Title: Xylem sap phosphorus sampling using microdialysis – a non-destructive high sampling frequency method tested under laboratory and field conditions

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Abstract

For a better understanding of plant nutrition processes, it is important to study the flux of nutrients within plants. However, existing xylem sap sampling methods are typically destructive and do not allow for repeated, highly frequent measurements of nutrient concentration. In this paper, we present a novel use of microdialysis (MD) for characterizing xylem sap phosphate (PO_4^{3-}) concentration as a possible alternative to destructive sampling. First, MD probes were tested under laboratory conditions in vitro, in a stirred solution test, and in vivo, using beech tree stem segments. Exponential decline in the relative recovery (RR) with an increasing MD pumping rate allows for determining an optimal sampling interval (i.e., the maximum amount of sample volume with the minimum required concentration). The RR changed only minimally, with a change in the simulated sap flow velocity during the in vivo stem segment test. This suggests that MD can be applied over a range of naturally occurring sap flow velocities. Differences in the ionic strength between the xylem sap and the perfusate pumped through the MD did not influence the RR. Then, MD was successfully applied in a 24h field campaign in two beech trees of different ages and allowed for in situ assessments of the diurnal variation of PO_4^{3-} concentration and (together with xylem flow measurements) flux variability in living trees. Both beech trees exhibited the same diurnal pattern in PO_4^{3-} concentrations with higher concentrations in the younger tree. The xylem PO_4^{3-} concentration measured with MD was in the same order of magnitude as that received through destructive sampling in the younger tree. The MD probes did not show a decline in RR after the field application. We showed that MD can be applied to capture the PO_4^{3-} concentration dynamics in the xylem sap with bihourly resolution under field conditions.

1. Introduction

Xylem sap sampling for chemical analysis is a problematic task due to large tension in the sap wood as reviewed e.g., by Schurr (1998). The sampling is usually done with destructive methods, such as the pressure chamber method (Netzer et al. 2017), the centrifugation method (Gourieroux et al.

2016), vacuum pump extraction (Regulski et al. 1982) or gravitational displacement (Glavac et al. 1990). Beis et al. (2009) sampled sap from leaves with a leaf pressurization technique with several pressure steps applied. They found that the pH of the sap differs for the lowest pressure applied, and hence the chemical composition of the sample changed due to the sampling procedure. According to Schurr (1998), a common problem of destructive as well as non-destructive methods is that they cannot prevent mixing of sampled xylem sap with other compartments of the plant tissue even though techniques (e.g. ATP content measurements) are available to determine the cytoplasmic contamination of xylem sap (c.f. Schneider et al. 1996).

Microdialysis (MD) is a technique well established in neuroscience and pharmacokinetics in order to sample compounds from the intercellular space with minimal tissue damage and perturbation (Davies, 1999). MD uses a small probe (4 cm in length and < 1 mm in diameter in our case) with a membrane that allows for a diffusive exchange between the substances that surround the membrane (here, the xylem) and the perfusate, a liquid that is pumped through the MD probe. MD is therefore a non-destructive (or a minimal tissue-damage causing) but invasive method (for more details on MD see section 2).

Recently, MD has been used in environment-related research (Miró et al. 2005), for instance in soil sciences to estimate plant-available N (Inselsbacher et al. 2011), P root uptake (Demand et al. 2017), or to study amino acid dynamics with high temporal resolution (Warren 2018) and in plant sciences to measure metal ions in tomatoes (Torto et al. 2002), pesticide residues in jade plants (Zhou et al. 2008, 2009), or ascorbic-acid concentration and antioxidant capacity in *Opuntia ficus-indica* (Pretti et al. 2014). To our knowledge, the application of MD in xylem wood of mature trees is novel and has not been carried out before.

The potential advantages of MD for xylem sap sampling are the following:

- 1) MD probes can be installed into the xylem of trees (i.e., plants with secondary growth) without causing substantial damage to the plant tissue and allows for subsequent sampling from a

single location on the plant body. Even though single xylem vessels might be damaged, lateral water transport in the xylem (Bull et al. 1972) is presumed to allow effective probing.

2) The passive diffusion principle of dialysis does not require the actual sap to be extracted.

As the perfusate is slowly pumped through the membrane to yield a sample (e.g., $10 \mu\text{L min}^{-1}$), the time for diffusive exchange through the MD membrane is limited, and only a fraction of the real concentration in the xylem wood is sampled. This fraction is called the relative recovery (RR). Among other factors, the RR largely depends on the pumping rate, which controls the flow of the perfusate through the probe (Stenken 1999) and is also affected by the transport properties of the material surrounding the probe (Bungay et al. 1990). The effect of the pumping rate and surrounding material transport properties on the RR needs to be characterized through calibration in order to derive concentration measurements with MD. In vitro (e.g., stirred solution test) and in vivo calibrations (e.g., stem segment tests) need to be undertaken separately to assess both the effect of the pumping rate and the surrounding material transport properties. The convection flux around the MD probe (e.g. revolutions per minute (RPM) of a magnetic stirrer), which is a function of the flow and transport properties in the surrounding material, may also affect the probe RR due to the changing concentration depletion zone around the probe (Stenken 1999, Zhou et al. 2008). In the case of xylem sap sampling, the convection flux of xylem sap around the probe membrane changes due to the daylight cycle and the meteorological conditions affecting the water pressure deficit between the leaf and atmosphere and the stomatal conductance. It is therefore necessary to perform an in vivo calibration to identify the effect of xylem sap flow velocity on the MD probe RR.

Although xylem sap PO_4^{3-} concentration was measured in our study, MD is applicable to other chemical substances. PO_4^{3-} , in particular it is essential for plant metabolism (Lang et al. 2016), and there is evidence that phosphorus (P) becomes a limiting factor in forest ecosystems (Goswami et al. 2018) due to the low rates of mineralization and its low mobility on the one hand and high anthropogenic nitrogen deposition in the northern hemisphere on the other, which may enhance the P demand of trees. (e.g., Jonard et al. 2015). The soil-plant-microbe interaction in respect of the

ecosystem P relationship is far from being understood (Lang et al. 2016), and characterizing and quantifying the P uptake by plants is necessary to understand the P fluxes in the entire forest ecosystem.

