RESPONSES OF BACILLUS SPORE GERMINATION TO SOIL EXTRACTS

Marine Biological Laboratory
Microbial Diversity 2016, MiniProject Report
Michael Braus, University of Wisconsin - Madison

INTRODUCTION

Viable spores of bacteria can be found in soils worldwide, and their activity contribute to complex soil communities (Barrios, 2007). *Bacillus* species are known to produce endospores, which can withstand pasteurization, as well as dessication, intense UV radiation, and long-term dormancy (McKenney et al., 2012). Germination of spores is less well-studied than sporulation of *Bacillus*, and this project explores potential differential effects of soil solutes (or "special sauce") on the germination of *Bacillus* endospores. During the first half of the Microbial Diversity 2016 summer course at MBL, soil samples were collected from the sand and dunes abutting Trunk River and the Atlantic, and endospores were later selected for using pasteurization and enrichment on 5YE media. Many species of bacteria-like colonies of different colors, growth rates, and growth patterns were found and isolated and their 16S rDNA sequenced, finding a diversity of *Bacillus* species as expected. To better understand these isolates, three species with different morphologies (*B. cereus, B. pumilus,* and *B. koreensis*) were chosen to perform a multifactorial germination assay and other tests to detect differences in how species of *Bacillus* respond to the presence of several soil extracts (SEs) composed of undefined solutes.

METHODS

Soils were collected from an oak forest, pine forest, and Trunk River soil, all of which yielded spore-forming bacterial growth after pasteurization and enrichment on 5YE media. Each SE was made by adding 1L of pure water to 1L of each soil, stirred for 5 minutes, then poured through several filters of decreasing pore size to remove large particles. Each soil solution was let to sit overnight at 4°C ("cold brew") then filtered through a 0.2µm filter into a sterile container. Experimental media was a mix of 25 parts 5YE and 75 parts SE, aliquotted across a 96-well plate, 125µL per well (see Table 1). Each well was inoculated with 10ul of endospore stock (scraped from 10-day-old plates, washed, and stored in PW at 4°C) before taking an initial count of spores using a hemacytometer under phase contrast. After 1.5 hours of incubation at 30°C and pasteurization at 75°C for 1.5 hours, a final count was made for each well. The following formula was used to calculate percent loss, and any percent losses with a Cook's distance (a metric that finds the change leverage a particular point has on an overall trend in a regression, ANOVA, or other statistical model) greater than 2 was discarded as outliers.

(initial spores – final spores) / initial spores = percent loss of spores

Chemical analyses used high-pressure liquid chromatography (HPLC) and ion chromatography (IC) to detect and measure solutes, anions, and cations in SEs as well as using scanning electron microscopy (SEM) energy dispersive X-ray (EDX) spectroscopy to detect and quantify relative percent molecular weights of elements in or on spore coats. Not all solutes were expected to be detected in these undefined SEs. SEM/EDX was performed on dried samples and again on a platinum sputter-coated sample of *B. pumilus*.

RESULTS

The phase contrast and scanning electron micrographs illustrate the different sizes and shapes of the cells and endospores of species of *Bacillus*. *B. cereus* has rounded rectangular spores that more often occupy the center of its vegetative cell (Figure 1). Spores of *B. pumilus* are smaller, and the volume its vegetative cell is mostly endospore (Figure 2). *B. koreensis* has longer rod-shaped vegetative cells and spherical spores developed at one end of the cell in a sporangium (Figure 3).

The germination assay showed no significant difference between any non-water media or *Bacillus* species percent loss of spores (Figure 5), and because of an interaction among bacterial species and media ($p=0.0129$, Table 2, Figure 6), pairwise t-tests were separated by factor to show that the comparisons of *B. cereus* and *B. koreensis* in water ($p=0.084$), *B. pumilus* and *B. koreensis* in water ($p=0.015$), and *B. pumilus* in all media (Figure 7) were the only cases of significantly or near-significantly different percent losses.

Acetate was found to be roughly equal across samples, but SE from Trunk River had far more anions and cations than the oak and pine SEs (Figure 4). This was expected because Trunk River is a brackish submerged marsh pond connected to the sea while the oak and pine soils are isolated microenvironments watered by rain and runoff only.

Results from SEM / EDX of calcium content on spore coats was inconclusive due to either inconsistent preparation or instrument error.

