# Discussion

Microbial contamination of drinking water poses a threat to public health, with thousands of hospitalizations yearly even in devolved countries (Leclerc, Collier). The current standard methods for detection of contamination require a long and labor-intensive culturing stage to get results. Many modern methods have been suggested to replace these standard methods, however most molecular methods are too costly to be applied in the food and water industries (Popp). Spectroscopy based methods such as Raman and Fluorescence spectroscopy have also been put forth as alternatives, although these have been mostly applied in the medical fields (Popp, ???) or in highly contaminated water such as boreholes in indian rural areas and wastewater treatment plants (Sorensen, Yang). The application of low resolution Raman spectroscopy or fluorescence spectroscopy for the detection of bacteria in the water industry requires defining the detection threshold of these approaches, and comparing those to the industry and regulatory requirements. Furthermore, an ability to differentiate between bacteria using these quick analytical methods may have industrial (ref) and medical (ref) applications. In this study we used low resolution Raman spectroscopy and fluorescence spectroscopy to try and detect bacteria, first in pure cultures suspended distilled water, and later in real drinking water.

## Raman spectroscopy for detection and differentiation of bacteria in water [2]

Low resolution Raman spectroscopy involved applying a laser beam of 785 nm light onto water samples inoculated by different concentration of either *E. coli* or *B. subtilis*. Unsurprisingly, no clear spectra could be obtained by this crude approach. This is because the Raman signal of bacteria is very weak and complex, and even in high concentrations the chance of a Raman reaction occurring at a frequency which can be clearly visible using a low-resolution instrument is remote (ref: Schmilovitch, Efrima). Nonetheless, with the application of multivariate analysis using PLS, we managed to confidently detect *E. coli* and *B. subtilis* in concentrations of 108 and 104 CFU/ml respectively (fig. 1). While the finding regarding *E. coli* is problematic, as the concentration detected is very high, an ability to detect *B. subtilis* at a concentration of 104 CFU/ml shows a significant improvement on current methods such as absorbance at 590nm. That being said, for industrial purposes, this does not appear to be a promising path for the rapid and accurate detection of bacteria in water.

Different approaches have been applied in an attempt to enhance the Raman signal of the bacteria, without the use of expensive reagents, procedures or instruments. Drying the samples, suspending the bacteria in saline instead of water, using aluminium foil to reflect light and cause a minor surface-enhancement effect, boiling and cooling the samples did not show any improvement on the signal of *E. coli*.

Interestingly, the spectral fingerprint that was detected for *B. subtilis* did occur in the expected functional group region, meaning that the signal was likely from biological molecules such as ??? (ref from Emir perhaps?). In *E. coli,* the spectral fingerprint was smeared, and appeared to be derived from a reduction in overall signal, such as may be expected in an absorbance reaction. This is supplemented by the fact that below the 108 CFU/ml, no clear signal is observed and the PLS model could not find any variation, much like in the use of standard absorbance photometers below the 108 CFU/ml threshold no absorbance is observed.

In a different perspective, differentiation of *E. coli* and *B. subtilis* was excellent at a concentration higher or equal to the detection threshold (Table 2). This may suggest a possibility for differentiation between these species in a dense culture quickly and accurately, but may also be the product of the *E. coli* spectral fingerprint being derived from absorbance rather than Raman effect.

It is difficult to explain why exactly *E. coli* does not show a spectral fingerprint while *B. subtilis* does. One hypothesis is that the *B. subtilis* cells are either larger (ref) or create large aggregates which may scatter light more effectively. Another is that the difference in the cell wall structures of these cells may cause a different scatter effect; since these species are gram positive (*B. subtilis*) and gram negative (*E. coli*), this may affect the transmittance of light into a cell or the scatter off the cell wall. Further research may explain the molecular differences in low resolution Raman spectroscopy.

Taken all together, these results signify a poor ability to detect bacteria in water using low resolution Raman. This finding is surprising since several studies have already demonstrated a promise for the application of this method for detecting bacteria (Schmilovitch, Mizrah, Popp, Premasiri…). One of the possible explanations for this finding is the use of an instrument that may not be appropriate for the job. The Raman instrument chosen for this study has not been designed for the detection of bacteria, and while it represents an example of a low-resolution instrument, it has not been used in any of the studies cited. This could not have been known in advance since the instrument has been calibrated to read ethanol and appeared to work perfectly. Then again, the Raman effect of ethanol is very strong (ref) and a more sensitive, yet still cheap, instrument may yield better results. It has been suggested that suspending the bacteria in distilled water would ruin their Raman spectra, due to osmotic-stress-related lysis, or that the laser used may damage the cells considerably and will actually reduce the signal, however, a simple viability test has shown that the cell viability has not been compromised after irradiation and suspension in distilled water. Moreover, the use of lower laser energy nor suspending the cells in a saline solution instead of water improved Raman signal.

