Toward Understanding Dynamic Microbiological Responses to Chemical Stress: Elucidating Biomarkers for Use in Upset Early Warning Systems

Metropolitan Water Reclamation District of Greater Chicago
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Nancy G. Love
nlove@vt.edu; 540-231-3980
Charles E. Via Dept. of Civil & Environmental Engineering
Adjunct, Department of Biological Sciences
Virginia Polytechnic Institute and State University
Blacksburg, Virginia, USA

Acknowledgements

Collaborators

Students:
Dr. Charles Bott
Dr. Inês Henriques
Ms. Kaoru Ikuma
Dr. Rick Kelly II
Dr. Joy Fraga Muller
Mr. Bob Wimmer

Post-Docs:
Dr. Kartik Chandran
Dr. Jane Duncan
Dr. Kathy Terlesky

Utilities and National Labs:
Dr. Laurie Locascio, NIST
Blacksburg-VPI WWTP

Faculty:
Dr. Diana Aga, Chemistry, Univ Buffalo
Dr. Brian Love, Materials Science, Virginia Tech
Dr. Pedro Mendes, Virginia Bioinformatics Institute
Dr. Bev Rzigalinski, VA College of Osteopathic Medicine
Dr. Ann Stevens, Biological Sciences, Virginia Tech
My 30-year research model

Understand Complex Biological Systems

Apply technologies to complex biological systems

Use basic observations to identify appropriate targets for technology development

Develop Technologies that Improve the Function of Complex Biological Systems
We can use a range of analytical tools to evaluate community structure and function

Our functional studies have focused on chemical stress of activated sludge systems
Our functional studies have focused on chemical stress of activated sludge systems
Survey indicates that stressed conditions at plants are common

Over 90% of respondents had experienced process upset.

83% of respondents thought that development of new sensor technologies was important or very important.

“Our monitoring technologies are primitive, at best.”

Ideally, our systems should be well instrumented to provide real-time information for improved operation and control that prevents upsets
Traditional indicators of activated sludge process stress are valuable, but provide limited information about corrective action.

We proposed a unifying concept for understanding the role of microbial stress in environmental monitoring.

**SOURCE**
Perturbation by Chemical(s)

**CAUSE**
Biochemical Physical Chemical

**EFFECT**
Poor Ecosystem Performance or Function
We proposed a unifying concept for understanding the role of microbial stress in environmental monitoring.

**Thesis:** An understanding of Source-Cause-Effect relationships is needed to enable:
- proactive design, operation and management
- development of “intelligent” sensors
- development of rapid prevention/corrective action strategies linked with decision support systems
My 30-year research model

Understand Complex Biological Systems

Apply technologies to complex biological systems

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Develop Technologies that Improve the Function of Complex Biological Systems

Hypothesis: Activated sludge bacteria rapidly adapt to chemical stressors, and the adaptation response influences system-level performance

Chemical Stressor

Cis/trans isomerization (immediate to minutes)

Gated efflux systems (immediate to minutes)

Catabolic Enzymes (minutes to days)

Stress proteins (seconds to minutes)

mRNA stability (minutes to hours)
Hypothesis: Activated sludge bacteria rapidly adapt to chemical stressors, and the adaptation response influences system-level performance.

We can use a range of analytical tools to evaluate community structure and function.
We focused on GroEL (Hsp60), a heat shock protein known to upregulate in response to chemical stressors and starvation.


Detecting stress proteins in communities
SDS-PAGE

1. Unstressed control
2. Heat Shock
3. Heat + Chloramphenicol

Duncan, Terlesky, Bott and Love, 2000, LAM
Detecting stress proteins in communities

SDS-PAGE

1. Unstressed control
2. Heat Shock
3. Heat + Chloramphenicol

Duncan, Terlesky, Bott and Love, 2000, LAM

Western blot (Anti-Hsp60)
Lessons learned from the protein work:

- It is possible to detect differential expression of a specific protein fingerprint in fresh activated sludge cultures exposed to toxins using traditional biochemical methods.

- Is the effort worth the climb using these methods?
Deflocculation of activated sludge due to toxic shock with ~IC$_{25}$ concentrations

Control  NEM  CDNB

Lessons learned from the protein work:

- It is possible to detect differential expression of a specific protein fingerprint in fresh activated sludge cultures exposed to toxins using traditional biochemical methods.
- Is the effort worth the climb using these methods?
- It is not clear that Hsp60 is a primary causal mechanism that explains the prevalent deflocculation observed.

