***The Influence of Biofilm Maturation on Fluoride's Anti-Caries Efficacy***

Hadeel M. Ayoub[1,2,3], Richard L. Gregory[2], Qing Tang[4], Frank Lippert[5*]

[1]Dental Health Department, College of Applied Medical Sciences, King Saud University, Riyadh, Saudi Arabia

[2]Department of Biomedical Sciences and Comprehensive Care, School of Dentistry, Indiana University, Indianapolis, Indiana, USA

[3]Bowen Center for Health Workforce Research and Policy, School of Medicine, Indiana University, School of Medicine, Indianapolis, Indiana, USA

[4]Department of Biostatistics, School of Medicine, Indiana University, Indianapolis, Indiana, USA

[5]Department of Cariology, Operative Dentistry and Dental Public Health, School of Dentistry, Indiana University, Indianapolis, Indiana, USA

*Corresponding Author

Frank Lippert

415 Lansing Street

Indianapolis, IN 46202-2876

USA

Email: flippert@iu.edu

Phone: +1(317)274-3983

Key words: Exposure Period. Fluoride. Fluoride Anti-caries Efficacy. Biofilm Recovery. Biofilm Maturation

---

This is the author's manuscript of the article published in final edited form as:

Ayoub, H. M., Gregory, R. L., Tang, Q., & Lippert, F. (2022). The influence of biofilm maturation on fluoride's anticaries efficacy. Clinical Oral Investigations, 26(2), 1269–1282. https://doi.org/10.1007/s00784-021-04100-6

**Acknowledgement**

This research study was supported by the Indiana University School of Dentistry Department of Cariology, Operative Dentistry and Dental Public Health, Oral Health Research Institute. It was funded by the Oral Health Research Institute Remineralization Research Program. The amine fluoride was donated by Colgate-Palmolive Europe.

Special thanks to Dr. E. Angeles Martinez-Mier (IU School of Dentistry), Dr. Richard Lynch (GlaxoSmithKline, UK), and Mr. George Eckert (IU School of Medicine) for their feedback in designing the study and reviewing the results.

**Author Contributions**

**Hadeel M. Ayoub:** Conceptualization; Data curation; Investigation; Methodology; Project administration; Visualization; Writing – original draft

**Richard L. Gregory:** Methodology; Writing – review & editing

**Qing Tang:** Formal analysis; Writing – review & editing

**Frank Lippert:** Conceptualization; Investigation; Methodology; Project administration; Resources; Supervision; Visualization; Writing – review & editing

**Compliance with Ethical Standards**

*Disclosure of potential conflicts of interest*

The authors declare that they have no conflicts of interest.

**Statement of Ethics**

This research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. Ethical approval for saliva collection was obtained from IUPUI Institutional Review Board (protocol #1406440799). The study did not include human subjects, and therefore informed consent was not obtained.

**Abstract**

**Objectives:** 1-To explore the influence of biofilm maturation and timing of exposure on fluoride anticaries efficacy; 2-To explore biofilm recovery post-treatment. **Methods:** Bovine enamel specimens were utilized in a pH cycling model (28 subgroups [n=18]). Each subgroup received different treatment [exposure]: (sodium fluoride [NaF]; stannous fluoride [$SnF_2$]; amine fluoride [AmF]; and de-ionized water [DIW]) at a specific period: early: days 1-4; middle: days 3-6; and late: days 7-10). During non-exposure periods, pH cycling including DIW instead of fluorides. Objective-1: part-1 (cycling for 4, 6, or 10-days). Part-2 (cycling for 10-days). Objective-2: early exposure: three sample-collection timepoints (immediate, 3-days, and 6-days post-treatment); middle exposure: two sample-collection timepoints (immediate, 4-days post-treatment).. The enamel and biofilm were analyzed ([surface microhardness; mineral loss; lesion depth]; [lactate dehydrogenase enzyme activity; exopolysaccharide amount; viability]). Data were analyzed using ANOVA ($p=0.05$). **Results:** Objective-1: Early exposure to fluorides produced protective effects against lesion progression in surface microhardness and mineral loss, but not for lesion depth. Objective-2: Early exposure slowed the demineralization process. $SnF_2$ and AmF were superior to NaF in reducing LDH and EPS values,

regardless of exposure time. They also prevented biofilm recovery. **Conclusion:** Earlier exposure to $SnF_2$ and AmF may result in less tolerant biofilm. Early fluoride treatment may produce a protective effect against demineralization. $SnF_2$ and AmF may be the choice to treat older biofilm and prevent biofilm recovery. **Clinical Relevance:** The study provides an understanding to biofilm-fluoride interaction with mature biofilm (e.g. hard-to-reach areas, orthodontic patients) and fluoride's sustainable effect hours/days after brushing.

## Introduction

Dental biofilm is an essential factor for the initiation and progression of carious lesions. The ability of bacterial biofilm to produce lactic acid as an end product of the glycolytic pathway is the key virulence factor related to caries. [1,2] Therefore, disrupting the ability of the biofilm to produce acid is one approach to control dental caries. Another virulence factor related to the caries process is the formation of the matrix, of which extracellular polysaccharides (EPS) are the main component. [3,4] EPS serve as a protective environment for the bacteria; they store nutrients when they become scarce in the environment, facilitate acid formation, and serve as a diffusion barrier against antibacterial agents. [3-7]

The interaction between biofilms and different fluoride compounds has been explored previously.[2,8--20] However, the impact of biofilm maturity (i.e. age) and how it affects the tolerance of the biofilm to fluoride compounds has not been studied before. Previous studies have focused mostly on analyzing biofilms at a one or two time points.[8,10,21] In the present study, we focused on the influence of timing of exposure to fluoride (i.e. early vs. late exposure) and biofilm maturation on the anticaries effect (i.e. on bacterial cariogenicity and carious lesion severity) of fluoride compounds.

Since the approach taken in most studies focused mainly on biofilm virulence factors and not bacterial viability [1,2,5,18,22], studying the biofilm recovery after fluoride treatment should also be taken into consideration. Some previous studies suggested that brief fluoride treatment through oral hygiene measures may allow the biofilm to recover after a certain time. [2,,4,22] Hence, we aimed in our study to evaluate the long-term recovery after exposing the cariogenic model to pH cycling, which included fluoride treatments.

Therefore, the two main aims we explored in our study were: first, to explore the influence of biofilm maturation and timing of exposure period to fluoride treatments on the anticaries efficacy of fluoride; second, to explore the recovery of biofilm cariogenicity after exposure to fluoride treatments using two exposure periods.

