Detection of Dichloromethane in Superfund Sites

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There are more than a thousand Superfund sites in the United States, and many of them are located near or in neighborhoods or areas prone to earthquakes, flooding, or erosion. Superfund sites are hazardous waste dumps that have been identified by the Environmental Protection Agency (EPA) as posing an unacceptable risk to human health and the health of other organisms. The EPA has identified several chemical substances present in Superfund sites, ranging from hazardous metals to harmful organic compounds. Our team decided to focus on dichloromethane (DCM), a widely used synthetic haloalkane that is present in high concentrations at a Superfund site close to our school. DCM has wide-ranging and serious health effects; it is carcinogenic, neurotoxic, and hepatotoxic. Our goal is to create a biosensor for the detection of DCM. We are utilizing synthetic biology to design a biological system that will display red fluorescence when DCM is detected. Our system uses *Escherichia coli* as the chassis and is composed of two connected devices: a DCM sensor and a DCM reporter. For the DCM sensor, we are using the *dcmR* gene, which was first identified in the genome of *Methylobacterium extorquens*, a methylotrophic bacterium capable of degrading DCM. The *dcmR* gene, which is regulated by a constitutive promoter, produces the DcmR protein, a transcriptional repressor for the *Red Fluorescent Protein* (*RFP*) gene. When DCM is present in the samples tested, it will bind to the DcmR protein and change its conformation, derepressing the *RFP* gene and triggering visible red fluorescence. We are adding a tuner in order to link the intensity of the red fluorescence to the varying DCM concentration. Compared with the conventional analytical methods for DCM detection, this biological system provides an inexpensive and easy-to-use DCM biosensor for testing water and soil samples from Superfund sites.

Keywords: Superfund sites, DCM, methylene chloride, detection, *dcmR*, *Methylobacterium extorquens*

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Watch a video introduction by the authors at https://bit.ly/2MnAfI2

Background

In 1980, the United States Congress passed the Superfund Act, also known as the Comprehensive Environmental Response, Compensation, and Liability Act, to address the dangers of abandoned hazardous waste dumps. The EPA's Superfund Program identified more than 1300 Superfund sites in the United States (EPA 2019 January). These sites, which contain toxic waste ranging from dangerous metals to harmful organic compounds, can be prone to earthquakes or flooding. Thousands of sites with contaminated soil and groundwater exist nationally due to hazardous waste being dumped, left out in the open, or otherwise improperly managed.

In 2017, approximately fifteen million people were living within one mile of a Superfund site (roughly five percent of the U.S. population) (EPA 2017 October). As changes in the global climate cause sea-level rise and a greater chance of flooding, more communities are being exposed to a serious health risk due to the contamination of the drinking water and soil by noxious substances present at Superfund sites.

Dichloromethane (DCM), also known as methylene chloride, is a volatile organic compound (VOC) widely used as a paint remover and industrial solvent. In addition to being highly volatile, DCM is water-soluble and can be present in aquifers, groundwater, or soil. The EPA has set a maximum contamination limit of DCM at five parts per billion (µg/L) for public or private well water sources (EPA 2009 May). According to the EPA's Toxic Chemical Release Inventory, DCM is a widespread contaminant in the environment. In the United States, from 1987 to 1993, the total amount of DCM on land and in water totaled over 2.1 million pounds. DCM is rated as potentially carcinogenic for humans and can cause liver problems and neurological symptoms after significant exposure (EPA 2017 June). It can be lethal in high doses. Additionally, DCM contributes significantly to the amount of chlorine in the atmosphere, having a similar effect as hydrochlorofluorocarbons (HCFCs), and it is being called "the new ozone booster" (Pearce 2017).

Systems Level

An important part of our system design is the *dcmR* gene, a regulatory gene found in *M. extorquens*, which is both a methylotroph and an extremophile. The *M. extorquens* strain DM4 has been isolated from industrial wastewater sludge in Switzerland as part of efforts to characterize microorganisms able to degrade DCM (Gälli and Leisinger 1985). A methylotrophic bacterium has the ability to grow on one or several reduced one carbon (C1) compounds (Muller et al. 2011) and use these compounds as its sole carbon and energy source. *M. extorquens* is able to grow on DCM and metabolize formaldehyde, a toxic product.

In the genome of *M. extorquens*, there are two genes responsible for metabolizing DCM: *dcmA*, which codes for the enzyme DCM dehalogenase, and *dcmR*, a regulatory gene. DCM dehalogenase is specific to DCM and catalyzes the following reaction (La Roche and Leisinger 1991) :

DCM (dichloromethane) + 2 H_2O ⟶ CH_2O (formaldehyde) + 2 HCl

The regulatory gene *dcmR* is induced by the presence of DCM in the environment, and it codes for the regulatory protein DcmR. The DcmR protein binds DCM and serves as a repressor for both the *dcmR* and *dcmA* genes (Leisinger et al. 1994).

