Monitoring of Bacterial Growth in Water using a Thickness Shear Mode Acoustic Wave Device

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1 Abstract

Acoustic wave devices have been successfully used for the sensing of various gases and vapors. A subset of these devices has the capability to interrogate liquids as well, making them attractive for biological sensing applications. In this work, heterotrophic plate count (HPC) measurements are considered with a thickness shear mode (TSM) acoustic wave sensor. Strains of *E. coli* K-12 were grown on the surface of agar growth medium coated TSM sensor surface. Growth rates were monitored for two concentrations of *E. coli* in water to establish the feasibility of utilizing acoustic wave devices in HPC measurements of water quality, and bacterial growth in general. It is found that faster HPC determinations are possible with the TSM device compared to the conventional microscopy, which can be further accelerated by utilizing higher frequency acoustic wave devices such as the shear horizontal surface acoustic wave device. Based on this work, we conclude that alternative matrix materials to hold the growth medium more effectively, statistical methods coupled to acoustic wave sensor array measurements, and the utilization of SH-SAW devices should yield rapid and reliable HPC measurement, and general bacterial growth monitoring sensors.

2 Introduction and Background

The quality of water intended for human consumption covers all aspects: drinking, cooking, bathing, and washing of devices that will come in contact with people. (WHO, 2003) Water is one of the most important substances required to sustain life. The average person requires approximately 20 liters of water each day (WHO, 2003). Bringing this fact to a larger scale means, for a population of a town or a city of 100,000 people, over two million liters of water is needed everyday. Obviously, with such large amounts required just to survive, water needs to be looked on as a precious commodity.

The pathogens listed in Table 1 are more than just common problems; they should be considered as possible biological threats of terrorism. These already well known bacteria would pose a threat, as the impact on a population would be significant. "…each nation's planning will have to take into account the laboratory capability

Table 1. Common bacteria found in water supplies as taken from WHO, 2003.

Bacteria	Health Significance	Persistence in water supplies[1]	Resistance to Chlorine[2]	Relative infective dose[3]
Campylobacter jejuni, C. coli	High	up to one month	Low	Moderate
Escherichia Coli	High	up to one month	Low	High
Salmonella typhi	High	up to one month	Low	High
Vibrio cholerae	High	up to one week	Low	High

[1] Detection period for infective stage in water at 20°C
[2] During infective stage with normal chlorine doses.
[3] Required amount to cause infection in 50% of adults tested.

required to minimize the impact and even to signal that an incident is occurring. The role of water testing will need to be re-evaluated…the HPC should be evaluated as a trigger for further investigation" (WHO, 2003)

It wasn't until after the invention of the microscope in the 17th century that the ability to look at cells and the development of microbiology lead to conclusions that diseases were caused by microorganism. As technology progressed, more conclusive studies were done that better identified specific organisms as the causative agent. Finally in the late 19th century, a general test for the biological load of water samples was developed, which is still used in the same fundamental method.

In 1883, Robert Koch published the first paper on methods for detection of microorganisms in water. Following this, Koch went on to do a number of studies on water quality. One of the most notable studies was performed on the Altona waterworks. This facility drew in contaminated water from the Elbe River and filtered it through sand. The results, demonstrated by Koch, were that by filtering the water and having bacterial count less than 100 colonies per milliliter, outbreaks of typhoid and cholera could be avoided. If the levels reached 1,000 colonies per milliliter due to filtration problems, an outbreak occurred (WHO, 2003).

Since the time of Koch, science has advanced considerably; however, the majority of techniques used today for the detection of microorganisms very closely resemble that of Koch's first work (WHO, 2003). There have only been modifications to the original method to enhance the results and specify particular organisms. Other improvements have been made by companies such as 3M™ that facilitate computer-assisted analysis of water samples. The major drawback of even the latest techniques is the need to incubate a sample from 24 hours to a week in time. Heterotrophic plate count (HPC) is a method that allows for the enumeration of live heterotrophic bacteria in water. Heterotrophs are broadly defined as microorganisms that require organic carbon for growth. They include bacteria, yeasts and molds. Heterotrophic plate counts are typically performed using a variety of nutrients and conditions intended to recover a wide range of micro-organisms. (WHO 2002)

"In general, HPC monitoring is used as a tool to gain information on the water treatment process and the general bacteriological quality of the water leaving the water treatment plant and within the distribution system" (WHO, 2003). HPC has advantages over simple coliform tests since not all pathogenic bacteria are a member of the coliform group (Deninger, 2001). It has numerous applications such as: process water monitoring, swimming pool quality monitoring, finished and raw water monitoring for drinking water systems. The method itself is relatively simple; a sample is applied to a nutrient enriched media that is then incubated at an elevated temperature for at least 24 hours. The media is then analyzed by counting the number of colonies present per unit area (Figure 1). This number is referenced back to the volume of sample originally used to generate a colony forming unit (CFU) per milliliter. A colony is essentially a

Figure 1. Culture plates with agar

dot on the surface of the media, where an organism was deposited and multiplied sufficiently that it can be observed visually (represents 10^6-10^9 individual cells).

