

Identification of footprints of microbial activity and their applicability for trace gas emission studies from soil after a rewetting event

Abstract

Microbes in soil play a major role in the release of NO into the atmosphere and thereby indirectly impact to the radiative forcing. In this study DAPI cell counting, Clone libraries for RNA and a H₂¹⁸O Stable Isotope Probing (SIP) for DNA were used to identify microbial activity over different soil moisture contents within a drying out. An optimum function for the DAPI cell counting was observed for the drying out. Within a series of clone libraries over the drying out footprints for several genera of the classes Alphaproteobacteria, Gammaproteobacteria and Bacilli, Planctomycetacia, Fusobacteria, of microbial activity could be identified. By the use of qPCR for the SIP fractions it could be shown that the DNA is significantly labelled with ¹⁸O - even in really short incubation times of ~9h. For further conclusions, in a future study the samples will be parameterized for their release of trace gases.

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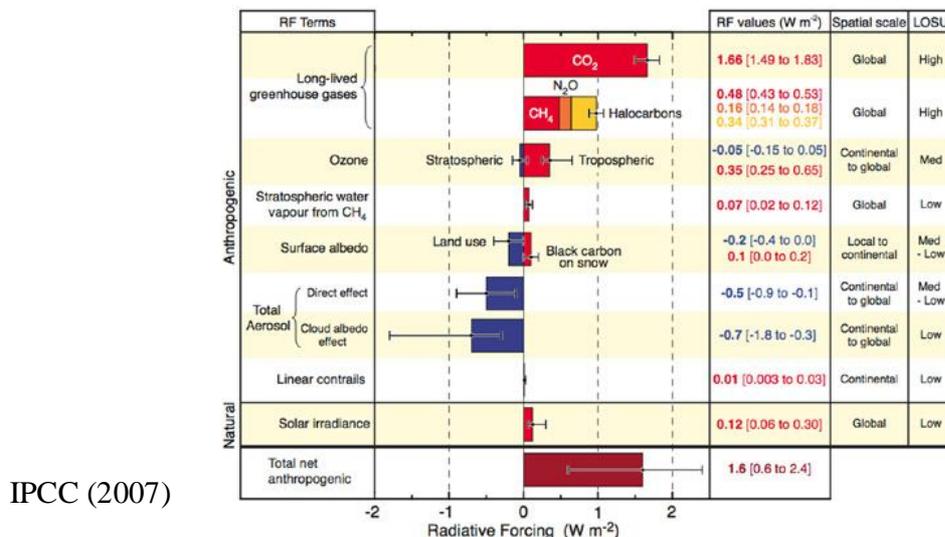
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1 Introduction

It is well known that within the metabolism of microorganisms in the upper soil layer nitric oxide (NO) is produced and consumed (Conrad 1996, Rudolph et al. 1996). The major processes are nitrification, denitrification and physicochemical processes – such as chemodenitrification. The major controllers of the processes rates are soil moisture, soil temperature and nutrients. According to the hole in the pipe model the release of NO and N₂O is controlled by the N-throughflow (in form of NH₄⁺ NO₃⁻ and N₂ (Firestone and Davidson 1989). Molecular diffusion leads to a release of NO into the atmosphere. Since NO_x (NO+NO₂) are reactive trace gases, once they reached the atmosphere photochemical reactions need to be considered. Basically NO can react with ambient O₃ to form NO₂. Under daylight conditions the NO₂ can lead to a formation of O₃. Thereby the biogenic release of NO from soils indirect influences the oxidative capacity of the troposphere.