## Materials and Methods

*Study Design*

After specimen preparation, baseline hardness measurements, and saliva collection, the study was initiated by growing biofilm for 24 hours in Brain Heart Infusion (BHI) media (with 0.2% sucrose). Then, the pH cycling phase began. The number of days of pH cycling for each subgroup and type of treatment each day (i.e. fluoride compound or de-ionized water [DIW]) were determined based on the aim of the study (Figure 1 [both aims]; Table 1**a** [aim 1]; Table 1**b** [aim 2]). The study included three major groups (Figure 1), representing the exposure periods to corresponding treatments (Figure 1): early exposure (T1; exposure on days 1-4), middle exposure (T2; exposure on days 3-6), and late exposure (T3; exposure on days 7-10). During non-exposure periods, the pH cycling continued, but corresponding treatments were replaced with DIW. A total of 28 subgroups (n=18 enamel specimens per subgroup) were included. These subgroups were distributed based on: the exposure period (T1, T2, T3); collection time (immediate, 3-day post-treatment, 4-day post-treatment, 6-day post-treatment, or 10-day old biofilm); and treatment type (Sodium fluoride [NaF], Stannous fluoride [$SnF_2$], Amine fluoride [AmF], and negative control [DIW]).

For the first part (i.e. condition) of aim 1, the pH cycling continued for 4 days (T1), 6 days (T2), or 10 days (T3), whereas in the second part (i.e. condition), the biofilm in all subgroups was allowed to grow for 10 days. The second aim of the study tested the ability of the biofilm to recover its cariogenicity after being exposed to fluoride for 4 days. In the first part, we allowed early exposure to treatments (T1; days 1-4), then collected the biofilm at three time points: immediate collection; 3-days post-treatment; 6-days post-treatment. In the second part, middle exposure to fluoride treatments was conducted (T2: days 3-6), then, two collection time points were selected: immediate collection and 4-days post-treatment.

At any collection time, enamel specimens were analyzed for caries lesion severity using Vickers microhardness and transverse microradiography; the biofilm was collected and analyzed for its cariogenicity (lactate dehydrogenase enzyme [LDH] activity, EPS amount, and bacterial viability).

*Specimen Preparation*

Extracted bovine incisors were sectioned to obtain 5 × 5 mm enamel specimens using a Buehler Isomet™ low-speed saw (Buehler, Ltd., Lake Bluff, IL, USA). A total of 679 enamel samples were prepared. During preparation, the teeth were stored in deionized water with thymol. Using a Struers Rotopol 31/Rotoforce 4 polishing unit (Struers Inc., Cleveland, PA, USA), all specimens were ground and polished to ensure flat parallel dentin/enamel surfaces. For the finishing process, the dentin side was ground using 500-grit silicon carbide grinding paper. Then, the enamel side was serially ground using 1,200, 2,400 and 4,000-grit papers. The accepted enamel thickness was in the range of 1.7-2.2 mm. After that, polishing of the specimens took place using a 1-µm diamond polishing suspension on a polishing cloth to obtain a 5 × 5 mm polished enamel surface. The final number of enamel samples was 522. All specimens were checked for cracks, white spots, or any other flaws that could lead to excluding the specimen from the study, using a Nikon SMZ 1500 stereomicroscope at × 20 magnification.

*Baseline Measurement (Surface Microhardness) and Experimental Groups*

A Vickers diamond identifier (Tukon 2100; Wilson-Instron, Norwood, MA, USA) was used with a load of 200 g for 15 s to obtain the surface microhardness measurements ($VHN_{sound}$) of sound enamel specimens. The average $VHN_{sound}$ measurement for each sample was obtained by creating three indentations, approximately 100 µm apart; the inclusion range was $VHN_{sound}$ between 300-380. Utilizing $VHN_{sound}$ values, specimens were randomly assigned into different groups and subgroups, as described below.

We used an active attachment model, following a previously described protocol.[10]. To allow true attachment between the bacterial biofilm and enamel specimens, specimens were mounted on the inside of a lid of a 6-well plate (FisherBrand, Fisher Scientific) using acrylic cubes. The model was disinfected using 70% ethanol prior to biofilm growth.[23]

Specimens were divided into three major groups, based on the exposure period to treatments as part of the pH cycling. In the early exposure period (T1), we exposed the samples to treatments on days 1-4 of pH cycling. The middle exposure period (T2) allowed the exposure for days 3-6. The late exposure period (T3) allowed the exposure

to treatments on days 7-10 of pH cycling. During the remaining days of pH cycling, fluoride treatments were replaced with deionized water. (Figure 1)

The first aim of the study included five collection time points (Table 1**a**). This aim had two parts (i.e. conditions). The first part (immediate collection) allowed collecting the biofilm-coated samples for analysis on the following days: (T1: day 4; T2: day 6; T3: day 10). The second part allowed full growth of the biofilm up to 10 days for all exposure periods.

The second aim tested the biofilm's recovery and compared it to immediate collection post-treatment (Table 1**b**); the collection time points were the following: (part 1(T1): days 4, 7, and 10; part 2 (T2): days 6 and 10).

For each collection time point in both aims, four subgroups were included based on the treatment type: Sodium fluoride (NaF) (ACROS Organics, Fair Lawn, NJ), Stannous fluoride ($SnF_2$) (ACROS Organics, Fair Lawn, NJ), Amine fluoride (AmF) (GABA, Grabetsmattweg, Therwil, Switzerland), and negative control (deionized water; DIW). All three fluoride compounds were used at a concentration of 287.5 ppm as solutions in deionized water (prepared fresh daily, used without pH adjustment), simulating a fluoride concentration of 1150 ppm found in toothpastes after 1:3 dilution occurring during toothbrushing. The total number of subgroups is 28; each subgroup included 18 samples.

*Salivary Bacterial Model*

*Saliva Collection.* Microcosm biofilm was used in this study, where the source is pooled wax-stimulated human saliva collected from three donors (ethical approval: IUPUI institutional review board [IRB #1406440799]). The inclusion criteria for the donors were: healthy participants (no systemic diseases) with normal salivary flow and no presence of active caries or periodontal disease. Participants refrained from oral hygiene measures overnight. Five ml of the pooled saliva and growth media mix (1:10 ratio) were incubated overnight, mixed with 10% glycerol and frozen immediately at -80° C, this microcosm bacterial mix was used as the source for bacterial inoculum.

