Chemische Methoden der Ökologie –

Analyse ökosystemarer Prozesse und Funktionen

Methods for analysis of terrestrial

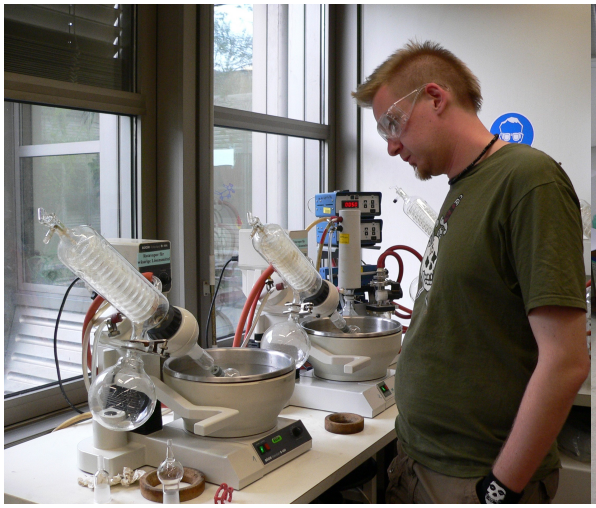
ecosystem processes and functions

300634 SE+UE MOE III-2

7 Stunde(n), 10 ECTS-Punkte

Gert Bachmann, Franz Hadacek, Andreas Richter, Wolfgang Wanek

Tutor: Birgit Wild



1. SAMPLE PREPARATION

1.1. Sample collection

The number of samples to be collected and the frequency thereof depends on site variability, soil parameters and the level of certainty one wants to achieve. Soil pools, moisture and transformation rates show great variability between seasons and weather conditions and therefore have to be measured frequently within a year or between years than, e.g. bulk soil density, soil pH or humus (soil organic matter) content. To measure a soil property with 90% confidence within 10% of the mean the following equation can be applied:

$$N = t^2 C^2 / E^2,$$

where n is number of samples to be collected, t is Student's t-statistics for the level of confidence and number of samples being collected, C is the coefficient of variation (standard deviation divided by the mean) and E the acceptable error as a proportion of the mean. E.g. to collect sufficient samples to be within 10% of the true population with a 95% probability, t statistic is 1.96 (95% confidence interval for a sample of indefinite large size) and E is 0.1. Values for C can be taken from pre-experiments or literature. Parameters that do not conform to normality in distribution are most commonly log-normally distributed and therefore have to be transformed log-normally accordingly before inserting into the equation above.

In this practical class, triplicate samples of fresh litter, Oh horizon (mull layer) and mineral soil (0-10 cm) are collected in an oak (*Quercus robur & Q. cerris*) forest in the Wiener Wald (Michaelerwald). The low number of replicates is selected to enable measurement of the most different pools and activities within the restricted time frame of this practical class. A first morphological soil assessment will be performed visually by a soil scientist in a soil pit, to provide information on pedogenic processes, texture, rooting depth, redox conditions etc.

Litter samples are collected by hand from a 1x1 m plot taking only intact, undecomposed samples of fresh beech litter. The depth of organic horizon is measured and samples are taken using a small shovel or knife, taking care not to mix the sample with mineral soil (distinguish by colour or reflecting mineral particles). Mineral soil is sampled from 20x20 cm plots using a shovel to a depth of 10 cm (measure, do not estimate). Coarse roots are removed in the field. The soil sample should represent all layers from soil surface to 10 cm depth in a ratio as found in the field. Each 2 kg fresh weight are collected in plastic bags and brought to the laboratory while keeping the samples at ambient soil temperature (10°C).

1.2. Bulk soil density

To determine soil bulk density (BD) the litter layer is removed and a steel soil corer (note diameter and height for calculation of soil volume) is driven into the soil until flat with surface. The core is carefully retrieved with a small shovel and both surfaces cut flat. The soil is transferred into a plastic bag and the fresh weight weighed in the lab. An aliquot of each core is weighed into an aluminium dish and dried at 85°C for 24 hours before determination of dry weight.

BD [g dry soil cm^{-3}] = W / V,

where W is soil dry weight of the soil core in grams and V the respective volume of the soil corer in cm^3. Values typically range between (0.6)1.0 and 1.4(1.8) g cm^{-3}. In case that coring is not feasible (moor layers, sandy or very coarse soils; significant soil compaction) bulk density can also be determined by cutting and retrieving a 10x10x10 cm soil cube. Fresh weight is determined and an aliquot of this is dried at 85°C for >24 hours. Carefully measure all lengths of the cube to accurately determine volume or fill the hole with sand. First determine the volume of an excess amount of sand that will be used to fill the hole. Fill the hole flush with the soil surface and determine the volume of sand remaining. Calculate the volume by subtracting remaining volume from the original volume.

1.3. Soil preparation

In the laboratory each soil sample is sieved to 5 (or 2) mm to obtain a representative mixed sample and to remove large particles (stones, mineral horizon), or roots and sticks (organic horizons). In some cases hand picking of roots and other large organic fragments might be necessary.

1.4. Plant/litter preparation

Fresh leaf litter and organic soil is hand sorted to remove woody parts and roots and then is cut in small pieces (less than 5 x 5 mm) before mixing.

1.5. Sample storage

The soil parameters to be measured determine how samples are stored best and whether they should be analyzed in field moist or air dried soil. Air dried soils can be used to determine particle size distribution, soil pH and total soil N and C. Sieved soil samples are either processed further or stored at 4°C in closed plastic bags to omit water loss. However, these bags must not be closed tightly, as this could induce anaerobic conditions. In general it is best to measure soil microbial activities directly after collection or keep soils at 4°C in a cold room for less than a week. Since sieving stimulates microbial activities (e.g. through oxygenation) it is best to store sieved soil samples for

>24 hours before measuring N transformation rates and soil respiration. To investigate soil pools such as nitrate, ammonium, phosphate and microbial biomass immediate extraction and processing of sieved soil samples is recommended. Extracts are best stored frozen at -20°C.

1.6. Soil moisture/water content

Soil moisture can be studied on a water potential, volumetric or gravimetric basis. In this course soil water content is measured by gravimetry.

WC_g [g H_2O g^{-1} dry soil] = (soil fresh weight – soil dry weight)/soil dry weight

Volumetric soil water content can be derived from gravimetric water content by:

WC_{vol} [g H_2O cm^{-3} soil] = WC_g x BD

It has been shown that water filled pore space (WFPS) represent a better indicator of microbial activities in terrestrial soils than WC_g and WC_{vol}. Microbial activities peak at ca. 60% WFPS, below and above this optimum value activities declined due to drought stress or oxygen depletion, respectively. To calculate WFPS soil porosity (S_t) has first to be calculated (particle density is 2.65 g cm^{-3} for most mineral soils):

S_t [%] = [1 – (BD/particle density)] * 100

WFPS [%] = [WC_g * 100 x (BD / S_t)] * 100

1.7. Preparation of soil/litter extracts

Almost all pool sizes of available nutrients are determined in soil or litter extracts. Chemicals and solvents used should be of highest purity (p.a.). For this each 1.5 g of fresh soil or 0.75 g of organic material (organic soil, litter) are weighed into polypropylene vials (scintillation vials, white caps; 20 mL volume) and the exact weight is noted to the nearest 0.01 g. Soil extractant (15 mL) is added and the soil suspended by vigorously shaking. Then soils are extracted on a horizontal shaker at 150 to 300 rpm for 60 min at room temperature, the vials lying flat on the shaker to ensure optimal suspension of the slurry. The following extracts are prepared:

Type	**Extractant**	**Application**
Water (H_2O)	Milli Q water (>18.2 MOhm)	Soil pH, nitrate
$CaSO_4$	10 mM $CaSO_4$ (1.36 g L^{-1})	Soil pH, nitrate
KCl	1 M KCl (74.5 g L^{-1})	Ammonium, microbial biomass by TOC/TN anal.
K_2SO_4	0.5 M K_2SO_4 (87.1 g L^{-1})	DON or microbial biomass by persulfate oxidation
NH_4OAc	1 M $NH_4CH_3CO_2$ (77.1 g L^{-1})	Available cations
Bicarbonate	0.5 M $NaHCO_3$ (42.0 g L^{-1}), pH 8.5	Available phosphate
MCW	Methanol: chloroform: water (3: 1:1, v: v: v)	Polyphenols, low-molecular weight substances (sugars)

Thereafter extracts are filtered using ash-free cellulose filter paper (7 or 9 mm diameter) and polypropylene funnels into new, properly labelled scintillation vials. About 10 mL of filtrate should be collected each. Use only dry filter paper and do not wash the filter with additional water! (Note: in case of highly inhomogeneous samples such as litter it may be advisable to use sample weights >0.75 or 1.5 g and upscale the extraction using Erlenmeyer flasks).

To extract soluble polyphenols and low-molecular weight compounds methanol: chloroform: water extractions are performed as follows: Aliquots of soil (1.0 g) or litter (0.5 g) are weighed and are frozen in liquid nitrogen in mortar where they are ground with a pestle to a fine powder. The homogenized materials are then transferred to 10 mL glass flasks (brown) and 2 mL chloroform is added. After 5 min extraction in the ultrasonic bath 8 mL methanol: water mixture (3:1, v/v) is added and the suspension is treated in the ultrasonic bath for further 5 min. Samples are heated at 60°C for 25 min in a water bath and after cooling to room temperature samples are centrifuged 10 min at 10.000 g. The supernatant is collected by decanting and reduced in volume on a rotary evaporator to 1 mL before dilution with 10 mL water.
Alternatively, methanol: chloroform: water extracts of plant powders (dry) can be prepared for e.g. determination of lipids, soluble sugars and amino acids. For this a slightly modified protocol is used. Grind the plant material to a fine powder (<10µm) in a ball mill or similar. Weigh approximately 100 mg of this plant material into 2 ml reaction vials, note the exact weight. Extract the plant material with 1.5 mL methanol/ chloroform/ water (MCW, 12:3:5, v: v: v) for 30 min at 70°C. Shake the vials occasionally. Let the samples cool down and centrifuge at 10,000 g for 2 min. Transfer 800 µL of the supernatant with a pipette into a 2.0 mL reaction vial. Remove the residual MCW and keep the pellet for preparation of starch and cellulose. For every five samples one blank should be processed (800 µL MCW, no sample). Add 800 µL water and 250 µL chloroform to induce phase separation and mix vigorously. Centrifuge at 10,000 g for 2 min to separate chloroform and aqueous phases. Transfer 1.2 mL of the upper aqueous phase into a 2.0 mL reaction vial, add 500 µL chloroform, mix vigorously and centrifuge at 10,000 g for two min to separate phases. Transfer 1.0 mL of the upper phase into a reaction vial and dry in a vacuum concentrator at approximately 100 mbar (Speed Vac). Re-dissolve the residue in 1.0 mL water. (Note: This drying step is necessary to remove traces of chloroform that dissolves in the aqueous phase (solubility ~1%) and solubilizes the DOWEX polystyrene resin). The chloroform phase can be used to determine total lipid content gravimetrically.

1.8. Soil pH

Soil pH is a measure of the hydrogen ion activity in soil solutions and is measured electrochemically or by ISFET technology. Soil pH is measured in water and in 10 mM $CaCl_2$ extracts (~10% w/v, fresh soil) using a glass electrode or ISFET. Before measuring the pH the electrode is calibrated using two reference buffers (4.0 and 7.0). Often soil pH is measured in a 1:2 (w:v) soil slurry in respective extractant after allowing to stand for 30 min.

2. QUANTIFICATION OF POOLS

2.1. Exchangeable cations (atomic absorption spectrometry)

Principle

Most nutrients are taken up by plants and microbes in an ionic form, as cations (e.g. ammonium, potassium, magnesium, calcium) and anions (e.g. phosphate, sulphate, nitrate), from soil solution. The ion pool size in soil solution is however minute compared to exchangeable and total ion contents. Soil organic matter and clay particles are charged, carrying negative and less positive charges that by ion-exchange forces bind cations or anions, respectively (exchangeable ions). The cation exchange capacity (CEC) and anion exchange capacity (AEC) varies greatly depending on mineral composition and soil organic matter content. Usually CEC by far exceeds AEC. To measure exchangeable cations soil and litter samples have to be extracted with salt solution to desorb bound cations, i.e. displace them from soil exchange sites into solution. The choice of salt for extraction depends on the target ion. The concentration must be high enough to quantitatively displace them from exchange sites and the form of salt must not interfere with subsequent quantification. KCl is the most common extractant for inorganic N. For total cation analysis ammonium acetate (NH_4 OAc) is useful because both ammonium and acetate are volatile and therefore do not interfere in the burner of the atomic absorption spectrometer. $BaCl_2$ can be used for simultaneous extraction of base cations and ammonium.

