g^{-1} needle FW, i.e. 0.10 ± 0.05 ng h^{-1} g^{-1} needle FW; $n = 4$) than detached shoots (Fig. 1). The very low $^{14}CO_2$ production was apparently dependent on light conditions. Supposing that the sterile seedlings have the same degrading enzyme set as adult spruces, the lower biodegrading activity observed can be explained by the absence of stress-induced enzymes (the activity of which can be measured in cut shoots) or by the absence of symbiotic or co-localized microorganisms. Also there can be difference in age-induced gene-expression. After 30 days of microbial root colonization in soil (the shoots being kept in an aseptic environment), different results were obtained when the seedlings were cut into root and shoot parts [B_2]. The root part degraded the added TCA with a rate characteristic of high microbial activity (400–1700 Bq h^{-1} g^{-1} needle FW, i.e. 9.51–40.43 ng h^{-1} g^{-1} needle FW) and the shoot part also showed intense degradation of TCA (200–2100 Bq h^{-1} g^{-1} needle FW, i.e. 4.76–49.95 ng h^{-1} g^{-1} needle FW). The rapid degradation of TCA in separated shoot parts after microbial colonization indicates the development and presence of phyllospheric microorganisms, which could originate from the bacterial, actinomycetous and microfungal communities in the soil (Matucha et al., 2003). Probably the microorganisms colonizing the phyllosphere proliferate there and degrade TCA.

4. Conclusions

There is a gradient in the accumulation of TCA in the tree crown after a one-shot dose as TCA is preferably transported into the current-year needles. A certain stability of TCA in spruce needles is, however, the cause of higher TCA concentrations in older needles of trees in a natural environment. TCA and/or its degradation products (measured as [1,2-^{14}C]TCA-derived radioactivity) are partly redistributed to the roots and with root exudates, they are also released into the soil.

Supposedly, both transport and microbial activity within the plant can have an influence on the degradation. TCA is presumably partly degraded in the needles, the only atmospheric degradation product being CO_2. The degradation of TCA to CO_2 in detached spruce shoots (or the CO_2 release) is most intense during the 48 h after uptake by the stem transpiration stream. Thereafter, the evolution of labeled $^{14}CO_2$ practically terminates. As antibiotics inhibit TCA degradation and TCA was almost not degraded in sterile spruce, the microbial colonization and TCA degradation in the needles were indicated and degradation by phyllosphere microorganisms was discussed.

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