*Biofilm Growth.* Prior to pH cycling, we grew the biofilm on the enamel specimens for 24 hours at 37° C in a growth medium that contained Brain Heart Infusion (BHI) broth, 0.5% (5 g/L) yeast extract (YE), 1 mM $CaCl_2.2H_2O$ and 0.2% sucrose.

*pH Cycling Model*

In this model, the growth medium was used as both the remineralization (remin) and demineralization (demin) solutions (Figure 2). Both the remin and demin medium contained BHI broth, 0.5% YE, and 1 mM $CaCl_2.2H_2O$.The pH of culture medium was adjusted to 7 (remin), and 4.5 (demin) with 1 mM acetic acid. The remineralization media did not contain sucrose, while the demineralization media contained 1% sucrose. The model utilized in this study was derived from Zhang et al. with modifications. [20] As illustrated in Figure 2, the daily pH cycling model started in the morning and ended at the end of the day with 5-minute treatment periods. Between the treatments, the model was exposed to alternating four 10-minute remin periods, and three 2:15-hour demin challenges. Overnight, we incubated the model in remin media. Sterile saline (0.9%) was used to wash the biofilm and enamel

specimens between treatments; we immersed the model in sterile saline for 2 minutes. We repeated the pH cycling model daily.

At the end of each pH cycling period, we collected the biofilm by carefully removing the biofilm-covered enamel specimens using tweezers. We then placed each specimen in an Eppendorf tube containing 1 ml sterile saline; sonicating at 30 W for 10 seconds, and vortexing immediately for 10 seconds for complete biofilm detachment from the enamel surface. The experiment was repeated three times; the same source for bacterial inoculum was used in all experiments.

*Post-treatment Analysis*

Enamel Substrate

*Surface Microhardness Change ($VHN_{change}$).* Post-treatment surface microhardness was measured following the same protocol used for the $VHN_{sound}$. The $VHN_{change}$ values were calculated using the formula $VHN_{change}$=100*(VHNsound - VHNpost)/VHNsound.

*Transverse Microradiography (TMR).* One section, approx. 100 µm thick, was cut from the center of each specimen and across the specimen using a Silverstone-Taylor Hard Tissue Microtome (Scientific Fabrications Laboratories, USA). All sections were placed in TMR-D1 v.5.0.0.1 system and X-rayed at 45 kV and 45 mA at a fixed distance for 12 s. An aluminum step wedge was X-rayed under identical conditions. Digital images were analyzed using TMR software v.3.0.0.18. Sound enamel was assumed to be 87% v/v mineral. The data obtained from this analysis were integrated mineral loss (ΔZ) and lesion depth (L).

Biofilm Analysis

*Lactic Acid Production: LDH Assay.* In order to determine the live biofilm metabolism, the lactate dehydrogenase enzyme activity was determined using a LDH cytotoxicity assay, following a previously published protocol. [24] For each sample, 45 µl of the collected, suspended biofilm was mixed with 5 µl of the LDH Assay Lysis Solution in a 96-well microtiter plate, and incubated at 37° C for 45 minutes. 100 µl of LDH Assay mixture was added to the cell lysate (LDH Assay Cofactor Preparation: LDH Assay Substrate: LDH Dye Solution = 1:1:1). The mixture was kept in the dark and incubated at room temperature for 30 minutes. To terminate the reaction, 50 µl of Stop Solution was added to the mixture. We measured the absorbance of the samples at $OD_{490nm}$ and the background absorbance at $OD_{690nm}$. The background absorbance values were subtracted from the sample readings.

*EPS amount: Phenol-Sulfuric Acid Colometric Assay.* The amount of EPS was determined using a previously described protocol [25]. Briefly, 50 µl of the biofilm of each sample was transferred to a 96-well microtiter plate. For each sample, 150 µl of concentrated sulfuric acid was added. Immediately after that, 30 µl of a 5% phenol solution was added to the mixture and heated to 90° C for 5 minutes. After cooling the plate at room temperature for 5 minutes the absorbance was measured at $OD_{750nm}$ [25].

All LDH and EPS values were normalized using the protein concentration of each biofilm sample, which was obtained using the RC DC Protein Assay (Bio-Rad Laboratories, Inc. Hercules, CA, USA) following the manufacturer's instructions.

*Bacterial Viability.* At the end of each time point, biofilm samples from all groups were serially diluted to $1{:}10^3$, $1{:}10^4$ and $1{:}10^6$ (using 0.9% sterile saline). Samples were plated on Blood Agar Plates (Thermo Scientific™, Remel, Lenexa, KS, USA) using a Spread Plate Procedure. [26] To determine bacterial counts, mean $\log_{10}$ CFU/ml values were calculated.

*Statistical Analysis*

All test results in both aims were analyzed using two-way ANOVA. For the first aim, factors for exposure period (i.e. T1, T2, and T3) and treatment type (NaF, $SnF_2$, AmF, and DIW) as well as the interactions between them were analyzed. For the second aim, all test results from the T1 exposure period collected at different time-points were analyzed with factors for collection time (immediate collection, 3-day post-treatment, and 6-day post-treatment) and treatment type as well as the interactions between them. Similarly, all test results from group T2 collected at different time-points were analyzed with factors for collection time (immediate collection, 4-day post-treatment) and treatment type as well as the interactions between them.

In all above two-way ANOVA models, the experimental unit was included as a random effect. All pair-wise comparisons from ANOVA analysis were made using Fisher's Protected Least Significant Differences to control the overall significance level at 5%. Statistical analysis was performed using SAS version 9.4 (SAS Institute, Inc., Cary, NC).

**Results**

**Aim 1: To explore the influence of biofilm's maturation and timing of exposure period to fluoride treatments on the anticaries efficacy of fluoride**

In both parts of this aim, the ANOVA analyses tested the effect of exposure period (T1, T2, and T3) and treatment type, as well as the interaction between them. The results from the immediate collection of the biofilm and enamel samples demonstrated that the change in surface microhardness was influenced by exposure period: early exposure (T1) to treatment resulted in significantly lower $VHN_{change}$ when compared to later exposure (T2 and T3). Exposure period also influenced the ΔZ and L values in all treatment types (Table 2**a**). The results of lesion progression over the different groups exhibited an increased lesion severity (ΔZ and L) with increased pH cycling days. This factor (pH cycling days) is also suggested to be a possible contributing factor in lesion progression and should be evaluated in future studies.

