# Discussion

Microbial contamination of drinking water poses a threat to public health, with thousands of hospitalization events yearly even in developed countries (Collier et al., 2012; Leclerc et al., 2002). The current standard methods for detection of contamination require a long and labor-intensive culturing stage to obtain results. Some alternatives have been suggested to replace these standard methods, however most molecular methods are too costly to be applied in the food and water industries (Stöckel et al., 2015). Spectroscopies, using Raman scattering and fluorescence emission have also been proposed, although these have been mostly applied in the medical fields (Eberhardt et al.; Krafft and Popp) or in highly contaminated water such as boreholes in Indian rural areas and wastewater treatment plants (Sorensen et al., 2015; Yang et al., 2015). The application of low resolution Raman spectroscopy or fluorescence spectroscopy for the detection of bacteria in the water industry requires knowledge of the detection threshold in order to compare those to the industry and regulatory requirements. Furthermore, an ability to differentiate between bacteria using these quick analytical methods may have sanitary, medical and industrial applications. In this study we used low resolution Raman spectroscopy and fluorescence spectroscopy to quantify bacteria, first present in pure cultures suspended in distilled water, and then in sampled groundwater intended for drinking use.

## Low resolution Raman spectroscopy: detection and differentiation of bacteria in water

. Nonetheless, with the application of multivariate analysis such as PLS, we managed to confidently detect *E. coli* and *B. subtilis* at concentrations of 108 and 104 CFU/ml respectively (Figure 7). Since detectable E coli concentrations are very high, the analytical performance of this method for this bacteria is generally not sufficient. However, in the case of *B. subtilis* a significant improvement of detection capability is expected when using low resolution Raman spectroscopy as compared with use of absorbance at 590 nm. However, the whole result does not seem promising for the rapid and accurate detection of bacteria in water.

Interestingly, the spectral fingerprint detected for *B. subtilis* was in the expected functional group region, meaning that the signal was likely from biological molecules such as sugars and lipid functional groups (Premasiri et al., 2005; Zeiri et al., 2004; Zeiri and Efrima, 2010). 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 absorption. This is supported by the fact that below the 108 CFU/ml, no clear signal was 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 4). 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 *E. coli* does not show a spectral fingerprint while *B. subtilis* does. Differences in the cell wall structures between two types of bacteria, i.e., gram positive (*B. subtilis*) and gram negative (*E. coli*), may cause a different scatter effect. Further research might be needed to explain the molecular nature of the differences in recognizing different bacteria by means of low resolution Raman spectroscopy.

Taken all together, these results indicate unfortunately a poor ability to detect bacteria in water using low resolution Raman spectrosscopy. This lack of success was surprising since several earlier studies have already demonstrated a potential of this spectroscopy for detecting bacteria (Mizrach et al., 2007; Schmilovitch et al., 2005; Stöckel et al., 2015). One of the possible explanations for this current discrepance is that the current instrument may not be appropriate for this 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 (Socrates, 2001) and a more sensitive, yet still cheap, instrument may yield better results. It has been hypothesized that suspending the bacteria in distilled water would weaken? The intensity of their Raman spectra, due to osmotic-stress-related lysis, or that the laser used might damage the cells thus reducing the signal. However, a simple viability test has shown that the cell viability has not been compromised due to suspension in distilled water and irradiation. Moreover, neither the use of lower laser energy nor suspending the cells in a saline solution instead of water improved Raman signal (data not shown). In conclusion, in the current study the use of low resolution Raman spectroscopy was not successful for the detection of bacteria in water.

## Fluorescence spectroscopy: use of a single excitation/emission wavelength pair for detection of bacteria in water

In fluorescence spectroscopy, one often approach in sample analysis is the "peak picking" or measuring emission intensity at a certain wavelength, after excitation by an another wavelength, lesser by magnitude. This method has been applied in many studies for the detection of microbial contamination of water, based on fluorescence of proteinaceous substances in the tryptophan-like-fluorescence (TLF) area (at the of 275±5 nm excitation and 360±10 nm emission (Baker et al., 2015; Bridgeman et al., 2015; Cumberland et al., 2012; Sorensen et al., 2018a; Sorensen et al., 2015; Sorensen et al., 2018b). In these studies the minimal detectable microbial concentration was equivalent to the 1-3 ppb concentration of free tryptophan. Different studies have defined this threshold as signifying >2 CFU/100ml of *E. coli* contamination in drinking water, >100CFU/100ml of *E. coli* in drinking water or 20-150 x 1,000 cells/ml using flow cytometry.