The exchangeable pool also contains H^+ and Al^{3+}, particularly in acid soils. They have to measured separately (plus sometimes Na^+) if CEC is estimated. CEC is affected by pH and ionic strength of soil solution, particularly in highly weathered soils and soils rich in Al and Fe oxides, hydroxides and amorphous clays. In these cases it is advisable to determine CEC from summing up exchangeable cations (base cations and acidic cations).

Atomic absorption spectrometry

The analytes (cations) are injected into the flame by an atomizer, the flame being based on air-acetylene (standard) or nitrous oxide-acetylene (calcium). In the heat of the respective flame (ca. 2300 °C) elements are atomized. Element-specific light is

generated by a hollow-cathode lamp and is sent through the flame. Hollow-cathode lamps are glow-discharge lamps where the cathode consists of the respective element and which therefore emits element specific light spectra. Free atoms of the element in the flame therefore absorb this light in a concentration-dependent manner. Light (energy) absorption results in excitation of (valence) electrons. The attenuation of light emitted from the hollow-cathode lamp is measured, analogously to photometric methods. Light absorbance (German: Extinktion) is proportional to the density of element atoms in the flame and therefore in the analyte solution. The monochromator is set to an element-specific wave length. For calibration a range of different standards are prepared and measured. Since at the high temperature valence electrons are easily stripped from the atom, caesium is added to all standards and samples in excess as an "ionisation buffer" effectively hindering thermal ionisation of atoms.

Procedure

Standards are prepared from 1000 mg L^{-1} stock solutions after producing a working solution of 100 mg L^{-1}. For convenience, four elemental standards are combined in the standards (see below) and each 100 mL are prepared. CsCl is added to an end concentration of 0.1% (stock solution 10% w: v). Due to differences in viscosity and therefore flow through the atomizer, the matrix of standards should mimic the one of the samples (e.g. water, ammonium acetate, acid).

Calculations

See ammonium determination, chapter 2.2.1.

mg L^{-1}	Standard 1	Standard 2	Standard 3	Standard 4
Magnesium	0.5	1.0	2.0	5.0
Calcium	10.0	5.0	1.0	2.0
Potassium	2.0	5.0	10.0	1.0
Sodium	0.5	1.0	2.0	5.0

2.2. Available nitrogen forms

General

Nitrogen occurs in various inorganic and organic forms in solutions collected from terrestrial ecosystems. Inorganic forms of N (NO_3^-, NH_4^+, and NO_2^-) are quickly measured by various methods (ion chromatography, flow injection analysis/colorimetry and other wet chemistry methods). The organic N fraction cannot be measured directly but is instead measured by subtracting the inorganic N concentration from the total dissolved N (TDN) concentration. Typical methods for determining TDN in soil solution include persulfate digestion, UV oxidation, and Kjeldahl TKN. These methods are all time consuming, tedious, use concentrated acids or bases, and may generate hazardous

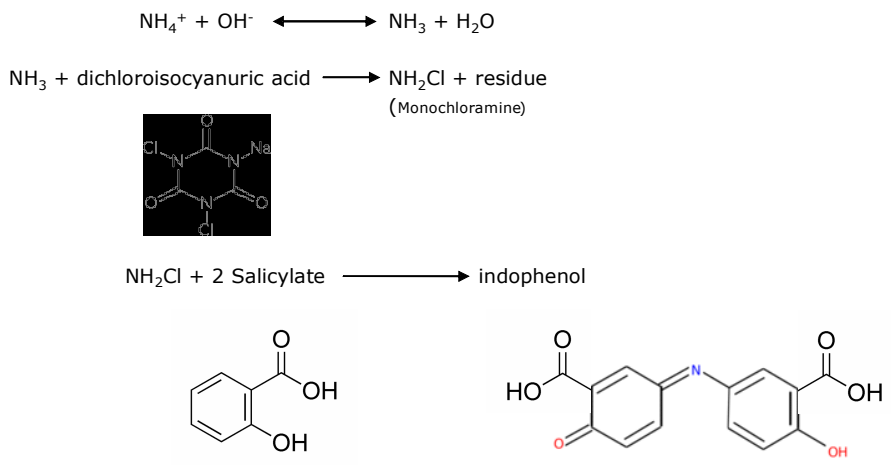
wastes such as Hg and Se. Each of these methods also requires at least two steps, degradation of organic N to an inorganic form and analysis of the resulting digest. Because of the difficulty in measuring TDN, DON is often overlooked or estimated. However, in many forests, DON is the largest pool of dissolved N. In a hardwood forest at Coweeta Hydrologic Laboratory, NC, USA, 94% of total N in soil solution leaching from the forest floor was DON. Dissolved organic N also can be the predominant form of N in runoff from forested basins. Measurement of TDN thus is critical for accurately estimating nutrient fluxes in forested ecosystems. We here use a catalytic oxidation at high temperatures to measure TDN and detection of the resulting NO by chemiluminescence after reaction with ozone.

The biggest challenge in the determination of soluble N forms in litter and soil, is not the quantification itself, but to choose the right and meaningful extractant. For example, ammonium in soils is usually extracted in 1 M KCl, yielding not only the plant available ammonium but also ammonium that is tightly bound in clay minerals. Extracting ammonium with water on the other hand strongly underestimates the available ammonium pool. As there is no easy and universal solution to this problem, it might often be necessary to use several extractions to get insight into the nutrient status of soil and litter samples.

2.2.1. Ammonium (colorimetry)

Principle

As described in chapter 2.1. salt solutions (commonly: 1 M KCl) have to be used to quantitatively extract inorganic N, particularly ammonium. Ammonium is quantified by an indophenol dye. Ammonia is oxidized to chloroamine by sodium dichloroisocyanuric acid and subsequently forms a bluegreen indophenol compound in the presence of salicylic acid (phenol) in alkaline medium (Berthelot reaction). Nitroprusside acts as a reaction catalyst. The absorbance is measured spectrophotometrically at 660 nm.



Chemicals and Solutions

0.3 M NaOH solution: Dilute 785 µl of 50% sodium hydroxide to 50ml Milli-Q water.
Sodium salicylate solution: Dissolve 8.5 g sodium salicylate and 63.9 mg sodium nitroprusside dihydrate in 50 ml Milli-Q water. Prepare fresh every day.

Colour reagent: Mix each 0.3 M NaOH solution, sodium salicylate solution and Milli-Q water 1:1:1. Prepare immediately before use.

Oxidation solution: Dissolve 0.1 g of dichloroisocyanuric acid sodium salt dihydrate in 100 ml Milli-Q water. Prepare freshly every day.

Calibration

Ammonium nitrogen stock standard solution (1000 mg N L^{-1}): Dissolve 0.382 g NH_4Cl in 100 ml 1 M KCl. Solution can be stored at 4°C for a several weeks.
Ammonium nitrogen working standard solutions: Dilute 0.5 ml ammonium stock standard solution in 100 mL 1 M KCl (5 mg L^{-1}).
Prepare seven 2 mL tubes with 0.8 mL 1 M KCl and add 0.8 mL ammonium working solution. Close the vial and mix. Transfer 0.8 mL of this 1:2 dilution into the next vial and repeat to produce a 1:2 standard dilution series. Concentrations range from 5 mg L^{-1} (undiluted working standard) to 0.0195 mg L^{-1}.

Procedure

Pipet each 600 µL aliquots of standards, reagent blanks (1 M KCl) and samples in 1.5 ml Eppendorf tubes. Add 300 µL of colour reagent and 120 µL oxidation solution. Mind the sequence!!! Shake the samples on a horizontal shaker at 300 rpm for half an hour. Measure the colour intensity at 660 nm. For microplate reader pipet each 250 µL sample or standard mixture in a 96 well microtiter plate, for "normal" spectrophotometers use transfer assays into polystyrene microcuvettes.

Calculation

Plot a calibration curve of measured absorption versus concentration. Perform a linear regression; determine sample and blank concentrations and subtract the mean of the blanks from sample values to give the corrected sample concentration (cs; mg NH_4^+-N L^{-1}, equivalent to µ NH_4^+-N mL^{-1}).
The concentration in the original soil samples per dry mass can be calculated as follows:

NH_4^+-N (µg g^{-1} fresh soil) = cs x V / W,

where V is the volume of the original KCl extract in mL and W is the sample fresh weight in grams. To convert to soil dry weight:

NH_4^+-N (µg g^{-1} dry soil) = cs x V / W x fw/dw,

where fw/dw is the fresh weight to dry weight ratio of the respective soil.

To convert to a molar basis divide by the mol mass M_r of the respective element (here N: 14 g mol^{-1}) or compound.

NH_4^+-N (µmol g^{-1} dry soil) = cs x V / W x fw/dw / M_r

2.2.2. Nitrate (anion chromatography)

Principle

The low anion exchange capacity of many soils allows extracting nitrate in those soils with water or with a low ionic strength solution such as 10 mM $CaSO_4$ for determination by anion chromatography. In soils with variable charge minerals (high AEC) nitrate has to be extracted by 1 M KCl and determined by Cadmium/copper reduction and subsequent nitrite detection by dye formation (see Appendix). Ion chromatography is based on liquid chromatography where sample ions are resolved on an ion-exchange resin. The column is eluted with a mobile phase that desorbs the different ions from the stationary phase. Anion chromatography can be applied to quantify a range of inorganic anions (e.g., nitrate, chloride, sulphate, phosphate) as well as organic acids (e.g., malic and citric acid). Ions are quantified by conductivity detection. Since the high conductivity of the eluent (for example KOH) would strongly interfere with analyte detection, the eluent conductivity is chemically suppressed before entering the detector cell.

Separation system

Column: anion exchange column IonPac AS11 (strong anion-exchange resin on latex-resin basis), 10 µm, 25 cm x 4 mm ID

Pre-column: IonPac AS11-guard, 13 µm, 5 cm x 4 mm

Eluent: 0.5 mM KOH to 37.5 mM KOH in 18 min

Flow rate: 2mL min^{-1}, column temperature 30 °C,

Conductivity detection after chemical suppression

Standards

A combined anion standard solution is prepared containing chloride, nitrate, sulphate and phosphate at a concentration von 100 mg L^{-1}. This solution is diluted to give standards of the following concentrations: 50, 25, 12.5, …., 0.391, 0.195 mg L^{-1}. For calculation see chapter 2.2.1.

2.2.3. Dissolved organic nitrogen

Principle

Dissolved organic nitrogen is extracted in water (water-extractable soil organic N, WSON) or by salt solutions (0.5 M K_2SO_4 or 1 M KCl). Total dissolved N is determined by TOC/TN-

analyzer. TDN is quantified by high temperature catalytic oxidation (HTCO) at temperatures around 680°C, and a catalyst to complete the oxidative conversion of all forms of N to NO. The NO is coupled with O_3, producing NO_2*, which is measured by chemiluminescence detection.

Analyzer: Shimadzu TOC-VCPH Total Organic Carbon Analyzer with TNM-1 Total Nitrogen Measuring Unit and ASI-V Autosampler; For further details on sample handling and standard preparation see chapter 2.5. (Microbial biomass N).

Calculation

DON is calculated by subtracting the inorganic N concentration from the total dissolved N (TDN) concentration i.e.

$$DON = TDN - (NH_4^+ + NO_3^-).$$

Before calculation of DON concentration all solute concentrations (mg L^{-1} or µg N g^{-1} dry soil) are converted to the Mol basis (µmol L^{-1} or µmol N g^{-1} dry soil).

2.3. Phosphate fractions - sequential extraction and resin P

Principle

Soil phosphorus is comprised by an inorganic (bound and dissolved) and an organic fraction. The multiple pools of P such as "available" P rather represent a functional concept than a measurable quantity. Here we will determine two measurable pools that are most closely linked to plant-available P, the bicarbonate extractable P and resin P. Only a very small part of phosphorus appears freely available in soil solution (0.01-1.0 mg P L^{-1}) which can be determined by anion chromatography.

2.3.1. Resin-extractable P

Phosphorus that is considered to be available to plants is commonly extracted by salt or acid solution. There is some relation between extracted P and the P supply and P content of plants; nonetheless, these P fractions are rather ill-defined. In contrast, a well defined fraction of P that is in equilibrium with soil solution may be determined by resin extraction. Soil is equilibrated with anion exchange resin in batch. Phosphorus adsorbed to the resin is eluted with HCl and inorganic P is determined by molybdenum blue method.

Preparation and conditioning of resin bags

Anion-exchange resin (DOWEX 1X8, chloride form, 50-100 mesh) is pre-conditioned in a large beaker containing 0.5 M $NaHCO_3$ (42 g NaHCO3, adjusted to pH 8.5 with 1 N NaOH or HCl, end volume 1 L) for one hour. The solution is exchanged once after which the resin is further equilibrated for 60 min and washed several times with Milli-Q water. The

resin is then collected on an ash-free paper filter. Nylon stockings are cut to produce small bags (4x4 cm) in which 0.4 g moist DOWEX 1X8 resin is weighed. The bags are then closed using a nylon string.

