Transpiration and metabolisation of TCE by willow plants – a pot experiment

Philipp Schöffner, Andrea Watzinger, Philipp Holzknecht, Bernhard Wimmer, and Thomas G. Reichenauer

AIT Austrian Institute of Technology GmbH, Health & Environment Department, Environmental Resources & Technologies, Konrad-Lorenz-Strasse, Tulln, Austria

ABSTRACT
Willows were grown in glass cylinders filled with compost above water-saturated quartz sand, to trace the fate of TCE in water and plant biomass. The experiment was repeated once with the same plants in two consecutive years. TCE was added in nominal concentrations of 0, 144, 288, and 721 mg l$^{-1}$. Unplanted cylinders were set-up and spiked with nominal concentrations of 721 mg l$^{-1}$ TCE in the second year. Additionally, $^{13}$C-enriched TCE solution ($^{13}$C = 110.3 %) was used. Periodically, TCE content and metabolites were analyzed in water and plant biomass. The presence of TCE-degrading microorganisms was monitored via the measurement of the isotopic ratio of carbon ($^{13}$C/$^{12}$C) in TCE, and the abundance of $^{13}$C-labeled microbial PLFAs (phospholipid fatty acids). More than 98% of TCE was lost via evapotranspiration from the planted pots within one month after adding TCE. Transpiration accounted to 94 to 78% of the total evapotranspiration loss. Almost 1% of TCE was metabolized in the shoots, whereby trichloroacetic acid (TCAA) and dichloroacetic acid (DCAA) were dominant metabolites; less trichloroethanol (TCHO) and TCE accumulated in plant tissues. Microbial degradation was ruled out by $^{13}$C measurements of water and PLFAs. TCE had no detected influence on plant stress status as determined by chlorophyll-fluorescence and gas exchange.

INTRODUCTION
Chlorinated hydrocarbons (CHCs), especially trichloroethene, tetrachloroethene, 1,1,1-trichloroethane and dichloromethane, are—together with hydrocarbons—the most frequent pollutants and can therefore be found at numerous industrial sites (Weisgram et al. 2012). Using plants for phytoremediation of CHCs is a cost-effective, noninvasive technology that uses sun energy to pump up contaminated groundwater. Phytoremediation of CHCs can be applied at sites that provide enough space for planting of vegetation, exhibit a shallow water table, and have a thin aquifer with low to medium discharge (Matthews, Massmann, and Strand 2003). Willow (Salix) and poplar (Populus) species have promising remediation properties due to their extensive rooting systems, high water consumption, high toxicity tolerance, rapid growth, and wide regional distribution (Miller, Khan, and Doty 2011). CHCs exhibit physical and chemical properties ideal for uptake by plant roots. These include $K_{ow}$ values (octanol-water partition coefficient) between 1.8 (Briggs, Bromilow, and Evans 1982) and 3.1 (Hsu, Marxmüller, and Yang 1990) as well as low ionization potential, strong polarity and water solubility (Dettenmaier, Doucette, and Bugbee 2009). Once organic xenobiotics such as CHCs are taken up, plants can metabolize and integrate them into their tissue to a certain extent according to the “green liver concept” of Sandermann (1992) or mineralize them to CO$_2$ and H$_2$O (Newman et al. 1997; Shang and Gordon 2002; Shang et al. 2001). Incomplete metabolization of CHCs can cause volatilization with the transpiration stream into the atmosphere (Ma and Burken 2004; Orchard et al. 2000; Wang et al. 2004; Winters 2008). In the case of TCE uptake, oxidative transformation has already been verified for poplar and willow trees. Trichloroethanol, dichloroacetic acid, and trichloroacetic acid were the main identified products (Gordon et al. 1998; James et al. 2009; Miller et al. 2011; Newman and Reynolds 2004; Newman et al. 1997, 1999). Willows and poplars are toxicity-tolerant species that can even grow at high TCE concentrations of around 200 mg l$^{-1}$ (Miller et al. 2011). We are unaware of further studies on the toxicity of chlorinated ethenes in willows. Studies on the physiologically similar poplars, however, revealed that 60 mg l$^{-1}$ TCE did not influence cell growth and that 8 months exposure to 50 mg l$^{-1}$ TCE hardly reduced plant growth of one-year-old poplar cuttings (Newman et al. 1997). Even at a TCE concentration of 550 mg l$^{-1}$, (Ma and Burken 2003) did not observe toxic effects in rooted poplar cuttings. In contrast, zero-growth of poplar cuttings was observed at 45 mg l$^{-1}$ PCE, 118 mg l$^{-1}$ TCE, 465 mg l$^{-1}$ 1,2-trans-DCE, 543 mg l$^{-1}$ 1,1-DCE, or 582 mg l$^{-1}$ 1,2-cis-DCE (Dietz and Schnoor 2001). All tested concentrations for poplars were far above expected plume concentrations at field sites. Moreover, bigger trees are probably even more tolerant than the tested cuttings. Thus, according to the present state of knowledge, phytotoxic effects at field sites are unlikely. To enhance CHC degradation in plants, they can be inoculated with endophytes (Weyens et al. 2010a; Weyens et al. 2009a; Weyens...
Plants can also be used to support microbial degradation in the rhizosphere (Anderson and Walton 1995; Godsy, Warren, and Paganelli 2003; James et al. 2009) by (i) release of plant exudates as nutrients, sugars, amino acids and secondary metabolites such as organic acids (Brigmon et al. 2003; Reichnauer and Germida 2008; Walton and Anderson 1990) and (ii) physical changes of soil structure such as gas exchange and soil moisture (McCully 1999). Since roots need oxygen for their growth, microbial degradation in the rhizosphere is assumed to largely follow an aerobic pathway. Microbial degradation leads to a shift in the signature of stable isotopes in the original contaminant towards heavier $^{13}$C isotopes. Accordingly, compound specific isotope analysis (CSIA) of the isotope ratio of TCE can be used to discriminate between plant uptake and microbial degradation of CHCs (Hunkeler, Aravena, and Butler 1999; Hunkeler and Aravena 2000; Meckenstock et al. 2004). This study was designed to assess the capability of willows to remediate TCE-contaminated sites. For this purpose, willows were grown in artificial aquifers; TCE uptake, TCE transformation in plant tissues, microbial degradation of TCE in the water and plant health status were investigated following a single TCE application. The experiment was repeated within two consecutive years using the same plants. CSIA was used to distinguish between microbial degradation and TCE elimination via plant uptake.

