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Methodological tests of the use of trace elements as tracers to assess root activity

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Abstract

Background and aims There is increasing interest in how resource utilisation in grassland ecosystems is affected by changes in plant diversity and abiotic conditions. Research to date has mainly focussed on aboveground responses and there is limited insight into belowground processes. The aim of this study was to test a number of assumptions for the valid use of the trace elements caesium, lithium, rubidium and strontium as tracers to assess the root activity of several grassland species.

Methods We carried out a series of experiments addressing the reliability of soil labelling, injection density, incubation time, application rate and the comparability of different tracers in a multiple tracer method.

Results The results indicate that it is possible to achieve a reliable labelling of soil depths. Tracer injection density affected the variability but not the mean level of plant tracer concentrations. Tracer application rates should be based on pilot studies, because of site- and species-specific responses. The trace elements did not meet prerequisites to be used in a multiple tracer method.

Conclusions The use of trace elements as tracers is potentially a very useful tool to give insight into plant root activity at different soil depths. This work highlights some of the main benefits and pitfalls of the method and provides specific recommendations to assist the design of tracer experiments and interpretation of the results.

Keywords tracer injection, grassland, Cs, Li, Rb, Sr, tracer uptake, root activity, soil depth, resource utilisation, mixtures, plant communities

Introduction

Across a range of terrestrial ecosystems, there is increased recognition that plant-soil interactions exert key influences on biogeochemical cycles, and there is growing attention to below- as well as above-ground ecosystem functioning (Scherber et al. 2010). How plant communities control nutrient fluxes, and how such control may be affected by a range of biotic and abiotic factors has far-reaching consequences that vary from improving predictions of the effects of climate change and species extinctions, to improved management decisions under current conditions and in response to changed conditions.

Here, we focus on grassland ecosystems, for which the responses of plants to changed environmental conditions have been the focus of a large number of studies e.g. abiotic factors such as drought or increased temperature and CO_2 levels, (e.g. De Boeck et al. 2008; Grime et al. 2000; Kahmen et al. 2005; Vogel et al. 2012), and is of considerable applied relevance in the context of climate change (Reichstein et al. 2013). Similarly, there is a lot of research on the effect of species richness in both natural and agricultural ecosystems on ecosystem services, including primary production, nutrient retention, resistance to weed invasion and stability in response to disturbance (Finn et al. 2013; Gubsch et al. 2011; Hector et al. 1999; Kennedy et al. 2002; Roscher et al. 2011; Tilman et al. 2002). The proposed mechanisms behind these responses include niche differentiation, positive interspecific interactions (e.g. nitrogen transfer from clover to grass (Nyfeler et al. 2011)) and a higher probability of mixtures containing a high-yielding species, (Hooper et al. 2005; Loreau and Hector 2001).

Despite significant progress in experimental measurement of the effects of biotic and abiotic factors on ecological processes in grassland vegetation, there has been an emphasis on yield and other physiological responses that are amenable to aboveground measurement (Finn et al. 2013; Gubsch et al. 2011; Tilman et al. 2002). However, there remain significant knowledge gaps about the role of belowground patterns and processes (Ehrmann and Ritz 2013), since it is hard to distinguish the roots of different species grown in mixtures in the field (see Mommer et al. 2010). Additionally, the presence of root biomass is not necessarily equivalent to root activity (Kulmatiski and Beard 2013), and the uptake of different chemical forms of nutrients cannot be derived from measures of tissue chemical content.

Tracer techniques can help to identify and quantify spatial, temporal and chemical patterns of nutrient and water uptake, and are usually applied by injecting tracers into the soil and, after a certain incubation period, harvesting the plant material and measuring tracer concentrations. Tracers are substances which naturally occur in very low quantities and are chemically equivalent to other nutrients that are studied. There are three different classes of tracers: a) radioisotopes (e.g., ^{32}P, ^{33}P, ^{35}S), b) stable isotopes (e.g., ^{15}N, ^{34}S, ^{18}O, D) and c) trace elements (e.g., Sr^{2+}, Li^{+}, Rb^{+}, Cs^{+}). The use

of radioisotopes was very popular a few decades ago; however, its use for agro-ecological studies is now limited, although still important regarding P-uptake. Recent studies have focussed on the use of stable isotopes, e.g. ^{15}N, to examine temporal, spatial and chemical resource partitioning (Kahmen et al. 2006; McKane et al. 2002; Pornon et al. 2007; von Felten et al. 2009). Similarly, deuterium, which is chemically equivalent to H or ^{18}O-labeled water, has been applied to assess the uptake of water in soil (Kulmatiski and Beard 2013; Moreira et al. 2000).

Other studies have used trace elements as tracers to monitor differences in root activity and nutrient uptake in soil space (horizontal and vertical) and over time (e.g. Casper et al. 2003; Fitter 1986; Mamolos and Veresoglou 2000; Pecháčková et al. 2003). Plants use the same transport carriers for potassium as for the trace elements caesium, lithium and rubidium, whereas strontium is physiologically analogous to calcium (Kabata-Pendias and Pendias 2000; Marschner 1995). Trace elements are non-toxic for plants in concentrations that are readily determinable, and occur in both soil and plants at low natural concentrations (Pinkerton and Simpson 1979), thus making them ideal tracers for nutrient uptake studies. There is renewed interest in the use of these trace tracers as there is evidence that niches are multi-dimensional (Harpole and Tilman 2007). The simultaneous application of different tracers may allow the quantification of resource partitioning along various spatial and temporal resource axes. Specifically, the use of multiple trace elements in a multiple tracer method (Fig. 1) in which several tracers are each injected to a different depth within a single sub-plot (Carlen et al. 2002; Fitter 1986) would have distinct benefits in terms of reduced logistical efforts and reduced between sub-plot variability.

Even though tracer methods that use trace elements have been used in many studies, there has been no rigorous testing of the method to date. This represents a significant knowledge gap about the reliability and validity of this methodology. The objective of this study was therefore to test the use of the trace elements Cs, Li, Rb and Sr as tracers to compare the root activity (here defined as activity or tracer uptake per unit ground area, Fitter 1986) or nutrient uptake dynamics of different species in grassland monocultures and mixtures. To address this objective, we present data from several experiments that focused on different methodological aspects of relating the uptake of trace elements from soils to aboveground plant materials. We use these data to test whether the following assumptions that underpin the reliability of the methodology are satisfied (summarised in Tables 1 and 2): 1) Tracers are not vertically mobile in soil for the duration of the experiment, 2) Injection density affects the variability but not the mean level of tracer uptake of different species, 3) Tracer incubation time does not limit opportunities for tracer uptake and relocation, 4) Tracer application rate should be adjusted to soil type, plant species, tracer and expected level of root activity, and 5) The use of trace elements satisfies a number of requirements for their valid use in a multiple tracer method. These requirements

include a strong relationship between plant uptake of the different trace elements (particularly Cs, Li and Rb) which is consistent across treatments and plant species (Table 2 and Fig. S1).

Materials and methods

Table 3 gives an overview of the site, materials and methods for each of the individual experiments on the different aspects of tracer methodology described above.

Site and experimental layout

Experiments A, B and G were part of trials located at two sites in Switzerland: A at Tänikon Research Station, Aadorf (47°48'N, 8°91'E), on a brown earth (topsoil sandy loam, subsoil clay), and experiments B and G at Reckenholz, Zürich (47°43'N, 8°53'E), on a cambisol (topsoil 20-30% clay, subsoil 30-40% clay). Monocultures and mixtures were sown in August 2010 and 2011 (Tänikon and Reckenholz, respectively) on 3 m × 5 m plots. At both sites, plots were cut six times per year. Plots received mineral fertilizers, with 145 to 200 kg N ha^{-1} yr^{-1} split over five applications, and enough P and K as to be non-limiting for intensively managed grassland. Four species, *Lolium perenne*, *Chicorium intybus*, *Trifolium repens* and *Trifolium pratense* were sown according to a simplex design (Kirwan et al. 2007), consisting of all four monocultures, all six binary stands (50% of two species), and one 4-species mixture with equal abundances (25% of each of four species), in three replicates. Half of the plots in experiment A were subjected to a 10-week summer drought by using polythene-covered shelters (3 m × 5.5 m) to exclude rainfall.

Experiments C and D were part of a larger trial that was established at Waldhof, Langenthal, Switzerland (47°12'N, 7°48'E) in August 2009, at a terminal moraine Cambisol, with a sandy texture. The 12 m × 3 m plots received 165 kg ha^{-1} yr^{-1} of nitrogen equally divided over six applications. Thirteen different stands were established consisting of different combinations of one, two or three of the following functional groups (FG): grasses (G), legumes (L) and forbs (F). Each FG was made up by two species: *Lolium perenne* and *Dactylis glomerata* (G), *Chicorium intybus* and *Plantago lanceolata* (F), and *Trifolium repens* and *Trifolium pratense* (L). Experiment C was carried out in July 2010, using all four replicates of the FG-monocultures (G, L, F), the FG-binary stands (G-L, G-F, L-F), legume- and forb-dominated stand (10% G, 45% L and 45% F, g-L-F) and the equal stand (G-L-F). Experiment D was carried out on four replicates of the G-L-F and the g-L-F stands in May 2011.

Experiments E and F were greenhouse studies, based at Freiburg University, Germany. Pots consisted of polyvinyl chloride and had a height of 60 cm and a diameter of 11 cm. The bottom 5 cm of the pots was filled with gravel, followed by 5 cm of sand, and the remaining part up to the top 2 cm was filled

with a mixture of sand and natural topsoil in a ratio 1:3 (experiment F) or topsoil collected from a grassland field site in Jena, Thuringia, Germany (experiment E). Seeds of *Anthoxanthum odoratum*, *Festuca rubra*, *Leucanthemum vulgare*, *Centaurea jacea*, *Plantago Laceolata*, and for experiment E *Onobrychis viciifolia* and *Trifolium pratense* were pre-germinated and sown successively to allow transplantion to the pots (four seedlings per pot) at equal stage of development (primary leaf stage). Experiment E was sown as monocultures, whereas experiment F consisted of 1-, 2-, and 4-species mixtures. The plants were grown in a growth chamber with 16 h light / 8 h dark at 23 / 15 °C and approximately 60 % relative humidity.

Tracer injection

Tracer solutions were injected using a 50 mL multipipette (Multipette plus, Eppendorf, Hamburg, Germany) or a dispenser (Ceramus-classic 1-5 mL, Hirschmann Laborgeräte, Eberstadt, Germany) attached via a silicone tube to a hollow steel needle with four holes at the bottom of the needle (see Fig. 2). The holes were arranged at equal distances in the same horizontal plane. The solutions were injected into pre-drilled holes at the required injection density, injection depths, injection volumes and tracer application rates as summarised in Table 3. Different needle lengths (ranging from 15 to 61 cm) were used, depending on the required injection depth and the inner needle diameter varied from 2 to 4 mm. For experiment F, the injection holes were drilled at three different angles (45°, 90°, 135°).

In experiments B, D, E and G, tracers were injected as cocktail solutions containing all or some of $SrCl_2$, LiCl, RbCl and CsCl. In experiment A, two tracer solutions containing a combination of either $SrCl_2$ and LiCl or RbCl and CsCl were alternately injected at 5 and 35 cm depth in two separate sub-plots (50 cm × 50 cm) per plot. In experiment C, single-tracer solutions of Li, Sr, Rb and Cs were injected at 5 cm, 15 cm, 25 cm and 60 cm depth, respectively, in one single sub-plot. In experiment F, tracers were injected as single-tracer solutions at three depths in each pot, in different tracer × depth combinations (see Table 3). Within each experiment, tracer solutions were based on either equal molarity (experiments A, C, D and E) or equal weight (experiments B, F and G) of the tracer elements (see Table 3).

