JOURNAL OF
EMERGING INVESTIGATORS

Article

Survival of *Escherichia coli* K-12 in various types of drinking water

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SUMMARY

For public health, drinking water should be free of bacterial contamination. Consumption of bottled water, especially in low-income countries has increased to avoid contamination. However, bottling, transportation, and handling procedures increase the risk of microbiological contamination, which is especially harmful to immunocompromised individuals. The objective of this research is to identify the fate of bacteria if drinking water becomes contaminated and inform consumers on which water type enables the least bacteria to survive. This research tested bottled mineral water, bottled spring water, and tap water inoculated with the *Escherichia coli* K-12 strain bacteria from times 0–72 hours. We hypothesized that bottled mineral water would provide the most sufficient conditions for the *E. coli* to survive. We found that if water becomes contaminated, the conditions offered by the three water types at room temperature allow the *E. coli* to survive up to three days. At 72 hours, the bottled spring water had the highest average colony forming units (CFUs), with tap and mineral water CFU values statistically lower than spring water but not significantly different from each other. The findings of this research highlight the need of implementing accessible quality drinking water for the underserved population and for the regulation of water sources.

INTRODUCTION

Quality drinking water is essential for public health and a development issue highlighted in Goal six of Sustainable Development Goals (SDGs) (1). More than 2 billion people lack access to safe drinking water (2). To eliminate the risk of disease, consumption of bottled water has increased, especially in developing countries as safe water sources are often not available. Annually, bottled water consumption increases by 7%, rising from 213 billion liters in 2011 to an estimated 513 billion liters by 2025 (3). However, bottling, transportation, and handling procedures increase the risk of microbiological contamination (4). Bacterial versatility and metabolic diversity may enable bacteria to proliferate and survive in tap and bottled water. The survival of bacteria in drinking water can be harmful, especially for immunocompromised individuals and children under seven years old because they do not have a developed immune system (5).

The purpose of this research is to identify the fate of the *Escherichia coli* K-12 strain in several types of water and inform consumers on which water type results in the least bacterial survival to enhance public health. Bottled water is potable water sealed in food-grade bottles designed for human consumption with no added ingredients except an antimicrobial agent when necessary (6). Daily there are eighty-five million bottles of water consumed in the United States (7). The increase in bottled water consumption is due to perceived convenience, health benefits, and purity. Bottled water may come from sources such as wells, springs, taps, and surface water. For instance, spring water is abundant in calcium, potassium, sodium, magnesium and obtained from naturally flowing water throughout the earth's surface or through a borehole (8). Mineral water must contain a minimum of 250 total dissolved solids (TDS) parts per million (ppm) and commonly includes zinc, calcium, sodium, and magnesium from natural mineral springs and underground reservoirs (9). Bottled water undergoes purification methods such as ozonation, absolute filtration, deionization, reverse osmosis, or distillation (10). Ozonation infuses ozone (an effective oxidant) into the water to dissolve contaminants (11). Filtration may occur naturally when water flows through layers of secured underground rock (12). Absolute filtration removes every particle larger than the absolute rating of a filter (pore opening size) and does not remove natural minerals (13). The captive deionization technology removes TDS through an electrical force on the ions between two electrodes to produce highly purified water (14). Reverse osmosis eliminates contaminants by applying pressure to force water through semipermeable membranes (15). Distillation transforms water into a vapor by heating the water to a boiling point and then is cooled to condense the vapor. In addition to bottled water, consumption of tap water is high (16). Moreover, in 2015, 77 million Americans obtained tap water that violated federal protection guidelines (17). Municipal tap water quality varies based on location as it may contain chlorine, lead, aluminum, and mercury (18).

