Comment on "Discrimination of Shifts in a Soil Microbial Community Associated with TNT-Contamination Using a Functional ANOVA of 16S rRNA Hybridized to Oligonucleotide Microarrays"

Eyers et al. (*1*) suggested that nonequilibrium thermal dissociation (NTD) curves could be used to discriminate shifts of soil microbial communities. Our own experience with gel pad array technology (*2* and refs within) suggests an alternative interpretation. In this correspondence, we consider the image acquisition and data processing algorithm they used, and, based on their results, question whether the NTD approach in conjunction with functional ANOVA (FANOVA) is capable of detecting community shifts. Testing the technology against a series of well-defined mixtures is needed before such claims can be made.

The image acquisition and filtering algorithm used by Eyers et al. (*1*) was evaluated in a previous paper (*2*), which showed that the grid placement significantly influenced initial signal intensities (SIs) and shapes of the curves (including T_d). In Figure S-1 and Table S-1 (Supporting Information), we show that only 15.6% of the grid frames are exactly placed in the center the pad.

Figure 1 shows what happened to raw SIs of NTD curves when the grid frames were displaced to simulate manual application of the sampling grid to the image. Our simulation was based on a NTD of one duplex, which was collected as an image stack. The image stack was analyzed by IstackX (*3*), which acquired raw SIs as well as filtered the data by the Fotin et al. (*4*) method. Each of the 12 NTD curves in Figure 1 represents an independent placement of the grid frame. Note that when the raw SIs are filtered and normalized, the shape of the curves was abruptly changed. Curves in Figure 1 are consistent with previous findings (see Figure 10 in ref *2*) that subtle displacements in the grid framing the pads significantly affect the shape of the filtered data (Figure 1, bottom panel), but not raw SIs collected from the inner frame (Figure 1A, top panel).

In their study, large standard deviations of the averaged curves and corresponding T_ds (shown in Figure 1B and Table S-3 of ref *1*) suggest that variations in NTD curves was due to the image acquisition and data processing algorithm used. Unfortunately, since the curve data are not compliant with MIAME standards (*5*), we are not able to conduct an independent analysis.

Given the high sensitivity of the Fotin et al. (*4*) method to grid placement, one can argue that some of the observed similarities of the curves are due to their differences being drowned by noise. Alternatively, the observed differences in curves could be due to specific grid placements. Since neither the original images nor the curves are available, it is not possible to reanalyze the data with a variety of grid placements to see if FANOVA results would be the same or different.

The authors stated that comparison of NTD curves from *in vitro* transcribed 16S rRNA of *P. putida* with *in vitro* transcribed 16S rRNA from two soil samples showed no differences in the shape of the curves for the *Bacteria* probe Eub338 and stated that this demonstrated that a robust and reproducible reference curve can be established for each probe. To the contrary, the authors revealed that FANOVA was able to detect differences in NTD curves for 11 broad-range probes obtained from the soil samples. Why do these curves differ between soil samples when they characterize broad microbial groups? Surely the treated site was not devoid of these groups. The curves should have been identical. We

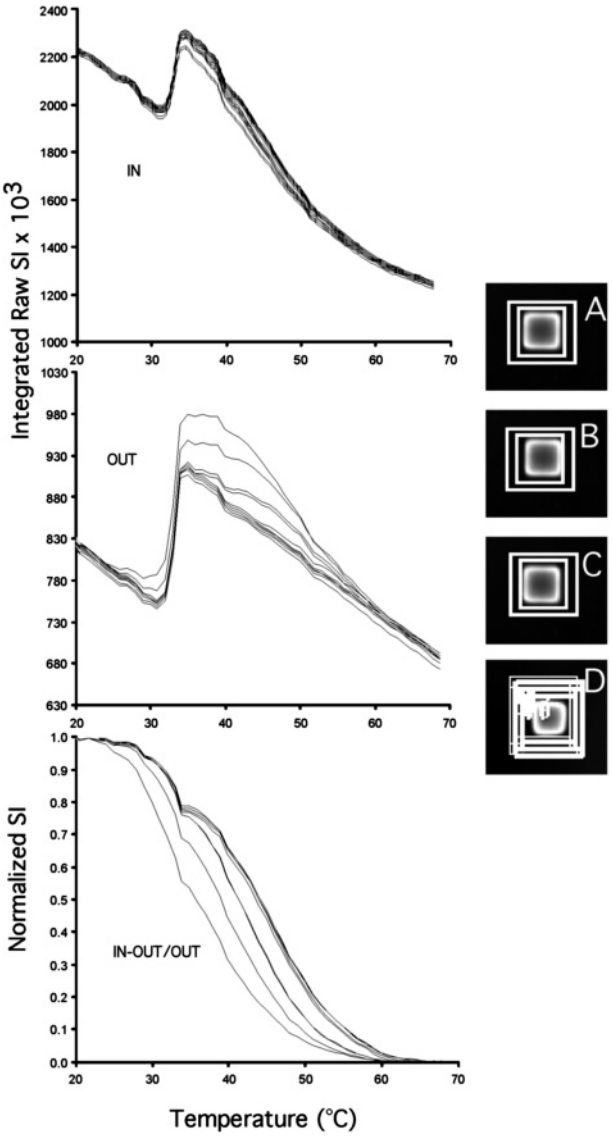

FIGURE 1. Effects of filtering the raw signal intensity (SI) data using the Fotin et al. (*4*) method (i.e., (IN − OUT)/OUT). Shown are SIs of native 16S rRNA from *Porphyromonas gingivalis* as it dissociates from probe Univ907 on one gel pad. Multiple samplings were made of the pad, each time manually varying the placement of the grid. Insets A−D, examples of grid frame placements at one temperature. Inset A, ideal placement of the inner (IN) and outer (OUT) frames; Inset B, subtle displacement of the frame to the lower left; Inset C, subtle displacement of the frame to the upper right; Inset D, composite of 12 random displacements used to produce figure. Number of the pad is faintly visible in the inset. Top panel, integrated sum of SIs from the inner grid frame. Middle panel, raw SIs obtained from the outer grid frame;. Lower panel, dissociation curves obtained using Fotin et al. (*4*) method. The bump in raw SIs has been routinely observed, see ref *2*. The same fragmentation/labeling method was used as in Eyers et al. (*1*). The data can be downloaded at http://staff.washington.edu/pozhit/pubs_rawdata.htm.

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suggest two alternative explanations: either the differences in the curves are due to the grid placement (see above) or, they are the result of an overlapped hybridization of specific and nonspecific duplexes since the relative abundance of specific and nonspecific duplexes determine the shape of a curve.

This contradiction suggests that, so far, the NTD approach with FANOVA is capable of detecting some undefined changes in curves; however, it is not capable for attributing the curves to particular groups of organisms. Alternatively, analysis of initial SIs provides the same ability to detect undefined changes (and it is simpler to perform), since statistical analysis of the data in Figure 2 (ref *1*) did not reveal any difference between initial SIs and FANOVA results in terms of their ability to detect undefined changes, and their agreement/disagreements (Tables S-2 and S-3).

Since Eyers et al. (*1*) did not use an alternative "gold" standard method to show that shifts of soil communities actually occurred in the samples examined, their findings could just as easily be due to an artifact of the method. If the authors knew what actual community shifts actually occurred, they could have then assessed if the differences or similarities of the SIs or NTD curves observed for particular probes made sense or not.

Acknowledgments

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Supporting Information Available

Figure S-1 and Tables S-1 to S-3. This material is available free of charge via the Internet at http://pubs.acs.org.

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