Sampling

For all field experiments, plant material on the sub-plots was harvested at 6 cm above soil level using electric shears. Harvesting occurred two to four weeks after tracer injection and the plant material was separated into species (see Table 3 for incubation times). For the pot experiments, the species were cut just above soil level on one to three days after tracer injection. For experiments A, B and G, soil cores

(20 mm diameter) were taken up to 50 cm depth (three cores per plot, split into 10 cm sections) to assess the concentration of the tracers in the soil. The soil cores were taken from the exact location of the tracer injections in a selection of sub-plots (*Lolium perenne* monoculture and the 4-species equal stand; for experiments A and G only).

Sample analysis

For experiments A-D and G, all plant and soil materials were oven-dried at 60 °C for 48 hrs prior to analysis. The concentrations of Li, Cs, Rb and Sr in plant and soil samples were determined with an inductively coupled plasma mass spectrometer (ICP-MS; 820 Varian, Santa Clara, California) equipped with a Meinhard vaporizer and Scott spray chamber. Analysis was conducted under standard conditions and at a plasma energy of 1.4 kW. Helium gas was used in the interference control cell. For calibration, ICP standards with 1000 mg l^{-1} were used to produce a standard curve of 2.5, 5, 10 and 40 $\mu g\ l^{-1}$ for each tracer.

For experiments E and F, plant material was dried at 70 °C for 48 hrs and ground prior to analysis. Lithium, Rb and Sr were analysed after acid digestion with 3 ml of water, 5 ml of HNO_3 (65 %) and 3 ml of $_{H2O2}$ (30 %) in a microwave acceleration system (CEM Mars 5, Matthews, NC, USA). Element concentrations were measured with an atomic absorption spectrometer (Perkin Elmer Analyst100, Waltham, MA, USA). Tracer concentrations could only be determined for sample sizes larger than 1 g dry matter (DM), resulting in missing values for some species × treatment combinations.

Data analysis

Concentrations of tracers in plant material were corrected for background levels. This correction was based on measurement of species-specific background concentrations of tracers in control plants from plots or pots within the same experiment that did not receive tracers, using the following equation:

$$\text{Excess tracer concentration} = TC_{Labelled} - TC_{Background} \quad \text{eqn. 1}$$

where $_{TCLabelled}$ is the tracer concentration of labelled plants or soils (mg kg^{-1} DM) and $_{TCBackground}$ is the tracer concentration of control (non-labelled) plants or soils. Background concentrations of tracers were not determined in experiment C in 2010, and we used the background concentrations from the same experimental site that was measured in 2011. We do not consider the lack of background concentration measurements in experiment F to be a major issue, due to the high tracer application rates (Table 3).

ANOVA was applied to assess whether excess tracer concentration was affected by either injection density or incubation time in interaction with plant species identity. To test whether lower injection

density resulted in higher variability, we calculated the coefficients of variation (CV) of individual species (stand × replicate, see Table S2 for more detail) for all tracer-depth × injection density combinations, and subjected these to ANOVA to determine the effect of species and tracer-depth on CV.

To determine the suitability of different tracer pairs for use in a multiple tracer method comparing the root activity at different soil depths, we assessed the following for individual species: 1) the strength of the regression between different tracer pairs (R^2 value) ; 2) whether there was a significant bias in the regression (intercept $\neq$ 0, and/or slope $\neq$ 1) and; 3) whether there was a significant treatment effect (drought in experiment A and injection depth in experiment D) on the regression between the different tracer pairs (Fig. S1). We only applied these tests to the datasets for which we had enough experimental plots (n > 3), i.e. experiments A and D. The regressions were based on excess tracer concentrations in either mmol g^{-1} DM or mg g^{-1} DM, depending on whether the injected tracer solutions were based on a molar or weight basis (see also Table 3). Additionally, we applied the same tests on the full datasets (containing all species) from experiments A, B, D and E, and assessed whether the slope and intercept of the tracer regressions were significantly different for the different species. All statistical analyses were carried out using the statistical software R version 2.12.2 (R Development Core Team 2012). When necessary, tracer concentrations were log-transformed to achieve normality.

Results

1) Tracer mobility in soil

In experiment A, injection of Cs, Rb and Sr at 5 cm soil depth significantly increased the tracer concentration in the 0-10 cm and the 10-20 cm soil layers (p<0.001, Fig. 3a). Tracers injected in paired plots that were protected from rainfall by using rainout shelters did not result in higher tracer concentrations in the 10-20 cm layer (data not shown). Therefore, movement of tracers to the deeper soil layer is probably due to leaching after relatively high rainfall (78 mm) during the four-week period between injection and harvest. Injection of Li did not result in higher Li concentrations in any of the soil layers (Fig. 3a), which may have been related to the relatively low Li application rate (see also Tables 3 and 5) and/or to leaching to deeper soil layers. Additionally, injection of Li in plots where rainfall was excluded did result in a significant increase in Li concentrations at the top soil layer (data not shown). Additional results from experiment A showed that concentrations of Cs, Rb and Sr in the soil located 3 cm from the centre of the injection hole were not higher than soil background concentrations (increases of 0.9, 0.5 and 0.3 % compared to the excess tracer concentration at the

centre of the injection hole for Cs, Rb and Sr, respectively, Table S1). Thus, there was very little horizontal mobility of tracers in the soil.

In experiment B, all tracers (injected to 5 cm) significantly (p<0.001) increased the tracer concentration of the 0-1 0 cm soil layer, with no significant movement to deeper layers in the 2-week period between injection and harvest (with 28 mm of rain) (Fig. 3b).

In experiment G, the concentrations of all four tracers were significantly (p<0.001) higher than background levels for all three targeted soil layers after the 1 .5 week period (with 47 mm of rain) between injection and harvest (Fig. 3c). For the 5 cm injection depth, only Li showed significant leaching to the 10-20 cm soil layer (p<0.001). Lithium injection at 20 cm depth also increased the Li concentration in the 20-30 cm soil layer (p<0.01), which is not unexpected, since the 20 cm is right at the border of the two soil depth layers. The other elements, however, did not show a similar increase in concentration in the 20-30 cm soil layer. The injection at 35 cm resulted in elevated tracer concentrations in the 20-30 cm soil layer for all tracers.

2) Injection density

Increasing the tracer injection density from 36 to 144 injections m^{-2} significantly (p<0.001) decreased the plant excess tracer concentration for Li applied at 5 cm soil depth (Table 4, experiment C). However, there was no significant effect of injection density or interaction of injection density with plant species for the other tracers. ANOVA of the coefficient of variation (CV) of the excess tracer concentration of individual species showed that the CV tended to be higher (p=0.069) for 36 compared to 144 injections m^{-2}. The CV was higher (p<0.01) for tracers applied at larger depths (Table 4). There was no significant species effect on CV and, therefore, no evidence that CV was larger for species with low abundance (e.g. *Plantago lanceolata*) (Table S2).

3) Incubation time

Delaying the harvest from 24 to 48 hours after tracer injection significantly (p<0.001) increased the concentrations of all tracers in plant material (Table 5, experiment E), but there were no significant interactions of incubation time with plant species or of incubation time with tracer (Table S3).

4) Tracer application rate

Soil tracer background concentrations ranged from 0.2 mg kg^{-1} DM for Cs to 24.7 mg kg^{-1} DM for Sr (Table 6). There was variation in tracer concentrations depending on the experimental site; for

example, the soil Cs concentration was 0.2 mg kg^{-1} DM in Tänikon (experiment A) and 4.1 mg kg^{-1} DM in Reckenholz (experiment B). Background concentrations of tracers in plant material tended to reflect trends in background levels of the different tracers in soil, and were lowest for Cs followed by Li, Rb and Sr (Table 6). In the majority of cases, background concentrations of tracers in plant material were significantly different for the different species (Table S4). Tracer application rates varied from 0.2 to 6.9 g m^{-2} and, in general, higher application rates tended to result in higher mean plant tracer uptake (Table 6). Tracer recovery rates (calculated as ratio of excess tracer uptake to application rate) were low and ranged from 0.06% (Cs, experiment D, 20 cm injection depth) to 3.6% (Rb, experiment D, 5 cm injection depth). There was a large range in the excess tracer concentration for the different species, reflecting their different root activities (Table S5). As expected for grassland species, the excess tracer concentration values for tracers injected at 5cm soil depth were a lot higher than those at 20 cm depth (experiment D, Table 6). Mean tracer enrichment (excess tracer concentration divided by tracer background concentration) ranged from 0.6 to 363 mg mg^{-1}.

5) The multiple tracer method

The regression between tracer pairs for individual species in experiments A and D was highly significant in all but one case (experiment D: Ci, Li × Rb, Fig. 4b) and R^2 for significant regressions ranged from 0.45 to 0.98 (Tables 7 and S6). The R^2 was higher than 0.75 in 23 out of 38 cases (15 and 8 cases for K-analogues and K-analogues × Sr, respectively; Table 7). There were only three cases out of 38 in which there was no significant bias in the regression between tracer pairs, i.e. only three cases where the intercept was not significantly different from 0 and the slope was not significantly different from 1 (e.g. Fig. 4a as opposed to Fig. 4c). In 18 out of 38 cases, the experimental factor (drought for experiment A and injection depth for experiment D) significantly affected the regression between tracer pairs (7 and 11 cases for K-analogues and K-analogues × Sr, respectively, Tables 7 and S6, Fig. 4d). In general, the effect of the experimental factor was significant across all species in at least one tracer pair, and was significant across all tracer pairs (except Li × Rb) for at least one species. In 12 out of 38 cases, we found a value of $R^2 > 0.75$ in combination with a lack of significant treatment effect, and this was the case more often for K-analogues than K-analogues × Sr (10 and 2 cases, respectively, Table S6).

To assess the suitability of the multiple tracer method for comparison of root activity of multiple plant species (Fig. S1 e), we performed a regression analysis across the whole dataset (containing the different species) for the individual experiments. The regressions of tracer pairs showed a significant effect of the different species on the slope and/or the intercept of the regression in 17 out of 21 cases (Tables 7, S6 and S7, Fig. 4e).

Discussion

Tracer mobility in soil (Assumption 1)

Tracer injection generally resulted in the effective and reliable labelling of the targeted soil depth. However, significant rainfall (78 mm in four weeks) resulted in leaching of all tracers to lower depths. Reducing the period between tracer injection and harvest or covering the plots will decrease the risk of leaching. Of all the tracers, Li seemed to be most prone to leaching, which is in agreement with other research (Kabata-Pendias and Pendias 2000). There was some evidence of contamination of soil layers above the target depth at deeper soil depths (Fig. 3), suggesting that this may be a problem either if the tracer solution is injected very quickly or in soils (or soil layers) with high bulk density. In these cases, the soil matrix surrounding the injection depth may not absorb the tracer solution quickly enough so that part of the solution rises within the drilling hole. This can be prevented by making sure that the tracer volume per injection does not exceed the volume of the drilled hole within the height of the soil layer to be labelled. The tracer volume per injection can be reduced by: decreasing the application rate (mg m^{-2}); increasing the injection density (number of holes m^{-2}) or; increasing the concentration of the tracer solution (mg l^{-1}). Altering the concentration of the tracer solution allows some flexibility without unduly affecting the reliability of the method; however, excessively high concentrations may increase the risk of toxicity to plants.

In order to minimise the vertical dispersion of tracers in soil we recommend to: 1) time the tracer application to limit or prevent the occurrence of significant rainfall amounts after tracer injection; 2) optimise tracer volume and injection speed (conduct pilot study with dye, e.g. Brilliant Blue); and 3) measure tracer concentrations at different soil depths at the end of the incubation period in order to verify the actual excess tracer concentration achieved at different soil depths.

Injection density (Assumption 2)

For most tracer-by-depth combinations, reducing the injection density from 144 to 36 injections m^{-2} tended to increase the variability (CV) of excess tracer concentrations, but did not affect the mean excess tracer concentration of the plant material for the different species. For Li injected at 5 cm depth, however, the reduction in injection density significantly increased excess tracer concentration, which may be related to the relatively low tracer application rate of Li compared to Li background concentrations in the soil. At these low application rates, concentrating the tracer solution in fewer

injection points may have increased the plant availability of Li disproportionately (Kabata-Pendias and Pendias 2000).