In a study by Lalumandier and Ayers, 57 bottled mineral water and 4 tap water samples from Cleveland were cultured to calculate the colony forming units (CFUs)/mL of unidentified bacteria to investigate the purity of water (19). They found that the CFU count for the 57 samples of bottled

mineral water ranged from less than 0.01 to 4900 CFUs/mL, while the tap water samples ranged from 0.2 to 2.7 CFUs/mL, showing that bottled mineral water bacterial counts were higher compared to tap water (19). Additionally, a different study by, Marie *et al.* compared the bacteriological quality of tap water to bottled mineral water (20). The study discovered that 76.6% of the bottled mineral water and 36.4% of the tap water were contaminated by a minimum of one pathogenic and coliform bacteria (20). In both studies, it was evident that bottled mineral water and tap water contained bacterial contamination.

In this research, we evaluated the survival of inoculated *E. coli* K-12 strain bacteria from times 0–72 hours in bottled spring, mineral, and tap water. *E. coli* are gram-negative, meaning that it has two membranes and is thus highly resistant to drugs and antibiotics as they cannot cross the outer membrane (21). When the non-pathogenic or pathogenic strain of *E. coli* are present in water, it is associated with fecal contamination (22). In the experiments, we utilized the *E. coli* K-12 strain as a model bacterium. The K-12 strain of *E. coli* is the primary model strain for the physiology of bacteria that has a fast growth rate of 1 generation per hour, which allows for efficient experiments (23) It is an ideal model organism because it is a safe non-pathogenic lab strain and a widely studied prokaryote (24).

In the present study, we utilized bottled spring water, bottled mineral water, and tap water. These varieties of water were used due to their distinct purification methods, sources, and minerals. With the prior knowledge that bottled mineral water is competent for bacterial contamination, we hypothesized that the *E. coli* K-12 strain would survive best in bottled mineral water. We found a statistical difference in the average CFUs/mL of the *E. coli* K-12 strain among the three types of water at each time point. We concluded that at 72 hours of growth, the bottled spring water had the highest average CFUs, with tap and mineral water CFU values statistically lower than spring water but not significantly different from each other. The ability of the *E. coli* K-12 strain to survive in these types of water indicates that tap water sources and water bottling practices must be kept free of contamination.

RESULTS

To culture the bacteria, we added liquid Luria Broth (LB) to the *E. coli.* We then conducted a 10^4 serial dilution of bacteria in LB to decrease the bacterial concentration. Subsequently, 90 mL of bottled mineral water, bottled spring water, and tap water were inoculated with 10 mL of the inoculum. These plates represented time 0 CFU. We spread equal volume (1 mL) of water from each water type with *E. coli* inoculum on each LB agar plates and incubated upside down at 37°C for 24 hours. To determine how each water affects the *E. coli* K-12 strain's survival and growth, we repeated the procedure at times 24, 48, and 72 hours post inoculation **(Figure 1).** Three trials were conducted to ensure consistent results.

When we observed the plates, it was evident that the *E. coli* survived in three varieties of water at times 0, 24, 48, and 72 hours **(Figure 1).** At the end of the 72-hour experimental period, the *E. coli* could still be recovered from each of the water samples **(Figure 2).** Initially, at time 0, the three water samples had a similar CFU average of 2616.89 CFUs/mL **(Figure 2).** Subsequently, after 24 hours of the water sample left at room temperature, the average CFUs/mL for each water sample consistently decreased, where mineral water produced statically fewer colonies than the bottled spring water and tap water plates **(Figure 2)**. The average CFUs/mL of the bottled spring water increased at time points 48 and 72, while bottled mineral water and tap water increased at time point 48 and then decreased at 72 hours.

