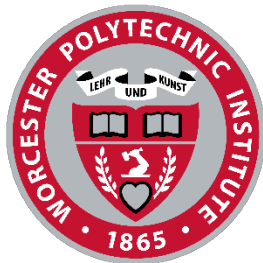


Using Biofilms for the Detection and Bioremediation of Arsenic



WPI

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ABSTRACT

The World Health Organization estimates that over 200 million people worldwide rely on water sources that are contaminated with arsenic at concentrations above the recommended maximum contaminant level of 10 ppb. Exposure to high concentrations of arsenic over prolonged periods has been proven to be detrimental to human health. Current arsenic remediation methods are prohibitively expensive in many areas where water sources have exceeded the maximum contaminant level. In order to detect and remediate arsenic in an efficient and yet cost effective manner, we designed two simple genetic circuits to both sense and absorb arsenic. When these circuits are incorporated into a *S. epidermidis* biofilm grown on nylon mesh, the biofilm will fluoresce in the presence of arsenic and chelate arsenic ions to remove them from water. This biofilm water filter has the ability to improve human health in an efficient and cost-effective manner, and open up possibilities for cleaner water for people around the globe.

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Introduction

Arsenic in Drinking Water

Over 780 million people in the world do not have access to safe drinking water resources, and 2 to 5 million people die yearly from water related diseases that are preventable (Gleick et al., 2014). Some of these annual mortalities can be attributed to arsenic, a heavy metal that enters water sources through erosion or industrial runoff (EPA). The World Health Organization estimates that over 200 million people worldwide rely on sources of water with arsenic concentrations in excess of the recommended 10 ppb maximum contaminant level (Naujokas et al., 2013). Table 1 below provides a breakdown for arsenic exposure through drinking water in 10 different countries.

Country	Estimated exposed population (millions) ^a	Arsenic concentration in drinking water (µg/L)
Argentina	2.0	< 1 to 7,550
Bangladesh	35–77	< 10 to > 2,500
Chile ^b	0.4	600 to 800
China	0.5–2.0	< 50 to 4,400
Ghana	< 0.1	< 2 to 175
India	> 1.0	< 10 to > 800
Mexico	0.4	5 to 43
Taiwan	NA	< 1 to > 3,000
United States	> 3.0	< 1 to > 3,100
Vietnam	> 3.0	< 0.1 to 810

Table 1: Arsenic exposure through drinking water around the world. Estimates for arsenic exposure and arsenic concentrations in 10 countries where arsenic contamination in drinking water is an issue (Naujokas et al., 2013).

Both short-term and long-term exposure to arsenic in concentrations exceeding 10 ppb can be detrimental to human health. Arsenic induces oxidative stress in different cells throughout the body, resulting in DNA damage and reduced DNA repair (Andrew et al., 2006). Short-term exposure to high concentrations of arsenic through drinking water can result in arsenic poisoning, which can affect digestive, cardiovascular, respiratory, neurologic, hepatic, and renal functions (ATSDR, 2010). Long-term exposure to high concentrations of arsenic, on the other hand, has been linked to developmental issues, diabetes, cardiovascular disease, and various cancers (WHO, 2012). Individuals who rely on sources of water contaminated with arsenic have an elevated risk of developing and dying from cancer, cardiovascular disease, and other health

complications listed below in Table 2 compared to individuals who have access to clean drinking water (Sohel et al., 2009).

Targets	Health effects
Skin	Skin lesions Skin cancer
Developmental processes	Increased infant mortality Reduced birth weight Altered DNA methylation of tumor promoter regions in cord blood and maternal leukocytes Neurological impairments in children Early-life exposure associated with increased cancer risk as adults
Nervous system	Impaired intellectual function in children and adults Impaired motor function Neuropathy
Respiratory system	Increased mortality from Pulmonary tuberculosis Bronchiectasis Lung cancer
Cardiovascular system	Coronary and ischemic heart disease Acute myocardial infarction Hypertension
Liver, kidney, and bladder	Liver cancer Kidney cancer Bladder and other urinary cancers
Immune system	Altered immune-related gene expression and cytokine expression Inflammation Increased infant morbidity from infectious diseases
Endocrine system	Diabetes Impaired glucose tolerance in pregnant women Disrupted thyroid hormone, retinoic acid, and glucocorticoid receptor pathways in mice and amphibians

Table 2: Long-term health effects of arsenic. Long term exposure to arsenic can result in variety of medical conditions (Naujokas et al., 2013).

Arsenic Remediation

Because arsenic poses a threat to public health, it must be remediated, or removed, from water prior to consumption or use in irrigation and food preparation (WHO, 2012). Arsenic remediation can be performed using a variety of physicochemical techniques, including ion exchange filtration, oxidation filtration, coagulation filtration, adsorption filtration, and membrane filtration (Mondal, 2006). Many of these filtration methods require the installation of expensive filtration membranes and can only be performed at water treatment facilities. Additionally, these treatments prove to be most effective when arsenic, which is most commonly found as As(III) in ground water, is oxidized to As(V). Oxidizing agents such as chlorine and ozone can be unsafe if handled improperly and further add to the cost of heavy metal remediation (“Technologies and Costs for Removal of Arsenic from Drinking Water,” 2000).

Many of the countries that have issues with arsenic contamination in water lack the funds, material, and trained personnel needed to properly treat drinking water with the remediation methods that are currently available. Bioremediation, or the use of a biological system or biological components to remove pollutants and contaminants from water, shows great potential as an alternative to traditional remediation methods. A simple method of bioremediation would eliminate the use of chemicals and provide a cheaper alternative to the expensive filtration membranes and the construction of water treatment facilities. Biofilms have shown great promise in the field of bioremediation over the past few decades because they are safe compared to planktonic organisms as well as efficient and cost effective compared to physiochemical methods of remediation (Singh et al., 2006).

Biofilms

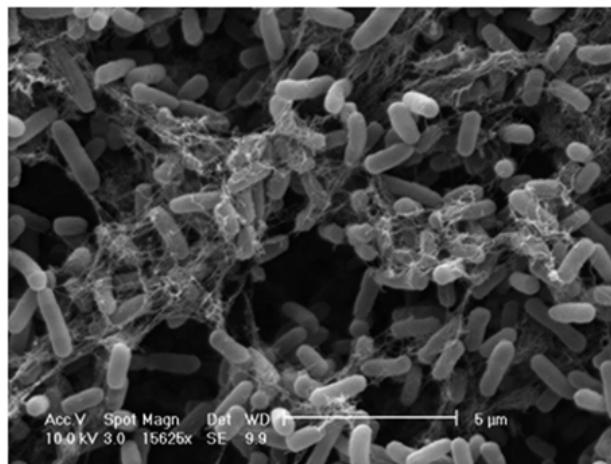


Figure 1: *E. coli* biofilm. An electron micrograph of an *E. coli* biofilm held together by fibrous EPS (Winn et al., 2012).

A biofilm, depicted in Figure 1, is a group of microorganisms that are adhered to a surface through secreted extracellular polymeric substance, or EPS (Singh et al., 2006). Biofilm formation is advantageous for microbes for a number of reasons. First of all, the anionic EPS matrix composed of extracellular DNA, glycoproteins, proteins, and glycolipids is able to retain water, bind cationic solutes, and nourish the biofilm (Flemming, 2007). Secondly, microbes in a biofilm can grow, communicate, and exchange genetic material horizontally with nearby microbes (Edwards and Birthe, 2013). Lastly, microbes within a biofilm are more resilient than free swimming cells under mechanical and chemical stress. Biofilms are up to 600 times more resilient under heavy metal stress than planktonic microbes (Teitzel, 2003) and can be resistant to

the mechanical stress of water flow if grown under hydrodynamic stress or another source of mechanical stress (Singh et al., 2006). These advantages of biofilm formation can be exploited to filter water contaminated with heavy metals such as arsenic in an efficient and cost-effective manner.