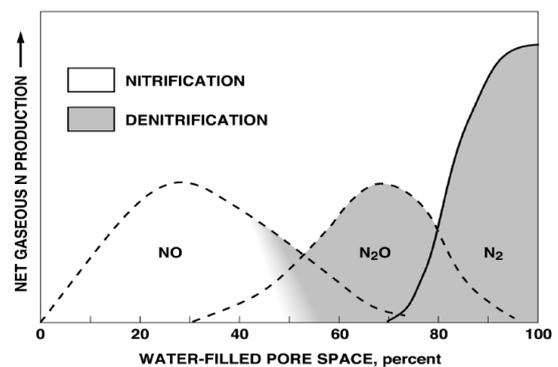
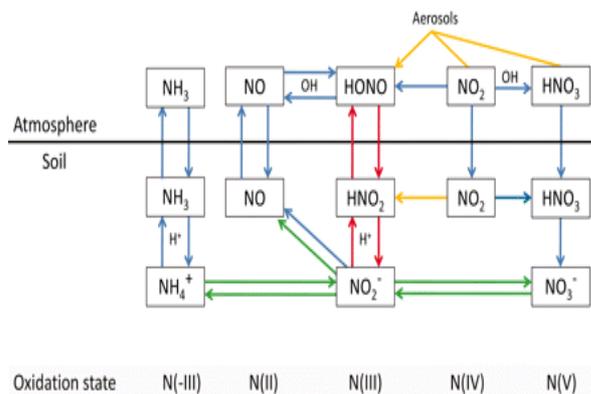
As the formation of tropospheric ozone is indirectly dependent on the NO-mixing ratio (Chameides et al. 1992, Crutzen 1987), NO act as indirect greenhouse gas. After CO₂ and CH₄, tropospheric O₃ is contributing with 0.35 (0.25 to 0.65) W m⁻² to the radiative forcing (IPCC 2007). The high uncertainty indicates that the major processes are not completely understood.



IPCC (2007)

To highlight the relevance of the footprinting assay, the principle of trace gas measurement will be shortly described for NO. In a laboratory incubation system the soil samples are wetted to field capacity and the release of NO is measured over a drying out experiment. After repeating the experiment for different temperatures and NO headspace concentrations the production (P) and consumption (k) of NO can be parameterized. There are indications that the microbes within a soil sample act in a functional community which results an optimum function for the P and k over the whole range of soil moisture. The release of NO seems to be diffusion limited (Skopp et al. 1990): In wet soil the release is limited by the molecular diffusion and in dry soil it is limited by the substrate availability. Therefore, usually a soil diffusion algorithm (Galbally and Johansson 1989) is applied, to convert P and k into a net potential NO flux.

Recently it was shown that HONO (the gaseous phase of HNO₂) can be released from soils (Su et al. 2011). Since HONO can react to OH, some soils seem to exhibit an interesting self regulation mechanism: While the NO is being released, OH can be formed, which is known as washing detergent of the atmosphere. Furthermore, there are indications for a release of VOCs from soils (Insam and Seewald 2010). A lot of the driving processes for their release are still unclear. It is speculated that some compounds might be released as intermediates of fermentation or signal compounds.



Su et al. (2011) SCIENCE

Firestone and Davidson (1989)

It seems that the release of trace gases from soil is much more complex than previously thought. Maximal release of different trace gases occurs at different soil moisture contents. And some trace gases show a multiple maxima. It is unclear whether this difference is caused by a change in

molecular diffusion or microbial activities over the drying out of the soil. Therefore this study focuses on the identification of footprints of microbial activity to understand the dominant process.

2 Material and Methods

“Soil is one of the most complex biomaterials on earth” (Young and Crawford 2004). Therefore, a simple sandy soil texture which is locally available at small dune sites close to the beach was sampled. The samples are expected to be low in organic matter and enriched with salts. These volitional characteristics allow comparing the results with samples from deserts which were studied by the author within the DEQNO-project (**D**esert **e**ncroachment in Central Asia – **Q**uantification of soil biogenic **N**itric **O**xide emissions by ground- and satellite-based methodologies). The sampling was performed at the July 1 and 2 in 2012 at the beach “The Knob” (KN1, N 41° 32.643’, W 70° 39.479’, 4m NN), in sand dunes close to the “Little Sippewisset Saltmarsh” (SIP2, N 41° 34.663’, W 70° 38.477’, 6m NN) and a beach in Woods Hole (SEC1, N 41° 31.981’, W 70° 40.113’, 4m NN). After the collection the soil samples were sieved (mesh size 0.002 m) for homogenisation. Within a test extraction for DNA (PowerBiofilm™ DNA Isolation kit, MO BIO, USA) and RNA (RNA PowerSoil® Isolation kit, MO BIO, USA) for each sample the SEC1 sample exhibits the highest yields of about 5 ng μl^{-1} DNA and 22 ng μl^{-1} RNA (nanodrop2000). Since the DNA PowerSoil kit usually results in a lower efficiency, the DNA PowerBiofilm kit was used.

