TOWARDS PRACTICAL BACTERIAL BIOSENSOR ASSAYS FOR ON-SITE ANALYTE DETECTION

THE AIR-DRYING OF *ESCHERICHIA COLI* REPORTERS IN NATURAL POLYMERS AND INCORPORATION INTO SIMPLE BIOASSAYS

By

ELIZABETH MARIE SALVO, B.Sc. (Honours)

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

MASTER OF SCIENCE (2017) McMaster University

(Chemistry and Chemical Biology) Hamilton, Ontario

TITLE: The air-drying of *Escherichia coli* reporters in natural polymers and incorporation into simple bioassays

AUTHOR: Elizabeth Marie Salvo, B.Sc. (Honours) (McMaster University)

SUPERVISOR: John D. Brennan

NUMBER OF PAGES: X, 47

Abstract

Microbial biosensor systems (MBS) are useful for analyte detection owing to their low cost, sensitivity, and selectivity for bioavailable analytes. Due to typically poor shelf-life and sensitivity to external conditions, there are few reports of MBS technology applied to simple analytical devices. The effectiveness of air-drying MBS in natural polymers was investigated as a novel preservation technique. Two colorimetric *Escherichia coli* MBS, a tetracycline-inducible reporter and an arsenate-inducible reporter, were dried on various substrates yielding novel MBS platforms. In proof-of-concept experiments performed in 96-well microplates, both systems demonstrated responsivity after air-drying in low concentrations of pullulan. However, the MBS were unresponsive following brief storage of 1 week. To improve the preservation of MBS, sensing strips were created by air-drying concentrated acacia gum-based MBS suspensions onto paper. Cells dried on these strips demonstrated responsivity upon solubilization in various tube-based assays. MBS sensing strip responsivity was demonstrated following storage for 6 weeks at 4 °C. Tetracycline-responsive sensing strips also performed well in assays using spiked lake water samples. Air-drying in natural polymers was an effective MBS preservation technique, and allowed for the creation of "mix and read" style assays which were simple, equipment-free and ready-to-use.

Acknowledgments

Thank you to Dr. John Brennan and Dr. Carlos Filipe for their ongoing support, as well as to Dawn White and the Biointerfaces Institute technicians for their kind assistance. Special thanks also to Dr. Bob Pelton for the use of his lab equipment during the project, and to Nisha Agarwal for her colour sensing work.

Table of Contents

List of Figures and Tables

List of Abbreviations

ABS – alternate binding site[10]

BSC – biosafety cabinet

CFU – colony forming units

DMSO – dimethyl sulfoxide

E. coli – *Escherichia coli*

GFP – green fluorescent protein

LB – Luria-Bertani

MBS – microbial biosensor

OD – optical density

PBS – phosphate-buffered saline

RH – relative humidity

X-gal – 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Chapter 1 | Introduction

The use of bacteria as reporters of chemical and physical stimuli has been a constantly evolving field over the last 30 years. These bacteria, termed microbial biosensor systems (MBS), are genetically engineered to elicit measurable responses to chemical compounds or external stresses. In 1982, Quillardet *et al.* introduced one of the earliest examples of an MBS known as the SOS Chromotest – an *E. coli*-based sensor in which the activation of synthetic SOS genes by DNA-damaging agents generated a yellow colour (mediated by a chromogenic substrate).[1] Advances in understanding of the genetic manipulation of bacteria has allowed for the generation of a variety of unique MBS. In addition to MBS reporting non-specific toxicity, a number of systems for single-compound detection have been introduced, utilizing more specifically activated genetic circuits. King *et al.* generated the first of these in 1990 – a bioluminescent *Pseudomonas fluorescens*-based sensor for naphthalene degradation in soil mixtures.[2] A key advantage of using a MBS for pollutant detection is the inherent specificity for bioavailable fractions of analyte, yielding more relevant toxicity results than many chemical tests.[3] Since these early systems, MBS engineering has advanced to yield a range of sensors which sensitively detect heavy metals, organic pollutants, and pharmaceuticals. However, the full potential of these sensors has not been realized, owing to a number of technical challenges that have yet to be overcome including long response time, lack of portability and in all cases, instability during long term storage.[4-6]

1.1 Reporter Gene-based MBS Assays

Non-specific MBS for toxicity detection often use “lights off” assays, where the inhibition of constitutive reporter gene activity is measured. In contrast, compound-specific MBS systems for toxic compounds exploit known bacterial resistance pathways. Bacterial resistance mechanisms

depend on specific interactions between a toxin and regulatory protein. Simple MBS designs operate using synthetic plasmids where a regulatory promoter is fused to a reporter gene. Interaction of the toxin and regulatory protein (a repressor) de-represses transcription – leading to a measurable reporter protein such as luciferase or green fluorescent protein (GFP). These are termed "lights-on" assays (Figure 1.1.1). A variety of analytes have been successfully detected using MBS operating in both modes, including but not limited to: cadmium[7], lead[7], copper[8], arsenic[9-10], tetracyclines[11-12], halogenated organic acids[13], hydrocarbons[14], polychlorinated biphenyl compounds[15], phenolic compounds[16], and organotin compounds[17].

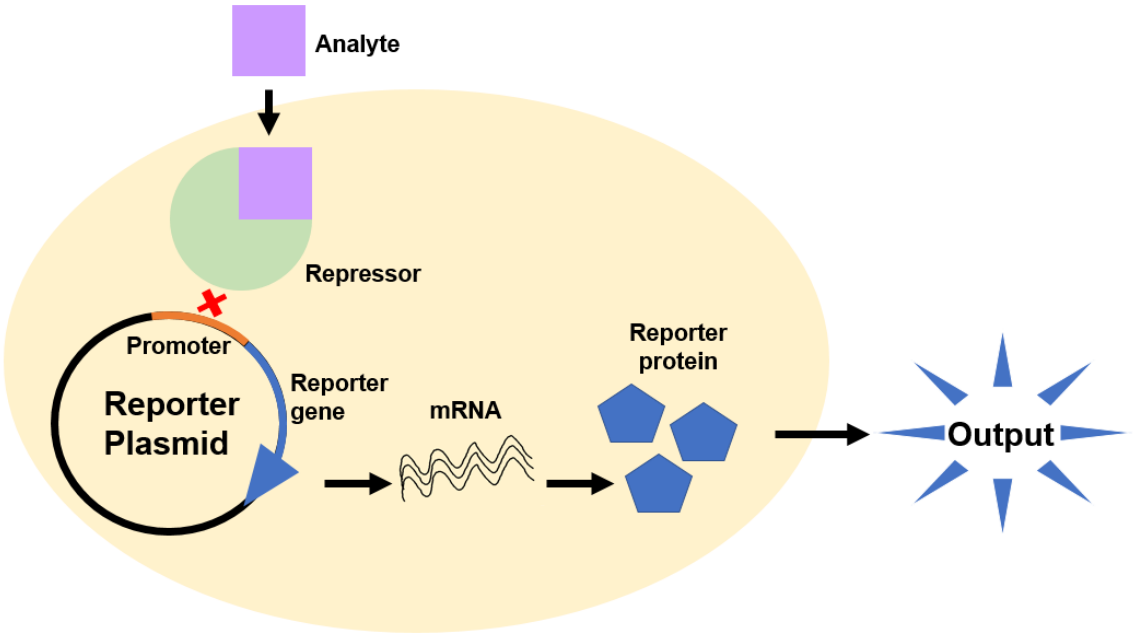

Figure 1.1.1 Schematic of a "lights on" reporter gene MBS assay. Analyte crosses the plasma membrane and binds to a repressor protein, allowing transcription of reporter gene and translation of reporter protein which can be quantified using optical methods.

Analytical parameters of these assays are dependent on regulation of the synthetic circuit, which allows for signal output by the reporter gene. Upregulation or downregulation of promoter activity, which is independent of analyte concentration, will reduce assay sensitivity or prevent responsivity overall. As promoter regulation is driven by the cell's metabolic machinery,

conditions which cause stress to the cells or negatively impact cell viability can be detrimental to responsivity.

1.2 MBS Preservation Strategies

The most pressing barrier to widespread usage of MBS in the field is instability during long-term storage.[18-21] An effective MBS preservation method should preserve viability as well as analytical performance, ideally under ambient conditions and without special equipment.[21] A major concern is the effect of preservation-related stress on genetic circuit regulation, especially when stress-responsive sensing promoters are used. A plethora of techniques have been reported which can increase bacterial tolerance to preservation methods – namely drying – most of which utilize protectant sugars or compatible solutes.[20,22-30] Some major MBS preservation methods, including their advantages and disadvantages are discussed below.

a) Freeze-drying (lyophilization). Freeze-drying is the most commonly used method for MBS preservation.[20-21] This method of bacterial preservation dates back to 1946, when Naylor and Smith reported the first matrix for the successful lyophilization of *Serratia marascens.*[31] In the freeze-drying process, cultures are grown to a growth phase optimal for the organism, mixed with drying protectants, and frozen in stages at low pressure.[21,32] Cells can then be rehydrated in various media for the desired application. The process is harsh, requiring initial cell suspensions with more than 10^8 cells per mL, and often resulting in 0.1% survival.[32] The initial freezing step is most detrimental to cell viability, as ice crystals formed during freezing can damage the cell wall.[32-33] Using a set of fluorescent and bioluminescent bioreporters with inducible stress promoters, a recent study concluded that the freeze-drying process induces DNA damage in *E. coli* and *Salmonella typhimurium.*[34] The same study verified mutagenic effects of the process using an Ames test. Viability effects can be reduced by rapidly freezing cells in liquid nitrogen.[35] During a slow

freezing process, the concentration of solutes increases gradually which lowers the freezing point. Further reduction of temperature towards this point yields more ice which pierces cell membranes. Inclusion of protectant additives is the most common approach used to reduce the harmful effects of lyophilization on bacterial viability.

The usage of cryoprotectant additives (CPAs) for the maintenance of bacterial viability through freeze-drying has been extensively studied, though CPA mechanisms are poorly understood. The suspending medium affects both bacterial survival through the process as well as survival during storage.[22] Font de Valdez *et al.* investigated protective effects of non-sugar additives through the freeze-drying of 14 *Lactobacillales* species.[23] Polyethylene glycol polymers, bovine albumin, asparagine, sodium glutamate, cysteine, glycerol, and β-glycerophosphate additives were evaluated. Protective effects were organism dependent, though sodium glutamate, cysteine and glycerol offered protection for most strains. Redway *et al.* assessed the effects of 21 carbohydrate and carbohydrate-related additives on the viability of various bacterial strains suspended primarily in horse serum prior to freeze-drying.[22] All compounds offered some protection, though *meso*-inositol, higher polyalcohols (dulcitol, mannitol and sorbital) and non-reducing disaccharides (sucrose and trehalose) were most effective. More recently, Leslie *et al.* also reported protected effects of sucrose and trehalose when freeze-drying *E. coli.*[26] Addition of trehalose and sucrose to drying matrices at 100 mM increased survival by 61.7% and 48.1%. Interestingly, only trehalose offered protection during storage. The researchers reasoned that sugar additives can decrease membrane phase transition temperature by replacing water molecules between lipophilic head group regions in the plasma membrane (previously termed “the water replacement hypothesis”).[26,36] Louis *et al.* reported similar results for *E. coli* freeze-dried in the presence of trehalose and sucrose, but also demonstrated protective effects of hydroxyectoine.[25]

Ectoine performed poorly compared to hydroxyectoine, further supporting the water replacement hypothesis as a mechanism for protection since the molecules differ only with respect to a hydroxyl group. Finally, Costa *et al.* showed that the rehydration media also affects viability of freeze-dried bacteria.[27] Rehydrating bacteria in non-fat skim milk or a peptone-tryptone-meat extract medium led to over 100% survival, compared to rehydration in water (less than 60% survival). Although a wide range of additives may be used to preserve bacterial viability, these compounds do not necessarily maintain activity of reporter bacteria.[21]

It is important to note that reporter bacteria are rarely the subject of studies investigating effects of freeze-drying, with the exception of shelf-life estimations. Freeze-dried reporter cells have been reported to retain responsivity for months.[16] In fact, the freeze-dried *Vibrio fischeri* isolates in commercially available Microtox kits have a shelf-life of 24 months when stored at -20 °C.[37] Unfortunately, shipment in chilled compartments and frozen storage significantly increases testing costs.[38] One *Photobacterium leiognathi*-based water toxicity kit retained responsivity for up to one week at ambient temperature, indicating that ambient temperature shipment may be possible, though it was not compatible with longer storage times.

Some additional disadvantages of freeze-drying reporter bacteria include the technique's complexity and associated costs.[20] Even the choice of storage container (glass ampoule or stoppered vial) can impact viability.[32] The process itself is also time-consuming – taking days for larger product loads. For reporter bacteria, performance of freeze-dried cells in extreme conditions (during storage or sensing) is rarely reported.[39] This is a major concern when designing sensors for on-site applications outside of the laboratory, as noted below.

b) Vacuum-drying (liquid-drying). Malik *et al.* introduced vacuum drying an alternative to freeze-drying for bacterial preservation in 1990.[40] In vacuum drying, samples are dried without

freezing. The method was effective for the preservation of sensitive microorganisms which were damaged due to freezing. Similar to the freeze-drying process, cell suspensions are first pelleted and resuspended in protective media. Instead of freezing, droplets are added to ampoules and briefly equilibrated in a room temperature water bath. Subsequent steps are also performed at room temperature. A centrifugal freeze-drying machine is used for drying steps, and ampoules are sealed under N_2. Similar to the freeze-drying process, skim milk and activated charcoal were used as suspending media in the first report of vacuum-drying. Glutamate, *meso*-inositol, honey, raffinose and adonitol additives improved bacterial survival. Many anaerobic and aerobic strains that were vacuum-dried and stored at 9 °C retained viability for 1-2 years. Pedahzur *et al.* compared the effects of vacuum-drying and freeze-drying on bioluminescent *E. coli* biosensor responsivity.[41] Lower survival rates were reported for vacuum-dried bacteria (1-2%), as compared to freeze-dried bacteria (19-20%), when bacteria were initially grown in LB media. Despite poorer survival through the process, vacuum-dried cells retained higher viability than freeze-dried cells upon storage at 37 °C. Similar relationships were reported when comparing the responsivity of the dried cultures, and the group concluded that vacuum-drying was more effective than freeze-drying for long-term preservation of reporter bacteria.

