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The Cleanliness of Reusable Water Bottles: How Contamination Levels are Affected by Bottle Usage and Cleaning Behaviors of Bottle Owners

ABSTRACT

Reusable water bottles are growing in popularity, but consumers regularly refill bottles without a corresponding effort at cleaning them. If the difficulties associated with various bottle designs and materials are added in, it is clear that improperly cleaned water bottles may present a potential contamination risk and thus be a risk for foodborne illness. The purpose of this study was to measure contamination levels of water bottles that are in use and to investigate bottle usage and cleaning behaviors by collecting survey data from the bottle owners. Total organic materials on the exterior surface and coliform and heterotrophic bacteria on the interior surface were enumerated, using ATP bioluminescence and the agar plate count method, respectively. The HPC and coliform results revealed a marked microbial contamination level among reusable water bottles that are in use, and the ATP levels suggest that the exterior bottle surfaces may serve as fomites that facilitate the transmission of infectious organisms. The contamination level can be affected by factors such as bottle material, refill frequency, beverage type, and cleaning behavior.

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INTRODUCTION

Demand for bottled water has consistently increased in recent years, making bottled water the fastest growing segment of the non-alcoholic beverage market worldwide *(11)*. However, massive consumption of water in disposable bottles has been connected to increased pollution and landfill waste. The EPA *(35)* reported that each year Americans throw away about 28 billion bottles and jars; notably, only 26% of plastic bottles were recycled. The environmental cost associated with bottled water has led to a social push to adopt reusable water bottles.

Reusable bottles are more environmentally friendly and economical because consumers can repeatedly refill them. This ability to refill and reuse water bottles comes with an implied mandate to clean the bottles on a regular basis. However, observation of consumer behaviors related to reusable water bottles suggests that users are regularly refilling bottles without making a corresponding effort at cleaning them. Moreover, the design of reusable water bottles may pose a barrier to their cleanliness. Bottle options are numerous and range from portable plastic or collapsible bottles to multi-purpose containers that can handle either hot or cold beverages. Some bottles come with built-in carbon filters that are replaceable, but not cleanable, while others include straws and areas that are difficult to clean properly. Some bottles have wide mouths that make it easy to clean the interior, while others have an opening that is only an inch or so in diameter, making it challenging to clean the inside. Users may think that it is sufficient to simply put the bottle into the dishwasher for cleaning, but not all reusable water bottles are dishwasher safe, and/or the diameter of the bottle mouth may not permit water and detergent to enter with sufficient force to coat the interior surface.

Improperly cleaned water bottles may present a potential contamination risk and thus be considered a risk for foodborne illness, particularly to those at higher risk such as immune compromised people, older adults, and young children. Microorganisms will normally grow in water and on surfaces in contact with water as biofilms *(3)*. The availability of nutrients and lack of residual disinfectant are some of the principal determinants of microbial growth in drinking water *(3)*. According to the FDA Food Code *(16)*, water is considered a food. Reusable bottles are therefore food-contact surfaces requiring proper cleaning and sanitizing. Unfortunately, consumers may not be aware of the potential hazards related to water bottles; thus, there is a possibility for complacency with regard to cleaning behaviors.

It is recognized that water can be a source of disease outbreaks *(27, 42)*. Despite worldwide efforts and the modern technology employed for production of safe drinking water, transmission of waterborne diseases is still a matter of major concern *(9, 37)*. Some common foodborne organisms associated with water include *Campylobacter*, *E. coli* O157:H7, *Salmonella*, and *Vibrio cholerae*, to name a few *(7)*. These can lead to severe illnesses and death. Clearly, there are health implications associated with unclean water consumption.