To test if the average CFUs/mL among the three types of water had a statistical difference, we utilized statistical tests. Initially, to identify if the variation between the data is equal before using ANOVA, we conducted a Levene's test that resulted in a p-value of 0.0627 ($p > 0.05$), indicating a homogeneity of variance. Since there are two independent variables consisting of water type and time point, we utilized a two-way ANOVA statistical test, with a statistical significance level of $p < 0.05$. The two-way ANOVA resulted in a p-value of 0.0329 for the water independent variable and a p-value of 0.0012 for the time point independent variable. In the two

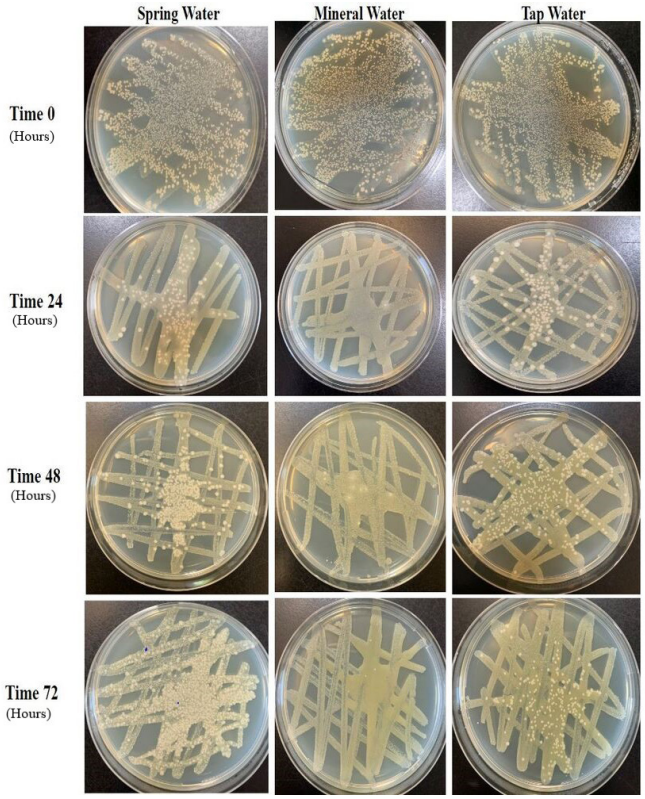

Figure 1. LB agar plates inoculated with water samples. The three water samples with inoculated E. coli plated on Luria Broth (LB) agar plates representing 0-, 24-, 48-, or 72-hours post-inoculation. Each plate contains 1 mL from the experimental group.