Considering biofilm cariogenicity (Table 2**b**), the results from the immediate collection of the biofilm demonstrated a significant interaction between exposure period and treatment type only in LDH activity and viability, but not for EPS amount. The more mature biofilm had higher viability when compared to early biofilm, even when the exposure to treatment was very close to collection time (T3). It should be noted that AmF was the only compound

that caused a significant reduction in biofilm viability when comparing T1 to T2 and T3 data. $SnF_2$ and AmF always exhibited a higher effect on bacterial viability when compared to NaF and DIW. Earlier exposure to treatment (T1 and T2) resulted in significant reduction in LDH activity than late exposure (T3). The EPS data demonstrated a significant effect of exposure period and treatment type ($p<0.0001$) but not the interaction between the two variables ($p=0.1270$). Both $SnF_2$ and AmF resulted in lower EPS amounts when compared to NaF and DIW.

The second part of this aim is the ANOVA analysis of 10-day old biofilms and enamel specimens exposed to treatments at different maturation stages (i.e. exposure periods) (Figure 1). The carious lesion severity was affected by the variables tested in this study. Samples in the "early exposure" groups (i.e. T1) exhibited a protective effect of fluoride when compared to control (Table 3**a**). Early exposure (T1) resulted in producing a protective effect in $VHN_{change}$ in all fluoride-treated groups, while later exposure (T2 & T3) did not always produce the same effect ($VHN_{change}$). The L data also indicated a protective effect in earlier exposure (T1 and T2) of the biofilm to treatments when compared to late exposure (T3). $\Delta Z$ was significantly affected as well by exposure period (Table 3**a**).

When testing biofilm cariogenicity, two-way ANOVA demonstrated a significant effect of both exposure period and treatment type. The interaction between these two variables was significant in bacterial viability and EPS amount. However, the LDH analysis exhibited a statistical significance in the treatment type only.

Regarding biofilm's viability, $SnF_2$ and AmF were always superior in their effect when compared to NaF and DIW. Similar to the first condition in this aim, AmF caused a significant reduction in biofilm's viability when comparing T1 and T2 to T3 exposure periods (Table 3**b**). The LDH activity was inhibited in the $SnF_2$ and AmF groups (when compared to NaF and DIW) regardless of the exposure period. The biofilm demonstrated an increased tolerance to NaF, in terms of lactic acid production, in all time periods. On the other hand, the EPS amount indicated a significant interaction between both variables ($p<0.0001$). Looking at exposure periods, late exposure to treatment still demonstrated more presence of EPS within the 10-day old biofilm when compared to earlier exposure (T1 and T2). Also, $SnF_2$ and AmF exhibited a significant effect when compared to NaF and DIW in all exposure periods (Table 3**b**).

**Aim 2: To explore the recovery of biofilm's cariogenicity after exposure to fluoride treatments using two exposure periods**

In the first part of this aim, we used early exposure to treatment (T1). We measured and compared lesion severity and biofilm cariogenicity at three time points: immediately after treatment; 3-day post treatment; 6-day post treatment.

The carious lesion severity data indicated that $VHN_{change}$ was influenced by treatment type with immediate collection. The further the lesion, the less significant $VHN_{change}$ was observed (Table 4**a**). Fluoride type did not create a significant difference in $\Delta Z$ and L data in any collection timepoint.

$SnF_2$ and AmF produced a lasting anticaries effect (LDH and EPS data) even up to 6 days after exposure, while NaF-treated biofilm started to produce higher EPS amounts 3 days post treatment and was able to fully recover (i.e. LDH and EPS data) after 6 days (Table 4**b**). In contrast, the viability data indicated a recovery of biofilm's viability post-treatment, especially 6-days post exposure in all treatment types (Table 4**b**).

In the second part of this aim, we used middle exposure to treatments (T2). We used two time points for comparison: immediate collection and 4 days post-treatment. The carious lesion severity (ΔZ and L data) was not significantly different between samples with immediate collection and after 4 days recovery in the three treatment tested. However, a significant difference was observed between treatments and controls in both ΔZ and L data (Table 5**a**). The LDH and EPS data of both $SnF_2$ and AmF indicate a sustainable effect up to 4 days when compared to NaF and DIW. Moreover, although AmF produced a significantly lower viability data than $SnF_2$ in both immediate collection and 4-day recovery, $SnF_2$ was able to produce a sustainable effect; the increase in viability 4 days post-treatment was nonsignificant (Table 5**b**).

**Discussion**

Two main aims were explored in this study. First, we evaluated how the timing of exposure to fluoride treatments and its interaction with biofilm maturation can alter fluoride's anticaries activity. Second, we tested the ability of the biofilm to recover after early (T1) and middle (T2) exposure periods to fluoride treatments. To our knowledge, this is the first study testing the interaction between biofilm maturation and different exposure periods to treatments. It is also the first study testing long-term biofilm recovery after treatment with different fluoride compounds.

Our study focused on 3 exposure periods of the biofilm to fluorides: early exposure (T1), middle exposure (T2); and late exposure (T3). In each exposure period, we tested how fluoride's activity was altered. We also tested whether the biofilm was able to recover (in terms of cariogenicity, as well). Based on the results from this study, both biofilm cariogenicity and its recovery are altered not only based on the exposure period, but also according to the fluoride compound used.

Fluoride is the agent of choice preventing the initiation and controlling progression of dental caries.[27] Several fluoride compounds are currently being utilized in oral hygiene products, including the studied NaF, AmF and $SnF_2$. The antibacterial effect of these fluoride compounds does not only come from the anion ($F^-$), but also from the cations ($Sn^{+2}$ and $NH_2^+$). [10,13,28,29]. This may explain the different outcomes observed on the biofilm cariogenicity and lesion severity when treated with different fluoride compounds.

The biofilm model and pH cycling model we used in this study were based on several previous pilot experiments conducted (data not shown). During these pilot experiments, we incorporated the microbial component as part of the pH cycling model to achieve more clinical relevance. The existing well-established models do not often incorporate a microbial component [30,31]; this suggests an increased demand for further studies exploring microbial caries models.

