Biochemical Mechanisms and Energy Strategies of *Geobacter sulfurreducens*

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Lab work performed by Laura Liermann and Camille Stephen

Research Objective

- Determine the survival characteristics of *Geobacter sulfurreducens* under prolonged starvation. The three aims are:
  - Characterize growth properties during prolonged starvation (does *Geobacter* undergoes dormancy or Growth Advantage in Stationary Phase (GASP))
  - Determine the energy requirements to maintain a growing *versus* a non-growing population (through chemostat studies)
  - Monitor gene expression patterns (using DNA microarrays) during growth and starvation phase to determine the metabolic strategy.
    - Determine gene expression patterns in biofilms relevant to iron reduction and survival.

Relevance to DOE

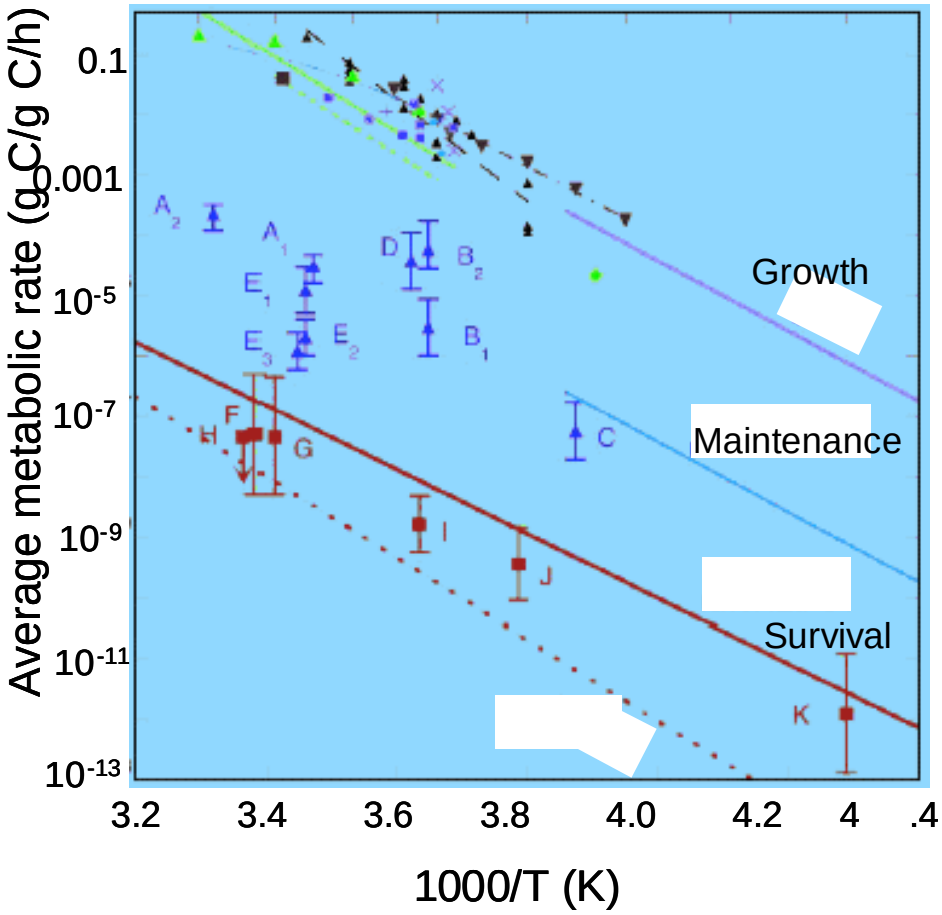
- Metal reducing microbes are of interest in maintaining U and Tc in the reduced state.
- The response of microorganisms to the varying nutrient conditions commonly found in the environment is therefore central toward the maintenance of the DOE sites

Feast or famine

- Morita* described the life of the typical bacterium as that of “feast or famine”
- Feast moments are rare and life is dominated by prolonged periods of famine.
  - How do non-sporulating microbes survive these long periods of nutrient deprivation.

*Morita, R.Y. (1988) Can. J. Microbiol. **34,** 436-441.

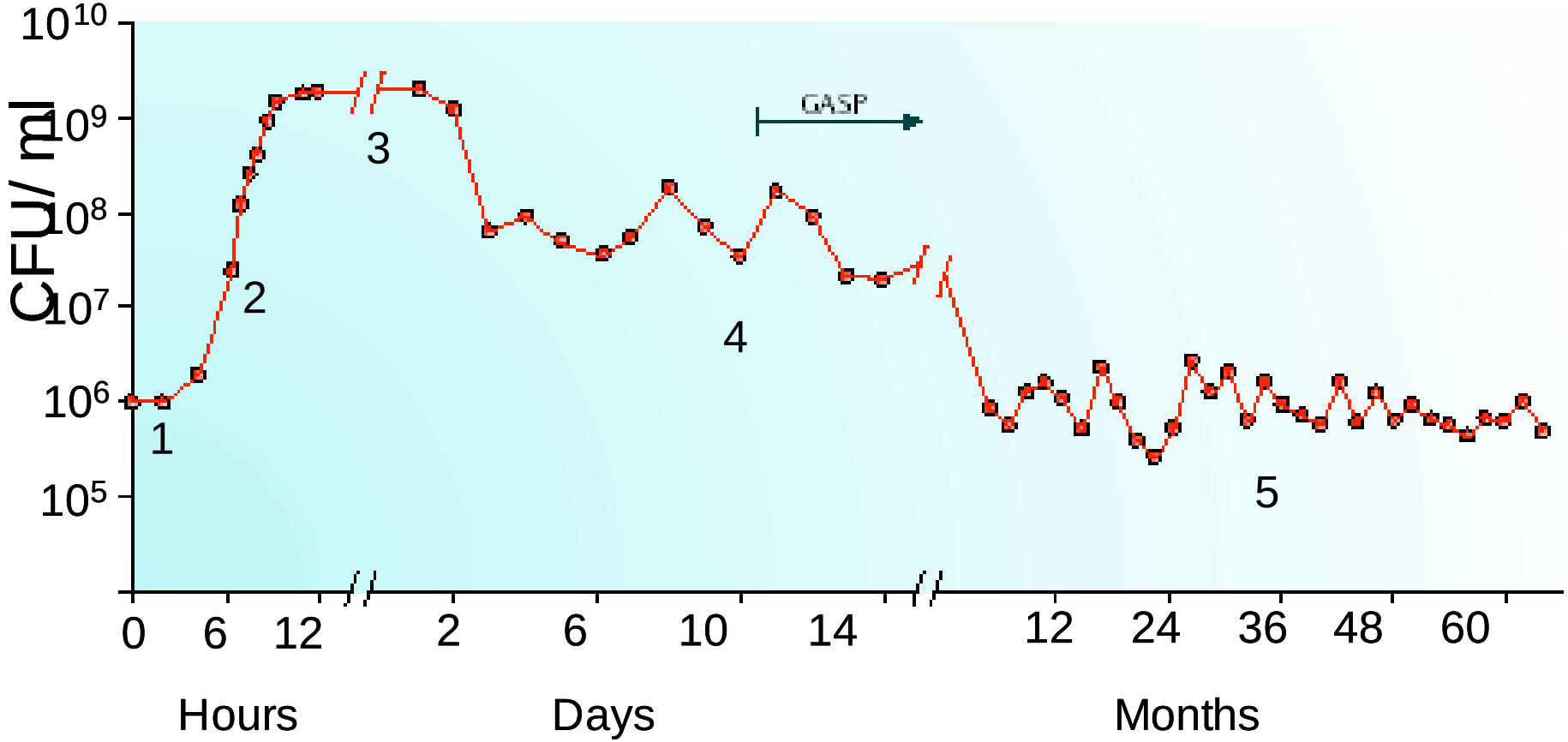
Comparison of metabolic rates of prokaryotes as a function of temperature in various ecosystems.



- Nutrient rich
  - Energy used for synthesis of cell biomass (reproduction) and for cellular maintenance (maintenance energy)
- Nutrient poor (Not enough energy for cell growth)
  - Energy utilization for only maintenance of cellular components
- Prolonged starvation
  - Change in metabolic strategy. Maintenance energy is needed, however, in addition to changes in gene expression patterns, may involve changes in genetic material (mutations)

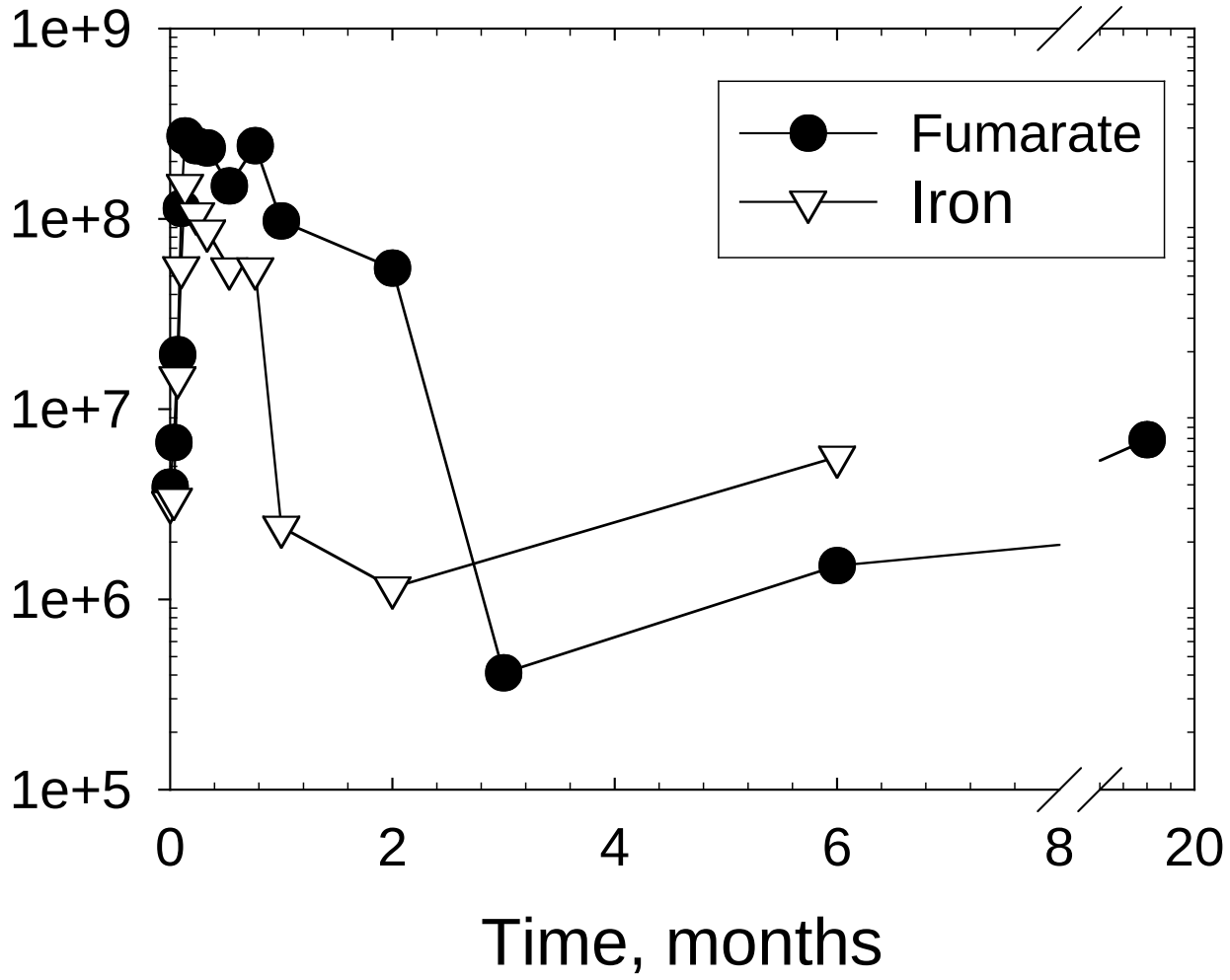
From Price and Sowers(2004) Proc. Natl. Acad. Sci.**101,** 4631-4636.

The five phases of the bacterial life cycle.

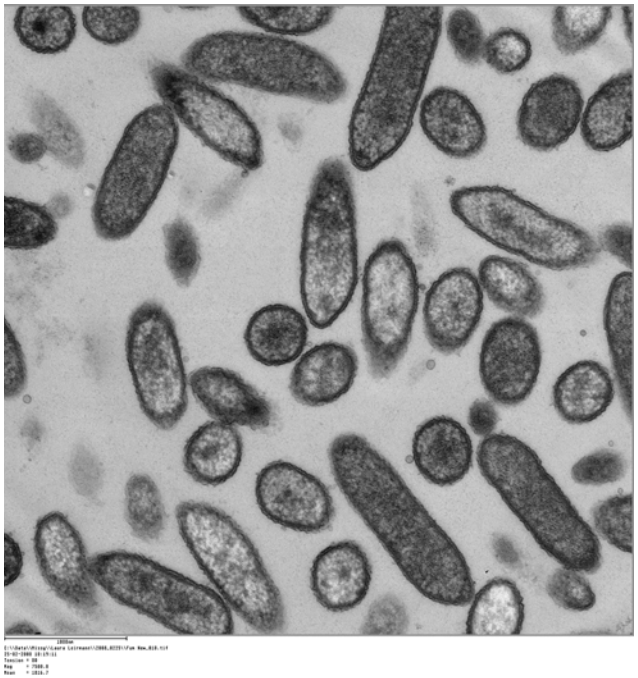


- *E. coli* was grown aerobically in shake batch cultures in LB. At indicated time points, aliquots were removed and colony forming units (CFU) were determined. Reproduced from Finkel (2006) Nat. Rev. Microbiol. **4,** 113-120.