DISCUSSION

The multifactorial germination assay designed in this project was a simple, efficient, and effective method for assessing the germination response of different spore-forming *Bacillus spp.* to different SE media. Species of *Bacillus* responded similarly to the presence of yeast extract, regardless of SE added, while *B. pumilus* seemed to germinate more readily than others. The likely primary germinant in yeast extract is L-alanine (Foerster & Foster, 1966), and this was validated in my germination assay. The enormous variance and negativity of percent loss, or percent gain, in spores in the water-only media was attributable to clumping that was evident on

the phase contrast images, but the clear conclusion of the assay was that yeast was the sole requisite germinant while SE solutes were not influential in the germination process.

The pasteurization step performed after incubation may have acted as more of a spore activation step than a vegetative cell deactivation step. Although cooling the media with ice while counting may have slowed confounding germination, it nonetheless may have also skewed the final counts because of the slowness of preparing the hemacytometer for phase contrast imaging. Though samples counted last would theoretically have higher percent losses of spores, the wells were counted in the same order and therefore had very similar total experimental run times.

Subsequent microscopic and chemical analyses of endospores were fruitful learning exercises that yielded some methodological considerations for future work of this nature. Estimates of chemical compositions of spore coats were hindered by the differences resulting from sputter coated and non-sputter coated samples (Figure 8). The former were filtered over 0.2µm nuclear pore filters, fixed with paraformaldehyde, critical point dried, and coated in 10nm of platinum while the latter were 10µl of spore stock dried directly on paper. Possible reasons for the difference are a false negative of calcium presence from the reflection of electrons off the platinum of the sputter coated sample or a false positive of calcium presence on the direct-application spores because the spore stock solution with undefined solutes dried to spore surfaces.

It is tempting to run a few samples through an *ad hoc* assay or protocol, lacking replicates for the sake of time and cost, but this will inevitably lead to false positives and false negatives, subsequently either wasting one's time experimenting on one-time flukes or not spending time exploring undetected phenomena. The experimental design of this project was intended to answer a simple question about the germination of *Bacillus* endospores in different SE media, which is very much outside the realm of new discovery. However, given future observations of other microbial species in environments with novel morphologies, metabolisms, and strategies for survival, a multifactorial empowers any microbiologist with a reliable and efficient tool that blends both hypothesis-testing and discovery in their science.

REFERENCES

Barrios, E. (2007). Soil biota, ecosystem services and land productivity. *Ecological Economics*, *64*(2), 269–285.

Foerster, H. F., & Foster, J. W. (1966). Response of *Bacillus* spores to combinations of germinative compounds. *Journal of Bacteriology*, *91*(3), 1168–1177.

McKenney, P. T., Driks, A., & Eichenberger, P. (2012). The *Bacillus subtilis* endospore: assembly and functions of the multilayered coat. *Nature Reviews Microbiology*, *11*(1), 33–44.

TABLES & FIGURES

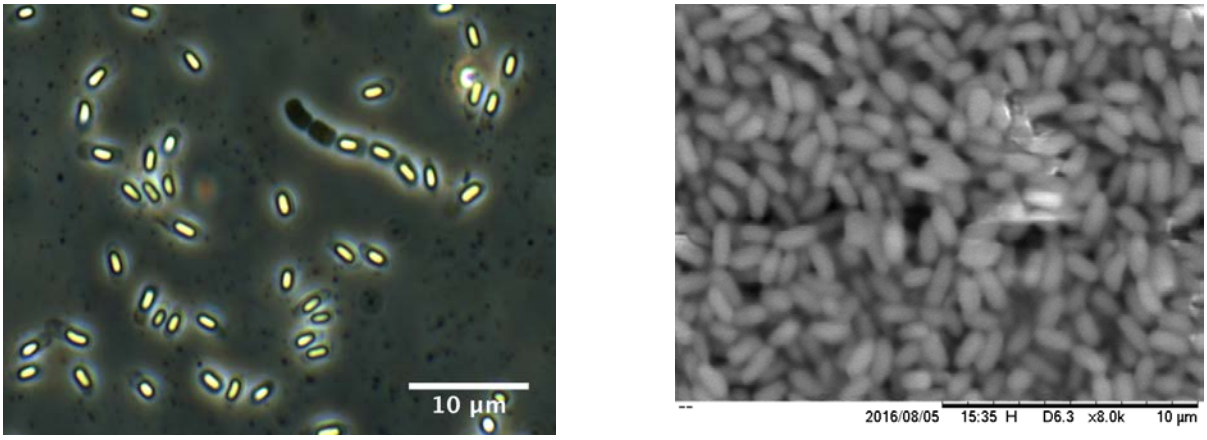


Figure 1. *Bacillus cereus* under phase contrast (left) and electron microscopy (right).