Extraction and measurement

Soil (1 g fresh weight, note exact weight) is weighed in 100 mL Erlenmeyer flasks that have been pre-washed with diluted HCl and Milli-Q. After adding 40 mL Milli-Q and a resin bags the flasks are closed with parafilm and shaken overnight on a horizontal shaker (10-12 hours). Resin bags are then removed, washed free of soil with Milli-Q (until wash water is uncoloured or not cloudy) and eluted with 20 mL ~0.5 M HCl (50 mL 32% HCl diluted to 1 L) for 60 min in scintillation vials. Inorganic phosphorus concentration is determined by molybdenum blue method. Standards should be prepared and diluted in 0.5 M HCl.

Calculation

The concentration in the original soil samples is calculated from sample concentration (cs; µg P mL^{-1})as follows:

PO_4^+-P (µg g^{-1} dry soil) = cs x V / W x fw/dw,

where V is the volume of the HCl solution used to desorb P from the resin in mL, W is the soil fresh weight in grams and fw/dw is the fresh weight to dry weight ratio of the respective soil.

2.3.2. Bicarbonate P

The alkaline bicarbonate extract (Olsen-P) provides a measure of relatively labile plant-available phosphorus i.e. which is loosely sorbed to soil surfaces or is exchangeable. Bicarbonate extracts exchangeable, soluble inorganic as well as organic phosphorus. As described above soil (1 g) is extracted with 0.5 M $NaHCO_3$ (pH adjusted to 8.5) for 60 min and inorganic (reactive) phosphorus is determined. To quantify dissolved organic phosphorus samples have to be digested to inorganic phosphorus. For calculation see chapter 2.2.1.

2.3.3. Alkaline persulphate digestion

Soluble (dissolved) organic phosphorus forms have to be converted to inorganic phosphorus (ortho-phosphate, P_i) to apply the colorimetric assay of phosphate concentration, see below. Persulfate oxidation therefore allows measuring total P, subtraction of inorganic P before persulfate oxidation gives the fraction of dissolved organic P. The method detailed below is suitable for soil solutions, surface water and precipitation samples. Soil extracts may be analyzed up to 0.5 M K_2SO_4. However, it is

not suitable for either solid samples or samples that contain a high amount of dissolved organic matter.

Prepare external standards from an organic standard solution: 0.10, 0.2, 0.4, 1.0, 2.0 and 4.0 mg P L^{-1}. For each batch of samples, at least three blanks without any phosphorus must be included. For every 10 samples at least one laboratory standard shall be processed. Add 10 mL of samples or standards to a 50 mL digestion flask (Only use Schott glass flasks with red caps that are autoclavable). If necessary, add water to reach a volume of 10 mL. Always keep a ratio of 15.0: 5.0: 0.25 (v: v: v) = sample: persulphate: NaOH. Add 3.5 mL of persulfate solution (0.148 M; dissolve 20 g $K_2S_2O_8$ in 500 mL of water, solution can be used for a week if kept at 4 °C) and 0.175 mL of NaOH (3 M; dilute 78.45 mL 50% NaOH with water to give 500 mL solution). Seal the flasks immediately and weigh. Autoclave flasks at 121 °C and 17 psi for 30 min. Cool the flasks to room temperature, wipe dry on the outside and weigh flasks. From the difference of the weight before and after the digestion, a loss of water can be calculated. Transfer samples to 30 mL plastic bottles and store at 4 °C for not more then 1 week. Determine phosphate concentration as given below.

Calculations

DOP (µg P L^{-1}) = TP – P_i

where, TP (total dissolved phosphorus) = phosphate-P in solution after digestion, based on a calibration curve constructed from organic standards after digestion, and Pi is dissolved inorganic phosphorus from the same sample before digestion. For further calculation see chapter 2.2.1.

2.3.4. Total phosphorus

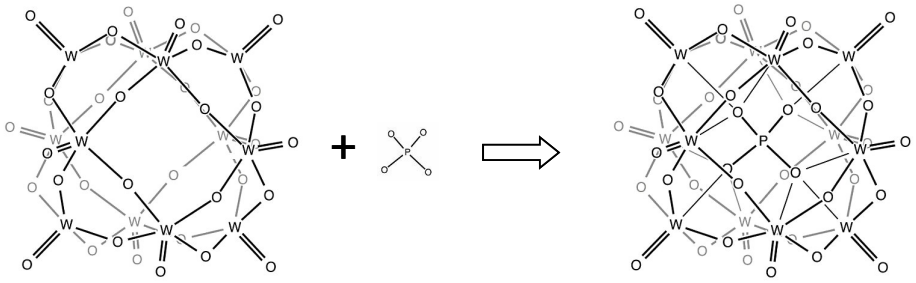
Acid digestion with HNO_3 and $HClO_4$ dissolves and releases P from organic and non-silicate inorganic forms (see 2.4.), inorganic phosphorus thereby produced is determined by colorimetry.

2.3.5. Phosphate determination (colorimetry)

Principle

Determination of phosphorus is based on the use of heteropoly-molybdenum blue. A sample containing phosphate is mixed with an acid solution of Mo(VI), for example ammonium molybdate, to produce a phosphoantimonyl-molybdenum complex ($PMo_{12}O_{40}^{3-}$), which has an α-Keggin structure (see below). This anion is then reduced by ascorbic acid to form the blue coloured mixed valence complex i.e. the β-Keggin ion, $PMo_{12}O_{40}^{7-}$. The amount of the blue coloured ion produced is proportional to the amount

of phosphate present and the absorption is measured using a photometer to determine the amount of phosphorus.



Keggin structure: The structure is composed of one heteroatom surrounded by four oxygen to form a tetrahedron. The heteroatom is located centrally and caged by 12 octahedral MO6-units linked to one another by the neighboring oxygen atoms.

Solutions

Ammonium heptamolybdate stock solution (0.01M): Dissolve 1.26g $(NH_4)_6Mo_7O_{24}.4H_2O$ in 40 mL Milli-Q. Add 14 mL conc. H_2SO_4 (beware: strong heat development) and let cool before adding 40 mL Milli-Q. Add 0.05g potassium antimony (III) oxide tartrate-hydrate ($C_8H_4K_2O_{12}Sb_2$. xH_2O) and fill to 100 mL end volume with Milli-Q.
Ammonium heptamolybdate working solution (0.001M): Dilute stock solution 1:10.
Ascorbic acid solution: Prepare fresh daily. Dissolve 0.044g in 10 mL Milli-Q.

Standards

Phosphate stock solution (0.1 g P L^{-1}): Dissolve 0.0439g potassium di-hydrogen phosphate in 100 mL Milli-Q, or the respective solvent such as 0.5 M $NaHCO_3$ or 0.5 M HCl.
Working solution (10 mg P L^{-1}): Dilute 10 mL phosphate stock solution with 20 mL 0.5 M $NaHCO_3$ and fill up to 100 mL with water. Dilute this working solution in a 1:2 dilution series in eight vials to 5, 2.5, …, 0.039 mg P L^{-1}.

Sample preparation in case of HCO_3^- extracts

The high concentration of bicarbonate buffers the extracts and standards at ca. pH 8.0 to 8.5, while the colorimetric reaction runs only at very acidic pH and therefore is strongly interfered by bicarbonate. Therefore, bicarbonate is first released as CO_2 by addition of an (>) equivalent amount of HCl. Pipette 2 mL 0.5 M bicarbonate extract or standard in wide plastic vials (e.g. 10 mL snap cap Greiner tubes) and add 1.1 mL 1 M HCl. Wait for ca. 60 min until the strong CO_2 development stops, and proceed with photometric measurement.

Photometric measurement

Pipet each 100 µL sample, standard or blank (0.5 M $NaHCO_3$ or acid) with 80 µL ammonium heptamolybdate working solution and 50 µL ascorbic acid solution. Read absorbance after 15 min at 882 nm.

Calculation

P_i [µg P g^{-1} dry soil] = cs (µg P mL^{-1}) x V (mL) / W (g dry soil)

For details see ammonium determination chapter 2.2.1.

2.4. Total cations and P (acid digestion)

Nutrients such as phosphorus, calcium and magnesium are to a large fraction bound in organic form (organic phosphorus), in complexes (chelates) or are tightly attached to organic molecules by ion-exchange forces (e.g. calcium to cell walls). To determine the total concentration of these elements organic matter first has to be oxidized before concentrations of phosphorus or calcium can be determined. Organic matter can be dry-oxidized in an air stream or in a muffle oven or be oxidized by acid digestion. Both procedures do not release P, calcium or magnesium from primary and many secondary minerals such as apatite and biotite in soils (HF digestion!).

Sample aliquots (200 mg litter and organic soil, 500 mg mineral soil) are weighed into narrow-bore Erlenmeyer flasks (50 mL) which have been pre-cleaned by rinsing with 1:10 diluted HCl and copious Milli-Q. Five mL of 1:4 solution of conc. $HClO_4$ and conc.HNO_3 (suprapure quality) is added and mixed. The Erlenmeyer flasks are then positioned on a heating plate (Ceran) and the temperature slowly increased to 160°C. This has to be done with uttermost care to avoid cooking, e.g. heat for 30 min at 100°C, 20 min at 120°C and then increase to 160°C. This temperature is kept for ca. 60-90 min until the fumes that are produced in the flask turn yellowish. (Caution: the fumes that develop are highly toxic! Perform acid digests in a fume hood). The temperature is then increased to 220°C until the fumes turn whitish (keep 10 more min). Take samples off the heating plate when this condition is met, samples close to the exterior of the plate take longer. Let samples cool to room temperature and fill to 25 mL with Milli-Q water in measuring bulbs. Filter the samples through ash-free cellulose filter paper.

Cations and phosphorus are measured as described in chapter 2.1. and 2.3.5.

2.5. Microbial biomass C, N and P (TOC/TN analyzer)

Principle

Microbial biomass represents 1-3% of total soil C and 3-5% of soil N. Though being a small fraction it is highly important as player in biogeochemical cycles, as a transient soil pool of C, N and P, and as a precursor of stable soil organic matter. Microbial biomass

can be determined by microscopy, by measurement of cell constituents released upon fumigation, by substrate-induced respiration or by quantification of biomarkers such as phospholipids fatty acids, ergosterol, or DNA. Here we will apply chloroform-fumigation extraction (CFE) to estimate microbial biomass C and N (C_{mic}, N_{mic}). Chloroform fumigation kills soil microbes by disintegrating their biomembranes and denaturing enzyme systems. Soluble intracellular components are then extracted and quantified. In parallel soluble organic carbon and total dissolved N is determined in unfumigated controls and is subtracted from fumigated samples. The difference in DOC and TDN between fumigated and non-fumigated samples (in chloroform fumigation extraction - CFE method) is related to the microbial biomass using k_{EN} factors (fraction of soluble and extractable N and C to total microbial N and C). Alternatively (extraction fumigation extraction, EFE method), DOC, TDN and TDP can be pre-extracted from unfumigated soils with 0.5 M K_2SO_4, and this pre-extracted soil then be taken to directly extract microbial C, N and P by addition of K_2SO_4 extractant + liquid chloroform.

Procedure CFE

Weigh duplicate samples of 2 g fresh soil, one replicate in 20 mL plastic vials (non-fumigated controls) and one replicate in aluminium dishes (fumigated sample) which have been labelled accordingly with sample codes engraved in the bottom using a pencil (Note: do not use Edding markers, which will dissolve in chloroform). Non-fumigated controls are extracted immediately with 15 mL 1 M KCl (see below). For fumigation-incubation fill about 100 ml of chloroform in a glass beaker and place it in a glass desiccator. To remove ethanol from $CHCl_3$, mix the commercially-available chloroform with Milli-Q water (1:1), separate phases in a separating funnel and discard water phase. Put samples in the desiccator, carefully evacuated until the chloroform boils or chloroform fumes can be smelled at the outlet of the vacuum pump (2 x 10 min), and then incubate for 48 h in the dark at room temperature. Transfer the fumigated soils in 20 ml plastic vials and add 15 mL 1 M KCl (74.5 g KCl in 1 L). Mix vigorously and shake for 1 h at room temperature. Filter extracts through ash-free filter paper. The extracts can be stored at -20°C.

Procedure EFE

Weigh samples of 2 g fresh soil in 20 mL plastic vials and extract with 15 mL 0.5 M K_2SO_4 for 60 min. Filter the extract through ash-free filter paper and keep this extract for DOC, TDN, and TDP measurements. Now transfer the rest of soil from the extraction vial into the filter by repeated addition of extractant to the vial, shaking and pouring the suspension into the filter. For direct "fumigation" i.e. liquid chloroform treatments take the filter and transfer it into a pre-weighed 45 mL plastic tube. Note the weight of the wetted filter plus wet soil, add 15 mL 0.5 M K_2SO_4 plus 1 mL chloroform and close the

vial tightly. Put these vials flat on a horizontal shaker and agitate for 2 hours. Filter the K2SO4 – chloroform extract through ashfree filter paper and keep for measurement of microbial N and P (and C). The extracts can be stored at -20°C.

