Applying High-Throughput Analysis to Biofilms

by

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Part I – Dentistry Mystery

Dr. S was a general dentist practicing in the western mountains of North Carolina, welcoming patients with a wide variety of needs into his office. The week of March 13th had been much like any other at DDS Family Dentistry, with the seats of the lobby filled daily with people anxiously waiting to receive care. However, the following week the office received a call that all practitioners dread—the health inspector was en route to assess the condition of the workplace. What was the reason for the unexpected inspection?

Let's backtrack to review some of the care that was administered during the week in question in order to establish the cause for the inspector's visit. The following patients were seen on Thursday, March 16th:

- Mark had been a smokeless tobacco user for nearly 25 years. His lower right molars had become so severely decayed that he was in excruciating pain. Dr. S offered the option to extract the teeth, and Mark decided to have the procedure performed immediately.

- Fourteen-year old Stephen had a biking accident. His front tooth hit the handlebars and knocked the bracket loose and bent the wire in his braces. Dr. S determined that no additional damage was done besides the one loose bracket and was able to complete the repair of the braces.

- Carla, Scott, Albert, and Samuel all came in for their bi-annual checkup. An oral examination revealed no disease, so all of these patients received routine teeth cleaning.

- Olivia had prom in two months and was not happy with her smile. She came in a week prior for impressions to be taken so that trays could be fabricated to deliver whitening product. Her trays were delivered on Monday, so she had come in to pick them up and receive instruction for use.

- Jaylen had been dealing with what he thought was an ulcer, but after using multiple over-the-counter (OTC) rinses for several weeks and seeing no improvement, he decided to make an appointment with Dr. S. After an oral examination, it was determined that a segment of metal had frayed on his bridge appliance. The stray end was clipped off using a pair of sterilized pliers, and Jaylen's pain was alleviated.

- Doris was scheduled for her annual checkup for her dentures. Dr. S observed signs of a malocclusion (in this case, crossbite) and made a minor adjustment by removing a small portion of several denture teeth using the high-speed handpiece. After rinsing off the appliance using water from the dental unit, the denture was placed back in the patient's mouth. A few bites on the articulating paper to check the contacts between the teeth made it clear that the problem had been solved.

- Brian had his braces removed last month and had already managed to lose his retainers. He came into the office in order to have new retainers made.

It was now a week later, and further discussion with the health inspector revealed that multiple patients had been admitted to the hospital with the same *Mycobacterium abscessus* bacterial infection. The factor they shared in common was a recent visit to DDS Family Dentistry. A thorough investigation of the facility was deemed necessary and patients treated within the same timeframe were notified about the apparent contamination and encouraged to visit their primary physician in order to be tested for the presence of this bacterial organism. *Mycobacterium abscessus* has been linked to the development of a "wide spectrum of skin and soft tissue diseases" (Lee *et al.,* 2015).

Questions

1. Using the information provided, complete the Treatment column in the following table and consider what the procedures have in common.

   Table 1: Treatment received by patient and presence of bacterial infection.

Patient	Treatment	+/- for *Mycobacterium abscessus*
Mark		+
Stephen		-
Carla		+
Scott		+
Albert		+
Samuel		+
Olivia		-
Jaylen		-
Doris		+
Brian		+

2. According to Table 1, what do you think could be the source of contamination leading to the infection? Use the following information about the various procedures the patients underwent to help you.

   - *Teeth extraction:* teeth extracted using sanitized instruments, area rinsed with water from dental unit waterline (DUWL), and gauze secured.
   - *Repair of broken braces bracket:* tooth lightly air-dried, bracket bonded into place, adhesive cured.
   - *Bi-annual checkup:* scaling, polishing, flossing, and rinsing of teeth from DUWL, along with complete oral examination with sterilized tools.
   - *Whitening tray pickup:* patient received whitening trays and supplied with whitening gel.
   - *Bridge repair:* anchor adjusted using sterilized instruments.
   - *Denture checkup:* appliance removed and adjusted, rinsed with water from DUWL, and re-inserted for patient.
   - *Fitting for retainer:* impressions taken of teeth and mouth rinsed with water from DUWL.