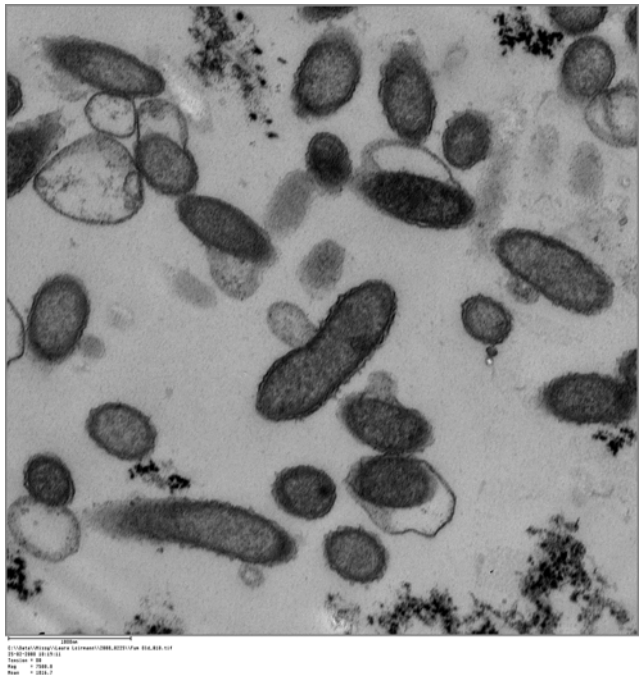
Growth phases of *G. sulfurreducens*



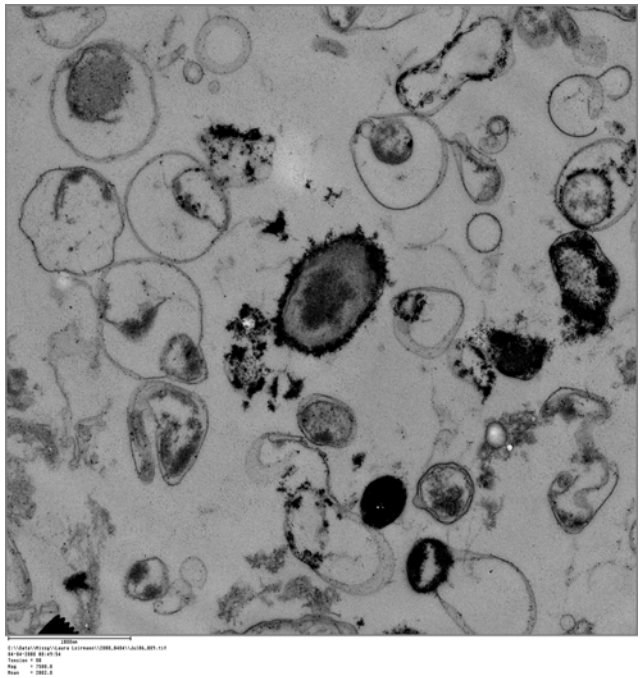
TEM imaging of active *versus* starving cells



2 days



6 months

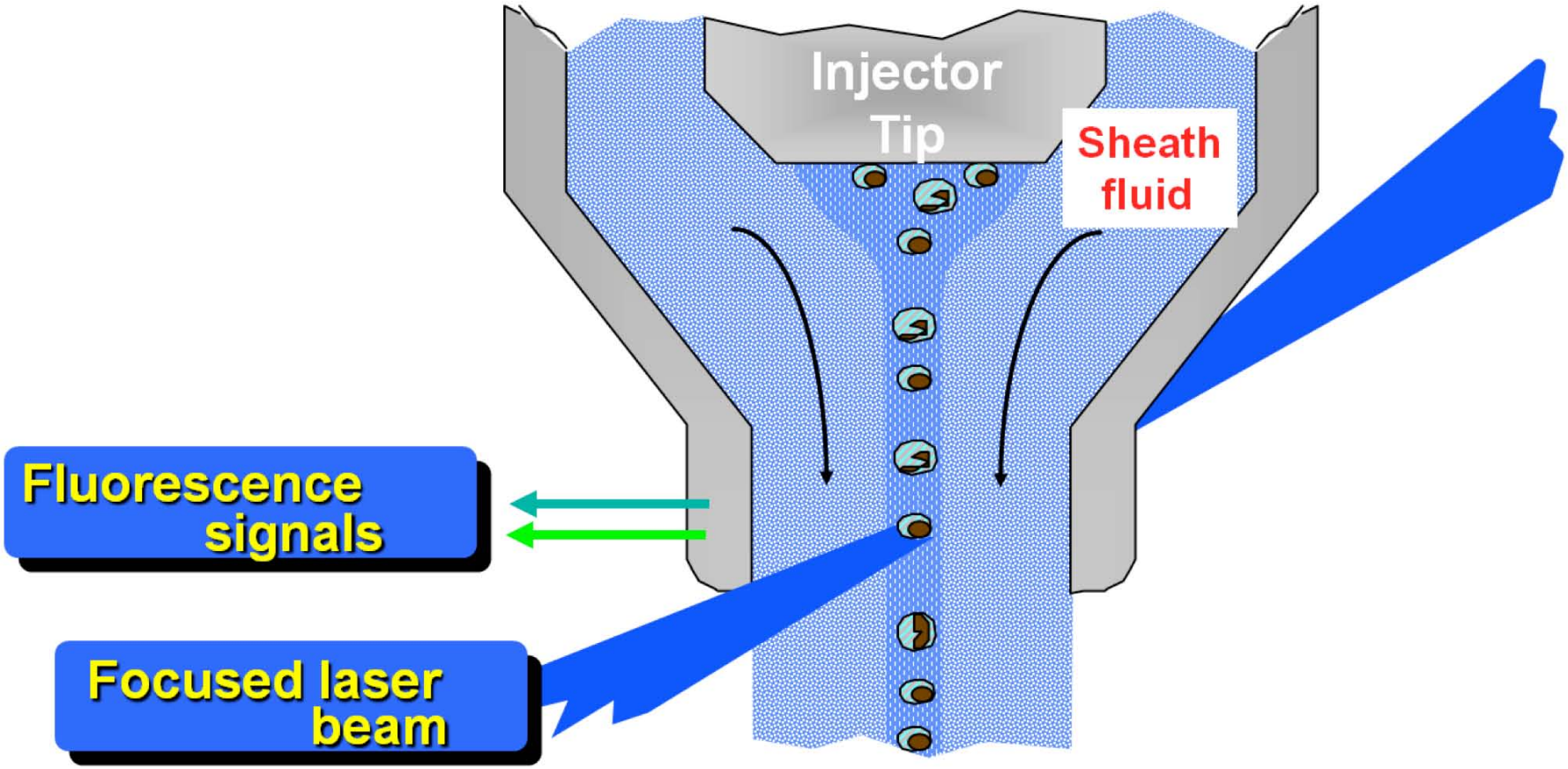


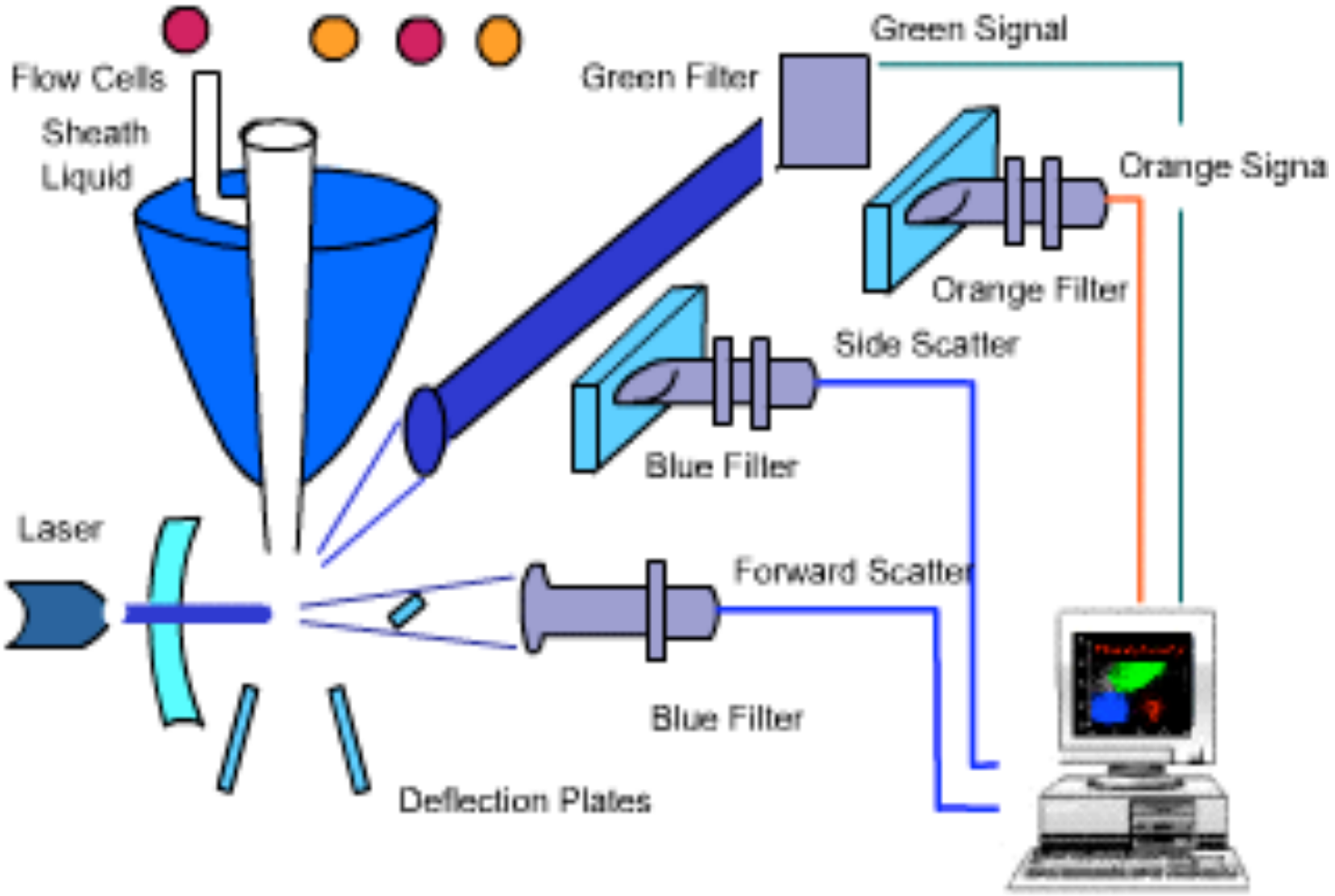
20 months

Dormancy in non-sporulating bacteria

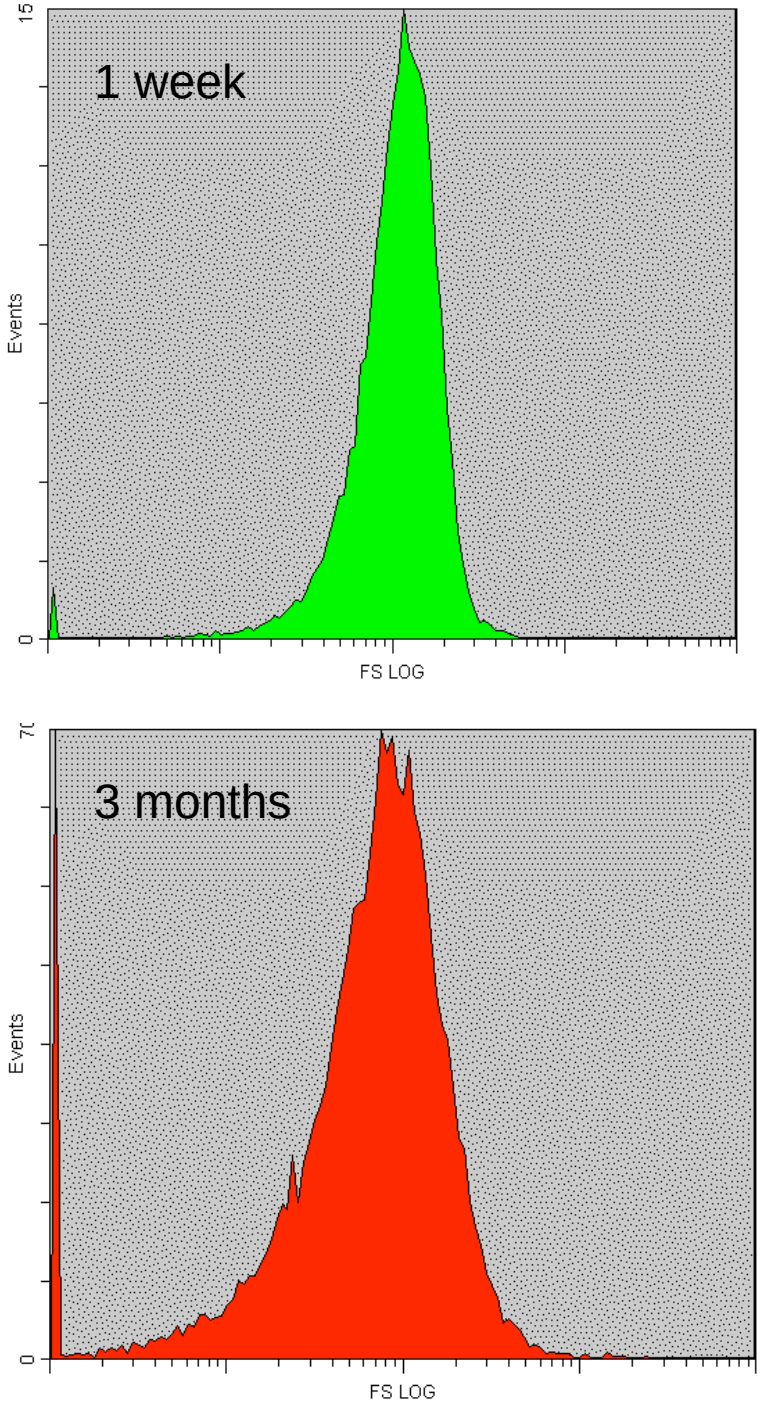
- Environmental dormant forms of non-sporulating bacteria observed in natural environments
  - More difficult to grow on enriched agar medium and need to be “revived”.
  - These “dormant” microbes were smaller* and have been referred to as ultramicrobacteria (terminology of Morita).
- Researchers have proposed that a decrease in size is an adaption to low nutrient conditions. Smaller bacteria have relative larger surface to size ratio; an advantage in nutrient depleted conditions.

