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Micro-segmented flow and multisensor-technology for microbial activity profiling†

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The combination of micro-segmented flow with miniaturized flow-through multisensor-technology has been utilized for metabolite profiling of soil bacteria. Series of sub-μl segments were generated containing soil sample slurry from historic copper mining sites and exposed to heavy metal salts of copper and nickel. Segments were examined for bacterial growth and spectral properties as well as for the effect of heavy metal-treatment after different incubation times. In order to evaluate microbial growth, extinction was recorded with 4 different spectral channels. Fluorescence was measured using a microflow-through fluorometer to detect both growth and production of fluorescent dyes or metabolites. The incidence of single segments with enhanced absorption in one of the spectral channels or enhanced fluorescence was scored to detect soil microorganisms with interesting properties for further screening. The study could show that the number of vegetated segments, the density of microorganisms in the segments after cultivation and the spectral response are different for separate soil samples and different metals. Thus, the highly parallelized and miniaturized segmented flow method is a promising tool for profiling of soil samples with regard to identifying micro-organisms with interesting profiles for secondary metabolite-production.

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Environmental impact

Soil accumulation of toxic heavy metals, derived from rapidly expanding industrial areas, abandoned mining areas and waste, represents a serious problem at the present time. Soil-micro flora, mainly containing bacteria, uses various mechanisms to adapt to the heavy metal pollution, *e.g.* intracellular accumulation and transformation of metals. This property can be specifically used in bioremediation; in this case heavy-metal tolerant microorganisms are used to detoxify contaminated soil. A recently observed phenomenon is the induction of secondary metabolites in soil bacteria which are exposed to heavy metals. In this work we report about a new strategy for monitoring soil samples in terms of their tolerance to heavy metals and (secondary) metabolite production by a droplet-based method combined with multisensor-technology.

Introduction

Soils contain large numbers of microorganisms and are very diverse habitats. Most microorganisms in soils are yet unknown, with only 0.1 to 10% of soil bacteria being cultivatable[1,2] and metagenomic data from soil samples implying a huge number of undiscovered species.[3] Thus, the biochemical capacity of the vast majority of soil microbes so far remains untapped, albeit an increasing necessity for new drugs has been acknowledged by, *e.g.* World Health Organisation.

Most soil bacteria can survive under difficult environmental conditions due to their evolutionary adaptation and their symbiotic coexistence in communities of organisms. For example, there are micro-organisms and communities that can exist in heavy metal-contaminated soils. Pollution of soils with heavy metals occurs *e.g.* from rapidly expanding industrial areas, abandoned mining areas, contaminated waste water or animal manures[4] and typical bacterial tolerance strategies against heavy metals include binding, complexation, accumulation, oxidation/reduction, precipitation and efflux of heavy metals.[5] These bacterial tolerance mechanisms can be utilized for bioremediation of heavy-metal-contaminated soils with dead or live microbial biomass to sequester metals from contaminated areas.[6] Recently, studies with such heavy metal-tolerant soil streptomycetes revealed that heavy metal-supplementation induces secondary metabolite production.[7] Thus, these bio-remediating bacteria have two very advantageous properties, which make them interesting research objects.

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It is well known that many soil microorganisms in particular the bacterial group of streptomycetes can produce a large variety of bioactive secondary metabolites including most antibiotics in use today and can, therefore, be considered an important source for new drugs and new biosynthetic pathways.[8] Both growth and production of secondary metabolites like antibiotics, pigments, *etc.* are strongly dependent on cultivation conditions. Different microbial species require special media formulations for growth and induction of secondary metabolite-production.[9] The screening of soil samples for unknown microorganisms and the optimization of the cultivation media for growth and induction of secondary metabolite production is a multi-dimensional problem. Therefore, traditional cultivation techniques have to be complemented by new approaches combining highly parallelized screening with minimal resource requirements, both for chemicals and space, while providing good statistics with manifold replicates. The applications of microfluidic techniques with droplet-based techniques[10,11] or microfluidic segments[12] are particularly well suited for such a miniaturized, multi-parameter approach.

The micro-segmented flow technique used here allows for the generation and handling of hundreds or thousands of samples in parallel, combined with an addressable order. The typical volumes are in the sub-μL and nL range. It could be shown that bacteria[13,14] and eukaryotic microorganisms[15,16] can be cultivated in these small volumes and the technique was successful for the determination of EC_{50}-values of antibiotics and other chemical effectors by highly resolved dose/response functions, as well as for the determination of combinatory effects in two- and three-dimensional concentration spaces.[17] Reading the bacterial growth inside segments can be realized by microflow-through photometry, measuring extinction which is nearly proportional to the density of bacteria. In addition, the detection of organism-derived fluorescence can provide information on growth; at the same time it might indicate the occurrence of additional, fluorescent metabolites produced during incubation within segments. Plant secondary metabolites have been identified already using micro-spectral fluorimetric studies.[18]