**Materials and methods**

**Experimental design**

Rooted cuttings of willow (*Salix viminalis* L.) were planted in a greenhouse in glass pots which were open to the atmosphere. Each of the pots was filled with a 25 cm layer of quartz sand (0.5–2 mm) that was covered by a 2 cm compost layer (Einheitserde ED 73, Germany), whereby 2/3 of the quartz sand layer was water saturated. Pots were wrapped with aluminium foil to avoid algae growth. A similar experimental set-up was successfully used by Newman et al. (1997). Two liters of TCE-containing solution was added via a Teflon (PTFE) tube into each quartz layer. Before applying the TCE, water was pumped out and residual water was taken up by the plants for half a day of transpiration. Hereafter, 2 1 solutions with nominal TCE concentrations of 0, 144, 288, and 721 mg l$^{-1}$ were applied once at each pot. The water level was kept between 18 and 22 cm during the experiment by repeatedly adding TCE-free water via the PTFE tubes described above, which diluted the TCE solutions. The amount of water that was added was measured gravimetrically. The compost layer was kept free of the TCE solution. The experiment was repeated once with the same plants and pots and with 5 replicates for each concentration in two consecutive years, starting on the 23 August 2010 and the 6 July 2011. The duration of the experiment was 45 days in 2010 and 29 days in 2011. In 2011, we added unplanted pots filled with a TCE solution with a nominal concentration of 721 mg l$^{-1}$. These were covered with moist compost (5 replicates) and with dry compost (5 replicates). Additionally, $^{13}$C-labeled TCE ($\delta^{13}$C $= 110.3 \%$) was used for the experiment in 2011.

