New CMEIAS Image Analysis Software for Computer-Assisted Microscopy of Microorganisms and Their Ecology

Frank B. Dazzo
Michigan State University
dazzo@msu.edu

Microscopy is one of the most important techniques in microbial ecology, since this is the most direct approach to examine the microbe's world from its own perspective and spatial scale. The value of quantitative microscopy in studies of microbial ecology can be increased even further when used in conjunction with computer-assisted image analysis. There are two main advantages of using digital image processing and pattern recognition techniques in conjunction with microscopy for quantitative studies of microbial ecology. First, automatic image analysis reduces the amount of tedious work with microscopes needed to accurately quantify *in situ* morphological diversity, abundance and metabolic activity of microbes. Secondly, these techniques provide an important quantitative tool that can significantly enhance the polyphasic analysis of the structure, diversity, spatial features, and functions of complex microbial communities *in situ* without cultivation.

One of the most important and yet most tedious tasks performed during microscopical analysis of microbial communities is the classification of observed cells into known morphological categories and recognition of new categories as well if new distinct characteristics are captured. Use of morphological diversity in evaluations of microbial community structure is more useful and valid if the cells are actively growing rather than in a non-growing state of quiescence, since the latter is more commonly associated with pleomorphic dwarf cells. This is because distinctive cell morphologies reflect the phenotypic expression of complex networks of genes involved in the synthesis and maturation of the shape-determining murein sacculus, plus other genes dedicated to the cell division cycle that are primarily expressed during active growth.

A major challenge in microbial ecology is to develop reliable and facile methods of computer-assisted microscopy that can analyze digital images of complex microbial communities at single cell resolution, and compute useful quantitative characteristics of their organization and structure without cultivation. Although several image analysis systems can classify microbes according to their cell size, automatic classification of cells according to their distinctive morphology (a dimensionless characteristic based on several shape features) represents a much more challenging task. Most commercial image analysis systems include some shape measurement features that compute the roundness or circularity of cells, and these characteristics are sufficient to distinguish regular rods and cocci, the most common shapes of bacteria. However, the difficulty increases with morphological diversity, since automatic classification of most other microbial morphotypes requires measurement of multiple shape and size features to resolve the distribution of their morphological space. Some custom image analysis systems are adequate for automatic shape classification of spheres, straight rods, and vibroids or prolate spheroids. This represents the morphological diversity of some marine bacterioplankton communities. However, comprehensive image analysis systems capable of automatically classifying much broader morphological diversity in complex bacterial communities, as commonly exists in nutrient-enriched habitats containing actively growing bacteria that are larger in size and typically monomorphic, did not exist prior to development of our new software application.

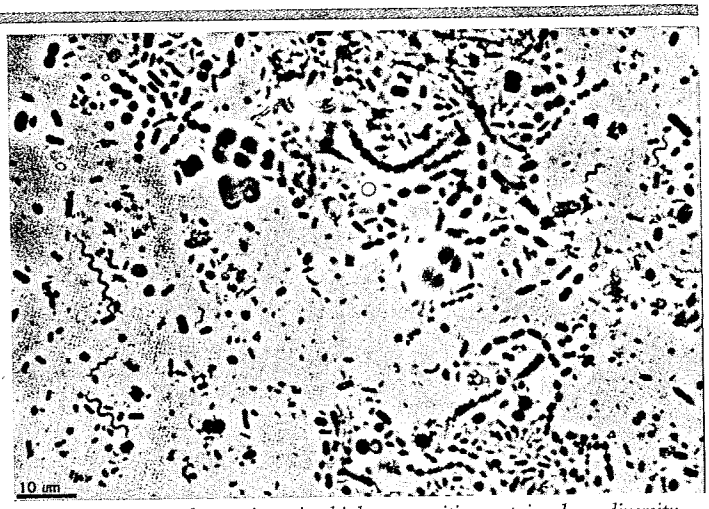


Fig. 1. *Actively growing microbial communities contain a large diversity of bacterial morphotypes, as shown directly by this phase contrast light photomicrograph of bovine rumen fluid. Acquiring an image similar to this image for the cover of Bergey's Manual was my spark of inspiration to develop CMEIAS.*

This recognition of the need to develop a comprehensive computer-aided image analysis system that could extract from images all the information needed to recognize and classify the morphological diversity component of microbial communities came to a pinnacle when I was preparing photomicrographs of the microbial community in the bovine rumen for the cover illustration of the 9th Edition of Bergey's Manual of Determinative Bacteriology (Fig. 1).