As plant nutrition exhibits day and night variations, diurnal measurement of chemical compounds in plant tissues are of particular interest. Köstner et al. (1998) reported higher organic sulfur during the night compared with daylight in the xylem sap of the Norway spruce (*Picea abies*), whereas the diurnal changes of inorganic sulfur were insignificant. Xylem sap composition for major ions in well-controlled conditions was measured diurnally in poplars (*Populus tremula* x *P. alba*) by Siebrecht et al. (2003), and these authors showed that ion concentrations were highest in the late morning and lowest at dusk. Major ions translocation (concentration multiplied by sap flow) was lowest in the middle of the night (Siebrecht et al. 2003). In addition, root uptake of nutrients shows diurnal variations; e.g., the highest ammonium uptake was observed at noon in both beech and spruce trees (Gessler et al. 2002).

In addition, seasonal differences were observed in nutrient fluxes in plants. Within the course of a year the highest concentration of ions in the xylem sap was reported in March and April in 42-year-old beech trees in central Germany (Glavac et al. 2006). Similarly, the highest concentration of total P and inorganic P was observed in spring in the xylem sap of mature beech trees in the Conventwald site in Germany (Netzer et al. 2017). Towards early summer, the concentration of total and inorganic P decreased while the concentration of organic P remained constant (Netzer et al. 2017).

All of these studies used destructive sampling techniques for xylem sap sampling. The main objective of this paper is to propose MD as a non-destructive alternative for the analysis of P in xylem sap that allows for high (i.e., hourly) temporal resolution sampling. We systematically tested the effect of the MD perfusate pumping rate and xylem flow velocity in the lab and show the successful application of MD to measure diurnal variability in phosphate concentrations in mature beech trees in the field, something that has not been reported in the literature so far.

2. Material and methods

2.1 The principle of microdialysis

Microdialysis (MD) is based on the dialysis principle where passive diffusion takes place between two liquids separated by a membrane (Stenken 1999). Figure 1 depicts the sketch of a MD probe. Through the inlet tubing the perfusate, a liquid with a known chemical composition, is pumped with a known rate (e.g., 10 $\mu\text{L min}^{-1}$) to the top of the cylindrical membrane. In the medium surrounding the probe, the substance of interest is typically present in higher concentrations and thus diffuses through the membrane into the probe. Depending on the flux rate of the surrounding medium and the diffusion rate of the substance between the surrounding medium and the perfusate, a depletion zone is potentially established around the membrane. The enriched solution in the probe (dialysate) is collected with the outlet tubing. The ratio of sampled dialysate concentration (C_{dial}) and the concentration of the substance in the surrounding medium, i.e., the analyte, (C_{∞}) is called the relative recovery (RR) and it is defined as

$$RR = \frac{C_{dial} - C_{per}}{C_{\infty} - C_{per}} \quad (1)$$

where C_{per} stands for the concentration in the perfusate, which is controlled by the user.

The RR is dependent on the contact time between the perfusate and the surrounding solution. The contact time is controlled by the pumping rate (Q_{pump}) of the perfusate. A typical parametric relationship between the RR and Q_{pump} was derived by Stenken (1999) as

$$RR = a - \exp(-k / Q_{pump}) \quad (2)$$

where a and k stand for dimensionless parameters that are probe-type specific (parameter a theoretically equals 1). The relationship (2) describes the RR as a function of Q_{pump} , which is the pumping rate of the perfusate. C_{∞} can be calculated with Eq. 1 if the calibration curve Eq. 2 and concentration C_{dial} and C_{per} are known.

It is hypothesized that the RR also depends on the flux rate of the medium around the probe; i.e. the xylem sap flow velocity in our case. Xylem sap flow velocity can affect the concentration depletion zone around the probe, and therefore the diffusion rate into the probe. We assessed whether and how the RR also depends on the xylem sap flow velocity (J_{xylem}) through the convection effect and transport resistance of the xylem sap for diffusion. In that case, the RR also becomes a function of J_{xylem} , $RR(Q_{pump}, J_{xylem})$. This function was measured in the laboratory, and the concentration of the *analyte* (C_{∞}) was calculated as

$$C_{\infty} = C_{per} + \frac{C_{dial} - C_{per}}{RR(Q_{pump}, J_{xylem})} \quad (3)$$

The distinct ionic strength of analyte and perfusate can also affect transport through the membrane (Miró and Frenzel 2004, Zhou et al. 2008, Demand et al. 2017). The ionic strength effect was analyzed in this paper during the *in vivo* measurement. Temperature also affects the RR since diffusion is temperature-dependent (Inselsbacher and Näsholm 2012), according to the Einstein-Stokes equation. The temperature effect, however, differs for chemical species with different molecular weight, as measured in the soil (Inselsbacher and Näsholm 2012). The temperature effect was not systematically tested in our study, since the complete chemical composition of the xylem sap in a living tree was not analyzed. We thus could not directly transfer the temperature effect obtained from the laboratory calibration to the field measurement. However, we discuss the potential effect of temperature and associated changes in the diffusion rate on MD measurements in general. It also was reported that pH has an effect on the RR (Sulyok et al. 2005); however, it was found that this effect is insignificant between pH 3.5 and 6.5 (Demand et al. 2017) and thus was not relevant for xylem probing (cf., Thomas and Eamus 2002).

In vitro stirred solution and *in vivo* stem segment calibration was undertaken in the laboratory before the xylem sap measurement in living beech trees was conducted in the field. With the calibration procedure we investigated the effect of the pumping rate Q_{pump} and xylem sap flow velocity J_{xylem} on the RR. Finally, a case study, utilizing MD was carried out in a beech forest field

site in southwest Germany, where the xylem sap PO_4^{3-} was sampled during a 24-hour measurement campaign.