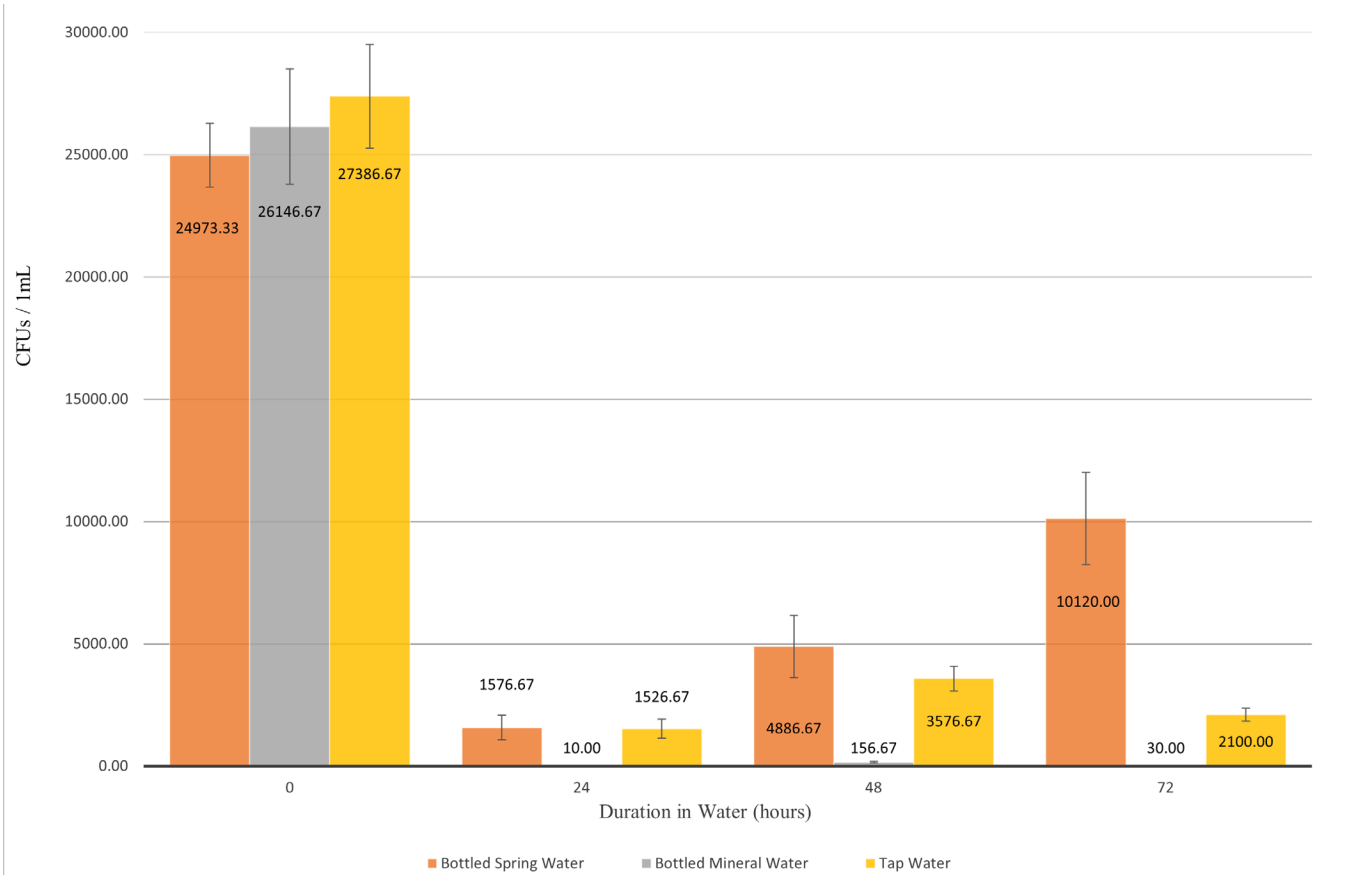

Figure 2. Survival of *E. coli* K-12 strain over time. *E. coli* K-12 strain average CFUs of three trials. Comparison of average Colony Forming Units (CFUs) of the *E. coli* K-12 strain in the three types of water. The error bars represent the SD across three trials. Levene's test with a *p*-value of 0.0627 ($p > 0.05$). Two-way ANOVA *p*-value of 0.0329 for the water independent variable and a *p*-value of 0.0012 for the time point independent variable.

independent variables, the $p < 0.05$ indicates that there is at least one statistical difference in the mean CFUs/mL between the three types of water throughout the different time points.

To identify which groups are significantly different from each other, we conducted a post-hoc Tukey Honest Significant Difference (HSD) test with a statistical significance level of $p < 0.05$ **(Table 1)**. At time 0, the Tukey HSD *p*-value was $p > 0.05$ between all the varying types of water. Time 24 and 48 resulted in a $p < 0.05$ between the spring and mineral water as well as between mineral and tap. However, time 24 also resulted in a $p > 0.05$ between the spring and tap water. Time 72 had a $p < 0.05$ between bottled spring and mineral water as well as between spring and tap water. Additionally, the *p*-value at time 72 was $p > 0.05$ between mineral and tap water. The null hypothesis states that the average CFUs/mL for each water type is equal. At each time point, a $p < .05$ indicates that the average CFUs/mL are statistically different between two types of water, while a $p > .05$ supports the null hypothesis.