The injection densities used in experiments A to G ranged from 36 to 144 injections m^{-2}, which is within the range reported for other such experiments (41-400 injections m^{-2}) (Carlen et al. 2002; Fitter 1986; Mamolos et al. 1995; Mamolos and Veresoglou 2000; Veresoglou and Fitter 1984). The injection densities in our study are somewhat lower than those used to quantify nitrogen complementarity with ^{15}N tracers (e.g. between 190 and 210 injections per m^2 in Kahmen et al. 2006; McKane et al. 2002; von Felten et al. 2009). Such densities result from injection grids of about 7 cm distances, which is commonly assumed to be within the main lateral rooting zone of many grassland species. Other tracer techniques, e.g. the application of ^{15}N solution to measure gross N fluxes in soil using the ^{15}N pool dilution technique, have injection densities that range from 543 to 8333 injection m^2 (Murphy et al. 2003). Assuming a maximum lateral spread of 3 cm around the point of injection in the soil types used in our experiments, even the highest density in the current study (experiment C) will have resulted in tracer hotspots rather than in a homogenously layered band. However, our results show that under appropriate application rates this will result in an increase in the level of variation, rather than affect the mean tracer concentrations in labelled plant material.

For grassland studies and soils with bulk densities that are not too high, we would recommend injection densities of approx. 100 to 200 injections per m^2. Pilot studies using dye could give some indication of the lateral spread and the degree of spatial homogeneity of the tracer.

Incubation time (Assumption 3)

Experiment E showed that as soon as 24 hours after injection, plants had already taken up significant amounts of the tracers. Even though the tracer concentrations in the plant material had increased after 48 hours, there was no indication that an incubation period of 48 compared to 24 hours would affect the comparison of root activity between species. The incubation times of the experiments in the current study ranged from 24 hours to 4 weeks, similar to other studies using trace elements where incubation times ranged from two weeks (Carlen et al. 2002; Casper et al. 2003; Mamolos and Veresoglou 2000) to six weeks (Fitter 1986). However, we do not have comparative data on the effect of prolonging the incubation time beyond 48 hours.

Incubation time will affect both the opportunity for plants to take up the tracer, and also the proportion of tracer that is allocated to the aboveground biomass that is harvested for later measurements. This is shown by Bristow *et al.* (1987) using double-labelled $^{15}NH_4{}^{15}NO_3$, where the proportion of total N uptake in the harvested plant varied from 2 % on the day of application to 16.8% after 27 days.

Additionally, the tracer availability for plants is affected by microbial immobilisation and fixation to the soil matrix (as discussed under Assumption 4, below).

Increased risk of leaching during prolonged incubation times may affect the reliability of soil labelling. In addition, very long incubation times are not recommended to avoid loss of tracers via senescence of plant material during the incubation period.

Tracer application rate and background levels (Assumption 4)

Tracer application rate should be targeted at ensuring a sufficiently high excess plant tracer concentration and enrichment (ratio of excess tracer concentration to tracer background concentration) for all species and treatments to give a reliable estimate of root activity. This depends both on background levels and on plant uptake of the injected tracer. The current study showed that background levels of tracers were highly variable for different tracers, sites and plant species. Background concentrations of tracers among different plant species varied by up to a factor of 7. This emphasises the need to measure background concentrations in plants for individual species, sites and conditions.

Plant uptake of injected tracers depends on a number of factors. The uptake of trace elements by plants is affected by soil factors such as pH, redox potential, water regime, bulk density and clay content, organic matter content, cation exchange capacity, nutrient balance and concentration of other trace elements (Kabata-Pendias and Pendias 2000). For example, the bio-availability of Cs has been shown to be greatly inhibited by the addition of lime and peat to soil (Kabata-Pendias and Pendias 2000). Additionally, plant availability of tracers may be affected by tracer uptake by soil micro-organisms (Avery 1995). However, research on the uptake of Cs, Li, Rb and Sr by microorganisms has mainly focused on the remediation of soils or industrial wastes that are contaminated with high concentrations (e.g. Li from lithium-ion batteries; Xin et al. 2009) or the radio-isotopes of these elements (Bossemeyer et al. 1989; Ohnuki et al. 2003), but offer little information on the magnitude of uptake in natural or agricultural systems.

This highlights the importance of testing the site-specific plant tracer uptake of the targeted species before the start of an experiment. The current study showed some variation in tracer recovery rates for the different sites; however, this was confounded by other factors such as season and application rate, and these data are therefore not suited to draw conclusions on the effect of soil type or site variability on tracer recovery rates.

Additionally, plant root presence and activity at the depth of injection affects how much of the available tracer is taken up and depends on species, conditions (e.g. drought, fertilisation level or season). For example, in experiment D, the excess tracer concentration of tracers injected at 5 cm

depth was up to 4 times larger than when injected at 20 cm. Thus, where tracer uptake is known to vary, tracer application rates should be designed to achieve sufficient enrichment even at the lowest level of expected tracer uptake.

As described above, incubation time affects the opportunity for plants to take up tracers, but also to allocate tracer to harvestable parts, and will therefore have an effect on the required application rate. For example, the shorter the incubation time, the higher the application rate that is needed to achieve a given level of tracer in plant material.

Application rates of trace elements as tracers range from 1.8 to 33.6 g m^{-2} (Mamolos et al. 1995; Veresoglou and Fitter 1984). In our study, application rates ranged from 0.2 to 9.5 g m^{-2}. The application rate of 0.2 g m^{-2} was for Li in a tracer cocktail with Rb, Sr and Cs on an equal molar basis, and was applied with the aim to optimise comparability of the tracers (experiments A and D). Due to the low molar weight of Li compared to the other elements, this resulted in very low levels of enrichment, which were too low to reliably assess root activity at deeper injection depths (deeper than 20 cm, data not shown).

Although we did not test specifically for toxicity effects, we found no indication of any toxicity effects on plants due to tracer application rates up to 9.5 g m^{-2}. Maximum plant concentrations in our study were 223, 422, 1100, and 968 mg kg^{-1}, for Cs, Li, Rb and Sr, respectively. For the trace elements used in this study, there is only limited data on toxic effects on plant growth. At high levels in the soil, lithium is toxic to all plants but sensitivity to lithium is species-dependent: for citrus leaves moderate to severe toxic effects were reported at plant concentrations 4 to 40 mg kg^{-1}, whereas the *Solanaceae* family can reach concentrations of above 1 g kg^{-1} (Aral and Vecchio-Sadus 2008; Kabata-Pendias and Pendias 2000). There are some reports of toxicity of Rb and Cs, as high concentrations in the soil can result in K starvation in plants (Hampton et al. 2004), but this is only relevant at low soil K levels. There is not much evidence for Sr toxicity in plants (Seregin and Kozhevnikova 2004).

In light of the several complicating factors highlighted in this section, we therefore recommend that pilot studies are conducted to assess the tracer application rate required to obtain sufficient enrichment for all treatments under the specific experimental conditions.

Multiple tracer method (Assumption 5)

Even though there tended to be a strong relationship between the plant excess tracer concentrations of the different tracer pairs for individual species (indicated by a high R^2 value of the regression), there was a significant bias ($a>0$, $b\neq1$) in the majority of regressions across all species and tracer combinations, indicating the need for a correction factor to make the tracer results directly comparable. Differences in plant excess tracer concentrations between the different tracers were not

unexpected as there is often a negative correlation between the uptake rate and the ion radius (Marschner 1995). This was shown to result in lower uptake of Li (hydrated ion radius 0.38 nm) compared to K (hydrated ion radius 0.33 nm). Despite its smaller diameter, Cs (hydrated ion radius 0.31 nm) was taken up at a much lower rate than K (Marschner 1995). Mamolos *et al.* (1995) reported that the correlation coefficients between relative root activities for Sr and Cs were highly significant for all treatments, but this was not the case for correlations between Sr and Li or Cs and Li.

The relation between the different tracers was significantly affected by the experimental factor (drought or injection depth) or by plant species in the majority of tracer pair comparisons across the different experiments. We were unable to identify a tracer pair that showed a consistently strong regression and was not affected by the experimental treatment or species. As expected, tracer pairs consisting of two K-analogues performed better than when combined with Sr. Mamolos *et al.* (1995) found that the rankings according to the relative root activity with depth in soil for different species assessed by Sr and Cs were correlated in five out of eight cases. Collander (1941) reported that the uptake of K, Rb and Cs from nutrient solutions by a range of plant species was very similar, as was the case for Sr and Ca; however, Li was taken up to a much lesser extent. The current study indicates that this close link does not hold when plants are cultivated in soil as opposed to nutrient solution.

As the trace elements did not meet the prerequisites to be used in a multiple tracer method, in which different tracers representing the same nutrient are injected to different soil depths within the same sub-plot, we cannot recommend their use in such a method.

Even though the plant transport mechanism for Li, Cs and Rb is similar to K, and that of Sr is similar to Ca, the quantitative plant uptake of these elements is not the same due to differences in physicochemical properties such as ion radius and differential interaction with the soil (Kabata-Pendias and Pendias 2000; Marschner 1995). As discussed above, this prohibits the use of these elements in a multiple tracer method; however, it also has implications for their reliability as tracers for K and Ca. Our study does not provide a direct comparison of plant uptake of Li, Cs and Rb with K and of Sr with Ca. Of the K-analogues, Rb and to a lesser extent Cs are most comparable to K (Collander 1941; Marschner 1995), whereas strong links between Sr and Ca have been reported (Collander 1941; Mamolos et al. 1995; Marschner 1995). However, the comparability is not as good as would be in the case of stable isotopes, which have known and very small deviations from the nutrient they are representing and, in contrast to trace elements, have the same physiological or metabolic roles in the plant (Marschner 1995). Even though this limits the use of the trace elements as direct K-analogues and Ca-analogues, their use as a measure of root activity is still valid, i.e. they do give a direct estimate of root activity. However, different tracers give different results, as they represent different activities of the root that may be affected by different factors. Therefore, it is important not

to use single trace elements in isolation to determine root activity, but in conjunction with other trace elements or stable isotopes.

Interpretation of tracer studies

The measurement unit for comparison has a large impact on the interpretation of tracer results. The tracer concentration in plant material (mg kg^{-1} DM) has been used as a proxy for root activity (Fitter 1986; Veresoglou and Fitter 1984). However, differences in growth rates among species can affect the measured concentrations, resulting in a dilution of the tracer in higher-yielding species or treatments and therefore an underestimation of their root uptake activity. This problem does not affect the plant tracer uptake or tracer yield per unit ground area (tracer concentrations multiplied with species aboveground biomass per unit ground area) (Carlen et al. 2002; Mamolos and Veresoglou 2000; Veresoglou and Fitter 1984). However, this measurement unit is not well suited to comparing species root activities in species mixtures with varying abundances, as root activity becomes confounded with the relative abundance of individual species in the sward. The ratio between the plant tracer concentration (or uptake) from the shallow and deep injection depths (Fitter 1986; Mamolos et al. 1995; Mamolos and Veresoglou 2000; Veresoglou and Fitter 1984) has the considerable benefit that it allows the comparison within and between species in different mixtures and treatments. However, even though it can show a shift in the depth of root activity, it does not indicate whether the absolute root activity increased or decreased.

Therefore, we recommend that tracer results are presented as tracer uptake per unit ground area for monocultures or as relative root activity in mixtures with varying species abundances. However, in the current study, we mainly use tracer concentrations, since our main aim was to assess the different methodological aspects and not to determine root activity per se.

The high variability in tracer background concentrations (Tables 6 and S4) highlights the need to correct tracer concentrations for the tracer background concentration. This is standard practice in isotope studies, but has been largely overlooked in previous studies using trace elements (e.g. Carlen et al. 2002; Fitter 1986; Mamolos and Veresoglou 2000). This correction can be very important, particularly when working with relatively low enrichment levels.

