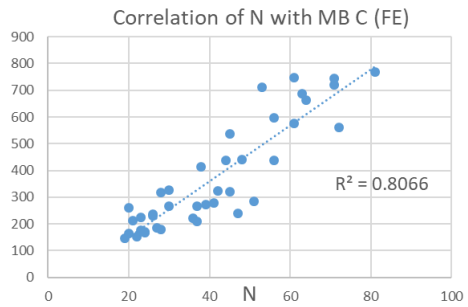
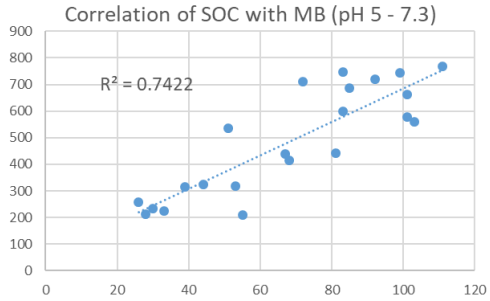
INTRODUCTION

Microbial biomass (MB) is the best single indicator of soil health (Doran, 2000). Microbes feed and protect plants, build soil structure which prevents erosion, increase water holding capacity, and build soil organic matter (SOC). MB is low in almost any situation that is harmful to plant growth (and vice versa) and protects against pathogens, thereby reducing the need for pesticides. MB can predict success before plant outcome because as shown below in data taken from Anderson et al 1989 MB as measured by Fumigation Extraction (CFE) correlates with soil nitrogen (N) and soil organic carbon (SOC).



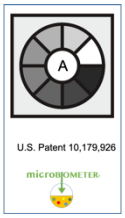


The Fungal:Bacterial ratio (F:B) of the MB provides crucial information regarding carbon sequestration, colonization by Arbuscular Mycorrhizal Fungi (AMF), and the recycling metabolic processes of saprophytic fungi (SpF).

microBIOMETER® Assay for MB and F:B

The microBIOMETER® is a patented, low-cost smartphone assay for MB and fungal:bacterial ratios that can be performed in-field on living soil. It is simple to use, free of hazardous materials, and produces rapid results. By following four easy steps, the results are available on your smartphone and stored in the cloud:

- Accurately and consistently measure soil by volume
- Extract microbes from soil particles using agitation in a precisely formulated solution
- Allow soil particles to settle, leaving microbes in suspension
- Measure MB by applying the microbial suspension to a test card and analyzing with a smartphone app



The smartphone app images the testcard (left) with extracted microbes applied to window (A) and calculates the intensity of the color present. Normally, bacteria are mostly transparent when viewed under a microscope, and stains are often employed to facilitate detection. Since bacteria, fungi, and other microbes in soil are colored, stains are not necessary with the microBIOMETER®. The color of the sample window is converted to µg microbial biomass carbon/gram of soil and the F:B ratio is determined.

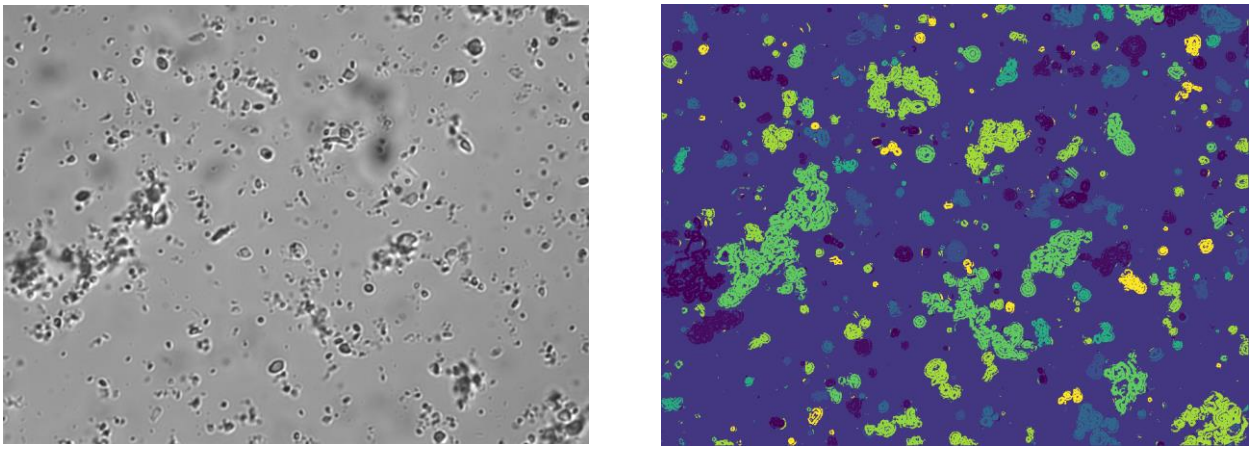
The company currently has relationships with several universities and is in the process of obtaining independent validation which will be shared once that data is available.

VALIDATION OF microBIOMETER® BY DIGITAL ANALYSIS OF MICROSCOPE IMAGES

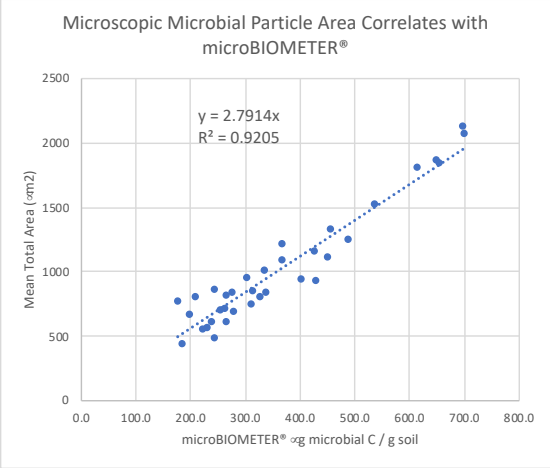
Methodology:

- Microbes were extracted from non-living soil particles using the microBIOMETER® test method
  - The assay was performed in triplicate for each soil
- 3 or 6 drops of extract was pipetted from each extract onto 3 microBIOMETER® Test cards and analyzed
  - The number of drops depends on the source of the soil samples but is the same for all samples from an individual source.
- 14 µl of the same extract was placed on a slide and covered with a slip, 3 samples per slide.
- 10 images in a spiral pattern around each coverslip were captured.
- The images were digitally analyzed to determine the total area of each particles, using a predetermined scale of 8.1 pixels / µm.

