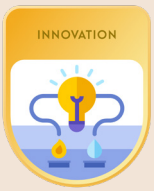
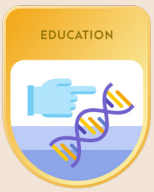
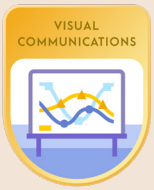
Bioluminescent Legionella detector







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Reviewed on 8 May 2021; Accepted on 28 June 2021; Published on 25 October 2021

Legionella spp. are waterborne bacteria that contaminate drinking water. *Legionella* spp. are opportunistic pathogens, meaning they do not usually infect healthy hosts. It often already exists in water systems, but only grows if water flows too slowly and stays in the pipes too long. This is dangerous because certain *Legionella* spp. cause a deadly form of pneumonia known as Legionnaires' disease. To reduce the threat of future outbreaks of Legionnaires' disease, we have designed a genetically engineered *Escherichia coli* that detects *Legionella* spp. in water and reports the presence of *Legionella* spp. using bioluminescence. The system detects *Legionella* spp. by sensing *Legionella*'s quorum sensing molecule with a chassis engineered to have *Legionella*'s quorum sensing system. Upon detection, a bioluminescence pathway is activated by turning on the promoter of the lux operon. This operon encodes genes for the production of an enzyme called luciferase, which oxidizes luciferin to produce light. If the production of light is observed, then *Legionella* spp. have been detected in the water. And, because it uses interchangeable parts, our system can be adapted to detect other pathogens. In alignment with the United Nations' Sixth Sustainable Development Goal, Clean Water and Sanitation, one of the goals of this system is to help ensure a world where everyone has access to clean and sanitary water.

Keywords: *Legionella* detection, bioluminescence, quorum sensing, clean water

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Background

Legionnaires' disease is a severe type of pneumonia that occurs when water droplets containing *Legionella* are inhaled and make their way into lungs (Centers for Disease Control and Prevention, 2021a). Normally, macrophages—a type of white blood cell—would be able to attack and break down invading bacteria and viruses; however, *Legionella* spp. avoids this by attacking the macrophages themselves (Centers for Disease Control and Prevention, 2021b). Inside these cells, *Legionella* survives and replicates, eventually taking over the macrophage and destroying it (Segal et al., 1998). This can cause tissue damage and inflammation in the lungs, which blocks the passage of oxygen, causing many negative effects. Initially, symptoms may include cough, fever, muscle aches, and headaches. People with the disease experience varying long-term effects as well. Sixty-six percent suffered from neurologic symptoms like memory loss and seventy-five percent from fatigue (Dooner, 2021). If not treated properly, the disease can cause multi-organ failure, eventually leading to death (Dooner, 2021). Because Legionnaires' is a lung disease, people who smoke, have underlying lung illnesses, or are over fifty years of age are more at risk (Dooner, 2021).

There are more than 58 species of *Legionella*, with 25 pathogenic species, the most common pathogenic species being *Legionella pneumophila*. However, some other *Legionella* spp. can cause Pontiac fever, a milder infection. This is not a type of pneumonia, so while it has most of the same symptoms as *Legionella*, it lacks those which are pneumonia related. Its symptoms are less severe, typically lasting less than a week (State of Hawaii, Department of Health, 2019).

Legionnaires' disease is often perceived as rare, but this is not the case. The false perception is caused by the rarity with which cases and even smaller outbreaks make the news (HC Info, 2021). According to the Center for Disease Control and Prevention (CDC), each year, between 8,000 to 18,000 people are hospitalized with Legionnaires' disease in the United States ("Legionnaires Disease", n.d.). Because approximately 5-10% of people who contract the disease die, between 400 and 1800 deaths occur in the US each year. The CDC also states that "many infections are not diagnosed or reported, so the number affected may be higher" ("Legionnaires Disease", n.d). It is often mistaken for another type of pneumonia, because recognizing it as Legionnaires' takes special testing (Centers for Disease Control and Prevention, n.d.).