Pooled saliva from three donors was used as the source of the salivary bacterial mix. It was reported previously in the literature that collecting different saliva samples from the same donor at different times or different donors, and also involving sucrose over time in growing the biofilm, all lead at the end to overcoming any differences between samples through the increased dominance of certain strains (mainly cariogenic) over time. [32-35]

Utilizing a pH cycling model, as we adopted in this study, has its significance. It allows mimicking daily activities: two treatments/day (toothbrushing); several demineralization challenges (mealtimes); several

remineralization times (between meals). In the study conducted by Zhang et al., a non-biofilm group was included and subjected to the pH cycling model.[20] The results indicated that the enamel surface demineralization was due to acid production from the bacterial biofilm and not the demineralization solutions. Therefore, utilizing a pH cycling model is appropriate in maintaining clinical relevance of the caries development process.[20]

Similar to Zhang et al, we utilized growth media as the demineralization and remineralization buffers. Because our goal in this biofilm model is creating a biofilm-induced carious lesion, our study differed from Zhang et al in which we excluded HEPES as pH buffer from the growth media to allow the lactic acid produced by the bacteria to create an effect. However, we still measured the pH level of the overnight media every morning. Although it dropped from pH 7, the pH level was maintained above 5.5 for all treatment groups (data not shown). This confirms that the demineralization of enamel was due to the acidic challenge produced the bacterial biofilm.

We used bovine enamel specimens in our model to grow biofilm. Lippert and Lynch [36] (2014) indicated that bovine enamel samples have the exact characteristics of human enamel except faster progressing lesions in bovine enamel. This is not confounding to the use of bovine enamel, especially considering that bovine enamel is cost-effective and more available than human enamel specimens. [36] We used an "active attachment" concept to ensure the bacteria was actively attached to the enamel surface; this prevents the accumulation of layers of bacteria over the sample if it was placed on the base of the well-plate, which can result in less clinical relevance.[10] The fluoride concentrations used in our study were 287.5 ppm F. This concentration has significance as it corresponds to the dilution of 1150 ppm fluoride found in toothpastes during toothbrushing (1:3 dilution ratio). Fluoride, when used as toothpaste or mouthwash, is expected to be diluted during expectoration and swallowing.[22,37] Finally, we used a pH cycling model, where we initially modified a previously published model [20], then optimized the conditions used in the study through pilot experiments measuring pH levels, pH cycling periods, and growth medium conditions (mineral saturation).

The first aim evaluated the variables under two different conditions: immediate collection, and 10-day old biofilm. We collected the biofilm-coated samples, in the first condition, after the last day of treatment, regardless of the biofilm's age (T1: day 4; T2: day 6; T3: day 10). In the second condition, we compared 10-day old biofilms that were exposed to treatments at different exposure periods. Carious lesion severity was influenced by both factors: treatment type and exposure period. The results from both, immediate collection (Table 2**a**) and 10-day old biofilm (Table 3**a**) show less variability between treatment types with early exposure.

When testing biofilm cariogenicity (LDH activity and EPS amount), $SnF_2$ and AmF always produced a superior effect when compared to NaF and DIW. With immediate collection of the biofilm (Table 2**b**), $SnF_2$ and AmF were able to inhibit LDH activity in early (T1) or middle (T2) exposure. NaF, on the other hand, lost its effectiveness when not introduced early (i.e. when introduced at T2 or T3) (Table 2**b**). In the second condition, similar LDH activity findings between the three exposure periods (T1, T2, and T3) were observed in all treatment types. Table 3**b**).

The amount of EPS was also influenced by exposure period and treatment type. With immediate collection, the later the exposure of the biofilm to treatment, the more EPS was formed (Table 2**b**). In fully mature biofilm (Table 3**b**), late exposure (T3) allowed more tolerance against fluoride compounds, even in $SnF_2$ and AmF (when compared to T1, T2). A possible explanation is how the biofilm (especially more mature biofilm) may serve as a diffusion barrier,

restricting the fluoride penetration to the outermost areas of the biofilm, preventing solutions and anticaries agents from penetrating deeper layers of the biofilm or reaching the enamel or dentin surface. This is an important area that should be investigated furthermore in future studies. [38,39]

The second aim tested the biofilm recovery after exposure to treatments. Findings from surface microhardness also demonstrated a protective effect of fluoride compounds after early exposure. The difference in the ΔZ and L values between immediate collection and 6 days of recovery were very slight (all treatment types), suggesting that early exposure may produce a long-term protective effect against more demineralization, regardless of treatment type (Table 4**a**). This, again, is a possible justification of the limited penetration of fluorides to more mature biofilms as described earlier.

All three fluoride compounds when introduced at a later stage (T2) were able to produce a slight reduction in ΔZ and L values (although non-significant) 4 days post-treatment (Table 5**a**). A possible explanation is that the biofilm stored amounts of fluoride that were released over time post-treatment and allowed for some remineralization.

Whether it was an early (T1) or middle (T2) exposure to treatment, both $SnF_2$ and AmF resulted in a sustained antibacterial effect up to 6 days post-treatment (T1) or 4 days post-treatment (T2). Regarding the superior effect of AmF on biofilm viability when compared to other fluoride compounds, this effect may come not only from the fluoride anion, but also from the $NH_2^+$ cation. [28,29] These results are consistent with a previous study that demonstrated a superior effect of AmF in terms of influencing biofilm viability. Naumova et al. (2019) concluded in a recent study that AmF toothpaste has a superior long-term fluoride-bioavailability effect when compared to NaF. [40] NaF and DIW-treated groups exhibited an increase in activity after treatment; the more time allowed for post-treatment, the higher the biofilm activity as well. (Tables 4**b** and 5**b**).

In all parts of this study, we found that irrespective of exposure period or collection time, full bactericidal effect was not observed. This is similar to what was found in several studies that explored fluoride and virulence factors of the biofilm, [2,1,18] and recommended in the end focusing on inhibiting biofilm cariogenicity rather than achieving a bactericidal effect.

Our previous studytested biofilm maturation when the biofilm was exposed to fluorides on a daily basis as part of the pH cycling protocol (2 fluoride treatments/day).[41] The pH cycling periods (and hence the number of days the biofilm was exposed to treatment) extended up to 12 days. In this study, we used a different approach, we kept the number of days of exposure to fluorides to 4 days, and consequently, modified the exposure period to early (T1), middle (T2), and late (T3) exposure periods. We found that the behavior of the biofilm changes depending on the fluoride compound and exposure period, and not necessarily the number of days of exposure to treatment.