In summary, using trace elements as tracers is potentially a useful tool to give insight in the root activity at different depths which is highly relevant in many research fields, including niche differentiation in ecological research and plant response to abiotic changes in climate change studies. This work highlights some of the main benefits and pitfalls of the method and provides specific recommendations to help with the design of tracer experiments and interpretation of the results.

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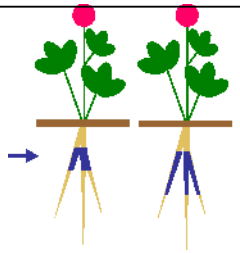
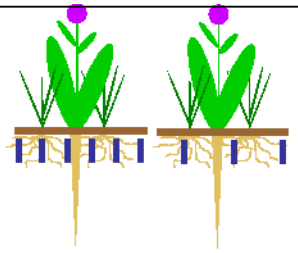
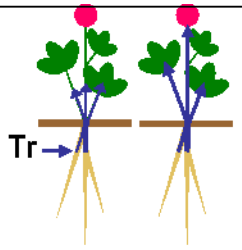
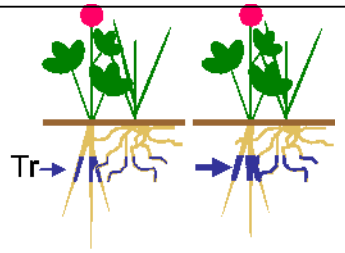
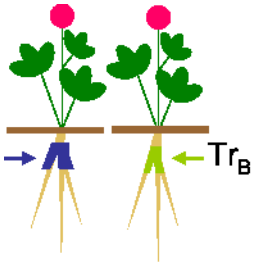
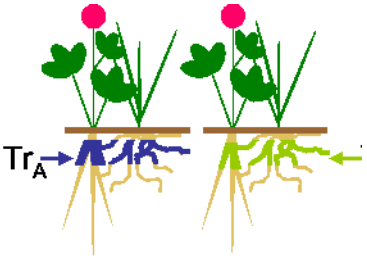
Tables

Table 1. Overview of methodological aspects of the tracer method. Assumption 5 (multiple tracer method) is not included here (see Table 2 and Figs. 1 and 1S)

	Assumption 1. Tracer mobility	**2. Injection density**	**3. Incubation time**	**4. Application rate**
				
Risk / problem	If tracer leaches down the soil profile, the measured root activity does not represent the targeted depth.	Low density: increased variability in plant dTC, especially for less abundant or narrow-rooting species. High density: risk of soil or sward disturbance, more labour intensive.	Short incubation times: may limit opportunity for uptake and (re-) location of tracers. Long incubation times: dilution, migration and loss of tracer through turnover processes.	Low rate (g m^{-2}): large effect from background signal, low precision. High rate: risk of affecting plants (unwanted fertilisation, toxicity) and not cost-effective.
Hypothesis	Tracers are not vertically mobile in soil for the duration of the experiment.	Injection density affects the variability but not the level of plant dTC of different species.	Incubation time affects the plant tracer concentration.	Application rate should be adjusted to soil type, plant species, tracer and expected level of root activity.
Experiment	**Exp A, B, G:** Measure dTC in soil depth increments (0-10, 10-20, 20-30, 30-40 cm) at time of tracer harvest.	**Exp C:** 36 or 144 injections per m^2.	**Exp E:** 24 and 48 hours incubation time.	**Exp A, B, D, E:** Measure background tracer concentration and dTC in relation to application rate.
Results	Fig. 1 Reliable marking possible, but evidence of leaching under very wet conditions, particularly for Li. Some rising tracer solution.	Tables 4 and S2 Reducing the number of injections did not affect the mean dTC, but tended to increase the variability.	Tables 5 and S3 Longer incubation time increased dTC, but did not affect comparison of species or tracers at this short time interval.	Tables 6, S4 and S5 Achieved dTC is affected by application rate, site, species, tracer and level of root activity.
Recommen-dations	1) Limit or prevent leaching due to significant rainfall after tracer injection. 2) Adjust tracer volume and injection speed to avoid rising tracer solution. 3) Measure soil dTC to assess actual labelling depth.	Injection densities of ~100 to 200 injections per m^2 are recommended for grassland studies.	Select incubation time to allow sufficient opportunity for uptake constrained by the need to limit the risk of leaching and loss of tracer through senescence of plant material.	Carry out site- and species- specific pilot study to assess required application rate. Should be based on the lowest expected root activity (e.g. deepest depth).

Tr = tracer, dTC = excess tracer concentration (see eqn 1), N.A. is not applicable

Table 2. The use of the multiple tracer method for assessing the root activity at multiple depths for single and multiple plant species swards (Test of assumption 5, see Introduction)

Aspect	Multiple tracer method for single species	Multiple tracer method for comparison of multiple species
Risk / problem	Unreliable comparison of plant dTC of tracer pairs, due to weak relation between a tracer pair (Fig. S1b), a bias in the relation (Fig. S1c) or treatment effect on the relation (Fig. S1d). 	Unreliable comparison of plant dTC of tracer pairs between different plant species (Fig. S1e) 
Hypothesis	a) There is a strong relation between the different tracers, particularly the K-analogues (Cs, Li, Rb), b), the relation of tracer pairs has no significant bias and c) is not affected by treatment.	d) The relation between tracer pairs is not affected by plant species.
Experiment	**Exp A, D:** apply tracer cocktails and measure the plant dTC of all tracers as affected by drought (exp A) or depth of injection (exp D).	**Exp A, B, D and F:** apply tracer cocktails and measure the plant dTC of all tracers.
Results	Tables 7 and S6, Fig. 4a-d	Tables 7, S6 and S7, Fig. 4e
	Regressions between tracer pairs within species had a high R^2 (>0.75) in 23 out of 38 cases, were not biased in 3 out of 38 cases and not affected by treatment in 20 out of 38 cases. K-analogues performed better than K-analogues × Sr.	Regression between tracer pairs was species dependent in 17 out of 21 cases.
Recommendations	We did not find a tracer pair that consistently met all the prerequisites and cannot recommend the use of these tracers in a multiple tracer method.	We do not recommend using the multiple tracer method for comparing multiple species for the species combinations tested in this study.

Tr_A and Tr_B = tracer A and tracer B, dTC = excess tracer concentration (see eqn 1).

Table 3. Summary information of the tracer experiments

Exp	Location	Date	Species[a]	Plot size (cm)	Injection grid	Injection density (number of injections m^{-2})	Injection depth (cm)	Injection volume (mL $injection^{-1}$)	Application rate (g m^{-2}) [b]				Incubation time	Assumption[c]
									Cs	Li	Rb	Sr		
A	Tänikon, CH	Aug-2011	Ci, Lp, Tp, Tr	50 × 50	5 × 5	100	5	1.5	3.4	0.2	2.2	2.2	4 weeks	1, 4, 5
B	Reckenholz, CH	Apr-2012	Ci, Lp, Tp, Tr	45 × 55	5 × 5	100	5	2	1.3	1.3	1.3	1.3	2 wks	1, 4, 5
									2.6	2.6	2.6	2.6		
C	Waldhof, CH	Aug-2010	Ci, Lp, Tp, Tr Dg, Pl	50 × 50	3 × 3 / 6 x 6	36 / 144	Li:5, Sr:15, Rb:25, Cs:60	1.5 / 3	4.9	0.3	3.1	3.2	4 weeks	2
D	Waldhof, CH	May-2011	Ci, Lp, Tp, Tr Dg, Pl	50 × 50	5 × 5	100	5, 20	1.5	3.4	0.2	2.2	2.2	3 weeks	4, 5
E	Uni Freiburg, DE	Mar-2011	Ao, Cj, Fr, Lv, Ov, Pl, Tp	Pots 60cm, ø11cm	1	53	5	10	na	0.5	6.7	6.9	24 / 48 hours	3
F	Uni Freiburg, DE	Jun-2010	Ao, Cj, Fr, Lv, Pl	Pots 60cm, ø11cm	3	79	5, 15, 35	8	na	9.5	9.5	9.5	3 days	5
G	Reckenholz, CH	Aug-2012	Ci, Lp, Tp, Tr	45 × 55	4 × 7	113	5, 20, 35	2	2.9	2.9	2.9	4.4	2 weeks	1

[a] Species abbreviations: *Chichorium intybus* (Ci), *Lolium perenne* (Lp), *Trifolium pratense* (Tp), *Trifolium repens* (Tr), *Dactylis glomerata* (Dg), *Plantago lanceolata (Pl), Anthoxanthum odoratum* (Ao), *Centaurea jacea* (Cj), *Festuca rubra* (Fr), *Leucanthemum vulgare* (Lv), *Onobrychis viciifolia* (Ov).

[b] Tracers were injected as a cocktail solution containing all the tracers in experiments B, D, E, F and G. In experiment A, two separate solutions were injected containing either Rb and Cs, or Li and Sr. In exp C, single-tracer solutions of Li, Sr, Rb and Cs were injected at 5 cm, 15 cm, 25cm and 60 cm depth, respectively. In exp F, all tracers were injected as single-tracer solutions, in 3 depth x tracer combinations (5-15-35: Li-Sr-Rb, Sr-Rb-Li, Rb-Li-Sr). Tracer cocktails were formulated based on equal molar (exp A, C, D and E) or weight (exp B, F and G) proportions of the tracers, which is reflected in the different application rates.

[c] See Tables 1 and 2: 1. Tracer mobility, 2. Injection density, 3. Incubation time, 4. Application rate, 5. Suitability for use in multiple tracer method.

Table 4. The effect of tracer injection density (number of injections m^{-2}) on mean (SE in parentheses) plant excess tracer concentrations (n = 48) and the coefficient of variation (CV = SD / mean) (n = 6) (Test of assumption 2, see Table 1). The injection density treatments received the same tracer application rate, but both the injection volume and tracer solution concentration were doubled for the 36 injections m^{-2} (see Table 3, experiment C). See Table S2 for species-specific results

Tracer	Mean excess tracer concentration (mg kg^{-1} DM)				CV (%)			
Injection density	36		144		36		144	
Cs, 60 cm[a]	5.8	(0.94)	4.6	(1.07)	86	(17.8)	74	(16.2)
Li, 5 cm	23.0	(1.88)	14.1	(1.27)	30	(5.6)	35	(2.5)
Rb, 25 cm	69.0	(13.72)	61.0	(5.54)	80	(19.6)	61	(7.4)
Sr, 15 cm	18.0	(1.79)	17.5	(2.13)	78	(7.7)	41	(4.0)

[a]Tracer application rate was 4.9, 0.3, 3.1 and 3.2 g m^{-2} for Cs, Li, Rb and Sr, respectively.