We quantified the detection limit of this method using our instrument for known bacterial species suspended in distilled non-fluorescent water, by using a 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 (. ). We could 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. Expressing this microbially-related TLF in terms of concentration of tryptophan having the same emission intensity at its maximum, this detection threshold was approximately equivalent to the presence of 2 ppb of tryptophan in solution. This suggests that the studies using microbial detection at a single excitation/emission wavelength and claiming the detection limit equivalent to 1-3 ppb of tryptophan are capable of detecting bacteria at the ~105 CFU/ml level. Furthermore, statistical analysis of our data has shown an ability to detect bacteria at a concentration of 103, 104 and 104 CFU/ml for *B. subtilis, E. coli* and *P. aeruginosa,* respectively, using this simple peak-picking approach.

This detection limit seems to be insufficient to the requirements of Israeli Ministry of Health regulations (2013), but it could have implications, e.g. in water, food, pharmaceutical and beverage industry for monitoring microbial contamination at a relatively early stage, and much earlier than most methods enable, using a quick, cheap fluorimeter.

However, in our further analysis of groundwater samples collected through the year, weak (albeith significant) correlation was found between emission at a certain point of the EEM map and HPC bacterial concentration (R2=0.23, p<0.01, Figure 11**Error! Reference source not found.**), It was possible to differentiate samples with >90 CFU/ml from those with less bacteria (Figure 12). 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 (Figure 13).

Examining the highly contaminated samples, it was clear that the emission was not limited to the TLF region but was actually spread over a large area of the EEM, including the entire proteinaceous region ( the 220-300 nm excitation wavelength range, the 300-400 nm emission wavelength range ,(Yang et al., 2015)), the edge of the humic substance region (excitation in the 210-300 nm range and the emission above 400 nm ,(Yang et al., 2015)) and also included a strong peak with excitation around 210 nm and emission at 370±10 nm emission, as described by Simelane (Simelane, 2013). This has led us to believe that examining the entire EEM could improve significantly the detection abilities. This was particularly relevant since samples with <500 CFU/ml showed very little overall fluorescence (Figure 14). Such weak signals could be properly analyzed using PLS multivariate analysis.

## Fluorescence spectroscopy: use of EEMs for detection and differentiation of bacteria in water

Another common approach for analysis of fluorescence data uses entire excitation-emission matrices (EEMs) ((Borisover et al., 2009; Carstea et al., 2016; Heibati et al., 2017)). This enables taking into account the large emission regions previously mentioned. PLS regression enables the formulation of a model for predicting the concentration of bacteria in water, based on the entire EEM data. In order to examine whether a PLS model is beneficial in data analysis and prediction, we compared the detection threshold of *E. coli* in distilled water using the peak-picking approach, with a PLS model built on either the spectrum of emission at 220-450 nm when excited by 280 nm light (which is typical for excitation of proteinaceous material), or on 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. The differences between using a peak-picking method, a PLS model of a single emission spectrum based on single excitation at 280 nm and a PLS model based on the entire EEM were significant in terms of correlation and detection thresholds (Table 7). Use of the PLS model reduced the detection threshold by 2.5-3.5 orders of magnitude. In addition, when applying PLS, a lesser variation between groups is observed thus allowing application of the Tukey-Kramer test and signifying a more robust result.

We extended this analysis toward *B. subtilis* and *P. aeruginosa*. These bacterial species were chosen because a) they represent the gram positive and gram negative groups, expected to have a different chemical composition (Jean and Simorre, 2018) and b) *P. aeruginosa* is a very common environmental pathogen which may be acquired from drinking water (Costa et al., 2015). The detection thresholds of these species differed when using the entire EEM or only the single emission spectrum: the differences reached 3-6 orders of magnitude (Table 8). This indicates that the single emission spectrum does not capture the entire spectral fingerprint of the bacteria. This is possible because a large fraction of the proteinaceous region in a fluorescence EEM is lost when a single 2-dimentional slice of it is analysed. The differences in protein contents, chemical composition and structures, including variable spatial distributions of aromatic amino acids within proteins and the different aromatic moieties in bacterial cells as well as the interactions of fluorophores with the surrounding may not be properly captured by examining a single emission spectrum or, certainly fluorescence emission at a single excitation-emission wavelength pair. It is teaching to see that when comparing use of PLS-analyzed emission spectrum with peak-picking, the detection threshold decreases by ~3 orders of magnitudes. Several more orders of magnitude of improvement are reached when 3D EEM data analyzed with PLS instead of 2D emission spectrum.

