Automatization of microbial cultures in deepwell plates for strain characterization

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Keywords: biofuels, *E. coli*, automation, decontamination

Abstract

There is an increasing need for biofuels to counteract climate change. Manipulating microorganisms and utilizing their membrane proteins will allow us to produce important standardized chemicals and biofuels in massive quantities. Such processes include the preparation, monitoring, and sampling of deep well plate cultures of a bacterial strain (e.g., *Escherichia coli*, *Saccharomyces cerevisiae*). Moreover, automatic liquid handlers are a key tool for scaling up biofuel production to an industrial level, presenting the possibility the fully automate the required procedures. Maximizing the robot's precision and culture reproducibility is essential for optimizing the expression of different proteins in cell metabolism. Recent efforts have explored using fixed-tip liquid handling systems as a more sustainable alternative to single-use plastic tips, effectively scaling up the biofuel output of liquid handlers is heavily contingent on completely disinfecting the fixed-tip pipettes of the liquid handler. In our study, we utilize a media volume of 500 µL, an array of air gap volumes, and three bleach concentrations to further study the necessary conditions for consistent and complete decontamination. Our findings reaffirm that an air gap greater than the process liquid volume is required for consistent and complete decontamination. Additionally, contamination increases along the experimental columns, with the first and seventh columns having a mean decontamination percentage difference of 16.67% (*p*-value 0.0148). This contrast in decontamination highlights the gradual system contamination that occurs within the liquid handling system when an insufficient air gap volume is implemented within the procedure.

Keywords: biofuels, *E. coli*, automation, decontamination

Introduction

In recent years, there has been increasing concern about the use of fossil fuels in energy production due to the looming onset of climate change, creating an immense need for more sustainable methods of energy production [1]. Through developments in metabolic engineering, synthetic fuels known as biofuels have been produced from biomass [1,2]. Biofuels are obtained by manipulating microorganisms and utilizing their membrane proteins to extract and develop important chemicals. Although biofuels are challenging to produce in massive quantities, they offer immense potential as a cleaner and more efficient energy alternative [3,4]. Currently, scaling biofuel production to an industrial level presents a significant challenge due to the need for precise procedures [5,6]. The gold standard framework for refining and generating microbial cultures is the design-build-test-learn (DBTL) cycle, which was devised to reduce the possibility of errors occurring on a large scale [7]. However, the testing phase of the DBTL cycle is severely under-optimized relative to the other phases, which have improved through developments in genetic engineering and machine learning [8-11]. To optimize the testing phase, recent efforts have explored using automation to streamline the extensive testing required to ensure precision on a larger scale [7].

Automation in chemistry was recognized early on for its benefits, including enhanced precision, extended operational hours, and reproducible procedures [12]. For example, automatic liquid handlers have been used to further study genetic engineering [13]. Although they are precise, current models are constrained to minuscule volumes (e.g., micro, nano) [13]. Many pipetting systems utilize single-use plastic tips to ensure a sterile environment, which results in significant plastic waste created during the lengthy testing phase of the DBTL cycle. The use of such plastics has become increasingly worrisome in laboratory work and many scientists have

motivated efforts to sterilize and reuse plastics to decrease the amount of waste generated by their work [14,15]. Consequently, there is a preference for the use of fixed-tip liquid handlers which mitigate the need for plastic pipette tips and have proven to be equally as effective with a thorough decontamination procedure [7].

While methods for biofuel production vary, the bacterium *Escherichia coli* (*E. coli*) has been well-studied for biofuel production [16]. Fortunately, the sterilization of *E. coli* has also been heavily documented, with various methods existing to eliminate this bacterium. For example, at temperatures of 50˚C or above it takes approximately an hour to eliminate the bacterium. However, this approach requires excessive energy and time, making this approach unviable for an optimized runtime [17,18]. Alternatively, sodium hypochlorite (bleach) can also be used as a decontaminant against *E. coli*, offering a simple and fast solution for decontaminating the fixed pipette tips [7]. Unfortunately, this approach lacks effectiveness, as a 12% bleach mixture has a roughly 50% decontamination rate within automatic liquid handlers [7].