A major advantage of the segmented flow-technique is the computer-controlled generation of high-resolution concentration gradients of chemicals in segments, or the precise dosing of different chemicals in segment sequences containing cells or micro-organisms. For application to soil, this specific segment generation might prove useful if every segment can be inoculated with soil sample slurry.[19] Due to the high number of segments a very large amount of soil microorganisms can be addressed simultaneously. Random encapsulation of soil-derived different microorganism species in small enclosed compartments leads to the formation of random mixed microorganism cultures with restricted cell numbers. For such mixed cultures the activation of gene promoters has been shown, which control the synthesis of secondary metabolites, which was not observed in pure cultures[20] and coculture studies with known bacterial species revealed induction or potentiation of metabolite production.[21] Using segmented flow-technology different agents can be readily co-administered to stimulate growth or secondary metabolite production. For example, heavy metals, supplied as ions or nanoparticles, are ecotoxicologically important stress factors that are able to induce secondary metabolite production.[7,22–24] For some *Streptomyces* producer strains (*S. tendae* F4, *S. acidiscabies* E13), the cultivation in segments and multi-heavy metal effects have been shown.[25] Based on these results, we wanted to investigate different soil samples using moderate concentrations of heavy metals to stimulate secondary metabolism. It is expected that different soil samples represent different microbial communities, most likely including potential new secondary metabolite producers. A profiling of soil samples by photometry and fluorometry after incubation would provide an elegant tool to select interesting microorganism-communities due to their spectral properties and would allow testing different sampling sites prior to intensive screening and isolation of pure cultures. In addition, the application of sensor sets or spectral sensing should help identifying samples with bacteria producing chromophores or fluorophores under different test conditions, here provided by multi-metal stress conditions.

Here, we present a methodological approach using micro-segmented flow-technology to test for growth and spectral response behavior of soil microorganisms in response to heavy metal stress. This strategy has the potential of screening significantly larger amounts of samples, already been shown for other segmented flow-based high-throughput-applications.[26,27]

Materials and methods

Materials

Perfluoromethyldecaline (PP 9) was from F2 Chemicals (Lancashire, UK). $CuSO_4 \cdot 5H_2O$ and $NiSO_4 \cdot 6H_2O$ were from Merck (Darmstadt, Germany). Cycloheximide was obtained from BioChemica (Düsseldorf, Germany). Fluorescein diacetate was from Acros organics (Geel, Belgium) and propidium iodide from Sigma-Aldrich (Steinheim, Germany). AM minimal medium for the incubation of soil sample slurry consisted of 0.5 g L^{-1} asparagine, 0.5 g L^{-1} K_2HPO_4, 0.2 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.01 g L^{-1} $FeSO_4 \cdot 7H_2O$ and 10 g L^{-1} glucose monohydrate. Agar plates were prepared by adding agar (BD Biosciences, Germany) at a final concentration of 1.8% to solid AM minimal medium followed by autoclaving and pouring.

Soil sample collection and preparation

Soil samples were collected from different former mining areas in Thuringia and Saxony-Anhalt, Germany. The idea behind was that multi-element contaminated soils have led to an enrichment of metal-responsive microorganisms. The contamination is due to late paleozoic Zechstein (Tartarian) and in particular copper shale mining that took place since the middle ages, with more intensive exploitation from the 15th to the 18th century.[28] The sulfidic ores lead to acid mine drainage when weathering which accelerated metal mobility.[29] All collection places, the date of collection, GPS coordinates and a short description of the sampling places are listed in Table 1.

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Table 1 Soil sample description

Name	Location	Date of collection	GPS coordinates	Description
G 7	Suhl-Goldlauter, Pochwerksgrund	25.10.2013	4 412 686/5 613 263	Early industrial mining
G 9	Suhl-Goldlauter, Pochwerksgrund	25.10.2013	4 412 576/5 613 035	Early industrial mining
G 12	Suhl-Goldlauter, Pochwerksgrund	25.10.2013	4 412 218/5 612 847	Early industrial mining
G 17	Ahlstaedt	25.10.2013	4 405 897/5 600 526	Small stone quarry
K 1	Doernfeld a.d.Heide	08.10.2013	4 434 395/5 613 870	Permian
K 6	Boehlen	08.10.2013	4 432 506/5 605 367	Middle age mining
M 7	Mansfeld–Benndorf	28.03.2013	4 463 891/5 714 917	Pre-industrial mining
M 10	Volkstedt	28.03.2013	4 468 772/5 712 885	Industrial smelting area

Soil samples were taken directly from the surface of the earth using a sterile falcon tube which was immediately sealed. Then they were air-dried under sterile conditions in the laboratory. For the experiments soil samples were treated as follows: 1 g soil was mixed with 40 ml sterile Aqua dest. and vortexed. After centrifugation for 20 minutes at 1200 rpm the supernatant was filtered through a sterile filter paper (GE Healthcare, Germany). The filtered undiluted solution containing mainly bacterial spores and vegetative bacteria was used for the experiments after addition of the eukaryotic translation inhibitor cycloheximide at a final concentration of 15 μg ml^{-1} to prevent growth of soil-derived fungi in segments.

Experimental setup

The experimental set-up for segment generation, incubation and optical characterization of spectral properties of the segment content is shown in Fig. 1.

