The Dental Fluoridation Potential of Drinking Water

by
Karissa Renyer

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in BioHealth Sciences
(Honors Scholar)

Presented May 29, 2020
Commencement June 2020

AN ABSTRACT OF THE THESIS OF

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Abstract approved:_____
Philip McFadden

Water fluoridation is considered one of the top ten greatest health achievements of the twentieth century due to its effectiveness in reducing the prevalence of dental caries. However, despite extensive research on the subject, there is little information currently available about how the constituents of drinking water affect the uptake of fluoride by tooth enamel. This study investigated how several key components and characteristics of drinking water influence fluoride uptake and developed a corresponding model system for testing fluoride uptake. The experimental variables included the concentration of calcium and magnesium ions, degree of water hardness, and pH and buffer strength; a final geographical experiment was also performed that analyzed the effectiveness of fluoride uptake in several different municipal water samples. Overall, standard levels of calcium and magnesium ions, water harness, and buffer strength did not affect fluoride uptake, and a pH of 6.0 was optimal for fluoride uptake. Notably, there was no significant difference in fluoride uptake between the water samples from different regions, indicating fluoride is a resilient ion likely be incorporated in enamel if originally present in the water.

Key Words: fluoride, water, dental, hydroxyapatite, calcium, magnesium, water hardness, pH
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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

_____

Karissa Renyer, Author

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Preface

To begin, I want to give you some background on my story, as I believe it will help you understand why I chose my project. I am a native Oregonian, and in the fall of 2016, I began my journey at Oregon State University. I had been accepted into the University Honors College (UHC) without fully realizing what taking on a thesis would entail. From the beginning, I knew I wanted to pursue a topic that was applicable to my future profession. However, as a pre-dental student at a university without a dental school, I knew I would need to be creative to find a research project in my field of interest.

I have grown up around dentistry my entire life, as my dad is a practicing general dentist. Initially, I found my love of this profession when I worked in his office for my first summer job, sterilizing instruments and cleaning rooms between patients. In my free time I would shadow different appointments, and the more I saw the more I was fascinated. I remember one particular patient who started to tear up when her veneers were placed. I had never realized before that such a small change could have such an immense impact on a person. That was when I knew I wanted to pursue dentistry as my profession.

By a stroke of good fortune in my sophomore year, I ended up stumbling upon another UHC thesis that was written by a student very similar to me. Audrey Riesen had also been a pre-dental student, and her thesis tackled the nuances of art and science and the relationship between the two, often separated, subjects. If you get a wild hair, a quick search for, “The Choice: Art or Science,” in the UHC thesis archive should take you to her paper. Her content resonated with me so profoundly because I had also been raised in a household where both art and science were valued subjects. For a science student, I believe I have had a much greater background in and appreciation for art than most of my peers, like Audrey. Once I read her paper, I decided to meet

with her mentor, Dr. Phil McFadden, Ph.D., a professor I myself had had in a UHC colloquia class, BB407H: Protein Portraits, the year prior.

When I originally met with Phil, we talked about the thesis in great depth. What was so wonderful was that he also shared the immense appreciation for art's place in science and vice versa, and it quickly became apparent that we shared many similar views. As the conversation progressed, Phil tentatively offered to provide mentorship for my own thesis, although neither of us had a specific project in mind at this point.

I reached out to a family friend, who was a general dentist and owner of a local dental product company, Dr. V. Kim Kutsch, D.M.D. He himself had conducted numerous dental-related research projects over the years, so he was able to give me several potential topics to investigate for my thesis. After doing some literature searching and discussing with Phil, I decided to go ahead with a topic related to fluoride's impact on teeth.

When I started my project in April 2018, I did not know where it would take me, both professionally and personally. Before, I thought I understood the basics of water fluoridation, through common knowledge and my exposure to dentistry. However, as I dug further into the literature and into conversations with Phil and dental health professionals, I found the subject was more complicated than I originally anticipated, and there were holes in the topic. I spent hours researching the mechanism of fluoride uptake, fluoride's impact on dental caries, ideal conditions for fluoride effectiveness, fluoride content in dental products, and so much more, with each specific topic generating thousands of search results. Eventually, as I went down a rabbit hole, I found a gap. There were no papers that discussed how the different components of drinking water affect fluoride uptake. I thought this was strange, considering how fluoridated

water is such a controversial topic. It seemed that every study that could have been done about drinking water and fluoride should have already been done, but that wasn't the case.

When I shared this revelation with Phil, we both knew the value of this missing information. Wouldn't people want to know how their local drinking water was affecting their oral health? I believe the experiments I performed and the content in this thesis are applicable to everyone. That is the brilliance of working with something that is a universal need—water. In fact, water fluoridation has been studied by biologists, chemists, physicists, epidemiologists, and so many other types of professionals (in addition to dentists), which I believe demonstrates how important this topic is to everyone, not just those in dentistry. As I progressed through this journey, I learned about many pieces of the fluoride puzzle that had previously been missing. I hope that you also gain a clearer picture regarding fluoridated drinking water and fluoride uptake into teeth as you delve into my work.

Chapter 1: Water you even talking about?

The degree of separation between the medical and dental professions is quite astounding because the two disciplines are undeniably intertwined. Increasingly, scientific research has been demonstrating the significant impact oral health has on overall health, and vice versa. Study after study shows how poor oral health and dental disorders have been linked to chronic systemic inflammation, bacteremia, insulin resistance and diabetes, cardiovascular disease, lung disease, neurologic disease, and pregnancy complications.(1) It is obvious we can no longer turn a blind eye by considering the oral cavity to be an isolated system.

Dental disease is more prevalent in those with lower socioeconomic standing as dental care is often unaffordable for this population.(1) This issue is magnified by the challenge of being able to consistently afford and consume a healthy diet.(1) Lack of a healthy diet and access to quality dental care creates many issues that negatively impact both oral and overall health, which snowball to create costly health problems. It is, therefore, imperative for affordable dental products to be available to the public. Although it is not a traditional dental "product," fluoridated water has been shown for decades to reduce the prevalence of dental caries, the most common chronic disease in children.(2) The Centers for Disease Control and Prevention (CDC) has found fluoridated water to be the most cost-effective and efficient way of providing the benefits of fluoride to the general population, regardless of age, income, or degree of education.(3) Overall, maintaining a healthy oral cavity is a key component of achieving overall health and well-being, and fluoridated drinking water is an essential way of helping protect the oral health of the public.

A History of Water Fluoridation:

While dental professionals began noticing the potential effects water could have on dental caries formation in the early 1900s, it was not until 1931 that fluoride was clearly identified as the cause.(4) Early research in the field revolved around excess fluoride and its association with dental fluorosis, but studies shifted to investigating fluoride's effects on dental caries and the minimum fluoride concentration that could reduce caries incidence while avoiding dental fluorosis.(5) The number that quickly came to light was 1 ppm fluoride, for caries prevention was not improved in concentrations higher than 1 ppm, and dental fluorosis became increasingly prevalent as the concentration increased past that point.(5) In 1945, trials began for adding fluoride to drinking water to the 1 ppm F^- threshold, with results appearing a few years down the road. Generally, caries incidence in children who consumed fluoridated water was cut in half.(5) While the incidence of dental fluorosis had also increased to 11 percent, most cases were very mild or questionable as to whether they existed at all.(5)

As decades passed, research led to models for testing fluoride uptake, information on caries prevention, and delivery of fluoride in ways beside drinking water. Toothpastes, mouth rinses, milk, and dietary salt all became commonly available products containing added fluoride, and fluoride tablets and lozenges also arose.(5) However, the adoption of these products brought another rise in dental fluorosis prevalence, as the exposure to fluoride-containing products, in addition to fluoridated water, results an increased amount of fluoride exposure.(5) Thus, the original water fluoridation guidelines needed revising to correct for the additional fluoride that had become commonplace. In 2015, the U.S. Public Health Service (USPHS) updated the recommendation for drinking water to contain an optimal level of 0.7 ppm F^-.(6)

Dental fluorosis is the hypomineralization of tooth enamel or dentin that results from the overexposure to fluoride during the formation of teeth, prior to their eruption.(7) The most common classification scheme of dental fluorosis is based on the Dean's classification system, developed in 1934.(5) Stages range from normal to severe, and geographical areas with more than 10 percent of the child population exhibiting mild to severe dental fluorosis are considered to have endemic dental fluorosis.(5) The Tooth Surface Index of Fluorosis (TSIF) may also be used to assess the risk of dental fluorosis.(7) Over the course of a person's life, the severity of dental fluorosis generally decreases compared to when the teeth first erupt.(8) This is due to use of the teeth and post-eruptive maturation, offering some relief to those who initially show fluorosis symptoms on their permanent teeth.(8)

A Background on Fluoride and Hydroxyapatite:

The fluoride ion, F^-, is a monatomic fluorine anion, and it is known to inhibit various enzyme systems and bind to calcium ions, Ca^{2+}.(9) It is naturally found in 0.06-0.9 percent of the Earth's crust and is also present in many minerals.(10) All natural sources of water contain some amount of fluoride, with sea water around 1 ppm F^-, rivers and lakes around 0.5 ppm F^-, and groundwater containing a variable amount.(10) Higher concentrations of fluoride are usually observed in groundwater from aquifers with less calcium.(10) The fluoride ion is also found in many different types of food, with higher levels in barley, rice, yams, and tea and lower levels in fruits, vegetables, and meat.(10)