TOC/TN analysis

Dissolved organic C and total dissolved N is determined by TOC/TN-analyzer. TDN is quantified by high temperature catalytic oxidation (HTCO) at temperatures around 680°C, and a catalyst (e.g., Pt) to complete the oxidative conversion of all forms of C to CO_2 and all forms of N to NO and NO_2. NO and NO_2 is then reacted with O_3, producing an excited state of NO_2 (NO_2*). Upon returning to ground state, light energy is emitted which is measured by chemiluminescence detection. It is possible to investigate the content of total organic carbon (TOC) in water samples using the difference method (TOC = TC – TIC) as well as the addition method (TOC = POC + NPOC). Here, TOC is measured as non-purgeable carbon (NPOC) where after in-syringe acid addition of acid the samples are purged with synthetic air to release inorganic carbon (TIC). CO_2 is quantified by Non-Dispersive Infrared (NDIR) detection. The NDIR detector consists of a light source, a cell, and a detection portion. The detector utilizes a movable diaphragm that is flexed with changes in CO_2 concentration. Light passes through the cell compartment and into the detector. Depending on the concentration of carbon dioxide generated from the sample and consequently the amount of light that passes through the quartz window, the movement of the trapped carbon dioxide within the detector compartment will flex the diaphragm. The flexing of the diaphragm creates electric signals which correspond to the concentration of TOC in the sample. Depending on the reactor makeup the extended working range for TOC is from 4 µg L^{-1} to 25,000 mg L^{-1}, for TN the working range is 0.1 up to 4,000 mg L^{-1}.

Analyzer: Shimadzu TOC-VCPH Total Organic Carbon Analyzer with TNM-1 Total Nitrogen Measuring Unit and ASI-V Autosampler

Calibration and sample analyses

Samples and standards (8 mL) are transferred into glass vials that have been pre-cleaned by heating in a muffle furnace at 450°C for 4 hours. Calibration of the system is performed with a solution containing 1000 mg C L^{-1} and 100 mg N L^{-1}. The solution is prepared from 268.11 mg glycine, 180.55 mg $(NH_4)_2SO_4$, 117.98 mg KNO_3, 2.173 mg sucrose L^{-1}, corresponding to ½ of total N contained in glycine and ¼ of total N in ammonium and nitrate each. The stock solution can be stored at 4 °C for about 2 months. Calibration is done two-fold: (1) automatic in-syringe dilution function of the analyzer system (internal calibration), and (2) external standards diluted from 10 to 0.1

mg N L^{-1} (external calibration). Additionally blanks are analyzed containing Milli-Q or the respective extraction solutions.

Calculation for Chloroform-fumigation extraction (CFE)

Microbial biomass N = $1/k_{EN}$ x (TDN_{FUM} – TDN_{NON})

Microbial biomass C = $1/k_{EC}$ x (DOC_{FUM} – DOC_{NON})

Calculation for pre-extraction method

Microbial biomass N = $1/k_{EN}$ x TDN

Microbial biomass C = $1/k_{EC}$ x DOC

where k_{EN}=0.54 (k_{EC}=0.35) and gives the extractable part of the biomass N (C), and TDN_{FUM} – TDN_{NON} (respectively, DOC) is the difference in total dissolved N (C) concentration between fumigated and non-fumigated soil extracts. TDN and DOC concentrations in the equations given above should be in µg g^{-1} dry soil!

2.6. Phospholipid fatty acids (GC)

Principle

Phospholipid fatty acids (PLFA) belong to the "whole-community" methods to analyze soil microbiota. The PLFA method is based on the extraction of phospholipids which are the core components of the biomembranes of all living organisms, and the analysis of their fatty acid composition. PLFAs can be used as biomarkers since some fatty acids occur only in specific, phylogenetically defined groups, such as certain bacterial or fungal classes (e.g., α-proteobacteria, actinomycetes, etc.). The occurrence and level of specific PLFAs therefore allows estimating the abundance and metabolic activity and tracer incorporation (e.g. ^{13}C) of such groups. Upon death of microbes PLFAs are quickly degraded and thus can be attributed to living organisms only.

General remarks

- Use only glassware throughout the procedure.
- Put clean glass vials and Pasteur pipettes into the muffle furnace at 500°C for 3 hours prior to use. Teflon seals and plastic caps are washed three times with Milli-Q water and dried. Rinse with hexane afterwards and dry them again.
- Used glass equipment, seals and caps shall be soaked in washing liquid overnight to remove fatty acids, rinsed several times with water and Milli-Q water and dried.
- All chemicals should be of highest purity available.
- Avoid using plastic vials or other plastic parts (such as pipette tips) throughout the procedure. If necessary to use them (for pipetting small amounts < 0.5 ml), rinse tip with the liquid you want to pipette.

- Un-powdered latex or vinyl gloves should be worn at all times.

Working procedure I (Soil extraction)

Soils: Determine water content of soils samples prior to extraction. Weigh samples of 0.5 – 2 g of soil in triplicate into 30 mL extraction tubes, i.e. 1 g (A horizon) to 2 g (Bg horizon) of mineral soils, and 0.5 g of organic soils (Ap or O). Note exact sample weight. Soils should be then quickly processed, as phospholipid fatty acids are degraded as soon as microbes die. One blanks should be done for each batch of samples (Blanks = reagent blanks; no soil, same procedure).

Extraction: Add citrate buffer (0.15 M, pH 4.0, with NaOH), so that the total volume of water in the vial is 1.5 mL. The water content of sample plus the amount of buffer should add to 1.5 mL in total. Tare the vial plus soil and add buffer with glass Pasteur pipettes. Add 1.9 mL chloroform, 3.7 mL methanol, and 2.0 mL CMB (chloroform: methanol: citrate buffer = 1: 2: 0.8 (v: v: v). Vortex for 30 s and let settle overnight at room temperature. Centrifuge at 1. 500 g for 7 min. The caps of the extraction tubes (PTFE/rubber) should be washed with hexane or acetone prior to use. Transfer the supernatant into a 30 mL glass vial. Use glass Pasteur pipettes. Re-extract the pellets with each 2.5 mL CMB (vortex 30s, incubate 30 min and centrifuge as above). Add the supernatant to the same glass vial as before.

Phase separation: Add 3.1 mL chloroform and 3.1 mL citrate buffer and vortex for 1 min. Allow the samples to stand for 24 hours for phase separation. Take out exactly 3 mL of the lower (organic) phase by means of a glass Pasteur pipette and transfer into a fresh 10 ml vial. Weigh the exact volume of sample, if you don't have the possibility to exactly pipette 3 mL. It is easier to pipette, if the upper phase is removed first.

Storage: Dry the extract at 40°C under a constant stream of N_2. Immediately after drying close the vial and store at –20°C. The samples are dried under N_2, to avoid oxidation of fatty acids. This takes approximately 30 min.

Working procedure II (Lipid fractionation)

Conditioning: Connect SI (silicate)-columns to the SPE block, add a blue connector and a reservoir (rinse all device components with hexane first and dry) and put 20 mL plastic under the outlet to collect the waste. Condition SI-columns with 6 mL chloroform. Let the liquid flow through by gravity only. The SI substance will shrink and give a clear band under the frit which won't interfere with the procedure. Pour the waste into the organic solvent waste bottle. Add 0.5 mL of chloroform to the sample and dissolve for 15 min at

room temperature. Disconnect the sample reservoirs and adaptors and apply the sample directly on top of the SI-column. Let the liquid run through by gravity.

Sample loading/elution: Elute neutral fatty acids with 5 mL of chloroform. Connect adapters with the syringes onto the SI-columns and put 20 mL plastic vials beneath. The neutral phase is discarded or can be saved for determination of ergosterol or polyhydroxybutyric acids (in which case this fraction is dried under N_2 and store at -20°C). Apply 20 mL acetone to the SI-columns. Put 20 mL plastic vials for waste under the SI-columns. You can attach the device to a water-jet vacuum pump to speed up the process of the liquid running through. Put the waste to the organic solvent flask. Apply 5 mL methanol to the SI-columns to elute the phospholipid fraction. Put 10 mL glass vials under the SI-columns. Let the liquid run through by gravity.

Storage: Dry the methanol eluate at 40°C under a constant stream of N_2. Immediately after drying close the vial and store at –20°C. See above!

Alkaline methanolysis: Prepare 1 mL 0.2 M methanolic KOH (3 KOH pellets in 50 mL methanol) for each vial, dissolve in an ultrasonic water bath. Add 100 µL internal standard to each sample. Prepare 1.2 mL methanol: toluene (1:1, v: v) for each sample. [Internal standard: Dilute the stock solution (20 mg mL^{-1} in methanol: toluene 1:1) 1:100 with methanol: toluene (e.g. 10 µL Standard + 990 µL mixture).] Add 1mL methanol: toluene mix and 1 mL 0.2 M methanolic KOH. Incubate at 37°C in the water bath for 15 min. Prepare 4 mL hexane: chloroform 4:1 per each sample (+reserve). Add 2 mL hexane: chloroform (4:1, v: v) and 2 mL Milli-Q and 0.3 mL pure acetic acid, shake vigorously for 1 min and centrifuge for 10 min (2500 rpm, Beckman centrifuge, rotor F 683). Put paper to the wells in the rotor to avoid breaking of the glass vials. Check pH (4-6) after shaking the vials. Transfer the upper, organic phase to a new 10 mL glass vial..

Storage: Dry the extract at 40°C under a constant stream of N_2. Immediately after drying close the vial and store at –20°C.

GC analysis

Add 100 µL iso- octane to the vial. Prepare external standard (BAME). Dilute the stock solution (10 mg mL^{-1}) by 1:10 and 1:5 (two replicates each). Transfer 50 µL of the sample to GC vials with 0.3 mL inserts.
Further details on the separation program are given at the GC.

2.7. Total N and C (elemental analyzer)

Principle

Most commonly total N and C contents of ecological samples such as soils, plants and animals are analysed by dry combustion at high temperature (~1000°C) and analysis of the gases produced thereby, i.e. CO_2 and N_2. Samples are prepared in tin capsules which drop into the oxidation reactor with an oxygen enriched helium stream where the samples combust immediately. Gases are transferred by a continuous helium stream through the oxidation and reduction oven (trap excess O_2 and reduce NO_x to N_2), a water trap and a GC column. The GC column is filled with a molecular sieve that allows entrance of CO_2 in the pores of the mol sieve but excludes N_2 from entering (size exclusion chromatography). Sample CO_2 and N_2 in the helium stream are quantified by thermal conductivity detection (TCD). A TCD detector consists of an electrically-heated wire or thermistor. The temperature of the sensing element depends on the thermal conductivity of the gas flowing around it. Changes in thermal conductivity, such as when organic molecules displace some of the carrier gas, cause a temperature rise in the element which is sensed as a change in resistance.

Preparation

Soils containing carbonate have to be treated with acid to remove carbonates as CO_2 before measuring soil organic C content, e.g. by adding small amounts of 1 M HCl until bubbling stops and drying in an oven (80°C) afterwards or by fuming with concentrated HCl in a desiccator for 48 hours. Since the sample amounts needed are very low samples have to be ground to a very fine powder to allow analysis of a representative aliquot. Soil, litter or plant samples are first dried at 80°C overnight and then homogenized in a ball mill (Retsch MM2000) to particle size <10 µm. Sample are weighed into tin capsules, the sample sizes being 1.5-2.0 mg for plant material, litter and organic soil and 5-10 mg for mineral soil. Tin capsules are closed tightly and folded to a small ball or cube and stored in microtiter plates. Sample weights, codes and positions in the MT plate are noted. Liquid samples can be pipetted into special tin capsules (placed in 1.5 mL plastic vials) and dried in the Speed Vac.

Analysis

Analyzer: Flash EA 1112 (CE Instruments), coupled to an isotope-ratio mass spectrometer (see below)
Oxidation reactor: chromium oxide, silvered cobaltous oxide, 1020 °C
Reduction reactor: copper oxide, copper wire, 640 °C
Helium flow rate: 120 mL min^{-1}
Oxygen flow rate: 120 mL min^{-1}
Purge flow (Helium, Autosampler): 200 mL min^{-1}

2.8. Stable isotope determination (IRMS)

Principle

Virtually all chemical elements of biological importance occur naturally in the form of multiple stable isotopes, including the light elements such as nitrogen (^{14}N, ^{15}N) and carbon (^{12}C, ^{13}C). The ratios of these stable isotopes show natural variability in biotic and abiotic compounds of ecological interest which are the result of isotope fractionation. Isotope variability therefore is the result of mixing of isotopically different sources, selectivity for isotopically different molecules (isotope discrimination), diffusional constraints and interactions between processes. Stable isotope investigations can therefore provide insights into fluxes of matter among organisms, between organisms and their abiotic environment, and between compartments of the abiotic environment.

