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14. ABSTRACT

Abstract: In the five years of this MURI program, we have made major advances in several areas, including: 1) power production optimization; 2) MFC design; 3) identification of the genes coding for the proteins involved with electricity production by Shewanella oneidensis (MR-1); 4) construction of conceptual models of extracellular electron transport (EET); 5) characterization and quantification of the per cell rates of EET to solid substrates; 6) characterization of a previously undescribed behavioral adaptation of microbes to charged surfaces called electrokinesis (and the impact of surface charge on bacterial attachment and biofilm formation); 7) the use of Vertical Scanning Interferometry for cell and biofilm analyses; 8) the design and implementation of a new type of (Deep Ultraviolet Light) microscope for non-invasive studies of microbes on surfaces; 8) detailed biophysical studies of the mechanism of electron transfer in conductive appendages called bacterial nanowires, produced by MR-1 and other Shewanella strains and species; 9) characterization of the selective advantages of Shewanella cells in late stationary phase, and the relationship o

15. SUBJECT TERMS
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Final Technical Report: (2007 – 2012)

MURI Program: Mechanism(s) of Electricity Production by *Shewanella* and other microbes: Understanding and Optimization

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This report is the last report for the MURI project that is now in its fifth and final year. It summarizes the progress made during the entire period, with a focus on the latest advances during the past year. We highlight the major accomplishments, some surprises, and new horizons and challenges that have arisen. We also point to the avenues that look the most promising, and speculate about the future of the field of microbial fuel cells and bioelectrical devices.

Abstract: In the five years of this MURI program, we have made major advances in several areas, including: 1) power production optimization; 2) MFC design; 3) identification of the genes coding for the proteins involved with electricity production by *Shewanella oneidensis* (MR-1); 4) construction of conceptual models of extracellular electron transport (EET); 5) characterization and quantification of the per cell rates of EET to solid substrates; 6) characterization of a previously undescribed behavioral adaptation of microbes to charged surfaces called electrokinesis (and the impact of surface charge on bacterial attachment and biofilm formation); 7) the use of Vertical Scanning Interferometry for cell and biofilm analyses; 8) the design and implementation of a new type of (Deep Ultraviolet Light) microscope for non-invasive studies of microbes on surfaces; 8) detailed biophysical studies of the mechanism of electron transfer in conductive appendages called bacterial nanowires, produced by MR-1 and other *Shewanella* strains and species; 9) characterization of the selective advantages of *Shewanella* cells in late stationary phase, and the relationship of this metabolic state to the long term function of MFC systems; and, 10) characterization of the activities of microbes acting as catalysts on the cathodes of MFC systems. During this time, we published over *** reviewed papers, presented over 100 talks at scientific meetings, and collaborated with colleagues from around the world.

Overview: The MURI Program in Microbial Fuel Cells, consisted of 5 laboratories, 4 located at the University of Southern California, and one at Rice University. The program had as its major goal, the understanding of how bacteria in the group *Shewanella* (specifically strain MR-1 of *S. oneidensisi*) were able to produce electrical current in microbial fuel cell systems. As a result of the multidisciplinary approach, many different problems were addressed by the group, and advances were made in a number of different areas, including microbial physiology and genetics, MFC design and operation, microbial adaptation and evolution, imaging techniques, the genetics of extracellular electron transfer (EET), and the biophysics of EET. The various areas will be highlighted below, with both overall progress and recent developments being noted.

MURI TEAM (etc.):

USC:	PI	Ken Nealson,	Earth Science & Biology
	CoIs	Steve Finkel,	Molecular and Computational Biology
		Moh El-Naggar	Physics
		Yuri Gorby	Marine and Environmental Biology (also Venter Institute)
Rice Univ.		Andreas Lüttge	Dept. of Earth Sciences
Etc:		Orianna Bretschger	J. Craig Venter Institute
		Jeff McLean	J. Craig Venter Institute
		Greg Wanger	J. Craig Venter Institute
		Shunichi Ishii	J. Craig Venter Institute
		Shino Ishii	J. Craig Venter Institute

Michael Cooney/Plamen Atanassov – MURI group
hybrid (enzyme/microbe) fuel cells

Photon Systems, Inc. – Los Angeles
Bill Hug, Ray Reid, Rohit
Deep UV lasers, and DUV Microscope: **DURIP**

The MURI team consisted of four groups of investigators from USC, and one from Rice University as noted above, all supported by the MURI grant, and collaborations with unfunded (by MURI) colleagues at the Venter Institute, at the University of New Mexico (MURI group on hybrid enzyme mediated fuel cells), and Photon Systems, a small company in Los Angeles, specializing in short wavelength (Deep UV) laser imaging systems. This led a very interactive group for 2010-2011, similar in content and activity to nearly all the years of the MURI program.