2.2 Laboratory tests

Stirred solution test

Calibration in the form of in vitro stirred solution tests was done in order to determine the parameters of the relationship between the RR and Q_{pump} (Eq. 2). CMA 20 probes (CMA, 2018) with a PAES (Polyarylethersulfone) membrane with a 20 KDa molecular cutoff were used in this study. Figure 2 depicts the setup of this measurement. The MD probe was placed into a stirred PO_4^{3-} solution (analyte) with a known chemical composition C_∞ . A standard solution of monosodium phosphate (NaH_2PO_4) with a concentration $C_\infty = 416.7 \mu\text{M}$ was used as an analyte. The perfusate consisted of de-ionized water containing no PO_4^{3-} ($C_{per} = 0 \mu\text{M}$). Potassium chloride with a concentration of 20 mM was added to both the analyte and the perfusate to reduce the ionic strength effect. A peristaltic pump (MINIPULS, Gilson, Inc., USA) and PVC tubes with inner diameters of 0.76 and 1.02 mm were used to pump the perfusate through the MD probes. Pumping rates Q_{pump} of 5, 7.5, 10, 15, and 20 $\mu\text{L} \cdot \text{min}^{-1}$ were chosen for the calibration. The Q_{pump} range was chosen in order to obtain adequate sample volume in a reasonable sampling time. All stirred solution tests were performed in an air-conditioned laboratory, with air temperature set to 21 °C.

In total, 51 samples were obtained with two different MD probes during the measurement (8 to 15 samples for each Q_{pump}). In a single measurement 0.3 to 1.2 mL of dialysate was collected. The samples were analyzed using the molybdenum blue method (Murphy and Riley, 1962; Drummond and Maher, 1995) utilizing a Unicam AquaMate photometer (Spectronic Unicam) with 700nm wavelength and a 1cm cuvette to detect phosphor-molybdate (DIN EN ISO 6878). The calibration curve (Eq. 2) was obtained with the nonlinear least square optimization in R software (R Core Team, 2019).

175 ***Stem segment test***

176 In vivo stem segment tests were performed in order to examine the effect of xylem sap flow
177 velocity and material properties on the RR. All measurements were undertaken with stem segments
178 of ca. 20-year-old beech trees (*Fagus sylvatica*). Figure 3 shows the setup of the procedure. Stem
179 segments with lengths ranging from 30 to 100 cm and diameters from 7 to 8.5 cm were collected in
180 a local forest and transported in a water barrel (30 cm stem segments) or with sealed ends (100 cm
181 stem segments) to the lab within two hours after collection and refrigerated. In the lab 5–10 cm of
182 the segments were cut off on both sides of each stem segment before vertically fixing the stem
183 segments with clamps. Both ends of the stem segment were equipped with tubing attached to the
184 stem segment by a heat shrinking tube and sealed with silicone. Sap flow through the stem segment
185 was simulated by introducing a pressure head by means of a water column on top of the upper end
186 of the stem segment. The sap flow velocity was calculated from the weighed analyte dropping from
187 the stem segment and stem segment cross-section area (all of the cross-section contributed to the
188 sap flow during the stem segment test). Each stem segment was flushed with the analyte, a PO_4^{3-}
189 solution (NaH_2PO_4 and KCl), for two hours (shorter stems) or overnight (longer stems) before
190 measuring. Longer stem segments were necessary to simulate slow sap flow velocities. MD probes
191 were inserted in a predrilled hole 1.7–2 cm deep (the MD chamber) with a drill bit of 1 mm in
192 diameter. A 14 mm long metal tube was installed 7–9 mm into the chamber in order to fix the shaft
193 of the probe during installation (see Figure 3). Probes were flushed with perfusate for 20 minutes
194 before sample collection began. Sampling time was 1 to 1.5 hours to yield 0.6–0.9 ml of sample. All
195 in vivo calibration measurements were done with a pumping rate of $10 \mu\text{L min}^{-1}$. This flow velocity
196 was chosen based on the in vitro results as an appropriate tradeoff between the sample volume and
197 sampling time. A total of 83 samples were collected. Different combinations of KCl and PO_4^{3-}
198 concentrations were used in the analyte and perfusate to consider a range of sap compositions and
199 ionic strengths (see Table 1). The simulated analyte xylem sap flow velocity ranged from 0.4 to 28.1
200 cm.h^{-1} (range estimates based on Hentschel et al. 2013). The stem segments were replaced every

first or second day to avoid influences by a potential decay of the wood or xylem embolism. All samples were measured under room temperature conditions (approx. 21 °C).

Effect of xylem flow rate

The effect of xylem sap flow velocity J_{xylem} on the RR was evaluated in order to relate MD measurements in a living tree to absolute PO_4^{3-} concentrations and to find the calibration relationship between the RR and J_{xylem} . All 83 samples were used to derive the relationship. The effect of xylem sap flow velocity was assessed by linear regression and by t-test for zero slope and intercept of the linear relationship.

In order to assess the effect of varying stem segment properties, subsets of the data were statistically analyzed with ANOVA. The J_{xylem} measured with 3 stem segments was classified in 2 levels with $J_{xylem} \leq 6.9 \text{ cm h}^{-1}$ and $6.9 < J_{xylem} \leq 10.04 \text{ cm h}^{-1}$ (based on 1/3 and 2/3 quantile of J_{xylem} data). Table 2 presents an overview of the data used. By this procedure a total number of 30 data points were obtained. This subset of the full dataset was selected in order to maximize the number of repetitions of tested stem segments and classified xylem sap flow velocity combinations (Table 2). As different stem segments may affect the RR, the interaction between J_{xylem} and the stem segment used was also tested.

Effect of ionic strength

The RR is potentially affected by the ionic strength of the perfusate relative to the surrounding medium, as large differences can lead to an osmotic potential. A test was performed with two concentrations of analyte (41.7 and 125 μM NaH_2PO_4 with ionic strengths of 250.5 and 750 μM) and perfusate (20 and 500 mM KCl with ionic strength of the same values). Those four concentration combinations were tested with three replications. A total of 12 data samples were collected. Three MD probes and two stem segments (one for each analyte concentration) were used. The final results were analyzed with ANOVA to test which variable (analyte or perfusate concentration) causes changes in the RR or as a consequence whether ionic strength affects the RR

226 of PO_4^{3-} in xylem sap. Differences in the stem segment properties lead to a variability of J_{xylem}
227 between 6.8 and 11.2 cm h^{-1} .

