

**The significance of the difference between the
Escherichia coli numbers in six different brands of
19 liters reusable plastic bottles of water**

*“Is there any difference in terms of the amount (colony number per petri plate) of
Escherichia coli in 19 liters reusable plastic water bottles from 6 different brands,
indicated by membrane filter method?”*

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ABSTRACT:

The purpose of this experiment was to see the amount of *Escherichia coli* in six different brands of 19 liter reusable bottles of water, and see if there is a significant difference between the amount of *Escherichia coli* they include or not. According to this, I came up with the research question: Is there any difference in terms of the amount of *Escherichia coli* in 19 liters reusable plastic bottles from 6 different brands, indicated by the membrane filter method? Six different brands of 19 liter reusable bottled water were chosen by considering their popularity and their prices. The ones with low popularity and price were chosen since they have a higher risk of contamination and named in alphabetic order as A, B, C, D, E and F. The amount of *Escherichia coli* in them was determined by the membrane filter method. The method can be briefly explained like this: The water is poured down the membrane filter system where the membrane filters don't allow the passage of bacteria. Then, the filter papers are put on different petri plates and incubated. If there is right type of colorizations then, further tests are done to determine if the colonies are *E. coli* or not. At the end of the process, the numbers of colonies that are found out to be *E. coli* is counted and a result is obtained. In this experiment, ANOVA test is used and it proved that there is a significant difference between the amounts of *E. coli* in different brands of water since the p value is approximately zero.

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1. INTRODUCTION

Water is the source of life for all of the living organisms in addition to being the source of many diseases. The World Health Organization says that every year more than 3.4 million people die as a result of water related diseases. H₂O may cause waterborne diseases by carrying pathogens. Microorganisms may get in human body by several ways, like bathing, washing nutrients, cooking or just by simply drinking. Also, in Turkey, some water brands have been accused of selling unsanitary water and have been sealed. However, they started to sell water again after a few weeks. This event shows the danger we are putting ourselves in by only choosing the wrong brand of water to drink.

The organisms that cause diseases via drinking water are classified as bacteria, viruses and parasites. The most abundant viruses are *Adenoviridae virus*, *Rotavirus* and *Polio* where the most abundant bacteria are *Vibrio choleraebacteria*, *Escherichia coli*, *Legionella pneumophila*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, *Clostridium perfringens*, *Staphylococcus aureus*, *Salmonella* and *Shigella*. In addition, the parasites that cause some waterborne diseases are known to be *Giardia lamblia*(protozoan parasite), *Entameba histolytica*, *Cryptosporidium parvum*, *Cyclospora cayetanensis*, *Balantidium coli*. The microorganisms given above can cause different diseases which mostly have one common symptom: diarrhea. Diarrhea is a sickness that causes dehydration due to some intestinal problems which may sometimes be fatal for children. This is a major danger of contamination in drinking water.¹

One of the most abundant bacteria that can spread through water is *Escherichia coli*. Physiologically, *E. coli* is a gram-negative, rod shaped bacteria which has high adaptation to its characteristic habitats. Wild-type *E. coli* has no growth factor requirements, and metabolically it can transform glucose into all of the macromolecular components that make up the cell. The bacterium can grow in the presence or absence of O₂. Since it can adapt to various conditions, when it is contaminated to water its reproduction is inevitable. Also, there are different serotypes of *Escherichia coli* bacteria that exist as part of the normal flora of the human gut and have many beneficial functions, such as the production of vitamin K. They also prevent harmful bacteria, from establishing themselves in the intestine.

The contamination risk, especially with *E. coli*, is high while filling water into the reusable plastic bottles because there is a cleaning process that needs to be done

¹ <http://www.azdhs.gov/phs/oids/epi/disease/waterborne/list.htm>

<http://www.lenntech.com/processes/disinfection/deseases/waterborne-diseases-contagion.htm>

http://water.wikia.com/wiki/Water-borne_disease

cautiously. Also, if the water source is not checked for microorganisms, the chance of contamination increases. Moreover, the bottles have different features that depend on the brand. The main matter used to produce such bottles is a chemical called “polycarbonate”. In order to make the polycarbonate bottles harder and more resistant so they can be used for longer time, Bisphenol A is used. BPA is a chemical that can cause harm in the human body (it could even lead to cancer) when used in high amounts.