To minimize the influence of variables that are not of interest, we utilized positive and negative controls **(Figure 3).** The positive control contained the inoculum and Liquid LB. We conducted a positive control at time 0 hours to display that the *E. coli* were viable. The positive control at time 0 showed growth on the LB agar plates **(Figure 3).** We conducted another positive control at the 72-hour time point to observe if the *E. coli* were still living. At 72 hours, it was evident that there was growth on the LB agar plates. We utilized three negative controls for each variety of water added with Liquid LB in three different test tubes that were plated on LB agar plates and then incubated overnight at 37°C **(Figure 3).** The negative control plates showed no growth on the LB agar plates for bottled mineral water and tap water. The LB agar plate of the bottled spring water negative control contained little to no growth.

DISCUSSION

We compared the number of *E. coli* CFUs in each type of water sample on LB agar plates after 0, 24, 48, and 72 hours of growth in bottled mineral water, bottled spring water, and tap water. At time 0 hour, the three types of water had a similar average CFU count because these initial plates represented the baseline for the number of bacteria added to each water sample. From 0 to 24 hours, the decrease of the average CFUs was consistent in each type of water. The increase in bacteria between 24 and 48 hours may indicate

Table 1. Post-hoc Tukey Honest Significant Difference Test.

Treatments Pair	Tukey HSD *p*-value	Tukey HSD Q Statistic	
Spring vs Mineral	0.7453699	1.0277	**Time 0** (Hours)
Spring vs Tap	0.3578155	2.1138	
Mineral vs Tap	0.7236115	1.0861	
Spring vs Mineral	0.0049532	7.3129	**Time 24** (Hours)
Spring vs Tap	0.8999947	0.2334	
Mineral vs Tap	0.0058092	7.0795	
Spring vs Mineral	0.0010053	10.3480	**Time 48** (Hours)
Spring vs Tap	0.1861535	2.8659	
Mineral vs Tap	0.0044170	7.4821	
Spring vs Mineral	0.0010053	15.8606	**Time 72** (Hours)
Spring vs Tap	0.0010053	12.6067	
Mineral vs Tap	0.1311860	3.2539	

NOTE: *Statistical difference between each varying type of water average CFUs/mL with a statistical significance threshold of* p < *0.05.*

that the conditions were adequate for the *E. coli* to proliferate. The CFU patterns reveal that bottled spring water continues to increase after the 48-hour time point, while bottled mineral water and tap water decrease.

The *p*-values of the two-way ANOVA test resulted in a $p < 0.05$, which rejects the three null hypotheses indicating that there is a difference in the average CFUs/mL for each type of water. There is a difference in average CFUs/mL at each of the 0–72-hour time points, and there is an interaction between the time points and water type of average CFUs/mL.

At time 0 hours, the Tukey HSD *p*-value was $p > 0.05$ between all the varying types of water because each had a similar initial number of bacteria added to each water sample and, thus, not statistically significant**.** At times 24 and 48 hours, the $p < 0.05$ between bottled spring and mineral water as well as between mineral and tap water displays that there is a statistical significance. Therefore, indicating that at time points 24 and 48 hours the mineral water had the lowest average CFUs/mL between the varying types of water. However, the $p > 0.05$ at times 24 and 48 hours between the spring and tap water indicates that the average CFUs/mL is not statistically significant. At time 72 hours, the post-hoc test indicates that the spring water had the highest average CFUs/mL with a $p < 0.05$ between bottled spring and mineral water as well as between spring and tap water. However, at time 72 hours, the $p > 0.05$ between mineral and tap water indicates

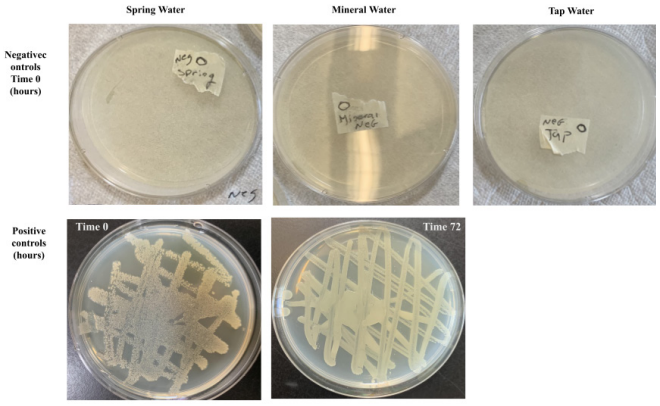

Figure 3. Positive and negative controls LB agar plates. Positive controls with inoculum and liquid Luria Broth (LB). Negative controls with liquid LB and samples from each variety of water.

that the difference in the average CFUs/mL is not statistically significant.