Hydroxyapatite, $Ca_5(PO_4)_3(OH)$, is a mineral that comprises 70 to 80 percent of tooth enamel (the outermost layer) and dentin (the middle layer).(11) It is a durable mineral that maintains its size under different temperature and pH conditions, and it has long been used in

medicine and dentistry for bone repair and dental therapies.(11) More recently, nano-hydroxyapatite particles have been produced that are close in size to the hydroxyapatite crystals naturally present in teeth. Recent dental research has shown these nano-hydroxyapatite particles to be effective in aiding the natural remineralization process of tooth enamel.(12) This has led to its incorporation in various dental products, such as toothpastes, to aid in caries-prevention.(13)

Fluoride in Action:

To know how fluoride can reduce the incidence of dental caries, we must first understand how caries form. The mouth undergoes cycles of pH changes throughout the day that correspond to eating and/or drinking.(4) These pH changes are caused by certain microbes in the mouth producing lactic acid when they are provided carbohydrates as a food source.(4) From there, the top layer of enamel starts to dissolve, with the nano-hydroxyapatite particles becoming incorporated into the surface biofilm. These episodes are followed by pH recovery in healthy individuals, the result of buffering agents found naturally in the saliva and provided by other bacteria; this process facilitates the reincorporation of nano-hydroxyapatite particles back into the enamel surface.(12) The remineralization is achieved via reoriented attachment of the hydroxyapatite crystals, not an ionic interaction, which is a common misconception of the process. This dynamic cycle occurs many times throughout the day, with demineralization caused by food and/or drink consumption always followed by remineralization, which creates an equilibrium. In individuals where this process is out of balance, abnormally long periods of exposure to the low pH environment results in net mineral loss from the enamel.(12) When the loss of enamel accumulates, a cavity develops.

There have been hundreds of articles over the years documenting the process of how fluoride protects enamel from the formation of dental caries, and the review "Fluoride Mode of Action: Once There Was An Observant Dentist…" by Cate and Buzalaf provides a nice overview. When fluoride is exposed to the oral cavity, it becomes incorporated into the plaque of the mouth, as a sort of reservoir. It then becomes part of the oral biofilm. From there, fluoride provides protection against the loss of enamel in two different ways. First, calcium and phosphate are both naturally present in saliva; when the oral pH is greater than 5.5 (the critical point), these compounds will precipitate as dental plaque and/or as patching material that fills the micropores in the enamel that are formed during periods of low oral pH. Fluoride aids in this process by coprecipitating the dissolved calcium and phosphate ions, which results in enhanced remineralization of the enamel. Second, at pH values above the critical point, fluoride tends to become incorporated in enamel by replacing the hydroxyl group of hydroxyapatite particles to form the mineral fluoroapatite. Fluoroapatite has a lower solubility than hydroxyapatite, which increases it range of acid-resistance to pH 4.5.(14) This means the fluoridated enamel has increased protection against demineralization. As an aside, fluoride has also been shown to have antimicrobial effects via its ability to interfere with enzymatic functions; this has also been proposed to lessen caries incidence by reducing the degree of acid formation by certain oral bacteria.(14)

The Road Ahead:

Today, we know that the consistent, topical application of fluoride is more important in maintaining oral health and caries prevention than the incorporation of fluoride into the enamel matrix during development.(4) As drinking water constitutes a significant source of oral fluoride,

it is necessary to understand how the drinking water itself affects the process of fluoride uptake into hydroxyapatite. This study will investigate how several components of drinking water affect this process. Specifically, we will be looking at the effects of calcium and magnesium ions, water hardness, and pH. Additionally, as water composition varies based on the source and the processes employed at each municipal water treatment facility, we will compare, in a single laboratory setting, how water samples from around Oregon and the United States affect fluoridation of nano-hydroxyapatite.

Chapter 2: The Model System

Overview:

As we begin our journey to examine fluoride uptake as an aspect of water quality and public health, we need a consistent method to test which components of drinking water affect the uptake of fluoride by tooth mineral. How can we experimentally model a tooth's exposure to drinking water? A model system will allow us to analyze the effects of various components of drinking water in a consistent way.

While studies may eventually be performed on live human teeth under exposure to fluoride, human subjects testing was not ideal given the available resources. An alternative, using extracted human teeth, requires storage in various solutions or autoclaving the teeth, both options that could disrupt the natural anatomy of the tooth.(15) Additionally, it is difficult to control for an "average" tooth. As many extracted teeth are partially or completely compromised, the lack of uniformity across samples poses a problem for being able to isolate a single independent variable for analysis.

As such, we turn our attention to the mineral that accounts for 97 percent of the weight of human tooth enamel, hydroxyapatite.(13) Hydroxyapatite provides a consistent and reliable way to test for fluoride uptake with a material that is very similar to human teeth, and it is an accepted model to represent the inorganic components of teeth.(16) While making hydroxyapatite from scratch is possible, it was advantageous to procure nano-hydroxyapatite in a pre-synthesized slurry form. Obtaining the material from a quality-controlled vendor ensured consistency of the samples of nano-hydroxyapatite, and it provided access to detailed information about the characteristics of each batch (see *Appendix, Section II*).

Starting with preliminary ideas and explorations, I reached a straightforward process by which nano-hydroxyapatite is used as a surrogate for teeth mineral under exposure to controlled compositions of synthetic drinking water. Synthetic water can be created as desired to test for various characteristics of water that could impact the fluoride uptake. Though minor details of the method evolved as the project progressed, the core elements remained consistent. Overall, the procedure is as follows (see *Appendix, Sections III & IV* for complete process):

As shown in *Figure 1*, a 1 mL sample of diluted nano-hydroxyapatite is added to a 1.5 mL centrifuge tube. The sample is centrifuged for five minutes at 10,000 rpm, and then the supernatant is removed via a Pasteur pipette. The supernatant is replaced by a 1 mL sample of synthetic drinking water. The synthetic water, prepared ahead of time, contains the chemical compounds under investigation. The process outlined thus far is repeated for the desired number of samples, which usually includes a control and duplicates or triplicates of each factor to test reproducibility. Next, all samples are placed on a Ferris wheel-like rotator and rotated end over end for a specified amount of time, usually between 20-90 minutes, depending on the experimental variables being investigated. This step is intended to mimic a long duration "swish" that teeth might encounter when exposed to drinking water in the mouth. The samples are then removed from the rotator, and 0.5mL of the supernatant is transferred to a larger centrifuge tube

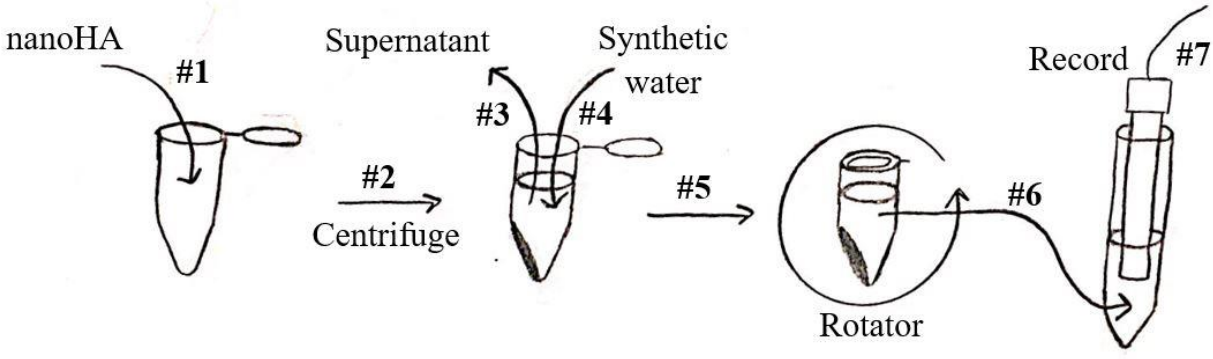

Figure 1: A generalized model for fluoride exposure testing.

that contains 1.5mL of ultrapure water and 2mL of a conditioning buffer used for measuring fluoride (see *Appendix, Section V* for information regarding the ultrapure water, and see *Conditioning Buffers* section below for further details on the buffers used). Finally, the samples are measured individually with a fluoride ion-selective electrode (ISE). Before each sample is measured, the electrode is rinsed with ultrapure water and gently dabbed dry with a delicate task wiper. The electrode is exposed to the solution for 60 seconds before recording the millivolt (mV) value. Once the millivolt reading has been obtained for a sample, a calibration curve can be used to determine the final concentration of fluoride remaining in the solution.

Calibration Curve Procedure:

The electrode reading for a solution with an unknown amount of fluoride is referenced to a calibration curve to determine the solution's concentration of fluoride ions. A calibration curve is produced from electrode readings for a series of water solutions containing known amounts of fluoride. See *Figure 2* for a representative calibration curve, one of many used in this study. The y-axis shows the response of the electrode to increasing concentrations of fluoride. Since fluoride is an anion, the more fluoride a solution contains, the lower the millivolt reading will be. The millivolt reading of the electrode (which can be a negative or positive voltage) can be adapted to a graph by adding 100 mV to each reading and then plotting the $\log_{10}$ of the result; adding the 100 mV eliminates calculations using negative values in logarithms while retaining the shape of the graph. A reading of 0 mV therefore is plotted as 2.0 on the y-axis. The electrode I used demonstrated good day-to-day consistency, with readings ranging between about +120 mV and -50 mV.

