RICE UNIVERSITY

Using gas-producing enzymes as outputs for synthetic genetic circuits to enable bacterial reporting within complex environmental materials

by

Hsiao-Ying Cheng

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE Doctor of Philosophy

APPROVED, THESIS COMMITTEE

Jonathan J. Silberg, Thesis Advisor
Associate Professor
Biosciences

Caroline A. Masiello
Professor
Earth, Environmental, and Planetary Sciences

Ka-Yiu San
E.D. Butcher Professor
Bioengineering

HOUSTON, TEXAS
MARCH 2018
Abstract

Using gas-producing enzymes as outputs for synthetic genetic circuits to enable bacterial reporting within complex environmental materials

by

Hsiao-Ying Cheng

Microbes drive processes in the Earth system far exceeding their physical scale, mediating significant fluxes in biogeochemical cycles. Microbial behavior also affects soil development, water quality, and crop yields. The tools of synthetic biology have the potential to significantly improve our understanding of the roles that microbes play in these processes and the effects of environmental fluctuations on microbial behaviors, which can advance our ability to engineer microbial system for environmental applications, such as bioremediation, waste water treatment, and rhizosphere engineering. However, synthetic biology has not yet been widely used within environmental materials (soils, sediments, and biomass). One of the challenges is that there is a lack of robust and simple-to-detect reporter proteins for nontransparent and heterogeneous materials. Common genetic reporters used to read out circuit status have limited utility for in situ measurements in Earth materials because environmental matrices display high absorbance and auto-fluorescence at the wavelengths of light used for visual reporters like GFP. This technical limitation has made it challenging to use programmed microbes to study
how variation in soil environmental parameters (moisture, nutrient status, mineralogy, structure, and temperature) affect real-time biological behaviors.

To overcome this limitation, my thesis research aims to develop a new reporting strategy using gas-producing enzymes, which generate diffusible gases that can be quantified in the headspace of soils using gas chromatography. First, I characterize the activities of two gas-producing enzyme, methyl halide transferase (MHT) and ethylene forming enzyme (EFE), in liquid media and an agricultural soil. Using these two enzymes, gas reporting strains were developed to monitor two dynamic soil microbial processes in situ, horizontal gene transfer and quorum sensing. These proof-of-concept applications demonstrate that the gas-reporting method is a generalizable alternative to study microbial gene expression within soil where visual reporters are not compatible. I envision that this easy-to-use gas reporting method would facilitate the development of more sophisticated synthetic genetic circuits for applications in Earth, environmental, and planetary science.
Acknowledgments

There are two people I am most grateful for in my graduate career, Joff and Carrie. Thanks to their guidance, support, and infectious enthusiasm in science, I enjoyed a productive yet joyful grad school experience. They not only trained me to become a scientist but also a more thoughtful person. They taught me how to present an idea and how to listen and learn from my audience at the same time. They are my role models not only as scientists but also mentors.

Thanks as well to Dr. George Bennett’s invaluable input to my project over the years, and Dr. Ka-Yiu San for serving on my committee and providing insightful advice. I also want to thank my labmates from both the Silberg lab and Masiello lab. I am so lucky to have you guys as a family away from home. Thank you for all the fun scientific/ non-scientific conversations and allowing me to steal your pipettes all the time. I am also grateful for the experimental help from the Matsuda lab on GC-MS and cloning and strain engineering advice from the Bennett lab and Tabor lab.

To my Rice friends, TSA friends, and the Catan group, thank you for enriching my life and helping me become a well-rounded person, so that when interviewers ask what my hobbies are, I have something clever and interesting to share. Thank you for your friendship and companionship throughout the laughter and tears of my life.

None of this would be possible without my parents’ love and constant encouragement. Thank you for trusting all my decisions and supporting me through every step I take. Finally, thank you Nic for being my writing coach, who always made sure I made good progress on writing and constantly inspires me to become a better version of myself.
Abstract ................................................................................................................................................. i
Acknowledgments ................................................................................................................................. iii
Contents ................................................................................................................................................ iv
List of Figures ......................................................................................................................................... vii
List of Tables ........................................................................................................................................ x
List of Equations .................................................................................................................................... xi
Abbreviations .......................................................................................................................................... xii

Chapter 1: Introduction ................................................................................................................................. 1
1.1. Engineering microbes with novel functions .................................................................................. 2
  1.1.1. Synthetic biology defines a conceptual framework to effectively engineer microbes ........................................ 2
  1.1.2. Engineered microbes have the potential to solve environmental issues .................................................. 5
    1.1.2.1. Whole-cell bioreporters enable measuring of bioavailable fraction of chemicals .................................... 5
    1.1.2.2. Using engineered microbes for bioremediation ............................................................................. 8
    1.1.2.3. Using engineered microbes in wastewater treatment .................................................................. 9
    1.1.2.4. Agricultural applications of engineered microbes ...................................................................... 11
  1.2. Engineered microbes remain challenging to deploy in environmental matrices ................................................. 14
    1.2.1. Overview of environmental matrices .......................................................................................... 14
    1.2.2. Soil is a matrix with varying particles, organic matter, and hydration .............................................. 15
      1.2.2.1. Minerals .......................................................................................................................... 15
      1.2.2.2. Soil organic matter ............................................................................................................ 16
      1.2.2.3. Soil structure .................................................................................................................... 16
      1.2.2.4. Hydration and osmotic pressure ...................................................................................... 17
      1.2.2.5. Soil pH .......................................................................................................................... 20
      1.2.2.6. Soil fauna ........................................................................................................................ 20
    1.2.3. Sediments are matrices with complex mineralogy, saturated with water, and exhibiting higher pressure ........................................... 21
      1.2.3.1. Redox gradients .............................................................................................................. 21
      1.2.3.2. Hydrostatic pressures ..................................................................................................... 22
    1.2.4. Gene expression dynamics are hard to study in matrices ............................................................ 23
1.2.4.1. Spectroscopically active proteins are common genetic circuit outputs. ................................................................. 24
1.2.4.2. The challenge of using existing reporters in soils and sediments 25
1.2.4.3. Ice-nucleating protein can be used in hard-to-image matrices..... 27
1.3. Gas reporter proteins as a new genetic output for use in hard-to-image matrices.................................................................................................................. 27
1.4. Thesis overview................................................................................................................................................................. 31

Chapter 2: Charcoal disrupts soil microbial communication through a combination of signal sorption and hydrolysis ......................................................... 32
  2.1. Summary .......................................................................................................................... 33
  2.2. Introduction ..................................................................................................................... 34
  2.3. Charcoal effects on AHL and pH ..................................................................................... 37
  2.4. Modeling charcoal effects on AHL................................................................................ 45
  2.5. Environmental Implications ......................................................................................... 50
  2.6. Materials and Methods ................................................................................................ 52

Chapter 3: Ratiometric gas reporting: a non-disruptive approach to monitor gene expression in soils ......................................................................................... 62
  3.1. Summary .......................................................................................................................... 63
  3.2. Introduction ..................................................................................................................... 64
  3.3. Gas reporter characterization ......................................................................................... 65
  3.4. Gas reporting of signaling compounds ........................................................................ 72
  3.5. Monitoring biological signal synthesis and degradation ............................................... 80
  3.6. Discussion ..................................................................................................................... 90
  3.7. Materials and methods ................................................................................................ 94

Chapter 4: Using volatile gas production by methyl halide transferase to report on microbial conjugation in a soil ................................................................. 104
  4.1. Summary .......................................................................................................................... 105
  4.2. Introduction ..................................................................................................................... 105
  4.3. Using CH₃X synthesis to report on gene expression ....................................................... 109
  4.4. Gas reporting from microbes in a soil .......................................................................... 113
  4.5. Non-disruptive reporting of horizontal gene transfer in a soil ...................................... 117
  4.6. Implications of gas reporting ....................................................................................... 125
  4.7. Materials and methods ................................................................................................ 129

Chapter 5: Future Directions ........................................................................................................ 140
5.1. Gas outputs would open the door for new applications of synthetic biology ................................................................................................................................. 141

5.2. Environmental microbial questions that can be answered using gas outputs ................................................................................................................................. 143

5.2.1. Ratiometric gas reporting can be used to study the effect of biochar on microbial and plant signaling in situ in soil .......................................................... 143

5.2.2. Ratiometric gas reporting can be used to study quorum sensing systems in *Rhizobium leguminosarum* ............................................................................. 145

5.2.3. Gas outputs can be used to study horizontal gene transfer in soils and sediments ............................................................................................................. 147

5.3. Further improvements are needed with gas reporting ..................................... 148

5.3.1. Current limitation of the gas outputs .............................................................. 148

5.3.2. Expanding the toolbox of gas-producing enzymes ....................................... 149

5.3.3. Increasing gas analysis sensitivity through analytical method improvement ......................................................................................................................... 149

5.3.4. Using continuous sampling methods to increase the resolution of information that can be obtained from gas-reporting microbes ...................... 150

5.3.5. Using orthogonal protein degradation tags to reduce the response time of gas outputs under fluctuating environmental conditions .................... 151

References .......................................................................................................................... 152

Appendix A ........................................................................................................................................ 202

Appendix B ........................................................................................................................................ 204
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>Common modular structure of a genetic device</td>
<td>3</td>
</tr>
<tr>
<td>1-2</td>
<td>Microbial activity under different soil water contents</td>
<td>19</td>
</tr>
<tr>
<td>2-1</td>
<td>Relationship between charcoal concentration and charcoal-induced pH changes</td>
<td>38</td>
</tr>
<tr>
<td>2-2</td>
<td>Charcoal-induced pH increases are inversely correlated to bioavailable AHL</td>
<td>39</td>
</tr>
<tr>
<td>2-3</td>
<td>Relationships between charcoal pH and AHL-dependent GFP expression</td>
<td>40</td>
</tr>
<tr>
<td>2-4</td>
<td>Charcoal acidification increases bioavailable AHL</td>
<td>42</td>
</tr>
<tr>
<td>2-5</td>
<td>Charcoals can convert AHL into soluble Acyl-HS prior to sorption</td>
<td>44</td>
</tr>
<tr>
<td>2-6</td>
<td>Effect of pH on AHL hydrolysis kinetics</td>
<td>47</td>
</tr>
<tr>
<td>2-7</td>
<td>A kinetic model captured the concentration change of AHL after reaction with the UKBRC charcoals for 1 hour</td>
<td>48</td>
</tr>
<tr>
<td>2-8</td>
<td>Modeling the effect of charcoals on AHL availability</td>
<td>49</td>
</tr>
<tr>
<td>2-9</td>
<td>Physical and chemical characterization of the UKBRC charcoals</td>
<td>54</td>
</tr>
<tr>
<td>2-10</td>
<td>Effect of charcoal concentration on AHL-dependent GFP expression within <em>E. coli</em></td>
<td>56</td>
</tr>
<tr>
<td>2-11</td>
<td>Recovery of AHL using acidification</td>
<td>58</td>
</tr>
<tr>
<td>3-1</td>
<td>Effect of temperature on volatile gas production rates</td>
<td>66</td>
</tr>
<tr>
<td>3-2</td>
<td>Effect of temperature on cell growth</td>
<td>68</td>
</tr>
<tr>
<td>3-3</td>
<td>Illustration of soil matrices and experimental setup for non-disruptive measurements of the gas reporter</td>
<td>69</td>
</tr>
<tr>
<td>3-4</td>
<td>Gas production rates in matrices</td>
<td>70</td>
</tr>
<tr>
<td>3-5</td>
<td>Bioreporter growth in soils</td>
<td>71</td>
</tr>
</tbody>
</table>
Figure 3-6. Using ratiometric reporting to quantify AHLs in soil .......................... 73
Figure 3-7. C_2H_4 production is proportional to extracted colony forming units .... 74
Figure 3-8. Robustness of the ratiometric signals in soil ................................. 76
Figure 3-9. The growth of MG1655-lux and MG1655-las in soil .......................... 77
Figure 3-10. Effect of low [AHL] on bioreporter signals in KBS soil .................. 78
Figure 3-11. Ratiometric gas reporting can be used in *Shewanella oneidensis* MR1 ........................................................................................................ 79
Figure 3-12. Monitoring *Bt* degradation of AHL in soil ................................. 81
Figure 3-13. Effect of different titers of *Bt* on AHL that had been added to soil .. 82
Figure 3-14. *Bt* extracted from KBS soil following incubations............... 83
Figure 3-15. Gas measured above soils containing *Bt*, AHL, and MG1655-las.. 84
Figure 3-16. Measuring *Rhizobium* AHL synthesis in soil............................. 86
Figure 3-17. Dynamics of *Rhizobium* AHL synthesis in soil............................ 87
Figure 3-18. Effect of *Rhizobium* AHLs on MG1655-lux and MG1655-las gas production............................................................................................................. 88
Figure 3-19. *Rl* does not alter the ratio of CH_3Br and C_2H_4 synthesized by *E. coli* constitutively expressing EFE and MHT ......................................................... 89
Figure 4-1. Using CH_3X production to report on gene expression .............. 108
Figure 4-2. Sodium halide concentrations in media affect CH_3X production.... 110
Figure 4-3. Effect of halides on *Escherichia coli* MG1655-mht growth .......... 111
Figure 4-4. Using CH_3X production to report on gene expression .................. 112
Figure 4-5. Inducible expression system used to compare GFP and MHT signals .................................................................................................................. 115
Figure 4-6. Monitoring constitutive gene expression in soil using CH_3Br ...... 116
Figure 4-7. Scheme illustrating the genetic program used to couple MHT transcription and gas production to conjugation ......................................................... 118

Figure 4-8. Altering the Tn10 mobile element within the F-plasmid ................. 119

Figure 4-9. Effect of soil on donor and transconjugant gas production .......... 120

Figure 4-10. Ratio of transconjugant to donor gas production in liquid culture . 121

Figure 4-11. Using CH₃Br production to report on horizontal gene transfer ...... 123

Figure 4-12. Donor CFU before and after incubations .................................... 124

Figure 4-13. Transconjugant strains arising from conjugation display similar gas production .......................................................................................... 133
List of Tables

Table 1. Gas output candidates synthesized by a single enzyme .......................... 30
List of Equations

Equation 2-1 .......................................................... 60
Equation 2-2 .......................................................... 60
Equation 2-3 .......................................................... 60
Equation 2-4 .......................................................... 60
Equation 2-5 .......................................................... 61
Equation 2-6 .......................................................... 61
Equation 3-1 .......................................................... 102
Equation 3-2 .......................................................... 103
Equation 4-1 .......................................................... 136
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACC</td>
<td>1-aminocyclopropane-1-carboxylate</td>
</tr>
<tr>
<td>Acyl-HS</td>
<td>Acyl-homoserine</td>
</tr>
<tr>
<td>AHL</td>
<td>Acyl homoserine lactone</td>
</tr>
<tr>
<td>Bt</td>
<td><em>Bacillus thuringiensis</em></td>
</tr>
<tr>
<td>BCA</td>
<td>Biocontrol agent</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>D</td>
<td>Donor</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EFE</td>
<td>Ethylene forming enzyme</td>
</tr>
<tr>
<td>EPS</td>
<td>Extracellular polymeric substances</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography mass spectrometry</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HSL</td>
<td>Homoserine lactone</td>
</tr>
<tr>
<td>HGT</td>
<td>Horizontal gene transfer</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography mass spectrometry</td>
</tr>
<tr>
<td>MHT</td>
<td>Methyl halide transferase</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PGPB</td>
<td>Plant growth promoting bacteria</td>
</tr>
<tr>
<td>P&amp;T</td>
<td>Purge and trap</td>
</tr>
<tr>
<td>QS</td>
<td>Quorum sensing</td>
</tr>
<tr>
<td>R</td>
<td>Recipient</td>
</tr>
<tr>
<td><em>Rl</em></td>
<td><em>Rhizobium leguminosarum</em></td>
</tr>
<tr>
<td>RBS</td>
<td>Ribosomal binding site</td>
</tr>
<tr>
<td>SOM</td>
<td>Soil organic matter</td>
</tr>
<tr>
<td>SA</td>
<td>Surface area</td>
</tr>
<tr>
<td>Tc</td>
<td>Transconjugant</td>
</tr>
<tr>
<td>UKBRC</td>
<td>UK biochar research center</td>
</tr>
<tr>
<td>KBS</td>
<td>W.K. Kellogg biological station</td>
</tr>
<tr>
<td>WHC</td>
<td>Water holding capacity</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction
1.1. Engineering microbes with novel functions

1.1.1. Synthetic biology defines a conceptual framework to effectively engineer microbes

Microbes are routinely modified through genetic engineering to exhibit novel functions. Building upon the wealth of knowledge from molecular biology and genetic engineering, synthetic biology provides effective genetic modification tools and conceptual frameworks to modularize design processes.¹ These advancements enable more sophisticated design of engineered microbes that can perform new functions in response to environmental stimuli. Novel microbial functions can be engineered by assembling three genetically-encoded modules²: sensors, regulators, and actuators. (Figure 1-1).

Within genetic circuits, sensors monitor environmental conditions using a range of mechanisms, such as ligand-dependent transcriptional regulators, RNA aptamers, membrane-bound receptors, and DNA damage sensory mechanisms. Researchers have constructed microbes that detect various stimuli by tapping into the diversity of naturally occurring sensory modules from non-native systems. In addition, they have used protein engineering to create novel substrate specificities by mutating existing sensors. Regulators use the information provided by the sensory module to perform computation and determine the signal produced by actuators.

Synthetic biology research has yielded a range of genetic architectures that can be used for calculations, such as logic gates (e.g., AND, OR, NOR) built from
Figure 1-1. Common modular structure of a genetic device
The modular structure of a simple programmable cell includes sensor, regulator, and actuator. Adapted from Kobayashi *et al.*, 2004.²
transcription factors,\textsuperscript{8,9} protein switches,\textsuperscript{10,11} CRISPRi,\textsuperscript{12} and DNA recombinases.\textsuperscript{13} These genetic components can be further assembled into more complex designs to achieve specific cellular functions, for instance, recombinase-based state machine,\textsuperscript{14} bacterial edge detector,\textsuperscript{15} and consortium oscillator.\textsuperscript{16} These complex genetic architectures have been used to engineer bacterial reporting systems that provide dynamic information on multiple environmental stimuli\textsuperscript{17} or even encode memories of previous environmental exposure.\textsuperscript{18} To assist the design of complex genetic circuits, computer-aided design tools, such as Cello,\textsuperscript{19} Genetic Constructor,\textsuperscript{20} and Clotho[SC10],\textsuperscript{21} have been developed. Using Cello, Nielsen et al. showed that combining with well characterized genetic parts, a computer algorithm can effectively design circuits with up to 12 promoters.\textsuperscript{19}

In genetic circuits, actuators transduce the signal from the regulatory module into a biological activity, such as molecules whose levels can be quantified using microscopy,\textsuperscript{22} or changes in biological functions, such as formation of biofilm,\textsuperscript{23} production of enzymes,\textsuperscript{24} sporulation,\textsuperscript{25} and swimming\textsuperscript{[direction]} changes.\textsuperscript{26} For example, a cancer targeting bacterium was engineered to synthesize and release cytotoxin when it senses tumor microenvironments.\textsuperscript{27–29} By regulating the expression of CheZ in flagella signaling pathway, \textit{E. coli} can also be engineered to seek a chemical.\textsuperscript{26} In general, most circuits initially use fluorescent proteins as outputs because these allow for rapid assessment of circuit function.

As the field of synthetic biology advances, more well-defined genetic parts are becoming available through better characterization of known genetic elements and the discovery of new parts by genome mining.\textsuperscript{30} Using the three-module
framework – sensors, regulators, and actuators – these parts have been assembled into genetic devices,\textsuperscript{31–33} which have shown great promise for tackling challenges in disease diagnosis,\textsuperscript{34} cancer treatment,\textsuperscript{27} feedback-controlled chemical production,\textsuperscript{35} and bioremediation.\textsuperscript{36} The following section will focus on the development of genetically-engineered bacteria for environmental applications, specifically in the context of whole-cell bioreporters, bioremediation, wastewater treatment, and agricultural applications.

1.1.2. Engineered microbes have the potential to solve environmental issues

1.1.2.1. Whole-cell bioreporters enable measuring of bioavailable fraction of chemicals

Microbes can be engineered to synthesize specific molecular output(s) when they encounter different chemical compounds within their environment. Because of their ability to report the conditions of the surrounding environment in a dose-response manner, these microbes are called bioreporters\textsuperscript{[J12]}. Thanks to the availability of diverse sensory modules (\textit{Appendix A}),\textsuperscript{37} bioreporters have been used to detect a wide range of molecules, such as trace toxins in human serum\textsuperscript{38} and urine,\textsuperscript{39} heavy metals in food\textsuperscript{40,41} and water,\textsuperscript{42} and pollutants in soils.\textsuperscript{43}

Researchers have implemented AND gate regulatory modules to integrate multiple inputs into an output with promoters that require multiple transcription factors for activation. For example, AND dates have been constructed using $P_{\text{lacI}}$ with Lacl/GalR family chimeras,\textsuperscript{8} $P_{\text{hrpL}}$,\textsuperscript{9} and $P_{\text{SicA}}$.\textsuperscript{44} More complex regulatory modules have been developed to achieve tunable sensitivity,\textsuperscript{45} ultra-sensitivity,\textsuperscript{46}
and feedback control. Common actuator modules used in bioreporters include fluorescent proteins, luciferase, β-galactosidase, and pigment production enzymes. The output of these proteins can be can be quantified visually through light or pigment production. Electrochemical methods have also been used to analyze gene circuit outputs, for instance, \( \beta \)-galactosidase can catalyze p-aminophenyl-β-galactopyranoside into p-aminophenol, which can be detected by amperometry.

There are notable advantages of bioreporters over traditional analytical chemistry methods. Traditional analytical methods use inorganic or organic solvents to chemically extract compounds from environmental samples. The extracted chemicals can be identified and quantified by chromatography, e.g., gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and high performance liquid chromatography (HPLC). These analytical methods provide information on the total extractable concentrations, which can differ from the levels experienced by organisms. On the other hand, bioreporters are better suited for determining the concentrations of chemicals that microbes encounter, e.g., the bioavailable fractions of toxins and nutrients. The bioavailable fraction of a chemical might vary according to the environmental conditions; for instance, sorptive material (such as, clay and organic matters) can decrease the availability of a chemical through ion exchange or elevating water content in a soil can increase the dissolved fraction of a chemical. Furthermore, since microbes can self-replicate, it is cost-effective to produce large quantities of bioreporters. They can be shared widely at low cost.
employed in multiple field sites to provide sensitive and rapid on-site measurements.$^{34,48}$

The application of bioreporters is greatly limited by their shelf lives. Live cell cultures can only remain functional for 2 days when stored at room temperature and for six weeks at 4°C.$^{54}$ With lyophilization, their storage time can be extended to more than a year, although additional re-hydration and culturing processes are needed prior to measurements.$^{55}$ Cell-free systems based on freeze-dried cell lysate or purified protein and DNA are attractive alternatives because they lack this limitation.$^{56}$ Additionally, the development of bioreporters using microbes that are long-lived in complex environmental conditions would extend their life.$^{[SC15]}$. For example, genetically tractable soil bacteria in the genus of *Agrobacterium* and *Bacillus* have been developed into bioreporters for monitoring quorum sensing molecules and nitrate in soil.$^{57,58,59}$

While diverse bioreporters have been developed to provide microbial perspectives on the levels of chemicals in environmental samples, most of these bioreporters remain in the proof-of-concept stage and are not suitable for *in situ* reporting in hard-to-image matrices, such as soils, sediments, and complex feedstock. This bottleneck occurs because existing visual bioreporter outputs are hard to detect in nontransparent materials like soils. More suitable outputs are currently needed that can be detected without disrupting the matrix. The reporting challenge in environmental samples will be further discussed in Section 1.3.
1.1.2.2. Using engineered microbes for bioremediation

The role of microorganisms in degrading or immobilizing environmental pollutants into non-toxic forms has been intensively studied. Natural microbial degraders have been found to use organic pollutants as carbon sources at contaminated sites. By culturing and screening microbial consortia at these sites, researchers have obtained isolates to degrade hydrocarbons from oil spills,\textsuperscript{60,61} 1,2-dichloroethane,\textsuperscript{62} and organophosphorus pesticides.\textsuperscript{63} Heavy metal contaminants (\textit{e.g.}, As, Cr, Pb, Hg, Cd and U), can be transformed by microbes through oxidation or reduction into less toxic forms.\textsuperscript{64} Additionally, microbes can transport them into the cytosol, or absorb them on to the cell wall and extracellular matrices.\textsuperscript{65,66} Commercially available bioremediation products consisting of bacterial or fungi consortia isolated from contaminated sites have been developed and are widely used to facilitate remediation of organic and heavy metal contaminations in soils and water bodies.\textsuperscript{60} These organisms have also been a treasure trove for new biological components, \textit{i.e.}, transcriptional regulators for synthetic biology.\textsuperscript{67,68}

With a better understanding of bioremediation pathways and the development of recombinant DNA technology, microbes with enhanced catalytic or adsorbing activity toward contaminants have been engineered. For instance, wild-type \textit{Burkholderia xenovorans} transformed with a dehydrogenase from \textit{Burkholderia} species MBA4 gained the metabolic capability of degrading halogenated alkanoic acids.\textsuperscript{69} Furthermore, the enzymatic activity was 30-fold higher than the original strain where the dehydrogenase was found.\textsuperscript{69} In addition,
Deinococcus radiodurans, the most radiation-resistant microbe, was engineered to degrade organic pollutants by expressing toluene dioxygenase (TDO). Other bioremediation strain engineering strategies include overexpressing transport proteins to increase contaminant sequestration, or up-regulating contaminant binding proteins displayed on the cell surface to create cellular biosorbents. More recently, a bioremediation strain designed with a mercury sensor regulating the remediation pathway was created. The engineered E. coli strain produced Hg⁺-sequestering amyloids in its biofilm only in the presence of Hg⁺ in the environment.