228 **2.3 Field application**

229 To explore MD probe performance under natural conditions, a 24h measurement campaign for
230 assessing PO_4^{3-} concentrations in the xylem sap was performed between 2 May and 3 May 2018 at
231 the Conventwald experimental site in the Black Forest, Baden-Württemberg, Germany. The
232 beginning of May was chosen, based on the long-term phenological data provided by the Climate
233 Data Center (Deutscher Wetterdienst 2018), as it is the beginning of the vegetation season where
234 high nutrition concentrations in xylem may be expected.

235 ***Site Description***

236 The Conventwald experimental site (CON) is located in the Black Forest, ca. 15 km east of
237 Freiburg, Baden-Württemberg, Germany (48°01'16"N 7°57'56"E). The site altitude is 766 m a.s.l.;
238 the climatic zone is temperate oceanic. The mean annual precipitation is 1749 mm with a mean
239 annual temperature of 6.8 °C. The geological parent material is Paragneiss with a soil-saprolite
240 depth between 280 and 300 cm. The soil type is loam in the topsoil and sandy loam in the subsoil
241 (WRB 2015). The soil exhibits a large portion of gravel fraction. Total P stock per unit area is 7.5
242 mol m^{-2} up to 1 m soil depth (Lang et al. 2017). Total P concentration in the A horizon is 30 mmol
243 kg^{-1} from which 0.77 mmol kg^{-1} is bioavailable (Lang et al. 2017). The site is covered with mixed
244 forest dominated by beech (*Fagus sylvatica*, 69%) and silver fir (*Abies alba*, 31%). The tree age is
245 about 130 years. The mean height is 27.6 m and mean breast height diameter is 40 cm. There were
246 312 beech trees per hectare determined at the CON site (Lang et al. 2017).

247 ***Sampling setup***

248 The measurements were undertaken in two beech trees with different ages (10 and ca. 130 years).
249 Three probes were installed in each tree as shown in Figure 4 (see details below). All probes were
250 installed one day prior to the experiment and flushed with perfusate for four hours prior to the

experiment. Two peristaltic pumps, Gilson Minipuls® 3, were used to push the perfusate through the probes. A potassium chloride solution (KCl) with a concentration of 20 mM was used as perfusate. The dialysate samples were collected into plastic vials and stored in a cooling box before bringing them all to the lab for analysis. The PO_4^{3-} concentrations were determined within six hours after the end of the 24h measurement. An in vitro stirred solution test of the MD probes was performed in the lab prior to and after the field test to assess the effect of a change in membrane properties due to the installation in living wood.

The 24h measurement started at 15:50 on 2 May 2018 and a sample was taken from each probe every 2h. In total the 6 MD probes yielded 72 samples. The perfusate was pumped with a pumping rate of $10.07 \pm 0.39 \mu\text{L min}^{-1}$. In addition, xylem sap flow velocities were measured in each tree using EAST30 heat pulse sensors (EAST 30 Sensors, Washington) (orientation of the heat pulse sap flow sensor in both trees is shown in Figure 4). Stem temperature data were extracted from the raw heat pulse sensors' sensor data (the initial temperature of the lower needle of the heat pulse sensors), and air temperature was measured by a logger temperature sensor located in the vicinity of both trees.

MD-Probe installation procedure in the field

The probes needed to be inserted into the rigid xylem tissue, where contact between the membrane and xylem sap had to be preserved. As it was noted during preliminary testing, the installation procedure is crucial for adequate contact between the probe membrane and the xylem sap, and thus for the probe's functionality. The following steps of the installation were optimized after experiencing several failures in the field the previous season:

- 1) 1 cm² of bark and phloem was removed from the mature beech tree before the probes were installed.
- 2) A 0.5–0.7 cm deep hole was drilled in the xylem wood with a drill bit of 1.2 mm in diameter.
- 3) The MD chamber was extended to 2.5 cm with a drill bit of 1 mm in diameter.

276 4) a 14 mm long iron shaft was inserted to a depth of 0.5–0.7 cm into the prepared MD
277 chamber in order to fix the probe during installation.

278 5) The MD chamber was flushed and filled with deionized water using a syringe within
279 seconds after the drilling (Figure 5A).

280 6) The MD probe was carefully inserted into the MD chamber immediately after the chamber
281 was filled with the deionized water (Figure 5BC).

282 7) The iron shaft with the MD probe was sealed with silicone (Figure 5D).

283 A fully installed silicone sealer probe is shown in Figure 5E.

284 Probes were installed the day before the experiment and flushed with perfusate for four hours
285 directly after installation and again prior to the experiment. Flushing was done in order to remove
286 possible air bubbles from the tubing system.

287 Probes were carefully removed from the MD chamber after the experiment. The diameter of the
288 MD, chamber, which was slightly larger than the membrane diameter, allowed for removal of the
289 MD probes from the MD chamber without damage. A knife was used to remove the silicon from the
290 bark of the tree; thus, the silicon stayed glued to the "head" of the MD probe.

291 ***Destructive xylem sap sample as control***

292 The younger beech tree was cut off after the measurement to collect samples from its sap as a
293 reference to evaluate the measured PO_4^{3-} concentrations with the new approach presented in this
294 paper. Tree stem segments of length of ca 50 cm were collected from a height of 20–70 cm, 70–120
295 cm, and from 120–170 cm above the ground. Stem segments were sealed with plastic sheets on both
296 sides and immediately transported to the lab.

297 The xylem sap was extracted and measured directly after the end of the 24h measurement with a
298 gravitational displacement method by applying a hydraulic potential (water column) to the top of
299 the stem segment and collecting the displaced water at the bottom. We followed the procedure
300 described by Glavac et al. (1989) modified by Weber et al. (1998). Bark and phloem were removed

from both ends of the stem segment (ca. 10 cm) and a sealed shrinking tube was put at the upper end of the stem segment where the water column pressure was applied. Parts that had been stripped of the bark-end phloem were tightly wrapped in paper towels in order to prevent and/or reveal potential contamination by leaking water (which did not occur). Since the initial fractions of the displaced xylem sap might have been contaminated with constituents of destroyed cells, the first 2–5 ml were discarded. Thereafter, two consecutive samples with a volume of 2–5 ml were collected and directly analyzed with the molybdenum blue method (see section 2.2 above). Weber et al. (1998) showed with berberine chloride as a tracer that sample dilution by the displacement solution is low for the first portions of the xylem sap, as sampled in our study. Moreover, they excluded significant contamination with cellular constituents by determining the ATP content of the xylem sap.