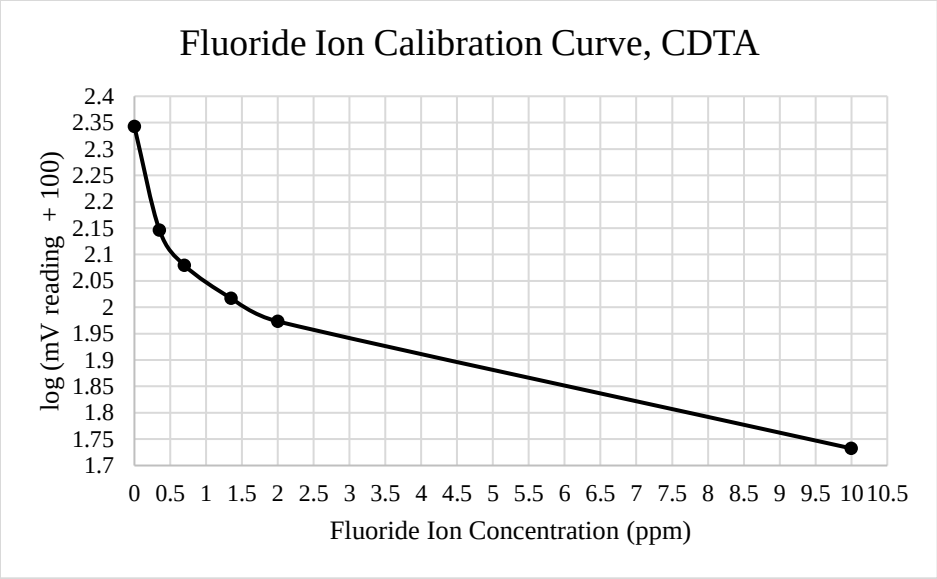

Figure 2: A representative calibration curve, used to determine the concentration of fluoride ions in a solution. Calibration curves were frequently remeasured and never appreciably differed from the sample shown here.

A side issue to be mindful of is taking ISE measurements of solutions having 0 ppm fluoride, for in this case, several readings may be needed before the electrode reaches a true "zero" value. If too few readings are taken, the values may read too low, such that the solution will appear to have more fluoride than it does. Caution is also needed when measuring solutions with high concentrations of fluoride and then moving directly to the solutions containing little to no fluoride. Ideally, experimenters should avoid using solutions with 0 ppm fluoride, and they should instead use solutions with at least small amounts of fluoride to establish a more accurate millivolt reading.

Conditioning Buffers for Fluoride Ion Measurements:

To properly measure fluoride ions, a conditioning solution must be used. A total ionic strength adjustment buffer (TISAB) helps maintain a constant pH and ionic strength in the solution during measurements. Additionally, a TISAB buffer that contains *trans*-1,2-

diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid monohydrate (CDTA), helps free fluoride ions from cations that may interfere with the measurement of fluoride ions.(17) CDTA is a chelating agent, so it binds excess cations that might affect the electrode reading by binding to fluoride ions, thereby allowing all fluoride to be accounted for in the measurement. For a detailed procedure for preparing the TISAB buffers, see the *Appendix, Sections VI & VII*.

Investigations of the Tooth-like Model System:

While the nano-hydroxyapatite pellet is far from being a replica of a genuine human tooth, there are ways I manipulated the nano-hydroxyapatite to make it more "tooth-like," including by switching from a homogeneous-suspension exposure to a pellet-form exposure and from a softer pellet to a more compact pellet. These distinctions came about as follows. When originally designing the experimental process, there was a step where the nano-hydroxyapatite sample was re-homogenized before the rotation step. This was intended to maximize the surface area of nano-hydroxyapatite available for binding to fluoride. However, further experimentation revealed there was no significant difference between fluoride uptake between nano-hydroxyapatite in the pellet form versus nano-hydroxyapatite in the re-homogenized form. Therefore, the nano-hydroxyapatite was kept as a pellet for the remainder of the experiments to reduce experimental error and simplify the experimental design. This revelation showed that nano-hydroxyapatite, even in pellet form, is very porous and able to easily bind fluoride ions. This held true whether the nano-hydroxyapatite pellet was formed by centrifugation at 5,000 rpm for one minute or 10,000 rpm for five minutes. The latter centrifugation condition prevented the nano-hydroxyapatite pellet form being disturbed during the removal of the supernatant while not significantly affecting fluoride uptake into the pellet.

Chapter 3: Fluoridation Factors

Having established a model system to test fluoridation factors, I turned to the main questions regarding which factors have an impact on the uptake of fluoride. To begin this section, we will look at some of the control experiments performed to set the stage for the remainder of testing.

Initial Studies:

In preliminary experiments, I exposed resuspended nano-hydroxyapatite to aqueous solutions of fluoride for 48 hours. After, I centrifuged the nano-hydroxyapatite in the solution back into a pellet form, and then I measured the fluoride content of the supernatant and nano-hydroxyapatite pellet. (Acid digestion was used for the pellets to release the fluoride for measurement.) The preliminary studies demonstrated that, at the end of a trial, all fluoride could be accounted for as being in either the supernatant or pellet. After numerous trials, it became clear the supernatant alone could be analyzed to show if an independent variable had a significant effect on the fluoride uptake. The acid digest step was, therefore, discontinued due to its redundancy and lack of necessity to quantify fluoride uptake by nano-hydroxyapatite.

Another set of controls was used to determine which conditioning buffer was optimal. I knew from the corresponding literature that a TISAB conditioning buffer is commonly used when working with fluoride electrodes.(17) However, I reasoned that a conditioning buffer that included CDTA could be useful to chelate calcium ions that are potentially released from the nano-hydroxyapatite pellet. As such, a control experiment was performed to determine if there was a significant difference between the millivolt readings of the fluoride ISE between solutions that contained TISAB buffer compared to CDTA buffer. In this experiment, sodium fluoride

(NaF) solutions with specific concentrations of fluoride ions were measured in the presence of either TISAB buffer or the CDTA buffer. As shown in *Figure 3*, there is no significant difference in the fluoride readings between the different buffers. This result agreed with my expectations and reinforces that later findings throughout this thesis are true fluoride readings, unobscured by background effects due to divalent cations.

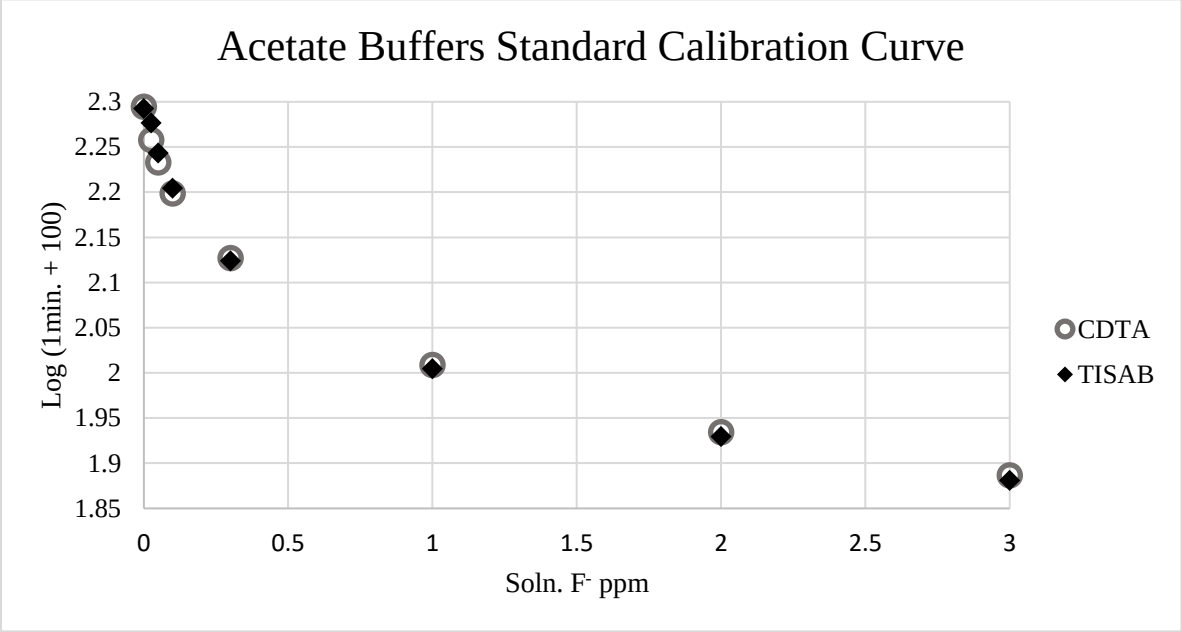

Figure 3: There is no significant difference in fluoride readings between using TISAB buffer and CDTA buffer.