Isotope ratio mass spectrometry

The natural abundance of light stable isotopes such as carbon and nitrogen is measured by isotope ratio mass spectrometry (IRMS). As the natural isotopic differences are very small, very precise instrumentation is needed. Isotopes of H, C, N, O and S are measured in gases e.g. CO_2 and N_2 which are introduced into the ion source of the mass spectrometer. In the ion source the gases are ionized by electron impact and the gas cations are then accelerated and focused to an ion beam. The ions are deflected in an electromagnetic field effectively separating cations of different mass/charge (m/z) ratio which are then counted. Measuring the absolute isotopic composition, particularly in the long term, is not as reliable and convenient as measuring isotopic differences between a sample and a defined standard. This differential analysis approach allows very small differences in the isotopic composition of two samples to be accurately and reliably determined. Isotope ratios therefore are measured against international reference materials (carbon: Vienna-Pee Dee Belemnite, V-PDB; nitrogen: atmospheric dinitrogen, at-air) and given in the delta (δ) notation. The delta unit expresses the relative (‰) deviation of the isotope ratio of a sample from that of the international standard. Therefore:

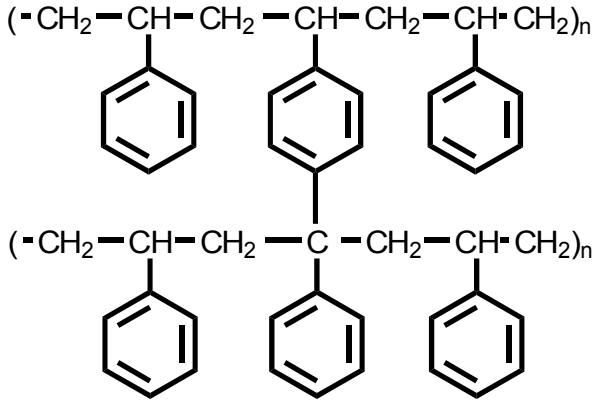
$\delta^{13}C$ [‰ vs. V-PDB] =
= $(R_{sample}/R_{ref}-1) *1000$, where R is the atomic ratio of $^{13}C/^{12}C$ of sample and reference, respectively, and

$\delta^{15}N$ [‰ vs. at-air] =
= $(R_{sample}/R_{ref}-1) *1000$, where R is the atomic ratio of $^{15}N/^{14}N$ of sample and reference, respectively.

2.9. Fractionation of soluble organic compounds

Principle

Several soluble organic compounds in soil and litter are bioactive, acting as a microbial substrate (sugars, amino acids) or affecting microbial processes (secondary plant compounds e.g. Polyphenols). Amberlite resins can be used to adsorb, isolate and pre-concentrate lipophilic compounds (e.g. Polyphenols) due to lipophilic interactions between compounds and the resin. (Note: Fatty acids and benzoic acid are – though lipophilic – not adsorbed to Amberlite XAD). Hydrophilic low-molecular weight compounds (LMWC) such as sugars, amino acids and organic acids are not retained by Amberlite XAD and therefore are recovered in the water phase. Lipophilic substances are thereafter desorbed with ethanol.



Amberlite™ resin

Preparation and conditioning of the resin

10 g Amberlite XAD 1180 is hydrated overnight in 200 mL water (Milli-Q). The material is sufficient to fill four columns. Each 50 mL suspended Amberlite is filled into glass columns. A plug of glass wool is put at the bottom of the column and one on the top to fix the resin bed. Before use the columns are rinsed with 50 mL water.

Fractionation

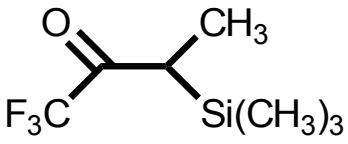
Samples (10 mL) are loaded onto the columns and rinsed first with 10 mL and then with 50 mL Milli-Q, the flow-through being collected in appropriate collection flasks (aqueous fraction). Columns are then eluted with 75 mL ethanol absolute (amended with 0.5% acetic acid) to collect the non-aqueous fraction. Each fraction is vacuum-concentrated to 1-2 mL on a rotary evaporator before being stored in sample vials at -20°C.

Analysis of low-molecular weight substances (LMWC) by GC/MS

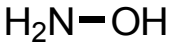
LMWC are analyzed by gas chromatography using a mass selective detector (GC-MS).

Preparation: The optimum concentration of samples is ~3 mg mL^{-1}. To obtain this concentration the fractions are transferred into pre-weighed glass micro inserts for

sample vials where they are dried overnight in a Speed Vac system. Sample dry weights are determined. To quantify hydrophilic non-volatile compounds by GC the substances are first chemically altered (derivatisation) to obtain volatile compounds. The following derivatisation is performed at room temperature:
1 hour at 75°C: 30 µL methoxylamine solution (20 mg mL^{-1})
1 hour at ambient temperature: (MSTFA)
Sample concentrations can be adjusted by adding pyridine.



MSTFA
(*N*-methyl-*N*-trimethylsilyl-trifluoroacetamide)



hydroxyl amine

GC-MS analysis
Oven: temperature 70 to 310°C, 5°C min^{-1}
Carrier gas Helium: 0.8 mL min^{-1}
Column: DB-5 (5% phenyl methyl silicone), 30 m, 0.25 mm ID, 0.25 µm film thickness
Transfer line: 250°C
Ion source: 200°C
Injection: 1 µL in splitless mode at 250°C

<u>Analysis of polyphenols (HPLC-PAD)</u>
Polyphenols are analyzed by liquid chromatography and UV-diode array detector (HPLC-PAD). The fractions are transferred into pre-weighed glass sample vials and are dried overnight in a Speed Vac system. Sample dry weights are determined. Samples are redissolved in methanol (Lichrosolv, HPLC grade quality), the amount optimized to reach a sample concentration of 10 mg mL^{-1}.

HPLC analysis
Column oven: 40°C
Mobile phase: 5% methanol in *o*-phosphoric acid (O.5%, v/v), increase to 100% methanol in 100 min
Column: Microbore, RP-18, 150x2 mm, 4 µm particle size
Injection: 5 µL
Detection signal: 229 nm
UV-spectra: 600–220 nm

3. DETERMINATION OF ACTIVITIES

3.1. Soil respiration and substrate induced respiration (IRGA)

Infrared gas analyzer (IRGA)

Heteroatomic gases such as CO_2 and H_2O absorb infrared light, the energy inducing or increasing molecular vibrations in all directions in space. The sum of molecules thereby experiences warming, inn a closed vessel pressure increases. In an infrared gas analyzer (IRGA) a CO_2 free reference gas and sample gas are simultaneously exposed to the same intensity of infrared light. Depending on CO_2 concentration a variable fraction of infrared light is absorbed, non-absorbed light is measured. The detector consists of a CO_2 filled chamber that is divided by a membrane. Residual infrared that is absorbed produces pressure differences that are measured electronically and presented in ppm CO_2.

Basal and substrate-induced soil respiration

IRGA allows continuous measurement of CO_2 production in soils *in situ* using soil chambers. Soil respiration in situ includes heterotrophic respiration (microbes feeding on soil organic matter; fauna) and autotrophic respiration (plant roots and rhizosphere microorganisms). Moreover, IRGA is also applied to measure basal soil respiration (BR) and substrate-induced soil respiration (SIR) in sieved soils. Both measures are highly dependent on microbial activity and SIR has been used to estimate soil microbial biomass. If substrates such as glucose or arginine are added to sieved soils, substrate-induced respiration can be quantified.

Analysis

SIR: 30-50 g field moist soil, sieved to 2 or 5 mm is weighed into plastic bags (2 L, hang into plastic beaker). Sugar (glucose, 0.2% of soil fresh weight) or amino acid (arginine, 0.6% f.w.) is pre-weighed and shortly before starting the measurement added to the respective bag. Mixing is achieved by filling the bag with air, and while keeping tightly closed, shaking the mixture vigorously. The SIR treatments are then filled in PVC soil cuvettes (bottom lined with tissue). For basal respiration untreated soil is filled into the soil cuvettes. Soil cuvettes (six can be measured at the same time) are connected to the open gas circuit by change-over switches and are kept at room temperature. The samples are continuously purged with CO_2-free air (or 100 ppm CO_2) at a flow rate of ~6 L h^{-1}. The CO_2 concentration that increases through soil respiration is measured at the outlet of each cuvette every 30 min by IRGA.

Calculations

Respiration rates are given in mg CO_2 h^{-1} kg^{-1} fresh or dry soil.

(Avogadro constant: 22.4 L mol^{-1} gas at normal conditions)
Kinetics of substrate-induction (slope by graphical extrapolation) and relative induction (Response: SIR-BR in % of BR = RESP) will be calculated.

3.2. MicroResp and BIOLOG systems (photometry)

Both systems can be used to assess community level physiological profiles (CLPP) in soils, litter and sediments, and therefore to estimate the functional diversity of soil microbial communities. This is done by assessing respiration or dehydrogenase activity is the presence a wide variety of different carbon sources. In contrast to MicroResp the BIOLOG system does not provide an estimate for the whole microbial community but only for the part that can be cultured under the conditions provided.

3.2.1. MicroResp

Principle

MicroResp is a microplate based respiration system which allows 96 whole soil, litter or sediment samples to be analysed simultaneously, for basal respiration or substrate-induced respiration testing a range of carbon sources. In contrast to the BIOLOG system which tends to select for fast-growing bacteria (gram-negative bacteria; less sensitive for gram-positive bacteria, no detection of fungi) and relies on the growth of organisms, MicroResp gives more immediate responses to the substrates, reflecting the respiratory activity rather than growth and represents the whole microbial community. CO_2 concentration (rather than the respiration rate) is measured in each well using a pH sensitive indicator (cresol red, buffered in bicarbonate solution) that shows discoloration (red → yellow) when the pH decreases in the bicarbonate buffer due to dissolution and hydration of CO_2. The MicroResp system consists of a 96-deep well microplate plate to hold soil samples, a 96-well detection plate, the blue MicroResp seal and a metal clamp to hold the three parts firmly together.

Preparation of soil samples

For comparison of carbon source use (CLPP) the filling device can be used. Otherwise it is advisable to weigh in triplicate samples per substrate (soil: 0.2-0.5 g, litter: 0.1-0.2 g fresh weight) in aluminium dishes and transfer them using a small plastic funnel into the tray. Tap the plate on the lab bench to pack the aliquots into the wells. Close the filled soil wells with small strips of Parafilm to hinder drying. Note the weights. Soil moisture should be within 30 and 60% of maximum water holding capacity (not drier or wetter).

Preparation of detection plates

Prepare 3% purified agar (3 g in 100 mL Milli-Q) and heat to 120 °C until the agar dissolves (autoclave or drying oven). Keep the agar in a glass beaker at 65 °C in a drying oven until further use. Prepare indicator solution (1 L: 18.75 mg cresol red, 16.77 g KCl, 0.315 g $NaHCO_3$) – to dissolve add first only 0.5 L water that has been warmed to 50 °C (may take 2-3 hours), then fill up to end volume. Combine in a glass beaker (pre-warmed) 1 part of agar with two parts of indicator solution that has been warmed to 65 °C. Mix thoroughly. Using a Multipette syringe (warmed to 65 °C) dispense each 150 µL into the wells of the detector plate, taking care to pipet the agar slowly into the centre of the well omitting inclined surfaces and trapping bubbles! (Practice first). The plates are then stored in the dark in a small desiccator or plastic box containing soda lime and wet tissue paper (CO_2 free atmosphere). For longer storage close the microplates with Parafilm. Directly before assembling the system the detection plate to be used is measured in a microplate reader at 570 nm.

Preparation of substrates

Substrates are prepared in a concentration of 400 mg in 10 mL and are added in 50 µL aliquots to the soils. Substrates can be stored at 4 °C for two weeks or frozen for up to six months. Substrate addition should be performed fast for each plate since adding carbon sources increases respiration within minutes.

Measurement

After substrate addition the blue MicroResp seal is applied to the deepwell plate. Then the indicator plate that has been measured at 570 nm immediately before (time 0, store electronically) is placed on the MicroResp seal – be aware that soil sample in slot A1 is measured by slot A12 of the indicator plate. Apply pressure until the seal sits correctly and closes the contact between both MT plates. Then close the metal clamps and incubate for 6 hours at room temperature in the dark. Disassemble, peel off the seal and repeat absorbance measurement at 570 nm. A new or regenerated indicator plate may be attached to continue respiration measurements over time. Indicator plates can be regenerated in a plastic box containing soda lime and a wet tissue paper, re-equilibration takes between 24 and 36 hours after which the indicator gel returns from yellow/orange to dark red/purple. Deepwell plates can be cleaned from soil and be reused.