Legionella are also Gram-negative bacteria, meaning they are less susceptible to antibiotics than Gram-positive bacteria because Gram-negative bacteria have an outer lipid membrane, making them more dangerous. Taken together, there is a need for continued, if not increased, testing for *Legionella*.

When they arrive at a host's alveolar macrophage via their polar flagellum, they inject effector molecules through the Dot/Icm T4SS system (Winn, 1996). This bacterial secretion system allows *Legionella* to deliver DNA or proteins into eukaryotic cells to carry out replication and certain activities to evade the immune response (Copenhaver et al., 2014). These effector molecules allow for the creation of a *Legionella*-containing vacuole that provides the ideal growing environment and protects *Legionella* against being destroyed. The *Legionella* bacteria then go on to multiply and spread within the host (Ziltener et al., 2016).

The table below summarizes the most common current solutions, their strengths, and their weaknesses.

Our goal was to engineer a system that can improve on these weaknesses: a system that is accurate, fast, and easy to use. Now that the design is completed, we hope that the use of *Legionella*'s quorum sensing system will provide high accuracy, reasonable speed, and that—through experimentation with the application of our system—implementing the system would be highly simplistic. Further descriptions of our system's advantages are in the discussion section.

Systems Level

This system is designed to detect the presence of *Legionella* spp. in water using a genetically engineered chassis. The system will be added to water samples with an experimentally determined amount of our modified chassis. Additionally, the system detects *Legionella*'s specific quorum sensing molecule (LAI-1) with *Legionella*'s own quorum sensing system (Lqs), which is included in our chassis (Personnic et al., 2018).

Quorum sensing (QS) is a bacterial system in which specific signal molecules are detected by corresponding receptor proteins at both intraspecies and interspecies levels. QS allows bacteria to determine population densities and alter gene expression accordingly (Rutherford & Bassler, 2012). Our QS pathway activates a bioluminescent reporter that produces light and induces the production of more LAI-1 to amplify the response.

Table 1. *The three widely used tests for Legionella.*

Current Solutions	Description	Pros	Cons
Culture	1. Sample of water is taken 2. Sample placed in controlled environment 3. Possible bacteria provided with Agar, to provide nutrients If bacteria are present, they will multiply to numbers so large that they are visible to the naked eye.	• Sensitive (with appropriate data) - can detect even a very small concentration	• Takes ten days • *Legionella* is often overgrown if there are other bacteria present
PCR	1. Sample of water is heated to appropriate temperature to separate DNA 2. Nucleotides and primers added 3. Specific portion of DNA replicated 4. Repeated When a sufficient amount of the DNA sequence is present, it can be detected, confirming the presence of *Legionella*.	• Specific - can differentiate between many different types of *Legionella* • Sensitive - can detect even a very small concentration • Fast - only a few hours	• False negatives are usually caused by any dirt or debris, almost always in samples exposed to the environment
Direct Fluorescent Antibody	1. Sample of water is taken 2. Antibody for a specific surface protein (that also reflects light at a lower wavelength) added 3. Loose antibodies removed 4. Specific wavelength shone on sample If *Legionella* is present, the test ends in light of a different wavelength being reflected.	• Specific - can differentiate between many different types • Sensitive - can detect even a very small concentration	• Requires a fluorescent microscope, which is expensive and difficult to procure • Difficult to develop the antibody • Possibility of cross-reactivity