Water samples were taken via a second perforated PTFE tube that was arranged in the middle of the quartz layer as a horizontal ring. Water samples were stored in PTFE-sealed vials at 4°C until analysis of TCE concentration and isotope analysis of $\delta^{13}$C. Leaves and bark (2 g moist weight) were sampled and stored immediately in liquid N$_2$ until extraction and measurement of TCE, TCOH, DCAA, and TCAA. Leaves and branches (1.5 cm diameter) were collected 45 days after TCE application in 2010. The branches were dissected into bark and wood. In 2011, samples were taken from shoots of the upper compartments (0.5 cm diameter) at days 4 and 9 and of the lower compartments (0.5–1 cm diameter) at days 19 and 29 after TCE application. Also in 2011, compost samples were taken with a soil auger (Ø 25 mm) from the quartz and the compost layer of each pot and were frozen at −20°C until analysis of composition and the $^{13}$C/$^{12}$C isotope ratio of phospholipid fatty acids (PLFAs). Finally, dark-adapted chlorophyll fluorescence was measured using a portable instrument on days 0, 1, 5, 6, 8, 9, 12, 13, 14, and 19 in 2010 and on day 17 in 2011 to evaluate plant stress status.

**Analytical methods**

Detailed information about the chemicals and materials used is listed in the supplementary data. Water samples (concentration of TCE and $\delta^{13}$C) were measured via Purge and Trap—Gas Chromatography—combustion—Isotope Ratio Mass Spectrometry (P&T-GC-c-IRMS). Here, a HP 5890 Series II GC (Hewlett-Packard, Wilmington, Delaware, USA) equipped with an oxidation oven was connected to a Delta S Isotope Ratio Mass Spectrometer (Finnigan, Bremen, Germany). Samples were injected via a VSP 4000 purge and trap unit (IMT, Innovative Messtechnik GmbH, Germany) using the following settings: 40°C sample temperature, 20 min purge time, 20 ml min$^{-1}$ purge flow, 80°C valve temperature, −50°C trap temperature, 200°C desorption temperature, 7 min desorption time, 50 sec transfer time, 0 ml min$^{-1}$ split flow, 200°C transfer line temperature, 1000 mbar constant helium pressure. Analytes were separated with an Agilent Technologies DB-VRX capillary column (60m, I.D. 0.32 mm, widebore film 1.8 µm) with He (5.0) as carrier gas (1.5 bar) and with the following temperature program: 200°C injection temperature, 40°C initial temperature hold for 10 min, ramp (40°C min$^{-1}$) to 100°C (hold for 2 min), ramp (10°C min$^{-1}$) to 200°C (hold for 2 min), ramp (20°C min$^{-1}$) to 240°C (hold for 2 min). The oxidation oven was operated at 940°C and was oxidized for 20 min with O$_2$ (0.4 bar) at 620°C prior to every measuring sequence. Within one measuring sequence a 2 s oxygen pulse was added to the oven after every sample at 940°C to prolong the oven’s reactive period.

TCE, TCOH, DCAA, and TCAA contents of plant tissues were extracted via solvent extraction according to James et al. (2009), Newman et al. (1997) and Shang et al. (2001) with minor adaptations. Detailed information regarding extraction procedure and its adaptations can be found in SI. Analytes were detected with an Agilent Technologies 6890 gas chromatograph coupled with an Agilent Technologies 5975 inert mass selective detector (MSD) in 2010; in 2011, analytes were detected with an Agilent Technologies (USA) G1223A electron capture...
detected by GC-FID (flame ionisation detector), and 13C fractionation was detected by GC-c-IRMS. PLFA extraction and measurement was conducted according to Watzinger et al. (2014). Dark-adapted chlorophyll fluorescence was measured with a Handy-PEA Fluorimeter (Hansatech Instruments, Norfolk, England). Leaves were dark adapted for 20 minutes prior to measurement. Chlorophyll fluorescence measurements were used to calculate the ratio $F_m/F_o$, as a parameter for “potential photochemical efficiency” of photosystem 2 (PS2). Here, $F_m$ represents the maximal fluorescence level at high light intensity and $F_o$ represents the variable fluorescence, which is derived from the difference between $F_m$ and minimal fluorescence $F_0$ at darkness ($F_m - F_o$).