The process that should be followed while filling these reusable bottles is explained below:

1. The bottle enters the filling machine and gets cleansed with special brushes inside and outside.
2. The bottle is sterilized with disinfectants chosen by the Ministry of Health.
3. The bottle is washed at 70°C with the appropriate detergents.
4. The rinsing is done in four steps. The last two steps include the cleaning of the bottle with natural spring water containing O₃.
5. The refilling process is completed.²

Although this is the main process, there might be some differences between brands when it comes to practice. The amount, type and concentration of the disinfectants affect the hygiene and the price of the brands. For economical profit, some of the brands might be using less than enough or dilute disinfectants or might be decreasing the temperature needed. If this process is not followed effectively, contamination may occur. Some brands of water, are not careful enough and do not take precautions. My intention is to find out which brand is healthier, and which type of water is contaminated. To measure healthiness of water number of *E. coli* in these different brand bottles will be counted. Therefore my research question is “Is there any difference in terms of the amount (colony number per petri plate) of *Escherichia coli* in 19 liters reusable plastic bottles from 6 different brands, indicated by membrane filter method?”

An earlier experiment similar to mine has been done in the University of Cumhuriyet in Tokat, Turkey. It is a research on drinking water in terms of Coliform bacteria. The research has been done on 2295 tap water and 200 source water. Sampling was done with 100 mL colorful glass bottles which were closed with cotton and covered with heat resistant paper. For the incubation of the samples, two etuves which were at 36-37°C and 44-44.5°C were used. The samples were taken under sterile conditions and brought to the laboratory within 8 hours where they were observed in the same day. In the observation of the samples multiple tube method was used. As a result of the experiments 12.7 % of the samples were found to be contaminated. The 34.7% of the contaminated water included *Escherichia coli*.

1. ² <http://www.hurriyet.com.tr/gundem/21098923.asp>

It can be concluded that different brands can cause different amounts of contamination. In that respect, my hypothesis is “There is a significant difference between reusable 19 liter water bottles from different brands in terms of *E. coli*.”

In order to perform a controlled and manageable investigation for the stated research question and hypothesis, experiment will be done in room temperature and in the same medium. I will use only water samples from different brands of reusable 19 liter plastic bottles and the method will be membrane filter method instead of multiple tube method. The reasons why I chose this method will be explained in my method development.

2. METHOD DEVELOPMENT

2.1. Preparation of the Experiment

The aim of experiment is to determine brands which have high risk for contamination with *Escherichia coli*. Therefore, the dependent variable is the *Escherichia coli* colony number per petri plate where the independent variable is the reusable bottled water brands. All other factors are kept constant, such as temperature, medium, size of the filter paper and the petri plate, the type of the petri plate, the volume of sample taken, the method used, etc. to get accurate results.

I chose *Escherichia coli* to work on because, bacteria are easier to work compared to viruses and parasites since bacteria are larger in size, approximately 1000 nanometers, compared to viruses and unlike viruses and parasites, they don't need a host to live. The reason I chose *E. coli* specifically is that its adaptation is high, it can survive in various conditions and cells are able to survive outside the body for a limited amount of time, which makes them ideal indicator organisms for fecal contamination. It can only cause diseases when a different serotype than we have is ingested orally as in case of drinking water. It can be grown easily and inexpensively in a laboratory setting. Moreover, if these bacteria are found in drinking water it serves as evidence that the water is contaminated by sewage which is quite disgusting and harmful. Lastly, diseases caused by *E. coli* are quite abundant.

The experiment will be done on 19 liter reusable bottles because their contamination risk is higher. The production and selling process of 19 liter bottles of water requires lots of care. I heard from the news that some brands weren't careful enough while refilling bottles. Due to the competition between brands, some of them might be using less than enough cleaners to clean the bottles before refilling or the tools used in cleaning process might not be well-kept in order to decrease the expenses and so decrease the price.

The brands that are used in the experiment are chosen from the declared list of the accused water brands to be contaminated. I have picked the ones with the lowest price and less preference rate because well-known brands would not be containing the bacteria I am looking for. Since I am not allowed disclose the brands, I tagged them as A, B, C, D, and E. I have put their names in alphabetic order and the first one got A, and the others followed respectively. The bottles will be taken to the laboratory with a special vehicle at 5 ± 2 °C and away from sunlight to prevent the growth of bacteria before reaching to the laboratory. Also, after the bottles arrive they should all be kept in the same dark room for same reasons.

Since 19 liter bottles will be heavy to carry to the membrane filter system, samples of 250mL water will be taken to sterile bottles. 250 mL is enough to do the experiment effectively, this amount of water will have proportional amount of bacteria to the whole bottle. The production dates of the bottles are the same so that if there are any bacteria in them, they will have the same amount of time to reproduce. For all of the brands, experiment will be done approximately at the same time. They will be kept in etuve for

the same amount of time (21 hours for colonial growth, 21 hours for oxidase test) after planting.