The optimization of vacuum-drying matrices for arsenate-inducible *E. coli* bioreporters was investigated by Kuppardt *et al.*[20] Primary matrix components such as gelatin or polyvinylpyrrolidine (PVP) were used to enhance viscosity. Raffinose, sucrose, or trehalose was then supplemented as a protectant. Responsivity after two weeks of storage at 4 °C – defined as a minimal response time and maximal response intensity – was greatest using a PVP-trehalose matrix. The authors also reported that preconditioning bacterial cultures by supplementing initial growth media with sodium chloride improved viability during storage in all cases, and improved

responsivity during storage in most cases. Manzanera *et al.* first reported viability improvements in vacuum-dried *E. coli* using this approach, and attributed the effect to osmotic stress-induced accumulation of trehalose in the cells.[28] Thus, it is not surprising that Kuppardt *et al.* reported limited effects of sodium chloride preconditioning in matrices containing trehalose.[20] Interestingly, the vacuum-drying method has also been extended to drying of bacteria on solid supports to allow MBS assays in remote locations. The Daunert group has reported two *E. coli*-based MBS platforms, for colorimetric reporting of arsenate and N-acyl homoserine lactones, which use the vacuum-drying method to dry cells onto paper strips.[10,42] The former was reported to retain full responsivity after 2 months at -20, 4 and 30 °C, and the latter was reported to retain full responsivity after 3 months at 4 °C.

Although the above reports indicate that the vacuum-drying method is superior to freeze-drying for the preservation of viability and responsivity in reporter bacteria, the practical disadvantages pertaining to freeze-drying are not avoided by vacuum-drying.[19] Furthermore, the conditions imposed in vacuum-drying are harsher than in freeze-drying and there is limited data detailing the performance of vacuum-dried reporter bacteria in sensing applications.

c) Encapsulation in polymeric matrices. Reporter bacteria may also be preserved without the use of drying steps, by encapsulating cells in organic or inorganic polymer gels. Protocols outlining the suspension of bacterial cells in polymer gels vary based on the gelation process.[21,43] These matrices allow for isolation of individual cells, and provide mild conditions such that bacterial viability and responsivity are not compromised.[21]

Immobilization of bacterial cells in organic matrices such as agar and alginates is relatively simple compared to the aforementioned preservation methods. Park *et al.* immobilized bioluminescent *Salmonella typhimurium* bioreporters in an LB agar matrix by mixing cell

suspensions with liquid LB-agar solution (1.5% agar) at 40 °C, and allowing gelation at room temperature.[44] Bioreporters retained responsivity to DNA damaging agents after 4 weeks at 4 °C. Alternatively, cells have been immobilized in calcium alginate spheres by mixing a cell suspension with sodium alginate solution and dripping this into a calcium chloride solution, where droplets form gel spheres instantly.[45] Alginate spheres are generally biocompatible, and their high porosity leads to greater diffusion rates for small molecules. Polyak *et al.* constructed novel biosensors using this method: bioluminescent *E. coli* reporters encapsulated in alginate-biotin spheres were introduced to optical fibers coated with streptavidin.[46] While such immobilization methods are relatively biocompatible and can be interfaced with biosensor surfaces, the organic matrices are susceptible to biodegradation and deformation over time, reducing long-term stability.[43]

Sol-gel derived inorganic materials comprised of porous silica or alumina have also been used as matrices for bacterial encapsulation. These materials proved effective previously for the entrapment of other labile biomolecules, including alkaline phosphatase and various antibodies.[47-48] In 2000, Fennouh *et al.* entrapped whole *E. coli* cells by rapidly mixing an aqueous bacterial cell suspension with a hydrolyzed solution of tetramethyl orthosilicate (TMOS), and allowing gelation over a few minutes.[49] Since the sol-gel process is performed at room temperature and neutral pH, cells are kept under mild conditions. Unlike organic matrices, the resultant gels are more rigid, transparent, chemically stable and thermally stable, making them a suitable choice for the preservation of optical MBS reporters.[21,43] However, a requirement for humid conditions during storage and handling makes the method less practical for MBS preservation.

In a separate study, Yu *et al.* encapsulated *Moraxella* bioreporters in sol-gel derived silica matrices, and reported 95% maintenance of activity after storage for 2 months at 4 °C.[50] Importantly, the authors used a sodium silicate precursor, instead of TMOS, so as to avoid the

liberation of cytotoxic alcohol byproducts. Amoura *et al.* demonstrated the effectiveness of sol-gel derived alumina matrices, although these proved more damaging to bacterial viability.[51] *E. coli* entrapped in sol-gel derived silica and alumina matrices retained 80% and 33% viability one hour after gelation. The shelf-life of bacteria entrapped in sol-gel derived silica materials is impressive under certain conditions – Alvarzez *et al.* reported cell viability over 1.5 years with the inclusion of LB media in the silicate gels.[52] Eleftheriou *et al.* further optimized the method using a lower-density silica matrix, which allowed entrapped *E. coli* bioreporters to divide and maintain sensing ability within pores (Figure 1.2.1).[53] Fluorescent *E. coli* bioreporters entrapped in this way have even been successfully printed onto microarrays, responding as expected when inducer solutions were placed over the array.[54]

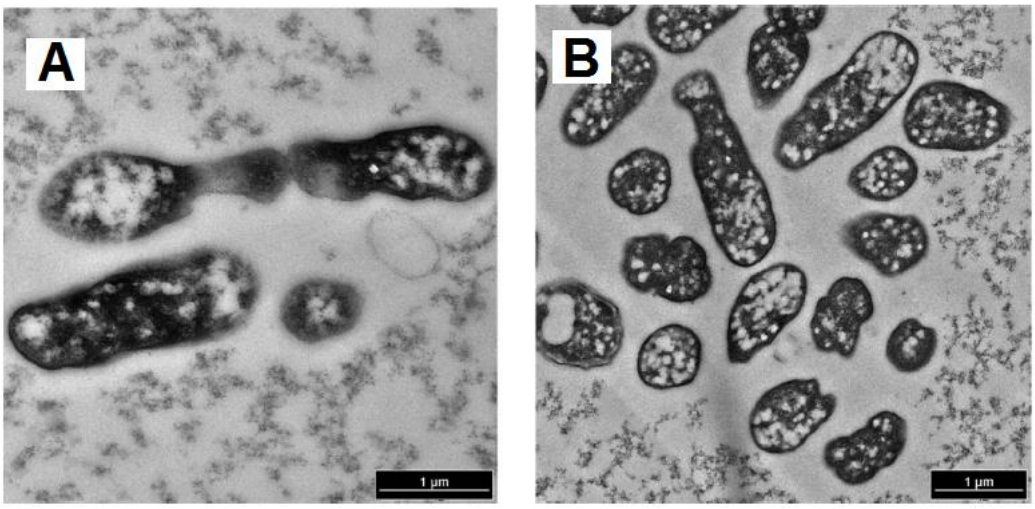

Figure 1.2.1. Thin cross-section transmission electron microscopy images of *E. coli* cells following entrapment in a sol-gel derived silica matrix. A) 24 hours after entrapment. B) 48 hours after entrapment.[54]

There are a few studies that have compared bacterial viability across several immobilization methods. For example, Kuppardt *et al.* compared the responsivity of vacuum-dried colorimetric *E. coli* bioreporters to the same bioreporters encapsulated in alginate, carrageenan, LB agar and TMOS.[20] All encapsulated cells yielded lower initial response intensities

relative to freeze-dried cells. However, maximum storage times were similar for vacuum-dried and encapsulated cells after 12 weeks, with the exception of carrageenan-encapsulated cells (2 weeks only). Bacteria encapsulated in TMOS and alginate preserved the induction response at a level comparable to fresh cells for six to nine weeks, whereas the induction response of cells vacuum-dried in certain matrices was doubled at least until 12 weeks. While such studies provide some insight into the potential of sol-gel entrapment to stabilize cells, further work is needed to deduce whether vacuum or other drying methods are superior to gel encapsulation methods, and whether other sol-gel derived materials may provide improved cell viability and stability.

d) Spore-based methods. Daunert *et al.* introduced a novel method for the preservation of reporter bacteria.[55] using spore-forming *Bacillus* and *Clostridium* bacteria. Under nutrient starvation, these bacterial cells are transformed into metabolically dormant structures known as spores.[56-57] Spores are hardy structures composed of various layers which protect DNA at the core from harsh conditions including desiccation, heat, oxidation, and radiation. Takamatsu *et al.* reported that the protein-based spore coat plays a key role in this resistance.[57] Spores not only survive starvation and extreme conditions, but rapidly recover from their dormant state in the germination process.[56-57] Germination typically entails a simple culturing step with heating and shaking.[55] Date *et al.* have demonstrated that germinated reporter cells retain viability and responsivity during storage. The protective function of the spore makes this method the most attractive yet for the preservation of bacteria through harsh environmental conditions, an important consideration when developing MBS-based assays. However, only a limited number of cell types can form spores, limiting the general applicability of this method.

For those bacteria that are spore-forming, Date *et al.* reported full maintenance of analytical performance for reporter cells for both arsenic and zinc when using *Bacillus subtilus* and *Bacillus*

megaterium bacterial spores, respectively. Such MBS remained stable for 6-24 months when stored at room temperature.[55] It should be noted that the germination and sensing process was not straightforward, as revival of the cells required both heating and shaking. To overcome this issue, the same group also developed a centrifugal compact disk microfluidic platform utilizing the same MBS systems.[21] Through centrifugal pumping, fluid was directed through defined channels of the disc allowing for germination and sensing in one step. This device offered miniaturization and increased simplicity without affecting analytical performance of the spore sensors. However, compact disc readers (which provide centrifugal motion) may not be available in resource-limited regions, and thus the use of these MBS-based assays may not always be cost effective.

1.3 Air-drying in Natural Polymers as a Potential MBS Preservation Method

Bacterial encapsulation and air-drying in natural polymers was introduced as an alternative method of bacterial preservation by Vodyanoy *et al*. in 2006.[58-59] The reported protocol was straightforward: bacterial culture was simply combined with pullulan (a fungal polysaccharide) or acacia gum polymers at 15% w/v, and typically placed in a 40 °C incubator until dried. Drying time varied based on the container and sample volume.[59] Figure 1.3.1 displays photographs of *Salmonella* bacterial culture throughout the process, showing that cells remain intact throughout drying.

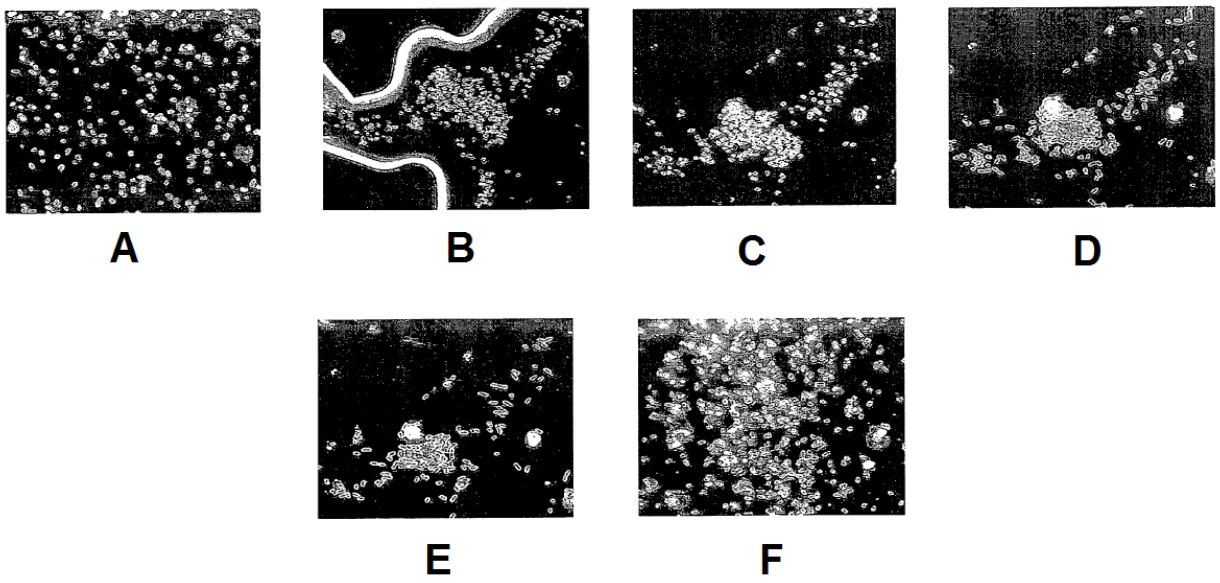

Figure 1.3.1. Photographs of *Salmonella* at various stages of the air-drying process in acacia gum polymer. A) Bacteria is immersed in polymer solution. B) Bacteria is immobilized in the solidified acacia gum solution. C-F) Bacteria cells at one, two, three and ten minutes after hydration.[59]

Krumnow *et al.* extended this method and reported the effective preservation of both *E. coli* and *B. subtilis* by air-drying in pullulan and acacia gum.[58-59] As for *Salmonella*, the bacteria dried in these materials retained viability during storage at a variety of temperatures and humidity levels. However, *E. coli* survival during storage was maximized using the acacia gum matrix with low temperature and medium humidity (76% RH), where viability (above 100 CFU per mL) was preserved for 17 months. Superior preservation of *E. coli* in acacia gum was attributed to the trapping of bound water molecules during drying, which prevented complete dehydration of the bacteria. While entrapment in pullulan and acacia gum has proven to be amenable to the bacterial species listed above, it should be noted that the method has not been rigorously tested using different bacterial strains, nor has it been employed for the preservation of reporter bacteria.

Key advantages of entrapment in natural polymers is that this process avoids many of the disadvantages associated with other bacterial preservation methods.[58] The method is simpler and

requires no equipment, thus minimizing costs. Furthermore, bacterial damage associated with extreme temperature is prevented entirely. Such damage is of particular concern when considering sensing applications following drying of the bacteria, where specific cellular functions must be retained in order to preserve responsivity to analytes.

More recently, Sorokulova *et al.* applied the air-drying preservation method to microorganism sample collection.[60] Effectively, the process involved drying acacia gum polymer onto swabbing materials such as Whatman paper. *Bacillus anthracis* spores or methicillin-resistant *Staphylococcus aureus* (*MRSA*) cells were inoculated onto these surfaces and stored. The authors demonstrated the stability of biological traits during storage – morphology of the cells under a microscope was unaffected, and the *MRSA* strains retained oxacillin resistance in all cases. Hence, the entrapment in natural polymers could be a versatile method for cell entrapment with retention of key biological functions.

1.4 Challenges for Field-Use of Use MBS-based Assays

As gold standard analytical tests are typically too complex and expensive for use in the field, effective alternatives must be developed to be used in their place. The "ASSURED" criteria for diagnostic tests was developed by the World Health Organization (WHO) to summarize ideal traits of diagnostic tests for the developing world.[61] The acronym stands for "Affordable, Sensitive, Specific, User-friendly, Robust and rapid, Equipment-free and Deliverable". These criteria are ideal for any point-of-need test required in a resource-limited setting, including pollutant monitoring applications.