Performance of this test can estimate the bacterial load on the water. Different countries give varying numbers for CFU, so it is difficult to state a definitive number. The EPA has avoided setting standards for plate counts most likely due to local variations in the bacterial densities observed (DuZuane, 1990). In the USA, guidelines for filtration systems state that a load of under 100 cfu/mL is achievable, 100-500 cfu/mL is a mediocre value and implies a seasonal fluctuation requiring attention, and above 500 cfu/mL is water of poor quality. All of this implies that the typical municipal water output at the tap should have fewer than 500 cfu/mL of bacteria (WHO, 2003).

HPC was originally used as a measure of the performance of filtration systems; today it still finds use as an indicator of water treatment effectiveness, a measure of re-growth organisms, and as a measure of possible interference with coliform measurements. Even with all of the uses of HPC, it is still not a sole indicator that a health risk is present. It is just one of many necessary measures to verify the content and safety of water.

Acoustic wave devices have been widely used primarily in the electronics industry to create stable frequencies for radio type devices. The fundamental principle of all acoustic wave devices is the same: An electrical signal from an electrode stimulates a piezoelectric material and creates a mechanical wave which is picked up by another electrode. This acoustic signal is then converted back into an electrical signal which can be compared to the input signal for frequency shift and power losses.

In a TSM device (Figure 3) an electrical signal is transmitted from one electrode to the one on the opposing side, where it is reflected. The transmission and reflection of the signal keeps reoccurring creating a standing resonance due to the physical properties of the substrate. The resulting signal can then be integrated with common electronics (Figure 2) to determine the optimum frequency, where the majority of the signal is reflected, referred to as the center or fundamental frequency (Ballantine et al, 1997).

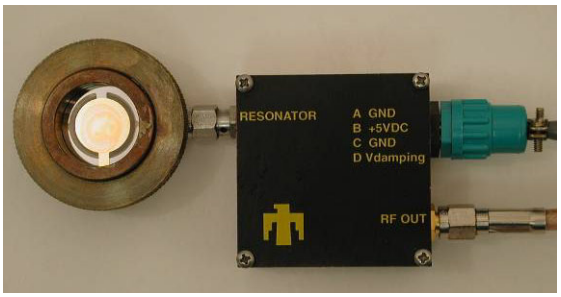


FIGURE 2. Sandia national laboratories' oscillator circuit and TSM fixture used in this work

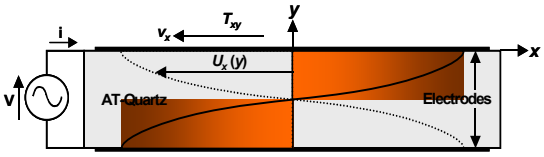


Figure 3. Schematic of TSM device with resonating wave

The Sauerbrey equation given in equation 1, relates the areal mass density deposited on the surface to resonant frequency shift for a TSM sensor.

$$\Delta f = -\frac{2 f_1^2 \rho_s}{\left(\mu_q \rho_q\right)^{\frac{1}{2}}} \qquad \textbf{Equation 1}$$

where f_1 is the fundamental mode oscillation frequency, ρ_s is the surface areal mass density, μ_q is the quartz shear stiffness, ρ_q is the quartz mass density and Δf is the expected frequency shift due to added mass ρ_s. (Ballantine et. al, 1997) The Sauerbrey equation does not hold true when viscoelastic properties of the film dominate, but could provide an initial estimate in this application.

The second important relation that is needed to complete the discussion of TSM sensors is the sensitivity of the device as given by equation 2:

$$S = \frac{df}{d\rho_s} = -\frac{f_1}{\left(\rho_q h_s\right)} \qquad \textbf{Equation 2}$$

where h_s is the thickness of the quartz. For a typical 5 MHz TSM device, the overall theoretical sensitivity (which is the total response to equal loading on both sides of the device) is approximately 58 Hz*cm^2/µg.