Although synthetic biology has the potential to enhance the performance of currently identified bioremediation isolates and develop novel microbial degraders for xenobiotic pollutants, the general concern of releasing genetically engineered microbes into the wild limits widespread application. In addition, it remains challenging to engineer robust microbes which can perform consistently under fluctuating field conditions. To overcome this challenge, the research community needs better tools for evaluating microbial gene expression dynamics in situ and microbial perception of their surroundings in response to changing environment conditions.

1.1.2.3. Using engineered microbes in wastewater treatment

Engineered microbes have the potential to facilitate wastewater treatment process in degrading pollutants and nutrient recycling. Wastewater treatment applies biological, chemical, and physical methods to remove solid waste, organic carbon and nitrogen, and toxic compounds in wastewater from
municipal, industrial, or agricultural sources. Conventional wastewater treatment plants use indigenous microbial communities to perform aerobic organic compound degradation. The resulting activated sludge is further treated with anaerobic digestion. Since the effluent from this type of process still contains a high nitrogen load, it can be treated with additional biological nitrification and denitrification processes. The latest technology uses partial nitrification followed by anaerobic ammonium oxidation. To enhance the quality of effluent after wastewater treatment and decrease the energy usage during this process, tremendous effort has been devoted to reactor design optimization, chemical addition, and enrichment of microbial degraders. In the scope of this thesis, the following section will be focused on the developments of using genetically engineered microorganisms that could be used to assist in wastewater treatment.

The idea of using genetically engineered microorganisms to enhance degradation of xenobiotic compounds in wastewater was first proposed in the 1980s. In an early experiment, a recombinant plasmid harboring catabolic genes was conjugated into a floc-forming *Pseudomonas lemoigne* strain isolated from activated sludge. The resulting strain not only formed stable flocs but showed enhanced degradation activity against salicylate and naphthalene. Although the stability of this strain in actual activated sludge was not examined, the floc formation suggested that this strain would not be washed out with the effluent and could remain in the treatment tank. Another example is the development of azo dye degrading bacteria. Specific azo dye degrading enzymes were identified and heterologously expressed in *E. coli*, which gained the catabolic activity of
decolorizing azo dyes. Furthermore, the dye degradation activity was stably maintained in a repeat-batch bioreactor. By harnessing the high horizontal gene transfer (HGT) rate in activated sludge, Venkata Mohan and coworkers showed that a conjugative TOL plasmid conferring toluene and benzyl alcohol degrading capacity could propagate through a mixed microbial community in a lab-scale wastewater treatment reactor. The degrading capacity persisted over a month of the experimental period.

In addition to chemical removal in wastewater treatment, genetically-engineered microorganisms have the potential to improve other aspects of wastewater treatment processes, such as converting organic matter in wastewater into valuable products and forming beneficial biofilms to prevent biofouling.

Additionally, recent advancements in synthetic ecology present new strategies to engineer microbial consortia. However, since a wastewater treatment plant is an open environment, biocontainment strategies for engineered strains and genetic material need to be established prior to the deployment of the synthetic microbes. Furthermore, real-time *in situ* monitoring of microbial activity in wastewater treatment processes remains challenging due to complex biotic and abiotic reactor conditions, which increases the difficulty of engineering microbes for wastewater applications.

1.1.2.4. Agricultural applications of engineered microbes

Since the early 19th century, biologists have recognized the importance of microorganisms to plant growth. Numerous biocontrol agents (BCA) and plant growth promoting bacteria (PGPB) have since been identified. BCA/PGPB
promote plant growth by increasing root nutrient uptake, releasing plant hormones, or protecting plants against pathogens. While there are many great successes of using BCA/PGPB in agriculture, the outcomes of applying them to a field could be inconsistent due to diverse environmental conditions, such as soil type, weather, inoculation technology, and crop-type.88 A better understanding of genetic regulation of microbial activities has shed light on how environmental cues influence beneficial microbial activities, thus providing strategies to enhance BCA/PGPB activities. By harnessing this knowledge, scientists have proposed engineering microbes to improve agricultural outcomes.89 In this sub-section, ideas and proof-of-concept experiments of genetically engineered microbes used in agriculture are reviewed.

Engineered plant protection in rhizospheric bacteria can be achieved by overexpressing anti-bacterial/fungal enzymes or compounds. In an early study, an E. coli strain was transformed with a plasmid harboring chitinase, chiA, to degrade hyphal tips. When this strain was sprayed on cotton plants in a greenhouse experiment, there was decreased damping-off disease caused by fungal infection.90 Pseudomonas fluorescens has been engineered to synthesize the anti-fungal compounds 2,4-diacetyl-phloroglucinol and phenazine-1-carboxylic acid, through plasmid transformation or chromosomal integration of biosynthetic pathways.86,91,92 Quorum sensing inhibition enzymes, such as lactonase and acylase, have been used to disrupt bacterial quorum sensing, thereby inhibiting virulence.93 Engineered Bacillus thuringeinsis with overexpressed lactonase (aiiA) was shown to inhibit tissue maceration by disrupting the quorum sensing
capabilities of the plant pathogen *Pectobacterium caratovora*. A recombinant rice endophyte, *Burkholderia* sp. KJ006 transformed with lactonase (*aiiA*), could reduce rice seedling rot caused by a pathogenic endophyte *Burkholderia glumae*.

Besides protecting crops from pathogens, crop yields can be improved by regulating plant hormones. Plant-associated bacteria naturally produce a wide variety of plant hormones to regulate plant tissue growth, such as root elongation, nodule formation, and floral development. Some bacteria are found to produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which degrades ACC, a metabolic precursor of ethylene. Ethylene is produced as a stress hormone and has inhibitory effects on plant growth. By degrading ACC, these bacteria can promote plant growth under stress conditions. ACC deaminase has been introduced into *Pseudomonas fluorescens*, and the resulting strain could enhance root elongation.

Increasing fertilizer efficiency is a prominent research area in the agricultural sciences. Researchers have been screening for biofertilizers, including biological nitrogen-fixation strains (either nodule-forming or free-living nitrogen fixers), phosphate-solubilizing microorganisms, and other microbes that help micro-nutrient uptake. There is also an increasing interest in developing genetically-engineered biofertilizers. However, developing robust strains that can integrate into the indigenous rhizosphere communities and perform their desired functions under diverse environmental conditions remains a challenge. To engineer strains that can regulate their activity according to the environment where they reside, we need to construct more sophisticated genetic devices using multiple
genetic parts. While the characterization of genetic parts in the suitable environmental context is imperative, there is no suitable reporting method to provide real-time information of their activities in soil.\textsuperscript{103}

In further exploration of how environmental context can affect microbial activities, Section 1.2 will discuss two environmental matrices, soils and sediments, in the context of bacterial physiology. Following that, Section 1.3 will introduce a novel reporting method to characterize gene circuits in complex matrices more accurately and efficiently than existing reporters used in environmental applications.

### 1.2. Engineered microbes remain challenging to deploy in environmental matrices

#### 1.2.1. Overview of environmental matrices

In this section, two types of environmental materials will be discussed, soil and sediment. Soils support life on Earth, they store and purify water, and they influence the gas composition of the atmosphere. Good soil resource management has been a perennial concern since the dawn of agriculture. The discovery of microorganisms inspired scientists to develop new soil management and agriculture enhancement strategies. Understanding how soil matrices affect microbial function can contribute to the better design of engineered microbes for agricultural and bioremediation applications.
Sediment is defined here as the matrix at the bottom layer of rivers, lakes, and oceans. These matrices often have abundant minerals at a similar level as soils but they experience low oxygen availability and high pressure due to the water mass above. Sediments are inhabited by \( >10^{29} \) prokaryotes\(^{104} \) on Earth, and these microbes contribute to the cycling of elements such as C, N, P, Fe, and S. Sediment microbiota also play an important role in water quality control and bioremediation of organic pollutants.\(^{105} \)

The following subsections will illustrate the complexity of these environments, which result in microbial activities different from what is observed in standard liquid culturing methods used in most research laboratories. Section 1.2.4 will further discuss the challenges in monitoring gene expression in environmental matrices.

1.2.2. Soil is a matrix with varying particles, organic matter, and hydration

1.2.2.1. Minerals

Most of the soil solid framework consists of mineral particles, which can be further categorized by size into sand (2~0.05 mm), silt (0.05~0.002 mm), and clay (<0.002 mm). The chemical composition of soil mineral is depending on the soil parent material and weathering history. Clay particles have high surface areas and may carry positive and/or negative surface charges, which contribute to soil's ion exchange capacity and its ability to sorb a wide range of inorganic or organic compounds.\(^{52,106} \) Soil minerals are also the source of essential elements to support life, e.g. Fe, Mg, Al, and trace metals.
1.2.2.2. Soil organic matter

Besides minerals, soil organic matter (SOM) is another important soil component, consisting of plant and animal residues from decomposition, soil biota, and organic compounds secreted by organisms. Depending on its stability, SOM is roughly categorized into two groups. First, labile SOM can be degraded through enzymatic processes and provide carbon, phosphorus, and nitrogen to plants and soil biota. Second, stable SOM with turnover time of >500 years is one of the major carbon sinks on Earth. SOM carries positive and negative charges and thus provides anion and cation exchange sites in soils, which retains nutrients (e.g., K⁺, NH₄⁺, Ca²⁺), pollutants, and cell-cell signaling compounds. Plant roots exude low molecular weight sugars, amino acids, and flavonoids to attract microorganisms; plant-associated microbes sense these compounds and swim toward plant roots through chemotaxis. The molecular mechanism of this process has been well studied in artificial systems but not in a natural soil where the root exudates might be sequestered by SOM or minerals.

1.2.2.3. Soil structure

In soils, minerals mix with SOM to form a complex structure with varying pore sizes. These soil pores hold air and water along with dissolved nutrients that support the growth of plants and soil biota. In soil macropores (>75 μm), solutes and microbes can be transported through soil water movement, which can form “biogeochemical hot spots” where higher microbial activity occurs. In smaller pores or on the surface of soil particles, diffusion is the dominant force driving
nutrient movement. In saturated soils, all pores are filled with water and form a relatively homogenous, connected environment. In unsaturated soils, however, pores are only connected by water films retained on particle surfaces, which results in fragmented aquatic habitats and heterogenous microbial and nutrient distribution. Furthermore, because of the low connectivity between pores, bacterial mobility and chemotaxis are greatly restricted in unsaturated soils.116–118

Soil water behavior is one of the most important parameters affecting microbial activity in soils, and it is constantly changing with climatic and hydrological conditions and biological activities, such as plant water uptake and biofilm formation. Soil water properties include total water content (θ, g/g or m³/m³) and water potential (ψ, kPa). Water molecules move from higher to lower water potential, and soil water potential is always less than zero (the potential of pure water) because of the presence of osmolytes and soil matrix. Soil water potential is the additive of osmotic potential (determined by the concentration of soil solutes) and matrix potential (caused surface tension between water and soil particle surface).116 Rapid hydration of soil through rainfall or irrigation increases water potential, while high fertilizer amendment rate, chemical contamination, and high soil evaporation rate due to dry climates or plant root water transportation can induce local hyper-osmotic stress thus limiting microbial growth and activity.119

1.2.2.4. Hydration and osmotic pressure

Microbes mitigate osmotic pressures caused by dynamic soil hydration conditions through: 1) secreting extracellular polymeric substances (EPS) to form biofilms that create relatively stable local water and nutrient conditions120,121 and 2)
balancing cytosolic and extracellular osmolytes by using membrane transport and osmoprotectant synthesis. Hypo-osmotic pressure causes water influx, which increases cell membrane tension and triggers mechanosensitive channels to release cytosolic osmolytes through diffusion. Under hyper-osmotic pressure, water flows out of cells and causes cytosolic ionic strength to increase, which initiates a series of physiological changes. Any ionic strength upshift first induces structural changes of membrane-bound transporters and import of activated ATP-dependent cations and small organic molecules. Global stress-response transcription factors, such as σs (or RpoS) in *E. coli*, σE (or AlgU) in *P. flourecens*, RpoE2 in *S. meliloti*, and KinD in *B. subtillus*, are then activated by increasing cellular potassium glutamate or membrane kinase-activated signal transduction pathways. These transcription factors up-regulate osmoprotectant (such as glycine betaine, trehalose, and proline) biosynthesis, lipid membrane composition change, biofilm formation, and sporulation. An osmosis-regulating promoter, *PproU* in *E. coli*, has been developed into a bioreporter to monitor water availability on plant leaves.

Soil water content also influences gas partitioning from the atmosphere into the soil. Since gas transport through liquid is 10,000X less effective than through air, microbial aerobic respiration decreases due to oxygen limitation under high soil water content conditions, despite potential increases in nutrient flux. The integral effect of nutrient supply and gas partition rate results in the convex relationship between aerobic microbial activity and soil water content (*Figure 1-2*).
Figure 1-2. Microbial activity under different soil water contents
This figure shows a conceptual illustration of the relationship between soil water content and macroscopic microbial activity. Two dash lines indicate the limits of substrate (transported through liquid) and oxygen (restricted by liquid) diffusion. $\theta_{max}$ represents the water content that maximizes microbial activity. Figure modified from Dani Or et al.\textsuperscript{116}
1.2.2.5. Soil pH

Soil pH is determined by mineral composition, soil amendment, climate, and biological activities. Soil pH can range from ultra-acidic (< 3.5) to very strongly alkaline (> 9.3) soils. Plants generally thrive around a neutral pH where most of the macronutrients (potassium, sodium, nitrogen, sulfur, calcium, magnesium) are presented in soluble forms and aluminum toxicity is minimal. To increase iron availability,\textsuperscript{133,134} plant roots release protons or organic acids to lower local pH by 1 to 2 units.\textsuperscript{135} pH is the master regulator of nutrient or toxin availability, and it affects the stability of cell-cell signaling compounds.\textsuperscript{136,137} For example, some plants respond to pathogen attacks\textsuperscript{[SC29]} by increasing extracellular pH (alkalization), which facilitates hydrolysis of acyl homoserine lactone used by \textit{Pectobacterium} to coordinate pectolytic enzyme production.\textsuperscript{138–142} Microbes sense external pH using membrane bound one-component or two-component systems and maintain pH-homeostasis in extreme pH environments through active proton transport and consumption or generation of proton by metabolic reactions\textsuperscript{[SC30]}.\textsuperscript{143}

1.2.2.6. Soil fauna

Besides plants and microbes, soil fauna, such as protozoa, nematodes, earthworms, insects, and small animals, is also an essential part of soil ecosystems, and their activities are constantly changing the soil environment where they reside.\textsuperscript{144} For instance, the feeding and burrowing of earthworms not only can increase soil porosity, which enhances soil aeration and drainage, but also can mix spatially separated microbial communities.\textsuperscript{145} In addition, studies
have shown that the intestinal track of earthworms is a hot spot for horizontal gene transfer between bacteria due to elevating microbial density and nutrient availability.\textsuperscript{145,146} Arthropods also create environments in their guts that are distinct from the bulk soil; for example, wood-feeding termites have anaerobic gut environments and maintain mainly anaerobic lignocellulose degrading bacteria in their microbiome.\textsuperscript{147}

1.2.3. Sediments are matrices with complex mineralogy, saturated with water, and exhibiting higher pressure

Sediments have diverse mineralogy, vertical chemical profiles, and ecology depending on the local geochemistry and the composition of the bottom water. Sediments are the destination of terrestrial and aquatic organic carbon, which fuels complex sediment ecosystems.\textsuperscript{148} There are an estimated $2.9 \times 10^{29}$ microbes residing in subsea-floor sediment,\textsuperscript{104} and they are important drivers of iron, sulfur, and carbon cycling. Their activity influences the stability of carbon burial and greenhouse gas production, \textit{e.g.} methane and carbon dioxide.\textsuperscript{149–153}

1.2.3.1. Redox gradients

Due to oxygen limitation, microbes residing in sediments use alternative electron acceptors in hypoxic or anoxic conditions. When the consumption and production of chemicals exceed diffusion rates, chemical gradients are formed along the vertical profile. Labile organic matter deposited from the water column is first processed aerobically, which depletes oxygen locally. Since the diffusion of oxygen is limited in aquatic environments, oxygen penetration depth in sediments
is restricted, varying from 1 mm to a few cm. Without oxygen, microbes respire using a cascade of electron acceptors including nitrate, Mn(III), Fe(III), and sulfate (mostly in marine sediments). The least thermodynamically unfavorable reaction uses carbon (carbon dioxide, carbon monoxide, and acetic acid) as acceptors to produce methane – methanogenesis, which is exclusively conducted by archaea.\textsuperscript{148,154,155}

In the complex sediment environment, diverse microbes evolved unique metabolisms and established niches in an ecosystem. Microbes not only compete for nutrients but also collaborate to allow thermodynamically unfavorable reactions to happen through intermediate depletion (syntrophy).\textsuperscript{156,157} Evidence of cross-feeding has also been found in lake sediment; for instance, the non-methanotroph \textit{Methylophilaceae}, survives on methanol produced by the methanotroph \textit{Methylococcaceae}.\textsuperscript{152,158,159} In addition to small molecules, electron movement can also link microbial metabolisms together. The recent discovery of microbial nanowires showed that microbes can form filaments to transport electrons and link reduction processes in the anoxic bottom layer to oxidation processes happening in the oxic top layer of sediment.\textsuperscript{160,161}

1.2.3.2. Hydrostatic pressures

Besides redox gradients, microbes also experience hydrostatic pressure (ranging from 0.1 to 140 MPa) in sediments. At the molecular level, pressure higher than 5 MPa has been shown to destabilize protein quaternary structures, decrease lipid membrane fluidity, influence DNA-protein interactions, and limit substrate transport.\textsuperscript{162} To overcome these changes, microbes evolved molecular strategies,
such as accumulating osmolytes (e.g., β-hydroxybutarate and glutamate), increasing unsaturated lipids and cholesterols in the cell membrane, regulating membrane protein expression, and protein structural adaptation. Microbes that are adapted to elevated pressure environments and have higher cellular activity in elevated pressure conditions over normal atmospheric pressure are called piezophiles. A deep-sea piezophile, Photobacterium profundum SS9, has been found to toggle the expression of two membrane proteins OmpL (low pressure) and OmpH (high pressure) through a transmembrane DNA binding protein, ToxR. The dimerization of ToxR is disrupted under elevated pressure and inversely regulates gene expression when hydrostatic pressure increases from 10 to 50 MPa. This finding also suggested a potential molecular mechanism for mesophiles (hydrostatic pressure-sensitive microbes) to sense hydrostatic pressure and further activate global cellular stress responses.

1.2.4. Gene expression dynamics are hard to study in matrices

While a wide variety of methods have been developed to examine DNA, RNA, and protein profiles in environmental samples in great detail, it remains challenging to understand microbial gene expression dynamics caused by environmental fluctuations in situ. The advancement of metagenomics can now provide insight into the microbial diversity found in different environmental settings. In addition, transcriptomics and proteomics can provide a snapshot of the genes and proteins that are made at any point of time in different settings. However, these approaches all required extraction of biomolecules out of environmental samples prior to analysis. The extraction procedures not only disrupts samples,
which prohibits continuous measurement, but also causes sampling bias.\textsuperscript{173} Therefore, these approaches are limited in their ability to provide dynamic information about the gene expression changes that underlie microbial processes in soils and sediments.

The development of genetically-encoded reporter proteins enables researchers to track promoter activities. Visual reporters, such as fluorescent proteins and luciferase, have been widely used to monitor cellular behaviors real-time in pure culture, and with the advancement in microscope technology, complex biofilm structures can be visualized.\textsuperscript{174,175} However, visual reporters are challenging to use in complex environmental matrices. The following section will discuss current statutes of reporter proteins for environmental applications.

1.2.4.1. Spectroscopically active proteins are common genetic circuit outputs.

Reporter proteins represent one of the most important tools used to understand natural and engineered genetic systems. They are widely used by biologists and engineers to obtain spatial and temporal information on protein translation, degradation, and cellular localization.\textsuperscript{176} In addition, they have been critical for synthetic biologists to assess the level of control achieved in designed genetic networks.\textsuperscript{33} A reporter protein requires several properties. First, a simple assay must be available to quantify the output generated by the protein. Second, the perturbation caused by reporter proteins must be minimal within the context of the cellular system being studied. Finally, the reporter protein should have minimal or no effect on normal cellular metabolism. The most commonly used proteins that meet these criteria include β-galactosidase, luciferase, and fluorescent proteins.
(Appendix B). β-galactosidase and luciferase catalyze chemical reactions that produce optically detectable output signals. The substrates of these chemical reactions must typically be added to the cellular system of interest, which increase the complexity of experimental procedures. In contrast, fluorescent proteins are intrinsically fluorescent because they have a chromophore that is formed by an autocatalytic post-translational modification or by ligand binding. Fluorescent proteins are easily detectable by optical methods, including spectroscopy for the total fluorescence from cell culture, epifluorescence and confocal laser scanning microscopy for single cell detection, and flow cytometry for quantitative single cell analysis.

1.2.4.2. The challenge of using existing reporters in soils and sediments

Although numerous genetically-encoded reporter proteins have been developed, with colors across the visible and near infrared spectrum, most of the signals produced by these proteins are limited to detection by colorimetry, bioluminescence, and fluorescence. These signals can be easily quantified in low extinction coefficient matrices (e.g., clear aqueous solution, single cells, thin-layer tissue culture, and microfluidic devices) to provide spatial and temporal information about biological reactions. In addition, proteins like near infrared fluorescent protein can be used in higher extinction materials, such as animal models. However, these spectroscopic reporters are hard to use within cells residing within matrices containing materials with high absorptivity and autofluorescence, such as bulk soils, complex feedstock, foods, and nanomaterial.
Researchers have used several approaches to leverage reporters in hard-to-image samples. One approach has been to extract analytes from the matrices prior to detection with microbial bioreporters. For instance, Baumann and van der Meer applied this approach using a bioluminance bioreporter developed to report on arsenic levels in rice.\textsuperscript{4} This approach has also been applied to the study of other pollutant concentrations in soils.\textsuperscript{42} However, the extraction step disrupts the matrix and can only collect single time point data, limiting the acquisition of temporal information. Engineered microbes with fluorescent proteins or luciferase output have also been added directly into soils or sediment matrices for biosensing purposes, yet due to the interference of matrix material,\textsuperscript{170,183} extraction steps are required to obtain quantifiable signals. Another approach has been to build special apparatuses that allow for imaging of bioreporters in the rhizosphere. Bringhurst and coworkers described a microcosm that forced a portion of plant roots to grow into a small compartment filled with Nylon mesh and covered with a piece of glass cover slip.\textsuperscript{184} After applying fluorescent bioreporters onto the root system, the distribution of β-galactosides activity can be visualized using an epifluorescence microscope. Other methods used to address the soil quenching issue include growing plants on agar or filter paper\textsuperscript{185,186} and separating bioreporters from the soil matrix before analysis.\textsuperscript{131} While these methods have enabled researchers to obtain some spatial information using visual output signals, they have failed to deliver quantitative data because of low image quality. In addition, since these methods either used artificial soil matrices or cleaned the root prior to analysis,
their observation might not truly represent natural phenomenon within bulk soils of ecological relevance.

1.2.4.3. Ice-nucleating protein can be used in hard-to-image matrices

A limited number of studies have used non-visual outputs to overcome challenges of applying synthetic biology in hard-to-image matrices. An ice nucleating protein (InaZ) was developed as a reporter protein for microbes in soil matrices. InaZ, a membrane protein that promotes nucleation of ice crystals, was applied to quantify nitrate level\(^{58}\) and AHL\(^{187}\) concentration in the rhizosphere. When cells programmed to generate InaZ as an output encountered one of these inputs, they expressed InaZ at their membrane. After the bioreporters were extracted out of the matrix, the gene expression was quantified using a droplet-freezing assay.\(^{188}\) Ice nucleating protein has advantages over visual reporter proteins in providing quantifiable data and signal amplification within bulk soils. However, this reporter can only be detected by disrupting the soil in where it resides, and it is not suitable for continuous monitoring.

1.3. Gas reporter proteins as a new genetic output for use in hard-to-image matrices

To overcome the limitation of existing reporters and enable a simple \textit{in situ} measurement of microbial gene expression, I have focused my thesis research on testing gas-producing enzymes as genetically-encoding reporters. With these
reporters, a protein is used to synthesize a small diffusible gas molecule, which is
detected in the headspace of hard-to-image samples to achieve dynamic reporting.

I hypothesized that proteins which produce rare volatile gases could have
widespread applications as outputs for genetic circuits in hard-to-image materials.
By measuring the concentration of gases accumulated in the headspace of
samples, I posited that gene expression could be quantified non-invasively.
Because the matrix would not require disruption to measure the gas signal, this
class of reporters would provide dynamic temporal information on biosensing in
any matrix that does not readily sorb the volatile gas. As I considered gas reporters
to test, I made a list of important characteristics of the enzymes. These included:

1) **The gas should be produced by a single enzyme.** To simplify the output
module, a single protein that catalyzes production of the gas would be ideal.

2) **The volatile compound should be uncommon.** The output gas needs to be
rare and have a low background in the environment for high sensitivity. Also
it cannot be consumed at high rates by organisms within the matrix.

3) **The substrate(s) used to produce the gas should be a ubiquitous metabolite.**
To ensure that the gas production wouldn’t require special cellular
resources, the reaction should depend on substrates that are synthesized
under diverse metabolic states and in many cell types.

4) **The gas should not be toxic, at least at the levels used for an output.** The
gas should not affect normal cellular metabolism and growth of the microbe
producing the gas or other microbes found in the complex samples where
biosensing is being performed.
5) **The gas should have a high vapor pressure (low boiling point).** We need a gas that readily partitions from liquid phase where it is synthesized to gas phase under normal growth condition to achieve distinctive signal.

6) **The volatile compound should readily diffuse out of many matrixes.** Porous solid materials (e.g., soil grains and charcoal) interfere with gas moving from the matrix to the headspace. The rate of effusion is inversely proportional to the molecular weight of gas and dependent upon the pore sizes within the matrix. If the gas is a large heavy molecule, it might take a long time for it to leave the matrix.