Flow cytometry (cell sorter)

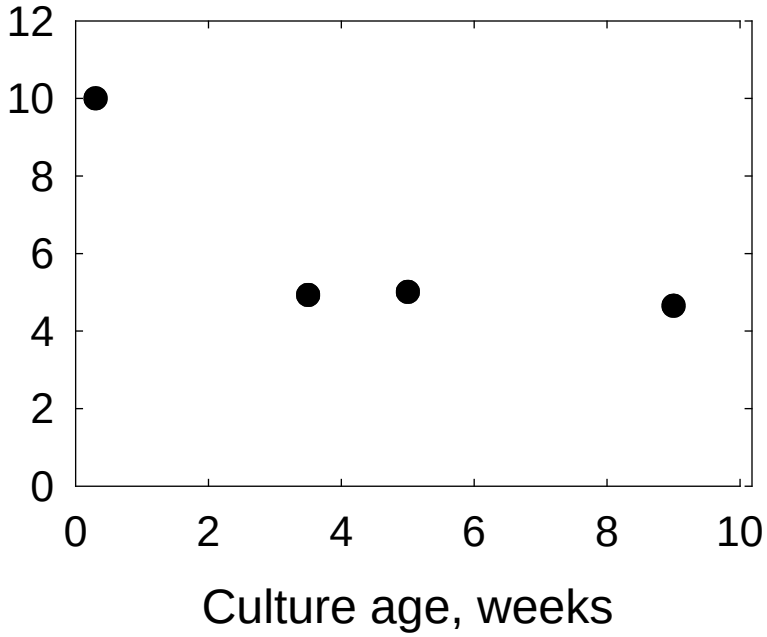




From Univ. Wisconsin flow cytometry facility

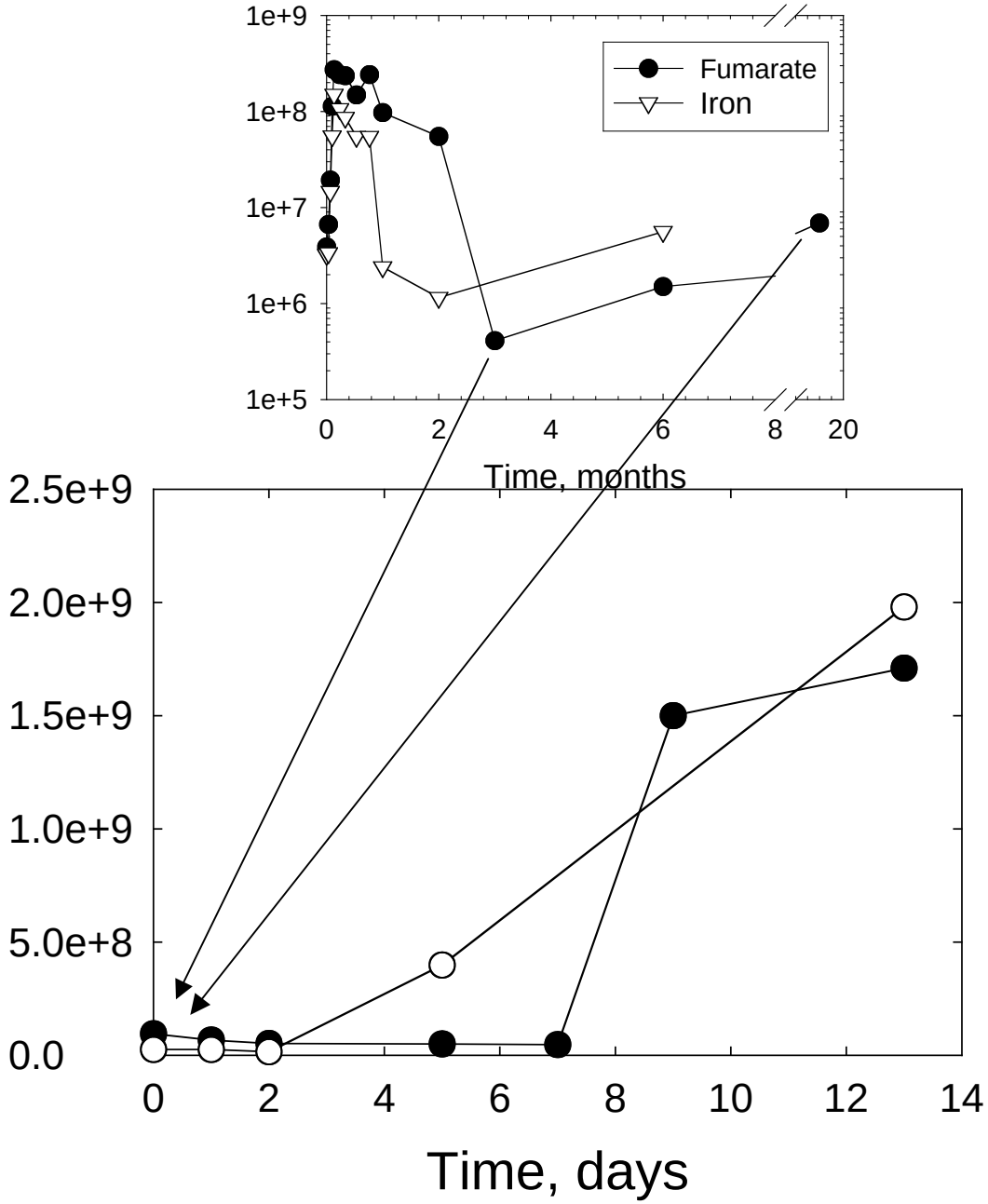


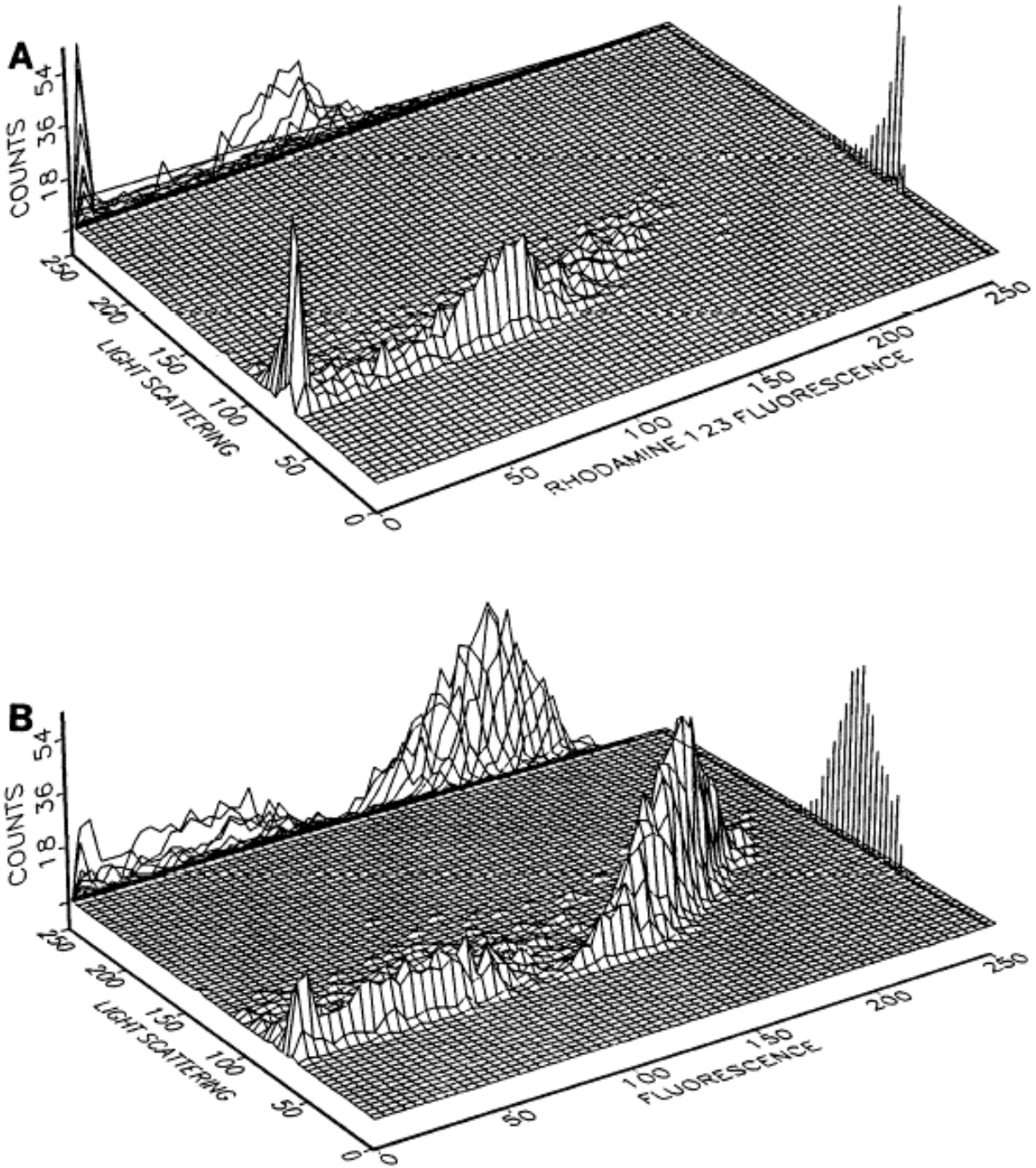
Cell size decreases upon prolonged starvation



- Decrease in size as cells age. The cell sorter has gated only live cells (BacLite live/dead staining)

Recovery of starved G. *sulfurreducens*





- Domancy and decrease in size has been observed with *Micrococcus luteus*. Cells were starved for 75 days, harvest and analyzed immediately and 45 h later.

From Kaprelyants et al. (1993) FEMS Microbiol. Rev. **104,** 271-286.

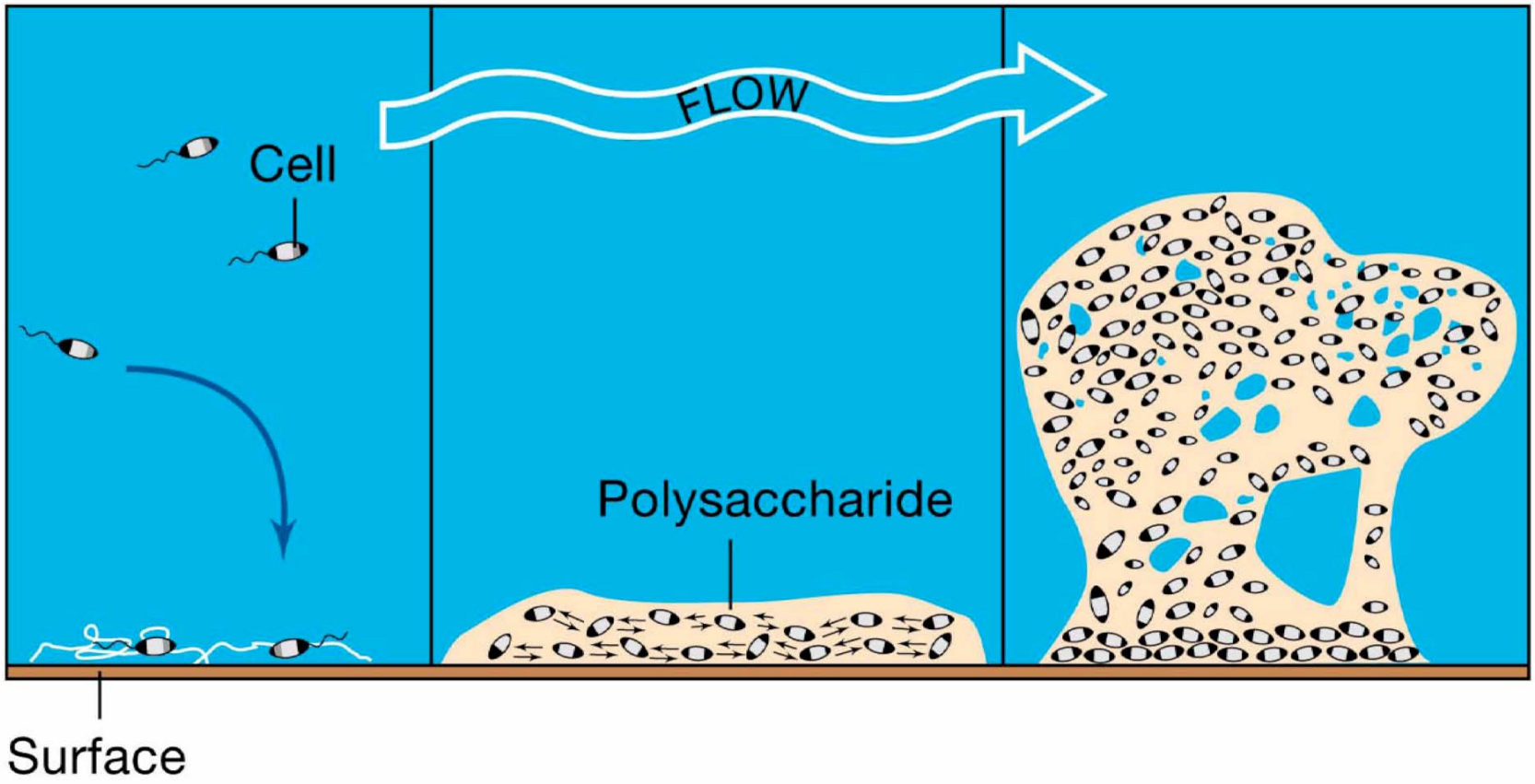
Conclusions thus far

- *G. sulfurreducens* can survive prolonged starvation
- Cells can be revived. Initial results suggest that older cells take longer to be revived
- Decrease in size is observed with prolonged starvation
- Growth conditions have been identified to study global gene expression analysis by DNA microarray
  - Identified mid long phase, stationary phase and prolonged starvation phase