3. Based on your answer above, is this source an ideal environment for the formation of microbial biofilms?

Part II – Method of Analysis

In this section, you will learn of a high-throughput (HT) approach that can be used to analyze biofilms such as those found in waterlines like those in Dr. S's practice. The use of such methods could play an instrumental role in the discovery of optimal techniques to be utilized to control the undesirable growth of bacteria in certain environments.

Dental-unit waterlines (DUWLs) are flexible pieces of plastic tubing that supply water to the different handpieces used in a dental office. Studies have shown that insufficient means to drain the waterlines makes for an ideal aquatic environment to harbor bacterial biofilms, some of which serve as an oasis for pathogens (Walker *et al.,* 2000). *Legionella pneumophila*, *Mycobacterium* spp., *Candida* spp., and *Pseudomonas* spp. are among the bacterial species found within DUWLs in regular practice. Although DUWLs have been identified as a reoccurring source of contamination, they are not the only way in which harmful microbes can be introduced into a dental office; it is also worth noting that cross-contamination can happen in a dental laboratory, such as when polishing dentures. "More than 60% of the prostheses delivered to clinics from laboratories are contaminated with pathogenic microorganisms…originating in the oral cavity of other patients" (Agostinho *et al.,* 2004). Such evidence indicates that there is still work to be done to completely eliminate the presence of unwanted bacteria in the realm of dentistry. Nonetheless, experiments are in progress to aid this cause.

It would be nearly impossible to completely inhibit bacteria from entering the water supply of the DUWLs, as the source of the bacteria is commonly from the "municipal water piped into the dental unit and suck-back of patient saliva into the line due to lack of anti-retraction valves"(Szymańska, 2003). Biofilm formation follows this bacterial contamination. For this reason, researchers have aimed to reduce DUWL contamination via filtration, use of biocides and chemical disinfectants, chlorination, and ultraviolet light, as well as many other approaches, but have found no definitive answer. Filters are ineffective and the biofilms are highly resistant to degradation by biocides, disinfectants, and hypochlorite (Zhang *et al.,* 2007). This is where high-throughput technology could play a vital role. Such an approach may allow researchers to screen numerous samples simultaneously to facilitate discovery of ways to inhibit DUWL contamination.

On the next page of this handout an experimental protocol is described that makes use of high-throughput technologies. The high-throughput scale of the protocol provides a suitable method for rapid antibiotic testing of biofilms "and offers a simple and flexible method for the identification of multiple parameters and factors influencing biofilm formation" (Müsken *et al.,* 2010). The researchers in this study also took the necessary precautions to show the reproducibility of the staining procedure, image acquisition, and data analysis. With such additional methods, the accuracy of the results is increased.

Besides antibiotics, other compounds exist to control microbial contamination. A specific sterilizing chemical that has been evaluated is Sterilox, a hypochlorous acid solution identified as an agent able to reduce biofilms in DUWLs (Zhang *et al.,* 2007). In a study using Sterilox treatment, dental tubing was evaluated for biofilms using a scanning electron microscope before and after treatment. In comparison to the control group in the experiment, CFU (colony forming unit) counts were eliminated in the tubes exposed to Sterilox, whereas tubes treated with distilled water still showed CFU levels similar to baseline values. However, earlier in this case study it was observed that biofilms are often not susceptible to hypochlorite solutions (Zhang *et al.,* 2007), so there seems to be a discrepancy in the reported results. Using the protocol described on the next page, it should be possible to determine the accuracy of the claims made within each of these studies (i.e., not susceptible to hypochlorite vs. effectiveness of Sterilox) to a quantifiable extent. The fact that biofilms have a known presence within the DUWLs of dental practices is a good reason to further explore this issue. It is within the responsibilities of the scientific community to find a system to completely eradicate the threat to patients posed by these harmful bacteria.