As to figure out number of *E. coli*, there are several ways both for testing existence and number of the coliform group. The standard test for coliform group may be carried out either by multiple-tube fermentation technique (through the presumptive-confirmed phases or completed test), by membrane filter (MF) technique, or by chromogenic substrate coliform test. Each technique is applicable within the limitations specified and with consideration of purpose of the investigation. When multiple tubes are used as the fermentation technique, results are found in terms of the Most Probable Number (MPN) of organisms present. This number, based on certain probability formulas, is an estimate of average density of coliforms in the sample. Coliform quantity gives the best assessment of water treatment effectiveness and the sanitary quality of water. The membrane filter (MF) technique is highly reproducible, can be used to test high volumes of sample, and yields numerical results quicker than the multiple-tube method. The membrane filter technique is extremely useful in monitoring drinking water and a variety of natural waters.

From all these methods, the membrane filter technique was chosen because counting of *E. coli* with the hydrophobic grid membrane filters has been accepted as the standard analyze method by American Official Analytical Chemist (AOAC) which is an international organization of standardization. According to the researches this technique has the lowest rate of contamination and lowest price.

I will do the experiment in a laboratory because the place of experiment needs to be disinfected and the membrane filter system can only be available in a laboratory setting. Cleaning of the laboratory and the system is explained in detail in the procedure. Briefly, the maximum number of colonies in a culture media that has waited with an open cap for 15 minutes should be 15kob/petri. The surface the experiment is to be held is cleaned with 70 % molar alcohol. The membrane filter system is heated with a flame thrower in order to prevent contamination within the lab. Temperature is constant in the laboratory because it is highly insulated. Besides, there isn't one constant temperature during experiment. Before the petri plates including samples are put in the etuve they are kept in room temperature and in the etuve during the expected colonial growth of bacteria at 36°C, during oxidase test at 44°C. The oxidase test, the β - glucuronidase test and indol tests are applied to indicate presence of *E. coli*. The fluorescent light used in β - glucuronidase test is 366nm. Oxidase reactive sheet and Kovacs reactive are also controlled variables. Due to the bacteria being oxidase negative, β - glucuronidase positive and production of indol the indicated colony number is determined. The same process will be applied to all brands so the culture medium, duration of experiment, method used to place the water into the medium will be constant. The controlled variables such as temperature, pH (6.2), culture medium (AGAR petri plate), duration of experiment, method used to place the water into the medium will be easier to control in a laboratory. Also, I need to use etuve during incubation which can be found in laboratory. The management of the controlled variables such as temperature, pH and duration of experiment are quite important because all three of these factors affect the rate of reproduction of the bacteria.

2.2. Material List:

- ✓ Membrane filter system
- ✓ 19 liter bottles of the brands A, B, C, D, E and F.
- ✓ Etuve
- ✓ 6 250mL sterile bottles
- ✓ 30 Filtration papers
- ✓ Flame thrower
- ✓ 30 TTC Agar with Tergitol petri plates
- ✓ Acetate pen
- ✓ Laboratory tweezers
- ✓ 30 petri plates having TSA
- ✓ Timer
- ✓ 30 thin glass tools
- ✓ 30 oxidase reactive sheets
- ✓ 30 petri plates including MUG and tryptophan
- ✓ A machine that gives light of 336nm wavelength.
- ✓ 7 mL of Kovacs reactive
- ✓ 30 tubes

2.3. Procedure:

1. Buy 6 different brands of water in the reusable 19-liter plastic
2. Clean the system and the surfaces you are going to work on with 70% molar ethyl alcohol. Also, heat the membrane filter system with a flame thrower for about 90 seconds per each part.
3. Name the Lactose *TTC* Agar with *Tergitol petri plates*. The first six will be used for the brand A. Give numbers to the trials. For example; *A1, A2, A3, A4, and A5*.
4. Clean the caps of the bottles with 70% molar ethyl alcohol solution.
5. Use membrane filter method starting with the 5th demand in the procedure. (See Appendix A.)