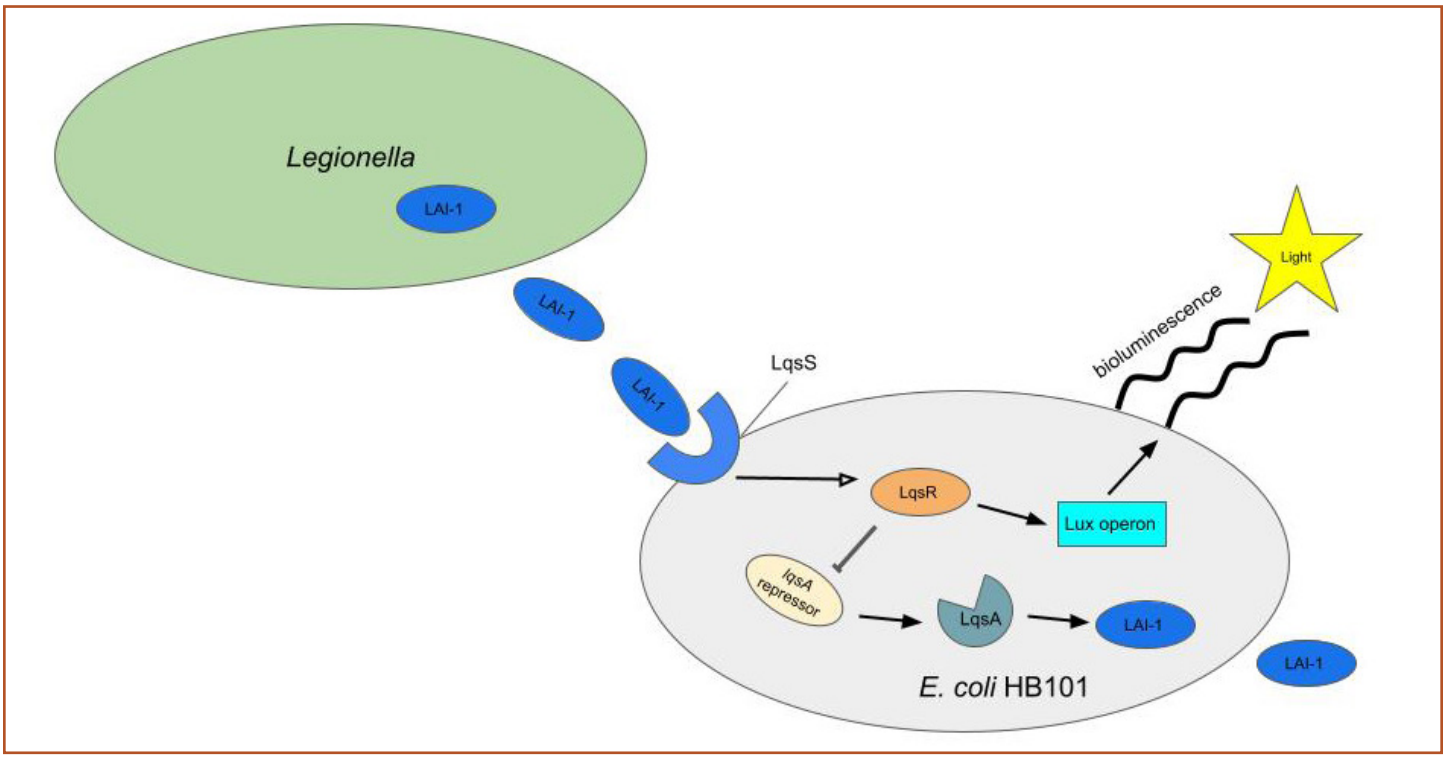

Figure 1. *Overview of the detection and reporting systems. The binding of signal molecule LAI-1 to the sensor kinase LqsS on the edited E. coli HB101 activates a signal transduction pathway through the response regulator LqsR resulting in bioluminescence, through the expression of the Lux operon. Additionally, LqsR inhibits the LqsA inhibitor, leading to signal amplification through production of additional LAI-1 that will stimulate other E. coli and Legionella.*

Device Level

We chose to use Escherichia coli HB101 as our chassis (Figure 1). It is a freshwater bacterium and laboratory strain that can be genetically engineered to express detection and reporting systems (Podstawka, 2020). *Legionella* also lives in freshwater, so having a freshwater bacterium as our chassis is ideal because it eliminates the possibility of our chassis drowning when in the same environment with *Legionella*. *E. coli* HB101 also does not produce bioluminescence naturally, which will reduce noise from natural bioluminescence during experimentation. A plasmid containing the Lqs system genes, which produce proteins involved in *Legionella*'s quorum sensing system (Lqs), and the *luxCDABEG* operon, which produces luciferase and the luciferin that react to create bioluminescence (Figure 3), will be inserted into *E. coli* HB101.