We hypothesized that mineral water would support the best bacterial survival. However, the result displayed that at 72 hours, the bottled spring water had the highest average CFUs, with tap and mineral water CFU values statistically lower but not significantly different from each other. Sulfate is an essential nutrient for the *E. coli* K-12 strain (25). An explanation for the distinct CFU patterns is that tap water has sulfate levels higher than bottled mineral and spring water **(Figure 4).** However, tap water also has chloride levels higher than bottled mineral water and spring water. A study by Li *et al.* found that elevated levels of sodium chloride, which is commonly found in drinking water, inhibits *E. coli* growth (26). We speculate that though tap water had the highest relative sulfate levels, the chloride limited the average CFUs. The bottled spring water has sulfate levels higher than mineral water. We hypothesize that, together, the high sulfate levels in bottled spring water and average CFUs suggest that sulfur may be a limiting nutrient in bottled mineral water due

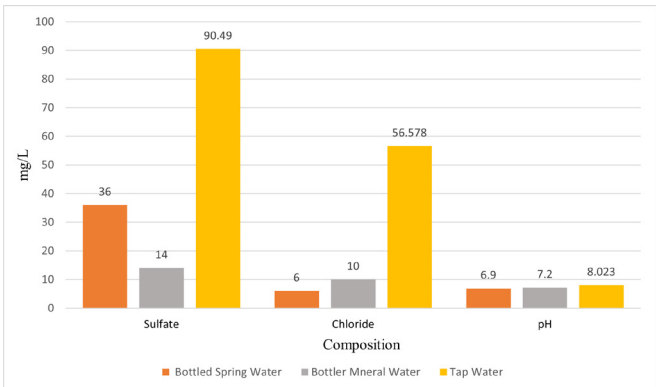

Figure 4. Key composition values of Table 2. Sulfate, chloride, and pH levels (mg/L) of the three varieties of water. Data for **Figure 4** has been taken from the information in **Table 2**.

to protein requirements. Thus, sulfate may have supported the continued proliferation of the *E. coli* CFUs in bottled spring water. Furthermore, the bottled mineral water had the lowest relative pH of 6.9 while the spring and tap water had a higher pH respectively (**Figure 4).** We also speculate that the survival of the *E. coli* is supported by lower pH levels (27). Future investigation should repeat this experiment with equal chlorine levels. If chlorine limited the survival of *E. coli*, then closer CFU values and patterns are predicted.

The composition levels of minerals and chemicals in drinking water indicate quality and contamination levels (28). Therefore, we speculate that the compositions and CFU patterns among the varieties of water are affected by the different purification methods **(Table 2)** (29; 30). The bottled mineral water is filtered and purified naturally through a source of protected underground layers of rock for over fifteen years before it reaches the consumer (31). The tap water and bottled spring water are artificially filtered with added minerals in the tap water (32). As a result, the lower CFU of bottled mineral water compared to bottled spring and tap water may have been due to the filtration and purification methods that affect its composition.

The negative controls showed little to no growth on the LB agar plates and test tubes for each type of water sample. As a result, the negative control displayed that the liquid LB, test tubes, LB agar plates, and the three varieties of water are sterile without contaminants. The findings of this research show that if bottled mineral, spring, and tap water get contaminated, then the *E. coli* K-12 strain can survive up to three days at room temperature. As a result, these findings highlight the importance of the bottled water industries and municipal tap water sources implementing significant regulation measures to exclude bacteria from their products to benefit public health.