Attachment
(adhesion of a few cells to a suitable solid surface)

Colonization
(intercellular communication, growth and polysaccharide formation)

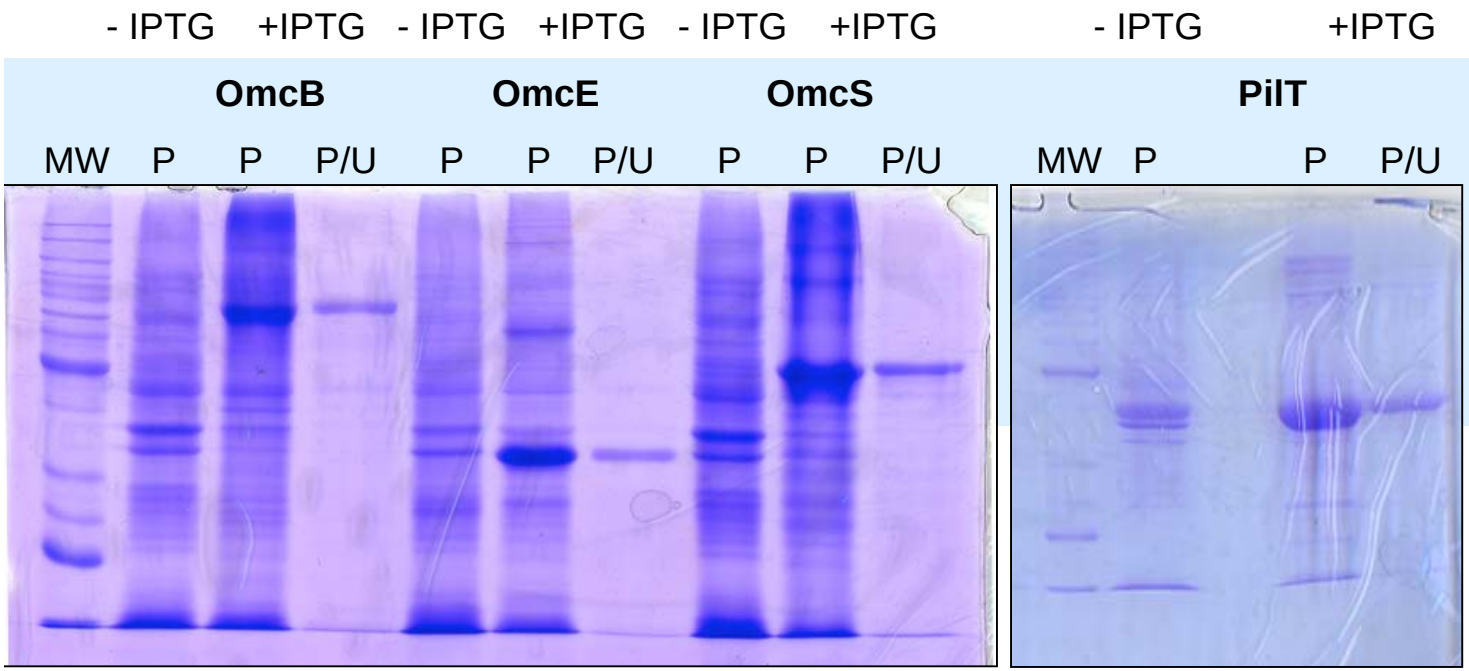
Development
(more growth and polysaccharide)



Geobacter proteins involved in iron reduction or biofilm formation

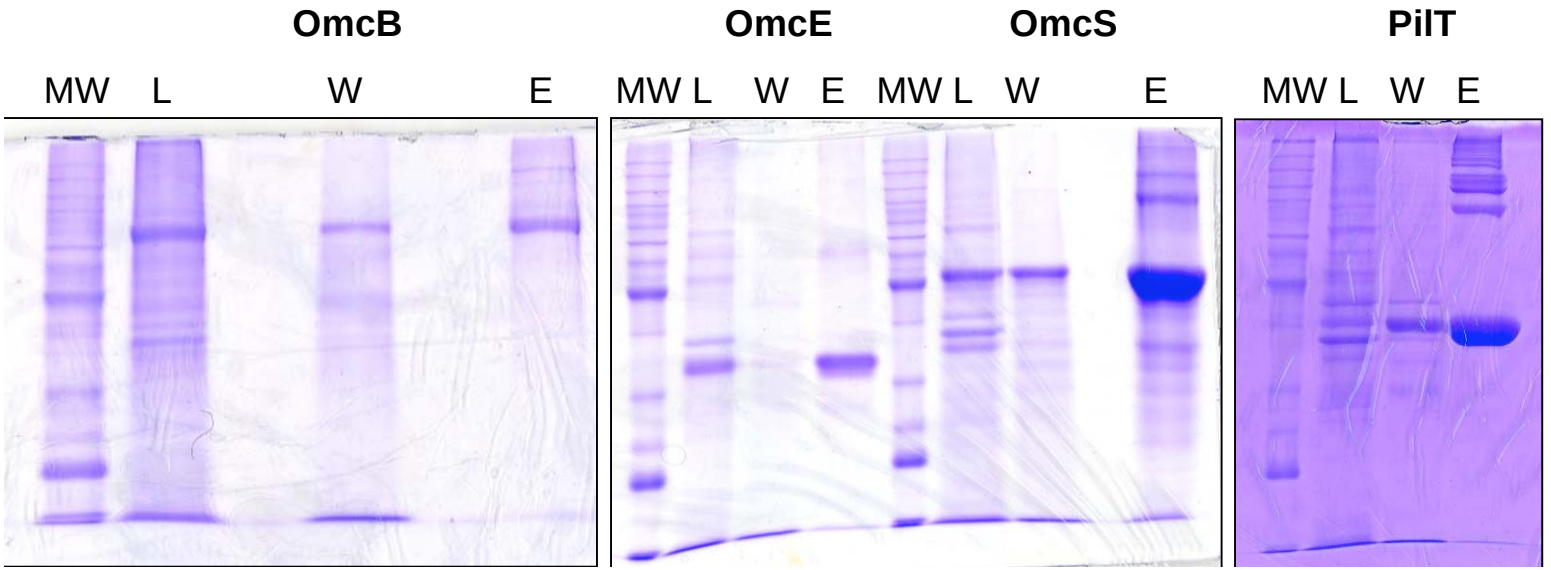
Protein	Properties
OmcB	85 kDa, polyheme **D. R. Lovley et al.** 2003. *J. Bacteriol.* 185.7.2096 -2103.
OmcE	30 kDa, tetraheme **D. R. Lovley et al.** 2005. *Appl. Environ. Microbiol.* 71.12.8634 – 8641
OmcF	9.4 kDa, monoheme **D. R. Lovley et al.** 2005. *J. Bacteriol.* 187.13.4505 - 4513.
OmcG **OmcH**	78.7 kDa 103 kDa Multiheme proteins **D. R. Lovley et al.** 2006. *J. Bacteriol.* 188.8.3138-3142.
OmcS	50 kDa, hexaheme **D. R. Lovley et al.** 2005. *Appl. Environ. Microbiol.* 71.12.8634 – 8641.
PilA	7.8 kDa, Pili protein **D. R. Lovley et al.** 2005. *Nature.* 435:1098-1101.
PilT	40.7 kDa, Pili protein **A. M. Spormann et al.** 2004. *J. Bacteriol.* 186.23.8096-8104.