For a community analysis with 454 NGS, DNA from all three soil samples was extracted. First 15 μl Phusion 2x HF Master Mix, 2.4 μl DMSO (100%), 0.6 μl 907R primer (25 μM) and 7.6 μl water were mixed with 2.4 μl 515F primer (6.25 μM). 2 μl DNA template were added and immediately the PCR was performed (protocol: 1x 98°C 60 s, 10x98°C 5 s, 68°C 10 s, 72°C 7 s, 12x 98°C 5 s, 58°C 10 s 72°C 7 s, 72°C 21s, then hold at 4 C). 5 μl of PCR product were transferred to a gel and the ladder was added into two lanes. The PCR product was quantified by using the KODAK Gel Logic 1500 Imaging system and ImageJ software. Finally the PCR product was send to the Pennsylvania State University. For the sample SEC 1 two drying out experiments were performed. The first drying out experiment consisted of 10 different subsamples (50g in petri dishes). After the coarse determination of the field capacity by the

Whatman-filter paper method, the fieldfresh soil was wetted to about 24% gravimetric water content. The petri dishes were incubated at 35°C slightly opened by fixing a piece of parafilm between the lid and dish. Since the drying out of a soil sample is a non-linear process, the sampling was performed after 6, 3 and 1 h. As indirect proxy for the microbial activity DAPI cell counts for each time point were performed. Therefore the cells of 1g soil were fixed in 1ml 1% formaldehyde solution and incubated in the 4°C fridge for several h. After 2 series of washing in 1ml PBS, the samples were sonicated 3 times for 20 s on ice followed by 30 s breaks. For the separation of the particles from the cells, after 15 min the supernatant was transferred into a new tube. After a centrifugation at 15,000 for 10 min, the pellet was resuspended in Ethanol PBS solution (50/50%) and stored in the fridge until filtration. For the filtration 100 µl were diluted in 100 ml PBS to distribute the sample homogeneously over the whole filter and passed a 16mm filter tower.

For the RNA extraction approximately 5g soil was bead beaded for 1 min. and the protocol of the RNA PowerSoil® Isolation Kit was used for 7 time points. To perform clone libraries of the RNA, the protocol of the reverse transcriptase kit (SuperScript®VILO™ cDNA) was used to transcribe it to cDNA. Since no RNase away was available, a 16S-PCR was performed with 2 µl RNA and 2 µl cDNA to show that the RNA was not contaminated with DNA. In the gel clear bands for the cDNA and no amplification for the RNA was observed. The amplified band of the cDNA was cutted out of the gel and spinned down at 5,000 g for 10 min (Millipore gel purification). The protocol of the TOPO® TA Cloning® Kit (invitrogen, USA) was used to insert 2 µl of the amplified PCR product into a plasmid vector. Since the amount of clone libraries was limited, for each time point a reaction was set up with 0.5 µl PCR product, 1.5 µl salt solution 0.5 µl vector and 3.5 µl water. To increase the efficiency of the cloning the incubation was extended to 3h at room temperature. 15 min. before the incubation time was reached, the E coli. cells were slowly thawed from the -80°C storage temperature on ice. After the incubation, the 4 µl of the cloning reaction was transferred into the vial with E .coli cells and gently mixed. After an incubation of 5 min. on ice, approximately 70 µl of the solution was transferred into a 0.1 cm cuvette (avoiding the formation of bubbles). A electroporation system (Gene Pulser Xcell electroporation system, biorad, Austria) was used to introduce the vector reaction into the E. coli cells by increasing the electrical conductivity and permeability of the cell plasma membrane with the Mdiv-protocol. Immideately 250 µl of SOC medium (prewarmed at room temperature) were

added into the cuvette and as much as possible ~50 μl were directly retransferred into the vial with the *E. coli* cells. If a noise and flash light occurs, the electroporation failed. The solution was incubated on a horizontal shaker at 200 rpm for 1h at 37°C. LB media was prepared and two plates (prewarmed at room temperature) with 15 μl and 100 μl were prepared containing 50 $\mu\text{g ml}^{-1}$ of ampicillin (select for growth of *E. coli*.) to get at least one plate with easy pickable colonies. To spread the solution homologous over the whole plate 5-8 sterilized beads were used and shaken over the plates in different orientations for approximately 2 min. The plates were stored at the bench for 15 min. until the surface of the plate seemed to be dry. The first colonies could be observed after ~14-15h. To minimize contamination, for each time point 46 colonies were picked and transferred into 96 well PCR plates, which were filled with 1.5 ml of liquid LB media containing 50 $\mu\text{g ml}^{-1}$ of ampicillin. After 24h incubation the wells were sequenced. Since the first time series results only a low amount of colonies, for some time points the whole process was repeated.