An epiphany at the 2000 Gordon Research Conference on Microbial Stress Responses

Hypothesis: Activated sludge bacteria rapidly adapt to chemical stressors, and the adaptation response influences system-level performance.

- Cis/trans isomerization (immediate to minutes)
- Gated efflux systems (immediate to minutes)
- Catabolic Enzymes (minutes to days)
- Stress proteins (seconds to minutes)
- mRNA stability (minutes to hours)
Hypothesis: the Glutathione Gated Potassium Efflux (GGKE) response causes deflocculation by thiol-reactive chemicals

Oxidative Chemical +

K+ Uptake Blocked

K+ + GSH → GS-SG (scavenging)

damage

Protection

H+ (acidification)

DNA

Protein

Bakker and Mangerich (1982); Meury and Robin (1990); Ferguson and Booth (1993 – 1998)

Hypothesis: High concentrations of GGKE-generated intrafloc K+ cations cause weak floc structure that leads to deflocculation

EPS

Bacteria

We can use a range of analytical tools to evaluate community structure and function.
**K⁺ effluxes from flocs to liquid**

<table>
<thead>
<tr>
<th>Sample and Time</th>
<th>CONTROL</th>
<th>NEM STRESSED - 50 mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Effluent VSS</strong></td>
<td>3.4 ± 0.5 mg/L</td>
<td>46 ± 2.1 mg/L</td>
</tr>
<tr>
<td><strong>K⁺ Concentration (mg/L of original sample volume)</strong></td>
<td>0</td>
<td>35</td>
</tr>
<tr>
<td>t=0</td>
<td>t=5 hours</td>
<td>t=0</td>
</tr>
</tbody>
</table>

- Soluble K⁺
- Floc Associated K⁺
- Calculated Total K⁺
- Measured Total K⁺


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**K⁺ efflux is reversible in pure culture**

*(Novosphingomonas capsulatum)*

<table>
<thead>
<tr>
<th>NEM (both)</th>
<th>DTT (both)</th>
<th>NEM (both)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mg/L (0.40 mM)</td>
<td>185 mg/L (1.2 mM)</td>
<td>450 mg/L (3.6 mM)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ISE Soluble K⁺ Concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>42</td>
</tr>
</tbody>
</table>

| Time (minutes) | 0 | 10 | 20 | 30 | 40 | 50 | 60 | 70 | 80 | 90 | 100 | 110 | 120 | 130 |

- Control/DTT/NEM
- NEM/DTT/NEM

K⁺ efflux is reversible with activated sludge

![Graph showing efflux of K⁺ over time with different stressors](image)

<table>
<thead>
<tr>
<th>Stressor</th>
<th>Concentration (mg/L)</th>
<th>Molar Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEM</td>
<td>50 mg/L (0.40 mM)</td>
<td>NEM/DTT/NEM</td>
</tr>
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Conclusion: GGKE is a causal mechanism for deflocculation

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<th>SOURCE</th>
<th>CAUSE</th>
<th>EFFECT</th>
</tr>
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<tbody>
<tr>
<td>Electrophilic Chemical Stressor</td>
<td>Potassium Efflux from Floc</td>
<td>Deflocculated biomass</td>
</tr>
</tbody>
</table>
My 30-year research model

Understand Complex Biological Systems

Apply technologies to complex biological systems

Use basic observations to identify appropriate targets for technology development

Develop Technologies that Improve the Function of Complex Biological Systems

We are converting source-cause-effect knowledge into technology development

Oxidative Chemical Stressor

Potassium Efflux from Floc

Deflocculated biomass

Early Upset Detection or Prevention

GGKE Biosensor

K⁺ K⁺ K⁺ K⁺
Is a glutathione-based, bacterial-based sentinel biosensor best for environmental health applications?