Calcium and Magnesium Ions:

Next, I investigated the effects of calcium and magnesium ions during a prolonged exposure of nano-hydroxyapatite to fluoride, simulating the effect of these divalent ions under chronic exposure of teeth to mineral-rich water. This experiment occurred early in the project, so the exposure time was approximately 48 hours, as opposed to shorter exposure times that ended up being optimal. The source of calcium ions was calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), and the source of magnesium ions was magnesium sulfate ($MgSO_4$). These ions were tested

individually for their effect on fluoride uptake, not concurrently. In designing this study, the concentrations of each divalent cation were selected based on documented information from the USDA Nutrient Data laboratory. I used the maximum concentration of Ca^{2+} (2.5 mM) and Mg^{2+} (1.9 mM) found in municipal water samples.(18) The concentrations of these ions were held constant, and their effectiveness was tested in the presence of 0.7 ppm F^-, 2 ppm F^-, and 10 ppm F^-. A control was also run with a solution containing no additional ions beside fluoride.

As shown in *Figure 4*, neither calcium nor magnesium ions had a significant effect (either positive or negative) on the uptake of fluoride into nano-hydroxyapatite at fluoride concentrations of 0.7 or 2 ppm. All fluoride ions from these starting points have been removed from the supernatant during the 48 hours, suggesting high effectiveness in the model system. Interestingly, in the samples containing 10 ppm F^-, both calcium and magnesium had a positive percentile effect on fluoride uptake. A single factor anova test revealed this finding was statistically significant. Thus, at fluoride concentrations at 10ppm or higher, standard

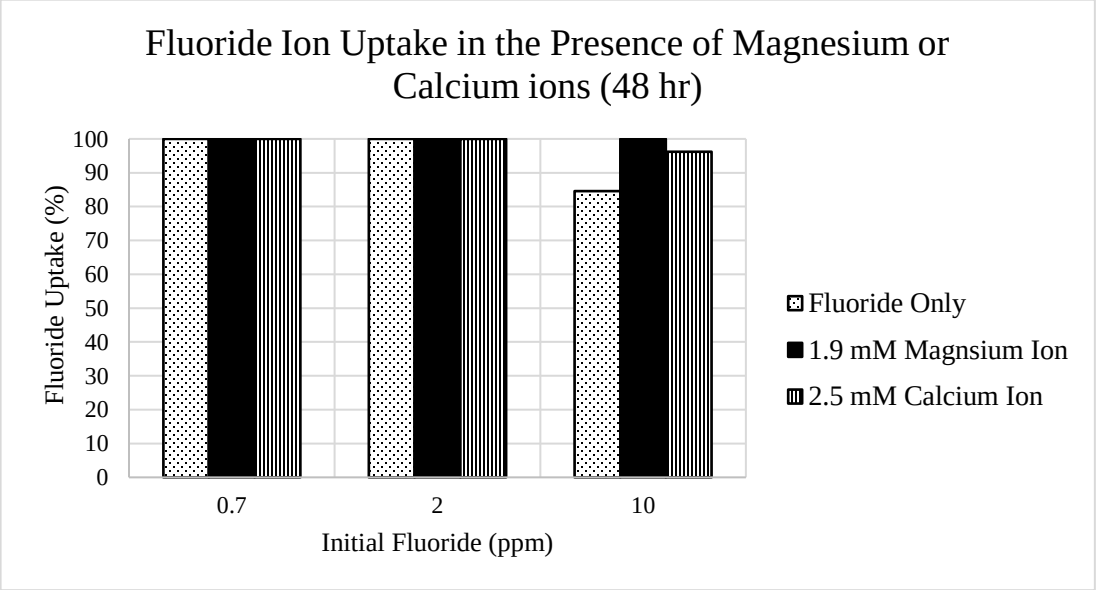

Figure 4: When fluoride ions are "swished" in the presence of magnesium or calcium, there is no significant difference in its uptake into hydroxyapatite at 0.7 or 2ppm fluoride. At 10 ppm fluoride, a slight positive effect is shown when calcium or magnesium is present.

concentrations of calcium and magnesium ions enhance fluoride uptake and are pro-fluoridation factors.

One possible mechanism behind this pro-fluoridation effect could be calcium or magnesium ions binding to fluoride ions, producing an ionic complex (such as calcium fluoride or magnesium fluoride) that settles into the matrix from the supernatant. Another possibility is that the calcium or magnesium ions draw out a hydroxyl group from the nano-hydroxyapatite, thus allowing the fluoride ion to take its spot. The pro-fluoridation potential of magnesium and calcium has possible relevance to dental fluorosis, the response of teeth to too much fluoride, as the concentration of these ions could influence the degree of dental fluorosis due to their effects on fluoride uptake. The pro-fluoridation effects of calcium and magnesium ions also have implications for the composition mouth rises and dental therapeutic products containing fluoride. If these ions facilitate fluoride uptake, then perhaps adding magnesium ions to these products or increasing the concentration of calcium ions could increase their overall effectiveness, either requiring less fluoride to be in the product or reducing the recommended exposure time.

Since there was complete uptake of all fluoride at 0.7 and 2ppm concentrations over 48 hours, future experiments could be conducted at a shorter time scale to determine whether calcium and magnesium ions have pro-fluoridation effects when the fluoride concentration is in the regulated range for drinking water (around 0.7-1 ppm F^-).

Varied Water Hardness:

Next, I investigated water hardness and its impact on fluoride uptake. Calcium carbonate ($CaCO_3$) is a key component of hard water, so its concentration was the independent variable in this experiment.(19) To create the hard water, I followed the procedure outlined by the United

States Environmental Protection Agency (US EPA) in their document, “Preparation of AOAC and OECD hard water and other diluents for preparation of antimicrobial products.” I decided to create the OECD hard water due to it having an overall concentration of 375 ppm $CaCO_3$, which made for simple dilutions to acceptable ranges within the scale of water hardness. Generally, the OECD water was comprised of two diluted solutions. “Solution A” contained 0.2084 M anhydrous magnesium chloride ($MgCl_2$) and 0.4163 M anhydrous calcium chloride ($CaCl_2$), and “Solution B” contained 0.4169 M sodium bicarbonate ($NaHCO_3$).(20) To obtain the final solution of 375 ppm $CaCO_3$, 0.3 mL of Solution A and 0.4 mL of Solution B were added to a 50 mL volumetric flask already filled with 30 mL of ultrapure water, and the final solution was diluted to the 50 mL mark with ultrapure water. For a more detailed procedure of how these solutions were synthesized, see *Appendix, Section VIII*.

In this experiment, 0.7 ppm F^- was tested for its uptake into nano-hydroxyapatite in the presence of 0 ppm calcium carbonate (control), 47 ppm calcium carbonate (soft water), 94 ppm calcium carbonate (moderately hard water), and 188 ppm calcium carbonate (very hard water). The total exposure to the hard water was 20 minutes. After this period, the supernatant was tested to reveal that there was no significant difference in the rate of fluoride uptake between the different samples of water hardness, which is shown in *Figure 5*. Therefore, it can be concluded that hard water does not impact fluoride’s uptake into nano-hydroxyapatite at standard concentrations of fluoride.

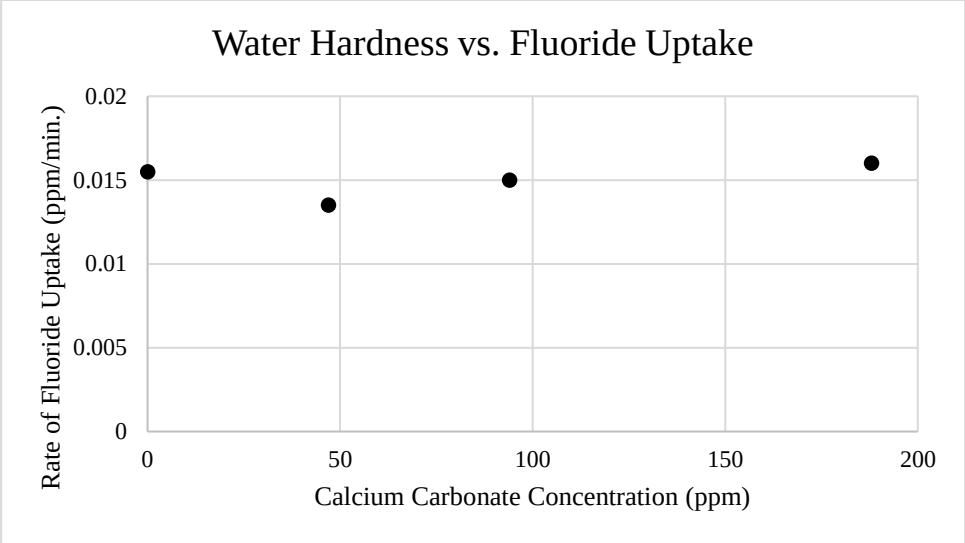

Figure 5: During a 20-minute exposure period, the water hardness does not significantly affect hydroxyapatite's uptake of fluoride ions. Note that, in addition to calcium carbonate, the synthetic hard water also contained magnesium chloride and sodium bicarbonate, as described in the text.

In a related control experiment, I tested calcium ions' binding potential to fluoride ions in the absence of nano-hydroxyapatite. This experiment revealed if the presence of calcium ions mask fluoride ions from being read by the fluoride ISE by binding to them, presumably forming a CaF_2 complex. Samples of 0.175 ppm fluoride ions were tested in solutions containing anywhere from 0 to approximately 50 ppm calcium ions and CDTA buffer. Duplicate samples were made for each concentration of calcium ion. The concentrations of ions were chosen to mimic the final concentrations of these ions when they are read by the fluoride ISE, after they are diluted by a factor of four. Additionally, the concentrations of calcium ions aligned with those of soft, moderately hard, and hard water. When all solutions were tested, there was no significant difference in fluoride electrode readings between any of the solutions. This trend is shown in *Figure 6*. It suggests the CDTA completed its role as a chelating agent by binding to calcium, allowing the fluoride ions to be free to be read by the electrode. With this in mind, we now move to another experimental factor, pH.

