



Supplement of

Physical constraints for respiration in microbial hotspots in soil and their importance for denitrification

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9 1. Supplementary information



10 1.1. Methods: Incubation

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Figure S1: (a) Half-pint incubation jar (230ml) filled with sand and porous glass beads that serve as microbial hotspots.
 (b) Respiration kinetics of different types of hotspots either inoculated with *Agrobacterium tumefaciens* or *Paracoccus denitrificans* are measured with 50 hotspots in an otherwise empty glass jar (120ml). Note that hotspots are packed densely
 with minimal distance, which might have affected aeration.

16 1.2. Methods: Product ratios

17 Ratios of gaseous denitrification products may vary strongly depending on the incubation period

18 for which they are computed. Moreover, in an experimental setup with a permanently close

19 headspace, intermediates may temporarily accumulate in the headspace and still be available as

an electron acceptor and diffuse back into the soil at a later incubation stage. This would not be

21 the case in a continuous flow setup with a carrier gas that lack NO and N_2O and also to a much

22 lesser extent under natural conditions were the NO and N_2O concentrations are immediately

23 diluted down to atmospheric concentrations. Still, apparent product ratios can still be computed

- 24 by adding up the release of intermediates and subtracting this amount from subsequent
- 25 denitrification product prior to computing ratios. To do so, we define

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$$NO^* := \int_0^T \Theta(no'(t)) dt, \ N_2O^* := \int_0^T \Theta(n_2o'(t)) dt, \ N_2^* := \int_0^T \Theta(n_2'(t)) dt$$

27 Where F^* is the cumulative release, *T* is the end of incubation, f' is the first time derivative of 28 concentration *f*, and Θ is defined as

$$\Theta := \begin{cases} x & \text{if } x > 0 \\ 0 & \text{else.} \end{cases}$$

We then have

30
$$NO = NO^*, N_2O = N_2O^* - NO^*$$
 and $N_2 = N_2^* - N_2O^*$.

31 We define the product ratios:

32
$$\mathbf{NO} := \frac{NO}{NO+N_2O+N_2}, \mathbf{N_2O} := \frac{N_2O}{NO+N_2O+N_2} \text{ and } \mathbf{N_2} := \frac{N_2}{NO+N_2O+N_2}.$$

Note that these estimated ratios disregard the actual effects that the presence of NO and N_2O may have on the regulation of enzymes for various denitrification steps. They should therefore only be considered as a rough approximation to product ratios in open systems. One benefit of this approach is that *T* is identical for all samples and does not have to be adjusted to individual denitrification kinetics.

38 1.3. Methods: Image processing and analysis

The inner volume of the glass jars filled with quartz sand, water and air constitutes the region of 39 40 interest (ROI) to be analyzed except for the porous hotspots which are considered as cavities in 41 the ROI. The ROI was determined by semi-automatic region growing in VG StudioMax 2.1 42 (Volume Graphics) based on gradient images, i.e. the first derivative of the original gray scale 43 image. The gradient was approximated by the Variance 3D filter in Fiji/ImageJ. Region growing 44 was initiated on the homogeneous glass wall of the jar and in the homogeneous head space of the 45 jar and stopped directly at the border of the repacked sand. The fully enclosed volume of both 46 region growing processes is considered as ROI. Likewise, region growing in the relatively homogeneous hotspots stopped directly at the border between hotspots and repacked sand. This 47 48 was used to demarcate the porous glass beads and subtract them from the ROI. The assignment to 49 At and Pd hotspots was done according to the vertical position in the layered architecture and 50 according to orientation of the flat and rounded end in the random architecture.

51 The raw image were filtered with a non-local means filter (Buades et al., 2005) for noise removal 52 at an estimated noise level of $\sigma = 7$ gray values using the non-local denoising plugin for ImageJ. 53 Edge enhancement was carried out with an unsharp mask filter (Sheppard et al., 2004; Schlüter et 54 al., 2014). A vertical drift in gray values due to uneven illumination was detected and corrected