Parts Level

Our *E. coli* HB101 is engineered with parts from Lqs to detect *Legionella*. *Legionella* produces a α-hydroxy ketone signaling molecule called LAI-1 (*Legionella* autoinducer-1, or 3-hydroxy pentadecane-4-one) through the autoinducer synthase LqsA (Hochstrasser & Hilbi, 2017). On the membrane of the engineered *E. coli*, the sensor kinase LqsS then detects LAI-1 (Figure 2).

The detection of *Legionella* using the LqsS sensor kinase will stimulate LqsR in the engineered *E. coli* HB101 through phosphorylation. LqsR then inhibits an *lqsA* gene repressor, resulting in the transcription of *lqsA* (Figure 2). *lqsA* produces LqsA proteins, which synthesize additional LAI-1 molecules that amplify the original signal (Hochstrasser & Hilbi, 2017).

Additionally, LqsR will be further designed to activate the transcription of *luxL,* to produce LuxL proteins, which synthesize an acylated homoserine lactone (AHL), specifically 3-oxo-C6-HSL molecules (Figure 3). These AHL molecules bind to the LuxR protein, which is produced by the *luxR* gene, to form a complex (Dunlap, 2009). The AHL and LuxR protein complex then binds to the *luxCDABEG* promoter to activate the transcription of the lux operon by RNA polymerase. The *luxA* gene and *luxB* gene lead to the production of the protein luciferase. Additionally, the *luxC*, *luxD*, and *luxE* genes code for Fatty Acid Reductase, and the *luxG* gene codes for Flavin Oxido-Reductase (Figure 3). The luciferase protein catalyzes the oxidation of the luciferin FMNH2, which is produced by Flavin Oxido-Reductase's reduction of FMN, and the long chain aldehyde RCOH, which is produced by Fatty Acid Reductase's reduction of RCOOH, to create bioluminescence (Brodl et al., 2018; Miyashiro & Ruby, 2012).