7) **The gas should be easy to detect using a common instrument.** For a gas bioreporter to be easily transferable to other labs and field/industrial applications, the output gas must be detectable by established analyzing method, such as a GC-MS or other detectors.\(^{189}\)

8) **Enzymes should be available that produce isosteres of the gas.** Ideally, genetic circuits would be constructed using multiple gas outputs with the same number of atoms and valence electrons (isosteres).

There are a small number of enzymes encoded by a single gene that produce gases which meet all or most of these criteria *(Table 1)* among all known volatile compounds produced by bacteria.\(^{190}\) In this thesis work, I explore the possibility of using methyl halide transferases (MHT) and ethylene forming enzyme (EFE) as gas reporters and identify optimal experimental conditions for using these two enzymes in parallel.
Table 1. Gas output candidates synthesized by a single enzyme

<table>
<thead>
<tr>
<th>Output Gas</th>
<th>Protein</th>
<th>Gene</th>
<th>Substrate</th>
<th>Boiling Point °C</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrous Oxide</td>
<td>Nitric oxide reductase</td>
<td>norB</td>
<td>2 nitric oxide + electron donor</td>
<td>-88.48</td>
<td>191</td>
</tr>
<tr>
<td>Methyl Halides</td>
<td>Methyl halide transferase</td>
<td>mht</td>
<td>SAM + halides</td>
<td></td>
<td>124, 125</td>
</tr>
<tr>
<td>Methanethiol</td>
<td>Dissimilatory sulfite reductase</td>
<td>dsr</td>
<td>SAM or methoxylated aromatic compounds + sulfite</td>
<td>5.95</td>
<td>149</td>
</tr>
<tr>
<td>Ethylene</td>
<td>Ethylene forming enzyme</td>
<td>efe</td>
<td>Oxygen + α-ketoglutarate</td>
<td>-103.7</td>
<td>188, 189</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>Alcohol dehydrogenase</td>
<td>adh</td>
<td>ethanol + NAD+</td>
<td>20.2</td>
<td>190, 191</td>
</tr>
</tbody>
</table>
1.4. Thesis overview

This thesis describes the first effort to utilize gas-producing enzymes as a reporting method for synthetic genetic circuits within hard-to-image soil samples. For my proof-of-concept applications, I apply gas reporters to study matrix effects on the concentrations of quorum sensing molecules and horizontal gene transfer through conjugation. In Chapter 2, I describe my studies examining the stability of quorum sensing molecules under the addition of a type of common soil amendments, biochar. This collaborative research provided evidence that two environmental parameters, pH and soil surface area, affect quorum sensing molecule bioavailability and thus influence microbial behaviors. In Chapter 3, I detail my efforts to engineer a ratiometric gas reporting method for whole-cell bioreporters. As proof-of-concept experiments, I constructed bioreporters that sense two different AHLs and applied them to measure AHLs production and degradation in situ in a loamy agricultural soil. In Chapter 4, I describe a second proof-of-concept application of gas reporters in the study of plasmid conjugation. This study demonstrates that gas production can be used to infer the viable transconjugant cell number in a soil sample, and this platform can be applied to screen environmental conditions that influence horizontal gene transfer. Lastly, in Chapter 5, I discuss future directions for refining gas reporting methods and potential research questions that can be answered using the new genetic outputs that I developed through my thesis work.
Chapter 2: Charcoal disrupts soil microbial communication through a combination of signal sorption and hydrolysis

(This chapter is based on the publication “Charcoal Disrupts Soil Microbial Communication through a Combination of Signal Sorption and Hydrolysis” and has been reformatted to departmental guidelines.)
2.1. Summary

Charcoal’s presence in soil triggers a range of biological effects not yet predictable, in part because it interferes with the functioning of chemical signals that microbes release into their environment to communicate. The mechanisms by which charcoal alters the biologically-available concentrations of these intercellular signals were not fully understood. Recently charcoal was shown to sorb the signaling molecules that microbes release, rendering them ineffective for intercellular communication. In this chapter, I describe the investigation of potentially more important mechanism of interference: signaling molecule hydrolysis driven by charcoal-induced soil pH changes. I describe the results from experiments examining the effects of ten charcoals on the bioavailable concentration of an acyl-homoserine lactone (AHL) used by many gram-negative bacteria for cell-cell communication. The results from these measurements show that charcoals decrease the level of bioavailable AHL through sorption and pH-dependent hydrolysis of the lactone ring. I then built a quantitative model that predicts the half-lives of different microbial signaling compounds in the presence of charcoals varying in pH and surface area. This model results suggest that the chemical effects of charcoal on pH-sensitive bacterial AHL signals will be fundamentally distinct from effects on pH-insensitive fungal signals, potentially leading to shifts in microbial community structures.
2.2. Introduction

Charcoal intentionally added to soil (called biochar) can trigger a wide range of biological effects, such as changing microbial community structure, soil nitrogen cycling, and plant-microbe symbiosis. Charcoal has also been shown to alter the rate at which microbes decompose soil organic carbon, inducing both increases and decreases in soil organic carbon mineralization. Interference with microbial cell-cell signaling (e.g., quorum sensing) is likely one of the mechanisms driving charcoal-induced microbial responses. In quorum sensing, microbes regulate physiological activities based on their population density. To sense population density, microbes synthesize chemical signals (called autoinducers) that diffuse across cell membranes and use the accumulation of these molecules to activate receptors that control gene expression. Early studies of quorum sensing linked this community-level decision making to bioluminescence, biofilm formation, virulence, plasmid transfer, and antibiotic synthesis. Increasing evidence points to roles for quorum sensing in the regulation of key processes in the biogeochemical cycling of carbon and nitrogen, such as development of the microbe-plant symbioses critical for nitrogen fixation, the secretion of enzymes that destabilize soil organic matter, and the production of methane by some archaea. Cell-cell signaling has also been shown to regulate the growth of ammonia oxidizing bacteria and the production of N2O after starvation, and it has been shown to downregulate denitrification. While charcoal has been proposed to alter microbial behaviors by interfering with quorum sensing, our fundamental understanding of charcoal’s chemical
reactivity with signaling molecules remains too limited to predict how the physicochemical properties of a given charcoal will impact signaling within a single microbial species or how charcoal will differentially affect bacteria and fungi that use distinct chemical signals for communication.

Processes that decrease the concentration of intercellular signaling molecules increase the population density required to trigger signal-dependent behaviors like $N_2O$ production, $N$ fixation, or production of some enzymes that decompose soil organic carbon.$^{211,214–216}$ The concentration of signaling molecules can be diminished ("quenched") by enzymatic degradation,$^{217}$ sorption to abiotic materials, abiotic degradation reactions,$^{136,139}$ and interference by other chemicals.$^{93}$ Charcoal has recently been proposed to contribute to the quenching of cell-cell communication in the environment,$^{203,218}$ displaying quenching activities that correlate with amendment amounts and production conditions.$^{209}$ The strong sorptive affinity that charcoal has for apolar organic compounds$^{219–222}$ suggests that it will efficiently sorb the diverse types of apolar signaling molecules synthesized by archaea, bacteria, fungi and plants. Indeed, recent work demonstrated that charcoal disrupts the cell-cell signaling mediated by $N$-3-oxo-dodecanoyl-L-homoserine lactone,$^{209}$ an AHL intercellular signaling molecule used by many gram-negative soil bacteria to regulate gene expression. In that study, charcoal quenching of cell-cell communication correlated with charcoal surface area (SA), which also covaried with charcoal production temperature.

While previous work showed a role for SA in charcoal quenching of quorum sensing, charcoal has many physicochemical properties that can vary with
pyrolysis temperature and feedstock, including alkalinity, aromaticity, density, hydrophobicity, and porosity.\textsuperscript{223–226} Among these properties, I hypothesized that alkalinity would also influence microbial quorum sensing because some autoinducers have structures that are sensitive to elevated pH.\textsuperscript{136} In the case of AHLs, alkalinity causes hydrolysis of the lactone ring, yielding an acyl-homoserine (Acyl-HS) product that can be inactive for cell-cell signaling.\textsuperscript{136,139} This susceptibility to hydrolysis at elevated pH suggests that the alkalinity of some charcoals may decrease AHL levels, perhaps even more efficiently than surface sorption. The effects of charcoal on AHL hydrolysis kinetics are expected to vary, because ash produced during pyrolysis can lead to a wide range of charcoal pH values (at least 5 to 10).\textsuperscript{227} The extent to which alkalinity contributes to charcoal quenching of AHL signaling has not been evaluated, and it is unclear how the rates of AHL inactivation through sorption and hydrolysis relate to one another.

The objective of this study was to determine the effect of charcoal alkalinity on microbial AHL signaling. Through a collaboration with others, I analyzed the effect of ten charcoals on AHL stability. I used the results to build a model that explores how charcoals with different SA and pH values will affect the half-life of an AHL signaling molecule. \textit{N}-3-oxo-dodecanoyl-L-homoserine lactone was used as a representative AHL because charcoal is known to influence the bioavailable levels of this signaling molecule.\textsuperscript{209} The experimental data and model provide evidence that the rate of AHL quenching varies by more than $10^4$-fold over a range of pH and SA values found in charcoals created from five different feedstocks. The
model also enables the comparison of the relative effects of charcoal on AHLs with its effects on a representative fungal signaling molecule, farnesol.

### 2.3. Charcoal effects on AHL and pH

Prior studies have reported that alkaline conditions accelerate hydrolysis of the AHL lactone ring,\textsuperscript{136,139} suggesting that some alkaline charcoals may destabilize these cell-cell signaling molecules through hydrolysis. To test this idea, the relationship between charcoal-induced pH changes and the bioavailable level of AHL after one hour of exposure to charcoal was examined. For this analysis, the pH of charcoal-treated AHL solutions was measured at the end of each exposure as well as the solutions' ability to induce a GFP signal in a microbial assay. In all cases, increasing charcoal concentrations was found to cause an elevated pH (Figure 2-1). To determine how pH relates to quenching of the AHL-dependent GFP signal across different feedstocks, the pH and GFP signal from every experiment (Figure 2-2) was compared. Below pH 8, the GFP signal was similar to that observed in the absence of charcoals, while the GFP signal decreased above pH 8. A linear fit to the data acquired using all of the charcoals yielded a strong correlation coefficient ($R = 0.95$) for the inverse relationship between charcoal pH and GFP signal. Strong linear correlations were also obtained when the charcoals from each pyrolysis temperatures was analyzed separately (Figure 2-3).

The inverse correlation between charcoal pH and the GFP signal suggested that AHL hydrolysis might at times account for a significant fraction of the decrease
For each of the experiments shown in Figure S2, we measured the solution pH at the end of reactions to establish how increasing concentrations of each charcoal influence solution pH. All measurements were performed in triplicate and are reported as the mean ±1σ.
Figure 2-2. Charcoal-induced pH increases are inversely correlated to bioavailable AHL

The relative fluorescence of *E. coli* mixed with charcoal-treated AHL is compared with the pH after exposure for 1 hour to 1, 5, 10, 25, and 50 mg/mL of wheat straw, oilseed rape, softwood, miscanthus straw, and rice husk charcoals. Measurements were performed using 550°C (open symbols) and 700°C (closed symbols) charcoals in triplicate and are reported as the mean ±1σ. An R = 0.95 was obtained from a linear fit (y = 9.9865 − 2.5809x) to the data. All measurements were performed in triplicate and are reported as the mean ±1σ.
Figure 2-3. Relationships between charcoal pH and AHL-dependent GFP expression

The relative fluorescence of *E. coli* mixed with charcoal-treated AHL is compared with the pH of each AHL-containing solution after reaction for 1 hour with charcoals generated through pyrolysis at (A) 550°C and (B) 700°C. Linear fits to the 550°C (Relative fluorescence = 3.4688 − 0.34357pH) and 700°C (Relative fluorescence = 3.7001 − 0.36549pH) charcoal data yield R = 0.947 and 0.958, respectively. Error bars represent ±1σ calculated using three independent measurements.
in bioavailable AHL. To evaluate the relative contributions of AHL surface sorption and hydrolysis under the conditions of our experiment, the ability of the bioreporter to detect AHL after the AHL had been exposed to acidified charcoal was tested.

Because the AHL hydrolysis rate becomes significant above pH 7\(^{136}\), whether charcoals have smaller effects on bioavailable AHL when adjusted to a lower pH value was investigated. For this experiment, the effect of pH-adjusted and untreated miscanthus straw charcoal (550°C) on the GFP signal was analyzed in the bioassay. Miscanthus straw charcoal had a very strong buffer capacity, presenting elevated pH values even after initial pH adjustment (Figure 2-4). The acid-treated miscanthus straw had pH values that ranged from 4 to 7 while the untreated miscanthus straw ranged from 6 to 10. Without pH adjustment, treatment of an AHL solution with 10 mg/mL charcoal decreased the GFP signal to a half-maximal value and yielded a pH ~9. In contrast, with pH adjustment, the GFP signal only decreased by ~20% at the highest charcoal concentration analyzed, 50 mg/mL. This charcoal-treated solution had a near-neutral pH. These results suggest that the large decrease in bioavailable AHL observed with the untreated miscanthus straw charcoal arises to a large extent because charcoal-induced pH changes accelerate AHL hydrolysis. The smaller loss of bioavailable AHL with acidified charcoal is interpreted as arising from sorption because this analysis was performed under pH conditions that are not predicted to hydrolyze AHL.

The experimental results indicated that charcoals decrease the bioavailable AHL during the time course of our experiments by two mechanisms. As previously
Figure 2-4. Charcoal acidification increases bioavailable AHL
(A) Varying concentrations of 550°C miscanthus straw charcoal were acidified to pH 3 and exposed to AHL. After 1 hour, the pH and the level of GFP expression induced by the AHL remaining in solution were measured. (B) Effects of untreated miscanthus straw charcoal (550°C) on GFP expression and pH. Error bars represent ±1σ calculated using three independent measurements.
suggested, charcoals decrease the bioavailable concentration of AHL through sorption. In addition, charcoals convert biologically-active AHL into soluble Acyl-HS, which is no longer biologically active. To directly investigate whether AHL is converted into Acyl-HS prior to charcoal sorption as predicted from the second mechanism (Figure 2-5 A), low and high pH charcoals was exposed to AHL for 1 hour, split the aqueous portion of each sample, acidified one fraction while leaving the other sample untreated, and measured the concentration of bioavailable AHL in each sample. When this analysis was performed with oilseed rape 550°C charcoal, one of the more alkaline charcoals, the acid-treated fractions yielded AHL concentrations that were significantly higher than that observed in the untreated fraction (Figure 2-5 B). Because acidic conditions promote the dehydration of Acyl-HS back to AHL, the increased yield of AHL with acid-treated fractions provides evidence for Acyl-HS accumulation in the presence of this charcoal. When this analysis was performed with the charcoal having the lowest pH (softwood 550°C), the acid-treated fractions yielded AHL concentrations that were not significantly different from the untreated fraction. Together, these results lead us to conclude that charcoal alkalinity contributes to the loss of AHL by hydrolyzing this signaling chemical.
Figure 2-5. Charcoals can convert AHL into soluble Acyl-HS prior to sorption

(A) AHL can be inactivated through pH-dependent hydrolysis ($k_{hyd1}$), pH-independent hydrolysis ($k_{hyd2}$), and sorption to charcoal ($k_s$). AHL can also be generated by dehydrating Acyl-HS ($k_{dehyd1}$ and $k_{dehyd2}$). (B) Oilseed rape and softwood 550°C charcoals were reacted with AHL for 1 hour and the fraction of soluble AHL was measured (untreated) as well as the total concentration of AHL and Acyl-HS. The latter was quantified by acidifying samples for 90 and 135 minutes (acid-90m and acid-135m) prior to analyzing the AHL levels. The AHL in the acidified samples from the oilseed rape reactions were significantly higher than the untreated fraction (two-tailed t test; $p < 0.01$), while no significance difference was observed between the untreated and acidified samples from softwood. Error bars represent ±1σ calculated using three independent measurements.
2.4. Modeling charcoal effects on AHL.

To predict how charcoals with different SAs and alkalinites affect AHL concentrations, I built a kinetic model that reports how AHL levels change with time upon exposure to charcoals having different physicochemical properties. In this model, the concentration of biologically-available AHL depends upon pH-dependent and pH-independent AHL hydrolysis reactions ($k_{\text{hyd}1}$ and $k_{\text{hyd}2}$), pH-dependent and pH-independent Acyl-HS dehydrations reactions ($k_{\text{dehyd}1}$ and $k_{\text{dehyd}2}$), and AHL and Acyl-HS sorption reactions ($k_s$). The model also considers the concentrations of soluble [AHL] and [Acyl-HS], pH, and charcoal SA. The rate constants describing hydrolysis and dehydration reactions were obtained by globally fitting the kinetics of AHL hydrolysis to our model (Figure 2-6).

As a frame of reference for the model, I analyzed the relative influence of pH and SA of the UKBRC charcoals on the AHL-dependent GFP signal using the microbial assay (Figure 2-7 A). At the one-hour time point, the GFP signal displayed a stronger correlation with pH compared with SA. When I fit the kinetic model to the data from this time point, using kinetic values for hydrolysis and dehydration measured in the absence of charcoal, I obtained a sorption rate constant $k_s = 0.0039 \text{ h}^{-1} \cdot \text{mM}^{-1}$ that allowed me to estimate the effects of charcoals on AHL activity over a greater set of pH and SA values (Figure 2-7 B). I used the model with this $k_s$ value to calculate time-dependent changes in AHL over the same range of physicochemical charcoal parameters (Figure 2-8 A). This analysis revealed that AHL half-life varies up to 5620-fold across the different pH and SA values analyzed (Figure 2-8 B). For these calculations, I used charcoal SAs that
overlap with the range that has been used for soil amendment.\textsuperscript{201} A smaller half-life range (273-fold) was observed when calculations were performed with farnesol (\textbf{Figure 2-8 C}), a fungal autoinducer that lacks the pH-sensitive lactone found in AHLs.\textsuperscript{228}
Figure 2-6. Effect of pH on AHL hydrolysis kinetics
Solution containing 50 µM AHL were reacted for different lengths of time (0.016, 0.25, 1, 2, and 24 hours) at four different pH conditions (6.7, 8.1, 9.6, 10.5). The lines represent a global fit of all kinetic data simultaneously to the ODE model that considers the two different mechanisms of hydrolysis, which yielded rate constants for pH-dependent ($k_{hyd1} = 8.36$ mM$^{-1}$hr$^{-1}$; $k_{dehyd2} = 2.03 \times 10^4$ mM$^{-1}$hr$^{-1}$) and pH-independent ($k_{hyd1} = 0.46$ hr$^{-1}$; $k_{dehyd2} = 0.085$ hr$^{-1}$) reactions.
Figure 2-7. A kinetic model captured the concentration change of AHL after reaction with the UKBRC charcoals for 1 hour
(A) The microbial assay results described in Figure S2 were plotted as a function of pH and SA to illustrate the fraction of AHL-dependent GFP signal remaining after incubation of different concentrations of each charcoal with AHL. Total SA was calculated as the product of the mass of a charcoal added in grams (g) and the value obtained from BET analysis (m^2/g). (B) A kinetic model was used to calculate the concentration of AHL that remains biologically available after 1 hour reaction with charcoals varying in a range of pH and SA properties. The color gradient indicates the concentration of biologically available AHL at the end of the reaction.
Figure 2-8. Modeling the effect of charcoals on AHL availability.
The kinetic model suggested that the charcoal pH has a more immediate effect on the half-life of AHL compared to charcoal surface area. (A) AHL concentration changes over time with different physiochemical charcoal parameters. The color gradient represents the AHL remaining after mixing with charcoal. The calculated half-lives of (B) AHL and (C) farnesol when reacted with charcoals having a range of SA and pH values. The colors represent the logarithm of the calculated half-lives in hours.
2.5. Environmental Implications

The experimental results provide evidence that charcoals can decrease the concentrations of autoinducers used for cell-cell signaling through multiple chemical mechanisms, including sorption and hydrolysis. Because these reactions occur through distinct mechanisms, their rates are controlled by distinct physicochemical properties of charcoals. With sorption, charcoal SA will control AHL sorption as observed with apolar chemicals.\(^{219-222}\) In contrast, hydrolysis is predicted to depend upon charcoal alkalinity, which varies with feedstock and pyrolysis conditions,\(^{224,225}\) and does not always correlate with SA. Microbes will likely respond to charcoal-induced AHL depletion, and this response will depend on soil type, ecosystem conditions, and charcoal amendment rate. Some soils may buffer the pH effects arising from charcoal addition, particularly those soils rich in organic matter and metal oxides (iron and aluminum) that have a strong buffering capacity.\(^{210}\) In these soils, the results predict that charcoals will quench AHL signaling primarily through sorption. In soils having limited buffering capacities, charcoals will quench AHL signaling through both hydrolysis and sorption. The observed AHL concentrations in a given environment may be influenced by the presence of lactonases, enzymes synthesized by soil microbes that catalyze AHL hydrolysis.\(^{217}\) Whether or not the activity of these enzymes is modulated by charcoal addition is not known.

The relative contributions that sorption and hydrolysis make to charcoal quenching of microbial communication are expected to vary across species due to the structural and chemical diversity in signaling molecules.\(^{93}\) Our findings suggest
that within a single soil, the rates of charcoal quenching of different cell-cell communication reactions will vary. I expect charcoal to sorb (and quench) all types of signaling compounds used for cell-cell communication as previously proposed, although the extent and rate of sorption may vary across with signaling molecule structure. However, only a subset of signaling compounds will be hydrolyzed by charcoal-driven pH-shifts. In the case of farnesol, a fungal autoinducer, I predict sorption to be the dominant mechanism by which charcoal could decrease the bioavailable concentration, since this chemical does not contain functional groups whose stability varies with pH like AHL. However, other classes of signals may be sensitive to charcoal-induced pH changes like AHL. For example, oligopeptide autoinducers display a charge that depends upon soil pH, since they contain functional groups whose protonation can change. This protonation is not expected to promote hydrolysis as observed with AHL, but could alter the charcoal sorption capacity by changing oligopeptide charge and altering ionic interactions as observed with other chemicals whose protonation changes with pH.

My modeling results suggest that the complex biological effects of charcoal in the environment, such as changes in microbial community structure, could arise because charcoals differentially affect microbial species by altering cell-cell signaling that enhances or inhibits growth. Indeed, both inhibitory and stimulatory effects on microbial physiological activities and population induced by charcoal have been reported. In one incubation study, the biomass of gram-negative bacteria was significantly increased as a result of soil charcoal addition, whereas
fungal and gram-positive bacterial biomass was less affected.\textsuperscript{1} In another field study, charcoal increased both bacterial and fungal populations. However, in this case, charcoal shifted the microbial community toward a greater relative amount of bacteria.\textsuperscript{2} In both studies, no clear mechanism was established for the charcoal-mediated microbial responses. Additional research is needed to establish how charcoal quenching of cell-cell communication relate to dynamic community level changes \textit{in situ}. The recent development of bioreporters that provide dynamic information on biological processes from within a soil should aid in future studies examining charcoal effects on cell-cell signaling.\textsuperscript{233}

\textbf{2.6. Materials and Methods}

\textbf{Materials.} \textit{Escherichia coli} XL1-Blue was from Stratagene, AHL (\textit{N}-3-oxo-dodecanoyl-L-homoserine, \textit{N}-3-oxo-C12 HSL) was from Cayman Chemical, and all other reagents were from Sigma-Aldrich, VWR or BD Biosciences. Luria-Bertani (LB) growth medium contained 100 mM 3-(N-morpholino) propanesulfonic acid (MOPS) pH 7. MOPS was included to buffer the growth medium against pH changes arising from reaction with the charcoal-treated solutions.

\textbf{Charcoal production.} We used charcoals obtained from the UK Biochar Research Center (UKBRC, University of Edinburgh, UK) prepared from oilseed rape, wheat straw, miscanthus straw, mixed softwood, and rice husk feedstocks. Each feedstock was subjected to slow pyrolysis at 550 and 700°C under N\textsubscript{2} in a pilot-scale pyrolysis unit with a continuous feed rotary kiln. All feedstock materials were pelletized to a size of 5 × 20 mm before pyrolysis except for the rice husk.
Charcoals were gently crushed by mortar and pestle and sieved to obtain the materials that were <1.4 mm.

**Charcoal characterization.** Samples were degassed in glass cells, vacuum dried overnight at 200°C, and analyzed using a Quantachrome Autosorb-3b Surface Analyzer. N\textsubscript{2} adsorption/desorption isotherms were obtained at 77 K by a 26-point analysis for relative pressures P/P\textsubscript{0} from 1.21 \times 10\textsuperscript{-4} to 0.99, where P is the adsorption equilibrium pressure and P\textsubscript{0} is the vapor pressure of bulk liquid N\textsubscript{2}. Specific SA was calculated using Brunauer-Emmett-Teller (BET) theory. Charcoal pH was measured using a 1:20 charcoal/water mixture (w/w) after a 1.5 hour reaction while shaking. The UKBRC charcoals exhibit a large variation in SA, 1.9 - 145.0 m\textsuperscript{2} g\textsuperscript{-1}, and pH, 7.88 - 10.69 (Figure 2-9). Those with the highest SA (softwood) displayed the lowest pH, while those with the lowest SA (oilseed rape) exhibited high pH.