41 Total Players in MFC Work This Year !

Co-I	Dept	P-doc	Grads	Staff	UGs
K Nealson	Geo	3	4	3	4
Y Gorby	Bio	2		1	1
S Finkel	Bio		4		3
M El-Naggar	Phys	1	3		2
A Luttge	Geo	1		1	
Photon Systems	ES/Bio	1	1		
JCVI		3		2	

5 PI/CoIs; 19 GS/PDs; 7 Staff Scientists; 10 undergrads

Brief History:

The program wasn't always like this. In the first two years of the program, under the guidance of Dr. Jennifer Grisham, we experienced some major changes in direction and in personnel, as noted below. The title of the MURI changed somewhat, and several personnel changes were instituted by Dr. Grisham. When Dr. Kozumbo took over, the program stayed on the same course set in the first two years.

MURI began as much more applied – multiscale modeling and engineering approaches to optimize MFC performance:
Bioengineered Fuel Cells: Optimization via genetic approaches and multi-scale modeling

Through interaction with the Program Manager, Major Jen Gresham, two program reviews, and lately, the input of Walter Kozumbo, it has evolved to a strongly mechanistic program, in search of mechanisms of microbial catalysis of MFC current production.

Now titled:
Mechanism(s) of Electricity Production by *Shewanella and* Other Microbes: Understanding and Optimization

Goals and Objectives:

Long Term Goals:

1. Understand how electron flow occurs between the biological catalysts and the fuel cell components (anode & cathode)
2. Use this knowledge to construct completely biologically catalyzed microbial fuel cells.

Relationship to other programs: This work intertwines with many other projects now underway in our various laboratories, and with various collaborators:

a. Corrosion control and remediation
b. Stress and corrosion recognition
c. Waste recycling and water regeneration
c. Pollution remediation, especially of metals

It also has **active interactions with a number of other programs and PIs**:

a. Plamen Atanassov and the enzyme-mediated fuel cell MURI
b. Brad Ringeisen of the NRL, and MFC work
c. Corrosion and Materials Science work funded by ONR
d. Nano-technology laboratory at LBNL
e. Recent collaboration with John Golbeck from Penn State University
f. Recent collaboration with L-P. Neilsen from Aarhus, Denmark

Power Production: (all PIs)

As instructed by the program manager, one of our goals of the MURI was NOT to focus on applications and increased power production. That being said it was an easy task, as we learned more about *Shewanella* and about microbial fuel cells, to see increases in power production. We began the work by designing a MFC that could be used for experimental purposes (Figure 1). This system had multiple inputs for electrodes so that a variety of electrochemical analyses could be done during the experiments (polarization, impedance spectroscopy, etc.). In addition, the cells (that were made by our shop in great numbers!) were composed of glass, so that they could be autoclaved, and reused. All labs involved with the MURI project used the same MFCs for study, and all experiments were started from frozen cell stocks of the same original culture.