Despite these limitations, Raj et al. (2021) found that incorporating an air gap between the process liquid and the system liquid increased the effectiveness of bleach decontamination to 100%. They discovered that to achieve complete and consistent decontamination, the airgap volume could not be smaller than that of the process liquid. Once an air gap satisfying this constraint was implemented in their automation, successful sterilization was less contingent on other factors such as wash time, decontaminant holding time, and decontaminant strength [7]. In their work, the process liquid occupied a volume of 200 μL and the air gap had a volume of 250 μL covering 45% of the total volume within the liquid handler. As indicated above, liquid handlers are often limited to smaller volume sizes, which has prevented metabolic biofuel

production from progressing toward industrial-level production. In this study, we aim to extend the findings of Raj et al. (2021) by utilizing a process liquid volume of 500 µL, this constrains the air gap size to match or succeed that of the process liquid volume. Moreover, we expect decontamination to remain inconsistent or incomplete for experiments where an insufficiently large air gap is used within the liquid handler. Finally, we explore how contamination occurs within the system and utilize extracted data to propose the behaviour of contamination through time.

Materials and Methods

Materials

Contamination was detected by the growth of wild-type *E. coli* K12 MG1655 strain and lysogeny broth (LB) media was used to support the growth of the bacterial strain. The LB medium was prepared with purified Milli-Q water and sterilized using an autoclave. Different concentrations of bleach were used to test decontamination in this procedure; 12% bleach was diluted to prepare the 3%, 6%, and 9% solution for this study. For the fixed-tip liquid handling system, the Tecan EVO 100 was used, offering the capability to automate the procedure. While the system was equipped with various instruments, the experiment required only the Tecan fixed-tip LiHa (liquid handling) arm, the Tecan RoMa (robotic manipulator) arm, and the Q Instruments Bioshake 3000. Throughout the experiment, the LB media was stored in 48-well microplates.

Methods

Thorough decontamination between pipette fillings is crucial to decrease the possibility of cross-contamination. Consequently, implementing a robust decontamination procedure within the Tecan Freedom Evo 100 is essential for designing a complete product.

Before the decontamination procedure, *E. coli* cell cultures were prepared and 500 µL of LB media was dispensed into a 48-well plate. In the first step of the procedure, the fixed-tip pipettes of the liquid handling system were initially decontaminated with 12% bleach to ensure sterility. This step involved aspirating 500 µL of the decontaminant, holding the bleach for 10 seconds, and then dispensing the 500 µL back into the decontaminant trough. An air gap of 250 µL was used to separate the system liquid from the bleach solution.

In the second step, the fixed-tip pipettes were contaminated with an *E. coli* cell culture solution (hereinafter referred to as waste). To contaminate, the pipettes aspirated 500 µL of the waste, held it for 10 seconds, and dispended it back into the waste trough. Different air gaps (0 µL, 50 µL, 100 µL, 250 µL, and 350 µL) between the system liquid and the waste solution were tested to optimize the minimal air gap needed for proper decontamination.

In the third step of this protocol, decontamination of the pipettes was tested using different concentrations of sodium hypochlorite (3%, 6%, and 9%). Regardless of the selected concentration, all experiments involved the aspirating of 500 µL, which was entirely dispensed after being held for 10 seconds. However, this decontamination step was repeated a different number of times depending on the selected air gap: it was repeated for 4 times for the lower air gaps (0 µL, 50 µL, 100 µL) and 6 times for the higher air gaps (250 µL, 350 µL). Additionally, this decontamination step was not performed in the negative control group, which was the column in the 48-well plate.

Once the pipettes went through the decontamination step with sodium hypochlorite, the results of the decontamination were then tested on the 48-well plate containing LB media. Approximately 500 µL of the LB media from each well of a column were aspirated, held for 10 seconds in the pipette, and dispensed back into their respective well. Different air gaps between

the system liquid and the media were also tested in this step of the procedure. Then, the plates were then incubated at 37˚C for a minimum of 8 hours to allow the *E. coli* cells to proliferate. Results were obtained through qualitative observation of the plates.