**Microbial assay.** Physiologically and environmentally relevant AHL concentrations are on the order of 100x lower than the detection limit of commonly available forms of analytical instrumentation, *e.g.*, GC-MS.\textsuperscript{234} Therefore, a microbial bioreporter was used to detect the presence of biologically active AHL, using an approach previously described by our group.\textsuperscript{209} This bioreporter has a detection threshold of 100 pM, while the detection threshold of our GC-MS was ~5 \textmu M, similar to previous studies.\textsuperscript{235} Varying concentrations of UKBRC charcoals (0, 1, 5, 10, 25, and 50 mg/mL) were added to water containing 2 \mu M AHL for 60 minutes at 23°C while shaking at 100 rpm. Charcoal was removed through centrifugation (13,000 g, 1 min), the pH of the supernatant fraction was analyzed
Figure 2-9. Physical and chemical characterization of the UKBRC charcoals

(A) N\textsubscript{2} sorption analysis was applied to charcoals generated at 550°C and 700°C, and surface area was calculated using BET. The charcoals were prepared from oilseed rape (OR), wheat straw (WS), miscanthus straw (MS), soft wood (SW), and rice husk (RH) feedstocks. (B) The pH of 1 mL solutions reacted with 50 mg charcoal (a 1 to 20 solid/liquid ratio) for 1.5 hours at 25°C. (C) Comparison of charcoal surface area and pH reveal that soft wood displays the highest surface area and lowest pH values, while oilseed displays the lowest surface area and higher pH values. All measurements were performed in triplicate and are reported as the mean ±1 standard deviation.
using a micro pH electrode, and aliquots of the supernatant were mixed in a 96-well plate (Corning Costar) with identical volumes of LB-MOPS containing _E. coli_ (at an OD\(_{600} = 0.05\)) transformed with the receiver plasmid and 50 µg/mL kanamycin. The receiver plasmid encoded a GFP that is only expressed in the presence of AHL.\(^{11}\) Cells were incubated at 30°C while shaking at 250 rpm for 18 hours, and whole cell fluorescence (\(\lambda_{ex} = 488\;\text{nm}\); \(\lambda_{em} = 509\) nm) and absorbance (600 nm) was measured using a TECAN M1000 plate reader. This long incubation was used to ensure that our assay was able to report on low levels of AHL remaining in solution after charcoal incubations. In all experiments, cultures grew to a similar maximal density as observed previously when this bioreporter was used to study charcoal effects on AHL bioavailability.\(^{209}\) To account for well-to-well variation in cell density, fluorescence was normalized to absorbance. Data are reported as the fraction of the signal obtained with AHL that had not been incubated with charcoal (Figure 2-10). All data represent the average of three or more independent incubation experiments. For each incubation, three replicate measurements were performed in parallel.

**Acidified charcoal.** We arrayed UKBRC charcoals in 24-well plates in 2 mL of sterile water at different concentrations (0, 1, 5, 10, 25, and 50 mg/mL). Each well was acidified with HCl to pH 3 and shaken at 100 rpm overnight at 23°C. Depending on the biochar, different HCl concentrations were used to minimize changes in liquid volume. The pH was measured a second time and samples were acidified again to pH 3 using HCl. AHL was then added to each sample to a concentration of 2 µM and held at 23°C for 60 min while shaking. At the end of
Figure 2-10. Effect of charcoal concentration on AHL-dependent GFP expression within *E. coli*

Varying concentrations of each charcoal were reacted with AHL for 1 hour, biochars were pelleted by centrifugation, and the soluble fractions were added to cells programed to produce GFP upon exposure to AHL. After overnight growth, AHL-induced GFP expression was quantified by measuring green fluorescence and normalizing the whole cell fluorescence signal to the cell density. The relative fluorescence values in the presence of charcoals were scaled against the signal obtained with untreated AHL. All measurements were performed in triplicate and are reported as the mean ±1σ.
these reactions, the concentration of soluble AHL and solution pH were measured as described above.

**AHL hydrolysis kinetics.** To develop a model that predict the rates of lactone hydrolysis in the presence of charcoals having a range of pH and SA properties, I needed the AHL hydrolysis kinetic parameters so that I could constrain the model. The pH-dependent hydrolysis of AHL was measured using GC-MS, which necessitated the use of a high AHL concentration (0.05 mM). Hydrolysis rates were determined at 25°C by adjusting solutions containing AHL concentrations suitable for GC-MS analyses to different pH values (6.7, 8.1, 9.6, 10.5) using 0.1 N NaOH. At different times after pH adjustment (1 minute, 15 minutes, 1 hour, 2 hours, and 24 hours), each sample was then split into two fractions. One fraction was mixed with an equal volume of chloroform to extract the soluble AHL, and the concentration of AHL (termed \([\text{AHL}]_{\text{soluble}}\)) was analyzed using GC-MS. The other fraction was acidified to pH 3 using HCl, shaken for 15 min to convert acyl-HS that formed during the reaction into AHL, and analyzed for AHL content using GC-MS. This 15 minute reaction time was chosen because we found that it was consistently sufficient to recover > 85% of the AHL after 24 hour reactions (**Figure 2-11**). The AHL measured in the latter fraction represents the sum of the AHL and acyl-HS at the end of each reaction, *i.e.*, \([\text{AHL}]_{\text{soluble}} + [\text{acyl-HS}]_{\text{soluble}}\). The fraction of non-hydrolyzed AHL at each time point was calculated by dividing the GC-MS signal obtained from the untreated sample by the signal obtained with the acid-treated sample.
Figure 2-11. Recovery of AHL using acidification
(A) Solution containing 50 μM AHL were adjusted to different pH values (6.7, 8.1, 9.6, 10.5), and reacted for 24 hours. At the end of the reaction, samples were split into two fractions, one fraction was adjusted to pH 3 while the other fraction was left untreated. (B) GC-MS signal obtained from acidified and untreated samples. As a control AHL was reacted in a solution at a low pH that favors conversion of Acyl-HS into AHL. Error bars represent ±1σ calculated using three independent measurements.
Gas chromatography-mass spectrometry. The rate of lactone hydrolysis was measured using GC-MS. Chloroform extractions were transferred to 2 mL amber vials and analyzed using a Agilent 6890 equipped with a capillary column (30 m × 250 μm ID and 0.25 μm film thickness) coated with 5% Ph Me siloxane in selective ion monitoring mode. Samples were injected at splitless mode using He as a carrier gas. The injector temperature was set at 200°C, and the oven was set to hold at 150°C for 1 min, and then to increase by 20°C min⁻¹ to 230°C, 10°C min⁻¹ from 230 to 260°C, and 20°C min⁻¹ from 260 to 300°C. A solvent delay (3 min) was used to prevent interference from the extraction solvent.

Accumulation of Acyl-HS. To establish whether Acyl-HS accumulates in the aqueous phase after exposing AHL to charcoals, oilseed rape and mixed softwood 550°C charcoals (5 mg/mL) were placed in petri dishes (60 x 15 mm) containing 2 µM AHL and 5 mL water. After 1 hour at 23°C while shaking at 50 rpm, the liquid in petri dishes was transferred to a sterile tube, and centrifugation was used to remove charcoal. One supernatant fraction was used to measure the pH, a second fraction was analyzed using the microbial bioassay to determine the AHL concentrations, and a third fraction was acidified to pH <3 using HCl and reacted for 90 and 135 minutes prior to analysis for AHL content. In each experiment, AHL concentration was measured using serial dilutions (1, 5, 25, 125, and 625x) of the acidified and untreated fractions. To calculate the AHL concentration, the signal from each experiment was compared with the signal in a standard curve generated by growing cells in the presence of different AHL concentrations and fitting the data to the Michaelis-Menten equation. The two dilutions that yielded signals within the
most sensitive range of the microbial assay (0.4 to 40 nM) were used to calculate AHL concentrations. This approach minimized error in estimates by avoiding GFP signals that were too near saturation or background.

**Modeling.** The model consisted of five first order ordinary differential equations that described the time-dependent concentrations of the different molecular species that can arise, including: (i) aqueous soluble AHL [AHL], (ii) aqueous soluble Acyl-HS [HS], (iii) insoluble charcoal-bound [AHL-BC], (iv) insoluble charcoal-bound Acyl-HS [HS-BC], and (v) charcoal binding sites [BC]. The time-dependent AHL and Acyl-HS concentrations are described by:

**Equation 2-1**
\[
\frac{d[AHL]}{dt} = -(k_{\text{hyd1}}[OH^-] + k_{\text{hyd2}}) [AHL] + (k_{\text{dehyd1}}[H^+] + k_{\text{dehyd2}}) [HS] - k_s[AHL][BC]
\]

**Equation 2-2**
\[
\frac{d[HS]}{dt} = (k_{\text{hyd1}}[OH^-] + k_{\text{hyd2}}) [AHL] - (k_{\text{dehyd1}}[H^+] + k_{\text{dehyd2}}) [HS] - k_s[Acyl-HS][BC]
\]

In this model, \(k_{\text{hyd1}}\) and \(k_{\text{hyd2}}\) represent the hydroxide dependent and independent rates of hydrolysis.\(^{237}\) The dehydration of Acyl-HS is described with the hydrogen ion-dependent term \(k_{\text{dehyd1}}\) and the independent term \(k_{\text{dehyd2}}\). Because of the chemical and structural similarities in AHL and Acyl-HS, their rates of association with charcoal were represented by a single rate constant, \(k_s\). This binding reaction was assumed to be irreversible. The concentrations of charcoal-bound AHL [AHL-BC] and Acyl-HS [HS-BC] are described by **Equation 2-3** and **Equation 2-4**:

**Equation 2-3**
\[
\frac{d[AHL-BC]}{dt} = k_s[AHL][BC]
\]

**Equation 2-4**
\[
\frac{d[HS-BC]}{dt} = k_s[HS][BC]
\]
The concentration of charcoal binding sites [BC] is described by Equation 2-5:

\[ \frac{d[BC]}{dt} = -k_s([AHL] + [HS])[BC] \]

I obtained the rate constants for AHL hydrolysis (\(k_{\text{hyd1}}\), \(k_{\text{hyd2}}\)) and Acyl-HS dehydration (\(k_{\text{dehyd1}}\), and \(k_{\text{dehyd2}}\)) by globally fitting the kinetic AHL hydrolysis data from GC-MS analysis (Figure S4) to this model. \(k_s\) was estimated by fitting the model to the result of the microbial assay. AHL half-life was calculated by fitting the time-dependent [AHL] for each SA and pH combination to the exponential decay equation (Equation 2-6):

\[ [\text{AHL}] = [\text{AHL}]_{t=0} \cdot 2\left(-t/t_{1/2}\right) \]

where \([\text{AHL}]_{t=0}\) is the initial concentration and \(t_{1/2}\) represents its half-life. Model predictions were generated using a defined range of SA (0.01 m\(^2\)ml\(^{-1}\) to 7.2 m\(^2\)ml\(^{-1}\)) and pH (6.25 to 10.5) values.
Chapter 3: Ratiometric gas reporting: a non-disruptive approach to monitor gene expression in soils

(This chapter is based on the publication “Ratiometric Gas Reporting: A Nondisruptive Approach to Monitor Gene Expression in Soils” and has been reformatted to departmental guidelines.)
3.1. Summary

Fluorescent proteins are ubiquitous tools that are used to monitor the dynamic functions of natural and synthetic genetic circuits. However, these visual reporters can only be used in transparent settings, a limitation that complicates non-disruptive measurements of gene expression within many matrices, such as soils and sediments. I describe a new ratiometric gas reporting method for non-disruptively monitoring gene expression within hard-to-image environmental matrices. With this approach, C\textsubscript{2}H\textsubscript{4} is continuously synthesized by ethylene forming enzyme to provide information on viable cell number, and CH\textsubscript{3}Br is conditionally synthesized by placing a methyl halide transferase gene under the control of a conditional promoter. I show that ratiometric gas reporting enables the creation of *Escherichia coli* bioreporters that report on acylhomoserine lactone (AHL) autoinducers used for quorum sensing by gram-negative bacteria. Using these bioreporters, I find that an agricultural soil decreases the bioavailable concentration of a long-chain AHL up to 100-fold. I also demonstrate that these bioreporters can be used in soil to non-disruptively monitor AHLs synthesized by *Rhizobium leguminosarum* and degraded by *Bacillus thuringiensis*. Finally, I show that this new reporting approach can be used in *Shewanella oneidensis*, a bacterium that lives in sediments.
3.2. Introduction

Genetically-encoded reporters that fluoresce or bioluminesce enable visualization of gene expression in diverse organisms.\textsuperscript{239,240} These reporters transformed biology by providing fundamental insight into mechanisms underpinning cellular behaviors. Visual reporters are also critical in synthetic biology, enabling the evaluation of engineered genetic circuits over a range of conditions.\textsuperscript{241} However, visual reporters can only be used in transparent settings, a limitation that prevents non-disruptive measurements in many hard-to-image environmental matrices, such as soils and sediments, where \( >10^{29} \) prokaryotes are estimated to live on Earth,\textsuperscript{104} and complex feedstocks used in bioreactors for the biological production of chemicals.\textsuperscript{242}

Enzymes that synthesize rare volatile gases represent an alternative strategy for monitoring gene expression in hard-to-image materials.\textsuperscript{243} With gas reporters, the activity of the promoter controlling enzyme expression is evaluated using the concentration of the volatile product that diffuses out of the matrix.\textsuperscript{233} Gas reporting is appealing for diverse environmental and engineering applications because it has the potential to provide information on gene expression without requiring cell extraction, which can vary in efficiency across matrices.\textsuperscript{173}

By enabling non-destructive monitoring of environmental microbiology, gas reporting could simplify studies examining the effects of matrix properties on the bioavailable levels of diverse molecules, such as pollutants,\textsuperscript{229} biological signals critical for agriculture,\textsuperscript{244} and organic matter,\textsuperscript{245} whose processing contributes to both soil fertility and to greenhouse gas emissions. Unfortunately, gas reporters
cannot yet accurately report on conditional gene expression, since it remains
difficult to distinguish between gas accumulation resulting from promoter activation
versus cell growth and metabolism. This problem also arises with visual reporters,
where it has been overcome by coupling pairs of reporters to conditional and
constitutive promoters (e.g., GFP and RFP) and evaluating the ratio of their signals
(GFP/RFP).246

I hypothesized that a ratiometric gas-reporting strategy could be developed
for use in environmental matrices by normalizing the gas signal obtained from a
conditionally-expressed methyl halide transferase (MHT) to the signal obtained by
constitutively-expressed ethylene forming enzyme (EFE). I tested this idea using
*Batis maritima* MHT, which synthesizes methyl bromide (CH$_3$Br), and
*Pseudomonas syringae* EFE, which produces ethylene (C$_2$H$_4$). These enzymes
were chosen because they can be heterologously expressed as active enzymes
in *Escherichia coli*.193,194

3.3. Gas reporter characterization

To determine if EFE and MHT yield stable cellular signals across a range
of microbial growth temperatures, I first evaluated gas production using
*Escherichia coli* strains that constitutively express EFE, MG1655-efe and MHT,
MG1655-mht (*Figure 3-1 A-B*). With MG1655-efe, the C$_2$H$_4$ production rate was
similar across six hour incubations at 30 and 33°C (*Figure 3-1 C*). At 37°C, the
C$_2$H$_4$ production rate was consistently lower across all time points, indicating that
Figure 3-1. Effect of temperature on volatile gas production rates
(A) In MG1655-efe, EFE uses α-ketoglutarate (AKG) and oxygen as substrates to produce C$_2$H$_4$. (B) In MG1655-mht, MHT uses S-Adenosyl Methionine (SAM) and bromide (Br$^-$) as substrates to synthesize CH$_3$Br and S-Adenosyl Homocysteine (SAH). (C) The C$_2$H$_4$ and (D) CH$_3$Br production rates of MG1655-efe and MG1655-mht in M63 liquid medium at 30°C, 33°C, and 37°C. Rates were calculated as the gas produced per hour per CFU following a 1 hour incubation. At 33°C, the C$_2$H$_4$ production rate did not significantly change across the different time points (one-way ANOVA, $p > 0.05$). Error bars represent ±1σ from three experiments and are only plotted when larger than symbols.
this enzyme is temperature sensitive. With MG1655-mht, in contrast, the CH₃Br production rate increased with time across all temperatures tested (Figure 3-1 D). MHT and EFE expression did not result in any apparent fitness burdens (Figure 3-2). All subsequent gas measurements were performed at 33°C to maximize both gas signals.

To demonstrate that EFE and MHT can be used as reporters in matrices, I first evaluated their signals using an artificial soil with a well-defined matrix mimicking soil texture, and then transitioned to an agricultural soil (Figure 3-3). For these incubations, I used environmentally-relevant hydration (75% water holding capacity) and constant nutrient levels. When MG1655-efe was incubated in the artificial soil, the C₂H₄ production rates changed by <25% over six hours (Figure 3-4 A). When MG1655-mht was incubated in the artificial soil, the rate of CH₃Br production was lower in the first hour and then increased to a constant level (Figure 3-4 B). Similar experiments performed using an agricultural soil from the NSF Kellogg Biological Station (KBS) Long Term Ecological Research (LTER) site (loamy Typic Hapludalf 0-5 cm Ap horizon) revealed that the C₂H₄ production rate stabilizes after two hours (Figure 3-4 C). In contrast, the CH₃Br production rate required three hours to stabilize (Figure 3-4 D). MG1655-efe and MG1655-mht grew to a similar extent in both soils (Figure 3-5).
Figure 3-2. Effect of temperature on cell growth
Following GC-MS measurements of C₂H₄ and CH₃Br, the optical density (OD₆₀₀) of (A) MG1655-efe, (B) MG1655-mht, and (C) MG1655 were evaluated hourly in M63 liquid medium. Error bars represent ±1σ calculated using three replicates and are only shown when larger than the symbol.
Figure 3-3. Illustration of soil matrices and experimental setup for non-disruptive measurements of the gas reporter
Artificial soil (top) was created using quartz (SiO₂) particles that have the same particle size distribution as the KBS soil (bottom), which is 65% sand (2 to 0.05 mm particles), 27% silt (0.05 to 0.002 mm), and 8% clay (<0.002 mm). With each soil, autoclaved and dried soil materials (800 mg) were added into each vial, and cells suspended in M63 medium (200 µL) were mixed into the soils (MG1655-efe, MG1655-mht, and MG1655). Vials were sealed with gastight caps and incubated at indicated temperatures for varying durations prior to analysis of headspace C₂H₄ and CH₃Br using GC-MS. With this workflow, distinct peaks of C₂H₄ and CH₃Br were obtained from each GC-MS measurement, which were quantified using Agilent MassHunter Workstation Software Quantitative Analysis software.
Figure 3-4. Gas production rates in matrices

(A) MG1655-efe and (B) MG1655-mht incubated at 33°C in an artificial quartz soil (80% matrix and 20% M63 medium) yielded C₂H₄ and CH₃Br that could be detected in the headspace. The C₂H₄ production rate showed no significant difference across all time points. With the CH₃Br production rates, only the first time point is significantly lower than the other time points. (C) MG1655-efe and (D) MG1655-mht grown in loamy agricultural soils held at environmentally-relevant hydration (80% soil and 20% M63 medium) also yielded strong C₂H₄ and CH₃Br production rates. The C₂H₄ production rates presented no significant differences from 120 to 360 minutes, while the CH₃Br production rate showed no significant differences between 180 and 360 minutes. Error bars represent ±1σ from three experiments and are only plotted when larger than symbols. n.s., not significant (one-way ANOVA followed by Tukey multiple comparison test, p > 0.05).
Figure 3-5. Bioreporter growth in soils
Following GC-MS measurements of C₂H₄ and CH₃Br in matrices at 33°C, the total cells were extracted from each soil, spread cells on agar plates, incubated overnight, and visually inspected to assess CFU. (A) MG1655-efe, (B) MG1655-mht, and (C) MG1655 CFU obtained from KBS soil are shown as open symbols, while those extracted from artificial soil are shown as closed symbols. Error bars represent ±1σ calculated using three replicates and are only shown when larger than the symbol.
3.4. Gas reporting of signaling compounds

I next used EFE and MHT to report on the output from synthetic genetic circuits that respond to acylhomoserine lactone (AHL) autoinducers used for quorum sensing in gram-negative bacteria. In these circuits (Figure 3-6 A), I constitutively expressed EFE, using C$_2$H$_4$ as an indicator of cell number (Figure 3-7), while AHL regulators were used to control MHT expression. I built two bioreporters. In MG1655-lux, MHT was regulated using *Vibrio fischeri* LuxR, a receptor for the short chain AHL 3-oxo-C$_6$-HSL. In MG1655-las, MHT expression was regulated using *Pseudomonas aeruginosa* LasR, a receptor for the long chain AHL 3-oxo-C$_{12}$-HSL.$^{247}$

With both AHL-sensing strains, C$_2$H$_4$ production in our standard loamy agricultural soil was not affected by AHL concentrations spanning five orders of magnitude (Figure 3-6 B-C), indicating that MHT expression does not influence EFE-dependent C$_2$H$_4$ production. In contrast, CH$_3$Br increased 14-fold in MG1655-lux (Figure 3-6 D) and ~200-fold in MG1655-las (Figure 3-6 E). I calculated the ratio of CH$_3$Br to C$_2$H$_4$ over all AHL concentrations. With this ratiometric signal, the highest concentration of 3-oxo-C$_6$-HSL resulted in a 15.3-fold increase in signal with MG1655-lux (Figure 3-6 F), while 3-oxo-C$_{12}$-HSL yielded an 81.5-fold increase in signal with MG1655-las (Figure 3-6 G). MG1655-las presented a half maximal response (EC$_{50}$) at a concentration of 3-oxo-C$_{12}$-HSL (2.6 ± 0.4 x 10$^{-7}$ M) that was >100-fold larger than previous reports,$^{248}$ while the response with MG1655-lux (EC$_{50}$ = 7.6 ± 1.3 x 10$^{-8}$ M 3-oxo-C$_6$-HSL) was more consistent with previous measurements.
Figure 3-6. Using ratiometric reporting to quantify AHLs in soil
(a) Scheme illustrating the genetic circuits used to program E. coli to report on 3-oxo-C6-HSL (MG1655-lux) and 3-oxo-C12-HSL (MG1655-las). In these circuits, EFE is expressed using a constitutive promoter ($P_1$), MHT is regulated by either the lux ($P_{lux}$) or las ($P_{las}$) promoters, and LuxR/LasR are constitutively expressed ($P_{con}$). Varying concentrations of 3-oxo-C6-HSL and 3-oxo-C12-HSL were added to the loamy agricultural soil, soils were incubated for 3 hours at 33°C with identical titers of (b, d) MG1655-lux and (c,e) MG1655-las, and the amounts of C$_2$H$_4$, (blue symbols) and CH$_3$Br (green symbols) were measured in the headspace of sample vials. This data was used to calculate the ratiometric signal (CH$_3$Br/C$_2$H$_4$) generated by (f) MG1655-lux and (g) MG1655-las in KBS soil (black symbols). Identical experiments performed using cells in M63 medium lacking soil (grey symbols) are shown for comparison. Error bars represent ±1σ calculated using three replicates and are only shown when larger than the symbol. Dashed lines represent a dose-response curve fit.
Figure 3-7. C$_2$H$_4$ production is proportional to extracted colony forming units
MG1655-efe was incubated for different times in KBS soil at 33°C, the C$_2$H$_4$ production rate (ng/hour) was measured, CFU were determined immediately following gas measurements, and the relationship between the C$_2$H$_4$ production per hour and the extracted CFU was evaluated. A linear fit to a semi-log plot yielded a correlation with $R^2 = 0.63$. 
To test whether the standard loamy agricultural soil affected the bioavailable levels of these autoinducers, AHL-induced gas production was also measured in liquid medium (Figure 3-6 F-G). With MG1655-lux, the 3-oxo-C6-HSL concentration that elicited a half maximal response was similar in liquid culture and in soil. In contrast, the 3-oxo-C12-HSL concentration required for a half maximal response ($EC_{50} = 2.1 \pm 0.6 \times 10^{-9} \text{M}$) with MG1655-las was two orders of magnitude lower in liquid culture. This finding is consistent with previous measurements that have shown that matrices decrease the bioavailable 3-oxo-C12-HSL through sorption and hydrolysis.$^{209,198}$

To investigate the reliability and robustness of the signals obtained from my bioreporters over the time required to run hundreds of samples, I measured the CH$_3$Br/C$_2$H$_4$ ratios following incubations of varying lengths with 1 µM AHL. With both bioreporters, CH$_3$Br and C$_2$H$_4$ continuously increased with time (Figure 3-8), as did the number of colony forming unit (CFU) (Figure 3-9). However, at all time points where CH$_3$Br could be detected, no significant change in the mean CH$_3$Br/C$_2$H$_4$ ratio was observed. A similar level of signal robustness was observed when an identical experiment was performed with a lower AHL concentration (Figure 3-10).