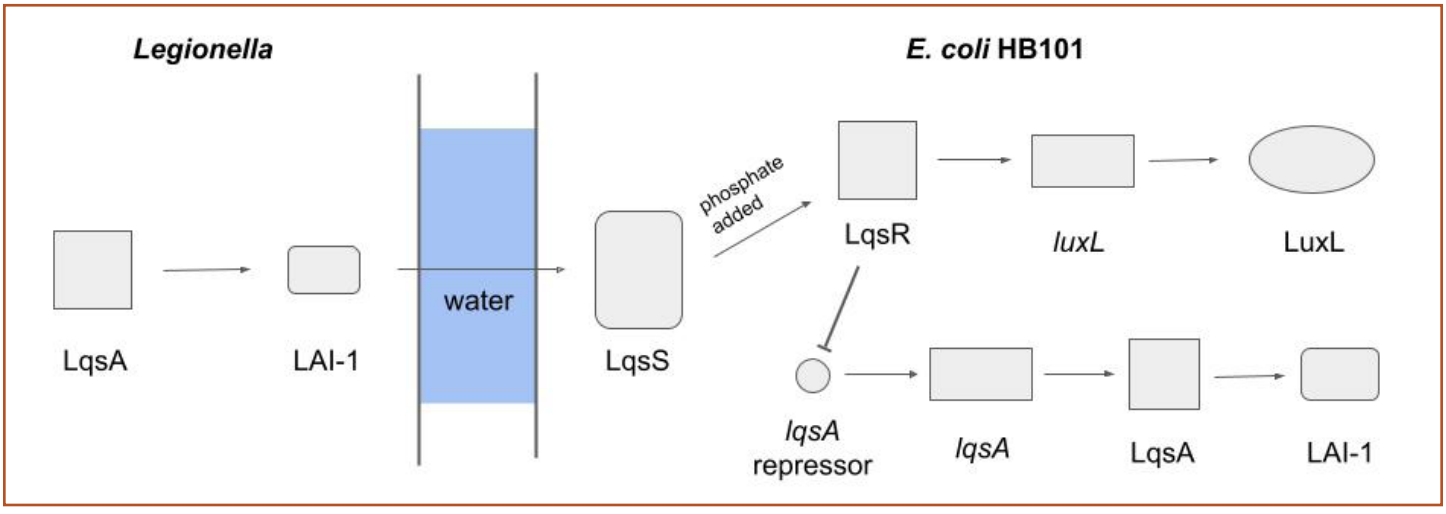

Figure 2: *The figure shows the parts involved with the detection pathway.The autoinducer synthase LqsA produces the autoinducer LAI-1 in Legionella. LAI-1 is released and stimulates LqsS (a membrane-bound sensor kinase on E. coli HB101). LqsS stimulates the protein LqsR, which activates the gene luxL to produce LuxL, an enzyme necessary for the bioluminescence pathway. LqsR also inhibits the lqsA repressor, allowing the gene lqsA to be expressed. Within the edited E. coli, lqsA produces LqsA, which then produces LAI-1. LAI-1 is released to stimulate other Legionella and edited E. coli HB101 in order to amplify the signal.*

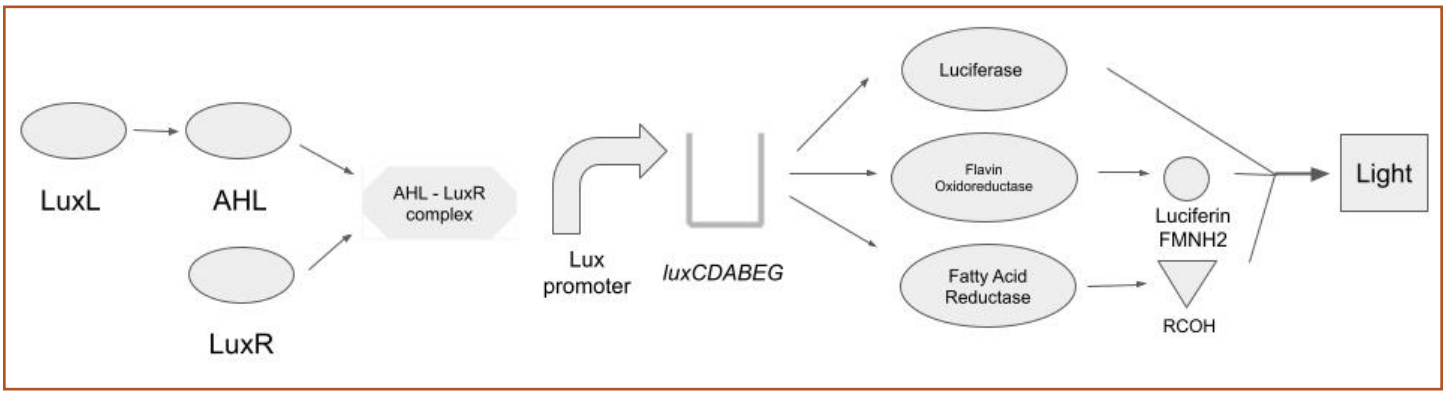

Figure 3. *The figure shows the parts involved in the bioluminescence pathway. The enzyme LuxL synthesizes AHL molecules, which bind to LuxR proteins. The AHL-LuxR complexes then activate the Lux promoter. The luxCDABEG operon is transcribed for the synthesis of Luciferase, Flavin Oxidoreductase, and Fatty Acid Reductase. Flavin Oxidoreductase leads to the production of FMNH2, and Fatty Acid Reductase leads to the production of RCOH. These three products (Luciferase, FMNH2, and RCOH) react to produce light.*

Safety

The most prevalent safety concern with the creation and testing of our system is the use of *Legionella pneumophila*. As stated in the "Background" section, exposure to this bacterium can cause Legionnaires' disease, which is an often deadly type of pneumonia. Most of its survivors are left with long-term symptoms as a result (Legionella.org, 2016). In the worst cases, the death rate can be as high as 80% for immunosuppressed people who are not treated, and overall it kills 5-10% of people infected (World Health Organization, 2018). We would follow lab safety procedures according to Biosafety Level 2 (BSL-2) guidelines to reduce the risk of infection.