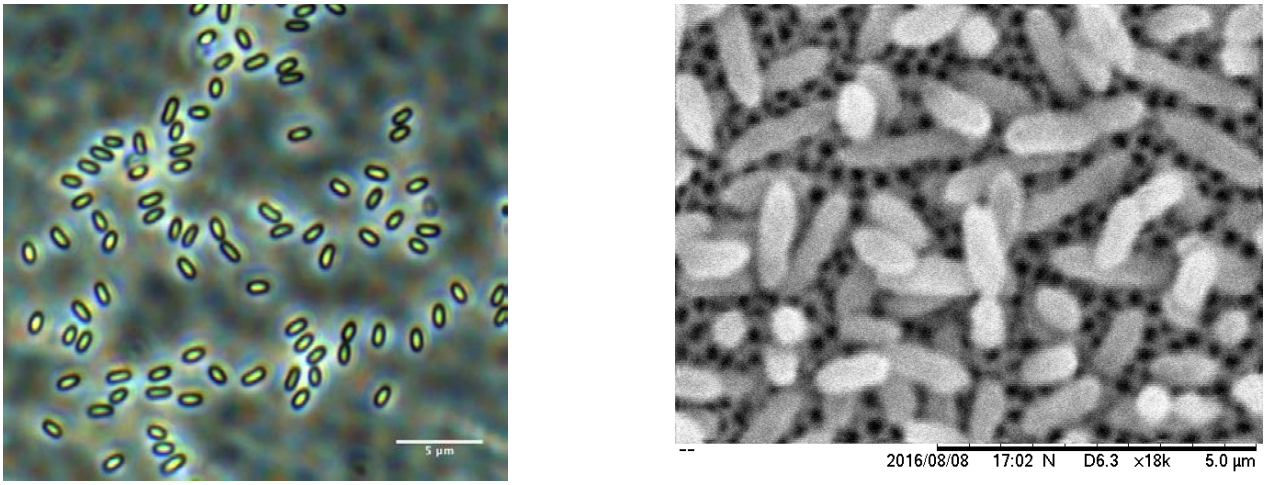


Figure 2. *Bacillus pumilus* under phase contrast (left) and electron microscopy after fixing, critical point drying, and sputter-coating with platinum (right).

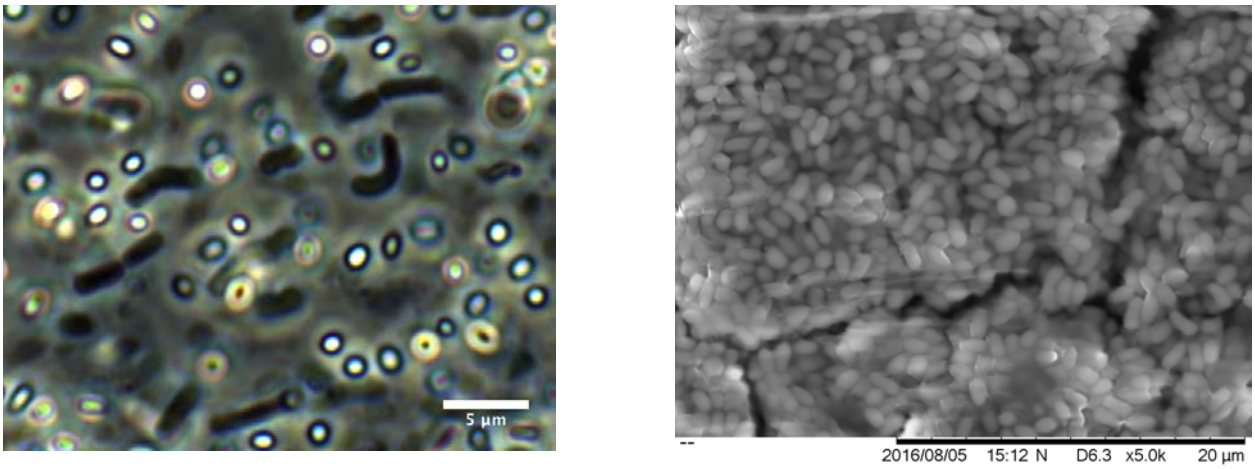


Figure 3. *Bacillus koreensis* under phase contrast (left) and electron microscopy (right)

