More than Meets the Dye: Evaluating Preferential Flow Paths as Microbial Hotspots

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Preferential flow paths have been suggested to function as microbial hotspots. This idea is speculative, based on biomass counts rather than assessments of the activity of the microbial population. In this study we used O$_2$-sensitive optodes, or optical sensor devices, to observe O$_2$ depletion by soil microbes as a reference for microbial activity along three artificially constructed preferential flow path geometries. The flow paths were constructed using contrasting fine (210–297 μm) and coarse (595–841 μm) sand textures in the geometries of (i) coarse sand in the center surrounded by fine sand, (ii) half coarse sand on the left and half fine sand on the right, and (iii) fine sand in the center surrounded by coarse sand. A soil slurry, containing soil microbes and glucose as an added source of C, was flowed through the sands to create nutrient gradients due to nonuniform flow. Results suggest that O$_2$ depletion is greatest along the boundary between preferential flow paths (coarse sand) and the bulk matrix (fine sand). While the results offer insight into the locations of microbial activity, the exact positioning remains unclear. Our work suggests that preferential flow plays a key role in the spatial distribution of microbial hotspots in soil and demonstrates the nexus between soil physical and microbial processes. We show that O$_2$-sensitive optodes were effective in monitoring O$_2$ depletion due to microbial activity along the paths. To address issues related to C cycling under the changing environment, biophysical processes in flow paths must be better understood.

Abbreviations: ROI, region of interest.
Because preferential flow paths have been shown to be stable, persisting for decades in some cases (Ritsema and Dekker, 2000; Hagedorn and Bundt, 2002; Leineman et al., 2016), assessing their biogeochemical function is necessary. In fact, Liu and Lin (2015) suggested that to accurately represent and predict hotspots and hot moments (high rates in time) of greenhouse gas emissions, we must model and identify large-scale preferential flow networks, which will aid in the elucidation of chemical fluxes. Additionally, Smith et al. (2017) proposed that C models should treat soil moisture as a network, emphasizing pathways for resource distribution and diffusion. Despite conclusions from the Bundt et al. (2001b) study and the significance of hotspots, few have attempted to examine the topic further (Rubol et al., 2014; Hagedorn et al., 2015). While the number of investigations on preferential flow has been on an upward trajectory since the 1980s, there continues to be a large number of unknowns associated with preferential flow resulting from theoretical and technological bottlenecks (Guo and Lin, 2018). These unknowns range from quantification to predictive modeling, and while not mentioned here, the biophysical properties of preferential flow paths.

To examine hotspot development along artificially constructed preferential flow paths, we used an O2-sensitive optode imaging system (Presens GmbH). This allowed us to visually observe the development of anoxic regions arising from O2 utilization by microbes along and adjacent to preferential flow paths. Our objectives were to: (i) determine if preferential flow paths function as microbial hotspots and determine hydrology’s role in hotspot development; and (ii) demonstrate the feasibility of using O2-sensitive optodes to monitor O2 depletion by microbes along artificially constructed flow paths. We hypothesized that anoxic regions would develop along the flow paths due to a C gradient created by preferential transport of input solution through coarse sand.

**Materials and Methods**

**Chamber Experiment**

A custom Hele–Shaw cell composed of three separate chambers and a removable front panel was fabricated from polycarbonate (Fig. 1). In this experiment, only the middle chamber was used. To ensure that the chamber functioned as a separate entity, a rubber gasket was glued to the dividing walls so that when the front panel was screwed on, an air-tight seal was achieved. The chamber dimensions were 6 by 1 by 17 cm, with a screened drain at the bottom. Sensor foils (SF-RP5U4, Presens GmbH) were glued to the inside of the removable front panel at three depths so that they would be in contact with the sand. They were arranged at a distance of 1 cm apart from each other and 1 cm from the edges of the chamber. To simulate preferential flow, we used contrasting fine (210–297 μm) and coarse (595–841 μm) sand so that flow would primarily take place in the coarse sand. The chamber was packed in three textural configurations while lying flat: coarse sand in the center surrounded by fine sand (Packing Geometry 1), half coarse sand on the left, half fine sand on the right (Packing
The next day, stirring was stopped and the soil suspension was made of 40.5 cm$^3$ fine and 52.5 cm$^3$ coarse. Glucose concentrations of 0.04 g would be created by preferential flow through the coarse sand, as was screwed on while still lying flat. After the panel was screwed on, the cell was turned upright and the barriers at the top were removed. The use of sand allowed us to control texture, and the sand functioned as a nutrient-free medium so that C gradients would be created by preferential flow through the coarse sand, as has been noted in previous literature. Additionally, while we have termed the 210- to 297-$\mu$m sand “fine,” it is still quite coarse and drained easily due to gravitational force at the conclusion of the flow event. Therefore, O$_2$ diffused readily into the chambers from the open-ings at the top and bottom (drain), and thus regions of anoxia could be attributed to high microbial activity.