At present, very few MBS-based assays are accredited or used commercially, and almost none have been employed for on-site applications.[62] The assays do not often meet the affordable,

user-friendly or equipment-free criteria. In 2005, Trang *et al.* was among the first to employ an arsenic-inducible bioluminescent *E. coli* reporter for field tests to evaluate groundwater contamination in Vietnam.[63] The MBS sensor proved much more reliable than the standard chemical tests used in the region. However, while the required reagents were inexpensive, the tests were performed in microplates which have a higher unit cost and higher disposal costs than paper-based chemical tests. In addition, the tests required a rotary shaker and luminometer, necessitating sample transport off-site prior to testing. Thus, it is not surprising that chemical tests are still the standard analytical technique used for arsenic quantification in the region.

1.5 MBS-based Assays on Paper

Paper is a popular choice for the fabrication of inexpensive analytical devices as it is abundant, low-cost, and easily manipulated.[64] Furthermore, paper is available in numerous thicknesses, porosities, and compositions which can be tailored to the specific application. The usage of paper as a substrate for analytical devices of varying complexity has been extensively reviewed elsewhere.[64,65] As paper is typically white in colour, there is also high contrast relative to colorimetric signals, allowing for highly sensitive assays.

There are very few examples of MBS-based assays performed on paper substrates.[10,42,66,67] All of these examples are colorimetric assays (incorporating a substrate for turnover by the β-galactosidase reporter) and utilize the paper as a lightweight support for live cells which have been freeze-dried or vacuum-dried onto the surface. For example, Stocker *et al.* vacuum-dried arsenate-inducible *E. coli* reporters onto paper.[10] Their sensing protocol entailed incubating the paper strips in a sample solution, then pipetting X-gal over the strip. The assay was highly sensitive with a 10 μM limit of detection, could be performed within an hour, and withstood storage 30 °C for two months. However, incubation required a heating step, and X-gal was provided as a liquid meaning

that the test required multiple steps. Struss *et al.* generated a similar sensing strip in 2010 for the detection of *N*-acyl homoserine lactones.[42] In contrast to the previous sensor, this assay could be performed entirely at room temperature without affecting sensitivity, but still required addition of external reagents to generate a signal. Alternatively, Fantino *et al.* generated a paper-based assay using *Bacillus subtilis* spores which they called a "sposensor".[66] Although the assay was robust and could be performed in only one step, the germination process to activate the test strip required heated incubation overnight. In 2016, Weaver *et al.* incorporated yeast biosensors onto paper substrates for tetracycline detection.[67] These sensors were extremely robust compared to bacterial sensors, retaining viability for more than one year at 4 °C. However, overnight heated incubation was still a requirement. Thus, additional developments are needed to produce paper-based MBS sensors that can provide results rapidly with minimal steps and without heated incubation.

1.6 Thesis Goals

The utility of MBS systems has not yet been fully realized for sensing applications in the field, and such systems remain mainly confined to academic research settings. While it is claimed that the technology is ready for environmental applications in the field from a technical stand point, there is risk that it cannot compete with existing technologies on the market owing to issues with assay complexity and shelf life.[62] It was hypothesized here that MBS can be made more competitive with existing tests by pairing MBS with more efficient preservation methods and using a simpler, equipment-free assay format.

To address these key issues, the first goal of the thesis project was to investigate the efficacy of the air-drying process on maintenance of MBS viability and responsivity, using the natural polymers pullulan and acacia gum as drying matrices and evaluating cell viability via reporter response rather than simple viability assays. The second goal was to develop a simple

tube-based sensing assay using cells that were stabilized via these preservation methods. To assess the applicability of the method for diverse sensing applications, both an arsenate-inducible and a tetracycline-inducible colorimetric *E. coli* reporter were used. The final goal was to create a simplified paper-based test for tetracycline could be useful for field applications. In all cases, stability and analytical performance were assessed to determine if the device met the ASSURED criteria.

Chapter 2 | Experimental

2.1 Materials

Desalinized pullulan was purchased from Polysciences Inc. (Mn ~ 200,000), and acacia gum or gum arabic was purchased from Sigma Aldrich (Mn ~ 250,000). X-gal was purchased from Thermo Fisher scientific. Whatman Grade 42 ashless filter paper was purchased from GE Healthcare Life Sciences. Coomassie Brilliant Blue G-250 was purchased from Sigma Aldrich (pure, solid).

2.2 Bacterial Strains and Growth Conditions

Three *E. coli* reporters were used, all of which were designed to produce β-galactosidase enzyme upon induction of a synthetic promoter. The arabinose-inducible pBAD-*lacZYA* (*E. coli* M182) was used in preliminary experiments as a model. The tetracycline-inducible pUT-*tetlac* (*E. coli* MT102) was used in all later experiments.[12] The arsenate-inducible pMV-*arsR*-ABS (*E. coli* DH5-α) was also used alongside pUT-*tetlac* in certain experiments to demonstrate suitability of the methods for a broad range of applications.[10]

pBAD-*lacZYA* cultures were prepared by inoculating 5 mL aliquots of LB with single colonies collected from agar plates. LB was supplemented with 34 μg/mL chloramphenicol for selection. Following overnight incubation with shaking (37 °C, 250 rpm), cultures were subcultured in M9 minimal media containing 34 μg/mL chloramphenicol to an OD_{600} of approximately 0.08 (Infinite M200 Pro, Tecan), then returned to the shaking incubator for 2 hours.

Cultures of pUT-*tetlac* or pMV-*arsR*-ABS were prepared by inoculating 5 mL or 100 mL aliquots of LB with single colonies collected from agar plates. LB was supplemented with

appropriate selecting antibiotics: 100 µg/mL ampicillin and 50 µg/mL kanamycin for pUT-*tetlac* cultures, and 100 µg/mL ampicillin only for pMV-*arsR*-ABS cultures. Cultures were placed in a shaking incubator overnight (37 °C, 250 rpm).

2.3 Selection of Equipment Used for Air-Drying of *E. coli*

Using the arabinose-inducible pBAD-*lacZYA* strain, a number of air-drying techniques were compared in order to select the optimal method for preservation of cell viability and responsivity. Pullulan solutions were sterilized by filtration only. pBAD-*lacZYA* subculture was combined 1:4 with 5% w/v pullulan stock (4% final pullulan concentration). Pullulan-reporter mixtures were then deposited into wells of a 96-well microplate at 100 µL. Five identical plates were prepared. One plate was dried in a static air incubator overnight (37 °C). One plate was dried inside a desiccator containing calcium sulfate (DrieRite) under vacuum for 3 hours then sealed overnight. Two plates were dried in the Platinous Sterling Series temperature and humidity chamber (Espec) overnight (23.5 °C, 55% RH) – one of which was partially covered by placing adhesive craft putty between the plate and its lid, leaving a 0.5 cm clearance. One plate was placed in a closed BSC overnight (reduced air flow, room temperature), but was discarded due to incomplete drying.

On each plate, duplicate wells were resuspended for viable cell counting purposes. Water was added at 100 µL over the dried films in these wells. Solution was pipetted up and down to resuspend cells. For each well, serial dilutions were prepared in PBS. The previous day, a fresh pullulan-reporter mixture was similarly diluted in PBS. All dilutions were immediately plated at 5 µL onto LB agar plate halves supplemented with 34 µg/mL chloramphenicol. Colonies were counted following overnight incubation of plates at 37 °C.

For each plate, the responsivity of dried cells was assessed in triplicate: three wells were resuspended in 100 µL of 1 mM X-gal and three wells were resuspended in 100 µL of 1 mM X-gal plus 0.1% arabinose. All plates were sealed and incubated at room temperature for 24 hours. OD_{610} measurements were then collected

2.4 Microplate-based Assays and Analysis

Natural polymer solutions were sterilized by filtration only. Stationary phase cultures of pUT-*tetlac* or pMV-*arsR*-ABS grown in LB were centrifuged in 25 mL aliquots (10 minutes, 4816xg) and each pellet was suspended in 500 µL of 5% w/v pullulan. On one occasion, pUT-*tetlac* reporters were prepared similarly in 5% w/v acacia gum. Suspension was deposited at 10 µL into wells of a 96-well microplate. Samples in the microplates were dried in the Platinous chamber as described above. The volume allowed for drying in 1-2 hours.

Each dried film was resuspended in water, to a final volume of 1 mL. Resuspensions were incubated at room temperature for 1 hour, then combined 1:1 with negative control solutions (17% v/v LB and 1 mM X-gal) or positive control solutions (former components plus 1 µM sodium arsenate or 100 µg/L tetracycline) in a 96-well microplate. Development was usually carried out at 30 °C. OD_{600} was measured at 10-minute intervals over 20 hours. Assays containing intermediate analyte concentrations were not performed.

The impact of the drying process on reporter viability was also assessed for both the pUT-*tetlac* and pMV-*arsR*-ABS reporters. Initial reporter suspensions (in 5% w/v pullulan) were diluted in PBS and spread onto LB agar plates. Dried films were resuspended in PBS, diluted further, and plated in the same way. LB agar was supplemented with appropriate selecting antibiotics as described previously. Plates were incubated overnight at 37 °C.

The effects of drying on responsivity were assessed by repeating the described microplate assay using fresh pUT-*tetlac* suspension in pullulan which had not been dried. The same 10^{-3} dilution was performed as had been used for rehydration previously. The diluted reporter suspensions were then used in the microplate assay as described previously.

2.5 Paper-based pUT-*tetlac* Sensing Strip Generation

Acacia gum solutions were prepared by dissolving in the required volume of milliQ water, filtering through a Buchner funnel lined with two coffee filter cutouts, then autoclaving (121 °C, 15 minutes). Pullulan solutions were prepared in the same way, but were not filtered. Whatman 42 paper was autoclaved (121 °C, 20 minutes) prior to use. Stationary phase cultures of pUT-*tetlac* grown in LB were centrifuged as before and the pellets resuspended in 15% w/v acacia gum or 15% w/v pullulan, concentrating 50-fold. Pieces of Whatman 42 paper (1 x 0.5 cm) were soaked in 15% w/v acacia gum or 15% w/v pullulan and placed onto Parafilm, where cell suspension prepared in the same polymer was added to each at 10 µL. Strips were left uncovered under a BSC fan until dried (1.5 hours).

Stocks of pullulan in LB for LB tablet casting were prepared by mixing solid pullulan into concentrated LB (standard reagent ratios dissolved in lower water volumes), then autoclaving (121 °C, 20 minutes). Droplets were deposited at ~50 µL onto a Parafilm-lined culture dish, and allowed to dry in a static air incubator (37 °C) overnight. Typically, 10% w/v pullulan in 7.5X LB was used. Tubes containing 2 mL of sample (typically milliQ water spiked with varying tetracycline concentrations) were supplied with an LB tablet and mixed by vortex until dissolved. Tubes were then supplied with dried sensing strips, mixed for 20 seconds, and incubated at room temperature for 1 hour.

Again, the impact of the drying process on reporter viability was also assessed for both the pullulan and acacia gum-based sensing strips. Initial reporter suspensions were diluted in PBS and spread onto LB agar plates. Additionally, dried sensing strips were solubilized in PBS using a vortex mixer, diluted further, and plated in the same way. LB agar was supplemented with appropriate selecting antibiotics as described previously. Plates were incubated overnight at 37 °C.

2.6 Calibration of Colour Intensity using CIELAB Coordinate Measurements

The use of the Nix colour sensor (Nix Sensor Ltd., Hamilton, ON) in place of a plate reader for colour intensity quantification was investigated. Specifically, the use of the L coordinate from the CIELAB coordinate set (measured using the Nix sensor) as an indicator of blue colour development was validated. Standard solutions of Coomassie Brilliant Blue G-250 (0.5-6 µM) in 70% ethanol were prepared. CIELAB coordinate measurements were collected in triplicate for each standard. For these measurements, 1 mL aliquots were transferred to a large custom well plate made of acetal (1 mL capacity wells). The Nix sensor was affixed over wells to minimize light interference, and paired with the Nix Pro Color Sensor iPhone application (Nix Sensor Ltd.) to obtain measurements. OD_{610} measurements were also taken for 100 µL aliquots of these standards, in triplicate.

2.7 All-in-one Tube Style Assay

In the all-in-one tube style assay, 2 mL of X-gal solution (final concentration 2 mM) was added to sensors pre-incubated with sample (see section 2.5). Triplicate assays were performed for tetracycline concentrations ranging from 10 µg/L to 200 µg/L. For all assays, negative control replicates were also prepared which did not contain inducer molecule. Development was carried out at room temperature, and photographs were taken using the iPhone SE camera application

periodically. Colour intensity was assessed 1.5 hours after X-gal addition. OD_{610} measurements were taken of 100 µL aliquots from individual tubes. Next, 1 mL aliquots from each tube were added to the acetal plate in triplicate, and CIELAB coordinates were measured as described previously. Both pullulan-based and acacia gum-based pUT-*tetlac* sensing strips were used in the assays, however assays using pullulan-based sensing strips were only quantified using OD_{610} measurements. Only acacia gum-based sensing strips were used in later experiments.

The effects of drying on responsivity of pUT-*tetlac* were assessed by repeating the described all-in-one tube assay using fresh reporter suspension which had not been dried (suspension previously spotted and dried onto sensing strips was added as a liquid to assay tubes). Colour intensity was quantified using OD_{610} measurements only.

In an effort to simplify the all-in-one tube assay, dried X-gal paper strips were used in place of X-gal solution (thus, final volume was halved). These strips were prepared by depositing 400 µL of X-gal stock (in pullulan) onto sterile rectangles of Whatman 42 paper (4 x 2 cm). Stock was created by mixing 30 mg/mL X-gal (in DMSO) with 10% w/v sterile pullulan stock, to final concentrations of 20.8 mM (or 8.5 mg/mL) X-gal and 7% w/v pullulan. X-gal paper strips were allowed to dry in a 37 °C static incubator for two days. Dried strips were rolled slightly and inserted into the top end of tubes before recapping and inverting. Colour intensity was quantified using OD_{610} as well as CIELAB measurements, as described previously.

2.8 Tube-Based Assays of Antibiotics in Lake Water

All-in-one tube assays using liquid X-gal and X-gal strips were repeated using lake water samples in place of milliQ water. Lake water was collected September 9, 2017 at Bayfront Park, (Hamilton, ON). Colour intensity was quantified using OD_{610} as well as CIELAB measurements,

as described previously, at 0.5 hours after liquid X-gal addition and at 1 hour after dried X-gal strip addition. Upon comparison of results obtained in the assays using lake water versus those using milliQ water, the assay was repeated in milliQ water adjusted to pH 5, pH 6, pH 7 and pH 8 (initially at pH 4.5). However, tubes were prepared in duplicate only, and analyzed using OD_{610} measurements only.