In detail, 500 nL segments were formed by coinjection of soil sample slurry (5 μl min^{-1}), cultivation medium with heavy metal salts at a final concentration of 0.25 mM or 0.30 mM (5 μl min^{-1}) into a flow of carrier solution PP 9 (40 μl min^{-1}) by using a PEEK™ 7-port manifold (YMC Europe GmbH, Dinslaken, Germany). This was done with the help of a computer-controlled syringe pump with 3 dosing-units (Cetoni GmbH, Korbußen, Germany), implemented with syringes (ILS, Stützerbach, Germany) with 500 μl (soil sample slurry and cultivation medium with heavy metals) and 5000 μl (carrier solution PP 9) volume. Connection of syringes with the manifold occurred by Teflon® tubes (0.5 mm id, 1.6 mm od, Bohlender GmbH, Germany) with suitable fittings (YMC Europe GmbH). After formation and after incubation segments were transported with a constant flow rate of 50 μl min^{-1} through an optical multisensor-detector unit for the simultaneous measurement of extinction and fluorescence of the segment content. Extinction was measured with 4 LEDs with peak wavelengths of 470 nm (Nichia, Japan), 505 nm (Agilent, United States), 615 nm (Agilent) and 660 nm (Kingbright, Taiwan) each with a photodiode (Osram, Germany) for the detection of light intensity after attenuation by microorganisms inside segments. Measurement of fluorescence induction was carried out with a LED with a peak wavelength of 470 nm (ledxon, Germany) with a combination of shortpass (480 nm) and longpass filters (500 nm) (Laser Components, Germany). The emitted photons were counted using a photomultiplier module (Hamamatsu, Japan). Values for fluorescence induction were normalized against the reference fluorescence of the tube subtracted with 1.

$$I_n = \frac{I_{fluo}}{I_{ref}} - 1$$

(I_n = normalized intensity; I_{fluo} = intensity of the measurement; I_{ref} = intensity of the tube).

Segments containing soil slurry from different collection places and different heavy metal treatments were stored in tube coils consisting of a PMMA plate with rolled transparent Teflon® (PTFE) tubes for 9 and 15 days in an incubator at 28 °C.

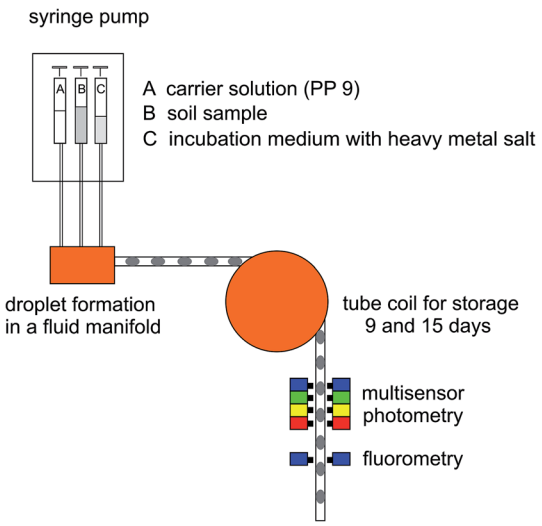

Fig. 1 Experimental setup: droplet formation, incubation and optical characterization by multisensor-technology.

Data processing-synchronization

Segment sequences were measured *online* at 100 Hz sampling rate shortly after formation and after incubation. Detection of segments occurred in a subsequent *offline* data analysis process using LabView™ software and provided segment-specific data, like segment size, distance between two neighbour segments and extinction or fluorescence. Segment detection was performed by the usage of three parameters: defining the upper and the lower threshold of the extinction range of the carrier liquid and defining the time interval corresponding to the minimum segment distance. Segments are recognized, when

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the segment-derived extinction exceeds the thresholds of the carrier liquid. The end of the segment is detected when all extinction values in this time interval are to be found within the thresholds. Since the microorganisms grew partially inhomogeneous inside segments, growth behaviour was analysed by forming the extinction signal integral with respect to the segment size. This value is termed in the diagrams as calculated signal (calc. signal). Due to possible false detections in the individual sensor channels, time-based synchronization for unique segment mapping was conducted (Fig. S1A and B†). The first, in all channels correctly detected segment, is used as the reference segment for time-based comparison of the following segments. False detection can occur if the extinction of the segment for the specified time interval corresponds to the extinction of the carrier liquid (increase in segment number) (Fig. S1C†) or if the distance between two segments is less than the defined time interval (decrease in segment number). Usually, only a few false detections occur (approx. 1%). These are recognized by the data synchronization and are not included in further data analysis. Thus, time-based synchronization allows unique assignment between individual segments (Fig. S1B†) and prevents analysis errors. The data obtained were then exported as tables and converted into graphics (Fig. 2). All measurement data from the individual segments were carefully compared shortly after segment generation and after incubation to verify that increases in the calculated signals were due to growth. Calculated signals after incubation which were equivalent to the calculated signals of the 0 hour-measurements were designated as "non-vegetated". Calculated signals of "vegetated" segments differed considerably from the calculated signals of "non-vegetated signals".

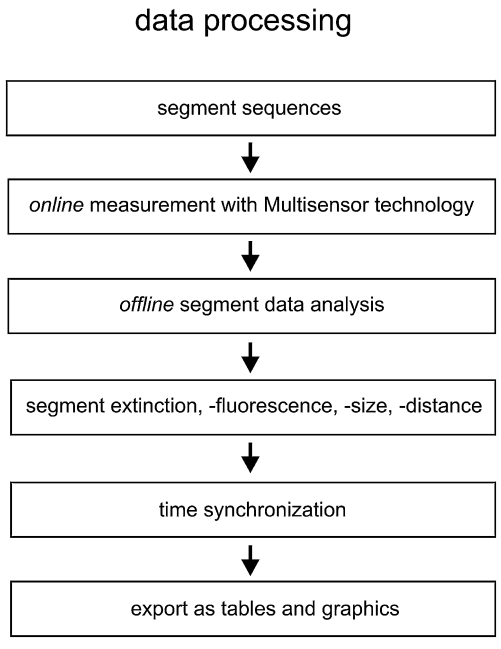

Fig. 2 Data processing: online-measurement of segment sequences and offline-data analysis with LabView™ software.