Combined, the clues point to the possibility of a large and growing problem. Reusable water bottles are growing in popularity, but consumers may not perceive the importance of cleaning water bottles as other food-contact surfaces must be cleaned, which can result in careless behaviors with regard to their cleaning. The difficulties associated with cleaning the bottles adequately, as well as the variability in designs and materials, make it easy to see the potential food safety hazard. Previous studies have conducted microbiological evaluation of bottled water *(18, 22, 33, 38)*, as well as cleaning protocols of infant feeding bottles *(24, 26)*. Oliphant, Ryan, & Chu *(30)* studied water quality in the personal water bottles of 75 elementary students and stated that the use of personal water bottles for students in elementary classrooms is not recommended because of the significant microbial contamination levels of the water in the bottles. However, limited information has been published on the cleanliness of reusable water bottles and consumer behaviors related to reusable water bottles. Therefore, the purpose of this study was two-fold: first, to measure contamination levels of water bottles that are in use, and second, to understand how contamination levels are affected by bottle usage and cleaning behaviors, by collecting survey data from the bottle owners.

Three methods were used to assess water bottle contamination. First was the use of adenosine triphosphate (ATP) bioluminescence on the exterior surface of water bottles. ATP tests provide evidence on the level of general cleanliness *(19)* by measuring organic materials *(41)*, with results reported in terms of RLUs (relative light units). Results are obtained rapidly but are considered only a generalized assessment that cannot provide information on the identity of organisms present in a sample *(25)*. Because of this, the second method quantified microbial contamination through a heterotrophic plate count (HPC) from the bottles' interior. "Heterotrophic bacteria" include all bacteria that consume organic nutrients for growth. These bacteria are universally present in all types of water, food, soil, vegetation, and air *(2)*. The heterotrophic plate count is a means of assessing the concentration of these bacteria in foods and water *(13)*. Enumeration of total heterotrophic counts is commonly used as an indicator of overall microbiological quality *(12, 32, 40)*, and results are reported as colony forming units per milliliter (CFU/mL). Finally, coliform testing was used to assess more potentially risky coliform bacterial contamination. Coliform organisms have long been recognized as a suitable microbial indicator of drinking-water quality, largely because they are easy to detect and enumerate in water *(9, 40)*.

MATERIALS AND METHODS

This study was piloted initially to ensure adequacy of the design, and a mixed methods approach was adopted in the full study. The bottle exteriors were assessed for organic contamination by ATP Bioluminescence testing. The bottle interiors were assessed for microbial contamination by use of Heterotrophic Plate Counts (HPC) and Coliform Plate Counts. Lastly, the bottle owners were asked to complete a digital questionnaire intended to document routine behaviors related to the use and cleaning of the reusable water bottles. Routine behaviors, bottle cleaning, and design of the bottles (documented during the surveys) were paired with the cleanliness assessment results through the use of anonymous coding numbers.

A total of 90 water bottles were collected from the participants. As shown in *Table 2*, 65 (72.22%) were hard plastic bottles, 22 of which had a straw or nozzle. The third commonly used type of water bottle was a squeezable bottle (13.33%), followed by metal bottle (11.11%), and glass bottle (3.33%). Built-in carbon filters were also investigated; nine out of ninety (10%) bottles had a built-in carbon filter.

Sample collection and surveying process

The sample collection and surveying process was set up in a linear fashion in a high traffic corridor of a Midwestern college campus building. Passersby were asked if they had a reusable water bottle with them and whether they would be willing to participate. Upon agreement, they handed over their water bottles, which were then emptied of any liquid. Gloved researchers took each bottle, labeled it with a sequential number, and, using an iPad set to the Qualtrics survey, took an embedded photo of the bottle and label. The iPad was then handed to the respondent, who completed the survey. Surveys were coded with an anonymous number, which was paired to the contamination testing by the use of adhesive labels with matching numbers.

Exterior surface testing by ATP bioluminescence

While the respondent was completing the survey, the researchers placed a 4" × 4" template over the middle of the bottle. This template was used to ensure that ATP swabbing was consistent across all bottles. Ultrasnap ATP test strips (Hygiena, Camarillo, CA) were removed from a refrigerator and were allowed to warm up to room temperature for ten minutes prior to use. The delineated space was swabbed vertically and horizontally while the swab was rotated and slight pressure was applied, after which the swabs were run through the SystemSURE plus luminometer (Hygiena, Camarillo, CA).