2.9 Storage and Shelf-Life Evaluation

Microplate-based assays were also conducted following storage at 4 °C. Immediately following the drying period, microplates were fitted with a lid, sealed with Parafilm at the edges, sealed again in a plastic zipper bag, and placed in the refrigerator. Microplate-based assays for both pUT-*tetlac* and pMV-*arsR*-ABS sensors were evaluated for performance after 1 week, using inducer concentrations of 100 µg/L tetracycline and 1 µM arsenate in triplicate. Negative control replicates were also prepared which did not contain inducer.

Dried acacia gum-based pUT-*tetlac* sensing strips enclosed in plastic culture dishes were sealed and stored at either room temperature or 4 °C. Performance of sensing strips was assessed after 1 week, using the all-in-one tube assay, over the 10-100 µg/L tetracycline range. In all cases, negative control replicates were also prepared which did not contain tetracycline. Furthermore, performance after 6 weeks was assessed using only 100 µg/L tetracycline, in triplicate. However, only photographs were obtained for the 6-week study.

Chapter 3 | Results and Discussion

3.1 Selection of Equipment Used for Air-Drying of *E. coli*

The effects of various drying conditions on the viability and responsivity to inducer is shown in Figures 3.1.1 and 3.1.2, respectively, for the pBAD-*lacZYA* strain that was dried in a 4% w/v pullulan material. The data show that the bacteria displayed maximal viability and responsivity when dried inside a controlled environment chamber operated at 23.5 °C and 55% RH with partial coverage of the microplate. Under these conditions, 25 ± 7% of cells remained viable. Removal of the lid during drying led to a reduction in viability to 15 ± 13%, while drying in a desiccator under vacuum yielded cell survival rates more similar to drying under static heat (1.4 ± 0.3% versus 0.7 ± 0.2% survival), yet entirely prevented responsivity of the cells to arabinose inducer. While earlier studies by Vodyanoy *et al.* suggested that cell survival was greatest when using a 40 °C static incubator,[59] their work utilized a 12% w/v pullulan material and did not investigate the use of a controlled environment chamber for drying. Based on the drying results, the use of the controlled environment chamber operated under ambient conditions with cells present in a partially covered microplate was selected as the optimal air-drying method for *E. coli* in future microplate inducibility assays.

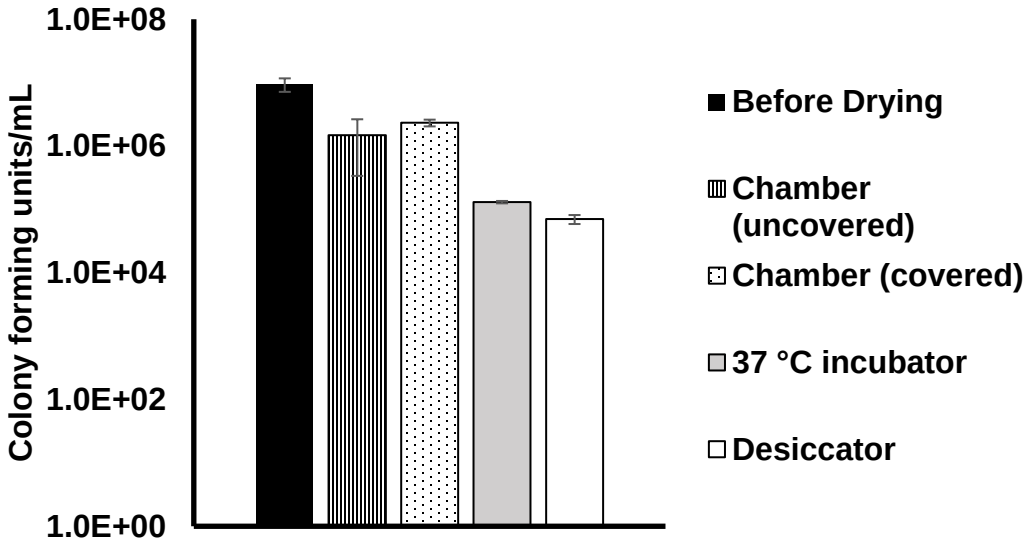

Figure 3.1.1. Survival of pBAD-*lacZYA* reporters through drying using various instrumentation.

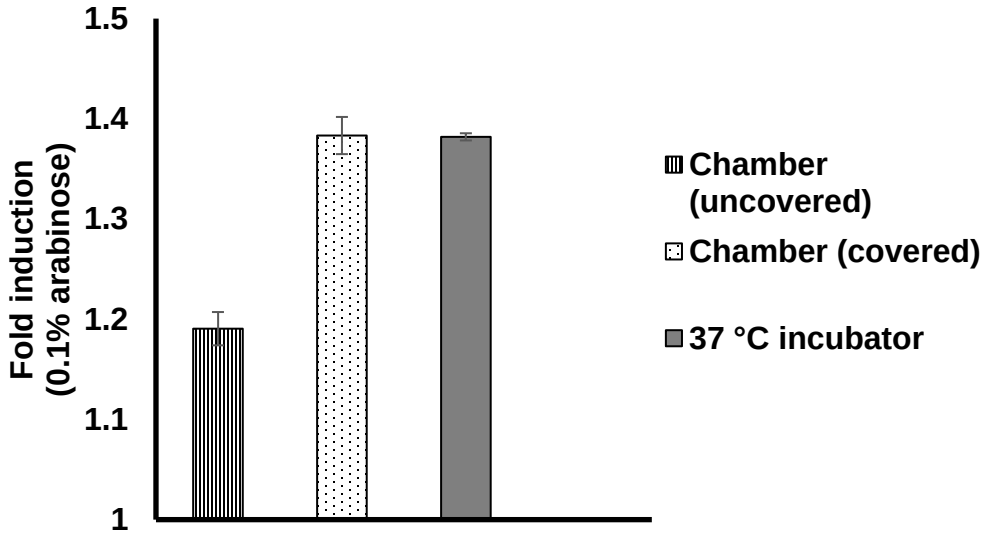

Figure 3.1.2. Responsivity of pBAD-*lacZYA* reporters following drying using various instrumentation. Fold induction is defined as the average signal intensity of induced reporters divided by that of un-induced reporters.

3.2 Microplate-based Assays

Microplate-based assays were conducted using reporters for tetracycline and arsenate, which were air-dried in 5% w/v pullulan. Cells were hydrated in sample solutions supplemented with X-gal and LB. Figure 3.2.1 shows an image of the induced pUT-*tetlac* response at 3 hours,

while Figure 3.2.2 shows optical density trends for induced pUT-*tetlac* and pMV-*arsR*-ABS reporters over 20 hours.

Figure 3.2.1. Photographs of pUT-*tetlac* response in the absence (left) and presence (right) of 100 µg/L tetracycline at 3 hours following X-gal addition in a microplate assay. Images were adjusted to improve brightness and contrast.

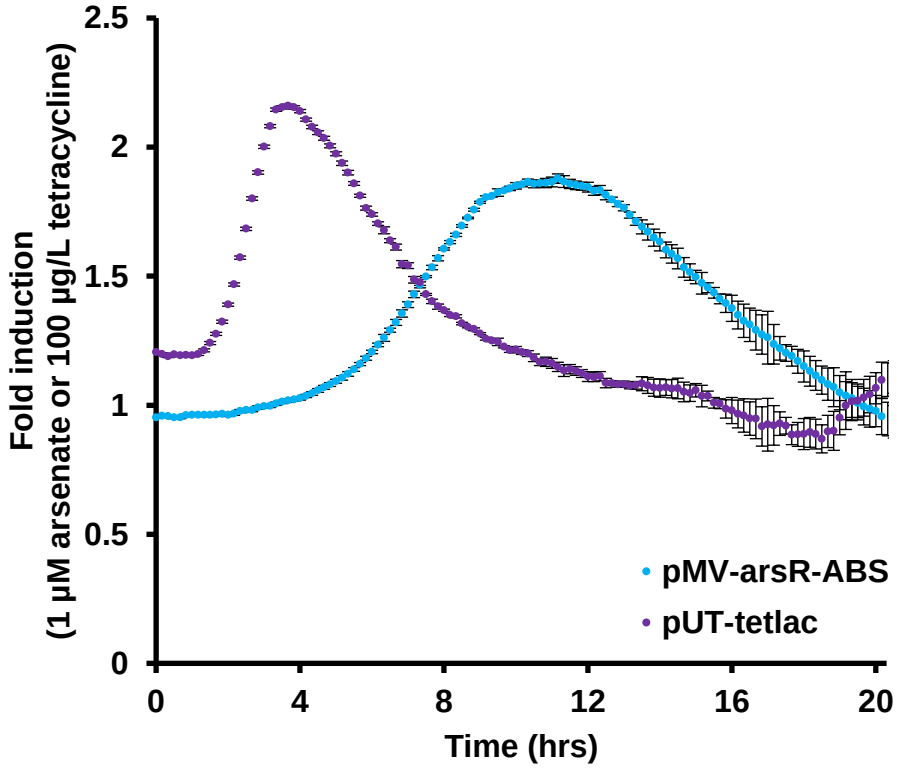

Figure 3.2.2. Time-dependent response of pMV-*arsR*-ABS and pUT-*tetlac* reporters over 20 hours following X-gal addition in a microplate assay.

In these experiments, a visually evident induction signal developed at about 5 hours for pMV-*arsR*-ABS reporters, and at about 2 hours for pUT-*tetlac* reporters. The difference between peak induction times for the two reporters was more drastic: peak induction signal was reached at 11 hours for pMV-*arsR*-ABS reporters and at 4 hours for pUT-*tetlac* reporters. The pUT-*tetlac*

reporters also responded more intensely than the pMV-*arsR*-ABS reporters, reaching maximal fold inductions of 2.16 and 1.88 respectively. Near the peak induction time, there was also no visually evident background signal for the pUT-*tetlac* reporter (Figure 3.2.1). Based on the results obtained for the pUT-*tetlac* reporter (ca. 4 h response time and >2-fold induction signal) relative to the pMV-*arsR*-ABS reporter (11 h response time, <2-fold induction signal) in these experiments, it was determined that the pMV-*arsR*-ABS reporter would be impractical for further development and thus further work utilized the pUT-*tetlac* reporter only.

Viable cell counting experiments were performed using the same dried reporters (Figure 3.2.3). The data show that in the case of either reporter, the there is no statistical difference in colony numbers between fresh and rehydrated cells, suggesting that survival rates approached 100% for rehydrated cells. For comparison, Kuppardt *et al.* reported 3.5% survival of the arsenate reporter used here through vacuum-drying, while Krumnow *et al.* reported 2% survival of non-reporting *E. coli* through their air-drying procedure.[20,58] Thus, the efficacy of the air-drying process performed here far exceeds published reports of various drying protocols applied to *E. coli*.

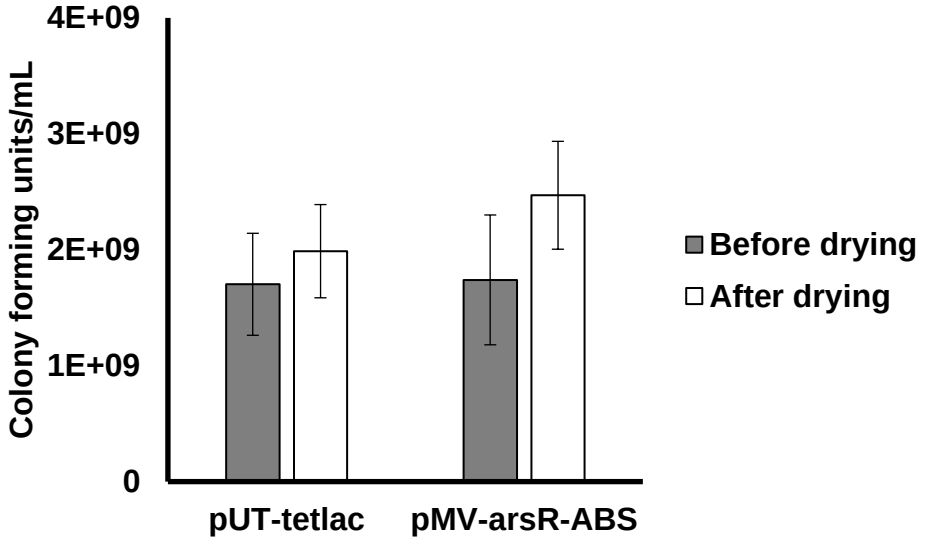

Figure 3.2.3. Survival of pUT-*tetlac* and pMV-*arsR*-ABS reporters through air-drying in a microplate.

Responsivity of the pUT-*tetlac* reporter was assessed using both dried/rehydrated and fresh cell suspensions in microplate assays. It was found that the fold induction of promoter activity was slightly decreased (ca. 20%) for rehydrated cells relative to fresh suspensions, as shown in Figure 3.2.4, with the peak induction signal being delayed 40 minutes for rehydrated cells relative to fresh cells suspensions. The delay is not likely due to the time needed for dissolution of the pullulan tablet (required only 20 seconds) or the increased viscosity of the sample when the pullulan is present, as the latter was controlled by adding pullulan to fresh cell suspensions. Growth-inhibiting stresses such as drying are known to induce a quiescent state in bacteria, where complex changes in regulatory processes including reductions in protein and RNA synthesis are known to occur, which is likely the source of the delayed response.[68]

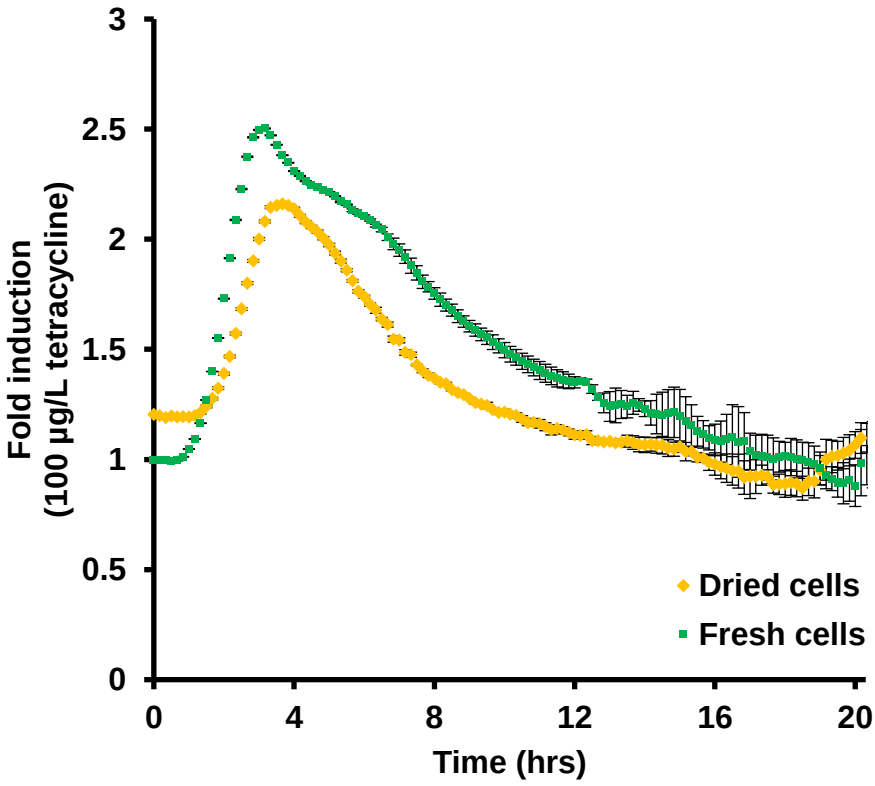

Figure 3.2.4. Time-dependent response of fresh and dried suspensions of pUT-*tetlac* reporters over 20 hours following X-gal addition in a microplate assay.