The biological system we have designed has DCM as its input and red fluorescence as its output (Figure 1). When DCM is present in the sample tested (either water or soil), the cells will fluoresce red and in the absence of DCM, there will be no color formed. The intensity of the color will reflect the concentration of DCM due to the presence of a tuner. The tuner will detect two levels of DCM concentration, each with a corresponding red fluorescence; a high concentration of DCM will result in a dark red color while a low concentration of DCM will result in a light red color.

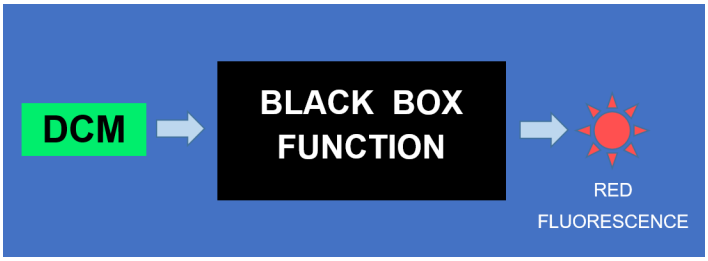

Figure 1. *System level design: When DCM is present, there will be a red fluorescence.*

Device Level

Chassis or host selection: We initially considered *M. extorquens*, a facultative methylotroph Gram-negative bacterium, as a wild chassis. However, we ultimately decided to use *E. coli*, a standard chassis, for the following reasons: *E. coli* is easy to grow in the lab, has been well characterized, and is safe (Kuldell 2015). It is also easily genetically engineered and provides better standardization; we found parts in the IGEM Registry that were standardized for use with *E. coli*. Additionally, *E. coli* is also more predictable. This is important because it has been suggested that *M. extorquens* has the potential for high genomic plasticity, which might lead to an unpredictable evolution of the designed system (Vuilleumier 2009). The Oxford IGEM 2014 team has found that the *M. extorquens* was very difficult to culture and grow in the

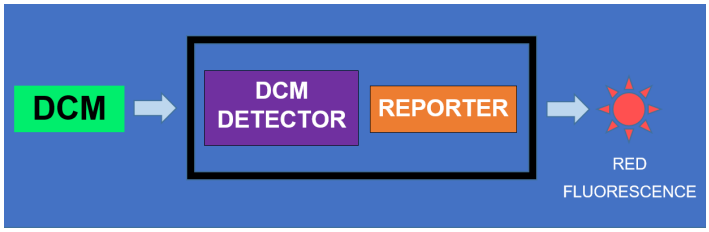

Figure 2. *Device level design: The DCM detector, acting as the sensor device, is connected to the reporter device, which generates the red color.*

lab (IGEM Team Oxford 2014 November). *E. coli* is most likely less resistant to DCM than *M. extorquens*, and it might not be able to survive at higher DCM concentrations. In order to address this potential problem, the tested samples should be diluted.

The device level design, which includes the detector and reporter, is shown (Figure 2).

Parts Level

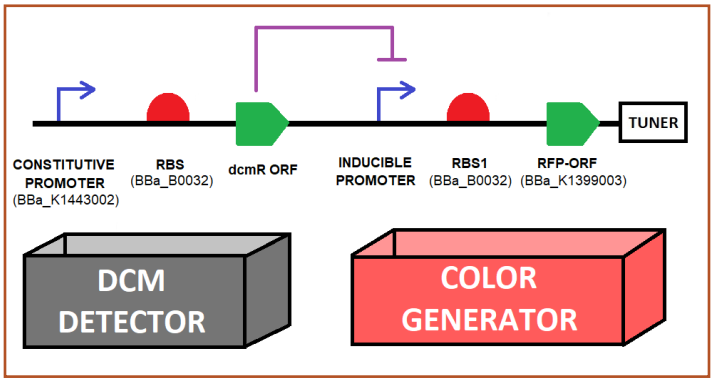

Figure 3. *Parts level design: The components for both the DCM sensor and the reporter device, in addition to a tuner that relatively quantifies the DCM in the environment.*

The first device, the DCM detector, is composed of a constitutive promoter, a ribosomal binding site (RBS), a *dcmR* open reading frame (ORF), and a forward terminator. The second device, the color generator, is composed of an inducible promoter, RBS, RFP ORF, and a tuner (Figure 3).

The output of the first device will be the DcmR protein, a transcriptional repressor that turns off the reporter. The repressor protein binds to the second promoter (PdcmR) and blocks the expression of the color-producing gene. When DCM is present in the environment, it will bind the DcmR protein (causing its conformation to change) and, as a consequence, it will remove the repressor protein from the DNA, allowing the *RFP* gene to be expressed.

We added a tuner to relatively quantify DCM in the environment; the intensity of the color output will vary with the concentration of DCM. For the tuner, we used a second reporter, which is the second copy of the PdcmR-RFP with a stronger RBS and a "novel" version of the promoter. This copy will be sensitive to a higher concentration of DCM, which will be reported with an intense red color (Table 1). The stronger RBS will ensure a higher amount of RFP protein when the promoter is expressed. The "novel" promoter will have two binding sites for the DcmR protein.