Using Synthetic Biology to Enhance Bioremediation

Although the negatively charged biofilm EPS already has the ability to bind cations such as heavy metals, chelation of heavy metals by a biofilm filter can be improved using synthetic biology. Synthetic biology utilizes the engineering of biological systems to improve upon existing functions or create novel functions for use in various applications (Andrianantoandro et al., 2006). In the context of this project, bacteria within a biofilm can be genetically engineered to express a protein that improves the natural chelating abilities of the biofilms. A class of proteins known as phytochelatins have shown promise in heavy metal bioremediation. Phytochelatins are a class of cysteine-rich heavy-metal binding proteins that are predominately found in plants (Cobbett and Goldsbrough, 2002). In 2000, Bae et al. constructed a synthetic phytochelatin consisting of 20 glutamic acid and cysteine (EC) amino acid repeats. When expressed on the cell surface of *E. coli*, synthetic phytochelatin was able to chelate cadmium ions in a 10:1 ratio (Bae et al., 2000). Synthetic phytochelatin could be introduced into a biofilm forming strain of bacteria via a plasmid to improve upon natural arsenic chelation by the biofilm EPS.

Engineering Biofilm Biosensors

In addition to being used as filters, biofilms can act as biosensors because they are made up of microorganisms that can be genetically engineered using recombinant DNA technology. Biosensors are able to detect a substrate in a short period of time, ranging from few seconds to a few hours. Genetic circuits for biosensors are composed of a detection element coupled with an output element. The detection element is typically a receptor or protein, while the output element is typically a detectable reporter protein, such as a fluorescent protein (Chappell and Freemont, 2011). The incorporation of a genetic circuit consisting of an arsenic detection element and a fluorescent output element into a biofilm forming strain of bacteria would allow individuals

using an arsenic biofilm filter to easily detect arsenic in their drinking water, thereby giving the filter a dual function.

Project Goal

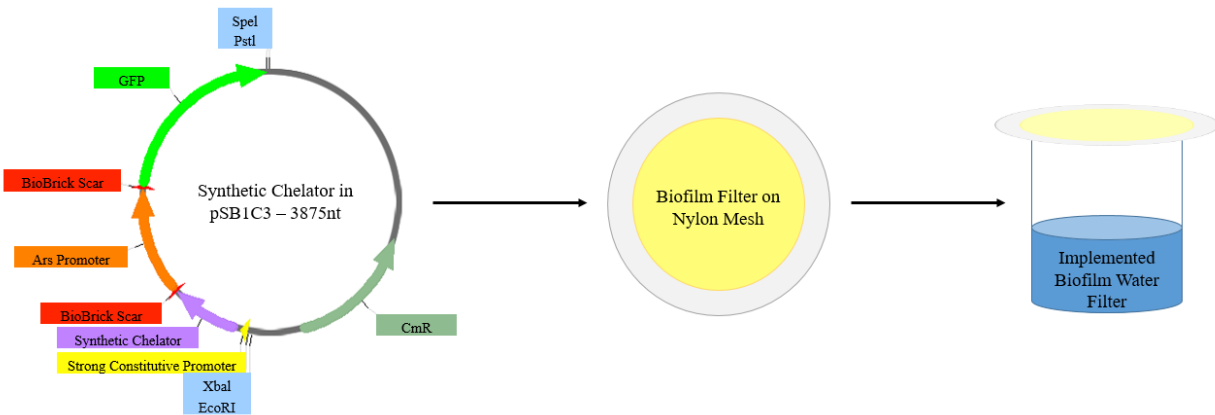


Figure 2: Schematic of the overall project goal.

The heavy metal arsenic is commonly found in sources of water worldwide and is a threat to public health. Current methods of arsenic remediation are expensive and unavailable to developing nations. Biofilms have shown promise both as filters for water contaminated with heavy metals and as biosensors. The goal of this project, which is illustrated above in Figure 2, was to construct a single genetic circuit with a dual arsenic bioremediation and biosensor function and express it in a biofilm forming strain of bacteria. The biofilm expressing the genetic circuit for bioremediation and biosensing of arsenic would then be used as a water filter. Over the course of this project, we were able to:

- Design a genetic circuit consisting of a GFP-based arsenic biosensor and a synthetic phytochelatin;
- Construct a genetic circuit for constitutive expression of synthetic phytochelatin;
- Test numerous laboratory strains of bacteria to identify an optimal host organism that forms a robust biofilm while posing little risk to water quality and human health; and
- Express and test the synthetic phytochelatin in *E. coli* EMG2:Kλ.

By incorporating both biosensing and bioremediation into a biofilm filter, inexpensive and efficient detection and remediation of arsenic in drinking water can be a possibility for people in all parts of the world who rely on sources of water that are contaminated with arsenic.

METHODS

Crystal Violet Biofilm Assay

The biofilm-forming properties of 11 different strains of bacteria, listed in Table 1, were tested using a crystal violet biofilm assay. Each of the 11 strains, along with a negative control strain and a positive control strain, was grown in a 5 ml LB liquid culture for 18-24 hours and diluted 100 fold to a total volume of 1 ml in both LB broth and M9 minimal media. The protocol for the preparation of M9 minimal media can be found in Appendix A. In a round bottom 96 well plate, 100 μ L of each dilution was plated in four wells. Once all of the dilutions were plated, the 96 well plate was incubated at 37°C for 48 hours.

Identifier	Strain
- control	<i>Escherichia coli</i> DH5 α
+ control	<i>Escherichia coli</i> EMG2:K λ
1	<i>Bacillus subtilis</i>
4	<i>Enterobacter aerogenes</i>
5	<i>Staphylococcus cohnii</i>
7	<i>Staphylococcus epidermidis</i>
8	<i>Escherichia coli</i> NCTC 9001
9	<i>Acinetobacter baylyi</i>
12	<i>Klebsiella pneumoniae</i>
13	<i>Proteus vulgaris</i>
14	<i>Staphylococcus aureus</i>
15	<i>Bacillus megaterium</i>
16	<i>Bacillus cereus</i>

Table 3: Strains Tested in the Biofilm Assay. *E. coli* DH5 α and *E. coli* EMG2:K λ served as the negative and positive control respectively. Experimental strains that were obtained from the Microbes to Molecules laboratory were labeled with previously assigned numerical identifiers.

Biofilms were stained by draining the media and rinsing the wells of the plate with water, then staining with 125 μL of 0.1% crystal violet for 10 minutes, followed by 4-5 additional rinses with water before drying overnight. For quantification, the crystal violet-stained biofilm was solubilized in 200 μL of 30% acetic acid. 100 μL of the crystal violet-acetic acid solution from each well was transferred to a new well in a flat bottom 96 well plate, then the OD_{595} of each well was measured using a plate reader (Biotek ELx800 Absorbance Reader). 100 μL of 30% acetic acid with no crystal violet was also plated in 4 wells for use as a blank. The average of four technical replicates was calculated for each strain in LB and M9 minimal media. The average of the blank was subtracted from each of the sample averages, then normalized to the average OD_{595} of the positive control strain, *E. coli* EMG2:K λ . Strains with normalized OD_{595} values greater than 1 formed strong biofilms relative to EMG2:K λ , while strains with normalized OD_{595} values of less than 1 formed poor biofilms relative to EMG2:K λ .