Several studies suggested a brief treatment (i.e. 1 minute) of the biofilm with fluoride to control virulence factors such as acidogenicity, acidurity and EPS formation to be a potential approach for preventing dental caries. [1,18,42] However, Dang et al. (2016) indicated full recovery of the biofilm post-treatment. [2] Pandit et al. (2015) indicated that fluoride's effect is concentration dependent up to 100 ppm, then reaching a plateau afterwards. [1] These studies focused on a single species biofilm. They also monitored biofilm for relatively short periods (compared to our study). Since increasing the concentration is not expected to produce a stronger anti-virulence effect, [1] we instead used the concentration that corresponds to over-the-counter oral hygiene products. We chose to test several fluoride

compounds on a microcosm biofilm, and longer treatment times during the day (5 minutes; twice/day). In our experiment, we extended the number of "treatment days" to 4 days at each exposure period. Then we allowed up to 6 days post-treatment recovery. NaF did allow a full recovery of the biofilm's cariogenicity, and this is consistent with the literature that reported how NaF lacks substantivity. [12,19]

In this study, some subgroups failed to significantly recover from fluoride treatment. We still do not fully understand the mechanism of biofilm recovery, but further research is needed in this area to also evaluate the influence of the other minerals (i.e. stannous and amine) on inhibiting biofilm's recovery.

Regarding carious lesion severity, ΔZ and L data in particular, this study demonstrated in general that with early exposure to fluoride, less progression of the lesion is observed. This suggests that biofilm may have stored some amounts of fluoride and served as reservoir over time. When we compare this study to our previous study (manuscript in progress), we may be able to state that whether the fluoride treatment was continuous as part of the daily pH cycling, or introduced at earlier periods of pH cycling, a protective effect of fluoride against demineralization is still observed.

The results from this study suggest that the earlier exposure of the biofilm to fluoride treatment, the less tolerant the biofilm may become over time, and this also depends on the fluoride compound used. Early treatment may suggest as well the sustainable release of fluoride treatment that penetrated the biofilm more effectively and was stored within the biofilm and allowed the enamel surface to be protected from further demineralization; this is an interesting area of research that should be explored more extensively in the future. Treating well-established biofilm with $SnF_2$ and AmF may achieve higher anticaries results when compared to NaF. Bacterial biofilm has the potential for full cariogenic recovery; to achieve a long-term antibacterial effect in oral hygiene products, $SnF_2$ and AmF may be the choice in this case.

Some limitations to this study include limiting the biofilm analyses to biofilm's function and not composition. Phenotypic changes within the biofilm may significantly influence biofilm's cariogenicity and therefore should be evaluated in future studies. Another limitation is the fluoride concentration used. The concentration selected for this study reflects the over-the-counter toothpastes. Testing varying concentrations of different fluoride compounds may give a better understanding of the fluoride-biofilm interactions. Finally, for the study's particular aims, we did not include other factors such behavioral factors and dietary variations (for example, different sucrose concentrations). This also should be considered in future studies.

Future studies may include studying the fluoride concentration within the biofilm and monitor its release in the environment (culture media) and at the biofilm-enamel interface (fluoride retention within the enamel). Another future approach in biofilm maturation studies is, as mentioned above, focusing on microbial composition and phenotypic changes- we focused our project on biofilm's cariogenic function. A third area of future research is studying the effect of fluoride on EPS architecture, since the variability in EPS architecture across the surface may modify the function of the EPS as a diffusion barrier.

Compliance with Ethical Standards

Conflict of Interest: Hadeel M. Ayoub declares that she has no conflict of interest. Richard L. Gregory declares that he has no conflict of interest. Qing Tang declares that she has no conflict of interest. Frank Lippert declares that he has no conflict of interest.

Funding: The work was supported by the Oral Health Research Institute Remineralization and Fluoride Research Programs at the Indiana University School of Dentistry in Indianapolis, Indiana in the USA.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent: For this type of study, formal consent was not required.

**References**


1. Pandit S, Cai JN, Jung JE, Jeon JG (2015) Effect of 1-Minute Fluoride Treatment on Potential Virulence and Viability of a Cariogenic Biofilm. Caries Res 49 (4):449-457. doi:10.1159/000434731
2. Dang MH, Jung JE, Lee DW, Song KY, Jeon JG (2016) Recovery of Acid Production in *Streptococcus mutans* Biofilms after Short-Term Fluoride Treatment. Caries Res 50 (4):363-371. doi:10.1159/000446408
3. Bowen WH, Koo H (2011) Biology of *Streptococcus mutans*-derived glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms. Caries Res 45 (1):69-86. doi:10.1159/000324598
4. Koo H, Falsetta ML, Klein MI (2013) The exopolysaccharide matrix: a virulence determinant of cariogenic biofilm. J Dent Res 92 (12):1065-1073. doi:10.1177/0022034513504218
5. Klein MI, Hwang G, Santos PH, Campanella OH, Koo H (2015) *Streptococcus mutans*-derived extracellular matrix in cariogenic oral biofilms. Front Cell Infect Microbiol 5:10. doi:10.3389/fcimb.2015.00010
6. Klein MI, DeBaz L, Agidi S, Lee H, Xie G, Lin AH, Hamaker BR, Lemos JA, Koo H (2010) Dynamics of *Streptococcus mutans* transcriptome in response to starch and sucrose during biofilm development. PLoS One 5 (10):e13478. doi:10.1371/journal.pone.0013478
7. Takahashi N, Nyvad B (2008) Caries ecology revisited: microbial dynamics and the caries process. Caries Res 42 (6):409-418. doi:10.1159/000159604
8. Arthur RA, Waeiss RA, Hara AT, Lippert F, Eckert GJ, Zero DT (2013) A defined-multispecies microbial model for studying enamel caries development. Caries Res 47 (4):318-324. doi:10.1159/000347050
9. Belli WA, Buckley DH, Marquis RE (1995) Weak acid effects and fluoride inhibition of glycolysis by *Streptococcus mutans* GS-5. Can J Microbiol 41 (9):785-791
10. Exterkate RA, Crielaard W, Ten Cate JM (2010) Different response to amine fluoride by *Streptococcus mutans* and polymicrobial biofilms in a novel high-throughput active attachment model. Caries Res 44 (4):372-379. doi:10.1159/000316541
11. Fernandez CE, Fontana M, Samarian D, Cury JA, Rickard AH, Gonzalez-Cabezas C (2016) Effect of Fluoride-Containing Toothpastes on Enamel Demineralization and *Streptococcus mutans* Biofilm Architecture. Caries Res 50 (2):151-158. doi:10.1159/000444888
12. Fernandez CE, Tenuta LM, Cury JA (2016) Validation of a Cariogenic Biofilm Model to Evaluate the Effect of Fluoride on Enamel and Root Dentine Demineralization. PLoS One 11 (1):e0146478. doi:10.1371/journal.pone.0146478
13. Ferretti GA, Tanzer JM, Tinanoff N (1982) The effect of fluoride and stannous ions on *Streptococcus mutans*. Viability, growth, acid, glucan production, and adherence. Caries Res 16 (4):298-307. doi:10.1159/000260612
14. Kondo KY, Buzalaf MA, Manarelli MM, Delbem AC, Pessan JP (2016) Effects of pH and fluoride concentration of dentifrices on fluoride levels in saliva, biofilm, and biofilm fluid in vivo. Clin Oral Investig 20 (5):983-989. doi:10.1007/s00784-015-1583-4
15. Kulshrestha S, Khan S, Hasan S, Khan ME, Misba L, Khan AU (2016) Calcium fluoride nanoparticles induced suppression of *Streptococcus mutans* biofilm: an in vitro and in vivo approach. Appl Microbiol Biotechnol 100 (4):1901-1914. doi:10.1007/s00253-015-7154-4