For the SIP experiment one petri dish with 20g soil was incubated with 1.5 ml H₂O and 2 g H₂¹⁸O (Cambridge Isotope, Inc., USA) at 35°C for 9h. A piece of parafilm was used to slightly open the petri dish, that the soil could slowly dry out. The soil moisture was monitored gravimetrically each h. Since the extractable amount of DNA for the SEC1 sample was low, about 2.5 g of soil were extracted by the protocol of the PowerBiofilm kit in 10 vials from the labelled incubation and about 2g from the unlabelled not incubated control. To concentrate the DNA only 100 μl water was added into the one tube of labelled and unlabelled DNA. After spinning the DNA through the column, it was transferred in the next tube. The final concentrations were measured by nanodrop 2000 at 2.5 mg/100 μl for the labelled and 1.3 mg/100 μl for the unlabelled. Usually at least 5 mg of DNA are recommended to perform a SIP fractionation. However, the time and DNA extraction reactions were limited and the lowest amount which can be used is about 1 mg. So the experiment was continued with a 16S PCR amplification the product was checked in a gel electrophoresis. A strong band of long DNA fragments was cutted out by a raser blade and transformed into a spin filter contained vial. After a gel purification (E.Z.N.A.® Gel Purification Kit, Omega bio-tek, USA) the DNA was evaporated to 30 μl for about 3 min. in a vacuum centrifuge at 45°C. Either a huge amount of DNA was lost due to cutting out the band to small or the nanodrop2000 readings of 1.25 mg for the labelled and 0.52 mg for the unlabelled were impacted by the high salt content. After the gradient media was

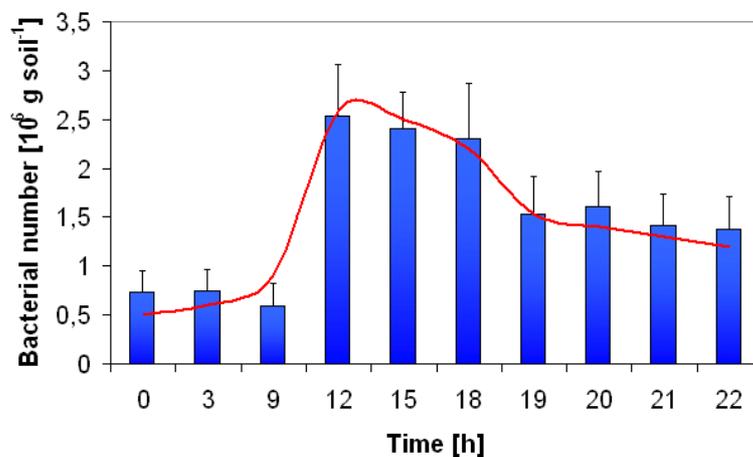
prepared (CsCl and gradient buffer fine tuned to a density of 1.762 g ml^{-1}), 4.3 ml of gradient media was filled into the ultracentrifuge tubes. The tubes were balanced to less than 0.01 g to each other. Then the DNA was added together with 400 μl of TE buffer. The total amount of DNA of both samples was pretty low and therefore the whole amount was added. However, usually it is recommended to add the same amounts of DNA for both samples. The tubes were filled to their necks with TE buffer and again balanced to each other. It is important that the corresponding tube on the other side in the ultracentrifuge is balanced as close as possible. Finally it should not be forgotten to mix the tubes by inverting several times until no refraction waves can be seen; otherwise the gradient media might reach a high density and the tubes might brake. Finally the ultracentrifuge is setup for 55,000 rpm at 20°C in a vacuum for at least 66h. After the centrifugation the tubes were transported extremely carefully to the setup fractionation system. Therefore 100 μl of tap water were filled into the top of the tube by a syringe pump in 1 min. At the same time on the bottom a syringe needle was put into the bottom of the tube to collect the fractions in a vial. Since the syringe pump creates a little pressure difference a 30sec delay was used after each 100 μl of tap water were pumped in, to catch as constant volumes per fraction as possible. In total 36 fractions per sample were collected. To remove the CsCl from the fractions, the 100 μl fractions were diluted in 300 μl water and 240 μl isopropanol and spinned at 14,000 g for 30 min at 4°C (since the precipitation of the DNA is enhanced in the ethanol at low temperature). After pouring of the supernatant the fractions were diluted twice in 1ml of 70% Ethanol, vortexed and spinned at 14,000g for 15min. Since the nanodrop2000 resulted low readings for the 230/260 nm value, which is an indication for the salt concentration in the sample, it should be washed more than twice in future experiments. Additionally the nanodrop2000 showed reading between 12 to 25 ng μl DNA for each fraction. Then the fractions were resuspended in 50 μl TE buffer. As a first check if the label was incorporated successfully, 6 fractions were pooled together to perform a “poor man 16S-PCR”. The gel electrophoresis showed two pooled fractions for the labelled sample which could not be observed in the unlabelled control. However, it was not possible to quantify the result.