**Input Solution**

To introduce a microbial community to the chambers, 100 g of wet soil was stirred in 1000 mL of deionized water overnight. The next day, stirring was stopped and the soil suspension was allowed to settle for 30 min. One-hundred milliliters (about three to four pore volumes) of the remaining solution was then collected. The average pore volumes were 32.10, 29.30, and 26.04 cm$^3$ corresponding to Packing Geometries 1 to 3, respectively. The volumes of fine and coarse sand used in Geometries 1 to 3 were 77.5 cm$^3$ fine and 15.5 cm$^3$ coarse, 46.5 cm$^3$ fine and 46.5 cm$^3$ coarse, and 40.5 cm$^3$ fine and 52.5 cm$^3$ coarse. Glucose concentrations of 0.04 to 0.06 g were added to the solution to serve as a C source for the microbes. This was done right before the initiation of each flow event so that microbial use of the glucose when in suspension was negligible. A single flow event of 100 mL was selected to have more control of the flow regime, and thus the nutrient gradient, with total flow lasting ~45 s. This limited the total flow in the fine-textured sand, providing a more representative simulation of preferential flow. Before initiating flow, the chambers were fully saturated. Once saturated, a Mariotte bottle applied the input solution at a constant head of 1 cm. Immediately after flowing the solution through the chamber, time series imaging at 15-min intervals was initiated to monitor O$_2$ depletion due to microbial consumption with time (Fig. 2). The experiment was conducted at room temperature (21–23°C), and the chamber was kept in the dark for the duration of the experiment.

**Image Capture and Processing**

A Universal Serial Bus (USB) microscope, VisiSens A1 (PreSens GmbH), was used to capture spatial heterogeneities in O$_2$ saturation from the O$_2$-sensitive sensor foils. The USB microscope was externally positioned so that it was in alignment with the sensor foil. The light-emitting diode (LED) lights contained in the microscope excite the luminescent O$_2$-sensitive and reference dyes contained in the sensor foil, which is translated into color images. For more detailed descriptions, see Rubol et al. (2016), Holst and Grunwald (2001), and Tschiersch et al. (2012). To calibrate the camera, a droplet of O$_2$-free water, made with 100 mL of deionized water, 1 g of Na$_2$SO$_3$, and 50 mL of cobalt nitrate standard solution, was pipetted onto the foil. Then regions of interest (ROIs) were selected for 0% O$_2$ saturation (the droplet) and 100% O$_2$ saturation (free air). The wide-view AT-4 adaptor tube was used to capture the trends of O$_2$ saturation. Time series images displayed in Fig. 1 were taken from the sensor foil location SF2 at 15-min intervals. Due to the frequency of image capture, we were only able to monitor one sensor foil at a time; however, our setup offers the possibility to monitor activity at all sensor foil locations if images are taken manually, but that was outside the scope of this study.

All images from the VisiSens AnalytiCal 1 software were converted to RAW image files using an application provided by the system manufacturer. This conversion assigned gray values for each pixel, resulting in files that could be uploaded to ImageJ open access software for analysis. To reveal the locations at which O$_2$ saturation percentage was the lowest, time series image sequences were averaged and analyzed at eight-bit grayscale. For Time Series 1, images 20 to 129 corresponding to the start and end of microbial activity (27.5 h) were averaged together. By excluding images prior to 20, the averages were not affected by periods when little to no O$_2$ consumption was occurring. Time Series 2 and 3 were analyzed following the same methodology over images 91 to 200 and 40 to 139, respectively, although we did not record periods long enough to document the end in activity. Custom look-up tables or color
scales were created to accentuate the variability in the averaged images. A selected region from the resultant averaged images was then used to plot the profile of O₂ saturation with distance (Fig. 3). These data were superimposed on idealized images of the textural configurations to clearly show the trends of O₂ saturation in space.