Below is an example of the analysis

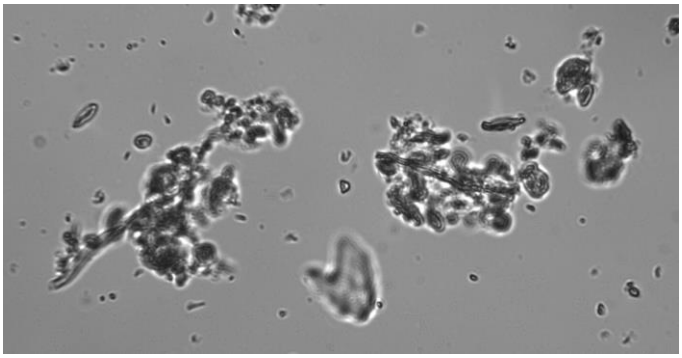


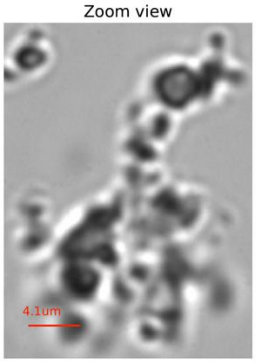
On the left is the original image, and on the right is the image after it has been segmented into individual contiguous particles. The area of each particle was determined by the area of the polygon that circumscribed it or more simply by the number of pixels it consumed. Both methods produced similar results. The total area of all particles was calculated for each of the 30 images and averaged. When compared to the average microBIOMETER® reading, the correlation is r = 0.96.



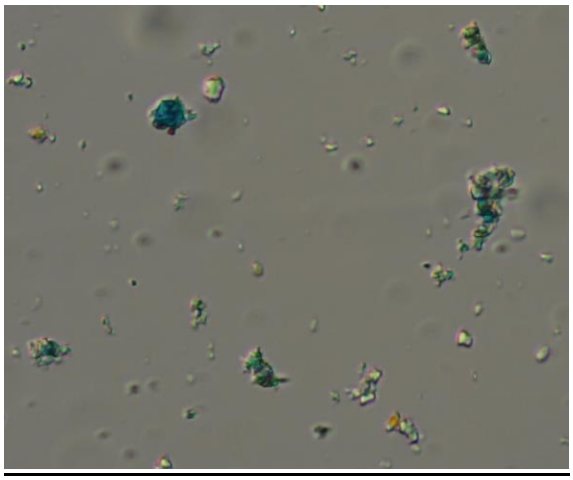
Determining the fungal : bacterial ratio by digital microscopy

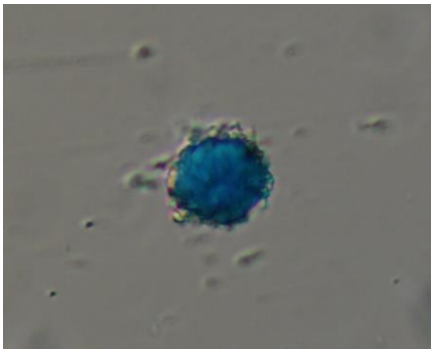
Microscopic examination of microBIOMETER® soil extracts revealed particles of a wide range of sizes, from <1 μm to much larger particles that appeared as conglomerates of filamentous and vesicular structures (see below). Since soil microbes are colored by uptake of dark organic molecules such as humic acid, no stains are necessary to image them.



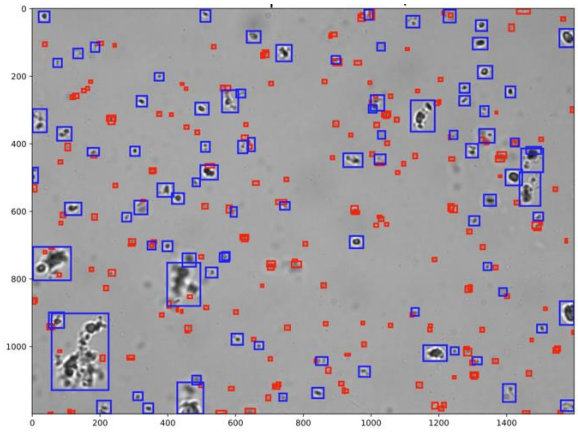


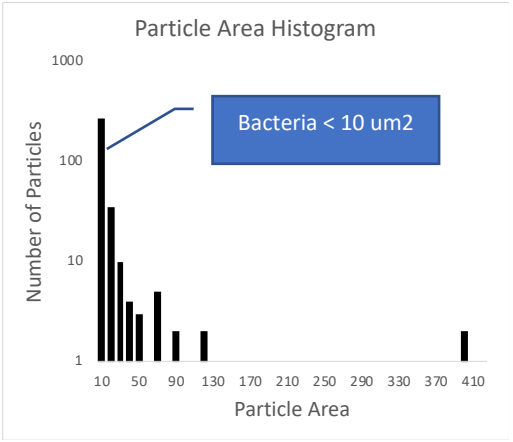
We hypothesized that the larger particles were fragments of fungal bodies that were disrupted/modified by the microBIOMETER extraction procedure. To determine their fungal identity, we stained with lactophenol blue, a stain commonly used to stain the chitin components of the fungal cell walls. The figures below illustrate the result of incubating soil extract with lactophenol blue at a 5:1 dilution. The large conglomerates are stained blue. The image on the right depicts a probable sporulated fungal particle.





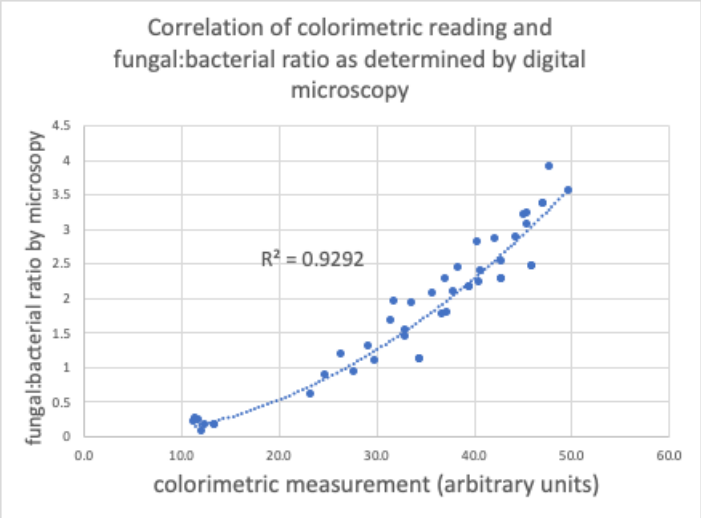
Considering that the largest soil bacteria is a rod that is 2x5 μm, and that fungi are larger, even after extraction, we used the threshold of 10 μm^2 to sort the particles into bacteria (≤10 μm^2) and fungal (>10 μm^2).