Calibration and calculations

Export absorbance values of time 0 and time 6 to Excel and transpose data in columns. To normalize the absorbance data for t6 divide by absorbance value at t0 and multiply by mean absorbance of the t0 plate.

$A_{corr\ t6} = A_{t6} / A_{t0} \times Mean_{plate\ t0}$

Absorbances can then be converted to CO_2 concentration using a rectangular curve fit (MicroResp manual, to be adapted in the ChECO laboratory):

$\%CO_2 = -0.2265 + -1.606 / (1 + -6.771 \times A_{corr\ t6})$

Respiration rate is calculated as following:

Resp (µg CO_2-C g^{-1} dry soil h^{-1})

$= H_{vol} /100 \times \%CO_2$ /gas constant x Mr /hours /soil d.w.

Headspace volume in the well, H_{vol} (945 µL), gas constant (22.4 L mol^{-1}), molecular weight of CO_2-C, M_r (12), time of incubation, hours (e.g. 6) and soil dry weight (soil d.w. = soil fresh weight per well in grams x f.w./d.w. factor).

The conversion of absorbance to $\%CO_2$ (or directly to Resp) has to be calibrated by parallel measurement of soil respiration by IRGA or Isermeyer (CO_2 absorption in NaOH, back-titration of residual NaOH), by exposing strips of indicator plates in flasks containing CO_2 reference gases of known concentration or in flasks with respiring soils with subsampling of CO_2 for GC quantification.

3.2.2. BIOLOG

Principle

Biolog Microplates monitor cell respiration. A cell that utilizes a substrate present in one of the 96 wells of the Microplate begins to respire thereby creating NADH. The production of NADH by the cells reduces a tetrazolium dye (TTC, triphenyl-tetrazolium chloride) to triphenyl-formazan, a kind of redox reaction. The microbes within the community will create characteristic patterns within the wells of the Microplate. These patterns are monitored over several days, and evaluated using Biolog software. Before, microbes are extracted from soils in buffered saline solution and a diluted suspension is added to the wells that contains a range of different substrates plus macro- and micronutrients. Colour formation is regularly measured with a microplate reader (every 6-12 hours).

Preparation

Soil aliquots (1 g fresh material) are suspended in each 10 mL Ringer solution (2.25 g NaCl, 0.105 g KCl, 0.12 g $CaCl_2$, 0.05 g $NaHCO_3$ per L Milli-Q) on a horizontal shaker for 30 min. Let the suspension stand on the lab bench for 30 min or centrifuge 1 min at 500 rpm. The supernatant is diluted 1:100 with Milli-Q or Ringer solution and 100 µL diluted medium is pipetted into the respective wells. The different substrates (60 µL) are added to the diluted soil suspensions while taking care not to contaminate the plates. Blanks are prepared by adding 160 µL Milli-Q, 160 µL Ringer solution or substrate plus water in wells of the MT plate. BIOLOG plates are incubated at 25 °C in the dark after taking the first absorbance reading at 542 nm directly after finishing the plates. Substrates are

prepared as 0.5% solutions: Asparagine, isoleucine, glycine, glutamine, glucose, sucrose, starch, methylcellulose, urea, bovine serum albumin (BSA), as well as mixtures of KCl, $CaCl_2$, $MgCl_2$, KNO_3, NH_4Cl or K_2HPO_4 with glucose. Take further readings every 12 hours.

Calculations

Absorbance shows a typical growth curve type kinetic, with a lag phase, log phase and stationary phase. Depending on the substrate the start time of log phase, the slope of log phase increases and maximum absorbance reached can differ greatly. Readings are normalized to the time 0 measurements (by subtraction). Positive substrate utilization is set to a threshold of absorbance increase of >0.1. For further calculations a time point where most carbon sources show detectable readings in the log phase is selected.

3.3. Trace gas isotope analysis (Gas Bench, IRMS)

Principle

To measure the stable isotope composition of atmospheric CO_2 or of CO_2 produced during respiratory processes headspace gas analysis with IRMS is used. Exetainers with sample gas are put into the headspace autosampler GC-PAL. During sample analysis a two-way needle is pushed into the vial and continuous addition of Helium at a low flow rate displaces sample gas from the sample vial. The sample gas is transferred into the GasBench where it is dried online through two Nafion traps and multiple injections of 100 µL sample on the GC are performed by a Valco 6-port valve. The Nafion trap is a hydrophilic membrane that absorbs water from the gas stream and transfers it to a drying gas flow passing over the outside of the membrane. Sample gas is injected onto a GC capillary column filled with molecular sieve to separate CO_2 from N_2 & O_2 and N_2O. The isotopic composition of CO_2 is measured by IRMS (Delta Advantage V) as described in chapter 2.8. A similar set-up using cryogenic pre-concentration of the whole volume of the gas sample in capillaries immersed in liquid nitrogen and chemical water and CO_2 removal (PreCon) can be used for stable isotope determination of nitrous oxide (N_2O) or methane (CH_4). The carbon isotope composition of CH_4 is measured after oxidation in an oxidation reactor to CO_2 and quantitative removal of sample CO_2 beforehand.

Procedure

Exetainer vials (12 mL vacutainer glass vials with screw caps) are evacuated, flushed with helium and re-evacuated using a manifold with three-way valve connected to vacuum pump and helium bag (fill freshly). Samples are collected by gas tight syringes (20 mL or 50 mL) with stop cock. After attaching a needle and piercing the exetainer septum the sample is drawn into the evacuated vial. Wait for 60 sec to hinder diffusional fractionation of isotopes and note the volume of gas that has been drawn into the

vacutainer (good way to ensure exetainer were really evacuated). A slight over pressure is applied by pushing the plunger and the syringe is pulled off. Samples are measured with a GasBench II coupled to isotope ratio mass spectrometer within two weeks. Store vials in 4 °C store room under water if necessary.

3.4. Gross N transformations (Pool dilution, IRMS)

Background

Nitrogen mineralization is one of the most important microbial processes in ecosystems, since it re-distributes nitrogen between high-molecular weight organic nitrogen forms and soluble inorganic nitrogen. The term "mineralization" is used to describe the process of re-mineralization of organic nitrogen (e.g., of proteins or amino acids) to inorganic nitrogen forms (e.g., ammonium and nitrate). The ammonium that is produced during the mineralization process is, however, not always accumulating in the soil, but may also be consumed by other processes. Ammonium may be taken up by plants (plant uptake), transformed to nitrate (nitrification), taken up by micro-organisms (microbial immobilization), lost by volatilization or immobilized in clay minerals, just to mention the most important processes that consume ammonium. Therefore, two different types of mineralization are usually distinguished: gross and net mineralization. Gross mineralization is the overall ammonification and is measured by a so-called "pool-dilution assay", while net mineralization is gross mineralization minus the ammonium fraction immobilized, transformed or lost, thus represents the change of the soil ammonium pool over time. Net mineralization can be either positive (then ammonium is accumulated in the soil over a certain period of time) or negative (ammonium concentrations are decreasing; net immobilization). In contrast gross mineralization is always positive (or 0 in rare cases).

Principle

"Pool dilution" assays are a type of assays, in which a certain pool is labelled and the dilution of the label by a certain process is followed over time. In our case we are labelling the soil ammonium (or nitrate) pool with $^{15}NH_4^+$ (or $^{15}NO_3^-$) and then follow the dilution of this pool by $^{14}NH_4^+$ (or $^{14}NO_3^-$) produced by mineralization of endogenous nitrogen-containing substrates (or in the case of nitrification by oxidation of ammonium) in the soil. This method assumes that all processes that consume ammonium do not discriminate between ^{15}N and ^{14}N (or only to an extent that does not interfere with the analysis, which usually is the case for labelled samples) while replenishing processes provide mostly ^{14}N to the target pool.

Soil labelling

For each treatment four 20-mL scintillation vials (labelled MS and ME for start and end of mineralization assays, NS and NE for start and end of nitrification assays) are prepared of each sample. In each vial 2 g of fresh soil is weighed. In one pair of vials each 0.5 mL 0.25 mM $(NH_4)_2SO_4$-solution is added while in the other pair each 0.5 mL 0.5 mM KNO_3^- solution (10 at % ^{15}N) is added. Each vial is then vigorously shaken to homogeneously distribute ^{15}N. The samples are incubated at room temperature for 4 hours (S, start) and 24 hours (E, end). The samples are stopped after the time indicated by addition of 15 mL 2 M KCl solution. The samples are horizontally shaken (about 200 rpm) for 1 hour and then filtered through ash-free filter paper. The extract is then transferred to pre-weighed 100-mL glass bottles. The exact weight (=the volume of the extract) is noted.

Acid traps

Acid-traps are prepared, by cutting off approximately 6 cm of Teflon tape. Two small filter paper discs (~0.5 cm diameter, ash-free paper) are cut and placed on the Teflon tape. Then each 7.5 µL 2.5 M $KHSO_4$ solution is pipetted onto the filter discs. Subsequently, the Teflon tape is closed and carefully sealed around the discs to form the acid trap. Acid traps can be kept in a clean (NH_3 free room) in closed glass bottles for a few days.

Mineralization

100 mg MgO is added to each bottle, the acid-trap is quickly added and the bottle is closed immediately. Hydratisation of MgO to $Mg(OH)_2$ leads to an increase in solution pH to >9.5. At alkaline pH the pH-dependent equilibrium between ammonium (NH4+) and ammonia (NH_3, gaseous) is shifted in the favour of NH_3. Released NH_3 is then collected from the gas phase into the acid traps where it again dissociates to NH_4^+. The bottle is now placed on a rotary shaker at 150 to 200 rpm at 35 °C for 4 days.

Nitrification

In a first step ammonium has be released from the extracts. Then nitrate is converted to ammonium by addition of a reducing catalyst (Devarda alloy) and at high pH collected in acid traps. 100 mg MgO is added to each bottle and the open bottles are placed on a heating plate (80 °C) for 240 min with frequent agitation to remove ammonium in the form of gaseous ammonia. To each bottle 0.5 g Devarda alloy is added, and after addition of the acid trap the bottle is closed immediately. The bottle is now placed on a rotary shaker at 150 to 200 rpm at 35 °C for 4 days.

Analysis

Thereafter the bottle is opened and the acid-trap removed. The acid-trap is carefully dried outside, put into a 1.5-mL reaction tube and put in a desiccator, containing a

beaker with concentrated sulphuric acid [Danger!! Please, use gloves and protective glasses, when working with a concentrated acid]. The desiccator is then evacuated and the samples dried for at least 12 hours. The acid trap is then opened and the filter paper disks folded and transferred to tin capsules and subjected to EA-IRMS.

Calculations

To calculate gross N mineralization the following equations are used (for nitrification substitute values accordingly for nitrate pools and atom % ^{15}N of nitrate):

$$m = \frac{M_1 - M_0}{t} * \frac{\ln \frac{AP_0 - AP_C}{AP_1 - AP_C}}{\ln \frac{M_1}{M_0}}$$

where m is the mineralization rate (mg N kg^{-1} h^{-1}), M_0 the initial N pool of ammonium (mg N kg^{-1}), M_1 the post-incubation N pool of ammonium (mg N kg^{-1}) at time t, AP_0 the initial atom % ^{15}N of ammonium, AP_1 the post-incubation atom % ^{15}N of ammonium at time t, AP_C the atom % ^{15}N of ammonium in unlabelled controls, and t is the incubation time.

3.5. Soil enzymes

General

Microbes can only use substrates if they are able to take them up. The bulk of plant and soil constituents (e.g. cellulose, lignin, proteins, humins and others) are too big to be taken up directly and thus need to be depolymerised extracellularly. Microbes, but also other organisms, therefore excrete enzymes to the environment, i.e. hydrolases and oxidases, to break down polymeric substances. Subsequently, they take up the oligo- and monomeric dissolved products into the organisms. In natural systems it has been proposed that the rate of generation of dissolved substrates from polymeric or condensed molecules limits microbial metabolism. Production of these extracellular enzymes is metabolically expensive and is highly regulated at the expression level. Once an enzyme is excreted into the environment the activity and lifetime is subject to environmental control by temperature, moisture, pH and the abundance of inhibitors and substrates. In soils enzymes are stabilized by adsorption to humic substances and clay minerals. Soil enzymes can be assayed under conditions close to their optimum (substrate concentration, pH) to investigate the effort of a microbial community allocated to decomposing or acquiring a particular resource, or close to ambient conditions.

The enzymes of broadest interest are those involved in decomposition of lignocellulose and its derivatives and in acquisition of organic nitrogen and phosphorus,

e.g. cellulases, hemicellulases, pectinases, phenol oxidases and peroxidases, chitinases, proteases, peptidases and phosphatases. Each of these functional categories includes multiple specific enzymes and therefore a range of enzyme assays are available to investigate the activity of high enzyme categories (e.g. cellulases by measuring glucose generation) or to measure the activities of specific hydrolase such as cellobiohydrolase.