3. Analysis

3.1 Raw Data Table

Brand of Water	Trials	Number of <i>E. coli</i> Colonies (± 1 per one filter paper)	Temperature of the medium of membrane filter system(± 0.5 °C)	Temperature of etuve in first incubation(± 0.5 °C)	Temperature of etuve in oxidase test(± 0.5 °C)	Temperature of etuve in β -glucuronidase test(± 0.5 °C)	pH(± 0.1)	Volume of sample taken(± 1 mL)	Diameter of the pore of membrane filter(± 0.01 μ m)	Wavelength of fluorescent light (± 0.5 nm)	Amount of Kovacs reactive(± 0.01 mL)	Duration of first etuve(± 0.01 hours)	Duration of second etuve(± 0.01 hours)	Duration of third etuve(± 0.01 hours)	Percentage concentration of ethyl alcohol	Heating time with the flame thrower(± 0.5 sec)
A	1	0	25	36	36	44	6.2	250	0.45	366	0.2	21+23	21	21	70	90
	2	0	25	36	36	44	6.2	250	0.45	366	0.2	21+23	21	21	70	90
	3	0	25	36	36	44	6.2	250	0.45	366	0.2	21+23	21	21	70	90
	4	0	25	36	36	44	6.2	250	0.45	366	0.2	21+23	21	21	70	90
	5	0	25	36	36	44	6.2	250	0.45	366	0.2	21+23	21	21	70	90
B	1	34	25	36	36	44	6.2	250	0.45	366	0.2	21+23	21	21	70	90
	2	23	25	36	36	44	6.3	250	0.45	366	0.2	21+23	21	21	70	90
	3	56	25	36	36	44	6.2	250	0.45	366	0.2	21+23	21	21	70	90
	4	48	25	36	36	44	6.1	250	0.45	366	0.2	21+23	21	21	70	90
	5	14	25	36	36	44	6.2	250	0.45	366	0.2	21+23	21	21	70	90
C	1	5	25	36	36	44	6.2	250	0.45	366	0.2	21+23	21	21	70	90
	2	7	25	36	36	44	6.2	250	0.45	366	0.2	21+23	21	21	70	90
	3	0	25	36	36	44	6.2	250	0.45	366	0.2	21+23	21	21	70	90
	4	6	25	36	36	44	6.2	250	0.45	366	0.2	21+23	21	21	70	90
	5	2	25	36	36	44	6.2	250	0.45	366	0.2	21+23	21	21	70	90

D	1	1	25	36	36	44	6.2	250	0.45	366	0.2	21+23	21	21	70	90
	2	0	25	36	36	44	6.2	250	0.45	366	0.2	21+23	21	21	70	90
	3	0	25	36	36	44	6.2	250	0.45	366	0.2	21+23	21	21	70	90
	4	1	25	36	36	44	6.2	250	0.45	366	0.2	21+23	21	21	70	90
	5	2	25	36	36	44	6.2	250	0.45	366	0.2	21+23	21	21	70	90
E	1	68	25	36	36	44	6.1	250	0.45	366	0.2	21+23	21	21	70	90
	2	53	25	36	36	44	6.2	250	0.45	366	0.2	21+23	21	21	70	90
	3	93	25	36	36	44	6.2	250	0.45	366	0.2	21+23	21	21	70	90
	4	62	25	36	36	44	6.2	250	0.45	366	0.2	21+23	21	21	70	90
	5	44	25	36	36	44	6.1	250	0.45	366	0.2	21+23	21	21	70	90
F	1	23	25	36	36	44	6.2	250	0.45	366	0.2	21+23	21	21	70	90
	2	17	25	36	36	44	6.2	250	0.45	366	0.2	21+23	21	21	70	90
	3	21	25	36	36	44	6.3	250	0.45	366	0.2	21+23	21	21	70	90
	4	10	25	36	36	44	6.2	250	0.45	366	0.2	21+23	21	21	70	90
	5	19	25	36	36	44	6.2	250	0.45	366	0.2	21+23	21	21	70	90