Growing *S. epidermidis* Biofilms on Nylon Mesh Discs

S. epidermidis biofilms were grown on Ted Pella nylon mesh with three different pore sizes. The 100, 200, and 300-gauge meshes had pore sizes of 140, 86, and 53 microns respectively. To grow the biofilms, a 5 ml *S. epidermidis* LB liquid culture was prepared and incubated in a shaking incubator at 37°C for 18-24 hours. The liquid culture was diluted 100-fold in M9 minimal media. The 100, 200, and 300-gauge nylon meshes were one-hole punched to create small discs (Figure 3). The discs were placed in wells of a 24-well plate and 500 μL of the diluted *S. epidermidis* culture was added to each of the wells. The 24-well plate was incubated at 37°C for 48 hours.



Figure 3: Nylon Mesh Discs. The discs were hole punched from sheets of 100, 200, and 300-gauge Ted Pella nylon mesh. Pore size decreases and mesh gauge increases from left to right.

Following the 48-hour incubation, the nylon mesh discs were carefully removed with tweezers and rinsed in water. The discs were placed on a paper towel to dry while the 24 -well plate was rinsed with water and dried. Discs were placed back into the wells of the plate and stained with 0.1% crystal violet for 10 minutes. The discs and wells were then rinsed with water

and dried overnight. For quantification, the crystal violet was solubilized in 200 μl of 30% acetic acid. 100 μL of the crystal violet-acetic acid solution from each well was transferred to a new well in a flat bottom 96 well plate, then the OD_{595} of each well was measured using a plate reader (Biotek ELx800 Absorbance Reader). 100 μL of 30% acetic acid was also plated in 4 wells for use as a blank. The average of four technical replicates was calculated for each mesh, then the average of the blank was subtracted from each of the sample averages.

Cloning the Arsenic Biosensor Construct

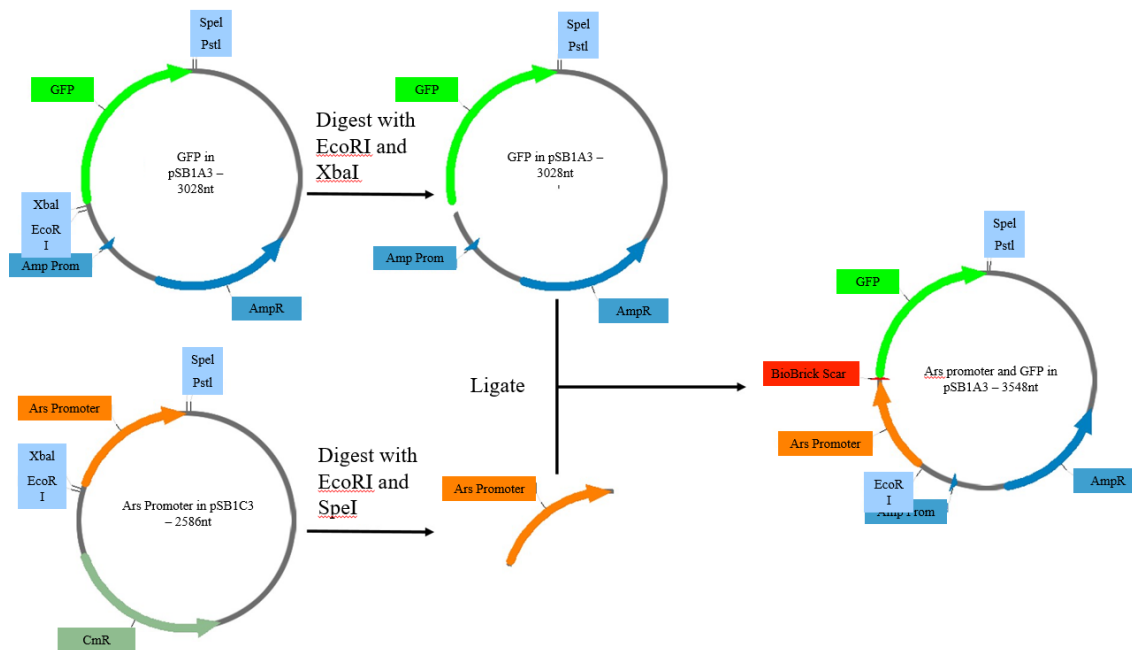


Figure 4: Arsenic Biosensor Cloning Strategy

To create the arsenic biosensor construct, the Ars promoter was cloned into the ampicillin resistant GFP plasmid through restriction digests and ligation with T4 DNA ligase (Figure 4). Plasmid DNA for the RBS-GFP-Double Terminator construct (BBa_I13504) and Ars promoter (BBa_J33201) was resuspended from the iGEM parts distribution plates in 10 μl of water. DH5 α *E. coli* was transformed with 2 μl of each plasmid resuspension. Plasmid DNA was isolated from the *E. coli* using a Macherey-Nagel NucleoSpin Plasmid Miniprep Kit. 50 μl restriction digests were prepared using 40 μl of each plasmid miniprep. The Ars promoter plasmid was digested with EcoRI and SpeI and the GFP plasmid was digested with EcoRI and XbaI. Both digests were incubated in a 37°C water bath overnight. Following the overnight incubation, the GFP plasmid

digest was treated with 5 μ l of shrimp alkaline phosphatase (SAP) for 30 minutes. Enzymes in the two digests were heat killed at 80°C for 20 minutes. The digests were then run on a 1.0% agarose gel and gel purified using a Macherey-Nagel NucleoSpin Gel and PCR Clean Up Kit. The gel purified digests were ligated together in 1:1, 1:2, 1:5, and 1:10 ratios of plasmid to insert with 2 μ l of T4 DNA ligase. The 20 μ l ligation reactions were incubated at room temperature for 1 hour. DH5 α *E. coli* was transformed with 5 μ l of each ligation and plated on an LB agar plate supplemented with ampicillin. Colonies were screened with colony PCR and picked from transformation plates and grown in LB liquid cultures supplemented with ampicillin. Plasmids were isolated from the *E. coli* liquid culture using a Macherey-Nagel NucleoSpin Plasmid Miniprep Kit and sequenced by Eton Bioscience.