Isolation of pigment-producing bacteria

Segmented soil sample G 17 was incubated in AM minimal medium containing 1% glucose and 0.3 mM nickel sulfate for 9 days in a Teflon tube rolled on a PMAA plate at 28 °C in an incubator. Subsequently, the segment sequence was measured *online* with the multisensor-detector unit and stored temporarily in another tube coil. After data analysis and time synchronization, the segment sequence was transported out of the Teflon tube by constant pumping of the carrier fluid. Meanwhile, segments were counted and segment no. 46 was collected in an Eppendorf tube. The segment content in the tube was mixed with 100 µl of the aforementioned medium and then transferred into 10 ml liquid medium with the same composition in an Erlenmeyer flask. This suspension was cultivated for 24 h at 28 °C at 120 rpm. 10 µl of the liquid bacterial culture was plated on an AM minimal medium agar plate containing 1% glucose and 0.3 mM nickel sulfate (solid).

Investigation of heavy-metal-resistance of the bacterial isolate under segmented flow-conditions

10 ml AM minimal medium containing 1% glucose was inoculated with a colony of the pigment-producing bacterial isolate and incubated at 28 °C at 120 rpm overnight. Continuous concentration gradients of nickel- and copper sulfate in segments containing the bacterial isolate G17/46 (inoculation cell number: 2500 bacteria/500 nL segment) were generated with the help of the LabView™ software controlling the flow rate of the syringe pumps. Segments containing the bacteria, incubation medium and the heavy metals in increasing concentrations were formed by variation of the flow rates. Increasing amounts of copper sulfate or nickel sulfate (flow rate from 0–5 µl min^{-1}) were compensated by a decreasing amount of incubation medium (5–0 µl min^{-1}). The final concentration of copper sulfate and nickel sulfate in the segments was 2 mM and 5 mM, respectively. The carrier liquid PP9 was transported with a constant flow rate of 40 µl min^{-1}. Segments were stored for 48 h in tube coils at 28 °C. After incubation, extinction of the segments at 505 nm was measured to determine heavy-metal tolerance of the bacterial isolate. The experiments were repeated two times.

Fractionation, ethanol treatment of soil sample and measurement of segment-derived fluorescence

10 ml soil sample slurry of G 9 was inoculated into 10 ml AM minimal medium containing 1% glucose reaching a final volume of 20 ml. This suspension was incubated for 9 days in an Erlenmeyer flask with constant slow stirring at 28 °C. The rest of the soil sample slurry was cooled at 4 °C in a fridge. Soil sample culture after 9 days growth was counted to determine the cell number. 10 ml of the soil sample was centrifuged for 10 min at 4500 rpm. The supernatant was transferred into a new falcon tube and the pellet was resuspended in fresh AM minimal medium 1% glucose. To obtain a dead soil sample again a 10 ml soil sample with the same cell number like before was centrifuged for 10 min at 4500 rpm and subsequently, the cell pellet

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was incubated for 1 h with 5 ml 70% ethanol. The cell pellet was washed 2× with 1× phosphate buffered saline and finally resuspended in 10 ml AM minimal medium/1% glucose. The starting soil sample, resuspended pellet (living), supernatant, resuspended pellet (dead) and AM medium/1% glucose (mixed 1 : 1 with soil sample slurry) were segmented in a flow rate ratio of 10 µl min^{-1} for cells or supernatant and 40 µl min^{-1} for the carrier liquid PP9 and the fluorescence of the segments was measured after excitation with 470 nm. Fluorescence values were normalized against the blank AM-medium/1% glucose mixed 1 : 1 with starting soil slurry solution.

FDA/PI staining

To prove whether ethanol treatment was successful in killing soil microorganisms FDA (fluorescein diacetate)/PI (propidium iodide) staining was performed with living and dead resuspended soil sample-pellets like described before. Non-treated and ethanol-treated soil samples were incubated with 10 µg ml^{-1} PI and 50 µM FDA in phosphate buffered saline for 30 min to stain living and dead microorganisms. Images of stained soil samples were taken with a Zeiss/Axioplan using filter sets of 510/580 nm for PI- and 450/510 nm for FDA-immunofluorescence.

Results and discussion

Cultivation directly from soil samples

First, we wanted to find out whether growth of microorganisms present in the soil sample can be detected by means of extinction measurements. Therefore, soil slurries were injected (without further dilution) and the segments were measured with excitation wavelengths of 470, 505, 615 and 660 nm after 9 days growth. The presence or absence of bacterial growth could be easily detected by the intensity of the signals; extinction differed considerably from segment to segment, from sample to sample and in the presence of different heavy metals. However, all four spectral channels behaved similarly in response to growth which indicates a lack of production of chromophores. An exemplary dataset is shown in Fig. 3A where soil sample G 9 was cultivated with copper sulfate. Thus, the high correlation of the signals indicates an increase in cell number inside every segment. To verify that the measured signals after cultivation are due to growth, calculated signals were compared for all sensor channels to the signals of the 0 hour-measurements (see description in data processing and synchronization). A comparison of sensor signals of the segment sequence from Fig. 3A at 505 nm wavelength before and after cultivation is shown in Fig. 3B.