16. Marsh PD, Bradshaw DJ (1990) The effect of fluoride on the stability of oral bacterial communities in vitro. J Dent Res 69 Spec No:668-671; discussion 682-663. doi:10.1177/00220345900690S129
17. Mei ML, Li QL, Chu CH, Lo EC, Samaranayake LP (2013) Antibacterial effects of silver diamine fluoride on multi-species cariogenic biofilm on caries. Ann Clin Microbiol Antimicrob 12:4. doi:10.1186/1476-0711-12-4
18. Pandit S, Kim HJ, Song KY, Jeon JG (2013) Relationship between fluoride concentration and activity against virulence factors and viability of a cariogenic biofilm: in vitro study. Caries Res 47 (6):539-547. doi:10.1159/000348519
19. Svanberg M, Rolla G (1982) *Streptococcus mutans* in plaque and saliva after mouthrinsing with $SnF_2$. Scand J Dent Res 90 (4):292-298
20. Zhang M, He LB, Exterkate RA, Cheng L, Li JY, Ten Cate JM, Crielaard W, Deng DM (2015) Biofilm layers affect the treatment outcomes of NaF and Nano-hydroxyapatite. J Dent Res 94 (4):602-607. doi:10.1177/0022034514565644
21. Nassar HM, Gregory RL (2017) Biofilm sensitivity of seven *Streptococcus mutans* strains to different fluoride levels. J Oral Microbiol 9 (1):1328265. doi:10.1080/20002297.2017.1328265
22. Jeon JG, Klein MI, Xiao J, Gregoire S, Rosalen PL, Koo H (2009) Influences of naturally occurring agents in combination with fluoride on gene expression and structural organization of *Streptococcus mutans* in biofilms. BMC Microbiol 9:228. doi:10.1186/1471-2180-9-228
23. Al-Ahmad A, Follo M, Selzer AC, Hellwig E, Hannig M, Hannig C (2009) Bacterial colonization of enamel in situ investigated using fluorescence in situ hybridization. J Med Microbiol 58 (Pt 10):1359-1366. doi:10.1099/jmm.0.011213-0
24. Huang R, Li M, Gregory RL (2015) Nicotine promotes *Streptococcus mutans* extracellular polysaccharide synthesis, cell aggregation and overall lactate dehydrogenase activity. Arch Oral Biol 60 (8):1083-1090. doi:10.1016/j.archoralbio.2015.04.011
25. Masuko T, Minami A, Iwasaki N, Majima T, Nishimura S, Lee YC (2005) Carbohydrate analysis by a phenol-sulfuric acid method in microplate format. Anal Biochem 339 (1):69-72. doi:10.1016/j.ab.2004.12.001
26. Hartman D (2011) Perfecting your spread plate technique. J Microbiol Biol Educ 12 (2):204-205. doi:10.1128/jmbe.v12i2.324
27. Marinho VC (2009) Cochrane reviews of randomized trials of fluoride therapies for preventing dental caries. Eur Arch Paediatr Dent 10 (3):183-191
28. Walker CB (1988) Microbiological effects of mouthrinses containing antimicrobials. J Clin Periodontol 15 (8):499-505
29. Kulik EM, Waltimo T, Weiger R, Schweizer I, Lenkeit K, Filipuzzi-Jenny E, Walter C (2015) Development of resistance of *mutans streptococci* and *Porphyromonas gingivalis* to chlorhexidine digluconate and amine fluoride/stannous fluoride-containing mouthrinses, in vitro. Clin Oral Investig 19 (6):1547-1553. doi:10.1007/s00784-014-1379-y
30. Featherstone JD, Stookey GK, Kaminski MA, Faller RV (2011) Recommendation for a non-animal alternative to rat caries testing. Am J Dent 24 (5):289-294