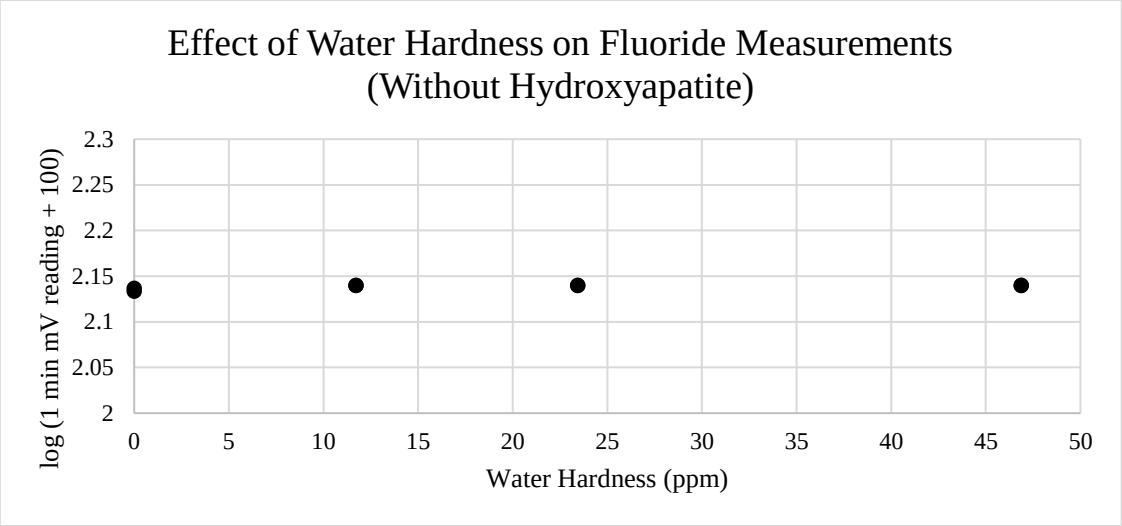

Figure 6: When fluoride ions are exposed to hard water without nano-hydroxyapatite, there is no significant difference in the readings of fluoride.

pH and Buffer Strength:

A common variable in drinking water is the pH, usually regulated between 6.5-8.5.(21) In this experiment, dibasic and monobasic sodium phosphate solutions were used to synthesize buffers at various pH values between 4.35 and 9.05, at a constant phosphate concentration. Each exposure solution of 1 mL contained 0.7 ppm fluoride and 5 μL of the appropriate buffer. When the nano-hydroxyapatite pellet was exposed to the solutions at different pH, the millivolt readings of the supernatants revealed a curious trend. After 90 minutes, the lower the pH, the more effective nano-hydroxyapatite was at taking up fluoride ions. This was true until around pH 6.0, and then fluoride uptake diminished again. This trend is shown in *Figure 7*.

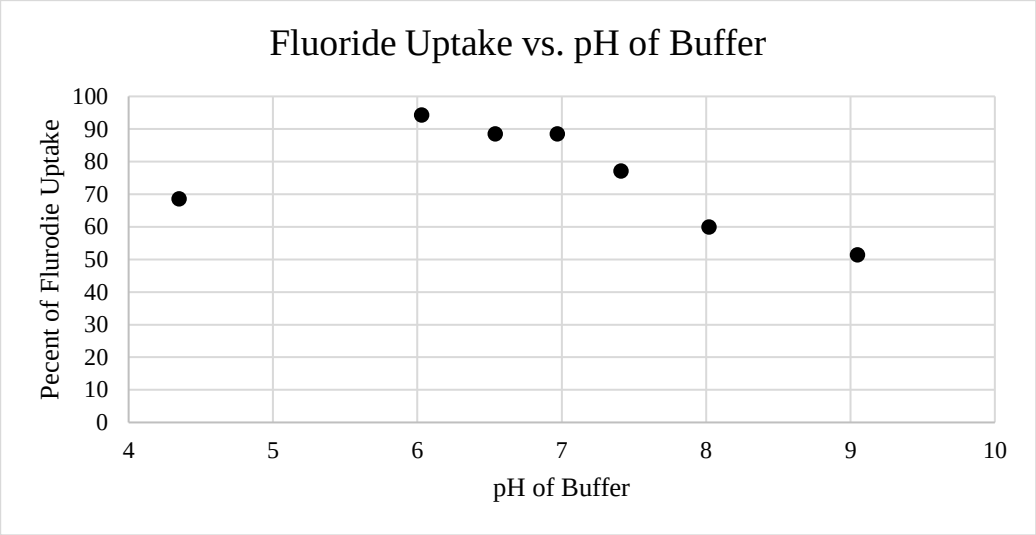

Figure 7: For a 90-minute swish, the ideal pH for fluoride uptake is around pH 6.0. As the pH gets farther from 6.0, the effectiveness of fluoride uptake diminishes.

However, once this trend is spotted, another explanation arises to potentially account for the difference in uptake in the pH experiment—buffer strength. This potential cause is due to the different proportions of the varying protonated forms of phosphate at each respective pH. See *Appendix, Section IX* for the specific compositions of each buffer at its respective pH. To investigate buffer strength as a potential contender, we need to employ an experiment where the pH of the buffer is not altered, but the proportion of buffer in the exposure solution (and therefore the overall buffer strength) is systematically increased. In this test, exposure solutions were made containing 2.5, 5.0, 10.0, or 20.0 μL of pH 6.0 buffer, in addition to the usual 1 mL of 0.7 ppm fluoride. A 90-minute swish against the nano-hydroxyapatite pellet was performed. Then, the supernatants were prepared via the standard method and tested for their remaining fluoride. As revealed in *Figure 8*, across all strengths of buffer at pH 6.0, the fluoride uptake was the same, 100 percent. Therefore, buffer strength is likely not the cause of the variation in fluoride uptake, and pH is.

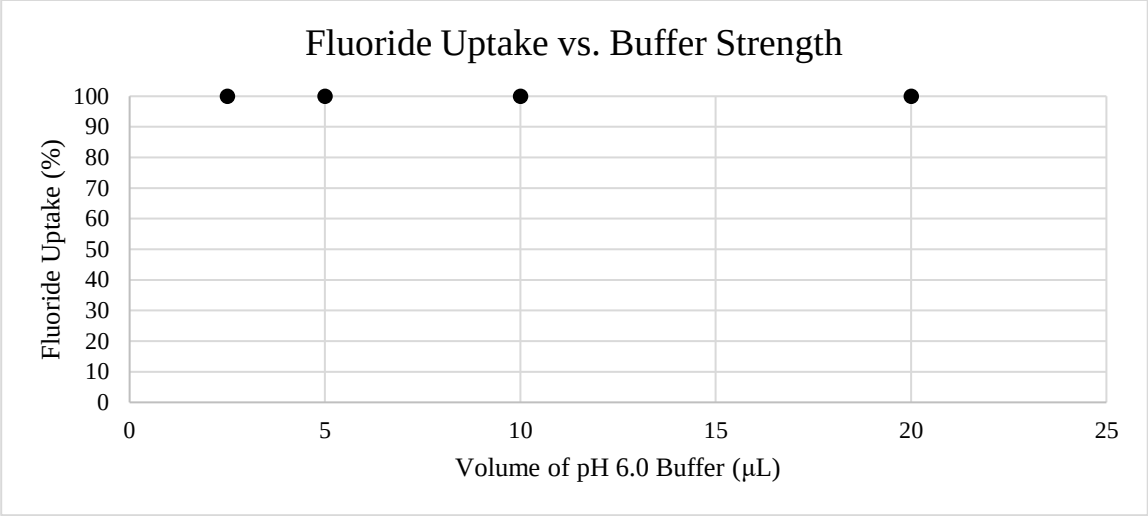

Figure 8: The buffer strength does not affect nano-hydroxyapatite's uptake of fluoride ions during a 90-minute exposure.

As a recap before heading into the next phase of these studies, we have learned some key takeaways about fluoride uptake. At everyday levels of fluoride in drinking water, magnesium and calcium do not affect its uptake, water hardness does not affect its uptake, and buffer strength does not affect its uptake. However, pH does impact fluoride uptake into nano-hydroxyapatite. The optimal pH for fluoride uptake is in the neutral range to slightly acidic, aligning with many drinking water supplies, which should be within a pH range of 6.5-8.5.(21) Now, since we have explored several factors individually that could affect fluoride uptake, we will move toward taking the geographical approach as to how fluoride uptake may differ among water from various locales.

Chapter 4: The Geographical Investigation

There are many ways to produce drinking water. Over time, water treatment methods have evolved and expanded to the wide range of diverse processes used today. Additionally, water has a range of acceptable guidelines for its content of various molecules, minerals, and other components.(21) In my thesis work, I therefore asked whether the wide range in drinking water composition influences the fluoridation of tooth enamel, as modeled by nano-hydroxyapatite.