We would start by testing the system with LAI-1 and not *Legionella* itself (outlined in the Next Steps section), which removes the need for the required BSl-2 lab until we want to test our system with actual *Legionella*. We would then need to partner with a university or industry lab, as these standards cannot be met by a high school level laboratory.

Our chassis *E. coli* HB101 is a laboratory strain, and thus would only need a BSL-1 lab rating to use. It is not as dangerous as using *Legionella*, but we would still have to follow lab safety precautions. Since both of these bacteria cannot be released into the environment, they should only be used in a lab setting or at water treatment plants to test for *L. pneumophila* with the proper precautions. To reduce risk to surrounding populations and users of our device, we will test multiple applications of our system to find the safest and most reliable one.

Discussions

This system is designed to quickly and easily detect *Legionella spp.* in locations commonly associated with its presence, like water towers, which will allow for better control and prevention of future outbreaks of the deadly Legionnaires' disease. This device would be a better solution in many cases compared to the current detection methods, as it may be quickly tested on contaminated water, without requiring a great amount of prior experience. As shown in Table 1, the current solutions for detecting *Legionella* each have one or more of the problems this device aims to solve, which will be determined through experimentation. For example, a dirty water sample messes up the PCR method (Table 1), while our engineered *E. coli* would not be affected by a dirty sample. This means less preparation of the sample would be needed before testing, which saves time and reduces necessary expertise.

Finally, using the quorum sensing method will allow for our device to detect all types of *Legionella* because all *Legionella spp.* use the Lqs system. This is an advantage over some current detection methods, because not all of them can detect all species of *Legionella*. We expect that our system would also be relatively accurate because this quorum sensing system is specific to *Legionella*, meaning bioluminescence would not occur if some other bacteria's QS molecule were to come near our chassis.

A possible issue with our implementation in using samples to detect *Legionella* is detecting low concentrations of *Legionella* in water. Since the amount of *Legionella* in water towers varies, a small sample

being tested from a large water tower may provide an inaccurate representation of the whole tower. Future research may be done to figure out the minimum amount of sample needed to provide accurate *Legionella* detection.

To sense LAI-1, we plan to use the LqsS membrane-bound sensor kinase engineered in *E. coli* HB101 (Figure 1). For our sensing to work correctly, *Legionella's* signaling molecule must be able to pass through the cell membrane of the *E. coli* HB101 cells. LAI-1 is mostly hydrophobic and small, and thus should be able to easily diffuse through the membrane (Personnic et al., 2018). This device contains genetic modular elements from the specific pathogen to be detected (the parts of the Lqs system in Figure 2). We aim to allow the detection of multiple common/deadly pathogens using the same device, but with different modules. This will allow the device to be used for a longer period of time at facilities, and as new pathogens arise. Additionally, this will allow costs to be reduced as facilities do not need to replace the whole device if new pathogens are to be tested, or if a part of the device malfunctions. Future improvements will focus on increasing accuracy and speed of detection.

Additionally, new deadly pathogens that require a quick test will automatically present an improvement opportunity. Future improvements to this device are linked to the modularity of the device; new improved modules may be added as an extra module, which will make future improvements easier to add. Further discussion of future improvements is in the "Next Steps" section.

Next Steps

Having done most of the background research, the next step would be to build the system and test it in a lab setting. As detailed in the "Safety" section, working with *Legionella* requires a BSL-2 lab rating. Thus, we would start by testing our system with only the quorum sensing product of the *Legionella* to bypass the need for a lab with a BSL-2 rating until we want to experiment with actual *Legionella*.