3 Experimental

All experiments were carried out at a temperature of 37 ºC with GPIB connectivity to a PC for data acquisition via custom LabView programs. Two sets of experiments are described.

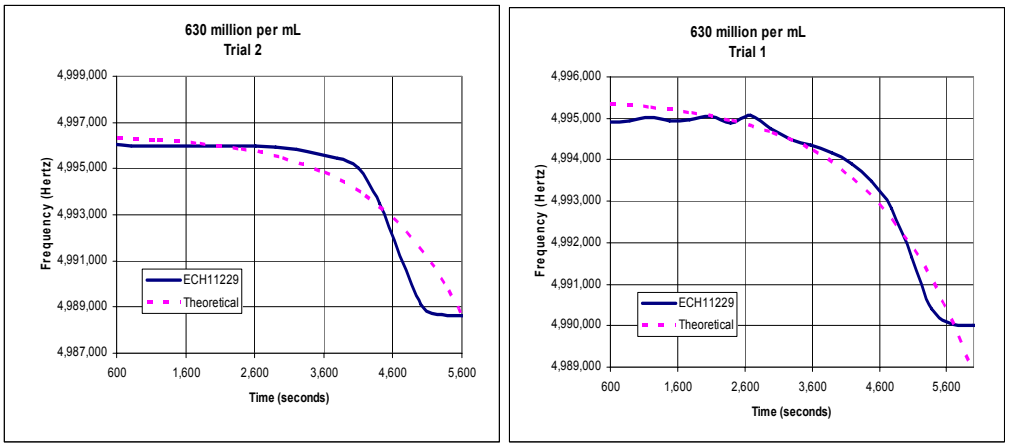
The first set of measurements were made using a Hewlett Packard P5384A frequency counter, an Agilent 34970A Data Acquisition/Switch unit equipped with voltage metering, and 50 ohm RF switching capabilities connected to a specially configured Lever oscillator as shown in Figure 2. Additional measurements were made using a Hewlett Packard 8751A Network Analyzer. The data from the sensor for both experiments were monitored and recorded using several LabView 6.1 programs written to incorporate all of the instruments and measurements. Time, frequency, damping voltage, and relative change in each was recorded from the various monitoring equipment, processed, and recorded in an ASCII tab delimited file. The temperature of the test cell was also recorded to monitor any temperature fluctuations.

In the second set of experiments, measurements were taken with a Maxtek Phase-Lock Oscillator-10i, Hewlett Packard 5334B universal counter and a Keithley 2010 multimeter. The TSM device and oscillator circuit were enclosed in fixtures designed to minimize the loss of signal between the crystal and oscillator circuit and minimize environmental interferences. Additional care was taken in cleaning of all equipment to prevent unwanted spreading of bacterial growth through the use of an autoclave and various disinfectants.

In the scope of this research, we operated with liquids or gels on the surface of the crystals, so special attention had to be paid to the circuitry. A Lever oscillator design was employed during the first set of experiments. This design was chosen due to the dampening effect of the agar on the sensor response. The Lever oscillator uses negative feedback to leverage the resonators impedance to maintain the gain and phase of the loop. Extra inductance was added to the circuit to compensate for the excessive load on the crystal and to maintain the circuit's phase and gain (Martin et. al, 1997).

Eosin methelyne blue (EMB) agar (Levine) was the primary growth medium coated on the TSM device. Spin coating was used to coat the devices with the agar solution. The agar solution was taken from just under a light boil and placed on the device surface within 30 seconds of removal from the heat and spun at approximately 2,500 revolutions per minute for 5 seconds. Once the sensors were coated, thickness measurements were made using a Dektak profilemeter. A clear step was achieved by masking the crystal with transparent tape prior to spin coating with agar. The tape was removed to generate a sharp edge to indicate the thickness of the film. Typically film thickness was kept to less then 10 µm. Due to the thinness of the agar film on the surface of the device, it is necessary to enclose the coated crystal in a high humidity chamber to prevent evaporative loss of water from the film layer.

The strain of *E. coli* K-12 that was selected for the first experiment was ATCC 11229, as this is a common and well-known strand. In the second set of experiments, a strain, ATCC 25922, was used. An initial broth was made and cultured with a count of 630 million organisms. In the second set of runs, Difco™ Nutrient Broth(beef extract 3.0 g, Peptone 5.0 g, 1 L de-ionized water) solution used to generate the bacterial samples used a different nutrient source from that found in EMB (Levine) agar (gelatin peptone 10.0 g, lactose 10.0 g, dipotassium phosphate 2.0 g, eosin Y 0.4 g, methylene blue 65.0 mg, agar 15.0 g, de-ionized water 1 L). In the initial runs, 10 µL of a broth solution were plated onto the sensor. In the second set of experiments, only 2.5 µL of the bacterial sample were plated. The first run used a moderate sample concentration of 155 million cells/mL, thus 375 thousand cells were placed on the sensor. The second run used a lower concentration, of 46 million cells/mL, so only 115 thousand cells were plated.