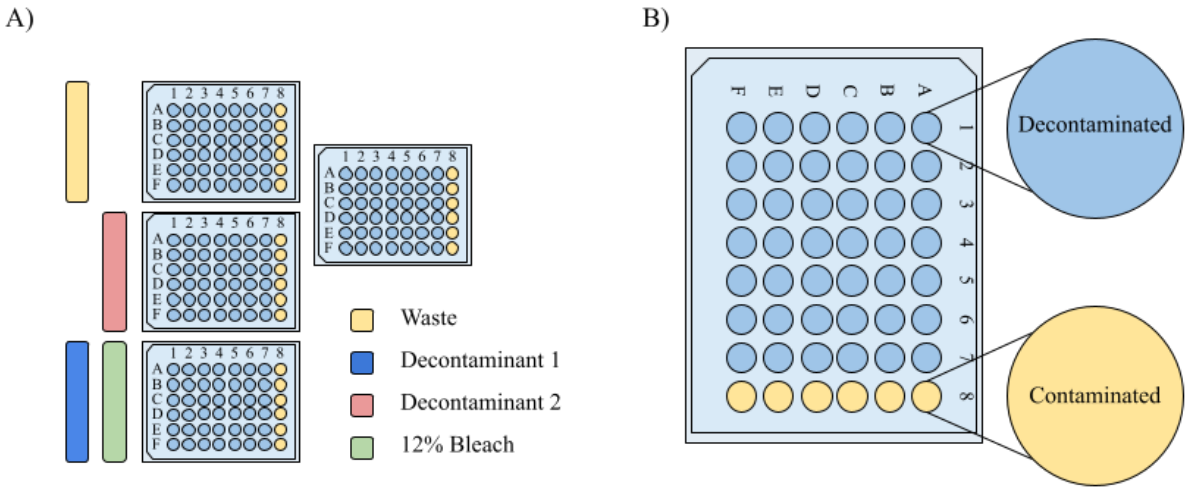

Figure 1. A) Experimental setup of microplates and the necessary troughs. The first and third microplates were decontaminated with the first decontaminant, whereas the second and fourth microplates were decontaminated with the second decontaminant. Columns are described using numbers (1 through 8), whereas rows with letters (A through F). B) Depiction of the ideal outcome, where each experimental column is decontaminated, and the control column is contaminated from the waste.

Data Analysis

Analyses were conducted using R version 4.3.0 [19] and plots were created using the ggplot2 package [20]. Although data was collected from every plate throughout this study, plates were only considered if the entire control column (8^{th} column) had bacterial growth. Specifically, if the control column lacked the presence of bacteria, there could be no certainty that the decontamination procedure was responsible for the sterilization of the experimental columns (1^{st} – 7^{th} columns) in the microplate.

If there were any signs of growth (i.e., cloudy, faint yellow colour) in the wells of the experimental group columns, the well was marked as contaminated. In contrast, wells that had no sign of *E. coli* growth (i.e., clear) were marked as successfully decontaminated. Contaminated

wells were assigned a value of 0 and decontaminated wells a value of 1. The sum of each row and column was then documented in a datasheet. The percentage of decontaminated wells was computed out of the 42 wells in the experimental columns. A significance level of $\alpha = 0.05$ was used throughout the analyses, though given the need for exceptional precision, a lower significance level would be preferable for future studies.

Results

A total of 69 plates were utilized throughout this study; however, 3 of these plates were omitted from analysis as they failed to pass the negative control (i.e., incomplete growth across the last column). Amid the plates that passed the negative control (n = 66), the mean decontamination percentage was 81.93% with a standard deviation of 27.18%. These percentages were computed out of the 42 experimental wells.