Interior surface sampling

Two 100 ml bottles of prepared 3M Phosphate Buffer solution plus Tween20 (PBS-T) were added to each water bottle. The solutions were prepared by taking purchased 99ml bottles of sterile Phosphate Buffer Solution (PBS) and aseptically pipetting 1 ml of 10% Tween20 to each bottle, thus filling the bottle to a full 100 ml. Prepared solutions were held under refrigerated conditions overnight until use.

Once PBS-T was added to each reusable water bottle, the lid was replaced and the bottle was shaken by hand 60 times, using a one-foot stroke. Subsequently, the sample was aspirated through the opening of the bottle (either poured or squeezed); a minimum of 80% of the PBS-T volume was collected in a new, sterile container. Two new water bottles were purchased from a local retailer and processed through the same ATP and microbial testing procedures as a means of control and comparison. Once all bottles were photographed, swabbed, and sampled, the PBS-T samples were shipped overnight in refrigerated conditions to the NSF International Applied Research Center (789 North Dixboro Road, Ann Arbor, MI 48105) for HPC and coliform counts analyses.

HPC and coliform counts

Membrane filtration and pour plating were used to enumerate heterotrophic bacteria and coliform bacteria from PBS-T samples, respectively. Samples were diluted in PBS as needed to ensure reliable colony counts. For HPC processing, aliquots were pour plated with Standard Plate Count (SPC) agar (one agar plate per dilution). For coliform processing, 100 mL of each sample was filtered through a 0.4 micron pore size membrane and plated onto mEndo agar. All plates were incubated at 35 ± 1°C for 24 ± 2 h. After incubation, colonies were enumerated and reported as CFU/mL of eluent for HPC and CFU/100 mL of eluent for coliforms.

Post-test cleaning

Once the respondent moved through the sample collection and surveying process, their water bottles were subsequently cleaned through the use of a standardized three compartment sink cleaning process, which included (1) washing in soapy water (Apex™ Presoak detergent, Ecolab Inc.), using a bottle brush to clean the interior of each bottle, (2) rinsing in clean tap water, and (3) immersing in a sanitizing solution of approximately 300 ppm Oasis 146 Multi-Quat sanitizer (Ecolab Inc.) for a minimum of five seconds, after which the bottle was inverted to allow the interior to air/drip dry, while the exterior was wiped dry. In addition to cleaning the bottle, the owner was offered a choice of cookie as a thank you for completing the testing and surveying process.

Survey design

The survey assessed respondents' behaviors in terms of bottle usage and cleaning *(Table 1)*. The first section of the survey was descriptive in nature, asking respondents to describe their bottle both in text and by selecting a graphic representation. Respondents were then asked to estimate the age of their bottle, and also to indicate what types of liquids (water, soda, juice, energy drinks, etc.) they had put into their bottle over the prior seven days. The following questions addressed the frequency of bottle use (both filling and refilling) as well as how often they emptied their bottle and whether or not they had shared their bottle with others. The second section of the survey focused on cleaning behaviors, with questions pertaining to frequency of cleaning and method of cleaning. The last section solicited demographic information, such as gender and age. The survey was submitted to the University's Human Research Protection Program for approval by the Institutional Review Board, which was granted prior to commencing the study

Statistical analysis

Upon completion of data collection, a database of ATP, HPC and coliform counts was compiled and sequenced by

TABLE 1. Survey about use and cleaning of reusable water bottles

*Which of the following best describes your water bottle?	• Hard Plastic • Straw/Nozzle • Collapsible	• Squeezable • Wide-mouth • Metal	• Other
Does your water bottle contain a built-in filter?	• Yes	• No	
How old is your water bottle?	• Less than 3 months • 3–6 months	• 6–12 months • More than 1 year	
*What was in your bottle in the past 7 days?	• Water • Soda • Juice	• Coffee/tea • Sports/energy drinks • Milk/soy-based drinks	• Sliced fruit • Other
How many days a week do you use your bottle?	• 1 day per week • 2 • 3	• 4 • 5 • 6	• Every day
How often do you refill your bottle each day?	• Times refill______		
When do you empty out the contents of your bottle?	• Never. I just add more to what was left from the day before • I don't have to dump it because I usually drink everything • At the beginning of the day when I fill it • At the end of the day	• More than once a day	
Have you ever shared your water bottle with someone else?	• Yes	• No	
*Do you clean your water bottle?	• No • Yes, I rinse it with water.	• Yes, I wash it with soapy water/cleaning tools/dishwasher.	
How often do you rinse your water bottle?	• Never • Once a day	• Twice a day • 2–3 times a week	• Once a month • 2–3 times a month
*Do you do any of the following when you wash your bottle?	• Wash with soapy water/baking soda/cleansing tablets • Use cleaning tools	• Dishwasher	