For this final part of my exploration, I collected nine municipal water samples from sources around Oregon and the rest of the country. From Oregon, I received water samples from Corvallis, Lincoln City, Medford (two sources), Portland, and Salem. The other three sources came from Denver, CO, Phoenix, AZ, and Milwaukee, WI. In some cases, fluoride had been synthetically added to the drinking water by local water treatment plants. In other cases, the samples did not contain added fluoride. To amend this difference, I first tested each sample to find its starting amount of fluoride. Concentrations of fluoride ranged from 0.015-0.80 ppm (see *Appendix, Section X* for the specific starting value for each sample). Then, I standardized each sample by adding a calibrated volume of 30 ppm NaF to bring the final fluoride value of each sample to 0.8 ppm. I also made a synthetic lab sample as a control that contained only 0.7 ppm NaF in ultrapure water. In total there were ten samples: six from Oregon, three from major cities in other states, and one laboratory control.

Once the samples contained the same starting value of fluoride, I followed my standard procedure to determine each sample's effect on fluoride uptake into nano-hydroxyapatite. To avoid sampling bias, I made triplicates of each sample in random order. As I progressed through each trial, I recorded the duration of each pellet's exposure to the fluoridated water sample, from

the moment the water sample came into contact with the pellet to the time the supernatant was removed from the centrifuge tube. Overall, each nano-hydroxyapatite pellet was exposed to the fluoridated solution for approximately 26 minutes. To further reduce measurement bias, I ran one measurement from each water source in randomized order, over three separate trials. This gave a total of 30 randomly assorted measurements. For this experiment, the data points are reported as the rates of fluoride uptake during time timed interval of 26 minutes. (This differs from expressing the total percent of uptake, which was reported for the longer interval experiments in the earlier parts of my study.) The results are shown in *Figure 9.*

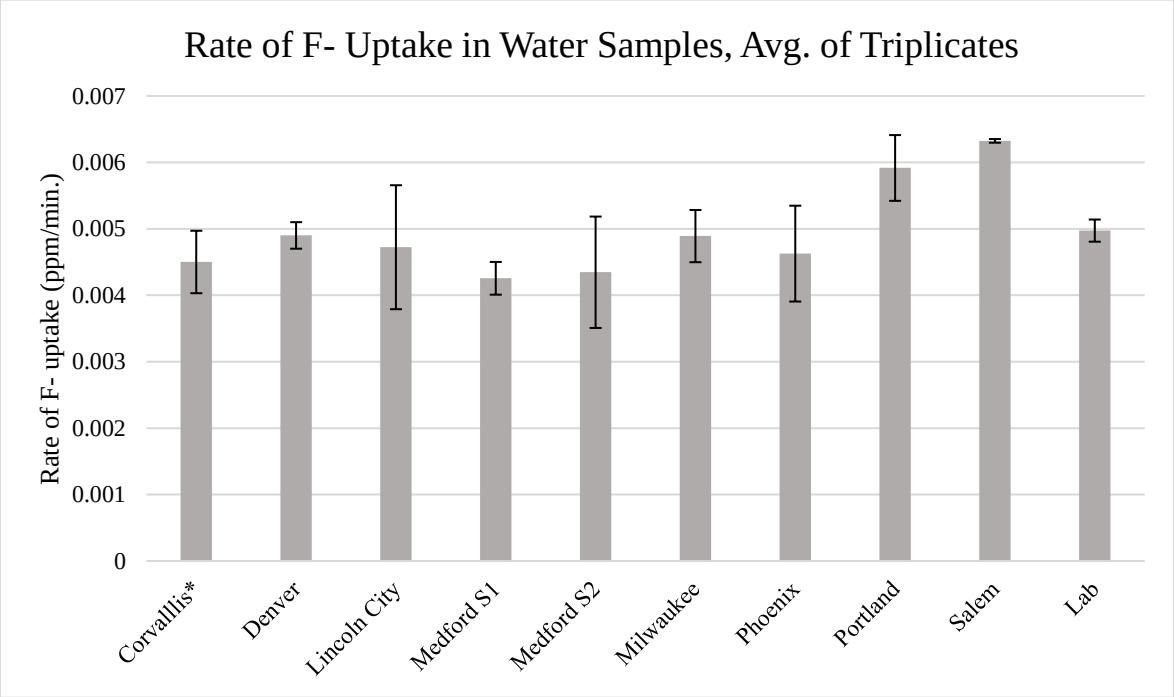

Figure 9: The rate of fluoride ion uptake does not significantly vary between water samples between different cities. Average values for each city are plotted, and the error bars represent the standard deviation for each set of samples.
**Note: Due to an unknown experimental error, Trial 1 of Corvallis' sample was thrown out.*

Though some variation was evident, a single-factor anova test revealed there was no significant difference in the rates of fluoride uptake between the different samples of water. This is notable because it suggests that for far-ranging drinking waters, if they contain 0.7ppm

fluoride, the rate of fluoride uptake by nano-hydroxyapatite, and presumably teeth, will be similar. While this experiment does not represent all sources of water a person could encounter, such as well water and bottled water, it does cover a variety of drinking water sources that each have a unique composition and production method. For each sample, the water could be subject to future tests to uncover more about its exact composition and characteristics. However, despite the differences between these samples, the rate of fluoride uptake was remarkably similar in each. Therefore, fluoride is a distinctively resilient ion that can complete its role of fluoridation in many different settings; in other words, it can act on many stages.

Chapter 5: Closing Thoughts

As we wind down from analyzing experiments, there are a few interesting points to be considered.

In 1962, the U.S. Public Health Service (PHS) issued its first recommendation that fluoride concentrations should range between 0.7-1.2 ppm.(6) However, in 2015, they replaced their recommendation to a flat value of 0.7 ppm fluoride; this was due in part to more fluoride-containing dental products being used and the finding that consumption of drinking water does not significantly vary with seasonal changes in outdoor temperature.(6) According to the World Health Organization (WHO), people typically receive 5-20 percent of their daily calcium and magnesium intake from drinking water.(22) Although the concentrations of these ions vary between each source of drinking water, the indication from my thesis work (see *Figure 4*) suggests less fluoride may be necessary in water with higher amounts of these ions due to their pro-fluoridation effects. Further research should be performed to address these conditions.

As mentioned previously, the pro-fluoridation effects that calcium and magnesium ions seem to have on fluoridation of nano-hydroxyapatite could also impact the composition of certain dental products. Many toothpastes and mouth rises contain fluoride in them. These are especially important for inidividuals who are at a high dental caries risk and/or live in a region with unfluoridated water. If these products also contain calcium and magnesium ions, these brands should investigate if they could reduce the amount of fluoride in their products or the recommended exposure time.

On a related note, some dental products have arisen recently that contain hydroxyapatite. Both hydroxyapaite and fluoride have been shown to prevent demineralization of teeth, but solutions that contain both components have the most significant preventative effect.(23) Thus,

companies making dental products that contain both hydroxyapatite and fluoride should consider product testing to determine the impact these have on preventing tooth demineralization in the presence of other pro-fluoridation factors, such as magnesium and calcium ions. They should also consider testing their products over time to determine how much hydroxyapatite is converted to fluoroapatite from the time the product is originally produced to when it is used by consumers at home or in the dental office. It is also possible that, by including both nano-hydroxyapatite and fluoride in a product, displacement of Ca^{2+} and formation of CaF_2 might occur. CaF_2 has been suggested to be a fluoride reservoir that can persist in the mouth between meals, enabling remineralization of enamel that was previously etched away by microbial-induced acidity.(4)

Another interesting avenue to explore is commercially bottled water. Bottled water generally has higher concentrations of certain molecules, such as calcium, magnesium, or sodium ions.(24) However, bottled water in the United States generally has lower concentrations of fluoride ion, less than 0.2 ppm on average.(25) Additionally, most bottled waters have an acidic pH, ranging as low as pH 5.15; this could be seen as a possible red flag, as any pH under 5.5 creates conditions for demineralization of enamel to occur.(26) As indicated from the experiments with calcium and magnesium ions and pH, the composition of bottled waters may have a positive or negative effect on enamel uptake of fluoride when consumed in large quanties, depending on the specific composition of each brand of water. Since the amount of bottled water consumption has increased over time in North America, further studies should be conducted to determine the effect on fluoride uptake of certain brands of bottled water.(24) This would also be appliable information for those who rely on bottled water as their primary source of clean

drinking water, as is the case for those living in areas without a municipal water system or with contaminated water.

Interestingly, many carbonated beverages contain around 0.5 ppm F^- on average, which is 0.3 ppm higher than the standard bottled water.(25) However, over 90 percent of carbonated beverages have a pH less than 4.0, which is bound to cause many issues for demineralization of enamel.(27) Additionally, flavored waters have pH values that hover around 3.0, and even sparkling mineral waters have a pH under 5.0, which is below the critical point. (27) Though these types of beverages do contain appreciable amounts of fluoride, the low pH is less conducive for fluoride uptake, and it is also likely to cause dental caries when there are prolonged and/or frequent exposures to such products. Carbonated water was not investigated in this study in regards to fluoride uptake but could be researched in the future.

While I did not procure a sample of well water to include with the geographical samples, I believe that, according to my prior experiment with hard water (see *Figure 6*), well water (which is often at the hard end of the spectrum) would be predicted to have similar results to the various municipal water samples I tested. Specificially, the water hardness and molecular composition of well water might not significantly impact fluoride uptake. This is something, however, that should be investigated in further experimentation.