To further examine extinction accuracy for growth detection, correlation plots were created where the optical signals from two sensors with different excitation wavelengths (470 nm; 660 nm) were correlated with each other. Using this analysis, differential responses by different wavelength measurements can be identified which would translate into potentially interesting, well-growing isolates inside the segments. The location of the segments in the graph also reflects their vegetation

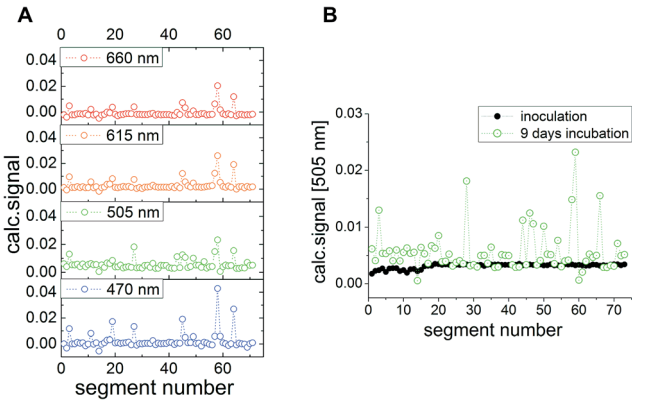

Fig. 3 Multisensor-measurement: (A) synchronized data from an online-measurement of a segment sequence of 71 segments, in which soil sample G 9 was incubated with 0.25 mM copper sulfate for 9 days in AM minimal medium/1% glucose. A multi-channel photometer set with excitation wavelengths of 470, 505, 615 and 660 nm was used. Every dot in the graph represents a segment. (B) Sensor signals (excitation 505 nm) of the same segment sequence measured at 0 hours and after 9 days were compared.

density. The example shown in Fig. 4A is typical for a cultivation study, where only some segments are prone to undergo growth, and the corresponding signals of the optical channels correlated with each other (see Fig. 4C). However, other soil samples showed a higher number of vegetated segments, indicating

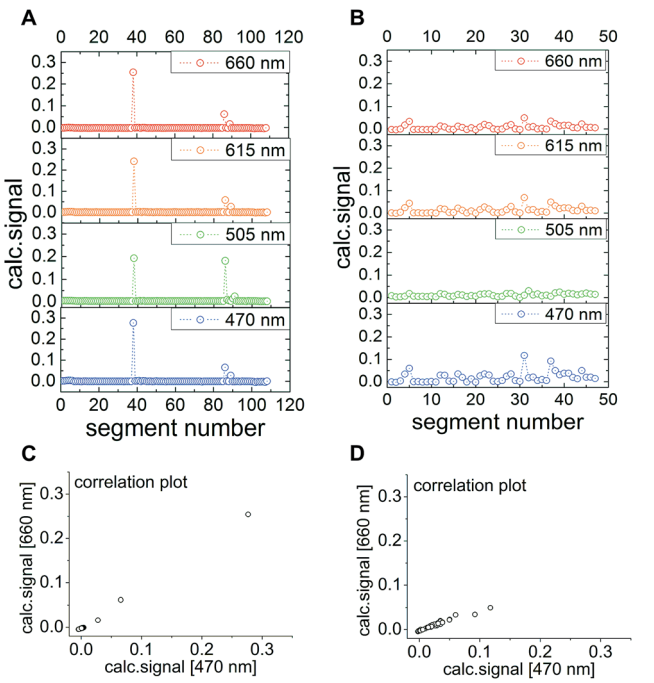

Fig. 4 Multisensor-measurement and correlation: synchronized data of online-multisensor-measurements of segment sequences of (A) soil sample G 7 treated with 0.25 mM copper sulfate and of (B) soil sample G 17 treated with 0.25 mM nickel sulfate for 15 days in AM minimal medium/1% glucose and the corresponding correlation plots for sensor 470 nm and 660 nm (C and D). Every dot in the graph represents a segment.

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higher proportions of bacteria being present in this soil. There, extinction measurements correlated well with each other again (Fig. 4B and D).

The method can also be applied to investigate the growth response of microbes in a soil sample to several metals, or various soil samples to one metal. As an example, two soil samples, K 1 and K 6, were segmented and incubated either with 0.30 mM copper sulfate or 0.30 mM nickel sulfate for 9 days, and extinction signals for 470 and 660 nm were analysed by correlation plots. A similar response for different soil samples to the respective metal indicated similar toxicity and hence microbial response to the respective metal. After nickel sulfate-treatment both soil samples showed more vegetated segments with higher extinction-values and generally a higher scattering of extinctions compared to copper sulfate-incubated soil microorganisms (Fig. 5). However, under nickel sulfate treatment there were also found segments with lower vegetation compared to copper-treated. This might well be due to the fact that copper, as an important trace metal and component of many bacterial enzymes, slightly stimulates growth but it is also a more potent inducer of oxidative stress in microbes than nickel.[30] Therefore it can be assumed that copper sulfate at this concentration stimulates but also suppresses higher growth of micro-organisms in both soil samples. In contrast, in the presence of nickel sulfate at the same concentration, segments with rising and scattering optical density indicated the presence of poor- and better growing microorganisms depending on their ability to tolerate nickel.

For a better classification and comparison of soil samples, ranking spectra were generated from the datasets. The graphs allow for a simple classification of response groups of segments in each sequence depending on the extinction signal (Fig. 6).