The most convenient enzyme assays for hydrolases are based on (1) hydrolysis of side groups linked to a chromogenic or fluorogenic moiety, such as methylumbelliferyl (MUF) and p-nitrophenyl (pNP) substrates or (2) on photometric detection of the natural product of an enzyme reaction, such as ammonium released from urea or amino acids by ureases or deaminases. Hydrolysis of MUF substrates yields fluorogenic umbelliferone, hydrolysis of NP substrates p-nitrophenol which has an intense yellow colour at basic pH. The fluorogenic substrates are more sensitive by at least an order of magnitude, but pNP-linked substrates are easier to work with in soils because of greater solubility and since humic substances exhibit auto-fluorescence or quenching effects on fluorescence.

Oxidative enzymes such as phenol/polyphenol oxidases and peroxidases and laccases are involved in the oxidative degradation of lignin and humus and have less substrate specificity than hydrolases. They are investigated based on the oxidation of a phenolic amino acid, L-3,4-dihydroxyphenylalanine (DOPA), which can be measured with or without addition of hydrogen peroxide.

3.5.1. Urease, amino acid deaminase: NH_4^+ detection

Measurement

Weigh samples of 0.5 - 1 g of frozen soil in triplicate into 20 mL plastic vials i.e. 1 g (A, Bg) of mineral, and 0.5 g of organic soils (Ap or O). Add 500 µL of amino acid or urea solution to the soil, mix vigorously and close the vial. Amino acid solutions (15 mM in water) should be prepared fresh every day (L-arginine: M=174.2 g/mol, L-glycine: M=75.1 g/mol). The urea solution (80 mM in water) should also be prepared freshly every day. Incubate flasks at 37 °C for 3 hours (deaminase) or 2 hours (urease). Blanks for deaminase are prepared by adding 500 µL of amino acid solution to the soils followed by immediate extraction. Blanks for urease are prepared by adding 500 µL of water to the soils. To extract ammonium produced 5 mL of 2 M KCl is added to each flask for deaminase and 10 mL 2 M KCl for urease assays. Mix vigorously and shake for 30 min at room temperature. All extracts will be filtered (ash-free filters, folded filters 595 ½). Samples can be kept at -20 °C until further analysis. Ammonium is determined as given chapter 2.2.1.

3.5.2. Lignin (per)oxidase: DOPA substrates

Soil suspension

Put 1g of fresh, sieved soil in a 250 mL Erlenmeyer flask. Add 100 mL of sodium acetate puffer (100 mM, adjust with acetic acid to pH 5.5). Homogenize in an ultrasonicator for 2 min at 10% amplitude (Department of Microbial Ecology). For the following analyses, put each soil suspension on a magnetic stirrer (located in the electrochemical lab-room) and withdraw under continuous stirring 1 mL of each soil suspension in a 2 ml screw-cap-vial, 3 replicates.

DOPA (L-3, 4-dihydroxyphenylalanin)

For a 20 mM DOPA solution dilute 394 mg DOPA (L-3,4-dihydroxyphenylalanin) in 100 ml sodium acetate buffer. Prepare fresh for everyday. Put in 2 mL screw-cap vials (pipette soil suspension under continuous stirring by a magnetic stirrer):
Samples: 1 mL soil suspension + 1 mL DOPA (20 mM, final concentration: 10 mM), 3 replicates per sample. Blanks: 1 mL sodium acetate buffer + 1 mL DOPA, 3 replicates. Shake for 10 min, then centrifuge (5 min, 10.000 U/min). Transfer 250 µl of the supernatant into transparent microtiter plates as outlined below. From each vial, two wells are filled, one for the detection of phenoloxidase, the other for peroxidase. Peroxidase wells receive additionally 10 µl of 0.3% H_2O_2. Absorption is measured photometrically at 450 nm (Start point). Then microtiter plates are incubated in the dark (room temperature) for 20 hours (note the time). After incubation, absorption is measured photometrically at 450 nm.

3.5.3. Chitinase, protease: MUF substrates and fluorescence detection

Soil suspension

Put 1g of fresh, sieved soil in a 250 mL Erlenmeyer glass. Add 100 mL of sodium acetate puffer (100 mM, adjust with acetic acid to pH 5.5). Homogenize in an ultrasonicator for 2 min at 10% amplitude (department of microbial ecology). For the following analyses, put each soil suspension on a magnetic stirrer and withdraw under continuous stirring 200 µl of each soil suspension in a black microtiter plate well, 3 replicates.

MUF (methylumbelliferone) labelled substrates

MUF-Substrate: Fluorimetrically labelled (4-Methylumbelliferone (MUF) and 7-amino-4-methyl coumarin (AMC)) substrates are used for the measurement of the following enzymes:

	Enzyme	Substrate
A	Exoglucanase	MUF-cellobioside
C	Exochitinase	MUF-N-acetyl ß D glucosaminide
D	Protease	Leucine-aminomethylcoumarin

For 1 mM substrate stock solution prepare as follows:

	Substrate	Water
A	5,0 mg	10 ml
C	3,79 mg	10 ml
D	3,25 mg	10 ml

Pre-dissolve all substrates except D in 1ml of ethylene glycol monomethylether (methylcellosolve, toxic!) and make up with deionised water to 10 ml. (Dilute D in 10 ml deionised water). Keep stock solution at 4°C. Working substrate solutions concentrations are 0.5 mM for enzyme A and 1 mM for C and D.

Standards: MUF/AMC standard is prepared as a 10 mM stock solution of pure MUF/AMC in methanol. Store the stock solution in the freezer. The MUF-Standard has to be used to calibrate for enzymes A and C, AMC Standard for calibrating enzyme D. For the MUF-degrading enzymes (A, C) it is necessary to make three different standard arrays, because the signal of different enzymes shows different intensities. Therefore it is necessary to prepare MUF-standard working solutions of two different concentrations:

MUF-standard working solution:
(1) 100 µM (for C, dilute MUF stock solution with sodium acetate puffer 1:100)
(2) 50 µM (for A, dilute 100 µM MUF solution with sodium acetate puffer 1:2)

AMC-standard working solution:
50 µM (for D, dilute AMC stock solution with sodium acetate puffer 1:200). Pipette in black microtiter plates as outlined below (two plates for the only-control-plot-sampling and four plates for the full-sampling). Soil sample wells (blue) receive 200 µl of soil suspension. Standard wells (green) also receive 200 µl of (any) soil suspension (quenched standard). Control wells receive 200 µl sodium acetate buffer. All sample and all control wells receive 50 µL of substrate (A: 0,5 mM - final substrate concentration 100 µM, C and D: 1 mM - final substrate concentration 200 µM). Standards receive a mixture of standard solution and buffer (making up 50 µL) as outlined below. Note the different standard solution concentration for each enzyme.

Incubation: Incubation time (at room temperature): approximately 140 min, note the exact time. Close the microtiter plates with a seal film (for incubation and transport). Stop A and C by adding 10 µL 1 M NaOH to each well (samples, controls and corresponding standards), but don’t add to D.

Measurement: Measure fluorescence with a fluorometer with the following settings. Excitation: 365 nm, Emission: 450 nm, Ex.Slit: 20 nm, Em. Slit: 10 nm, Av.time: 0.2.

4. APPENDIX (ALTERNATIVE METHODS)

4.1. Ninhydrin method for α-amino N (N_{mic})

Principle

Ninhydrin, which is originally yellow, reacts with amino acid and turns from yellow to deep purple. Ninhydrin reacts with a free α-amino group, NH_2-C-COOH. This group is contained in all amino acids, peptides, or proteins. This reaction between α-amino acids and ninhydrin represents a multistep process. Step 1 is an oxidative deamination reaction that removes two hydrogen from the α-amino acid to yield an α-imino acid. Simultaneously, the original ninhydrin is reduced and looses an oxygen atom with the formation of a water molecule. Hydrindantin serves as catalyst for the reaction, which is formed by reductive coupling from two molecules of ninhydrin. In Step 2, the NH_2 group in the α-imino acid is rapidly hydrolyzed to form a α-keto acid with the production of an ammonia molecule. This α-keto acid further undergoes a decarboxylation reaction (Step 3) under heated conditions to form an aldehyde and a carbon dioxide molecule. These first three steps produce the reduced ninhydrin and ammonia that are required for the production of colour (Step 4).

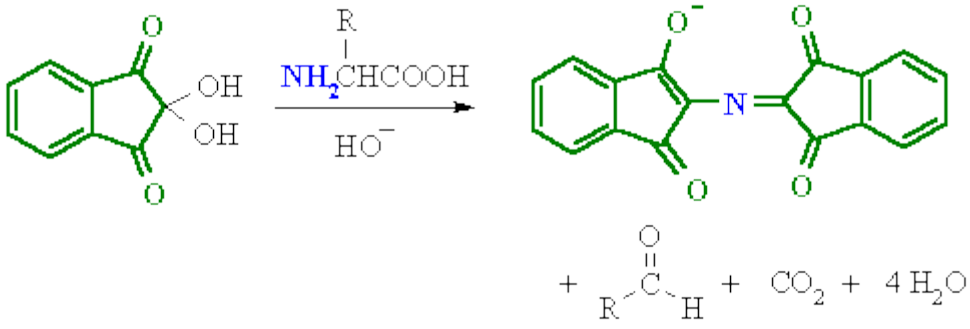
Whereas, the decarboxylation reaction will proceed for a free amino acid, it will not happen for peptides and proteins. Thus, theoretically only amino acids will lead to the color development. However, ninhydrin also reacts with sugars and ammonium, and the colour intensity developed is dependent on the type of amino acid.

α-amino acid + ninhydrin → hydrindantin (reduced ninhyd.) + α-imino acid + H_2O

α-imino acid + H_2O → α-keto acid +NH_3

α-keto acid + NH_3 → aldehyde + CO_2 + NH_3

ninhydrin + NH_3 + hydrindantin ---> Ruhemanns´ Purple



Standards

A leucine standard solution is prepared as follows (28 mg leucine-N L^{-1}): 2.62 mg L-leucine is first dissolved in 10 mL 0.1 M HCl and then diluted to 100 mL with Milli-Q or 1 M KCl, depending on the type of extract (do not use K_2SO_4 extracts which produce a precipitate of the dye). This solution is diluted each 1:2 until a concentration of 0.109 mg leucine-N L^{-1} is reached (8 dilution steps). Reagent blanks are prepared from 1 M KCl or Milli-Q water.

Procedure

Since the ninhydrin reagent also reacts with ammonium 0.5 mL aliquots of samples may be pre-treated with 5 mg MgO overnight on a shaker (keep vials open) to release NH_4^+ form the sample as NH_3 and centrifuge then. The MgO treatment has not to be done for determination of microbial biomass N (only if α-amino N is determined). The ammonium pool is the same in fumigated and unfumigated samples and therefore is automatically deleted in the calculation of N_{mic}.

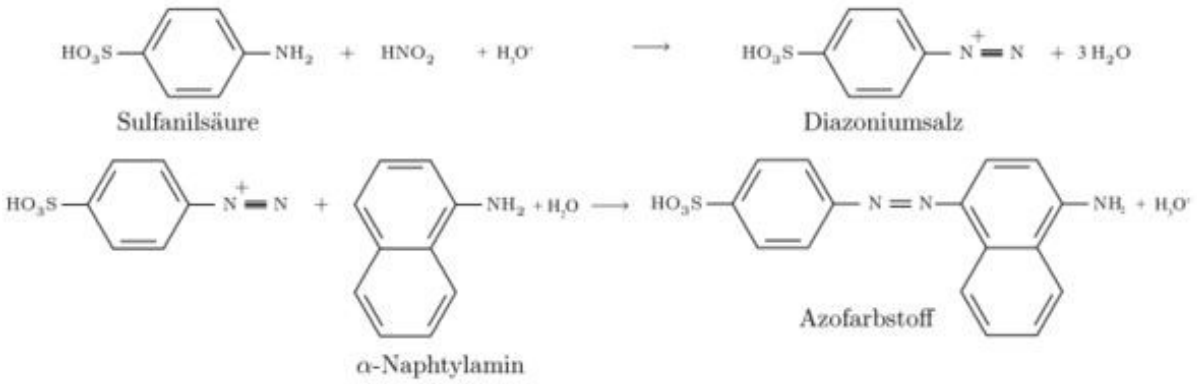
Pipet 250 µL soil extract, standards or blanks into 1.85 mL screw cap vials and add 250 µL ninhydrin reagent (Sigma-Aldrich, store at 4 °C). Handle ninhydrin reagent and samples after reagent addition only with gloves since ninhydrin produces nice red staining on your skin. Vortex. Close the vials and incubate in the water bath exactly 10 min at 100 °C. Cool the samples quickly to room temperature by keeping them in cool water. Add 625 µL 50% ethanol und mix. Pipet each 250 µL of samples, blanks and standards in a microtiter plate and measure absorbance at 570 nm.