As was revealed in the pH experiments, the ideal pH for fluoride uptake centers around a pH of 6.0. However, most water sources have a pH between 6.5-8.5.(21) Due to the lower effectiveness of fluoride uptake into nano-hydroxyapatite at the higher pH values, more fluoride may need to be added to drinking waters as the pH increases. This could help ensure that the presumed full benefit of fluoride is taken up by hydroxyapatite of the teeth. While there is currently no strict consensus view of the amount of fluoride uptake that is optimally beneficial,

the results of my thesis suggest that pH adjustment could provide a method to adjust the uptake of fluoride.

My geographical study demonstrated how the rate of fluoride uptake remains similar across multiple samples of drinking water. Despite the different composition of each sample, fluoride uptake proved to be a nearly unwavering process when the initial concentration of fluoride is 0.7 ppm. While some of the factors mentioned previously impact the magnitude of fluoride uptake, the bottom line is that, when fluoride is present, it is taken up by nano-hydroxyapatite and likely tooth enamel as well.

As I progressed through this journey, the properties of drinking water unfolded for me to create a vivid bigger picture. Water is a universal need. There are many ways drinking water can be created, and while many components can individually alter fluoride's uptake into nano-hydroxyapatite, the holistic composition does not strongly impact fluoride uptake. While this uniformity across the drinking water of different regions may be related with the need to adhere to specific compositional guidelines, there are many components that have a wide range of their potential concentration in water. Despite this compositional diversity, fluoride remains a robust ion in regulatory-approved drinking water. The results of my thesis therefore show the resilience and wide applicability of water fluoridation as a biochemical phenomenon, an important realization that speaks to the matter of whether a municipality should fluoridate its drinking water. While many dismiss the importance or scope of oral health, fluoride plays an important role in caries prevention that cannot be ignored. Its versatility makes it a remarkable ion, with the potential to do so much good in our communities. In fact, the CDC considers water fluoridation to be one of the top ten greatest public health achievements of the 20th century.(28) Although there may be other ways for the general public to obtain topical fluoride, water fluoridation is

considered the most efficient and cost-effective way to protect oral health across the whole population.(3) Fluoride may have been a significant achievement from the last century, but its substantial and enduring contributions to human health merits continued study.

Appendix

I. Abbreviations & Acronyms:

Abbreviation or acronym	**Full word or phrase**
Avg.	Average
Ca^{2+}	Calcium ion
$CaCl_2$	Anhydrous calcium chloride
$CaCl_2 \cdot 2H_2O$	Calcium chloride dihydrate
$CaCO_3$	Calcium carbonate
CDTA	*Trans*-1,2-Diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid monohydrate
F^-	Fluoride ion
g	Gram
hr	Hour
ISE	Ion-selective electrode
L	Liter
log	Logarithm
M	Molarity (mol/L)
mg	milligram
Mg^{2+}	Magnesium ion
$MgCl_2$	Anhydrous magnesium chloride
$MgCl_2 \cdot 6H_2O$	Magnesium chloride hexahydrate
$MgSO_4$	Magnesium sulfate

µL	Microliter
mL	Milliliter
mM	Millimolar
mV	Millivolt
N	Normal
NaF	Sodium fluoride
NaOH	Sodium hydroxide
nm	nanometer
PHS	Public Health Service
ppm	Parts per million; equivalent to mg/L
rpm	Rotations per minute
S1	Source 1 of Medford's water supply
S2	Source 2 of Medford's water supply
TISAB	Total ionic strength adjustment buffer
TSIF	Tooth surface index of fluorosis
WHO	World Health Organization

II. Product Information from Certificate of Analysis, Hydroxyapatite:

- Produced by: Fluidinova
- Trade name: nanoXIM•Hap102
- Chemical name: hydroxyapatite
- Chemical formula: $Ca_{10}(PO_4)_6(OH)_2$
- Molecular weight: 1004.6 g/mol
- Particle size: less than 50 nm
- EINECS#: 215-145-7
- CAS #: 1306-06-5
- Lot number: I367C5
- Appearance: white aqueous paste
- Phase purity: 100%
- Hydroxyapatite content: 15.0±1.0 %wt

III. Shortened Version of Nano-Hydroxyapatite Testing:

1. Add 1 mL of diluted nano-hydroxyapatite to a 1.5 mL centrifuge tube.
2. Centrifuge sample at 10,000rpm for five minutes.
3. Remove supernatant from the centrifuge tube via a Pasteur pipette.
4. Replace original supernatant with 1mL of synthetic drinking water. (Repeat steps 1-4 for the desired number of samples.)
5. Place sample(s) on a rotator. Rotate on maximum speed for desired length of time.
6. Remove 0.5mL of the now exposed synthetic drinking water and add to a 15mL plastic tube containing 1.5 mL ultrapure water and 1 mL CDTA-containing TISAB buffer.
7. Record mV of solution using a fluoride ISE.
8. Compare millivolt reading to calibration curve to determine concentration of fluoride ions remaining in synthetic drinking water after exposure to hydroxyapatite.

IV. Detailed Procedure of Nano-Hydroxyapatite Testing:

1. Find and record mass of 50-mL polyethylene conical centrifuge tube.
2. Add 1.00g of nano-hydroxyapatite slurry to 50-mL centrifuge tube via a 1mL disposable pipette.
3. Add 30.0 mL of ultrapure water to 50-mL centrifuge tube. Find and record new mass.
4. Vortex the contents of the 50-mL centrifuge tube for approximately 30 seconds to ensure re-homogenization.
5. Place diluted nano-hydroxyapatite sample on laboratory rocker. Transfer 1.00 mL samples of the solution into 1.5-mL polypropylene centrifuge tubes.
6. Run samples in centrifuge at 10,000rpm for five minutes.
7. Remove the supernatant using a Pasteur pipette. Discard supernatant.
8. Replace supernatant with 1.00 mL of desired synthetic drinking water solution.
9. Add samples to rotator. Rotate samples at max. speed for desired amount of time.
10. Remove samples from rotator.
11. Transfer 0.50 mL of supernatant to a 15-mL polyethylene conical centrifuge tube.
12. Add 1.50 mL of ultrapure water to 15-mL centrifuge tube.
13. Add 2.00 mL of CDTA buffer to 15-mL centrifuge tube.
14. Gently swirl solution for approximately five seconds.
15. Use a fluoride ISE to record millivolt value of solution.
    a. Before testing each sample, rinse electrode with approximate 1 mL of ultrapure water. Gently pat dry with a delicate task wiper (i.e. Kimwipe).
    b. Record mV reading after 1 min. of the electrode's submersion in the solution.
16. Use calibration curve to determine approximate concentration of F^- in supernatant.

V. Ultrapure Water:

- Definition: water that has purified to only contain H_2O and H^+ and OH^- ions, all in equilibrium
- Water purifier used:
    - Brand: EMD-Millipore Synergy
    - Thermo number: SYNSVHFUS

VI. Buffer-Making Procedure:

1. Obtain proper mass of sodium chloride and add to a 100-mL glass media bottle.
2. Obtain proper mass of sodium citrate or CDTA and add to glass media bottle.
3. Add approximately 70mL of ultrapure water to glass media bottle.
4. Obtain proper mass of acetic acid (if in liquid form, use specific gravity to find corresponding volume) and add to glass media bottle.
5. Add another 10-15mL of ultrapure water.
6. Begin monitoring solution's pH with a pH meter.
7. Gradually add NaOH pellets to bring pH up to 5.5.
8. Add remaining ultrapure water to bring final volume up to 100mL.

VII. Conditioning Buffers for Fluoride Measurements:

TISAB Buffer (100mL)	**CDTA Buffer (100mL)**
5.8g sodium chloride	5.8g sodium chloride
0.03g sodium citrate	0.4g CDTA
5.7g acetic acid	6.0g acetic acid
Add sodium hydroxide until pH=5.2	Add sodium hydroxide until pH=5.5

Table 1: Formulas of the acetate buffers used during measurements with the fluoride ISE. TISAB was initially used but was eventually replaced by CDTA to account for loose calcium ions that could "hide" some of the fluoride in the supernatant via binding.

VIII. OECD Hard Water Preparation:

Solution A:

1. Dissolve 0.992 g $MgCl_2$ (or 2.118 g M, $MgCl_2 \cdot 6H_2O$) and 2.312 g $CaCl_2$ in ultrapure water in a 50 mL volumetric flask. Dilute to 50 mL mark with ultrapure water.
2. Transfer solution to glass media bottle. Store in refrigerator*.

Solution B:

3. Dissolve 1.751 g $NaHCO_3$ in ultrapure water in a 50 mL volumetric flask. Dilute to 50 mL mark with ultrapure water.
4. Transfer solution to glass media bottle. Store in refrigerator*.

OECD Hard Water:

5. Place around 30 mL of ultrapure water in a 50 mL volumetric flask.
6. Add 0.3 mL of Solution A to flask.
7. Add 0.4 mL of Solution B to flask.
8. Dilute solution with ultrapure water to the 50 mL mark.
9. Check that the pH is 7.0 +/- 0.2. If not, adjust solution using 1 N NaOH or 1 N HCl.
10. Transfer solution to glass media bottle. Store in refrigerator**.

*Solution A and Solution B can be stored in the refrigerator for up to one month.