MD probes durability under field conditions

To assess the potential decay of the *MD* probe as a result of being used in a living tree, an in vitro membrane test in stirred solution with known PO_4^{3-} concentration was performed before and after the field campaign. The test undertaken before the experiment also served as a control for the correct functionality of the probe and membrane, and to reveal possible leakage of the tubing. The same test was performed after the experiment to show potential decay effect of the probes and to check for a possible decrease in relative recovery.

3. Results

3.1 Laboratory tests: Effect of the pumping rate on the relative recovery

The PO_4^{3-} RR decreased exponentially with the increasing pumping rate (see Figure 6). The obtained parameters for Eq. 2 are: $a = 1.017$ [-] and $k = 1.265$ [-] and the exponential function explains 95% of the data variability. The RR values were between 7.5% to 25% for pumping rates between 5 to 20 $\mu\text{L min}^{-1}$, which corresponds to the values reported in the literature (Stenken 1999,

Demand et al. 2017). The variability of RR within the samples of each pumping rate was smallest for the $5 \mu\text{L min}^{-1}$ pumping rate (Interquartile Range (IQR) = 1.38%) and highest for the $20 \mu\text{L min}^{-1}$ pumping rate (IQR = 2.46%).

3.2 Laboratory tests: *Effect of xylem sap flow velocity (J_{xylem}) on the RR*

The effect of xylem flow velocity J_{xylem} on the RR is shown in the Figure 7. A linear relationship was fitted through the data to obtain a calibration curve, which was used to interpret the diurnal measurement data in the field shown below. The data points exhibit large variability, and therefore the linear regression yields a low R^2 value ($R^2 = 0.17$). The slope of the linear function fitted to the data is 0.179 and thus small. This means that the RR only slightly increases with xylem sap flow velocity. The hypothesis of zero slope and intercept of the linear expression (shown in Figure 7) was tested using the student t-test (in Table 3). The null hypothesis was rejected in both cases with a level of significance $\alpha=0.05$.

The effect of both the varying stem segment transport properties and J_{xylem} was tested with ANOVA (Table 4). The results indicated no significant effect of the used stem segment and J_{xylem} on the RR. No significant interaction between those effects was observed (Table 4). Only the intercept effect was significant (Table 4). Results of this test indicated that different stem segments and potential differences in transport properties do not override the effect of the J_{xylem} .

Effect of ionic strength on the RR

In order to assess the effect of varying PO_4^{3-} concentration in the analyte and KCl concentration in the perfusate during the stem segment measurements, an ANOVA was performed. Mean RR and standard deviation (in brackets) with three replications are displayed in Table 5. Higher concentration of KCl in perfusate increased the RR. Higher PO_4^{3-} analyte concentration increased the mean RR and RR standard deviation.

Results of ANOVA for varying PO_4^{3-} and KCl concentration are shown in Table 6. Only 29% of the variability of the RR is explained by PO_4^{3-} and KCl concentration, and both effects are not significant. Only the intercept (Table 6), which represents a constant offset, was significant. It was therefore concluded that all other factors did not significantly affect the RR.

3.3 Field testing: diurnal measurement

Xylem PO_4^{3-} concentrations calculated from C_{dial} using Eq. 3 was higher for the young compared to the mature beech tree but both trees showed the same diurnal pattern of C_{dial} and PO_4^{3-} variability (Figure 8). PO_4^{3-} concentrations decreased during the late afternoon on day 1 along with a decrease in sap flow velocities from 2 cm h^{-1} to almost zero cm h^{-1} . During the night PO_4^{3-} concentrations increased and reached a peak of $456 \text{ }\mu\text{M}$ and $292 \text{ }\mu\text{M}$ PO_4^{3-} in the young and mature tree, respectively, at the end of the night. After sunrise, PO_4^{3-} concentrations started to decrease again down to $121 \text{ }\mu\text{M}$ and $51 \text{ }\mu\text{M}$ in the young and mature beech tree, respectively. When the sky cleared up at midday PO_4^{3-} concentrations in both trees experienced a second peak of $475 \text{ }\mu\text{M}$ and $162 \text{ }\mu\text{M}$ PO_4^{3-} concentrations (young and the mature tree, respectively) followed by a decline during the late afternoon, similar to the pattern observed on day 1.

PO_4^{3-} mass fluxes were dominated by the change in sap flow velocities between day and night (Figure 8). PO_4^{3-} mass fluxes were generally low during night and did not show a peak towards the end of the night as observed in C_{dial} and PO_4^{3-} concentrations (see above). Night PO_4^{3-} mass fluxes were slightly higher for the mature (max.: $2.2 \text{ }\mu\text{mol dm}^{-2} \text{ h}^{-1}$) than the young tree (max.: $0.2 \text{ }\mu\text{mol dm}^{-2} \text{ h}^{-1}$) due to differences in sap flow velocities between both trees. During day when the sky was clear PO_4^{3-} mass fluxes increased with increasing sap flow velocities in both trees (max.: $15.8 \text{ }\mu\text{mol.dm}^{-2}.\text{h}^{-1}$ young and $4.1 \text{ }\mu\text{mol dm}^{-2} \text{ h}^{-1}$ mature beech tree) on day 1 and (max.: $7.4 \text{ }\mu\text{mol dm}^{-2} \text{ h}^{-1}$ and $5.9 \text{ }\mu\text{mol dm}^{-2} \text{ h}^{-1}$) on day 2.

The temperature in the xylem fluctuated between 8.5 and $11 \text{ }^\circ\text{C}$ in the mature and between 7.5 and $11.5 \text{ }^\circ\text{C}$ in the young beech tree with minimum temperature at 8 a.m. and maximal temperature in

the afternoon of day 1 and 2 (Figure 9). Air temperature exhibit similar temperature pattern as the one measured in the young beech tree.