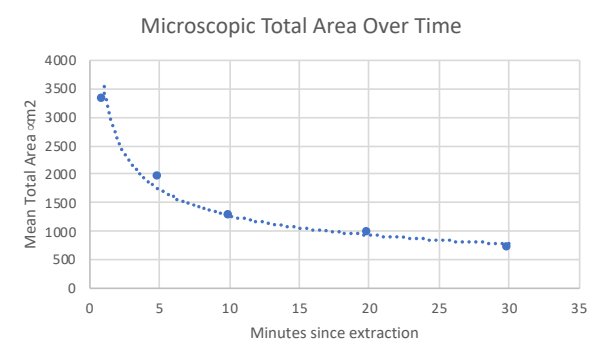
Blue boxes surround fungal particles (≥10 µm^2) and red boxes surround bacterial particles (< 10 µm^2). In this example the total areas attributed to bacteria and fungi are 675.37 um2 and 1733.1 um2, respectively. The ratio of fungal:bacterial areas is 2.57.

With the microBIOMETER® app, the fungal:bacterial ratio is calculated based on absorbance differences, and agrees (r = 0.96) with the microscopic method. The accuracy of the app is ± 0.5 to 0.6 units.



Absolute Difference from predicted	*Frequency*	*Cumulative %*
0.1	14	31.11%
0.2	10	53.33%
0.3	12	80.00%
0.4	1	82.22%
0.5	4	91.11%
0.6	2	95.56%
0.7	2	100.00%
0.8	0	100.00%
0.9	0	100.00%
1	0	100.00%
More	0	100.00%

The extraction fluid is formulated to aid in extraction of the microbes and rapid precipitation of the non-living soil particles. Below is a series of total areas derived from microscopic analysis over 30 minutes, showing the stability of the microbial suspension after 10 minutes of settling.



COMPARISON WITH OTHER METHODS

Overview of current methods run for correlation

- Current methods extract and measure a small component of the MB (e.g. Carbon, phospholipid fatty acids, or nucleic acids) and then multiply the result by 2 factors:
  - An estimate of the fraction of that component in the microbial population.
  - An estimate of the efficiency of the extraction method(s).
- Uncertainties about extraction efficiencies create uncertainties in measurements as does the fact that a single component does not compose the same % in every microbe.
- Current methods are technique dependent and, in many research labs, are not routine. Therefore, run-to-run variation can greatly affect consistency, leading to large study-to-study and lab-to-lab variability.

Method of estimating Microbial Biomass (MB)	% of sample evaluated	Avg Cost	Avg Analysis Time (hours)	Disadvantages	Reproducibility
Carbon fumigation	~40%	$500	336	Cost & Time	Reasonable but not consistent lab to lab
Phospholipid Fatty Acid Analysis (PLFA)	~10%	$80	168	Cost & Time, small % of sample measured	Reasonable but not consistent lab to lab
Microscopy	<10%	$30	168	Dependent on skill of microscopist	Too technique dependent
microBIOMETER®	>50%	$10	0.2		CVs range from 3.1% to 11.8%.

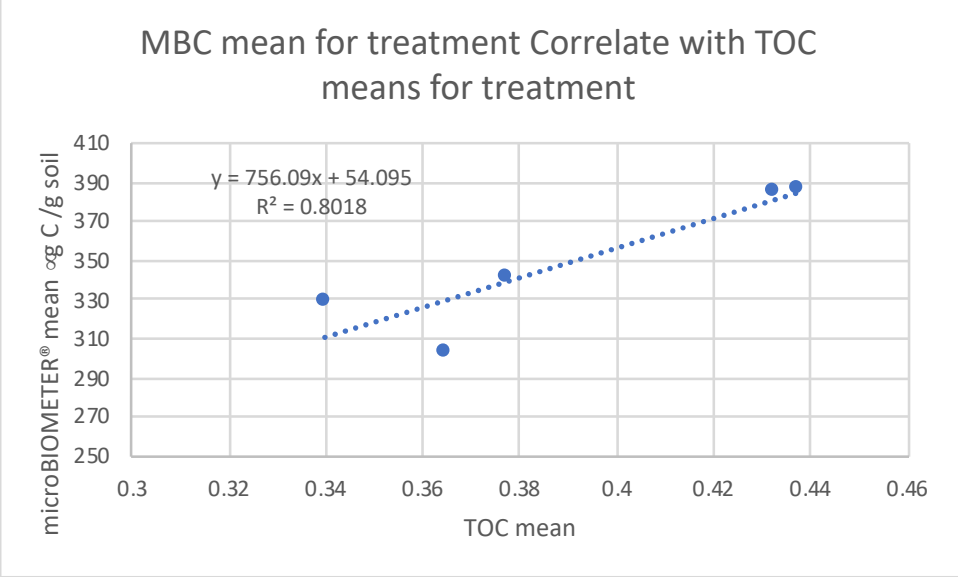
Although carbon fumigation is considered by some to be the "gold standard" of measuring soil microbial biomass, we have found widely ranging correlations in the literature between carbon fumigation and other soil microbial biomass tests, such as flow cytometry, ATP, quantitative PCR, and PLFA analysis, with Pearson coefficients ranging from ~-0.01 to ~0.95. While our literature search is by no means exhaustive, it appears that soil type, time of sampling, and geography play a role in these test results and correlations between test types.