Cloning the Synthetic Chelator Construct

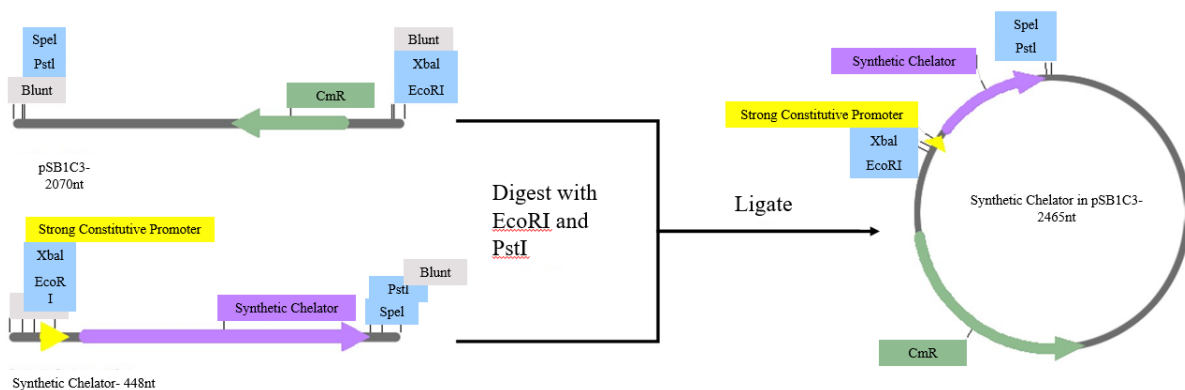


Figure 5: Synthetic Chelator Cloning Strategy

The synthetic chelator construct was cloned into the chloramphenicol-resistant plasmid pSB1C3 through restriction digests with EcoRI and PstI and ligation with T4 DNA ligase (Figure 5). The synthetic chelator construct, consisting of a high strength constitutive promoter (BBa_J23101), a ribosome binding site (BBa_B0034), synthetic phytochelatin (BBa_K1321005) with repetitive regions broken up by linkers, and a double terminator (BBa_B0015) was synthesized as a gBlock by IDT. The full sequence of this synthetic chelator can be found in Appendix C. The DNA was resuspended in 50 μ l of elution buffer (5mM Tris/HCl, pH 8.5). 100 ng of resuspended DNA was used to prepare a 25 μ l EcoRI and Pst I restriction digest. The digest was incubated in a 37°C water bath overnight. Linearized pSB1C3 from the iGEM parts distribution was also digested with EcoRI and PstI overnight. The restriction digest contained

125 ng of pSB1C3 DNA and had a total volume of 25 μ l. Once the synthetic chelator construct and chloramphenicol-resistant plasmid backbone were digested, the two digests were ligated together in a 20 μ l reaction using T4 DNA ligase. The ligation was left at room temperature for 1 hour. DH5 α *E. coli* was transformed with 5 μ l of the ligation and plated on LB agar plates supplemented with chloramphenicol. Colonies were picked from transformation plates and grown in LB liquid cultures supplemented with chloramphenicol. Plasmid DNA was isolated from the liquid culture using a Macherey-Nagel NucleoSpin Plasmid Miniprep Kit. Insertion of the synthetic chelator construct into pSB1C3 was verified by gel electrophoresis.

Testing the Natural Chelating Abilities of *S. epidermidis* and *A. baylyi*

Two *S. epidermidis* liquid cultures and two *A. baylyi* liquid cultures were grown in 10 ml of M9 minimal media containing arsenic. 10 ml of M9 minimal media containing only arsenic served as a control. The materials needed to prepare M9 minimal media with arsenic are listed in Appendix B. The four cultures and the control were incubated in a 37°C shaking incubator for 24 hours. After the 24-hour growth period, one *S. epidermidis* culture and one *A. baylyi* culture were centrifuged for 10 minutes at 1000 x g. The M9 control, the media from the spin down cultures, and samples from the other two cultures were then tested using the Hach Low Range Arsenic Test Kit. 5ml of each liquid culture was placed into the test kit reaction vessel and diluted with 45ml of DI water. The procedure for using the test kit, which can be seen in Appendix D, was then followed to test the natural chelating abilities. Test results were compared to the test strip standard (Figure 6).

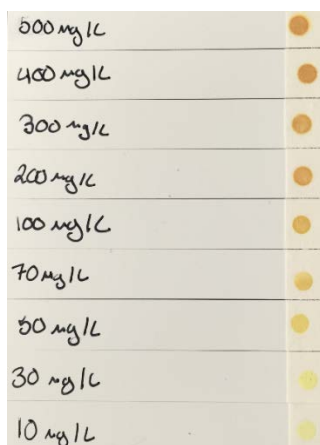


Figure 6: Hach Arsenic Test Kit Standards. Water with arsenic concentrations ranging from 10 μ g/L to 500 μ g/L were tested to generate color standards for the test strips.

Measuring Arsenic Chelation by *E. coli* Expressing the Synthetic Chelator

DH5 α and EMG2:K λ *E. coli* were transformed with approximately 200 ng of synthetic chelator and high strength constitutive promoter (Bba_J23101) plasmid minipreps. Both strains of *E. coli* were transformed with the high strength constitutive promoter BioBrick to determine if the chloramphenicol resistant plasmid had any effect on chelating properties. Liquid cultures of J23101 in EMG2:K λ , the synthetic chelator in EMG2:K λ , and *E. coli* EMG2:K λ were prepared in 10 ml of M9 minimal media containing arsenic (Appendix B). 10 ml of M9 minimal media containing arsenic served as a control. All cultures and the control were incubated in a shaking incubator at 37°C for 24 hours. Following the 24-hour growth period, the cultures were centrifuged at 1000 x g for 10 minutes. 5 ml of media from the spin down cultures and the control were transferred to the Hach Low Range Arsenic Test Kit reaction vessel and diluted with 45 ml of DI water. The procedure for test kit, which can be seen in Appendix D, was followed and test results were compared to the test strip standard (Figure 6).

RESULTS

Crystal Violet Biofilm Assay

In order to select the optimal bacterial strain for our biofilm filter, we screened several common laboratory bacterial strains for their ability to form biofilms. Of the 11 strains of bacteria tested in the crystal violet biofilm assay, *S. epidermidis*, *E. coli* NCTC 9001, *A. baylyi*, and *K. pneumoniae* demonstrated the strongest biofilm formation relative to *E. coli* EMG2:K λ . Figure 7 below shows that *K. pneumoniae* consistently exhibited the strongest biofilm formation, with an average OD₅₉₅ over 4 times greater than that of *E. coli* EMG2:K λ . *E. coli* NCTC 9001 formed biofilms that were about 3 times stronger than *E. coli* EMG2:K λ biofilms, but with greater variability between biological replicates. *S. epidermidis*, and *A. baylyi* formed biofilms that were twice as dense as *E. coli* EMG2:K λ .

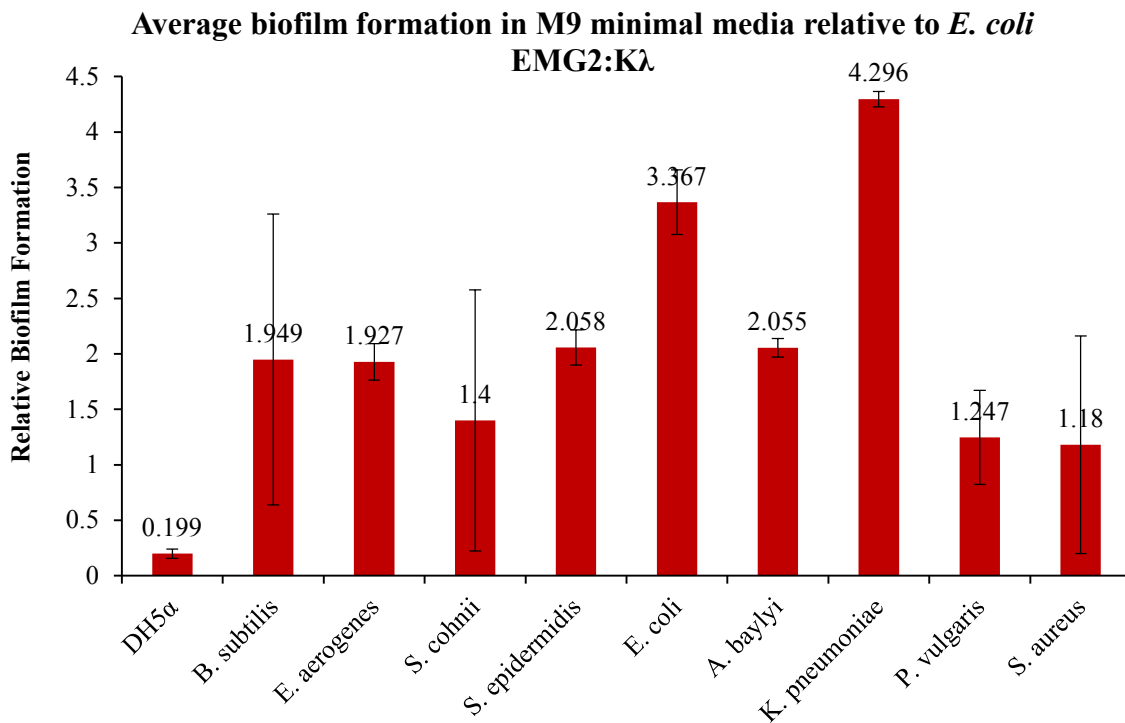


Figure 7: Average Biofilm Formation. Average biofilm formation of 11 strains of bacteria relative to EMG2:K λ . Error bars indicate +/- standard error for three biological replicates.