Table 5. The effect of incubation time (24 and 48 hours) on mean (SE in parentheses) plant excess tracer concentration (Test of assumption 3, see Table 1). Results presented here are the mean of seven plant species, n = 22 (24 hours) and n = 20 (48 hours), see Table S3 for species-specific results. See Table 3, experiment E for more information

	Plant excess tracer concentration (mg kg^{-1} DM)			
Tracer	24 hours		48 hours	
Li	34.1	(6.46)	49.1	(8.29)
Rb	264.6	(47.32)	379.7	(51.93)
Sr	128.8	(31.53)	216.6	(50.90)

Table 6. Soil and plant background tracer concentrations ($TC_{Background}$, SD in parentheses) and the effect of tracer application rate on plant excess tracer concentration (dTC, SD in parentheses), plant tracer uptake (mg m^{-2}), enrichment (dTC / $TC_{Background}$) and recovery (tracer uptake / application rate) for different sites, treatments and tracers (Test of assumption 4, see Table 1). For species-specific background and excess tracer concentrations see Tables S4 and S5, respectively. For more information see Table 3

	Application rate (g m^{-2})	Soil $TC_{Background}$ (mg kg^{-1} DM)		Plant $TC_{Background}$ (mg kg^{-1} DM)		Plant dTC (mg kg^{-1} DM)		Plant tracer uptake[a] (mg m^{-2})		Enrichment (mg mg^{-1})	Recovery (%)
Exp A, Tänikon[b]											
Cs	3.4	0.2	(0.03)	0.24	(0.33)	58.4	(40.39)	10.6	(7.05)	244.9	0.31
Li	0.2	6.2	(0.88)	0.34	(0.18)	3.6	(3.03)	0.56	(0.51)	10.6	0.28
Rb	2.2	12.3	(1.17)	7.0	(1.91)	359.1	(169.48)	66.2	(32.82)	51.3	3.01
Sr	2.2	25.0	(4.30)	21.8	(8.92)	12.7	(11.67)	2.35	(2.49)	0.6	0.11
n		6		46		59		33			
Exp B, Reckenholz											
Cs	2.6	4.1	(17.68)	0.5	(0.19)	31.3	(13.18)	6.6	(0.8)	62.4	0.25
Li	2.6	8.1	(2.38)	2.0	(2.28)	207.0	(53.10)	47.5	(8.76)	102.9	1.83
Rb	2.6	13.8	(11.84)	10.2	(4.93)	158.5	(81.54)	41.2	(11.47)	15.5	1.58
Sr	2.6	19.0	(16.07)	18.0	(7.69)	42.5	(21.31)	9.0	(1.29)	2.4	0.34
n		4		16		16		4			
Exp B, Reckenholz, low tracer application rate											
Cs	1.3	4.1	(17.68)	0.5	(0.19)	12.9	(11.57)	5.3	(0.93)	25.8	0.40
Li	1.3	8.1	(2.38)	2.0	(2.28)	98.1	(51.00)	38.9	(7.48)	48.7	2.99
Rb	1.3	13.8	(11.84)	10.2	(4.93)	79.2	(42.67)	27.3	(3.83)	7.7	2.10
Sr	1.3	19.0	(16.07)	18.0	(7.69)	13.2	(6.83)	7.9	(1.41)	0.7	0.61
n		4		16		16		4			
Exp D, Waldhof, 5cm injection depth											
Cs	3.4	0.4	(0.23)	0.3	(0.25)	102.5	(49.16)	11.3	(3.82)	401.3	0.33
Li	0.2	4.5	(0.41)	0.3	(0.17)	11.5	(6.81)	1.3	(0.32)	39.8	0.65
Rb	2.2	8.0	(0.58)	15.6	(7.10)	605.8	(186.60)	78.4	(27.12)	38.8	3.56
Sr	2.2	10.5	(3.81)	24.9	(15.46)	59.4	(33.45)	6.3	(1.44)	2.4	0.29
n		3		15		41		9			
Exp D, Waldhof, 20cm injection depth											
Cs	3.4	0.4	(0.23)	0.3	(0.25)	24.6	(21.62)	2.2	(1.14)	96.2	0.06
Li	0.2	4.5	(0.41)	0.3	(0.17)	4.3	(5.25)	0.4	(0.12)	15.0	0.18
Rb	2.2	8.0	(0.58)	15.6	(7.10)	184.1	(113.05)	20.1	(14.31)	11.8	0.91
Sr	2.2	10.5	(3.81)	24.9	(15.46)	25.4	(17.90)	2.2	(0.78)	1.0	0.10
n		3		15		41		9			
Exp E, Pot experiment, 24 hrs											
Li	0.5	N.A.		0.7	(0.27)	34.1	(30.28)	4.3	(3.14)	48.2	0.86
Rb	6.7	32.0			(18.15)	264.6	(221.94)	33.6	(24.57)	8.3	0.50
Sr	6.9	62.0			(35.85)	128.8	(147.89)	17.1	(16.86)	2.1	0.25
n				7		22		22			
Exp E, Pot experiment, 48 hrs											
Li	0.5	N.A.		0.7	(0.27)	49.1	(37.07)	7.3	(4.93)	69.3	1.47
Rb	6.7	32.0			(18.15)	379.7	(232.26)	58.1	(36.04)	11.9	0.87
Sr	6.9	62.0			(35.85)	216.6	(227.63)	35.2	(34.99)	3.5	0.51
n				7		20		20			

[a]Tracer uptake is calculated on field plot level or pot level: i.e. as the species tracer concentration multiplied by the species aboveground dry matter yield, summed over all species in one plot or pot. This is to avoid confounding species tracer uptake with the proportion of species in mixtures with varying abundances (see also Discussion on interpretation of tracer studies).

[b]Exp F is not included in this Table due to the lack of tracer background concentrations.

N.A. = not available

Table 7. Summary of the suitability of different tracer pairs in a multiple tracer method. Summaries are either for individual species under different experimental conditions, or for the comparison of multiple species in monocultures and mixtures (Test of assumption 5, see Table 2). To be suitable for use with individual species, the regression between two tracers should have a high R^2 (>0.75) and there should be no significant effect of the experimental treatment on the tracer regression. If there is a significant absolute (intercept ≠ 0) or relative (slope ≠ 1) bias in the regression, [illegible] (further details for individual species are provided in Table S6). Additionally, in order for the multiple tracer method to be suitable for use in species comparisons, there should be no significant species effect on the regression (i.e. no differences in the slope or intercept of the tracer regression for the different species) (for further details see Table S7)

		Individual species				Species comparison		
				Proportion of species for which there is				
Exp	Tracers	Nr of species	R^2 >0.75	no significant treatment effect[a]	no significant bias	R_2	Treatment effect[b]	Species effect
K-analogues								
Exp A	Cs x Rb	4	3/4	3/4	0/4	0.84		***
Exp B	Cs x Li	4				0.38		ns
	Cs x Rb	4				0.90		*
	Li x Rb	4				0.66		**
Exp D†	Cs x Li	5	4/5	2/5	1/5	0.77		***
	Cs x Rb	5	5/5	3/5	0/5	0.45		**
	Li x Rb	5	3/5	5/5	0/5	0.41		*
Exp F	Li x Rb	5				0.42	ns	*
Subtotal K-analogues			15/19	12/19	1/19			
K-analogues x Sr								
Exp A	Li x Sr	4	0/4	2/4	0/4	0.82		***
Exp B	Cs x Sr	4				0.86		**
	Li x Sr	4				0.81		*
	Rb x Sr	4				0.86		***
Exp D	Cs x Sr	5	3/5	1/5	1/5	0.63		**
	Li x Sr	5	4/5	3/5	1/5	0.72		***
	Rb x Sr	5	1/5	2/5	0/5	0.00		ns
Exp F	Li x Sr	5				0.13	ns	ns
	Rb x Sr	5				0.00	ns	ns
Subtotal K-analogues x Sr			8/19	8/19	2/19			
Total			23/38	20/38	3/38			

[a]Treatment is drought in exp A and injection depth in exp D and F.

[b]We did not include the results of *Plantago lanceolata* from exp D, because of the large number of missing values

Figure captions

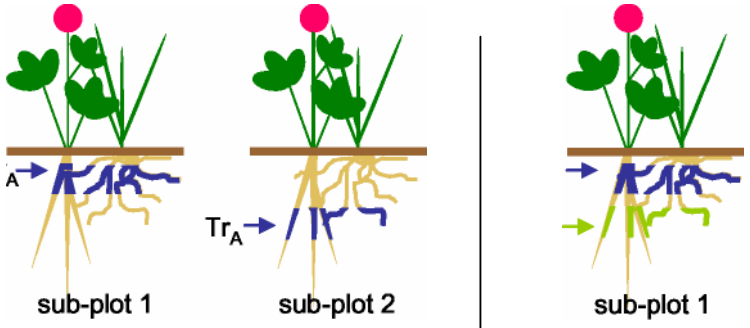
Fig. 1 Overview of the single and the multiple tracer methods

Fig. 2 Tracer injection using a multipipette with a four side-port steel needle attached via a silicone tube.

Fig. 3 Excess soil tracer concentration (mg kg^{-1} dry soil) of Cs, Li, Rb and Sr throughout the soil profile, for: a) Experiment A: four weeks after injection at 5 cm depth, *n* = 6; b) Experiment B: two weeks after injection at 5 cm depth, *n* = 4 and c) Experiment G: two weeks after injection at 5, 20 and 35 cm in three separate sub-plots, *n* = 6 for 5 and 35 cm, *n* = 3 for 20 cm (Test of assumption 1, see Introduction). See Table 3 for more information. Injection depth: = 5 cm, = 20 cm and = 35 cm. Error bars are SE. *, ** and *** indicate that the excess tracer concentration is significantly different from 0 at P = 0.05, 0.01 and 0.00 1, respectively

Fig. 4 Examples of the regression between plant tracer excess concentrations (dTC) from different tracers, and how this affects the suitability of tracer pairs for use in the multiple tracer method. For details, see Tables S6 and S7.

a) Experiment D, Tp, Rb × Li: High R2 (0.88), no significant bias and no significant treatment effect (depth of injection). b) Experiment D, Ci, Rb × Li: no significant regression - tracer pair is unsuitable for use in the multiple tracer method in this case. c) Experiment A, Ci, Rb × Cs: High R2 (0.92), significant relative bias (slope = 0.14) - correction factor required for quantitative comparison of plant tracer concentrations. d) Experiment A, Tp, Rb × Cs: High R2 (0.89), significant bias and significant effect of treatment . e) Experiment A, Ci and Lp, Rb × Cs: Significant effect of species on the regression means that the tracer pair is unsuitable for use in a multiple tracer method comparing multiple species

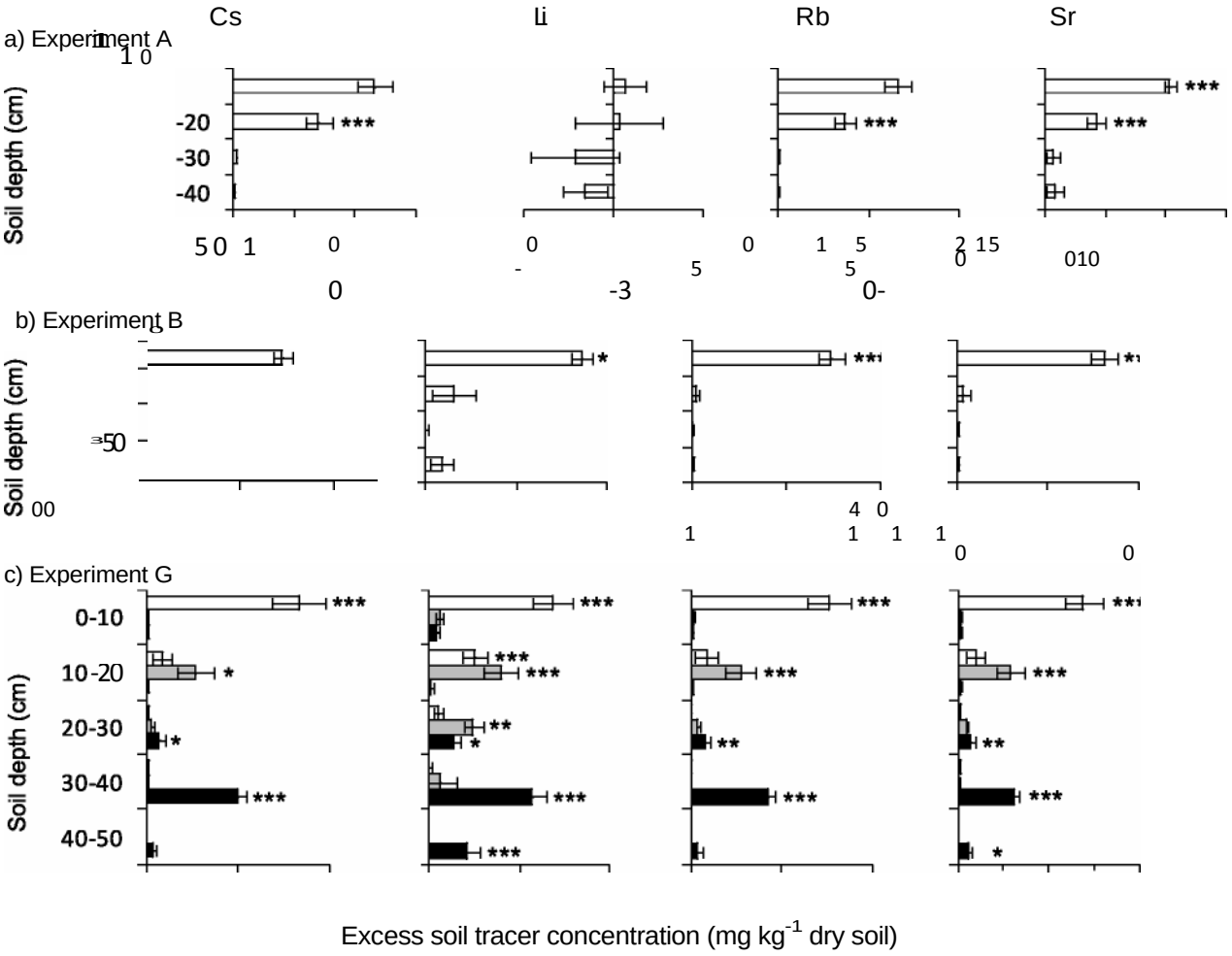


Single tracer method
- Multiple sub-plots (sub-plot 1and 2) per experimental plot
- One soil depth per sub-plot
- One tracer (Tr_A) for all depths