Carbon fumigation methods measure the difference in carbon between soil samples that have or have not been exposed to chloroform. The amount of carbon is determined by chemical ***e***xtraction (CFE) or the volume of CO_2 released by respiration during extended ***i***ncubation (CFI). Microbial biomass carbon (MBC) is calculated as E_C/K_C (CFE) or F_C/K_C (CFI) where E_C or F_C is the difference in organic carbon extracted/evolved between fumigated and nonfumigated soil. K_C is a factor used to determine the extent of fumigation and is determined by addition of known amounts bacteria and fungi labeled with ^{14}C and quantification of the extracted ^{14}C or evolved $^{14}CO_2$.

Vance et al 1987 investigated the surprising finding that MBC in acidic soils was underestimated by CFI when compared to microscopic observations. They found that K_C was much lower in acidic soils than for other more neutral soils. With the lack of reliable correlation among the various tests listed above and the trusting of microscopic observations as a basis for determining K_C in carbon fumigation studies, we chose to validate the microBIOMETER® via microscopy.

Other Methods in testing for correlation:

- Jill Clapperton with Rhizoterra is currently field testing the Solvita CO_2 Burst field test against the microBIOMETER® and finding good correlation.
- The Carbon Sponge Project at City University of NY (CUNY) is using microBIOMETER® to track carbon sequestration and to evaluate effective sequestration efforts.
- CUNY demonstrated high correlation of microBIOMETER® results with agricultural outcome at 20 agricultural sites in Ecuador.
- Forbes Walker and his associates at the University of Tennessee have found that microBIOMETER® correlates highly with soil health from a study of 32 plots, each of which have 30 years or more productivity data. microBIOMETER® is the only soil test they have found with such a correlation.
- In addition, microBIOMETER shows excellent correlation with soil Total Organic Carbon measurements from these fields. See below.

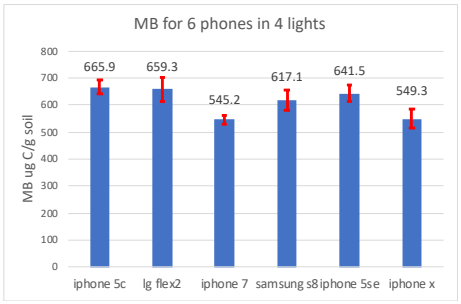


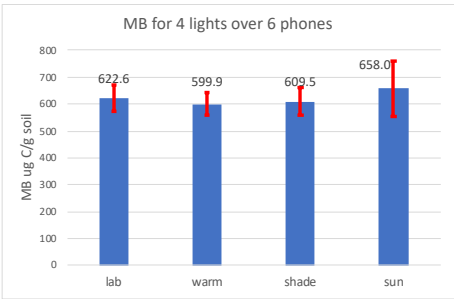
REPRODUCIBILITY OF THE TEST AMONG PHONES AND LIGHTING CONDITIONS

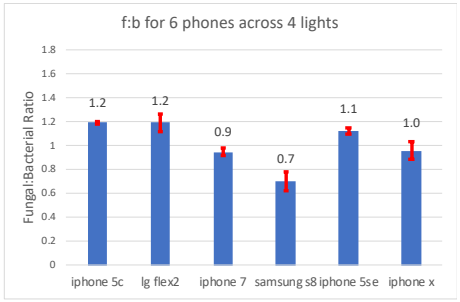
One of the most difficult aspects of analyzing with smartphone cameras is differences in color temperature used to image the testcard. The microBIOMETER® app and testcard format control for differences in color temperature and allows for consistent readings in all lighting conditions.

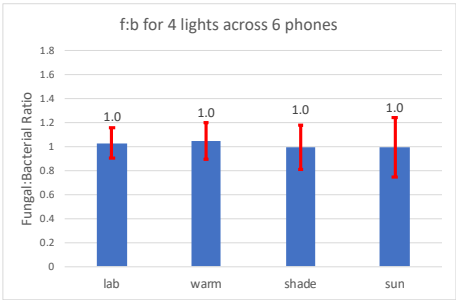
Here we demonstrate using 6 phones and 4 different lighting conditions the consistency of the 6x4=24 test results for 2 different soils. Soil A MB was 613 ± 61.6 (SD) with a CV of 10.04%. Soil B MB was 1194.8 ± 114.9 (SD) with a CV of 9.62%. Soil A F:B was 1.02 ± 0.18 with a CV of 18.19%. Soil B F:B was 2.39 ± 0.34 with a CV of 14.05%. The increase in variability in direct sun can be attributed to variance in the angle of the sun and the slight but unavoidable reflectivity of the testcard color-comparator surround.

A)

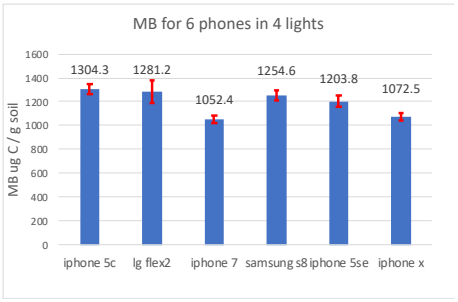


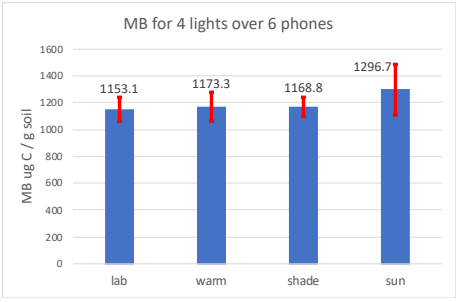


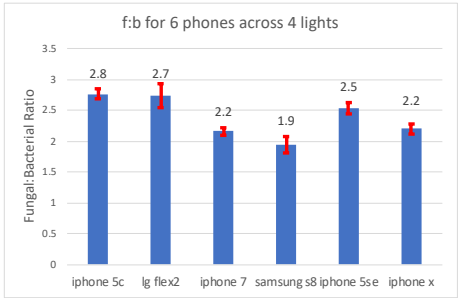


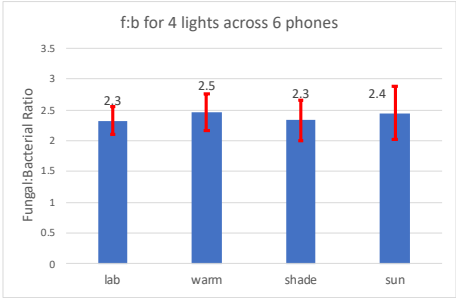


B)