***S. epidermidis* Growth on Nylon Mesh Discs**

Considering issues of biosafety and reproducibility, we selected *S. epidermidis* as a potential host strain for our biofilm filter. We next sought to characterize *S. epidermidis* growth on potential filter materials. From the three nylon mesh gauges tested, the weakest *S. epidermidis* biofilm grew on the 100-gauge mesh while the 300-gauge allowed for the formation of the strongest *S. epidermidis* biofilm. Figure 8 shows average biofilm formation by *S. epidermidis* on the discs of the three different nylon meshes. Results were normalized to a 100 μ l acetic acid control. Figure 9 shows *S. epidermidis* grown on the three different nylon mesh at 15X magnification over a 24-hour growth period. The 200 and 300-gauge have a tight network of *S. epidermidis* cells between the pores while the 100-gauge mesh appears to have little to no cell growth between the pores.

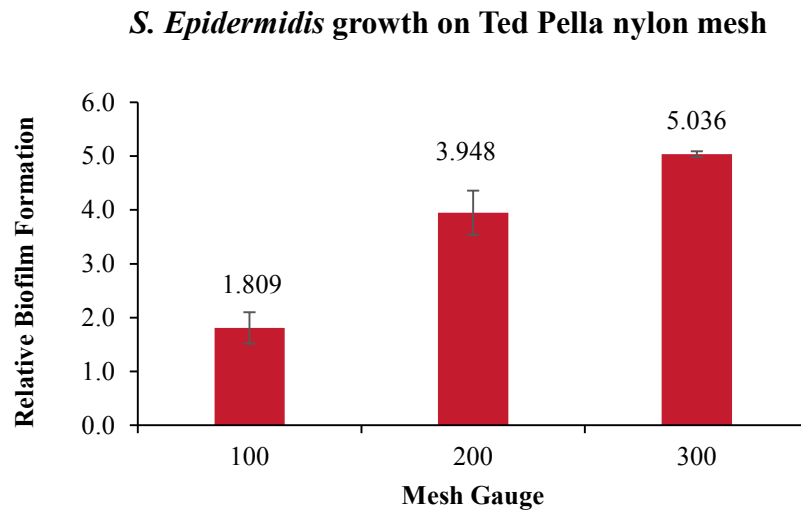


Figure 8: *S. epidermidis* growth on nylon mesh. Average growth of *S. epidermidis* on Ted Pella 100, 200, and 300-gauge nylon mesh discs. Error bars indicate +/- standard error for three biological replicates.

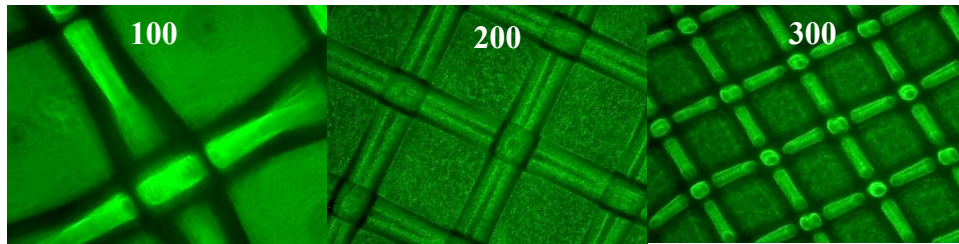


Figure 9: *S. epidermidis* growth under an inverted compound microscope. Growth of *S. epidermidis* on three different nylon gage sizes over a 24-hour period at 15X magnification on an inverted compound microscope.

Arsenic Biosensor Cloning

We next sought to clone and characterize the arsenic biosensor construct. This construct would place the arsenic repressor protein and pArs promoter upstream of a GFP reporter, thus regulating GFP expression in response to arsenic. Unfortunately, cloning of the arsenic biosensor construct was unsuccessful. On several occasions, colony PCR of colonies on Ars promoter-GFP ligation plates and test digests of clone minipreps indicated successful cloning of the Ars promoter and GFP into the same plasmid. The colony PCR gel in Figure 10, for example, shows that the PCR products for all of the Ars promoter-GFP colonies lack a 0.8 kb band that is present in the PCR product for the two control colonies. Additionally, the PCR product for colonies 2, 3, and 4 contains a fragment that appears to be 1.5 kb in length, or the approximate length of the Ars promoter and GFP combined. However, when minipreps from colonies that produced a 1.5 kb band on the colony PCR gel were sequenced, the sequences did not align whatsoever with the expected Ars promoter-GFP sequence. When the miniprep sequences were aligned to the pSB1C3 vector sequence, as shown below in Figure 11, the miniprep sequences aligned with 106 nucleotides of the pSB1C3 vector.

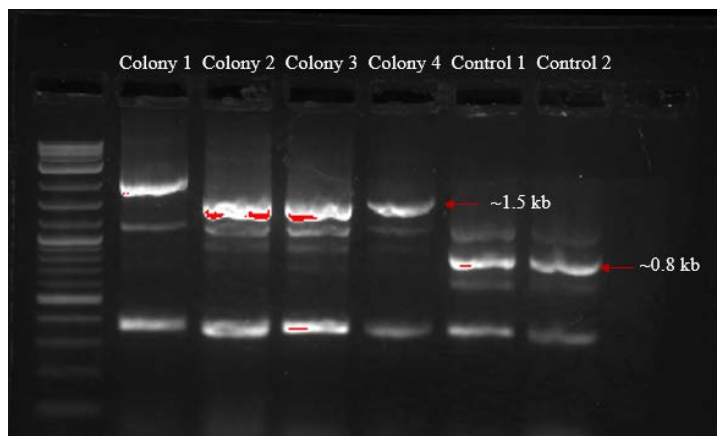


Figure 10: Ars promoter-GFP colony PCR. Band patterns on a colony PCR gel for 4 colonies from Ars promoter-GFP ligation plate (Colonies 1-4) differed from the band pattern for colonies from the ligation control plate (Control 1 and 2).