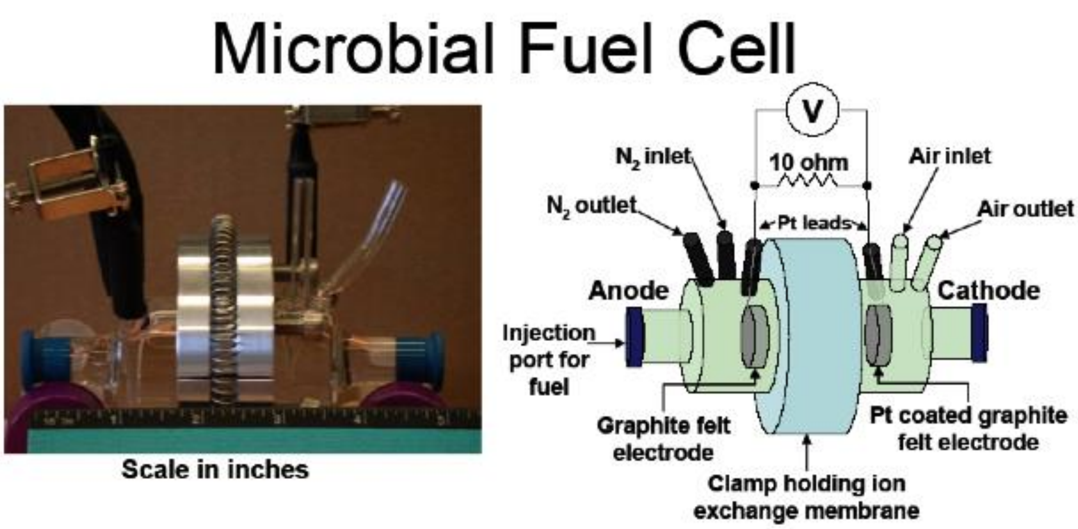

Figure 1: Picture of the MFC design used by our laboratories for the MURI project, and, on the right, a diagram of the MFC components.

Using this system, we realized a remarkable increase in power production during the years of the program. Increases that were not MFC design dependent, but were a function of the increased biological performance of the system. Our own results are shown in Figure 2, below, showing about a 10X increase per year.