Statistical analysis

For the PLFAs, all analytical results were calculated based on oven-dry (105°C) weight of soil. Statistics were performed with SPSS 17.0 for Windows. Impacts were tested by ANOVA and Tamhane post-hoc test because inhomogeneity of variance was confirmed. Significance was accepted at $p < 0.05$. Additionally, the factors substrate (sand - compost), plants (planted - unplanted pots) and TCE concentration (0, 144, 288, and 721 mg l$^{-1}$) were tested in a multivariate general linear model (full factorial model III, intercept included). Significant differences of PLFAs between TCE concentrations were determined separately for each substrate of the planted pots.

Results and discussion

Uptake of TCE by willows

The average initial TCE concentrations of replicates were 390 ± 192, 11 ± 2, and 6 ± 3 mg l$^{-1}$ in 2010 and 76 ± 50, 21 ± 11, and 12 ± 5 mg l$^{-1}$ in planted pots in 2011 and 28 ± 9 and 26 ± 11 mg l$^{-1}$ in unplanted pots (Fig. 1 a, b). These values are lower than the nominal added 721, 288, and 144 mg l$^{-1}$ TCE. Additionally, slightly increasing TCE concentrations were observed within the first days in 2011. The potential reasons likely were (i) TCE loss during filling of the pots via volatilization, (ii) incomplete dissolution of TCE in the solutions. Two sinks with minor influence may have been (iii) dilution by residual water in the sand, or (iv) adsorption of TCE on organic matter (Brigham et al. 2003) impurities in the sand layer. TCE solutions were filled directly into the inorganic sand layer, but thereafter minor amounts of organic matter moved from the top soil layer into the quartz sand layer. The TCE concentration in water decreased with time (Fig. 1 a, b). More than 95% of TCE was eliminated during the experiment in the planted pots, whereas TCE concentrations in unplanted pots with wet and dry compost did not decrease. Willows took up TCE from the water phase. The TCE concentrations decreased according to the calculated dilution effect caused by watering with TCE-free water (Fig. S1). The dilution was computed based on the initially measured TCE concentrations. In conclusion, the TCE decrease was controlled by transpiration. Loss of TCE by evaporation was negligible, independent of the water content of the compost layer (wet or dry).

Evapotranspiration (ET) rates of planted pots were constant at 0.42 ± 0.05 l day$^{-1}$ during the whole experimental period in 2010. In 2011, pots with initial concentration of 76 mg l$^{-1}$ TCE showed significantly reduced ET of 0.38 ± 0.05 l day$^{-1}$, whilst ET of other planted pots was constant at 0.50 ± 0.09 l day$^{-1}$ in the first 9 days. This probably reflects the coincidentally lower leaf area of 76 mg l$^{-1}$ TCE-treated plants. Independent of the TCE concentration, the ET rate dropped after 9 days to 0.22 ± 0.04 l day$^{-1}$, probably the result of the reduced leaf area due to sampling of plant tissues as well as a continuously decreasing air temperature. Maximum temperature outside the greenhouse dropped from ~31°C to 20°C and minimum temperature from ~17°C to 13°C (ZAMG - Zentralanstalt für Meteorologie und Geodynamik, Austria 2012). Leaf area data of single willow plants were unknown, hindering a more precise evaluation of the influence of TCE concentration on ET and a normalization of transpiration rates. Evaporation in unplanted pots in 2011 was 0.071 ± 0.007 l day$^{-1}$ when the overlying compost layer was wet and 0.035 ± 0.005 l day$^{-1}$ when the compost layer was dry. Hence, compared to the ET of planted pots, total evaporation (liters) in unplanted pots accounted for 13% of ET (wet compost) and 6% of ET (dry compost) in total.