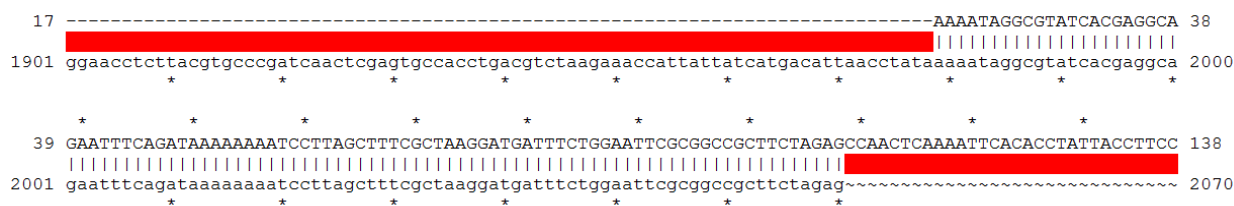


Figure 11: Sequence alignment to pSB1C3. Nucleotides 17 through 138 of the sequenced Ars promoter-GFP minipreps (top sequence) aligned with 106 nucleotides of the pSB1C3 vector sequence (top sequence).

Synthetic Chelator Cloning

The chelating portion of the plasmid is the second half to our synthetic bioremediation and biosensing construct. We used gene synthesis to design a synthetic chelator protein based on the phytochelatin construct (BBa_K1321005) from the Registry of Standard Parts (parts.igem.org). Due to the highly repetitive nature of phytochelatin, it was not an amenable sequence for gene synthesis. We therefore inserted several flexible linker domains throughout the protein to interrupt the repetitive sequence such that it could be created by gene synthesis. We then cloned this new synthetic chelator (SC) insert into pSB1C3. A test digest of the two synthetic chelator minipreps revealed that the SC was successfully cloned into the pSB1C3 vector. The gel in Figure 12 shows two bands in the SC Clone 1 and SC Clone 2 lanes. The top band in these two lanes is approximately 2 kb in length, which is the same size as the band in the pSB1C3 digest lane. The bottom band in these two lanes is approximately 0.4 kb in length and is the same size as the band produced by the SC gBlock (gene synthesis product) digest.

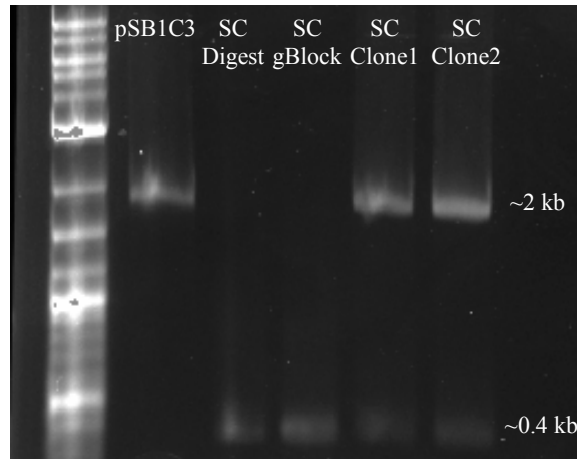


Figure 12: Synthetic chelator test digest. The pSB1C3 lane contains the linearized pSB1C3 backbone digested with EcoRI and PstI. The SC digest lane contains the synthetic chelator gBlock digested with EcoRI and PstI. The SC gBlock lane contains the synthetic chelator DNA. The SC Clone lanes contain synthetic chelator plasmid minipreps digested with EcoRI and PstI. Both synthetic chelator miniprep digests released bands that were approximately 2 kb and 0.4 kb in size.

Testing the Natural Chelating Abilities of *S. epidermidis* and *A. baylyi*

S. epidermidis and *A. baylyi* were grown in cultures containing arsenic to test their natural chelating abilities and create an arsenic testing protocol that could be both useful within a lab setting as well as in practice. Testing of the natural chelation of arsenic by *S. epidermidis* and *A. baylyi* revealed that both strains have natural chelating properties. Figure 13 shows the arsenic test strip results for each culture and the approximate arsenic concentrations estimated by comparing test strip coloration to the kit standard. These results demonstrate that both strains have natural chelating abilities in their planktonic form, with *A. baylyi* absorbing approximately 17ppb arsenic from the media, and *S. epidermidis* absorbing approximately 27ppb.






	<i>A. Baylyi</i>	≈35ppb
	<i>A. Baylyi</i> "Spin Down"	≈20ppb
	<i>S. Epidermidis</i>	≈35ppb
	<i>S. Epidermidis</i> "Spin Down"	≈10ppb
	Control	37ppb

Figure 13: Natural chelation of arsenic by *S. epidermidis* and *A. baylyi*. Arsenic test strip results and estimated arsenic concentrations for cultures of *S. epidermidis* and *A. baylyi* grown in media with an initial arsenic concentration of 37 ppb.

Measuring Arsenic Chelation by *E. coli* Expressing the Synthetic Chelator

Next we tested the effectiveness of our synthetic chelator in arsenic chelation. The effectiveness of the construct would be directly correlated to the ability of the biofilm to remove arsenic from water. Testing of the J23101 negative control plasmid and the synthetic chelator with EMG2:Kλ revealed that both have additional chelation abilities on top of the chelation from untransformed EMG2:Kλ. Figure 14 shows the test strip from each culture and the approximate arsenic concentrations estimated by comparing test strip coloration to the kit standard. In the case of both J23101 and the synthetic chelator, each transformed strain absorbed an additional approximately 5ppb more arsenic than the untransformed EMG2:Kλ. Thus the synthetic chelator did not appear to increase arsenic chelation in this experiment.





	EMG2:Kλ	≈25ppb
	J23101 & EMG2:Kλ	≈20ppb
	Synthetic Chelator & EMG2:Kλ	≈20ppb
	Control	37ppb

Figure 14: Chelation of arsenic by promoter J23101 and synthetic chelator complex. Arsenic test strip results and estimated arsenic concentration for cultures of EMG2:Kλ, J23101, the synthetic chelator complex created grown in media with an initial arsenic concentration of 37ppb.

DISSCUSION

In the crystal violet biofilm assay, *S. epidermidis*, *E. coli* NCTC 9001, *A. baylyi*, and *K. pneumoniae* all formed stronger biofilms than the positive control, *E. coli* EMG2:Kλ. Each of these strains would undoubtedly be able to generate a durable biofilm filter. However, safety of the biofilm filter is a major concern that needs to be taken into consideration. Although *K. pneumoniae* formed the strongest biofilms of the 11 strains of bacteria, it is a pathogen that causes pneumonia, bloodstream infections, and meningitis (CDC, 2012). The second best biofilm former was the NCTC 9001 strain of *E. coli*. This strain of *E. coli* is found in human urine and is a biosafety level 1 organism (ATCC). While the virulence of NCTC 9001 has not been characterized and infections have not been reported to date, this strain of *E. coli* shares a serovar with extraintestinal pathogenic *E. coli*, which causes meningitis, urinary tract infections, and septicemia (Mora et al., 2009). The third strongest biofilm forming strain of bacteria was *A. baylyi*, a biosafety level one organism commonly found in soil. Contrary to *E. coli* NCTC 9001, *A. baylyi* is known to cause opportunistic infections in immunocompromised individuals (Chen et al., 2008). Lastly, the fourth strongest biofilm former was *S. epidermidis*, a strain of bacteria that naturally colonizes the human skin. Like *A. baylyi* and *E. coli* NCTC 9001, *S. epidermidis* is an opportunistic pathogen. Opportunistic infection with *S. epidermidis* can result in antibiotic resistant skin and bloodstream infections (Otto, 2009). Because most strains of bacteria can cause an opportunistic infection, we decided to select a strain that naturally colonizes humans for the biofilm filter. We therefore proceeded with growing *S. epidermidis* biofilms on nylon mesh and testing the natural arsenic chelating abilities of *S. epidermidis*.