That work clearly indicated the following key points:

- Contrary to current popular thinking about microbial community analysis, microscopy does reveal significant morphological diversity in complex, actively growing microbial communities.
- Automatic morphotype classification of complex communities exhibiting high morphological diversity will require development of a more flexible and robust computer-assisted image analysis system than those currently available.
- Phase-contrast light microscopy of dispersed samples immobilized on agarose-coated slides is a simple yet effective direct method to acquire images with the resolution and range of object brightness at high magnification that are sufficient to reveal the rich morphological diversity of actively growing microbial communities. Its essential requirements to detect microbes are that their size exceeds the ~0.2 µm limit for light microscopy, their refractive index differs from that of the surrounding medium, and high-quality optics are available to acquire the images.

A team of microbiologists, mathematicians, and computer

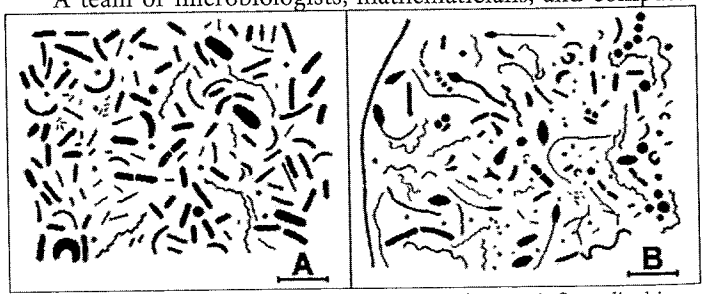


Fig. 2.) A high quality 8-bit grayscale digital image is first edited in an image editing program such as Adobe Photoshop so that all foreground objects of interest have a brightness range outside that of background, and then converted into a binary image using UTHSCSA ImageTool Ver. 1.27. In this example, two binary composite images were made, each contain 170 microbes representing the distribution of morphotype abundance in different (A and B) anaerobic bioreactor communities. Bar scales equal 10µm.

V.12 no.3

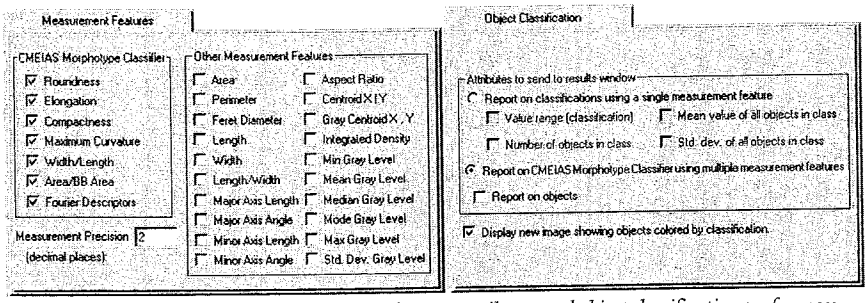
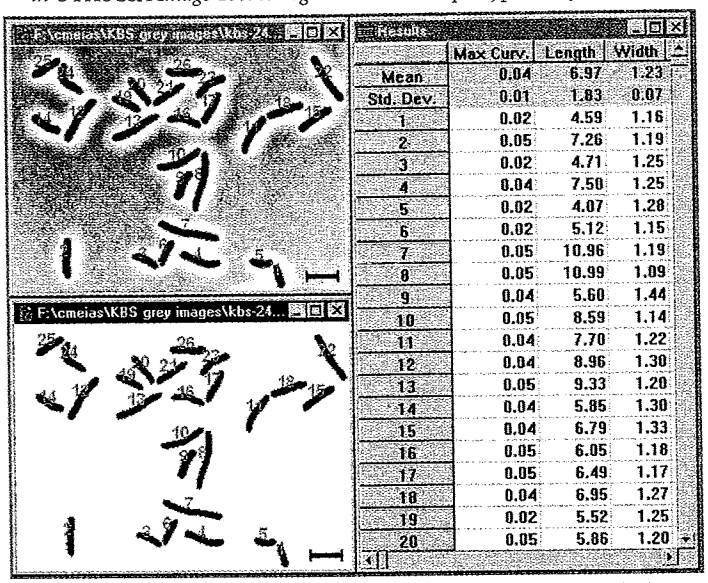