Protein Expression and Purification



Induction of protein expression, BL21(DE3) pEC86 cells

P= pellet, **P/U**= pellet resuspended in 50mM Tris Cl with 8M Urea, pH8



Protein Purification

L= load

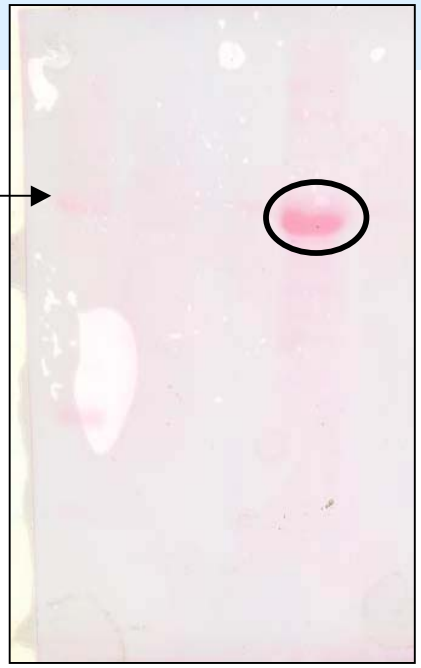
W= wash

E= eluate

Western Blot

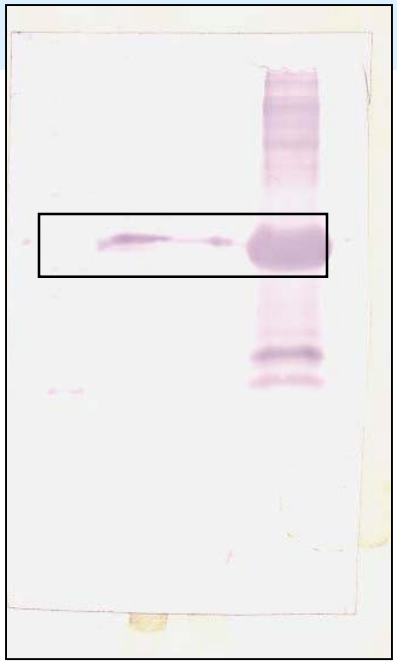
MW cells OmcS

50 kDa



Nitrocellulose membrane stained with Ponceau S

MW cells OmcS

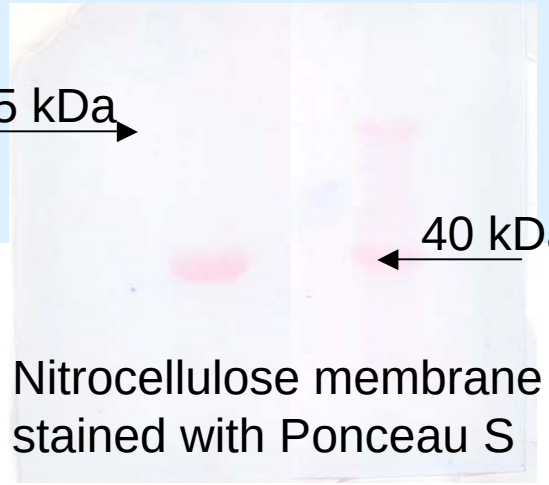


Western blot

MW OmcB cells PilT cells

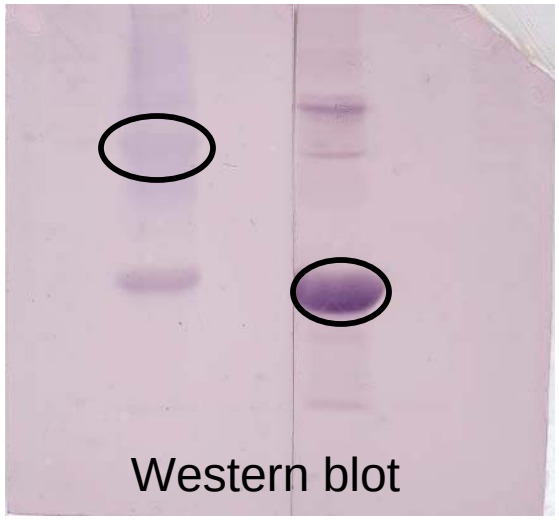
85 kDa

40 kDa



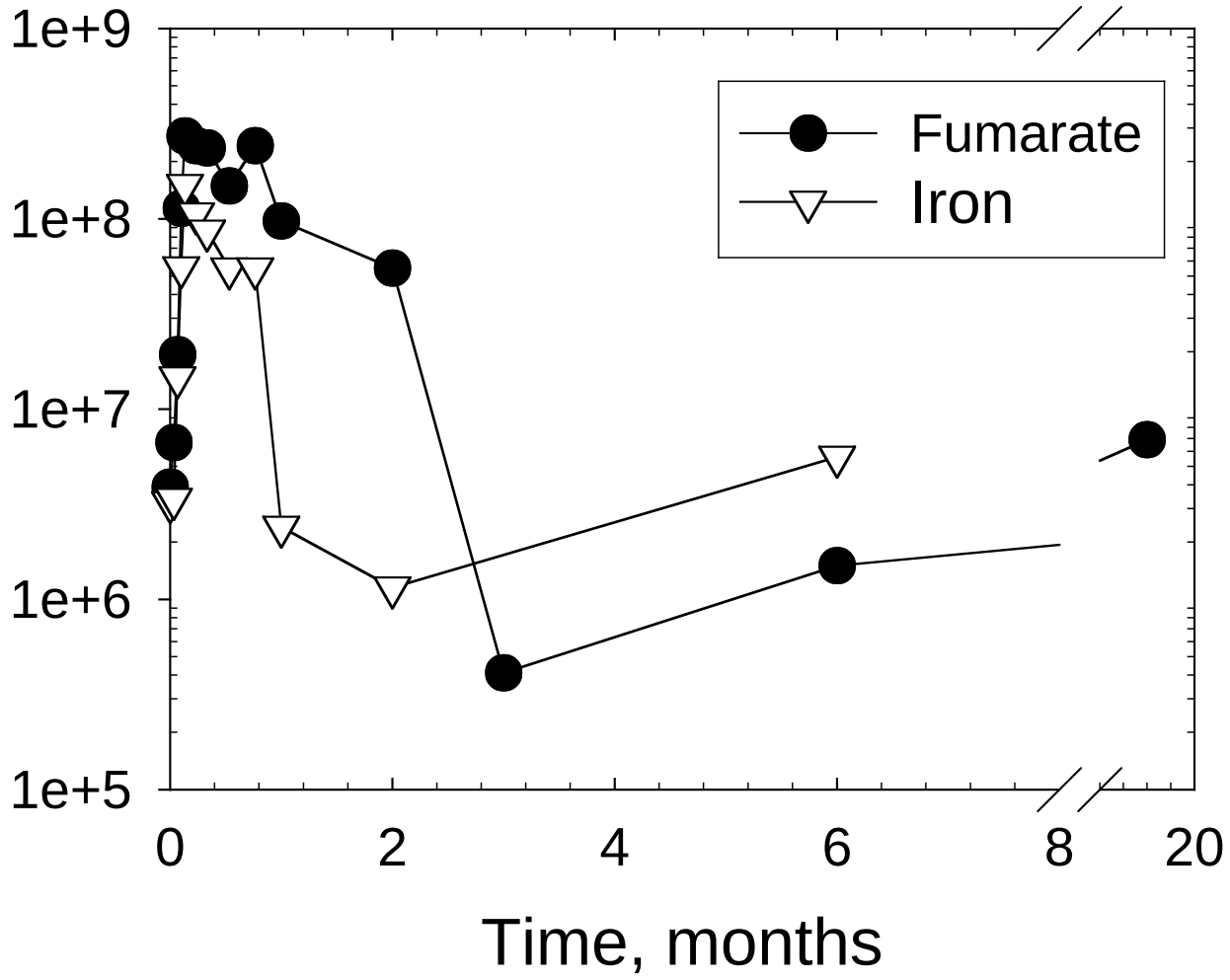
Nitrocellulose membrane stained with Ponceau S

MW OmcB cells PilT cells



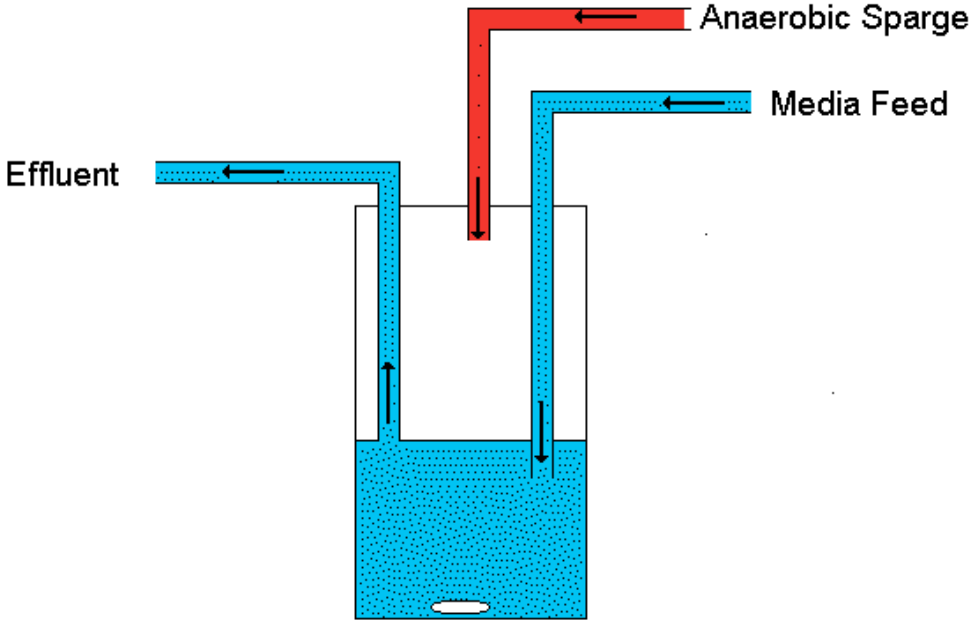
Western blot

Growth phases of G. *sulfurreducens*



- To determine maintenance energy, chemostat studies are planned. These studies will yield energy values for healthy growing cells
- Maintenance energy of starving cells can be determined by using a retentostat (developed to eliminate cell loss by dilution during zero growth)

Maintenance Energy and chemostats

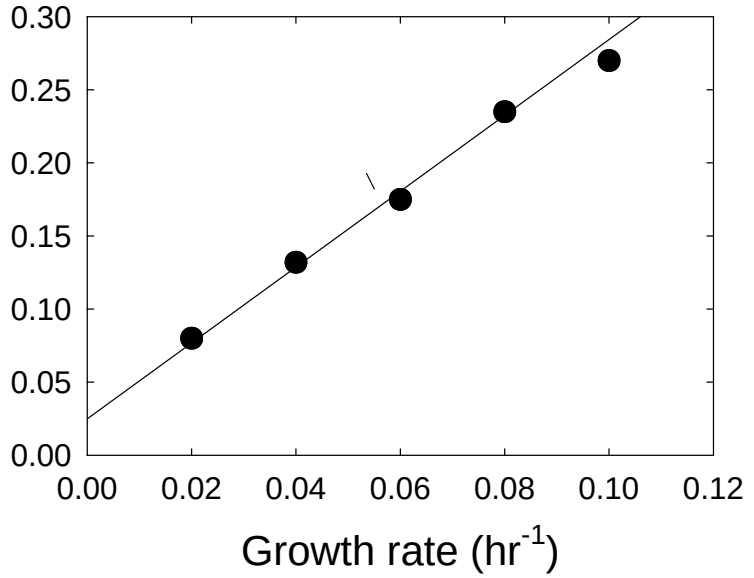


- This concept of maintenance energy was also observed independently by microbiologist working on growth yield using chemostats
- In a chemostat, micro-organisms are grown within a flow-through reactor under conditions of constant flow. The cell density in a reactor, *x*, is observed to be a function of the substrate concentration in the reactor.
- A linear relationship is often observed between the specific growth rate (μ) and the specific rate of substrate consumption (q). For such conditions, the cell yield, *Y*, can be defined:

$$Y = \frac{\mu}{q} = \frac{\frac{1}{\text{hr}}}{\frac{\text{mol of substrate utilized/hr}}{\text{gm dry weight of cells}}} = \frac{\text{gm dry weight}}{\text{mol of substrate utilized}}$$

Maintenance energy cont'd

- A plot of growth rate versus substrate utilization rate yields a straight line. However, at growth rates extrapolated to zero, in most cases, the substrate utilization rate, *q* typically exhibited a finite non-zero value.
- Researchers reasoned that this value is the so-called maintenance energy, energy utilized for house keeping functions.