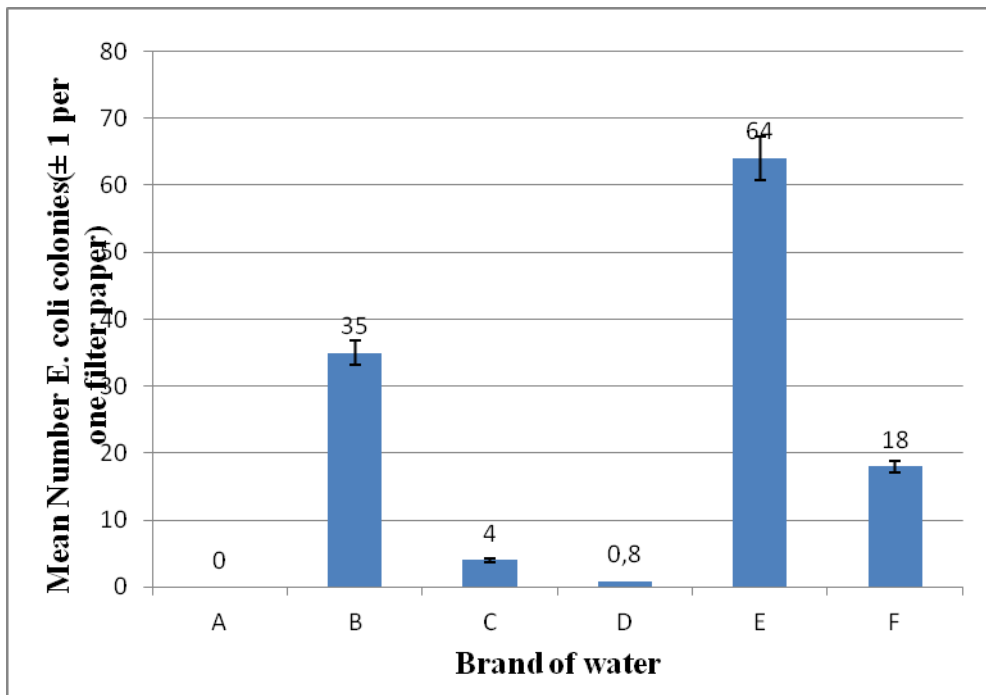
Table1: The numbers of E. coli colonies of the brands A, B, C, D, E, and F per one filter paper.

3.2 Descriptive Table

Groups	Number of trial	Sum	Mean	Variance
A	5	0	0	0
B	5	175	35	299
C	5	20	4	8,5
D	5	4	0,8	0,7
E	5	320	64	345,5
F	5	90	18	25

Table 2: Mean, median, mode and the standard deviation of the trials done to indicate the colony numbers on the brands A, B, C, D, E and F.

The table above shows the total number of *E. coli* colony found per brand. Samples taken for one trial are 250mL so the sum value indicates the approximate number of *E. coli* colony if the samples were 1250mL. Brand E has the highest sum, mean and variance value, where brand A has that the lowest.



Graph 1: The mean number of *E. coli* colonies per one filter paper of the water brands A, B, C, D, E and F are shown.

Difference between the amounts of *E. coli* within different water sources from different brands can be clearly seen from the graph as well. Brand A has the lowest average number of *E. coli* colonies per one filter paper with the number 0 where the brand E has the highest with the number 64. Then follow the brands B, F, C and D with the average colony numbers of 35, 18 and 4 respectively.

3.3 The ANOVA Table

<i>Variance Source</i>	<i>Standard Deviation</i>	<i>df</i>	<i>MS</i>	<i>F ratio</i>	<i>P-value</i>	<i>F-probability</i>
Between Groups	15945,5	5	3189,1	28,19301606	2,62666E-09	2,620654148
Within Groups	2714,8	24	113,1166667			
Total	18660,3	29				

Dependent Variable: Number of *E. coli*

a Computed using alpha = 0,05

b R Squared = 0,882 (Adjusted R Squared = 0,858)

Table 3: ANOVA results of the experiment done to indicate the *E. coli* amounts in the brands A, B, C, D, E and F.

There are two methods that can be used to analyze the data above. These possible methods are T-test and ANOVA. T-test is used to assess whether mean values of Z groups are different or not. However, T-tests are unreliable when more groups are compared so to check if this difference is significant, ANOVA test is done. The results of ANOVA are represented above. The p value is $2,63 \times 10^{-9}$ where α is equal to 0,050. It can be seen that $p < \alpha$ which means there is a significant mean value difference between the amounts of *E. coli* within different water sources from different brands. The null hypothesis is rejected.

3. Conclusion and Evaluation:

The experiment was done to determine the existence of *E. coli* bacteria in drinking water brands used by the people in Turkey and evaluate reasons for the water contamination. By considering the news that show people infected because of drinking water sold by the brands with low prices which weren't preferred by most people. My research question is "Is there any difference in terms of the presence of *Escherichia coli* in 19 liters reusable plastic bottles from 6 different brands, indicated by membrane filter method?" The aim of experiment is to determine brands which include *Escherichia coli* bacteria and whether there is a significant difference between brands. The hypothesis indicates, there is a significant difference between reusable 19 liter water bottles from different brands in terms of *E. coli* which is determined and counted by membrane filter method.

This is a statistical experiment so Anova test was used in order to find the result by using Microsoft Excel. The reason Anova is chosen is there are more than two categories. My p value is $2,63 \times 10^{-9}$ and α value is 0,050. Since p is smaller than α , there is a significant difference between brands statistically so my hypothesis was accepted.