In conclusion, low resolution Raman may not be a good method for the detection of bacteria in water, according to the findings of this study, and the conditions of our experiment.

## Single Wavelength fluorescence spectroscopy for detection of bacteria in water [2]

In fluorescence spectroscopy, 2 main approaches are commonly applied in order to analyze samples, the first is the "peak picking" or single wavelength approach. This method involves measuring the emission of light at a single wavelength, after excitation by a single wavelength. This method has been applied in many studies for the detection of microbial contamination of water, based on fluorescence of proteinous substances in the tryptophan-like-fluorescence (TLF) peak of 275±5 nm excitation and 360±10 nm emission (Sorensen, baker, X4). In these different studies the minimal concentration for detection was 1-3 ppb of tryptophan equivalent, which is the calibrated measure of fluorescence in tryptophan units. Different studies have defined this threshold as signifying >2 CFU/100ml of *E. coli* contamination in drinking water (Sorensen 2015), >100CFU/100ml of *E. coli* in drinking water (Baker 2015) or 20-150\*1,000 cells/ml using flow cytometry (Bridgeman 2015). This means that the commonly observed detection threshold for bacteria in water, using the TLF peak single wavelength measurement is ~2 ppb of tryptophan equivalent.

We tried to quantify the detection limit of this method using our instrument in known bacterial species suspended in distilled non-fluorescent water, in tryptophan equivalent. We first calibrated our instrument by creating a calibration curve, using different wavelengths found in the literature and our own maximum emission wavelength in the TLF region (fig. 3). We found that, using our local maxima as the proxy for tryptophan equivalence, we can see a significant increase in TLF in *B. subtilis* and *E. coli* suspensions at a concentration of 105 CFU/ml and in *P. aeruginosa* suspensions at 106 CFU/ml. The increase is roughly to the known 2 ppb of tryptophan equivalence, signifying that the single wavelength method applied by Sorensen, Baker and Bridgeman is capapble of detecting bacteria in ~105 CFU/ml. Furthermore, statistical analysis has shown that using our lab spectrofluorometer we were capable of detecting bacteria at a concentration of 103, 104 and 104 CFU/ml for *B. subtilis, E. coli* and *P. aeruginosa* respectively using the single wavelength approach.

This finding signifies that using single wavelength fluorescence spectroscopy, at the TLF region, bacteria can be detected in clean water at a concentration of 103-104 CFU/ml. While not keeping up with the ministry of health regulations, this could have industrial implications, since it enables containment of contaminations at a relatively early stage, and much earlier than most methods enable, using a quick, cheap fluorimeter.

On the grounds of this finding, we collected water samples over a year from different drinking water wells in the north of Israel. The raw drinking water were transferred immediately after sampling to our lab for fluorescence scanning, and to the main microbial lab of the water authorities for standard microbiological testing which includes *E. coli,* coliform, faecal coliform counts and heterotrophic plate counts (HPCs). We then tried to apply our method for the enumeration of HPCs in drinking water and while little correlation was measured between fluorescence and bacterial concentration (R2=0.23, p<0.01, fig. 5), we could statistically differentiate samples with >90 CFU/ml from those with less bacteria (fig. 6). Furthermore, even though a very small sample size (n=2) was found of samples with over 500 CFU/ml, those samples showed a significant increase in fluorescence which could be visually observed (fig. 7).

When observing these highly contaminated samples, it was obvious that the signal is not limited to the TLF peak area of 275±5 nm excitation and 360±10 nm emission but is actually spread over a large area of the EEM, including the entire proteinous region (220-300 nm excitation, 300-400 nm emission, Yang), the edge of the humic substance region (210-300 nm excitation, 400+ emission, Yang) and also included a strong peak in the region of 210 nm excitation and 370±10 nm emission, as described by Simelane (Simelane). This has led us to believe that perhaps by observing the entire EEM we could improve significantly our detection abilities. This was particularly relevant since samples with <500 CFU/ml showed very little overall fluorescence (fig. 8), which may be clarified using multivariate analysis by PLS.