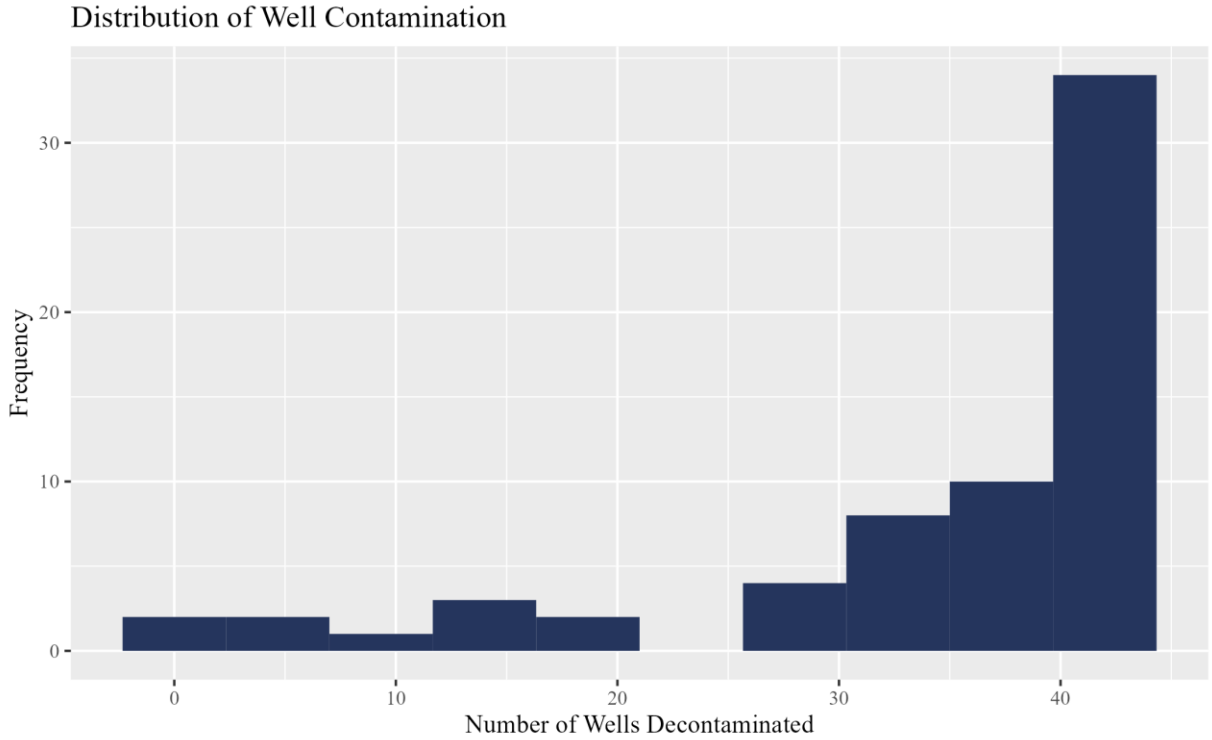

Figure 2. Histogram depicting the distribution of the number of wells decontaminated across the experimental columns. Notably, the distribution is right-skewed, with most plates showing 38 or more decontaminated wells (approximately 90% to 100% decontaminated).

Furthermore, we grouped the plates by decontaminant concentration and air gap volume. When grouped by decontaminant concentration, plates with 3% bleach (n = 11) had a mean decontamination percentage of 72.94% with a standard deviation of 26.90%, plates with 6% bleach (n = 30) had a mean decontamination of 79.13% with a standard deviation of 30.79%, and plates with 9% bleach (n = 25) had a mean decontamination of 89.24% with a standard deviation of 21.24%. We conducted an ANOVA (*F*-value 1.7, *p*-value 0.191) to determine whether the decontaminant concentration affected the decontamination percentage (Table 1).