DCM CONCENTRATION	REPORTER 1	REPORTER 2
0	0	0
LOW	1	0
HIGH	1	1

Table 1. *Truth table: A low DCM concentration will turn on the first reporter, while a high DCM concentration will also turn on the second reporter, which will create a stronger red signal.*

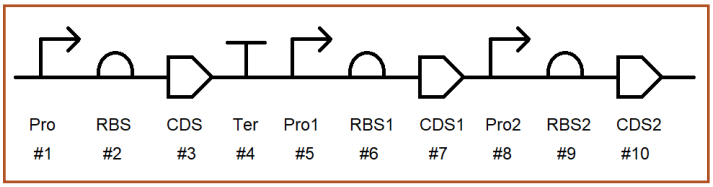

Figure 4. *Summary of parts*

The parts of the DCM detection system are summarized (Figure 4) and detailed (Table 2).

Design diagram number	Part
1	**Constitutive promoter** Part: BBa_K1443002
2	**RBS** (medium RBS) Part: BBa_B0032
3	**dcmR ORF** We could not find this part in the registry, but we can get the *dcmR* coding sequence from https://www.ncbi.nlm.nih.gov/nuccore/M32346.1, Under "CDS", (pos. 1508-2374)
4	**Forward terminator** Part: BBa_B0010
5	**Inducible promoter**: PdcmR
6	**RBS** (medium RBS) Part: BBa_B0032
7	**RFP ORF** Part: BBa_K1399003
8	**"Novel" promoter**: Two binding sites for the DcmR protein
9	**RBS** (strong RBS) Part: BBa_B0034
10	**RFP ORF** Part: BBa_K1399003

Table 2. *Design parts*

Safety

Our design will use *E. coli* strains that are safe and do not present any risks to healthy humans. Any sequences that can introduce pathogenicity will not be used. Possible exposure to DCM will be prevented by following standard safety precautions: wearing protective clothing, chemical splash goggles, and safety gloves. The area will be well ventilated with a fume extraction system. We will be using sterile techniques in order to avoid any contamination.

Discussions

The conventional methods for DCM detection are analytical. After the separation of organic compounds by gas chromatography (GC), DCM is detected by one of several types of instruments: a flame ionization detector (FID), electron capture detector (ECD), electrolytic conductivity detector (ELCD), halogen specific detector (HSD), or mass spectrometer (MS). High-resolution gas chromatography, using either an MS or an ELCD, is also reliable.

The analytical methods have a high level of sensitivity; however, they have a few limitations. All these methods require specialized equipment, which is expensive (tens or even hundreds of thousands of dollars), and trained technicians. Another problem is that purging and trapping DCM in the sample preparation can create problems affecting the sensitivity and the speed of the analysis. "While the ELCD appears to be most sensitive, detection limits for all these methods are well below levels of health concern, but are not below EPA calculated cancer risk levels or all MRLs [maximum residue limits]" (HHS 2000). Certain conditions, such as high temperature or humidity, can decrease the adsorption of methylene chloride vapor on the solid absorbent and affect the analytical accuracy.

In the literature, we found one article on a bioluminescent (lux) bacterial bioreporter that has *M. extorquens* as the chassis and a lux cassette as the reporter (Lopes et al. 2012). Compared with the conventional analytical methods, the biosensor designed by our team will be much less expensive and easier to use. Unlike the bioluminescent (lux) bacterial bioreporter, our DCM bioreporter will not need a luminescence plate reader. Additionally, as explained under the "Parts Level" section, the *E. coli* chassis is easier to manipulate and grow in the lab than *M. extorquens*.

"In most cases, bioreporter assays are extremely simple; cells are maintained in some kind of buffered and equilibrated suspension and are brought into contact with a sample or calibrating solution for a particular time period, after or during which the reporter signal is recorded" (Van der Meer 2010). The measurements obtained with the bioreporter assays are usually relative (not absolute) because they depend on the number of cells in the assay and the incubation time. This should not be much of a problem when the bioreporter's role is a first-line rapid detector. If needed, we can add a set of calibration assays in order to obtain more precise measurements of the DCM concentration.

The red fluorescence in the bacteria cell culture should be visible with the naked eye or the help of an epifluorescence microscope; in general, a spectrophotometer would not be needed. However, in the case that a spectrophotometer is used to better quantify the amount of DCM, the optical density (OD) should be measured at 700 nanometers (nm) instead of the customary 600 nm because "RFPs can strongly absorb light at 600 nm" (Hecht et al. 2016).

We found that a biological system to detect DCM is easier to construct than a bioremediation system, which would need to include the gene that codes for the enzyme DCM dehalogenase, *dcmA*. Additionally, there would need to be a method to control the effects of the genotoxic reactants (Kayser 2001) formed during the enzymatic transformation of DCM to formaldehyde.

In a future project, we would like to embed the DCM reporter cells in small agarose strips; if successful, this would be an extremely accessible and easy-to-use biosensor.

We believe that bioengineering can provide solutions for many environmental problems, helping with both the detection and bioremediation of pollutants.

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