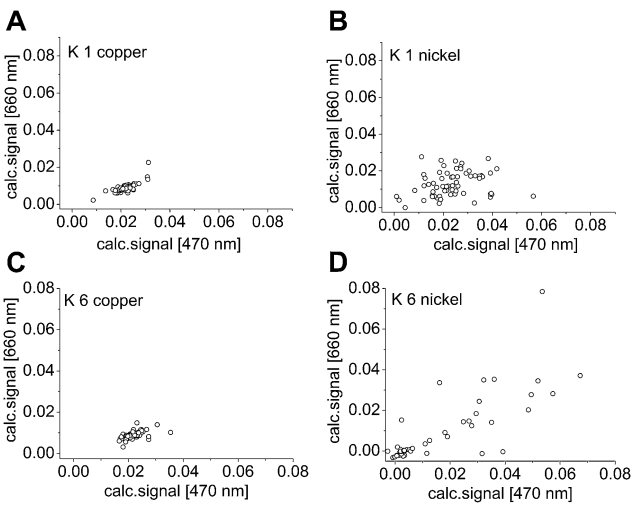

Fig. 5 Metal tolerance of soil samples: metal tolerance of microbial communities of soil samples K 1 and K 6 was analysed by correlation plots from calculated extinction data from sensors 470 nm and 660 nm. Segment sequences were incubated for 9 days with 0.30 mM copper sulfate (A and C) and 0.30 mM nickel sulfate (B and D) in AM minimal medium/1% glucose.

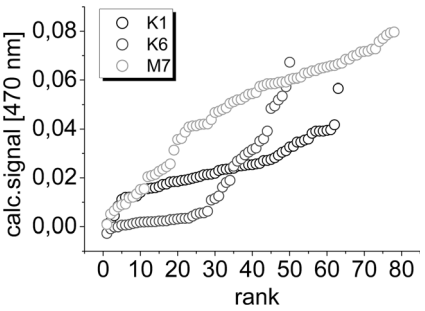

Fig. 6 Analysis of soil sample-derived growth of microorganisms by extinction ranking spectra: extinction ranking spectra prepared from data of online-multisensor-measurement (470 nm) of soil samples K 1, K 6 and M 7 treated with 0.3 mM nickel sulfate for 9 days in AM-minimal medium/1% glucose.

The ranking spectrum of soil sample K 6 reflects the presence of a number of segments with low extinction (low scattering and hence low growth) and other groups of segments with increasing optical response and higher extinction indicating "good growth". In contrast, the ranking spectra of soil sample K 1 and M 7 are marked by a smooth or stepwise, respectively, increase of extinction which indicates a more uniform distribution of vegetated segments and hence can be interpreted as a population of micro-organisms with metal-tolerance levels present that allow for a wider variety of metals in the droplets to still obtain growth. Segments that were inoculated with soil sample M 7 showed the highest extinction values in the presence of nickel sulfate indicating better nickel tolerance or resistance for this soil sample. Typically, all four photometric sensors showed a similar or identical type of ranking spectra, again underlining lack of chromophore production (data not shown). The number of ranks in each class of signals and their mean extinction level are specific parameters for the undiluted soil community response to heavy metal exposition. Obviously, these parameters can be used as general parameter to distinguish different soil sample-microfloras under the same stress conditions.

Identification of optical indicators for secondary metabolite production

Besides a general classification of growth from soil samples, the detection of the production of secondary metabolites was of particular interest. The application of multi-sensor technology can help to identify production scenarios. Therefore, *online* detection with optical sensors was used. To validate our assumption, we first searched for pigmented soil bacteria. The formation of chromophores can easily be identified by altered correlation between the photometric signals, depending on the spectral features of the coloured compound. An enhanced absorption signal of the short-wavelength sensors reflects an increase of blue light absorption and hence a yellow colour of the segment content. In contrast, the enhancement of long-wavelength absorption is an indicator of the formation of a brownish, red, green or even blue colour in a segment. These deviations in the correlation between different wavelengths

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could be recognized from correlation diagrams. An exemplary dataset is shown in Fig. 7 where the calculated signals (extinction signal integral with respect to the segment size) of *online*-measurements at 505 nm and 470 nm of soil sample G 17 grown under nickel sulfate-stress were correlated (Fig. 7A). Most segments did not allow for growth or were not fully grown, visible with low extinction values (*e.g.*, see segment no. 47, Fig. 7B). Segments with strong bacterial growth were identified by high extinction values (see segment no. 57, Fig. 7C). Of particular interest was segment number 46, which plotted outside of the assumed correlation line. This was due to an enhanced absorption signal of the short-wavelength sensor at 470 nm (Fig. 7A and D) compared to the sensor with 505 nm excitation wavelength.

The subsequent inoculation of this potential chromophore-producing microbial community into liquid solid media, followed by plating on a minimal medium agar plate revealed the growth of a strongly yellow-coloured bacterial isolate, verifying that the method can be applied for initial screening (Fig. 8A). In addition, the bacterial isolate was tested for its tolerance against the heavy metals nickel (Fig. 8B) and copper (Fig. 8C) under segmented flow-conditions. Two independent experiments revealed mean-EC_{50}-values of 1.35 mM for nickel sulfate and 0.66 mM for copper sulfate-treatment showing that the tolerances of the bacterial isolate against the tested heavy metals reside in the moderate range. Thus, using multisensor-technology, a moderately heavy-metal-tolerant bacterial isolate could be gained from a larger number of soil slurry-loaded segments, which secretes a yellow pigment as secondary metabolite, most likely, to defend itself against nickel-associated oxidative stress.