When grouped by air gap volume, plates with a 0 µL airgap (n = 8) had a mean decontamination percentage of 83.93% with a standard deviation of 17.62%, plates with a 50 µL airgap (n = 12) had a mean decontamination percentage of 92.06% with a standard deviation of 17.62%, plates with a 100 µL airgap (n = 10) had a mean decontamination percentage of 57.14% with a standard deviation of 30.43%, plates with a 250 µL airgap (n = 3) had a mean decontamination percentage of 76.98% with a standard deviation of 39.86%, and plates with a 350 µL airgap (n = 33) had a mean decontamination percentage of 85.71% with a standard deviation of 28.23%. We conducted an ANOVA (F-value 3.028, p-value 0.0242) to determine whether the air gap volume affected the decontamination percentage (Table 2).

Lastly, we performed a two-way ANOVA to assess whether the interaction of the decontaminant concentration and the air gap volume affected the decontamination percentage. Only the air gap volume had a statistically significant effect (Table 3).

Table 1. Summary statistics for the ANOVA conducted to determine whether the decontaminant concentration affected the decontamination percentage.

	Degrees of Freedom	Sum of Squares	Mean Square Difference	*F*-value	*p*-value
Concentration	2	2459	1229.6	1.7	0.191
Residuals	63	45556	723.1		

Table 2. Summary statistics for the ANOVA conducted to determine whether the air gap volume affected the decontamination percentage.

	Degrees of Freedom	Sum of Squares	Mean Square Difference	*F*-value	*p*-value
Air Gap	4	7954	1988.6	3.028	0.0242
Residuals	61	40061	656.7		

Table 3. Summary statistics for the two-way ANOVA conducted to determine whether the air gap volume affected the decontamination percentage.

	Degrees of Freedom	Sum of Squares	Mean Square Difference	*F*-value	*p*-value
Concentration	2	2459	1229.6	1.870	0.1642
Air Gap	4	7450	1862.4	2.832	0.0334
Interaction	6	3250	541.7	0.824	0.5567
Residuals	53	34857	657.7		

Next, we aimed to assess decontamination by column and by row to determine when contamination was most likely occurring during the procedure (note that the liquid handling operates column-wise). Additionally, since the sample comprises of 66 plates, there is a total of 528 columns and 396 rows. To determine whether the column number affects the decontamination percentage we conducted an ANOVA (*F*-value 73.12, *p*-value 2e-16). Similarly, to determine whether the row number affects the decontamination, we conducted an ANOVA (*F*-value 0.465, *p*-value 0.802). The results for column number are summarized in Table 4, and those for row number are summarized in Table 5. Further analyses using a Tukey HSD Test revealed that each experimental column had a statistically significant difference in decontamination percentage compared to the control column. However, within the experimental columns, only the difference in decontamination percentage between the first and the seventh column was statistically significant ($\bar{x}$ = 16.67%, *p*-value 0.0148). Additionally, we conducted a separate ANOVA (*F*-value 0, *p*-value 0) that omitted the control column observations (Table 6).

Table 4. Summary statistics for the ANOVA conducted to determine whether column number affected the decontamination percentage.

	Degrees of Freedom	Sum of Squares	Mean Square Difference	*F*-value	*p*-value
Column Number	7	39.81	5.687	73.12	< 2e-16
Residuals	520	40.44	0.078		

Table 5. Summary statistics for the ANOVA conducted to determine whether row number affected the decontamination percentage.

	Degrees of Freedom	Sum of Squares	Mean Square Difference	*F*-value	*p*-value
Row Number	5	10.5	2.102	0.465	0.802
Residuals	390	1762.7	4.520		

Table 6. Summary statistics for the ANOVA conducted to determine whether column number affected the decontamination percentage. The control column was omitted from the statistical test.