**USACEHR**

Collaboration with Dr. Beverly Rzigalinski, VCOM Pharmacologist

Bacterial and rat brain cells show similar dose-dependent damage in response to NEM

Combined dose response curve: NEM
We proposed a unifying concept for understanding the role of microbial stress in environmental monitoring

**SOURCE**
Perturbation by Chemical(s)

**CAUSE**
- Biochemical
- Physical
- Chemical

**EFFECT**
- Poor Ecosystem Performance or Function

Thesis: An understanding of Source-Cause-Effect relationships is needed to enable:
- proactive design, operation and management
- development of “intelligent” sensors
- development of rapid prevention/corrective action strategies linked with decision support systems
Summary of process effects for a nitrifying biomass using a pseudo-quantitative scale

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Observed Process Effect Relative to Control</th>
<th>Hypothesized Causal Mechanism</th>
<th>Ammonium ~ 400 mg/L</th>
<th>Cadmium</th>
<th>CONB</th>
<th>Cyanide</th>
<th>DNP</th>
<th>Octanol</th>
<th>pH 11</th>
</tr>
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<tr>
<td>Effluent TSS/VSS</td>
<td>Increase in effluent TSS/VSS</td>
<td>Flocs deteriorate and cause smaller, less dense particles (deflocculation)</td>
<td>↓↓↓↓</td>
<td>↓↓↓</td>
<td>0</td>
<td>↓↓</td>
<td>+</td>
<td>↓↓↓↓</td>
<td></td>
</tr>
<tr>
<td>Effluent COD</td>
<td>Decrease in COD removal</td>
<td>Inhibition of metabolic pathways</td>
<td>0</td>
<td>↓↓↓</td>
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<td>+</td>
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<tr>
<td>SOUR</td>
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<td>Inhibition of catabolic pathways</td>
<td>0</td>
<td>↓↓↓</td>
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<td>↓↓/++</td>
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<td>X</td>
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<tr>
<td>Inorganic N effluent conc. and NGR</td>
<td>Nitrification inhibition</td>
<td>Varies</td>
<td>↓</td>
<td>↓↓↓</td>
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<td>↓↓</td>
<td>↓</td>
<td>↓↓↓↓</td>
<td></td>
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<tr>
<td>SVI</td>
<td>Increase in SVI</td>
<td>Poor biosolids compression, or settleability</td>
<td>0</td>
<td>++</td>
<td>+</td>
<td>↓</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>CST</td>
<td>Increase in CST</td>
<td>Retention of bound water, leading to poor dewaterability</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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*The qualitative scale reflects the intensity of the effect for IC₅₀ shocked reactors and the indicated NH₃ and pH shock level, in comparison to a negative control. The intensity scale ranges from ↓↓↓↓ (most intense process deterioration effect), 0 (no effect), and ++++ (most intense process improvement effect). X means inconclusive results.*

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Effluent TSS and COD removal deterioration almost always correlated

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An epiphany at the 2004 Gordon Research Conference on Environmental Chemistry

(Remember: Ardem and Lockett were chemists!)

We can use a range of analytical tools to evaluate community structure and function

Dr. Ines Henriques
Dr. Diana Aga
Dr. Pedro Mendes
Can metabolomics’ techniques be applied to complex environmental matrices?

Fingerprint the effluent COD from chemically-stressed SBRs

Sampling at:
- < 5 mins
- 45 mins
- 3 hours
- 5 hours

From: Dunn and Ellis (2005). *Trends in Analytical Chemistry*


Virginia Tech
Fingerprint the effluent COD from chemically-stressed SBRs

Sampling at:
< 5 mins
45 mins
3 hours
5 hours

Chromatograms from stressed and control reactors were complex but differences could still be visually identified.
The intensity of the peaks from shocked reactor samples increased in a time-dependent manner.

NEM immediately after the shock

NEM 5 hrs after the shock
Mass spectra data were used to run statistical analysis.

**Chromatogram VS. Mass spectrum**

Peak retention times are not the same for all the samples (alignment problems)

m/z ratios provide a global picture of the substances present in the sample

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**Principal Component Analysis (PCA)**
- Unsupervised method
- Maximizes variance between samples
- Does not allow identification of differences between samples

**Multiple Discriminant Function Analysis (DFA)**
- Supervised method
- Maximizes variance between classes and minimizes variance within a class
- DFA with GA, allows identification of main differences (m/z) between classes
Mass specta data were used to perform Multiple Discriminant Function Analysis with Genetic Algorithm Variable Selection (GA-DFA). Multiple runs of GA-DFA allow identification of the m/z ratios responsible for the differences between the samples.
Multiple runs of GA-DFA allow identification of the m/z ratios responsible for the differences between the samples.