Additionally, different species were found to have different detection thresholds, varying between 10-1,000 CFU/ml (Table 8). 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 (Jean and Simorre, 2018) and may block the light from reaching the inside of the cells. IGram negative bacteria are known to have an outer layer which is abundant with proteins, some of which might contain aromatic side-chains and structures (Madigan et al., 2006) thus increasing the whole emission. 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 groundwater intended for drinking use by means of fluorescence spectroscopy

Since the threshold for the detection of bacteria in water was equal to or lower than 1,000 CFU/ml (Table 8), this method may be applied for the detection of heterotrophic bacteria in drinking water. The detection limit required by the Ministry of Health and other regulatory bodies is 1,000 CFU/ml for heterotrophic plate counts (2013; Allen et al., 2004).

In order to test this hypothesis, we scanned entire EEMs of raw 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 0-300 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 (**Error! Reference source not found.**), the method did not show high classification ability (Figure 17). This means that while examining the entire EEM improved the sensitivity of fluorescence spectroscopy for the detection of bacteria in real water samples, 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.

Currently, based on this study, it is difficult to conclude which approach - the use of EEMs, single emission spectrum or single excitation/emission wavelength pair fluorescence measurements - will be best applied for detection of low concentrations of heterotrophic bacteria in drinking water. However, based on the experience with identification and quantification of pure culture bacteria, one may expect that use of the whole EEM data might be more efficient for successful detection of microbial contamination of water.

## Differentiation of bacterial species

Since different detection thresholds were calculated for different bacterial species, it is expected that each species has a different spectral fingerprint. In order to examine the spectral fingerprints of different bacteria, the VI of the PLS models used to quantify bacteria were plotted and analysed (Figure 18 and Figure 19). All the bacterial strains, as well as heterotrophic bacteria have a spectral fingerprint in the TLF region (at 225–237 nm excitation with 340–381 nm emission and 270–280 nm excitation and 330–368 nm emission) however, this fingerprint is variable across species. Furthermore, it is surprising to see the importance of the Rayleigh 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 low resolution Raman spectrum was measured with excitation by near-infrared light (785 nm), the EEM were obtained using UV light (200-400 nm) which has much higher energy levels. It is difficult to explain currently how chemical composition present in different bacterial cells may be responsible for different fluorescence spectra of species. It is clear that proteinaceous substances demonstrating tryptophan-like fluorescence form a large part of the spectral fingerprint, but they may represent entire cells, live or died, cell wall components or dissolved proteins and peptides which were excreted by the bacteria. Further studies are needed to elucidate the chemical and biological components leading ti the observed fluorescence fingerprints. It is clear however that intensity of these fluorescence fingerprints correlates with the concentration of species, and may thus be further employed in industrial environments.

Considering the spectral fingerprints of different species, we hypothesised that a PLS discriminant model can be designed to classify microorganisms. The EEMs of *E. coli, B. subtilis* and *P. aeruginosa* pure cultures in distilled water were used alongside the EEMs of clean water to 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 9). 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. waswasAll together, the data analysis suggests that fluorescence spectroscopy of EEMs can be used to differentiate species of bacteria in pure, dense cultures.

## The importance of multivariate statistics

This study demonstrated the breakthrough 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 15, Table 7). 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 (Rösch et al., 2005), others use Parallel Factor Analysis (PARAFAC) to resolve overlapping peaks in fluorescence spectra (Baghoth et al., 2011; Borisover et al., 2009; Nebbioso and Piccolo) and few even use complex machine learning algorithms which include sparse coding and neural networks to understand the obscure interplay between variables (Frolich et al., 2017; Stöckel et al., 2010).