	Degrees of Freedom	Sum of Squares	Mean Square Difference	*F*-value	*p*-value
Column Number	6	1.05	0.17450	1.963	0.0695
Residuals	455	40.44	0.08889		

Discussion

Although we achieved a high decontamination rate in this study, decontamination overall was neither consistent nor complete, which is expected given the insufficient air gap volumes [7]. The ANOVA revealed no statistically significant effect of decontaminant concentration on decontamination percentage. However, graphically, there appears to be a slight positive relationship (Figure 3). Similarly, the ANOVA for the effect of air gap volume on decontamination percentage was also not statistically significant**;** nonetheless, there is no clear relationship between these two measurements (Figure 4). Previous studies have shown a positive relationship between air gap volume and decontamination percentage, which was not observed in our data. The upside-down parabola in Figure 4 is likely due to the minimal data and outliers

within the 100 µL and 250 µL groupings.

While these results do not directly support the hypothesis that an air gap volume greater than that of the process liquid volume is required for consistent and complete decontamination, we observed that perfect decontamination was not achieved while lacking this criterion. Given that the total volume of the liquid handler's pipette was 1000 µL, it was not possible to implement a sufficiently large air gap (≥ 500 µL), highlighting concerns about the current limitations in pipette volume for liquid handlers [13]. Ultimately, for biofuel production to become commercially viable, it will be necessary either to optimize processes for smaller liquid volumes (e.g., 200 µL) or to advance liquid handlers to accommodate larger pipette volumes. However, achieving either of these goals must not compromise precision.

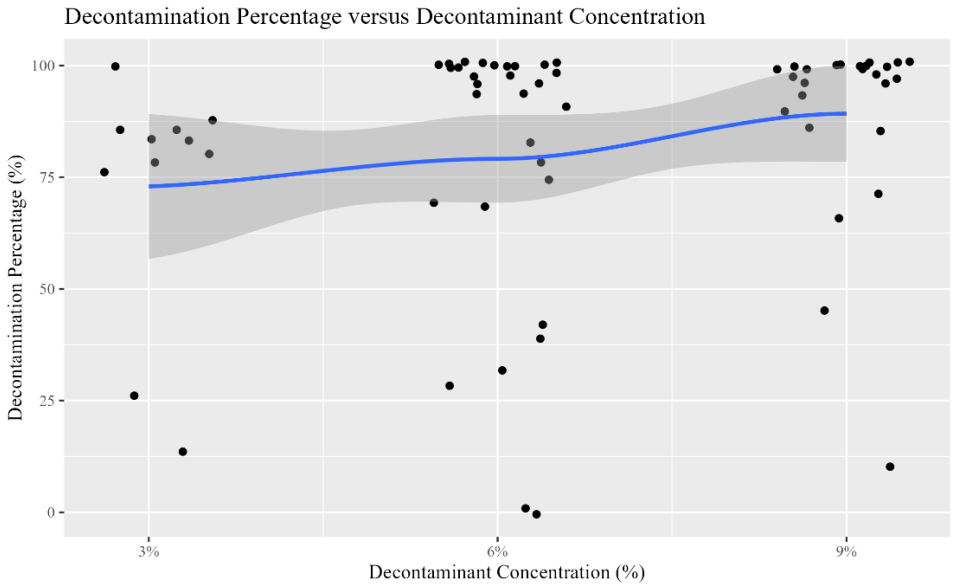

Figure 3. Scatter plot representing the decontamination percentage of plates for each decontaminant concentration. The blue line indicates the line of best fit across the groups, with the shaded area representing the 95% confidence interval.