The microplate assay was also performed using the natural polymer acacia gum in place of pullulan. Figure 3.2.5 displays time-dependent response of reporters as well as cell viability changes due to the drying process. Reporters dried in acacia gum achieved a greater signal intensity than those dried in pullulan. However, there was no statistical difference in colony numbers following the drying process using either polymer. Although published reports of *E. coli* survival through air-drying have suggested that acacia gum is superior to pullulan for the preservation of viability, pullulan was as efficient as acacia gum here, despite slightly reduced MBS responsivity.[58]

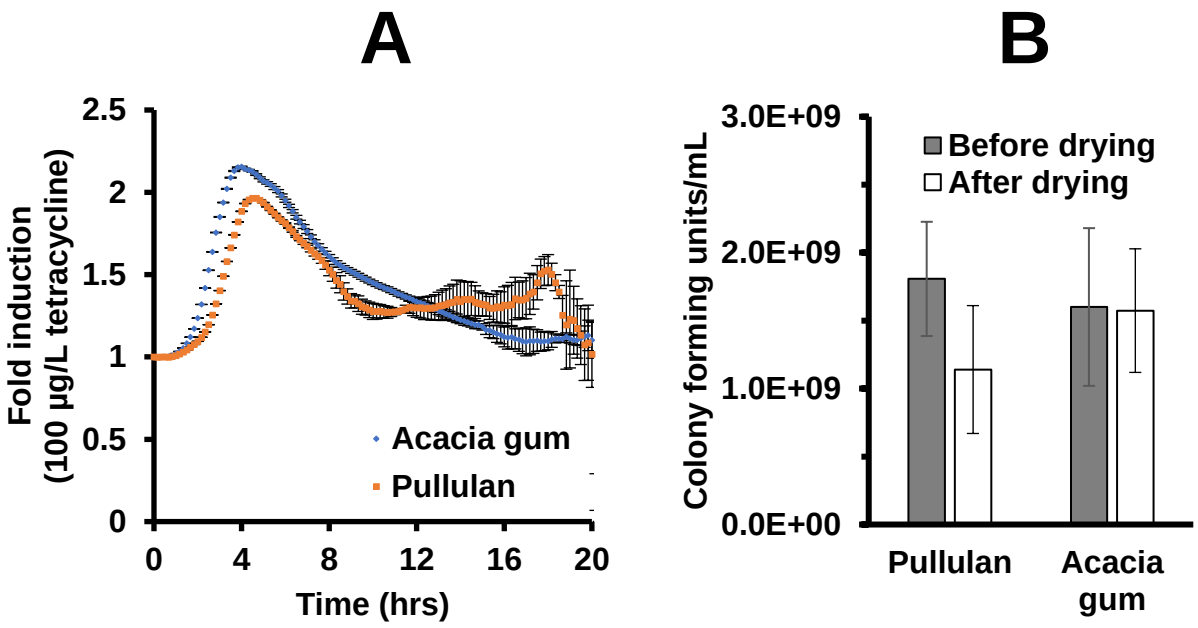

Figure 3.2.5. (A) Time-dependent response pUT-*tetlac* reporters dried in acacia gum and pullulan over 20 hours following X-gal addition in a microplate assay. (B) Survival of pUT-*tetlac* reporters through air-drying in pullulan and acacia gum.

3.3 Paper-based pUT-*tetlac* Sensing Strip Generation

It was thought that paper might provide a practical vehicle for the use of air-dried MBS systems in a platform that was compatible with use of such assays in the field. Concentrated pUT-*tetlac* suspensions were prepared in 15% w/v acacia gum or pullulan and dried onto small Whatman 42 paper strips. Due to the potential for aerosol production, the use of the temperature and humidity controlled chamber for air-drying was avoided (the well depth of the microplate prevented this previously). Instead, strips were dried uncovered inside a BSC. The process allowed for rapid drying of the suspensions in only 1.5 hours, similar to microplate assays dried in the controlled chamber. Figure 3.3.1 shows a schematic of the process.

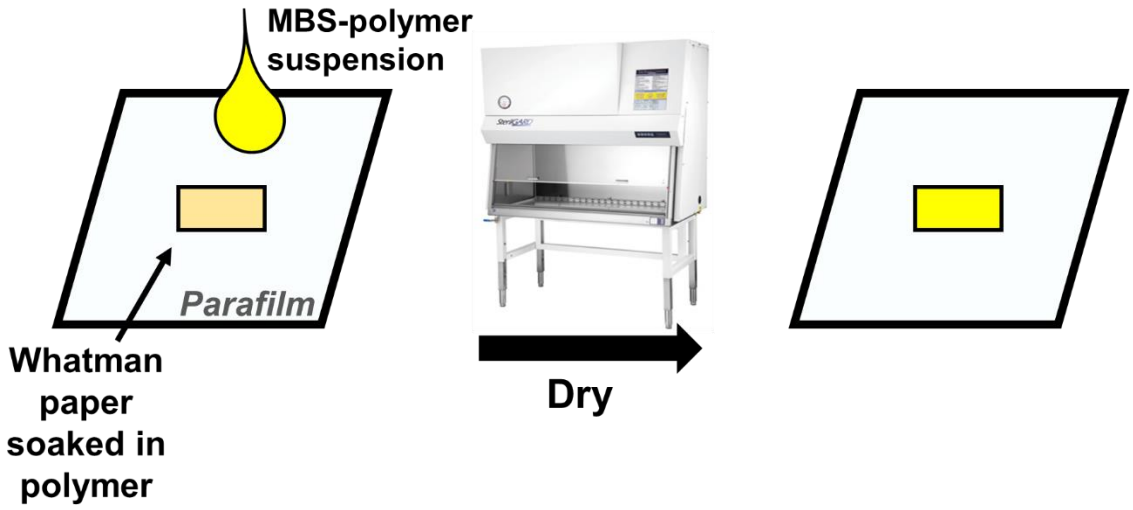

Figure 3.3.1 Schematic describing preparation of paper-based MBS sensing strips.

Viable cell counting experiments were performed to assess effects of the drying procedure on bacterial viability, the results of which are displayed in Figure 3.3.2. As in the previous microplate experiments, the drying matrix did not impact cell survival through the process. Despite a large degree of error in the plate counting experiments, these survival rates were again well above those currently published for air-dried and vacuum-dried *E. coli* samples.[20,58]

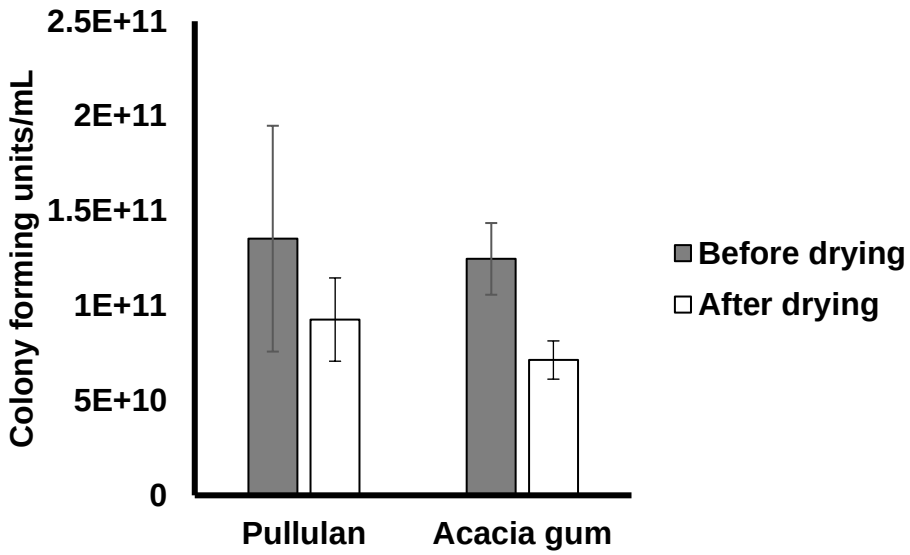

Figure 3.3.2. Survival of pUT-*tetlac* reporters following drying on acacia gum and pullulan-based sensing strips.

3.4 Calibration of Colour Intensity using CIELAB Coordinate Measurements

Colour analysis on paper strips was done using a Nix colour matching reader paired to an iPhone App to quantify colour intensity. Prior to obtaining data for MBS-related colour changes, the Nix sensor system was calibrated against a standard absorbance method to ensure that the readings were linear over a broad range of OD values. Coomassie Brilliant Blue G-250 standard solutions in microplates were examined using both the Nix sensor and OD_{610} measurements. Nix readings were converted to CIELAB coordinates using the App, and the L coordinate describing colour darkness was plotted against increasing dye concentration (Figure 3.4.1 A). OD_{610} measurements for the same samples are shown in Figure 3.4.1 B. The limits of detection for the colour sensing and optical density methods were 0.6 µM and 0.04 µM, respectively, and both methods showed excellent linearity in the response (r^2 = 0.98 – 0.99). Thus, the Nix colour sensor should provide a convenient method for assessing color changes in MBS paper sensors.

A particular advantage of the Nix sensor is the ability to place the reader over the sample and block any ambient light. Previous analysis methods typically involved photography of the

sensor strip followed by image processing using software such as ImageJ.[69] However, such analyses are time-consuming and are greatly affected by lighting conditions.[70] The Nix colour sensing method is rapid, simple, and insensitive to lighting conditions, and only requires the colour sensor and a smartphone

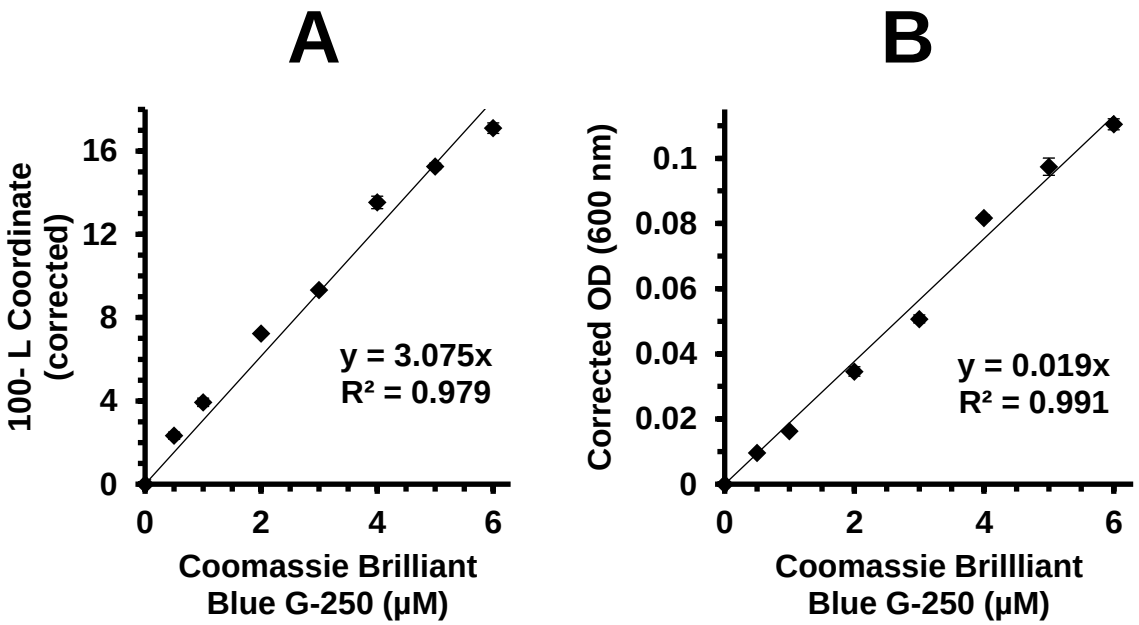

Figure 3.4.1 Calibration curves depicting dependence of L coordinate (A) and optical density (B) measurements on Coomassie Brilliant Blue G-250 dye concentration. The blank signal is subtracted from all data points, and regression lines are adjusted to pass through y=0.

3.5 All-in-one Tube Based Assay

The initial assay format investigated in this work was an all-in-one tube assay wherein two dried reagent systems were added to a single Eppendorf tube along with a sample to initiate a colorimetric assay. In this work, the MBS was present on a paper strip, while LB media was present in a pullulan tablet. As shown in Figure 3.5.1, the LB tablet and paper strip were first added to a sample solution, which was then incubated at room temperature for one hour. Liquid X-gal substrate was then added directly to the sample tube and the colour was read after a set incubation time.

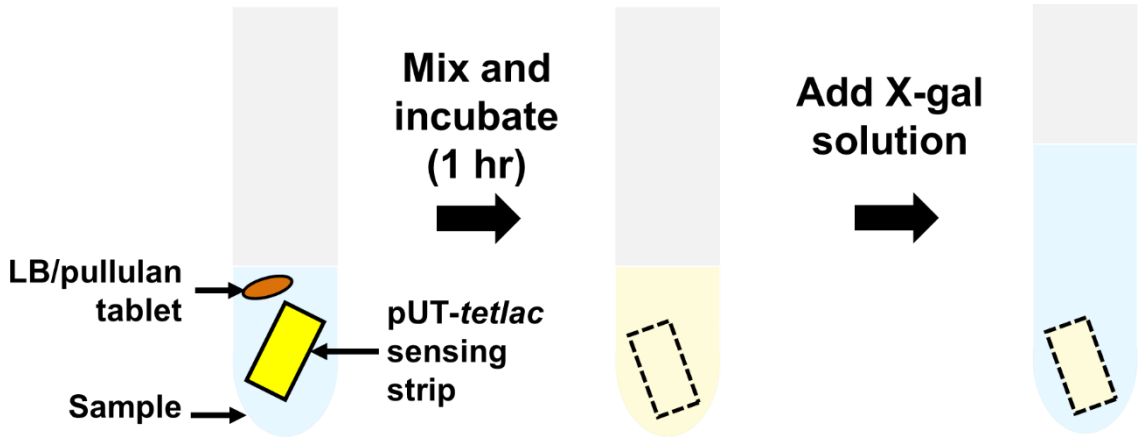

Figure 3.5.1. Schematic of all-in-one tube assay performance using pUT-*tetlac* dried onto sensing strips for the detection of tetracycline in water samples.