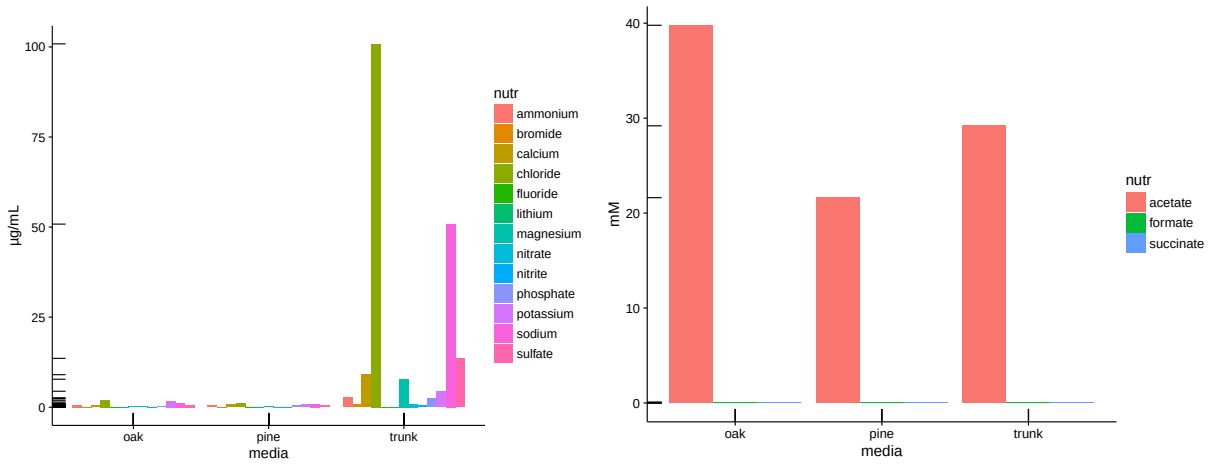


Figure 4. Chemical composition of SE media using a HPLC (right) and IC (left).

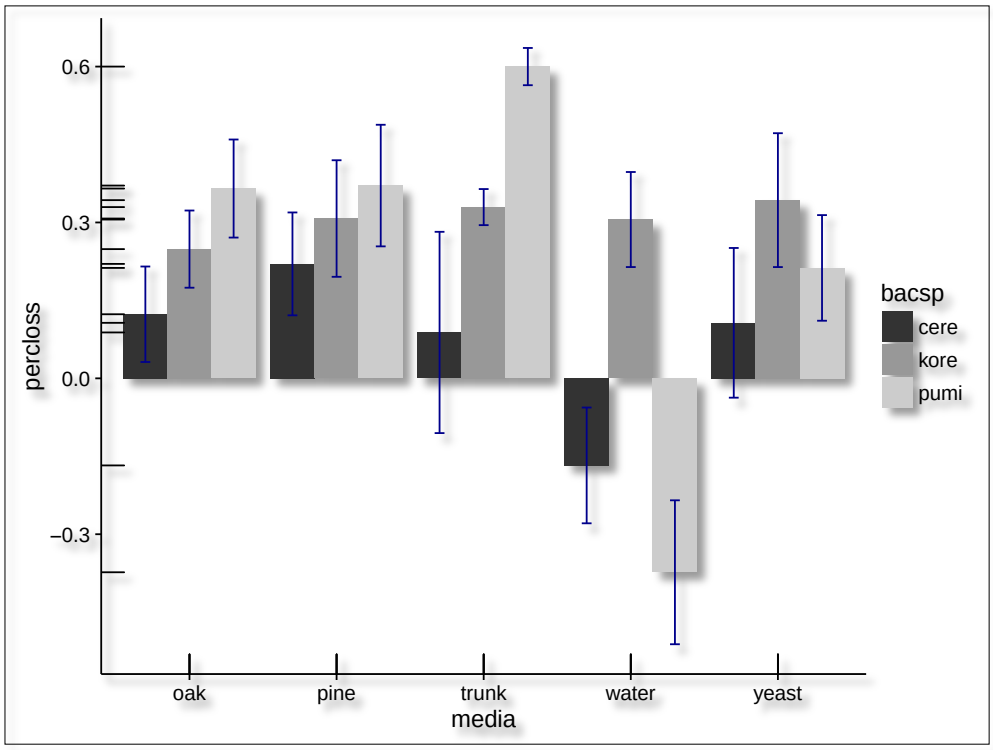


Figure 5. Results of a *Bacillus spp.* germination assay, where “percloss” is the percent loss from the initial spore count to the final spore count after incubation and pasteurization.