Fig. 3.) Set the required measurement feature attributes and object classification preferences in UTHSCSA Image Tool to begin CMEIAS morphotype classification.



	Max Curv.	Length	Width
Mean	0.04	6.97	1.23
Std. Dev.	0.01	1.83	0.07
1	0.02	4.59	1.16
2	0.05	7.26	1.19
3	0.02	4.71	1.25
4	0.04	7.50	1.25
5	0.02	4.07	1.28
6	0.02	5.12	1.15
7	0.05	10.96	1.19
8	0.05	10.99	1.09
9	0.04	5.60	1.44
10	0.05	8.59	1.14
11	0.04	7.70	1.22
12	0.04	8.96	1.30
13	0.05	9.33	1.20
14	0.04	5.85	1.30
15	0.04	6.79	1.33
16	0.05	6.05	1.18
17	0.05	6.49	1.17
18	0.04	6.95	1.27
19	0.02	5.52	1.25
20	0.05	5.86	1.20

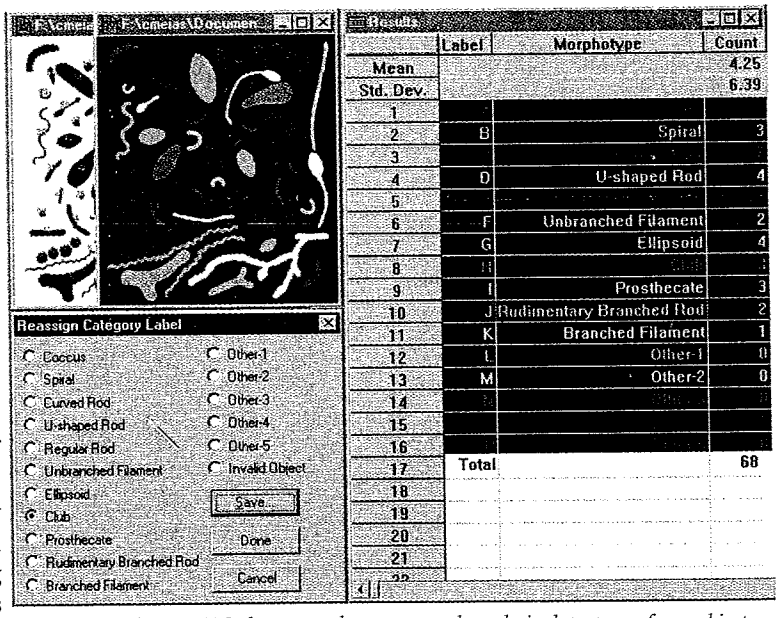
Fig. 4.) CMEIAS/ImageTool finds the foreground objects (microbes) in the images using a thresholding procedure, assigns a numbered annotation to each microbe found, and reports the total object count in the image. It then performs an object analysis on the image and displays the values for each shape measurement feature selected in a Results window worksheet.