The initial goal was to use the tube-based assay to compare the responsivity of sensing strips prepared using pullulan and acacia gum. Induction was visually obvious at 10 µg/L tetracycline by 1.5 hours, at which time performance was quantified using optical density measurements. Dose response curves are displayed in Figure. 3.5.2 A. Overall, reporters dried in acacia gum exhibited up to 4-fold more intense signals, and also produced more obvious dose-dependent responses. The results clearly show that acacia gum provided far better performance that pullulan when used as a coating material on paper. Thus, only acacia gum-based sensing strips were used in further experiments. Note that while assays prepared using acacia gum-based sensing strips were initially analyzed via ImageJ processing of photographs, the level of noise was very high owing to issues with lighting variations (see differences in background intensity levels in Figure 3.5.2 B), making it difficult to obtain useful quantitative data. Therefore, colour intensity quantification was used in all remaining tube assays.

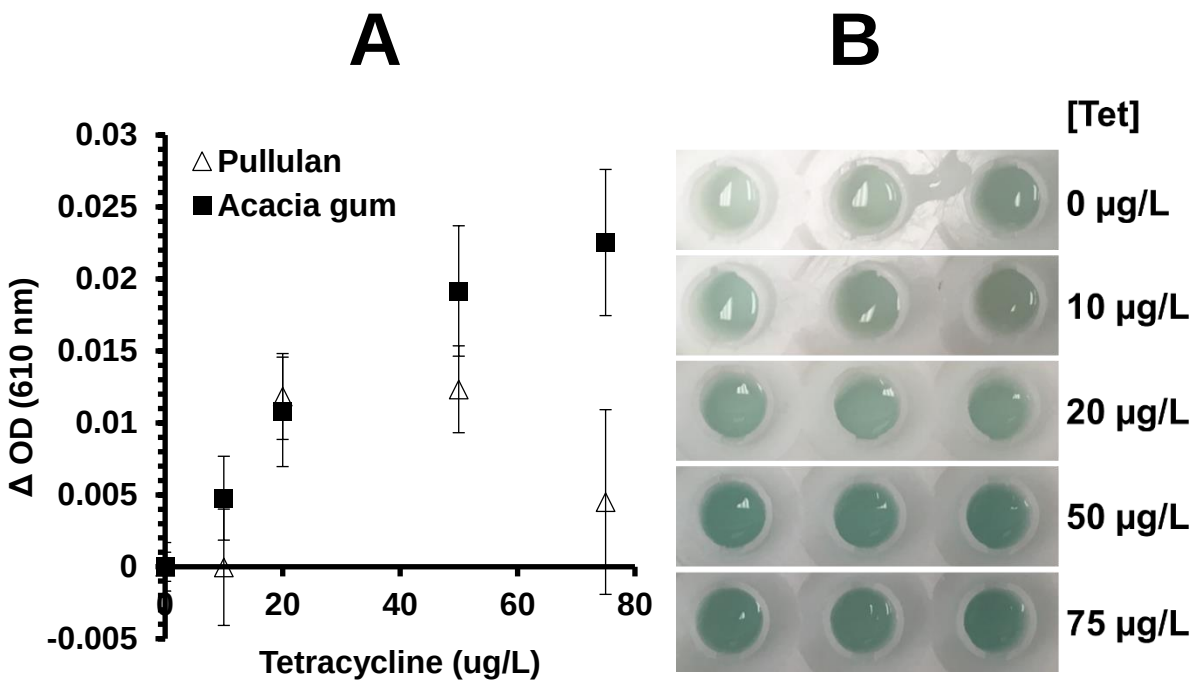

Figure 3.5.2. pUT-*tetlac* reporter response in milliQ water samples spiked with varying tetracycline concentrations in the all-in-one tube assay. (A) Optical density trends for assays prepared using pullulan-based and acacia gum-based sensing strips. (B) Top-view photograph of a well plate containing assays prepared using acacia gum-based sensing strips (triplicate wells shown).

As with the microplate assay, pUT-*tetlac* sensing strip performance in the tube-based assay was compared to the same assay using fresh cell suspensions. As observed above, colour development was evaluated for samples containing 10 µg/L tetracycline after 1.5 h incubation, when an induction signal was visibly evident. Though dried cells exhibited a higher background, optical density measurements indicated a similar response slope to fresh cells (Figure 3.5.3). It is possible that the higher signal in dried/rehydrated cells may be due to a specific stress response that alters the pUT-*tetlac* sensitivity to tetracycline – additional studies utilizing a library of stress-inducible promoters may provide further insight into this hypothesis.[71]

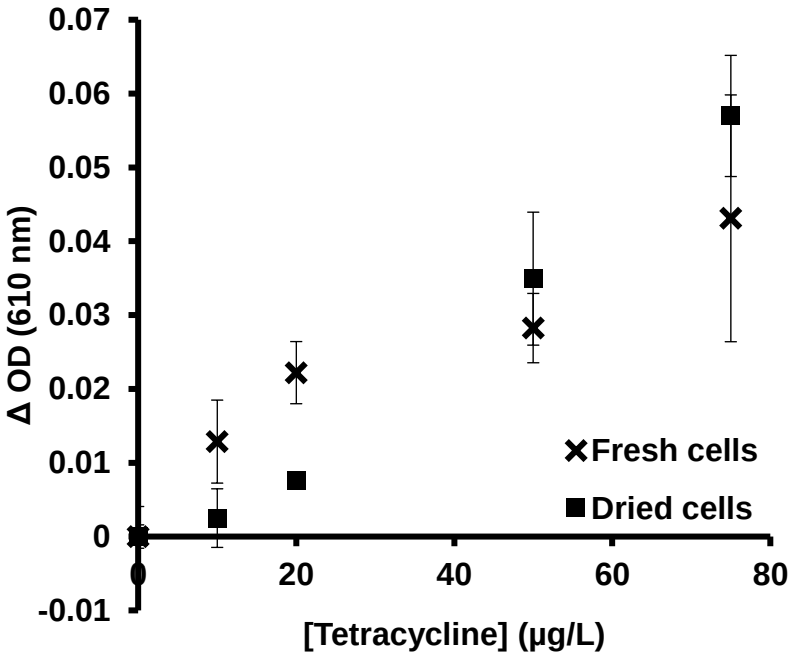

Figure 3.5.3. pUT-*tetlac* reporter response in milliQ water spiked with varying tetracycline concentrations in the all-in-one tube assay using fresh and dried cell suspension. Optical density measurements taken at 1.5 hours following X-gal addition, on different days.

As a simplification, the tube-based assay was also performed using MBS-coated paper strips containing a dried mixture of X-gal in pullulan, in place of liquid X-gal (Figure 3.5.4). A dose response curve obtained in this assay is compared to a similar curve obtained for the tube-based assay using liquid X-gal in Figure 3.5.5. The use of the X-gal strip in place of liquid X-gal offered greater sensitivity and a faster response time of only 1 hour. Importantly, the dried X-gal strips could be easily fit into assay tubes to provide a more easy-to-use "kit-format" test, and this also avoided the need to handle organic solvents. Pullulan has been used previously by our group for the long-term stabilization of enzymes and unstable reagents as tablets which dissolve in aqueous solution.[72]

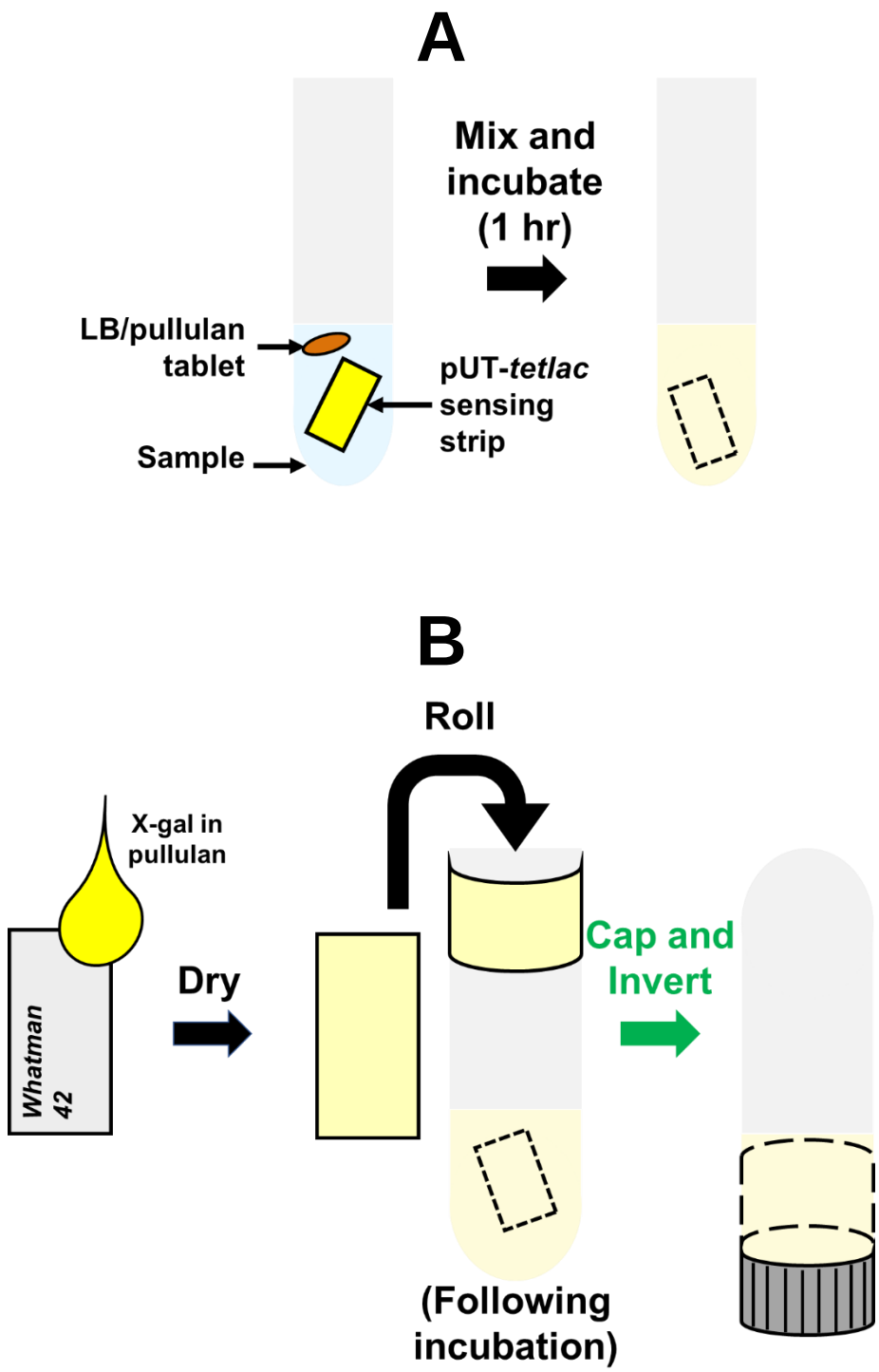

Figure 3.5.4. Schematic of the simplified all-in-one tube assay using dried X-gal strips for the detection of tetracycline in water samples. A) Sample preparation and incubation step is unchanged. B) X-gal is dried onto paper and added to sample tubes.

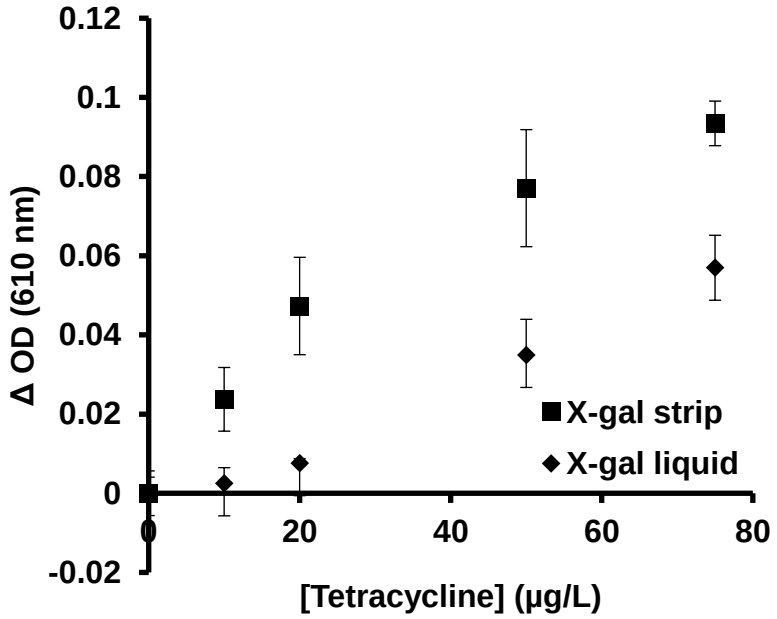

Figure 3.5.5. pUT-*tetlac* reporter response in the all-in-one tube assay using liquid X-gal and dried X-gal strips, in milliQ water spiked with varying tetracycline concentrations. Optical density measurements taken 1 hour following X-gal strip addition, and 1.5 hours following X-gal liquid addition.

As a preliminary assessment of potential shelf-life of sensing strips, the tube-based assay was repeated with liquid X-gal using sensing strips which had been stored for one week at room temperature and at 4 °C. Dose response curves were constructed using optical density measurements (Figure 3.5.6 A). Responsivity was preserved with cold storage, but was substantially reduced for strips stored at room temperature. Test strips were further investigated after six weeks of storage at 4 °C using 100 µg/L of tetracycline (Figure 3.5.6 B). These results indicate that strips have the potential to survive longer-term cold storage, without declines in assay signal intensity.