**OECD must be used within five days of preparation.

IX. Phosphate Buffer Composition at Varying pH:

pH at 25°C	**[Na_2HPO_4] (M)**	**[NaH_2PO_4] (M)**
4.35	0.0000	0.2000
6.03	0.0246	0.1754
6.54	0.0750	0.1250
6.97	0.1220	0.0780
7.41	0.1620	0.0380
8.02	0.1894	0.0106
9.05	0.2000	0.0000

Table 2: Concentrations of Na_2HPO_4 and NaH_2PO_4 in the pH buffers at each respective pH. All buffers were made in volumes of 50 mL in 50-mL polypropylene volumetric flasks.

X. Initial Fluoride Concentration of Water Samples:

Sample Location	Starting Amount of Fluoride (ppm)
Corvallis, OR	0.65
Denver, CO	0.45
Lincoln City, OR	0.02
Medford, OR (Source 1)	0.085
Medford, OR (Source 2)	0.06
Milwaukee, WI	0.43
Phoenix, AZ	0.80
Portland, OR	0.015
Salem, OR	0.53

Table 3: The respective initial amount of fluoride in each source of water in the geographical investigation, alphabetically (Chapter 4)

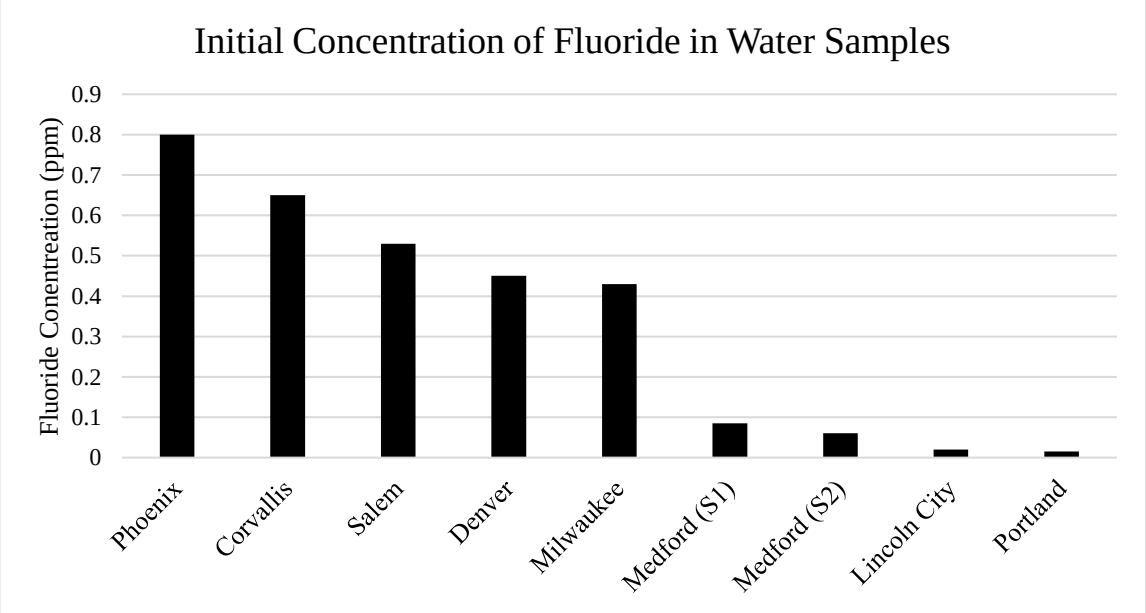

Figure 10: The respective initial amount of fluoride in each source of water in the geographical investigation, listed from highest to lowest initial value (Chapter 4)

References

1. Fiorillo L. Oral Health: The First Step to Well-Being. Medicina (Mex) [Internet]. 2019 Oct 7 [cited 2020 Jun 4];55(10). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6843908/

2. Benjamin RM. Oral Health: The Silent Epidemic. Public Health Rep. 2010;125(2):158–9.

3. CDC. Community Water Fluoridation [Internet]. 2020 [cited 2020 Apr 17]. Available from: https://www.cdc.gov/fluoridation/index.html

4. Cate JM ten, Buzalaf MAR. Fluoride Mode of Action: Once There Was an Observant Dentist... J Dent Res. 2019 Jun 20;98(7):725–30.

5. Whelton HP, Spencer AJ, Do LG, Rugg-Gunn AJ. Fluoride Revolution and Dental Caries: Evolution of Policies for Global Use: J Dent Res. 2019 Jul 8;98(8):837–46.

6. U.S. Department of Health and Human Services Federal Panel on Community Water Fluoridation. U.S. Public Health Service Recommendation for Fluoride Concentration in Drinking Water for the Prevention of Dental Caries. Public Health Rep. 2015;130(4):318–31.

7. Horowitz HS. Indexes for measuring dental fluorosis. J Public Health Dent. 1986;46(4):179–83.

8. Burger D. Study: Dental fluorosis generally less noticeable over time [Internet]. American Dental Association. 2020 [cited 2020 Apr 17]. Available from: https://www.ada.org/en/publications/ada-news/2020-archive/march/dental-fluorosis-generally-less-noticeable-over-time

9. PubChem. Fluoride ion [Internet]. [cited 2020 Apr 17]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/28179

10. Fawell JK, Bailey K, editors. Fluoride in drinking-water [Internet]. London; Seattle: IWA Pub; 2006. 134 p. (WHO drinking water quality series). Available from: https://www.who.int/water_sanitation_health/publications/fluoride_drinking_water_full.pdf

11. Habibah TU, Salisbury HG. Hydroxyapatite Dental Material. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2020 [cited 2020 Apr 17]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK513314/

12. Kutsch VK, Kois J, Chaiyabutr Y, Milicicc G. Reconsidering Remineralization Strategies to Include Nanoparticle Hydroxyapatite. Compendium. 2013 Mar;34(3):1–6.

13. Fluidinova. Hydroxyapatite properties, uses, and applications [Internet]. [cited 2020 Feb 9]. Available from: https://www.fluidinova.com/hydroxyapatite-properties-uses-and-applications

14. Kanduti D, Sterbenk P, Artnik B. Fluoride: A Review of Use and Effects on Health. Mater Socio-Medica. 2016 Apr;28(2):133–7.

15. UW School of Dentistry. Safe Handling of Extracted Teeth [Internet]. UW School of Dentistry. [cited 2020 Feb 10]. Available from: https://dental.washington.edu/policies/clinic-policy-manual/safe-handling-extracted-teeth/

16. Mathew M, Takagi S. Structures of Biological Minerals in Dental Research. J Res Natl Inst Stand Technol. 2001;106(6):1035–44.

17. Competence Center Titration at Metrohm International Headquarters. Determination of fluoride with an ion-selective electrode [Internet]. Metrohm. [cited 2020 Feb 10]. Available from: https://www.metrohm.com/en-us/applications/AB-082

18. Patterson K, Pehrsson P, Perry C. The mineral content of tap water in United States households. J Food Compos Anal. 2013 Aug 1;31(1):46–50.

19. USGS. Hardness of Water [Internet]. [cited 2020 Feb 20]. Available from: https://www.usgs.gov/special-topic/water-science-school/science/hardness-water?qt-science_center_objects=0#qt-science_center_objects

20. US EPA. Preparation of AOAC and OECD hard water and other diluents for preparation of antimicrobial products [Internet]. US Environmental Protection Agency Office of Pesticide Programs; 2017 [cited 2020 Mar 30]. Available from: https://www.epa.gov/sites/production/files/2018-01/documents/mb-30-01.pdf

21. US EPA. Drinking Water Regulations and Contaminants [Internet]. US EPA. 2015 [cited 2020 Feb 20]. Available from: https://www.epa.gov/sdwa/drinking-water-regulations-and-contaminants

22. Joseph Cotruvo, Fawell J, Giddings M, Jackson P, Magara Y, Ngowi AVF, et al. Hardness in Drinking-water [Internet]. World Health Organization; 2011. Available from: https://www.who.int/water_sanitation_health/dwq/chemicals/hardness.pdf

23. Hill R, Gillam D, Chen X. The ability of a nano hydroxyapatite toothpaste and oral rinse containing fluoride to protect enamel during an acid challenge using 19F solid state NMR spectroscopy. Mater Lett. 2015 Oct 1;156:69–71.

24. Azoulay A, Garzon P, Eisenberg MJ. Comparison of the Mineral Content of Tap Water and Bottled Waters. J Gen Intern Med. 2001 Mar;16(3):168–75.

25. Nutrient Data Laboratory. USDA National Fluoride Database of Selected Beverages and Foods, Release 2 [Internet]. USDA; 2005. Available from: https://data.nal.usda.gov/system/files/F02.pdf

26. Wright KF. Is Your Drinking Water Acidic? A Comparison of the Varied pH of Popular Bottled Waters. Am Dent Hyg Assoc. 2015 Jun 1;89(suppl 2):6–12.

27. Reddy A, Norris DF, Momeni SS, Waldo B, Ruby JD. The pH of beverages available to the American consumer. J Am Dent Assoc 1939. 2016 Apr;147(4):255–63.

28. CDC. Ten Great Public Health Achievements -- United States, 1900-1999 [Internet]. [cited 2020 Apr 17]. Available from: https://www.cdc.gov/mmwr/preview/mmwrhtml/00056796.htm