To evaluate whether ratiometric gas reporting of AHLs can be used in an organism whose native habitat is a sediment, I evaluated whether this approach can also be used with Shewanella oneidensis MR1.$^{249}$ For these experiments, I constructed a plasmid (pHC01) that constitutively expresses EFE and use 3-oxo-C12-HSL to regulate MHT expression (Figure 3-11 A). This plasmid was
The relationship between the total time where the gas was allowed to accumulate following bioreporter addition and \( \text{C}_2\text{H}_4 \), \( \text{CH}_3\text{Br} \), and the ratiometric signals for (A) MG1655-lux and (B) MG1655-las that had been added into KBS soil at 33°C containing 1 \( \mu \text{M} \) of 3-oxo-C6-HSL and 3-oxo-C12-HSL, respectively. The ratios of \( \text{CH}_3\text{Br} \) to \( \text{C}_2\text{H}_4 \) present no significant differences across all incubation times (one-way ANOVA, \( p > 0.05 \)). Error bars represent ±1σ calculated using three replicates.
Figure 3-9. The growth of MG1655-lux and MG1655-las in soil
Following GC-MS measurements of C₂H₄ and CH₃Br at 33°C, (A) MG1655-lux
and (B) MG1655-las were extracted from the KBS soil matrices that had been
incubated for varying lengths of time (1, 3, 5, 7, 9, 11, and 13 hours), and the
number of CFU was determined. Error bars represent ±1σ calculated from three
replicates.
Figure 3-10. Effect of low [AHL] on bioreporter signals in KBS soil
(A) The amount of \( \text{C}_2\text{H}_4 \) and \( \text{CH}_3\text{Br} \) produced (top) by MG1655-lux following addition to KBS soil containing 50 nM 3-oxo-C6-HSL at 33°C. At each time point (1, 3, 5, 7, 9, 11, and 13 hours), the ratio of \( \text{CH}_3\text{Br} \) to \( \text{C}_2\text{H}_4 \) (bottom) was calculated. No significant difference was observed across all incubation times (one-way ANOVA, \( p > 0.05 \)). (B) MG1655-las was added to KBS soil containing 100 nM of 3-oxo-C12-HSL. The \( \text{C}_2\text{H}_4 \) and \( \text{CH}_3\text{Br} \) at each time point (top) was used to calculate the ratio of \( \text{CH}_3\text{Br} \) to \( \text{C}_2\text{H}_4 \) (bottom). With this sensor, the ratiometric was unchanged between the 3 and 13 hour incubation times (one-way ANOVA, \( p > 0.05 \)). Error bars represent ±1σ calculated using three replicates.
Figure 3-11. Ratiometric gas reporting can be used in *Shewanella oneidensis* MR1

(A) A ratiometric bioreporter plasmid, pHCO1, was constructed that constitutively expresses a bicistronic EFE/RFP cassette and uses LasR to regulate MHT expression. This plasmid was transformed into *S. oneidensis* MR-1. (B) Gas production was measured in 2 mL vials containing liquid medium or a soil slurry at 33°C. The slurry was 70% soil and 30% LB medium. (C) *S. oneidensis* MR-1 transformed with pHCO1 was added into liquid medium and the soil slurry that had been pre-inoculated with various concentration of 3-oxo-C12-HSL, and C2H4 and CH3Br were measured 4 hours and 8 hours, respectively. The gas ratio was calculated, normalized to the highest value obtained, and a dose-response curve was fit to the data. Error bars represent ±1σ calculated using three replicates and are only shown when larger than the symbol.
transformed into *S. oneidensis*, and the effect of different concentrations of 3-oxo-C12-HSL on gas production was measured in liquid medium and a soil slurry (*Figure 3-11* B). As observed with our *E. coli* bioreporter, the AHL concentration required to achieve a half maximal response in liquid culture was lower than the concentration required for the same signal in the presence of soil (*Figure 3-11* C). These finding provides evidence that ratiometric gas reporting method can be used in different microbes to study the effects of a matrix on the bioavailable level of an AHL autoinducer.

### 3.5. Monitoring biological signal synthesis and degradation

To test whether the ratiometric gas reporters are sufficiently sensitive to monitor biological degradation of an AHL, I measured the effect of adding a lactonase-synthesizing gram-positive bacterium (*Bacillus thuringiensis, Bt*) on the stability of 3-oxo-C12-HSL in the KBS soil (*Figure 3-12* A). A range of *Bt* titers (10⁴, 10⁵, and 10⁶) were added to soil matrices that contained 3-oxo-C12-HSL. At different times following incubation of *Bt* in AHL-containing soil, MG1655-las was added to read out the effect of *Bt* on 3-oxo-C12-HSL. After 24 hours, MG1655-las presented a CH₃Br/C₂H₄ ratio that was inversely correlated with the titers of *Bt* added (*Figure 3-13*). The highest titer of *Bt* (10⁶ CFU) yielded a gas ratio that decreased exponentially with a 16.8 hour half-life (*Figure 3-12* B). At the end of the incubations, the *Bt* CFUs correlated with incubation time and titer added (*Figure 3-14*). A comparison of the individual gases at each time point attributed the signal change to a decrease in CH₃Br (*Figure 3-15*). The C₂H₄ production
Figure 3-12. Monitoring Bt degradation of AHL in soil

(A) Scheme illustrating how the E. coli MG1655-las bioreporter (cyan) was used to spy on Bt (yellow) degradation of 3-oxo-C12-HSL in soil. Bt synthesizes an AHL-degrading lactonase. Soils containing 3-oxo-C12-HSL and Bt were incubated for the indicated durations at 30°C, MG1655-las was added and the temperature was shifted to 33°C, and the gas ratio was measured 3 hours following MG1655-las addition to assess the level of 3-oxo-C12-HSL remaining in the soil. (B) The ratiometric signal generated by MG1655-las that had been added to soils containing 1 µM 3-oxo-C12-HSL and either 0 or 106 cfu of Bt. The incubation time represents how long Bt and 3-oxo-C12-HSL were allowed to incubate in the loamy agricultural soil prior to MG1655-las addition. Error bars represent ±1σ calculated using three replicates. The line represents a fit of the data to a single exponential model.
Figure 3-13. Effect of different titers of *Bt* on AHL that had been added to soil

The ratiometric signal generated by MG1655-las that had been added to soils containing 1 μM 3-oxo-C12-HSL and the indicated titers of *Bt*. The incubation time represents how long *Bt* and 3-oxo-C12-HSL were incubated with one another in the KBS soil prior to MG1655-las addition. The individual gas concentrations that were used to calculate the ratiometric signal are shown in Figure 3-15.
Figure 3-14. *Bt* extracted from KBS soil following incubations
Following C$_2$H$_4$ and CH$_3$Br measurements for each incubation at 33°C, the number of viable *Bt* cells was determined by extracting total cells, spreading cells on LB-agar plates, incubating plates overnight, and counting colonies with a *Bt*-type morphology and color. Data is shown for soil incubations initiated using $10^4$, $10^5$, and $10^6$ CFU of *Bt*. Error bars represent ±1σ calculated using three replicates and are only shown when larger than the symbol.
Figure 3-15. Gas measured above soils containing Bt, AHL, and MG1655-las
(A) The C\textsubscript{2}H\textsubscript{4} produced by MG1655-las following addition to KBS soil that had been pre-incubated with Bt and 3-oxo-C12-HSL for the indicated durations at 33 °C. (B) The C\textsubscript{2}H\textsubscript{4} accumulation divided by viable cell counts of MG1655-las on LB-agar plates shows that there is no significant difference between bioreporter CFU in the presence and absence of Bt (one-way ANOVA followed by Tukey multiple comparison test, p > 0.05). (C) The CH\textsubscript{3}Br produced by MG1655-las decreases when Bt is incubated for longer periods of time with 3-oxo-C12-HSL in KBS soils prior to bioreporter addition. (D) The ratio of CH\textsubscript{3}Br to C\textsubscript{2}H\textsubscript{4}. Error bars represent ±1σ calculated using three replicates.
remained constant across all measurements.

I next investigated whether our ratiometric bioreporters could monitor the dynamic production of multiple AHL molecules (Figure 3-16) synthesized by the soil bacterium *Rhizobium leguminosarum* (*Rl*), a nitrogen-fixing bacterium that forms symbioses with agricultural crops. Among the AHLs produced by *Rl*, MG1655-lux is specific for 3-oxo-C8-HSL, while MG1655-las is activated by 3-OH-C14:1-HSL (Figure 3-17 A-B and Figure 3-18). MG1655-las can also be activated by 3-oxo-C8-HSL, but this strain has a lower sensitivity than MG1655-lux.

To determine if *Rl* affects bioreporter gas production, I incubated *Rl* with an *E. coli* strain that constitutively produces both gases (MG1655-mht-efe). With MG1655-mht-efe, the ratiometric signal was not affected by the presence of *Rl* (Figure 3-19). I next used our bioreporters to monitor dynamic AHL production by *Rl* in the loamy agricultural soil. Using the more specific MG1655-lux bioreporter, I found that the CH$_3$Br/C$_2$H$_4$ ratio increased over a four-day incubation to a level that corresponds to $4 \times 10^{-7}$ M 3-oxo-C8-HSL (Figure 3-17 C). This 3-oxo-C8-HSL concentration is not sufficient to activate MG1655-las (Figure 3-18), indicating that the signal arising from our second bioreporter results primarily from the accumulation of 3-OH-C14:1-HSL. Assuming that this ratiometric signal from MG1655-las is generated by 3-OH-C14:1-HLS, our measurements suggest that this longer chain AHL accumulated at a greater rate and to a higher concentration over the incubation, $\approx 5 \times 10^{-5}$ M (Figure 3-17 D).
Figure 3-16. Measuring *Rhizobium* AHL synthesis in soil
Scheme showing how *E. coli* MG1655-lux and MG1655-las (cyan and blue, respectively) were used to spy on *Rl* (orange) synthesis of 3-oxo-C8-HSL and 3-OH-C14:1-HSL. Following different incubations of *Rl* in soil at 30°C, the bioreporters were added, the temperature was shifted to 33°C, and the ratiometric signal was used to read out the accumulation of each AHL.
Figure 3-17. Dynamics of *Rhizobium* AHL synthesis in soil
(A) Ratiometric signals arising from adding MG1655-lux and MG1655-las to a loamy agricultural soil containing different concentrations of 3-oxo-C8-HSL and (B) 3-OH-C14:1-HSL. A fit of a dose response curve to the MG1655-lux data yielded an EC$_{50}$ = 3.3 ±1.9 x 10$^{-7}$ M for 3-oxo-C8-HSL, while a fit of the curves for MG1655-las data produced EC$_{50}$ values of 2.6 ±0.8 x 10$^{-6}$ and 1.1 ±0.2 x 10$^{-5}$ for 3-oxo-C8-HSL and 3-OH-C14:1-HSL, respectively. (C) The ratiometric signals arising from adding MG1655-lux and (D) MG1655-las to loamy agricultural soils at different times after addition of medium containing *Rl* (shaded circles) or lacking *Rl* (open circles). AHL concentrations produced by *Rl* were estimated by comparing these ratiometric signals to the standard curves generated in soil. The concentration of 3-oxo-C8-HSL detected by MG1655-lux is not sufficient to activate CH$_3$Br production in MG1655-las, indicating that the signal obtained from MG1655-las arises solely from 3-OH-C14:1-HSL, even though this sensor can be activated by 3-oxo-C8-HSL. Error bars represent ±1σ calculated using three replicates and are only shown when larger than the symbol.
Figure 3-18. Effect of *Rhizobium* AHLs on MG1655-lux and MG1655-las gas production

*Rl* synthesizes multiple AHLs and uses different AHL receptors to read out the relative concentrations of each signal. I evaluated how (A) MG1655-lux and (B) and MG1655-las respond to the different AHLs synthesized by *Rl* at 33°C. A range of concentrations of each AHL (diluted in 100 μL of M63 medium) were added into KBS soil (800 mg), each bioreporter (100 μL) was mixed into the samples, CH₃Br and C₂H₄ were measured following a 3 hour incubation, and the ratio of CH₃Br to C₂H₄ was calculated. The data for each AHL was fit to a simple dose response model (lines) to identify the AHL concentration required for half maximal signal. MG1655-lux is specific for 3-oxo-C₈-HSL (EC₅₀ = 3.3 x 10⁻⁷ M), while MG1655-las can be activated by 3-oxo-C₈-HSL (EC₅₀ = 2.6 x 10⁻⁶ M) and 3-OH-C₁₄:1-HSL (EC₅₀ = 1.1 x 10⁻⁵ M). Error bars represent ±1σ calculated using 3 replicates and are only shown if larger than the symbol.
Figure 3-19. *Rl* does not alter the ratio of CH$_3$Br and C$_2$H$_4$ synthesized by *E. coli* constitutively expressing EFE and MHT.

The signal generated by MG1655-mht-efe at 33°C upon addition to KBS soil that had been incubated with (closed circles) and without (open circles) *Rl* for varying durations. No significant differences were observed between two groups at each time point (student *t*-test, two-tailed, *p*>0.05). Error bars represent ±1σ calculated using three replicates.
3.6. Discussion

Ratiometric gas reporting represents a simple method for monitoring the temporal activities of promoters, yielding a large dynamic range (>80 fold) when coupled to a conditional promoter in *E. coli* without presenting an apparent fitness burden. When used in an environmental matrix, this method was able to provide information on the bioavailable concentrations of different cell-cell signaling molecules. A comparison of the effects of a loamy agricultural soil on the bioavailable concentration of short- and long-chain AHLs revealed that the concentration of the long-chain AHL is decreased to by the matrix, while the short chain AHLs remain unaffected. This finding suggests that ratiometric gas reporting will be useful in future studies that seek to evaluate the effects of different soils on a wide range of chemicals that regulate promoters. One advantage of this approach is that it produces a consistent bioreporter signal for over half a day in capped vials, even if cells are growing in the matrix. This finding is significant because it represents the period of time that it takes to analyze gas within a [fully populated](#) 150-vial autosampler on a GC-MS, thereby enabling a simple, efficient workflow.

Our proof-of-concept ratiometric gas reporter will have immediate applications that expand the utility of synthetic biology in environmental science. Our results show that this approach can be used in short incubations that seek to examine how the bioavailable concentration of chemicals within a soil or sediment differ from the concentrations that are determined following extraction of molecules from a matrix. Environmental scientists frequently use analytical chemistry and
bioreporters to quantify the concentrations of chemicals in soils.\textsuperscript{22,47} However, these methods only provide insight into the total chemical concentrations following extraction, rather than the bioavailable concentrations \textit{in situ}. Our ratiometric gas reporter overcomes these limitations by enabling measurements without the need for extraction. In the case of chemicals used for cell-cell signaling in soils, our approach will be useful for studying how the composition of a matrix affects the fidelity of intercellular signaling. In the case of toxic compounds in the environment, \textit{e.g.}, organic and inorganic pollutants, our ratiometric reporter can be used to study how the composition of a matrix modulates the bioavailability of toxic compounds. Beyond chemical sensing applications, our new ratiometric reporter will also be useful for studying the effects of matrix composition on horizontal gene transfer,\textsuperscript{233} and it will be useful in studies with artificial soils that examine how matrix heterogeneity affects microbial behaviors.\textsuperscript{251}

When using ratiometric reporting to study the activity of conditional promoters, it is critical to consider how the rate of production of each gas is influenced by environmental conditions and the duration of the incubation. The concentration of each gas used to generate a ratiometric signal depends upon the expression and stability of the EFE and MHT, the availability of the EFE and MHT substrates, and gas partitioning out of the soil matrix following synthesis. In this work, I found that EFE activity decreases at 37\textdegree{}C as previously observed,\textsuperscript{195} indicating that it is best to perform measurements at lower temperatures where both gases accumulate to level that can be easily detected. When ratiometric measurements were performed at 33\textdegree{}C, a robust gas ratio signal was obtained
with our AHL bioreporters that remained constant even when the signal was measured following different incubation lengths, ranging from three to thirteen hours in duration (Fig. 4). Thus, the relative accumulation of both gases remains constant when vials remain capped for different periods of time before GC-MS analysis, even though the absolute amount of each gas increases with time. I avoided performing longer incubations with our sensors because oxygen required for EFE activity can become depleted in the capped vials used for these measurements.

Even though a consistent ratiometric gas signal was obtained with our AHL bioreporters over a period of half a day, our measurements with strains that constitutively produce EFE and MHT at high levels suggested that the individual gas signals could be influenced by incubation time and environmental conditions. With strains that constitutively produced the individual gases, I measured the rate of gas accumulation by uncapping the vials following each measurement and then recapping for one hour before the next measurement. With this higher resolution temporal data (Fig. 2), the signal did not vary significantly from three to six hours for all conditions tested, which included a synthetic and real soil. However, the average CH$_3$Br production rate decreased over this time period. The underlying cause of this latter trend is not known. This trend could arise because of time-dependent changes in MHT substrate concentrations within the cell. The MHT used to synthesize CH$_3$Br requires SAM and bromide as substrates. To minimize substrate limitation issues, I supplied bromide at a concentration that is $10^4$ higher than total CH$_3$Br produced in our experiments as previously
SAM could not be similarly controlled since it is synthesized from ATP and methionine.\textsuperscript{253} With further development, our ratiometric gas reporter should have even wider applications. In the case of commensal bacteria that are being developed as human gut diagnostics and therapeutics, ratiometric gas reporting could be useful for studying how synthetic microbes behave in hard-to-image environmental matrices that they enter following their exit from host organisms.\textsuperscript{18,254} With further refinement, this new approach should be useful for monitoring dynamic changes in metabolites within bioreactors that use non-transparent feedstocks to produce green chemicals,\textsuperscript{242} and it should be valuable for studies that examine how soil amendments disrupt symbiosis that are important for agriculture.\textsuperscript{203}

Current limitations of ratiometric gas reporting include a need for oxygen and halides as substrates and a minimum bioreporter titer to obtain a reliable signal. The development of a pair of enzymes that produce rare volatile gases anaerobically would enable broader applications,\textsuperscript{190} e.g., uses in anaerobic soils and fermenters, as would the application of more sensitive detection measurements.\textsuperscript{255} In the future, ratiometric gas reporting could be used for longer-term incubation studies. However, these longer incubations will require the use of microbes that grow more stably in matrices than \textit{E. coli} and continuous gas sampling, similar to the gas sampling approached used by ecologists to study greenhouse gas production.\textsuperscript{256}
3.7. Materials and methods

Materials. AHL autoinducers were purchased from Cayman Chemical, antibiotics were from Research Products International, and all other chemicals reagents were from Sigma-Aldrich, VWR, or BD Biosciences. Kits for DNA and plasmid purification were from Zymo Research and Qiagen. Vials for gas measurement were from Phenomenex.

Growth medium. Lysogeny Broth (LB) and LB-agar plates containing antibiotics were used to propagate plasmids and strains including *E. coli*, *Bt*, and *S. oneidensis* MR-1. Unless stated otherwise, all gas production assays were conducted using a modified M63 medium (referred to as M63) supplemented with 200 mM NaBr, which serves as substrate by MHT for the CH$_3$Br production. To generate our modified M63 medium, I generated 1 L of medium by mixing 50 mL 20% glucose, 50 mL 4 M NaBr, 1 mL 1 M MgSO$_4$, 100 µL 0.1% thiamin, 200 mL M63 salt stock, and water. The M63 salt stock contained 75 mM ammonium sulfate, 0.5 M potassium phosphate monobasic, and 10 µM ferrous sulfate. The salt stock and water were autoclaved, and all other components were sterile filtered. The M63 was adjusted to pH 7 prior to use. For simplicity, I refer to this modified M63 medium simply as M63 medium. Yeast Mannitol Broth (YMB) and Yeast Mannitol Agar (YMA) were used to culture *Rl*. In cases where *E. coli* bioreporters were added to soils already harboring *Rl*, the *Rl* in soil contained YMB medium and the *E. coli* added to soil was in M63 medium. *S. oneidensis* MR1 experiments utilized LB medium containing 200 mM NaBr and 50 µg/mL kanamycin.
Bacterial strains and plasmids. *E. coli* XL1-Blue (Stratagene) was used for cloning and plasmid amplification, while *E. coli* MG1655 was used as a chassis for building a ratiometric gas bioreporters. This latter strain was used to develop sensors because it does not present detectable C$_2$H$_4$ or CH$_3$Br production. In a previous study, I integrated DNA containing the *Batis maritima* MHT$^{187}$ into the 186 attB site in MG1655 to obtain MG1655-mht.$^{227}$ In MG1655-mht, MHT expression is driven by the strong constitutive promoter/RBS pair P2.BCD2.$^{258}$ MG1655-efe was constructed by integrating *P. syringae* EFE$^{247}$ into the phage 186 attB site using Clonetegration.$^{252}$ This EFE has previously been expressed in *E. coli* as a functional enzyme.$^{194}$ In MG1655-efe, EFE expression is controlled by the strong constitutive promoter/RBS pair P1.BCD2.$^{258}$ MG1655-mht-efe was modified from MG1655-mht by integrating P1.BCD2.EFE into P21 attB site using Clonetegration.

MG1655-lux and MG1655-las were constructed by integrating LuxR and LasR sensing modules into the λ attachment site in MG1655-efe using the λ recombination system.$^{259}$ The LuxR sensing module includes the gene encoding LuxR (BBa_C0062) under the control of the strong constitutive promoter/RBS pair P1.BCD2 and the MHT gene under control of the wildtype $P_{lux}$ promoter harboring a C to T mutation (BBa_K658006). The LasR sensing module includes the gene encoding LasR (BBa_C0078) driven by the constitutive promoter $P_{lacIq}$ (BBa_J56015), and the MHT gene regulated by $P_{las}$. *Rl* was from ATCC (10004™).$^{260}$ *Bt* serotype israelensis AM65-52 was isolated by dissolving Mosquito Bits (Summit Chemical) in LB medium, streaking the slurry on LB plates,
and isolating an individual colony. This strain was verified by PCR amplifying the AiiA gene encoding a lactonase that degrades AHL, and sequence verifying the amplicon.

*Shewanella oneidensis* MR-1 was kindly provided by Dr. Timothy Palzkill. I constructed a plasmid, pHCO1, harboring the lasR sensing module and a bicistronic message encoding EFE and red fluorescent protein (RFP) under control of *P_{tet}*. This plasmid contains a ColE1 origin and kanamycin resistance marker. I transformed pHCO1 into *S. oneidensis* MR-1 using electroporation and selected for transformed cells on LB-agar plate containing 50 μg/mL kanamycin

**Headspace gas analysis.** All gas analysis was performed using an Agilent 7820a gas chromatograph (GC) with PoraPLOT Q capillary column (24 m, 0.25 mm ID, and 8 mm film) and a 5977E mass spectrometer (MS). An Agilent 7693A liquid autosampler with a 100 μL gastight syringe (Agilent G4513–80222) was used to inject 50 μL of headspace gas from sealed 2 mL vials containing soils and microbes into the GC. The autosampler is equipped with a cooling/heating tray (Agilent 7693A) that connects to a heated water bath with PID temperature controller (Thermo Scientific SC100-S7 4L). To quantify C$_2$H$_4$ and CH$_3$Br, the oven temperature was programmed to ramp from 85°C to 105°C at 12°C/min, followed by a fast increase (65°C/min) to 150°C and hold for 144 seconds. The total run time was five minutes. The 5977E was configured for selected ion monitoring mode, specifically to C$_2$H$_4$ (MW = 27 and 26 for C$_2$H$_3$ and C$_2$H$_2$, respectively) and CH$_3$Br MW = 93.9 and 95.9 for CH$_3$$^{79}$Br and CH$_3$$^{81}$Br, respectively). Agilent MassHunter Workstation Quantitative Analysis software was used to evaluate the amount of
gas in each sample; the peak areas of the major ions (C$_2$H$_3$ and CH$_3$Br$^{79}$) were used to quantify the amount of each gas while the minor ions were used as qualifiers.

The peak area from the GC-MS measurement was converted to the total mass of C$_2$H$_4$ and CH$_3$Br in each experimental vial using standard curves. Two CH$_3$Br standard curves generated. A standard curve for liquid studies was built using serial dilutions of an analytical standard (100 µg/mL in methanol, purchased from Sigma-Aldrich) that had been diluted 100x into M63 medium. A standard curve for soil studies was generated by mixing the serial dilutions (10 µL) with M63 medium (190 µL) and soil (800 mg). Two C$_2$H$_4$ standard curves were produced using the decomposition of ethephon (Sigma-Aldrich) in alkaline solution. An ethephon stock solution was prepared at 100 µg/mL in 0.1 M HCl. To build a C$_2$H$_4$ standard curves for liquid, serial dilutions of ethephon solution (100 µL) were added to M63 medium (800 µL), 6 M NaOH (100 µL) was added, and the vial was immediately crimped. To build a C$_2$H$_4$ standard for soil, serial dilutions of ethephon solution (100 µL) were added to soils (800 mg), 6 M NaOH (100 µL) was added to the mixture, and the vial was immediately crimped. All standards were incubated in 2 mL gastight vials (Phenomenex) for 2 hours at 45°C with shaking at 290 rpm and then moved to static 30, 33, or 37°C incubators for 2 hours to allow each gas to reach equilibration between the aqueous and gas phases prior to the GC-MS analysis. Standard curves were calculated in Excel using liner regression and a y intercept of zero.
**EFE and MHT temperature dependence.** MG1655-efe and MG1655-mht were used to inoculate M63 medium and incubated at 37°C while shaking at 250 rpm. At OD$_{600}$ = 0.5, cultures were spun down and resuspended at an OD$_{600}$ = 0.1 (2 x 10$^7$ cell/ml) using fresh M63 medium. These cultures (1 mL) were added to 2 mL gastight vials and incubated on the autosampler tray that was programmed to either 30, 33, or 37°C. Every hour, the gas in the headspace of each vial was measured using the GC-MS, the culture was transferred to a cuvette, and an OD measurement was performed using a Cary 50 spectrophotometer. Following the OD measurement, the culture was transferred back to the vial, re-capped, and returned to the tray for the next incubation period. OD$_{600}$ readout was converted to CFU using a fit to a standard curve that revealed CFU = OD$_{600}$ x 2 x 10$^8$ (*data not shown*).

**Soil matrices.** Two soils were used for measurements. First, I used a soil from the NSF Kellogg Biological Station Long-term Ecological Research station (KBS LTER) located in Hickory Corners, Michigan. The 0–5 cm depth from the Ap horizon of a Typic Hapludalf (USDA soil taxonomic nomenclature) that was part of a switchgrass biofuels trial experiment was collected. I used this soil as our standard loamy agricultural soil. The soil particle distribution was measured using chemical dispersion followed by gravity precipitation and found to be 64.9 ± 0.6% sand (2 to 0.05 mm particles), 27.2 ± 0.5% silt (0.05 to 0.002 mm), and 7.9 ± 0.1% clay (<0.002 mm). With this soil, the water holding capacity (WHC), defined as the amount of water held after saturation and free drainage for 6 hours, was 0.33 g water/g soil. All the soil incubation experiments in this work were conducted at 0.25
g water/g soil (75.7% WHC) to represent agriculturally-relevant hydration conditions.

The particle size distribution of the loamy agricultural soil was used to guide the creation of an artificial soil by mixing varying sizes of quartz particles (U.S. Silica) corresponding to the sizes of sand (NJ 2, 1.19 mm to 1.68 mm), silt (Min-U-Sil 40, median size 8.71 µm), and clay (Min-U-Sil 5, median size 1.7 µm). Aggregates were created for artificial soil to mimic soil structure of natural soil using multiple wet-dry cycles. Briefly, artificial soil was wet to its water holding capacity and dried in oven at 105 °C overnight. The dried material was gently crushed and passed through 2 mm sieve. The step was repeated 3 times. All soils were sieved to 2 mm and autoclaved twice prior to incubation measurements.