31. White DJ, Chen WC, Nancollas GH (1988) Kinetic and physical aspects of enamel remineralization--a constant composition study. Caries Res 22 (1):11-19. doi:10.1159/000261077
32. Cenci MS, Pereira-Cenci T, Cury JA, Ten Cate JM (2009) Relationship between gap size and dentine secondary caries formation assessed in a microcosm biofilm model. Caries Res 43 (2):97-102. doi:10.1159/000209341
33. Filoche SK, Soma KJ, Sissons CH (2007) Caries-related plaque microcosm biofilms developed in microplates. Oral Microbiol Immunol 22 (2):73-79. doi:10.1111/j.1399-302X.2007.00323.x
34. Rudney JD, Chen R, Lenton P, Li J, Li Y, Jones RS, Reilly C, Fok AS, Aparicio C (2012) A reproducible oral microcosm biofilm model for testing dental materials. J Appl Microbiol 113 (6):1540-1553. doi:10.1111/j.1365-2672.2012.05439.x
35. Wong L, Sissons C (2001) A comparison of human dental plaque microcosm biofilms grown in an undefined medium and a chemically defined artificial saliva. Arch Oral Biol 46 (6):477-486
36. Lippert F, Lynch RJ (2014) Comparison of Knoop and Vickers surface microhardness and transverse microradiography for the study of early caries lesion formation in human and bovine enamel. Arch Oral Biol 59 (7):704-710. doi:10.1016/j.archoralbio.2014.04.005
37. Koo H, Jeon JG (2009) Naturally occurring molecules as alternative therapeutic agents against cariogenic biofilms. Adv Dent Res 21 (1):63-68. doi:10.1177/0895937409335629
38. Robinson, C., J. Kirkham, R. Percival, R. C. Shore, W. A. Bonass, S. J. Brookes, L. Kusa, H. Nakagaki, K. Kato, and B. Nattress (1997) A method for the quantitative site-specific study of the biochemistry within dental plaque biofilms formed in vivo. Caries Res 31 (3): 194-200
39. Pessan, Juliano Pelim, Karina Mirela Ribeiro Pinto Alves, Flávia de Moraes Italiani, Irene Ramires, José Roberto Pereira Lauris, Gary Milton Whitford, Kyriacos Jack Toumba, Colin Robinson, and Marilia Afonso Rabelo Buzalaf (2014) Distribution of fluoride and calcium in plaque biofilms after the use of conventional and low-fluoride dentifrices. Int. J. Paediatr. Dent. 24 (4): 293-302
40. Naumova EA, Weber L, Pankratz V, Czenskowski V, Arnold WH (2019) Bacterial viability in oral biofilm after tooth brushing with amine fluoride or sodium fluoride. Arch Oral Biol 97:91-96. doi:10.1016/j.archoralbio.2018.10.013
41. Ayoub, Hadeel M., Richard L. Gregory, Qing Tang, and Frank Lippert. (2020) The Anti-Caries Efficacy of Three Fluoride Compounds at Increasing Maturation of a Microcosm Biofilm. Arch Oral Biol: 104781
42. Pandit S, Jung JE, Choi HM, Jeon JG (2018) Effect of brief periodic fluoride treatments on the virulence and composition of a cariogenic biofilm. Biofouling 34 (1):53-61. doi:10.1080/08927014.2017.140458

Fig. 1. pH cycling days. Highlighted days: pH cycling using fluoride treatments; non-highlighted days: pH cycling with DIW instead of fluoride treatments. Aim 1, evaluated all exposure periods (T1; T2; T3) and two conditions (immediate collection and 10-day old biofilms). Aim 2, included early exposure: T1 (immediate; 3-day post treatment; 6-day post treatment) and middle exposure: T2 (immediate; 4-day post treatment).

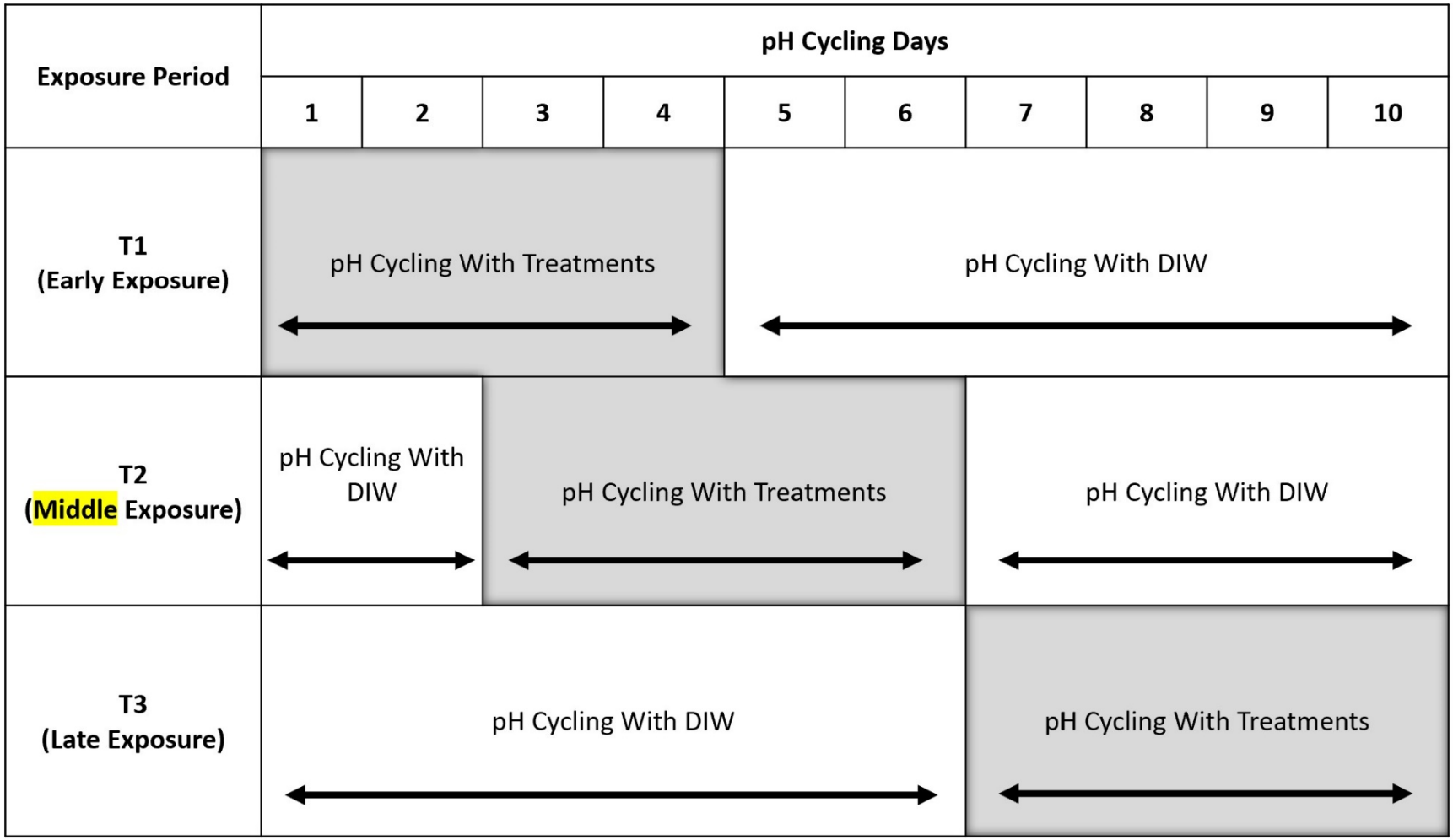

Fig. 2. Daily pH cycling protocol. Four treatment types were tested: Sodium fluoride (NaF), Stannous fluoride ($Snf_2$), Amine fluoride (AmF), and negative control (De-ionized water [DIW])