Multiple tracer method
- One sub-plot (sub-plot 1) per experimental plot
- Multiple soil depths per sub-plot
- Different tracers (Tr_A, Tr_B) for each depth

Fig. 1

Fig. 2



Excess soil tracer concentration (mg kg^{-1} dry soil)

Fig. 3

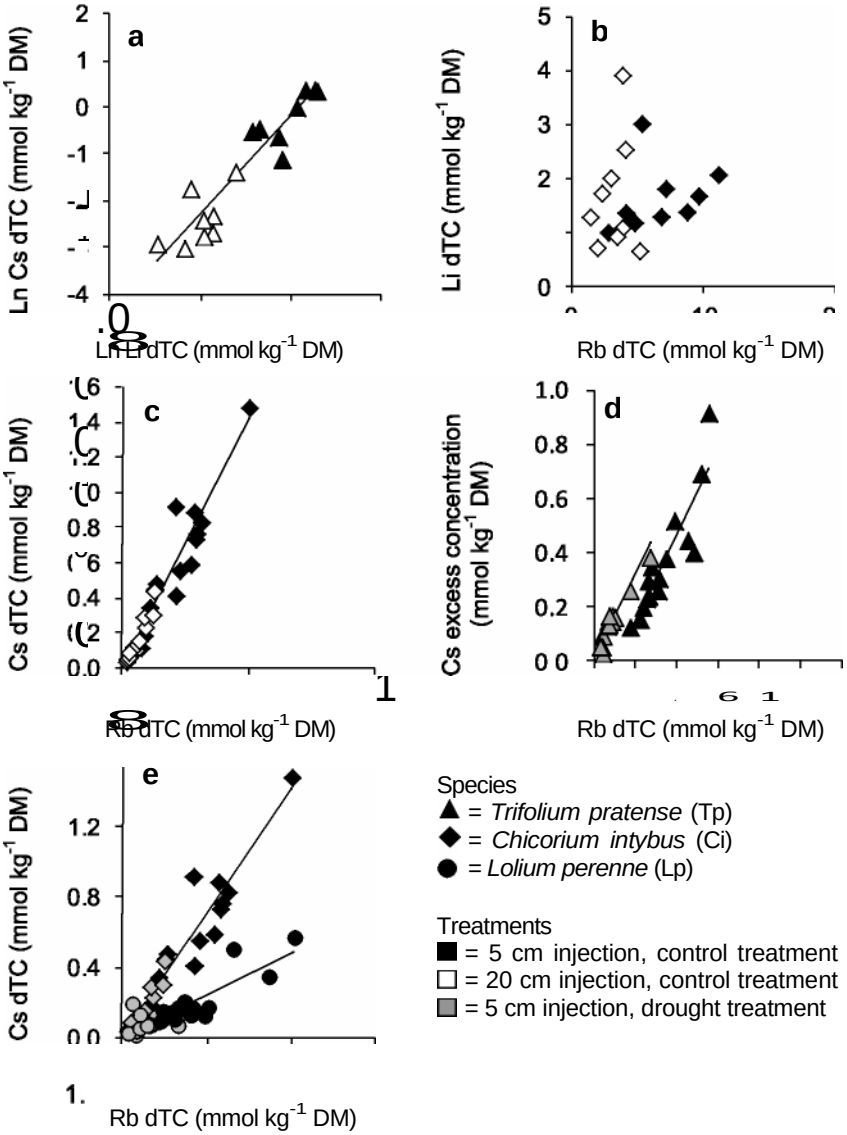

a
b
c
d
e
Ln Cs dTC (mmol kg-1 DM)
Li dTC (mmol kg-1 DM)
Cs dTC (mmol kg-1 DM)
Cs excess concentration (mmol kg-1 DM)
Rb dTC (mmol kg-1 DM)
Species
= Trifolium pratense (Tp)
= Chicorium intybus (Ci)
= Lolium perenne (Lp)
Treatments
= 5 cm injection, control treatment
= 20 cm injection, control treatment
= 5 cm injection, drought treatment

Fig. 4

Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1) Excess concentration (mean ±SE, mg kg DM^{-1}) of soil tracer at 0-10 cm soil depth measured at 0 cm and 3 cm horizontal distance from the injection site, after tracer injection at 5 cm depth (*n*= 6 for Cs and Rb, *n* = 5 for Sr). See Table 3, experiment A for more details

Table S2 The effect of tracer injection density (ID, number of injections m^{-2}) on mean plant tracer concentrations (mg kg^{-1} DM) and the coefficient of variation (CV = SD / mean, %) (±SE) of individual plant species (Test of assumption 2, see Table 1). The injection density treatments received the same tracer application rate, but both the injection volume and tracer solution concentration were doubled for the 36 injections m^{-2} (see Table 3, exp C). Mean and CV of individual species are based on the different replicates and plant communities (*n* = 10 for Dg and Lp, *n* = 9 for Ci and Tp, *n* = 7 for Pl and *n* = 3 for Tr). The mean results across species are reported in Table 4

Table S3 The effect of tracer incubation time (24 and 48 hours) on mean (±SE) plant excess tracer concentration (mg kg^{-1} DM) for individual species (*n* = 3) (Test of assumption 3, see Table 1). The mean results across species are reported in Table 5; for more information see Table 3, experiment E

Table S4 Plant background tracer concentrations (±SD) for individual species at the different experimental sites (test of assumption 4, see Table 1). For more details see Table 3. Mean values across species are presented in Table 6

Table S5 Mean plant excess tracer concentrations (mg g^{-1} DM, SD in parentheses) for individual species at the different experimental sites as affected by treatment and tracer application rate (test of assumption 4, see Table 1). For more details see Table 3. Mean values across species are presented in Table 6

Table S6 Assessment of the suitability of tracer pairs for use in a multiple tracer method for individual species (test of assumption 5, see Table 2). The R^2 value of the regression between the plant tracer concentrations of two tracers should be high; if there is a significant absolute (intercept $\neq 0$) or relative (slope $\neq 1$) bias in the regression, a correction factor is needed for tracer comparison (values in bold indicate no significant bias). There should be no significant effect of the experimental treatment (exp A: drought, exp D: injection depth) on the tracer regression. Tracers were injected in a cocktail containing equal molar quantities of each tracer and tracer concentrations are expressed in mmol kg^{-1} DM. Analysis for individual species were only performed for n>3, i.e. exp A and D. For exp D, all

regression models with Cs were based on log-transformed tracer concentrations to achieve normality. These data are summarised in Table 7, see Table 3 for more information

Table S7 Assessment of the suitability of different tracer pairs for use in a multiple tracer method comparing different species (test of assumption 6, see Table 2). Tracer pairs are only suitable when 1) there is a strong regression (R^2) of the plant excess tracer concentration on the different tracers (R^2 value of Model I should be high), 2) the intercept and/or the slope of the regression are not different for individual species (Model II and Model III should not be significantly better than model I) and 3) there is no treatment effect on the regression (Model IV should not be significantly better than the previous models). Tracers were injected in a cocktail containing equal molar or mass quantities of each tracer for exp A, B and D. For exp F, single tracers Li, Rb and Sr were injected at 3 separate and alternating depths in each pot. For exp D, all regression models with Cs were based on log-transformed tracer concentrations to achieve normality. See Table 3 for more information. These data are summarised in Table 7

Fig. S1 Different scenarios for the regression between plant excess tracer concentrations (dTC) from different tracers (Tracer 1 and Tracer 2). These illustrate the relative suitability of tracer pairs for use in a multiple tracer method assessing different treatments for single (a-d) or multiple (e) species (Table 2). a) A good regression between tracers, this illustrates the ideal scenario for the use of different tracers in a multiple tracer method, b) no good regression between tracers, unsuitable for use in multiple tracer method, c) bias in regression between tracers, correction factor required for use in multiple tracer method, d) treatment effect on regression, unsuitable for use in multiple tracer method, e) regression between tracers is species dependent, unsuitable for use in multiple tracer method comparing different species

Table S1 Excess concentration (mean ±SE, mg kg DM^{-1}) of soil tracer at 0-10 cm soil depth measured at 0 cm and 3 cm horizontal distance from the injection site, after tracer injection at 5 cm depth (*n*= 6 for Cs and Rb, *n* = 5 for Sr). See Table 3, experiment A for more details

	Distance from injection hole			
Tracer	0 cm		3 cm	
Cs	116.0	(14.78)	1.0	(0.60)
Rb	66.1	(7.30)	0.3	(0.92)
Sr	105.8	(4.71)	0.3	(2.44)

Table S2 The effect of tracer injection density (ID, number of injections m^{-2}) on mean plant tracer concentrations (mg kg^{-1} DM) and the coefficient of variation (CV = SD / mean, %) (±SE) of individual plant species (test of assumption 2, see Table 1). The injection density treatments received the same tracer application rate, but both the injection volume and tracer solution concentration were doubled for the 36 injections m^{-2} (see Table 3, exp C). Mean and CV of individual species are based on the different replicates and plant communities (*n* = 10 for Dg and Lp, *n* = 9 for Ci and Tp, *n* = 7 for Pl and *n* = 3 for Tr). The mean results across species are reported in Table 4

	ID	Cs, 60 cm				Li, 5 cm[a]				Rb, 25 cm				Sr, 15cm			
Species[b]		Mean (mg kg^{-1} DM)		CV (%)		Mean (mg kg^{-1} DM)		CV (%)		Mean (mg kg^{-1} DM)		CV (%)		Mean (mg kg^{-1} DM)		CV (%)	
Ci	36	9.2	(2.06)	67%		17.3	(2.70)	47%		20.0	(5.37)	80%		167.0	(60.61)	109%	
	144	11.6	(5.12)	133%		10.3	(1.08)	31%		10.7	(2.48)	70%		106.0	(18.31)	52%	
Dg	36	5.9	(1.94)	104%		27.9	(2.85)	32%		17.6	(2.60)	47%		62.1	(12.41)	63%	
	144	2.7	(0.37)	43%		17.7	(2.01)	36%		14.2	(1.62)	36%		53.0	(6.67)	40%	
Lp	36	5.1	(1.24)	77%		31.2	(3.94)	40%		18.7	(2.50)	42%		70.7	(14.01)	63%	
	144	3.6	(0.39)	34%		15.1	(1.30)	27%		15.8	(2.21)	44%		66.4	(7.30)	35%	
Pl	36	7.9	(4.62)	155%		37.0	(1.66)	12%		26.0	(3.25)	33%		24.2	(6.61)	72%	
	144	4.9	(1.33)	72%		27.1	(3.16)	31%		34.1	(10.54)	82%		23.4	(4.25)	48%	
Tp	36	2.3	(0.68)	89%		7.4	(0.82)	33%		11.8	(5.37)	137%		28.2	(6.27)	67%	
	144	1.4	(0.51)	111%		3.1	(0.47)	44%		19.1	(4.86)	76%		45.8	(7.24)	47%	
Tr	36	3.0	(0.40)	23%		11.2	(1.02)	16%		10.0	(8.13)	140%		19.1	(10.23)	93%	
	144	2.3	(0.69)	52%		12.9	(2.80)	38%		10.7	(3.48)	56%		67.2	(9.90)	25%	
Mean	36	5.8	(0.94)	86%	(17.8%)	23.0	(1.88)	30%	(5.6%)	69.0	(13.72)	80%	(7.7%)	18.0	(1.79)	78%	(19.6%)
	144	4.6	(1.07)	74%	(16.2%)	14.1	(1.27)	35%	(2.5%)	61.0	(5.54)	61%	(4.0%)	17.5	(2.13)	41%	(7.4%)

[a]Tracer application rate was 4.9, 0.3, 3.1 and 3.2 g m-2 for Cs, Li, Rb and Sr, respectively.