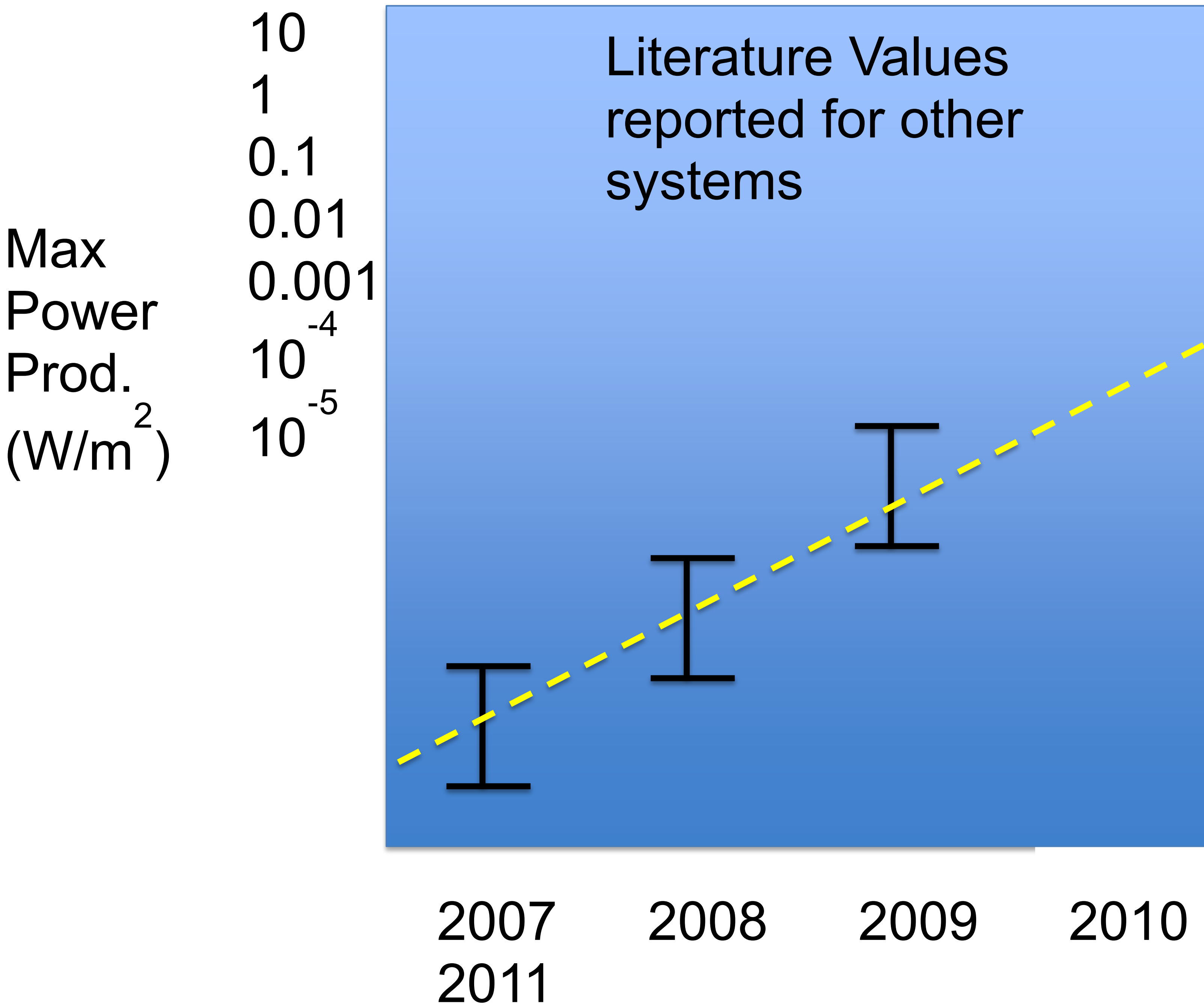

Figure 2. Increases in power production in the MURI MFC system during the entire program period. As seen, the increase was about 10-fold per year in terms of watts per square meter of anode surface.

During the MURI, we learned some important lessons that can be summarized as "No two MFCs can be meaningfully compared with regard to power production unless they are identical. They differ with regard to internal resistance, external resistance, material properties, actual electrode surface areas, and other properties. This has led to many unnecessary conflicts between laboratories.

Over the course of the years, we worked first with pure cultures (mostly MR-1), then with mutants of MR-1 and other *Shewanella* strains, and finally with mixed cultures. The bulk of what we accomplished has been done with MR-1 and our standard system, but when other systems were used, they will be noted.

Using the standard system, a number of accomplishments were seen:

1. Installation, set-up, calibration and use of a reproducible MFC system (14-16)
2. Comparison of all *Shewanella* strains for current production (6)
3. Identification of the major genes involved with current production (7)

Genetics and Genomics and Current Production: (Nealson/Finkel)

In her Ph.D. Thesis work, Orianna Bretschger, in the Nealson lab, with collaboration from many others, identified the major enzymes involved with electricity production in MFCs, and confirmed which of these genes were required for optimal current production. This work was done via the use of deletion mutants with lowered activity, and verification of the mutant via complementation experiments. The identification of these genes confirms that EET to metal oxides is similar to EET to electrodes, with a few subtle differences. The work set the stage for many other projects in the MURI program, and for other workers in the field (2). The figure below show the so-called mtr operon (bottom), containin the mtrA-C genes that are required for any *Shewanella* strain that produces electricity. Other genes that are required are shown in the conceptual model, including an intact electron transport chain, cymA, mtrA-C, and an outer membrane protein called omcA. During this work, the Nealson lab participated in the sequencing and analysis of more than 20 strains of *Shewanella*: only one did not contain this set of genes, and it was incapable of current production (2). The lower figure shows a general depiction of EET, with the involvement of nanowires (refs), soluble electron shuttles, and direct EET.

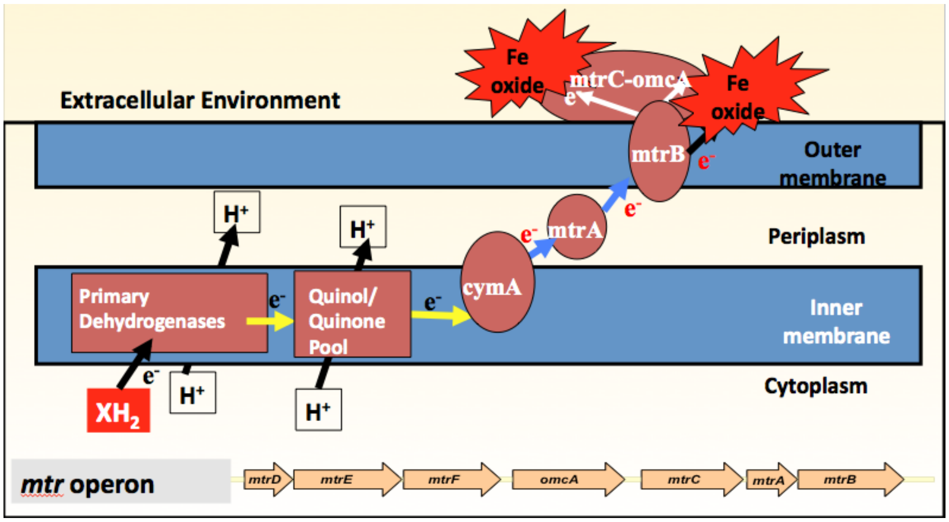

Figure 3. Working model of EET for *Shewanella.* This model diagram depicts the major genes and electron transport for MR-1, showing the connection to iron oxides at the outer cell wall. The inner membrane electron flow is where energy conservation takes place, and then cyma, mtrA,B,C, and omcA work together to accomplish EET, and allow electron flow in the absence of soluble electron acceptors.