As a proof of evidence that the method worked, a qPCR was performed. Therefore the copy number of the clone library plasmids was calculated (<http://www.uri.edu/research/gsc/resources/cndna.html>). The plasmids from the clone library were suspended in 2ng μl irradiated sheared salmon sperm to reduce binding of DNA to microfuge tube walls. For the conversion of copy

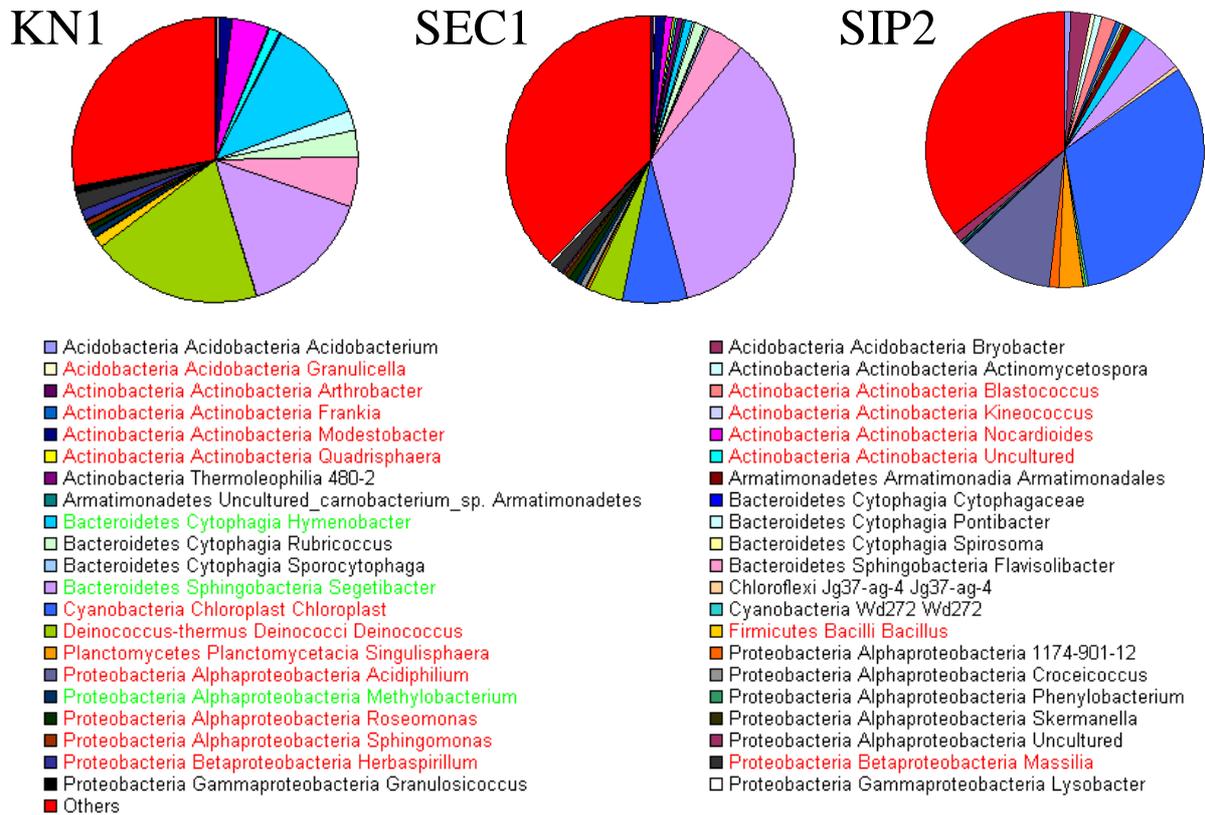
number per ng into copy number per μl the standard solution was measured by the nanodrop2000 98.2 ng μl . The copy number per ng resulted $1.67 \cdot 10^{10}$. For the qPCR reactions 12.5 μl 2X QuantiFast SYBR Green, 1 μl 515F and 907R primer, 1 μl template and 9.5 μl water were setup. Additionally a standard dilution series 10^6 to 10^1 and two negative controls were prepared. After spinning the plate for $\sim 30\text{sec}$ at 1000g to avoid bubbles in the reaction solution, the qPCR should be started immediately, since the SYBR Green is sensitive to light.