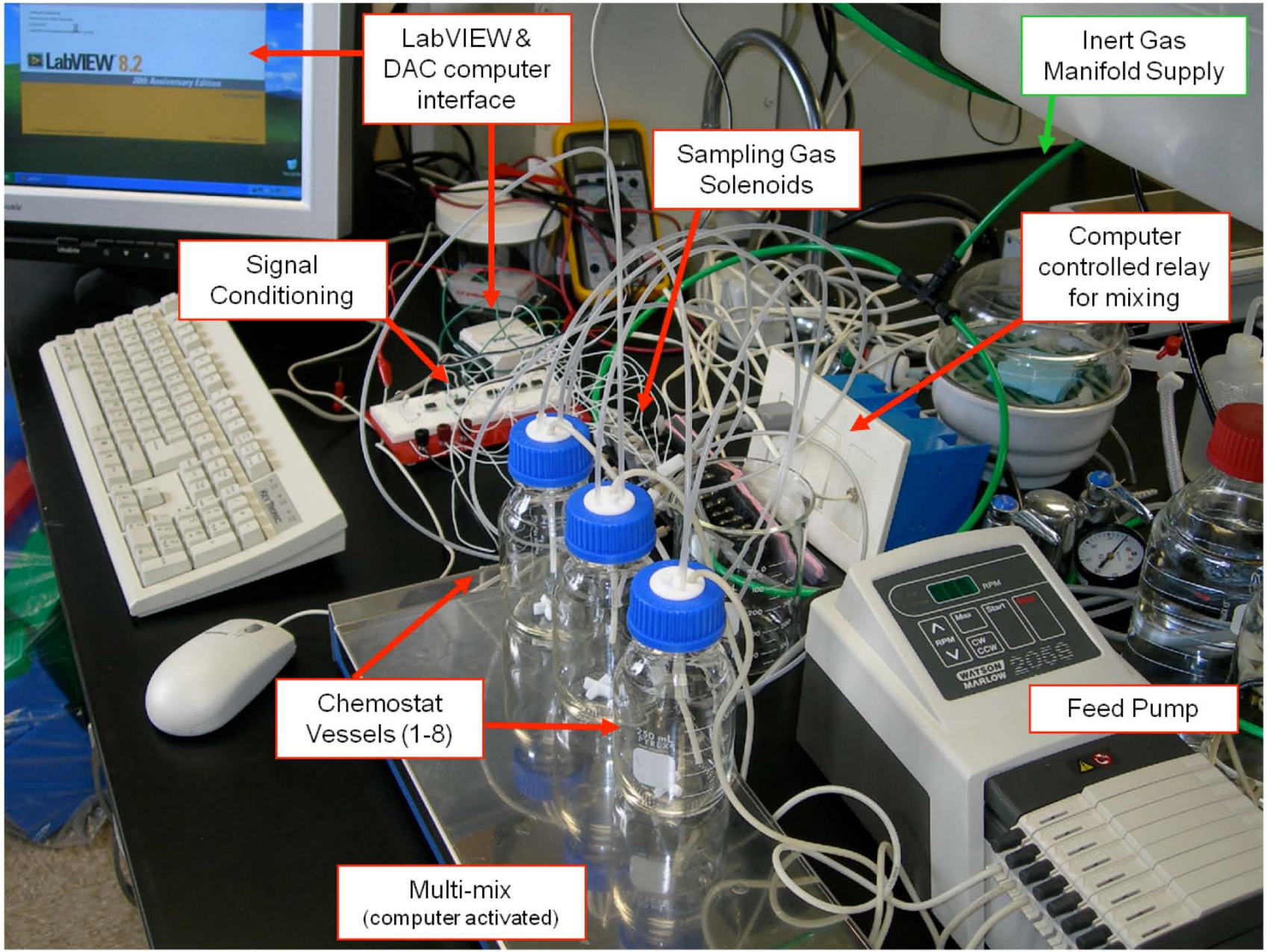


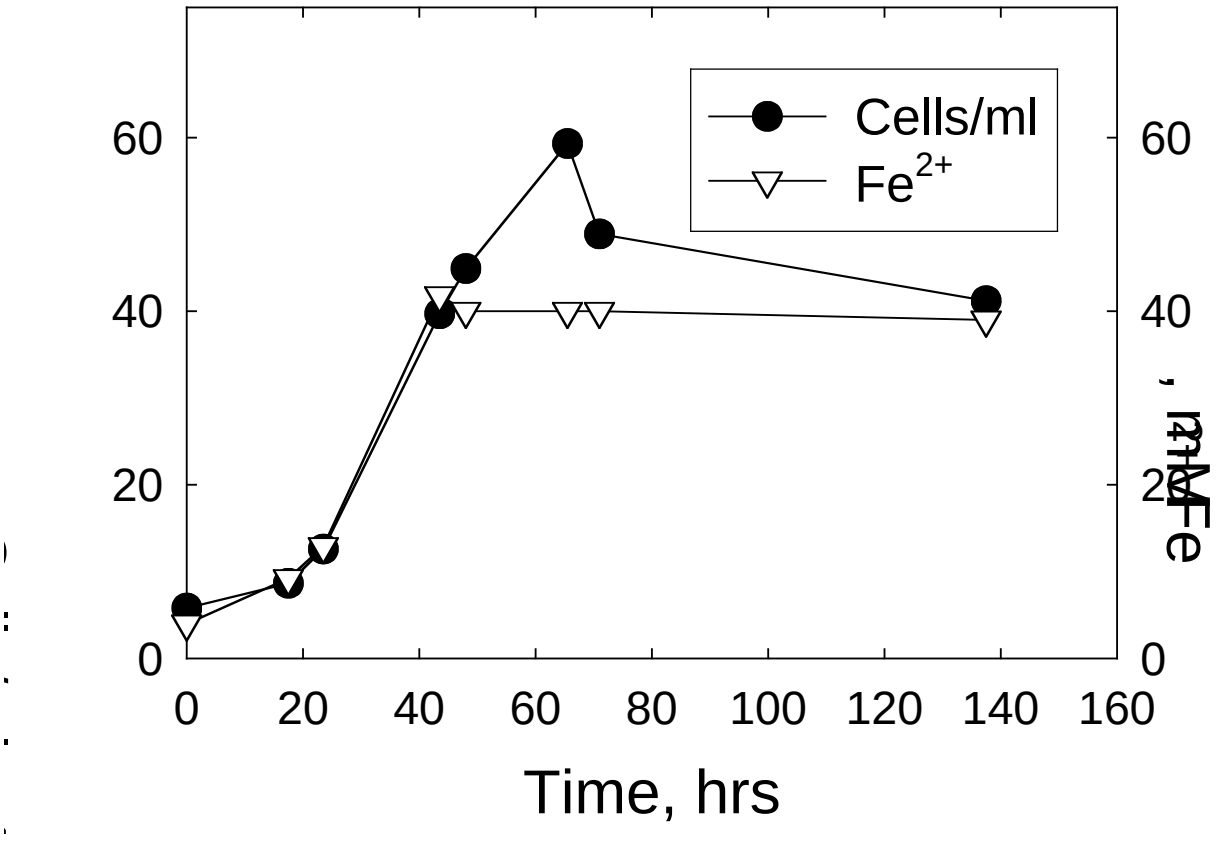
Perform chemostat studies to determine growth yields and maintenance energy and correlate these parameters with gene expression patterns to determine metabolic strategy.

Chemostat studies

- Compare growth yield and ME with iron and Mn terminal electron acceptors (soluble and insoluble forms)
- Use growth yield results as a window into the biochemistry
  - Variation in growth yield per mole of substrate utilized should be reflected in expression of new proteins

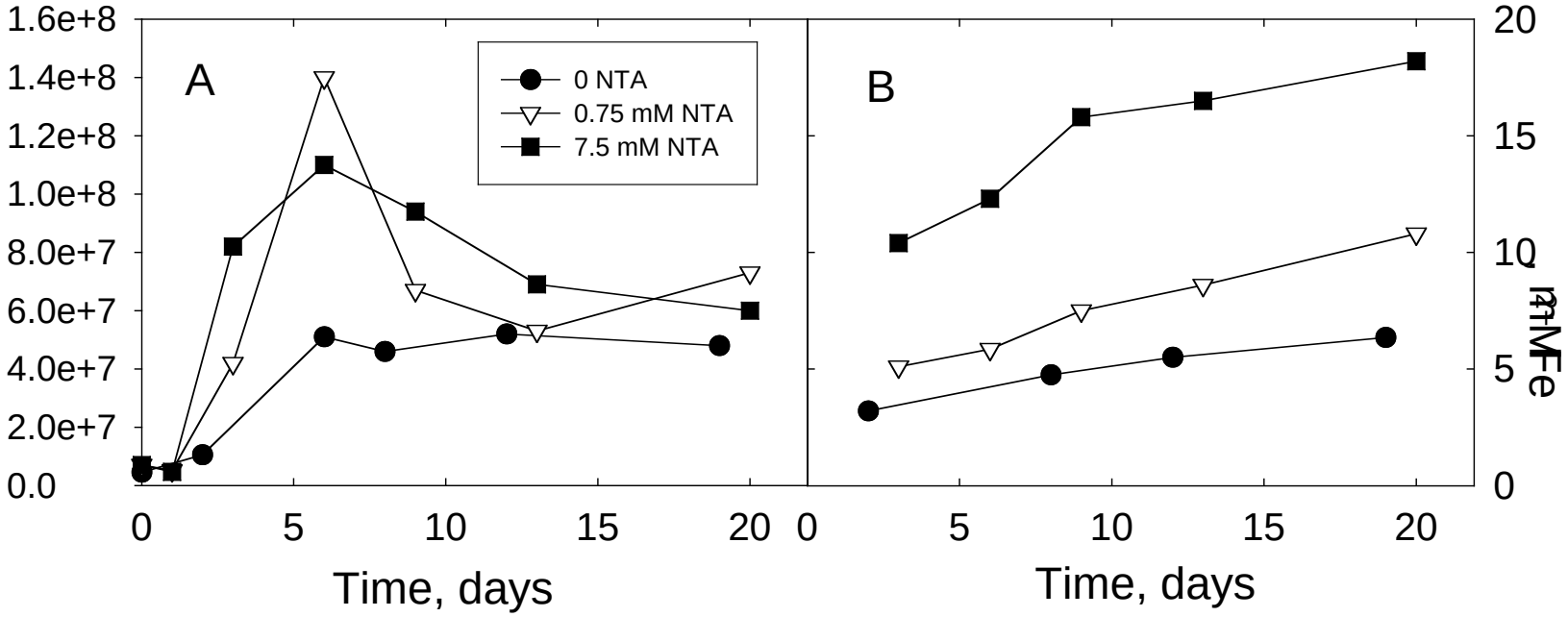
Chemostat Prototyping & Experimental Set-up



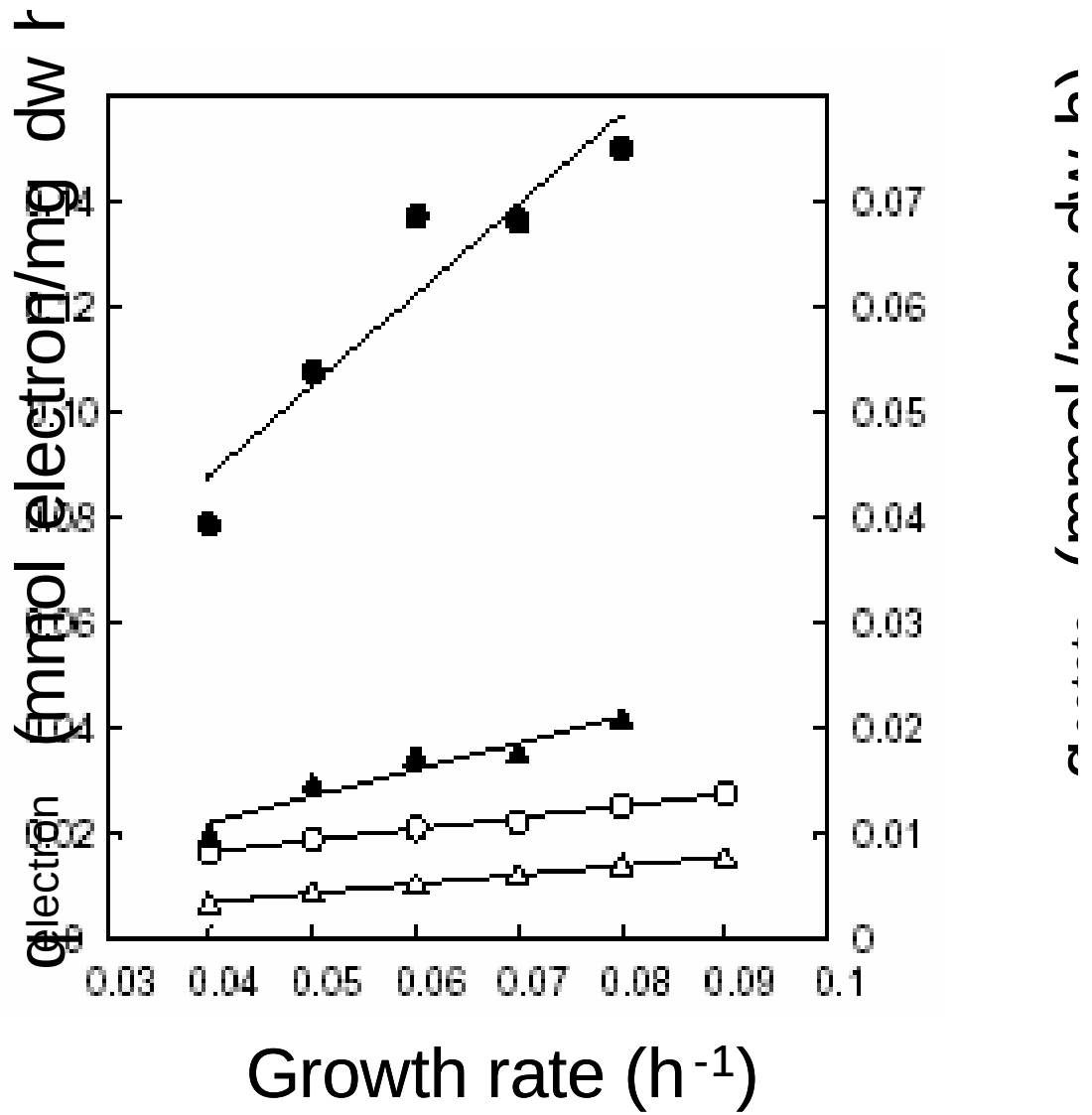


- Growth yield is approximately 1 x 10^{10} cells/mmol of Fe^{3+}

Growth of *G. sulfurreducens* on ferrihydride



- While growth rates are higher at increased NTA concentration, the growth yields are similar with and without NTA



Influence of growth rate on specific acetate consumption (triangles) and respiration (circles) of *Geobacter*. Cells were chemostat grown under acetate limitation with either fumarate (open symbols) or iron (closed symbols) as electron acceptor. Reproduced from Esteve-Nunez(2005) Environ. Microbiol. **7,** 641-648.