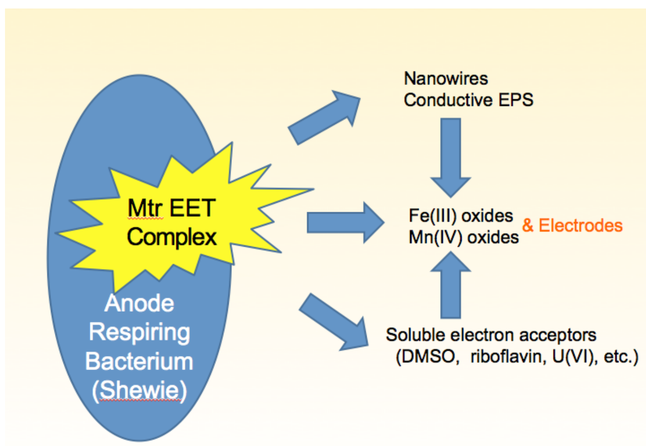

Figure 4: A simplified model for EET in MR1. This diagram depicts the suspected pathways for EET, which include nanowires or conductive extracellular matrices, as well as soluble electron shuttle compounds, all thought to be involved with electron flow to solid substrates, such as metal oxides and/or MFC electrodes.

Adaptation and evolution of *Shewanella oneidensis* MR-1:

In the laboratory of Dr. Steve Finkel, the issue of how cells adapt and evolve in the MFC environment has been studied. For the use of MFC systems as long-term power sources, such knowledge is critical. Do the cells adapt to the anode envioronment, and if so does this involve genetic changes and cell evolution, or simply adaptive (physiological changes)? This work has involved an intensive study of *Shewanella* cells in long term stationary phase culture, to examine a mechanism that in *E. coli* is termed GASP, of Growth Advantage in Stationary Phase.   In brief, it was shown that the planktonic growth form of MR-1 was capable of adapting to stationary phase, and improving its survival ability. This work, which was the thesis work of Meaghan Ribbens, is discussed in detaile in the recent accomplishments section below. The situation with regard to biofilm formation is far more complex, as also discussed below. In short, cells become more adaptive with regard to growth in biofilms, but these advantages come and go, suggesting that the adaptation is physiological rather than genetic. This work is the precursor to work on charged surfaces, which is now beginning in the Finkel laboratory.

Imaging cells on surfaces: (Luettge; Nealson)

One of the big challenges of working with microbes on electrodes is that many of the best electrodes for MFCs are composed of solid substrates that are not transparent, making cell visualization difficult. We have taken two "new" approaches to the solution of this problem: 1) VSI (Vertical Scanning Interferometray); and 2) DUV (Deep UltraViolet) light microscopy. The first approach (VSI), which had, to our knowledge, never been used for the study of microbes on surfaces, is discussed below in the Luettge section. This work has revealed many new aspects of microbes on surfaces, including some novel ways of looking at the microbe/mineral interface (18, 19).

The second approach (DUV microscopy) grew out of an idea for looking in a minimally invasive way at microbes on surfaces, and involved the development of a new type of microscope using laser illumination at 224 nm wavelength. This system was funded via a DURIP addition to our laboratory, and resulted in the design and building of a microscope with unique capabilities of imaging bacteria on mineral (and electrode) surfaces (1, 2) without the need for stains or sample preparation. This work is continuing under the funding of the ARO (Army Research Office).

Conductive nanowires produced by bacteria: (El-Naggar/Gorby)

Conductive appendages, called nanowires, were first discovered in *Shewanella* by Gorby (11), and have been studied extensively in the Gorby and El-Naggar laboratories over the course of the MURI program (8-10). Experiments were done via the more standard approach using the Atomic Force Microscope (8) and then via the multi-electrode approach in collaboration with the Molecular Foundry at UC-Berkeley and the Berkeley National Laboratory (9)

Collaborative work with other laboratories:

Jet Propulsion Laboratory/Caltech: The development of the DUV microscope was done in collaboration with the optical physics group at the Jet Propulsion Laboratory in Pasadena, CA.