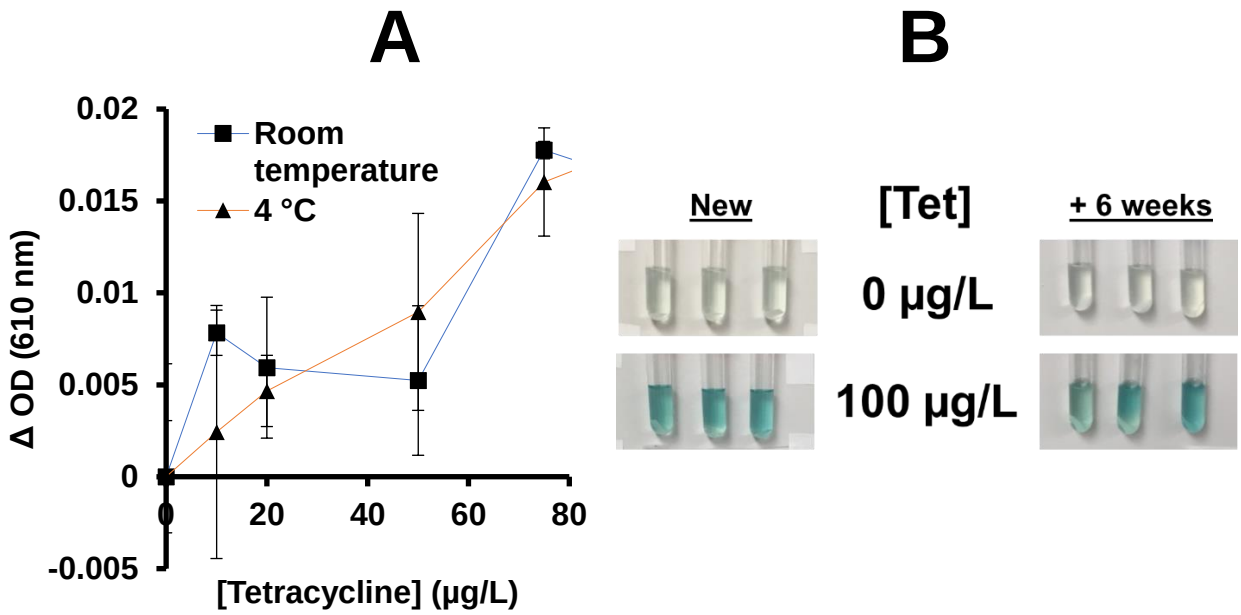

Figure 3.5.6. pUT-*tetlac* reporter response in the tube-based assay using stored sensing strips. (A) Optical density trends for assays using 1 week old strips stored at room temperature or 4 °C. (B) Photograph of assays performed using fresh and 6 week old strips (stored at 4 °C); triplicate tubes shown. All data collected 1.5 hours following X-gal addition.

3.6 Tube-Based Assays of Antibiotics in Lake Water

As an initial test of the use of the tube-assay for more complicated "real world" samples, the assay was used for the detection of tetracycline in lake water. Such antibiotics are widely used both for control of infections and in sub-therapeutic doses for growth promotion in livestock.[73-74] As such, they are becoming more and more prevalent as contaminants in in environmental water samples, where they may ultimately promote the production of bacterial with significant antimicrobial resistance. Hence, a simple method is needed to evaluate tetracycline levels in environmental water samples at the source.

The initial goal of this study was to evaluate the performance of the MBS tube assay for detection of tetracycline that was spiked into filtered lake water, which may contain a variety of microbes, various metal ions, nanoscale particulate matter, dissolved organics and salts, which could interfere with either the microbial biosensor system or the X-gal reaction. To evaluate the

performance of the MBS in lake water, dose response curves were obtained using optical density measurements and compared for response curves obtained for spiked milliQ water, as shown in Figure 3.6.1. Interestingly, colour development was accelerated in lake water as compared to milliQ water, hence colour intensity resulting from induction by 10 µg/L tetracycline was recorded at 0.5 hours for lake water assays and at 1.5 hours for milliQ water assays. Overall, the curves are similar, although the lake water samples showed a much better linear response (r^2 = 0.98 vs 0.87 for lake water and milliQ water, respectively). These results indicate that the tube-based assay can be used successfully to detect tetracycline in a complex matrix.

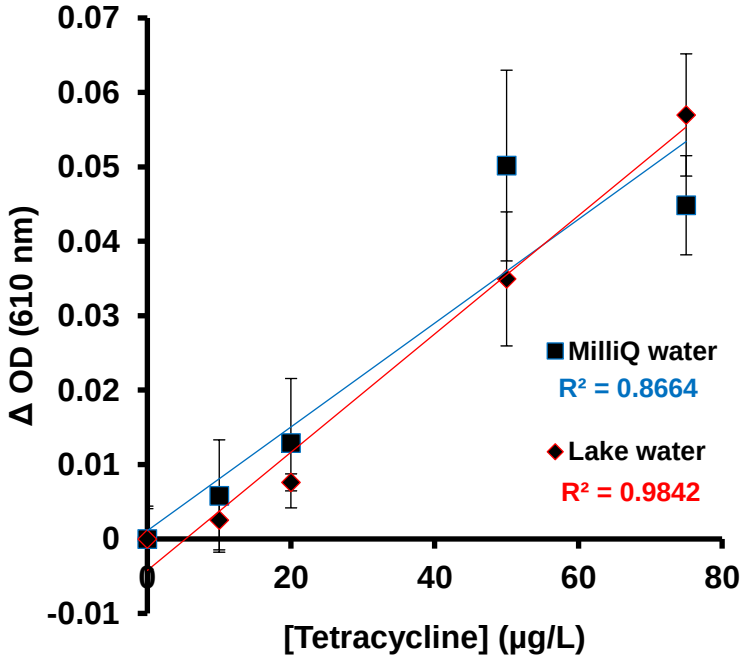

Figure 3.6.1 pUT-*tetlac* reporter response in the all-in-one tube assay in milliQ water and lake water spiked with varying tetracycline concentrations. Optical density measurements taken 1.5 hours and 0.5 hours following X-gal addition for milliQ water and lake water assays.

Test strips containing dried X-gal were also investigated in tube-based assays with lake water. Dose response curves were obtained using both optical density measurements and colour sensing for both lake water and milliQ water, as shown in Figure 3.6.2. Unlike the assays which used liquid X-gal, colour development required a similar time for both matrices, with readings for

samples containing 10 µg/L tetracycline being recorded at 1 hour. Again, the curves were very similar between matrices. The two methods generated very similar dose response curves, although the colour sensing method yielded more linear curves with better correlation coefficients relative to optical density readings.

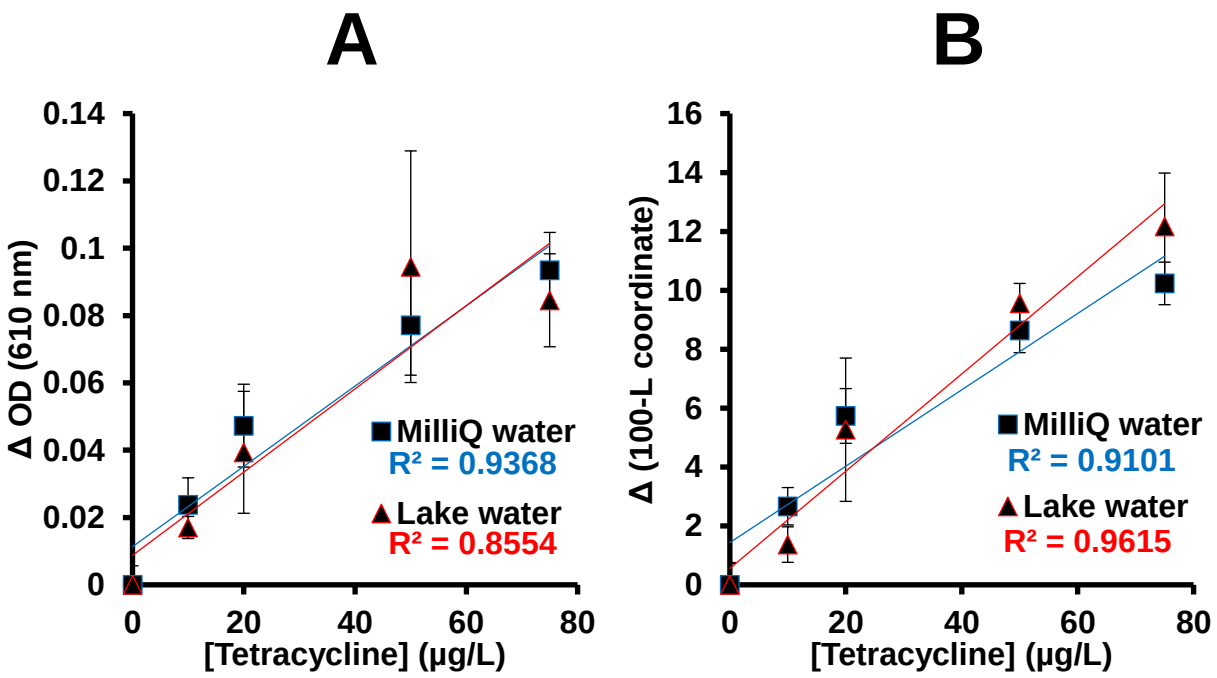

Figure 3.6.2 pUT-*tetlac* reporter response in the all-in-one tube assay using X-gal strips in milliQ water and lake water spiked with varying tetracycline concentrations. (A) Optical density trends. (B) L coordinate trends (from CIELAB set). All measurements taken 1 hour following X-gal strip addition.

As noted earlier, an ideal point-of-care test should meet the ASSURED criteria (affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free and deliverable). The tube-based assay using liquid X-gal was relatively expensive owing to the need for large amounts of substrate, and thus may not meet the affordability criteria, though use of the strip that incorporated X-gal partially addressed this concern. No constituent of the lake water matrix appeared to yield significant background signals, which is not surprising as MBS are well known for their specificity

for target analytes (or groups thereof).[62] Hence the assay is selective, and the detection limit of 10 μg/mL makes the test sufficiently sensitive. The dried X-gal strip made assay performance very user-friendly – all materials excepting the sample solution were premeasured and present on the paper strip, minimizing sample handling steps. Given that assays were colorimetric, they could be performed entirely equipment-free, though precise quantitation would not be possible without a portable reader. Total assay time in lake water was minimal at two hours. Since performance was not adversely affected by the lake water composition, or by large variations in acidity, these assays can be classified as fairly robust. More rigorous experiments need to be conducted in order to assess deliverability – storage experiments conducted here were only preliminary. Overall, the test meets most of the ASSURED criteria and could be further developed to meet all required criteria, making these tests suitable for POC applications.

Chapter 4 | Conclusion

Air-drying in natural polymers is an effective preservation method for MBS systems which allows for rapid responsivity following the process, and maintenance of this responsivity even six weeks later. The air-drying process here can be performed using no equipment and preserves a large proportion of viable reporters, unlike traditional drying methods. Air-dried MBS systems can be applied to simple sensing assays for diverse analytes – including but likely not limited to tetracycline and arsenate. It is not unreasonable to consider the application of these methods to the wide range of MBS systems available for the detection of diverse small molecules. Practicality of the air-dried MBS systems is further demonstrated by their performance in the more complex lake water matrix, where results were obtained rapidly and simply.

Chapter 5 | Future Work

This work provides useful background for a successful application of MBS-based assays in the field. Future work should include a more in-depth assessment of the shelf-life of MBS systems preserved in this way. Ideally, room temperature storage should be possible. The inclusion of protectant additives such as sugars or polyalcohols, similar to traditional freeze-drying methods should be explored.

In addition, increased simplicity could be achieved with the generation of paper-based assay formats which do not require a pseudo-culturing step. That is, an assay where reporters are minimally hydrated, and remain immobilized on the paper substrate throughout the sensing and colour development processes. Furthermore, the use of colour sensing to quantify signal intensity in flat paper assays could offer more quantitative measurements for these types of assays.

Perhaps most importantly, the application of a wider range of bacterial bioreporters should be attempted. In these experiments, an arsenate sensor was applied preliminarily, but excluded later due to longer sensing times. It is likely that a number of differences in responsivity will be encountered when a new MBS system is used, and assays will require optimization based on these effects. With further optimization and development, air-dried MBS-based assays may become a viable option for rapid and simple analyte detection in a number of fields, from pollutant monitoring to point-of-care diagnostics.

Chapter 6 | References

1) Quillardet, P., Huisman, O., D'Ari, R., and Hofnung, M. (1982) SOS Chromotest, a direct assay of induction of an SOS function in *E. coli* K-12 to measure genotoxicity. *PNAS 79,* 5971-5975.
2) King, J.M.H., DiGrazia, P.M., Applegate, B., Burlage, R., Sanseverino, J., Dunbar, F., et al. (1990) Rapid, Sensitive Bioluminescent Reporter Technology for Naphthalene Exposure and Biodegradation. *Science 249,* 778-781.
3) Tecon, R., and van der Meer, J.R. (2008) Bacterial Biosensors for Measuring Availability of Environmental Pollutants. *Sensors 10,* 4062-4080.
4) D'Souza, S.F. (2001) Microbial biosensors. *Biosens. Bioelectron. 16,* 337-353.
5) Lim, J.W., Ha, D., Lee, J., Lee, S.K., and Kim, T. (2015) Review of micro/nanotechnologies for microbial biosensors. *Front. Bioeng. Biotechnol. 3,* 61.
6) Kim, M., Lim, J.W., Kim, H.J., Lee, S.K., Lee, S.J., Kim, T. (2015) Chemostat-like microfluidic platform for highly sensitive detection of heavy metal ions using microbial biosensors. *Biosens. Bioelecton. 65,* 257-264.
7) Shetty, R.S., Deo, S.K., Shah, P., Sun, Y., Rosen, B.P., and Daunert, S. (2003) Luminescence-based whole-cell-sensing systems for cadmium and lead using genetically engineered bacteria. *Anal. Bioanal. Chem. 376,* 11-17.
8) Shetty, R.S., Deo, S.K., Liu, Y., and Daunert, S. (2004) Fluorescence-based sensing system for copper using genetically engineered living yeast cells. *Biotechnol. Bioeng. 88,* 665-670.
9) Tauriainen, S.M., Virta, M.P.J., and Karp, M.T. (2000) Detecting bioavailable toxic metals and metalloids from natural water samples using luminescent sensor bacteria. *Wat. Res. 34,* 2661-2666.
10) Stocker, J., Balluch, D., Gsell, M., Harms, H., Feliciano, J., Daunert, S., et al. (2003) Development of a Set of Simple Bacterial Biosensors for Quantitative and Rapid Measurements of Arsenite and Arsenate in Potable Water. *Environ. Sci. Technol. 37,* 4743-4750.
11) Kurittu, J., Lonnberg, S., Virta, M., and Karp, M. (2000) A group-specific microbiological test for the detection of tetracycline in raw milk. *J. Agric. Food. Chem. 48,* 3372-3377.
12) Hansen, L.H., Sorensen, S.J. (2000) Detection and quantification of tetracyclines by whole cell biosensors. *FEMS Microbiol. Lett. 190,* 273-278.
13) Tauber, M., Rosen, R., and Belkin, S. (2001) Whole-cell biodetection of halogenated organic acids. *Talanta 55,* 959-964.
14) Tecon, R., Beggah, S., and van der Meer, J.R. Bacterial Bioreporter Assays to Measure Hydrocarbons. In *Handbook of Hydrocarbon and Lipid Microbiology*; Timmis, K.N.; Springer: Heidelberg, 2010; p 3642.
15) Turner, K., Xu, S., Pasini, P., Deo, S., Bachas, L., and Daunert, S. (2007) Hydroxylated polychlorinated biphenyl detection based on a genetically engineered bioluminescent whole-cell sensing system. *Anal. Chem. 79,* 5740-5745.
16) Shin, H.J., Park, H.H., and Lim, W.K. (2005) Freeze-dried Recombinant Bacteria for On-site Detection of Phenolic Compounds by Color Change. *J. Biotechnol. 119,* 36-43.
17) Durand, M.J., Thouand, G., Dancheva-Ivanova, T., Vachon, P., and DuBow, M. (2003) Specific detection of organotin compounds with a recombinant luminescent bacteria. *Chemosphere 52,* 103-111.