Important People

Susan Brantley
co-PI

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Third year graduate student

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Research Associate

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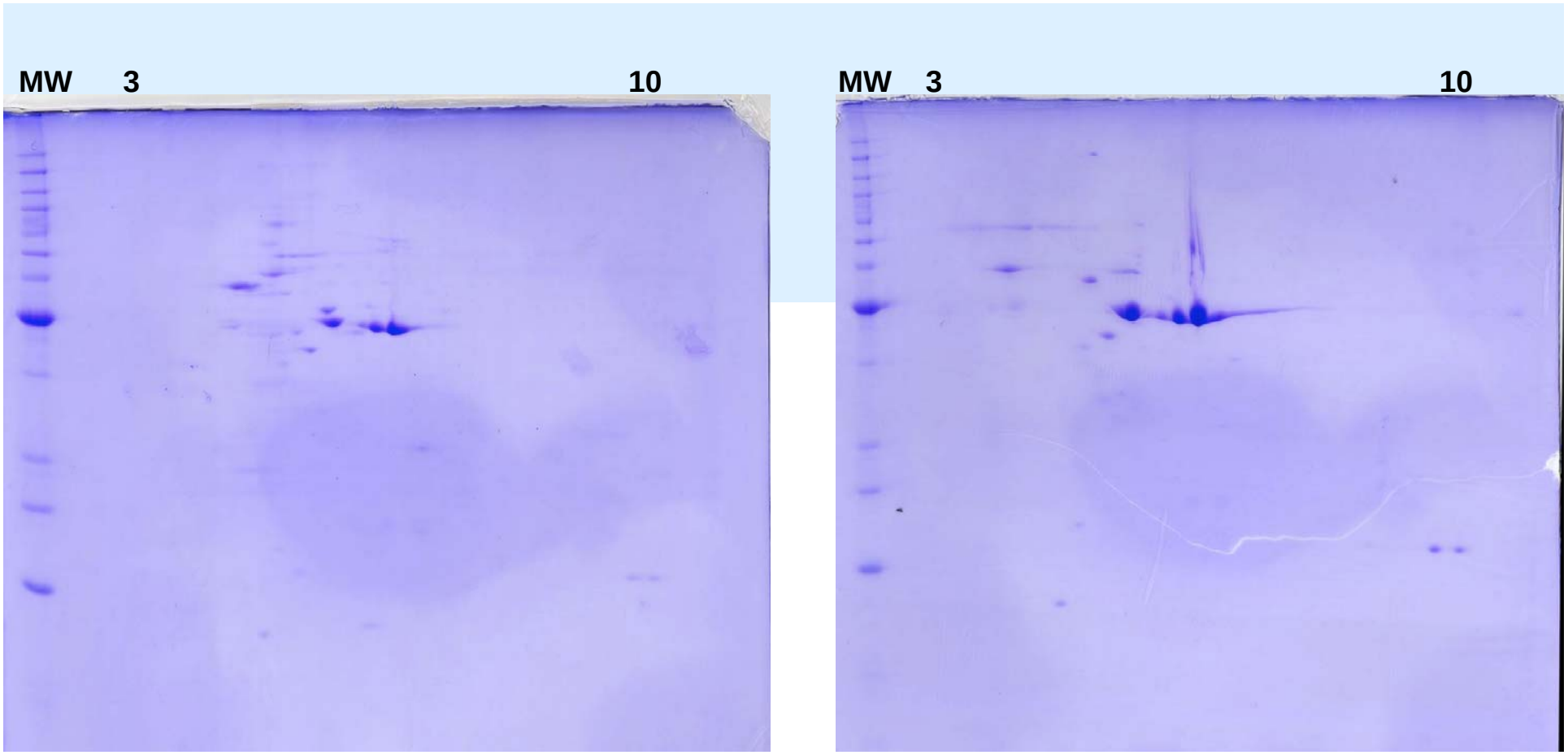
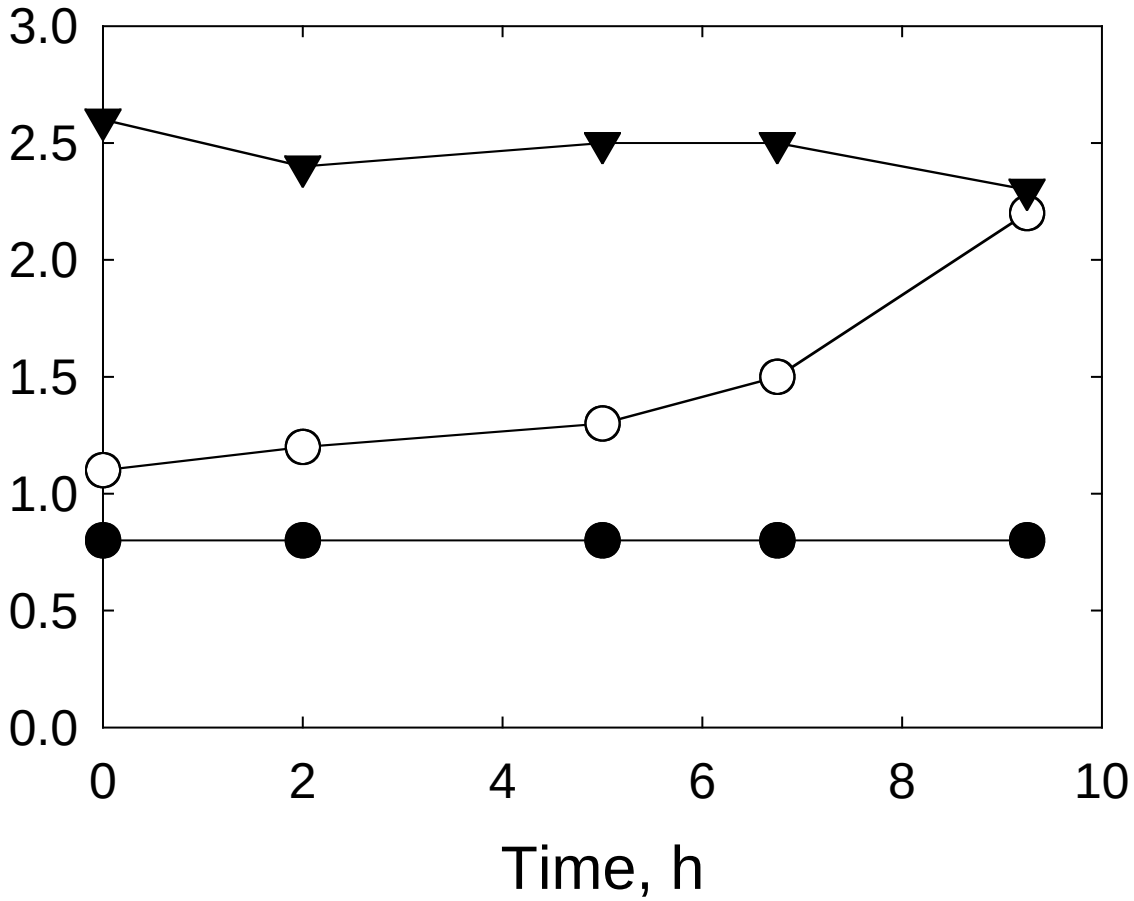
DOE-ERSP Program



TM

OM



- Resuscitation of *M. luteus*. Cells were grown in carbon-limited chemostat , harvest, and resuspended in lactate minimal media. At specified times, aliquots were removed and analyzed for total bacterial cell counts, (▼) viable cell (cfu) (○) and optical density (•). Reproduced from Kaprelyants and Kell (5).

To do list

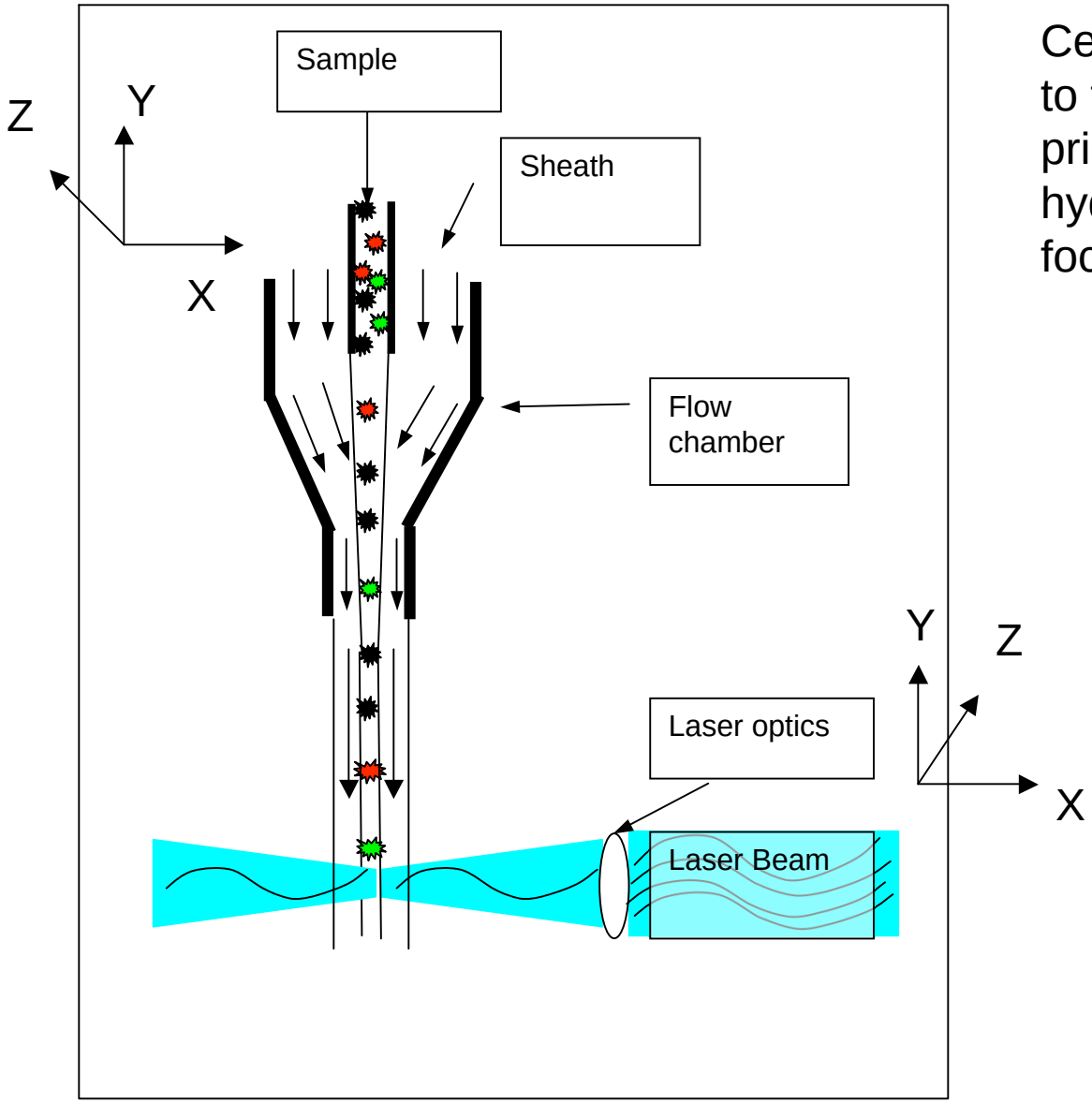
- DNA microarrays
- Chemostat studies

Chemostat studies

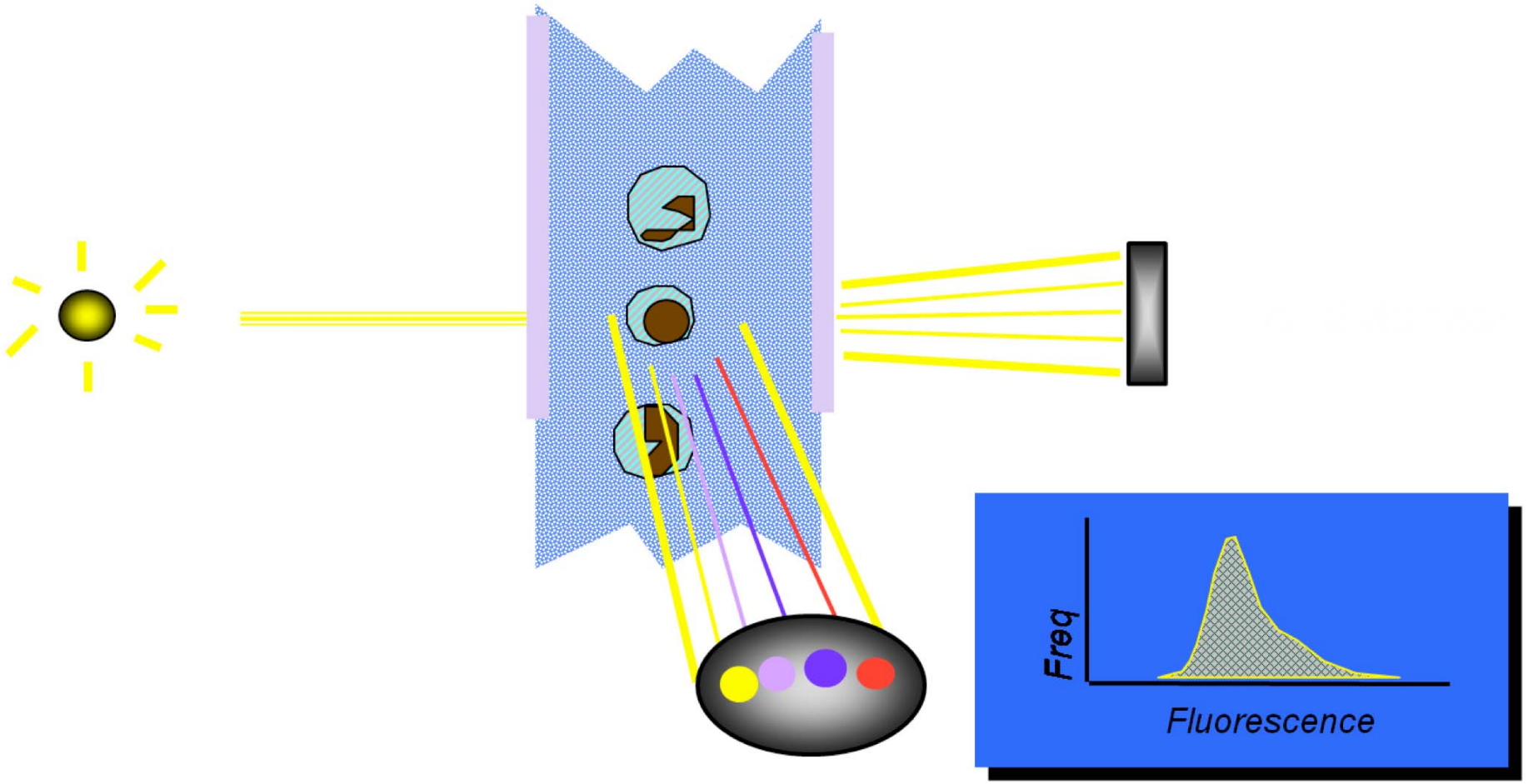
- Data thus far indicate that like *E. coli, Geobacter* has a prolonged starvation phase that may last for years with little consumption of energy.
- DNA microarray experiments will reveal metabolic strategies during these different phases.
- To determine the energy consumption profile (revealing growth yield and maintenance energy parameters),chemostat studies will be performed.
  - For starved cells, time and money permitting, retentostats will be constructed (no time to talk about this)

Global analysis yields no information on local enviroments

- Global gene expression analysis does not yield survival strategies that may be relevant in biofilm communities
- For such systems, specific probes are required due to stratification of the community