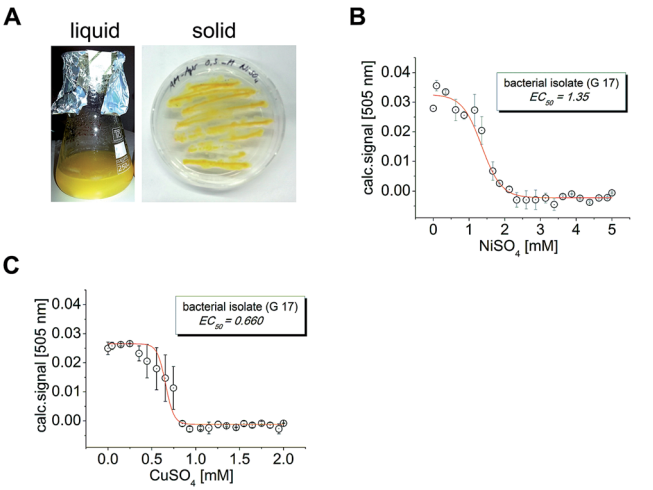

Fig. 8 Isolation of pigment-producing and heavy metal-tolerant bacteria: (A) after data analysis segment 46 was isolated by counting of the segment sequence of soil sample G 17 after incubation with 0.3 mM nickel sulfate for 9 days. The segment content was transferred into liquid AM minimal medium followed by plating on an AM minimal medium agar plate (solid) containing 0.3 mM nickel sulfate. Microscopic analysis showed rod-shaped bacteria. (B) and (C) Metal tolerance of isolated bacterial microorganisms.

Monitoring fluorescence

The presence of fluorophores may even better indicate secondary metabolite production. While some antibiotics are known to be coloured (*e.g.*, actinorhodin),[31] many drugs (including antibiotics) consist of fluorescent molecular structures.[32,33] Since primary metabolites are known to include UV-

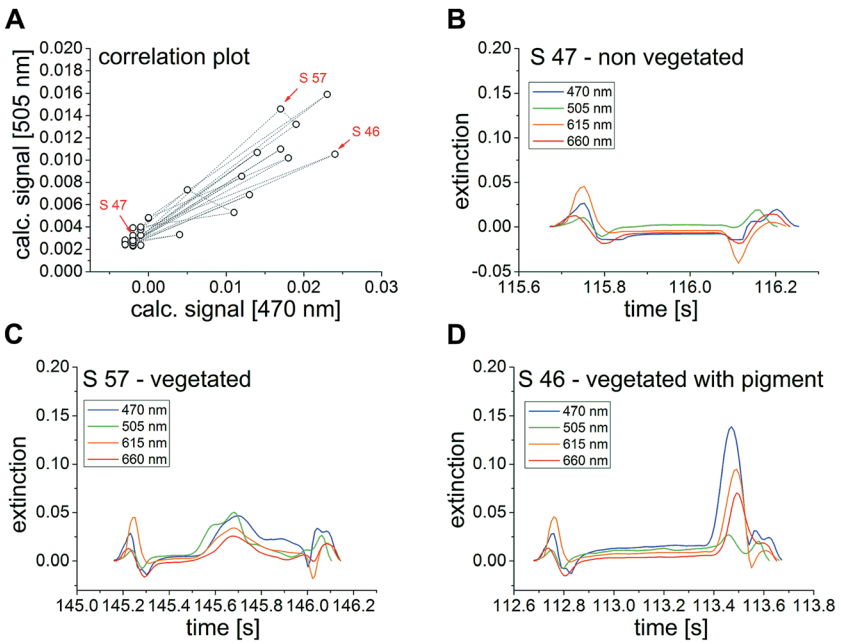

Fig. 7 Optical indicator of pigment production: (A) correlation plot indicates growth response of soil sample G 17 incubated with 0.30 mM nickel sulfate for 9 days in AM minimal medium/1% glucose and the formation of a dyed product in a single segment (no. 46); (B–D) extinction diagrams for single segment-photometric measurements: (B) non-vegetated segment no. 47, (C) vegetated segment no. 57 without optical conspicuousness, (D) vegetated and pigment-bearing segment no. 46.

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fluorescent metabolites like NAD(P)H, flavins and amino acids tryptophan and tyrosine, the fluorescence measurements will also allow for well detectable signals indicating growth at low cell densities by autofluorescence.[34]

Thus, we measured fluorescence signals (excitation wavelength: 470 nm) of the segments containing different soil inocula (excitation wavelength: 470 nm). Fluorescence diagrams as well as correlation plots were created. Soil samples treated with different heavy metals showed varying degrees of fluorescence (Fig. 9A and B).