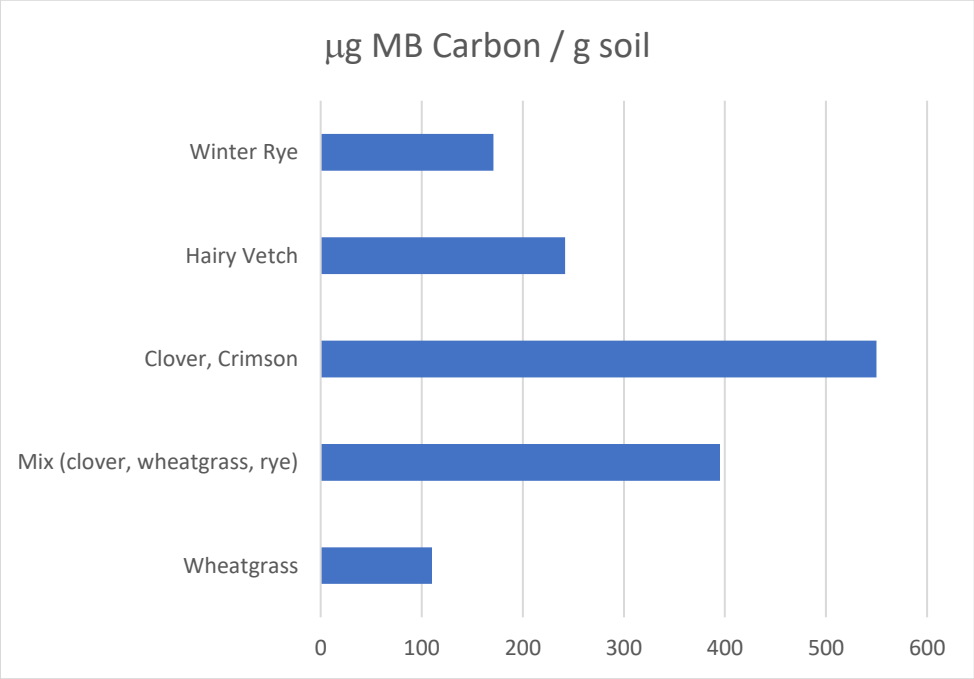




The light blue rectangles in the MB plots show the mean and standard deviation for the whole population, whereas in the F:B plots they represent the accuracy of the method, which is ± 0.6 units around the mean.

USES FOR THE microBIOMETER®

- Determining the efficacy of a cover crops: microBIOMETER® is excellent for short term (<1 month) potted evaluation of the efficacy of a cover crop. The below graph shows the increase in MB in the soil of a vacant New York City lot with various cover crops.



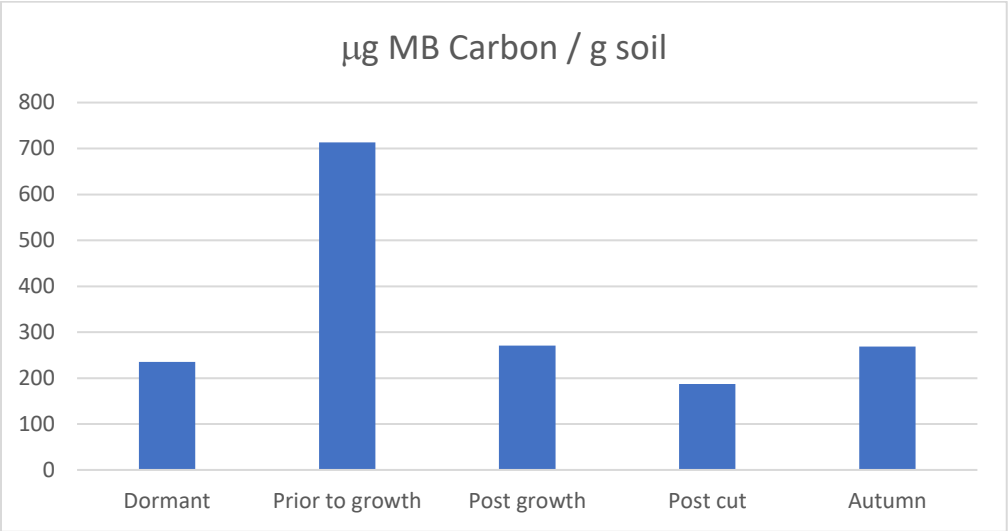
- Assay health of soil before planting, applying fertilizer, amendment or regenerative regimen. As an example, a plot in Englewood, NJ was planting very expensive trees ($2,000 evergreens) and it was discovered that three (3) had died in a row in the same spot. The microBIOMETER® was used to determine MB of the soils. Thus, the landscaper determined that the spot where they had been planted has <100 µg MBC/g of soil, while trees planted in locations on either side with MB >400 µg MBC/g have thrived.

- Assay progress of remediation or efficacy of treatment. The test quickly, sometimes within days, shows microbial response to treatment providing information before plant outcome.

- The test could be used to discriminate between chemically treated and organically treated soil. Soil that has been treated with chemical fertilizer will generally have MB that is below our level of detection, i.e. < 200 µg MBC/g soil. It is usually around or less than 100. Soil would be considered on a path to health at 200 or above.

- Quality control of compost and compost teas and for evaluating quality of compost and titration of tea.

- Research: replaces expensive laboratory test for microbial biomass. The graph below plots the MB in a Texas field over a growing season.



- Microbial biomass has been shown to be a reliable short-term predictor of accumulation of soil organic matter, so should have use in evaluating regenerative methods.
  - Maeder, et al 2002, Science 296 1694.
  - SAFFIGNA et al. 1989. INFLUENCE OF SORGHUM RESIDUES AND TILLAGE ON SOIL ORGANIC MATTER AND SOIL MICROBIAL BIOMASS IN AN AUSTRALIAN VERTISOL Soil Biol. Biochem Vol. 21. No. 6, pp. 759-763.
  - WANG et al. 2012 Fertilization increases paddy soil organic carbon density. Biomed & Biotechnology 13(4):274-282.
  - Valpassos et al. 2001 Effects of soil management systems on soil microbial activity, bulk density and chemical properties. Pesquisa Agropecuária Brasileira.
  - Fontaine et al. 2004. Carbon input to soil may decrease soil carbon content Ecology Letters, 7: 314–320.
  - Parihar et al. 2016. Long term effect of conservation agriculture in maize rotations on total organic carbon, physical and biological properties of a sandy loam soil in north-western Indo-Gangetic Plains. Soil and Tillage Research 161:116-128.
  - Balota et al. 2004. Long-term tillage and crop rotation effects on microbial biomass and C and N mineralization in a Brazilian Oxisol. Soil Tillage Res. 77:137–145.