A major limitation is that the experimental plates may have had lawns due to the high concentration of bacteria which may have impacted the bacterial counts. Therefore, future experiments should conduct a higher dilution of approximately six 1:10 serial dilutions before plating for less bacterial growth. Further dilution would lead to discrete colonies that can be proportionally compared with less background from the lawn effect. Additionally, utilizing beads is more conventional than streaking as it spreads suspensions of bacteria to plate the samples. It is advantageous that future works conduct the experiment using a liquid without nutrients such as deionized (DI) water or Phosphate Buffered Saline (PBS). This would cause the Luria Broth nutrients to be negligible in the inoculum for the improved survival of *E. coli.* In addition, an alternate method is also utilizing a centrifuge to conduct a wash step for the bacteria to form a pellet, take off the liquid supernatant with a vacuum, and resuspend in PBS or DI water. Moreover, the standard deviation of the three trials indicated that there was slight variation between each trial, demonstrating that the data collected is reliable. However, to improve statistical analysis, the number of trials could be increased. Future experiments should have negative and positive controls at every trial and time point to minimize variables outside of the experiment. Additionally, using an autoclave on the water before inoculation with *E. coli* would be suitable for further prevention of bacterial contamination. Furthermore, future experiments should utilize other categories of water beyond what was tested in this research to broaden the knowledge of bacterial survival in different water types. Moreover, future studies could assess the varieties of water at different temperatures to identify the storage temperatures that are most effective in reducing or suppressing bacterial growth. The findings of this research found that bottled spring water had the highest average CFUs/mL compared to tap and bottled mineral water. For public health, considering the increase of bottled water, especially in low-income communities, future studies can expand this research from an environmental and economic perspective to determine if funding should be allocated for bottled water or to establish quality municipal sources.

Table 2. Characterization of water types.

Water Type	Composition (mg/L)	Purification Method
Bottled Spring Water	Calcium — 27 Magnesium — 6 Potassium — 2 Sodium — 13 Bicarbonates — 0 Sulfates — 36 Chlorides — 6 pH — 6.9	- Ozonation - Absolute 0.1-micron filtration
Bottled Mineral Water	Calcium — 80 Magnesium — 26 Potassium — 1 Sodium — 6 Silica — 14 Bicarbonates — 360 Sulfates — 14 Chlorides — 10 Neutrally balanced pH — 7.2	- Purified naturally through glacial sand layers
Tap Water (ppm)	Groundwater / Surface water Calcium — 60.2 / 66 Magnesium — 9.4 / 2.6 Potassium — 2.7 / 4.6 Sodium — 50.6 / 96 Sulfates — 53 / 216 Chloride — 45.4 / 94 Fluoride TR — 0.79 / 0.7 Uranium (pCi/L) — 3.15 / 2 Lead (ppb) — 7 Copper — 0.18 pH — 8 / 8.1	- Local water supply is a combination of 77% groundwater blended 23% with imported treated water from Northern California and the Colorado river

NOTE: *Composition and purification methods for the characterization of the three varieties of water (29, 30). Data was gathered from open-source data on Huntington Beach drinking water quality and unrestricted access composition of bottled drinking water calculator. pCi/L = picoCuries per liter. TR = treatment related. ppm = parts-per million. ppb = parts-per billion.*

MATERIALS AND METHODS

Preparation and collection of samples

Sample collection took place in Huntington Beach, California, in which the sterile bottled mineral water and bottled 100% natural spring water were purchased from local stores. The bottled mineral water has dissolved solids at

180° C, 345 ppm mg/L, which meets the TDS requirement of bottled mineral water. The tap water was collected from the municipal tap source of the laboratory at Huntington Beach High School and placed in a sterile flask covered with cheesecloth to prevent particulate contamination. The living liquid *E. coli* K-12 strain was purchased from Carolina Biological Supply (33).