**Constitutive gas production in soils.** To ensure that the amount of nutrient per cell was the same across experiments, I inoculated soil matrices with the same cell density as the liquid culture experiments (2 x 10⁷ cells in 1 mL). Thus, with 200 µL of the medium volume in soil matrices, I used 5-fold lower cells for inoculation (4x10⁶ cells). For each sample, dry artificial or loamy agricultural soil (800 mg) was weighed and transferred to a 2-mL vial. M63 medium (200 µL) containing 2 x 10⁷ CFU of MG1655, MG1655-efe or MG1655-mht was added into the vial, and a sterile toothpick was used to homogenize the mixture. Vials were sealed with Parafilm to prevent evaporation and incubated on the autosampler tray set at 33°C for up to six hours. For each condition, 18 identical vials were prepared. After different durations (0, 60, 120, 180, 240, and 300 minutes), three vials were crimped for an hour before C₂H₄ and CH₃Br was measured by GC-MS. After each
GC-MS measurement, cells from these samples were extracted to determine CFU following the incubation.

**Soil bacteria extraction from soil.** After GC-MS measurements, LB (800 µL) was added to each vial, which increased the total liquid to 1 mL. Each vial was covered with Parafilm, vortexed for 30 seconds, and allowed to stand for 10 seconds so that the larger particles settled to the bottom. The liquid-soil mixture was serially diluted into LB medium, dilutions were spread on LB agar plates, and colonies were counted after incubating 16 hours at 37°C.

**Measuring AHL following addition to soils.** Experiments evaluating AHL effects on bioreporter functions were performed by adding M63 medium (100 µL) containing the various AHLs to the loamy agricultural soil (800 mg), and adding M63 medium (100 µL) containing 4 x 10⁶ bioreporter cells (MG1655-las or MG1655-lux) into the soil. The final soil water content of each sample was 0.25. Samples were incubated statically at 33°C for three hours prior to using GC-MS to measure C₂H₄ and CH₃Br.

**AHL degradation by Bacillus.** Overnight cultures of *Bt* were used to inoculate fresh LB, and cells were grown to OD₆₀₀ = 0.5 at 30°C with 250 rpm shaking. The *Bt* culture was spun down and resuspended in fresh M63 supplemented with 0.01% casamino acid to support *Bt* growth²⁶² and ZnSO₄ (10 µM) to support lactonase activity.²⁶³ Varying titers of Bt (0, 10⁴, 10⁵, or 10⁶ CFU) were mixed with 3-oxo-C₁₂-HSL (1 µM) in modified M63 (100 µL) and immediately added to 800 mg of the soil in 2 mL vials. Twelve vials were prepared for each *Bt* titer. Vials were sealed with Parafilm and incubated at 30°C for up to 36 hours. After different
durations, Parafilm was removed from three vials in each group and M63 (100 µL) containing MG1655-las (4 x 10⁶) was added to the soil. The vials were immediately crimped and incubated at 33°C for 3 hours prior to the gas measurement. The number of MG1655-las and Bt were measured after each incubation by extracting cells, diluting the extracted cells, and spreading dilutions on LB-agar plates. After 16 hours at 37°C, Bt formed white colonies with rough edge while MG1655 formed colonies with smooth edges that were more yellow in color. This colony-morphological difference allowed us to enumerate the titer of each cell type following the gas measurements. Under the experimental conditions, Bt alone did not produce detectable C₂H₄ and CH₃Br.

**Rhizobium AHL production dynamics.** YMB (2 mL) was inoculated with a single colony of Rl grown on a YMA plate at 30°C for four days. The Rl culture was incubated at 28°C with 250 rpm shaking for 48 hours until it reached an OD₆₀₀ = 0.4. The culture was diluted 10x in fresh YMB and then incubated for 24 hours while shaking at 28°C. At the end of the incubation the OD₆₀₀ was ~0.5. This culture was spun down and resuspended in a modified YMB which had NaCl substituted by NaBr (0.2 g/L). Rl (10⁷ cells) in 150 µl of the modified YMB were added into the KBS soil (800 mg) in 2 mL vials. Vials were sealed with Parafilm, and incubated at 30°C. A YMB only control group was also set up in the same manner. After varying incubation times (0, 12, 24, 48, 72, and 96 hours), MG1655-las, MG1655-lux, or MG1655-mht-efe (4 x 10⁶ CFU each) were added into separate vials in triplicates. Both C₂H₄ and CH₃Br were measured following a 3 hour incubation at 33°C. Rl did not produce detectable amount of C₂H₄ and CH₃Br.
**AHL-sensing by *Shewanella oneidensis* MR-1.** An overnight culture of *S. oneidensis* transformed with pHCO1 was used to inoculate fresh LB medium containing NaBr, and the resulting culture was grown in a shaking incubator at 30°C to an OD$_{600}$ = 0.5. The culture was pelleted, resuspended in fresh medium, and used for sensing in liquid culture and soil slurries. To evaluate AHL detection in liquid culture, 2 mL gastight vials containing 1 mL of LB medium containing NaBr and varying concentration of 3-oxo-C12-HSL were inoculated with 2 x 10$^7$ *S. oneidensis* cells. To evaluate AHL in soil slurries, 150 µL of LB medium containing NaBr and varying concentrations of 3-oxo-C12-HSL was first mixed with 700 mg of KBS soil. Then, 150 µL of medium containing 6 x 10$^6$ cells was mixed into the matrix. Vials were incubated at 33°C without shaking for 4 hours and 8 hours, respectively, prior to the gas measurement.

**Statistical analysis.** GraphPad Prism® 7 software was used to conduct ANOVA and AHL dose-response curve fitting. The gas production data was fitted to a dose-response curve (**Equation 3-1**):

**Equation 3-1**

$$y = b + \frac{x^h(a-b)}{x^h+EC50^h}$$

where $y$ is the gas production of a bioreporter, $x$ is the AHL concentration, $a$ represents the maximal response, and $b$ is the basal response. $h$ indicates the hill slope, which represents the steepness of a response curve and $EC50$ is the concentration of AHL that results in the half-maximum response of a system. The one-phase decay model (**Equation 3-2**) was used to describe the half-life of AHL co-culture with *Bt*.
Equation 3-2

\[ y = (y_0 - \text{Plateau})e^{-kx} + \text{Plateau} \]

where \( y \) is the gas production of a bioreporter, \( x \) is the incubation time, \( y_0 \) is the \( y \) value at \( x \) is zero, \( \text{Plateau} \) is the gas production at infinite times (the value must be greater than zero), and \( k \) is the rate constant. The half-life was calculated with \( \frac{\ln(2)}{k} \). Student \( t \)-test and the linear regression of the \( \text{C}_2\text{H}_4 \) and \( \text{CH}_3\text{Br} \) standard curves were calculated in Excel.
Chapter 4:
Using volatile gas production by methyl halide transferase to report on microbial conjugation in a soil

(This chapter is based on the publication “Volatile Gas Production by Methyl Halide Transferase: An in Situ Reporter of Microbial Gene Expression in Soil” and has been reformatted to departmental guidelines.)
4.1. Summary

Traditional visual reporters of gene expression have only very limited use in soils because their outputs are challenging to detect through the soil matrix. This severely restricts our ability to study time-dependent microbial gene expression in one of the Earth’s largest, most complex habitats. Here I describe an approach to report on dynamic gene expression within a microbial population in a soil under natural water levels (at and below water holding capacity) via production of methyl halides using a methyl halide transferase. As a proof-of-concept application, I couple the expression of this gas reporter to the conjugative transfer of a bacterial plasmid in a soil matrix and show that gas released from the matrix displays a strong correlation with the number of transconjugant bacteria that formed. Gas reporting of gene expression will make possible dynamic studies of natural and engineered microbes within many hard-to-image environmental matrices (soils, sediments, sludge, and biomass) at sample scales exceeding those used for traditional visual reporting.

4.2. Introduction

Cells are routinely programmed to report on their behaviors by producing molecular outputs that can be visually quantified, such as colored, fluorescent, and luminescent molecules. These reporters transformed biology by enabling the real-time tracking of dynamic biological processes, such as cell localization and migration, enzyme activation, macromolecular interactions, and horizontal
gene transfer\textsuperscript{268}. Simultaneously, innovations in DNA sequencing\textsuperscript{269}, microchip biotechnologies,\textsuperscript{270} and mass spectrometry\textsuperscript{271} transformed our understanding of complex microbial communities by enabling us to catalog what organisms are present in a soil\textsuperscript{272}, including microbes we cannot yet cultivate, and the ensemble of RNA, proteins, and metabolites synthesized by communities. This explosion of microbiome data has generated many hypotheses about the connections between soil microbial behaviors and ecosystem-scale phenomena. These hypotheses would be easy to test in a transparent matrix using the palette of colored reporter proteins. However, soils and sediments display high absorbance at the wavelengths of light required for visual reporting, restricting the application of existing gene expression reporters to organisms in specialized microcosms that are compatible with imaging or cells extracted from soils\textsuperscript{22,273}.

I sought to develop a reporting approach that could be used nondisruptively to study microbial gene expression in bulk soils under conditions that reflect those commonly used for laboratory soil incubations\textsuperscript{206,274,275}. I hypothesized that methyl halide transferases (MHT) could provide real-time, nondestructive reporting of microbial processes within hard-to-image matrixes like soils and sediments (Figure 4-1). MHT synthesize volatile methyl halides (CH\textsubscript{3}X) using halide ions (Cl\textsuperscript{-}, Br\textsuperscript{-}, or I\textsuperscript{-}) and S-adenosyl methionine, a common cellular metabolite\textsuperscript{276}. Lowell Hager’s initial discovery and characterization of an MHT suggested that these enzymes might be well suited as microbial reporters in hard-to-image conditions like soils because they synthesize a volatile gas that is easy to detect using gas chromatography-mass spectrometry (GC-MS).\textsuperscript{277} Hager also showed that MHT
are encoded by a single polypeptide and demonstrated that these enzymes can be produced in a functional form in non-native bacteria like *Escherichia coli*. Since Hager's detection of MHT in algae, fungi, and plants, a range of organisms and soils have been found to encode MHT and produce this volatile metabolite. While CH$_3$X are produced by soils and plants, these gases are expected to be present at very low levels in sterile soils. For this reason, I hypothesized that microbes introduced into sterile soils could be programmed using synthetic biology to report on promoters whose activities change dynamically with time and soil conditions.

Here I describe how an MHT-expressing *Escherichia coli* can be used to report on gene expression in a microbe within a moist soil under a range of growth conditions in a lab setting. In proof-of-concept experiments, I used this gas reporting approach to monitor *E. coli* conjugation within an agricultural soil and examined how hydration affects horizontal gene transfer. These experiments demonstrate that gas reporting can be used to non-disruptively monitor a dynamic microbial behavior within natural soil conditions.
Figure 4-1. Using CH3X production to report on gene expression
The identity of the methyl halides synthesized by an MHT depends upon the halides taken up by cells and the level of S-adenosyl methionine.
4.3. Using CH$_3$X synthesis to report on gene expression

To establish the range of microbial growth conditions where an MHT can be used as a gene expression reporter, I chromosomally incorporated *Batis maritima* MHT into *Escherichia coli* under control of a constitutive promoter. This MHT can accept different halides as substrates to synthesize CH$_3$Cl, CH$_3$Br, and CH$_3$I$^{193}$. I first analyzed the effect of halide concentrations on gas production and cell viability in liquid culture to establish the halide concentrations compatible with gas reporting. Concentrations of chloride, bromide, and iodide (Figure 4-2) spanning five orders of magnitude yielded detectable methyl halides in the headspace of liquid cultures. In addition, this range of halide concentrations did not significantly alter cell growth in our parental and modified strains (Figure 4-3). The lowest concentrations tested (10 µM), which represent levels found in many soils,$^{284,285}$ yielded strong signals with all three halides. In addition, the minimal inhibitory concentration (MIC) of each halide varied. I found that the MIC for chloride and bromide were 800 mM. In contrast, the MIC for iodide was 200 mM.

To determine which CH$_3$X yields the best signal for reporting on *Escherichia coli* gene expression, I compared the levels of each gas produced by cells containing and lacking a MHT (Figure 4-4 A). When unmodified cells were grown in medium supplemented with 100 mM chloride or bromide, no CH$_3$X was detected after a 24-hour incubation, as observed with growth medium lacking cells. Under the same conditions, cells expressing an MHT yielded CH$_3$Cl and CH$_3$Br signals
Figure 4-2. Sodium halide concentrations in media affect CH₃X production
The effects of different (A) NaCl, (B) NaBr, and (C) NaI concentrations on gas production by *Escherichia coli* constitutively expressing an MHT.
Figure 4-3. Effect of halides on *Escherichia coli* MG1655-mht growth

*E. coli* growth was monitored continuously for 24 hours in M63 medium containing the indicated concentrations of halide ions, and the data was fit to a simple model for exponential growth to obtain the growth delay and growth rate. Experiments were performed in triplicate and error bars represent ±1 standard deviation.
Figure 4-4. Using CH₃X production to report on gene expression

(A) *E. coli* MG1655-mht (+MHT) produce volatile methyl halides at levels that greatly exceed the amount observed in the headspace of cultures containing unmodified cells (*E. coli*) or growth medium alone (M63). (B) Gas levels in culture headspace after short incubations (1 hour) of *E. coli* MG1655-mht at the indicated titers of cells. (C) Gas production reports on gene expression under anaerobic conditions. MG1655 harboring pHCP.las:MHT:GFP was induced with 100 nM of AHL and incubated for 24 hours under aerobic or anaerobic conditions. For each sample, GFP and CH₃Br signals are normalized to the optical density of the sample, and the resulting values are reported as relative signals to cells grown aerobically and without AHL induction. CH₃Br production per cell is significantly higher under the anaerobic condition than the aerobic condition (two tailed t-test; p < 0.05). Error bars represent ±1σ calculated using 3 replicates.
that exceeded our detection limit by $10^4$ and $10^5$, respectively. In medium containing iodide, unmodified cells yielded a background CH$_3$I signal. However, this signal was $10^3$ fold lower than that obtained with MHT-expressing cells. NaBr was used for gas reporting in all subsequent measurements because this salt yielded the strongest signal. To evaluate how the sensitivity of MHT reporting relates to the concentration of microbes in soils, which has been estimated to contain as many as $10^9$ microorganisms per gram, I analyzed the gas production by different numbers of MHT-expressing cells (Figure 4-4 B). Gas measurements after 1 hour incubations provided evidence that a signal can be detected from samples containing $\geq 10^5$ cells per gram of soil.

**4.4. Gas reporting from microbes in a soil**

Some visual reporters like GFP require O$_2$ to function, because their chromophore matures through an oxidative mechanism$^{286}$. This potentially limits their application within soils that experience anoxic conditions. To evaluate the relative performances of MHT and GFP reporters under anoxic conditions, I analyzed the effects of O$_2$ on their reporting activities (Figure 4-4 C). For these experiments, I transformed *E. coli* with an expression vector that produces GFP and MHT in the presence of inducer (Figure 4-5) and analyzed the signal obtained in the presence and absence of O$_2$. Induction of protein expression in the presence of O$_2$ resulted in 55-fold and 44-fold increases in GFP and MHT expression, respectively. In N$_2$ purged samples, a large increase in MHT signal (135-fold) was also observed. However, a 10-fold smaller increase in GFP signal (4-fold) was
obtained. The finding that MHT generates a strong signal under anoxic condition suggests that MHT will be useful for reporting in soils and sediments that lack O$_2$ or experience O$_2$ gradients.

To investigate whether MHT can be used to report on gene expression within a soil under natural soil water levels, I mixed 10$^8$ cells containing or lacking an MHT with a soil hydrated to differing extents and measured the CH$_3$Br production after 24 hours. These experiments were performed in sealed aerobic vials containing an identical total mass of soil and liquid (1 gram) generated by mixing varying amounts of dry soil and liquid growth medium (Figure 4-6 A). After overnight incubations, the CH$_3$Br signal from MHT-expressing cells was 10$^3$ higher than that obtained from unmodified cells across all soil water conditions analyzed (Figure 4-6 B), including soils at or below the WHC (75% of this soil by mass). To determine how the CH$_3$Br signal relates to cell titer in each incubation measurement, I extracted cells and quantified the number of colony forming units (CFU) (Figure 4-6 C) and used this to calculate the cell-normalized gas signal (Figure 4-6 D). This analysis revealed that the gas signal per CFU at 100% WHC is not significantly different from that observed in drier soil conditions, including 75, 50, and 33% WHC.
Figure 4-5. Inducible expression system used to compare GFP and MHT signals

*E. coli* was transformed with a plasmid that uses the *lasR* promoter to regulate transcription of a bicistronic message that encodes a MHT and GFP. When cells are grown in the presence of an acylhomoserine lactone (AHL), LasR binds AHL and activates transcription from the *lasR* promoter. This induces expression of both MHT and GFP.
Figure 4-6. Monitoring constitutive gene expression in soil using CH₃Br

(A) Soil (Kellogg Biological Station switchgrass plot H2-404, 0-5 cm depth) hydrated to different water holding capacities was inoculated with 10⁸ CFU of Escherichia coli containing (closed symbols) or lacking a MHT (open symbols). M63 liquid medium containing 100 mM NaBr was used to hydrate 0.1 to 0.9 grams of soil to the indicated WHC within 2 mL sealed vials. After an incubation for 24 hours at 37°C while shaking at 290 rpm, I measured (B) the level of CH₃Br present in 50 µL of the headspace, and (C) the number of extractable CFU. (D) The cell-normalized gas signal was calculated at each water holding capacity. The gas signal obtained at 100% WHC was not significantly different from that observed at lower soil water contents (two tailed t-test; p > 0.05). Error bars represent ±1σ calculated using 3 replicates.
4.5. Non-disruptive reporting of horizontal gene transfer in a soil

To directly demonstrate the utility of gas reporting for non-disruptive studies of gene expression in soil, I used an MHT to monitor bacterial conjugation, a dynamic horizontal gene transfer process that is prevalent in the environment. I built a F-plasmid for *E. coli* that expresses the *mht* gene using a promoter that is repressed by the tetracycline repressor TetR and monitored the transfer of the F-plasmid between cells using CH₃Br production under aerobic conditions. Within the context of donor cells (Figure 4-7, Figure 4-8), which express TetR, MHT transcription from the F-plasmid is repressed. In contrast, transconjugant cells arising from F-plasmid transfer transcribe and translate MHT at elevated levels because they do not express TetR. This design strategy was chosen to minimize cellular stress arising from MHT expression, which could negatively impact cell growth. To allow for parallel detection of conjugation using antibiotic selections of soil-extracted cells, a common approach for quantifying conjugation in soils, I used an F-plasmid that confers kanamycin resistance (Km⁺), a donor strain that is spectinomycin resistant (Spec⁺), and a chloramphenicol-resistant (Cm⁺) receiver strain.

I first determined the extent to which CH₃Br production can be coupled to conjugation by comparing the rate of CH₃Br produced by donor, recipient, and transconjugant cells (10⁵, 10⁶, 10⁷, and 10⁸ CFU) after a 1-hour incubation in a static liquid culture (Figure 4-9). Transconjugants displayed the strongest gas signal, yielding CH₃Br levels that exceeded donor and recipient cells by up to 1000-fold (Figure 4-10). Experiments performed using a soil at 100% and 50% of the
Figure 4-7. Scheme illustrating the genetic program used to couple MHT transcription and gas production to conjugation.
Figure 4-8. Altering the Tn10 mobile element within the F-plasmid
An F plasmid (AF162223) containing the Tn10 transposon that confers resistance to the antibiotic tetracycline was modified by knocking in a linearized plasmid (ptetBCD2MHT.mcherry) using the sites indicated in red and orange. The resulting plasmid (pF-mht) lacks the gene encoding the tetracycline efflux pump (tetA) and the gene encoding the tetracycline repressor (tetR).
Figure 4-9. Effect of soil on donor and transconjugant gas production
Comparison of donor (D) and transconjugant (Tc) *E. coli* gas production in liquid culture (open bars), soil hydrated to 100% WHC (gray bars), and soil hydrated to 50% WHC (black bars). In each experiment, the indicated number of colony forming units (cfu) was incubated for 1 hour, and CH$_3$Br in the sample headspace was measured at the end of the incubation. Error bars represent ±1σ calculated using 3 replicates.
Figure 4-10. Ratio of transconjugant to donor gas production in liquid culture
The ratio of the gas produced by different cfu of donor and transconjugant cells enabled calculation of the extent to which conjugation enhances transcription of the mht gene upon transfer from a donor to recipient cell. Asterisks indicate ratios of experimental conditions where no gas could be detected in the headspace of donor cell cultures. In these cases, a value of 1 CH$_3$Br count was given to these samples so that I could calculate a lower bound on the ratio of transconjugant to donor gas production.
WHC yielded similar results. The large change in CH$_3$Br production upon conjugation suggests that CH$_3$Br levels can be used to report on conjugation dynamics in soils containing donor (<10$^6$) and recipient (<10$^8$) concentrations that do not yield a detectable signal.

To evaluate gas reporting of conjugation, I mixed 10$^5$ donor and 10$^7$ recipient cells and compared CH$_3$Br production with the number of cells that are chloramphenicol and kanamycin resistant (transconjugant cells). I performed measurements in liquid culture (Figure 4-11 A), a soil at 100% WHC (Figure 4-11 B), and at 50% WHC (Figure 4-11 C). The number of recipient and transconjugant CFU increased with time across all conditions tested (>100-fold), albeit to differing extents, while viable donor cells were unchanged (or decreased) with time (Figure 4-12). Calculation of the conjugation frequency using these data (Figure 4-11 D) revealed that the frequency is highest in liquid culture and decreases significantly when soil is added. In addition, a soil held at 50% WHC displays a lower conjugation frequency compared with soil at 100% WHC. I observed a similar trend when gas production was compared across these experimental conditions (Figure 4-11 E). In addition, a log-log correlation exists between transconjugant CFU and CH$_3$Br production rate (Figure 4-11 F), indicating that CH$_3$Br can be used to estimate dynamic changes in transconjugants CFU within a soil.
**Figure 4-11. Using CH$_3$Br production to report on horizontal gene transfer**

Soil extracted CFU on LB-agar plates containing chloramphenicol (gray) and plates containing both chloramphenicol and kanamycin (blue) after growing mixtures of donor ($\sim 10^5$ CFU) and acceptor ($\sim 10^7$ CFU) cells in static (A) liquid culture, (B) soil hydrated to 100% WHC, and (C) soil hydrated to 50% WHC. The number of transconjugants obtained in liquid was significantly greater than that obtained in soil at all time points except at 3 hours (two tailed $t$-test; $p < 0.05$).

(D) The conjugation frequency at each time was calculated as the ratio of transconjugants to receiver cells measured using CFU from liquid cultures (white), soil hydrated to 100% WHC (gray), and soil hydrated to 50% WHC (black). The conjugation frequency in soil was significantly lower than in liquid culture at all time points exceeding 5 hours, and the conjugation frequency in soil at 50% WHC was significantly lower than soil at 100% WHC at the 2, 3, 8, 10, and 24 hour time points (two tailed $t$-test; $p < 0.05$). (E) Headspace CH$_3$Br measurements prior to CFU analysis is shown for each condition. The gas production in soil was significantly lower than that in liquid culture at six time points (2, 3, 4, 6, 8 and 10 hours), and the gas production from soil at 50% WHC was significantly lower than 100% WHC after 3 hours (two tailed $t$-test; $p < 0.05$). (F) Gas production from soil is proportional to the number of extractable transconjugant CFU. A fit of the data to the line log(Tc CFU) = 3.97 + 1.04·log(CH$_3$Br) yields a R value = 0.95. Error bars represent ±1σ calculated using 3 replicates.
Figure 4-12. Donor CFU before and after incubations

The donor CFU were obtained prior to the incubations (0 hours) and after the incubations (24 hours) with recipient (~10^7 CFU) cells in static liquid culture (black), soil hydrated to 100% WHC (gray), and soil hydrated to 50% WHC (white). Across all conditions, donor CFU remained below 10^6, a level that is not sufficient to generate a detectable CH_3Br signal. Error bars represent ±1σ calculated using 3 replicates.
4.6. Implications of gas reporting

Our results show that MHT gas reporting can be used to monitor dynamic changes in the composition of a microbial community within hard-to-image soils hydrated at environmentally-relevant levels. Gas reporting has the advantage over visual\textsuperscript{239,240,264} and ice nucleation reporters\textsuperscript{58,292} in its ability to yield a signal that can be measured in the headspace of matrices without requiring microbial extraction prior to analysis. MHT should also be compatible with many environmental materials (sediments, wastewater, sludge, and biomass) at sample scales exceeding those used for visual reporting in microcosms, including sealed vessels widely used by environmental scientists to monitor the temporal dynamics of greenhouse gas production\textsuperscript{206,274,275}. Unlike GFP-type reporters, MHT activity does not require O\textsubscript{2}\textsuperscript{286} and is therefore compatible with microbial studies under environmentally common low-O\textsubscript{2} conditions. However, MHT require halides to generate a CH\textsubscript{3}X signal. Our studies examining the halide concentration dependence of gas reporting revealed a strong signal at 10 µM halide, which reflects the halide ion concentration found in many soils\textsuperscript{285,293}. In soils where halide ions are not sufficiently abundant to facilitate a strong MHT signal, halides can be provided with water.

MHT reporting of conjugation should be useful for studying how variation in other soil environmental parameters (nutrient status, mineralogy, structure, and temperature) impact conjugation within a range of soil microbial species. For example, gas reporting could be used to study how the physicochemical properties of a soil affect conjugation among rhizosphere bacteria, which use horizontal gene
transfer to exchange symbiotic plasmids that encode genes required nodule formation and nitrogen fixation. Gas reporting will also be useful in many other situations, such as studying conjugation frequencies within soils under nutrient limiting conditions, which have been implicated in reducing the intensity of horizontal gene transfer, deciphering the role that conjugation plays in blooms of antibiotic resistant bacteria following different land use practices such as manure amendment, and analyzing whether charcoal soil amendment quenches these blooms through interactions with veterinary antibiotics. Furthermore, gas reporting will be useful for studying how soil conditions affect horizontal gene transfer through other mechanisms, such as phage transduction and the uptake of DNA from the environment.