**Example**

m/z ratios 828 and 1306 are discriminating biomarkers that reveal the presence of inhibiting levels of 2,4-DNP.

We have correlated 2,4 DNP biomarkers from activated sludge with *Pseudomonas aeruginosa* exudates.
How can this be applied or used?

- Key m/z fingerprints from mass spectrometry data

- Deploy mini mass spectrometers for real time field monitoring with micro total analysis systems (μTAS) ([http://aemc.jpl.nasa.gov/activities/mms.cfm](http://aemc.jpl.nasa.gov/activities/mms.cfm))

- Develop and deploy antibody microarrays that target key fingerprint biomolecules

Identify fingerprints and compare to those in sequenced pure cultures (true metabolomic studies)

Ideally, our systems should be well instrumented to provide real-time information for improved operation and control that prevents upsets

- UPSTREAM
- IN PROCESS
- Instrumentation output
- automated responses
- operator actions
- operator decisions
Hypothesis: Stress response-focused sensors will provide useful functional information and will be applicable in a range of environments.

Is there a link between stress and antibiotic resistance?
The Role of Multidrug Efflux Pumps in the Stress Response of *Pseudomonas aeruginosa* to Organic Contamination

J. Fraga Muller, Ph.D. Dissertation

Muller, Stevens, Craig, and Love. In press. *Applied and Environmental Microbiology*.
Muller, Stevens and Love. In review. *Science*.

We can use a range of analytical tools to evaluate community structure and function

- community composition (structure)
- gene expression (function)
- metabolic footprinting (function)
- protein upregulation (function)

Dr. Jocelyn Fraga Muller
Dr. Ann Stevens
A *Pseudomonas aeruginosa* chemostat was sampled over time for changes in cell physiology & gene expression after perturbation with pentachlorophenol (PCP).

60 transcripts increased, with transport the most represented functional class.
60 transcripts increased, with transport the most represented functional class

<table>
<thead>
<tr>
<th>Functional Category</th>
<th>% of Total Transcripts Increasing</th>
</tr>
</thead>
<tbody>
<tr>
<td>transport/membrane proteins</td>
<td>30</td>
</tr>
<tr>
<td>antibiotic resistance</td>
<td>0</td>
</tr>
<tr>
<td>metabolism</td>
<td>10</td>
</tr>
<tr>
<td>adaption, protection</td>
<td>0</td>
</tr>
<tr>
<td>other</td>
<td>10</td>
</tr>
<tr>
<td>hypothetical</td>
<td>40</td>
</tr>
</tbody>
</table>

**DNA Microarray**

**P. aeruginosa** upregulates multi-drug efflux pumps and demonstrates an antibiotic resistance phenotype in the presence of PCP

13 hour exposure to PCP
“Antibiotic resistance has been called one of the world’s most pressing public health problems”

http://www.cdc.gov/drugresistance/community/qa.htm

Environmental factors contributing to antibiotic resistance:

- **Overuse of antibiotics (human and animal)**

  Occurrence and Relatedness of Vancomycin-Resistant Enterococci in Animals, Humans, and the Environment in Different European Regions

  Inger Kriisa,1,* Ales Pernin,1 Martin Fic,1 Christina Gesels,2 Laci G. Barnum,2 Annett R. Hamböck,4 Xavier Vilanova,5 Alfred Moors4,5; Eric Etienne,6 Jonathan Coplan,4 Lucin Dingemanse,7 Tommaso A. Novella,8 Miguel A. Moreno,8 and Roland Nolty

- **Natural soil community structure**

  Sampling the Antibiotic Resistome

  Vanessa M. D’Costa,1 Katherine M. McGraw,1 Donald W. Hughes,2 Gerard D. Wright1,*

- **Anthropogenic contamination**

  Does pollution in the environment contribute to antibiotic resistance?

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**Toward Understanding Dynamic Microbiological Responses to Chemical Stress:**

Elucidating Biomarkers for Use in Upset Early Warning Systems

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nlove@vt.edu; 540-231-3980

Charles E. Via Dept. of Civil & Environmental Engineering

Adjunct, Department of Biological Sciences

Virginia Polytechnic Institute and State University

Blacksburg, Virginia, USA
Thank You!