Continued on the next page

TABLE 1. Survey about use and cleaning of reusable water bottles (cont.)

When was the last time you rinsed your bottle?	• Today • Yesterday	• 2–3 days ago • 4–7 days ago	• More than 7 days ago
When was the last time you washed your bottle?	• Today • Yesterday	• 2–3 days ago • 4–7 days ago	• More than 7 days ago
When you are sick, do you change the frequency of bottle cleaning?	• Yes, I clean my bottle less frequently • Yes, I clean my bottle more frequently • No, the frequency is about the same	• Other	
What is your gender?	• Female	• Male	
What is your year of birth?	• Year ________		
What is the highest degree you have completed?	• Less than high school • High school or equivalent • Associate's degree	• Bachelor's degree • Master's degree • Professional school degree	• Doctorate degree

**Question marked with a "*" has a statement "check all that apply."*

TABLE 2. Effect of bottle material on ATP readings on exterior bottle surfaces

Bottle Material	*n*	ATP readings in RLUs				F-value	*P*-value
		Mean	Standard Deviation	Minimum	Maximum		
Glass	3	281.70[a]	66.11	211	342	2.35	0.08
Hard Plastic	65	375.70[ab]	351.70	43	2165		
Soft Plastic	12	391.10[ab]	258.10	47	1034		
Metal	10	732.90[b]	789.50	32	2510		

**Means with the same letter do not differ significantly at $P < .05$.*

bottle number. Survey responses were downloaded from Qualtrics, and behavior and frequency data were merged into the database for analysis. Relative cleanliness and demographic data were gathered separately; however, bottle numbers permitted the association of this data with their respective cleanliness data.

The ATP, HPC and coliform counts of 90 water bottles were analyzed statistically to compare the effect of bottle design, bottle usage, bottle cleaning behaviors, and demographic information. Statistical analysis was carried out using the one-way or two-way analysis of variance ANOVA in SAS, version 9.4, statistical program (SAS Institute, Cary, NC) to assess significant influence ($P < 0.05$).

RESULTS

One of the overall goals of this study was to quantify the contamination levels of 90 water bottles collected from the participants. Results of ATP swabbing ranged from a minimum of 32 RLU to a maximum of 2510 RLU, with a median of 300 RLU. HPC results ranged from a minimum of < 1 CFU/mL to a maximum of 8.03×10^6 CFU/mL, with a median of 1115 CFU/mL and a SD of 8.46×10^5. The value "< 1" here indicates undetectable heterotrophic bacterial contamination and was used to differentiate from the value zero, which indicates a sterile sample. For the purpose of calculation, the "< 1" value was coded as "0.5." Coliform counts ranged from < 1 CFU/100mL to a maximum of > 150 CFU/100mL, with a median of 0.5 CFU/100mL.

Effect of bottle design

Bottle design was coded according to survey results and photos. To further investigate the effect of different bottle designs on contamination level, ATP, HPC, and coliform counts were analyzed by bottle type, built-in filter and their interaction, using a two-way ANOVA. Two bottles with straw/nozzle, six squeezable bottles, and one metal bottle were found to have a built-in filter. The F-test concluded that no significant difference of HPC and coliform counts was observed between bottles with and without built-in filters ($P = 0.10$ and 0.95, respectively). However, when bottle type was recoded according to material (hard plastic, soft plastic, metal, and glass), an F-test using one-way ANOVA found that the impact of bottle material on ATP readings approached significance ($P = 0.08$). While 0.08 does not meet the stated significance threshold of 0.05, it is still worth noting for future studies and reference. As shown in *Table 2*, the ATP readings of glass bottles were found to be significantly lower than the readings of metal bottles.