[b]Species abbreviations: *Chichorium intybus* (Ci)*, Dactylis glomerata* (Dg)*, Lolium perenne* (Lp)*, Plantago lanceolata* (Pl)*, Trifolium pratense* (Tp)*, Trifolium repens* (Tr).

Table S3 The effect of tracer incubation time (24 and 48 hours) on mean (±SE) plant excess tracer concentration (mg kg^{-1} DM) for individual species (n = 3) (test of assumption 3, see Table 1). The mean results across species are reported in Table 5; for more information see Table 3, experiment E

Incubation time	Species[a]	Li		Rb		Sr	
24h	Ao	55.6	(23.5)	460.0	(135.2)	170.9	(123.9)
	Cj	51.4	(17.6)	398.5	(143.7)	174.2	(82.9)
	Fr	16.7	(9.3)	126.5	(61.8)	5.9	(46.7)
	Lv	57.6	(17.4)	425.6	(133.1)	235.1	(106.5)
	Ov	13.1	(4.6)	113.0	(29.0)	49.4	(15.6)
	Pl	22.9	(4.8)	186.0	(59.7)	192.0	(44.8)
	Tp	14.3	(0.0)	77.2	(13.3)	60.0	(16.7)
48h	Ao	83.3	(51.7)	533.7	(232.3)	236.6	(190.1)
	Cj	45.0	(3.1)	356.7	(39.1)	110.0	(30.9)
	Fr	17.0	(4.2)	200.0	(15.2)	-11.9	(18.4)
	Lv	91.0	(30.5)	666.4	(243.4)	539.6	(215.6)
	Ov	24.9	(9.4)	317.6	(92.4)	180.3	(87.4)
	Pl	40.3	(13.1)	276.0	(76.5)	187.4	(24.9)
	Tp	53.7	(10.4)	358.7	(67.0)	280.9	(93.5)

[a]Species abbreviations: *Anthoxanthum odoratum* (Ao), *Centaurea jacea* (Cj), *Festuca rubra* (Fr), *Leucanthemum vulgare* (Lv), *Onobrychis viciifolia* (Ov), *Plantago lanceolata* (Pl), *Trifolium pratense* (Tp)

Table S4 Plant background tracer concentrations (±SD) for individual species at the different experimental sites (test of assumption 4, see Table 1). For more details see Table 3. Mean values across species are presented in Table 6

	Species[a]											
	Ci		Dg		Lp		Pl	Tp		Tr		
Experiment A, Tänikon												
Cs	0.2	(0.17)			0.2	(0.30)		0.3	(0.50)	0.3	(0.23)	
Li	0.5	(0.20)			0.3	(0.07)		0.2	(0.08)	0.4	(0.10)	
Rb	8.0	(1.29)			6.1	(1.96)		8.0	(1.77)	5.9	(1.60)	
Sr	30.7	(5.95)			10.1	(1.50)		26.0	(4.81)	22.8	(4.17)	
n	10				13			13		10		
Experiment B, Reckenholz												
Cs	0.5	(0.09)			0.7	(0.23)		0.3	(0.06)	0.5	(0.14)	
Li	0.8	(0.25)			5.1	(3.01)		1.3	(0.35)	0.8	(0.19)	
Rb	13.8	(1.65)			15.0	(4.90)		5.8	(0.77)	6.3	(0.24)	
Sr	16.1	(1.23)			8.1	(0.29)		26.4	(6.08)	21.4	(3.58)	
n	4				4			4		4		
Experiment D, Waldhof												
Cs	0.3	(0.27)	0.1	(0.05)	0.3	(0.35)	0.3	0.4	(0.39)	0.1	(0.06)	
Li	0.4	(0.08)	0.1	(0.14)	0.3	(0.07)	0.2	0.2	(0.15)	0.4	(0.35)	
Rb	19.9	(10.29)	10.7	(2.80)	15.8	(10.00)	16.4	16.6	(8.47)	14.6	(1.21)	
Sr	30.5	(11.08)	6.7	(0.41)	10.6	(3.28)	45.3	39.3	(8.63)	33.2	(6.88)	
n	3		3		3		1	3		2		

	Ao	Cj	Fr	Pl	Tp	Lv	Ov
Experiment E, Pot experiment							
Li	0.86	0.84	0.62	0.17	0.73	0.74	1.01
Rb	25.7	19.9	45.5	19.7	17.6	67.1	28.2
Sr	25.5	80.3	29.2	119.5	90.2	33.1	56.3
n	1	1	1	1	1	1	1

[a]Species abbreviations: *Chichorium intybus* (Ci)*, Dactylis glomerata* (Dg)*, Lolium perenne* (Lp)*, Plantago lanceolata (Pl), Trifolium repens* (Tr)*, Trifolium pratense* (Tp), *Anthoxanthum odoratum* (Ao), *Centaurea jacea* (Cj), *Festuca rubra* (Fr), *Leucanthemum vulgare* (Lv), *Onobrychis viciifolia* (Ov).

Table S5 Mean plant excess tracer concentrations (mg g^{-1} DM, SD in parentheses) for individual species at the different experimental sites as affected by treatment and tracer application rate (test of assumption 4, see Table 1). For more details see Table 3. Mean values across species are presented in Table 6

	Appl. rate ($g\ m^{-2}$)			Species							
Cj				Dg		Lp		Pl		Tp		Tr	
Exp A, Tänikon†													
Cs	3.4	83.3	(46.8)			27.9	(19.5)			48.5	(28.2)	75.4	(39.0)
Li	0.2	5.8	(3.2)			4.4	(2.5)			0.8	(0.4)	3.4	(2.8)
Rb	2.2	380.9	(197.7)			382.6	(198.4)			286.6	(100.0)	388.1	(160.8)
Sr	2.2	13.2	(11.2)			15.1	(11.0)			8.0	(7.3)	14.6	(15.7)
n		14				15				15		15	
Exp B, Reckenholz													
Cs	2.6	31.4	(22.1)			21.2	(3.9)			35.0	(10.2)	37.5	(7.5)
Li	2.6	161.6	(59.5)			188.4	(23.9)			254.2	(46.5)	224.0	(35.4)
Rb	2.6	74.9	(54.0)			261.9	(59.2)			124.9	(26.4)	172.3	(28.2)
Sr	2.6	43.2	(21.3)			22.7	(4.7)			67.1	(14.5)	36.9	(14.6)
n		4				4				4		4	
Exp B, Reckenholz, low tracer application rate													
Cs	1.3	23.1	(19.9)			7.5	(2.5)			7.9	(2.9)	13.3	(7.0)
Li	1.3	128.4	(98.2)			107.8	(7.3)			64.3	(7.9)	91.8	(18.1)
Rb	1.3	51.9	(38.7)			129.5	(41.1)			49.4	(11.1)	86.0	(13.2)
Sr	1.3	17.7	(11.5)			12.7	(2.7)			10.0	(6.9)	12.3	(2.0)
n		4				4				4		4	
ExpD, Waldhof, 5cm injection depth													
Cs	3.4	132.3	(63.0)	77.9	(11.2)	68.4	(10.1)	81.9	(13.8)	119.3	(59.7)	105.6	(49.6)
Li	0.2	11.5	(4.3)	12.2	(3.1)	10.3	(4.4)	26.8	(14.7)	6.9	(3.6)	11.0	(4.7)
Rb	2.2	567.9	(235.8)	663	(121.7)	689.3	(246.2)	497	(102.7)	581.1	(157.1)	547.8	(132.2)
Sr	2.2	42.2	(33.7)	38.3	(11.1)	54.7	(21.1)	103	(63.8)	78.1	(26.9)	74.8	(23.0)
n		9		8		8		3		9		4	
Exp D, Waldhof, 20cm injection depth													
Cs	3.4	51.3	(27.9)	11.2	(4.9)	13.4	(5.6)	25.1		14.1	(9.5)	16.9	(12.4)
Li	0.2	11.5	(7.3)	2.43	(0.7)	2.1	(0.6)	8.81		1.0	(0.5)	3.0	(1.2)
Rb	2.2	267.8	(101.5)	111	(49.8)	209.0	(132.9)	258		156.5	(109.5)	152.8	(111.4)
Sr	2.2	41.2	(23.2)	11.3	(3.6)	21.0	(8.2)	61.3		23.1	(11.6)	23.5	(16.6)
n		9		8		8		1		8		7	

Fr				Ao		Cj		Pl		Tp		Ov		Lv	
Exp E, Pot experiment, 24 hrs															
Li	0.5	55.6	(46.9)	51.4	(30.4)	16.7	(16.0)	22.9	(8.3)	14.3	(0.1)	13.1	(8)	57.6	(30.2)
Rb	6.7	460.0	(270.4)	398.5	(248.8)	126.5	(107.1)	186.0	(103.4)	77.2	(23.0)	113.0	(50.2)	425.6	(230.5)
Sr	6.9	170.9	(247.7)	174.2	(143.5)	5.9	(80.9)	192.0	(77.6)	60.0	(29.0)	49.4	(27)	235.1	(184.5)
n		4		3		3		3		3		3		3	
Exp E, Pot experiment, 48 hrs															
Li	0.5	83.3	(73.2)	45.0	(5.3)	17.0	(7.3)	40.3	(22.6)	53.7	(18.0)	24.9	(16.4)	91.0	(52.9)
Rb	6.7	533.7	(328.5)	356.7	(67.7)	200.0	(26.2)	276.0	(132.5)	358.7	(116.0)	317.6	(160.1)	666.4	(421.6)
Sr	6.9	236.6	(268.9)	110.0	(53.5)	-11.9	(31.8)	187.4	(43.1)	280.9	(162.0)	180.3	(151.4)	539.6	(373.5)
n		2		3		3		3		3		3		3	

Table S6 Assessment of the suitability of tracer pairs for use in a multiple tracer method for individual species (test of assumption 5, see Table 2). The R^2 value of the regression between the plant tracer concentrations of two tracers should be high, if there is a significant absolute (intercept ≠ 0) or relative (slope ≠ 1) bias in the regression, a correction factor is needed for tracer comparison (values in bold indicate no significant bias). There should be no significant effect of the experimental treatment (exp A: drought, exp D: injection depth) on the tracer regression. Tracers were injected in a cocktail containing equal molar quantities of each tracer and tracer concentrations are expressed in mmol kg^{-1} DM. Analysis for individual species were only performed for n>3, i.e. exp A and D. For exp D, all regression models with Cs were based on log-transformed tracer concentrations to achieve normality. These data are summarised in Table 7, see Table 3 for more information