J. Craig Venter Institute: Considerable collaborative work has been done with the JCVI with regard to the study of rates of electron transport (17) and the study of cell-cell interactions and optimization of systems for current production (13).

MURI group of Dr. Plamen Atanassov, UNM: Throughout the MURI project, we have been in contact with the group of Dr. Plamen Atanassov, who have a MURI funded in enzymatic fuel cells. This has resulted in one publication of a hybrid fuel cell (12), and another recent submission dealing with the issue of riboflavin as an electron shuttle (paper in review).

NRL Research Group of Dr. Bradley Ringeisen: We have worked with Justin Biffinger from the NRL in Washington DC, to optimize fuel cells, and with regard to other types of organisms, and different types of fuel cells: all with the goal of optimizing power production of the MFCs (3-5)

The Loker Hydrocarbon Institute at USC: We have done a lot of joint work on MFCs with the fuel cell group at the USC Loker Hydrocarbon Institute, to understand and optimize MFC production (15, 16).

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Final Addenda:

In the final note, the USC MURI produced a great amount of new data, and considerable new insights with regard to the functioning of microbial fuel cells. It spawned a wealth of new data, new methods, and new ideas.

Some of these are game-changing findings, while others are in the category of incremental knowledge. Some of them include:

Accomplishments Related to Imaging:

1. Development of VSI for biology and MFC work
   (Waters et al. 2008. Geobiology 6:254-262)
   (Waters et al., 2009. AEM. 75:1445-1449)
   (GO paper)
2. Development of the DUV imaging system (DURIP)
   (Bhartia et al., 2010. AEM. 76:7231-7237)***
3. Development of the Flow-Thru Biofilm Imaging System
   (McLean et al., ES&T, 44:2721-2717)***
4. Development of the anaerobic poised electrode method
   (Harris et al., PNAS, 107:326-331*

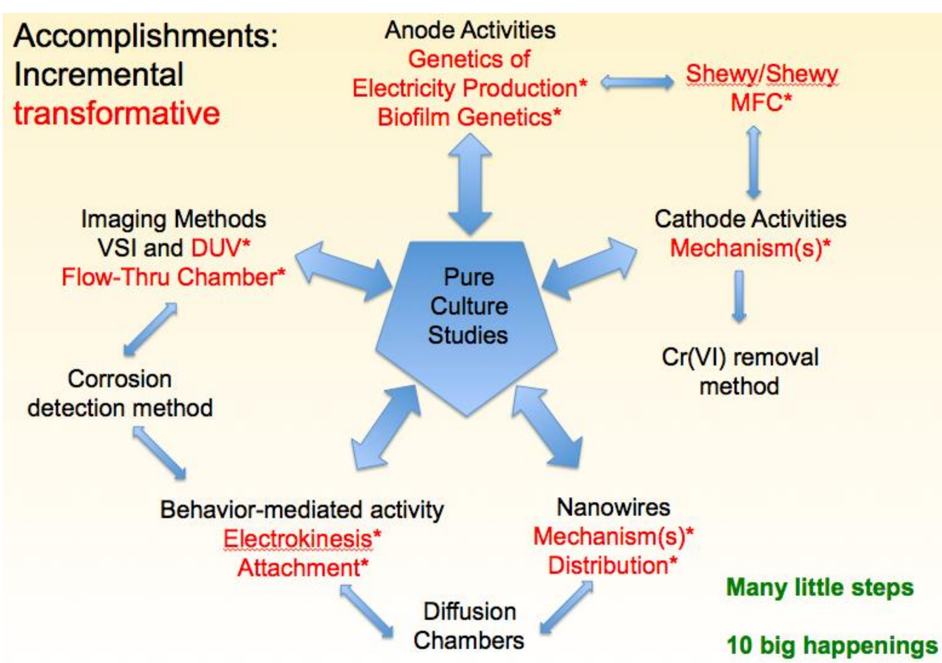
Accomplishments Related to Microbial Behavior and Ecology:

1. Electrokinesis – surface charge and swimming
   (Harris et al., 2010. PNAS107:326-331)****
2. Early detection of oxidized surfaces and corrosion
   (Waters et al., 2009. Biofouling:25:163-172)
3. Impact of surface charge on biofilm formation
   (McLean et al., 2010. ES & T. 44:2721-2717.