Stability of Soil Samples for microBIOMETER®

Conclusion: Soil samples can be tested immediately or stored at 4C, 20C, or 37C for 10 days before testing by microBIOMETER® for microbial biomass (MB) and fungal to bacterial ratio (F:B)

Background: microBIOMETER® test requires field moist fresh samples for accurate analysis: estimates of MB on dried soil is can drop by almost 90%. The primary aim of this analysis was to determine storage conditions for sample analysis by microBIOMETER®. Analysis of microbial biomass by some carbon fumigation methods (CF), phospholipid fatty acid analysis (PLFA) and respiration use dried soils for analysis: the secondary aim was to gain information on the changes to the microbial biomass under drying conditions so as to better understand discrepancies between microBIOMETER® and assays calculating MB and F:B in dried soils.

Summary: Analysis of sample stability for testing by microBIOMETER® showed that field moist samples stored at 4C RT, or 37C for 10 days delivered the same results as samples analyzed upon the same days as sampling, and remained suitable for estimation of microbial biomass (MB) and Fungal to Bacterial Ratio (F:B). Air drying of samples caused up to 80% loss of MB over 4 days and a sharp decrease in the F:B ratio indicating that fungi were preferentially lost during drying. Reconstitution of dried samples by rehydrating to 20% did not reliably restore the MB or F:B and cannot be used for testing.

Methods: Soils from 3 locations were sampled and analyzed by both the microBIOMETER® app and digital microscopy. The first was from an area of cropland that is alternatively planted with soybean or corn (denoted as "field" herein). The second is from a residential lawn that has been covered by fescue for over 35 years (denoted as "grass" herein). The third is from a wooded area that surrounds the wetland formed by runoff of the surrounding sloping property (denoted as "wood" herein).

Storage conditions: Soils were collected 3/30/2020, stored in Ziploc bags and subjected to varying treatments for 24 hours to several months.

- 24 days
  - Refrigeration (4 C)– minimal air flow — rose to room temp each day analyzed before returning to refrigeration (< 2 hrs. at room temp during analysis).
  - Room Temperature (RT)– sealed bag at 65 F
  - Drying out – open tray at 65 F. Sample was probably not entirely dry for days.
- 14 days
  - Reconstituted (rehydrated) -- dried soils (10 days dried) were reconstituted according to Haney method (20% water –> 8 g soil + 2 ml H_20) and
- 7 days
  - 37 C in sealed bags to prevent evaporation
- 24 hours
  - 70 C in sealed bags to prevent evaporation

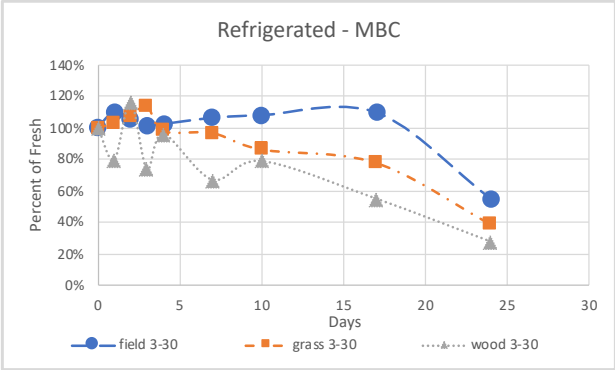
ANALYSIS

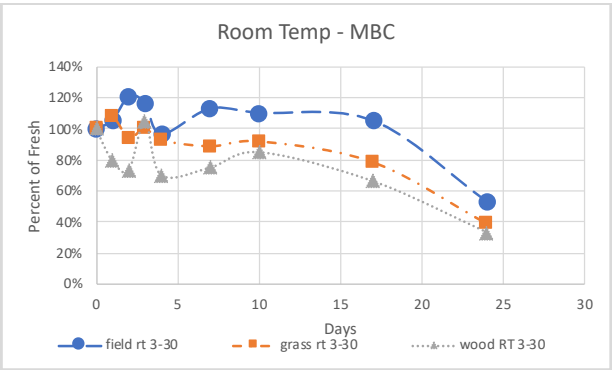
The microBIOMETER® extraction procedure was used to isolate soil microbes. Briefly ½ cc of sifted field moist soil was added to 9.5 ml of microBIOMETER extraction fluid and whisked for 30 seconds with the microBIOMETER® whisker. After settling for 20 minutes, the extract was analyzed by placing 6 drops on the microBIOMETER® test card and soil microbial biomass carbon (MBC, in units μg C g^{-1} soil) and fungal:bacterial ratios (F:B) were estimated using microBIOMETER® smartphone application. With the microBIOMETER® app, F:B is determined colorimetrically.

RESULTS

4C and RT CHANGES IN MICROBIAL BIOMASS BY microBIOMETER® "MBC" OVER TIME

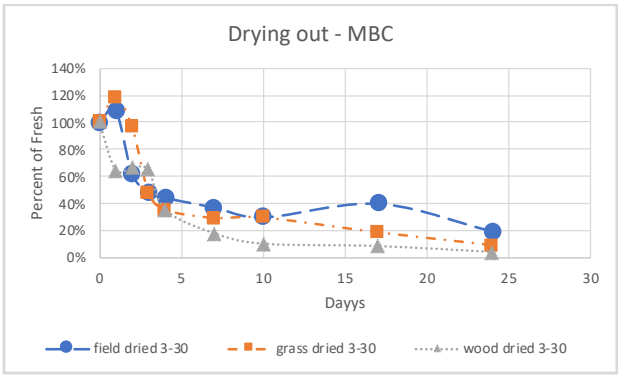
Below are the results of soils measured with microBIOMETER® as microbial biomass carbon " MBC". Day zero is when the soils were collected and percent change over 24 days is displayed. The graphs illustrate that the soil sample reads within the assay variation for microBIOMETER® for 10 days at 4C and RT. We conclude that samples may be assayed for MB in samples stored at RT and 4C for 10 days.