					Parameter estimates								
					Intercept			Slope			Treatment		
Exp	Species[a]	R^2	P	df	Estimate (SE)		P	Estimate (SE)		P	Estimate (SE)		P
K-analogues													
A	Cs x Rb (mmol kg^{-1} DM)												
	Ci	0.92	<0.001	25	**0.00**	(0.030)	ns	0.14	(0.008)	<0.001	-	-	ns
	Lp	0.73	<0.001	28	**0.00**	(0.020)	ns	0.05	(0.005)	<0.001	-	-	ns
	Tp	0.89	<0.001	26	-0.16	(0.049)	<0.01	0.16	(0.014)	<0.001	0.18	(0.043)	<0.001
	Tr	0.82	<0.001	28	**0.04**	(0.037)	ns	0.12	(0.011)	<0.001	-	-	ns
D	lnCs x lnLi (mmol kg^{-1} DM)												
	Ci	0.67	<0.001	15	-0.44	(0.177)	<0.05	**0.68**	(0.226)	<0.01	-0.86	(0.206)	<0.001
	Dg	0.95	<0.001	13	-0.83	(0.147)	<0.001	0.54	(0.218)	<0.05	-1.16	(0.377)	<0.01
	Lp	0.92	<0.001	13	-0.80	(0.123)	<0.001	0.37	(0.223)	ns	-1.11	(0.381)	<0.05
	Tp	0.88	<0.001	15	**-0.17**	(0.149)	ns	**1.06**	(0.100)	<0.001	-	-	ns
	Tr	0.79	<0.001	9	-0.99	(0.200)	<0.001	**1.22**	(0.212)	<0.001	-	-	ns
	lnCs x lnRb (mmol kg^{-1} DM)												
	Ci	0.80	<0.001	16	-2.17	(0.213)	<0.001	**1.10**	(0.138)	<0.001	-	-	ns
	Dg	0.98	<0.001	14	-2.74	(0.067)	<0.001	**1.07**	(0.046)	<0.001	-	-	ns
	Lp	0.98	<0.001	13	-1.81	(0.167)	<0.001	0.56	(0.078)	<0.001	-0.97	(0.124)	<0.001
	Tp	0.88	<0.001	14	-1.70	(0.553)	<0.01	**0.78**	(0.282)	<0.05	-1.06	(0.464)	<0.05
	Tr	0.91	<0.001	9	-2.64	(0.159)	<0.001	**1.20**	(0.125)	<0.001	-	-	ns
	Li x Rb (mmol kg^{-1} DM)												
	Ci	0.02	ns	16	-	-	-	-	-	-	-	-	-
	Dg	0.90	<0.001	14	**0.08**	(0.110)	ns	0.21	(0.020)	<0.001	-	-	ns
	Lp	0.81	<0.001	14	**-0.08**	(0.151)	ns	0.18	(0.024)	<0.001	-	-	ns
	Tp	0.51	<0.01	15	**-0.01**	(0.183)	ns	0.14	(0.034)	<0.01	-	-	ns
	Tr	0.80	<0.001	9	**0.03**	(0.167)	ns	0.24	(0.039)	<0.001	-	-	ns
K-analogues x Ca-analogue													
A	Li x Sr (mmol kg^{-1} DM)												
	Ci	0.68	<0.001	21	0.45	(0.095)	<0.001	2.63	(0.451)	<0.001	-0.37	(0.105)	<0.01
	Lp	0.63	<0.001	28	0.17	(0.074)	<0.05	2.64	(0.386)	<0.001	-	-	ns
	Tp	0.61	<0.001	26	0.13	(0.045)	<0.01	0.38	(0.100)	<0.001	-0.10	(0.046)	<0.05
	Tr	0.72	<0.001	27	0.16	(0.044)	<0.01	1.91	(0.229)	<0.001	-	-	ns
D	lnCs x lnSr (mmol kg^{-1} DM)												
	Ci	0.73	<0.001	15	**0.37**	(0.186)	0.06	0.50	(0.131)	<0.01	-0.98	(0.185)	<0.001
	Dg	0.94	<0.001	13	**-0.31**	(0.205)	ns	0.27	(0.200)	ns	-1.70	(0.283)	<0.001
	Lp	0.92	<0.001	13	-0.52	(0.152)	<0.01	0.28	(0.212)	ns	-1.43	(0.250)	<0.001
	Tp	0.85	<0.001	14	**-0.16**	(0.179)	ns	0.41	(0.230)	0.09	-1.62	(0.403)	<0.01
	Tr	0.66	<0.01	8	**-0.31**	(0.350)	ns	**1.32**	(0.334)	<0.01	-	-	ns
	Li x Sr (mmol kg^{-1} DM)												
	Ci	0.59	<0.001	16	0.70	(0.236)	<0.01	2.01	(0.416)	<0.001	-	-	ns
	Dg	0.90	<0.001	13	0.83	(0.345)	<0.05	**2.12**	(0.761)	<0.05	-0.75	(0.269)	<0.05
	Lp	0.96	<0.001	12	**-0.05**	(0.180)	ns	2.45	(0.271)	<0.001	0.23	(0.254)	ns[b]
	Tp	0.82	<0.001	15	**-0.18**	(0.110)	ns	**1.30**	(0.155)	<0.001	-	-	ns
	Tr	0.79	<0.001	9	**0.02**	(0.178)	ns	1.73	(0.300)	<0.001	-	-	ns

Table S6 Continued

				Parameter estimates					
				Intercept		Slope		Treatment	
Exp Species[a]	R^2	P	df	Estimate (SE)	P	Estimate (SE)	P	Estimate (SE)	P
D Rb x Sr (mmol kg^{-1} DM)									
Ci	0.45	<0.01	15	6.09 (1.064)	<0.001	1.16 (1.634)	ns	-3.50 (1.017)	<0.01
Dg	0.94	<0.001	13	4.72 (1.172)	<0.01	6.94 (2.585)	<0.05	-4.32 (0.915)	<0.001
Lp	0.73	<0.001	14	**0.18** (0.965)	ns	11.76 (1.919)	<0.001	- -	ns
Tp	0.73	<0.001	14	6.40 (1.666)	<0.01	**0.45** (1.764)	ns	-4.69 (1.370)	<0.01
Tr	0.73	<0.001	9	**0.41** (0.758)	ns	6.36 (1.282)	<0.001	- -	ns

[a]Species abbreviations: *Chichorium intybus* (Ci)*, Dactylis glomerata* (Dg), *Lolium perenne* (Lp)*, Trifolium pratense* (Tp), *Trifolium repens* (Tr). We did not include the results of *Plantago lanceolata* from exp D, because of the large number of missing values.
[b]There was a significant (P<0.05) dTC by Treatment interaction

Table S7 Assessment of the suitability of different tracer pairs for use in a multiple tracer method comparing different species (test of assumption 6, see Table 2). Tracer pairs are only suitable when 1) there is a strong regression (R^2) of the plant excess tracer concentration of the different tracers (R^2 value of Model I[a] should be high), 2) the intercept and/or the slope of the regression are not different for individual species (Model II and Model III should not be significantly better[b] than model I) and 3) there is no treatment effect on the regression (Model IV should not be significantly better than the previous models). Tracers were injected in a cocktail containing equal molar or mass quantities of each tracer for exp A, B and D. For exp F, single tracers Li, Rb and Sr were injected at 3 separate and alternating depths in each pot. For exp D, all regression models with Cs were based on log-transformed tracer concentrations to achieve normality. See Table 3 for more information. These data are summarised in Table 7

		K-analogues						K-analogues x Ca-analogue					
Tracer pair		Cs x Li		Cs x Rb		Li x Rb		Cs x Sr		Li x Sr		Rb x Sr	
Exp.	Model[a]	R^2	P**t**	R^2	P	R^2	P	R^2	P	R^2	P	R^2	P
A	I			0.64						0.55			
	II			0.79	<0.001					0.70	<0.001		
	III			**0.88**	**<0.001**					0.74	<0.001		
	IV			0.88	ns					**0.76**	**<0.05**		
B	I	**0.38**		0.00		0.06		0.55		0.48		0.07	
	II	0.60	ns	0.64	<0.01	**0.66**	**<0.01**	**0.86**	**<0.01**	**0.81**	**<0.05**	**0.86**	**<0.001**
	III	0.60	ns	**0.90**	**<0.05**	0.76	ns	0.91	ns	0.82	ns	0.90	ns
D	I	0.70		0.82		0.40		0.52		0.41		0.33	
	II	0.82	<0.001	0.89	<0.001	0.59	<0.001	0.64	<0.001	0.75	<0.001	0.41	0.06
	III	0.83	ns	0.90	ns	**0.65**	**<0.05**	0.69	<0.05	**0.82**	**<0.001**	0.60	<0.001
	IV	**0.89**	**<0.001**	**0.91**	**<0.001**	0.66	ns	**0.86**	**<0.001**	0.82	ns	**0.76**	**<0.001**
F	I					0.10				0.13		0.00	
	II					**0.42**	**<0.05**			0.37	ns	0.24	ns
	III					0.53	ns			0.44	ns	0.27	ns
	IV					0.43	ns			0.44	ns	0.39	ns

[a]Model I: $Tr1 = a + b \cdot Tr2$. Model II: $Tr1 = a_{i\,04\,40Ej} + b \cdot Tr2$, intercept of regression between tracer 1 and tracer 2 different for species i-j. Model III: $Tr1 = a_{i04\,40Ej} + b_{iERII404\,404bi} \cdot Tr2$, intercept and slope of regression between tracer 1 and tracer 2 different for species i-j. Model IV: Best of Model I, II and III + $a_{Inj\ Depth}$ or + $a_{Drought}$: the intercept of the regression between tracer 1 and tracer 2 is affected by injection depth or drought.

[b]P value of F-test for comparison of the current model with the previous model, the best model is indicated in bold. ns not significant

Supplementary Figures

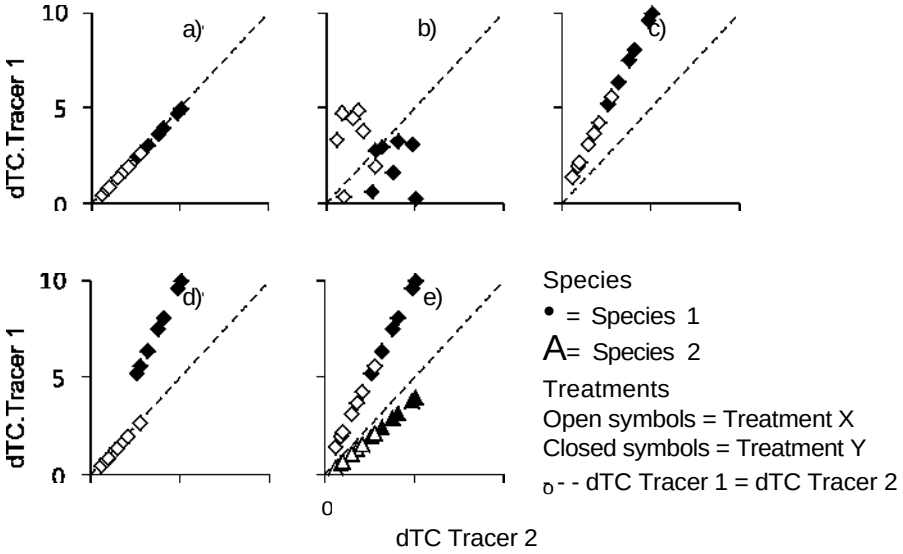


Fig. S1 Different scenarios for the regression between plant excess tracer concentrations (dTC) from different tracers (Tracer 1 and Tracer 2). These illustrate the relative suitability of tracer pairs for use in a multiple tracer method assessing different treatments for single (a-d) or multiple (e) species (Table 2).

a) A good regression between tracers, this illustrates the ideal scenario for the use of different tracers in a multiple tracer method, b) no good regression between tracers, unsuitable for use in multiple tracer method, c) bias in regression between tracers, correction factor required for use in multiple tracer method, d) treatment effect on regression, unsuitable for use in multiple tracer method, e) regression between tracers is species dependent, unsuitable for use in multiple tracer method comparing different species