	1	2	3	4	5	6	7	8	9	10	11	12
A	tc1	tu1	tk1	tc2	tu2	tk2	tc3	tu3	tk3	tc4	tu4	tk4
B	oc1	ou1	ok1	oc2	ou2	ok2	oc3	ou3	ok3	oc4	ou4	ok4
C	pc1	pu1	pk1	pc2	pu2	pk2	pc3	pu3	pk3	pc4	pu4	pk4
D	yc1	yu1	yk1	yc2	yu2	yk2	yc3	yu3	yk3	yc4	yu4	yk4
E	wc1	wu1	wk1	wc2	wu2	wk2	wc3	wu3	wk3	wc4	wu4	wk4

Table 1. Well position code = media-bacsp-rep. “t” = 1% YE base with pine SE. “o” = 1% YE base with oak SE. “p” = 1% YE base with Trunk River SE. 1% YE base only. PW only. “c” = *B. cereus*. “u” = *B. pumilus*. “k” = *B. koreensis*. Each number indicates each replicate (four to get a decent standard error).

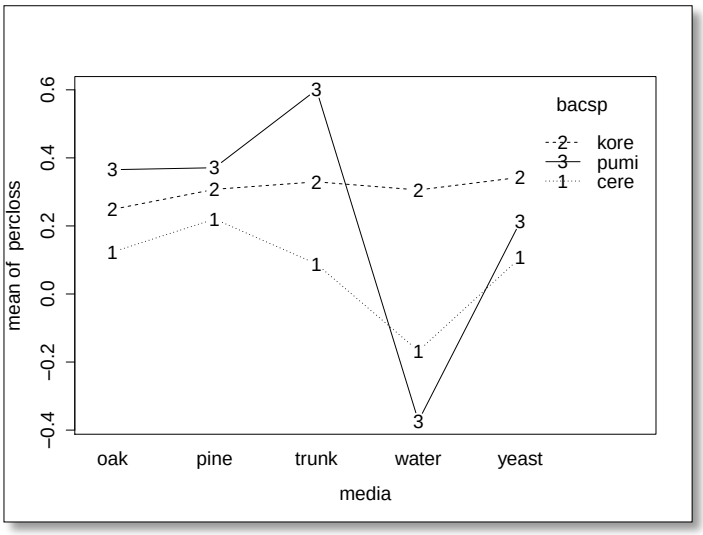
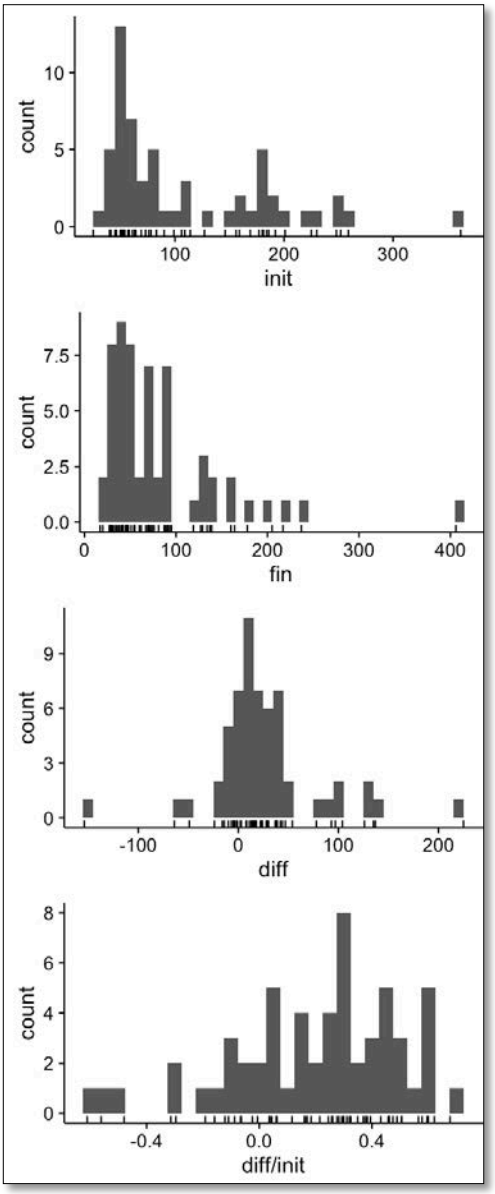


Figure 6. Interaction plot of means resulting from a *Bacillus spp.* germination assay. Note the non-parallel lines, which indicate an interaction between treatments of all media and species except pure water, the negative control.