The ninhydrin reagent is prone to oxidation by air and therefore is supplied bottled in N2 atmosphere. The life time of a bottle of ninhydrin reagent is therefore <1 year after opening.

4.2. Nitrate determination by Cd reduction and colorimetric detection of nitrite

Principle

This method can be used for 1 M KCl extracts and is based on a two-step procedure, (1) reduction of nitrate (NO_3^-) to nitrite (NO_2^-) with a cadmium-copper catalyst at basic pH, and (2) colorimetric detection of nitrite by azo dye formation. Nitrate is quantitatively converted to nitrate only at pH 8.5 and greater, lower pH ranges result in side products such as NH_4^+, hydroxylamine, nitrous oxide etc. The nitrite is determined by diazotizing with sulphanilamide and coupling with N-(1-naphthyl)-ethylenediamine dihydrochloride to form a highly coloured azo dye which is measured colorimetrically. The applicable range of this method is 0.05 to 10.0 mg L^{-1} nitrate-nitrite nitrogen.



Procedure 1 (nitrate reduction)

Prepare cadmium reactors from 1x1 cm pieces of cadmium foil which are rolled to small open tubes. Wash the reactors with 6 M HCl for 1 min and excessively with Milli-Q thereafter (7-8 times). The cadmium reactors are then coated with copper by immersion in 0.04 M $CuSO_4$ (0.499 g in 50 mL Milli-Q) and cleaned by several washes with Milli-Q. The cadmium foil thereby turns from shining silvery to black. Reactors can be kept in water at room temperature for 2-3 weeks.

Prepare nitrate standards (1 mM KNO_3) in 1 M KCl and a 1:2 dilution series thereof with 1 M KCl (7 dilution steps to 7.8 µmol L^{-1}) and blanks (1 M KCl). Pipette 1 mL of soil extract or standard into 2 mL plastic vials, add 20 µL 1 M imidazol (0.6808 g into 10 mL Milli-Q, pH 9) and a copperized cadmium reactor. Let react the samples and standards in an ultrasonic bath for one hour. Remove the Cd reactor (wash, reusable for 2-3 times) and proceed with nitrite determination.

Procedure 2 (nitrite determination)

Prepare the following reagents:

Reagent A: 1% sulphanilamide (sulfanilic acid) in 3 N HCl

Reagent B: 0.02% N-(1-naphthyl)-ethylenediamine dihydrochloride in Milli-Q

Standards: 1 mM potassium nitrite (KNO_2, highly toxic!) in 1 M KCl

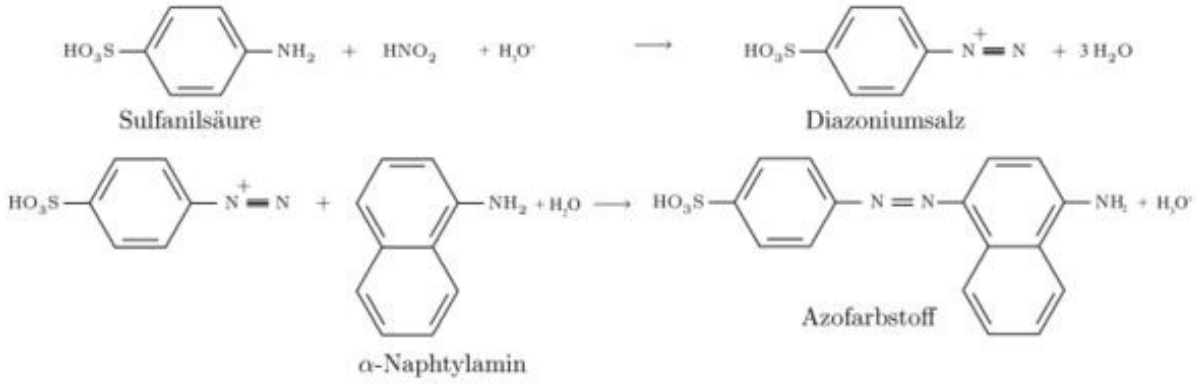
Prepare a 1:2 dilution series of this working solution with 1 M KCl (7 dilution steps to 7.8 µmol L^{-1}) and blanks (1 M KCl)

Transfer 250 µL reagent A in a series of 1.5 mL plastic tubes and add either 250 µL blank, sample or nitrate standard from procedure 1. Mix and add 250 µL reagent B and mix again. Pipet 250 µL aliquots of the assay into microtiter plates. The absorbance of the purple dye can be measured after 30 min at 540 nm.

4.3. Nitrate (VCl_3-Griess method)

Principle

The low anion exchange capacity of many soils allows extracting nitrate in those soils with water or with a low ionic strength solution such as 10 mM $CaSO_4$ for determination by anion chromatography. In soils with variable charge minerals (high AEC) nitrate has to be extracted by 1 M KCl and determined by VCl_3 reduction and subsequent nitrite detection by dye formation (see below). The latter method can be used for 1 M KCl and 0.5 M K_2SO_4 extracts and is based on a two-step procedure, (1) reduction of nitrate (NO_3^-) to nitrite (NO_2^-) with a Vanadium III chloride at acid pH, and (2) colorimetric detection of nitrite by azo dye formation. The nitrite is determined by diazotizing with sulphanilamide and coupling with N-(1-naphthyl)-ethylenediamine dihydrochloride to form a highly coloured azo dye which is measured colorimetrically. The applicable range of this method is 0.05 to 10.0 mg L^{-1} nitrate-nitrite nitrogen.



Method based on: Miranda, Espey and Wink, 2001

Chemicals

VCl_3 Reagent: 400 mg VCl_3 in 50 mL 1 M HCl – filtered

Griess Reagent: 1:1 mixture of 50 mg N-Naphtylethylenediamine in 250 ml MQ and 5 g sulfanilic acid in 500 ml 3 M HCl

A saturated vanadium reagent solution was prepared fresh daily by dissolving 400 mg vanadium (III) chloride in 50 mL 1 M HCl and excess solids were filtered through ashless Whatman filter paper. Griess reagent 1 was made up by dissolving 50 mg N-napthylethylenediamine dihydrochloride in 250 mL of deionised water. Griess reagent 2 was prepared by dissolving 5 g of sulfanilamide in 500 mL 3 M HCl. The latter solutions are stable for several months when stored separately in the dark at 4° C. Prepare mixture of both Griess reagents immediately before use.

Standards: Prepare a 1:2 dilution series of working solution (1 mM KNO_3) in 1 M KCl or 0.5 M K_2SO_4, 7 dilution steps to 7.8 µmol L^{-1} and blanks (1 M KCl, 0.5 M K_2SO_4)

Procedure

Pipette 500 µL sample, standard or blank into 2.0 mL plastic vials. Add 500 µL reagent 2 and then 100 µL reagent 1 (keep the sequence since VCl3 not only produces nitrite but also gaseous products (NO, NO2) that escape and are lost for determination. Using a multipette accelerates the addition of reagents. Incubate the samples at 37°C for 30 minutes. Measure absorbance of the purple dye at 540 nm.

4.4. Dissolved organic nitrogen and alkaline persulfate digestion

Principle

Dissolved organic nitrogen is extracted in water (water-extractable soil organic N, WSON) or by salt solutions (0.5 M K_2SO_4 or 1 M KCl). Total dissolved N is determined by TOC/TN-analyzer or alkaline persulfate digestion to NO_3^- with subsequent NO_3^- quantification by VCl_3-Griess method (see chapter 2.2.2.).

Alkaline persulfate digestion

Total soluble nitrogen (TSN) is quantified by alkaline persulphate digestion (oxidation) (Cabrera and Beare, 1993; Doyle et al., 2004) of ammonium and organic N to nitrate which is subsequently quantified by VCl_3-Griess method (see above). Persulphate oxidation depends on peroxodisulphate decomposition to persulphate radicals which represents the oxidizing agent. Persulphate has a half-life of ca. 30 s at 125°C and 4 h at 75°C (Peyton, 1993). It is this decomposition step that is rate-limiting while further oxidation steps are rapid relative to free radical production (Peyton, 1993).

Reagent, standards and procedure

The persulphate reagent is prepared by dissolving 50 g $K_2S_2O_8$, 16.8 g NaOH and 30 g H_3BO_3 and making up to 1 L deionised water. For digestion each 2.5 mL K_2SO_4 extract is mixed with 2.5 mL persulphate reagent in 6 mL GC headspace vials (Perkin Elmer N930-2134, with PTFE butyl septa) which are immediately crimped thereafter. Samples are autoclaved for 40 min at 120° C or are digested in a drying oven at 100 °C for 4 hours. Septa can be re-used three times, thereafter materials corrode and became leaky. Amino acid standards (glycine, 0.16-5 mg N L^{-1}) are prepared in 0.5 M K_2SO_4 and digested as above to test digestion efficiency. Nitrate is consequently quantified by VCl_3-Griess method (see above). Nitrate standards (0.078 to 5 mg N L^{-1}) are prepared in 0.5 M K_2SO_4 but calibration is performed with glycine standards, ranging from 0.078 to 5 mg N L^{-1}.

Calculation

DON is calculated by subtracting the inorganic N concentration from the total dissolved N (TDN) concentration i.e.

$$DON = TDN - (NH_4^+ + NO_3^-).$$

Before calculation of DON concentration all solute concentrations (mg L^{-1} or µg N g^{-1} dry soil) are converted to the Mol basis (µmol L^{-1} or µmol N g^{-1} dry soil).

4.5. Soil respiration by chamber method/NaOH absorption

Principle

Soil respiration (root and microbial respiration) releases CO_2 that is emitted from the soil surface. NaOH effectively absorbs the CO_2 and is thereby consumed.

$$2\ NaOH + CO_2 \rightarrow Na_2CO_3 + H_2O$$

Therefore the greater amounts of CO_2 are produced the more NaOH is consumed in this neutralisation reaction and the less NaOH is left in the residue. NaOH consumption is quantified after precipitation of carbonates as $BaCO_3$ (addition of $BaCl_2$) by acid-base titration using phenolphthalein as an indicator and HCl. The moles of HCl used to neutralize the residual NaOH are equivalent to moles NaOH left.

$$Na_2CO_3 + BaCl_2 \rightarrow 2\ NaCl + BaCO_3\ (precipitate)$$

Method

The soil is cleaned from litter and herbs. A PVC tube (8 cm diameter, 15 cm length) is inserted into the soil to ca. 2 cm depth. A 20 mL polypropylene vial containing 10 mL 0.25 M NaOH is inserted into the PVC tube and fixed to a rod using cable ties. Thereafter the tube is immediately closed with a suitable lid and sealant and left for 1 week outdoors. Thereafter the vials are disconnected, closed and brought to the laboratory. 5 mL 1 M $BaCl_2$ is added to precipitate carbonates for 15 min. The mixture is then taken, transferred to a glass beaker, diluted with water to about 25 mL volume and 3 drops of ethanolic solution of the indicator added, which turns the solution purple. Put a sheet of white paper underneath the beaker to allow more sensitive detection of colour changes. The equivalence point of phenolphthalein is at ca. pH 9.0. Using a calibrated burette filled with 0.1 M HCl the sample is titrated back until the indicator turns colourless. Five blanks i.e. 10 mL 0.25 M NaOH (initial solution) + 5 mL 1M $BaCl_2$ are titrated first to obtain the zero value, the amount/volume of HCl needed to neutralize NaOH in the absence of soil respiration. Any soil respiration will decrease this amount since NaOH is consumed by the CO_2 absorption reaction. Additional blanks are performed using the PVC tubes but instead of being inserted into the soil they are closed off from the atmosphere at both ends, containing the same amount of NaOH in a polypropylene vial as the others.

Chemicals

0.25 M NaOH, prepare from titrisol by diluting to 1 L with water
0.1 M HCl, prepare from titrisol by diluting to 1 L with water
1 M $BaCl_2$: dissolve 208.2 g $BaCl_2$ in water and make up to 1 liter
Indicator: 0.5% phenolphthalein in 50% ethanol:water

Calculations

The difference between NaOH blanks and exposed samples is related to the intensity of soil respiration; therefore the volume of HCl used to neutralize the sample is subtracted from that used for the NaOH blanks. For this blank-sample difference, each mL of 0.1 M HCl difference corresponds to 100 µmol HCl equivalent to 100 µmol NaOH consumed. Since 1 mole CO_2 consumes 2 moles of NaOH, this also has to be taken into account. The values are then back-calculated to time in days and soil surface area (m^2).

Soil respiration (µmol CO2 m^{-2} d^{-1}) =

$(HCl_{blank} - HCl_{sample})*100$ (µmol(mL)/7 (days)/0,005 (m^2)

Where HCl_{blank} (mL), HCl_{sample} (mL), area (surface area of PVC tube, in m^2), time (days), 22.4 L/mole gas