Accomplishments Related to MFC Functioning and EET

1. Installation and set up of a reproducible MFC system (manufacture of MFCs for all PIs – and many others!) (Manohar et al., Electrochem. Acta. 3508-3513)
2. Comparison of all *Shewanella* strains for current production (Bretschger et al., 2010. Electroanalysis 22:883)
3. Identification of genes involved with current production*** (Bretschger et al., 2007. Appl. Environ. Microbio. 73:7003)
4. Evidence for cathode catalysis by *Shewanella**** (Hsu et al., ISME J. in revision)
5. Development of a Cr(VI) removal system*** (Hsu et al., ISME J. in revision)
6. Discovery of nanowires (chemostat cultures, biofilm imaging) (Gorby et al., PNAS, 1996, 103:11358-11363)****

A summary of activities is shown in the last figure, below, with the incremental discoveries shown in black, and the transformative ones shown in red. All of this was done using pure cultures and our "standard" MFC system.



August 23, 2013

Final Progress Report:
Submitted by Dr. K. H. Nealson
Wrigley Professor of Environmental Sciences
University of Southern California

Summary Overview Final Report:
AFOSR MURI
PI: Dr. Kenneth Nealson
Co-Is: Dr. Mohamed El-Naggar (USC)
Dr. Steve Finkel (USC)
Dr. Yuri Gorby (USC)
Dr. Andreas Luettge (USC)
Air Force Office of Scientific Research
Proposal #: FA9550-06-10292

Title: Mechanism(s) of Electricity Production by *Shewanella* and other microbes: Mechanisms and Optimization.

In the 5 years of the MURI (plus the extended year) we have accomplished an immense amount. The summary reads like this:

Intellectual achievements:

1. identification of the genes responsible for electricity production
2. optimization of electron flow in MFC systems
3. the role of extracellular electron transport (EET) in current production
4. the role of EET in corrosion processes
5. the use of EET as a measure of community respiration
6. the role of surface charge in bacterial attachment and growth
7. the importance of surface charge in the development of biofilms
8. the ability of biofilm bacteria to evolve and resist "invaders"
9. the importance of electron flow to the development of biofilms
10. the use of anodes to enrich EET-capable bacteria
11. the use of bacteria to catalyze oxygen reduction at the cathodes
12. demonstration of nanowire conductivity
13. modeling electron transfer as a physical electron-hopping process
14. use of deep ultraviolet light to detect unstained microbes on surfaces

Applications:

1. optimization of current production through MFC design
2. optimization of current production through microbe "improvement"
3. method for removing Chromium from water (Patent submitted)
4. method for removing Selenium from water (Patent being written)
5. method for cleaning human wastewater (Patent pending)
6. development of a new type (deep ultraviolet – DUV) of microscope

Ph.D. Students Obtaining Degrees:

1. Bretschger, Orianna
2. Barge, Laurie
3. Waters, Michael
4. Salas, Everett
5. Ribbens, Megan
6. Corzett, Chris

7. Hsu, Lewis
8. Kus, Esra
9. Manohar, Aswan
10. Bhartia, Rohit
11. Harris, H. Wayne
12. McLean, Jeffrey
13. Chellamuthu, P.

Postdoctoral Scholars Trained:

1. El-Naggar, Mohamed
2. Kan, Jinjun
3. Jang, Jun
4. Ishii, Shunichi
5. Suzuki, Shino
6. He, Zhen
7. Wanger, G.

Undergraduates trained:

Five to 10 undergraduates were involved with the PIs each year, so that around 50 undergraduates had the opportunity to be mentored and learn about research methods. Several of these students have gone on to fruitful research careers because of the experience.

Collaborations with other Institutions:

1. University of New Mexico – Atanassov's MURI group (biophysics)
2. Pacific Northwest laboratories – Fredrickson group (microbiology)
3. Tokyo University – Hashimoto electrochemistry group (electrochemistry)
4. Naval Research Laboratory, D.C. – Ringeisen group (MFC)
5. Naval Research laboratory, Florida – Johnson group (MFC)
6. Berkeley DOE laboratory – Molecular Foundry (nanowires)
7. Jet Propulsion Laboratory – Optics group (microscope development)

Collaborations with business:

1. Photon Systems, Inc. – collaboration for building new (DUV) microscope

Publications:

Workers being supported by the MURI grant were authors on more than 50 major publications, 20 of which were in "high-impact" journals. Given that we were not in the MFC "business" when this proposal was funded, and that it took close to two years to begin serious publishing, this was a very productive endeavor.