Effect of bottle usage

As shown in *Fig. 1*, the age of water bottles was approximately evenly distributed, with 27.8% of the respondents indicating that their bottles were less than three months old. The majority (81.82%) of respondents indicated that water was the most common liquid placed in the bottles, followed by coffee/tea (6.82%), sports/energy drinks (4.55%), and soda (4.55%) *(Table 3)*. The HPC and coliform readings in bottles that had beverages (namely coffee/tea, sports/energy drinks, soda, juice, and sliced fruit) were significantly higher than those in bottles used only for water ($P = 0.02$ and $P < .01$, respectively).

Most respondents indicated that they used their bottles frequently, with 63.33% reporting that they use their bottles every day of the week, followed by 14.44% who indicated that they use their bottles five days a week. About a third of the respondents (27 out of 90, or 30%) reported refilling their bottles three times per day, followed by 20 (22.22%) refilling it twice a day. Results showed that the frequency of bottle use had a significant impact on the ATP readings (P = 0.04), and an F-test using simple linear regression indicated that the ATP readings were positively associated with daily

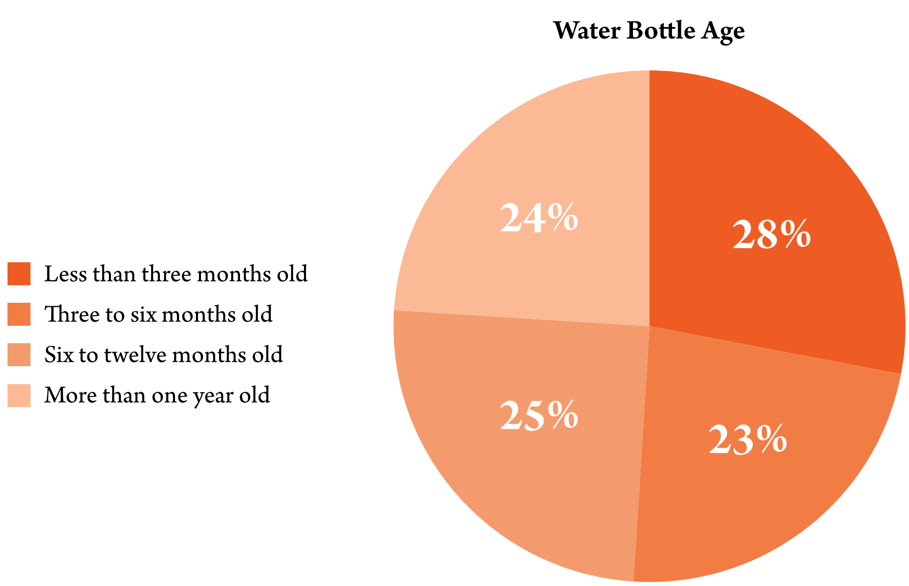

FIGURE 1. The distribution of water bottle age

TABLE 3. The effect of beverage in the bottle in the past 7 days

Beverage	n	Proportion	HPC Results in CFU/mL					Coliform
			Mean	Standard Deviation	Minimum	Maximum	P-value (Mann–Whitney U test)	P-value (Mann–Whitney U test)
Water	72	81.82%	7.07×10^3	1.44×10^4	< 1	6.62×10^4	0.02	< .01
Other beverages, include coffee/tea, sports/energy drinks, soda, juice, and sliced fruit	16	18.18%	5.54×10^5	2.10×10^6	4	8.03×10^6		

**Two respondents indicated there was nothing in the bottle and were excluded from beverage analysis.*
**The "< 1" values were coded as "0.5" in order to calculate means.*

refill frequency ($P < .05$). However, the effect of weekly bottle use frequency and daily refill frequency on HPC and coliform results was not significant.

With regard to when people empty their bottles, 25 (27.78%) respondents indicated that they don't dump their bottles because they usually drink everything. On the other hand, 14 (15.56%) respondents indicated that they never dump the contents of their bottles and just add more to what was left from the day before. In addition, 16 (17.78%) indicated that they empty out their bottles more than once a day. A one-way ANOVA revealed that the impact of dump frequency on ATP, HPC, and coliform results was not significant ($P = 0.63$, 0.34, and 0.64, respectively; data was not included in the table).