scientists at Michigan State University have improved on existing image analysis systems of computer-assisted microscopy by introducing new measurement features and robust object classifiers capable of automatically classifying most of the predominant microbial morphotypes encountered in digital micrographs of complex microbial communities growing in nutrient-enriched habitats, and have implemented these features in a flexible, user-friendly and robust semi-automatic image analysis system to strengthen microscopy-based methods for understanding microbial ecology. We named the program "CMEIAS®", an acronym for the Michigan State University Center for Microbial Ecology Image Analysis System. CMEIAS Ver. 1.27 is not a stand-alone program, but rather consists of several custom plug-ins designed to operate in the host program UTHSCSA ImageTool® Ver. 1.27, a free downloadable open-architecture software operating on a personal computer. Two of these CMEIAS 1.27 plugins (objanal.dll and objclass.dll) are derived work, representing modified plugin versions of object analysis and object classification plugins and not the original UTHSCSA ImageTool distributed by the University of Texas. The accuracy of microbial morphotype classification has been thoroughly tested against ground truth data using CMEIAS v. 1.27 in UTHSCSA ImageTool running in Windows NT 4.0 (Liu et al. 2001). Similar results have been obtained using the Windows 2000 Professional operating system. Also, limited operational testing has indicated compatibility with Windows 95/ME/XP, but quantitative measurements of CMEIAS accuracy have not been evaluated for CMEIAS/ImageTool 1.27 using these alternative operating systems.

To perform a morphotype classification using CMEIAS 1.27 in ImageTool, the operator first finds the objects of interest in the image by using a thresholding procedure, then conducts an Object Analysis to extract various size and shape measurements from each microbe present, and finally uses these Object Analysis data to perform an Object Classification that automatically assigns the appropriate morphotype to each microbe found. This object classification procedure uses a series of pattern recognition algorithms optimized by us for 11 major microbial morphotypes represented by 98% of the genera described in the 9th Edition of Bergey's Manual of Determinative Bacteriology. Extensive testing using large ground truth data sets indicate that CMEIAS performs with an overall morphotype classification accuracy of 97% on properly edited images.

The CMEIAS data output is a results window that reports on the types of different morphotypes found and the abundance among each of them. These data can be used to compute various indices of morphological diversity and distribution of numerical abundance in microbial community analysis. An interactive edit feature is included to address the main sources of error in automatic morphotype classification, enabling the operator to inspect the morphotype assigned to each microbe in the image based on visual recognition of its distinctive pseudocolor, reassign it to another morphotype class if necessary, and add up to five other morphotypes to the supervised classification scheme.

Comprehensive descriptions of CMEIAS Ver. 1.27, including its rationale for development, algorithms of size and shape measurements, statistical rules of pattern recognition for each morphotype classification, results of thorough accuracy testing, sources of error and limitations, and an example of how CMEIAS can augment the polyphasic analysis of growing, complex microbial communities are presented in our publication Liu *et al.*, 2001. Microbial Ecology



	Label	Morphotype	Count
Mean			4.25
Std. Dev.			6.39
1			
2	B	Spiral	3
3			
4	D	U-shaped Rod	4
5			
6	F	Unbranched Filament	2
7	G	Ellipsoid	4
8	H	Club	1
9	I	Prosthecate	3
10	J	Rudimentary Branched Rod	2
11	K	Branched Filament	1
12	L	Other-1	0
13	M	Other-2	0
14	N	Other-3	0
15			
16			
17	Total		68
18			
19			
20			
21			

Fig. 5.) CMEIAS then uses these extracted analysis data to perform object classification (identify morphotypes), tabulates the classification data in the Results window, and creates a new image in which each microbe is distinctively pseudocolored *according to its assigned morphotype.*