A list of the publications follows, with up-to-date references. Because some of this work is still being finished and written up, the list will grow somewhat, but this provides and idea of what was done and where it was published. As noted in the project final report, this work goes far beyond just the study of microbial fuel cells

Summary Publications List:

1. Barge, L.M., D.E. Hammond, M.A. Chan, S. Potter, J. Petruska,, and K.H. Nealson. 2011. Precipitation patterns formed by self organizing processes in porous media. Geofluids, no. doi: 10.1111/j.1468-81232010.00324.x
2. Barge, L.M., K.H. Nealson, and J. Petruska. 2010. Organic influences on inorganic patterns of diffusion-controlled precipitation in gels. Chem. Physics Lett. 493: 340-345.
3. Bhartia, R., E.C. Salas, W.F. Hug, R.D. Reid, A.L. Lane, K.J. Edwards, and K.H. Nealson. 2010. Label-free imaging with deep UV laser induced native fluorescence. Appl. Environ. Microbiol. 76:7231-7237

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5. Biffinger, J.C, B. R. Ringeisen; M. Ribbens; S, Finkel; K. Nealson, and J. J. Pietron. 2009. Characterization of electrochemically active bacteria (EAB) utilizing a high-throughput voltage-based screening assay Biotechnology and Bioengineering 102: 436-444.
6. Biffinger, J.C., J. Pietron, O. Bretschger, L. J. Nadeau, G.R. Johnson, C.C. Williams, K.H. Nealson, and B.R. Ringeisen. 2008. The influence of acidity on microbial fuel cells containing *Shewanella oneidensis*. Biosensors and Bioelectronics 24:906-911.
7. Biffinger, J.C., L.A. Fitzgerald, R. Ray, B.J. Little, S.E. Lizewski, E.R. Petersen, B.R. Ringeisen, W.C. Sanders, P.E. Sheehan, J.J. Pietron, J.W. Baldwin, L.J. Nadeau, G.R. Johnson, M. Ribbens, S.E. Finkel, K.H. Nealson. 2010. The utility of *Shewanella japonica* for microbial fuel cells. Bioresource Technol. 102:290-297.
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9. Bretschger, O., et al., 2007. Current production and metal oxide reduction by *Shewanella oneidensis* MR-1 wild type and mutants. Appl. Environ. Microbiol. 73:7003-7012.
10. Bretschger, O., A.C.M. Cheung, F. Mansfeld, and K.H. Nealson. 2010. Comparative microbial fuel cell evaluations of *Shewanella* spp. Electroanalysis 22: 883-894.
11. Bretschger, O., L. Hsu, M. Ghassemian, P. Chellamuthu, A.C.M. Cheung, C.M. Frantz, M. Pirbazari, and K.H. Nealson. 2012. Differential redox activity and protein expression as function of extracellular electron transfer in microbial fuel cells using *Shewanella oneidensis* MR-1. DOI:a10.1002/cssc.200
12. Chang, I.S., H. Moon, O. Bretschger, J.K. Jang, H.I. Park, K.H. Nealson, and B.H. Kim. 2006. Electrochemically active bacteria (EAB) and mediator-less microbial fuel cells. J. Microbiol. Biotechnol. 16:163-177.
13. Davis K.J., A. Lüttge, and K. H. Nealson. 2007. Calcite and dolomite dissolution rates in the context of microbe-mineral surface interactions. *Geobiology* 5:191-205
14. El-Naggar, M. , G. Wanger, K.M. Leung, T.D. Yuzvinsky, G. Southam, J. Yang, W.M. Lau, K.H. Nealson, and Y.A. Gorby. 2010. Electrical Transport Along Bacterial Nanowires from *Shewanella oneidensis* MR-1 Proc. Nat. Acad. Sci. USA 107:18127-18131.
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16. Fredrickson, J.K., et al., 2008. Towards Environmental Systems Biology of *Shewanella*. Nature Rev. Microbiol. 6:592-603
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