As predicted, the 300-gauge nylon mesh supported the formation of the strongest *S. epidermidis* biofilms. There are a number of ways to improve this experiment in the future. First, a wider variety of mesh sizes should be tested above the 300-gauge. There should also be a comparison to the strength and formation of the biofilm with the ability of water to flow through the mesh itself. Additionally, larger pieces of mesh, similar to the size of the full filter, should be tested to determine how well the biofilm can withstand the flow of water. Finally, there should be a frame or holder for the biofilm to be pulled over so it does have the actual ability to filter water.

Successful cloning of the arsenic biosensor is crucial for the development of the complete biofilm filter. The arsenic detection circuit was incorporated into the overall filter design to make arsenic testing simple and available to individuals who are using the filter. The visual output of a fluorescent protein or chromoprotein would allow filter users to determine if their water is contaminated with detectable levels of arsenic. Alternative cloning strategies may have to be tested to successfully construct the arsenic biosensor, as ligation of the pArs promoter into the GFP plasmid produced high background, despite treating the destination vector with Shrimp Alkaline Phosphatase (SAP). The colonies on the ligation plates that were screened and sequenced only produced copies of the pSB1C3 plasmid or the GFP plasmid. Because this problem may stem from the pArs promoter and GFP plasmid minipreps throughout the cloning process, DNA synthesis may be the most viable option for the construction of the arsenic biosensor in the future.

A test digest of the plasmid minipreps isolated from synthetic chelator clones indicated that the synthetic chelator gBlock was successfully cloned into the chloramphenicol resistant plasmid pSB1C3. Completing this cloning step allowed us to test the synthetic chelator in *E. coli*, but further steps would have to be taken to determine where in the cell the synthetic chelator is expressed and express the synthetic chelator in other strains of bacteria. Achieving the correct subcellular localization of the synthetic chelator protein is important for arsenic bioremediation. If the synthetic chelator protein that we designed naturally localizes to the cytoplasm, then the entire construct would have to be re-cloned to add a cell surface localization tag. Cell surface expression of the chelator protein would allow for the chelation of all surrounding arsenic rather than the arsenic that is absorbed and internalized by the cells within the biofilm. Expression of the synthetic chelator protein in strains of bacteria other than *E. coli* is also an important aspect of this project. In order to express the synthetic chelator in *S. epidermidis*, our strain of choice for the biofilm filter, the synthetic chelator gBlock would have to be codon optimized and cloned into a plasmid with an origin of replication that is used by *S. epidermidis*. Much additional characterization and research will need to be done in order to produce a synthetic chelator construct that can be used in the final filter product.

It is important to clone the goal plasmid into a bacterial strain that also has natural chelation properties for arsenic. *S. epidermidis* itself can naturally chelate arsenic, making it an excellent strain to use for arsenic bioremediation. In fact, our results suggest that just using a

biofilm of *S. epidermidis* as a simple biological filter potentially has the ability to reduce drinking water arsenic down to a safe level. *A. baylyi* in comparison only was able to chelate about 17ppb of the 37ppb within the solution. This could be due to the internal machinery of the bacteria being unable to deal with heavy metal in high quantities. There was also decreased bacteria growth within the *A. baylyi* liquid culture compared to *S. epidermidis*, meaning there could be heightened cell death when *A. baylyi* comes into contact with toxic levels of heavy metals. This experiment could be improved by using a more quantitative test kit to measure exact levels of arsenic that remain within these cultures.

In our arsenic chelation assay, both the strong constitutive promoter alone and the synthetic chelator were able to remove a portion of the arsenic from the water. Therefore our synthetic chelator did not confer any additional chelating properties in this assay. It is possible that the DNA plasmid itself binds some arsenic ions, or that the antibiotic resistance proteins have some chelating properties. It is also possible that the position of the linkers in the phytochelatin sequence decreased the efficiency of chelation. In the future, it will be necessary to investigate alternative synthetic chelator designs that are both amenable to gene synthesis and maintain chelating properties.

From the results, there are a number of concerns with the implementation of this product. First, there is some concern in creating a biofilm that would be able to withstand the flow of water containing while still allowing it to pass through. Any break within the biofilm could potentially contaminate water depending on which strain of bacteria is chosen. There is also a concern with balancing the biosafety of the bacterial strain with the strength of the biofilm it creates. It will be difficult to promote a bacterial biofilm water filter if the strain of bacteria has any potential of being harmful pathogen. Secondly, there is some concern regarding the size of the genomic circuit being proposed. Depending on which strain it is cloned into, size of the circuit could cause the cell to be damaged therefore not being able to act as the goal biofilm water filter. Overall, this water filter design has some great potential to create a new and cheaper option of the filtration of arsenic with the field of synthetic biology. With some improvements in testing, synthesis of different portions of the genetic circuit, expanding the search for favorable bacterial strains, and finally, a well strong water filter design, there is a potential to open a new option for many communities with unsafe water.

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APPENDICES

Appendix A: M9 Minimal Media

M9 Salts

1. Measure 800 ml H₂O
2. Add 64g Na₂HPO₄-7H₂O
3. Add 15g KH₂PO₄
4. Add 2.5g NaCl
5. Add 5.0g NH₄Cl.
6. Stir until dissolved
7. Adjust to 1000 ml with distilled H₂O
8. Separate into 5 200 ml aliquots. Sterilize by autoclaving 15 minutes on liquid setting.

M9 Liquid Media

1. Measure 700ml of distilled H₂O (sterile)
2. Add 200ml of M9 salts
3. Add 2ml of 1M MgSO₄ (sterile)
4. Add 20 ml of 20% glucose (or other carbon source- 20% glycerol) (This is 0.4% final)
5. Add 100µl of 1M CaCl₂ (sterile)
6. Add 1 ml of sterile filtered 1000x L-arginine.
7. Adjust to 1000ml with distilled H₂O

Appendix B: M9 Minimal Media with Arsenic

1. Measure 7 ml 500 µM sodium arsenite
2. Add 2 ml M9 salts
3. Add 20 µl sterile 1M MgSO₄
4. Add 200 µl 20% glucose
5. Add 1 µl 1M CaCl₂
6. Add 10 µl 1000x L-arginine
7. Adjust to 10 ml with 500 µM sodium arsenite

Appendix C: Sequence of Synthetic Chelator (includes promoter, ribosome binding site, phytochelatin with interspersed linkers, and double terminator sequence)

TATAGAATTCGCGGCCGCTTCTAGAGTTTACAGCTAGCTCAGTCCTAGGTATTATGCTA
GCAAAGAGGAGAAAATGGAATGTGAATGTGAGTGCTCCGCTTGTTACTGTGAGCTTTC
CGAATGCGAGTGTGAATGTGAGTGTGAGTGTGGTGAAAATTTGTATTTTCAATCTGGT
GGTGAGTGTGAGTGTGGTAGCGGCAGCGGTAGCGAATGCGAGTGTGGCAGCTGTGAA
TGCGAGTGCGAAGGTAGCTGCGAATGCGAGTGCGGCCACCATCACCATCATCACTAAT
AACCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCT
GTTGTTTGTCCGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCC
TTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAGTATATA

Appendix D: Hach Low Range Arsenic Test Kit Instructions

Arsenic Test Kit 0–500 ppb (0, 10, 30, 50, 70, 300, 500 ppb)

28000-88

WARNING: Hydrogen and arsine gases are generated during the test. Work in a well-ventilated area away from open flames and other sources of ignition. Review the Material Safety Data Sheets before handling any chemicals.