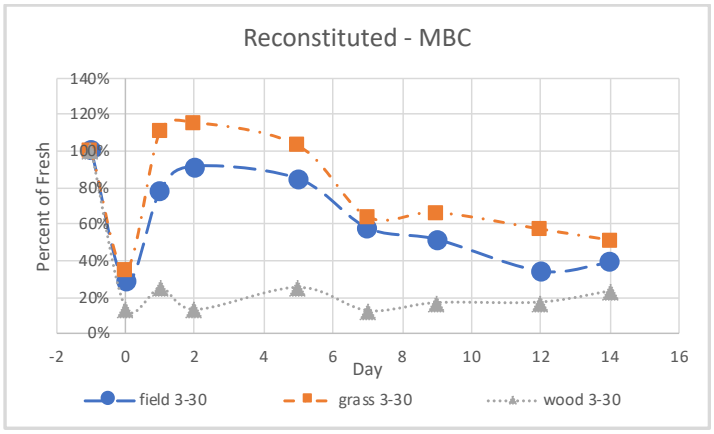
DRIED SOIL CHANGES IN MICROBIAL BIOMASS BY microBIOMETER® "MBC" OVER TIME

Soil was placed in plastic weigh boats and allowed to dry at 20 C over the indicated time. The % water of the samples was never measured, but as there was much rain at the time of collection all samples were moist and presumably they dried at various rates over 5-7 days. microBIOMETER® shows a steep drop in MB over the first 4 days and both record a MB loss of 40% or greater. We conclude that microBIOMETER® testing should **not** be performed on dryed out samples.



RECONSTITUTION OF DRIED SOILS DOES NOT RESTORE THE ORIGINAL POPULATION

Because respiration assays claim that respiration over 24 hours significantly correlates with MB, on day 10 we reconstituted the dried soils to 20% water. As shown below this resulted in a highly variable return of microbial biomass as assayed microBIOMETER®. Below is depicted the recovery after reconstitution. Day -1 represents the MBC measured on the day the soils were sampled. Day 0 is the value after 10 days of drying at room temperature.

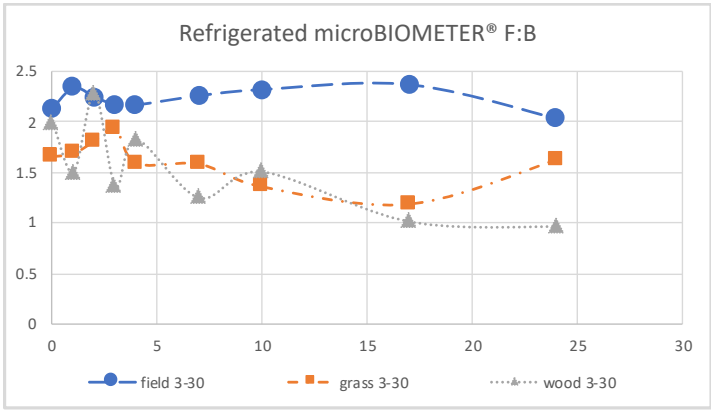


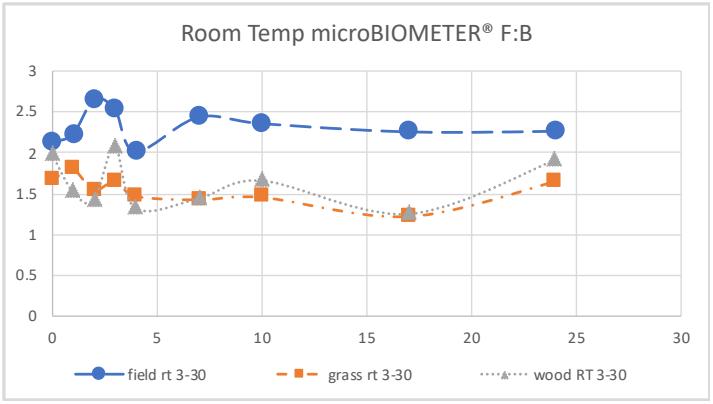
Recovery of grass soil was the greatest, with MBC overshooting the value for fresh soil within one day of rehydration. Field soil recovered to 80% of the fresh value within one day, the usual time allowed for soil reconstitution when used in the Haney test. Soil from the wooded area did not recover at all. We conclude that drying significantly affects the ability to accurately measure microbial biomass. Because both the carbon fumigation and phospholipid fatty acid methods of estimating MB use dried soil, we question whether these methods are measuring the actual MB present in field moist conditons.

4C, and RT CHANGES IN FUNGAL:BACTERIAL RATIOS BY microBIOMETER® "F:B" AND DIGITAL MICROSCOPY OVER TIME

Fungal:bacterial ratios (F:B) were determined using the microBIOMETER® app. With the microBIOMETER® app, F:B is determined colorimetrically.

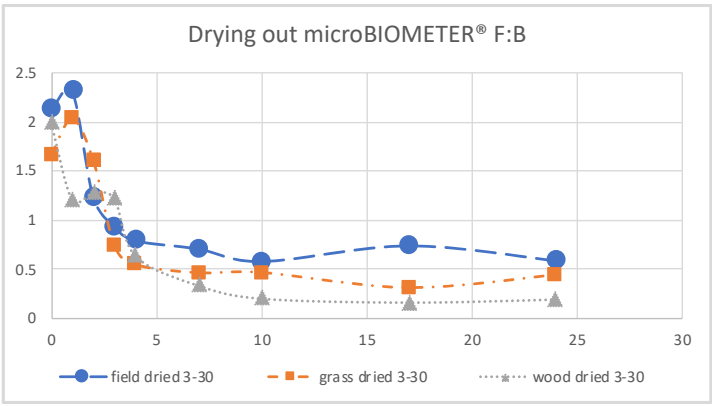
As shown below, microBIOMETER® estimates for F:B stayed near the value for fresh soil for both refrigerated and room temperature treatments for 10 days. There is ±0.5 variability in the microBIOMETER® F:B estimate.