## Multispectral fluorescence spectroscopy for detection and differentiation of bacteria in water [2]

Analysis of entire fluorescence excitation emission maps (EEMs) is a common approach to characterize organic matter in water samples (ref: Yang, Heibati, Borisover, Stedmon). This enables taking into account the large regions previously mentioned, and with advanced multivariate statistics also to weigh their different contribution to the overall spectrum. PLS regression enables the formulation of a prediction model to predict the concentration of bacteria in water according to the entire EEM. In order to measure the benefit of using a PLS model we compared the detection threshold of *E. coli* in distilled water using the single wavelength approach, with a PLS model designed on either the spectrum of emission at 220-450 nm when excited by 280 nm light, or the entire EEM measured in the excitation range of 210-400 nm and emission of 220-450 nm. The reason for this comparison is because we believe that perhaps the emission spectrum of a single wavelength could be enough for the detection of bacteria in water, which will make future instrument design simpler since only a single light source will be required. We chose the 280 nm wavelength since it falls within the proteinous fluorescence zone and has shown good results. The difference in correlation and detection threshold between using a single wavelength, a PLS model based on single excitation of 280 nm and a PLS model based on the entire EEM was significant (Table 5). We found that by applying the PLS model on either the single excitation or the entire EEM we could lower the detection threshold by 2.5, 3.5 orders of magnitude. Furthermore, this method had less variation between groups which meant the Tukey-Kramer test can be applied, signifying a more robust result.

We followed up this finding by testing out the different detection thresholds of *B. subtilis* and *P. aeruginosa*. These bacterial species were chosen because a) they represent the gram positive and gram negative groups, which we expect to have a different chemical composition (ref) and b) because *P. aeruginosa* is a very common environmental pathogen which may be acquired from drinking water (ref). The detection thresholds of the different species were surprisingly different when using the entire EEM or only the 280 nm excitation spectrum; with differences of between 3-6 orders of magnitude of difference (table 6). This indicates that the 280 nm excitation spectrum does not capture the entire spectral fingerprint of the bacteria. This is likely because the proteinous region is a large 2-dimentional space and a large proportion of it is lost when a singly 1-dimentional slice of it is analysed. The variation in protein structures, the different positions of aromatic amino acids within proteins and the different aromatic moieties in bacterial cells are far more diverse than what can be captured by looking at the spectrum of emission from excitation at 280 nm, or likely any other single wavelength. It is interesting to note that the increase in sensitivity from 1-dimentional single wavelength (280 nm excitation, 360 nm emission) to a 2-dimetional spectrum at a single excitation wavelength (280 nm excitation) is ~3 orders of magnitude, and the increase from single excitation spectra to full 3-dimensional EEM is again several orders of magnitude (depending on the bacterial species).

Additionally, different species were found to have different detection thresholds, varying between 10-1,000 CFU/ml (Table 6). The difference, surprisingly, corresponds to the bacterial gram type; gram negative bacteria (*E. coli, P. aeruginosa*) were detected at a concentration of 10 CFU/ml, while gram positive bacteria (*B. subtilis*) were detected only at 103 CFU/ml. This may be due to the different nature of the cell wall of these bacteria; since gram positive bacteria have a thick layer of peptidoglycan which does not contain aromatic amino acids ([ref](https://glycopedia.eu/Peptidoglycan-Molecular-Structure)) and may block the light from reaching the inside of the cells. In addition, gram negative bacteria are known to have an outer layer which is abundant with proteins, some of which might contain aromatic side-chains and structures (ref). This difference in detection threshold hints that each bacterial species is likely to have its own spectral fingerprint, which may be used to differentiate different bacteria in water samples.

## Detection of heterotrophic bacteria in drinking water using fluorescence spectroscopy

Since the detection threshold for the detection of bacteria in water was as equal to or lower than 1,000 CFU/ml (table 6), this method may be applied for the detection of heterotrophic bacteria in drinking water, since the detection limit required by the ministry of health and other regulatory bodies is 1,000 CFU/ml for heterotrophic plate counts (MinOH).