Beyond horizontal gene transfer, MHT should be useful for reporting on the timing of microbial gene expression in soils, the environmental factors that affect this expression, and the relationship between dynamic gene expression and soil fluxes of greenhouse gases (CO₂, CH₄, and N₂O) and dissolved organic matter (carbon and nitrogen). MHT gas reporting of these reactions will require the construction of additional genetic programs that use different conditional promoters to regulate the transcription of the MHT gene, similar to those constructed to diversify visual bioreporter functions. Correlations between microbial gene expression and biogeochemical cycling have been challenging to establish using existing methods which require soil disruption to quantify gene expression. In the case of N cycling, gas reporting will be useful for relating the timing of fertilizer application with greenhouse gas production (N₂O), the
expression of genes that encode denitrification enzymes, and the expression of proteins that mediate acquisition and assembly of the metal cofactors that these enzymes require for catalytic activity.\textsuperscript{302} In addition, gas reporting can be used to help understand the dynamics of gene expression that contribute to the cryptic biogeochemical cycles in sediments, such as sulfur and iron.\textsuperscript{303–305} Metagenomic studies can identify the microbial communities involved in the fast fluxes that drive cryptic cycles, but it remains challenging to measure time-dependent gene expression that contributes to this cycling and the environmental parameters that control these reactions.\textsuperscript{306}

The results described here demonstrate that an MHT gas reporter can be used to report on conjugation in a sterile soil, which suggests that this approach might be useful in other soils to study how the physical and chemical properties of those soils affect conjugation. Gas reporting has the advantage that it allows for rapid measurements, and thus, this approach enables the analysis of large combinations of these soil conditions. However, additional studies are needed to determine the diversity of soil conditions where gas reporting can be effective. In the case of organic rich soils, \text{CH}_3\text{Br} produced by the abiotic reaction of iron and organic matter could complicate the signal observed,\textsuperscript{307} especially in cases where exogenous halides are added to the soil. If this occurs, MHT levels can be increased through synthetic biology by enhancing the rate of MHT translation initiation using a well-established method for designing synthetic ribosomal binding sites of defined strengths.\textsuperscript{308} Transitioning this approach to live soils could be more complicated, however, since challenges associated with \text{CH}_3\text{Br} consumption will
need to be overcome. In the case of CH$_3$Br, consumption by soil bacteria has been observed,$^{309-312}$ suggesting that the signal from a synthetic microbe could be attenuated when introduced into a live soil. In cases where methyl halides are not compatible with gas reporting, alternative gas-producing enzymes$^{190}$ can be evaluated for studies in those conditions.

In future studies, gas reporting can quickly be tested across a wide range of microbial species by using MHT as outputs from cross-species expression systems,$^{313}$ which do not rely on the genetic parts from microbial hosts to function. In addition, gas reporters can be developed as outputs for more sophisticated microbial genetic programs. For example, gas reporters would be useful as outputs from synthetic gene circuits that report on quorum sensing and used to study how changes in the physical and chemical characteristics of a soil affect the cell density where microbes make coordinated decisions (quorum sensing) through the accumulation of diffusible chemicals.$^{209,247}$ In addition, gas reporters would be useful as outputs from circuits that encode cellular memory, such as toggle switches,$^{314}$ which will be useful for reading out the historic record of conditions that microbes experience in a soil, similar to that recently achieved in the gut microbiome.$^{18}$ By expanding the palette of gas reporters beyond the MHT described herein, gene expression can also be monitored across multiple cell types, similar to that recently achieved with fluorescent reporters in synthetic microbial communities.$^{16}$
4.7. Materials and methods

**Bacterial Growth Medium.** Luria Broth (LB) and LB-agar plates containing the indicated levels of antibiotics were used to amplify strains and plasmids. M63 defined medium, a low osmolarity medium that lacks halide ions, was used in cultures studying cellular gas production. To create M63 growth medium for studying the effects of halide salt concentration on gas production, I diluted a M63 salt stock (5x), 1 M MgSO$_4$ (1000x), 20% glucose (100x), 0.5% Thiamin (10,000x), 20% casamino acids (200x), and 4M sodium halide ions (varying dilutions) into autoclaved water. The M63 salt stock (75 mM ammonium sulfate, 0.5 M potassium phosphate monobasic, and 10 µM ferrous sulfate) was adjusted to pH 7. All soil measurements were performed by hydrating soil to the indicated levels using 1x M63 medium containing 100 mM NaBr. The M63 salt stock (5x) was prepared by mixing 10 g (NH$_4$)$_2$SO$_4$, 68 g KH$_2$PO$_4$, 2.5 mg FeSO$_4$·7H$_2$O in 1 L water, adjusting to pH 7 using KOH, and autoclaving.

**Plasmids.** A Km$^R$ plasmid, which expresses *Aequeora victoria* green fluorescent protein (GFP), which is encoded by IGEM registry part number BBa_E0040, using the lasR promoter ($P_{lasR}$) and *Pseudomonas aeruginosa* LasR using a constitutive promoter, was amplified using Phusion® High-Fidelity DNA polymerase, and the Golden Gate assembly was used to insert the gene encoding *Batis maritima* MHT upstream of the GFP gene to create a bicistronic message. LasR is a transcriptional regulator that induces expression from the $P_{lasR}$ when it binds to N-(3-oxodecanoyl)-L-homoserine lactone, an acylhomoserine lactone (AHL). In this plasmid (pHC.plas:MHT:GFP), MHT and GFP translation initiation are
regulated by the strong RBS, BBa_B0034 and BBa_B0030 (from the IGEM registry), respectively. A conjugative plasmid (pF-mht) was created by modifying the F-plasmid from XL1-Blue (Stratagene) using the Datsenko-Wanner method\textsuperscript{315} so that it lacks the tetracycline-efflux pump (TetA) and contains a kanamycin-resistance cassette (Km\textsuperscript{R}) and a MHT-mCherry fusion protein. The MHT-mCherry was placed under control of a TetR-repressible promoter (\(P_{\text{tet}}\)), so that expression is repressed by TetR but constitutive in the absence of TetR. This modified F-plasmid was built by synthesizing a linear DNA that encodes Km\textsuperscript{R} adjacent to a fusion made up of \(P_{\text{tet}}\), BCD2 RBS,\textsuperscript{258} and an open reading frame encoding MHT-mCherry. The termini of this DNA contained 40 base pair sequences that are homologous to the regions of the F-plasmid that encode the C-terminus of TetR and C-terminus of TetA. The linear DNA was electroporated into \textit{E. coli} XL1-Blue that contained pKD46, a plasmid that encodes the recombinase RepA\textsubscript{101},\textsuperscript{315} and expression of RepA\textsubscript{101} was induced with 0.2% arabinose. Cells containing the pF-mht were obtained by growing cells on LB-agar plates containing 25 \(\mu\text{g}/\text{mL}\) of kanamycin and then screening those cells for variants that did not grow on LB-agar plates containing 5 \(\mu\text{g}/\text{mL}\) tetracycline plates. Plasmids were verified by commercial DNA sequencing (Lone Star Labs).

**Bacterial strains.** \textit{E. coli} XL1-Blue was used for molecular biology to construct and amplify plasmids. \textit{E. coli} MG1655 was used as a control for all measurements analyzing methyl halide production by cells lacking an MHT. An MHT-expressing \textit{E. coli} strain (MG1655-mht) was built using Clonetegration,\textsuperscript{257} a one-step cloning and chromosomal integration method. This was achieved by creating a DNA that
encodes the *B. maritima* MHT gene under transcriptional and translational control of a previously described promoter-RBS fusion (P14:BCD2),\textsuperscript{258} cloning this DNA into a previously described plasmid (pOSIP-KO) using Gibson Assembly,\textsuperscript{315} transforming the resulting plasmid (named pOSIP-KO-mht) into *E. coli* MG1655 using heat-shock, and selecting for transformants on LB-agar plates containing 25 \( \mu \)g/mL of kanamycin. A colony obtained from this selection, which has the DNA integrated into phage 186 *attB* site within the chromosome, was transformed with the plasmid pE-FLP to remove the Km\textsuperscript{R} cassette. Chromosomal integration was verified by colony PCR as previously described.\textsuperscript{257} An *E. coli* MHT-donor strain (MG1655-tetR) was created by chromosomally integrating a spectinomycin resistant cassette (Spec\textsuperscript{R}), the gene encoding tetracycline repressor (TetR) and the gene encoding lac repressor (**lacI**) into the \( \lambda \) attachment site of *E. coli* MG1655 using the \( \lambda \) recombination system.\textsuperscript{259} An integration plasmid, pZS4Int-lacl/tetR, from the pZ Expression System, manufactured by Expressys, that constitutively expresses TetR, was PCR amplified using primers that introduce a second AvrII restriction site adjacent to the pSC101 origin. The plasmid was digested with AvrII to remove the pSC101 origin, and the remaining DNA fragment was circularized using T4 DNA ligase and transformed into MG1655 harboring the integrase-expressing plasmid pLDR8\textsuperscript{259} using heat-shock. The resulting cells were plated on LB-agar plates containing spectinomycin (50 \( \mu \)g/mL) to select for cells that contain the TetR gene integrated into the \( \lambda \) attachment site. Integration was verified by PCR amplifying the TetR gene. An *E. coli* recipient strain (MG1655-chl) was generated by chromosomally integrating a chloramphenicol-resistant cassette
(CmR) cassette into the phage 186 attB site in *E. coli* MG1655 using Clonetegration. This was achieved by amplifying a chloramphenicol-resistance cassette (CmR), cloning the product of this reaction into pOSIP-KO using classical cloning with BamHI and SpeI restriction sites, transforming the resulting plasmid into MG1655 using heat shock, and plating the cells on LB-agar containing kanamycin (25 µg/mL) and chloramphenicol (17 µg/mL). Colonies obtained from this selection were transformed with pE-FLP to remove KmR, and integration of the CmR at the 186 attB site was verified using colony PCR as described. An *E. coli* transconjugant strain (MG1655-Tc) was generated by mating MG1655-tetR and MG1655-chl. MG1655-tetR (10^5 CFU) transformed with pF-mht was incubated with MG1655-chl (10^7 CFU) in LB medium (20 µL) for 4 hours at 37°C without shaking, and plated on LB-agar containing kanamycin (25 µg/mL) and chloramphenicol (17 µg/mL). After overnight growth at 37°C, colonies were visually inspected to assess mCherry expression, and twenty colonies appearing red were used to inoculate 1 mL M63 cultures containing 100 mM NaBr, 25 µg/mL kanamycin and 17 µg/mL chloramphenicol. After 24 hours of growth in 2 mL gastight vials, CH₃Br production was analyzed using GC-MS (Figure 4-13) as described below. A CmR and KmR transconjugant strain obtained from conjugation in liquid culture, which displayed representative gas production, was used for all studies comparing donor and transconjugant gas production.

**GC-MS analysis of CH₃X production.** The analytical system consisted of an Agilent 7820 gas chromatography (GC) with DB-VRX capillary column (30m, 0.15 mm ID, and 1 µm film) and a 5977E mass spectrometry (MS). Samples were
Figure 4-13. Transconjugant strains arising from conjugation display similar gas production

After conjugation between donor and receiver strains, the mixture was plated on selective medium to obtain colonies that represent different transconjugant clones. The gas production from twenty different transconjugant clones was measured in M63 medium supplemented with 100 mM NaBr and compared to gas production from a culture containing the E. coli donor strain. In each experiment, 10^6 CFU of each strain was incubated for 24 hours prior to measurement of CH3Br in the culture headspace.
injected into the GC using an Agilent 7693A liquid autosampler with either a 100 μL (Agilent G4513-80222) or 250 μL gastight syringe (Agilent G4513-60560). The oven temperature was programmed to hold at 45°C for 1 minute, followed by an increase to 60°C. The total run time was 1 minute and 32 seconds. The 5977E MSD was configured for selected ion monitoring (SIM) mode, specifically to CH₃Cl (MW = 50 and 52 for CH₃³⁵Cl and CH₃³⁷Cl, respectively), CH₃Br (MW = 93.9 and 95.9 for CH₃⁷⁹Br and CH₃⁸¹Br, respectively), or CH₃I (MW = 127 and 142 for ¹²⁷I and CH₃¹²⁷I). The peak area of a sample was integrated using Agilent’s MassHunter Workstation quantitative analysis; the major ions (CH₃³⁵Cl, CH₃⁷⁹Br, and CH₃¹²⁷I) were used for quantifying peak areas while the minor ion was used as a qualifier. Standard curves for CH₃Cl and CH₃Br were obtain by mixing methanol (100 μL) containing serial dilutions of analytical standards provided in methanol (Sigma) with M63 medium (900 μL) containing either 100 mM NaCl or NaBr. These mixtures were incubated in 2 mL gastight vials for 3 hours at 37°C with shaking at 290 rpm to allow gas to equilibrate between the aqueous and gas phases, and CH₃X levels were measured within a 50 μL sample of the headspace gas. All experiments except the conjugation measurements used the 100 μL syringe to inject and analyze CH₃X levels in 50 μL of headspace gas; the conjugation experiments used the 250 μL syringe to inject and analyze CH₃Br levels in 125 μL of headspace gas. Our standard curve for CH₃Br revealed that our maximum signal from our longest incubations (~10 ppm) was 10³ lower than the level used for fumigation in agriculture (~10⁴ ppm) and higher than atmospheric background (~10⁻⁵ ppm). Our maximum
CH₃Br signal observed in conjugation experiments is also lower than the levels of methyl halides (CH₃Cl and CH₃I) previously found to induce expression of the adaptive response protein (Ada) in *Escherichia coli*, which senses and responds to methylation induced by these compounds.³²¹,³²²

For soil experiments that used varying amounts of water and soil, I calculated the total mass of CH₃Br in each vial using the standard curve and Henry’s law for adjusting the gas partitioning between liquid and gas phase as previously described.³²³ For this calculation, I estimated the total CH₃Br mass (W_total) as the product of the concentration measured in the gas phase (C_gas), the sum of the partition coefficient and the ratio of the volume (K + V_gas/V_liquid), and the volume of the liquid phase, *i.e.*, W_total = C_gas·(K + V_gas/V_liquid)·V_liquid. The volume of the gas phase is estimated by subtracting the volume of the liquid phase and the volume of the soil calculated by dividing soil mass to its density (KBS top soil gravel free bulk density is reported at 1.5 g/ml) from the total volume of the vial, *i.e.*, V_gas = V_total – V_liquid – (W_soil/ D_soil). All of the data is now reported as the total gas mass.

**Cell viability.** A stationary phase culture of MG1655-mht (1 mL) was washed twice with 10% glycerol and resuspended in 10% glycerol to an optical density (OD) of 0.05, measured at 600 nm. M63 medium (200 µL) containing varying concentrations of halides (0.01 to 1600 mM NaCl, NaBr, or NaI) was arrayed in a flat bottom 96-well plate, and each well was inoculated with 2 µL MG1655-mht from the resuspended culture, and growth was assessed in unsealed wells. Growth was continuously monitored at 37°C for 24 hours while shaking at 216 rpm with 1.5 mm amplitude using a TECAN M1000 Pro Microplate Reader by measuring
absorbance at 600 nm every five minutes. These OD values were fit to Equation 4-1:

**Equation 4-1**

\[
OD = \frac{A}{1 + \exp\left(\frac{4\mu_m}{A}(\lambda - t) + 2\right)}
\]

(Eq 1)

where \( t \) is time, \( \lambda \) represents the growth delay, \( \mu_m \) is the maximum growth rate, and \( A \) represents the asymptote and the maximum OD. \(^{324}\) Data shown for each growth experiment represents the average of three independent measurements. To examine the effect of methyl halide accumulation on cell viability, MG1655-mht and its parental strain were also incubated in sealed cuvettes using rich medium containing 10 g/L tryptone, 5 g/L yeast extract, and 100 mM sodium bromide. Overnight cultures were diluted to an OD = 0.01 with 1 mL fresh medium, and the OD of each vial was measured until cells reached stationary phase. *E. coli* MG1655 and MG1655-mht presented no significant differences in cell growth under these conditions.

**Liquid culture analysis of CH₃X production.** Stationary phase MG1655-mht (10 µL) that had been washed and resuspended in 10% glycerol was used to inoculate M63 medium (1 mL) containing varying concentrations of halides (0.01 to 1600 mM NaCl, NaBr, or NaI). These halides are required as substrates for the MHT in addition to S-adenosyl methionine, a metabolite that is synthesized from methionine and ATP. While methionine can be synthesized by *E. coli*, it was also provided in the growth medium in the form of casamino acids. Each culture was incubated at 37°C in 2 mL glass vials that were capped with gastight seals (Phenomenex) while shaking at 290 rpm. After 24 hours, CH₃X levels were analyzed using GC-MS. In the case of measurements comparing gas production
from different titers of MG1655-mht, I first estimated the cell number of the cultures using OD$_{600}$ measurements, washed cells twice with 10% glycerol and diluted cells to the indicated titer using 1 mL M63 medium containing 100 mM NaBr. Each sample was placed in a 2 mL vial, crimped, and incubated at 37°C. After 1 hour incubations, the headspace gas (125 µL) in each sample was measured with the GC-MS. Data shown for each liquid culture experiment represents the average of three independent measurements.

**Anaerobic CH$_3$X analysis.** MG1655 transformed with pHC.plas:MHT:GFP was grown to stationary phase in LB medium containing 50 µg/L kanamycin. This culture was washed twice with 10% glycerol, and diluted to an OD$_{600}$ = 0.05 in M63 medium containing 100 mM NaBr and 50 µg/mL kanamycin. Cultures (5 mL) were grown in capped Hungate vials (15 mL) containing or lacking 100 nM N-(3-oxodecanoyl)-L-homoserine lactone, AHL (Sigma). Anaerobic cultures were generated by purging the air with nitrogen gas in the Hungate vials using a vacuum gas manifold. After incubating for 24 hours at 37°C while shaking at 250 rpm, CH$_3$Br levels were measured by manually injecting headspace gas into the GC-MS. GFP levels were measured by uncapping vials, arraying 200 µL of each culture in a flat bottom 96-well plate, and analyzing green fluorescence ($\lambda_{ex} =$ 488 nm; $\lambda_{em} =$ 509 nm) and optical density using a TECAN M1000 microplate reader. The small GFP induction in cells derived from anaerobic cultures is attributed to a brief cellular exposure to air (<2 min) during the transfer of samples to aerobic 96-well plates, since previous studies have shown that the p-hydroxybenzylidene-imidazolidone chromophore in GFP cannot mature in anaerobic conditions that
lack oxygen. Three replicates were performed for each experiment.

**Soil incubation conditions.** All soil was from Kellogg Biological Station, collected at 0-5 cm depth from the Ap horizon of a Typic Hapludalf (USDA soil taxonomic nomenclature) and consisted of 43% sand, 38% silt, and 19% clay. This soil was sieved to 2 mm and autoclaved twice. The water holding capacity (WHC) was determined by measuring the amount of water retained by 20 g of the soil sample after saturated and drained for 6 hours. For all analysis involving MG1655-mht, conditions reflecting different WHC were generated by mixing different amounts of soil and M63 medium to a total mass of 1 gram. To prepare each sample, dry soil was weighed out and transferred into 2 mL vials. M63 medium containing or lacking cells (10^8 CFU of MG1655 or MG1655-mht) was added to the soil, and a sterile toothpick was used to homogenize the M63-soil-cell mixture. All samples were incubated in 2 mL gastight vial for 24 hours at 37°C while shaking at 290 rpm, and then headspace gas (50 µL) was injected into the GC-MS using the autosampler.

**Bacterial extraction from soil.** After gas measurements, soils were hydrated using LB so that their soil content was a minimum of 33% by mass. Vials were vigorously shaken by vortexing for 30 seconds, the liquid within each vial was serially diluted, and serial dilutions were spread on LB agar-plates lacking or containing antibiotics. After incubation at 37°C for 16 hours, the number of colonies on each plate was manually counted, and the titer of cells in each soil was calculated by considering dilutions. Three replicates were performed for each experiment. When experiments were performed using sterilized soils that lacked
added *E. coli*, colonies were not obtained from this extraction procedure.

**Conjugation experiment.** Overnight LB cultures of the donor and receiver strain were washed with fresh M63 medium and diluted into M63 medium containing 100 mM NaBr to $2 \times 10^6$ and $10^8$ cells/mL, respectively. Receiver cells ($10^7$ cells) were added to soil within 2 ml glass vials, and these mixtures were homogenized by mixing with a sterile toothpick. After mixing, 50 µL donor cells ($10^5$ cells) were added to the sample, and a toothpick was used to homogenize the matrix a second time. Samples were sealed with Parafilm to minimize evaporation and incubated at 37°C without shaking. For each matrix condition, 36 identical vials were prepared. At each time point, three vials were crimped for one hour, and CH$_3$Br was analyzed within 125 µL headspace gas using GC-MS. Immediately after each gas measurement, I extracted bacteria from each vial by adding additional LB to 1 mL of total liquid and vortex for 30 seconds, plating dilutions of extracted cells on LB-agar plates containing chloramphenicol (17 µg/mL) and a mixture of chloramphenicol (17 µg/mL) and kanamycin (25 µg/mL), and counting colonies after 16 hours of growth at 37°C. The number of transconjugants in each vial was calculated using CFU on plates containing two antibiotics, while the total number of cells derived from the original receiver strain was calculated as the number of CFU on plates containing chloramphenicol.
Chapter 5: Future Directions
5.1. Gas outputs would open the door for new applications of synthetic biology [J42]

With the ability to report non-invasively in hard-to-image conditions, gas reporters will allow scientists to conduct in situ measurements on microbial gene expression in various environmental matrices. Furthermore, gas reporters will enable synthetic biology to be leveraged for various environmental applications which are currently difficult to analyze with conventional reporting techniques. Gas reporters could be applied to report on a variety of microbial activates in high extinction materials, including:

1) **The transcriptional activities of individual genes within a cell.** Promoter activities can be measured in complex matrices by placing gas reporter genes under the control of any promoter. Information obtained from such reporters would be useful for assessing the robustness of genetically-modified organisms in complex conditions.

2) **The level of chemical compounds in a complex environmental sample.** Gas bioreporters can be used to elucidate chemical levels in soils or other matrices being remediated, such as superfund sites and oil spills, which would help with evaluating the benefits of different remediation strategies.

3) **The presence of microbes in the environment.** Rapid and sensitive detection of microbes in food is urgently needed to avoid foodborne illness, as well as detection of changes in the levels of beneficial and deleterious microbes upon amendment of soils with various materials (e.g., biochar, fertilizers, pesticides,
etc.). Also, simple monitoring methods which track bacterial consortia changes in active sludge in waste water treatment are needed to improve treatment processes.

4) The coordinated decision making within a population. Quorum sensing controls beneficial (nodulation)\textsuperscript{294} and deleterious (plant root infection)\textsuperscript{325} behavior of microbes. By understanding the mechanisms by which soil manipulations affect microbial signaling, we can make land use decisions that promote beneficial signaling systems while eliminating or minimizing deleterious systems.

5) The metabolic status of biochemical reactions. By analyzing the metabolic status of cells, researchers can use this information to optimize microbial strains for converting complex feedstocks like beet molasses into high-value chemicals and biofuels. Such reporters could be used to provide temporal information on cellular levels of redox cofactors, \textit{e.g.}, NADPH/NADP levels using SoxR regulation,\textsuperscript{326} to assess and tune redox flow as microbes transition between the use of different sugars.
5.2. Environmental microbial questions that can be answered using gas outputs

5.2.1. Ratiometric gas reporting can be used to study the effect of biochar on microbial and plant signaling *in situ* in soil [J43]

Biochars can increase crop productivity, improve plant pest resistance, and decrease nitrogen loss from soils. However, sometimes biochar amendments induce agricultural costs, including decreased crop yields, sorption of pesticides, shifts microbial composition, and disruption of important fungal-plant symbiosis. In addition, the amendment of biochars to soil has been shown to promote the loss of non-charcoal organic matter (priming) in some studies. This increase in soil decomposition causes an enhanced flux of CO$_2$ into the atmosphere from soils, potentially decreasing the carbon sequestration benefits of biochars. These results vary with the biomass feedstock used to create biochars as well as the production temperature. The underlining mechanism of how biochar interferes with soil biota is unclear.

Based on my results, I hypothesize that biochars influence soil microbes through interfering cell-cell signaling molecules. Mechanistic studies of biochars' effect on cell-cell signaling molecules have been done in artificial matrices, such as liquid and soil. These studies have validated that biochars sequester AHL through hydrolysis and sorption. However, biochars’ effect on cell-cell signaling molecules has not been studied in a natural soil matrix. With the development of my ratiometric reporting strategy described in Chapter 4, we can
now better evaluate biochar’s effect on cell-cell signaling molecules in soils. By correlating biochars’ biological effect and their physical properties (surface area, pH value, and mineral content), these types of measurements will provide the fundamental knowledge needed to engineer a biochar that maximizes benefits for carbon sequestration and agriculture [J44].