Results and Discussion

Since soil samples usually exhibit a high background signal for each timepoint 1000 cells were counted at the same time of day on two following days. This method seems useful to avoid a counting bias and reach a reliable statistic. In general, a low amount of cells in the order of 10^6 was observed. This seems to be in a good agreement with the low extractable amount of DNA. Considering the error in both directions an optimum function – as observed for the trace gas emissions – can be overlaid the counts. Since only one dilution was used for all filters, some filters show a high and others a really low concentration of material. For future studies different dilutions should be made to decrease the background signal and improve the results. Since a program could be trained for automatic cell counting, the DAPI method seems to be a fast method with a high resolution in time and soil moisture, respectively. In a higher time resolution it might be possible to identify corresponding peaks in the DAPI cell counting corresponding to the release of trace gases.



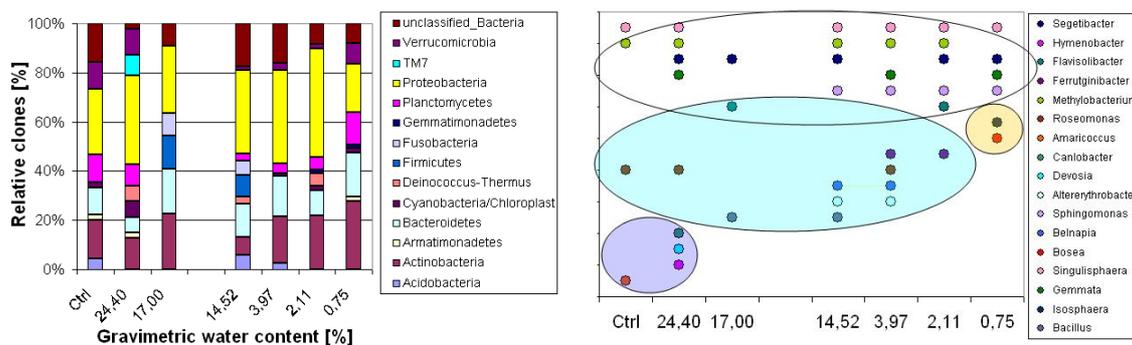
The taxonomy for the 454 sequences was analyzed by the alignment with the RDP classifier. As confidence threshold 50% was used, to compare the 454 reads with the clone libraries on the genus level. KN1 results 7991 reads (5741 classified), SEC1 6872 (4275 classified) and SIP2 8218 (5370 classified) came back. Basically all sequences which were found in the clone libraries are found as well in the 454 data. Only the TM7 is found in the clone libraries with a confidence of 100%. A lot of genera occur in different abundance in all three samples (red coloured). However, the samples show a huge diversity (genera in black). The most abundant in all three samples (coloured green) are *Segetibacter*, *Hymenobacter* and *Methylobacterium*. There is some indication that these genera might be involved in the release of NO (Kulichevskaya et al. 2008, Zhang et al. 2009, Zhang et al. 2011).



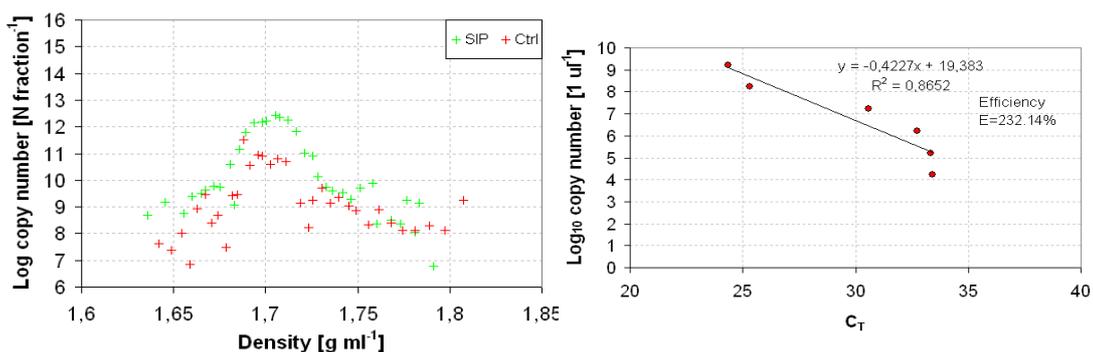
Beside time point 2 for each clone library about 60 sequences came back. Therefore, it is assumed that the possibility for a huge change within the composition of each library is low. This seems plausible, because the extractable amount of DNA and the DAPI cell counts in a lower order of magnitude. It is assumed that the change in relative clones is caused by the drying out