To demonstrate spatial heterogeneities in O₂ saturation with time, the VisiSens AnalytiCal 1 software was used to select three ROIs on individual images from each time series (Fig. 4, top). The data were then exported and plotted (Fig. 4, bottom).

**Results and Discussion**

Time series image sequences in Fig. 2 show formation of anoxic zones due to O₂ consumption by microbes (see supplemental video). The coarse sand flow paths conducted a higher volume of input solution than the fine sand, as expected. This resulted in a nonuniform spatial distribution of microbial activity and O₂ saturation, probably due to an uneven distribution of glucose and microbes. Rapid flow through the coarse sand coupled with limited surface area offered poor conditions for the accumulation of glucose and microbes in the flow paths themselves, and in our study the flow paths remained fully oxygenated throughout the experiment. Higher surface area and frictional forces at the textural interface caused slower velocities, allowing accumulation in the fine sand. Both Time Series 1 and 2 show that similar O₂ depletion patterns occurred near and along the textural interface. In Time Series 1, O₂ depletion began directly at the textural interface of the fine and coarse sands. This was again seen in Time Series 2. Higher flow velocities in Time Series 2 due to half the chamber being coarse sand probably caused an increased occurrence of lateral transmission of the input solution into the fine sand, therefore widening the area of O₂ depletion. However, in the averaged images, we can still note that O₂ depletion was highest near the textural interface and became more oxygenated moving toward the edge of the frame of view for Time Series 1 and 2 (Fig. 3), confirming our hypothesis. In other words, activity was concentrated near and along the interface and did not continue outside of the frame of view.

Time Series 3 followed a different formation pattern as O₂ was depleted from the center first. While counterintuitive based on the O₂ depletion pattern, it is likely that microbial activity was in fact localized near the textural interface, as was the case with Time Series 1 and 2, but due to the presence of microbes on both sides of the fine sand, O₂ in the center was depleted first. While we did not directly measure microbial parameters, previous literature is in support of our conclusion. The observed O₂ depletion pattern we observed is nearly identical to O₂ gradients measured in soil aggregates by Sexstone et al. (1985). This effect was demonstrated again by Borer et al. (2018) when examining C and O₂ gradients in an artificial pore network, where it was found that aerobic microbes consumed O₂ at the edge, resulting in an anoxic center. Other work has stated that aggregate surfaces are microbial hotspots (Kuzyakov and Blagodatskaya, 2015), and furthermore, a study examining the transport and fate of *Escherichia coli* in the presence of preferential flow simulated using contrasting sand textures found significant cell retention at the textural interface (Wang et al., 2013). Furthermore, we provide additional images from the developmental stages of the experiment that highlight...
Fig. 3. The average of a select 110 images from each time series. Color scales were made to accentuate the most O₂-depleted regions. The color images correspond to the graphs below them. Each black rectangle is the selected area from which the graphs were created, showing O₂ saturation percentage with distance.

Fig. 4. Trends in O₂ saturation with time for a select three regions of interest (ROIs). The ROIs are shown by the numbers on each image. Images A, B, and C correspond to the packing geometries 1 to 3, respectively. The most representative image from each time series is shown. Image A was taken at 1590 min, B was taken at 2250 min, and C was taken at 2925 min for each individual time series.
the occurrence of O\textsubscript{2} depletion just off of the textural interface to further support our conclusion that microbial activity was concentrated in one area (Supplemental Fig. S1).