As a result of the experiment, five of six brands are found out to be involving *E. coli* bacteria. However, brand D had much less number of colonies than the other contaminated ones and at the two of the trials no colonies were found at all. This shows that there has probably been an error. Therefore we might conclude that four of six brands of 19 liter reusable bottles contained *E. coli* bacteria.

A graph is used to show these results clearly. As seen from the graph, the brand with the highest number of colonies is brand E with a mean *E. coli* number of 64. This indicates that the brand E is very harmful to human health and further researches should be done on this brand. The reasons of the intense existence of the microorganisms in this brand of water will be investigated. Also, the data obtained from the experiment trials done on the brand E has the highest standard deviation as it can be seen from the table. This indicates that different bottles of brand E contain variable numbers of *Escherichia coli* bacteria. Therefore, the source of the problem may be the 19-liter reusable bottles. The ones that are highly damaged might involve a better environment for the bacteria. The damage rate of the bottles would be different and this would explain the different rates of the bacteria colonies. When the scratches at the inside of the bottle see sunlight, they form an appropriate environment for not only *Escherichia coli* but also for the other microorganisms. Also, the other colonies found in the petri plate prove that there are other types of microorganisms in brand E water. Apparently, the bottles of brand E are being used over a long time and they are not taken good care of.

The brand that has the second highest number of average colonies is brand B with an average of 35 colonies. In addition, this brand also has the second highest standard

deviation so it can be considered as this brand has the same problem with brand E, but it is in a previous level. The cause of scratches at the inside of the bottle is likely to be the problem in cleaning process before the bottles are refilled. First step of the cleaning process is that the bottle is washed with special brushes from inside out. The brushes may be old and damaged causing the bottles to be scratched and damaged. The reason of contamination may also be the way of filling the bottles. The pipes used to carry the water might be involving microorganisms and they should be regularly disinfected. The bottles should be filled by following a special process, as mentioned in the introduction part. Since the detergents used to clean the bottles chemically are chosen carefully so that they won't be harmful to human health, they may not be also strong enough to kill resistant bacteria.

Brand F has the third highest amount of colonies with an average colony number of 18 and brand C has an average colony number of 4 and then followed by the brands D and A with average colony numbers of 0.8 and 0 respectively. As discussed before, the number of colonies in the brand D is very low, and in some of the trials no colonies were found at all, as it can be seen from the raw data table. Therefore, we can conclude that a mistake has been done in some of the trials and brand D is clean too. The errors can be that the petri plates used for the brand D were not properly disinfected so there could be a problem at the materials causing a systematic error to be done. To overcome this kind of error, the package of the materials used should be checked properly and make sure they are disinfected.

There are also some limitations related to nature of the experiment. One of the limitations was when the colony number in a petri plate is too many; it is hard to count the colony number without making a mistake. In order to overcome this limitation, a photo of the petri plate could be taken and uploaded to a computer so that the counted colonies could be marked and they would be counted without any mistake.

Another limitation is when a petri plate includes different types of organisms' colonies it is harder to distinguish *E. coli* from the other ones. Although all suspected colonies were checked if it was *E. coli* or not, there might have been mistakes. In order to fix this, the experiment could be done with smaller filter papers and smaller petri plates so that the area that concerns us would be smaller and colonies would be easier to distinguish.

The third limitation is brands cannot be trusted about the refilling process date of the 19 liter bottles. They might have done mistakes with dating and keeping conditions of the bottles, causing bacteria to reproduce. To fix this the experiment can be done this way: Six 19 liter bottles of the same material which were not used before can be obtained and they could go through different refilling processes. For example, each would be cleaned with brushes of different material and of different kind or each would be cleaned with different chemicals. After this process is done for a period of time the bacterial investigation of the water inside the different bottles could be held. This way we would be able to determine the ideal refilling process.

Furthermore, the scratches inside the bottles are appropriate environment for not only *E. coli* bacteria but for also other microorganisms. Although the cause of the scratches might be the cleaning before the refilling, the effect of the amount of the scratches on reproduction of microorganisms should also be investigated. In real life, the amount of scratches inside the bottles can be checked regularly and the bottles with dangerous amount of scratches could be recycled.

According to the result of this experiment, buying the reusable 19-liter plastic bottles could be harmful to human health due to the risk of contamination, unless the brand is reliable, mostly preferred and not too cheap. The safest consumption of water would be the usage of glass bottles. Some brands have recently started selling glass bottles with high amount of water in them. Glass is a material which does not make an appropriate environment for the microorganisms. Other result is to use smaller plastic bottles of water like 5 liter ones which are not reusable. Since these ones are disposable they won't cause damage to human health. Although similar experiments including water investigation have been done, there is no literature value for this experiment specifically.