Plant metabolism of TCE

Plants, even plants from control pots from the experiment in 2011, contained between one and two nmol g$^{-1}$ TCE without showing temporal changes (Fig. S2 a, b). We assume that TCE that was incorporated in control plants derived from TCE that was transpired by plants with TCE addition because all plants were grown within the same greenhouse chamber. A stomatal uptake of TCE was already shown by Su et al. (2010). TCE cross contamination during the extraction procedure was ruled out by blank extractions without plant material. Total TCOH concentrations in leaf and bark samples of plants spiked with 76 mg l$^{-1}$ TCE varied between 1.5 and 0.3 nmol g$^{-1}$ without a distinguishable temporal pattern (Fig. S2 a,b). TCOH concentrations in plants that were spiked with 21 mg l$^{-1}$ TCE decreased from day 9 to day 29 from 0.56 to 0.06 nmol g$^{-1}$ in leaf samples ($p < 0.05$) and from 0.25 to 0.02 nmol g$^{-1}$ in bark samples (insufficient data for statistical analysis). TCOH was not detected in leaf samples of control plants and was negligible (<0.08 nmol g$^{-1}$) in bark samples of control plants. TCOH was mostly glycolized; non glycolized TCOH accounted for 22 ± 15% of TCOH in leaves and for 4 ± 3% in bark. In 2010, plant tissue was sampled 45 days after TCE addition. No TCE was found in these samples, and TCOH was detected in small amounts (<0.5 nmol g$^{-1}$) solely in leaves and bark of plants that were spiked with high TCE concentrations (Fig. S2 a, b).

We assume that residual volatile analytes - TCE and non-glycolized TCOH—were further metabolized as proposed by
Shang et al. (2001) and vaporized out of plant tissues. This was congruent with TCE water concentrations, which approached near-zero values after 30 days (Fig. 1 a, b). This explains why TCE was hardly detectable after 45 days in 2010 and why TCOH concentrations decreased in 2011.

In contrast, concentrations of the non-volatile metabolites DCAA and TCAA in leaf samples of 2011 increased with time (Fig. S2 a), albeit not significantly. Bark sample DCAA concentrations increased between days 9 and 19 and decreased between days 19 and 29, whereas TCAA bark concentrations decreased (but not significantly) between days 9 and 29. DCAA concentrations in leaf and bark samples were between 0.5 and 3.0 nmol g$^{-1}$ and TCAA concentrations between 0.5 and 8 nmol g$^{-1}$, whilst control sample concentrations were < 0.3 nmol g$^{-1}$ in 2011 (Fig. S2). Differences between DCAA and TCAA concentrations of controls and TCE-spiked samples were significant for DCAA bark samples and TCAA leaf samples, but not for DCAA leaf samples or TCAA bark samples. In 2010, 45 days after TCE addition, DCAA and TCAA were mainly found in leaves and hardly in bark samples. Detailed concentrations of samples from 2010 are illustrated in (Fig. S2 a, b).

Using solvent extractions, only a small amount of incorporated analytes are extractable (Orchard et al. 2000). Regarding TCE extraction, efficiencies between 50 and 90 % can be assumed (Gopalakrishnan, Werth and Negri 2009). The sum of TCE and its metabolites were compared to the initially dosed molar TCE equivalents. The measured amounts in plant biomass represented a very minor part of the TCE eliminated from the aqueous phase, which agrees with previous studies (James et al. 2009; Newman et al. 1997, 1999; Shang et al. 2001). In 2010, summed metabolites of leaves and bark accounted for 0.57 % in the case of 11 mg l$^{-1}$ TCE dosage and for 0.04% at 390 mg l$^{-1}$ TCE dosage. In 2011, extracts accounted for 0.9% at 21 mg l$^{-1}$ and for 0.28% at 76 mg l$^{-1}$. Total amounts per willow (nmol) were determined by extrapolating of TCE and its metabolite concentrations from the last extractions on day 45 in 2010 and on day 29 in 2011 to total biomass. To calculate total TCE and metabolite amounts, a total leaf mass per willow of 0.1 kg and total bark mass of 1 kg were estimated.

Previous studies showed great differences regarding transformation and transpiration of chlorinated hydrocarbons by plants, whereby differences were even obtained between different genotypes of willow and poplar (Miller et al. 2011). Some