To monitor biochar sequestration of cell-cell signaling molecules in soils, experiments similar to those described in Section 3.4 can be used. For instance, synthetic 3-oxo-C6-HSL and biochars can be added into 2 mL vials containing soils with a MG1655-lux bioreporter applied to read out the AHL concentration change over time. The results would allow us to calculate the half-lives of 3-oxo-C6-HSL under various conditions. Since the ratiometric reporting strategy enables mid-throughput analysis – up to 300 samples a day using one GC-MS, it’s possible to screen a wide range of biochar and soil combinations in a couple of weeks. This kind of experiment would provide two layers of information. First, it would identify the biochars most effective at sequestering 3-oxo-C6-HSL in a particular soil. Since 3-oxo-C6-HSL has been shown as a master regulator that coordinates the attack of Pectobacterium carotovora to potato roots (soft rot disease), this biochar could be applied as a disease control agent in the fields after further validations with greenhouse experiments. Second, by analyzing the properties of biochars and their effect on the half-life of 3-oxo-C6-HSL in different soil samples, we could apply this information to engineer biochars that have maximum ability to degrade 3-oxo-C6-HSL in targeted soil types.
I foresee that the sorptive nature of biochars\textsuperscript{230,332} could interfere with the partitioning of gas reporting signals, CH\textsubscript{3}Br and C\textsubscript{2}H\textsubscript{4}, from the soil matrix into the headspace. To mitigate this problem, individual standard curves that convert GC-MS peak areas of methyl bromide and ethylene into absolute mass values need to be generated for each biochar and soil combination. Alternatively, strains that constitutively express both MHT and EFE, such as MG1655-efe-mht described in Section 3.4, can be used to benchmark the matrix effect of gas production and portioning. By normalizing gas production from bioreporters to MG1655-efe-mht in each type of sample matrix, we can compare gas signal production across different matrices.

5.2.2. Ratiometric gas reporting can be used to study quorum sensing systems in

\textit{Rhizobium leguminosarum}

\textit{Rhizobium leguminosarum}, a nodule-forming nitrogen fixer, has been shown to use elaborate quorum sensing systems to regulate symbiotic plasmid transfer, population density, and nodule formation.\textsuperscript{244,335-343} It produces a wide range of AHLs, ranging from short chain (C6-HSL) to long chain (3-OH-C14:1-HSL) AHLs. The master autoinducer, N-(3-hydroxy-7-cis-tetradecenoyl)-l-homoserine lactone (3-OH-C14:1-HSL), is synthesized by CinI located on the \textit{Rl} chromosome, whose expression is normally repressed by BisR, a luxR-type regulator located on the symbiotic plasmid.\textsuperscript{336} In \textit{Rl} cells without the symbiosis plasmid, CinI is expressed and produces 3-OH-C14:1-HSL, which then triggers a cascade of reaction in \textit{Rl} cells harboring the symbiotic plasmid and leads to
plasmid transfer. 3-OH-C14:1-HSL activates Tra QS system, which produces mainly 3-oxo-C8-HSL and regulates symbiotic plasmid transfer. 3-OH-C14:1-HSL also activates Rhi QS system (produces C6-, C7-, and C8-HSL) located on the symbiotic plasmid that controls the gene cluster related to nodulation processes.335,344,345

This complex QS network coordinates communications within rhizobium population and between rhizobium and plants, and it relies on the concentration of each autoinducer.244,335–343 Perturbation to the autoinducers by soils could influences nodulation, for instance, environmental sequestration of autoinducers208 and autoinducer inhibitors secreted by competitors.217 The molecular mechanisms of these QS systems have been well studied in the lab, but little is known about the temporal dynamics of these autoinducers in soils. I hypothesized that this knowledge gap is due to the difficulty of quantifying autoinducers in soils. As discussed in Section 1.1.2.1, bioreporters have been developed to sense autoinducers,187,346,347 but previous genetic reporters are difficult to detect directly from soil samples, which results in laborious procedures to extract bioreporters and high uncertainty of quantitation. The gas reporting strategy I developed in this thesis can overcome these issues and provide quantitative information of autoinducer concentrations in soils.

In Section 3.4, I applied MG1655-lux and MG1655-las to measure 3-oxo-C8-HSL and 3-OH-C14:1-HSL produced by Ri in an agricultural soil. After four-day incubation, increased AHL concentrations were detected by both bioreporters (Figure 3-17). Additionally, the activation of MG1655-las happened earlier than
MG1655-lux. Although this is just a qualitative and preliminary observation, the trend coincides with the hypothesis of 3-OH-C14:1-HSL as a master regulator, which activates the production of short chain AHLs. This experiment could be improved using more specific and sensitive bioreporters for Rl AHLs, such as a 3-OH-C14:1-HSL specific transcriptional activator, CinR-based bioreporter. In the future, qPCR can be used to monitor the ratio between the symbiotic plasmid and total Rl population while applying gas bioreporters to monitor autoinducer concentrations. By examining the temporal dynamics of autoinducer concentration and symbiotic plasmid transfer under different matrix conditions, we could better understand the link between short-term environmental conditions and long-term nodulation and nitrogen-fixing efficiency.

5.2.3. Gas outputs can be used to study horizontal gene transfer in soils and sediments

Studying environmental effects on the rate of horizontal gene transfer (HGT) is not only important in understanding the fundamental mechanism of microbial evolution but also essential for environmental engineering applications, such as mitigating antibiotic gene transfer and bioremediation. The synthetic F-plasmid E. coli conjugation system that I constructed in Chapter 4 could be easily applied to screen the effect of different environmental parameters on conjugation rate. This would be useful for environmental engineers to identify strategies to prevent the spread of antibiotic genes surrounding animal farms.
The gas reporting strategy described in Chapter 4 could be expanded to other HGT systems. The F-plasmid conjugation system has a high conjugation rate (1%) but narrow host range – limited to Enterobacteria.\textsuperscript{350} Other conjugation systems, such as the RP4 plasmid\textsuperscript{351} or IncQ plasmid,\textsuperscript{352} have much broader host range but lower conjugation rate (0.1 to 0.01%). When using gas as the transconjugant reporting strategy, the most important consideration is the background gas production by the donor strain, especially at the low conjugation rate condition since longer incubation times might be needed. In Chapter 4, I used TetR to repress the expression of MHT in the donor cells, which achieved 1000-fold dynamic range. To further increase the dynamic range, we could apply more stringent repression strategy, such as CRISPRi,\textsuperscript{12} or adding orthogonal degradation tag to MHT in the donor cells.\textsuperscript{353} Besides conjugation, we could also construct gas reporters in phage transduction system or linear environmental DNA to explore other HGT mechanisms.

5.3. Further improvements are needed with gas reporting

5.3.1. Current limitation of the gas outputs

In this thesis research, I demonstrated the utility of MHT and EFE as gas reporters. However, there are still limitations:

1) \textbf{Only one of the two gases are anaerobic.} EFE requires oxygen to function, which limits its utility in sediments or soils with low oxygen content.

2) \textbf{MHT and EFE use different metabolites} (SAM and $\alpha$-ketoglutarate,}
respectively) as substrates. Since their substrates are coming from different metabolite pools, the ratio could be influenced by cellular metabolic states.

3) The current gas analytical method has a high detection limit. Current detection limit requires at least of $10^5$ gas-producing cells to obtain a quantifiable signal after one-hour accumulation, which limits the application of the gas bioreporter.

5.3.2. Expanding the toolbox of gas-producing enzymes

Bacteria naturally produce a wide diversity of volatile compounds. Here, I explored the possibility of using MHT and EFE as reporter proteins. Other candidates that satisfy some of the criteria discussed in Section 1.3 were listed in Table 1. In addition, with further protein engineering, it is possible to create specialized MHT – MHT that only produce $\text{CH}_3\text{Cl}$, $\text{CH}_3\text{Br}$, or $\text{CH}_3\text{I}$. The crystal structure and catalytic site of *Arabidopsis thaliana* MHT are known, which could be used to guide saturation mutagenesis experiments to create specialized binding pockets for different halides. Also, the 89 identified MHT homologous that have different affinity toward halides could be potential gas reporter candidates.

5.3.3. Increasing gas analysis sensitivity through analytical method improvement

The high detection limit problem could be addressed through adding a purge-and-trap (P&T) system to a GC-MS, such as the Teledyne Tekmar Atomx XYZ P&T system. A P&T system concentrates headspace gas prior to injecting into the GC-MS. In general, a P&T system is three orders of magnitude more sensitive over normal headspace analysis. Therefore, by implementing a P&T
system, we could potentially reduce the minimal number of gas-producing cells required for a detectable signal from $10^5$ to 100 cells.

5.3.4. Using continuous sampling methods to increase the resolution of information that can be obtained from gas-reporting microbes[45]

In this thesis, I have demonstrated the utility of using gas-reporting microbes with a static headspace-measuring workflow. To achieve continuous monitoring of gas reporting, a method of continuous gas sampling needs to be developed. To build this dynamic headspace analyzer, a gas sampling valve can be connected to an inlet of an Agilent 7820A gas chromatograph. The sampling volume of this valve will be governed by the size of sampling loop and the sampling rate controlled through the MassHunter software. For sample incubation, 125 ml glass serum vials sealed with rubber septa or incubation chambers designed for soil and plant gas emission can be used. Clean air will flow through the sample vial to the valve, and a fixed volume of air will be injected into the GC at a constant rate. The main challenge of developing this method will be tuning the sampling rate, sampling volume, and air flow rate to achieve biologically relevant sampling rates (minutes-hours) with quantifiable signal intensity. Such dynamic sampling will be important for linking time-dependent changes in gene expression to other observable processes, such as respiration in soils, which can already be monitored through continuous measurements of CO$_2$ production.
5.3.5. Using orthogonal protein degradation tags to reduce the response time of gas outputs under fluctuating environmental conditions

To increase the response dynamics of MHT, further tuning on MHT stability would be needed. In Section 3.2, I showed that constitutive MHT expression leads to a monotonically increasing gas production rate (ng/CFU/hr) in the first six hours (Figure 3.1). This result indicated that MHT is accumulating faster than its degradation and dilution (due to cell division) rate, which suggested that MHT is a very stable protein. The advantage of a stable reporter protein is that it can amplify input signal and provide a constantly detectable output. However, a stable output could mask the dynamics of the input signal. For instance, MG1655-las could produce a consistent methyl bromide/ethylene ratio even when 3-oxo-C12-HSL has been depleted from the sample due to remaining MHT function in the cells. Therefore, to create a gas bioreporter that responds to fluctuating environmental cues, the stability of MHT needs to be reduced.

Protein stability can be adjusted through adding protein degradation tags to a protein sequence. Well characterized degradation systems in *E. coli*, such as the ssrA tag recognized by *E. coli* indigenous ClpX protease or tags recognized by the orthogonal *Mesoplasma florum* mf-Lon protease, have been used to tune the stability of reporter proteins to decrease gene circuit response delays. By adding a suitable protein degradation tag to MHT, a gas bioreporter with faster on-off transition could be created. Together with the dynamic headspace analyzer, this bioreporter could provide *in situ*, real-time monitoring of chemical degradation and production in soil samples.
References


25. Vishnoi, M. *et al.* Triggering sporulation in *Bacillus subtilis* with artificial two-component systems reveals the importance of proper Spo0A activation


57. Kobras, C. M., Mascher, T. & Gebhard, S. Application of a Bacillus subtilis Whole-Cell Biosensor (Plial-lux) for the Identification of Cell Wall Active
Antibacterial Compounds. in *Methods in molecular biology (Clifton, N.J.)*


88. Dobbelaeere, S. *et al.* Responses of agronomically important crops to


142. Moroz, N. *et al.* Extracellular Alkalinization as a Defense Response in


174. Remus-Emsermann, M. N. & Leveau, J. H. Linking environmental


183. Dorn, J. G., Frye, R. J. & Maier, R. M. Effect of temperature, pH, and initial


16th August 2014)


198. Gao, X. *et al.* Charcoal Disrupts Soil Microbial Communication through a


232. Noyce, G. L., Winsborough, C., Fulthorpe, R. & Basiliko, N. The


239. Tsien, R. Y. THE GREEN FLUORESCENT PROTEIN. Annu. Rev.


248. Winson, M. K. *et al.* Construction and analysis of luxCDABE-based plasmid sensors for investigating N-acyl homoserine lactone-mediated quorum


280. Redeker, K. R. *et al.* Emissions of Methyl Halides and Methane from Rice


305. Holmkvist, L., Ferdelman, T. G. & Jørgensen, B. B. A cryptic sulfur cycle driven by iron in the methane zone of marine sediment (Aarhus Bay,


313. Kushwaha, M. & Salis, H. M. A portable expression resource for


352. Tauch, A. et al. The complete nucleotide sequence and environmental
distribution of the cryptic, conjugative, broad-host-range plasmid pIPO2
isolated from bacteria of the wheat rhizosphere. *Microbiology* 148, 1637–
1653 (2002).


fabrication, and application of a dynamic chamber for measuring gas

forest soil CO2 efflux: an in situ comparison of four techniques. *Tree

proteases degrade proteins with carboxy-terminal peptide tails added by

357. Gur, E. & Sauer, R. T. Evolution of the ssrA degradation tag in


359. Shingler, V. & Moore, T. Sensing of aromatic compounds by the DmpR
transcriptional activator of phenol-catabolizing Pseudomonas sp. strain


366. Leveau, J. H. & Lindow, S. E. Appetite of an epiphyte: quantitative


389. Daunert, S. *et al.* Genetically engineered whole-cell sensing systems:


Appendix A

Sensory modules for bacterial bioreporter construction. Adapted from reference (22).^{22}

<table>
<thead>
<tr>
<th>Sensor proteins</th>
<th>Host chassis</th>
<th>Promoter–reporter fusion</th>
<th>Chemical targets</th>
<th>Detection sensitivity</th>
<th>ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>XylR of <em>Pseudomonas putida</em></td>
<td><em>Escherichia coli</em></td>
<td><em>Pu</em>&lt;sup&gt;+&lt;/sup&gt;–<em>luxFF</em></td>
<td>Benzene, toluene and xylene</td>
<td>40 mM</td>
<td>358</td>
</tr>
<tr>
<td>DmpR of <em>P. putida</em></td>
<td><em>P. putida</em></td>
<td><em>Po</em>&lt;sup&gt;−&lt;/sup&gt;–<em>luxAB</em></td>
<td>Phenol</td>
<td>3 mM</td>
<td>359</td>
</tr>
<tr>
<td>TbuT of <em>Ralstonia pickettii</em></td>
<td><em>E. coli</em></td>
<td>*tbuA1p–<em>luxAB</em></td>
<td>Benzene, toluene and xylene</td>
<td>0.24 mM</td>
<td>360</td>
</tr>
<tr>
<td>HbpR of <em>Pseudomonas nitroreducens</em></td>
<td><em>E. coli</em></td>
<td><em>hbpCp–luxAB</em></td>
<td>Hydroxylated biphenyls</td>
<td>0.4 mM</td>
<td>3639</td>
</tr>
<tr>
<td>PhnR of <em>Burkholderia sarsisoli</em></td>
<td><em>B. sartisoli</em></td>
<td><em>phnSp–luxAB</em></td>
<td>Naphthalene and phenanthrene</td>
<td>0.17 mM</td>
<td>360</td>
</tr>
<tr>
<td>IbpR of <em>P. putida</em></td>
<td><em>E. coli</em></td>
<td><em>ibpAp–luxCDABE</em></td>
<td>Various aromatics</td>
<td>1 mM</td>
<td>361</td>
</tr>
<tr>
<td>NahR of <em>P. putida</em></td>
<td><em>P. putida</em></td>
<td><em>nahGp–luxAB</em></td>
<td>Naphthalene and salicylate</td>
<td>10 nM</td>
<td>362</td>
</tr>
<tr>
<td>AlkS of <em>Pseudomonas oleovorans</em></td>
<td><em>E. coli</em></td>
<td><em>alkBp–luxAB</em></td>
<td>C6–C10 alkanes</td>
<td>10 nM</td>
<td>363</td>
</tr>
<tr>
<td>TodST of <em>P. putida</em></td>
<td><em>P. putida str. F1</em></td>
<td><em>todXp–luxCDABE</em></td>
<td>Toluene, benzene, phenol, p-xylene, m-xylene and trichloroethene</td>
<td>0.3 mM</td>
<td>364</td>
</tr>
<tr>
<td>SepR of <em>P. putida</em></td>
<td><em>P. putida str. F1</em></td>
<td><em>sepAp–luxCDABE</em></td>
<td>Solvents</td>
<td>~0.5 mM</td>
<td>365</td>
</tr>
<tr>
<td>FruR of <em>Erwinia herbicola</em></td>
<td><em>E. herbicola</em></td>
<td><em>fruBp–gfp[AAV]&lt;sup&gt;i&lt;/sup&gt;</em></td>
<td>Fructose and sucrose</td>
<td>~2 mM</td>
<td>366</td>
</tr>
<tr>
<td>AraC of <em>E. coli</em></td>
<td><em>E. coli</em></td>
<td>*pBAD–gfpuv&lt;sup</td>
<td>i&lt;/sup&gt;*</td>
<td>L-Arabinose</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>ArsR of <em>E. coli</em></td>
<td><em>E. coli</em></td>
<td><em>arsRp–luxAB</em></td>
<td>Arsenite and antimonite</td>
<td>5 nM</td>
<td>41</td>
</tr>
<tr>
<td>MerR of <em>E. coli</em></td>
<td><em>E. coli</em></td>
<td><em>merTp–luxCDABE</em></td>
<td>Hg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>1 nM</td>
<td>368-369</td>
</tr>
<tr>
<td>CadC of <em>Staphylococcus aureus</em></td>
<td><em>Bacillus subtilis</em></td>
<td><em>cadCp–luxFF</em></td>
<td>Cd&lt;sup&gt;2+&lt;/sup&gt;, Pb, Sn and Zn</td>
<td>3 nM</td>
<td>370</td>
</tr>
<tr>
<td>ZntR of <em>E. coli</em></td>
<td><em>E. coli</em></td>
<td><em>zntAp–luxCDABE</em></td>
<td>Zn, Pb and Cd</td>
<td>5 mM, 0.7 mM and 10 nM, respectively</td>
<td>371</td>
</tr>
<tr>
<td>TetR of <em>E. coli</em></td>
<td><em>E. coli</em></td>
<td><em>tetAp–luxCDABE</em></td>
<td>Tetracyclines</td>
<td>45 nM</td>
<td>372</td>
</tr>
<tr>
<td>MphR of <em>E. coli</em></td>
<td><em>E. coli</em></td>
<td><em>mphAp–lacZ</em></td>
<td>Macrolides (such as erythromycin)</td>
<td>~10 mM</td>
<td>373</td>
</tr>
<tr>
<td>SOS response proteins of <em>B. subtilis</em></td>
<td><em>B. subtilis</em></td>
<td><em>yorBp–luxFF</em></td>
<td>Various antibiotics (for example, ciprofloxacin)</td>
<td>60 nM</td>
<td>374</td>
</tr>
<tr>
<td>SpolIID and sigma E of <em>B. subtilis</em></td>
<td><em>B. subtilis</em></td>
<td><em>yhel–luxFF</em></td>
<td>Various antibiotics (for example, linezolid)</td>
<td>0.1 mM</td>
<td>374</td>
</tr>
<tr>
<td>NisRK of <em>Lactococcus lactis</em></td>
<td>L. lactis</td>
<td>nisAp–gfpuv[</td>
<td></td>
<td>]</td>
<td>Nisin</td>
</tr>
<tr>
<td>LuxR of <em>Aliivibrio fischeri</em></td>
<td>E. coli</td>
<td>luxIp–gfp[ASV][</td>
<td>]</td>
<td>N-Acyl homoserine lactones</td>
<td>1–10 nM</td>
</tr>
<tr>
<td>Ada of <em>E. coli</em></td>
<td>E. coli</td>
<td>alkAp–luxCDABE</td>
<td>DNA-alkylating agents</td>
<td>70 nM N-methyl-N'-nitro-N-nitrosoguanidine, for example</td>
<td>377</td>
</tr>
<tr>
<td>DnaK and S32 of <em>E. coli</em></td>
<td>E. coli</td>
<td>dnaKp–luxCDABE</td>
<td>An increase in the level of intracellular misfolded proteins</td>
<td>0.25 M methanol, for example</td>
<td>378</td>
</tr>
<tr>
<td>Crp–cAMP transcriptional dual regulator of <em>E. coli</em></td>
<td>E. coli</td>
<td>grpEp–luxCDABE</td>
<td>An increase in the level of intracellular misfolded proteins</td>
<td>0.14 mM pentachlorophenol, for example</td>
<td>378</td>
</tr>
<tr>
<td>OxyR of <em>E. coli</em></td>
<td>E. coli</td>
<td>katGp–luxCDABE</td>
<td>Intracellular production of oxygen radicals</td>
<td>3 mM H₂O₂, for example</td>
<td>379</td>
</tr>
<tr>
<td>SoxRS of <em>E. coli</em></td>
<td>E. coli</td>
<td>micFp–luxCDABE</td>
<td>Intracellular production of oxygen radicals</td>
<td>Detection sensitivity not indicated</td>
<td>380</td>
</tr>
<tr>
<td>RecA–LexA of <em>E. coli</em></td>
<td>E. coli</td>
<td>cdap–gfp</td>
<td>Single-stranded DNA that arises as a consequence of inhibition of DNA replication</td>
<td>5 nM N-methyl-N'-nitro-N-nitrosoguanidine, for example</td>
<td>381</td>
</tr>
<tr>
<td>RecA–LexA of <em>Salmonella enterica</em> subsp. enterica serova r Typhimurium</td>
<td>S. Typhimurium</td>
<td>sfiA–lacZ</td>
<td>Single-stranded DNA that arises as a consequence of inhibition of DNA replication</td>
<td>4 nM mitomycin C</td>
<td>382</td>
</tr>
<tr>
<td>RecA–LexA of <em>Salmonella enterica</em> subsp. enterica serova r Typhimurium</td>
<td>S. Typhimurium</td>
<td>recNp–luxCDABE</td>
<td>Single-stranded DNA that arises as a consequence of inhibition of DNA replication</td>
<td>46 nM mitomycin C</td>
<td>383</td>
</tr>
<tr>
<td>RecA–LexA of <em>Salmonella enterica</em> subsp. enterica serova r Typhimurium</td>
<td>S. Typhimurium</td>
<td>umuDp–lacZ</td>
<td>Single-stranded DNA that arises as a consequence of inhibition of DNA replication</td>
<td>10 nM mitomycin C</td>
<td>384</td>
</tr>
</tbody>
</table>

*AraC*, arabinose operon regulatory protein; *cdap*, promoter of the colicin D gene; *cAMP*, cyclic AMP; *Crp*, cAMP regulatory protein; *katGp*, promoter of the catalase–peroxidase gene; *lacZ*, *B*-galactosidase gene; *luxFF*, firefly luciferase gene; *lux*, bacterial luciferase biosynthesis gene; *Rec*, recombination and repair; *sfiA*, SOS cell division inhibitor gene (also known as *sulA*); *tbuA1p*, promoter of the toluene monooxygenase A-subunit gene. *A XylR-responsive promoter of *P. putida*. *A DmpR-responsive promoter of *P. putida*. § Unstable variants of GFP. ||A GFP variant that is optimized for maximal fluorescence when excited by ultraviolet light.
Appendix B

Common visual reporter proteins and their method of detection. Adapted from reference (22).²²

<table>
<thead>
<tr>
<th>Protein</th>
<th>Gene</th>
<th>Source</th>
<th>Substrate(s)</th>
<th>Detection</th>
<th>Notes</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial luciferase</td>
<td>luxAB* or luxCDABE</td>
<td>Bioluminescent bacteria*</td>
<td>O₂, FMNH₂ and long-chain aldehydes Bioluminescence</td>
<td>Bioluminescence</td>
<td>Requires O₂; aldehyde addition is required if only luxAB is used</td>
<td>384, 385</td>
</tr>
<tr>
<td>Firefly luciferase</td>
<td>lucFF</td>
<td>Firefly (Photinus pyralis)</td>
<td>O₂, ATP and luciferin</td>
<td>Bioluminescence</td>
<td>Requires O₂</td>
<td>386</td>
</tr>
<tr>
<td>Click beetle luciferase</td>
<td>lucGR</td>
<td>Click beetle (Pyrophorus plagiophthalmus)</td>
<td>O₂, ATP and pholasin</td>
<td>Bioluminescence</td>
<td>Requires O₂</td>
<td>387</td>
</tr>
<tr>
<td>Renilla luciferase</td>
<td>Rluc</td>
<td>Renilla reniformis</td>
<td>Coelenterazine and Ca²⁺</td>
<td>Bioluminescence</td>
<td>Requires O₂</td>
<td>388</td>
</tr>
<tr>
<td>Galactosidase</td>
<td>lacZ</td>
<td>Escherichia coli</td>
<td>Galactopyranoside‡</td>
<td>Chemiluminescence, colorimetry, electrochemistry and fluorescence</td>
<td>External substrate addition (may require cell permeabilization)</td>
<td>389</td>
</tr>
<tr>
<td>Fluorescent proteins gfp, etc.</td>
<td>gft, etc.</td>
<td>Aequorea victoria and additional marine invertebrates</td>
<td>N/A</td>
<td>Fluorescence</td>
<td>O₂ is required for maturation; different colour varieties exist</td>
<td>390</td>
</tr>
<tr>
<td>Spheroidene monooxygenase</td>
<td>crtA</td>
<td>Rhodovulum sulfidophilum</td>
<td>Spheroidene</td>
<td>Colorimetry</td>
<td>None</td>
<td>391</td>
</tr>
<tr>
<td>Infrared fluorescent proteins</td>
<td>Various</td>
<td>Bacteriophytocrome family</td>
<td>N/A</td>
<td>Fluorescence</td>
<td>None</td>
<td>392</td>
</tr>
<tr>
<td>FMN-based fluorescent proteins</td>
<td>Various</td>
<td>Engineered from Bacillus subtilis and Pseudomonas putida</td>
<td>N/A</td>
<td>Fluorescence</td>
<td>Functional in both oxic and anoxic conditions; requires endogenous FMN</td>
<td>318</td>
</tr>
</tbody>
</table>

N/A, not applicable. *Most commonly used species include Aliivibrio fischeri (also known as Vibrio fischeri), Vibrio harveyi and Photorhabdus luminescens. ‡ For example, O-nitrophenyl-β-D-galactoside (ONPG), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 4-methylumbelliferyl-β-D-galactopyranoside, 4-aminophenyl-β-D-galactopyranoside and D-luciferin-O-β-galactopyranoside.