Destructive xylem sap sample

For comparison with the in situ MD measurements, a destructive sample of xylem sap from the young beech tree was collected by gravitational displacement at the end of the field campaign. The PO_4^{3-} concentrations were $115 \pm 45.7 \mu\text{M}$, $100 \pm 3.14 \mu\text{M}$ and $73.6 \pm 4.9 \mu\text{M}$ for the stem segments taken in 20-70 cm, 70-120 and 120-163 cm height above surface, respectively. The PO_4^{3-} concentration in the lower segment (where the MD probe was installed) was only slightly lower than the PO_4^{3-} concentration of the last in situ MD measurement of the field campaign for the young tree which was $146.5 \pm 40.4 \mu\text{M}$.

MD probes durability under field conditions

The MD probes were tested prior and after the 24 h measurement campaign using the stirred solution test described in the method section. In Figure 10 values of RR before and after the field campaign for all used probes are shown. Probes 1 to 3 were installed in the young beech tree, probes 4 to 6 in the mature beech tree. The field measurements did not substantially affect the RR of the probes. The within probe variability was higher than the prior/after single probe variability. The within probe variability was in the same order of magnitude than the variability observed during the in vitro measurement.

4. Discussion

Variables affecting relative recovery

In vitro laboratory stirred solution tests showed that the MD probes exhibited an exponential decay of the RR with increasing Q_{pump} ; something that was also reported in other studies using MD probes in stirred solution tests (Stenken 1999, Miró et al. 2005, Demand et al. 2017). For practical

applications the presented relationship between the RR and Q_{pump} allows for an optimization of the sampling interval. However, the minimum sampling interval is determined by the RR, and thus it is the Q_{pump} that guarantees the required amount of sample with the minimum concentration necessary to perform the chemical analysis (i.e., PO_4^{3-}). All in vivo measurements with stem segments in the lab were performed with a Q_{pump} of $10 \mu L \min^{-1}$. The RR for stem segment measurements varied between 2.5% and 7.5% while the RR from the in vitro stirred solution tests using the same Q_{pump} rate ranged between 10% and 15%. The pumping rate could be reduced in order to obtain a higher RR. However, pumping rate reduction would lead to a coarser temporal resolution. In general, the RR from the stirred solution and stem segment were relatively low but realistic when compared to the RR in other studies that used a slower Q_{pump} . Demand et al. (2017), for instance, used a Q_{pump} of $4 \mu L \min^{-1}$ and reported a RR of 14% in a laboratory stirred solution test. We might conclude that the discrepancy between the in vivo and in vitro measured RR in our study was caused by the resistivity of the surrounding material which increases the concentration depletion zone around the probes, this leading to lower recovery for the given Q_{pump} . Significant effects of the outer medium transport properties on the RR were also reported in the literature (Bungay et al. 1990).

The in vivo laboratory measurement exhibited a weak linear relationship between the RR and J_{xylem} ($R^2 = 0.17$, p-value for slope < 0.01) associated with a large variability in RR (standard deviation = 2.89% with mean RR = 4.09%). The slope of that relationship between the RR and J_{xylem} was small (i.e., 0.179), suggesting that the RR varies only to a minor extent with changing J_{xylem} . The variability of $RR \sim J_{xylem}$ relationship was further investigated by adding a different used stem segment in the model next to the classified J_{xylem} (several stem segments were used during testing). In this case neither classified J_{xylem} nor used stem segment showed significant effects in RR. This has important implications for practical applications of MD in living trees, which naturally experience changes in diurnal and seasonal sap flow velocities (e.g., Granier 1987, Zeppel et al. 2004). It was also shown that varying stem segment properties (here expressed by different stems used) do not influence the relationship between the J_{xylem} and RR, indicating that the method can be

applied in different trees of the same species and still yield reliable results. Our work also showed that potential differences in ionic strength between the solution pumped through the MD probe and the matrix surrounding the probe, which is not known before MD probe installation or during measurements, has also no significant effect on the RR. Warren (2018) also failed to find any ionic strength difference effect for stirred solution measurement, with artificial soil solution, water and several organic nitrogen compounds used. Our results suggest that MD can be applied over a range of realistic xylem flow velocities and potential differences in ionic strength found in living trees (Zeppel et al. 2004, Lopushinsky 1986) without strong effects on the RR.

Xylem sap sampling in the field

The MD probe installation procedure turned out to be crucial for successful PO_4^{3-} sampling in living trees. We therefore suggest following the detailed protocol given in the method section. It is advisable to avoid periods of summer dryness, where the xylem water potential in trees can be very low (< -10 MPa (e.g., Martínez-Vilalta and Garcia-Forner 2017) and the risk is high that the MD probe has no contact with the sap. During the measuring campaign in May with moderate moisture deficits and air temperature, however, it was possible to successfully install all probes. Earlier attempts with a simpler procedure and under dry summer conditions were not successful in enabling proper functioning of the MD sampling.

We need to assume that, even though our method is minimally invasive, placing the MD probes into the xylem might cause damage to living cells in the parenchymatic tissue of the stem and might also cause wound reactions. In the European beech parenchymatic cells make up between ca. 16% and 32% of the woody stem tissue (Morris et al. 2016). As a consequence, cellular constituents of destroyed cells might contaminate the xylem sap and thus lead to incorrect estimates of the xylem PO_4^{3-} concentration. However, the other xylem sap sampling methods, which are even more destructive, also share the same problem. Schneider et al. (1996) tested the contamination with cellular constituents by determining the ATP content following the method of Schupp (1991) for cut

twigs of beech, where the xylem sap was sampled with the help of a pressure chamber. ATP is assumed not to occur in the apoplastic space of the xylem and is thus a contamination marker. After discarding the first portion of the xylem sap they determined cytoplasmatic contamination to be as low as 0.2–0.6%. We might assume that the destruction of living cells upon insertion of the MD probes could lead to an initial increase in PO_4^{3-} concentration in the perfusate. Later, however, the concentrations should decrease again due to dilution with the ascending xylem sap. Given that an initial PO_4^{3-} concentration peak was not seen in the field, and assuming that cytoplasmatic contamination upon insertion is comparable or even lower with our minimally invasive technique, we consider our results to be robust with respect to contamination. To get more quantitative insights into the degree of cytoplasmatic contamination, we suggest flushing stem segments with a solution without PO_4^{3-} and determining the PO_4^{3-} concentration in the perfusate.