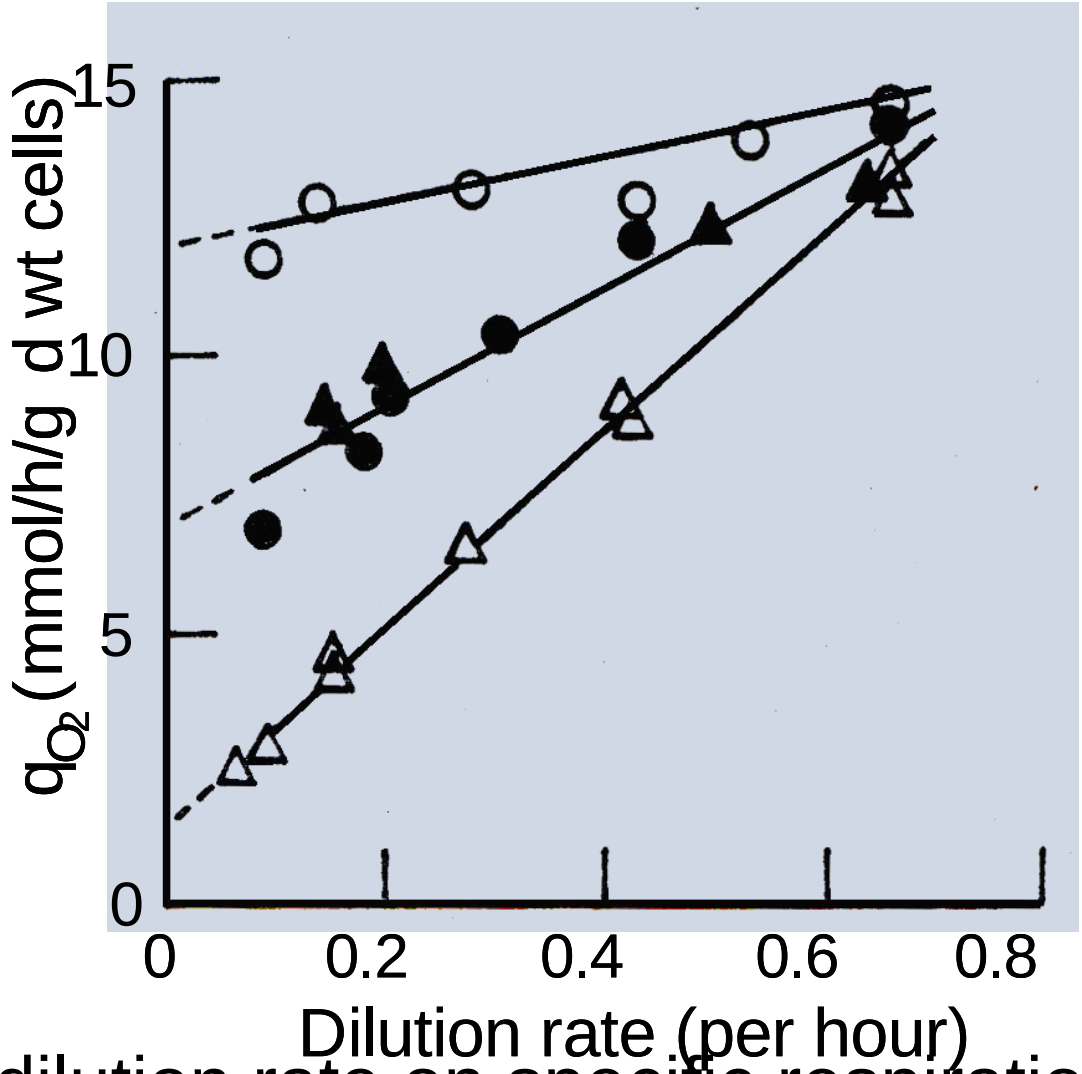
Cells are presented to the laser using principles of hydrodynamic focusing


Freq
Fluorescence

Flow cytometry: forward light scattering is indicator of cell size

- This behavior offers the combined benefit of decreasing the biomass and, correspondingly, the ME, while also offering a higher ratio of membrane surface area (across which substrate transport occurs) to cell mass. Indeed, the maximum volume-normalized rate of metabolism in a cell varies inversely with the square of the cell radius, and cells have been shown to respond to nutrient limitation by shrinking (34). Conceivably, cell-specific maintenance energies could approach zero if there were no minimum limits on biomass. Clearly, however, a minimal complement of genetic material, proteins, and membrane materials are needed to constitute a functional living unit. A National Research Council panel that convened to address minimum size limits for viable cells conservatively estimated that a cell radius of about 100 nm is minimally necessary to constitute the simplest of living cells (35). This size limit corresponds directly to a minimum limit on the cell-specific ME demand that can be achieved by virtue of cell shrinkage.

Maintenance Energy and Growth Yield



Influence of dilution rate on specific respiration rate of chemostat cultures of *Klebsiella aerogenes* in minimal medium plus glucose: (△) glucose limited, (▲) ammonia limited, (●) sulphate limited, and (○) phosphate limited. Reproduced from (4).

- The dormant forms of bacteria observed in chemostat cultures at low dilution rates are smaller and similar in size to bacteria isolated from oligotrophic environments (6). The smaller bacteria are also referred to as ultramicrobacteria, terminology of Morita (13). Environmental dormant forms of vegetative non-sporulating bacteria were first argued to exist when researchers found that in most natural microbial environments, only a small fraction of the microbes could be cultivated on agar plates (23). Large differences are consistently reported between results of plating counts (colony forming units, cfu), defined as viable and total direct microscopic counts (fluorochrome staining of physically-intact bacteria (24). This discrepancy has been observed in bacteria isolated from soil (13, 25) and water environments (13, 26). Researchers have proposed that such physically-intact but nonculturable bacteria represent dormant forms of non-sporulating bacteria which are formed in adaptation to low nutrient conditions. These putative forms of dormant bacteria have also been referred to as “viable but non culturable” (VBNC) forms of gram negative bacteria. These forms, again, are not revealed by agar plating but can be monitored in a starved bacteria population by their metabolic activity as shown by direct viable count (27).
- If dormancy is a strategy used by many bacteria to survive prolonged nutrient deprivation, then as indicated by Kaprelyants et al. (2), dormancy must be a reversible state. While resuscitation of ultramicrobacteria (or resuscitation from dormancy) has been reported, such studies are few in number. These studies are also viewed skeptically since it is difficult to discriminate between resuscitation of truly dormant cells or of resuscitation of a limited number of nondormant, “viable” cells in the starved population. MacDonell and Hood (28) were able to recover ultramicrobacteria from estuarine waters using dilute nutrient broth (over a limited range of nutrient concentrations). Consistent with the dormancy theory, the recovery of cells and adaptation to nutrient-rich agar was accompanied by an increase in size (from 0.5 to 2-2.5 mm). However, the percentage of ultramicrobacteria that were able to be resuscitated was not determined. Morita concluded that most ultramicrobacteria are not able to multiply simply because proper conditions for their resuscitation have yet to be determined (13). An opposing view to the ultramicrobacteria-dormancy hypothesis suggests that ultramicrobacteria are not small forms of the typical cells but are cells of novel genera and species that happen to be small and that they cannot be reverted back to normal-sized cells.

Energy Utilization

Biological Energy Quantum

$$\Delta G_{min} = \frac{\Delta G_{ATP \rightarrow ADP}}{n \cdot f}$$

- To survive, all life requires a minimum driving force to synthesize ATP from ADP (sometimes called the biological energy quantum, BEQ;(12, 16)). Because many organisms store energy by generating a transmembrane ion potential (17), this minimum driving force is dependent upon the number of ions (*n*) that must be translocated across the membrane to synthesize ATP from ADP. Schink and Stams (16) defined the minimum free energy that must be available in a given environment to sustain life as the Biological Energy Quantum:

  where *f* is the thermodynamic efficiency factor. The BEQ can be thought of as the voltage or potential required to maintain life (the ΔG of the environmental reaction catalyzed by the organism).

Growth yield studies

- The conceptual realization that micro-organisms have measurable energy requirements arose from growth yield studies, first in batch reactors and then with chemostats. In a chemostat, micro-organisms are grown within a flow-through reactor under conditions of constant flow (*Q*). The cell density in a reactor, *x*, is observed to be a function of the substrate concentration in the reactor, *s*. For example, a relationship is often observed between the specific growth rate (*m = 1/x dx/dt*) and the specific rate of substrate consumption (*q = -1/x ds/dt*). For such conditions, the cell yield, *Y*, can be defined:

$$Y = \frac{\mu}{q} = \frac{\frac{1}{\text{hr}}}{\frac{\text{mol of substrate utilized/hr}}{\text{gm dry weight of cells}}} = \frac{\text{gm dry weight}}{\text{mol of substrate utilized}}$$

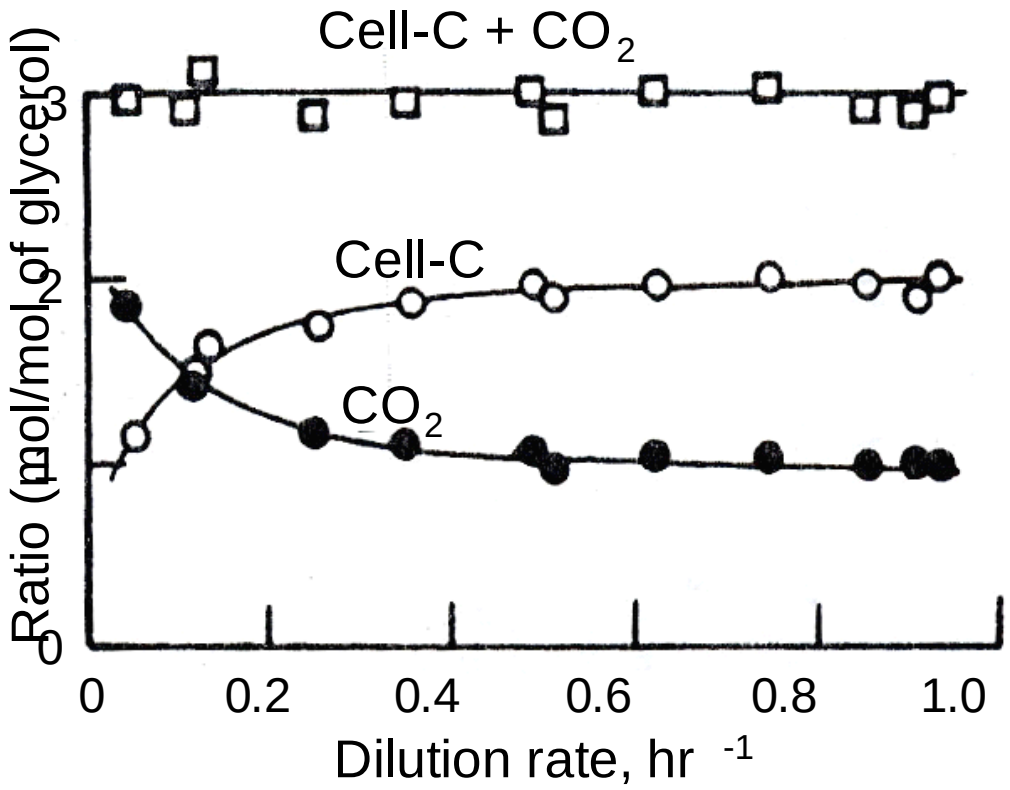
$$D = Q / V$$

Chemostats

- When chemostats are run under steady state conditions such that cell numbers remain constant in the reactor, then the specific growth rate (μ) is equivalent to the dilution rate (*D* and μ both have units of per unit time, hr-1). For example, a *D* value of 0.1 hr-1 equals the addition of 100 ml to a 1 l chemostat per hr and can be thought of as the specific growth rate for the chemostat under steady state conditions. The rate of substrate utilization, *q*, has units of moles substrate consumed per unit time per gm of cell (mol of substrate consumed/hr/gm dry weight of cells).

- Tempest and Neijssel (18) reasoned that if the yield (*Y*) was a constant, then *q* should vary linearly with the dilution rate and Y should equal zero at zero dilution rate ($q = D/Y$). In fact, a plot of *q versus D* (at values above 0.1 hr-1) is observed to be generally linear (at high D values) and the slope is interpreted to equal $1/Y$. In most cases, however, upon extrapolation to zero dilution rate, *q* typically demonstrates a finite non-zero value as seen in next slide.

- Energetically speaking, as the specific growth rate decreases, a larger fraction of the influent carbon substrate is catabolized to CO2 and less is assimilated into biomass. To explain these findings, it has been argued that some of the carbon substrate that is consumed is portioned into growth-related and non-growth-related functions. Thus, this concept of energy usage for non-growth functions, originating from mathematical modeling of chemostat fermentation data, is identical to the concept used to describe substrate fluxes under survival or stationary mode (Fig. 1). In both cases, there is no net cell growth but there is a metabolic rate of energy expenditure: by definition, this energy is used for maintenance.



- Influence of dilution rate on distribution of substrate carbon between cells and CO2 by *Klebsiella aerogenes*. Reproduced from (2).

Table 2. rpoS-Controlled Genes in *E. coli*.

Gene/operon	Gene product	Function
bola	Regulatory protein	Morphogen, control of PBP6 synthesis
appY	Regulatory protein	Control of *cyxAB-appa* expression
Dps	DNA binding protein	DNA protection; control of gene expression
cyxAB-appA	Third cytochrome oxidase and acid phosphatase	?
csgA	Curli subunit protein	Fibrinogen binding
treA	Periplasmic trehalase	Trehalose uptake in high osmolarity medium
osmB	Lipoprotein	?
katE	Catalase HPII	H_2O_2 detoxification
xthA	Exonuclease III	DNA repair
otsBA	Trehalose-6-phosphatase	Trehalose synthesis, osmoprotection, thermotolerance
glgS	GlgS	Glycogen synthesis
csi-5	Periplasmic protein	?
pex genes	Six polypeptides	?
mcc	Several proteins	Synthesis/secretion of microcin C7