Interestingly enough, more than half (51.11%) of the respondents indicated that they have shared their bottles with others. Again, no significance was found on the effect of sharing behavior ($P = 0.76$, 0.52, and 0.52, respectively) at the 0.05 level.

Effect of bottle cleaning behavior

With regard to cleaning behavior, the majority of respondents (84.44%) indicated that they cleaned their bottles either by rinsing, washing, or both. Only 14 (15.56%) indicated that they never clean their water bottles. Of those people who indicated they rinse their bottles, 46.34% indicated that they rinsed every day and 39% indicated that they had most recently rinsed their bottles on the previous day. Of those who wash their bottles, 40% indicated that they wash once a month, with 14 (31.11%) reporting that the most recent time that they washed was more than seven days ago. The dominant method of bottle washing was washing with soapy water/baking soda/cleansing tablets (75.56%), followed by the use of cleaning tools (e.g., brush, sponge) (46.67%). When they were sick, 45.56% of the respondents reported bottle cleaning more frequently, while 52.22% reported that their bottle cleaning behaviors were about the same.

Clearly, there was a relationship between bottle cleanliness and whether or not people rinsed/washed their water bottles. However, when bottle cleaning behaviors were correlated with contamination results, findings were surprising. Results showed that cleaning behavior has a significant impact on HPC and coliform counts ($P < .05$ and $P = 0.03$, respectively). However, factors such as cleaning frequency, last time of cleaning, cleaning method, and whether behavior changed during illness were not significant *(Table 4)*.

Demographics

Gender, age, and education level were not significantly associated with contamination level of water bottles. Of the ninety respondents approached, 68.89% of respondents were female, and the majority (80%) reported being 18–29 years of age. Overall, all of the respondents had at a least high school level education. 43.33% had a high school diploma or equivalent as the highest degree they received, followed by 31.11% with a bachelor's degree earned.

DISCUSSION

Bottled water consumption has steadily increased over the past 15 years, according to a report published by Business Wire *(6)*. The global bottled water market, which

TABLE 4. The effect of cleaning behaviors

				ATP	HPC	Coliforms
Whether people clean their bottles	**Mean**	1. Never		404.10	9.67×10^3	0.75
		2. Rinse		464.00	2.88×10^4	5.63
		3. Wash		385.90	7.78×10^3	9.73
		4. Rinse & wash		374.10	8.12×10^5	38.10
			F-statistic	0.23	2.73	3.18
			***P*-value**	0.88	0.05	0.03
Rinse frequency & last time rinsed			**F-statistic**	0.07	0.17	1.34
			***P*-value**	0.98	0.92	0.28
Wash frequency & last time washed			**F-statistic**	0.28	0.51	0.21
			***P*-value**	0.84	0.68	0.89
Wash method	**Mean**	1. Soapy water		358.60	8.46×10^3	36.42
		2. Tools		217.00	1.67×10^4	0.50
		3. Dishwasher		394.60	3.49×10^3	0.56
		4. Soapy water & tools		480.90	6.67×10^5	1.04
		5. Soapy water & dishwasher		197.00	1.63×10^3	22.83
		6. All of above		402.50	1.12×10^4	26.83
			F-statistic	0.59	0.51	1.16
			***P*-value**	0.70	0.76	0.34
Bottle cleaning behaviors while sick	**Mean**	1. I clean less frequently		-	-	-
		2. I clean more frequently		414.60	4.46×10^3	2.93
		3. Frequency is the same		411.60	4.41×10^4	15.97
		4. Other		473.00	1.89×10^5	18.00
			F-statistic	0.02	0.55	1.80
			***P*-value**	0.98	0.58	0.17

is dominated by Europe and the United States, is expected to reach $70 billion by 2017 and will be stimulated by rising population and consumer spending patterns *(14, 31)*. However, that increase came at the expense of increased pollution and landfill waste, which has led to a growing trend toward reusable water bottles. These bottles come in all shapes and sizes and are made of a variety of materials, ranging from plastic to glass to aluminum. Bottle features include straws, folding spouts, and filters, all of which can influence cleanability. Cleanability aside, if people do not make a significant effort to clean their bottles, then clearly the bottles can become a marked source of microbial contamination. The propensity for sharing bottles can cause reusable bottles to represent a marked means of transmitting foodborne illnesses, not to mention other illnesses that are spread through saliva, such as influenza.