Comparison of fluorescence diagrams for soil sample G 9 treated with copper- or nickel sulfate at 0.3 mM concentrations revealed that copper induces a higher degree of fluorescence in segments than nickel correlated with the higher toxicity observed earlier. Theoretically, this could be attributed either to more fluorescent cells or, more likely, to a stronger induction of (secondary) metabolites. To find out where the measured fluorescence originates, experiments with soil sample G9 in conventional flask-culture were performed. Separation of soil sample culture into particulate and supernatant fraction after 9 days incubation revealed that almost all the fluorescence is derived from the supernatant (Fig. 9B). This nicely shows that secreted metabolites of soil microorganisms must be fluorescent in some way. Pelleted microorganisms from the soil sample only showed a little fluorescence which can be referred here as autofluorescence. To verify whether dead cells are fluorescent, a "living" soil pellet was treated with 70% ethanol to produce cell death. This pellet of "dead" soil microorganisms showed the lowest fluorescence. It can therefore be assumed that the measured fluorescence in segments is derived mostly from secreted metabolites, but a smaller proportion also stems from living and dead microorganisms. Live/dead staining was performed to see the amount of live and dead microorganisms in the differently treated soil pellets. In the "dead" soil sample (after ethanol treatment) FDA-staining was completely absent and only propidium iodide-staining for the detection of released DNA from dead cells could be detected. In the non-treated soil sample living as well as dead cells could be found with live/dead staining (Fig. 9C).

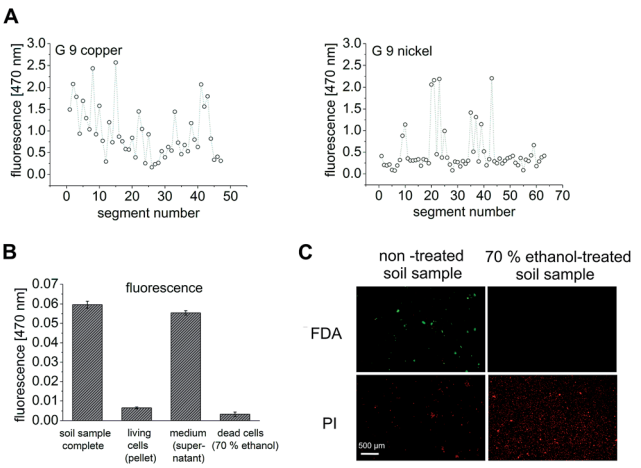

Fig. 9 Detection of soil sample-derived fluorescence in segments by micro-flow-through fluorometry at 470 nm: (A) segment sequence inoculated with soil sample G 9 was incubated with 0.25 mM copper sulfate and with 0.25 mM nickel sulfate and segment-derived fluorescence was measured. (B) Fluorescence of segments filled with soil sample completely, living soil cells (pellet), incubation medium (supernatant) or dead soil cells (70% ethanol) is shown. (C) Fluorescein diacetate/propidium iodide immunofluorescence staining of the non-treated soil cell pellet and 70% ethanol-treated soil sample pellet.

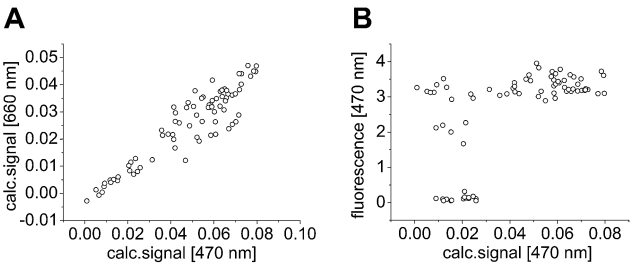

Fig. 10 Correlation of sensor signals (A) correlation plot of calculated extinction signals at 470 and 660 nm shows a uniform distribution and growth of microbial communities in segments of soil sample M 7 incubated with 0.30 mM nickel sulfate for 9 days, (B) controversially, correlation of fluorescence signals with extinction signals indicates non-uniformity.

The potential of multi-sensor-technology for classification of soil communities in response to stress under given conditions is well reflected by correlation plots for soil sample M 7 under nickel exposition (Fig. 10). The photometric signals at 660 nm and 470 nm are well correlated (Fig. 10A). However, high scattering was observed at higher extinction values. If fluorescence levels are considered, segments can be subdivided into three groups representing high, moderate and low fluorescence at low overall extinction levels (Fig. 10B). Thus, four types of response groups could be identified.

Conclusion

In the current study, an attempt was made to characterize soil samples from different metal-contaminated sampling sites with respect to spectral properties by means of multi-sensor technology. For this purpose, a high number of segments containing soil sample slurry incubated at known heavy metal concentrations were analysed. Growth of microbes in segments could easily be detected by measuring extinction with good correspondence between all four wavelengths. Correlation plots could show that microbial communities of different origin show similar behaviour with respect to metal toxicity. Additional information could be obtained when analysing over-representation of extinction at one wavelength. Moreover, the potential for the discovery of secondary metabolite-producing microorganisms in segments was demonstrated by the detection and isolation of a yellow-pigmented bacterial isolate which showed tolerances against nickel (EC_{50}: 1.35 mM) and copper (EC_{50}: 0.66 mM). Thus, it could be shown that the segmented flow technique and multisensor-technology are suited for the evaluation of physiological activity of microbial communities in the presence of heavy metals and therefore of their bioremediation potential. Monitoring of fluorescence induction in the soil

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sample may be used as an additional approach to detect secondary metabolites. Analysis of a long term-cultivated soil sample revealed the strongest fluorescence for the metabolite-containing culture medium. Further studies are necessary to verify that the observed fluorescence is associated with an induction of secondary metabolite-production, in our case by exerting stress through addition of heavy metals. In the future our miniaturized strategy will be used for the characterization of a larger number (100–1000) of soil samples from historical mining areas with different environmental conditions.

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