over time. Over the complete drying out Proteobacteria, Verrucomicrobia, Planctomycetes and Actinobacteria are present at all time points. However several footprints can be identified: Bosea, Amaricoccus and Isospaera are active in the dry soil, Canlobacter, Devosia and Hymenobacter in the wet soil. Bacillus, Altererythrobacter, Belnapia, Roseomonas and Ferrutginibacter cluster in the intermediate range of water content. As generalists over the whole range of water content, Flavisolibacter, Sphingomonas, Gemmata, Segetibacter, Methylobacterium and Singulisphaera cluster together. In case some of this bacteria are able to produce NO the release of trace gases from soil might be more dominated by different activities of microbes than molecular diffusion. There is a strong need to measure the release of NO for a more comprehensive conclusion. Since no microsensors for NO was available, a test measurement with the O₂ sensor was performed. It seems that the sensor is sensitive enough to resolve the boundary layer above the soil and different layers of microbial activity in the soil.

However, since time and soil moisture change it is not clear which parameter dominates. In future experiments a second control should be incubated at constant soil moisture to separate the change over time from the change caused by soil moisture. Thereby it could be shown whether a “history of drying out” changes the microbial community and thereby the release of trace gases. Another indication for the dominance of different microbial activities over molecular diffusion is that the exchange of NO through the cell wall is not only dominated by molecular diffusion. There are several regulatory mechanisms (e.g. detoxification of the cell) which are driven by other factors (Moir 2009).



The results of the SIP experiment will be only described briefly. For further reading some papers are referenced (Adair and Schwartz 2011, Buckley et al. 2008, Buckley et al. 2007) in this report. For future experiments it should be thought about incubate the control as well. For me it seemed to make no difference since the incubation is really short. Since the labelled $H_2^{18}O$ is pretty expensive, only one experiment could be performed. It was not possible to perform clone libraries of the labelled fractions, because the fractionation took longer than expected, the first pooled PCR was contaminated and the first qPCR did not work out. The R^2 reached only 0.75 and the efficiency was only 46%. Therefore the final result of the SIP experiment in this study is to give a proof of evidence whether the technique is working in short incubation times. After observing the amount in the pipette tips for the standard dilution series, the efficiency was 232% and the R^2 to 0.86. After exclusion of highest dilution, R^2 reaches 0.91. Since the pipetting error can influence the result significantly, it is recommended to do at duplicates of the standards on the optical plate. It is unclear, why the efficiency is 232%. However, the labelled sample shows an enrichment in DNA – visible in a double peak feature. The second peak is about 0.04 g ml shifted to the right compared to the first peak at ~ 1.71 g ml. The theoretical shift for ^{18}O should be 0.03. Since the refractometer was damaged by CsCl which was not cleaned properly in the last year, it might explain the slightly difference. This suggests that the incorporation worked out. For the future PCRs 2 μ l template should be used to minimize pipetting errors. Usually the results are all calculated back to g of dry soil and the fractions are normalized to the area of the curve. However, since time was running, the log copy number was only converted as N per fraction.



Conclusion and Outlook

DAPI cell counting in a high temporal resolution for different soil moistures and RNA clone

libraries are powerful techniques to study footprints of bacterial activity during a drying out of soil. In the future the data will be combined with trace gas measurements and it will be tried to solve the question about the dominant limitation either by molecular diffusion or different microbial activities. Instead of DNA SIP it might be more useful to use RNA SIP maybe in combination with TRFLP. However, these methods are really consuming in time and effort. For the parameterization of the release of trace gases, the samples will be send to the Max Planck Institute for Chemistry, Mainz, Germany. It is expected that further conclusions can be drawn out of the data.

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