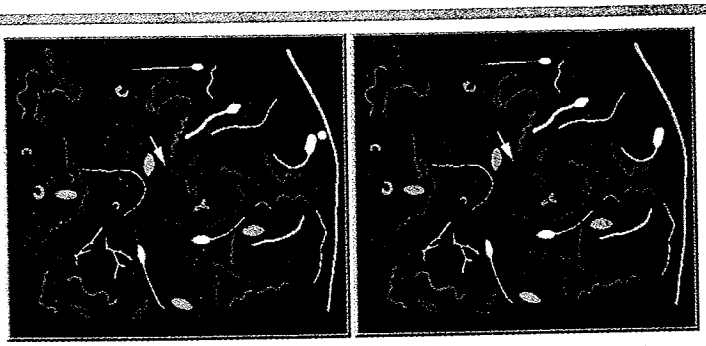


Fig. 6.) CMEIAS then allows you to inspect the classification assignments in the pseudocolored image, and if necessary (errors occur at a 3% rate), to manually edit individual morphotype classifications using the Reassign Category Label interactive edit feature, as illustrated here using the pseudocolored classification result images of community B. The red coccobacillus (white arrow, center of left image) is reclassified as a regular straight rod (blue, right image) and the corresponding classification data automatically update.

41(3):173-194 and 42:215 . An operator manual and interactive tutorial are also provided in the CMEIAS 1.27 download.

CMEIAS/ImageTool can also measure various object size attributes (cell length,width, area, perimeter, Feret diameter, major axis length, minor axis length) and classify the cell size distribution in microbial populations and communities. A unique feature of the length and width measurement attributes of CMEIAS is that the algorithms used to compute them are adaptive to object shapes, and therefore the values reported are more accurate over a wider range of object shapes than alternate algorithms that are invariant to object shape. In theory, these measurement attributes and shape classifications could be used to analyze and classify any foreground object in the digital image.

Abbreviated CMEIAS Demo:

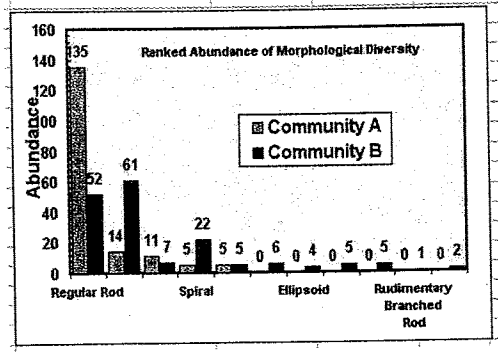
In this example, the major steps to analyze the morphological diversity of two community images using CMEIAS/ImageTool 1.27 are presented in figures 2 through 7. A more detailed version of these image analysis steps is available in the operator manual and training tutorial website downloads.

In summary, CMEIAS© is an accurate, robust, flexible semi-automatic computing tool that fills a major gap by significantly strengthening microscopy-based quantitative approaches to understanding microbial ecology at spatial scales relevant to the microbe's niche, and should serve as a useful adjunct in the analysis of microbial community structure *in situ* without cultivation. A free download of CMEIAS ver. 1.27 is available for educational and research purposes at <http://cme.msu.edu/cmeias/>.

If you acquire image analysis data using CMEIAS/ImageTool, we ask that you cite us in the following way:

"Digital images were analyzed using CMEIAS Ver. 1.27 (Liu et al. 2001) operating in UTHSCSA ImageTool Ver. 1.27 (Wilcox et al. 1997)."

Descending Sort Rank Abundance Graph		
Morphotype	Community A	Community B
Regular Rod	135	52
Coccus	14	61
Curved Rod	11	7
Spiral	5	22
U-shaped Rod	5	5
Unbranched Filament	0	6
Ellipsoid	0	4
Club	0	5
Prosthecate	0	5
Rudimentary Branched Rod	0	1
Branched Filament	0	2



Community morphotype diversity indices		
Parameter	Community A	Community B
Morphotype Richness =	5	11
Total cell abundance =	170	170
Simpson Dominance (l) =	0.641	0.241
Simpson Diversity (D) =	0.359	0.759
Max of D =	0.805	0.914
Simpson evenness =	0.446	0.830
Inverse dominance (d) =	1.560	4.152
Max of d =	5.121	11.692
d evenness =	0.305	0.355
Log used for H' =	2.718	2.718
Shannon Diversity (H') =	0.773	1.726
H'max =	1.609	2.398
H' evenness(J%) =	0.480	0.720
Brillouin diversity =	0.728	1.622
Brillouin max =	1.551	2.351
Brillouin evenness =	0.469	0.690
Hill's N1 =	2.167	5.618
Hill's N2 =	1.560	4.152
Hill's evenness =	0.720	0.739