The averaged images enabled us to determine what locations were most anoxic and measure the distances into the fine sand at which anoxia was occurring. For Time Series 1, O\textsubscript{2} depletion was greatest at 3 mm from the textural interface, and activity clearly lessened moving farther away from the textural interface. In Time Series 2, O\textsubscript{2} depletion was greatest at 6 mm off the textural interface and then began to become more oxygenated. In Time Series 3, O\textsubscript{2} depletion was greatest at the center of the fine sand at first and then became relatively uniform when reaching a quasi-stable state. We note that there is an additional centimeter of sand outside of the imaged frame view in Time Series 1 and 2, which should be highly oxygenated based on the trend of the averaged data. Additionally, with more significant textural contrasts than what was tested in this study, we would expect to see hotspots taking place more directly at the textural interfaces due to reduced transport into the fine material.

Our averaged time series results are supported by the ROI data (Fig. 4, bottom). Images selected from Time Series 1 and 2 show that O\textsubscript{2} saturation with time was lowest near the textural interface. The depletion of O\textsubscript{2} was not as intense when moving farther away from the textural interface. Again, consistent with our averaged images, the ROIs selected for an image from Time Series 3 show that O\textsubscript{2} depletion was nearly uniform across the fine sand. Although the duration of O\textsubscript{2} depletion varied among the time series, it appears that O\textsubscript{2} consumption began to rapidly decrease between 1300 and 1500 min in each case in our study.

Our results indicate that the effects of preferential flow extend beyond the physical processes of water flow and contaminant and nutrient transport and are likely to play important roles in biogeochemical processes in soil. Work from Hagedorn et al. (1999) showed that preferential flow paths exhibit enhanced rates of denitrification when wet and early onset of nitrification and at higher intensity than that of the matrix soil. At microbial hotspots, the resultant O\textsubscript{2} gradients create micro-niche environments (Rubol et al., 2016). Carbon and O\textsubscript{2} gradients such as these have been shown to create intimate spatial segregation of aerobes and facultative anaerobes (Borer et al., 2018), which may offer an explanation for what was seen by Hagedorn et al. (1999). Given that C and N in flow paths are often much fresher than that of the matrix (Bundt et al., 2001a; Chabbi et al., 2009), we can infer that it is more labile and thus more susceptible to microbial utilization. Nonetheless, we are aware of only one study that has attempted to link CO\textsubscript{2} emissions and preferential flow (Hagedorn et al., 2015), although results from greenhouse gas studies seem to offer analogous conclusions (Jarecke et al., 2016; Frouz and Bujalský, 2018).

We show that hydrology plays an important role in the formation and distribution of microbial hotspots. This is in agreement with other work, as water flow has been noted to play an important role in the distribution of microbes and hotspots of reactivity at varying scales. A small-scale laboratory experiment examining O\textsubscript{2} dynamics at a fluctuating capillary fringe found that E. coli was predominantly deposited in the transition region of the fringe (Jost et al., 2015). A review termed ecohydrological interfaces hotspots (Krause et al., 2017), drawing conclusions similar to those of McClain et al. (2003). Modeling results for a heterogenous aquifer system has also suggested that zones of higher conductivity result in hotspots of chemical reactivity due to enhanced mixing (Pool and Dentz, 2018). Microscale modeling work has again suggested that biofilm distribution and biogeochemical activity are increased along preferential flow paths (Yan et al., 2017).

Conclusions

We provide visual evidence that microbial hotspots are localized along and adjacent to preferential flow pathways. The use of the AT-4 wide-view adaptor tube enabled observations of microbial hotspot development throughout a relatively large frame of view (3 by 2.5 cm), giving general trends of O\textsubscript{2} saturation in the system. It should be noted that we were able to offer only an estimation of the spatial distribution of microbial activity using this methodology. This is apparent from Time Series 3, where active microbes are probably coating the outside of the fine sand on opposing sides, thus causing O\textsubscript{2} depletion to be highest in the center of the fine sand similar to what is seen in soil aggregates. Hydrology played a significant role in the spatial distribution of microbial hotspots. We do not discount the importance of root exudates and biopores in hotspot formation, but our results suggest that flow patterns arising from structural heterogeneities can also play a significant role in the spatial distribution of hotspots. Additional studies following our experimental design are necessary to assess how the microbial community arranges in the presence of preferential flow, and more direct measurements of other microbial parameters should be made. Preferential flow paths function as dynamic biogeochemical entities, but the biophysical aspects of preferential flow paths remain largely unknown and deserve further investigation.

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