This study revealed a marked contamination level among reusable water bottles that are in use. According to the ATP manufacturer, surfaces are considered clean if the readings are at or below ten RLUs. Readings that range from 11 to 29 RLUs are considered inadequately cleaned, while those exceeding 30 RLUs are considered dirty. ATP readings indicated that the exterior surfaces of all the water bottles collected (including two new bottles that were purchased from a local retailer as controls) were considered dirty and failed the ATP test. In addition, heterotrophic plate counts were tested as an overall indicator of the quality of water that had been in the water bottle. According to Allen, Edberg, & Reasoner *(2)*, the number of HPC bacteria in drinking water varies widely, and it is not surprising that different countries establish different upper HPC limits in drinking water. The Netherlands, Sweden, and Germany have set a regulation of 100 CFU/mL for drinking water *(3)*. The maximum HPC level of bottled water legally permitted in Taiwan is 200 CFU/mL *(33)*. The microbiological standard of bottled water in Massachusetts in the United States follows a guideline of 500 CFU/mL for HPC *(21)*. The HPC results of the two control bottles were less than 1 CFU/mL. Of the PBS-T solutions collected from the interior surfaces of 90 water bottles, 63 (70%) were above the limit of 100 CFU/mL, 60 (66.67%) were above 200 CFU/mL, and 54 (60%) had a HPC result higher than 500 CFU/mL. Moreover, coliform bacteria were also found on the interior surfaces of respondents' water bottles. According to the standard of microbiological quality established by FDA *(15)* for total coliforms in bottled water, coliform counts shall not exceed 1 CFU/100mL. Of the 90 samples analyzed for total coliforms, 21 (23.33%) contained more than 1 CFU/100mL. Four samples were reported with coliform counts greater than 150 CFU/100mL.

While microbial counts were not determined for exterior bottle surfaces, ATP levels suggest these surfaces may serve as fomites that facilitate the transmission of infectious organisms *(36)*. The data clearly demonstrate that the exterior cleanliness of reusable water bottles is associated with bottle materials. As this study showed, among the four bottle materials examined (hard plastic, soft plastic, metal, and glass), glass bottles have the lowest reading of organic residue on the exterior surface. This might be attributed to the fact that glass is nonporous and easy to clean, but perhaps more importantly, it is easy to see when it is sufficiently clean, compared to plastic or metal bottles.

The exterior cleanliness of reusable water bottles was also affected by the frequency of bottle use per week and the frequency of bottle refilling per day. Generally speaking, more frequent bottle refilling was associated with higher levels of contamination. Possible reasons include that each time people refill the bottle, the bottle might touch a dispenser or have it drip onto the outside of a bottle, providing a good source of moisture and nutrients for the growth of microorganisms. A bottle owner's hands could also be a source of contamination. Even if people wash their hands on a regular basis, these uncleaned surfaces may potentially re-contaminate hands and thus cause health issues, especially with use of those bottles with a straw or nozzle that require touching the drinking surface with fingers in order to open and close it.