Please review the protocol used in the experiment below while considering how this method could be applied in this case study.

Summary of methods used in "A 96-well-plate–based optical method for the quantitative and qualitative evaluation of *Pseudomonas aeruginosa* biofilm formation and its application to susceptibility testing" (2010) by Mathias Müsken, Stefano Di Fiore, Ute Römling, and Susanne Häussler. *Nature Protocols* 5(8): 1460–9.

Preculture Preparation

1. Inoculate bacteria into 2 ml of LB medium and incubate in an orbital shaker at 37 °C overnight.

2. Prepare subculture from overnight culture by diluting with fresh LB to an OD600 of 0.02.

Biofilm Growth

3. Use 100 µl diluted overnight culture per well of 96-well plate to inoculate wells. Seal plate with an air-permeable cover foil and incubate for 24 hour at 37 °C with humid atmosphere.

Antibiotic Treatment of Biofilms

4. Prepare stock solutions of chosen antibiotics in LB medium and perform 6–8 twofold serial dilutions per antibiotic in fresh LB using a multichannel pipette.

5. After 24 hours of growth, add 40 µl of desired antibiotic dilution to all wells. Consider testing different antibiotics and dilutions at a minimum of two replicates for each combination.

Staining of Biofilms

6. Prepare staining solution.

7. Add 20 µl of staining solution to each well directly after the addition of antibiotic solution using multichannel pipette.

8. Cover the plate with an air-permeable foil and return plate to incubator for 24 hours at 37 °C.

9. Immediately before microscopy, remove 96-well plate with treated biofilms from incubator.

Microscopy

10. Using the automated confocal Opera system, choose two positions at center of each well to acquire z-stacks of the biofilms. Select the desired wavelength to excite the dye and detect using band-pass emission filter at appropriate wavelength.

Data Analysis

11. Save image stacks.

12. Batch-process all images using background subtraction tool (i.e., ImageJ software).

13. Reduce appearance of planktonic bacteria and outlying pixels with a noise filter.

14. Reassemble image stack from individual binary images for each position and channel with Auto-PHLIP-ML software.

15. Use MATLAB to calculate descriptive parameters of biofilms from the integrated total of each individual slice of thresholded z-stack.

16. Calculate (with Microsoft Excel) the different proportions of green (live bacteria) as well as red and yellow (dead bacteria) biovolumes from analyzed stack using "colocalization in 3D" value and the parameters "red," "green," and "total biovolume" generated by PHLIP software. A biofilm is considered affected by antibiotic within given concentration range when there is a constant increase in the red + yellow (RY) biovolume fraction within given antibiotic concentration range and this fraction is at least 80% of total biovolume.

It is anticipated that the results from this protocol will provide insight into the susceptibility of biofilms to the antibiotics being studied. It is expected that, for instances where the biofilm is susceptible, the proportion of dead bacteria should increase with increasing concentrations and the fraction of viable bacteria should decrease.

Questions

1. Using the information provided, diagram a high-throughput method for testing the effectiveness of Sterilox on the *Mycobacterium abscessus* biofilms that existed in the case at Dr. S's office.

2. After reviewing the diagram your instructor will provide, how does your method compare? Do you believe the diagram represents a plausible technique for analyzing the effectiveness of Sterilox in reducing *Mycobacterium abscessus* biofilms in DUWLs? Explain your answer.

3. Besides the mentioned sources (DUWLs and prostheses), what could be another source of contamination in a dental office?

Figure 2. The arrow indicates a possible source of contact with water from DUWLs. A hose connects the piece indicated to the water bottle shown in Figure 1 and a button is pressed to release water through the water tip. *Credit:* PD, <https://pixabay.com/en/dental-chair-dentist-clinic-1491237/>.

Figure 1. Standard dental chair with the DUWL system circled. The water bottle is often times filled with water from the sink, coming from the municipal water supply. *Credit:* Andrew Horne, <https://commons.wikimedia.org/wiki/File:Dental_Chair_UMSOD.jpg>.

References

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