Community similarity analyses comparing communities A vs. B	
Prop. similarity (%) =	48.824
Jaccard coefficient =	0.455
Sorenson coefficient =	0.625
Morisita index =	0.634
Horn index =	0.758
Sneath/Sokal distance =	0.663
Bray/Curtis distance =	0.512
Chord distance =	0.769

Fig. 7.) Community morphotype analysis data can then be copied to the clipboard and exported to Windows-compatible spreadsheet programs (e.g. Excel, EcoStat) where they can be graphed and analyzed statistically. CMEIAS analysis of this pair of images indicates that communities A and B have 48.82% proportional similarity in morphological diversity, with Community B being 2.2-fold higher in morphotype richness and diversity indices and 1.6-fold higher in distribution of morphotype evenness.

Citations:

1) Liu J., F.B. Dazzo, O. Glagoleva, B. Yu, A.K. Jain. 2001. CMEIAS: A computer-aided system for the image analysis of bacterial morphotypes in microbial communities. Microbial Ecology 41(3):173-194 and 42:215. http://cme.msu.edu/cmeias/

2) Wilcox C.D., S.B. Dove, W. Doss-McDavid, and D.B. Greer. 1997. UTHSCSA ImageTool Ver. 1.27, http://www.ddsdx.uthscsa.edu/dig/itdesc.html. Univ. of Texas Health Science Center, San Antonio, TX, USA.

Below is a list of published studies that include data acquired by CMEIAS. We will periodically update this list with references sent to us by CMEIAS users. Please send references in the format shown to cmeiasfd@msu.edu or dazzo@msu.edu.

Dazzo, F., and J. Wopereis. 2000. Unraveling the infection process in the *Rhizobium*-legume symbiosis by microscopy. In E. Triplett (ed.), Prokaryotic nitrogen fixation: a model system for the analysis of a biological process; Chap. 19; pp. 295-347. Horizon Scientific Press, UK.

Dazzo, F.B., Y.G. Yanni, R. Rizk, F. de Bruijn, J. Rademaker, A. Squartini, V. Corich, P. Mateos, E. Martinez-Molina, E. Velazquez, J. Biswas, R. Hernandez, J.K. Ladha, J. Hill, J. Weinman, B. Rolfe, M. Vega-Hernandez, J.J. Bradford, R.I. Hollingsworth, P. Ostrom, E. Marshall, T. Jain, G. Orgambide, S. Philip-Hollingsworth, E. Triplett, K. Malik, J. Maya-Flores, A. Hartmann, M. Umali-Garcia, and M.L. Izaguirre-Mayoral. 2000. Progress in multi-national collaborative studies on the beneficial association between *Rhizobium* leguminosarum bv. trifolii and rice. In J.K. Ladha and P.M. Reddy (eds.), The Quest for Nitrogen Fixation in Rice. International Rice Research Institute, Manila, The Philippines, pp. 167-189.

Fernandez, A., S. Hashsham, S. Dollhopf, L. Raskin, O. Glagoleva, F.B. Dazzo, R. Hickey, C. Criddle, and J.M. Tiedje. 2000. Flexible community structure correlates with stable community function in methanogenic bioreactor communities perturbed by glucose. Appl. Environ. Microbiol. 66: 4058-4067.

Hashsham, S., A. Fernandez, S. Dollhopf, F.B. Dazzo, J.M. Tiedje, R. Hickey, and C.S. Criddle. 2000. Parallel processing of substrate correlates with greater functional stability in methanogenic bioreactor communities perturbed by glucose. Appl. Environ. Microbiol. 66: 4050-4057.

Liu, J., F.B. Dazzo, O. Glagoleva, B. Yu, and A. Jain. 2001. CMEIAS: a computer-aided system for the image analysis of bacterial morphotypes in microbial communities. Microbial Ecology 41: 173-194 and 42:215.