Figures 4 and 5. Frequency response to 630 million unit concentration of *E. coli* growth on TSM device with agar thin film

4 Results

Two initial trials were made using a high concentration of approximately 630 million units per milliliter of *E. coli* 11229. The concentration was determined through a standard culture and counting techniques over a 48 hour period. Results are shown in Figures 4 and 5.

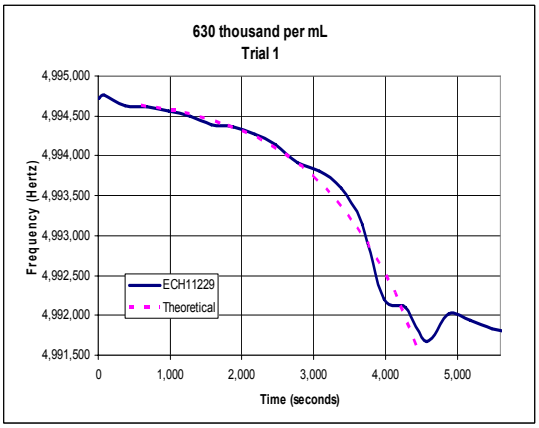


Figure 6. Frequency response to 630,000 unit concentration of E. coli growth on TSM device

In an attempt to study lower concentrations, 6.3 million bacteria were deposited onto the surface of the agar, with results shown in Figure 6. Through basic curve fitting procedures using the above equations and an Arrenihus rate law, these results indicate that bacteria with a mass of 1 pico-gram per unit show a count of approximately 700 million units per milliliter with a doubling time of approximately 17 minutes. The measured doubling time corresponds well to literature values of 20 minutes. These results indicate that at high levels of contamination results can be generated in less than an hour using our TSM sensor. The ½ way point (between starting and maximum response times) occurs at an average of the two at ~4900 seconds. The frequency response to a concentration of 630,000 bacteria per milliliter is illustrated in Figure 6. The ½ way point (between starting and maximum response) occurs at ~3800 seconds.

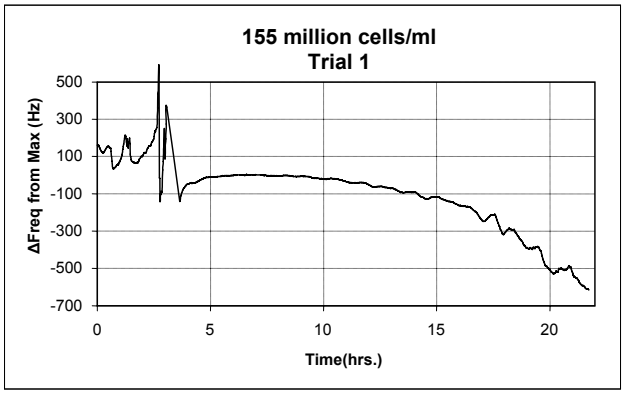


Figure 7. Frequency response to 2.5 mL of 155 million cells/mL sample

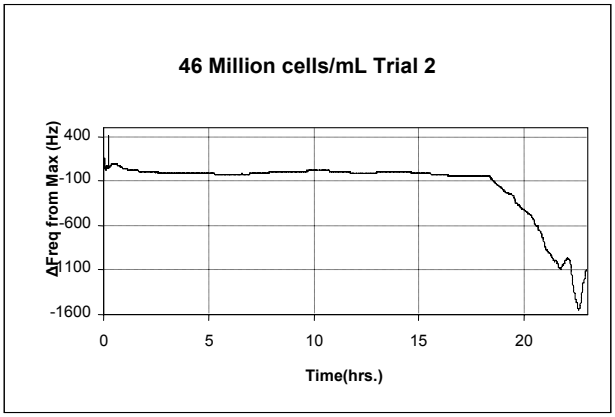


Figure 8. Frequency response to 2.5 mL of 46 million cells/mL sample.

Results from two trials of the second set of experiments are shown in Figures 7 and 8. Response times are larger than the first set of trails. The first of these trials that was loaded with 155 million units/mL only shifted 500 Hz over a 22 hour period. The second trial did not start showing signs of a growth curve until nearly 18 hours after plating. The shift for the second trial was nearly 1.1 kHz.