In order to test this hypothesis, we scanned entire EEMs of raw drinking drinking water collected in wells in the north of Israel, and tried to train a PLS model to predict the concentration of heterotrophic bacteria in them. Although we collected water over a yearly period, in drinking water wells which were expected to have occasional microbial contaminations, only one sample over the entire sampling period had more than 1,000 CFU/ml and another over 500 CFU/ml. The rest of the samples (n=97) contained between 0-250 CFU/ml of heterotrophic bacteria as measured by standard methods. A model was then designed to differentiate between samples with either more or less than 90 CFU/ml. This threshold was chosen as it approximated 100 CFU/ml, but still contains several samples which had 99 or 98 CFU/ml. While the model did manage to significantly differentiate samples with either over or under 90 CFU/ml (fig. 11), the method did not show very good classification ability (figure 12). This means that while using the entire EEM for the detection of bacteria in real drinking water improves the sensitivity of fluorescence spectroscopy for the task, it is still not accurate enough for reliable differentiation (κ<0.6). We considered training a PLS model for the detection of higher concentration, but since only 2 samples had more than 500 CFU/ml over the entire measuring period, no prediction model could be calculated. Collecting a larger, more diverse data set, which involves more contamination events is likely to yield significantly better results.

It is difficult to tell which approach, the use of EEMs, single excitation wavelength spectrum or single wavelength fluorescence measurements, will be best applied for the detection of low concentrations of heterotrophic bacteria in drinking water based on this study. It is however, highly probable that fluorescence spectroscopy can be used for the detection of microbial contamination of water.

## Differentiation of different species of bacteria

Since different detection thresholds were calculated for different bacterial species, it is expected that each species has a different spectral fingerprint. In order to understand the spectral fingerprint of different bacteria, the VI of the PLS models used to quantify bacteria were plotted and analysed (fig. 13, 14). It is interesting to note that all bacterial strains, as well as heterotrophic bacteria have a spectral fingerprint in the TLF region (???), however, this fingerprint is different across species. Furthermore, it is surprising to see the importance of the scatter region (???) and the Raman scatter region for the quantification of bacteria. It appears that all strains have some effect on light scattering, which was surprising since Raman spectrometry did not yield good results. One explanation for this is that while the Raman spectrum measured in earlier experiments was based on excitation by near-infrared light (785 nm), the spectrofluometer excited the samples using UV light (200-400 nm) which has much higher energy levels. It is difficult to explain what molecular moieties are responsible for the different fluorescence specra of different species. It is clear that proteinous substances form a large part of the spectral fingerprint, but the source of these could be entire cells, cell wall components or dissolved proteins and peptides which were excreted by the bacteria and it is not clear from the spectra which of these actually contributes to the spectral fingerprint. Further studies are needed to elucidate the precise mechanism of the fluorescence reaction. It is clear however that these fluorescence fingerprints are correlated to colony-forming-units, and may thus be further employed in industrial scenarios.

Considering the different spectral fingerprints of different species, we hypothesised that a PLS discriminant model can be designed to classify different bacterial species. The EEMs of *E. coli, B. subtilis and P. aeruginosa* pure cultures in distilled water were used alongside the EEMs of clean water to try and classify the samples according to their spectra. Only samples at a high concentration (>104 CFU/ml) were used, and a good classification model was created (table 7.). This model has substantial agreement with real classifications (K=0.78), and shows near perfect classification of both *E. coli* and *B. subtilis* (16/17 correctly classified samples in the validation set). The model poorly distinguishes *P. aeruginosa* samples from water, although it does not confuse these with other species. This strongly suggests that fluorescence spectroscopy full EMM analysis can be used to differentiate between species of bacteria in pure, dense cultures. It is not clear exactly why *P. aeruginosa* is poorly distinguished. A bias is not expected since water samples were taken evenly from all sampling days where bacteria were tested equally.

## The importance of multivariate statistics [1]

This study demonstrates the breakthroughs that can be achieved when using advanced multivariate statistical models like PLS, instead of "peak picking" or visual assessment, on spectral data. In different experiments, data which seemed to have no significance to the naked eye was calibrated and modelled to enable higher sensitivities by several orders of magnitude (Figure 2, table 6). It is obvious that with the advancement in computer power, the speed of calculation and the accessibility of smart algorithms, spectral data can now be much better understood. Better models can probably be made with more advanced algorithms than PLS, some studies employ support vector machine (SVM) algorithms to better describe non-linear phenomena (ref), others use PARAFAC to resolve overlapping peaks in fluorescence spectra (ref: stedmon, Borisover) and few even use complex machine learning algorithms which include sparse coding and neural networks to understand the obscure interplay between variables (ref: Frolich 2017, Stockel 2010, Popp review).

## Conclusions [1]