Dollhopf S., S.A. Hashsham, F.B. Dazzo, C. Criddle, and J.M. Tiedje. 2001. The impact of fermentative organisms on carbon flow in methanogenic systems under constant low substrate conditions. Appl. Microbiol. Biotechnol. 56: 531-538.

Liu, J., F-I. Liu, E. Marshall, and F.B. Dazzo. 2000. CMEIAS© Software for Computer-Assisted Microscopy of Microbial Communities. Annual Mtg., Long-Term Ecological Research in Row-Crop Agriculture. Michigan State Univ. East Lansing, MI.

Dazzo, F.B., J. Liu, F-I. Liu, and G.P. Robertson. 2001. *In situ* analysis of microbial communities on the 2 day-old white clover rhizoplane and the mature BT-corn phylloplane using CMEIAS innovative software for computer-assisted microscopy. Annual Mtg. Long-Term Ecological Research in Row-Crop Agriculture. Michigan State Univ. East Lansing, MI.

Y.G. Yanni, R.Y. Rizk, F.K. Abd El-Fattah, A. Squartini, V. Corich, A. Giacomini, F. de Bruijn, J. Rademaker, J. Maya-Flores, P. Ostrom, M. Vega-Hernandez, R.I. Hollingsworth, E. Martinez-Molina, P. Mateos, E. Velazquez, J. Wopereis, E. Triplett, M. Umali-Garcia, J.A. Anarna, B.G. Rolfe, J.K. Ladha, J. Hill, R. Mujoo, P.K. Ng, and F.B. Dazzo. 2001. The beneficial plant growth-promoting association of *Rhizobium* leguminosarum bv. trifolii with rice roots. Austr. J. Plant Physiol. (now Functional Plant Biology) 28: 845-870.

McDermott, T.R., and F.B. Dazzo. 2002. Use of fluorescent antibodies for studying the ecology of soil- and plant-associated microbes. In C. Hurst, R.C. Crawford, G.R. Knudsen, M.J. McInerney, and L.D. Stetzenbach (eds.), Manual of Environmental Microbiology 2nd ed., Chapter 28, p. 615-626, American Society for Microbiology Press, Washington, DC.

C. Reddy, J. Liu, M. Wadekar, A. Prabhu, D. Trione, E. Marshall, J. Zurdo, F-I. Liu, J. Urbance, and F. B. Dazzo. 2002. New features of CMEIAS: innovative software for computer-assisted microscopy of microorganisms and their ecology. 2002 Annual Mtg., Long-Term Ecological Research in Row-Crop Agriculture. Michigan State Univ. East Lansing, MI.

F. B. Dazzo, J. Liu, A. Prabhu, C. Reddy, M. Wadekar, R. Peretz, R. Bollempalli, D. Trione, E. Marshall, J. Zurdo, H. Hammoud, J. Wang, M. Li, D. McGarrell, A. Gore, J. Maya-Flores, S. Gantner, and N. Hollingsworth. 2003 CMEIAS v. 3.0: Integrative software package to strengthen microscopy-based approaches for understanding microbial ecology. 2003 Annual Mtg., Long-Term Ecological Research in Row-Crop Agriculture. Michigan State Univ. East Lansing, MI. (Summary of CMEIAS upgrade currently under development.) <http://lter.kbs.msu.edu/Meetings/2003_All_Inv_Meeting/Abstracts/dazzo.htm>

Dazzo, F.B., A.R. Joseph, A.B. Gomaa, Y.G. Yanni, and G.P. Robertson. 2003. Quantitative indices for the autecological biogeography of a *Rhizobium* endophyte of rice at macro and micro spatial scales. Symbiosis 34: 147-158.

Dazzo, F.B. 2004. Applications of quantitative microscopy in studies of plant surface microbiology. In: A. Varma, L. Abbott, D. Werner, and R. Hampp (eds.), Plant surface microbiology. Springer-Verlag, Germany, pp. 503-550.