Moreover, the results obtained for bottle usage behaviors show that the interior cleanliness of water bottles is affected by different types of beverage. Bottles that had beverages such as coffee/tea and soda in the past seven days were found to be more contaminated than bottles that had only water. This might be partially due to the fact that coffee and tea leave residues that are hard to remove from food-contact surfaces, and that soda contains sugar, which cannot be removed easily by rinsing. Although studies have shown that caffeine at high or low concentrations was effective at inhibiting several strains of bacteria *(1, 28)*, the sugar and cream that may be added to coffee and tea could provide nutrients for bacterial growth. A study that tested microbial contamination in unfinished beverages, including coffee with milk, green tea, apple juice drinks, and carbonated drinks, found that pH, temperature, additives or ingredients (such as carbon dioxide) are important for microbial growth in beverages. The same study found that unfinished beverages support microbial growth and can contain foodborne pathogens and bacterial toxins *(39)*. Bacterial cells are more likely to adhere to and interact with surfaces that are improperly cleaned and sanitized *(5)*. It is possible that residual nutrients are the cause of the attachment by sugar and other additives. Moreover, coliform bacteria are normal inhabitants of plant material such as tea leaves *(43)*. If tea is brewed at inadequate temperatures, or if it is stored for too long or in an improperly cleaned container, coliform bacteria may grow in it. In addition, bacteria grow most rapidly in the range of temperatures between 40°F and 140°F, doubling in number in as little as 20 minutes *(34)*. It could be that the temperature of slowly cooling coffee/tea could encourage bacterial growth. Therefore water bottles which had coffee/tea/soda/energy drinks inside may have been more susceptible to microbial contamination.

More than half (51.11%) of the respondents indicated that they shared their bottles with others, a possible cross-contamination risk. Researchers have suggested that hundreds of bacteria species (around 700) exist in the human oral cavity *(8, 23)*. Harmful bacteria from saliva will reproduce inside the bottle. When taking a drink, reflux can occur into the liquid in the bottle, which is also known as "backwash." Saliva not only provides the bacteria for transmission but may also provide nutrients allowing the microorganisms to multiply. In the possibility of virus transmission from saliva means that sharing water bottles with someone else could potentially lead to health issues.

When the impact of people's cleaning behavior on the interior bioburden of water bottles was examined, it appeared that whether or not people rinsed/washed their water bottles affected the contamination level. However, factors such as cleaning frequency, last time of cleaning, cleaning method, and whether behavior changed during illness when sick were not predictive of contamination levels. There are some possible explanations regarding the ineffectiveness of different cleaning behaviors. First, it may have been caused by limitations of a self-report questionnaire. Participants may over or under-report their performance, either because they cannot remember clearly or because they wish to present themselves in a socially acceptable manner, which is referred to as social desirability bias *(20)*. Another limitation of the self-report questionnaire is the unequal sample sizes between variables that the questionnaire is assumed to measure. For instance, only 17 respondents reported that he/she used the dishwasher to clean bottles, while 34 reported that they wash with soapy water. Such unequal sample sizes may potentially affect the validity of analysis. This might be an area for further research with a larger sample size. Lack of significance may also have been caused by other factors, for instance, the cleaning methods that people adopted may have been insufficient to reduce contamination to safe levels. Bacteria are naturally present in drinking water and can reproduce in the presence of nutrients in saliva and backwash *(3, 10)*. Significant bacterial growth has been shown to occur in treated, chlorinated water left at room temperature *(17, 30)*. It is possible that cleaning the day before may not be adequate to result in a low bacterial count. Reusable water bottles need to be thoroughly sanitized to reduce the number of harmful microorganisms to safe levels. Many residential dishwashers have sanitization cycles that achieve this goal *(29)*. Future studies may wish to sample the water bottles after cleaning procedures have been performed. Emptying out the contents and rinsing/washing the bottle each time before refilling could also be a suggestion and an area for further research.

In conclusion, although reusable water bottles offer a green and healthful way to drink more water without increasing landfill waste, improperly cleaned water bottles may present a potential reservoir for bacterial colonization and thus be a risk for foodborne illness. Safe use of reusable water bottles should include cleaning that effectively removes all residues and disinfects the bottles on a regular basis. For water bottles that are not dishwasher safe, or for residential dishwashers that do not have sanitization cycles, future studies should determine "best practices" for effectively cleaning and sanitizing water bottles in a home environment with minimal special tools or procedures, in order to maximize user adoption.

Significant levels of contamination were clearly evident for ATP, HPC, and coliform tests in this study and should be a cause for concern. In addition, one of the most interesting results of this study was that use of water bottles for other beverages results in significantly higher levels of contamination. Apparently, water bottles are actually very well-named, and they should be used only for water.

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