Inoculation, serial dilution, and bacterial plating

To culture the *E. coli* K-12 strain, 100 mL of sterile liquid Luria Broth (LB) was inoculated with 30 mL of the *E. coli* and left sealed at room temperature in a sterilized flask for three days. Next, four 1:10 dilutions were conducted to lower the concentration of the analyte (*E. coli*) to 1:10,000 its original concentration (10^4 dilution). The dilution consisted of 10 mL of the saturated culture added to 90 mL of liquid LB for a total volume of 100 mL. Throughout the serial dilution, the tubes were gently inverted several times to ensure equal distribution of the samples. Subsequently, 10 mL of the inoculum was added to 90 mL of each type of water experimental group **(Figure 5).** A total of three trials were performed with the same conditions. In each trial, the experimental group consisted of twelve sterile LB agar plates. Utilizing a sterile syringe, 1 mL from the three water samples, each containing 10 mL of inoculum and 90 mL of water was plated using a sterile cotton swab on the LB agar plates and incubated upside down at 37˚C for 24 hours. To obtain CFU values, *E. coli* colonies were counted on the plates utilizing a magnifying glass and a dissection microscope. To simplify the counting process some LB agar plates were divided into eight sections. Throughout this experiment, the experimental groups were left at room temperature to replicate the common storage conditions for bottled and tap water. Subsequently, this process was repeated for the experimental groups at 24, 48, and 72 hours.

Positive and negative controls

Positive and negative controls consisting of five LB agar plates were utilized. The positive control contained 10 mL of the inoculum added to 90 mL of liquid LB. The positive control was conducted at time points 0 and 72 hours. The three negative controls consisted of 10 mL of liquid LB added to 90 mL of each type of water sample in three different tubes and then plated to display the absence of contamination.

STATISTICAL ANALYSIS

Data analysis for figures was performed utilizing Microsoft Excel. "For the Levene's test, two-way ANOVA, and Tukey Honest Significant Difference Test, the significant threshold used was $p < 0.05$. The standard deviation was calculated among the three trials.

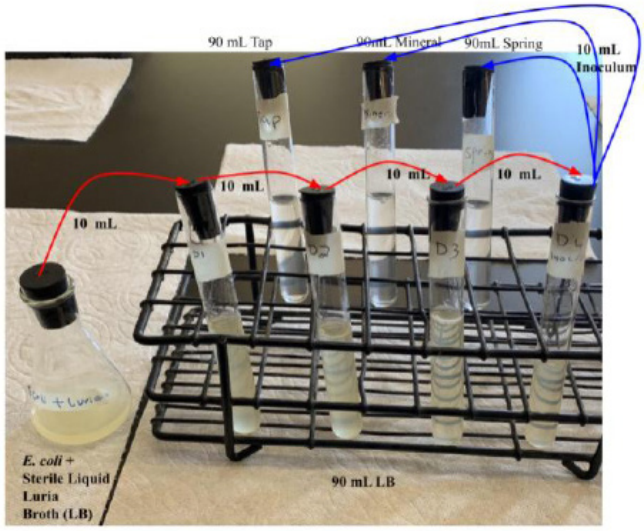

Figure 5. Inoculation and 1:10 serial dilution of the *E. coli* K-12 strain. The *E. coli* K-12 strain cultured in a nutritionally rich medium of Liquid Luria Broth (LB) in a sealed flask. A 1:10 serial dilution in test tubes labeled D1–D4, in which the D4 test tube is the inoculum. Three test tubes containing the experimental group (90 mL of each water type with 10 mL of inoculum).

ACKNOWLEDGMENTS

The lab area and scientific equipment for this research were provided by Tr. David Renfrew and Mr. Tim Floyd of Huntington Beach High School. Tr. Renfrew's invaluable guidance and oversight for this research is greatly appreciated.

Received: September 22, 2021
Accepted: August 14, 2022
Published: September 25, 2022

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