If the experiment was to be redone, further investigations could take place. The contamination of water by *Vibrio cholera* bacteria can be investigated. *Vibrio cholera* causes cholera which is a disease that has been seen on 140 000 people and caused 5000 people's death (World Health Organization). The existence of these bacteria can be questioned at water sources especially in Africa due to the high death rate caused by cholera. As a result, the new research question could be "Is there a significant difference between the number of *Vibrio cholera* in source waters of five different countries of Africa, indicated by the SMARTTM II. CHOLERA Water Test, while keeping the temperature, pH, and duration of experiment constant?".

Appendix A:

Membrane Filter Method:

1. Buy 6 different brands of water in the reusable 19-liter plastic bottles and make sure they are brought to the laboratory in a special vehicle at 5 ± 3 °C and away from sunlight. Label them as A, B, C, D, and E in alphabetic order.
2. Make sure the laboratory is clean and organized. Clean the system and the surfaces you are going to work on with 70% molar ethyl alcohol. Also, heat the membrane filter system with a flame thrower for about 90 seconds per each part.



Figure 1: The sterilization process with the usage of the flame thrower

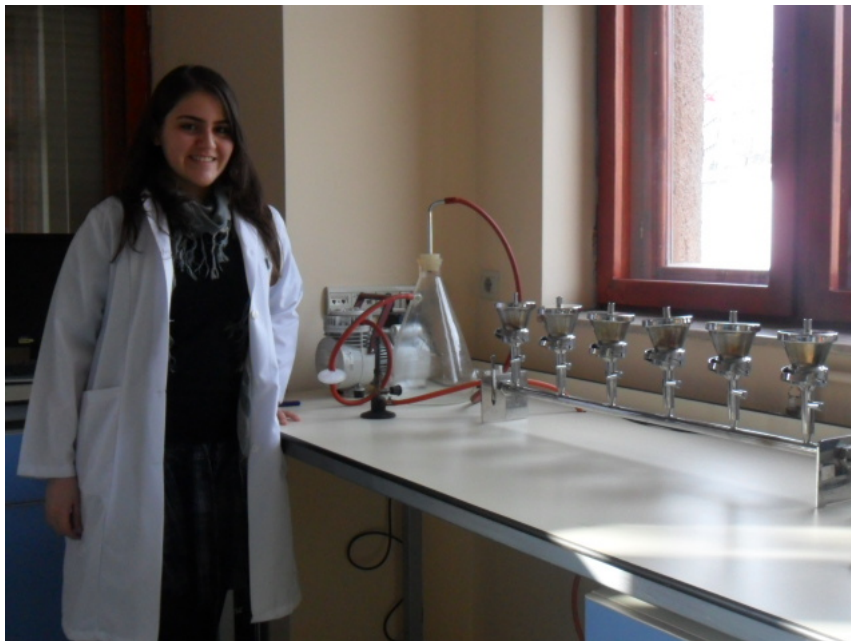


Figure 2: The membrane filter system

3. Name the Lactose TTC Agar with Tergitol petri plates. For example; A, B, C, D, etc.



Figure 3: The Lactose *TTC* Agar with Tergitol petri plate

4. Clean the cap of the first bottle with 70% molar ethyl alcohol solution.
5. Place the sterile membrane filter of 0.45 μ m pore diameter. This membrane will keep the bacteria on it and let the water flow. Put the metal funnel parts of the system on the filters, which were sterilized with ethyl alcohol and the flame thrower before.



Figure 4: Placing the membrane filter to the system



Figure 5: The sight of the filter with the funnel

6. After opening the bottle pour 250 mL of water to a sterile bottle unless you can carry the 19 liter reusable bottle close enough to pour it to the membrane filtration system.
7. Using the membrane filtration system, shown in figure, pour 250 mL of water to the funnel shaped part of the system.



Figure 6: Water is put in the funnel which has a filter at the bottom

8. After the water has flown by and carried to a collecting bottle with the pipes of the system, take the membrane filters one by one and place each to the petri plates so that the face without the squares will be completely in touch with the culture medium.