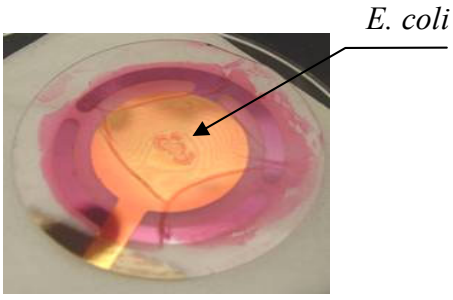


Figure 9. 2x magnification of bacterial growth on sensor.

Figures 9 and 10 demonstrate that colonies formed on the surface of the sensor but did not proliferate as extensively as the growth from the first set of experiments. Particularly also observed in the frequency shifts was the effects of temperature on the sensor response. Towards the end of the second trial there is drop in temperature in the test cell of one degree Celsius. The drop in temperature coincides with drop in frequency of nearly 500 Hz. No significant drop in temperature was observed in the first run.

5 Discussion

It is interesting to note that in the first set of experiments using the same starting concentration had a faster time interval to maximum response on the device. This could be an

indication that the organisms are in a severely overcrowded state at high deposited concentrations where there are less total available nutrients to be used for reproduction (and subsequently a sensor response). Looking at the shapes of the curves it appears that the lower concentration deposition started changing very quickly after deposition whereas the higher concentrations had longer lag times before significant changes were observed which would support this hypothesis.

The results of the two sets of experiments do not agree. The time frames for detection of bacteria were different by magnitudes even though the number of cells plated was similar. Several differences between the two experiments could have caused the difference in results. First, the EMB agar film coated on the sensor in the first set of experiments was significantly thinner than the film coated in the second trials. This could have reduced the dampening effect of the film on the sensor's response thus increasing the sensitivity of the sensor to changes in the material properties of the film. Although the agar film was thin enough to be treated as an ideal mass layer, it is possible that the material is more elastic than anticipated and thus it requires thinner coating.

Secondly, the nutrient broth used to culture the bacterial sample is a rich media with various nutrient sources. In the process of culturing, the *E. coli* adapt to the nutrient sources found in the broth. Once plated on the sensor, the bacteria must change their physiology to metabolize the new nutrient source. In the second set experiments, the *E. coli* had to switch from various nutrients in beef extract to lactose alone. The bacterial sample could have remained in lag phase for several hours. In the first trial of the second set, that lag phase was in the excess of 10 hours. EMB (Levine) agar was selected for this experiment because of its selectivity for *E. coli*. Unfortunately, the change from one nutrient source in the broth solution to another in the agar coating of the sensor encourages any lag phase that the bacteria would experience. This can be avoided by using a Difco™ nutrient agar(beef extract 3.0 g, peptone 5.0 g, Sodium Chloride 8.0 g, agar 15.0 g, de-ionized water 1 L) so the agar would provide the same nutrient source as the broth does.

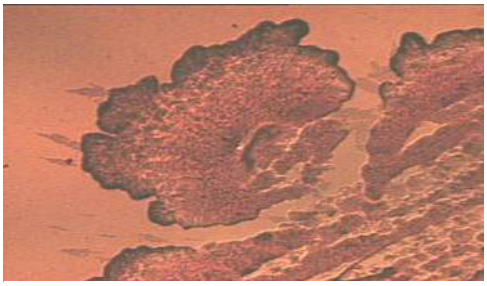


Figure 10. 20x magnification of *E. coli.* magnification of bacterial growth on sensor.

6 Conclusions

This work has shown that organisms can be cultured and detected on a QCM coated with growth media. By comparing the results of the two sets experiments one can conclude that optimization of the coating and testing procedure is still pending. Also, further inquiry into the behavior of the bacteria as they are introduced onto the sensor material would be beneficial. Whether or not the bacteria experience lag phase when plated needs to be determined. Additionally, for the purpose heterotrophic applications a general medium should be employed for the growth of a bacterial sample. Nevertheless, the initial results of this experiment show great promise for generating a powerful sensor capable of detecting and quantifying low density starting solutions (1000-100 CFU/mL). From this study, it was shown that there is merit to measuring the bacteria load in water using acoustic wave sensors. The determination of the number of organisms can be found in a fraction of the time needed for normal plating techniques. Further experiments are required to reinforce these initial data and

to develop a scheme for a more selective sensor in water bacteria load monitoring. The continuation of the project to incorporate higher frequency sensors to provide an even lower detection limit is necessary as well.

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