Diurnal variability in PO_4^{3-} in living trees

For the first time to our knowledge we show high-frequent in situ measurements of PO_4^{3-} concentrations of xylem sap of living trees using MD probes. Independent of the tree's age, two concentration peaks were observed during a 24h measuring campaign. One peak occurred in the night between 04:00 and 06:00, a second one between 12:00 and 14:00. In general, the measured PO_4^{3-} concentrations were in a comparable range as shown by Siebrecht et al. (2003) for poplar xylem but almost one order of magnitude lower than in kiwifruit vines (Clark et al. 1986). Herdel et al. (2001) showed, however, for the castor bean that even with a constant nutrient supply, phosphate concentrations in the xylem can vary between 0.1 mM and 3 mM and thus within one order of magnitude within a few weeks.

Bimodal concentration patterns in the xylem with one peak at daytime and one at nighttime as observed for PO_4^{3-} in our study have been reported for NO_3^- in poplar (Siebrecht et al. 2003) and castor bean xylem (Peuke et al. 2001). Siebrecht et al. (2003) also determined diurnal dihydrogen phosphate concentrations, but a bimodal day-night pattern was not clearly expressed. The first peak

472 in our study occurred at very low sap flow velocity (at night) and we might speculate that phosphate
473 release from living cells in the xylem parenchyma might have contributed to that peak, since
474 dilution with the ascending xylem sap would be low.

475 From the afternoon to the end of the night PO_4^{3-} concentrations increased with decreasing xylem
476 flow velocity. After sunrise, the onset of xylem flow coincides with a decrease in concentration.
477 These patterns point to a dilution of the PO_4^{3-} pool by root water uptake and subsequent water
478 transport in the xylem indicating that P uptake into and transport in the plant is not proportional to
479 the water flux. Nutrient uptake is assumed to be an active and regulated process for which specific
480 transport proteins are responsible (e.g., Santi et al. 2003). For phosphate, however, changes in the
481 capacity of the P-uptake system in the roots are often assumed to have only small effects on the
482 overall uptake (see e.g., Lambers et al. 2006) as the ability of the roots to mobilize sorbed
483 phosphate, involving the release of carboxylates (e.g. Lambers et al. 2018) is the key limiting factor
484 for uptake. We might assume that with a higher water flux in the soil-plant-atmosphere continuum
485 (as indicated by the higher xylem flow velocity), mobilization of PO_4^{3-} might become limited, and
486 thus the concentration of PO_4^{3-} decreases. The peak after midday seems to contradict this
487 assumption, but it has been shown that organic acid (citrate) exudation from roots that allows
488 phosphate mobilization shows diurnal oscillations with a midday peak (Watt and Evans 1999). This
489 is consistent with the finding that organic acids acting as iron-chelating phytosiderophores are also
490 preferentially released for several hours during the light period (Takagi et al. 1984). The increase in
491 PO_4^{3-} concentration after midday could thus be explained by a higher mobilization in the soil that
492 allows more intensive transport to, into and within the plant. Considering the higher sap flow
493 velocities during the light-period, the total flux of PO_4^{3-} was largest during the daytime and smallest
494 at night. This dependency of nutrient fluxes in the xylem is also in agreement with other
495 observations on phosphate (Siebrecht et al. 2003) and also matches the findings of higher nutrient
496 uptake by the roots during periods of higher diurnal transpiration (Gessler et al. 2002).

As mentioned above, temperature can also change the diffusion rate into the MD probe (Inselsbacher and Näsholm 2012). The RR is therefore higher at higher temperatures and vice versa. As the laboratory calibration was done under higher temperature conditions compared with the field measurement, the actual RR under field conditions might have been lower and as a consequence the PO_4^{3-} concentrations might have been higher than presented. As the temperature in the field changed over the course of a campaign, this might have also affected the diurnal courses of RR. However, in our case the temperature differences during the field campaign was in the order of 2 to 5 °C, and the PO_4^{3-} concentrations determined from destructive sampling and from the in situ MD measurements at the end of the experiment were similar. Small differences in PO_4^{3-} concentration between the two methods may be caused by the different sampling period (the gravitational displacement was done a few hours after the last MD measurement), or by different spatial integration of the measurements. While the MD probes determine the concentration in a small area of the trunk, the displacement method integrates the whole sapwood. Still, since the destructive sampling and MD-measurements yielded very similar PO_4^{3-} concentrations we expect that the difference in temperature between lab and field and between night and day in our study had a limited influence on the PO_4^{3-} -concentrations derived.

5. Conclusions

This paper presents a laboratory testing and field application of microdialysis (MD) for non-destructive in situ xylem sap sampling for subsequent PO_4^{3-} analysis in beech trees. Laboratory in vitro stirred solution tests and in vivo stem segment tests were performed in order to assess the MD probe's performance and to identify potential influencing factors when using MD in xylem wood. MD probes were further tested in a 24h measuring campaign under field conditions in a young (10 years) and a mature (130 years) beech tree. Our results show that MD is a technique that is suitable to probe the xylem of trees for PO_4^{3-} concentration assessments with the potential to also be applied to other nutrients. MD has the

advantage to be minimally invasive and to provide concentration information with high temporal resolution so that diurnal patterns can be assessed. The laboratory testing allowed for an investigation of the relationship between simulated xylem flow rate (J_{xylem}), perfusate pumping rate (Q_{pump}) and relative recovery (RR) and ruled out potential interactions with tree specific xylem properties, and potential differences in ionic strength of the perfusate and the xylem sap. MD application in a field campaign exemplified the reliability of the method under natural conditions in living trees. Initial failures to measure PO_4^{3-} in situ with MD showed that the installation procedure of the MD probes is crucial. For this reason, we have included a detailed description of our approach.

MD opens new avenues to understand the variability of nutrient transport in trees at the sub-daily but also at the seasonal time scale. If combined with other techniques, such as systems that allow to detect the depths of root water uptake by analyzing water isotopologues (Volkman et al. 2016), MD has the potential to give insight into nutrient source depth and nutrient transport dynamics in living trees. The MD probes used in this study were originally designed for use in soft tissue and therefore are delicate. One possible future enhancement could therefore be to design more robust MD probes.