**Figure 1.** Measured TCE concentrations of the experiments in (a) 2010 and (b) 2011. In (a) 2010 the insert shows control pots without TCE (stars), pots with initially measured TCE concentrations of 6 mg l$^{-1}$ (○) and pots concentration of 11 mg l$^{-1}$ (●). In (b) 2011 the insert shows unplanted pots with initial TCE concentrations of 28 mg l$^{-1}$ (gray = dry compost layer) and 26 mg l$^{-1}$ (black = wet compost layer). Concentrations stated in the legend refer to initial measurements of TCE concentrations. Symbols show mean ± standard deviation (n = 5).
In 2010, the microbial degradation of TCE in the rhizosphere on PLFA amounts was not con-
tests. 2004; Doty 2008; Kang, Khan, and Doty et al. endophytic microorganisms may be necessary to ensure suf-
et al. (Brigmon 2000; Wang et al. 2004), especially if supporting soil amendments as fertilizers or vegetable oil were applied (Brigmon et al. 2003). Furthermore, inoculating trees with endophytic microorganisms may be necessary to ensure sufficient degradation of chlorinated hydrocarbons (Barac et al. 2004; Doty 2008; Kang, Khan, and Doty 2012; Khan and Doty 2011; Weyens et al. 2010a,b; Weyens et al. 2009a,b,c).

Microbial degradation of TCE in the rhizosphere

In 2010, the δ¹³C-values of TCE in water were −29.9 ± 0.9 %o and in 2011 113.7 ± 0.7 %o. No temporal trend was evident. In addition, no degradation metabolites, such as dichloroethene, were found in the water. Consequently, microbial degradation is negligible as an elimination pathway in the water of our experimental pots. This conclusion was strengthened by constant δ¹³C values of soil microbial PLFAs (phospholipid fatty acids). ¹³C/¹²C ratios of single PLFAs were between −25 and −30 %o, i.e., close to the natural δ¹³C values of plant-derived organic carbon. No ¹³C-labeled TCE (δ¹³C = 110.3 %o) was incorporated into the microbial biomembranes.

The PLFA composition was mainly influenced by the set-up time and/or planting of pots (unplanted pots were set-up one year later) and by the different layers (sand and compost, wet and dry compost); TCE concentration had only minor effects (Fig. 2). The amounts of PLFAs in the sand and compost layer were significantly higher in planted (older) versus unplanted (newer) pots (Fig. 2). No significant differences in PLFA concentration were recorded between unplanted pots with wet or dry compost, and PLFA values were significantly higher in the compost than in the sand layer, independent of other factors (Fig. 2). With increasing TCE concentration, PLFA values in the compost layer decreased, but increased in the sand layer (Fig. 2, Fig. 3). Significant effect of various TCE concentrations on PLFA amounts was not confirmed by Tamhane post doc tests.

Figure 2. Amount of total microbial biomass (total PLFAs) in compost (black) and sand (gray) in 2011 in replicates with 0, 12, 21, and 76 mg l⁻¹ TCE. "Dry" represents compost and sand layers of unplanted pots with dry compost layer. "Wet" represents compost and sand layers of unplanted pots with wet compost layer. Error bars represent standard deviation.

Summarizing, (i) TCE was apparently not degraded by microbes and (ii) the slight influence of TCE on PLFA amounts probably reflected secondary effects in our pot experiment.

Phytotoxicity of TCE

Chlorophyll fluorescence was used as a method for measuring plant stress at the leaf level. Values of Fv/Fm (i.e. actual photochemical potential of PS2) were 0.82 ± 0.03 in 2010 and 0.84 ± 0.02 in 2011, indicating non-stressed leaves at all TCE concentrations. Fv/Fm values did not differ significantly under different TCE concentrations, neither in 2010 nor in 2011 on any measuring day. Hence, TCE had no influence on photosynthesis or on plant stress status, which is consistent with the observation that TCE did not influence transpiration substantially. Six days after TCE application in 2010, however, fluorescence values decreased significantly, but values still indicated non-stressed leaves (Fig. 4).

Conclusion

Willows took up TCE without excluding TCE during the transpiration process, whereby even high TCE concentrations of about 390 mg l⁻¹ did not cause detectable toxic effects in plants. In our experiments, TCE was only minimally metabolized by willows. The degradation efficiency of TCE in the plants may be higher at the field scale due to bigger tree size and lower TCE concentrations. Nevertheless, our results suggest that, metabolization by willows may be insufficient what can cause transition of high amounts of CHC from water to the atmosphere via the transpiration pathway. To prevent such transition and to ensure total decontamination within the phytoremediation process, enhancing microbial degradation in the rhizosphere and by CHC-degrading endophytes might be a future promising strategy.
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Conflict of interest

The authors declare that they have no conflict of interest.

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