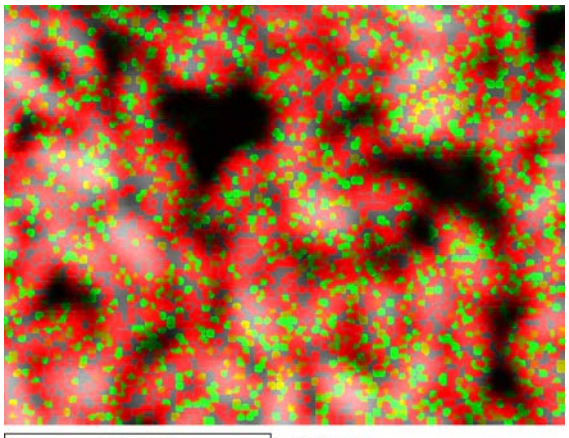
```
> mod2 <- aov(percloss ~ media*bacsp, data=data); summary(mod2)
             Df Sum Sq Mean Sq F value   Pr(>F)
media         4 1.4383  0.3596   7.340 0.000134 ***
bacsp         2 0.4932  0.2466   5.034 0.010857 *
media:bacsp   8 1.1089  0.1386   2.830 0.012918 *
Residuals    43 2.1065  0.0490
```

Table 2. ANOVA table displaying the results of a *Bacillus spp.* germination assay in several soil extract media and positive and negative controls.

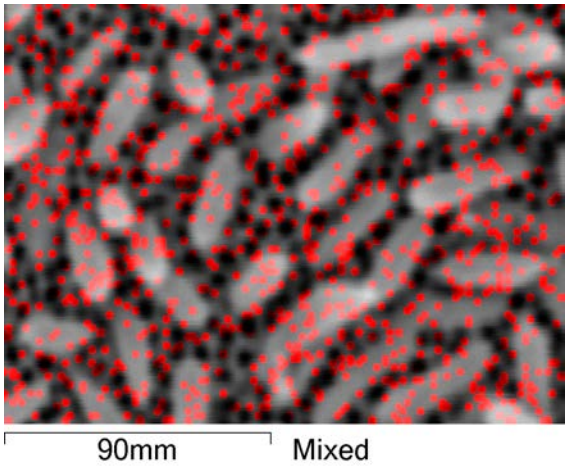



```
# Pairwise t-tests (adj = Bonf.)

B. cereus
      oak  pine trunk water
pine  1.00 -    -     -
trunk 1.00 1.00 -     -
water 1.00 0.58 1.00  -
yeast 1.00 1.00 1.00  1.00

B. koreensis
      oak pine trunk water
pine  1   -    -     -
trunk 1   1    -     -
water 1   1    1     -
yeast 1   1    1     1

B. pumilus
      oak    pine   trunk    water
pine  1.0000 -      -        -
trunk 1.0000 1.0000 -        -
water 0.0013 0.0024 7.4e-05  -
yeast 1.0000 1.0000 0.1584   0.0099

Oak
     cere kore
kore 1.00 -
pumi 0.25 1.00

Pine
     cere kore
kore 1    -
pumi 1    1

Trunk
     cere  kore
kore 0.521 -
pumi 0.036 0.396

Water
     cere  kore
kore 0.084 -
pumi 0.734 0.015

Yeast
     cere kore
kore 0.65 -
pumi 1.00 1.00
```

Figure 7. Histograms of all initial spore counts, all final spore counts, all differences, and all percent losses, or “diff/init” (left). Pairwise t-tests among and between bacterial species and media (left). Arrow added to show limited significant differences only between water and the other media.



Project: edx-pumi-somespores-Ca-map-2.ipj
Acc. Voltage: 5.0 kV
Resolution: 128 x 96 pixels
Viewed Resolution: 100%
Process Time: 5
Image Width: 188.513 mm
Mixed map: Calcium Ka1(red), Magnesium Ka1_2(green)



Project: edx-pumi-somespores-sputtered2.ipj
Acc. Voltage: 5.0 kV
Resolution: 128 x 96 pixels
Viewed Resolution: 100%
Process Time: 5
Image Width: 188.513 mm
Mixed map: Calcium Ka1(red)

Figure 8. Maps of chemical spectra using EDX on SEM images of *B. pumilus* spores. The top image is from spores dried on a filter, and the bottom image is the same spore stock but platinum sputter-coated.