6. Conflict of interest

None declared.

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9. Authors' Contributions

JJ, MR, AG, MW developed the experimental design, and JJ tested the method in the lab, conducted the field measurements and laboratory analysis. JJ processed the data and performed the statistical analysis including graphs and tables. JJ and MR wrote the manuscript; AG provided the plant physiological interpretation of results. JJ and MR incorporated review comments of AG, MW and external reviewers.

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11. Figures

Figure 1. Sketch of MD probe and its function, (C_{dial} , concentration in the dialysate; C_{per} , concentration in the perfusate; C_{∞} , concentration in the surrounding medium).

Figure 2. The setup of the stirred solution test (C_{dial} , concentration in the dialysate; C_{per} , concentration in the perfusate; C_{∞} , concentration in the surrounding medium).

Figure 3. Setup of the in vivo laboratory measurement. (C_{dial} , concentration in the dialysate; C_{per} , concentration in the perfusate; C_{∞} , concentration in the surrounding medium). The flow through the stem segment was controlled by the pressure head difference "h". The specific sap flow velocity was calculated from the amount of effluent from the stem segment (weighed with a scale) and the stem segment cross section area.

Figure 4: Schematic figure of the orientation of MD probes and heat pulse sap flow sensor. Drawings are not to scale.

Figure 5: Details of the installation procedure. A) flushing of the MD probe chamber with deionized water. B) and C) insertion of the MD probe in the chamber. D) sealing of the MD probe with silicone. E) fully installed probe.

Figure 6. Relation between pumping rate (PR) and relative recovery (RR) derived from the in vitro test. The exponential relationship (2) with high R^2 shows a good fit with the data. Sample size is indicated by n, in the graph. Boxplots show median, 25th and 75th percentile, whiskers indicate ± 1.5 IQR and point symbols show outliers beyond this range.

Figure 7. The effect of xylem sap flow velocity J_{xylem} on the relative recovery (RR). The linear relationship fitted to the dataset has a small slope, considerable variability and therefore low R^2 . Q_{pump} was set to $10 \mu\text{L} \cdot \text{min}^{-1}$.

Figure 8: Results of the 24h measurement campaign applying the MD probes in a mature (circle) and a young beech tree (triangle). A: xylem sap flow velocity; B: dialysate concentration; C: PO_4^{3-}

concentration calculated with formula (3) and the RR calibration curve shown in Figure 7; D mass flux (PO_4^{3-} concentration multiplied by xylem sap flow velocity). Each data point represents the mean of all 3 probes installed in each tree. Whiskers show the standard deviation.

Figure 9: Temperature in the tree xylem during field measurements extracted from the xylem sap flow sensors (see method section for details) and air temperature from the logger next to the trees.

Figure 10: Test of the MD probes performance prior and after the 24h field campaign. The prior measurement of probe 2 was excluded due to failure.

12. Tables

Table 1. Number of in vivo experiments under various analyte and perfusate concentrations.

Table 2. Stem segment number and used classified xylem sap flow velocity combinations

Table 3: t-student test for zero slope and intercept of the $\text{RR} \sim J_{\text{xylem}}$ relationship

Table 4: Results of the ANOVA testing the influence of using different stem segments on the relationship between $\text{RR} \sim J_{\text{xylem}}$.

Table 5. Effect of varying analyte and perfusate NaH_2PO_4 and KCl concentration on the RR. Values shown indicate mean RR with the standard deviation of RR in brackets.

Table 6: Results of the ANOVA testing the importance of PO_4^{3-} and KCl concentrations on the measured RR.

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748 Figure 1

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Figure 3

Figure 4

Figure 5

Figure 6

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Figure 8

Figure 9

Figure 10

Table 1. Number of in vivo experiments under various analyte and perfusate concentrations.

		analyte PO_4^{3-} conc. μM			No. of measure.
		41.7	83.3	125	
perfusate KCl con. (mM)	5	<i>n/a</i>	<i>n/a</i>	20	20
	20	3	<i>n/a</i>	3	6
	500	2	31	24	57
No. of measure.		5	31	47	83

Table 2. Stem segment number and used classified xylem sap flow velocity combinations

		Xylem sap flow vel. (cm h^{-1})		No. of measurements
		≤ 6.93	≤ 11.04	
Stem segment	1	6	4	10
	2	6	6	12
	3	4	4	8
No. of measure.		16	14	30

Table 3: t-student test for zero slope and intercept of the $\text{RR} \sim J_{\text{xylem}}$ relationship

Model	Sample size	Intercept value)	β_0	(p- value)	Slope value)	β_1	(p- value)
$\text{RR}(J_{\text{xylem}}) = \beta_0 + \beta_1 J_{\text{xylem}}$	83	2.38 (3e-5*)			0.18 (5e-5*)		

Table 4: Results of the ANOVA testing the influence of using different stem segments on the relationship between $\text{RR} \sim J_{\text{xylem}}$.

	Sum of Sq. (T. III)	DF	F value	P(>F)
Intercept	685.86	1	116.74	< 0.0e-5
Used stem seg.	31.8	2	2.7	0.08
Classified J_{xylem}	2	1	0.34	0.56
Interaction	26.6	2	2.27	0.12
Residuals	141	24		

Table 5. Effect of varying analyte and perfusate NaH_2PO_4 and KCl concentration on the RR. Values shown indicate mean RR with the standard deviation of RR in brackets.

		perfusate KCl con. (mM)	
		$(\text{NaH}_2\text{PO}_4^{3-} = 0 \text{ mM})$	
		500	20
analyte			
$\text{NaH}_2\text{PO}_4^{3-}$	125	4.39 (1.9)	3.36 (2.2)
con. (mM)			
(KCl = 0 mM)	41.7	12.19 (10.6)	8.45 (6.6)

Table 6: Results of the ANOVA testing the importance of PO_4^{3-} and KCl concentrations on the measured RR.

	Sum of Sq. (T. III)	DF	F value	P(>F)
Intercept	604.56	1	16.4	0.003
PO_4^{3-} conc.	124.55	1	3.37	0.1
aKCl conc.	17.11	1	0.46	0.51
Residuals	332.55	9		