We plan on testing the detection and bioluminescence pathways (Figure 2 & Figure 3 respectively) separately so that it would be easier to determine which pathway does not work as expected. We would start by adding our chassis—engineered to only express the necessary parts up until the production of AHL and LAI-1 (Figure 2)—into a sample of water. This method may need the additional production of green fluorescent protein (GFP) to observe, but this would be a simple addition to the system since making LqsR also activates a GFP pathway. We would first test the detection pathway by itself for the production of AHL and LAI-1. In this way, we will detect false negative outcomes (i.e. no production of AHL and LAI-1 in a sample that has *Legionella*'s QS molecule LAI-1) are due to problems with the detection pathway and not the bioluminescence pathway. Generally, we would test this by adding our engineered *E. coli* into positive control samples (those which we know have LAI-1), and once it works on those samples, we would test the system on unknown water samples that potentially contain real *Legionella*.

Once the detection system can accurately produce AHL in both scenarios, we will combine the bioluminescence and detection pathways to test if our bioluminesce pathway works. We expect to be able to see the bioluminescent output with our bare eyes, however we may find that the strength of the signal may need to be increased. We could do this by increasing the output of our amplification system by making each *E. coli* produce a larger number of new LAI-1 molecules after it detects *Legionella*.

After testing that the system works, we plan to experiment on improving the sensitivity of the detection and the time detection takes. There are non-pathogenic strains of *Legionella* that may cause our system to detect false positives. Thus, in future, we will determine a method of differentiating the false positives from samples which actually contain pathogenic *Legionella*. Additionally, we would experiment with making the strength of the bioluminescent light proportional to the concentration of *Legionella*: the higher the concentration of *Legionella*, the brighter the light should be. To determine the time our detection system takes, we would first test our system with only LAI-1 molecules and then with *Legionella*. Once we have established a baseline with our original system, we would tweak the system, possibly by changing the parts in the pathway or the environment of our application, to optimize the time detection takes.

When creating the application of our system, we also need to determine how many *E. coli* HB101 would need to be present in the system, and the relationship between the number of *E. coli* HB101 present and light intensity produced by the accumulation of luciferase. We are also considering a cell-free system, which would not dilute our samples as much as adding many *E. coli* cells, giving us a higher concentration of *Legionella*, which could improve our accuracy in testing. Once the *Legionella* is detected, we would then need to eliminate it. The simplest way to do this would be to boil the water

and kill the *Legionella*, but we plan on exploring methods of bacterial elimination as well.

Besides detecting *Legionella*, there are other quorum sensing bacteria that we could easily detect by switching out the membrane-bound sensors and response regulators to match the quorum sensing molecule of the bacteria we are targeting. *Vibrio cholerae* is a great example because it has a similar quorum sensing system to *Legionella*, with similar parts that could be switched out.

Author Contributions

All authors contributed to the "Abstract." X.J., A.M., and H.Ts. wrote the "Background" section. A.A., S.D., A.J., and A.Z. researched the detection system, and S.D. wrote the "Systems Level," "Device Level," and "Parts Level" for the detection system. A.A., X.J., R.K., S.L., H.Ta., and A.Z. researched the bioluminescence pathway, and A.A. wrote the "Device Level," and "Parts Level" for the bioluminescence pathway. S.L. wrote the "Safety" section. M.H. wrote the "Discussions" section. H.Ta. wrote the "Acknowledgements" section. A.J., H.Ta. wrote the "Next Steps" section. S.D. made the "References" section.

Acknowledgements

We would like to thank our teacher Mr. Mathieu and our mentor William Beeson for providing us with expert advice and giving us their immense support throughout the year.

This project was accomplished through participation in the BioBuilderClub, an after-school program organized by the BioBuilder Educational Foundation. The BioBuilderClub engages high school teams around the world to combine engineering approaches and scientific knowledge to design, build, and test their project ideas using synthetic biology.

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