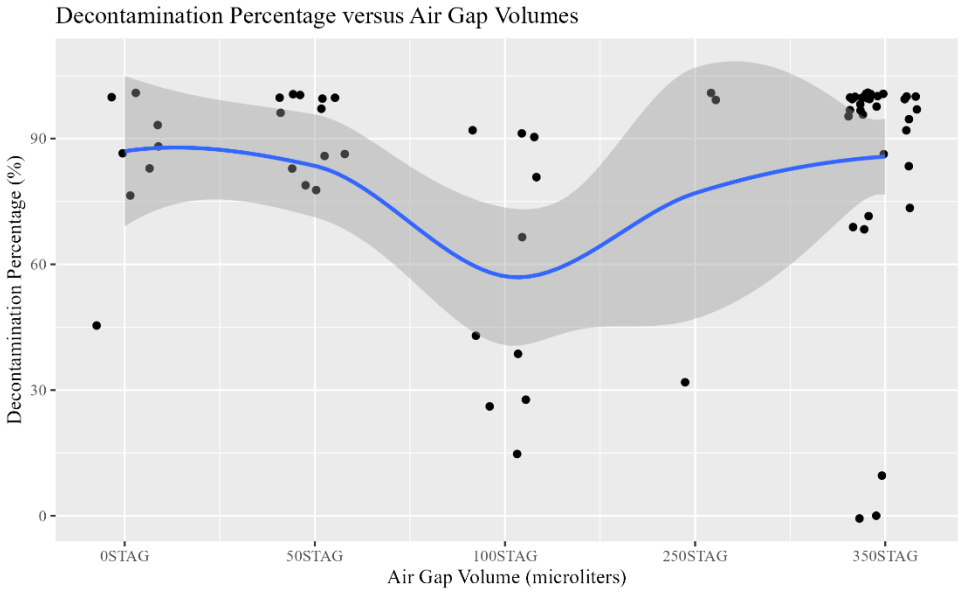

Figure 4. Scatter plot representing the decontamination percentage of plates for each air gap volume. The blue line indicates the line of best fit across the groups, with the shaded area representing the 95% confidence interval.

Our assessment of contamination patterns, both column-wise and row-wise, revealed notable insights into bacterial growth occurrences. The difference in decontamination percentage between each experimental column and the control column was statistically significant. While decontamination was not consistently achieved, this finding indicates that the procedure does have functional aspects. Notably, statistical differences were observed only between the first and seventh experimental columns, suggesting that contamination may be distributed gradually throughout the process.

Analysis using a linear model demonstrated a negative relationship between decontamination percentage and column number further supporting the hypothesis that contamination occurs incrementally rather than in a singular step (Figure 6). In contrast, the comparison of row-wise decontamination percentages showed no statistically significant differences (Figure 7). Given that the liquid handling system operates column-wise, the

similarity in decontamination between rows was anticipated as the contrary implies a potential technical issue, such as coding errors or malfunctioning machinery.

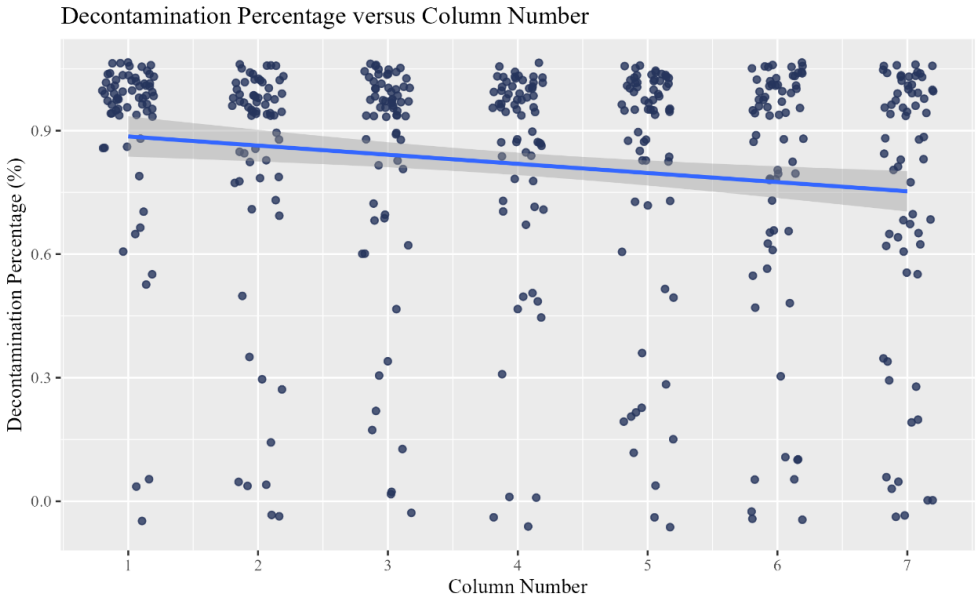

Figure 5. Scatter plot representing the decontamination percentage of plates for each experimental column. The blue line indicates the line of best fit across the groups, with the shaded area representing the 95% confidence interval. Generally, there is a negative relationship between the two parameters.