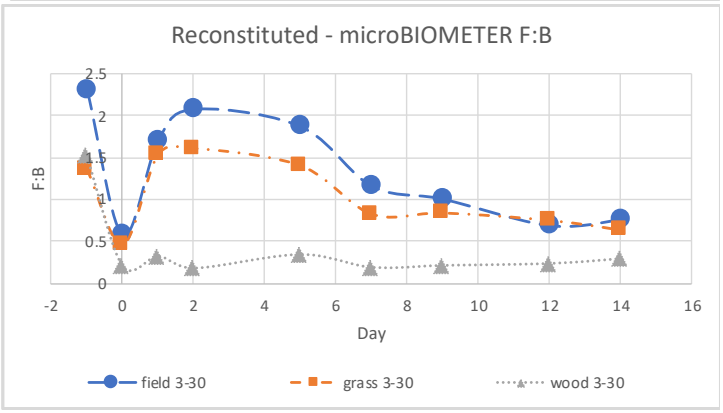




Dried and Reconstituted CHANGES IN FUNGAL:BACTERIAL RATIOS BY microBIOMETER® "F:B" OVER TIME

As shown below, the loss of 80% of MBC as measured by microBIOMETER® appears to change the microbial population that can recover upon reconstitution to 20% wetness: the woods sample did not recover to fresh fungal levels after reconstitution.

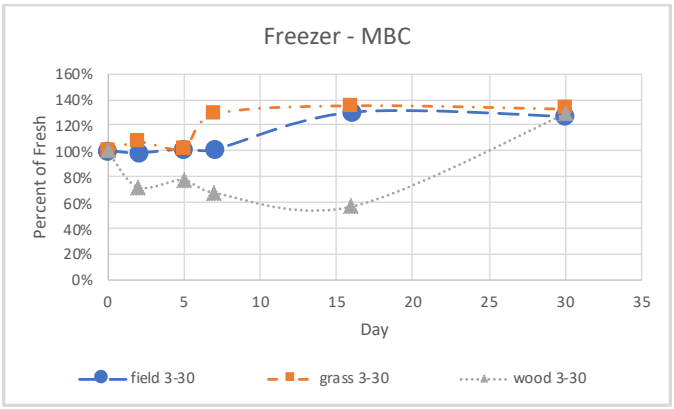


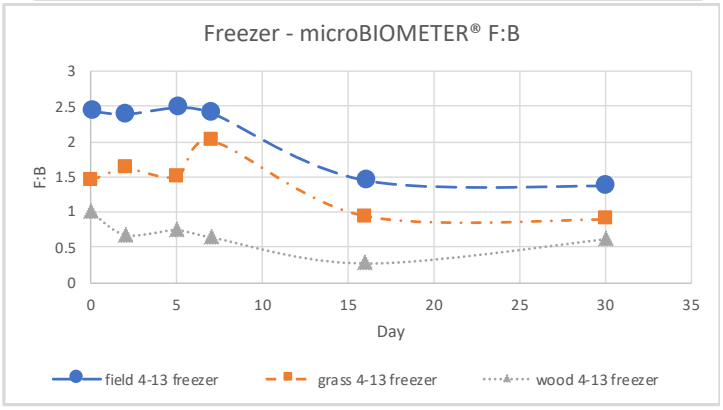


We conclude that fungi are more sensitive to storage conditions than bacteria and, due to great variation in MB recovery during rewetting, that dried samples do not report field soil microbial mass or composition.

FREEZE THAW CHANGES IN MICROBIAL BIOMASS AND FUNGAL:BACTERIAL RATIOS BY microBIOMETER® OVER TIME

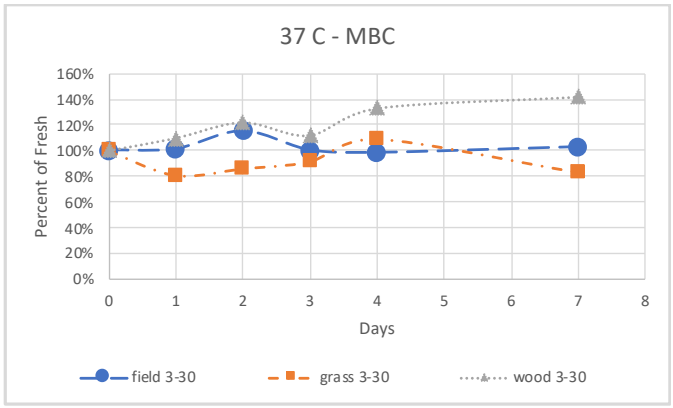
Effect of freezing and thawing on sample integrity for microBIOMETER® assay of soils. The freezer samples showed a decline in F:B ratio by microBIOMETER® after day 7. Since refrigerated, RT and 37C samples were stable for 10 or more days for both MB and F:B, we conclude that intermitant freezing and thawing is not a viable solution for storing soil samples for microBIOMETER®.

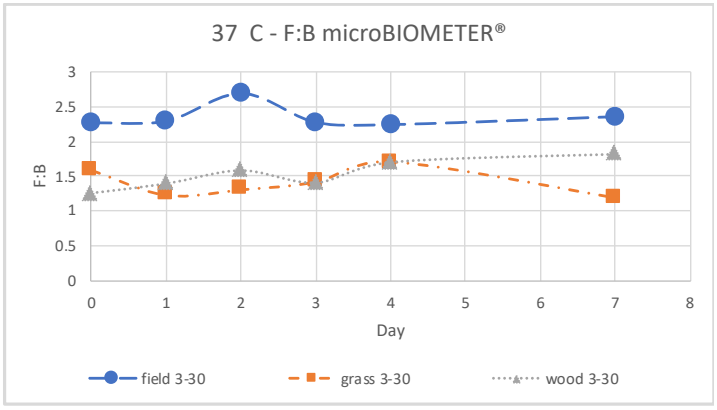




AT 37C, MICROBIAL BIOMASS AND FUNGAL:BACTERIAL RATIOS WERE STABLE FOR 7 DAYS

Samples were placed in bags at 37C for 7 days. Within the limits of the assays, both MB and F:B ratio read consistently over the 7 days. This is probably not surprising since this a common temperature for these soils and microbes.





CONCLUSION:

Soil samples can be tested immediately or stored at 4C, 20C, or 37C for 7-10 days before testing by microBIOMETER® for microbial biomass (MB) and fungal to bacterial ratio (F:B). For accurate estimation we recommend running the assay in triplicate, which is the usual method for soil MBC and F:B analysis.