Figure 7: Placing the membrane filter to the Lactose *TTC* Agar with *Tergitol* petri plate

9. Do from 3 to 9 for each of the brands and trials.
10. To check if there is an error or a contamination in your experiment. Follow these steps:
 - a. Obtain 250 mL of distilled water and be sure that it is sterile.
 - b. Do from 3 to 9 for this type of water as well. (Name the petri plate you use for the sterilized water CONTROL.)
 - c. This process is called internal quality control.
11. Place the petri plates of all the brands to the etuve at $36\pm 2^{\circ}\text{C}$.
12. Set the timer to 21 hours.
13. After 21 hours, check if you can see little dots on the petri plates, which indicate the bacterial colonies. If there aren't any, wait for 23 hours more and check again.
14. After 44 hours, if there is no sign of bacterial growth in any of the trials, then that brand is clear.
15. Also, if there is bacterial growth on the control petri plate then redo the experiment for the brands that have colonies in the culture media.
16. If there is bacterial growth in a brand and there is no error or contamination, then check how many of them is *E. coli* in several ways.
17. Firstly, the colour of the filter should be yellow around the colonies.

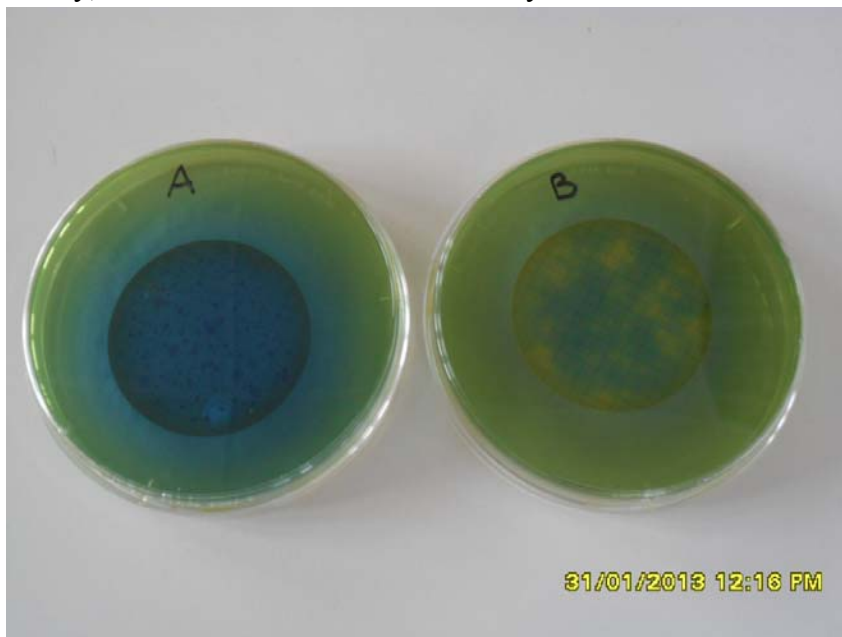


Figure 8: The plate A has also bacteria but not *E. coli* so it won't take the further tests. The plate B is named as a suspicious colony because it is yellow around the colonies.



Figure 9: Suspicious colonies of bacteria

18. Oxidase testing should be done:

- ♥ Take the colony and put on TSA in a petri plate.
- ♥ Incubate it in a $36\pm 2^{\circ}\text{C}$ etuve for 21 hours.
- ♥ Take the colony with a glass tool and place it on the oxidase reactive sheet.
- ♥ *E. coli* is an oxidase negative bacteria so there shouldn't be a shift in colour.



Figure 10: Oxidase positive bacteria (on the left) and oxidase negative bacteria (on the right)

19. If the colony is yellow in colour, gram negative and oxidase negative, then indol and β -glucuronidase test should be done:

- ♥ Put the suspicious colony to a petri plate including MUG and tryptophan
- ♥ Incubate them at $44\pm 0.5^{\circ}\text{C}$ in an etuve for 21 hours.
- ♥ The tubes which give fluorescence under 366nm wavelength are marked. These ones are β -glucuronidase positive and are used in the next step of the test.



Figure 11: β - glucuronidase negative(left) and β - glucuronidase positive(right) bacteria

- ♥ 0.2-0.3 mL Kovacs reactive is put to the fluorescence giving tubes, and checked if there is any red circles on the culture media. If so, it means that there is indol production.
- ♥ *E. coli* is β - glucuronidase positive and produces indol.



Figure 12: Indol producing bacteria is on the right.

20. Then the final colony number is counted and stated as number of colony per petri plate.³

4. References:

³ T.C. SAĞLIK BAKANLIĞI TÜRKİYE HALK SAĞLIĞI MÜDÜRLÜĞÜ. (2012), *Su Mikrobiyolojisi ve Uygulamaları El Kitabı*, Ankara.

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