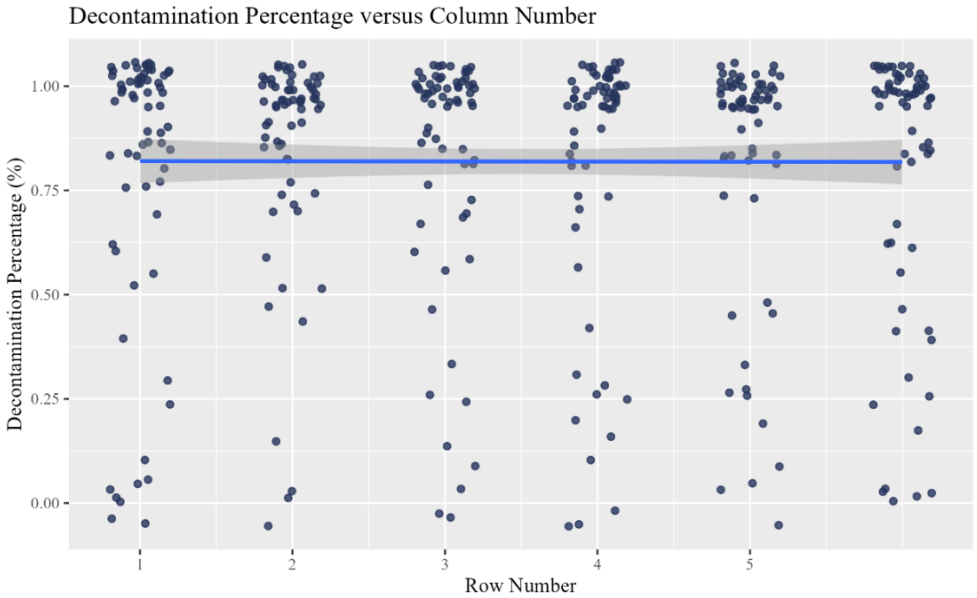

Figure 6. Scatter plot representing the decontamination percentage of plates for each row. The blue line indicates the line of best fit across the groups, with the shaded area representing the 95% confidence interval. Generally, there is no relationship between the two parameters.

The behaviours observed with the column-wise contamination demonstrate the necessity of complete and consistent decontamination for biofuel production. Even minimal contamination in the liquid handler's pipettes can lead to systemic contamination. These findings underscore the importance of the learning phase in the DBTL cycle. Our results confirm the necessity for a larger air gap to prevent contamination, as established in previous studies. Consequently, to scale up a previously successful procedure (e.g., [7]), future efforts should focus on optimizing the procedure's runtime or advancing pipette capacity in liquid handlers without compromising precision.

Conclusion

The devastating effects of climate change have created a necessity for synthetically produced biofuels. Harnessing the powers of microorganisms such as manipulating microorganisms and utilizing their membrane proteins is a promising approach to extract and develop important chemicals and biofuels to counteract the effects of climate change. However, the success of this approach depends on the precision of the techniques used. Robots can provide remarkable accuracy for this task, but the quality of the product largely depends on the reliability of the automation process. By using cultures of *E. coli*, coding the Tecan Freedom Evo 100, and testing varying concentrations of decontaminants, we were able to conclude that employing an air gap smaller than the maximum process volume achieved incomplete decontamination, resulting in gradual contamination within the pipette. The methods and procedures used in this project could potentially serve as a commercially viable process for producing membrane proteins to help counteract the effects of climate change. In summary, the proposed study can contribute to the progress of synthetic biology and bioengineering research, moving us towards establishing a sustainable and carbon-neutral future.

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