- 55 for all voxels by subtracting the difference between the average gray values in sand grain voxels
- 56 per slice with the average gray value of all sand grain voxels (Iassonov and Tuller, 2010; Schlüter
- 57 et al., 2016). Thresholds were chosen manually and adjusted to the predefined water saturation
- 58 within test regions. Simple thresholding was carried out to segment the ROI voxels into air water
- and solid followed by a majority filter with a cubic 3³ kernel to remove partial volume effects
- 60 (Schlüter et al., 2014).
- 61 These segmented images were analyzed with respect to different morphological properties of the
- 62 air-filled and water-filled pores space. All properties are reported separately for the complete ROI
- and the direct neighborhood of the hotspot boundaries to assess the local conditions experienced
- 64 by the hotspots. The reduced ROI for the hotspot boundaries was created by dilation and
- 65 subsequent subtraction of the hotspot ROI.
- 66 Dimensionless air connectivity was determined through the volume fraction of air-filled pores
- 67 with a continuous path to the headspace. To do so, a connected components labelling was
- 68 performed with the MorpholibJ plugin in Fiji/ImageJ (Legland et al., 2016) to mask out all air
- 69 clusters without a connection to the headspace.
- Air tortuosity was determined in the connected air cluster using the Geodesic Distance map 3D in
- the MorpholibJ plugin (Legland et al., 2016). This transform writes the shortest path lengths fully
- 72 within the air cluster from the headspace to any location belong to the air cluster. The
- 73 dimensionless tortuosity number is then determined for each voxel as the ratio between geodesic
- 74 distance and depth-dependent, Euclidean distance.
- 75 Air distance is determined as the shortest, geodesic distance within the water-filled pore space
- from any water voxel to the closest air voxel belonging to the connected air cluster. This air
- distance cannot be normalized in a meaningful way and is therefore reported as a length in mm.



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Figure S2: Depth profiles (n=5) of (a) air saturation, (b) air connectivity, (c) air tortuosity and (d) air distance. Individual
profiles are shown in (a) two highlight the differences between layered and random architectures that are caused by
packing gaps around hotspots. All other profiles (b-d) are shown as averages (lines) with min-max bands (transparent areas).





Figure S3: (a) Visible porosity and (b) pore diameter of individual hotspots (n=4) and the incubated sand samples (n=9)
reported separately for the full sample or the only the hotspot boundaries affected by packing gaps. (c) The pore size
distributions have an overlap at a diameter range of 80-150µm, which explains why liquid is sucked out of the hotspots,
when pores <150µm in the sand are air-filled. (a-b): Data shown as box-whisker plots: Whiskers- min-max, middle lines –
median, dots: outliers. (c) Lines represent average and transparent bands the min-max range.



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91 92 93 94 95 Figure S4: Gas kinetics in all treatments at high saturation in the sand (90% WFPS) and an initial O₂ concentration of 20% in the headspace for three different hotspot architectures containing a total of 100 hotspots, in which the hotspots either had maximum separation distance (random) or were densely packed in layers either with all At hotspots (layered At/Pd) or all Pd hotspots (layered Pd/At) in the upper layer : (a) O₂, (b) CO₂, (c) NO, (d) N₂O, (e) N₂. Different lines styles

represent replicates.

Results: Incubation at 60% saturation



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98Figure S5: Gas kinetics in all treatments at medium saturation in the sand (60% WFPS) and an initial O2 concentration of9920% in the headspace for three different hotspot architectures containing a total of 100 hotspots, in which the hotspots100either had maximum separation distance (random) or were densely packed in layers either with all At hotspots (layered101At/Pd) or all Pd hotspots (layered Pd/At) in the upper layer : (a) O2, (b) CO2, (c) NO, (d) N2O, (e) N2. Different lines styles102represent replicates.

1.6.



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- 107 had maximum separation distance (random) or were densely packed in layers either with all At hotspots (layered At/Pd)
- 108 or all Pd hotspots (layered Pd/At) in the upper layer : (a) O_2 , (b) CO_2 , (c) NO, (d) N_2O , (e) N_2 . Different lines styles
- 109 represent replicates.
- 110



Figure S7: (a) transport of brilliant blue dye from aggregates into sand at different saturations after≈10min. At 30%
 WFPS transport is dominated by convection, whereas at higher saturation only diffusion remains. (b) dye loss after one day.

- 115 1.7. Results: Nitrogen balance
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- Figure S8: Sum of denitrification products (N₂O, N₂) at the end of incubation (300h) for all architectures and saturations (n=3). The amount of initially present nitrate estimated from the internal porosity of hotspots was 96 µmol NO₃-N per jar, with some additional nitrate in growth medium adhering to the hotspot boundaries by week capillary forces during packing.

122 **1.8. References**

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