Scope and Application: For natural waters, drinking water, and groundwater

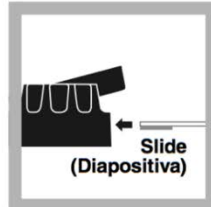
Introduction

Hach's new Arsenic test kit provides a simple, effective way to test for arsenic in the range of 0–500 ppb. The visual comparison test is ideal for use almost anywhere that trace amounts of total inorganic arsenic must be quantified. This new kit uses safe, easy-to-handle reagents packaged in unit doses, with a test strip to determine the final result. Up to 5 mg/L hydrogen sulfide in the sample can be tolerated. The design of the apparatus offers increased sensitivity (down to 10 ppb) and also minimizes exposure to arsine gas.

Tips and Techniques

1. Do not expose the **reacted** test strips to direct sunlight. The reaction products are photosensitive and will tend to darken, which may cause difficulty in color matching.
2. At no time should the solution in the reaction vessel come into direct contact with the test strip. The test strip reacts with gases released from the chemical reaction, not with the solution in the reaction vessel.
3. It is **critical** that the pad on the test strip **face downward**, centered over the hole in the black cap. If the placement of the test strip is incorrect, the generated gases may not contact the pad correctly and the final reading may be low.
4. Two reaction vessels and two black caps are provided to allow for the simultaneous analysis of two samples.

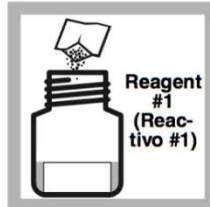
Procedure



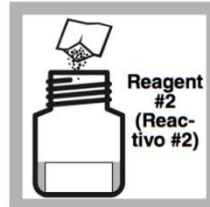
English
1. Lift the flap on the black cap and **slide** a test strip into the groove so that the reactive pad faces the small opening and completely covers it; secure by pressing the flap back in place.



2. Fill the reaction vessel with sample water to the fill line (50 mL).



3. Add the contents of 1 Reagent #1 powder pillow to the sample and swirl to dissolve.



4. Add the contents of 1 Reagent #2 powder pillow to the sample and swirl to dissolve.

Note: Solution may be cloudy at this point.

Español
1. Levante la trampilla encima del tapón negro y deslice una tira de prueba en la ranura, cuidando que la almohadilla reactiva mire hacia la abertura y la cubra completamente; cierre la trampilla presionándola.

2. Llene el frasco de reacción con la muestra de agua hasta la marca (50 mL).

3. Agregue el contenido de 1 cápsula de polvo Reactivo #1 a la muestra y revolver para disolver.

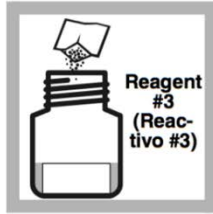
4. Agregue el contenido de 1 cápsula de polvo Reactivo #2 a la muestra y revuelva para disolver.

Nota: La solución se verá opaca en este punto.



English 5. Wait at least 3 minutes.

Español 5. Espere por lo menos 3 minutos.



6. Add the contents of 1 Reagent #3 powder pillow to the sample and swirl to mix.

Note: Not all of the powder will dissolve.

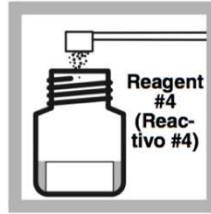
6. Agregue el contenido de 1 cápsula de polvo Reactivo #3 a la muestra y revuelva para disolver.

Nota: No todo el polvo entrará en solución.



7. Wait at least 2 minutes and swirl again to mix.

7. Espere por lo menos 2 minutos y revuelva de nuevo para mezclar.



8. Using the plastic scoop, add 1 level scoop of Reagent #4 to the sample and swirl to mix.

Note: Most of the powder will dissolve at this time.

8. Empleando la cuchara plástica, agregue 1 cucharada rasa de Reactivo #4 a la muestra, y revuelva para mezclar.

Nota: Ahora se disolverá la mayor parte del polvo.



English 9. Add the contents of 1 Reagent #5 powder pillow to the sample.

Español 9. Agregue el contenido de 1 cápsula de polvo Reactivo #5 a la muestra.



10. Immediately attach the black cap, with the test strip inserted, to the reaction vessel. **Do not shake or invert!**

Swirl to mix. Do not allow sample to contact the test strip pad.

10. Inmediatamente vuelva a taponar el frasco de reacción con el tapón negro con la tira de prueba inserta. **¡No sacuda ni invierta el frasco!**

Revuelva para mezclar. No permita que muestra tenga contacto con tira de prueba.



11. Allow vessel to react for 30 minutes, but no more than 35 minutes; swirl twice during the reaction period.

11. Deje que la reacción proceda por 30 minutos, pero no más de 35 minutos; revuelva 2 veces durante el período de la reacción.



12. Remove the test strip and immediately compare the developed color to the chart on the test strip bottle.

Note: For best results, read the strip outdoors in a shady place. Direct sunlight will change the color of the strip.

12. Retire la tira de prueba y compare inmediatamente el color revelado con la carta de color pegada al recipiente de las tiras de prueba.

Note: Para lograr resultados más exactos, lea la tira de prueba afuera, pero en un sitio sombreado. La luz directa del sol alterará el color de la tira.

Interferences

The following were found to interfere:

Ion or Substance	Concentration
Sulfide	> 5 ppm
Selenium	> 1 ppm
Antimony	> 250 µg/L
Tellurium	Likely to interfere, but not tested

The following did not interfere at the levels tested:

Ion or Substance	Concentration
Hardness	1000 ppm as CaCO ₃
Alkalinity	1000 ppm as CaCO ₃
Iron	10 ppm
Temperature	10 to 40 °C

Other interferences are unlikely.

Summary of Method

Hydrogen sulfide is first oxidized to sulfate to prevent interference, and the oxidizing environment is then neutralized. Sulfamic acid and powdered zinc react to create strong reducing conditions in which inorganic arsenic is reduced to arsine gas (AsH₃). The arsine gas then reacts with mercuric bromide in the test strip to form mixed arsenic/mercury halogenides that discolor the test strip. The color ranges from yellow through tan to brown, depending on the concentration.

Organic Arsenic

Organic arsenic represents a small proportion of the arsenic in most systems. The instructions, as written for this test, are designed to detect inorganic arsenic. Organic arsenic compounds, such as dimethylarsenic acid, are not detected. To quantify inorganic and organic arsenic (total arsenic) with this kit, the following modification is needed: Collect 50 mL of sample in a glass beaker. Add the first two reagents according to the instructions. Place the beaker in a boiling water bath for 30 minutes. Remove the beaker from the water bath and transfer the contents to the reaction vessel. Allow the sample to cool to room temperature. Complete the procedure, beginning with step 6.

Required Reagents

Description	Unit	Cat. No.
Arsenic Test Kit Reagent Set.....	each.....	27999-00
Includes: Arsenic Test Strips and Arsenic Reagents #1 – #5		
Arsenic Test Strips.....	100/pkg.....	*
Arsenic Reagent #1, Powder Pillows	100/pkg.....	*
Arsenic Reagent #2, Powder Pillows	100/pkg.....	*
Arsenic Reagent #3, Powder Pillows	100/pkg.....	*
Arsenic Reagent #4	250 g.....	*
Arsenic Reagent #5, Powder Pillows	100/pkg.....	*
Cap, Santoprene	2/pkg.....	49348-00
Reaction Vessel, 50-mL fill line	2/pkg.....	28002-00
Scoop, 2 g, for 454-29	each.....	27998-00

* These items are not sold separately. Please order the complete reagent set (Cat. No. 27999-00) as a replacement.



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