18) Nivens, D.E., McKnight, T.E., Moser, S.A., Osbourn, S.J., Simpson, M.L., and Sayler, G.S. (2004) Bioluminescent bioreporter integrated circuits: potentially small, rugged and inexpensive whole-cell biosensors for remote environmental monitoring. *J. Appl. Microbiol. 96,* 33-46.
19) Bjerketorp, J., Hakansson, S., Belkin, S., and Jansson, J.K. (2006) Advances in preservation methods: keeping biosensor microorganisms alive and active. *Curr. Opin. Biotechnol. 17,* 1-7.
20) Kuppardt, A., Chatzinotas, A., Breuer, U., van der Meer, J.R., and Harms, H. (2009) Optimization of Preservation Conditions of As (III) Bioreporter Bacteria. *Appl. Microbiol. Biotechnol. 82,* 785-792.
21) Date, A., Pasini, P., and Daunert, S. Fluorescence and bioluminescent cell-based sensors - strategies for their preservation. In *Whole Cell Sensing Systems I: Reporter Cells and Devices*; Date, T., Belkin, S., and Gu, M.B.; Springer-Verlag: Berlin, 2010; Vol. 117; p 57-75.
22) Redway, K.F., and Lapage, S.P. (1974) Effect of carbohydrates and related compounds on the long-term preservation of freeze-dried bacteria. *Cryobiology 11,* 73-79.
23) Font de Valdez, G., Savoy de Giori, G., Pesce, A., Holgado, R., and Oliver, G. (1983) Comparative study of the efficiency of some additives in protecting lactic acid bacteria against freeze-drying. *Cryobiology 20,* 560-566.
24) Caesar, A.J., and Burr, T.J. (1991) Effect of conditioning, betaine, and sucrose on survival of rhizobacteria in powder formulations. *Appl. Environ. Microbiol. 57,* 168-172.
25) Louis, P., Truper, H.G., and Galinski, E.A. (1994) Survival of *E. coli* during and storage in the presence of compatible solutes. *Appl. Microbiol. Biotechnol. 41,* 684-688.
26) Leslie, S.B., Israelim E., Lightheart, B., Crowe, J.H., and Crowe, L.M. (1995) Trehalose and Sucrose Protect Both Membranes and Proteins in Intact Bacteria during Drying. *Appl. Environ. Microbiol. 61,* 3592-3597.
27) Costa, E., Usall, J., Teixido, N., and Vinas, I. Effect of protective agents, rehydration media and initial cell concentration on viability of *Pantoea agglomerans* strain CPA-2 subjected to freeze-drying. *J. Appl. Microbiol. 89,* 793-800.
28) Manzanera, M., de Castro, A.G., Tondervik, A., Rayner-Brandes, M., Strom, A.R., and Tunnacliffe, A. (2002) Hydroxyectoine is superior to trehalose for anhydrobiotic engineering of *Pseudomonas putida* KT2440. *Appl. Environ. Microbiol. 68,* 4328-4333
29) Streeter, J.G. (2003) Effect of trehalose on survival of *Bradyrhizobium japonicum* during desiccation. *J. Appl. Microbiol. 95,* 484-491.
30) Carvalho, A.S., Silva, J., Ho, P., Teixeira, P., Malcata, F.X., and Gibbs, P. (2004) Effects of various sugars added to growth and drying media upon thermotolerance and survival throughout storage of freeze-dried *lactobacillus delbrueckii* ssp. *bulgaricus. Biotechnol. Prog. 20,* 248-254.
31) Naylor, H.B., and Smith, P.A. (1946) Factors affecting the viability of *Serratia marascens* during dehydration and storage. *J. Bacteriol. 52,* 565-573.
32) Morgan, C.A., Herman, N., White, P.A., and Vesey, G. (2006) Preservation of micro-organisms by drying; a review. *J. Microbiol. Method. 66,* 183-193.
33) Carpenter, J.F., Crowe, J.H., and Arakawa, T. (1990) Comparison of solute-induced protein stabilization in aqueous solution and in the frozen and dried states. *J. Dairy. Sci. 73,* 3627-3636.

34) Rosen, R., Buchinger, S., Pfander, R., Pedahzur, R., Reiffersheid, G., and Belkin, S. (2016) SOS gene induction and possible mutagenic effects of freeze-drying in *E. coli* and *Salmonella typhimurium. Appl. Microbiol. Biotechnol. 100,* 9255-9264.
35) Gu, M.B., Choi, S.H., Kim, S.W. (2000) Some observations in freeze-drying of recombinant bioluminescent *E. coli* for toxicity monitoring. *J. Biotechnol. 88,* 95-105.
36) Crowe, J. H., L. M. Crowe, J. F. Carpenter, and C. Aurell Winstrom (1987) Stabilization of dry phospholipid bilayers and proteins by sugars. *Biochem. J. 242*, 1–10.
37) Johnson, B.T. Microtox Acute Toxicity Test. In *Small-scale Freshwater Toxicity Investigation Volume 1 - Toxicity Test Methods*; Blaise, C., and Ferard, J.; Springer: Dordrecht, 2005; p 73.
38) Ulitzur, S., Lahav, T., and Ulitzur, N. (2002) A novel and sensitive test for rapid determination of water toxicity. *Environ. Toxicol. 17*, 291-296.
39) Date, A., Pasini, P., Sangal, A., and Daunert, S. (2010) Packaging sensing cells in spores for long-term preservation of sensors: a tool for biomedical and environmental analysis. *Anal. Chem. 82*, 6098-6103.
40) Malik, K.A (1990) A simplified liquid-drying method for the preservation of microorganisms sensitive to freezing and freeze-drying. *J. Microbiol. Method. 12,* 125-132.
41) Pedahzur, R., Rosen, R., and Belkin, S. (2005) Stabilization of recombinant bioluminescent bacteria for biosensor applications. *Cell Preserv. Technol. 2,* 260-269.
42) Struss, A., Pasini, P., Ensor, M.C., and Daunert, S. (2010) Paper Strip Whole Cell Biosensors: A Portable Test for the Semiquantitative Detection of Bacterial Quorum Signaling Molecules. *Anal. Chem. 82*, 4457-4463.
43) Premkumar, R.J., Rosen, R., Belkin, S., and Lev, O. (2002) Sol-gel luminescence biosensors: encapsulation of recombinant *E. coli* reporters in thick silicate films. *Anal. Chim. Acta 462*, 11-23.
44) Park, K.S., Baumstark-Kahn, C., Rettberg, P., Horneck, G., Rabbow, E., and Gu, M.B. (2005) Immobilization as a technical possibility for long-term storage of bacterial biosensors. *Radiat. Environ. Biophys. 44*, 69-71.
45) Smidsrod, O., and Skjak-Braek, G. (1990) Alginate as immobilization matrix for cells. *Trends Biotechnol. 8,* 71-78.
46) Polyak, B., Geresh, S., and Marks, R.S. (2004) Synthesis and characterization of a biotin-alginate conjugate and its application in a biosensor construction. *Biomacromolecules 5,* 389-396.
47) Braun, S., Rappoport, S., Zusman, R., Avnir, D., and Orrolenghi, M. (1990) Biochemically active sol-gel glasses: the trapping of enzymes. *Mater. Lett. 10,* 1-5.
48) Roux, C., Livage, J., Farhati, K., and Monjour, L. (1997) Antibody-antigen reactions in porous sol-gel matrices. *JSST 8,* 663-666.
49) Fennouh, S., Guyon, S., Livage, J., and Roux, C. (2000) J. Sol-gel entrapment of *E. coli. JSST 19,* 647-649.
50) Yu, D., Volpani, J., Chhabra, S., Brinker, C.J., Mulchandani, A., and Singh, A.K. (2005) Aqueous sol-gel encapsulation of genetically engineered *Moraxella* spp. cells for the detection of organophosphates. *Biosens. Bioelectron. 20,* 1433-1437.
51) Amoura, M., Nassif, N., Roux, C., Livage, J., and Coradin, T. (2007) Sol-gel encapsulation of cells is not limited to silica: long-term viability of bacteria in alumina matrices. *Chem. Commun.,* 4015-4017.

52) Alvarez, G.S., Foglia, M.L., Copello, G.J., Desimone, M.F., and Diaz, L.E. (2009) Effect of various parameters on viability and growth of bacteria immobilized in sol-gel-derived silica matrices. *Appl. Microbiol. Biotechnol. 82,* 639-646.
53) Eleftheriou, N.M., Ge, X., Kolesnik, J., Falconer, S.B., Harris, R.J., Khursigara, C., *et al.* (2013) Entrapment of living bacterial cells in low-concentration silica materials preserves cell division and promoter regulation. *Chem. Mater. 25,* 4798-4805.
54) Ge, X., Eleftheriou, N.M., Dahoumane, S.A., and Brennan, J.D. (2013) Sol-Gel-Derived Materials for Production of Pin-Printed Reporter Gene Living-Cell Microarrays. *Anal. Chem. 85,* 121088-12117.
55) Date, A., Pasini, P., and Daunert, S. (2007) Construction of spores for portable bacterial whole-cell biosensing systems. *Anal. Chem. 79,* 9391-9397.
56) Setlow, P. (1995) Mechanisms for the prevention of damage to DNA in spores of *Bacillus* species. *Annu. Rev. Microbiol. 49,* 29-54.
57) Takamatsu, H., and Watabe, K. (2002) Assembly and genetics of spore protective structures. *CMLS 59,* 434-444.
58) Krumnow, A.A., Sorokulova, I.B., Olsen, E., Globa, L.,Barbaree, J.M., and Vodyanoy, V.J. (2009) Preservation of bacteria in natural polymers. *J. Microbiol. Methods 78,* 189-194.
59) Vodyanoy, V.J., Barbaree, J. M., Chin, B.A., Neely, W.C., Pathurana, S.T. and Braden, T.D. (Auburn University, Auburn, AL, USA). Use of pullulan to isolate and preserve biological material. US Patent 7,604,807, January 27, 2006.
60) Sorokulova, I., Watt, J., Olsen, E., Globa, L., Moore, T., Barbaree, J., *et al.* (2012) Natural biopolymer for preservation of microorganisms during sampling and storage. *J. Microbiol. Method 88,* 140-146.
61) Kettler, H., White, K., and Hawkes, S. (2004) Mapping the Landscape of Diagnostics for Sexually Transmitted Infections. Geneva: WHO/TDR.
62) Harms, H., Wells, M.C., and van der Meer, J.R. (2006) Whole-cell living biosensors – are they ready for environmental application? *Appl. Microbiol. Biotechnol. 70,* 273-280.
63) Trang, P.T.K., Berg, M., Viet, P.H., Mui, N.V., and van der Meer, J.R. (2005) Bacterial bioassay for rapid and accurate analysis of arsenic in highly variable groundwater samples. *Environ. Sci. Technol. 39,* 7625-7630.
64) Martinez, A.W., Phillips, S.T., Whitesides, G.M., and Carrilho, E. (2010) Diagnostics for the developing world: microfluidic paper-based analytical devices. *Anal. Chem. 82,* 3-10.
65) Cate, D.M., Adkins, J.A., Mettakoonpitak, J., and Henry, C.S. (2014) Recent Developments in Paper-based Microfluidic Devices. *Anal. Chem. 87,* 19-41.
66) Fantino, J.R., Barras, F., and Denizot, F. (2009) Sposensor: a whole-bacterial biosensor that uses immobilized Bacillus subtilis spores and a one-step incubation/detection process. *J. Mol. Microbiol. Biotechnol. 17,* 90-95.
67) Weaver, A.A., Halwag, S., Joyce, M., Lieberman M., and Goodson, H.V. (2016) Incorporating yeast biosensors into paper-based analytical tools for pharmaceutical analysis. *Anal. Bioanal. Chem. 407,* 615-619.
68) Rittershaus, E.S.C., Baek, S., and Sassetti, C.M. (2013) The Normalcy of Dormancy: Common Themes in Microbial Quiescence. *Cell Host Microbe 13,* 643-651.
69) Zakir Hossain, S.M., and Brennan, J.D. (2011) β-galactosidase-based colorimetric paper sensor for determination of heavy metals. *Anal. Chem. 83,* 8772-8778.

70) Sicard, C., Glen, C., Aubie, B., Wallace, D., Jahanshahi-Anbuhi, S., Pennings, K, et al. (2015) Tools for water quality monitoring and mapping using paper-based sensors and cell phones. *Water Res. 70*, 360-369.
71) Zaslaver, A., Bren, A., Ronen, M., Itzkovitz, S., Kikoin, I., Shavit, S., *et al.* (2006) A comprehensive library of fluorescent transcriptional reporters for *Escherichia coli. Nat. Methods. 3,* 623-628.
72) Jahanshahi-Anbuhi, S., Pennings, K., Leung, V., Liu, M., Carrasquilla, C., Kannan, B., *et al.* (2014) Pullulan encapsulation of labile biomolecules to give stable bioassay tablets. *Angew. Chem. Int. Ed. 53,* 6155-6158.
73) Wei, R., Ge, F., Huang, S., Chen, M., and Wang, R. (2011) Occurrence of veterinary antibiotics in animal wastewater and surface water around farms in Kiangsu Province, China. *Chemosphere 82*, 1408-1414.
74) Joakin Larsson, D.G. (2014) Antibiotics in the environment. *Ups. J. Med. Sci. 119*, 108-112.